



OPEN Targeting dual-specificity phosphatase 23 to overcome chemoresistance and stem cell-like behavior in non-small cell lung cancer cells

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Chemoresistance remains a significant challenge in the treatment of non-small cell lung cancer (NSCLC) and is often associated with poor clinical outcomes. This resistance is closely linked to cancer stem cell (CSC)-like properties, such as the ability to evade anoikis. In this study, we identify dual-specificity phosphatase 23 (DUSP23) as a critical regulator of CSC-like traits and chemoresistance. DUSP23 expression was significantly elevated in cell clusters grown under ultra-low adhesion conditions that mimic CSC-promoting environments. *DUSP23* knockdown impaired cluster formation, and suppressed the expression of *SOX2*, a representative marker for CSC-like property, and lung tumorigenesis in vivo. In cisplatin-resistant NSCLC cells, *DUSP23* knockdown further decreased invasive behavior and induced apoptosis. Mechanistically, DUSP23 promoted STAT3 phosphorylation, thereby enhancing *SOX2* transcription in cisplatin-resistant cells. Clinically, a DUSP23–*SOX2* target gene signature stratified patients into high- and low-risk groups, with high-risk patients showing significantly shorter overall and disease-free survival. Collectively, our findings indicate that DUSP23 plays a central role in maintaining CSC-like properties and chemoresistance through the regulation of *SOX2*. Targeting DUSP23 may provide a novel therapeutic approach to overcome treatment resistance and improve outcomes for patients with NSCLC.

Lung cancer is the most commonly diagnosed cancer and the leading cause of cancer-related deaths worldwide, with approximately 2.5 million new cases and 1.8 million deaths reported in 2022¹. Histopathologically, lung cancer is classified into two major types: small-cell lung carcinoma (SCLC) and non-small cell lung cancer (NSCLC), which account for 85% of all cases². NSCLC includes subtypes such as squamous cell carcinoma, large-cell carcinoma, and adenocarcinoma, making it the most frequently diagnosed form of lung cancer³. Early-stage NSCLC is typically treated with surgical resection, while advanced or unresectable cases often require chemotherapy, such as cisplatin, in combination with radiation therapy. However, despite these interventions, the prognosis for advanced NSCLC remains poor, largely due to the development of treatment resistance, including chemoresistance⁴.

Chemoresistance, a significant challenge in lung cancer treatment, is closely associated with cancer stem cell (CSC), a small population of tumor cells with self-renewal capacity and the ability to drive tumor formation⁵. CSCs are known to promote tumor progression, metastasis, and therapy resistance by surviving treatments that eliminate most cancer cells, ultimately leading to recurrence and relapse^{6,7}. These cells exhibit a mixed epithelial-mesenchymal phenotype, enhancing their invasiveness and metastatic potential⁸. Moreover, CSCs display resistance to anoikis, a form of programmed cell death triggered by detachment from the extracellular matrix. This anoikis resistance is particularly critical for CSC survival and tumor dissemination in fluid culture conditions that mimic the tumor microenvironment⁹. Therefore, elucidating the molecular targets that support CSC survival under CSC-promoting conditions is essential for developing effective strategies to overcome chemoresistance and prevent tumor recurrence. Dual-specificity phosphatase 23 (DUSP23) is a member of the dual-specificity phosphatase family, which dephosphorylates phosphoserine/threonine and phosphotyrosine residues in the target proteins¹⁰. Unlike other DUSPs, DUSP23 lacks a MAP kinase-targeting motif, making it the

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smallest catalytically active DUSP¹⁰. Previous studies have linked DUSP23 to the regulation of various cellular processes, including dephosphorylation of ERK1¹¹, activation of c-Jun N-terminal kinase (JNK) and p38 under osmotic stress¹², and modulation of cell–cell adhesion and migration through β -catenin dephosphorylation¹³. Our previous work identified *DUSP23* as upregulated in malignant lung cancer and demonstrated its negative correlation with prognosis¹⁴. Transcriptomic analysis further suggested that DUSP23 is involved in cancer-related pathways such as the cell cycle and filopodium assembly under adherent conditions¹⁵. Recent studies have linked DUSP23 to cisplatin-resistant lung adenocarcinoma (LUAD)¹⁶, acute myeloid leukemia¹⁷, and breast cancer, where it enhances cell growth and survival¹⁸. However, its role in maintaining CSC-like properties and supporting cell survival under non-adherent, CSC-promoting condition remains unclear.

In this study, we demonstrated that DUSP23 plays a critical role in regulating CSC-like properties and chemoresistance in NSCLC. By promoting stem cell-related pathways and supporting the survival and invasiveness of cisplatin-resistant cells, DUSP23 contributes to the persistence of treatment-resistant cancer cells. Our findings highlight DUSP23 as a potential therapeutic target for addressing chemoresistance and improving outcomes in NSCLC.

Results

DUSP23 expression is associated with NSCLC progression and CSC-like features

To further investigate the clinical significance of DUSP23 in lung cancer, we analyzed its mRNA expression levels and associated clinical outcomes using two independent datasets. Consistent with our previous findings identifying DUSP23 as a prognostic marker¹⁵, its expression was significantly higher in NSCLC tissues compared to normal lung tissues in both The Cancer Genome Atlas (TCGA) and Okayama cohorts (Fig. 1a). Kaplan–Meier survival analysis further demonstrated that NSCLC patients with high *DUSP23* expression exhibited significantly shorter disease-free survival compared to those with low expression levels (Fig. 1b). These findings highlight the critical involvement of DUSP23 in NSCLC progression and recurrence.

CSCs are critical drivers of chemoresistance and tumor recurrence^{5,6}. Based on this, we hypothesized that DUSP23 may be associated with CSC-like properties in cancer cells. Gene set enrichment analysis (GSEA) of the TCGA-LUAD cohort revealed a strong correlation between high *DUSP23* expression and the upregulation of genes involved in stemness pathways, including embryonic stem cells and CSC-related signaling (Supplementary Fig. 1a). To experimentally validate this association, we cultured A549 NSCLC cells under ultra-low adherent conditions in serum-free media supplemented with epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF), which are known to promote the formation of floating cell clusters with CSC-like properties⁹ (Fig. 1c). Under these conditions, the expression of *SOX2*¹⁹ and *ALDH1*^{20,21}—representative markers associated with CSC-like characteristics and self-renewal potential—were significantly elevated in floating cell clusters compared to adherent cells (Fig. 1d). Notably, DUSP23 expression was also significantly increased at both the mRNA and protein levels in these clusters (Fig. 1d, e and Supplementary Fig. 1b), suggesting the potential involvement of DUSP23 in regulating CSC-like properties under non-adherent conditions.

DUSP23 is critical for cell proliferation under non-adherent conditions and tumorigenesis in vivo

We investigated whether DUSP23 influences cell proliferation under non-adherent conditions that mimic stem cell-like behavior in cancer cells by performing a cell cluster formation assay using A549 cells with *DUSP23* knockdown (KD; Supplementary Fig. 1c and d). *DUSP23* KD significantly reduced both the number and size of cell clusters, with smaller cluster diameters compared to shMock control cells (Fig. 1f). In contrast, overexpression of *DUSP23* in H1299 cells, which exhibit relatively low basal DUSP23 expression, resulted in a notable increase in cluster formation rates and cluster diameters (Fig. 1g, Supplementary Fig. 1e and f). Furthermore, to assess the in vivo tumorigenic potential of DUSP23, we subcutaneously injected murine Lewis lung carcinoma (LLC1) cells with stable *Dusp23* KD (Supplementary Fig. 1g). Loss of *Dusp23* markedly reduced tumor growth and tumor weight (Fig. 1h–k). These observations suggest that DUSP23 supports cell proliferation and cluster growth under non-adherent conditions and in vivo models that promote stem cell-like features in lung cancer cells.

DUSP23 promotes SOX2 expression and cancer stemness-related pathways

To investigate the role of DUSP23 in cancer stem-like characteristics, we performed RNA sequencing (RNA-seq) analysis on A549 cell clusters with *DUSP23* KD (sh*DUSP23*) and compared them to control cluster (shMock). This analysis identified 788 genes that were downregulated and 475 genes that were upregulated in the *DUSP23* KD cell clusters ($|\text{Log}_2(\text{fold change})| > 1$ and $p\text{-value} < 0.05$; Fig. 2a). GSEA of the differentially expressed genes (DEGs) showed that stem cell-related gene signatures were significantly enriched in shMock compared to sh*DUSP23* cells (Supplementary Fig. 2a–c). In addition, HALLMARK gene sets analysis of DEGs revealed that cell proliferation-related processes, including the G2M checkpoint, E2F targets and Myc targets, were markedly enriched in shMock clusters, whereas immune-related processes were enriched in *DUSP23* KD clusters (Supplementary Fig. 2d). Collectively, these findings suggest that DUSP23 supports proliferative and stemness-associated processes, thereby helping to sustain CSC-like features in cell clusters.

To further examine the signaling pathways affected by *DUSP23* KD, we performed NetPath analysis using the RNA-seq data. This analysis revealed the downregulation of pathways associated with the EGF receptor (EGFR), T cell receptor, and interleukin-4 as well as pathways linked to transforming growth factor β , receptor activator of nuclear factor κ B ligand, and thyroid-stimulating hormone (Fig. 2b). Gene ontology analysis also supported the involvement of DUSP23 in pathway regulating stem-like traits, reinforcing its role in maintaining cancer stem-like characteristics.

Notably, the leading-edge analysis of GSEA results revealed significant downregulation of SOX2 target genes in *DUSP23* KD cells (Fig. 2c). Reverse transcription-quantitative PCR (RT-qPCR) analysis further showed a

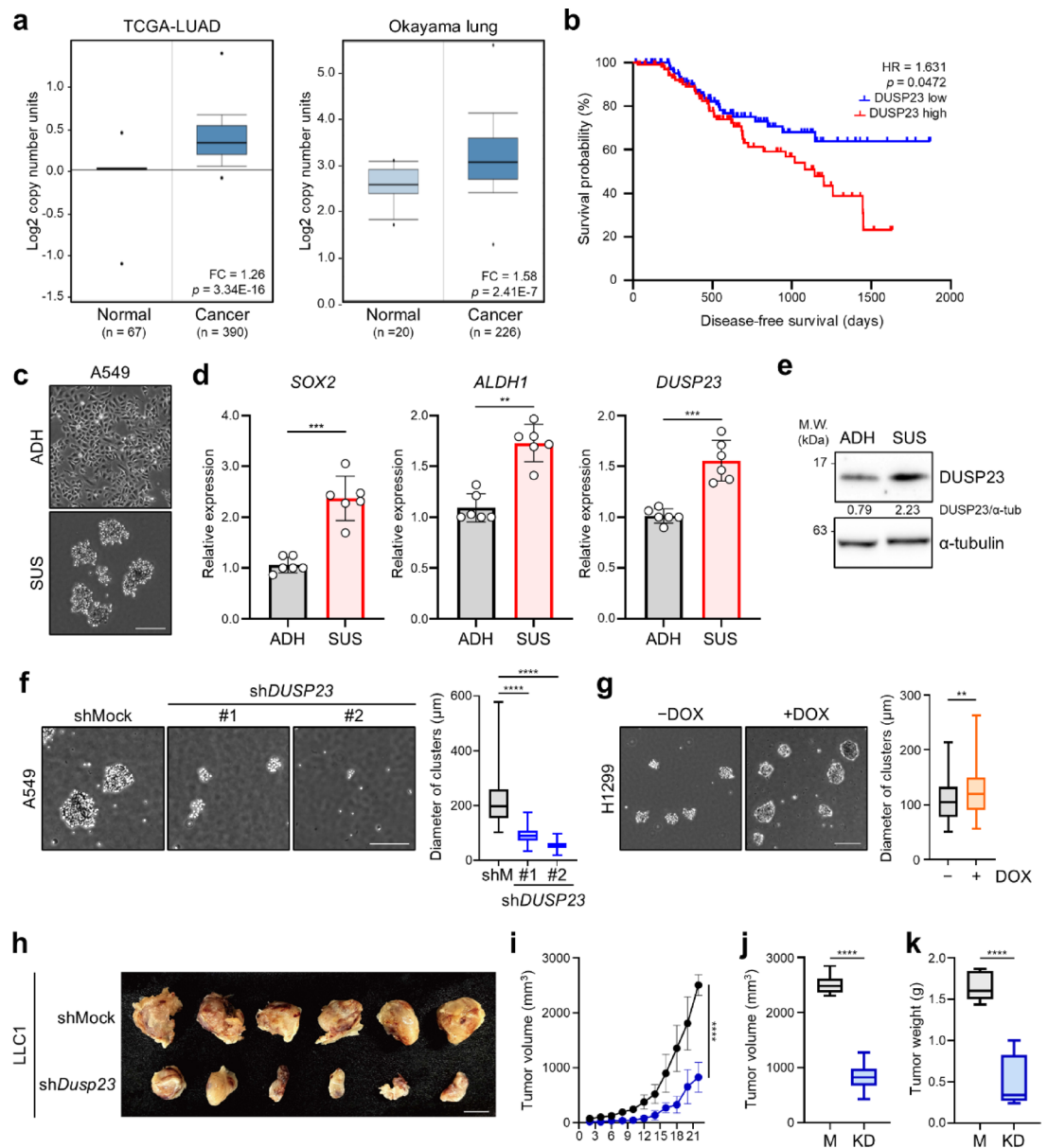


Fig. 1. Elevated DUSP23 expression in NSCLC contributes to cell cluster formation and tumorigenesis in vivo. **(a)** Analysis of *DUSP23* using the Oncomine database. Box plot shows mRNA levels of *DUSP23* in LUAD compared with normal tissues, based on TCGA and Okayama LUAD datasets, respectively. **(b)** Disease-free survival curves were plotted using the Kaplan–Meier method. Patients with low of *DUSP23* expression are shown in blue lines and those with high expression in red lines. **(c)** Representative images of A549 cells under adherent (ADH) and fluid culture (suspended; SUS) conditions. Scale bar: 250 μ m. **(d)** Bar graph of *SOX2*, *ALDH1*, and *DUSP23* mRNA expression in ADH and SUS A549 cells determined by RT-qPCR. Statistical analysis was performed using Welch's t-test (*SOX2* and *DUSP23*) and Mann–Whitney test (*ALDH1*). **(e)** Western blots analysis of *DUSP23* expression in ADH and SUS A549 cells. **(f)** Cluster formation assay of *DUSP23* KD A549 cells. Scale bar: 250 μ m. Box plot shows clusters diameters > 50 μ m, (minimum to maximum). Statistical analysis by one-way ANOVA with Tukey's post hoc test ($F_{2,10758} = 798$). **(g)** Cluster formation assay of DOX-untreated and -treated H1299 cells. Scale bar: 250 μ m. Box plot shows cluster diameters > 50 μ m (minimum to maximum). Statistical analysis by Mann–Whitney test. **(h)** Representative tumors images from shMock and shDusp23 groups. Scale bar: 1 cm. **(i)** Tumor growth curves of shMock and shDusp23 groups. **(j, k)** Tumor volume and weight of shMock and shDusp23 groups at end point. Statistical analysis by Welch's t-test. Data are presented as mean $\pm$ standard deviation. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; NSCLC, Non-small cell lung cancer; FC, fold change; LUAD, lung adenocarcinoma; TCGA, The Cancer Genome Atlas.

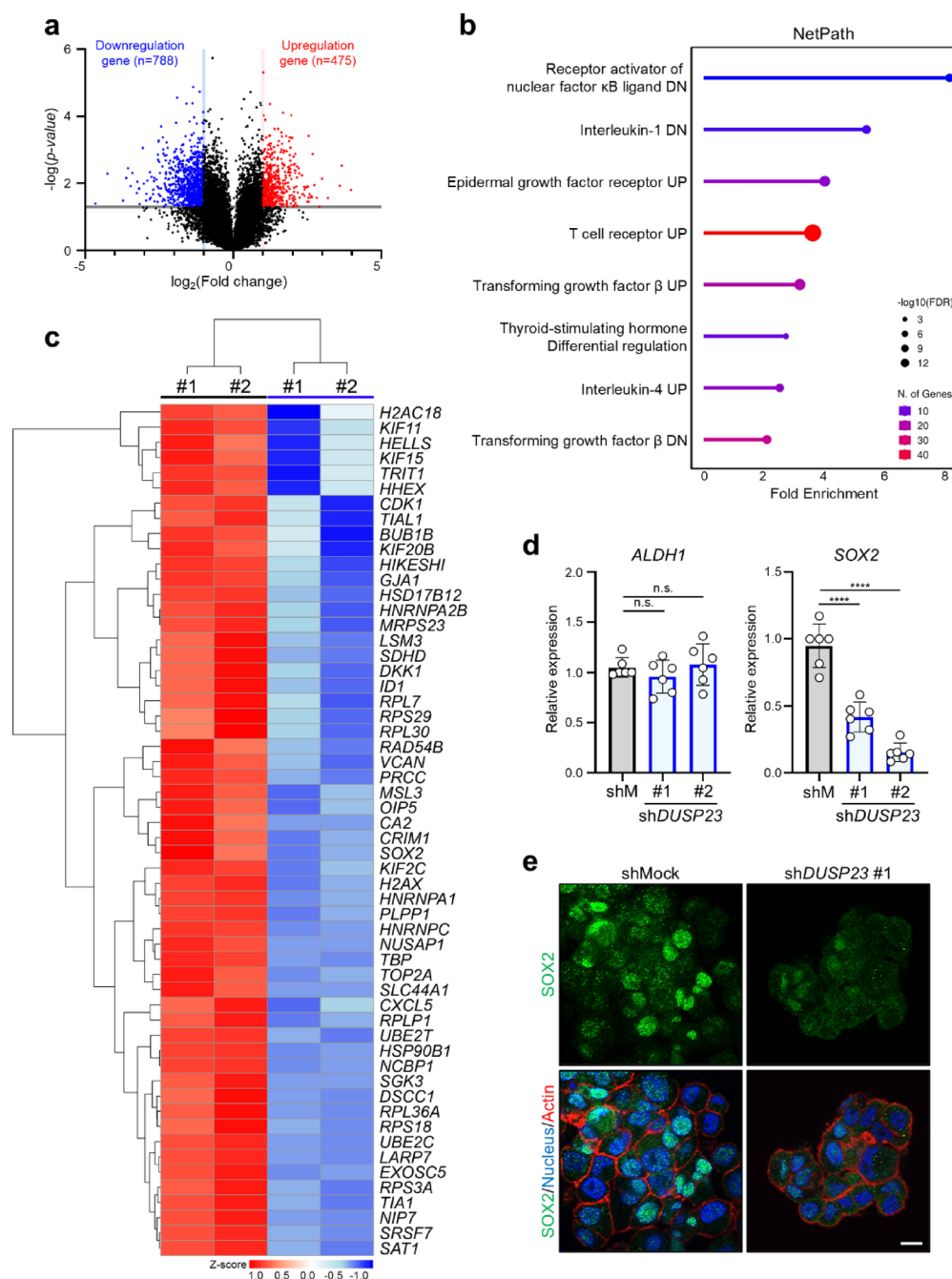


Fig. 2. Downregulation of DUSP23 reduces SOX2 expression. **(a)** Volcano plot of differentially expressed genes between shMock and shDUSP23 #1 A549 clusters based on RNA-seq results. In the shDUSP23 #1 A549 clusters ($|\log_2\text{FC}| > 1$, $p < 0.05$), 788 and 475 genes were downregulated and upregulated, respectively. Genes with significantly decreased expression are shown in blue; genes with significantly increased expression are shown in red; genes with non-significant changes in expression are shown in black. **(b)** Genes that were downregulated in RNA-seq data were analyzed using ShinyGO 0.77 to identify relevant signaling pathways based on the NetPath database. **(c)** Heatmap showed the downregulation of SOX2 target genes in shDUSP23 #1 A549 clusters based on RNA-seq data. **(d)** RT-qPCR analysis of *ALDH1* and *SOX2* expression in DUSP23 KD A549 cluster cells. Statistical analysis was performed using a one-way ANOVA with Tukey's multiple comparisons. One-way ANOVA, $F_{2,15} = 0.907$ for *ALDH1*; $F_{2,15} = 67.53$ for *SOX2*. **(e)** Immunofluorescence images of SOX2, F-actin, and nuclei of cell clusters from shMock and shDUSP23 #1 A549 cells. Scale bar: 10 μm . Data are presented as mean $\pm$ standard deviation. **** $p < 0.0001$, n.s. not significant; FC, fold change; NES, normalized enrichment score; FDR, false discovery rate.

marked reduction in *SOX2* mRNA levels following *DUSP23* KD, while *ALDH1* expression remained unchanged (Fig. 2d and Supplementary Fig. 2e). Consistently, western blot analysis confirmed a decrease in *SOX2* protein levels in *DUSP23* KD cells (Supplementary Fig. 2f and g). In contrast, overexpression of *DUSP23* in H1299 cells led to an increase in *SOX2* expression (Supplementary Fig. 2h). Furthermore, immunofluorescence analysis demonstrated a significant reduction in nuclear localization of *SOX2* in *DUSP23* KD cell clusters, supporting the role of *DUSP23* in regulating *SOX2* activity (Fig. 2e).

DUSP23 expression is elevated in cisplatin-resistant cells

CSC-like cells are known to be key contributors to chemoresistance^{5,6}. To investigate the potential role of *DUSP23* in this process, we conducted GSEA, which revealed a strong positive correlation between *DUSP23* expression and multiple drug resistance (MDR)-related genes in NSCLC patients (Supplementary Fig. 3a). Given that cisplatin is one of the most commonly used chemotherapeutic agents for NSCLC⁴, we further analyzed whether *DUSP23* expression levels influence cisplatin resistance using GSEA. The analysis revealed a positive correlation between *DUSP23* expression and cisplatin resistance gene signature in LUAD patients (Fig. 3a and Supplementary Fig. 3b). In addition, *DUSP23* mRNA expression in lung cancer cell lines, as reported in the Human Protein Atlas, was positively correlated with the half-maximal inhibitory concentration (IC_{50}) values of cisplatin (below 20 μ M), based on Genomics of Drug Sensitivity database analysis (Supplementary Fig. 3c). These findings suggest that *DUSP23* plays a critical role in the development of cisplatin resistance in NSCLC.

To test this hypothesis, we established a cisplatin-resistant (CisR) cell line by gradually exposing H1299 cells to increasing concentrations of cisplatin. CisR cells displayed distinct morphological differences compared to the parental cells and had an IC_{50} value for cisplatin that was approximately four-fold higher (Fig. 3b and c). These cells also exhibited increased expression of the ABC transporter *ABCC2*, a well-documented contributor to cisplatin resistance^{22,23} (Supplementary Fig. 3d). Consistent with previous studies^{24,25}, the CisR cells demonstrated enhanced invasive capacity in a three-dimensional (3D) spheroid invasion assay (Supplementary Fig. 3e). Furthermore, we observed elevated phosphorylation of STAT3 (tyr705) and AKT (ser473), downstream effectors of EGFR signaling associated with drug resistance, in CisR cells (Fig. 3d and Supplementary Fig. 3g)²⁶.

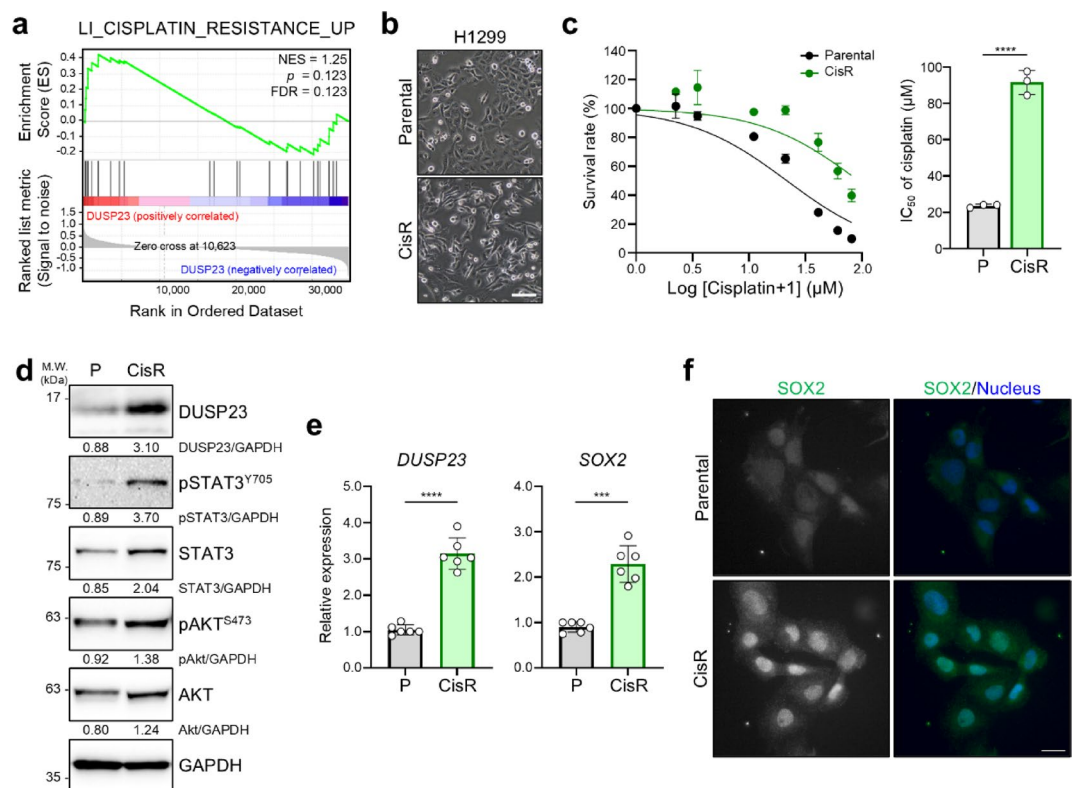


Fig. 3. *DUSP23* is upregulated in cisplatin-resistant (CisR) H1299 cells. **(a)** Association of *DUSP23* with cisplatin resistance was determined using GSEA datasets. **(b)** Morphology of H1299 parental and CisR cells. Scale bar: 25 μ m. **(c)** H1299 parental and CisR cells were treated with cisplatin at indicated dose for 72 h, and cell survival rate was examined using the WST-8 assay. Bar graph shows the IC_{50} of cisplatin in parental and CisR H1299 cells. **(d)** Western blot analysis of the proteins in parental and CisR H1299 cells using the indicated antibodies. **(e)** RT-qPCR analysis of *DUSP23* and *SOX2* expression in parental and CisR H1299 cells. Significance of data was examined using Welch's t-test. **(f)** Immunofluorescence images of *SOX2* and nuclei of parental and CisR H1299 cells. Scale bar: 10 μ m. Data are presented as mean $\pm$ standard deviation. *** $p < 0.001$, **** $p < 0.0001$; NES, normalized enrichment score; FDR, false discovery rate.

Remarkably, DUSP23 expression was significantly upregulated in CisR cells compared to parental cells, as evidenced by both mRNA and protein levels (Fig. 3d and e). In contrast to DUSP23, the expression of other atypical DUSPs in CisR cells was either unchanged or only modestly increased, underscoring the striking upregulation of DUSP23 among this family (Supplementary Fig. 3f.). In parallel, SOX2 expression and its nuclear localization were markedly elevated in CisR cells (Fig. 3e and f). Together, these findings suggest that DUSP23 contributes to the maintenance of CSC-like properties and plays a pivotal role in cisplatin resistance, likely through the regulation of SOX2 activity.

DUSP23 promotes proliferation and invasion, and prevents apoptosis of CisR cells

The upregulation of DUSP23 in floating clusters and CisR cells prompted us to examine whether targeting DUSP23 could serve as a therapeutic strategy for overcoming cisplatin resistance. In line with its role in CSC-like traits, DUSP23 KD impaired cluster formation under non-adherent conditions, as reflected by a marked reduction in cluster size (Fig. 4a). Conversely, doxycycline-inducible overexpression of DUSP23 promoted cluster formation in CisR cells (Fig. 4c). Consistent with these findings, DUSP23 also enhanced the invasive capacity of CisR cells in 3D collagen matrices, underscoring its importance in maintaining the invasive potential of chemoresistant cells (Fig. 4b and d). Furthermore, KD of DUSP23 markedly reduced cell survival both in the presence and absence of cisplatin, whereas overexpression conferred a modest but reproducible survival advantage (Supplementary Fig. 4a and b). Further analysis revealed that DUSP23 KD induced apoptosis in CisR cells, as evidenced by increased levels of cleaved caspase-3 (Fig. 4e and Supplementary Fig. 4c). Moreover, DUSP23 KD led to a reduction in the phosphorylation of STAT3 and AKT, both of which are associated with survival and resistance mechanisms in cancer cells. (Fig. 4e and Supplementary Fig. 4c). Interestingly, DUSP23 overexpression selectively activated STAT3, but not AKT, suggesting that DUSP23 promotes STAT3 signaling to sustain CSC-like properties in cisplatin-resistant cells rather than through the AKT pathway (Fig. 4f and Supplementary Fig. 4d). Consistent results were observed in another cisplatin-resistant NSCLC line, H460 (Supplementary Fig. 5a and b), where DUSP23 KD reduced cluster formation and STAT3 phosphorylation, led to only a modest decrease in Akt phosphorylation, and induced cleaved caspase-3 (Supplementary Fig. 5c–e). Previous studies have shown that phosphorylated STAT3 (pSTAT3) can drive SOX2 expression by binding to promoter regions located ~3.9 kb and ~0.3 kb upstream of the transcription start site^{27–29}. To test whether this regulatory mechanism depends on DUSP23, we performed ChIP-qPCR in DUSP23 KD H1299 CisR cells (Fig. 4g). Our ChIP-qPCR analysis showed reduced STAT3 binding at the SOX2 promoter in DUSP23 KD CisR cells, indicating that DUSP23 is necessary for STAT3 to drive SOX2 transcription (Fig. 4g). Accordingly, downregulation of DUSP23 reduced SOX2 at both the transcript and protein levels in H1299 and H460 cisplatin-resistant cells (Fig. 4h, Supplementary Figs. 4e, 5f, g), while DUSP23 overexpression led to increased SOX2 expression (Fig. 4i). Together, these results indicate that DUSP23 is required for pSTAT3-driven SOX2 expression in cisplatin-resistant cells and, in turn, promotes their cluster-forming ability and invasive capacity.

DUSP23 serves as a potential therapeutic target for NSCLC patients under chemotherapy

To investigate the clinical relevance of DUSP23 in NSCLC patients receiving adjuvant chemotherapy, we performed a univariate Cox regression analysis using DUSP23 together with SOX2 target genes identified from our DUSP23 KD RNA-seq dataset (Fig. 2c). A risk score was then calculated for each patient, allowing stratification into low- and high-risk groups (Fig. 5a). Scatter plots and Kaplan–Meier curves showed that patients in the high-risk group had significantly shorter progression-free and overall survival under chemotherapy (HR = 5.50, $p = 0.0013$ in Fig. 5c; HR = 16.17, $p < 0.0001$ in Fig. 5d; Fig. 5b–d). Consistently, disease-free survival was significantly longer in low-risk patients than in high-risk patients across two independent datasets (HR = 5.57, $p < 0.0001$ in TCGA-LUAD; HR = 4.04, $p < 0.0001$ in GSE8894; Fig. 5e and f). Together, these findings underscore that the DUSP23–SOX2 axis is linked to poor prognosis in NSCLC and support the idea that DUSP23 may serve as a prognostic biomarker in chemotherapy-treated patients.

Discussion

CSCs are key drivers of tumor recurrence, progression, and therapy resistance, making them important targets for cancer treatment^{5,6,8,30,31}. Although many CSC-associated biomarkers have been identified³¹, the molecular mechanisms that regulate CSC-like behavior remain incompletely understood, posing a major barrier to the development of effective therapies. In this study, we identified DUSP23 as a novel regulator of CSC-like properties in NSCLC and demonstrate its critical role in chemoresistance.

We found that DUSP23 expression was significantly elevated in NSCLC tissues, with higher levels correlating with shorter disease-free survival. DUSP23 expression was particularly enriched in cell clusters grown under non-adherent conditions that mimic the tumor microenvironment and promote CSC-like traits. DUSP23 KD marked reduction in cluster formation and size, whereas overexpression enhanced cluster-forming ability. In vivo, DUSP23 KD suppressed tumor growth, supporting its role in sustaining CSC-like properties. RNA-seq analysis further implicated SOX2, a key transcription factor in CSC biology, as a downstream effector of DUSP23. Indeed, DUSP23 KD reduced SOX2 expression and nuclear localization, suggesting a potential SOX2-dependent mechanism, although further studies will be needed to confirm the direct molecular interaction between DUSP23 and SOX2.

Beyond its role in CSC-like properties, DUSP23 was significantly upregulated in CisR cells, where it promoted cluster formation, enhanced invasive potential, and protected cells from apoptosis. These observations indicate that DUSP23 regulates multiple pathways associated with both CSC-like features and chemoresistance. Unlike other well-characterized phosphatases such as DUSP4, and DUSP6, which are primarily through MAPK signaling and have been linked to chemoresistance^{32–34}, DUSP23 appears to operate through a distinct mechanism, underscoring its unique role in cancer biology.

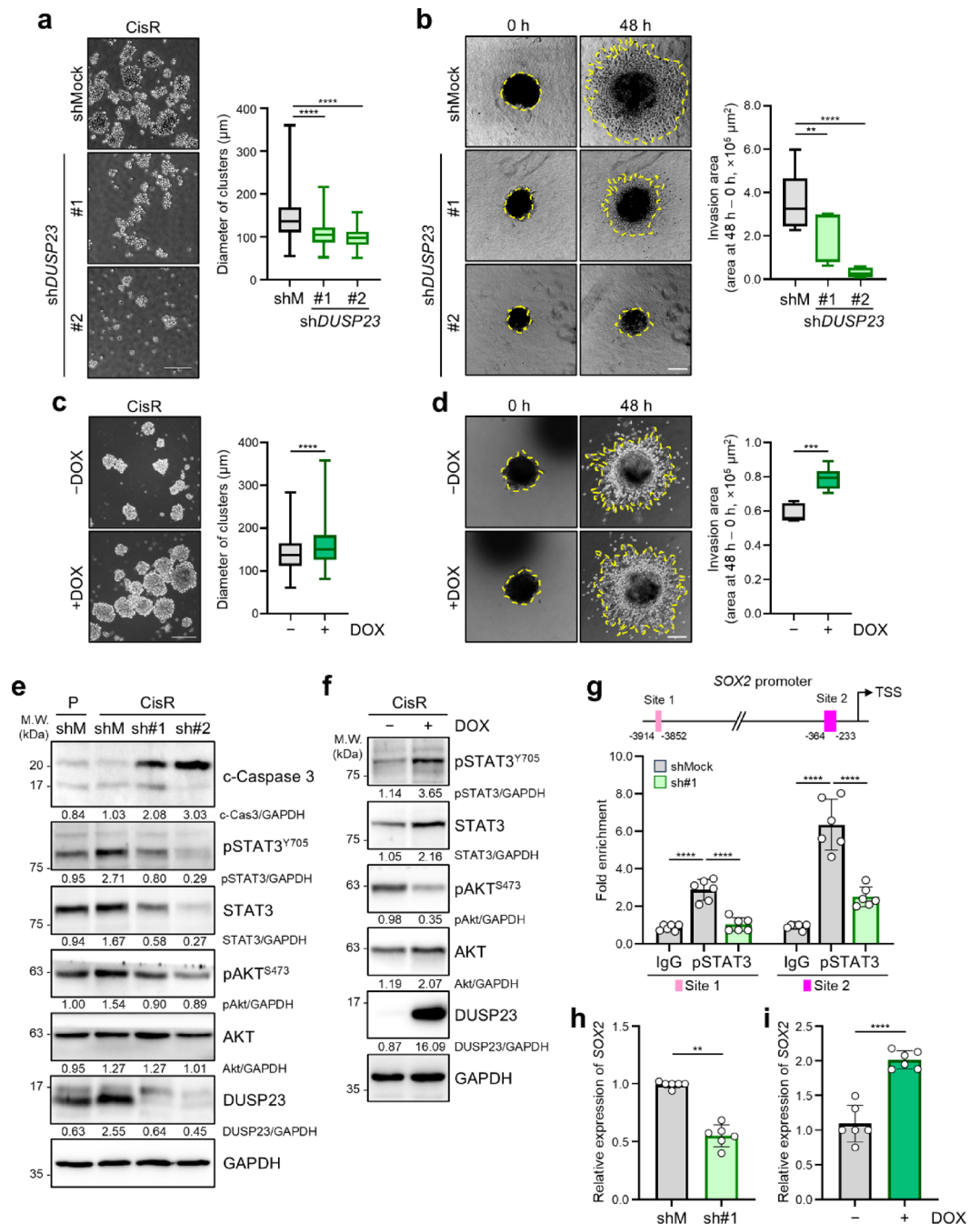


Fig. 4. DUSP23 enhances phosphorylated STAT3-mediated SOX2 expression, promoting cluster formation and invasion in CisR cells. **(a)** Cluster formation assay of *DUSP23* KD H1299 CisR cells. Scale bar: 250 μ m. Box plot shows cluster diameters > 50 μ m (minimum to maximum). Statistical analysis by one-way ANOVA with Tukey's post hoc test ($F_{2,1628} = 242$). **(b)** Representative images of the 3D spheroid invasion assay in *DUSP23* KD CisR cells. Yellow circles indicate the distribution of invasive cells at 0 and 48 h. Box plot depicts invasion area relative to baseline (0 h) (minimum to maximum). Scale bar: 250 μ m. Statistical analysis by one-way ANOVA with Tukey's post hoc test ($F_{2,16} = 16.31$). **(c)** Cluster formation assay of doxycycline (DOX)-inducible *DUSP23*-overexpressing CisR cells. Scale bar: 250 μ m. Statistical analysis by Mann-Whitney test. **(d)** 3D spheroid invasion assay of DOX-inducible *DUSP23*-overexpressing CisR cells. Scale bar: 250 μ m. Statistical analysis by Student's t-test. **(e)** Western blot analysis of *DUSP23* KD H1299 CisR cells with the indicated antibodies. **(f)** Western blot analysis of *DUSP23*-overexpressing H1299 CisR cells with the indicated antibodies. **(g)** ChIP-qPCR analysis of pSTAT3 binding to the SOX2 promoter region. Statistical analysis by one-way ANOVA with Tukey's post hoc test ($F_{2,15} = 47.44$ for Site 1; $F_{2,15} = 65.4$ for Site 2). **(h)** Bar graph showing SOX2 mRNA expression in *DUSP23* KD CisR cells. Statistical analysis by Mann-Whitney test. **(i)** Bar graph showing SOX2 mRNA expression in *DUSP23*-overexpressing CisR cells. Statistical analysis by Student's t-test. Data are presented as mean $\pm$ standard deviation. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

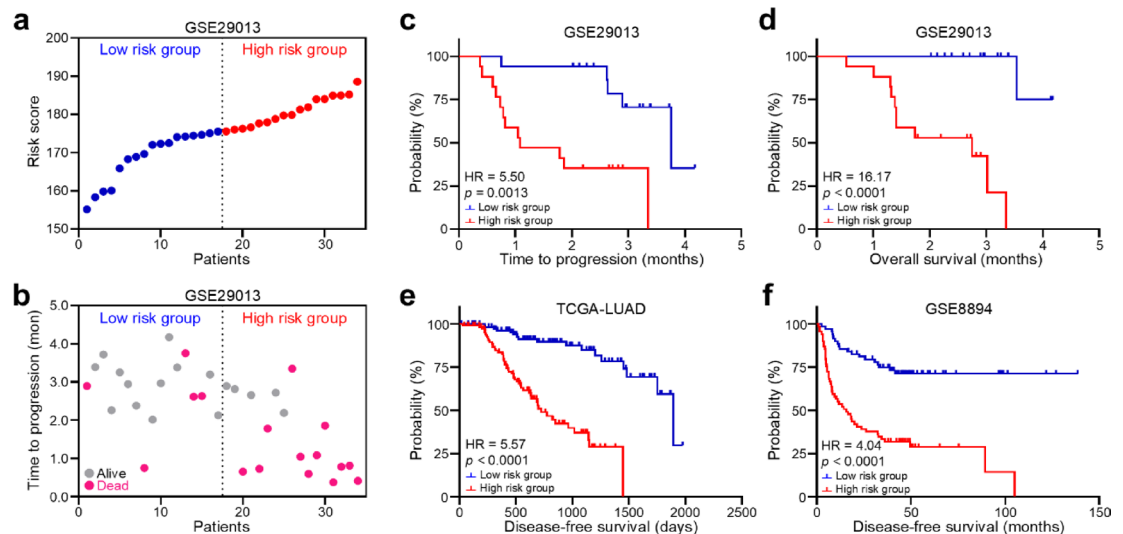


Fig. 5. DUSP23 upregulation is associated with poor prognosis in chemotherapy-treated NSCLC patients. **(a)** Distribution of risk scores in chemotherapy-treated NSCLC patients from the GSE29013 dataset. Patients were stratified into low- and high-risk groups based on the median risk score, with blue and red dots indicating low- and high-risk patients, respectively. **(b)** Scatter plot showing progression-free survival status of NSCLC patients. Gray dots represent surviving patients and pink dots represent deceased patients. **(c, d)** Kaplan–Meier curves of progression-free survival and overall survival in chemotherapy-treated NSCLC patients stratified by risk scores. **(e, f)** Disease-free survival of NSCLC patients (high- vs. low-risk groups) in two independent datasets: TCGA-LUAD **(e)** and GSE8894 **(f)**. NSCLC, Non-small cell lung cancer; TCGA, The Cancer Genome Atlas; LUAD, lung adenocarcinoma.

SOX2, a hallmark transcription factor of CSCs, controls pluripotency and stem-like behavior together with OCT4, NANOG, and KLF4³⁵. In NSCLC, SOX2 is frequently upregulated in advanced or recurrent tumors through pathways such as EGFR/Src/Akt signaling^{36,37}. Elevated SOX2 levels contribute to therapy resistance and tumor survival³⁷, making it an attractive therapeutic target³⁸. Our data show that DUSP23 KD not only reduced SOX2 expression but also downregulated EGFR-related pathways, including STAT3 and AKT phosphorylation, which are critical for chemoresistance and CSC maintenance. Conversely, DUSP23 overexpression selectively activated STAT3, but not Akt, thereby enhancing SOX2 expression. Although PI3K/Akt signaling is generally thought to activate STAT3, in some contexts it can suppress STAT3 activity^{39,40}. Taken together, these results indicate that DUSP23 promotes SOX2 expression in CisR cells primarily by activating STAT3 rather than the AKT pathway.

Through in vivo experiments and bioinformatic analysis, we further demonstrated the clinical significance of DUSP23 in chemotherapy-treated NSCLC patients. As a phosphatase, DUSP23 likely functions by dephosphorylating specific substrates, and identifying these substrates will be essential to fully understand how it governs CSC-like traits and chemoresistance. Given the broad substrate range of phosphatase⁴¹, DUSP23 may also influence additional biological processes beyond CSC regulation. These considerations highlight the need for further mechanistic studies, particularly to clarify how DUSP23 regulates STAT3 phosphorylation in CisR cells. It should also be noted that our experimental system captures CSC-like characteristics but does not fully model *bona fide* CSCs. Thus, our findings provide evidence that DUSP23 promotes CSC-like properties, while further studies using more definitive CSC models and additional chemoresistant in vivo systems will be required to validate its therapeutic potential.

Methods

Cell culture

Human non-small cell lung adenocarcinoma cell lines A549, H1299 and H460 were purchased from the Korean Cell Line Bank (Seoul, South Korea). A549, H1299 and H460 cells were cultured in Roswell Park Memorial Institute 1640 medium (RPMI 1640; #23400021; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; #US-FBS-500; GW Vitek, Seoul, South Korea), 100 units/ml penicillin, and 100 µg/ml streptomycin (#LS202-02; WelGENE, Daegu, South Korea). Murine Lewis lung carcinoma LLC1 cell line, derived from C57BL/6 mouse, were generously provided by Prof. Yoosik Yoon (Chung-Ang University, South Korea). Human embryonic kidney 293 T (HEK293T) cells were obtained from the American Type Culture Collection (#CRL-3216; Manassas, VA, USA). LLC1 and HEK293T cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM; #12800017; Gibco) supplemented with 10% FBS, 100 units/ml penicillin, and 100 µg/ml streptomycin. All cell lines were incubated at 37°C under humidified atmosphere of 95% air and 5% CO₂.

Cell cluster formation assay

Approximately 2×10^4 – 1×10^5 cells per well were seeded into ultra-low-attachment six-well plates (SPL 3D™ Cell Floater Plate; #39706; SPL Life Sciences, Gyeonggi-do, South Korea). The cells were cultured in Advanced DMEM/F-12 (#12634010; Gibco) supplemented with 10 mM HEPES (pH 7.4; #7365-45-9; Sigma-Aldrich, Saint Louis, MO, USA), 1% GlutaMax supplement (#35050; Gibco), 100 units/ml penicillin, 100 µg/ml streptomycin, 20 ng/ml EGF (#E9644; Sigma-Aldrich), 20 ng/ml human recombinant bFGF (#13256-029; Gibco), and B-27 supplement (#17504044; Gibco). The cells were incubated under these conditions for 4 days to allow tumor cluster formation. All clusters were photographed using a Nikon ECLIPSE Ti2 inverted microscope equipped with a digital camera (DS-Qi2; Nikon, Tokyo, Japan), and the diameters of the clusters were measured using the NIS-Elements image analysis software (NIS-Elements Advance Research 5.20.00; Nikon). To minimize measurement bias, the images were randomly renamed before analysis and then independently reviewed by two investigators who were blinded to the experimental conditions.

RNA extraction and RT-qPCR

Total RNA was isolated from cells using the RNAiso Plus reagent (#9109; TaKaRa, Tokyo, Japan) according to the manufacturer's instructions. After adding chloroform and performing centrifugation at $15,000 \times g$, the solution was separated into three phases; total RNA was present in the aqueous phase. The aqueous phase was transferred into 1.5 ml microtubes containing isopropanol to precipitate the total RNA. RNA pellets were washed with 75% ethanol in ultrapure diethyl pyrocarbonate-treated (DEPC)-treated water (#750023; Invitrogen, Carlsbad, CA, USA), completely dried, dissolved in DEPC-treated water, and quantified at 260/280 nm using an Epoch spectrophotometer (BioTek Instruments, Winooski, VT, USA). One microgram of total RNA was reverse-transcribed into complementary DNA using 100 mM oligo (dT) and PrimeScript reverse transcriptase (#2680; TaKaRa). RT-qPCR was performed using the SYBR Premix Ex Taq II (#RR820, TaKaRa) and QuantStudio 3 (Applied Biosystems). Gene expression levels were calculated using the $2^{-\Delta\Delta C_q}$ method and normalized to the C_q value of *GAPDH*⁴². The primers used for RT-qPCR are listed in Supplementary Table 1.

Western blotting

The cells were seeded in 35 mm culture dishes. After 72 h, the cells were washed with cold phosphate-buffered saline (PBS). Total proteins were extracted from the cells by adding a phosphate-based lysis buffer (pH 7.0) containing 1% sodium deoxycholate, 2% sodium dodecyl sulfate, 1% NP-40, 150 mM sodium chloride, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 2 mM ethylene diamine tetraacetic acid, 1 mM dithiothreitol, 1 mM phenylmethane sulfonyl fluoride, 6 mM sodium hydrogen phosphate, and 4 mM sodium dihydrogen phosphate. Equal amounts of proteins were electrophoresed on 8–15% of sodium dodecyl sulfate–polyacrylamide gels. Resolved proteins were transferred onto a polyvinylidene difluoride membrane (#IPVH00010; Millipore, Burlington, MA, USA), which was blocked with 5% skim milk. The membrane was incubated with primary antibodies, including mouse anti-DUSP23 (#TA503944; Origene Technologies, Rockville, MD, USA; 1:2000), rabbit anti- α tubulin (E-19; #sc-12462; Santa Cruz Biotechnology, Dallas, TX, USA; 1:2500), mouse anti-STAT3 (F-2; #sc-8019; Santa Cruz Biotechnology; 1:1000), mouse anti-GAPDH (6C5; #sc-32233; Santa Cruz Biotechnology; 1:2500), Rabbit anti-SOX2 (#3579; Cell Signaling Technology, Danvers, MA, USA; 1:1000), mouse anti-phospho-STAT3 (Y705; #4113; Cell Signaling Technology; 1:1000), rabbit anti-cleaved caspase-3 (#9661; Cell Signaling Technology; 1:1000), rabbit anti-phospho-AKT (S473; #9271; Cell Signaling Technology; 1:1000), and rabbit anti-AKT (#9272; Cell Signaling Technology; 1:1000). Several antibodies were diluted using the SignalBoost Immunoreaction Enhancer Kit (#407207; Millipore) to amplify the antibody reaction. Membranes were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies, including HRP-conjugated goat anti-mouse (#115-035-006; Jackson ImmunoResearch Laboratories, West Grove, PA, USA; 1:5000) and HRP-conjugated goat anti-rabbit (#111-035-006; Jackson ImmunoResearch Laboratories; 1:5000). The blotted proteins were detected using enhanced chemiluminescence reagents (#XLS071, XLS0142, and XLS3; CYANAGEN, Bologna, Italy) and the Fusion Solo S imaging system (VILBER, Collégien, France). Band intensity was quantified using Fusion Solo S imaging system and normalized to internal control in each blot.

Short hairpin RNA (shRNA)-mediated KD experiment

DUSP23 KD human non-small cell lung adenocarcinoma cell lines were generated using a lentiviral system. The target sequences (sh*DUSP23* #1: 5'-GCTGAAATCCGACGACTACGA-3'; sh*DUSP23* #2: 5'-CCTATGAGCAG GAGAAAGCAG-3', both targeting the coding sequence region [CDS]) were cloned into pLKO.1-TRC cloning vector (#10878; Addgene, Watertown, MA, USA) and pLKO.1-GFP vector, replaced puromycin resistant gene into GFP expression gene. Additionally, the target sequences of *Dusp23* CDS region in LLC1 cells (sh*Dusp23*: 5'-CCAGTTCTACCAGCGAACAAA-3') were cloned into pLKO.1-TRC cloning vector (#10878; Addgene). The shRNA-targeting region was packaged with helper plasmids such as psPAX2 and pMD2.G (#12260 and #12259; Addgene), which were co-transfected into HEK293T cells. Lentiviral particles were harvested from HEK293T cells after 72 h. Lentiviral particles were allowed to infect the cells treated with 8 µg/ml polybrene. The expression of *DUSP23* was confirmed using RT-qPCR and western blotting. Infected cells were used for further analysis.

Generation of DUSP23-overexpressing cell lines using a doxycycline (DOX)-inducible overexpression system

The CDS of *DUSP23* was cloned into pCW57.1 (#41313; Addgene) using the Gateway LR Clonase II enzyme mix (#11791020; Invitrogen) following the manufacturer's protocol. For packaging, helper plasmids psPAX2 and pMD2.G (#12260 and #12259; Addgene) were co-transfected into HEK293T cells. Lentiviral particles were harvested from HEK293T cells after 72 h. Human NSCLC cells were seeded into 100 mm cell culture dishes and allowed to attach overnight. Following attachment, lentiviral particles were allowed to infect the cells

treated with 8 µg/ml polybrene. After 48 h, infected cells were selected using growth media containing 1 µg/ml puromycin. For DUSP23 overexpression, the infected cells were treated with DOX. The expression of DUSP23 was confirmed using RT-qPCR and western blotting. Infected cells were used for further analysis.

Subcutaneous injection of LLC1 cell line

For subcutaneous injection, 1×10^5 *Dusp23* KD LLC1 cells in 50% Matrigel solution (#356231; Corning) were injected into the flank of 6-week-old female C57BL/6 mice. Tumor volume was assessed every two days by using a caliper and calculating with the formula: $V = (L \times W^2)/2$, where V represents tumor volume, L as tumor length and W as tumor width ($L \geq W$). Mice were maintained under 18–22 °C temperature with a 12-h light–dark cycle and fed normal diet. All mice were sacrificed at the experimental endpoints after anesthesia with Avertin (2,2,2-Tribromoethanol; #T1420; TCI, Tokyo, Japan) by i.p. injection.

All animal experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Chung-Ang University (Approval Number: 2019-00133). All methods were carried out in accordance with relevant guidelines and regulations, and are reported in compliance with the ARRIVE guidelines.

RNA-seq

Total RNA was extracted from A549 shMock and shDUSP23 tumor cluster cells. RNA quality was assessed using a bioanalyzer (RNA 600 Nano kit; Agilent Technologies, Santa Clara, CA, USA). Library construction was performed using an mRNA-seq Library Prep Kit (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. High-throughput sequencing was performed using the NovaSeq 6000 platform (Illumina). For pathway analysis, ShinyGo 0.77 (<http://bioinformatics.sdstate.edu/go/>) was used to perform NetPath analysis. Additionally, FPKM values from each sample were utilized for Gene Set Enrichment Analysis (GSEA version 4.4.0).

Immunocytochemistry

Cells were seeded on 50 µg/ml collagen-coated 12 mm coverslips for 24 h, fixed with 3.7% cold paraformaldehyde for 15 min, and permeabilized with 0.5% Triton X-100 in PBS for 10 min. After blocking with 2% bovine serum albumin in 0.1% Triton X-100 in PBS for 1 h, samples were incubated with the mouse anti-SOX2 antibody (E-4; #sc-365823; Santa Cruz Biotechnology; 1:200) for 1 h. Next, the coverslips were washed thrice with 0.1% Triton X-100 in PBS and incubated with an Alexa Fluor-conjugated secondary antibody (#A-11001; Invitrogen; 1:100) for 1 h at 22 °C. Nuclei were stained with 4',6-diamidino-2-phenylindole. Coverslips were mounted on glass slides using Fluoromount-G (#0100-01; Southern Biotechnology Associates, Birmingham, AL, USA). The images were acquired using an Eclipse 80i fluorescence microscope and DS-Qi2 (Nikon). Tumor clusters were fixed with 3.7% paraformaldehyde for 15 min and transferred onto adhesive microscope slides (HistoBond microscope slides; #08110001; MARGINFELD, Lauda Königshofen, Germany). The immunocytochemical procedures were performed as mentioned above. Images were acquired using a C2+ confocal microscope equipped with a DS-Qi2 camera (Nikon).

Water-soluble tetrazolium (WST)-8 and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay

For the WST-8 assay, 5×10^3 cells in 100 µl medium were seeded in duplicate into 96-well culture plates. To assess cell viability, 10 µl of WST-8 reagent (#W0200; GenDEPOT, Barker, TX, USA) was added to each well, and the plates were incubated at 37 °C for 2 h. Absorbance was measured at 450 nm using an Epoch spectrophotometer (BioTek Instruments). For the MTT assay, each well was treated with 500 µg/ml MTT solution (#MC1029-001-02; Biosesang, Gyeonggi-do, South Korea) in culture medium and incubated for 2 h at 37 °C. After incubation, MTT solution was removed, and intracellular formazan crystals were solubilized in dimethyl sulfoxide (DMSO; #DMS555-1; Biopure, Ontario, Canada). Absorbance was measured at 570 nm using the same spectrophotometer.

Generation of cisplatin-resistant H1299 and H460 cell lines

A cisplatin-resistant (CisR) H1299 cell line was derived from its original parental cell line through continuous exposure to 16 µM cisplatin (#P4394; Sigma-Aldrich). The cisplatin-resistant cell line was initially treated with 6 µM cisplatin for 72 h. The cells were maintained in increasing concentrations of cisplatin. Additionally, cisplatin-resistant H460 cell line was initially treated with 2 µM cisplatin, followed continuous exposure to 6 µM cisplatin. This developmental period lasted for approximately 6 months. The IC₅₀ was reassessed using the WST-8 assay (H1299 CisR cells) and MTT assay (H460 CisR cells) in the resistant cell line treated with increased concentrations up to 16 µM and 6 µM for 72 h, respectively.

3D spheroid invasion assay

Cells (5×10^3) were seeded into ultra-low-attachment 96-well plates (SPL 3D™ Cell Floater Plate; #34896; SPL Life Sciences) and incubated for 24 h. Then, the cells were coated with growth factor-reduced Matrigel matrix (#356231; CORNING, Bedford, MA, USA) for 24 h. Next, a 1 mg/ml rat tail type I collagen (#ALX-522-435-0100, Enzo Life Sciences, Farmingdale, NY, USA) mixture was poured into the ultra-low-attachment 24-well plates (SPL 3D™ Cell Floater Plate; #39724; SPL Life Sciences) and incubated for 1 h to allow polymerization; then, RPMI 1640-containing serum was added. Spheroids were allowed to invade for 48 h, and invasion was determined by measuring the area of the spheroid normalized to the original area of the spheroid using the NIS-Elements image analysis software (Nikon). For live imaging, images were captured at 30 min intervals for 30 ms using a Nikon ECLIPSE Ti2 inverted microscope system (Nikon, Tokyo, Japan) equipped with a digital camera, DS-Qi2 (Nikon).

Chromatin immunoprecipitation (ChIP)-qPCR assay

Cells were cross-linked with 1% formaldehyde for 10 min at room temperature (RT) and quenched by 125 mM glycine for 5 min at RT. ChIP was performed according to the manufacturer's instruction (#17-295; Sigma-Aldrich). Rabbit anti-phospho-STAT3 (Y705; #9145; Cell Signaling Technology) and normal rabbit IgG (#12-370; Sigma-Aldrich) were used as the primary antibody and negative control, respectively. Immunoprecipitated DNA was analyzed by qPCR using primers targeting the human *SOX2* promoter (listed in Supplementary Table 2).

Bioinformatic analysis

mRNA expression of *DUPS23* was examined in TCGA and Okayama lung cohorts using the Oncomine database (<https://www.oncomine.com/>). Kaplan–Meier survival analysis was performed with TCGA and Gene Expression Omnibus (GEO) datasets. GSEA was conducted using both TCGA and our RNA-seq data. Gene sets were searched using GSEA (<https://www.gsea-msigdb.org/gsea/>). Data on *DUPS23* expression in various cell lines were obtained from the Human Protein Atlas (<https://www.proteinatlas.org/>), and the IC₅₀ values for cisplatin were downloaded from Genomics of Drug Sensitivity in Cancer (<https://www.cancerrxgene.org/>). To construct a prognostic risk score prognosis model, coefficient values of identified genes were derived from univariate Cox regression analysis. Risk scores were calculated according to the formula:

$$\text{Risk score} = \sum_{i=1}^n \text{coef}_i \times \exp(G_i),$$

where n is the number of identified genes, coef_i represents the regression coefficients and $\exp(G_i)$ denotes the expression level of each gene. NSCLC patients were then stratified into high and low-risk groups based on the median risk score, and progression-free and overall survival were evaluated using Kaplan–Meier curves and log-rank test.

Statistical analysis

All data are presented as the mean $\pm$ standard deviation from two or three independent experiments. Statistical analyses were performed using GraphPad Prism 10.5.0 (GraphPad Software, San Diego, CA, USA). Data normality was first assessed using the Shapiro–Wilk test. Depending on the results, Student's t-test (for equal variances) or Welch's t-test (for unequal variances) was used when normality was satisfied, and the Mann–Whitney test when it was not. For comparisons among more than two groups, one-way ANOVA followed by Tukey's post hoc test was applied. One-way ANOVA F-values are reported in the figure legends as $F_{(DFn, DFd)}$, where DFn and DFd indicate numerator and denominator degrees of freedom, respectively. A p -value < 0.05 was considered statistically significant (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

Data availability

The public datasets analyzed during the current study are available in the Oncomine database (<https://www.oncomine.com/>), The Human Protein Atlas (<https://www.proteinatlas.org/>) and Genomics of Drug Sensitivity in Cancer (<https://www.cancerrxgene.org/>). Raw and processed RNA-sequencing datasets generated and analyzed during the current study are available in Gene Expression Omnibus (GEO) databases (GSE288032). Further inquiries are available from the corresponding author on reasonable request.

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Author contributions

Conceptualization and experimental design: S. K., Y. H., M. L., J. J., and S. R. Manuscript writing: S. K., Y. H., M. L., and S. R. Conduction of experiments and data collection: S. K., Y. H., M. L., S. S., Y. E. H. Transcriptomic analysis: J-W. K. Analysis of results: S. K., Y. H., M. L., S. S., J. J., J-H. C., Y. E. H., J-W. K., and S. R. Supervision and administration: S. R. All the authors critically revised and approved the final version of the manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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