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Original Articles

Targeting of TMPRSS4 sensitizes lung cancer cells to chemotherapy by impairing the proliferation machinery

Francisco Exposito[a,b,c], Maria Villalba[a,b,c], Miriam Redrado[a], Arrate L. de Aberasturi[a], Cristina Cirauqui[a], Esther Redin[a,b], Elizabeth Guruceaga[d], Carlos de Andrea[b], Silvestre Vicent[a,b,c], Daniel Ajona[a,c,e], Luis M. Montuenga[a,b,c], Ruben Pio[a,c,e], Alfonso Calvo[a,b,c,*]

[a] IDISNA and Program in Solid Tumors, Center for Applied Medical Research (CIMA), University of Navarra, 31008, Pamplona, Navarra, Spain
[b] Department of Pathology, Anatomy and Physiology, School of Medicine, University of Navarra, 31008, Pamplona, Navarra, Spain
[c] CIBERONC, ISC-III, Spain
[d] Bioinformatics Platform. Center for Applied Medical Research (CIMA), University of Navarra, Pamplona, Spain
[e] Department of Biochemistry and Genetics, School of Science, University of Navarra, Pamplona, Spain

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ABSTRACT

High mortality rates caused by NSCLC show the need for the identification of novel therapeutic targets. In this study we have investigated the biological effects and molecular mechanisms elicited by TMPRSS4 in NSCLC. Overexpression of TMPRSS4 in LKR13 cells increased malignancy, subcutaneous tumor growth and multiorganic metastasis. In conditional knock-down (KD) experiments, abrogation of TMPRSS4 in H358 and H2170 cells altered proliferation, clonogenicity, tumor engraftment and tumor growth. Reduction in S and G2/M phases of the cell cycle, decreased BrdU incorporation and increased apoptosis was also found. Transcriptomic analysis in KD cells revealed downregulation of genes involved in DNA replication, such as MCM6, TYMS and CDKN1A (p21). In patients, expression of a signature of MCM6/TYMS/TMPRSS4 genes was highly associated with poor prognosis. Downregulation of TMPRSS4 significantly increased sensitivity to chemotherapy agents. In experiments using cisplatin, apoptosis and expression of the DNA-damage marker γ-H2A was higher in cells lacking TMPRSS4. Moreover, *in vivo* assays demonstrated that tumors with no TMPRSS4 were significantly more sensitive to cisplatin than controls. These results show that TMPRSS4 can be considered as a novel target in NSCLC, whose inhibition increases chemosensitivity.

1. Introduction

Lung cancer bears the highest mortality rate worldwide among all malignancies, representing 24% of cancer-related deaths and showing a survival rate of 15%, 5 years after diagnosis [1]. Approximately 85% of lung cancer cases are classified as non-small cell lung cancer (NSCLC), with adenocarcinomas (ADC) and squamous cell carcinomas (SCC) representing 50% and 30% of the cases, respectively. In patients with early NSCLC, surgery is an appropriate treatment option but in advanced cases without treatable oncogenic alterations, first-line platinum-based chemotherapy remains as the gold standard treatment. Treatments with pemetrexed and the anti-angiogenic antibody bevacizumab in combination with platinum increase response rates in comparison with chemotherapy alone, in patients with non-SCC histology [2]. Nonetheless, response rates are only within 25–35% range and the median survival time remains between 8 and 12 months [3].

Metastasis is present in most lung cancer patients at the time of diagnosis, a condition that worsens prognosis. For cancer cells to escape from the primary tumor and develop a new tumor mass within the lung or in other organs, they have to acquire a migratory phenotype, survive in lymphatic or blood vessels and proliferate [4]. Proteases play an important role in primary tumor growth and metastasis, as they contribute to degradation of the basement membrane and the extracellular matrix (ECM), facilitate cancer cell invasion and modify tumor microenvironment [5,6]. Some proteases have also shown to activate intracellular signaling cascades that favor tumor cell survival and resistance to chemotherapy [7]. The type II transmembrane serine proteases (TTPs) contain a large extracellular domain that includes the catalytic activity and a short cytoplasmic domain that can interact with cytoskeletal and cellular signaling molecules [8]. Deregulation of proteases is a common finding in cancer. TMPRSS4 is a TTP member that is highly expressed in different solid tumors including liver [9], thyroid,

* Corresponding author. Program in Solid Tumors, CIMA of the University of Navarra, Laboratory 203, Pio XII, 55, 31008, Pamplona, Spain.
E-mail address: acalvo@unav.es (A. Calvo).

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colon, pancreas and lung (reviewed by Ref. [10]). Expression of this protease is associated with loss of E-cadherin and increased expression of vimentin and other epithelial to mesenchymal transition (EMT) markers. TMPRSS4 upregulates integrin-α5 to promote cell migration and invasion of colon cancer cells [11]. Activation of its proteolytic activity has also been shown to cleave pro-urokinase type plasminogen activator (pro-uPA) which, upon cleavage, binds to its receptor to increase invasion [12]. Enhanced expression of TMPRSS4 in hepatocellular carcinoma increases angiogenesis through RECK [9]. In this same tumor type, radiation causes EMT through TMPRSS4 and sustains long-term metastasis [13]. Our previous work in lung cancer has demonstrated that TMPRSS4 promotes cancer stem cell features and depleted expression of this gene impairs tumorsphere formation and reduces the population of ALDH + cells [14]. All these data indicate that TMPRSS4 can be a promising therapeutic target. In addition, translational studies have shown that high TMPRSS4 mRNA and protein levels are associated with worse prognosis in a variety of cancers, which suggests a consolidated role as a biomarker [10]. Up-regulation of TMPRSS4 is mediated, at least in lung cancer, by DNA hypomethylation of the promoter region [15]. Moreover, methylation status has also a prognostic value and can be used as a biomarker to predict reduced relapse-free survival [15].

Because of the potential importance of TMPRSS4 as a target in lung cancer, we explored in this study how the modification of endogenous levels of this protease affected lung cancer malignancy, using *in vitro* and *in vivo* assays. In knock-down experiments and microarray analysis we found numerous changes in replisome-related genes. When exposed to chemotherapy, cells with reduced TMPRSS4 levels were highly sensitized to its cytotoxic effect. This supports the role of TMPRSS4 as a therapeutic target for lung cancer in combination with chemotherapy.

2. Material and methods

2.1. Cell culture

The human lung cancer cell lines H358 and H2170 were obtained from the American Type Culture Collection (ATCC). Murine cell lines were donated by J. Sage (LKR10, LKR13), S. Vicent (LSZ1, LSZ2, LKP1ary), J.M. Kurie (334SQ and 393P) and M. M. Winslow (368T1, 389T2,482N1, 482T1, 802T4). These cell lines were derived from $Kras^{LA1/+}$ [16], LSL-$Kras^{G12D/+}$ [17], $Kras^{LA1/+}$;$p53^{R172H\Delta G}$ [18] and LSL-$Kras^{G12D/+}$; $p53^{flox/flox}$ [19], respectively. Human cells were cultured in RPMI (Lonza) and murine cells in DMEM (Gibco). Cultures were supplemented with 10% HyClone serum (Thermo Scientific) and 1% penicillin-streptomycin (Lonza), and grown at 37 °C in 5% CO_2 humidified atmosphere.

2.2. Doxycycline-inducible silencing of TMPRSS4 in human lung cancer cell lines

The RNAi Consortium Collection tool (Broad Institute, Public TRC Portal) was used to design shRNA sequences to silence TMPRSS4 expression in H358 and H2170 cells in order to be cloned into the doxycycline-inducible Tet-pLKO-puro plasmid (Addgene, Cambridge, MA, USA). A plasmid containing a shRNA against GFP (shCtrl) was used as control. More detailed protocols are explained in Supplementary Materials and Methods. To produce lentiviruses, HEK293T cells were cultured in HyClone-supplemented DMEM and transfected with the shRNA-containing plasmids, using MISSION Lentiviral packaging mix (Sigma) and X-Tremegene HP DNA transfection reagent (Roche diagnostics). Cells were selected with puromycin (5 μg/mL, Sigma-Aldrich) and qPCRs and Western blots were used to evaluate TMPRSS4 levels.

2.3. Overexpression of murine TMPRSS4 in mouse lung cancer cell lines

Murine (m) TMPRSS4 was cloned from the vector pmPII-mCap2 (kindly donated by Dr. Edith Hummler, Université de Lausanne, Switzerland) into the retroviral vector pBABE-puro (Addgene). Briefly, the insert was obtained from the original plasmid by digestion with Eco-RI and Sal-I and ligated into the pBABE-puro plasmid. One Shot INVaF' bacteria (Thermo-Fisher) were used for transformation and colonies were grown in LB with ampicillin (100 μg/mL). Amphopack-293 cells were used for virus production and infection was done in LKR13 cells with supernatants and polybrene (8 μg/mL, Sigma-Aldrich). LKR13 cells containing the vectors were selected with puromycin (1 μg/mL).

2.4. Quantitative real-time PCR and Western blotting

qPCR and Western blot methods were performed as previously described [14]. Sequences of the primers are shown in Supplementary Table 1. The primary antibodies for Western blotting were as follows: anti-TMPRSS4, 1:5000 (Ingenasa); anti-γ-H2A, 1:1000 (Cell Signaling); anti-PARP, 1:1000 (Cell Signaling); anti-cleaved caspase-3, 1:1000 (Cell Signaling); anti-MCM6, 1:500 (Santa Cruz); anti-p21, 1:1000 (Santa Cruz,), anti-TYMS, 1:200 (Chemicon) and anti-β-actin at 1:10000 (Sigma).

2.5. MTT and clonogenic assays

For MTTs, cells (1500 per well) were seeded in 96-well plates (BD Falcon) and grown for 96 h. MTT solution (Sigma) was added to cells and incubated for 4 h. Then, crystals were solubilized and plate's absorbance was read at 570 nm with a TECAN (Sunrise) machine using the Mallegan Plate Reader software.

For clonogenic assays, 500 H358 or 1000 H2170 cells per well were plated in 6-well plates (BD Falcon) and cultured for 14 and 8 days, respectively, with doxycycline-containing medium. Cells were fixed with 4% formaldehyde (Panreac) and stained with 1% crystal violet (Sigma-Aldrich), and the number of colonies was counted.

2.6. Cell cycle and apoptosis

For cell cycle, cells were fixed with 70% ethanol after doxycycline-induced TMPRSS4 silencing and then incubated with RNase A (0.2 mg/mL, Sigma) for 30 min at 37 °C and stained with 7AAD (0.02 mg/mL, Sigma). A FACSCalibur Flow cytometer was used for quantification based on 2n or 4n DNA content. Data were analyzed with the FlowJo software. BrdU analysis is explained in Supplementary Materials and Methods.

Apoptotic cells were detected with the Annexin-V assay. Briefly, cells were collected, washed with PBS and resuspended in 100 μL of Annexin binding buffer (BD, Pharmingen) containing Annexin-V Dye634 (ImmunoStep). Afterwards, cells were incubated in darkness for 15 min at RT. Annexing binding buffer was added to a final volume of 500 μL and 0.5 μL of Sytox green was used to detect dead cells. Samples were analyzed in a FACS Canto II cytometer (BD Bioscience) and data processed with FlowJo software.

2.7. Microarray and gene pathway analysis

Transcriptomic profiles of H358 parental (Par) cells, shCtrl cells and TMPRSS4-depleted sh1 and sh4 clones were compared upon treatment with 1 μg/mL doxycycline for 4 days. Total RNA was extracted with the Maxwell system (Promega), quantified by Qubit (ThermoFisher Scientific) and analyzed for purity and integrity with Experion (BioRad). The Claryom S Human Whole Transcriptome Expression Profile expression microarray (ThermoFisher Scientific) was used for hybridization. Microarrays were performed at CIMA Lab Diagnostics and data normalization and processing at CIMA Bioinformatics Platform. Background correction and normalization were carried out with RMA (Robust Multichip Average) algorithm and a filtering process was applied to eliminate probe sets with low expression intensities. The

analysis of significant gene expression changes was done with Z-score transformation of fold-change distributions. To categorize differentially expressed genes, Ingenuity Pathway Analysis (IPA) (www.qiagenbioinformatics.com) and Gene Ontology (GO) (www.geneontology.org) were used. Prediction of transcriptional activation and up-stream regulators with IPA was calculated by means of Z-score.

The functional enrichment of the gene rankings obtained based on the logFC of the biological comparisons was analyzed with Gene Set Enrichment Analysis (GSEA) [20]. Functional categories were selected from MsigDB database (C2 gene sets) and the non-parametric Kolmogorov-Smirnoff rank test was performed with 4000 permutations.

2.8. Cytotoxicity and apoptotic assays after chemotherapy

The following chemotherapy drugs were used *in vitro* to evaluate cytotoxicity in the different cell clones: cisplatin, docetaxel, paclitaxel, etoposide, pemetrexed, methotrexate and 5-fluorouracyl (5-FU). Drugs were obtained from Sigma-Aldrich except for pemetrexed (Activate Scientific) and methotrexate (Disproquima life Science).

For cytotoxicity assays, cells were plated in 96-well plates (1500 cells per well) and treated with serially diluted drugs. Seventy two hours after incubation, MTT assays were conducted as described before. For apoptosis experiments, cells were exposed to 2.5 μM cisplatin for 72 h prior to evaluation of apoptosis by annexin-V/Sytox, as described before.

2.9. In vivo experiments

For primary tumor growth, LKR13 control or (m)TMPRSS4-overexpressing cells (5×10^6 per cell type) were subcutaneously injected into immunocompromised mice (n = 5 per group). For the metastatic model, intracardiac injection of cells (10×10^3 per cell type) was used (n = 5 mice per group). For experiments using H358 cells, we first tested tumor engraftment ability. Cells (5×10^6 per cell type) were injected subcutaneously (n = 5 per group) and animals were administered with doxycycline in the drinking water (1 μg/mL) starting from day 1 post-cell injection, which was replaced every 72 h. Doxycycline was removed at week 4 and tumor volume was monitored until week 10. In a second experiment, cells (10×10^6; n = 4 mice per group) were injected and tumors were grown until they reached 100 mm^3 before animals received doxycycline (1 μg/mL). In the third *in vivo* assay, experimental procedures were similar to the ones carried out in the second experiment but the goal was the evaluation of the combined effect of cisplatin treatment with TMPRSS4 inhibition. Four groups of animals were established: a) vehicle-treated shCtrl; b) cisplatin-treated shCtrl; c) vehicle-treated sh1; d) cisplatin-treated sh1. Cisplatin was injected intraperitoneally, weekly at a dose of 3 mg/kg. All animal procedures were carried out in accordance to the ethical guidelines established by our Institution under an approved ethical protocol.

2.10. Histology, immunohistochemistry and immunofluorescence

Tumors were extracted and immediately fixed in 10% formaldehyde for 24 h. After standard histological processing, tissues were sectioned (4 μm in thickness) and stained with haematoxylin-eosin. Methods for quantification of the metastatic area can be found in Supplementary Materials and Methods. For immunohistochemistry, sections were hydrated and endogenous peroxidase was blocked with a 3% hydrogen peroxide solution. Antigen retrieval was done with Tris-EDTA (pH = 9, 6 min at 98 °C). After incubation with normal goat serum to block unspecific binding, anti-TTF1 (thyroid transcription factor-1, 1:100, Dako) and the Envision system (Dako, Denmark), followed by DAB (3,3′-diaminobenzidine; Dako) and counterstaining with haematoxylin were applied prior to observation. The detailed protocol for immunofluorescence can be found in Supplementary Materials and Methods.

2.11. Statistical analysis

Statistical differences between groups were examined with the Student's *t*-test (for comparison of two groups) or ANOVA (when comparing several groups). Survival analysis was conducted on the selected genes (MCM6, TMPRSS4 and TYMS) or gene sets using public datasets Km plot [21], GSE3141, GSE8894 and GSE31210. In the case of Km plot, optimal cut-off values suggested by the software were used to stratify the patients in high and low risk. In the case of the other databases, a summation index of the genes for a particular sample was calculated as previously described [22] and the median (GSE3141 and GSE8894) or 70th percentile (GSE31210) was used to stratify the patients. Log-rank test was used to calculate the statistical significance of differences observed among Kaplan-Meier curves. Survival analyses were performed with R [23]. The rest of the data were analyzed with GraphPad Prism 5 software (GraphPad). Values are expressed as means ± SD, and statistical significance was defined as $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

3. Results

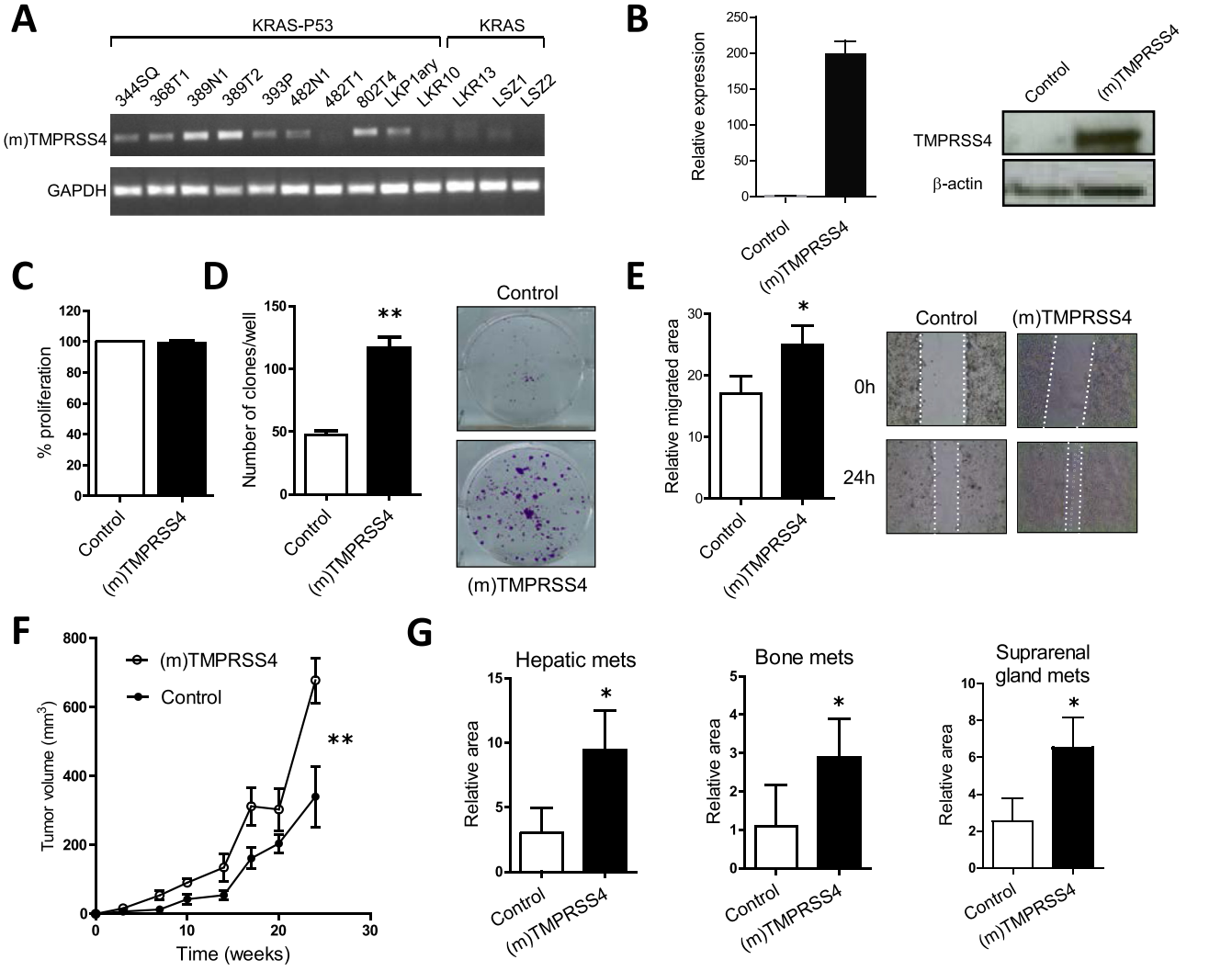
3.1. Increased expression of TMPRSS4 promotes cell malignancy, primary tumor growth and multiorganic metastasis

Our first goal was to modify expression levels of TMPRSS4 in lung cancer cells to assess *in vitro* and *in vivo* malignancy as well as metastasis potential. We evaluated the expression of TMPRSS4 in a series of *Kras* and *Kras*/p53 mutant cell lines, some of which exhibit metastatic potential. *Kras* mutant cells have been shown to be poorly metastatic, whereas *Kras*/p53 mutant cells exhibit higher aggressiveness and metastatic potential [16–19,24]. Fig. 1A shows weak or no detectable levels of TMPRSS4 mRNA in the *Kras* mutant cells LKR10, LKR13, LSZ1 and LSZ2. On the contrary, expression was observed in 8 out of 9 *Kras/p53* mutant cells. This suggests that expression of TMPRSS4 may be associated with a more aggressive and malignant phenotype in these mutant *Kras*/p53 models. We overexpressed murine (m)TMPRSS4 in LKR13 cells and analyzed the biological effect *in vitro* and *in vivo*. Increased expression of (m)TMPRSS4 (Fig. 1B) did not modify proliferation over a period of 96 h (Fig. 1C), as assessed by MTT assays, but resulted in higher clonogenic potential ($p < 0.01$, Fig. 1D). High levels of (m)TMPRSS4 also increased cell migration ($p < 0.05$, Fig. 1E) and tumor growth ($p < 0.01$) in a subcutaneous model (Fig. 1F).

We next evaluated the metastatic potential of (m)TMPRSS4 in a multiorganic model of metastasis initiated by intracardiac injection of LKR13 cells (Fig. 1G and H). Increased levels of (m)TMPRSS4 in LKR13 cells significantly enhanced ($p < 0.05$) metastases in the liver, bone and suprarenal glands, typical locations of lung cancer metastasis in NSCLC patients.

3.2. Knock-down of TMPRSS4 decreases proliferation and clonogenicity of human lung cancer cells

Silencing of TMPRSS4 expression was performed with lentiviral particles containing 4 different shRNA sequences that target the human TMPRSS4 gene (Supplementary Table 2). After inducing the shRNAs with doxycycline, we analyzed TMPRSS4 mRNA and protein levels by qPCR and Western blotting, respectively, in H358 and H2170 lung cancer cells (Fig. 2A–D). Parental cells as well as cells carrying the Tet-pLKO-puro plasmid containing a control shRNA (shCtrl) were used as controls. As expected, whereas no change was observed in control cells after administration of doxycycline (1 μg/mL) a dramatic drop in TMPRSS4 mRNA levels were found in clones carrying the TMPRSS4-targeting shRNAs in comparison with untreated cells (Fig. 2A and B). For Western blotting analysis and subsequent functional experiments, only sh1 and sh4 were used, as the sequence of these two shRNAs did not have any predicted off-target. No protein expression of TMPRSS4



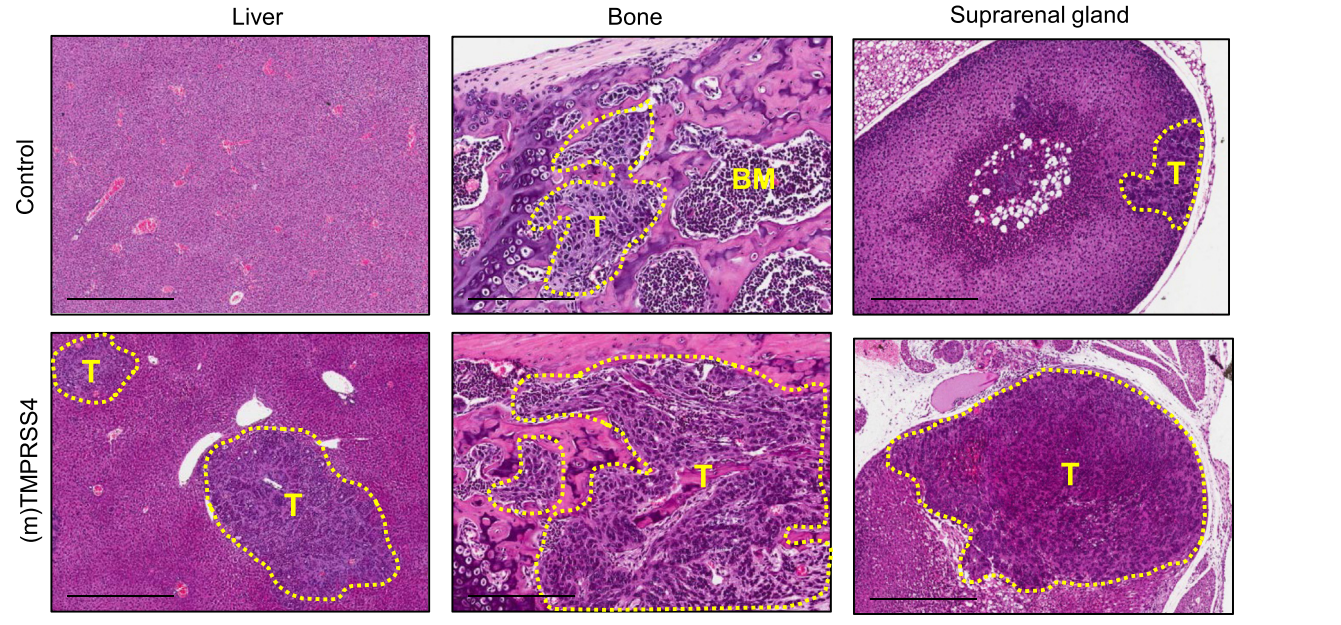


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Fig. 1. Increased expression of TMPRSS4 in murine lung cancer cells promotes malignancy. A. Expression of TMPRSS4 in murine (m) lung cancer cell lines isolated from either *Kras* or *Kras/p53* mouse models. **B.** Overexpression of (m)TMPRSS4 in LKR13 cells analyzed by qPCR and Western blotting. **C.** Proliferation rates in cells with (m)TMPRSS4 overexpression in comparison with controls. **D.** Quantification of clonogenic assays showing that cells with high (m)TMPRSS4 are more clonogenic than control cells. Representative image of a clonogenic assay. **E.** Quantification and representative images of migration evaluated in controls and cells with high (m)TMPRSS4 levels. **F.** Subcutaneous tumor growth is larger in (m)TMPRSS4-overexpressing cells than in controls. **G.** Quantification of multiorganic metastases caused by intracardiac injection of (m)TMPRSS4-overexpressing cells. **H.** Representative images of liver, bone and suprarenal gland metastases in controls and (m)TMPRSS4-overexpressing groups. T: tumor; BM: Bone marrow. *: $p < 0.05$; **: $p < 0.01$. Bars: liver and suprarenal gland: 700 μm; bone: 200 μm.

was observed in these clones upon treatment with doxycycline (Fig. 2C and D). In a time-course experiment, we first treated the cell clones with doxycycline for 3 days and then removed doxycycline and let the cells grow untreated over a period of 7 days. TMPRSS4 expression remained inhibited 7 days after removal of doxycycline (not shown).

H358 and H2170 cells with reduced TMPRSS4 expression showed a significant decrease in cell proliferation ($p < 0.001$), when compared with parental and shCtrl cells (Fig. 2E, H). Effect on cell clonogenicity in adherent conditions was dramatic for both H358 (Fig. 2F, G) and H2170 cells (Fig. 2I and J), as virtually no colonies were observed in sh1/sh4 clones. Representative images are shown in Fig. 2G, J.

3.3. Alteration of cell cycle and induction of apoptosis in cells lacking TMPRSS4

Decrease in proliferation suggested an impairment of the cell cycle and/or increase in cell death. Analysis of the cell cycle in H358 cells revealed a significantly higher percentage of cells in the sub-G0/G1 (indicative of cell death) in the sh1/sh4 clones, in contrast with controls (Fig. 3A). Moreover, sh1/sh4 clones had a lower proportion of cells in the G2/M phase and in the S phase. To further substantiate the impairment in cell proliferation and incorporation of the synthetic nucleotide BrdU into the DNA, we performed immunofluorescence experiments. A shown in Supplementary Fig. 1, a very significant reduction in BrdU incorporation was found in cell clones lacking TMPRSS4. We next conducted flow cytometry assays with annexin-V and Sytox staining to quantify apoptosis (Fig. 3B and C). The percentage of apoptosis in the cell clones with low TMPRSS4 levels was significantly higher than that of controls (Fig. 3B). Representative images of the flow cytometry plots are shown in Fig. 3C. Taken together all these results show that lack of TMPRSS4 impairs proliferation and induces cell death.

3.4. Conditional downregulation of TMPRSS4 hinders tumor engraftment and tumor growth

To test the effect of TMPRSS4 depletion *in vivo*, two experiments were carried out. In the first one, aimed mainly to assess engraftment, animals were administered with doxycycline from day 1 after subcutaneous injection of either control cells (H358 parental or shCtrl) or cells carrying the shRNAs targeting TMPRSS4 (sh1 or sh4). After removal of doxycycline, tumors from the control groups grew rapidly, unlike those corresponding to the sh1/sh4 groups (Fig. 4A), which remained without growing for the entire length of the experiment. This result is in keeping with the reduced levels of TMPRSS4 upon doxycycline removal that we have observed *in vitro*. Representative pictures of tumor-bearing mice and macroscopic images of tumors corresponding to each group are shown in Fig. 4B. Histological examination of the small tumors harvested from sh1/sh4 groups revealed focal areas of fibrosis and/or necrosis within the cancer cell population, positive for the adenocarcinoma marker TTF1 (thyroid transcription factor-1) (Fig. 4C). In the second experiment, tumors were let to grow until they reached 100 mm^3 and doxycycline was administered to the animals. As shown in Fig. 4D, tumors from TMPRSS4-depleted clones were significantly smaller ($p < 0.001$) in comparison with controls.

3.5. Gene expression changes in cells with TMPRSS4 downregulation

To elucidate the molecular mechanisms that might cause the biological effects previously described, we performed gene expression microarray analyses comparing transcriptomic profiles of control cells with those of sh1/sh4 clones ($n = 2$ per cell clone). Using a 1.5-fold change in at least one of the cell clones as a cut-off value, a total of 136 genes were differentially expressed in cells with reduced TMPRSS4 levels. From these, 64 were up-regulated and 72 down-regulated. The list of deregulated genes can be found in Supplementary Table 3. Analysis of gene categories using quantitative methods, such as Ingenuity Pathway Analysis (IPA) (https://www.qiagen.com) and Gene Ontology (GO) (http://geneontology.org/) revealed that approximately 20% of those genes were involved in cell replication, cell cycle or DNA repair. The IPA z-score was statistically significant ($z > 2$,) for regulation of cell cycle at the G1/S checkpoint, p53 signaling and cell cycle regulation. Gene set enrichment analysis (GSEA) using MSigDB revealed a significant enrichment of our TMPRSS4-down (TMPRSS4-) gene set in genes categorized as cell cycle, transition from G1 to S, DNA synthesis and DNA repair (Supplementary Fig. 2). The list of genes found at the leading edge of each of these categories can be found in Supplementary Table 4. All these results suggested that lack of TMPRSS4 was altering molecular pathways related to cell proliferation.

Fig. 5A shows a hierarchical cluster analysis of a selected group of genes related to DNA replication, DNA repair, cell cycle and cytoskeleton/ECM in clones lacking TMPRSS4 in comparison with controls. Most deregulated genes known to be critical for DNA replication and progression through the cell cycle, such as E2F1, MYB, MYBL2, thymidylate synthase (TYMS), and the minichromosome maintenance complex (MCM) proteins with helicase activity MCM3 and MCM6 were down-regulated, whereas the cyclin-kinase inhibitor CDKN1A (p21) was up-regulated. Alteration of these genes suggested that lack of TMPRSS4 affects the replisome and incorporation of nucleotides into the nascent DNA. Then we used the pathway upstream regulator analysis from IPA: a tool that, based on prior knowledge of expected effects between transcriptional changes, examines how many known targets of each transcription regulator are present in our dataset, and also compares their direction of change to what is expected from the literature, in order to predict likely relevant transcriptional regulators. This analysis predicted that E2F1 was an upstream regulator for the decreased expression of 12 target genes identified in our microarray analysis (Supplementary Fig. 3).

Some of the genes identified by microarrays were validated by qPCR. A significant decrease in MCM6, MYB and TGF-β was observed (Fig. 5B). A more modest reduction was found for TYMS, CHEK1 and E2F1. On the contrary, CDKN1A (p21) levels were clearly increased in cells depleted of TMPRSS4. We performed Western blot analysis for MCM6, CDKN1A (p21) and TYMS, and found concordant results with those observed by qPCR (Fig. 5C).

3.6. A three-gene TMPRSS4-related signature is predictive of poor prognosis

We have previously demonstrated that high TMPRSS4 expression predicts poor prognosis in early NSCLC [15]. We asked whether genes identified by microarrays could constitute a TMPRSS4-associated signature with prognostic value. To this aim we used published data from Gyorffy et al., a lung cancer public database that includes patient's

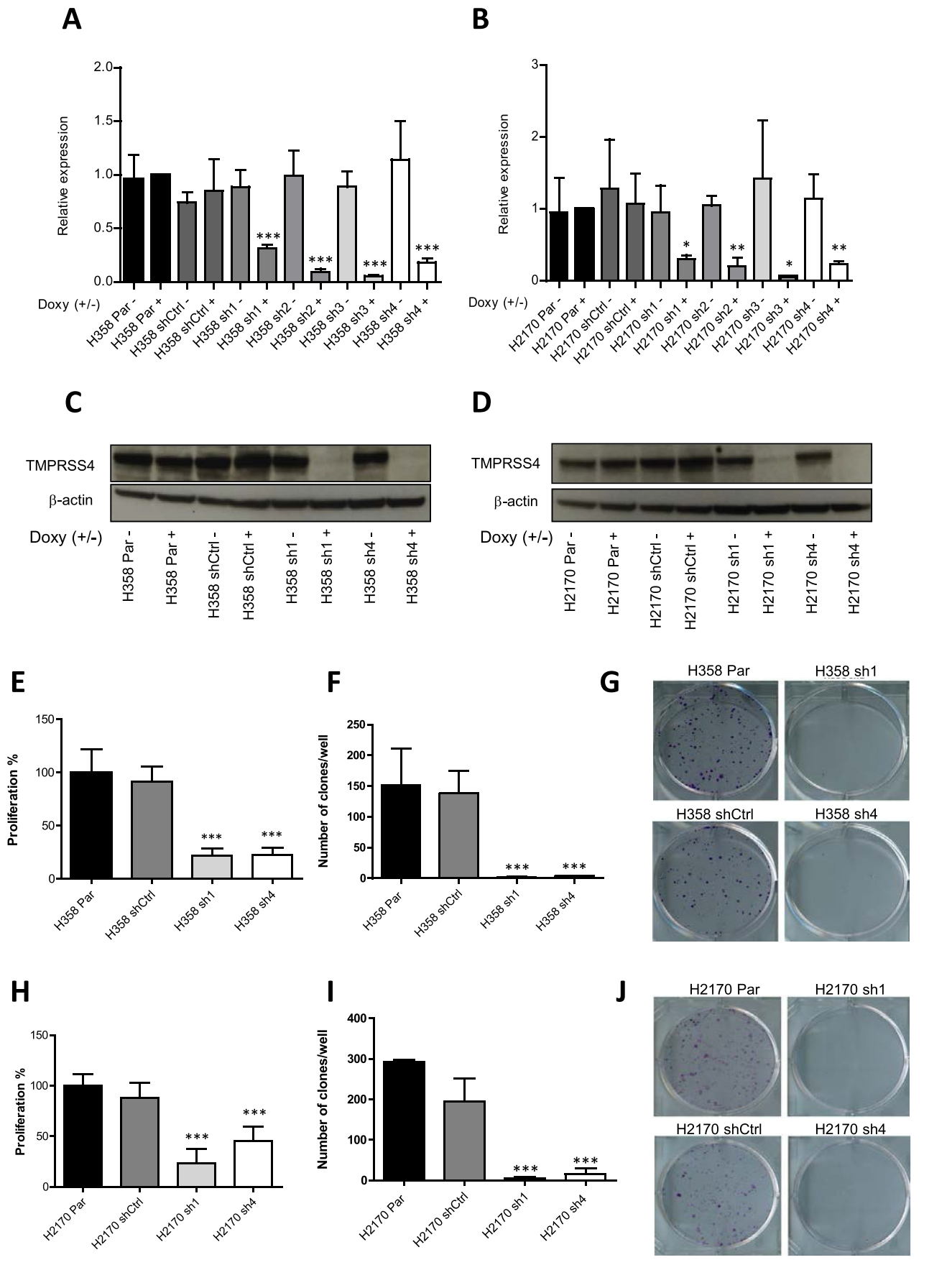

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Fig. 2. Effect of reduction of TMPRSS4 levels on proliferation and clonogenicity. mRNA levels of TMPRSS4 in the different H358 (**A**) and H2170 (**B**) clones transduced with shRNA lentiviral particles. Administration of doxycycline in the shTMPRSS4 clones reduced significantly expression of TMPRSS4. Statistical analysis corresponds to comparisons between untreated and treated clones by Student's T test. Western blotting in H358 (**C**) and H2170 (**D**) cells to show conditional reduction of TMPRSS4 upon treatment with doxycycline in sh1/sh4 TMPRSS4 clones. Proliferation was significantly reduced in sh1/sh4 clones in H358 (**E**) and H2170 cells (**H**) in comparison with control cells. Cells depleted of TMPRSS4 showed a dramatic reduction of clonogenicity (**F**, H358; **I**, H2170). Representative images are shown in **G, J**. *: $p < 0.05$; **: $p < 0.01$;***: $p < 0.001$.

information from The Cancer Genome Atlas (TCGA, http://cancergenome.nih.gov), Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and Cancer Biomedical Informatics Grid (caBIG, http://cabig.cancer.gov/) [21]. We used the optimal probes and the optimal cut-off values suggested by the software. MCM6, TYMS, CDKN1A (p21), as well as TMPRSS4, were interrogated alone or in combinations in early stage NSCLC. CDKN1A (p21) had no prognostic value, whereas high levels of TMPRSS4, TYMS and MCM6 were significantly associated with reduced survival (results not shown). A gene signature including these 3 genes had the best prognostic value in comparison with each of the genes alone or combinations of two genes. Fig. 5D shows Kaplan Meier curves for progression-free survival (PFS, HR = 2.32 [1.45–3.72]; $p < 0.001$) and overall survival (OS, HR = 3.33 [2.33–4.76]; $p < 0.0001$) for the 3-gene signature in stage I NSCLC. Values for HR and p-values for each individual gene are shown in Supplementary Table 5. To validate the prognosis of this signature we analyzed three previously published microarray expression datasets: Lee (GSE8894), Okayama (GSE31210) and Bild (GSE3141). Supplementary Fig. 4 shows the prognostic value of the signature evaluated by the Logrank test in the Lee ($p = 0.007$), Okayama ($p = 0.002$) and Bild ($p = 0.043$) databases, which was in the three cases superior than the p-values found for the individual genes (not shown).

3.7. Downregulation of TMPRSS4 increases significantly sensitivity to chemotherapy

Microarray data suggested that alteration of proteins involved in the replication machinery could sensitize cells to chemotherapy. In addition, a bioinformatic analysis using IPA showed that pathways modified by changes in the expression of TMPRSS4 significantly overlapped with those altered by cisplatin (Supplementary Fig. 5). We tested the effect of cisplatin, docetaxel, etoposide, paclitaxel, pemetrexed, 5-FU (Fig. 6), and methotrexate (Supplementary Fig. 6A) on H358 control and shTMPRSS4 groups and found that sh1 and sh4 cell clones were much more sensitive to these drugs than control cells. The effect of cisplatin was also tested in H2170 control/shRNAs cells and we found similar results, with sh1 and sh4 clones being significantly more affected than controls (Supplementary Fig. 6B). These results demonstrate that TMPRSS4 targeting causes chemosensitization to different cytotoxic drugs.

We then focused on cisplatin for further studies, as this drug is the

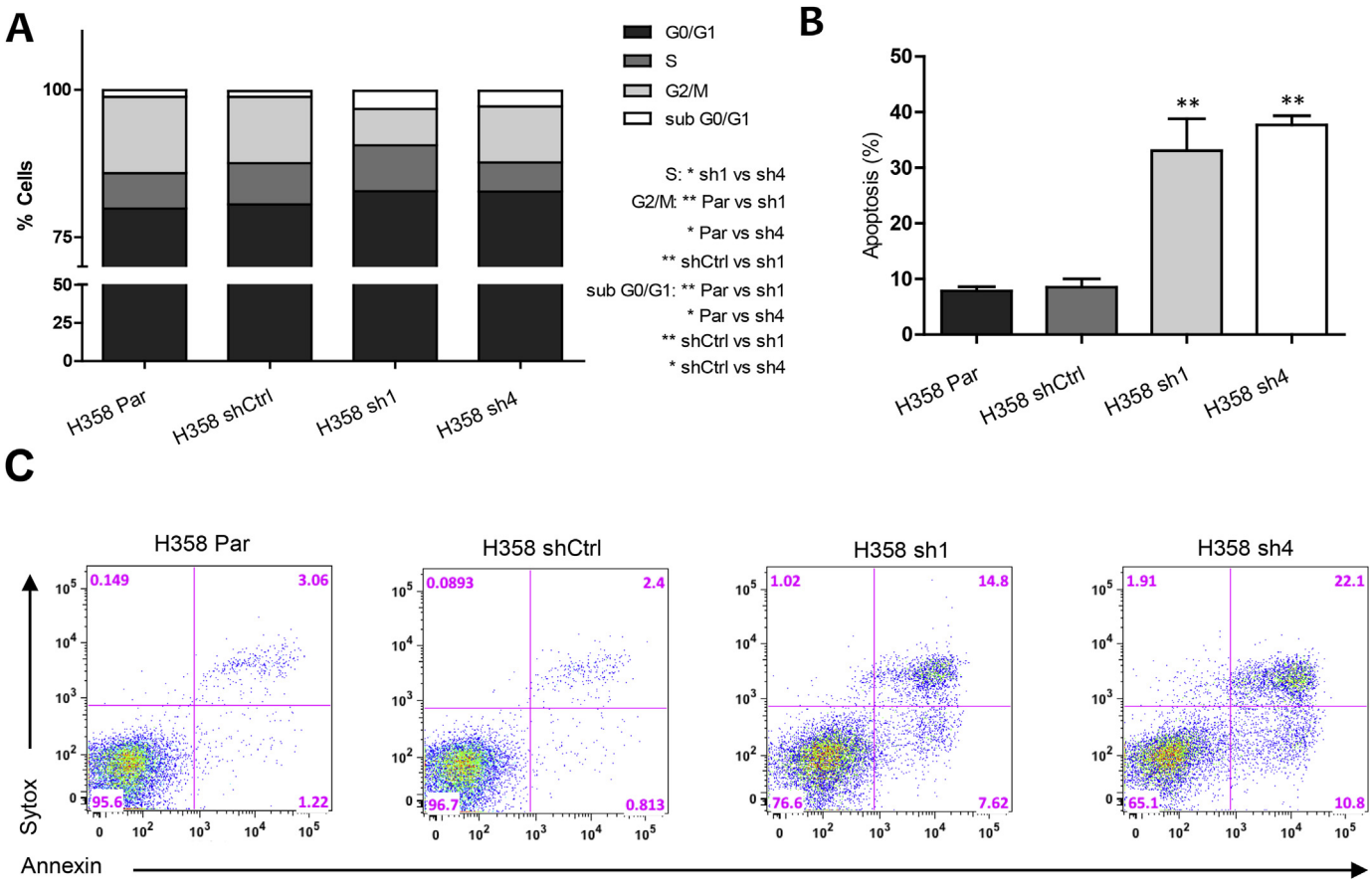

Fig. 3. Effect of reduction of TMPRSS4 levels on cell cycle and apoptosis. A. Significantly higher percentage of cells in the sub-G0/G1 (indicative of cell death), lower proportion in the G2/M phase (especially for sh1) or in the S phase (in the case of sh4) was found in clones with reduced TMPRSS4. **B.** Quantification of apoptosis using annexin-V/Sytox and flow cytometry. A high percentage of apoptotic cells was observed in the sh1/sh4 clones in comparison with controls. **C.** Representative images of the flow cytometry plots. *: $p < 0.05$; **: $p < 0.01$ sh1 or sh4 vs controls; # sh1 vs sh4.

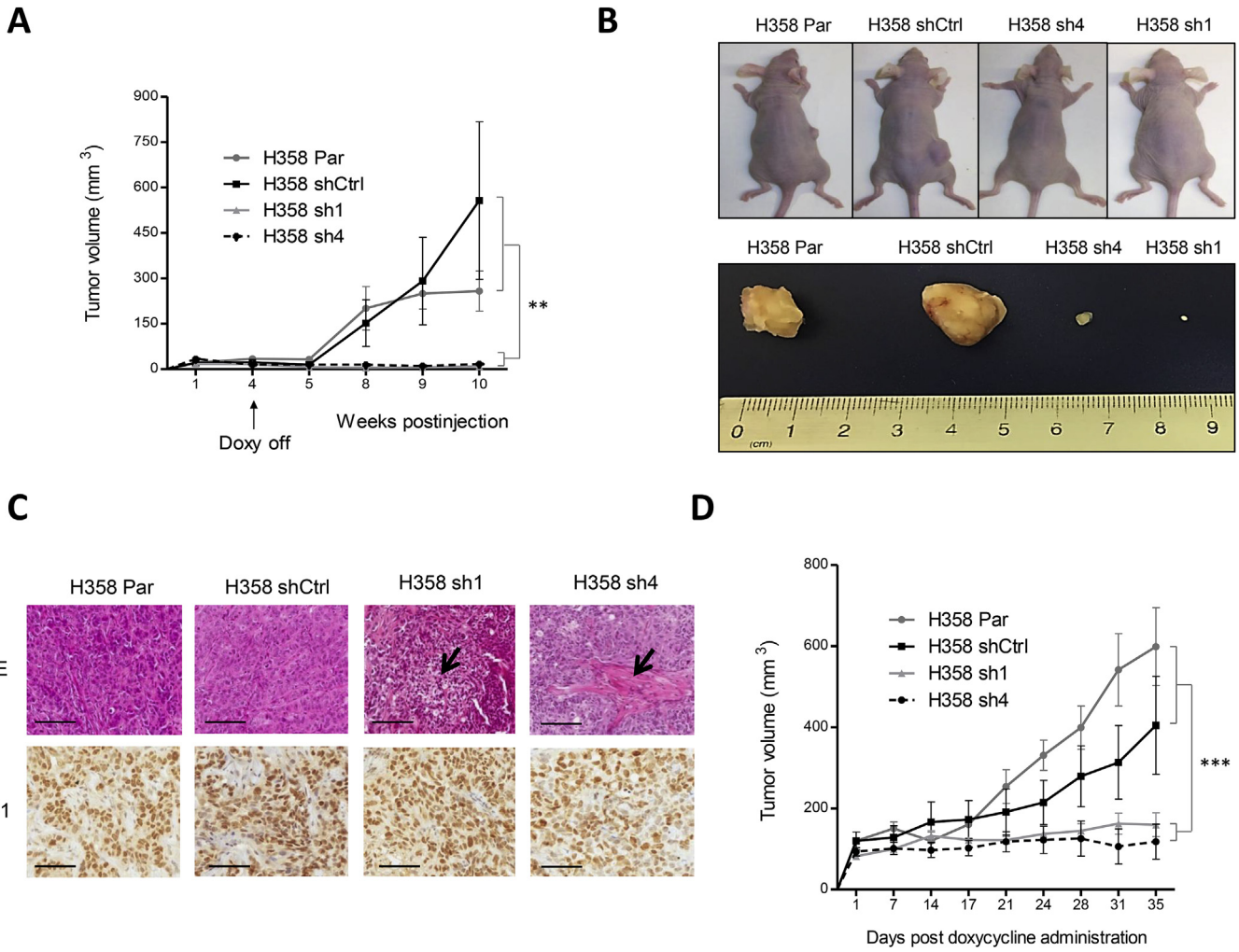

Fig. 4. Effect of TMPRSS4 inhibition on tumor growth. A. Experiment to evaluate engraftment. Animals were injected with H358 control cells or sh1/sh4 clones and administered with doxycycline from day 0. At week 4, doxycycline was removed and tumor volume was monitored until week 10. A significant reduction in tumor volume was observed in animals injected with clones devoid of TMPRSS4 in comparison with controls (lines corresponding to tumor volumes of sh1 and sh4 overlap). **B.** Representative images of tumor-bearing mice for each group and macroscopic images of the tumors. **C.** Representative histological images and immunohistochemistry for TTF1 in tumors of each group. Arrows indicate areas or necrosis or fibrosis. Bar: 100 μm. **D.** *In vivo* experiment where doxycycline was administered when tumors reached 100 mm^3. Tumor growth was significantly lower in animals injected with sh1/sh4 clones than in controls. **: $p < 0.01$, ***: $p < 0.001$.

standard chemotherapy used for the treatment of NSCLC patients. Apoptotic analysis with annexin-V/Sytox using cisplatin in the different clones showed that cells with reduced levels of TMPRSS4 underwent apoptosis in a significantly higher proportion than controls (~50–60% for sh1/sh4 clones versus < 30% for controls) (Fig. 7A and B). Western blot analyses in treated cells showed high levels of phosphorylated histone H2A (demonstrating DNA damage), as well as active caspase-3 and cleaved PARP (revealing apoptosis) in sh1/sh4 clones in comparison with controls (Fig. 7C).

To assess *in vivo* the effect of cisplatin and down-regulation of TMPRSS4, we measured tumor growth in animals inoculated with shCtrl cells treated or untreated with cisplatin, as well as in animals inoculated with sh1 cells treated or untreated with cisplatin. Fig. 7D shows that, in the group of mice administered with cisplatin in the absence of TMPRSS4, tumors were significantly smaller ($p < 0.01$) than in the other groups. Therefore, all these experiments demonstrate that TMPRSS4 targeting increases sensitivity to cisplatin.

4. Discussion

The limited clinical benefit of chemotherapy in NSCLC shows the need to identify novel genes with a relevant protumorigenic and pro-metastatic effect that, in addition, may constitute novel targets. TMPRSS4 is a type II serine protease overexpressed in NSCLC and other tumor types (reviewed in Ref. [10]), whose high levels are associated with reduced disease-free and overall survival [15]. In the present study we have overexpressed TMPRSS4 to show its role in promotion of both primary tumor growth and multiorganic metastasis. In agreement with these results, depletion of TMPRSS4 expression in cell lines with high endogenous levels of this protease significantly alters the cell cycle and the expression of multiple genes involved in proliferation and apoptosis, leading to impairment of tumor engraftment and tumor growth. Moreover, cells lacking TMPRSS4 are highly sensitized to the cytotoxic effect of different chemotherapy drugs.

To test the effect of TMPRSS4 in metastasis we took advantage of the metastatic potential of murine LKR13 cells. Using an intracardiac injection model we have demonstrated that overexpression of TMPRSS4 enhances metastasis in bone, liver and suprarenal glands, typical metastasis sites in lung cancer patients. In these cells, overexpression led to increased clonogenic and invasive potential. To our knowledge, this is the first report showing the malignant phenotype conferred by murine TMPRSS4. In human cell lines, increased metastasis caused by high

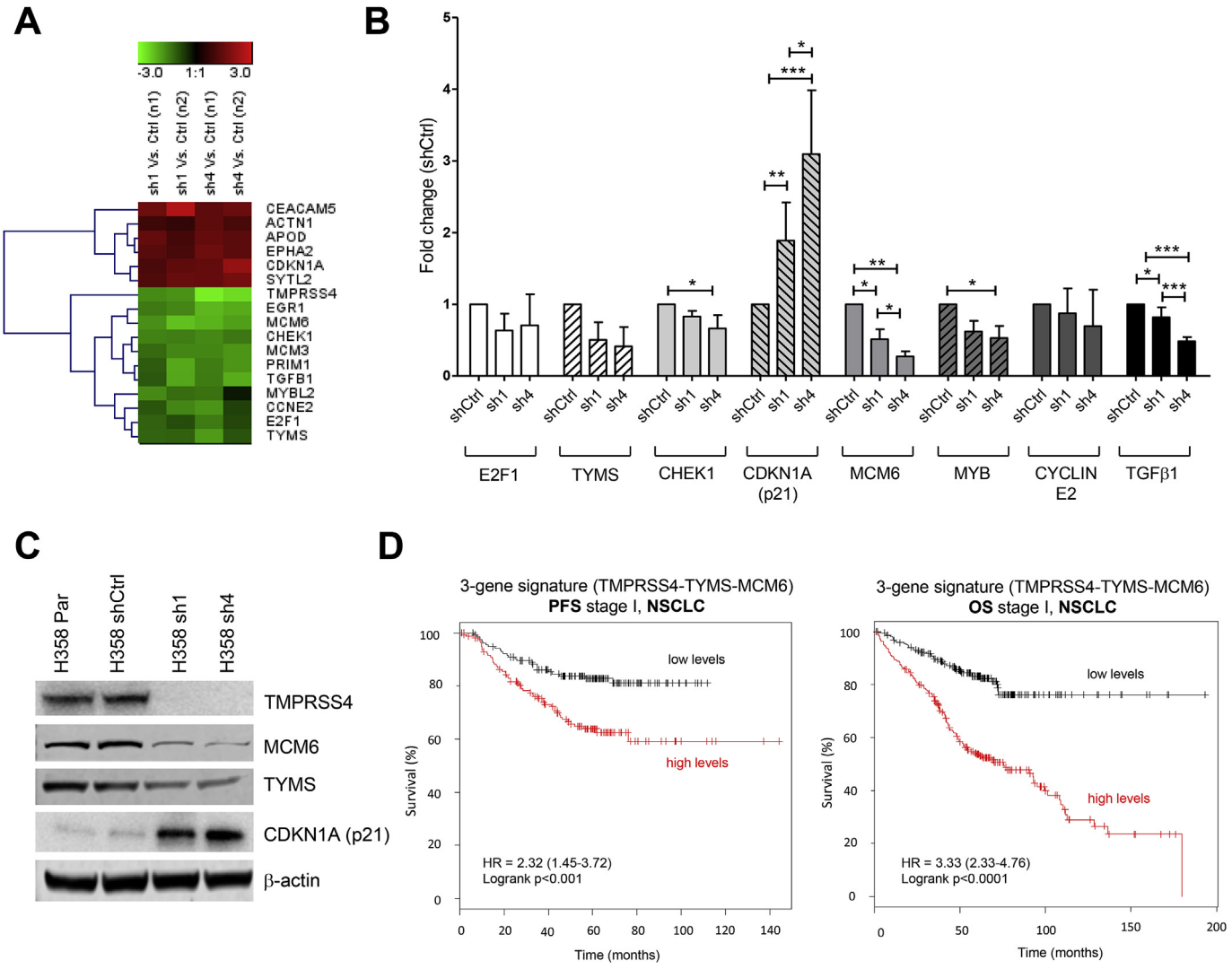

Fig. 5. Transcriptional profiles associated with the downregulation of TMPRSS4. **A.** Cluster analysis of a selected group of genes related to proliferation and interaction of cancer cells with the ECM. Many down-regulated genes in the sh1/sh4 clones corresponded to genes involved in cell proliferation. **B.** Validation of expression changes of some of the proliferation-related genes in sh1/sh4 clones by qPCR. **C.** Western blot showing reduced levels of MCM6 and TYMS, as well as increase in CDKN1A (p21) in clones lacking TMPRSS4. **D.** Kaplan Meier curves showing that high expression levels of the 3-gene signature TMPRSS4-TYMS-MCM6 are very significantly associated with reduced PFS and OS in stage I NSCLC patients. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.01$.

TMPRSS4 levels has been shown for SW480 colon [25], H358 lung [26] and BEL-7402 hepatic carcinoma cells [9]. This effect is likely due to the concomitant effect of enhanced proliferation and invasion elicited by TMPRSS4. Previous publications from different groups including ours have shown that this protease causes EMT in cancer cells, with loss of E-cadherin and gain in mesenchymal markers, such as vimentin and N-cadherin, together with increased cell motility [11,14,27].

Our knock-down models in H358 and H2170 cells reveal that proliferation and clonogenicity are highly affected when TMPRSS4 levels are genetically reduced. Cell cycle and apoptosis analysis showed lower number of cells in the S/G2M phases and higher in the subG0/G1 phase, as well as an increase in apoptosis. Transcriptomic analysis identified downregulation of numerous genes involved in DNA synthesis and repair, including E2F1, thymidylate synthase (TYMS), MYB, MYBL2, cyclin E2, CHECK1 and several members of the minichromosome maintenance complex (MCM). On the contrary, the CDK inhibitor CDKN1A (p21) was found to be highly up-regulated. DNA replication requires the activity of members of the MCM family, a hexameric complex in the replication origins that is necessary for cell division [28]. These proteins are also essential in the S phase, as they constitute part of the helicase complex that is necessary for the maintenance of the replication forks [29]. In hepatocellular carcinoma cells, MCM6 has been shown to promote S to G2/M cell cycle progression and serves as a prognostic maker [30]. Expression of this protein is directly related with the cell replication ability [29].

TYMS is another gene involved in DNA replication and repair, as it plays a role in the synthesis of dMTP from dUMP [31]. Reduced levels of TYMS have been associated with decreased proliferation and increased apoptosis [31]. Antimetabolites such as 5-FU and folate analogs target TYMS, and high expression of this enzyme is related to lack of response to pemetrexed in NSCLC patients [32]. Moreover, high TYMS expression is associated with poor prognosis [33].

We have also found using public available data that high levels of a 3-gene signature constituted by TYMS, MCM6 and TMPRSS4 predicts worse prognosis in early NSCLC patients. It is possible that these genes cooperate to promote both proliferation and metastatic properties in cancer cells, increasing cell malignancy. Interestingly, expression of these 3 genes has been implicated in the acquisition of an EMT phenotype and metastasis exacerbation. Thus, in hepatocellular carcinoma, metastasis promoted by MCM6 has been related to increased mesenchymal phenotype in experimental models [34]. In NSCLC, knockdown of TYMS in cancer cells reduces the EMT phenotype and

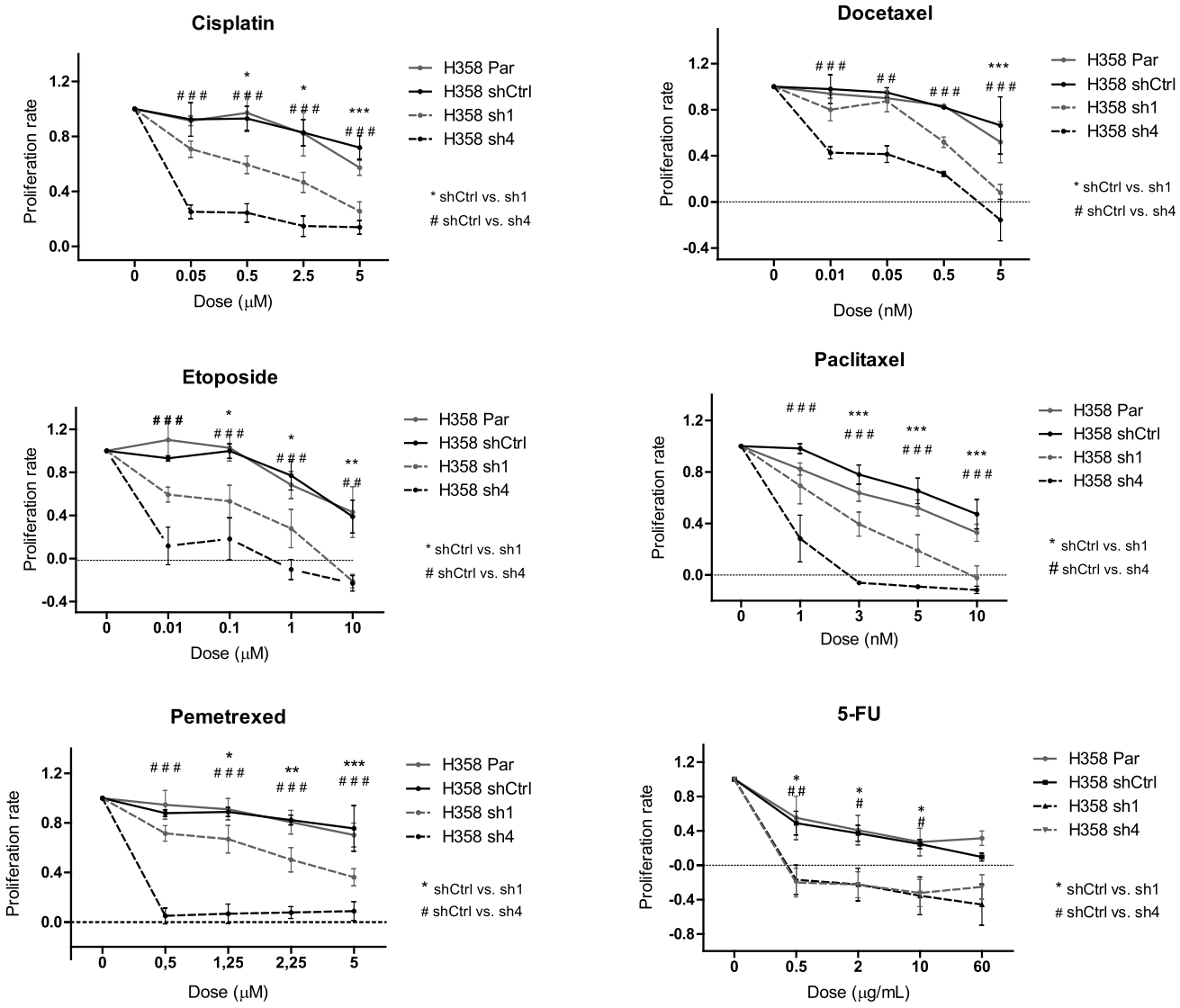

Fig. 6. Inhibition of TMPRSS4 increases sensitivity to chemotherapy. Cisplatin, docetaxel, etoposide, paclitaxel, pemetrexed and 5FU at different doses in H358 parental cells (Par), shCtrl, sh1 and sh4 cells. The cytotoxic effect is in all cases higher in cells lacking TMPRSS4. */#: $p < 0.05$; **/##: $p < 0.01$; ***/###: $p < 0.01$.

migratory ability, the expression of stem-like markers and chemoresistance [35].

The fact that many genes down-regulated by TMPRSS4 depletion were involved in replication/DNA repair led us to hypothesize that targeting this gene could sensitize cells to the cytotoxic effect of chemotherapy drugs. We have used different drugs and found that cells lacking TMPRSS4 were significantly more sensitive to the cytotoxic effect of all of them than controls. Such drugs work through different mechanisms on cancer cells, including an inductor of DNA damage (cisplatin), inhibitors of microtubule dynamics (paclitaxel, docetaxel), a topoisomerase-II inhibitor (etoposide) and antimetabolites (pemetrexed, methotrexate, 5-FU). Therefore, the sensitization process seems to be a general mechanism, rather than a pathway-specific effect. It is likely then that targeting TMPRSS4 leads to a sensitization status that facilitates cytotoxicity. Our further *in vitro* and *in vivo* experiments using cisplatin show that cells with no TMPRSS4 treated with this drug undergo apoptosis with activation of caspase-3 and PARP proteolysis, as well as DNA damage. This effect could be mediated by the impairment in DNA replication efficiency, but this hypothesis needs further demonstration in future studies.

Our results suggest that pharmacological compounds to inhibit TMPRSS4 should be developed and tested in combination with chemotherapy, as a possible novel treatment for TMPRSS4-positive tumors, which are associated with worse prognosis. TMPRSS4 may be a good target candidate, since it is a druggable membrane-bound protein. The tripeptide tyroserleutide (YSL) has been shown to inhibit the invasive and metastatic potential of HCC induced by irradiation through downregulation of TMPRSS4 and inhibition of EMT, in a model of hepatocellular carcinoma [36]. A first family of small molecules consisting of 2-hydroxydiarylamide derivatives, identified by screening of a large library of chemical compounds, has been shown to inhibit TMPRSS4 activity and invasiveness of colon cancer cells [37]. However, no data are yet available on these or other compounds *in vivo*.

In summary, we show in this study that genetic inhibition of TMPRSS4 implies changes in the genetic program of cancer cells related to cell cycle disruption, apoptosis and increased sensibility to chemotherapy.

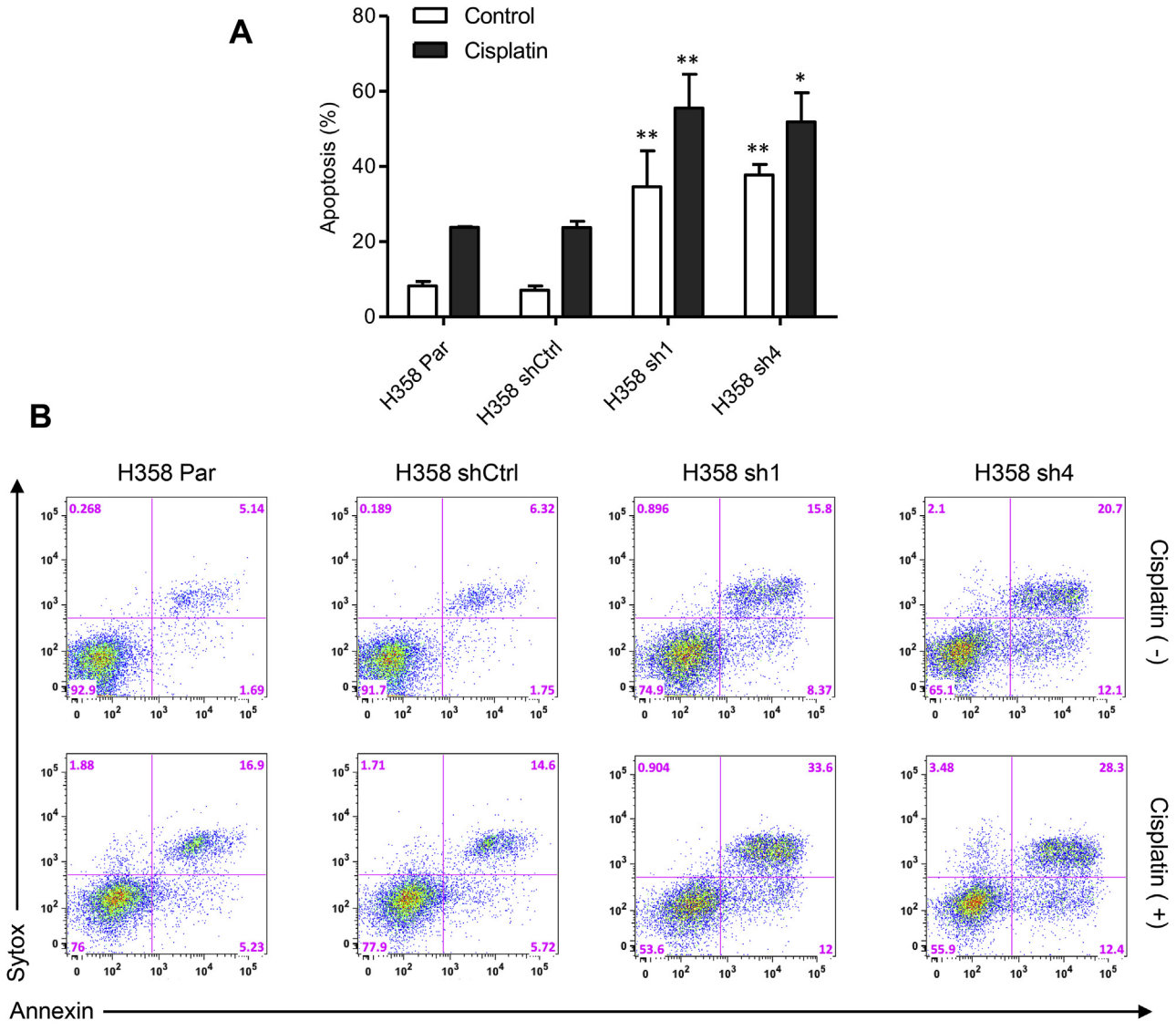

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Fig. 7. Increased apoptosis and decreased tumor growth triggered by cisplatin in cells lacking TMPRSS4. A. Apoptosis was higher in sh1/sh4 clones than in control cells, but the effect was much higher when these clones were administered with cisplatin, reaching 50–60% of the cell population. **B.** Representative images of flow cytometry plots. **C.** Cisplatin treatment caused phosphorylation of H2A (revealing DNA damage) and activation of caspase-3 and PARP (showing apoptosis). **D.** Cisplatin treatment *in vivo* (n = 4 mice per group): tumor size in mice injected with sh1 cells was smaller than that found in the group of untreated sh1, shCtrl treated and shCtrl untreated mice. *: $p < 0.05$; **: $p < 0.01$.

Conflicts of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.03.013.

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