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SERPINA3 is a key modulator of HNRNP-K transcriptional activity against oxidative stress in HCC

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ABSTRACT

Most studies about serpin peptidase inhibitor, clade A member 3 (SERPINA3) has been limited to its inhibitory functions and mechanisms. Herein, we report a novel role of SERPINA3 in transcriptional regulation of HCC progression-related genes. Among 19 selected genes through HCC cell isolation system based on telomere length, microarray analyses, and cell-based studies, SERPINA3 was the strongest determinant of increases in telomere length, HCC cell proliferation, survival, migration, and invasion. We also found that SERPINA3 strongly interacted with heterogeneous nuclear ribonucleoprotein K (HNRNP-K) under H_2O_2 exposure, and the oxidation-elicited SERPINA3-HNRNP-K complex enhanced the promoter activities and transcript levels of a telomere-relating gene (*POT1*) and HCC-promoting genes (*UHRF1* and *HIST2H2BE*). Intriguingly, the inhibition of SERPINA3 oxidation rendered the transcriptional activity of the SERPINA3-HNRNP-K complex suppressed. Moreover, the co-immunoprecipitated HNRNP-K with SERPINA3 quantitatively correlated with not only the level of SERPINA3 oxidation but also the level of *POT1*, *UHRF1,* and *HIST2H2BE* transcripts and telomere length in HCC tissues. Therefore, the upregulated transcriptional activity of HNRNP-K mediated by SERPINA3 promotes HCC cell survival and proliferation and could be an indicator of poor prognosis for HCC patients.

1. Introduction

Liver hepatocellular carcinoma (HCC) is the second leading cause of cancer-related death worldwide, with a mortality rate exceeding 37% in Asia for patients ≥50 years of age and approximate 5-year survival rates of 15% in the United States, and 12% in Europe [1,2]. The incidence and mortality rates of HCC are expected to increase steadily [1,2]. Despite the increasing number of treatment options against advanced HCC (GII and GIII HCC), the selection of a suitable treatment strategy can be difficult [1,2]. This is not only because HCC is a very heterogeneous disease induced by multiple underlying etiologies, molecules, and signaling pathways, but also the drug-mediated hepatotoxicity can occur during treatment [1,2]. Major risk factors for HCC such as the chronic viral infections (hepatitis B or C virus), alcoholic hepatitis, and non-alcoholic fatty liver disease increase the level of reactive oxygen species (ROS) [3,4]. The ROS levels are strongly correlated with the progression of human HCC from early-to advanced-grade [5,6]. HCC cell lines under the elevated ROS environment also show similar malignant phenotypes with the advanced HCC tissues [5–7]. These phenotypes encompass the decreased level of antioxidant enzymes [5,7], the increased epithelial-to-mesenchymal transition (EMT) [5], the increased activity of cell invasion and migration [5–7], the increased expression of telomerase reverse transcriptase (TERT), the enhanced telomerase activity, and the elongated telomere length [6].

It has been known that the heterogeneous nuclear ribonucleoprotein-K (hnRNP-K) was upregulated in human HCC tumor tissues, compared to the corresponding non-tumorous tissues [8]. Similarly, the upregulated hnRNP-K expression has been correlated with poor clinical outcomes and HCC malignancy [8]. The hnRNPs comprise a large family of proteins that play important roles in telomere biogenesis, DNA repair, and the regulation of expression at both the transcriptional and translational levels [9,10]. The hnRNP-K, which is one of the most extensively studied hnRNP family members, recognizes the C-rich binding motif in gene regulatory and telomeric DNA [8–10]. It has been suggested that the binding of hnRNP-K to the specific C-rich motifs could be essential in diverse signal transduction pathways, including

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growth regulation and cellular transformation [8,9], but the modulatory mechanisms regarding the hnRNP-K transcriptional activity in HCC still remain unclear.

Recently we reported that the serpin peptidase inhibitor, clade A member 3 (SERPINA3) was a transcriptional activator of PI3Kδ expression in HCC progression [11]. ROS induced oxidative modification of specific residues in SERPINA3, thereby leading to PI3Kδ signaling activation and HCC proliferation [11]. In this paper, we provide the mechanism for the SERPINA3-mediated transcriptional expression of genes responsible for HCC progression. SERPINA3 modulates hnRNP-K transcriptional activity through interaction with the HNRNP-K-protein-interactive (KI) domain of hnRNP-K in human HCC cell lines. For the interaction of SERPINA3 with hnRNP-K through KI domain, the ROS-dependent oxidation of SERPINA3 protein was found to be the prerequisite step. Moreover, the strong interaction of SERPINA3 with HNRNP-K promoted HCC cell survival and proliferation via regulating the gene transcriptions involved in telomere-maintenance and HCC malignancy. Thus, blockade of the association of SERPINA3 with HNRNP-K protein could be valuably used for HCC therapy.

2. Materials and methods

All detail experimental procedures have been described in the supplementary information. Please see details in *SI Appendix*.

3. Results

3.1. Identification of a novel role of SERPINA3 as a molecular linker between telomere maintenance factors and HCC tumorigenic factors

We previously documented that H_2O_2 treatment induced a telomere elongation and cell invasion [5,6,12] in HCC cells. In this study, to further examine the molecular linkage between telomere maintenance and cell invasion, we isolated cells with H_2O_2-mediated long telomeres (cells with top 6% of telomere intensity) and short telomeres (cells with bottom 66% of telomere intensity), respectively (Fig. 1A). HCC cells with long telomeres showed a higher invasion ability, as well as better *in vivo* tumor growth compared to the short telomere-containing cells (Fig. 1B and C). While the BIBR1532, a telomerase inhibitor, alone was not enough to shorten telomeres in HCC cell lines even after two-weeks (SI Appendix, Fig. S1), co-treatment with BIBR1532 and H_2O_2 facilitated the telomere shortening (SI Appendix, Fig. S1). This is consistent with the previous reports describing severe telomeric damage caused by H_2O_2 in HCC cells under telomerase inhibition [6]. After the induction of telomere shortening, HCC cells with long telomeres also exhibited decreased telomere length, cell migration, invasion, and *in vivo* tumor growth (Fig. 1B and C). Taken together, these results suggest that the telomere length positively associates with the HCC cell migration, invasion, and tumor growth, and the telomere machinery may be linked with the HCC-progression factors.

To identify the regulatory factor(s) which links the telomere maintenance factors with HCC-progression factors, we analyzed the microarray data of HCC cells with long and short telomeres (GEO accession number GSE114004), and found that *UHRF1, HIST2H2BE*, *ARL14*, *PTGS2*, *MYLIP*, *HINT3*, *PTGER4*, *UGCG, ZNF121, HSD3B1, TMEM27,* and *SULT2A1* were upregulated, while *SERPINA3*, *MT1X*, *SCL6A14*, *AHSG*, *ITIH2*, *ORM1,* and *ORM2* were downregulated in long telomere-containing HCC cells (Fig. 1D). To determine the subcellular localization of the candidate proteins, the candidates were tagged with EGFP at the individual N-terminus and expressed in Huh7 cells (Fig. 1D and E; SI Appendix, Fig. S2). Among the 19 candidates, 10 genes (*SERPINA3*, *UHRF1*, *HIST2H2BE*, *ARL14*, *PTGS2*, *MT1X*, *SCL6A14*, *MYLIP*, *AHSG*, and *HINT3*) were frequently distributed in telomeres after H_2O_2 treatment (Fig. 1E; SI Appendix, Fig. S2). Approximately 90–95% of HCC tumor cells showed telomerase-dependent telomere maintenance [13–15]. Telomere maintenance is required for evading the cellular crisis induced by the shortened telomeres during liver cirrhosis [3,4,6]. Thus, we hypothesized that telomere maintenance might be essential for HCC progression and the genes, which represent an altered frequency of telomere localization under H_2O_2 treatment, could affect telomere maintenance-mediated HCC tumor progression.

We next dissected the roles of 10 selected genes based on the H_2O_2 treatment dependent changes in the telomere localization frequency using Ingenuity Pathway Analysis (IPA) (SI Appendix, Fig. S3A). The IPA of differentially expressed genes including the 10 selected genes, predicted the prominent function of tumor progression signaling networks (SI Appendix, Fig. S3A). We next quantified telomere lengths, the transcript levels of telomerase subunits, and invasion-associated genes in HCC cells during gene overexpression (Fig. 1F; SI Appendix, Figs. S3B and S3C). In addition, we measured cell growth rates, cell migration and invasion abilities, and the high-dose chemical-mediated mortality rates in the HCC cells following gene overexpression (Fig. 1F; SI Appendix, Figs. S3B and S3C). Overall, we concluded that *SERPINA3* was the most determining gene candidate responsible for telomere maintenance, HCC cell proliferation, survival, migration, and invasion (Fig. 1F; SI Appendix, Figs. S3B and S3C).

3.2. Involvement of SERPINA3 in HNRNP-K's transcriptional activity

SERPINA3 activity to promote both telomere elongation and HCC progression was upregulated under H_2O_2 exposure (Fig. 1). To disclose the underlying mechanism of SERPINA3-mediated telomere maintenance and HCC malignancy, we aimed to identify interacting partners with SERPINA3 under H_2O_2 treatment in HCC cells. Co-immunoprecipitation (co-IP) and LC-MS/MS analyses showed 21 protein candidates for SERPINA3 interactome (Fig. 2A; SI Appendix, Fig. S4). Via label-free quantification analysis of co-IP LC-MS/MS data against those from the mock- and H_2O_2-treated Huh7 cells, we further identified HNRNP-K as an H_2O_2-sensitive binding partner of SERPINA3 (Fig. 2A and B; SI Appendix, Fig. S4). The KI domain corresponding to amino acids 210–340 in HNRNP-K, was the binding part to SERPINA3 (Fig. 2B).

We previously reported the increased oxidation levels of methionine residues including M137, M141, M284, and M290 in SERPINA3 induced by H_2O_2 treatment and the lack of oxidation levels induced by mutating the methionine to leucine [11]. H_2O_2 increased the nuclear levels of both SERPINA3 and HNRNP-K proteins (Fig. 2C left) and induced the strong association of SERPINA3 with HNRNP-K in HCC cells (Fig. 2C right). In particular, the SERPINA3 variant exhibiting low oxidation levels markedly failed to coimmunoprecipitate with HNRNP-K (Fig. 3A). To determine whether the oxidation-defective SERPINA3 influenced HNRNP-K transcription activity, we compared expression levels of protection of telomeres 1 (POT1), which is the target gene of the transcription factor, HNRNP-K [16], between HCC cells, following overexpression of the SERPINA3 WT and SERPINA3 oxidation-defective variant (Fig. 3B left). The promoter activity and transcription level of POT1 was reduced by the SERPINA3 oxidation variant (Fig. 3B left). The promoter activity and transcription level of PI3Kδ, which was changed by SERPINA3 expression in regard to transcription level [11], was also decreased by the oxidation variant (Fig. 3B middle left). Based on data showing that SERPINA3 only influenced the expressions of *UHRF1* and *HIST2H2BE* (SI Appendix, Figs. S5 and S6) among the 19 genes selected in Fig. 1 and SI Appendix, Figs. S2–S3, we also tested the effect of the SERPINA3 oxidation defect on *UHRF1* and *HIST2H2BE* expressions. SERPINA3 oxidation variant decreased promoter activity and transcription level of *UHRF1* and *HIST2H2BE* (Fig. 3B middle right and right). Accordingly, oxidation of SERPINA3 is responsible for the POT1, UHRF1, HIST2H2BE, and PI3Kδ transcription levels in HCC cells.

We next determined that the transcriptional repression effect by the SERPINA3 oxidation defect involved a blockade of HNRNP-K activity. To examine the effect of this defect by the removal of endogenous activity, we established HCC cell lines stably expressing the SERPINA3

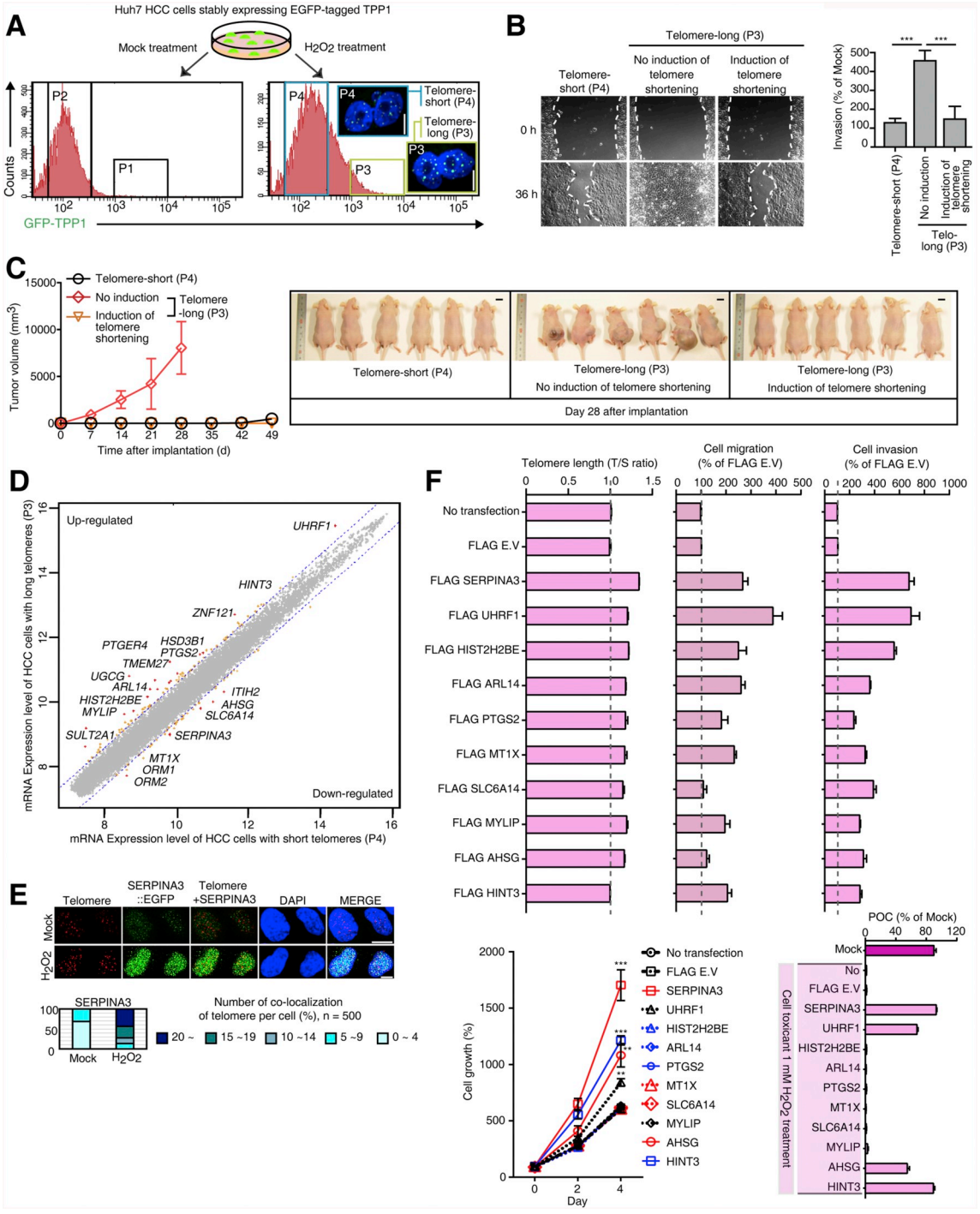

Fig. 1. SERPINA3 induces telomere elongation, cell migration and invasion, cell proliferation, and resistance to cell toxicity in HCC cells. (A) Strategy for sorting Huh7 HCC cells according to telomeric intensity. The telomere marker TPP1 and nuclei are evident as green and blue fluorescence, respectively. Scale bars, 10 μm. (B–C) Migration (B left) and invasion assay (B right) and tumor growth of mice (C) of Huh7-cells with short telomeres or long telomeres with or without the induction of telomere shortening by treatment with both 300 μM H_2O_2 and 15 μM of the telomerase inhibitor BIBR 1532. (D) Microarray analysis of gene expression of short telomere-exhibiting cell fractions and long telomere-exhibiting cell fractions in H_2O_2-treated Huh7 HCC cells. (E) Fluorescence images of SERPINA3 protein localization in mock- and H_2O_2-treated Huh7 cells (top) and percentage of co-localization of SERPINA3 with telomeres per cell (bottom). Scale bars, 5 μm. (F) Functional profiling of candidate genes by analyses of telomere length (top left), cell migration (top middle), invasion (top right), cell proliferation (bottom left), and cell survival in Huh7 cells treated with the cell toxicant (1 mM H_2O_2) (bottom right). HCC, hepatocellular carcinoma; H_2O_2, hydrogen peroxide; EGFP, enhanced green fluorescent protein; T/S, the ratio of telomere repeat copy number (T) to single copy number (S); E.V, empty vector; POC, percent of control; Mean ± s.e.m. (n = 3), except for Fig. 1C (Mean ± s.d., $n = 6$). $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, Student's *t*-test. Sterile distilled water (pH 7.0) was used as the negative control (mock treatment). . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

oxidation-defective variant plasmid, which is not degraded by SERPINA3 siRNA, and then administered SERPINA3 siRNA to the stable cell lines. The SERPINA3 deficiency using SERPINA3 siRNA remarkably decreased POT1, UHRF1, HIST2H2BE, and PI3Kδ transcription levels in the HCC cell lines stably expressing SERPINA3 oxidation-defective mutant (Fig. 3C). Similarly, siRNA-mediated HNRNP-K silencing

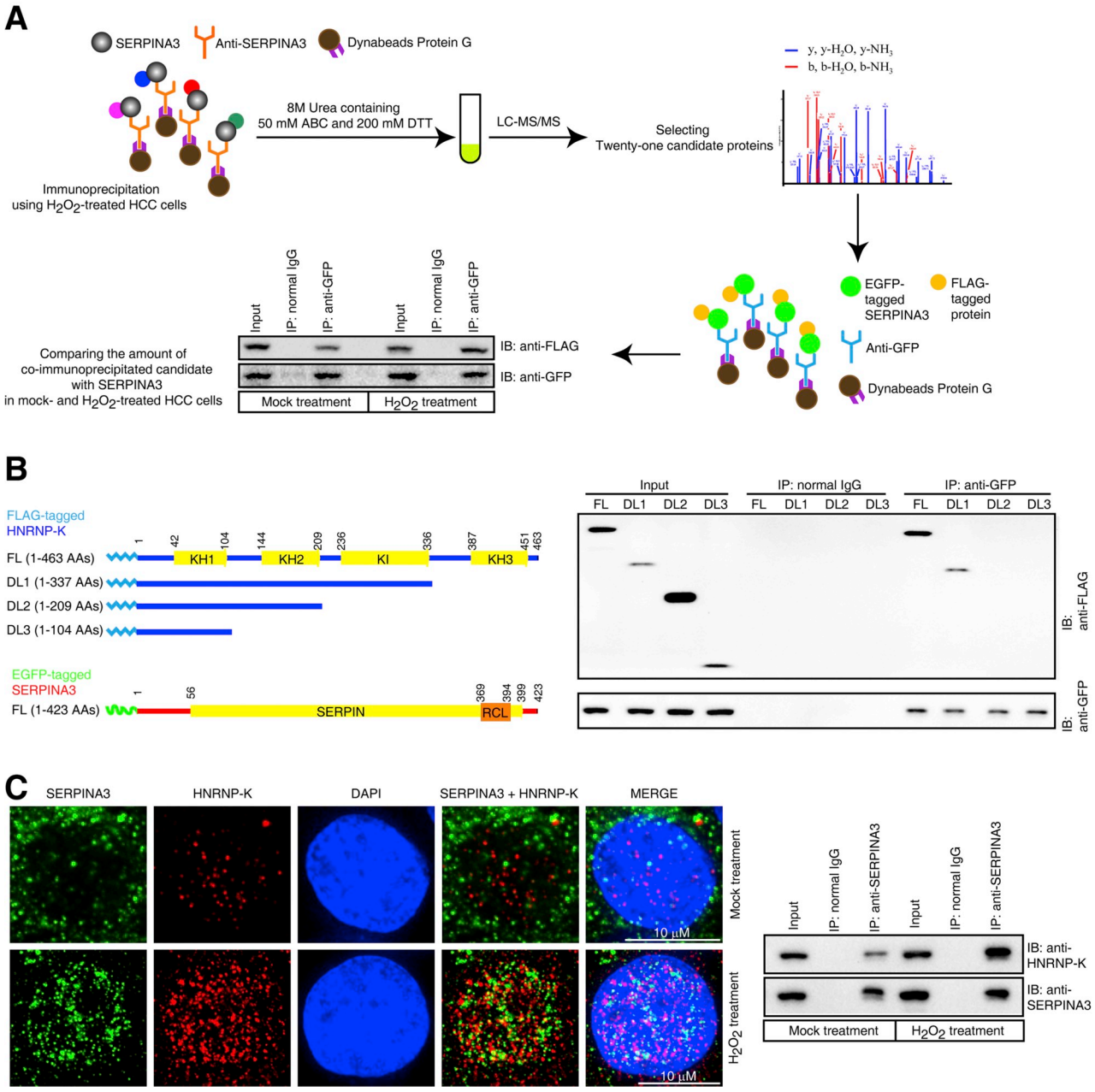

Fig. 2. SERPINA3 interacts with HNRNP-K and H_2O_2 induces increased association between SERPINA3 and HNRNP-K. (A) Experimental overview of the immunoprecipitation and LC-MS/MS analyses for the identification of interacting proteins with SERPINA3 in Huh7 HCC cells. (B) Diagrams of the FLAG-tagged HNRNP-K structure and its deletion mutants and EGFP-tagged SERPINA3 structure (left) and GFP immunoprecipitation followed by immunoblotting analyses using antibodies against FLAG in Huh7 cells expressing full-length FLAG-tagged HNRNP-K or its deletion mutants and full-length EGFP-tagged SERPINA3 (right). (C) Immunofluorescence of endogenous SERPINA3 and HNRNP-K (left) and immunoprecipitation with anti-SERPINA3 antibody followed by immunoblot analyses with anti-HNRNP-K antibody (right) on Huh7-cells with mock or H_2O_2 treatment. Sterile distilled water (pH 7.0) was used as the negative control (mock treatment). EGFP, enhanced green fluorescent protein; FL, full-length; DL, deletion mutant; KH, HNRNP-K-homology; KI, HNRNP-K-interactive; SERPIN, serine protease inhibitors; RCL, Reactive Center Loop; AAs, amino acids; IP, immunoprecipitation; IB, immunoblot; IgG, immunoglobulin G. . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

significantly diminished transcription levels of the above-mentioned genes in the stable cell lines and human primary HCC cells (Figs. 3C and SI Appendix, Fig. S7A). This indicates that SERPINA3 functions in oxidized form to enable the transcriptional activity of HNRNP-K. Overall, these results suggested that decrease in oxidatively-modified SERPINA3 weakens the interaction between SERPINA3 and HNRNP-K and results in transcriptional downregulation of genes that are influenced by the SERPINA3-HNRNP-K complex.

3.3. Action of SERPINA3-HNRNP-K complex in transcriptional activation of POT1, UHRF1, and HIST2H2BE genes

To address the mechanism of action of the oxidized-SERPINA3 in regulation of POT1, UHRF1, and HIST2H2BE transcription, we performed the ChIP assay using HCC cells stably expressing SERPINA3 WT or the oxidation-defective variant. We found that the impaired oxidation of the SERPINA3 protein abolished the binding of SERPINA3 to the regulatory DNA in the *POT1, UHRF1,* and *HIST2H2BE* genes (Fig. 4A). We next determined whether SERPINA3 and HNRNP-K co-occupy the

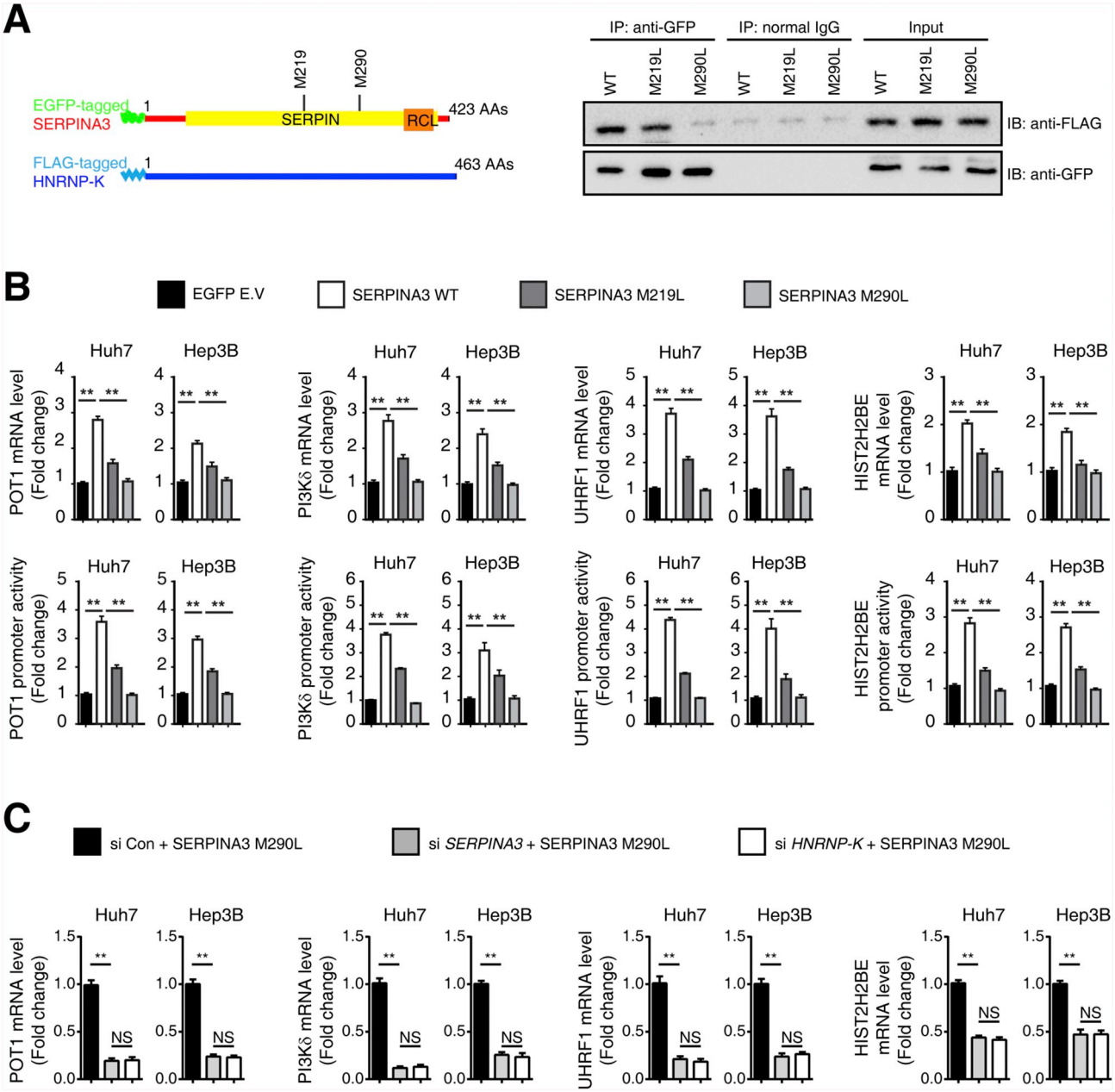

Fig. 3. Lack of interaction between SERPINA3 and HNRNP-K reduces promoter activities and mRNA expression levels of *POT1 PI3Kδ, UHRF1,* and *HIST2H2BE.* (A) A view of the location of the oxidation modified residues M219 and M290 on SERPINA3 protein (left) and GFP immunoprecipitation followed by immunoblotting analyses using antibodies against FLAG in Huh7 cells expressing FLAG-tagged HNRNP-K and EGFP-tagged SERPINA3 WT, M219L, or M290L (right). (B) Transcription levels (top) and promoter activities (bottom) of *POT1, PI3Kδ, UHRF1,* and *HIST2H2BE* in HCC cells, following overexpression of EGFP E.V, SERPINA3 WT, SERPINA3 M219L, or SERPINA3 M290L plasmid. (C) Transcription levels of *POT1, PI3Kδ, UHRF1,* and *HIST2H2BE* in HCC cells stably expressing the SERPINA3 M290L plasmid, which is not degraded by *SERPINA3 siRNA,* followed by administration of siControl (siCon), si*SERPINA3,* and si*HNRNP-K.* EGFP, enhanced green fluorescent protein; IP, immunoprecipitation; IgG, immunoglobulin G; WT, wild-type; E.V, empty vector; siCon, non-targeting siRNA; siRNA, short-interfering RNA; Mean ± s.e.m. (n = 3). $^{**}p < 0.01$, Student's t-test. NS denotes non-significance. . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

same regions as the regulatory DNA in *POT1*, *UHRF1,* and *HIST2H2BE* genes, and found that SERPINA3 formed a complex with HNRNP-K on the regulatory DNA in these genes (Fig. 4B). While H_2O_2 induced a marked increase in the binding of HNRNP-K coimmunoprecipitated with SERPINA3 on these regulatory DNA, the ROS scavenger reagent *N*-acetylcysteine (NAC) abolished the H_2O_2-mediated binding of the SERPINA3-HNRNP-K complex (Fig. 4B). These results suggest that SERPINA3 that was oxidatively modified by H_2O_2 treatment increased the binding of the HNRNP-K-SERPINA3 complex in regulatory DNAs in *POT1*, *UHRF1,* and *HIST2H2BE* genes.

We next determined the effect of the SERPINA3 oxidation defect on the HNRNP-K binding to the regulatory DNA in *POT1*, *UHRF1,* and *HIST2H2BE* genes. ChIP data revealed that SERPINA3 oxidation impairment induced a sharp reduction in HNRNP-K binding to these regulatory DNA (Fig. 4C). This data indicated that the impaired oxidation on SERPINA3 limits the function of HNRNP-K as a transcriptional regulator. Further, we showed that the oxidation-defective SERPINA3 mutant displayed decreased telomere lengths, invasion ability, and cell viability as well as increased Caspase-3/7 activity (Fig. 4D). This is consistent with the observation of siRNA-mediated

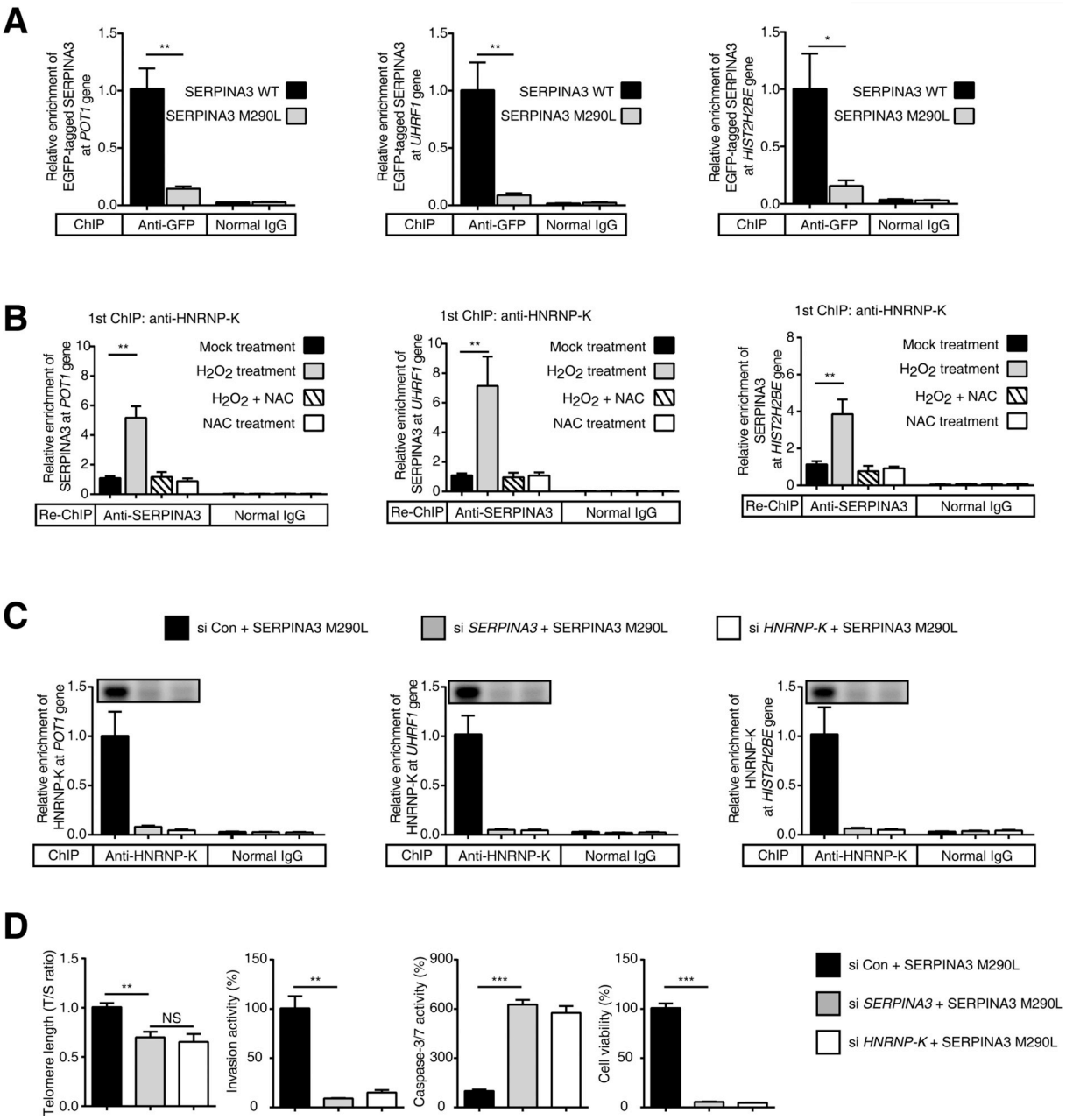

Fig. 4. Oxidatively-modified SERPINA3 promotes transcriptional activity of HNRNP-K through the increasing interaction between SERPINA3 and HNRNP-K (A) Enrichment of EGFP-tagged SERPINA3 at the *POT1 (left), UHRF1 (middle), and HIST2H2BE (right)* gene regulatory DNAs in Huh7 cells expressing stably EGFP-tagged SERPINA3 WT or SERPINA3 M290L. (B) Enrichment of SERPINA3 using anti-SERPINA3 antibody after the first ChIP assay using anti-HNRNP-K antibody at the *POT1 (left), UHRF1 (middle), and HIST2H2BE (right)* gene regulatory DNAs in Huh7 cells with and without H_2O_2 treatment. Sterile distilled water (pH 7.0) was used as the negative control (mock treatment). (C) Enrichment of HNRNP-K at the *POT1 (left), UHRF1 (middle), and HIST2H2BE (right)* gene regulatory DNA in Huh7 cells stably expressing the SERPINA3 M290L plasmid not degraded by si*SERPINA3* followed by administration of siControl (siCon), si*SERPINA3,* and si*HNRNP-K.* Gel images show the amount of HNRNP-K in the indicated gene regulatory DNA. (D) Telomere length, invasion activity, caspase-3/7 activity, and cell viability of Huh7 cells stably expressing the SERPINA3 M290L plasmid not degraded by si*SERPINA3* followed by administration of siControl (siCon), si*SERPINA3,* and si*HNRNP-K.* siCon, non-targeting siRNA; siRNA, short-interfering RNA; WT, wild-type; ChIP, chromatin-immunoprecipitation; IgG, immunoglobulin G; H_2O_2, hydrogen peroxide; NAC, *N*-acetylcysteine; Mean ± s.e.m. (n = 3). $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, Student's t-test. NS denotes non-significance.

HNRNP-K silencing in human primary HCC cells (SI Appendix, Fig. S7B). Collectively, these results suggest that oxidatively-modified SERPINA3 interacts with HNRNP-K, which results in the up-regulation of *POT1*, *UHRF1,* and *HIST2H2BE* promoter activities and their transcription, thereby promoting HCC cell telomere elongation, invasion, proliferation, and survival.

3.4. SERPINA3-HNRNP-K complex as the target in the treatment of liver tumor

The SERPINA3 oxidation-defective mutation interfered with HNRNP-K binding to SERPINA3 and then inhibited HNRNP-K transcriptional activity (Figs. 3 and 4). We administered subcutaneous

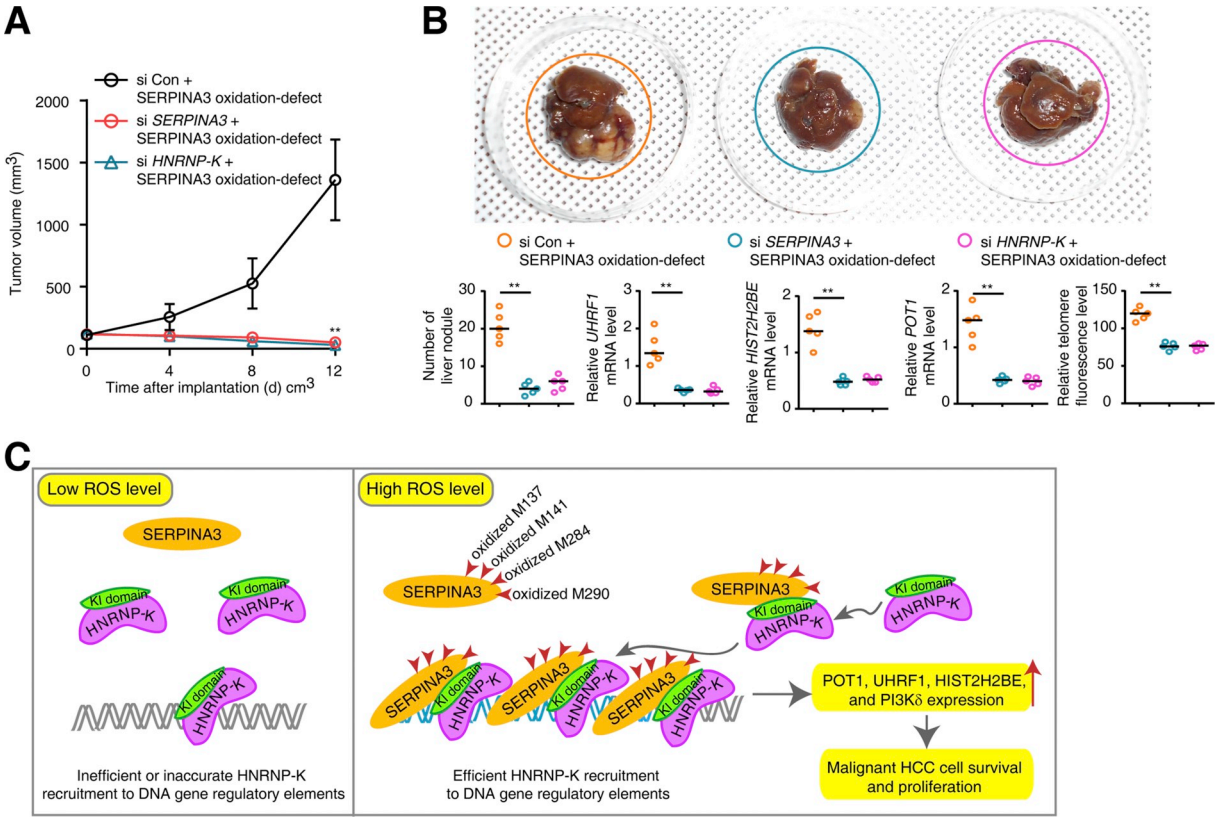

Fig. 5. The blockade of SERPINA3 binding to HNRNP-K inhibits murine tumor growth *in vivo* (A) Subcutaneous tumor volumes from mice that were subcutaneously injected with Huh7 cells stably expressing the SERPINA3 oxidation-defect plasmid not degraded by si*SERPINA3* followed by intra-tumoral injection of si*SERPINA3* and si*HNRNP-K* ($n = 5$). Student's *t*-test; Mean ± s.e.m. (B) Liver tumor growth inhibition in mice after intrahepatic injection of Huh7 cells stably expressing the SERPINA3 oxidation-defect plasmid not degraded by si*SERPINA3* followed by tail injection of si*SERPINA3* and si*HNRNP-K* ($n = 5$). Photographs of liver tumors (top) and median number of tumor nodules (> 4 mm), mRNA levels of *UHRF1*, *HIST2H2BE*, and *POT1*, and telomere fluorescence intensity (bottom). Horizontal bars indicate median values. $^{**}p < 0.01$, Mann–Whitney *U* test. (C) Molecular model illustrating action of oxidized-SERPINA3 complexed with HNRNP-K protein as a transcriptional activator for HCC cell survival and proliferation. siCon, non-targeting siRNA; siRNA, short-interfering RNA; ROS,reactive oxygen species; KI, HNRNP-K-interactive; HCC, hepatocellular carcinoma.

(Fig. 5A) and intrahepatic injections (Fig. 5B) of Huh7 cells stably expressing SERPINA3 oxidation-defect to immunodeficient mice and tested whether the SERPINA3 oxidation-impairment inhibited tumor growth *in vivo*. Notably, we showed that SERPINA3 oxidation-defect decreased growth of subcutaneous (Fig. 5A) and liver tumors (Fig. 5B top). Moreover, the SERPINA3 oxidation-defect reduced the number of liver nodules, the mRNA levels of *UHRF1, HIST2H2BE*, and *POT1*, as well as telomere lengths (Fig. 5B bottom). Similar phenotypes were observed in the case of *HNRNP-K* knock-down through siRNA administration (Fig. 5A and B); supporting data showed that the SERPINA3-HNRNP-K complex acts as a transcriptional regulator (Figs. 3 and 4). Together, our results indicate that oxidative-modification of SERPINA3 is critical for the functioning of the SERPINA3-HNRNP-K complex as a transcriptional regulator in liver tumor progression. Furthermore, the increased association of SERPINA3 with HNRNP-K could promote malignant liver progression by modulating the expression of genes involved in HCC malignancy and telomere maintenance (Fig. 5C).

In human GIII HCC tissues, protein expression levels of SERPINA3, HNRNP-K, UHRF1, HIST2H2BE, and POT1 were higher than those in paired non-tumor tissues (Fig. 6A). SERPINA3 and HNRNP-K proteins were observed to be more distributed in the nuclei of GIII HCC tissues (Fig. 6A), supporting the transcriptional regulator activity of the SERPINA3-HNRNP-K complex (Figs. 3–5). We next analyzed the correlation between the levels of the oxidized-SERPINA3 protein and the amount of coimmunoprecipitated HNRNP-K with SERPINA3 in GII and GIII HCC tissues (Fig. 6B and C). Linear regression analysis revealed a significant positive correlation between the oxidized-SERPINA3 levels and the SERPINA3-HNRNP-K complex levels (Pearson correlation coefficient: R = 0.8457; 95% confidence interval (CI) = 0.7864 to 0.8805; $p < 0.0001$, Fig. 6C). The high amount of coimmunoprecipitated HNRNP-K with SERPINA3 was significantly associated with the high mRNA levels of *UHRF1*, *HIST2H2BE*, *POT1*, and longer telomeres (Fig. 6D). Moreover, HCC patients with a high level of the SERPINA3-HNRNP-K complex showed significantly frequent HCC recurrence compared to those with low level of the complex (Hazard ratio = 10.36, 95% CI = 3.289 to 32.64, $p < 0.0001$; Fig. 6E). This result suggests that the strong interaction between SERPINA3 and HNRNP-K is a poor prognostic indicator of HCC. Next, we checked whether the upregulation of genes activated by recruitment of HNRNP-K to the gene loci, of which SERPINA3 binding to HNRNP-K can increase its efficiency, is related to HCC patient's survival using The Cancer Genome Atlas (TCGA) data. In agreement to our results, patients with high UHRF1 expression showed poor survival (Fig. S8).

4. Discussion

Oxidative modification induced by ROS alters the stability and function of proteins [17,18]. ROS triggers several signal transduction pathways through the oxidation modification of protein kinases and tyrosine phosphatases, including protein kinase C and phosphatase and tensin homolog deleted on chromosome 10 (PTEN) in tumorigenesis [19–21]. ROS also incrementally affects nuclear localization and the affinity of regulatory DNA-interaction of transcription factors, such as nuclear factor kappa B, p53, activating protein-1, and protein-disulfide

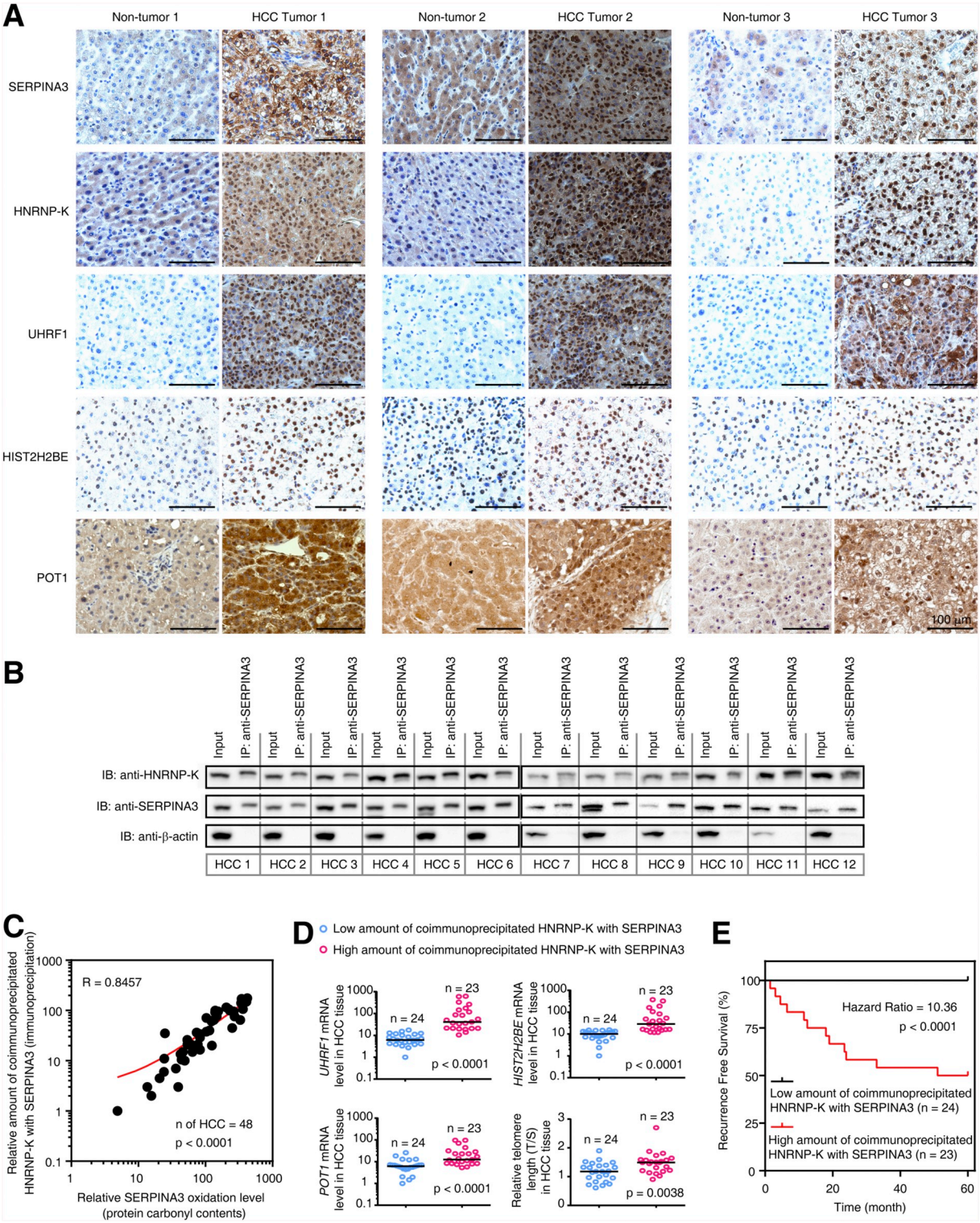

Fig. 6. High-amount of coimmunoprecipitated HNRNP-K with SERPINA3 is associated with high levels of UHRF1, HIST2H2BE, and POT1 expressions as well as with high HCC recurrence in human HCC tissues. (A) Representative immunohistochemical staining of SERPINA3, HNRNP-K, UHRF1, HIST2H2BE, and POT1 in GIII HCC tumor samples and non-tumor samples. (B, C) Immunoprecipitation (IP) using anti-SERPINA3 antibodies followed by immunoblotting analyses using antibodies against HNRNP-K in GII and GIII HCC tissue lysates (B), and correlation analysis between the oxidized-SERPINA3 and the amount of coimmunoprecipitated HNRNP-K with SERPINA3 determined by linear regression (C). n = 48 (D, E) Median mRNA levels of *UHRF1*, *HIST2H2BE*, and *POT1*, and median telomere length (D) and recurrence-free survival rates (E) in GII and GIII HCC tissues with low- and high-amount of coimmunoprecipitated HNRNP-K with SERPINA3 (n = 47). Horizontal bars indicate median values. IP, immunoprecipitation; IB, immunoblot; GII, Grade-II; GIII, Grade-III; R, Pearson correlation coefficient; HCC, hepatocellular carcinoma.

isomerase-associated 3 in tumor cell proliferation and survival [12,22,23]. In addition, ROS influences the DNA damage response and repair systems through the phosphorylation of ataxia-telangiectasia mutated protein kinase, p53, and checkpoint kinase 2 [24]. These observations indicate the effects of ROS-mediated oxidative modification on the regulation of protein activity in multiple cellular processes. In this study, we demonstrated that oxidative modification of SERPINA3 accentuates the role of the SERPINA3-HNRNP-K complex on target gene regulatory DNA.

Serine protease inhibitors (SERPIN, also known as serine-type endopeptidase inhibitors) are a functionally diverse family of proteins with highly evolutionarily conserved structure from prokaryotes to placental mammals [25]. Encoded by a member of the SERPIN gene family, SERPINA3 has been known to be increased in plasma during proteolysis induced by infection, surgery, and tissue injury based on several reports dissecting the protein interactome in which SERPINA3 participates [26–30]. In some prokaryotes, reduction–oxidation reactions stabilize SERPIN protein but do not influence its protease inhibitory mechanism, suggesting another role of SERPINA3 than as a serine protease inhibitor [31]. Our current findings demonstrate the role of SERPINA3 as a novel transcriptional regulator of HCC progression, irrespective of the conventional role of SERPINA3 as a protease inhibitor.

Telomeres often form structures, such as T-loop, to protect it from DNA damage and to regulate telomere length [32]. One of the shelterin proteins, POT1 is an evolutionarily conserved telomere-binding protein that is essential in T-loop structure and telomere maintenance [33]. POT1 reportedly modulates telomere length by binding to a 3′ single-strand overhang telomeric DNA [34,35]. Importantly, its deficiency causes telomere loss and damage, genomic instability, and cell death [32]. Similarly, tumor cells overexpressing POT1 display telomere elongation, while inhibition of POT1 decreases telomere length [34,35]. Several papers have reported the positive association of POT1 expression and tumorigenesis in human cancers [36,37]. Here, we showed that *POT1* transcription elicited by the SERPINA3-HNRNP-K complex induced telomere elongation. We also showed that the SERPINA3-HNRNP-K complex increased *UHRF1* and *HIST2H2BE* transcriptional levels. UHRF1 functions as a regulator of DNA methylation and its overexpression drives HCC progression [38]. HIST2H2BE is a replication-independent histone variant [39] and to our knowledge, until now, there has been no functional study on HCC-associated HIST2H2BE. Our data and prior data from the TCGA [40] and GENT [41] databases show that HCC tumors have high mRNA levels of *POT1*, *UHRF1*, and *HIST2H2BE* compared with non-tumors. Moreover, we observed enhanced cell growth, EMT-promoting gene expression, migration, and invasion through UHRF1 and HIST2H2BE. Collectively, these results suggest that HNRNP-K increases *POT1*, *UHRF1*, and *HIST2H2BE* transcription via the ROS-mediated interaction between SERPINA3 and HNRNP-K in malignant HCC. *SERPINA3* mRNA levels were reported to be lower in HCC tumors compared with non-tumors [42]. We also observed lower *SERPINA3* mRNA levels in HCC cells with long telomeres relative to those with short telomeres. Notably, HCC tumors and HCC cells with long telomeres displayed higher SERPINA3 protein levels, suggesting that SERPINA3 activity is determined by the level of SERPINA3 protein, rather than by the SERPINA3 transcription level. These results suggest that SERPINA3 protein oxidation induced by ROS contributes to liver malignancy.

In conclusion, we screened for genes that were essential for telomere maintenance, cell migration and invasion, and cell proliferation in HCC, and identified the novel function of the SERPINA3-HNRNP-K complex as the elevated ROS-promoted transcriptional regulator. Thus, the inhibition of interaction between SERPINA3 and HNRNP-K possibly via the use of a non-toxic reductant that prevent the oxidation of SERPINA3 or a specific inhibitor of SERPINA3-HNRNP-K interaction could block HCC survival and proliferation.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2019.101217.

Conflicts of interest

The authors declare no conflict of interest.

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