PNO1 promotes cell proliferation in prostate cancer

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Abstract

Background Prostate cancer (PCa) is one of the most commonly diagnosed cancers. The functions of PNO1 in yeasts were involved in regulating ribosome and proteasome biogenesis. However, its roles in PCa remained largely unclear. Methods The present study evaluated the expression levels of PNO1 in PCa by using GSE45016, GSE55945 and GSE17951 datasets. Then, in vivo and in vitro assays were conducted to detect the biological functions of PNO1 in PCa. BALB/c mice were used for in vivo assay in this study. Microarray and bioinformatic analysis were carried out to detect the downstream targets and pathways regulated by PNO1. Results The present study for the first time demonstrated PNO1 was up-regulated in PCa samples compared to normal tissues. ShRNA mediated knockdown of PNO1 significantly suppressed PCa proliferation and clone formation, however, induced PCa apoptosis. Microarray analysis and bioinformatics analysis revealed PNO1 was involved in regulating multiple cancer related biological processes, such as regulation of DNA repair, single organismal cell-cell adhesion, translational initiation, RNA splicing, transcription, and positive regulation of mRNA catabolic process. OF note, in vivo results showed PNO1 knockdown remarkably reduced the PCa growth rate. Conclusions Despite more in-depth research is still required, this study showed PNO1 could serve as a potential biomarker for PCa.

Background

Prostate cancer (PCa) is one of the most commonly diagnosed cancers and caused about 9% of cancer-related deaths in males in US [1]. PCa is a highly heterogeneous cancer. Although there has been a series of studies showed Androgen Receptor signaling [2], PI3K/mTOR signaling [3, 4] and TGF-β signaling [5, 6] played crucial roles in PCa progression, the detail mechanisms underlying PCa tumorigenesis had not been fully understood. Identifying novel PCa progression regulators are critical to improve the understanding of the biological progression of PCa and to provide novel biomarkers for PCa.

PNO1 localized on chromosome 2p14, which contained seven exons [7]. The functions of PNO1 in yeasts were mainly involved in regulating ribosome and proteasome biogenesis [8]. However, the functions of PNO1 in mammalian cells remained unclear. knockout of PNO1 in mice caused early lethality by stopping embryo development before compaction stage [8]. Human PNO1 is crucial to the site 3 cleavage at the 3′-end of 18S pre-rRNA [9]. Zhou et al. found PNO1 was expressed in liver, lung, spleen and kidney, but not in heart, brain, skeletal muscle, placenta, pancreas, and prostate [7]. NOB1 was identified as a PNO1 binding partner in both yeasts and human cells [10]. Previous studies showed NOB1 played as an oncogenetic gene, whose expression was found to up-regulated in multiple cancer types, including laryngeal cancer[11], and ovarian cancer[12]. Silencing of NOB1 suppressed laryngeal cancer [11] and oral cancer proliferation[13]. These results suggested the potential functions roles of PNO1 in human cancers. However, the function of PNO1 in prostate cancer has not been studied in vitro and in vivo so far.

So far, the expression levels and molecular functions of PNO1 in PCa remained unknown. This study for the first time showed PNO1 played as a tumor promotor in PCa. Our results showed PNO1 may be a

potential therapeutic and diagnostic marker for PCa.

Methods

Cell culture.

The human PCa cell lines, DU145 and PC-3, were purchased from the American Type Culture Collection (Manassas, VA, USA). All cells were cultured in RPMI-1640 medium (HyClone Laboratories; USA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc. USA) at 37°C in a humidified atmosphere with 5% CO2.

Lentiviral constructs and transfections.

The PNO1 shRNA sequences were got from GeneChem Co., Ltd. (Shanghai, China). Recombinant lentiviral vectors carrying PNO1 shRNA were constructed in accordance to manufacturer's instruction [14]. PNO1 shRNA-1 was CCGGCCCATGATTGACCAGTCAAATTTCAAGAGAATTTGACTGGTCAATCATGGGTTTTTG.

RT-qPCR.

Total RNA was extracted from PCa cells using TRIzol reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Reverse transcription was performed using the RevertAid First Strand cDNA Synthesis kit (Promega Corporation, USA), according to the manufacturer's protocol. RT-qPCR was performed using SYBR Master mix (Takara Biotechnology Co., Ltd., Dalian). The thermal cycle included: 95°C for 15 sec, 45 cycles of 95°C for 5 sec, and 60°C for 30 sec. The following primers were used for qPCR: CEBPA-F, CCAGAAAGCTAGGTCGTGGGT and -R, TGGACTGATCGTGCTTCGTGT; GAPDH-F, ATGGTCAGGTTATACTCCTCCTC and -R, CACATGCCACTTGATACAATCC. The 2-ΔΔCt method was conducted to calculate the relative expression levels of targets.

Celigo analysis

Celigo® Image Cytometer (Nexcelom, Lawrence, MA, USA) was used to evaluate the number of cells by scanning green fluorescence daily for 5 days at room temperature according to our previous reports [15].

CCK-8 assay

CCK-8 assay was used to analyze the effect of PNO1 knockdown on cell proliferation according to our previous reports [15].

FACS analysis

Fluorescence-activated cell sorting (FACS) was used to analyze cell apoptosis according to our previous reports [15].

In vivo tumorigenicity

shPNO1-transfected or ctrol PC-3 cells were suspended in phosphate-buffered saline (PBS) and injected subcutaneously into the backs of six-week-old male BALB/c nude mice (Shanghai SLAC Laboratory Animals Co., Ltd. Shanghai, China). Animals were divided into 2 groups（NC and KD group individually）. Tumor growth was measured using calipers over the course of 49 days. Tumor volume was calculated according to the formula: volume = 0.5 × length × width2. The mice were euthanized with CO2 and sacrificed on day 49. Tumor weight was measured and compared between two groups. All in vivo studies protocols were approved by the Shanghai Medical Experimental Animal Care Commission (Approval ID: ShCI-14-008).

Western blot analysis

In accordance with standard Western-blot protocols, proteins were separated in 10% SDS-PAGE and transferred to PVDF membrane though Bio-Rad systems (Bio-Rad, Hercules CA, USA). Rabbit anti-PNO1, and mouse anti-GAPDH antibodies were used in this study. The Quantity One software package (Bio-Rad, USA) was used for the quantitation of signal intensities.

Statistical analysis

The SPSS 19.0 software (IBM Corp, Armonk, NY, USA) was used to perform statistical analysis. Each experiment was performed for three times. Differences between 2 groups was calculated using student' T-test. For more groups, one-way ANOVA followed by Newman–Keuls posthoc test was used. $P<0.05$ was considered to indicate a statistically significant difference.

Results

PNO1 was overexpressed in PCa samples.

Three independent GEO datasets, GSE45016 [16], GSE55945 [17] and GSE17951 [18], were used to determine the expression levels of PNO1 in PCa samples. Our results revealed PNO1 was markedly up-regulated in PCa compared to normal tissues. Moreover, we detected FAM64A expression in PCa cell lines, including LNCaP, PC-3 and DU145 cells, and found FAM64A was highly expressed in metastatic cell lines PC-3 and DU145 than that in LNCaP.

Silencing of PNO1 suppressed PCa cell proliferation

In present study, we used lentiviral vectors mediated knockdown to suppress PNO1 expression in PC-3 and DU145 cells. By using RT- qPCR, we found that 80% and 65% PNO1 endogenous expression was knockdown in DU145 and PC-3 cells, respectively. western blot assay also showed the protein levels of PNO1 in DU145 and PC-3 cells were also successfully knockdown using a special shRNA.

Celigo analysis was used to detect the effect of PNO1 knockdown on PCa proliferation. Five days' post transfection, we found the cell numbers in PNO1 knockdown groups decreased by 88.3% and 65% compared to control group in both DU145 and PC-3 cells. Similar with Celigo analysis results, the CCK-8 assay also demonstrated that PNO1 knockdown suppressed proliferation compared to control ($P<0.01$; Fig. 3D and E).

In addition, we found that knockdown of PNO1 suppressed DU145 and PC-3 cell colony formation. The relative colony number in PNO1 knockdown decreased by 85- and 78- percent in DU145 and PC-3 cells, respectively.

PNO1 knockdown induced apoptosis in PCa cells.

To investigate the effect of PNO1 knockdown on PCa cell apoptosis, cells transfected with shPNO1 and shCtrl were subjected to FACS analysis. We revealed the apoptosis of PC-3 and DU145 cells was significantly increased after PNO1 knockdown compared with control groups. These results suggested that PNO1 suppressed PCa cell apoptosis.

Bioinformatics analysis revealed the targets regualted by PNO1 in PCa.

Microarray analysis was conducted to identify PNO1 targets in PCa. Totally, 291 genes were found to be up-regulated and 498 genes were found to be suppressed after PNO1 knockdown in PC-3 cells. The top 10 up- and down-regulated genes were shown in Table1. Bioinformatics analysis revealed PNO1 induced genes was involved in regulating positive regulation of DNA repair, single organismal cell-cell adhesion, cellular amino acid metabolic process, response to stress, preassembly of GPI anchor in ER membrane, membrane to membrane docking, regulation of angiogenesis, viral process, post-translational protein modification, and response to oxidative stress. Meanwhile, the PNO1 reduced genes were involved in regulating translational initiation, RNA splicing, transcription, DNA-templated, positive regulation of mRNA catabolic process, regulation of energy homeostasis, cellular response to hypoxia, rRNA processing, mRNA processing, energy reserve metabolic process, and response to unfolded protein.

Identification of key targets of PNO1 in PCa using PPI network analysis

Furthermore, PPI networks were constructed to reveal the protein-protein interaction among PNO1 induced and reduced genes using String database. A Mcode plugin in Cytoscape was used to identify hub genes in these networks. As shown in Figure 6, we showed the top 3 PNO1 up-regaulted and down-regualted genes mediated hub networks.

Five PNO1 down-regaulted genes (PRPF8, CDC5L, RPL36, RPL23, RPL28) and 10 PNO1 up-regaulted genes (TNF, EGFR, RNF213, CLTCL1, AP2B1, CXCL1, KLHL5, UBE2J1, CXCL8, PLAU) were identified as key targets of PNO1 in PCa. These genes interacted with more than 10 nodes in PPI network.

Knockdown of PNO1 suppressed prostate cancer growth in vivo

We further conducted in vivo tumor growth assay to determine the effect of PNO1 on tumor growth. A luciferase-expressing DU145 cell line transfected with shPNO1 or shNC were constructed to race tumors in live animals. In vivo tumor growth were detected using caliper measurements. The tumor growth curve analysis showed the PNO1 knockdown tumor xenografts had an obvious reduction of tumor volume relative to that in control groups. On day 41, the luciferase expression in all mice were detected and the results showed the luciferase levels in shPNO1 group was down-regulated compared to normal group. Then mice were sacrificed and the tumor xenografts were excised and weighed according to the manufacturer's instruction. It was observed that the weight of tumor xenografts in shPNO1 group was significantly lower than that of shNC group (Figure 7C, $P<0.05$).

Discussion

The functions of PNO1 in human cancers remained unclear. Previous studies showed PNO1 was involved in regulating ribosome and proteasome biogenesis [8]. NOB1, a PNO1 cofactor, was overexpressed in multiple cancer types, including laryngeal cancer [11], and ovarian cancer [19]. These reports suggested PNO1 may be also involved in regulating progression of human cancers. In present study, we for the first time demonstrated PNO1 was up-regulated in PCa samples compared to normal tissues. ShRNA mediated knockdown of PNO1 significantly suppressed PCa proliferation and clone formation. Moreover, our results showed PNO1 knockdown induced PCa apoptosis. OF note, in vivo results showed PNO1 knockdown remarkably reduced the PCa growth rate. Until to now, this is the first study revealed PNO1 played as an oncogene in PCa.

The detail mechanism of PNO1 underlying PCa growth remained to be further investigated. With the development of high-through methods, microarray and RNA-sequence were widely used to identify functions and molecular mechanism of novel genes in human cancers. For example, Yao Li et al conducted microarray to reveal downstream targets of Androgen Receptor (AR) [20]. Sunkel et al identified CREB1/FoxA1 transcriptional targets using ChIP-seq and RNA-seq method[21]. In this study, we identified 291 up-regulated and 498 down-regulated targets of PNO1 in PCa. Bioinformatics analysis showed PNO1 was involved in regulating multiple cancer related pathways, such as DNA repair,

regulation of angiogenesis, translational initiation, RNA splicing, and cellular response to hypoxia. Emerging studies showed these pathways played crucial roles in PCa progression and treatment. Defective DNA repair induced tumour evolution and progression. DNA repair pathway was widely observed to be mutated in primary and advanced-stage PCa[22]. Angiogenesis was considered one of the hallmarks of tumor initiation, growth and development. Emerging studies had demonstrated angiogenesis also plays a fundamental role for PCa growth [23]. Changes in mRNA splicing patterns have been associated with key pathological mechanisms in prostate cancer progression[24].

A few regulators were identified as key targets of PNO1, including PRPF8, CDC5L, RPL36, RPL23, RPL28, TNF, EGFR, RNF213, CLTCL1, AP2B1, CXCL1, KLHL5, UBE2J1, CXCL8, and PLAU. PRPF8 was a spliceosomal protein [25]. RPL36, RPL23, and RPL28 were ribosomal proteins, which were involved in regulating protein synthesis. TNF was involved in regulating cancer apoptosis though multiple kinases. Including ASK1 and MEK4 [26]. EGFR played crucial roles in regulating PCa proliferation and apoptosis [27, 28]. Targeting EGFR was considered as a potential therapeutic target. CXCL1 was increased in high-grade PCa. In PCa, CXCL1 was reported to induce cell growth and metastasis though activating a secretory network [29]. KLHL5 knockdown was able to increase the sensitivity of cancer cells to anticancer drugs[30]. These reports and our finding showed PNO1 played key roles in PCa though regulating these important cancer regulators.

In conclusion, this study for the first time showed PNO1 was up-regualted in PCa. PNO1 played its oncogenetic role in PCa progression though inducing cell proliferation and reducing cell apoptosis. Despite more in-depth research is still required, this study showed PNO1 could serve as a potential biomarker for PCa.

Abbreviations

PCa, prostate cancer

shRNA, Short hairpin RNA

PNO1, Partner Of NOB1 Homolog

FACS, Fluorescence-activated cell sorting

CCK-8, cell counting kit-8.

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Declarations

Acknowledgements

None.

Authors' contributions

FC conceived the experiments and drafted the manuscript. JH, ZX, JT conducted the experiments. ZW helped to analyze and interpret the data. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

This study was approved by the ethics committee of the people's Hospital Affiliated to Jiangsu University. (NO. K-20190083-Y)

Consent for publication

Not applicable.

Competing Interests

The authors declare that they have no conflict of interest.

Figures

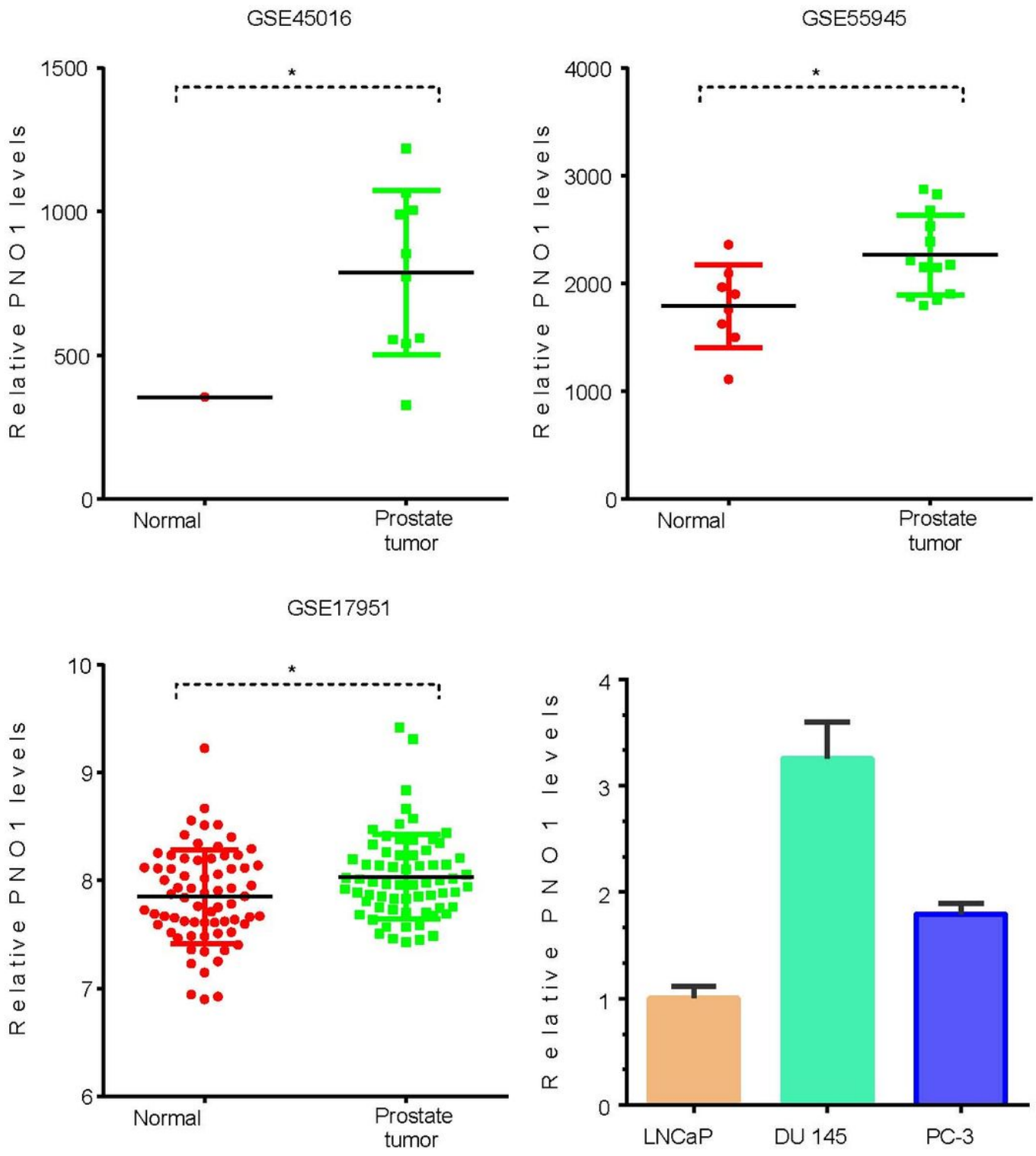

Figure 1

PNO1 was up-regulated in prostate cancer. (A-C) PNO1 was significantly overexpressed in PCa compared to normal samples using GSE45016 (A), GSE55945 (B), GSE17951 (C). (D) The expression pattern of PNO1 in LNCaP, PC-3 and DU145 cells. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

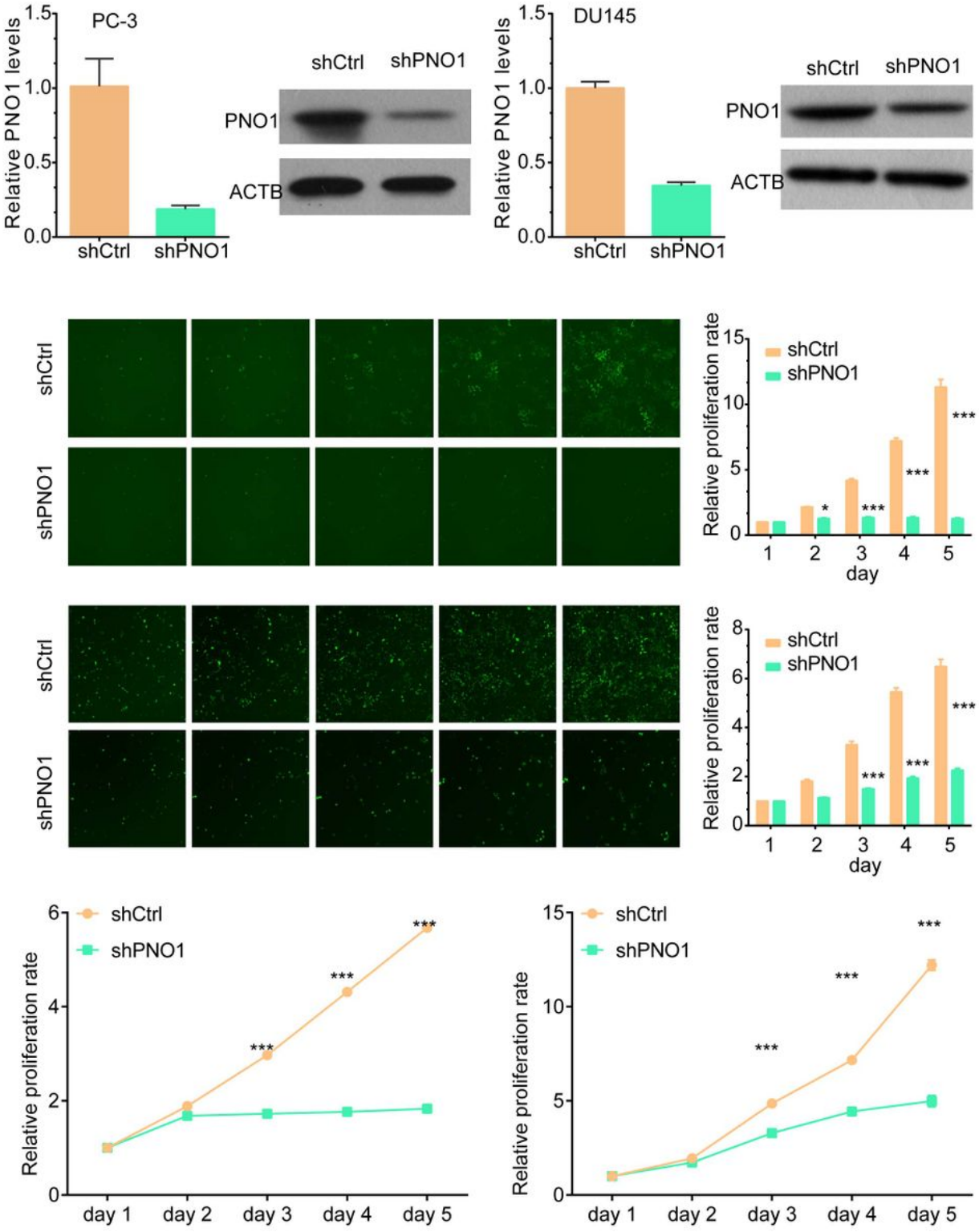

Figure 2

Knockdown of PNO1 suppresses cell proliferation in PC-3 and DU145 cells. (A-B) RT-PCR and western blot assay were used to detect the mRNA (A) and protein (B) levels of PNO1 after transfecting with shRNA or NC in DU145 cells. (C-D) RT-PCR and western blot assay were used to detect the mRNA (C) and protein (D) levels of PNO1 after transfecting with shRNA or NC in DU145 cells. (E-F) The Celigo® system showed knockdown of PNO1 inhibited cell proliferation in PC-3 cells. (G-H) The Celigo® system showed

knockdown of PNO1 inhibited cell proliferation in DU145 cells. (I-J) The CCK-8 assay showed knockdown of PNO1 inhibited cell proliferation in PC-3 and DU145 cells. p < 0.05; **, p < 0.01; ***, p < 0.001).

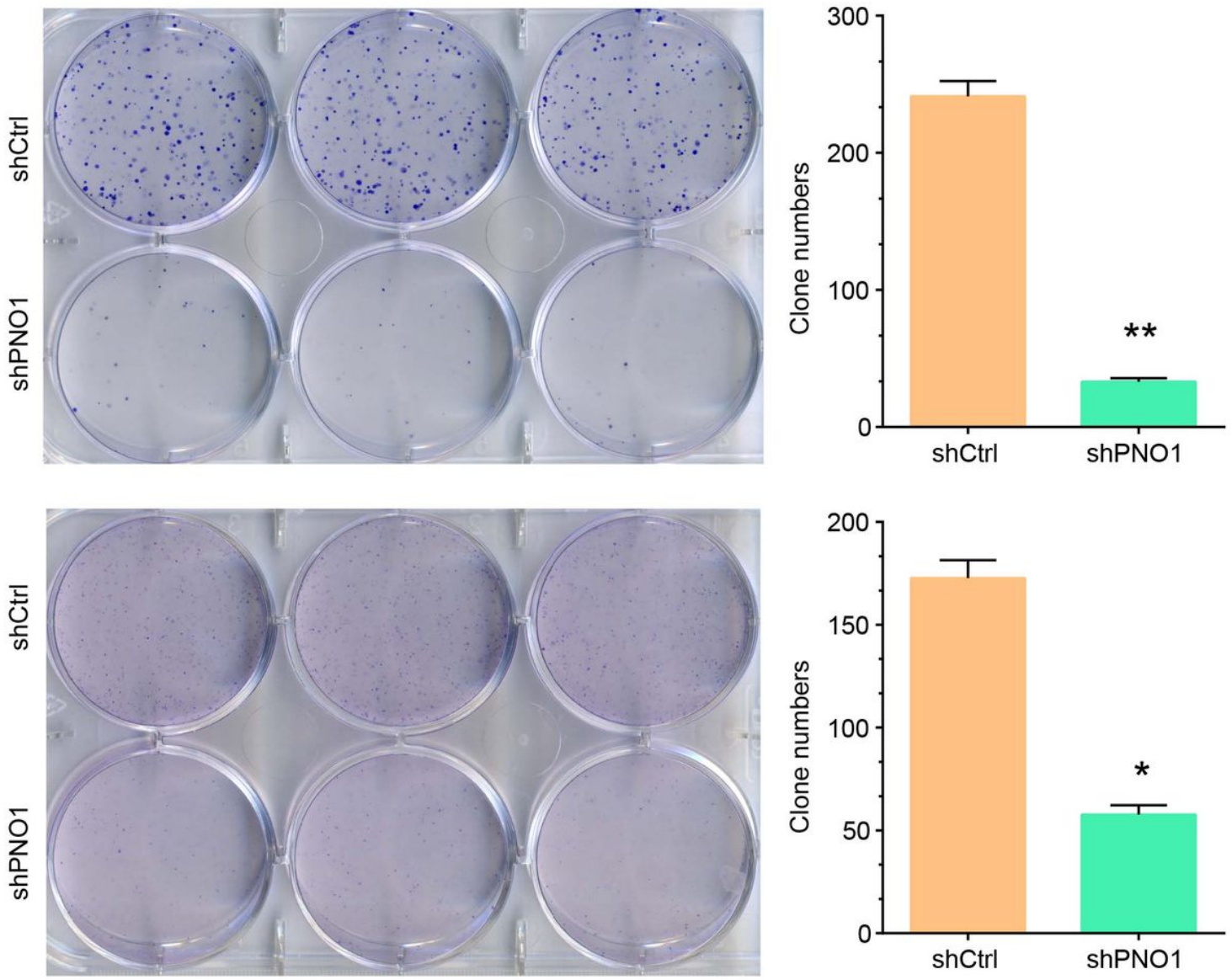

Figure 3

PNO1 knockdown inhibits cell colony formation in PCa cell lines. (A-B) knockdown of PNO1 inhibited cell colony formation in PC-3 cells. (C-D) knockdown of PNO1 inhibited cell colony formation in DU145 cells. The cell apoptosis analysis results presented as mean ± SD (n = 3). Significance was defined as p < 0.05 (*, p < 0.05; **, p < 0.01; ***, p < 0.001).

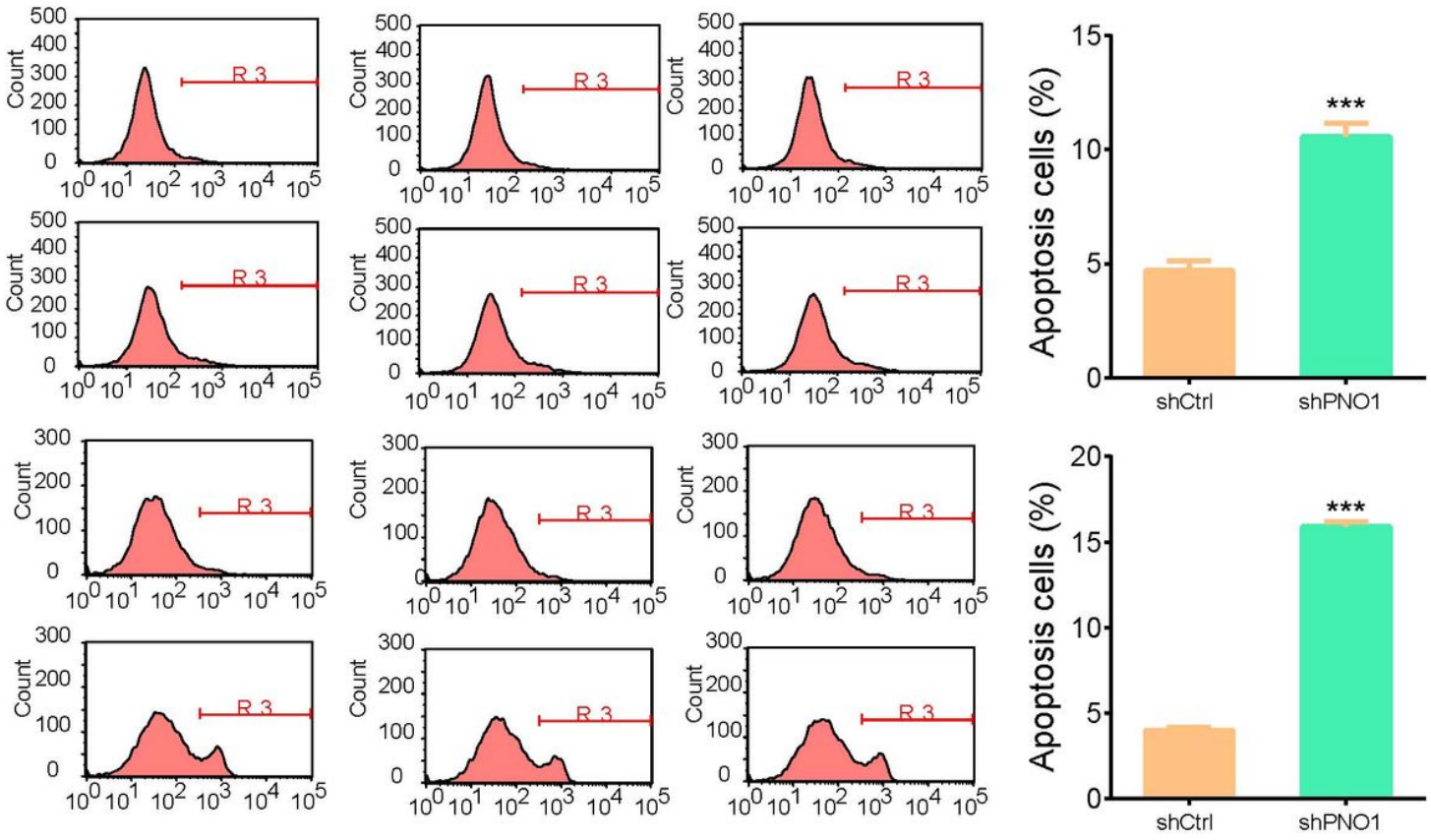

Figure 4

PNO1 knockdown promotes apoptosis of prostate cancer cells. (A-D) Treatment with shPNO1 promotes apoptosis of prostate cancer cells. The cell apoptosis analysis results presented as mean ± SD (n = 3). Significance was defined as p<0.05 (*, p < 0.05; **, p < 0.01; ***, p < 0.001).

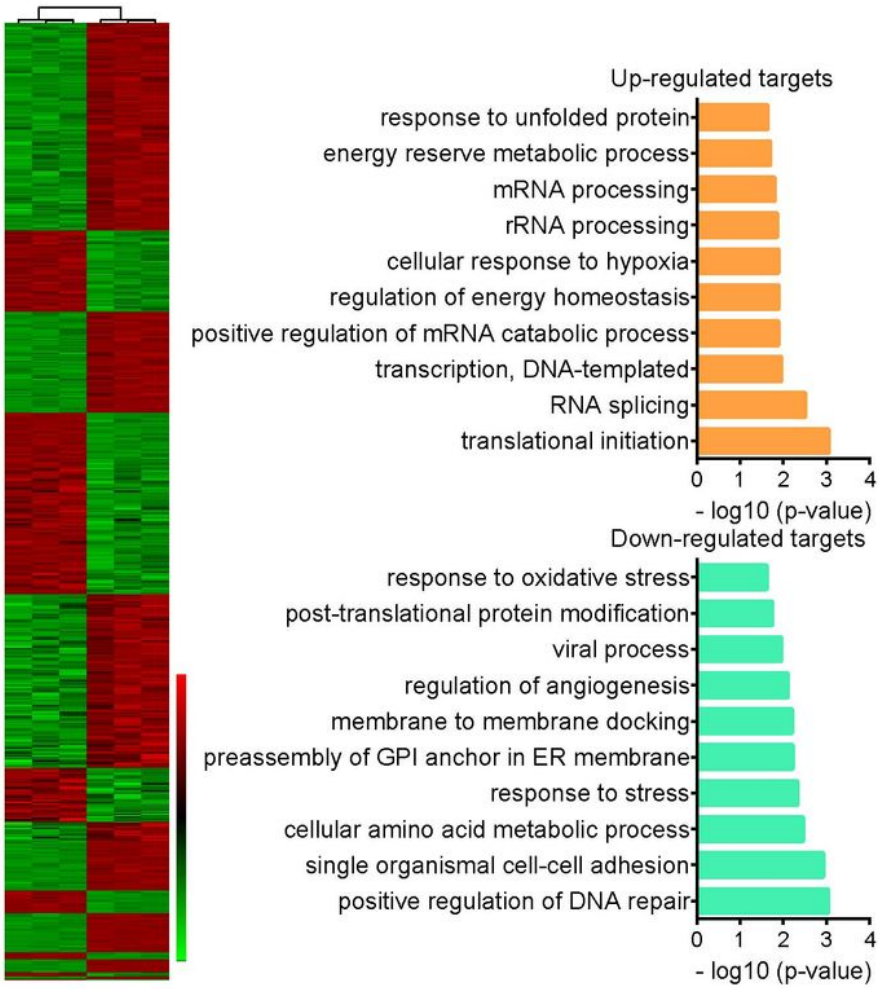

Figure 5

Bioinformatics analysis revealed multiple functional roles of PNO1 in PCa. (A) Heat map shows differential genes expression after PNO1 knockdown. (B-C) GO analysis revealed the potential pathways regulated by PNO1 up-regulated genes and down-regulated genes in PCa.

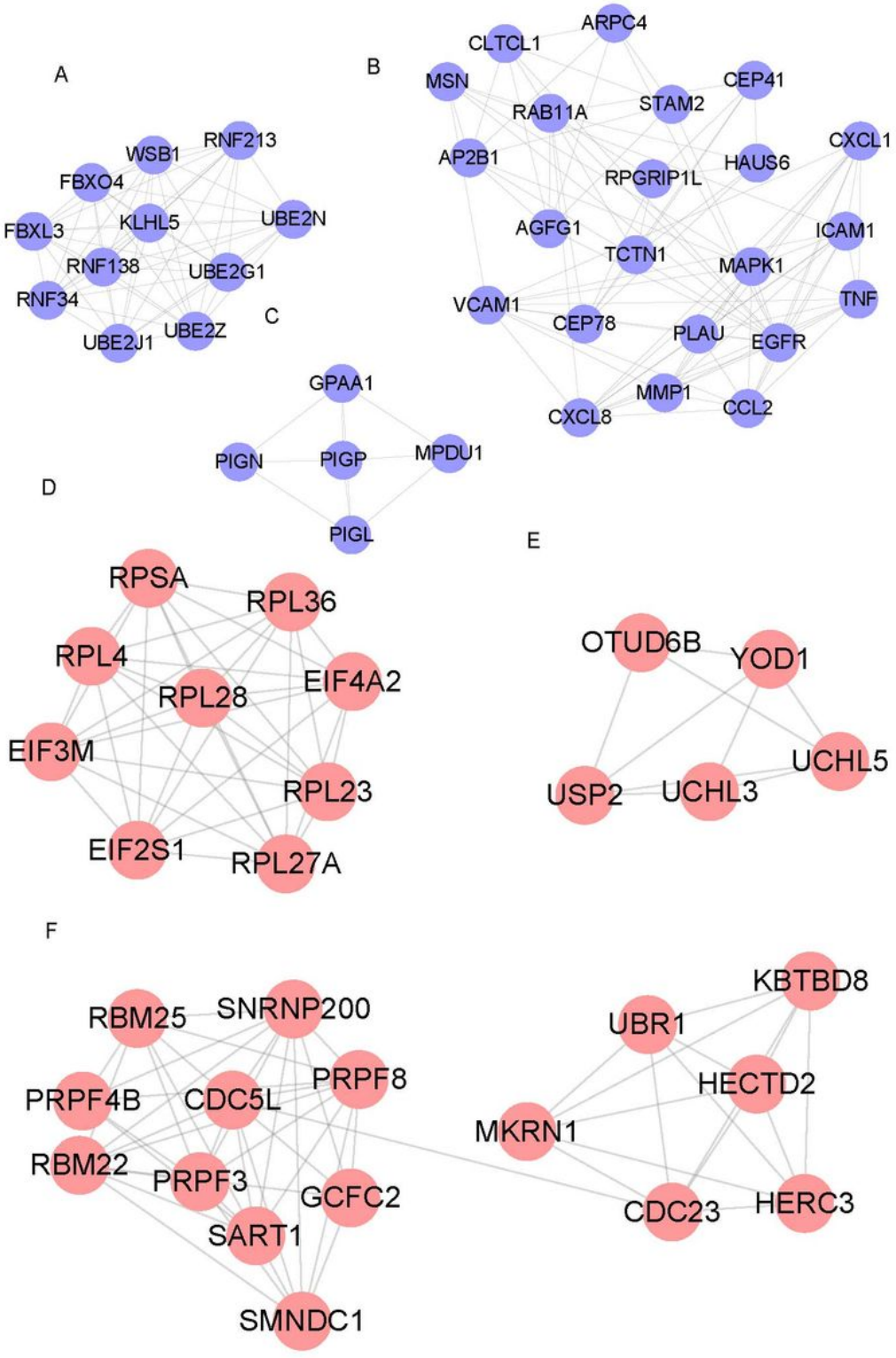

Figure 6

Identification of key regulators of PNO1 in PCa. (A-C) The top 3 hub networks were identified in PNO1 up-regualted genes mediated PPI networks. (D-F) The top 3 hub networks were identified in PNO1 down-regualted genes mediated PPI networks.

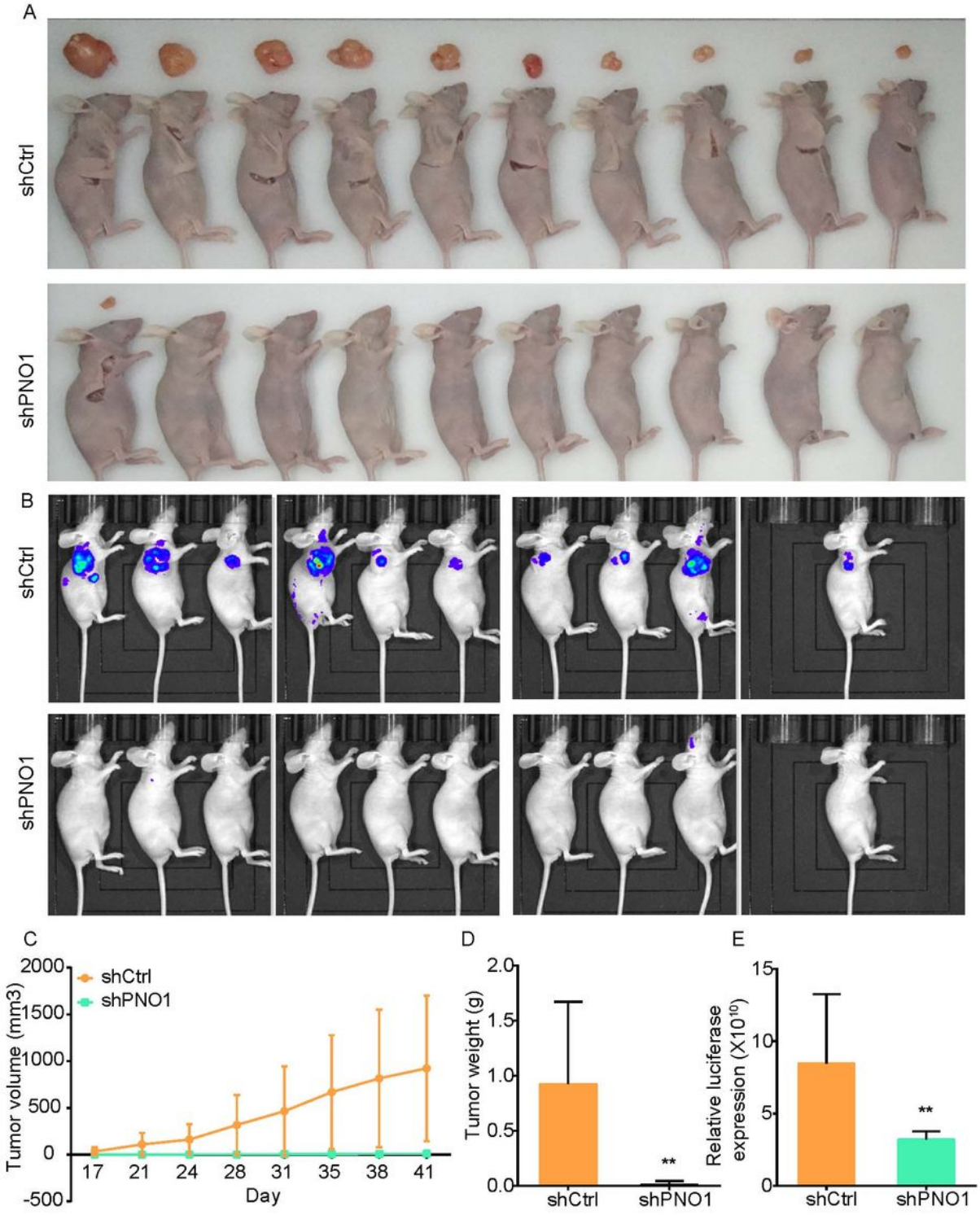

Figure 7

PNO1 knockdown inhibits prostate cancer growth in vivo. (A, D) PNO1 knockdown significantly inhibits PCa growth in vivo. (B, E) The luciferase signaling in PNO1 knockdown group was significantly lower than that in control groups. (C) The growth curve revealed PNO1 knockdown significantly inhibits PCa growth in vivo. Data are presented as the mean ± SD (n = 3) *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.