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14. ABSTRACT

I found the first time that ERG could interact with SND1/MTDH protein complex in prostate cancer cell. Here I used co-immunoprecipitation and proximity ligation assays to demonstrate that ERG interacts with SND1/MTDH complex in prostate cancer cells. I also identified the protein domains in both ERG and SND1 responsible for binding between ERG and SND1/MTDH complex. Overexpression of ERG promotes transformation of RWPE-1 immortalized human prostatic epithelial cells. I found that SND1 is necessary for ERG-mediated transformation of RWPE-1 cells. In prostate cancer VCaP cells, both ERG and SND1 are required for normal rates of VCaP cell proliferation; however, they do not show an additive effect and overexpression of SND1 partially rescues the effects of ERG knockdown, suggesting that in these cells ERG and SND1 are working in the same signaling pathway. Our in vivo mouse studies also revealed that SND1 is required for ERG/Pten KO-mediated prostate tumorigenesis. I also performed RNA-Seq experiments to analyze if SND1 is involved in ERG-regulated transcriptional program. I observed highly statistically significant overlap between genes regulated by ERG and SND1 in VCaP cells and mouse prostates in our genetic mouse model. Moreover, SND1 was necessary for ERG-mediated activation of some of its gene targets. Utilizing mouse prostate organoid model, I also found that SND1 is necessary for ERG-mediated growth of mouse cells in 3D cultures. While ERG is a nuclear protein, endogenous SND1/MTDH are primarily localized to the cytoplasm. Our nuclear/cytosol fractionation experiments revealed that nuclear SND1/MTDH is increased and cytoplasmic SND1/MTDH is decreased in RWPE-1 cells overexpressing ERG. Moreover, using CRISPR/Cas9-mediated knockout of endogenous SND1 and re-expression of exogenous SND1 protein variants in RWPE-1-Control and RWPE-1-ERG cells, I found that SND1 with exogenous nuclear localization signal (NLS) bypasses the requirements for ERG in transformation of RWPE-1 cells, suggesting that the primary function of ERG in promoting growth of RWPE-1 cells is to increase nuclear localization of SND1/MTDH. Therefore, I conclude that ERG-SND1 interaction is necessary to translocate some of SND1/MTDH to the nucleus, where they can promote cell transformation. Taken together, these observations suggested that SND1 is involved in ERG-mediated cell transformation and it may be considered as a potential therapeutic target in ERG-positive prostate cancer.

15. SUBJECT TERMS					
Prostate cancer, ERG, MTDH, SDN1, neoplastic transformation					
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INTRODUCTION

Background: The ETS family transcription factor ERG is recombined and upregulated in approximately half of all metastatic castration resistant prostate cancer (PC). The molecular mechanisms responsible for ERG mediated transformation and therapeutic vulnerabilities of ERG positive PC are not completely understood. In this proposal, I will investigate novel mechanisms of ERG mediated oncogenesis in PC and analyze potential vulnerabilities of ERG positive prostate cancers. Transcriptional co-activator Staphylococcal nuclease domain containing 1 (SND1) and its critical binding partner Metadherin (MTDH) are multifunctional proteins strongly implicated in the formation of cancer-initiating cells, metastasis and drug resistance. MTDH and SND1 are frequently amplified and overexpressed in PC; however, the role of these genes in ERG-mediated prostate tumorigenesis remains unknown. In my preliminary data, I determined that MTDH/SND1 proteins physically interact with ERG and they drive very similar transcriptional programs in metastatic human PC. The objective of this proposal is to determine the role of MTDH/SND1 protein complex in ERG-mediated transformation and therapeutic resistance. I hypothesize that: (1) MTDH/SND1 are necessary for ERG-mediated transformation and therapeutic resistance of ERG-positive metastatic PC; (2) drug-mediated inhibition of MTDH/SND1 alone or in combination with existing treatment modalities will result in the development of new therapeutic approaches for the treatment of metastatic drug-resistant PC.

KEYWORDS

Prostate cancer, ERG, MTDH, SDN1, neoplastic transformation

ACCOMPLISHMENTS

To summarize the research accomplishments to date, the tasks described in the proposed Statement of Work are itemized here with a brief update for each task.

Aim 1: Determine the functional role of ERG/MTDH/SND1 protein interaction in human and mouse prostate cell transformation and PC progression (months 1-19).

In this aim, I will examine the functional significance of ERG/MTDH/SND1 interaction, map the functional domains responsible for binding between ERG and MTDH/SND1, and investigate the role of MTDH-SND1 complex in ERG-mediated neoplastic transformation.

Task 1: Examine the protein interaction between ERG/SND1/MTDH and map the protein domains responsible for interaction (months 1-4). Our lab previously performed Mass Spectrometry analysis to identify novel ERG-interacting proteins in VCaP prostate cancer cells, we revealed that MTDH-SND1 complex proteins as putative candidates of ERG interacting proteins with high confidence in VCaP cells. To validate our mass spectrometry finding, I performed the reciprocal immunoprecipitation (IP) assay using anti-SND1, anti-MTDH and anti-ERG antibodies to analyze the protein interaction between endogenous ERG and MTDH/SND1 complex in VCaP cells. The IP experiments with anti-MTDH and anti-SND1 antibodies revealed interaction between endogenous MTDH/SND1 and ERG in VCaP cells **(Fig. 1A-C)**. Moreover, as expected, our data showed that SND1 and MTDH form the protein complex which is consistent with previous finding [1-2]. However, IP experiments with anti-ERG antibodies revealed only weak interaction between ERG and MTDH/SND1 **(Fig. 1C)**. Since VCaP cells express large amounts of ERG, these data indicate that while significant part of SND1/MTDH protein complexes interacts with ERG, only a small part of all ERG proteins is engaged in SND1/MTDH interaction at any given time. Immunofluorescence staining for endogenous ERG, SND1 and MTDH revealed that only a small proportion of SND1/MTDH is present in the nucleus in VCaP cells, while the bulk of ERG protein is in the nucleus in these

cells **(Fig. 1D-E)**. Moreover, I performed the *in situ* proximity ligation assay to confirm the in situ interaction and to determine the cellular compartments involved in ERG-SND1/MTDH binding. I found that ERG interacted with MTDH and SND1 in both the nucleus and the cytoplasm in VCaP cells **(Fig. 1F)**. To further analyze the binding between ERG and MTDH/SND1 complex, I overexpressed HA-tagged ERG, Flag-tagged SND1 and V5-tagged MTDH in HEK293 cells and found that ERG could form the protein complex with SND1/MTDH proteins **(Fig. 1G)**.

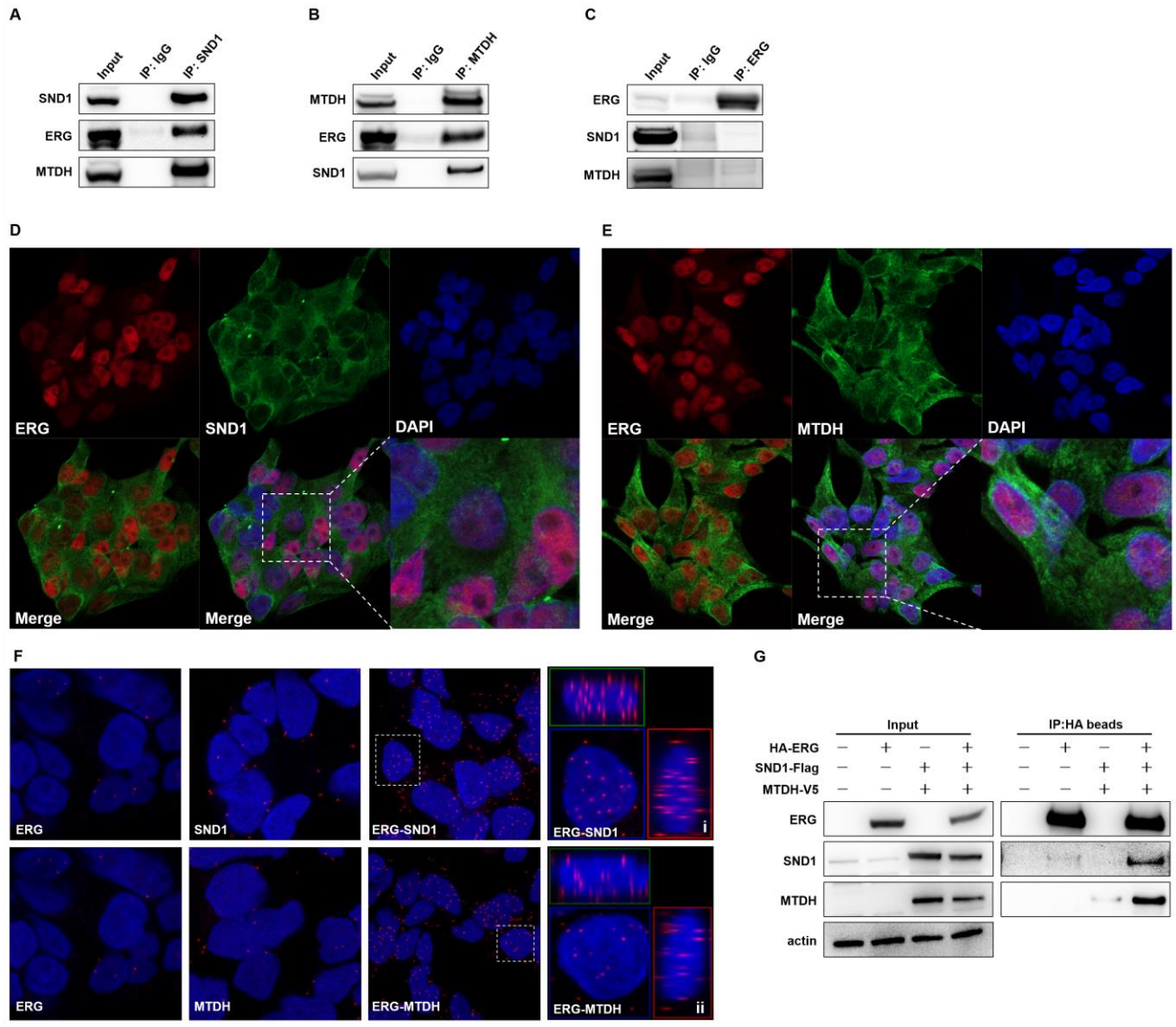

Figure 1. MTDH/SND1 protein complex interacts with ERG in VCaP prostate cancer cells. (A-C) Cell lysates from VCaP cells were subjected to IP using normal rabbit IgG, rabbit anti-MTDH (A), anti-SND1 (B) or anti-ERG (C) antibodies and protein lysates were analyzed by western blot analysis with indicated antibodies. (D-E) Immunofluorescent staining of VCaP cells with anti-ERG (red), and anti-SND1 (green, left panel) and anti-MTDH (green, right panel) antibodies. DAPI indicates nuclear counterstain (blue). (F) Representative proximity ligation assay (PLA) results of ERG, SND1 and MTDH interaction using combinations of anti-ERG, anti-SND1 (upper panel) and anti-MTDH (lower panel) in VCaP cells. An interaction or close proximity between two proteins is revealed by the appearance of red fluorescent spots. The nuclei were stained with DAPI (blue). Z-stack analysis of confocal microscopy sections revealing that some of ERG/SND1 (i) and ERG/MTDH (ii) interactions occur in the nucleus of VCaP cells. (G) Cell lysates from HEK293 cells transfected with indicated HA-tagged ERG, Flag-tagged SND1, V5-tagged MTDH expression plasmids were subjected to anti-HA IPs and analyzed by western blotting with indicated antibodies.

To map the ERG-MTDH/SND1 interacting domains, I created HALO-tagged full length ERG and a serious of truncated ERG fragments including: C-terminal deleted ERG (Δ-C-ERG), N-terminal deleted ERG (ΔN-ERG) and six individual fragments spanning the entire ERG protein: N terminus (N-Term); pointed domain (PNT); the central alternative exons (CAE); central domain (CD); ETS DNA binding domain (ETS) and C terminus (C-Term), as described in **Fig. 2A**. I performed IP assays to identify which ERG domains interact with SND1/MTDH complex. The results showed that both N-terminal and C-terminal region of ERG can interact with SND1/MTDH complex. Moreover, N-terminal region of ERG possesses strong binding ability to SND1/MTDH complex **(Fig. 2B)**. Furthermore, I determined which ERG functional domains involved in ERG-SND1/MTDH interaction. The IP result showed that ERG-SND1/MTDH interaction occurred through N-terminus, pointed domain and ETS DNA-binding domain of ERG **(Fig. 2C)**.

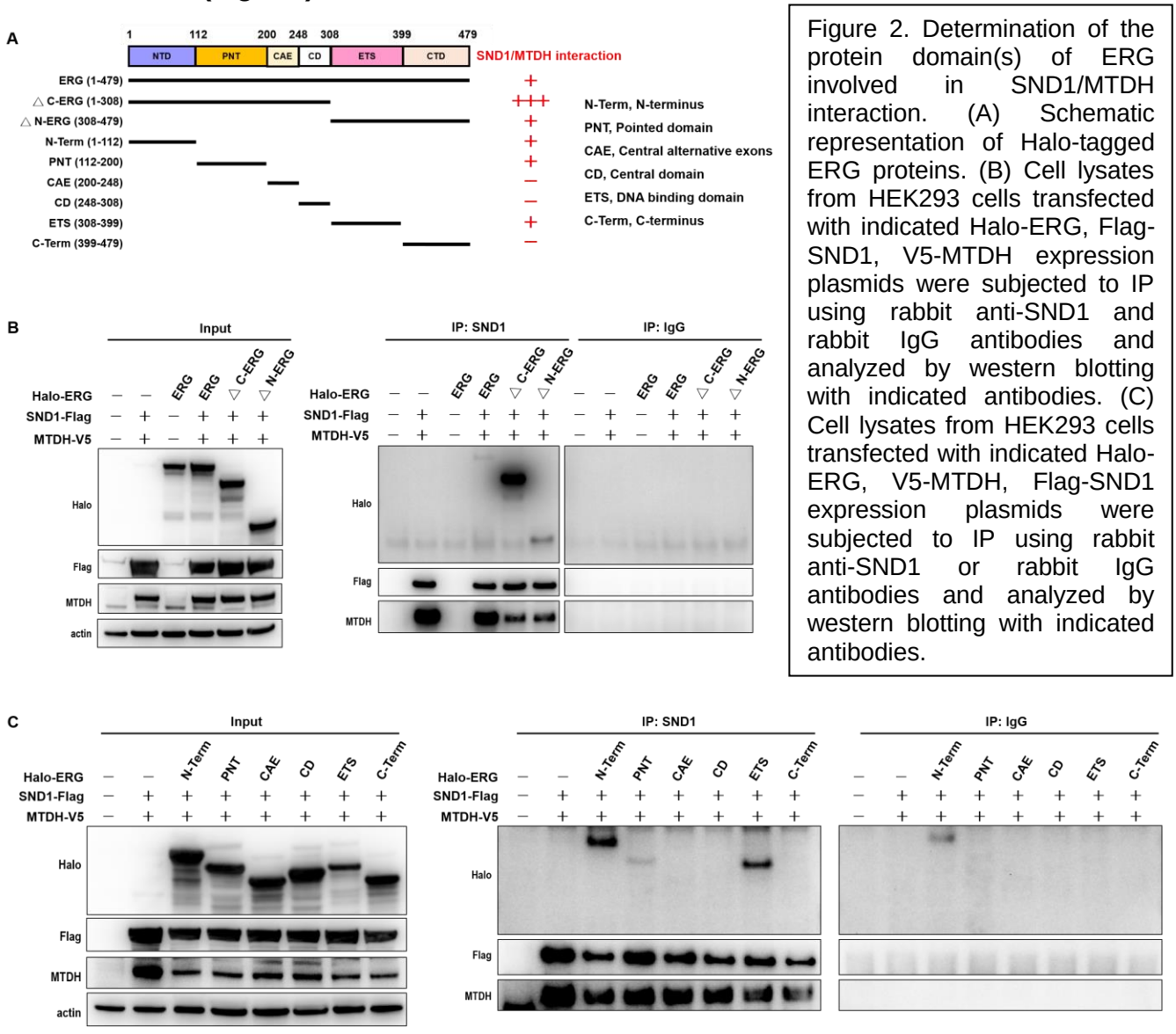

Figure 2. Determination of the protein domain(s) of ERG involved in SND1/MTDH interaction. (A) Schematic representation of Halo-tagged ERG proteins. (B) Cell lysates from HEK293 cells transfected with indicated Halo-ERG, Flag-SND1, V5-MTDH expression plasmids were subjected to IP using rabbit anti-SND1 and rabbit IgG antibodies and analyzed by western blotting with indicated antibodies. (C) Cell lysates from HEK293 cells transfected with indicated Halo-ERG, V5-MTDH, Flag-SND1 expression plasmids were subjected to IP using rabbit anti-SND1 or rabbit IgG antibodies and analyzed by western blotting with indicated antibodies.

SND1 contains four tandem repeats of Staphylococcal nuclease (SN)-like domains at the N terminus (SN1-4), and a fusion Tudor (TD) and SN domain at the C terminus, whereas MTDH is largely unstructured in its entire sequence except a trans-membrane domain near the N terminus[2]. To identify protein domains of SND1 and MTDH involved in interaction with ERG, we created V5-tagged SND1 full length and truncated fragments including C-terminal deleted SND1 (Δ-C-SND1), N-terminal deleted SND1 (ΔN-SND1), SN domain and TD domain; as well as, V5-tagged MTDH fragments including full length, C-terminal deleted MTDH (Δ-C-

MTDH), N-terminal deleted MTDH (ΔN-MTDH) as described in **(Fig. 3A),** and performed IP assays in HEK293 cells. IP results showed that C-terminus of SND1 which harbored SN3/4 and TD domain bind to ERG. Moreover, the C-terminal TD domain of SND1 displayed the strongest interaction with ERG **(Fig. 3B-C)**. Furthermore, I found that while full-length MTDH prominently interacted with ERG, C-terminal fragment of MTDH did not interact and N-terminal fragment interacted only weakly. The C-terminal fragment of MTDH contained previously mapped SND1 interaction domain[3] **(Fig. 3D).**

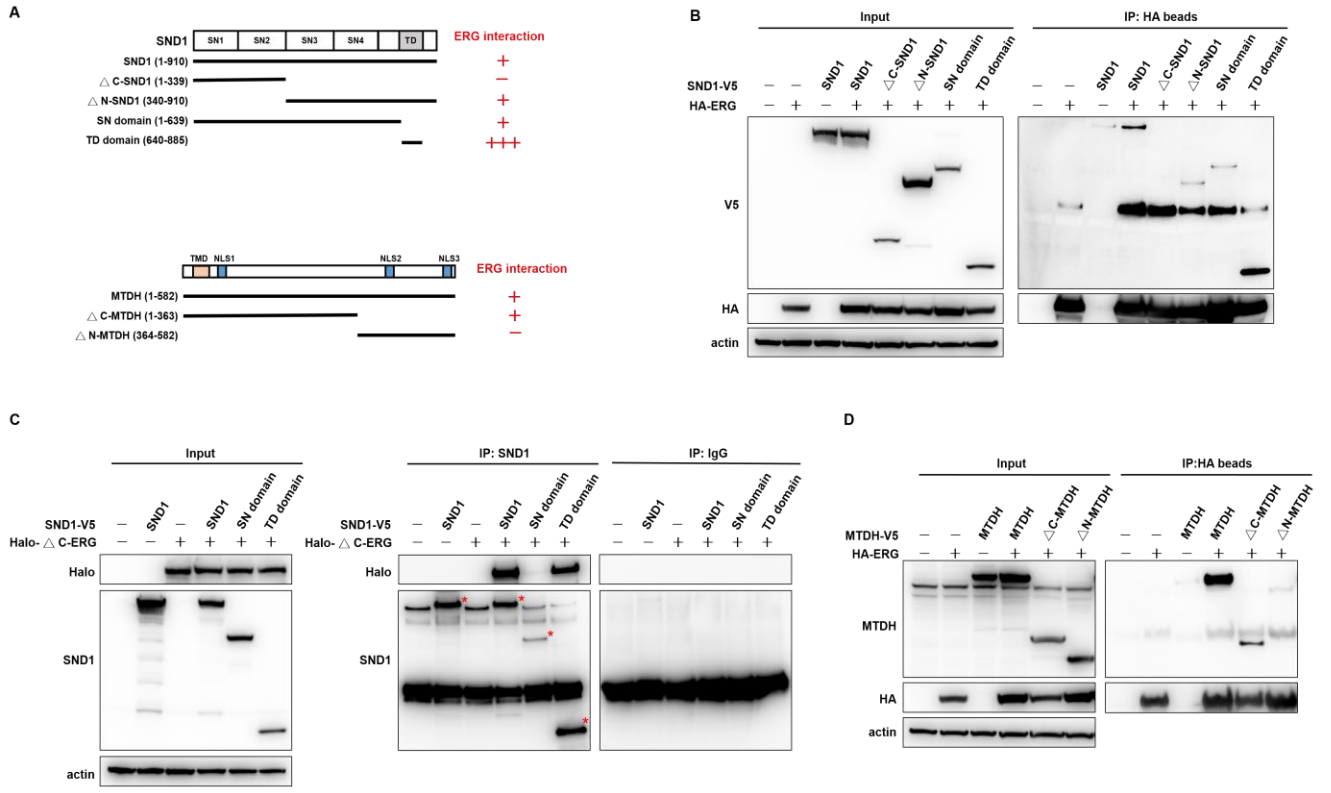

Figure 3. Determination of the protein domain of SND1/MTDH in mediating SND1/MTDH-ERG interaction. (A) Schematic representation of V5-tagged SND1, MTDH proteins. (B) Cell lysates from HEK293 cells transfected with indicated V5-SND1 and HA-ERG expression plasmids were subjected to IP using anti-HA agarose beads and analyzed by western blotting with indicated antibodies. (C) Cell lysates from HEK293 cells transfected with indicated V5-SND1 and Halo-ΔC-ERG expression plasmids were subjected to IP using rabbit anti-SND1 and rabbit IgG antibodies and analyzed by western blotting with indicated antibodies. (D) Cell lysates from HEK293 cells transfected with indicated V5-MTDH and HA-ERG expression plasmids were subjected to IP using anti-HA agarose beads and analyzed by western blotting with indicated antibodies.

To further decipher the configuration of ERG/SND1/MTDH interaction, I determined if SND1 is required for ERG/MTDH interaction and vice versa. IP experiments demonstrated that the interaction between endogenous ERG/MTDH was attenuated by the knockdown of SND1 in VCaP cells, while knocking down of MTDH did not affect ERG/SND1 interaction **(Fig. 4A)**. Previous studies showed that SN1/2 domains of SND1 bind stoichiometrically with MTDH, whereas the SN3/4-TSN5 domains did not interact with MTDH [2]. Consistently, IP results showed weak interaction between MTDH and TD domain of SND1 comparing to full length SND1, whereas ERG still strongly interacted with TD domain **(Fig. 4B)**. Furthermore, I found that the binding of MTDH to ERG is increased in the presence of SND1. C-terminal region of MTDH cannot directly bind to ERG without SND1 **(Fig. 4C)**. The result is consistent with previous report showing that MTDH has been reported to interact with SND1 through its C-terminal region[1,2]. These data suggested that SND1 is required for ERG-MTDH interaction.

Taken together, these findings for the first time demonstrate that ERG interacts with MTDH/SND1 proteins in human prostate cancer cells and I identify the critical domain for ERG/MTDH/SND1 interaction, will help to understand the mechanisms of MTDH/SND1 in ERG-mediated transformation and facilitate the design of the dominant negative constructs that can disrupt ERG - MTDH/SND1 interaction.

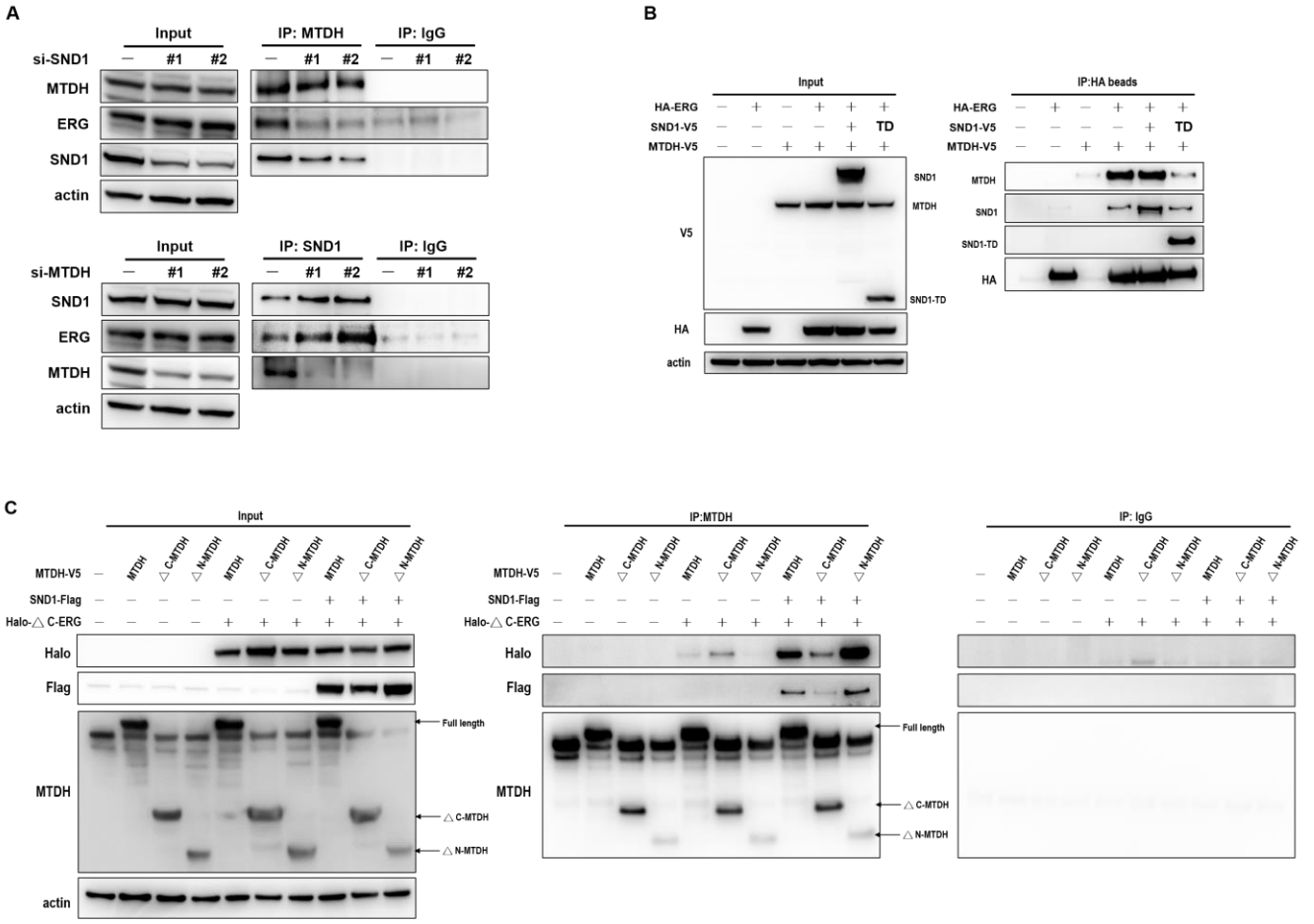

Figure 4. SND1 is required for ERG-MTDH interaction. (A) Cell lysates from VCaP cells transfected with indicated siRNA oligos were subjected to IP using normal rabbit IgG, rabbit anti-MTDH (upper panel) or anti-SND1 (lower panel) antibodies and protein lysates were analyzed by western blot analysis with indicated antibodies. (B) Cell lysates from HEK293 cells transfected with indicated HA-ERG, V5-SND1 or V5-MTDH expression plasmids were subjected to IP using anti-HA agarose beads and analyzed by western blotting with indicated antibodies. (C) Cell lysates from HEK293 cells transfected with indicated V5-MTDH, Halo-ΔC-ERG or Flag-SND1 expression plasmids were subjected to IP using rabbit anti-SND1 and rabbit IgG antibodies and analyzed by western blotting with indicated antibodies.

Task 2: Examine the functional significance of MTDH/SND1 in ERG-mediated cell transformation and cancer progression using human cell lines in culture. (months 1-8). To determine the biological significance of ERG and MTDH/SND1 protein interaction in prostate tumorigenesis. We previously found that overexpression of ERG significantly promotes growth of RWPE-1 cells in the 3D organoid prostate culture system. Here I generated lentiviral short hairpin RNA (shRNA) constructs targeting SND1 or MTDH in RWPE-1 cells overexpressing exogenous ERG (RWPE-ERG) and control (RWPE-Ctrl) cells (**Fig. 5A-B**). I found that overexpression of ERG significantly promoted growth of RWPE-ERG cells in the 3D organoid prostate culture system. Importantly, SND1 and MTDH were necessary for this ERG-mediated transforming phenotype, knockdown of SND1 and MTDH using two independent lentiviral shRNA constructs erased the growth differences between RWPE-Ctrl and RWPE-ERG cells (**Fig.**

5C-D). Similarly, I also observed that overexpression of ERG prominently stimulated formation of RWPE-1 cell colonies in clonogenic growth assay, the colony size of RWPE-ERG cells is bigger than RWPE-Ctrl cells. The stable knockdown of SND1 or MTDH significantly erased the differences between RWPE-Ctrl and RWPE-ERG cells (**Fig. 5E-F**). These results suggested that both of SND1 and MTDH played the important role in ERG-mediated transforming phenotype.

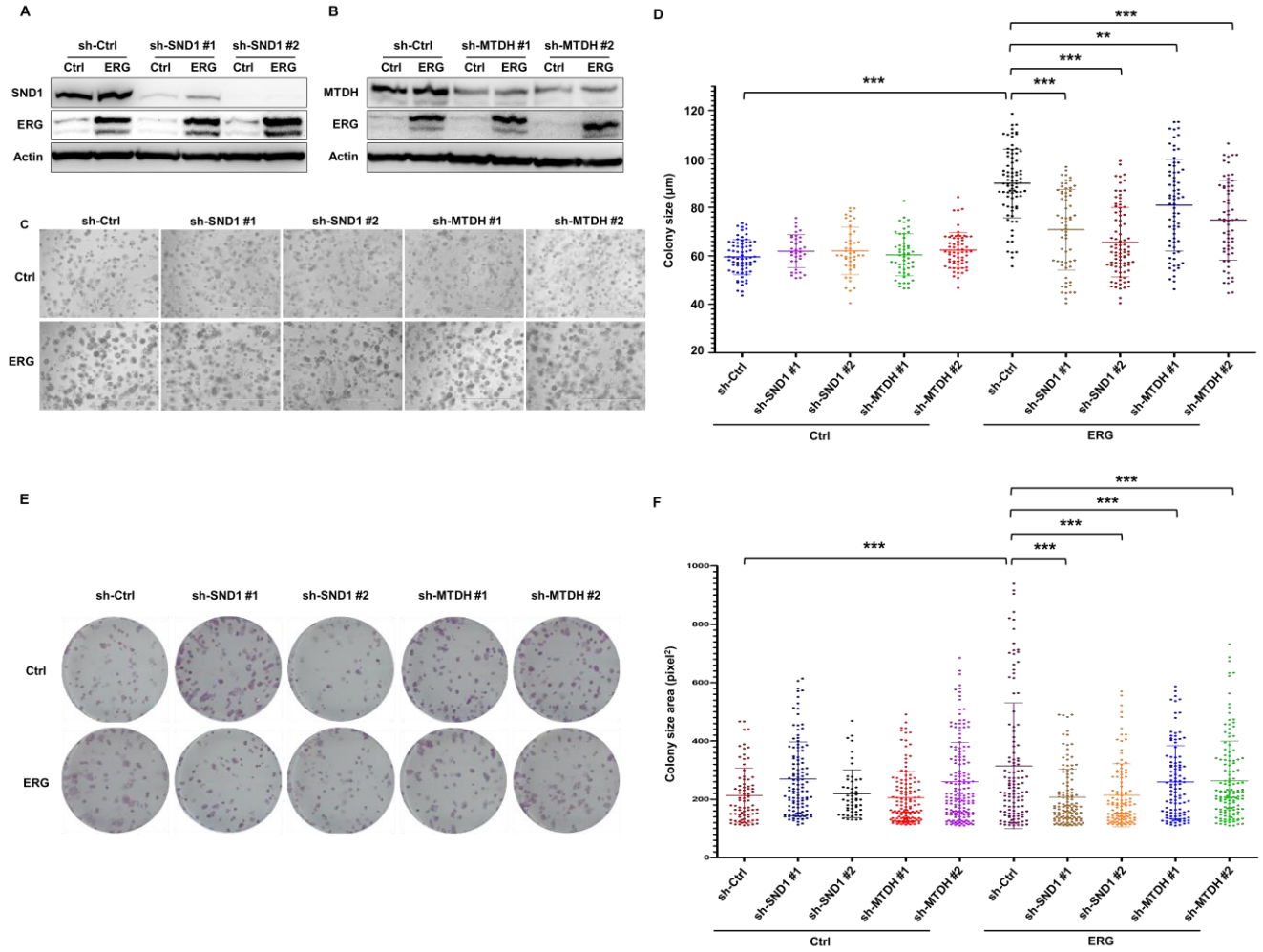

Figure 5. Role of SND1/MTDH in ERG-mediated transformation of RWPE-1 prostate epithelial cells. (A) Western blot analyses of RWPE-Ctrl (Ctrl) and RWPE-ERG (ERG) cells stably transduced with control pGIPZ, sh-SND1 or sh-MTDH lentiviral vectors and analyzed with indicated antibodies. (B-D) Brightfield images (B-C) and colony size quantitation (D) of RWPE Ctrl and ERG cells with or without sh-SND1 and sh-MTDH colonies formed after 5 days in 3D organoid culture system. The graph shows mean ± SD. Student's t test was used to determine statistical significance. (E-F) Images of clonogenic growth assay by crystal violet staining (E) and colony size quantitation (F) of RWPE Ctrl and ERG cells with or without sh-SND1 or sh-MTDH colonies formed after 10 days expansion of cells. Colony sizes were measured and analyzed using ImageJ. The graph shows mean ± SD. n≧30. Student' s t test was used to determine statistical significance.

Importantly, I observed that the impact of SND1 on ERG-mediated transforming phenotype is stronger than MTDH. Thus, I further investigated the effect of SND1 on ERG-mediated transformation, I generated SND1 knockout (KO) cells in both RWPE-Ctrl and RWPE-ERG cells by using CRISPR/Cas9-mediated knockout system (**Fig. 6A**). Consistently, knockout of SND1 remarkably decreased the growth of RWPE-ERG cells both in 3D organoid prostate culture system and clonogenic growth assay, while SND1 re-expression in SND1 KO cells rescued the knockout effect of SND1 (**Fig. 6B-E**). Due to the expression level of exogenous

SND1 was not equivalent to endogenous SND1 level (**Fig. 6A**), I only observed the partial rescue effect in SND1 re-expressing cells. Taken together, our results suggesting that SND1 was necessary for ERG-mediated transformation of RWPE-1 cells.

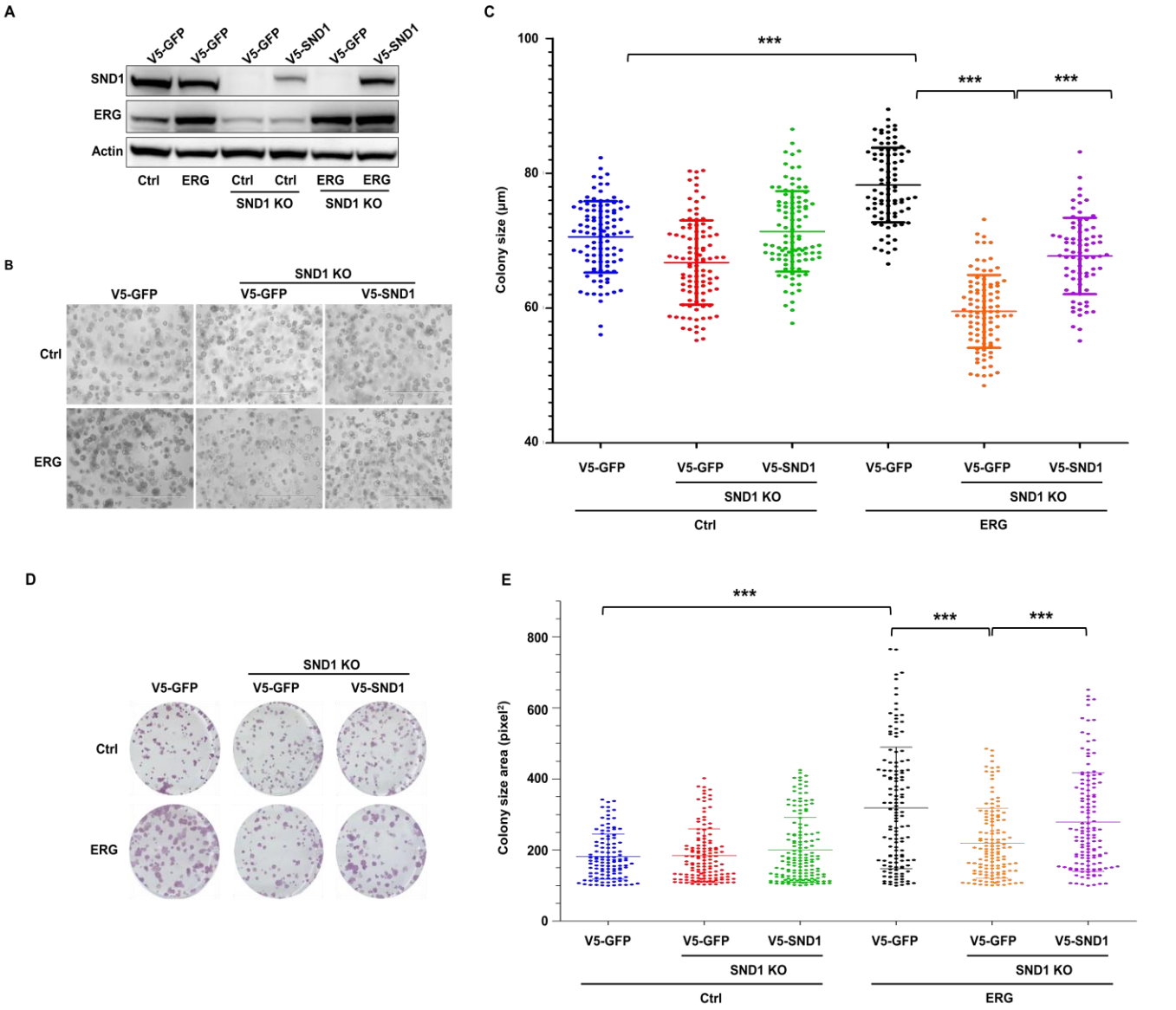

Figure 6. Role of SND1 in ERG-mediated transformation of RWPE-1 prostate epithelial cells. (A) Western blot analyses of RWPE-Ctrl and RWPE-ERG cells with or without SND1 KO and re-expressed with V5-GFP or V5-SND1 and analyzed with indicated antibodies. (B-C) Brightfield images (B) and colony size quantitation (C) of RWPE-Ctrl and RWPE-ERG cells with or without SND1 KO and re-expressed with V5-GFP or V5-SND1 colonies formed after 5 days in 3D organoid culture system. (D-E) Images of clonogenic growth assay by crystal violet staining (D) and colony size quantitation (E) of RWPE-Ctrl and RWPE-ERG cells with or without SND1 KO and re-expressed with V5-GFP or V5-SND1 colonies formed after 10 days expansion of cells. Colony sizes were measured and analyzed using ImageJ. The graph shows mean ± SD. n≧30. Student's t test was used to determine statistical significance.

To analyze the functional importance of SND1/MTDH in mediating ERG-associated malignant phenotypes in prostate cancer, I examined the role of SND1 and MTDH in ERG fusion positive VCaP cells. For this purpose, VCaP cells were transfected with si-Ctrl, si-ERG, si-SND1 or si-MTDH oligos **(Fig. 7A-C)**. The results showed that knockdown of ERG and SND1 decreased VCaP cell proliferation while the knockdown of MTDH did not have an affect **(Fig. 7D-F)**. Combined knockdown of ERG and SND1 did not show an addictive effect on VCaP cell proliferation, suggesting that ERG and SND1 are working in the same signaling pathway

and deletion of one of them is sufficient to inhibit this pathway **(Fig. 7G)**. Moreover, I also found that overexpression of SND1 promoted cell proliferation in VCaP cells **(Fig. 7H)**. SND1 overexpression could rescue the effect of ERG knockdown on VCaP cell proliferation **(Fig. 7I)**. These results suggested that SND1 plays an important role in ERG-mediated prostate cancer.

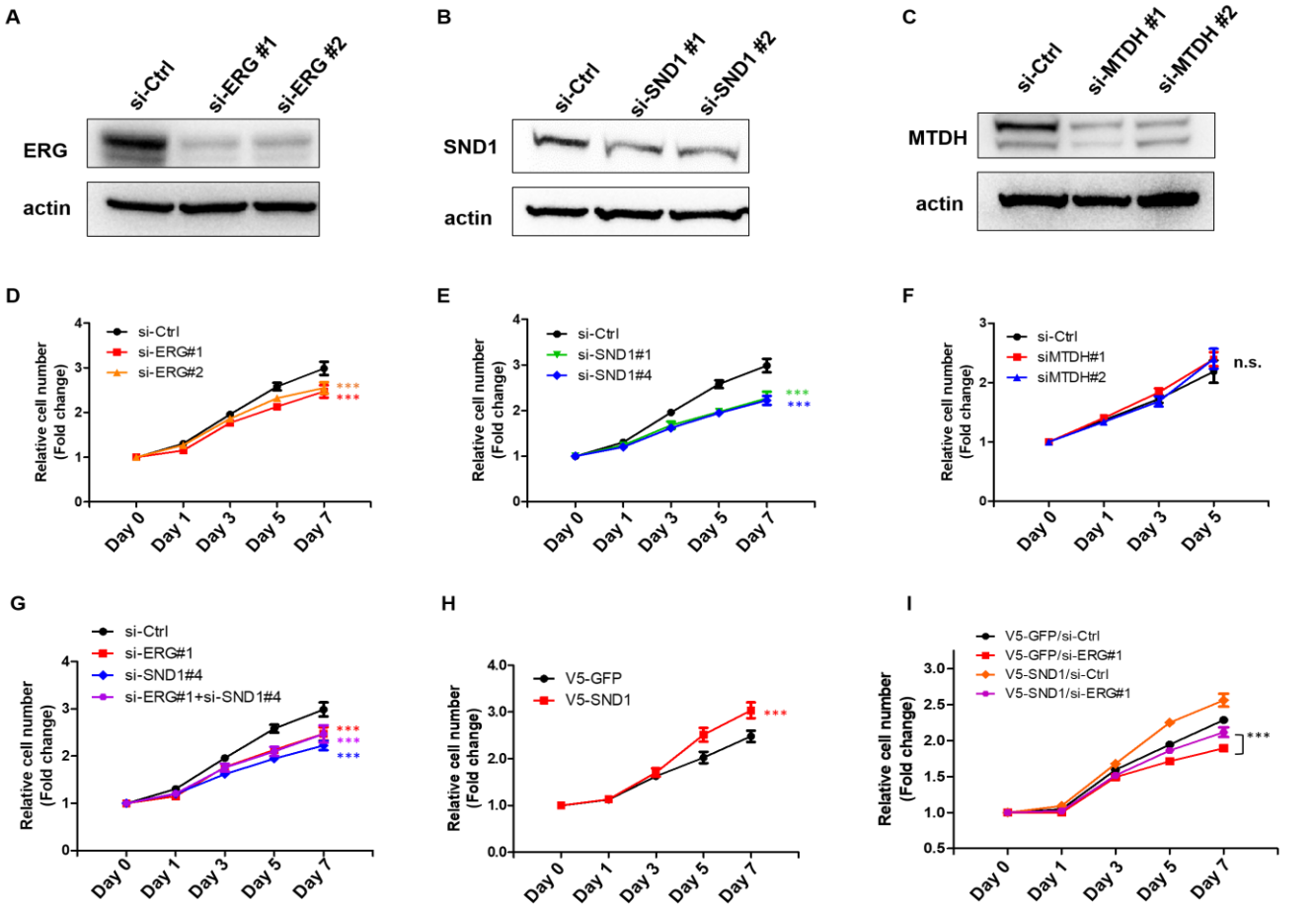

Figure 7. Role of SND1/ MTDH in mediating ERG-associated malignant phenotypes in VCaP cells. (A-C) Western-blot analyses of ERG, SND1, MTDH and actin expression in VCaP cells transfected with si-Ctrl, si-ERG, si-SND1 or si-MTDH oligos. (D-G) VCaP cells transfected with indicated siRNA oligos were grown on 96 well plate and analyzed by CellTiter-Glo 2.0 Assay. (H) VCaP cells stably expressing with V5-GFP and V-SND1 were grown on 96 well plate and analyzed by CellTiter-Glo 2.0 Assay. (I) VCaP cells stably overexpressing with V5-GFP and V-SND1 were transfected with indicated siRNA oligos and grown on 96 well plate and analyzed by CellTiter-Glo 2.0 Assay. P-values determined using two-tailed Student's t-test. Data represent mean ± standard deviation (n = 3). P-values determined using two-tailed Student's t-test. Data represent mean ± standard deviation.

ERG is a transcription factor, which is majorly expressed in the nuclear. SND1 is reported to localize both in the cytoplasm and nuclear. I considered the possibility that the ERG-SND1 interaction could translocate some of SND1 into the nuclear. To test this, I performed nuclear/cytosol fractionation experiments in RWPE-Ctrl and RWPE-ERG cells. Interestingly, I observed that nuclear SND1 is increased and cytoplasmic SND1 is decreased in RWPE-ERG cells (**Fig. 8A-C**). I next hypothesized that ERG could bring some SND1 into the nuclear where it could promote cell transformation. To determine the physiological significance of nuclear SND1 in cell transformation, I generated the SND1 expression vectors with exogenous nuclear localization signal (NLS-SND1) (**Fig. 8D**). The nuclear/cytosol fractionation experiments confirmed that the amount of NLS-SND1 is expressed in the nuclear comparing to wild type SND1 (WT-SND1) in 293T cells (**Fig. 8E**). I next analyzed the effect of NLS-SND1 on cell transformation. Using CRISPR/Cas9-mediated knockout of

endogenous SND1 and re-expression of exogenous SND1 protein variants in RWPE-Ctrl and RWPE-ERG cells (**Fig. 8F**), I found that the overexpression of NLS-SND1 in SND1 KO cells possess stronger oncogenic effect on colony formation ability comparing to WT-SND1 both in 3D organoid prostate culture system (**Fig. 8G-H**). Importantly, NLS-SND1 could bypass the requirements for ERG in transformation of RWPE-1 cells. Taken together, these results indicated that the primary function of ERG in promoting growth of RWPE-1 cells is to increase nuclear localization of SND1 (**Fig. 8I**).

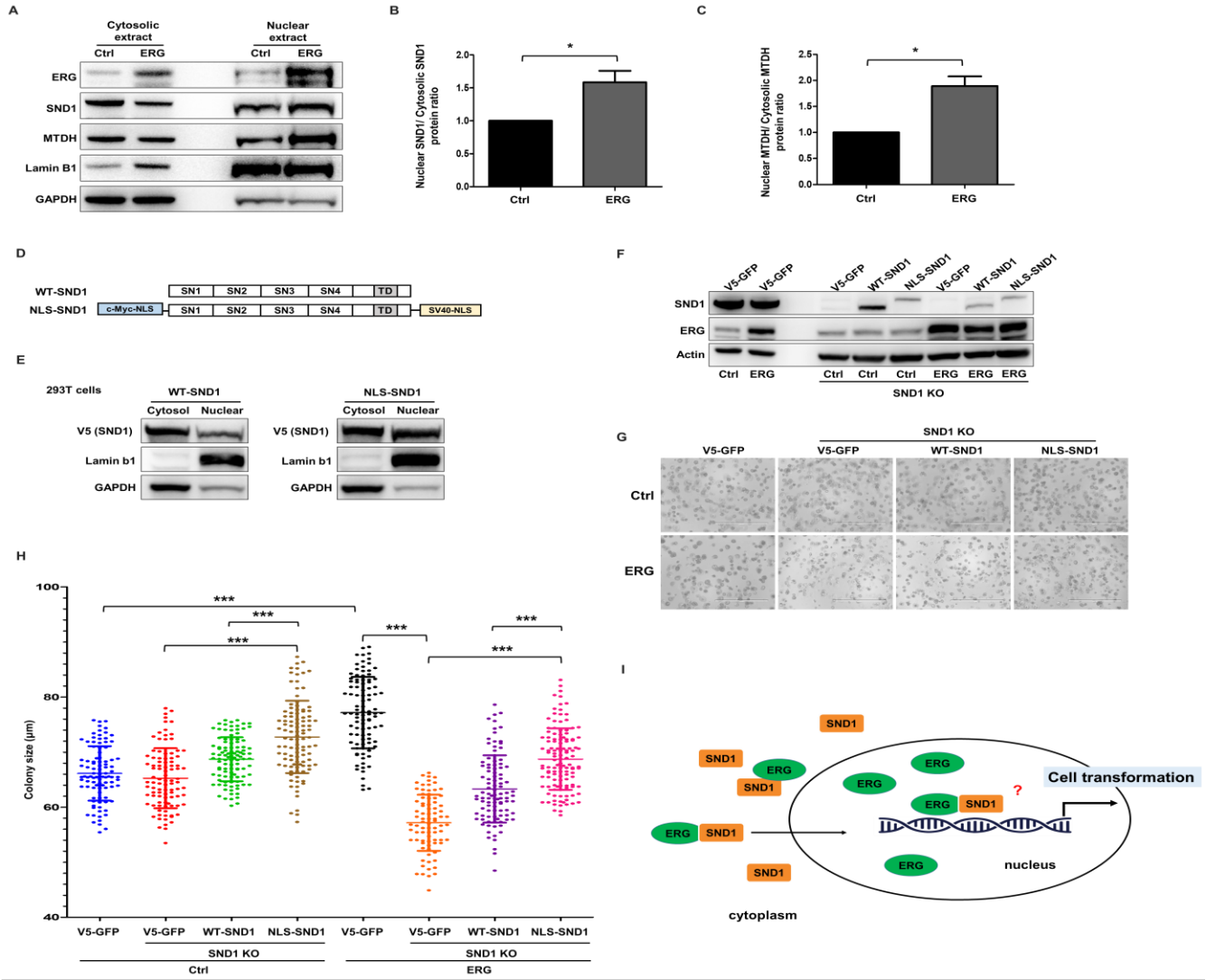

Figure 8. ERG promotes nuclear translocation of SND1 in transformation of non-tumorigenic prostate epithelial cells (A) Cytosolic and nuclear fraction protein lysates from RWPE-Ctrl and RWPE-ERG cells were subjected to western-blot analyses and analyzed with indicated antibodies. (B-C) Quantification of nuclear SND1 versus cytosolic SND1 protein ratio (B) or nuclear MTDH versus cytosolic MTDH protein ratio (C) in RWPE-Ctrl and RWPE-ERG cells by ImageJ. Protein expression ratio were quantified and presented on the graph in arbitrary units with values in RWPE-CTRL cells adjusted to 1. P-values were determined using two-tailed Student's t-test. *, $P < 0.05$. (D) Schematic representation of SND1 expression vectors with exogenous nuclear localization signal. (E) Cytosolic and nuclear fractions protein lysates from HEK293 cells expressing with V5-tagged WT-SND1 (upper panel) or NLS-SND1 (lower panel) were subjected to western-blot analyses and analyzed with indicated antibodies. (F) Western blot analyses of RWPE-Ctrl and RWPE-ERG cells with or without SND1 KO and re-expressed with GFP, WT-SND1 and NLS-SND1 and analyzed with indicated antibodies. (G-H) Brightfield images (G) and colony size quantitation (H) of RWPE-Ctrl and RWPE-ERG cells with or without SND1 KO and re-expressed with GFP, WT-SND1 and NLS-SND1 colonies formed after 5 days in 3D organoid culture system. Colony sizes were measured and analyzed using ImageJ. The graph shows mean ± SD. $n \geqq 30$. Student's t test was used to determine statistical significance. (I) Model showing that the primary function of ERG in promoting growth of RWPE-1 cells is to increase nuclear localization of SND1.

Task 3: Examine the functional significance of MTDH/SND1 in ERG-mediated cell transformation and cancer progression using in vivo mouse genetics approach (months 1-18). To further evaluate the oncogenic effect of SND1 in prostate tumorigenesis *in vivo*, I generated genetic mouse models with prostate-specific knockout of *Snd1* either alone or in combination with gain of function of *ERG* and loss of function of *Pten* (PB-Cre/Snd1$^{fl/fl}$, PB-Cre/ERG/Snd1$^{fl/fl}$ and PB-Cre/ERG/Pten$^{fl/fl}$/Snd1$^{fl/fl}$ mice). First, I examined the effect of *Snd1* knockout in mouse prostate. Cre-mediated recombination of *Snd1* floxed allele was analyzed by genotyping PCR and western blot results also confirmed the loss of Snd1 protein in mouse prostate (**Fig. 9A-C**). As expected, prostate-specific knockout of *Snd1* alone (PB-Cre/Snd1$^{fl/fl}$ mice) did not affect mouse prostate size and did not develop the tumor in prostate (**Fig. 9D-F**). In addition, the expression of development and differentiation marker of prostate cancer was not significantly changed in *Snd1* KO mice comparing to wild-type littermates by immunohistochemistry staining (**Fig. 9G**). Next, I analyzed the effect of *Snd1* knockout in genetic mouse with overexpression of *ERG* and knockout of *Pten*. Cre-mediated recombination was confirmed by genotyping PCR and the protein expression of Snd1, Pten and ERG in mouse prostate was analyzed by western blot (**Fig. 10A-C**). Interestingly, I found that PB-Cre/ERG/Pten$^{fl/fl}$ mice developed prostate tumors in one year old mice, while knockout of Snd1 (PB-Cre/ERG/Pten$^{fl/fl}$/Snd1$^{fl/fl}$) significantly suppressed prostate tumor formation. The prostate weight were significantly decreased in Snd1 KO mice (**Fig. 10D-F**). Consistently, immunohistochemistry staining results showed that the expression of cell proliferation marker, Ki67, and phospho-histone 3 (PH3), a marker of mitosis was significantly decreased in PB-Cre/ERG/Pten$^{fl/fl}$/Snd1$^{fl/fl}$ mice comparing to PB-Cre/ERG/Pten$^{fl/fl}$ mice. In contrast, the expression of cleaved Caspase-3, an apoptosis marker, was increased in Snd1 KO mice (**Fig. 10G**). These results suggested that knockout of *Snd1* suppressed prostate tumorigenesis, the results were consistent with our in vitro cell-based studies. Taken together, our *in vivo* study demonstrated the oncogenic role of SND1 in prostate tumorigenesis and SND1 is required for ERG/PTEN-mediated prostate tumorigenesis.

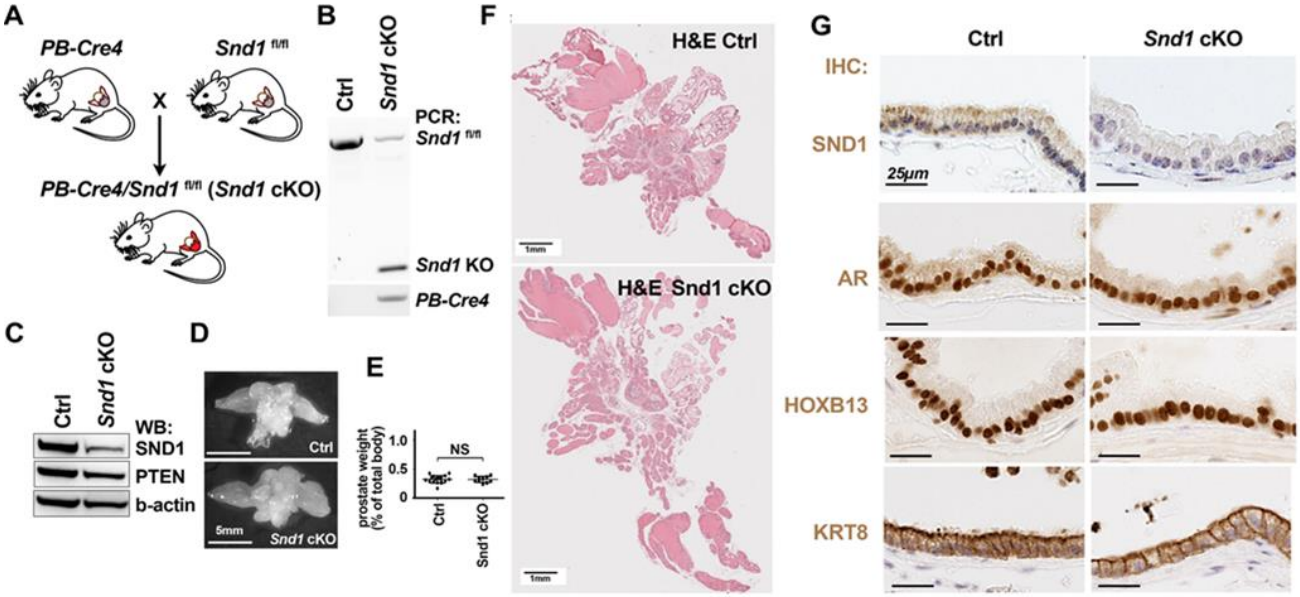

Figure 9. Role of SND1 in prostate tumorigenesis. (A) Model showing the development of prostate epithelium-specific Snd1 KO mice. (B) Genotyping PCR reaction examined the Snd1 floxed and KO alleles using DNA isolated from tails snips. (C) Western-blot analysis of Snd1 protein expression in mouse ventral prostates from mice as indicated. (D) Gross appearance of prostate tissue from mice as indicated. (E) The quantitation of the prostate tissue weights. The graph shows mean ± SD. n≧10. Student' s t test was used to determine statistical significance. (F)Histology of prostate gland from mice as indicated. (G) Serial sections of the prostate tissue from mice as indicated stained with Snd1, luminal cell markers: anti-Androgen Receptor (AR) and anti-cytokeratin-8 (KRT-8), and prostate cancer maker, anti-HOXB13.

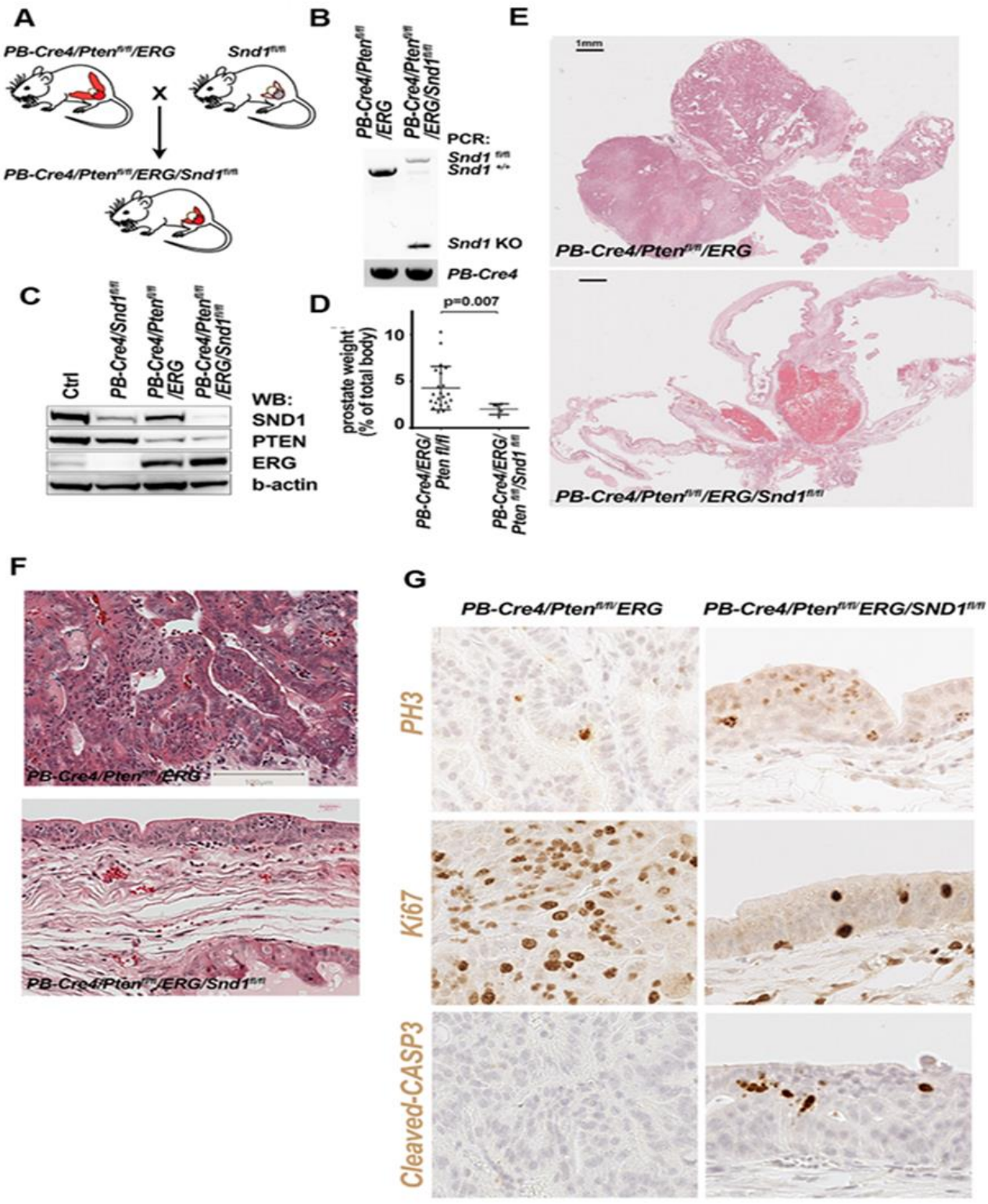

Figure 10.Knockout of Snd1 suppresses ERG/Pten KO-mediated prostate tumorigenesis. (A) Model showing the development of prostate epithelium-specific Snd1 KO in ERG/Pten$^{fl/fl}$ mice. (B) Genotyping PCR reaction examined the Snd1 floxed and KO alleles using DNA isolated from tails snips. (C) Western-blot analysis of Snd1, ERG, PTEN protein expression in mouse ventral prostates from mice as indicated. (D) Gross appearance of prostate tissue from mice as indicated. (E) The quantitation of the prostate tissue weights. The graph shows mean ± SD. n≧10. Student’ s t test was used to determine statistical significance. (F) Histology of prostate gland from mice as indicated. (G) Serial sections of the prostate tissue from indicated mice stained with Snd1; cell proliferation marker, Ki67; mitosis marker, phospho-histone 3 (PH3) and cleaved Caspase-3, an apoptosis marker.

Task 4: Analyze the role of MTDH/SND1 using mouse prostate epithelial cell organoids (months 8-18).

To further examine the oncogenic role of SND1 in prostate transformation. I isolated mouse primary prostate cells from wild type and *ERG* mouse and stable knocked down of SND1 in these cells and analyzed the phenotypical change of these cell lines in 3D culture system. I found that *ERG* GOF increased mouse prostate organoid growth, whereas knockout of *Snd1* erased ERG-induced organoid growth phenotype, indicating that SND1 is necessary for ERG-mediated growth. **(Fig. 11A-C)**. To further confirm the effect of Snd1 in mouse cell model, I isolated primary prostate epithelial cells from PB-Cre/Snd1fl/fl and PB-Cre/ERG/Snd1fl/fl mice and created paired floxed/knockout lines by infecting cells with adenovirus carrying Cre (or GFP as control) to induce recombination. The western blot results revealed high knockout efficiency of SND1 **(Fig. 11D)**. I next performed the 3D organoid culture to analyze the phenotypical change in these cells. Consistently, ERG GOF increased mouse prostate organoid growth, whereas knockout of Snd1 erased ERG-induced organoid growth phenotype **(Fig. 11E-F)**, indicating that SND1 is necessary for ERG-mediated growth.

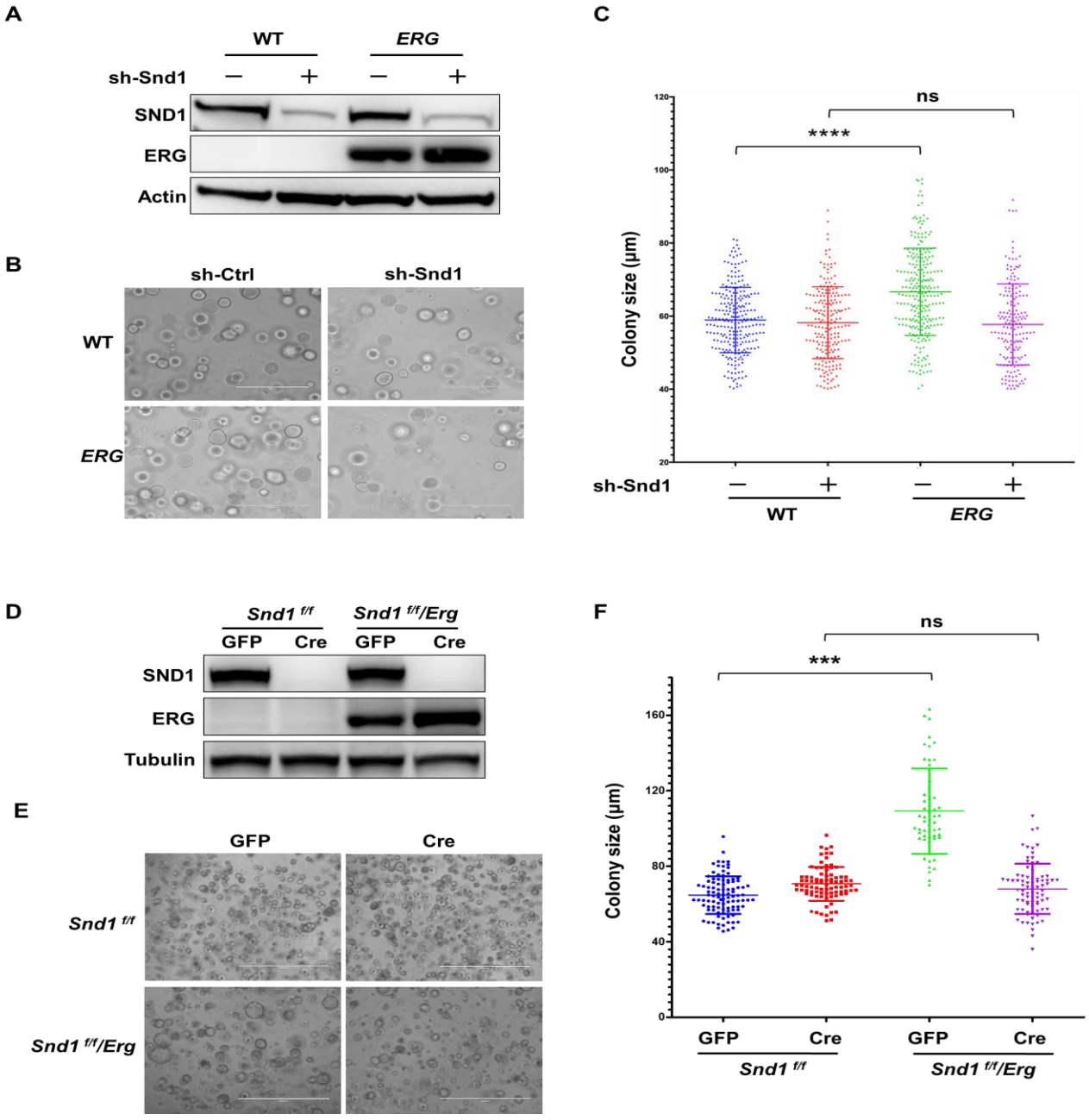

Figure 11. Role of Snd1 in ERG-mediated transformation of *mouse prostate epithelial cell organoids*. (A) Western blot analyses of mouse wild type, and ERG cells transduced with control pGIPZ and sh-Snd1 lentiviral vectors and analyzed with indicated antibodies. (B-C) Brightfield images (B) and colony size quantitation (C) of mouse wild type, and ERG cells transduced with control pGIPZ and sh-SND1 lentiviral vectors colonies formed after 5 days in 3D organoid culture system. Colony sizes were measured and analyzed using ImageJ. The graph shows mean ± SD. n≧30. Student's t test was used to determine statistical significance. (D) Western blot analyses of mouse PB-Cre/Snd1fl/fl and PB-Cre/ERG/Snd1fl/fl cells infected with adenovirus carrying Cre (or GFP as control) to induce recombination and analyzed with indicated antibodies. (E-F) Brightfield images (E) and colony size quantitation (F) of mouse PB-Cre/Snd1fl/fl and PB-Cre/ERG/Snd1fl/fl cells infected with or without adenovirus carrying Cre colonies formed after 5 days in 3D organoid culture system. Colony sizes were measured and analyzed using ImageJ. The graph shows mean ± SD. n≧30. Student's t test was used to determine statistical significance.

Aim 2: To investigate the MTDH/SND1 transcriptional program and role of MTDH/SND1 in ERG-mediated gene transcription and chromatin interaction (months 4-18).

Task 5: *Perform RNA-Seq to analyze the role of MTDH/SND1 in ERG-mediated transcription. (months 4-18).* To determine the consequences of ERG/SND1 interaction on ERG-mediated transcription program, I performed the RNA-Seq experiments using VCaP cells with siRNA-mediated knockdown of ERG and SND1 to identify the gene signature of ERG and SND1 in prostate cancer. Our RNA-Seq analysis identified a highly statistically significant overlap between genes altered by knockdown of ERG and SND1 in VCaP cells. (**Figure 12A-C**). Gene set enrichment analysis (GSEA) analysis revealed that genes downregulated in both si-ERG and si-SND1 VCaP cells were enriched in cell cycle and E2F pathways (**Figure 12D-E**). These results supported our finding showing that knockdown of ERG and SND1 decreased prostate cancer cell proliferation.

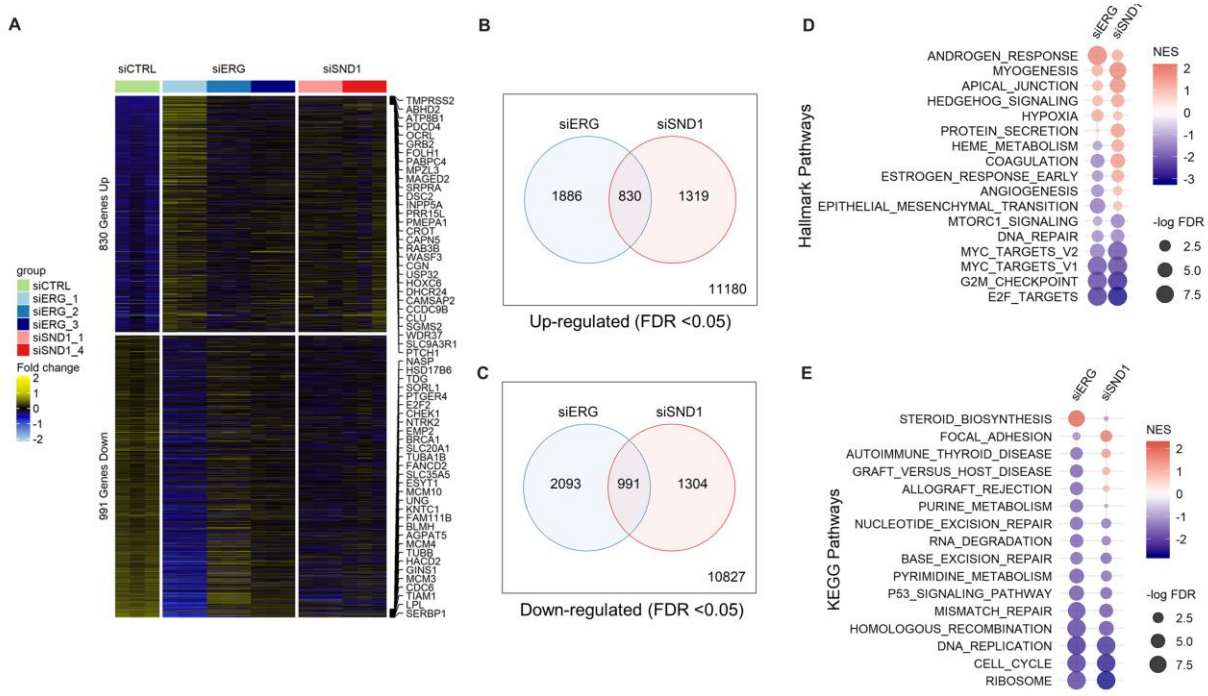

Figure 12. Significant concordance between ERG and SND1 gene expression programs in VCaP cells. (A) Heatmap of gene expression determined by RNA-seq of VCaP cells transfected with indicated siRNA oligos (n=3, using three independent si-ERG oligos and two independent si-SND1 oligos). (B-C) Venn diagram showing common significant up-regulated genes and down-regulated genes (FDR< .05) between si-ERG and si-SND1 cells determined by edgeR3 analysis. (D-E) Gene set enrichment analysis plots of ERG and SND1 common regulated genes.

To determine the role of Snd1 in ERG-mediated transcription program *in vivo*. I performed RNA-Seq experiments using mouse prostate tissue collected from *Pten KO, Pten KO/ERG-overexpression (OE)* and P*ten KO/Snd1 KO/ERG-OE mice*. Our RNA-Seq analysis identified a highly statistically significant overlap between ERG and Snd1 regulated genes in mouse prostates. (**Figure 13A-C**). GSEA analysis also revealed that genes downregulated by Snd1 in mouse prostate were enriched in cell cycle and cell proliferation pathways (**Figure 13D**). These results were consistent with our cell-based studies, suggesting that SND1 is required for ERG-mediated tumorigenesis.

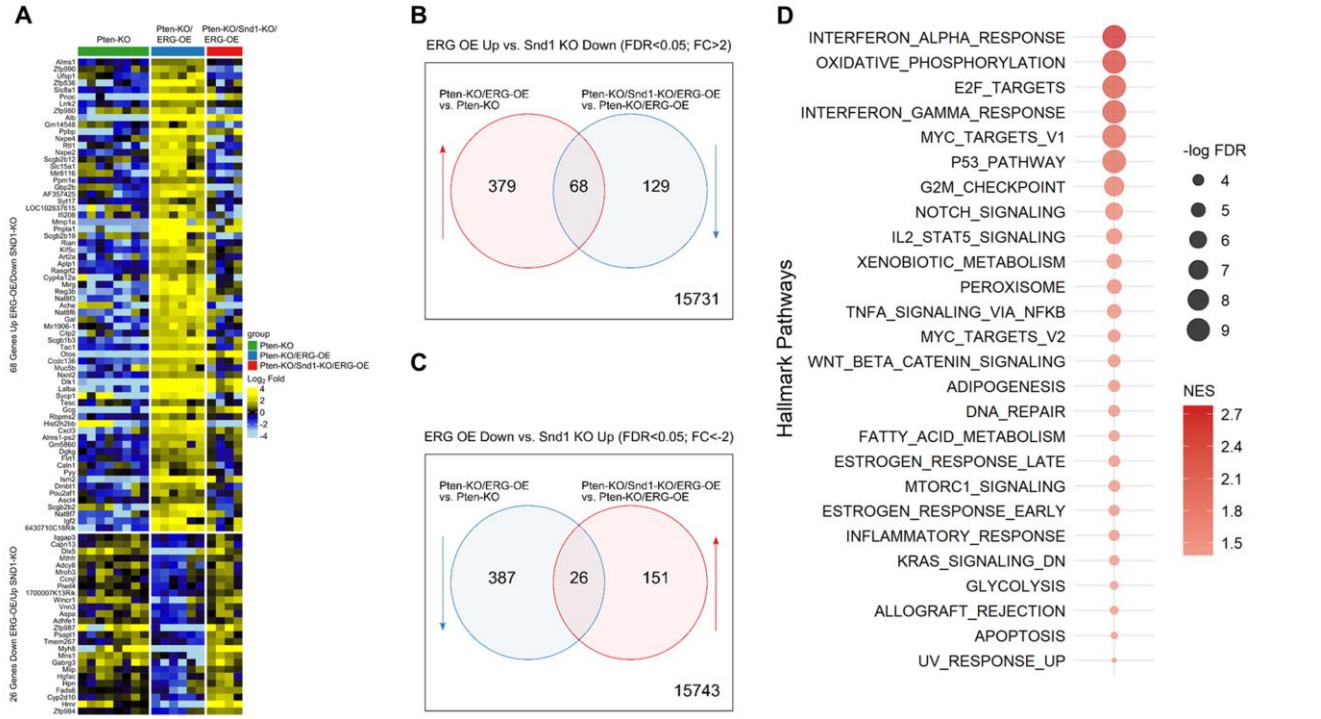

Figure 13. Significant overlap between ERG and SND1 regulated genes *in vivo*. (A) Heatmap of gene expression determined by RNA-seq of mouse prostate from *Pten KO, Pten KO/ERG-OE* and *Pten KO/Snd1 KO/ERG-OE mice* (B-C) Venn diagram showing common significant up-regulated genes and down-regulated genes (FDR< .05) between *Pten-KO/ERG-OE vs. Pten-KO* and *Pten-KO/Snd1-KO/ERG-OE vs. Pten-KO/ERG-OE* by edgeR3 analysis. (D) Gene set enrichment analysis plots of SND1 regulated genes from *Pten-KO/Snd1-KO/ERG-OE* vs. *Pten-KO/ERG-OE* comparision.

Task 6: Perform ChIP-Seq to identify the genome-wide chromatin binding regions of MTDH/SND1 and determine the role of MTDH/SND1 in ERG chromatin occupancy (months 8-13). To determine whether ERG and SND1 bind to the same chromatin sites in prostate cancer cells, I firstly performed the chromatin immunoprecipitation (ChIP) assay in VCaP cells using ERG and SND1 antibodies to examine the binding of ERG and SND1 on *target gene* promoters. I did not observe the chromatin interaction of SND1 in ChIP assays. Later genome-wide analysis using ChIP-Seq approach demonstrated that SND1 antibody did not work for ChIP and failed to identify any specific chromatin binding peaks. As an alternative to Chip-Seq, I performed the 'cleavage under targets and release using nuclease"-Seq (CUT&RUN-seq) experiment to determine whether ERG and SND1 bind to the same chromatin sites of their target genes. RNA-Seq experiment identified some of ERG and SND1 regulated genes such as CDK2, CCNE1, CDC6 and E2F2. qRT-PCR results also confirmed that knocking down of either ERG or SND1 in VCaP cells significantly decreased the expression of CDK2, CCNE1, CDC6 and E2F2 genes **(Fig. 14A-F)**. I next analyzed if ERG and SND1 could bind to these target gene promoter. CUT&RUN Seq results showed that both ERG and SND1 bind to the

same DNA regulatory elements in *some of their target* gene promoters such as CDC6 and CDK2. The results revealed partial overlap between ERG and SND1 peaks in VCaP cells **(Fig. 14G-H)**. These data suggested that ERG/SND1 could bind to same chromatin regions and cooperatively regulate gene expression. However, the detail mechanism of ERG and SND1 in regulation of target gene expression need to be further addressed.

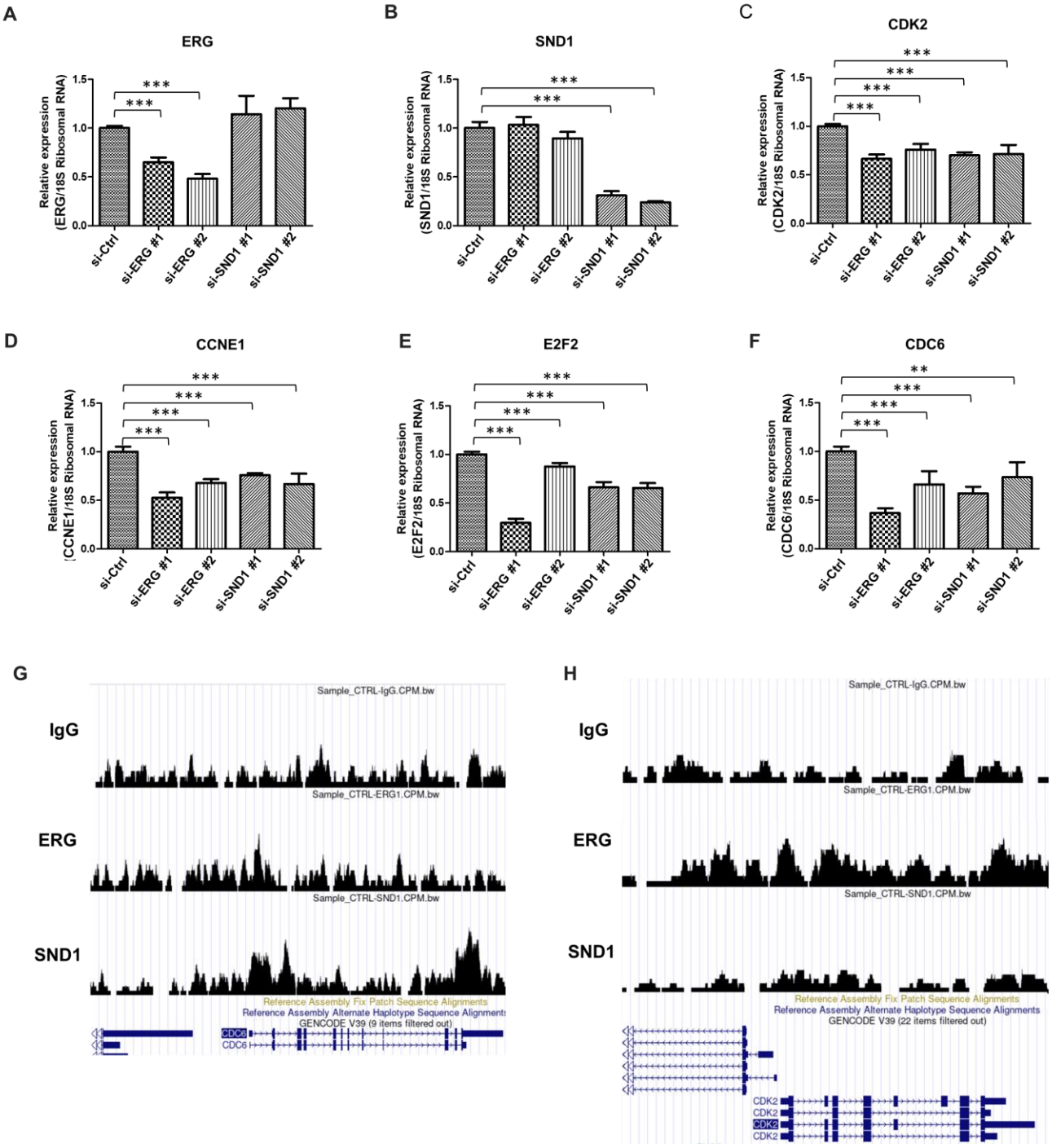

Figure 14. ERG and SND1 could bind to their target gene promoter (A-F) qRT-PCR analysis of expression of ERG/SND1 positively-regulated genes CDK2, CCNE1, E2F2 and CDC6 in VCaP transfected with si-control (si-Ctrl) or si-ERG or si-SND1 oligo. Gene expression data were normalized using combined values for GAPDH, ACTIN, RPS16 housekeeping genes. P-values were determined using two-tailed Student's t-test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (G-H) UCSC genome browser views of CUT&RUN-Seq data from VCaP cells using IgG, anti-ERG and anti-SND1 antibodies.

Aim 3: Determine if ERG-positive prostate tumors respond to inactivation or drug-mediated inhibition of SND1/MTDH alone or in combination with existing therapeutic modalities. (months 12-24)

Task 7: To investigate whether ERG-positive PC cells are hypersensitive to SND1 inhibition using PC cell models. (months 12-16) . Our results suggested that SND1 is involved in ERG-mediated cell transformation and it may be considered as a potential therapeutic target in ERG-positive prostate cancer. Recently, a selective inhibitor of SND1, the 3'5'-deoxythymidine bisphosphate (pdTp), was shown to reduce hepatocellular carcinoma cells proliferation, as well as *in vivo* tumor growth[4, 5]. To investigate if inhibition of SND1 by pdTp could suppress the growth of VCaP human metastatic PC cells. VCaP cells were treated with escalating concentrations of SND1 inhibitor (pdTp), our results showed that chemical inhibition of SND1 indeed decreased VCaP cell growth, but the effect was not as strong as siRNA treatment (**Fig. 15A-B**). In addition, I also found that SND1 inhibitor treatment in si-ERG cells did not show an addictive effect on VCaP cell growth (**Fig. 15B**). These results were consistent with Figure 7, suggesting that ERG and SND1 are working in the same signaling pathway and inhibition of one of them is sufficient to inhibit this pathway. The function of pdTp is to inhibit the nuclease activity of SND1. I found that SND1 inhibitor treatment in si-SND1 cells did not show an addictive effect on VCaP cell growth (**Fig. 15B**), suggesting that the endonuclease activity of SND1 is responsible for SND1 growth promoting role in VCaP cells.

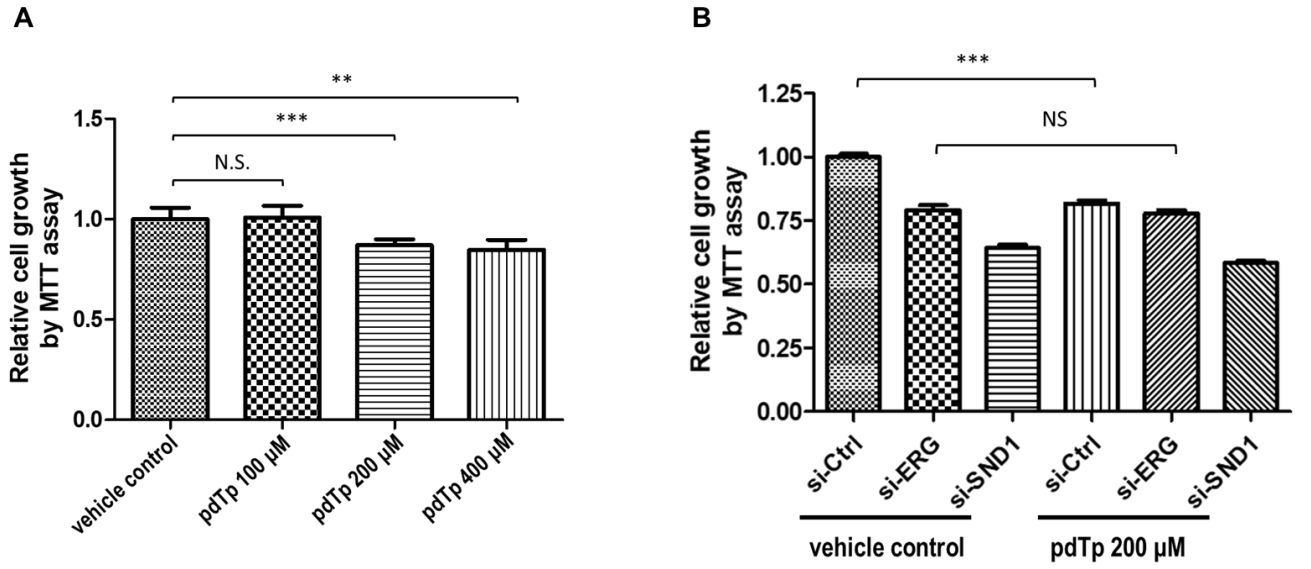

Figure 15**.** Drug-mediated inhibition of SND1 negatively impacts growth of human metastatic PC cells. (A) VCaP cells were treated with escalating concentrations of SND1 inhibitor (pdTp) or vehicle control for 4 days and cell viability analyzed using MTT. (B) VCaP cells knock down with ERG and SND1 were treated with pdTp or vehicle control for 4 days and cell viability analyzed using MTT. P-values were determined using two-tailed Student's t-test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Task 8: To investigate whether ERG-positive PC cells are hypersensitive to SND1 inhibition using patient-derived metastatic PC xenograft models (months 15-24). Given that we found that SND1 inhibition could decrease the cell growth of VCaP cells. I firstly analyzed the effect of SND1 inhibitor (pdTp) on ERG positive LuCaP 35CR and 35CS cells *in vitro*, which were derived from castration-resistant patient-derived xenografts (PDX). Similar to VCaP cells, I found that inhibition of SND1 decreased cell growth of LuCaP cells but the effect was not very strong (**Fig. 16A-B**). Maybe pdTp is not sufficient to inhibit SND1 activity in prostate cancer cells comparing to hepatocellular carcinoma cells [4,5]. Therefore, *the in vivo effect of SND1 inhibitor in xenograft models were not performed. Taken together, my observations*

demonstrated that SND1 is involved in ERG-mediated cell transformation and it still can be considered as a potential therapeutic target in ERG-positive prostate cancer.

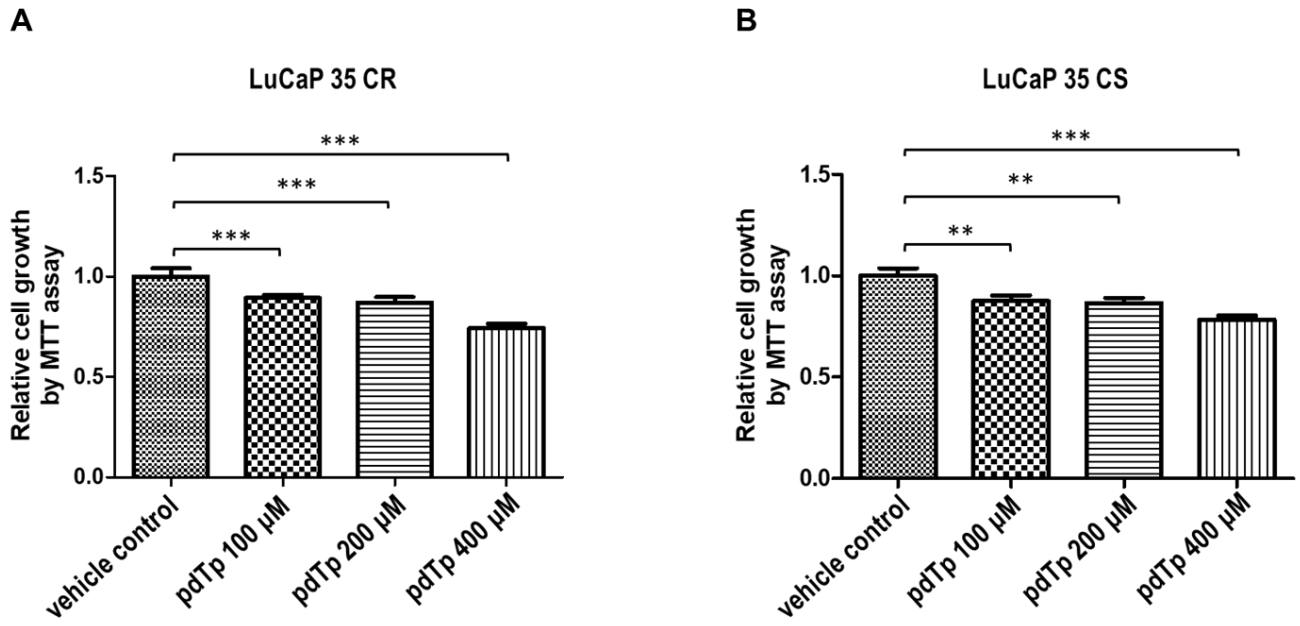

Figure 16**.** Drug-mediated inhibition of SND1 negatively impacts growth of patient-derived xenografts LuCaP cells. (A-B) LuCaP 35 CR (A) and 35 CS (B) cells were treated with escalating concentrations of SND1 inhibitor (pdTp) or vehicle control for 4 days and cell viability analyzed using MTT. P-values were determined using two-tailed Student's t-test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Opportunities for Training and Professional Development:

During the reporting period, I continued to meet with my mentors Drs. Valeri Vasioukhin and Peter Nelson to discuss study results and research goals. My co-mentors helped me learn how to effectively communicate research findings and enhance my ability to write grant proposals and manuscripts, critically review research publications. I presented the study results in our weekly Program for PC Research (PPCR) meeting where all the students in the program present their research. These meetings are very useful to hone presentation skills, obtain feedback and learn new information. As part of the training, at our lab meetings I also presented and discussed recently published papers that are relevant to my research. Our PC program has close collaboration with Pacific Northwest PC Specialized program for Research Excellence (SPORE) which includes the University of Washington, Oregon Health and Sciences University, the University of British Columbia. The SPORE researchers hold weekly webcast research meetings, with Seattle laboratories meeting at the Fred Hutchinson Cancer Research Center (FHCRC). Moreover, there are many seminar series, such as: Friday night talks (weekly), current biology seminar series (weekly), distinguished speakers series (monthly), PC SPORE (weekly), and division retreats (yearly) that are held at the FHCRC. I had many opportunities to interact with a diverse range of scientific faculties. My research project integrate the most advanced *in vivo* novel mouse genetic models of prostate cancer, patient derived xenograft tumors) and *in vitro* (PC organoids, cell lines) modeling of prostate cancer. For my research skill development, I learned variety of methods and techniques including the analysis of genetic mouse models of prostate cancer, LuCaP patient-derived xenograft models, organoid culture, genomic and bioinformatics analysis. With these research training and excellent mentorship and research environment, I expect that I will be in a good position to become an outstanding and independent prostate cancer researcher.

Dissemination of Results:

The findings of this work have been shared locally with the Fred Hutch Prostate Cancer Program through the weekly lab meeting. In addition, I also presented these study in the Friday night talk's presentation which is held at the FHCRC.

Plan for the Next Reporting Period:

Nothing to Report.

IMPACT

ERG gene is the key driver of human prostate cancer. Therefore, it's important to identify novel therapeutic strategies for treatment of ERG-positive prostate cancer. The results of this project will help to understand how overproduction of ERG transforms prostate gland and helps prostate cancer cells resist drug treatment. I will analyze the role and mechanisms of MTDH/SND1 proteins in ERG-mediated transformation and drug resistance of ERG-positive and ETS-negative prostate tumors. To accurately model PC growing in patients, I will extensively use both *in vivo* (novel genetic mouse models of PC, patient-derived LuCaP xenograft tumors) and *in vitro* (human and mouse PC cell lines, tumor organoids) research systems. If my hypothesis is correct and MTDH/SND1 do play an important role in ERG-positive PC and/or the development and maintenance of drug resistance, this study will stimulate further development of even more potent inhibitors of ERG signaling pathway and support new clinical trials for ERG-targeted therapies in advanced prostate cancer.

CHANGES/PROBLEMS

No changes to the project are anticipated at this juncture.

PRODUCTS

This study was presented in the Friday night talk which is held at the FHCRC and the manuscript of this study is in preparation.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name:	Sheng-You, Liao
Project Role:	*Project PI*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	23
Contribution to Project:	Dr. Liao designs and performs the experiment and data analysis for this project
Funding Support	

Special Reporting Requirements

Nothing to report.

REFERENCES

1. Wan L, Lu X, Yuan S et al. MTDH-SND1 interaction is crucial for expansion and activity of tumor-initiating cells in diverse oncogene- and carcinogen-induced mammary tumors. Cancer Cell 2014; 26:92-105.

2. Guo F, Wan L, Zheng A et al. Structural insights into the tumor-promoting function of the MTDH-SND1 complex. Cell Rep 2014; 8:1704-13.
3. Blanco, M.A., Aleckovic M, Hua Y et al. Identification of staphylococcal nuclease domain-containing 1 (SND1) as a Metadherin-interacting protein with metastasis-promoting functions. J. Biol. Chem 2011. 286 (22); 19982–19992.
4. Jariwala N, Rajasekaran D, Mendoza RG et al. Oncogenic Role of SND1 in Development and Progression of Hepatocellular Carcinoma. Cancer Res 2017; 77:3306-3316.
5. Yoo BK, Santhekadur PK, Gredler R et al. Increased RNA-induced silencing complex (RISC) activity contributes to hepatocellular carcinoma. Hepatology 2011; 53:1538-48.

APPENDICES

None.