AWARD NUMBER: W81XWH-20-1-0082

TITLE: MTDH/SND1 protein complex in ERG-mediated transformation and therapeutic resistance

PRINCIPAL INVESTIGATOR: Sheng-You Liao, PhD

CONTRACTING ORGANIZATION: Fred Hutchinson Cancer Research Center
Seattle, WA

REPORT DATE: March 2021

TYPE OF REPORT: Annual Report

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
March 2021	Annual Report	01Feb2020-31Jan2021

4. TITLE AND SUBTITLE
MTDH/SND1 protein complex in ERG-mediated transformation and therapeutic resistance

5a. CONTRACT NUMBER
W81XWH-20-1-0082

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Sheng-You Liao, PhD

E-Mail: sliao@fredhutch.org

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Fred Hutchinson Cancer Research Center
1100 Fairview Ave N. J6-300
Seattle, WA 98109-4433

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
In this reporting period, I found the first time that ERG could interact with SND1/MTDH protein complex in prostate cancer cell. I also identified the functional domains responsible for binding between ERG and SND1/MTDH complex. Our RNA-Seq experiment found a highly statistically significant overlap between genes regulated by ERG and SND1/MTDH proteins in VCaP cells. ERG and SND1 act in a concert and SND1 is necessary for ERG-mediated activation of some of its gene targets. Furthermore, CUT&RUN assay results suggested that ERG and SND1 bind to the same DNA regulatory elements in *FZD8* and *HPN* gene promoters. These results suggested that SND1/MTDH complex is involved in ERG-mediated transcriptional regulation. I found that SND1/MTDH were both necessary and sufficient for ERG-mediated transformation of RWPE-1 cells. In addition, ERG, SND1 and MTDH are required for prostate cancer cell proliferation and invasion, and overexpression of SND1 rescues the effects of ERG knockdown in VCaP cells. Our results suggested that SND1/MTDH complex is involved in ERG-mediated prostate cancer and it may be considered as a therapeutic target in ERG-positive prostate cancer.

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

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	17	19b. TELEPHONE NUMBER *(include area code)*

TABLE OF CONTENTS

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 progress.

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, 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) Completed. 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)**.

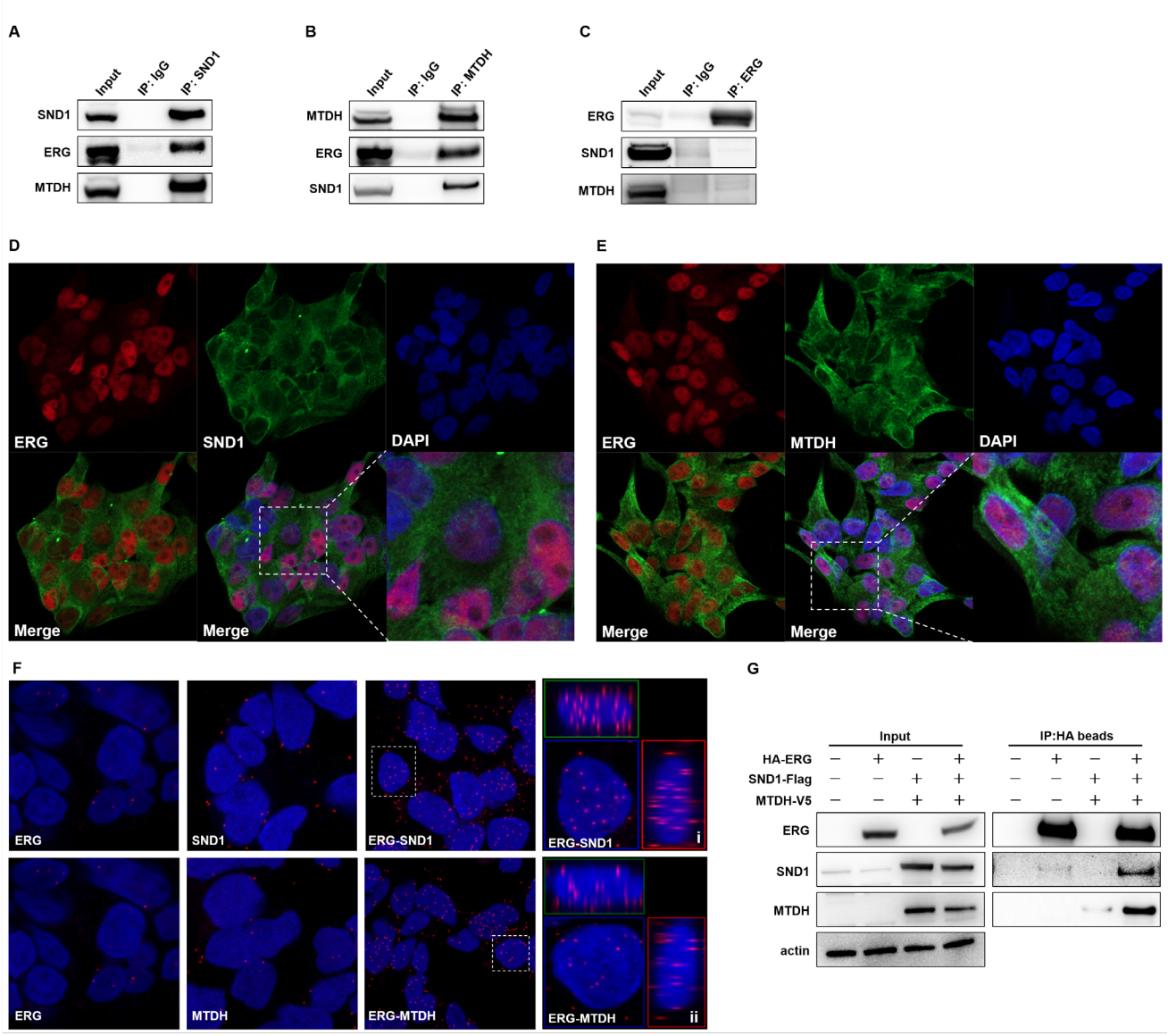

Figure 1. MTDH/SND1 protein complex interacts with ERG in VCaP prostate cancer cells. (A-C) Cell lysates from VCaP cells were subjected to IP using normal rabbit IgG, rabbit anti-MTDH (A), anti-SND1 (B) or anti-ERG (C) antibodies and protein lysates were analyzed by western blot analysis with indicated 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)**.

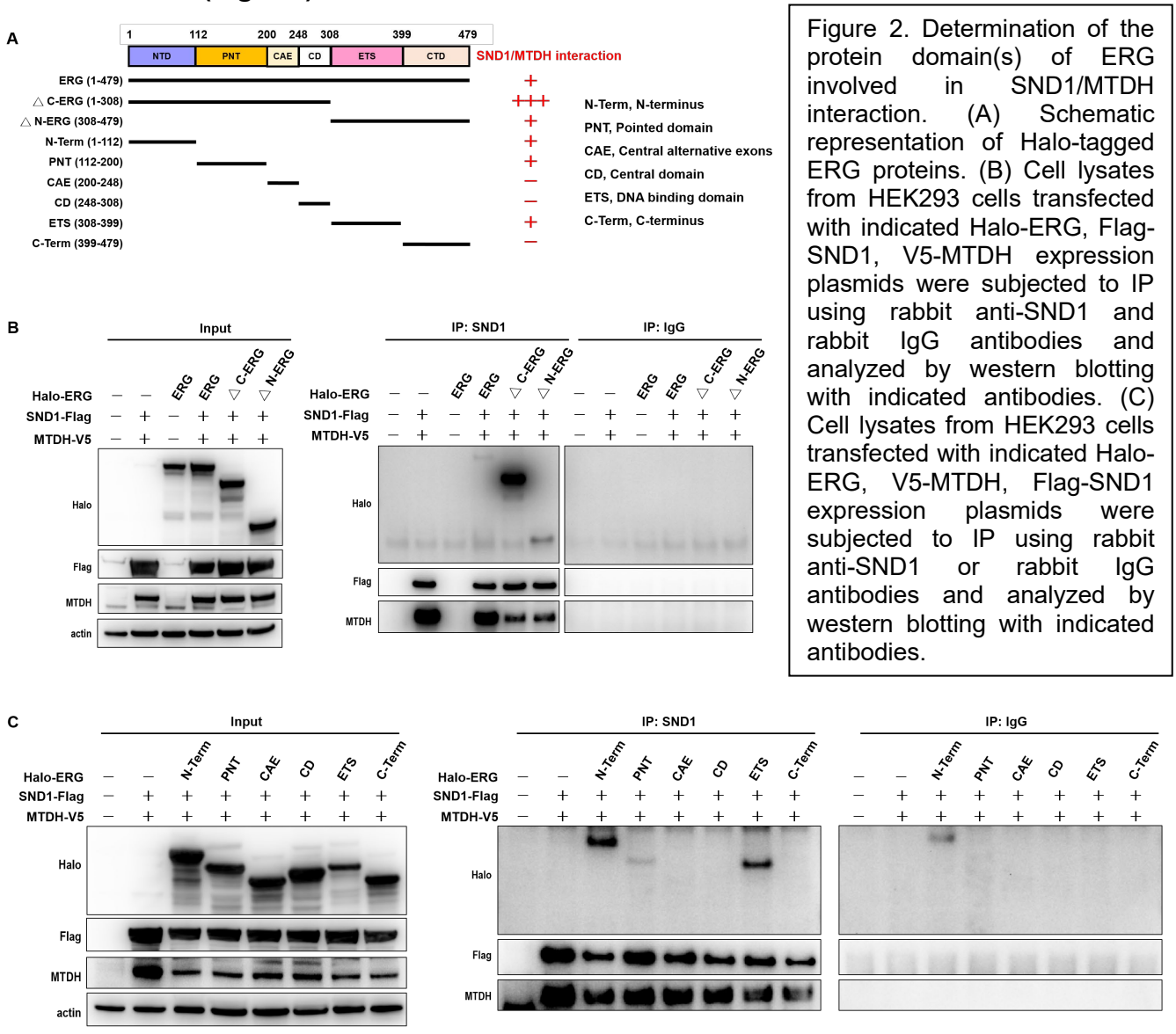

Figure 2. Determination of the protein domain(s) of ERG involved in SND1/MTDH interaction. (A) Schematic representation of Halo-tagged ERG proteins. (B) Cell lysates from HEK293 cells transfected with indicated Halo-ERG, Flag-SND1, V5-MTDH expression plasmids were subjected to IP using rabbit anti-SND1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

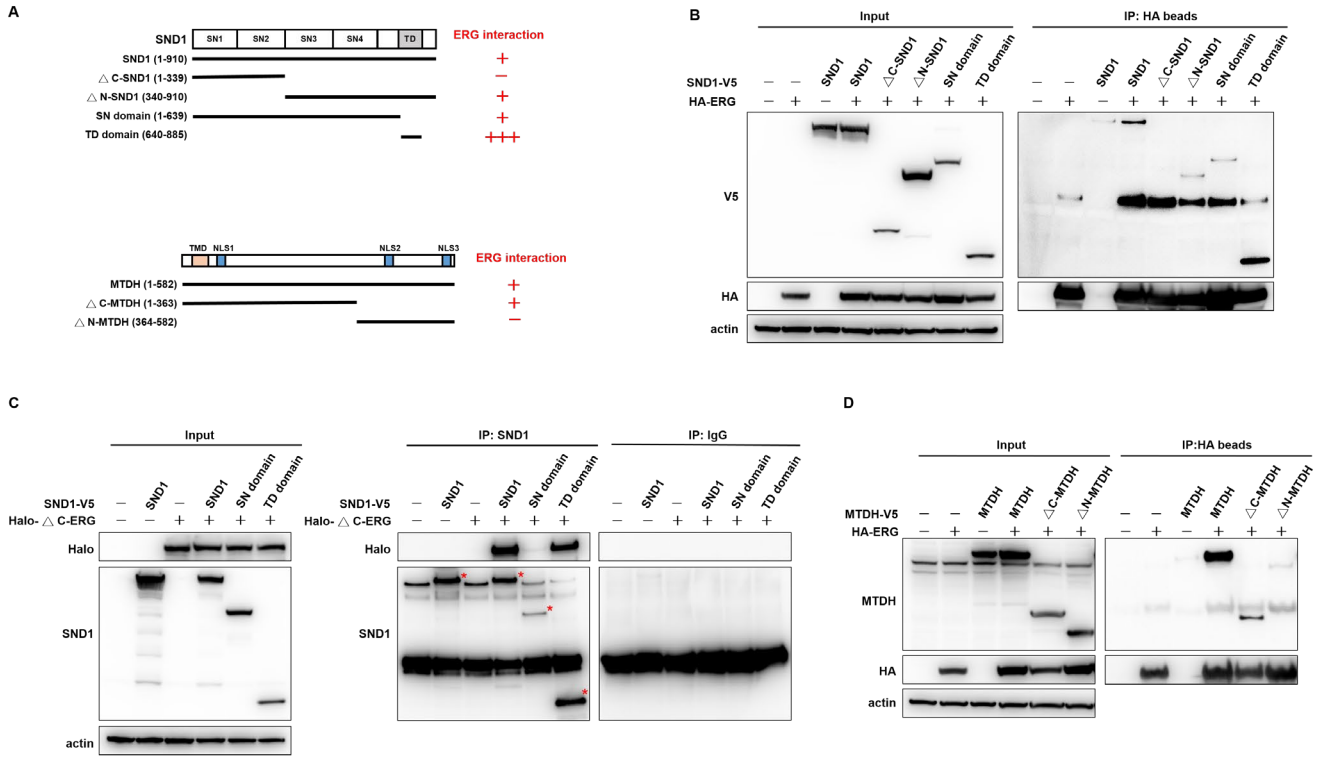

Figure 3. Determination of the protein domain of SND1/MTDH in mediating SND1/MTDH-ERG interaction. (A) Schematic representation of V5-tagged SND1, MTDH proteins. (B) Cell lysates from HEK293 cells transfected with indicated V5-SND1 and HA-ERG expression plasmids were subjected to IP using anti-HA agarose 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.

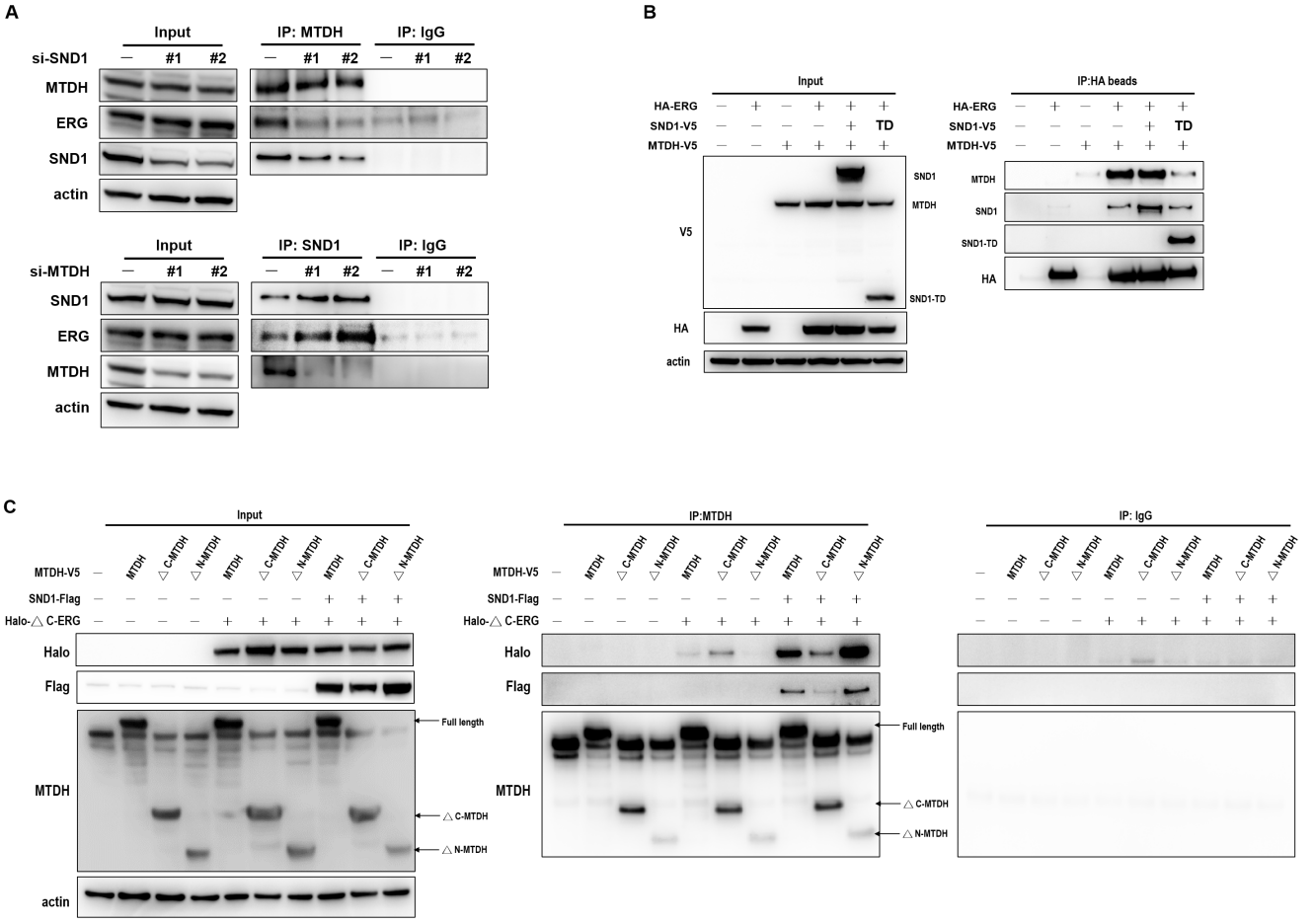

Figure 4. SND1 is required for ERG-MTDH interaction. (A) Cell lysates from VCaP cells transfected with indicated siRNA oligos were subjected to IP using normal rabbit IgG, rabbit anti-MTDH (upper panel) or anti-SND1 (lower panel) antibodies and protein lysates were analyzed by western blot analysis with 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) *In progress, 60% completed.* To determine the biological significance of ERG and MTDH/SND1 protein interaction in prostate tumorigenesis. I firstly analyzed the functional importance of SND1/MTDH in mediating ERG-associated malignant phenotypes in RWPE-1 cells. I used RWPE-1 immortalized human prostate epithelial cells to generate ERG gain of function cell model. After that, I generated lentiviral shRNA constructs targeting SND1 or MTDH and knocked down SND1 and MTDH in RWPE-1 cells overexpressing exogenous ERG (RWPE-ERG and control RWPE-Ctrl) cells **(Fig. 5A)**. I found that overexpression of ERG significantly promoted growth of RWPE-1 cells (RWPE-ERG) in the 3D organoid prostate culture system **(Fig. 5B)**. Importantly, SND1 and MTDH were necessary for this ERG-mediated phenotype,

because the knockdown of SND1 and MTDH using two independent small hairpin RNA (shRNA) constructs erased the growth differences between RWPE-Crtl and RWPE-ERG cells **(Fig. 5C-E)**. The impact of SND1 on ERG-mediated transforming phenotype is stronger than MTDH. Similar results were also observed in anchorage independent colony formation assays **(Fig. 5F)**. These data indicate that SND1 and MTDH were both necessary for ERG-mediated transformation of RWPE-1 cells. To determine if MTDH/SND1 gain of function (GOF) is sufficient for transformation, I generated SND1, MTDH overexpressing RWPE-Ctrl, RWPE-ERG cells. I will analyze these cell lines in my future experiments.

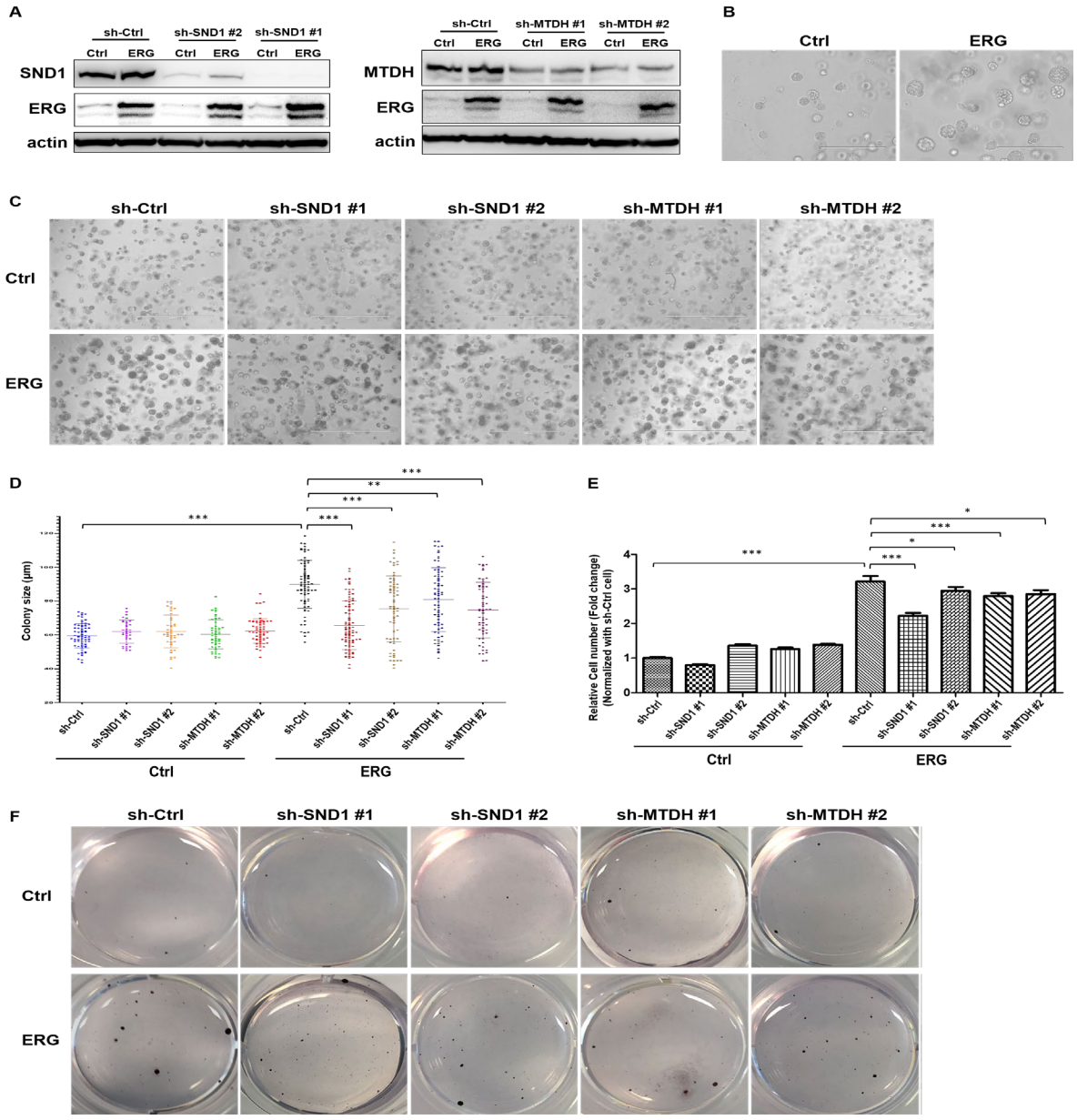

Figure 5. Role of SND1/MTDH in ERG-mediated transformation of RWPE-1 prostate epithelial cells. (A) Western blot analyses of RWPE-Ctrl (Ctrl) and RWPE-ERG (ERG) cells stably transduced with control pGIPZ, sh-SND1 or sh-MTDH lentiviral vectors and analyzed with indicated antibodies. (B-D) Brightfield 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) Cell proliferation of RWPE Ctrl and ERG cells with or without sh-SND1 and sh-MTDH colonies formed after 5 days in 3D organoid culture system and analyzed by CellTiter-Glo 3D cell viability assay. P-values determined using two-tailed Student's t-test. Data represent mean ± standard deviation (n = 3). (F) Representative image of RWPE Ctrl and ERG cells with or without sh-SND1 and sh-MTDH colonies in soft agar.

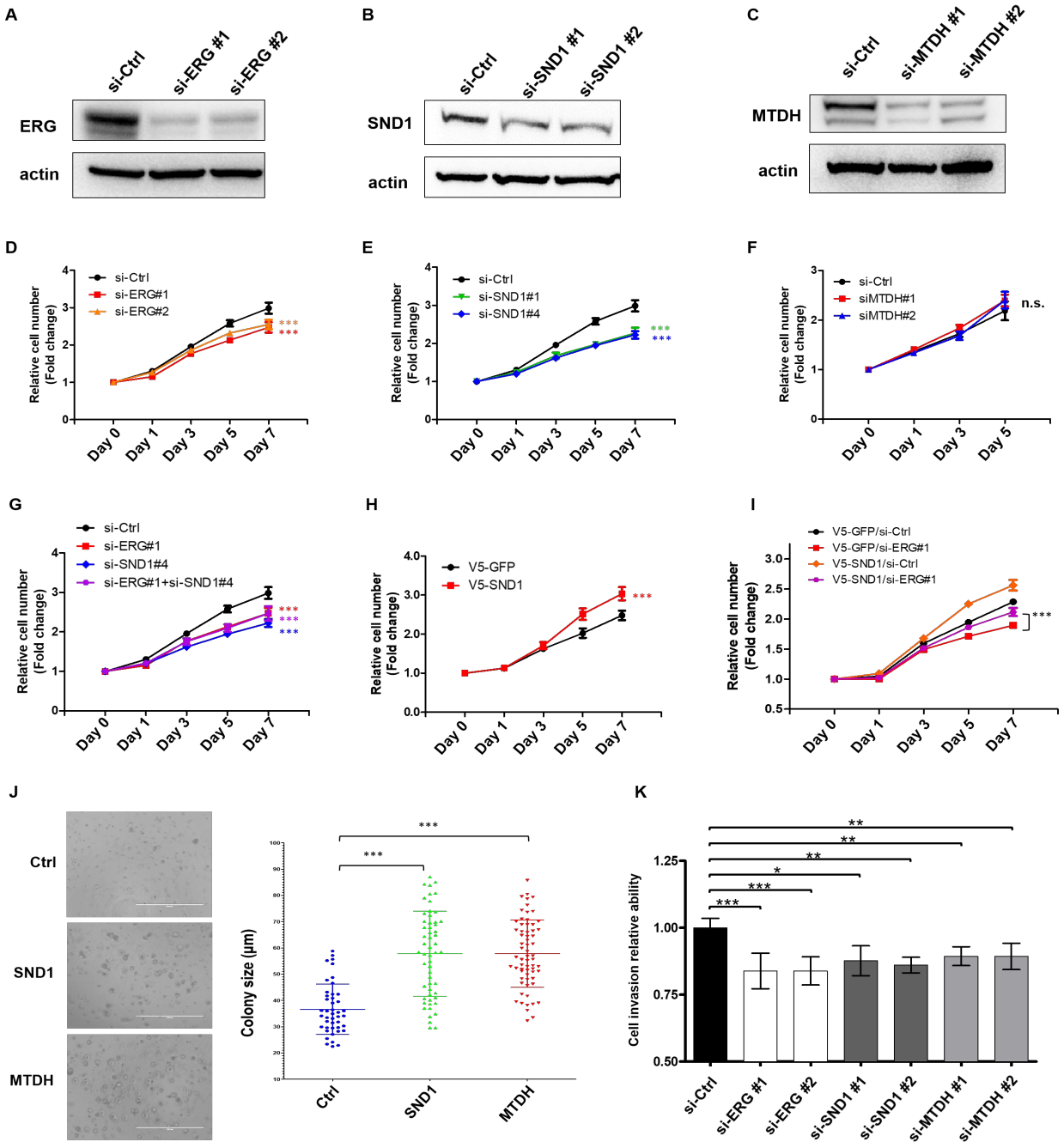

Figure 6. Role of SND1/ MTDH in mediating ERG-associated malignant phenotypes in VCaP cells. (A-C) Western-blot analyses of ERG, SND1, MTDH and actin expression in VCaP cells transfected with si-Ctrl, si-ERG, si-SND1 or si-MTDH oligos. (D-G) VCaP cells transfected with indicated siRNA oligos were grown 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). (J) Brightfield images and colony size quantitation of VCaP cells stably overexpressing with V5-GFP, V-SND1, V5-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. (K) VCaP cells were transfected with indicated siRNA oligos and invasion assays were performed using matrigel invasion assay kit. Invasion data were quantified and presented on the graph in arbitrary units with values in siCtrl cells adjusted to 1. P-values determined using two-tailed Student's t-test. Data represent mean ± standard deviation.

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. 6A-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. 6D-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. 6G)**. Moreover, I also found that overexpression of SND1 promoted cell proliferation in VCaP cells **(Fig. 6H)**. SND1 overexpression could rescue the effect of ERG knockdown on VCaP cell proliferation **(Fig. 6I)**. In addition, overexpression of SND1 and MTDH could promote growth of VCaP cells in the 3D organoid prostate culture system **(Fig. 6J)**. Furthermore, I found that the knocking down of either ERG, SND1 or MTDH decreased invasion ability of VCaP cells **(Fig. 6K)**. These results suggested that SND1/MTDH plays an important role in ERG-mediated prostate cancer.

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) In progress. To further evaluate the oncogenic effect of MTDH/SND1 in ERG-mediated prostate tumorigenesis *in vivo*, I will generate genetic mouse models with prostate-specific knockout of Snd1 either alone or in combination with GOF of ERG (PB-Cre/Snd1fl/fl, PB-Cre/ERG/Snd1fl/fl and PB-Cre/ERG/Ptenfl/fl/Snd1fl/fl mice). Mice will be monitored for tumor growth by palpitation and fluorescence imaging for TdTomato. Groups of 24 mice per single mutant Snd1 (Pb-Cre/Snd1fl/fl), double and triple mutant (PB-Cre/Ptenfl/fl/Snd1fl/fl and PB-Cre/ERG/Ptenfl/fl/Snd1fl/fl) will be generated, and prostates from 6 mice per genotype will be harvested at 4 and 8 months, and potentially longer (12-18 months). Conditional SND1 knockout mice have been generated and these mice are presently monitored for tumor development and used for primary cell isolation. I will collect prostate tissue from mice to perform RNA-Seq analysis and IHC staining.

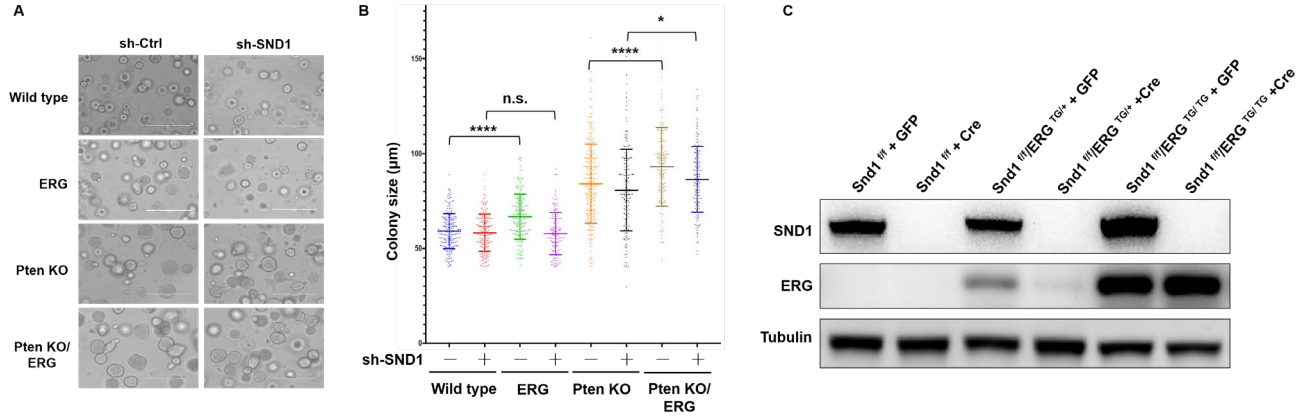

Figure 7. Role of SND1 in ERG-mediated transformation of *mouse prostate epithelial cell organoids*. (A-B) Brightfield images (A) and colony size quantitation (B) of mouse wild type, ERG, Pten KO and ERG/Pten KO cells transduced with control pGIPZ and sh-SND1 lentiviral vectors colonies formed after 5 days in 3D organoid culture system. The graph shows mean ± SD. Student's t test was used to determine statistical significance. (C) 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.

Task 4: Analyze the role of MTDH/SND1 using mouse prostate epithelial cell organoids (months 8-18) In progress. First, I isolated mouse primary prostate cells from wild type, *ERG* and *ERG/Pten* KO 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. Interestingly, the SND1 dependence is weakened in cells with combination of *ERG* GOF and loss of *Pten* **(Fig. 7A-B)**. 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 reveal high knockout efficiency of SND1 **(Fig. 7C)**. I will use these cell lines in my future studies.

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). In progress.

Task 5: *Perform RNA-Seq to analyze the role of MTDH/SND1 in ERG-mediated transcription. (months 4-18). In progress.* To determine the consequences of ERG/SND1/MTDH interaction on ERG-mediated transcription program, I performed the RNA-Seq using VCaP cells with siRNA-mediated knockdown of ERG, MTDH and SND1. Each gene was knocked down using 2 independent siRNA oligos and the efficiency of the knockdown was confirmed using qRT-PCR. SND1/MTDH function primarily as transcriptional activators. Indeed, I found that most differentially expressed genes in si-SND1 and si-MTDH cells were downregulated, with only a few genes upregulated. Importantly, RNA-Seq results showed a highly statistically significant overlap between genes positively regulated by ERG and MTDH/SND1 in VCaP cells. As much as ~1/3 of all ERG gene targets were also similarly regulated by SND1 or MTDH **(Fig. 8A-B)**. These results suggested that MTDH/SND1 proteins regulate similar to ERG transcriptional program. Next, I performed qRT-PCR experiments and validated the RNA-Seq data for several genes (FZD8, HPN) that were similarly downregulated by the knockdown of either ERG, SND1 or MTDH. **(Fig. 8C-F)**.

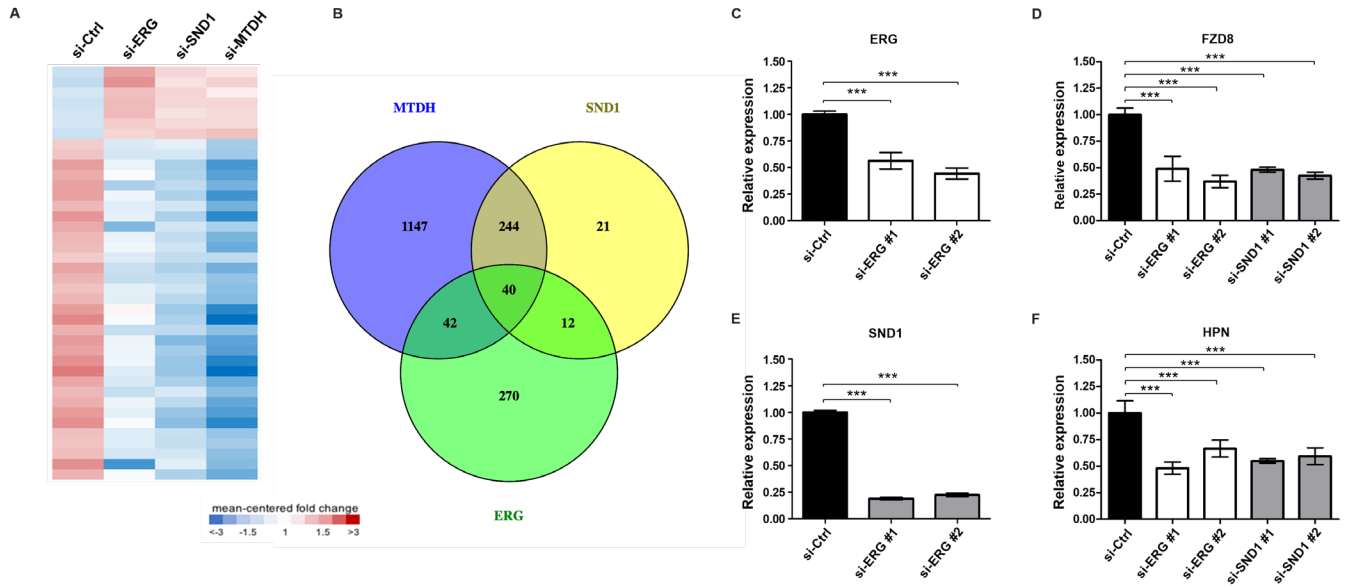

Figure 8. Significant concordance between ERG/MTDH/SND1 gene expression programs in VCaP cells. Gene expression determined by RNA-seq of VCaP cells transiently transfected with non-targeting siRNA (siCtrl, n = 2), siRNAs targeting ERG, MTDH and SND1 (using two independent siRNA oligos (A) Heatmap. (B) Overlap between significantly (Q < 0.05) downregulated genes. Two-tailed Chisquare was used to determine statistical significance in (B). (C-F) qRT-PCR analysis of expression of ERG/SND1 positively-regulated genes FZD8, HPN 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.

To determine whether ERG and SND1 regulate the same genes independently or in a concert with each other, I performed qRT-PCRs experiments to analyze the gene expression changes in VCaP cells with either a single knockdown of *ERG* or *SND1* or a combined knocking down of both *ERG* and *SND1*. The results showed that combined knockdown of *ERG* and *SND1* did not show additive effect on *FZD8* and *HPN* gene expression comparing to the single knockdown by either siERG or siSND1, suggesting that ERG and SND1 act in a concert and SND1 is necessary for ERG-mediated activation of some of its gene targets **(Fig. 9)**.

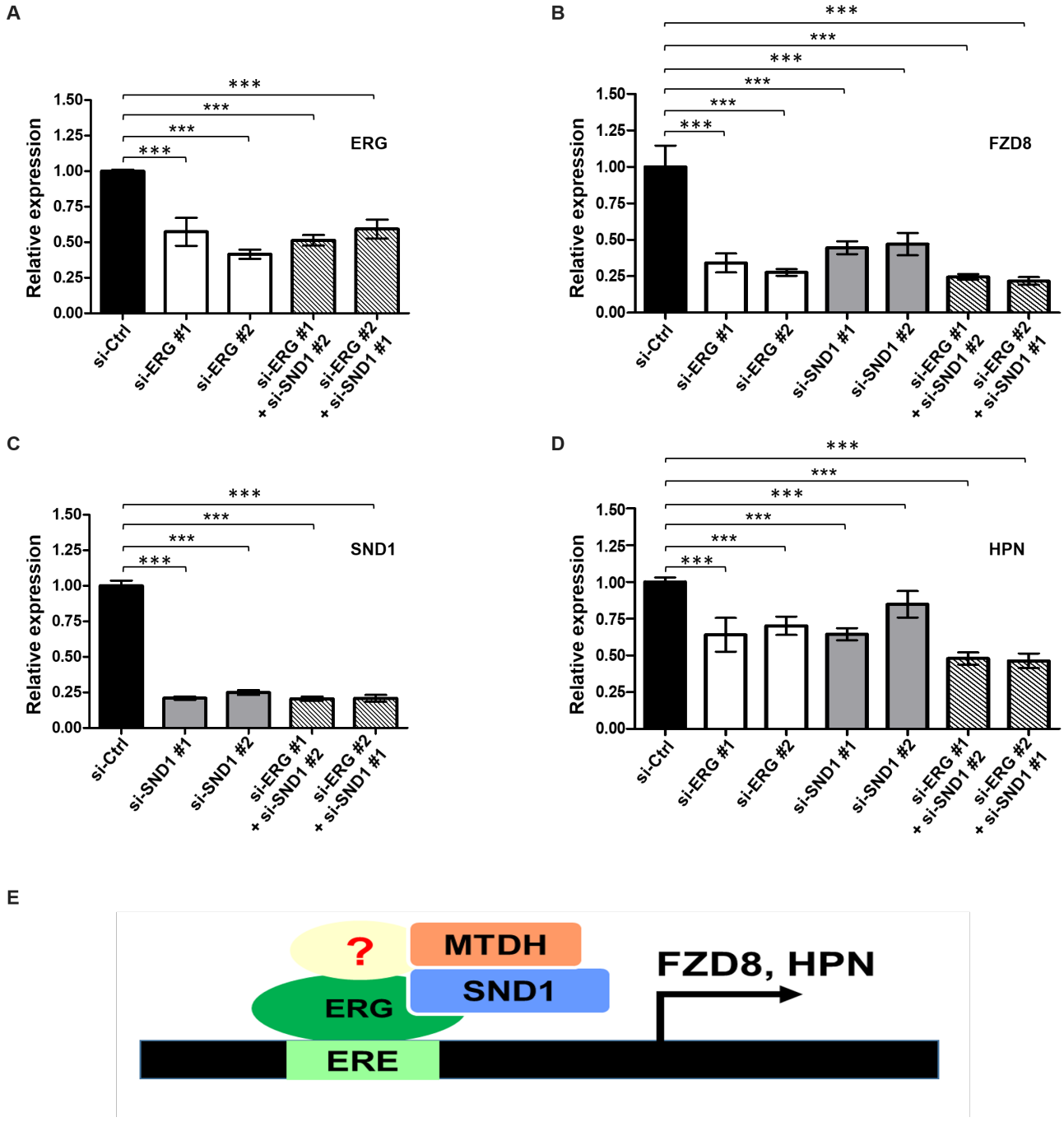

Figure 9. SND1 is necessary for ERG-mediated activation of its target genes. (A-D) qRT-PCR analysis of the expression of ERG/SND1 positively-regulated genes FZD8 and HPN in VCaP cells transfected with si-control (si-Ctrl) or si-ERG or si-SND1 oligos. Gene expression data were normalized using combined values for GAPDH, ACTIN, RPS16 housekeeping genes. P-values were determined using two-tailed Student's t-test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (E) Hypothetical model showing that ERG and SND1 form the transcriptional complex to regulate gene expression. ERE, ERG response element.

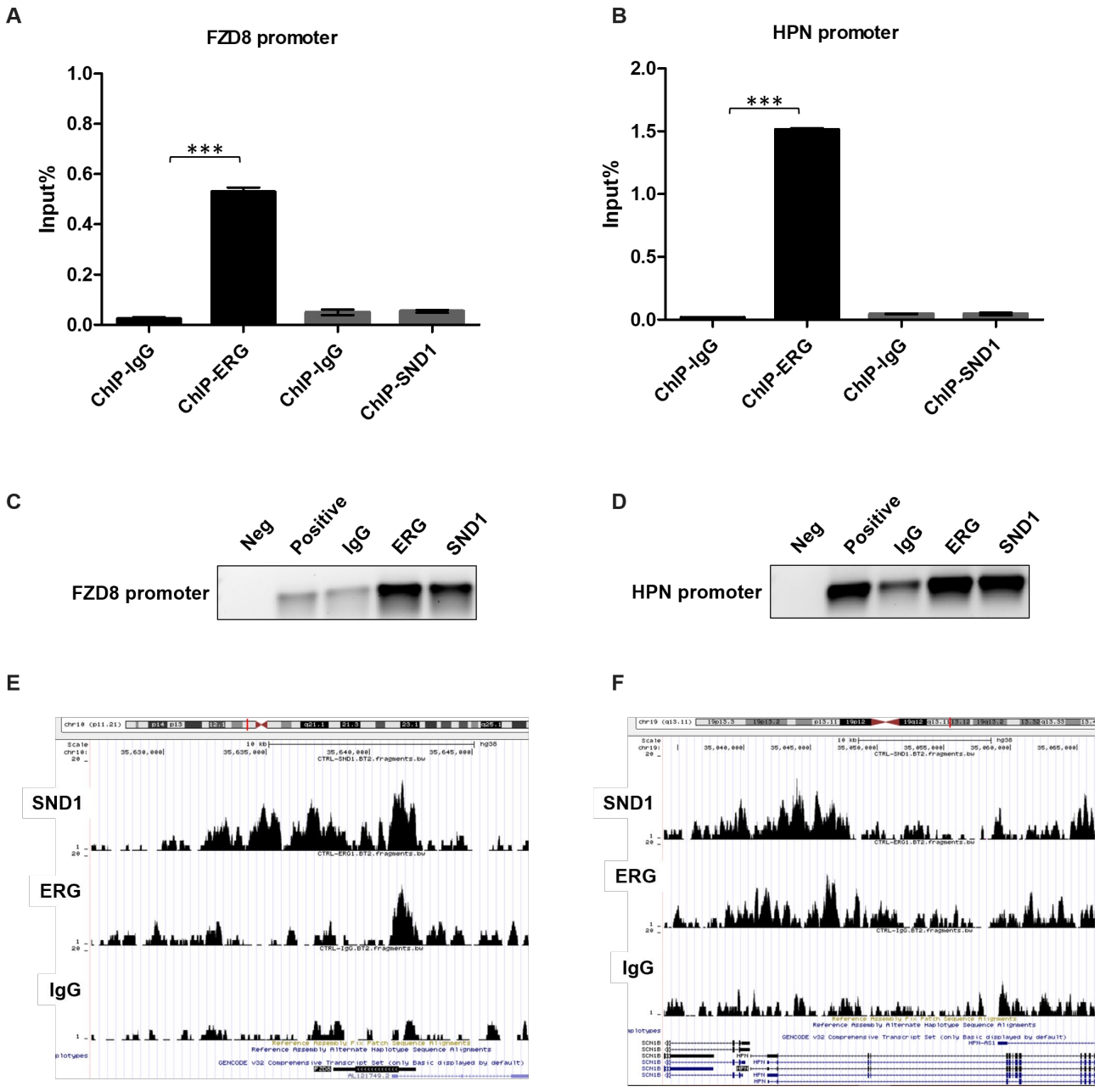

Figure 10. ERG and SND1 could bind to FZD8 and HPN promoter. (A-B) qPCR analysis of ChIP experiments from VCap cells using ERG and SND1 antibodies. Data represent mean ± SD. Student's t test was used to determine statistical significance. (C-D) CUT&RUN assay using anti-IgG, anti-ERG and anti-SND1 antibodies to analyze the binding of ERG and SND1 to FZD8 and HPN gene promoters in VCaP cells. PCR was performed to amplify FZD8 and HPN promoter regulatory elements. Water was used as a negative control and prostate cancer cell genomic DNA was utilized as a positive control. (E-F) UCSC genome browser views of CUT&RUN-Seq data from VCaP cells using IgG, anti-ERG and anti-SND1 antibodies

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) In progress. 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 *FZD8* and *HPN* promoter. The results showed that ERG binds to FZD8 and HPN promoters, whereas no SND1 interaction was detected in these assays **(Fig. 10A-B)**. 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 and Chip-Seq, I performed the 'cleavage under targets and release using nuclease" (CUT&RUN, and CUT&RUN-seq) experiments. CUT&RUN PCR results showed that both ERG and SND1 bind to the same DNA regulatory elements in *FZD8* and *HPN* gene promoters **(Fig. 10C-D)**. Moreover, I also performed CUT&RUN Seq to analyze the binding of ERG/SND1 to their target gene promotes. The experiments revealed partial overlap between ERG and SND1 peaks in VCaP cells **(Fig. 10E-F)**. These data suggested that ERG/SND1 could bind to same chromatin regions and cooperatively regulate gene expression.

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) Not yet started.

Task 7: To investigate whether ERG-positive PC cells are hypersensitive to SND1 inhibition using PC cell models. (months 12-16) Not yet started.

Task 8: To investigate whether ERG-positive PC cells are hypersensitive to SND1 inhibition using patient-derived metastatic PC xenograft models (months 15-24) Not yet started.

Opportunities for Training and Professional Development:

During this reporting period, I continue to meet with my mentors Drs. Valeri Vasioukhin and Peter Nelson to discuss study results and research goals. My co-mentors help me learn how to effectively communicate research findings and enhance my ability to write grant proposals and manuscripts, critically review research publications. I present 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 present and discuss 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.

My research project integrates 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 learn 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. Through attending the meetings, I will have many opportunities to interact with a diverse range of scientists and present my research via talks and posters to develop my presentation skills and receive the feedback on my research. In addition, I will attend a national conference at least once a year, to learn the most recent scientific advances and present my research finding. I will have many opportunities to interact with experts in cancer research that should help me broaden my horizons and build up the national and international networks.

Dissemination of Results:

The findings of this work have been shared locally with the Fred Hutch Prostate Cancer Program through the weekly lab meeting. I will plan to share additional results through the Friday night talks which is held at the FHCRC in the future.

Plan for the Next Reporting Period:

In the next reporting period, I will complete the functional analysis of the role of SND1/MTDH in ERG-mediated cell transformation and cancer progression using human cell lines in culture. I will continuously breed the genetic mice, monitor tumor growth in mice and isolate the mouse prostate primary cells to reveal the role of Snd1 in autochthonous models of ERG-mediated PC *in vivo.* I will collect the mouse prostate tissues and perform the RNA-Seq analysis to determine the *in vivo* role of Snd1 in ERG-mediated prostate tumorigenesis. To further identify the ERG and SND1 chromatin regulatory regions, I will perform the CUT&RUN Seq analysis to examine if there is the overlap between ERG and SND1 chromatin binding elements and determine how ERG/SND1 corporately regulate their target gene expression. To determine whether ERG-positive prostate cancer is hypersensitive to SND1 inhibition, I will analyze the significance of siRNA-and drug-mediated inhibition of SND1 alone or in combination with existing therapeutic modalities such as docetaxel in eradication of ERG-dependent and ERG-independent metastatic PC. I will analyze sensitivity of human metastatic PC tumors to SND1 inhibition using ERG-positive and ETS-negative LuCaP patient-derived PC xenograft models. I will treat LuCaP96 (ERG-negative) and LuCaP35 (ERG-positive) xenografts with SND1 inhibitor pdTp, docetaxel or combination of pdTp and docetaxel.

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

None.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name:	Sheng-You, Liao
Project Role:	*Project PI*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	12
Contribution to Project:	Dr. Liao designs and performs the experiment and data analysis for this project
Funding Support	W81XWH-20-1-0082

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.

APPENDICES

None.