**Distribution Agreement**

In presenting this thesis as a partial fulfillment of the requirements for a degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis in whole or in part in all forms of media, now or hereafter now, including display on the World Wide Web. I understand that I may select some access restrictions as part of the online submission of this thesis. I retain all ownership rights to the copyright of the thesis. I also retain the right to use in future works (such as articles or books) all or part of this thesis.

Kathy Pei Li April 3, 2019

# KLF5 activates ALPP to promote the proliferation of AR-null prostate cancer cells

by

Kathy Pei Li

Dr. Jin-Tang Dong

Adviser

Biology Department

Dr. Jin-Tang Dong

Adviser

Dr. Gregg Orloff

Committee Member

Dr. Kevin Karnes

Committee Member

2019

# KLF5 activates ALPP to promote the proliferation of AR-null prostate cancer cells

By

Kathy Pei Li

Dr. Jin-Tang Dong

Adviser

An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Sciences with Honors

Biology Department

2019

Abstract

KLF5 activates ALPP to promote the proliferation of AR-null prostate cancer cells
By Kathy Pei Li

Prostate cancer is the second most lethal type of cancer for men in the United States, claiming almost 30,000 lives annually. Most prostate cancers initially depend on the presence of androgens for cell survival and proliferation, making them targetable by androgen deprivation therapy. However, prostate cancer at later stages often develop into androgen independent, including androgen receptor (AR) pathway-independent, variants, which are resistant to hormonal therapy and no other agents have yet been shown to be effective targets for treatment of these tumors. Ultimately, our goal is to identify prospective novel targets for the development of targeted therapies in AR pathway-independent prostate cancer to improve patient survival. Krüppel-like Factor 5 (KLF5) is a transcription factor that is expressed at lower levels but is rarely mutated in prostate cancers. In this study, we investigated the Placental Alkaline Phosphatase (*ALPP*) gene as a potential transcriptional target KLF5. RT-PCR and western blotting showed ALPP upregulation by KLF5. ChIP PCR and sequencing suggested that KLF5 bound directly to the promoter region of *ALPP*. To characterize the effects of the KLF5/ALPP axis on cell growth and chemosensitivity, we performed colony formation and cytotoxicity assays and found that ALPP was involved in cell proliferation, and that knockdown of *ALPP* reduced the pro-proliferative effects of KLF5. While KLF5 knockout also caused a decrease in cell proliferation and colony formation, such a decrease was rescued by ALPP overexpression. Preliminary cytotoxicity assays also suggest that ALPP expression may sensitize AR-null prostate cancer to docetaxel treatment. These results suggest that KLF5-regulated ALPP promotes cell growth and is involved in chemosensitivity.

# KLF5 activates ALPP to promote the proliferation of AR-null prostate cancer cells

By

Kathy Pei Li

Dr. Jin-Tang Dong

Adviser

A thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Sciences with Honors

Biology Department

2019

Acknowledgements

I would like to thank Dr. Dong for taking me into his lab and being a wonderful and supportive mentor for the past 3 years. I would like to thank Yixiang Li, Dr. Baotong Zhang, and Dr. Jamie King for being incredible mentors, teachers, and friends to me. I look up to you all as models for the kind of scientist and person I strive to become. I appreciate your immense patience and generosity with me.

I would also like to thank Drs. Jenny Ni, Ran Tao, and Lin Xie for making my time in lab truly enjoyable. You have become my second family.

I would like to thank Dr. Orloff and Dr. Karnes for taking the time to be a part of my committee as well as for being two of the most engaging professors I've had at Emory. I've truly enjoyed my time in each of your classes.

Finally, I would like to thank my parents for their unconditional love and support, and my roommates, Megan Jiang, Mone Anzai, and Elshaddai Girma, for keeping me sane and well fed through this process.

Table of Contents

Figures and Tables Table of Contents

## Introduction

Prostate cancer (PCa) is the second most common cause of cancer-related death among men in the United States. The American Cancer Society projects almost 175,000 new cases of PCa in 2019, which will account for almost 20% of all new cancer diagnoses in American males, as well as 31,620 deaths in 2019 [1]. Both healthy prostate cells and prostate cancer cells rely on active androgen receptor (AR) signaling cascade for cell proliferation and survival. This occurs when AR is bound by androgens, including testosterone, and acts as a transcription factor to regulate many genes [2]. In their beginning stages, most forms of prostate cancer are responsive to and treated with androgen deprivation therapy (ADT), which interrupts AR signaling and causes prostate cancer cell apoptosis. However, after extended periods of ADT, these cancers often develop AR signaling pathway-independence through one or multiple mechanisms. Prostate cancers may acquire mutations in AR and/or other genes in the AR signaling pathway to keep AR signaling activated regardless of exogenous androgen levels [3]. PCa may also develop an AR-null phenotype, meaning that they are no longer dependent on AR signaling for survival. These carcinomas have been shown to possess with increased activity of MAPK and EGF signaling pathways [4]. AR pathway-independent prostate cancers are aggressive and resistant to ADT. Patients with this type of prostate cancer usually have poor prognoses as there are currently no effective therapies, although some chemotherapies can prolong a patient's life [2]. Therefore, it is important to identify novel therapeutic targets for AR pathway-independent PCa.

The zinc finger transcription factor Krüppel-like Factor 5 (KLF5) acts as both a tumor suppressor and promoter of PCa, even though *KLF5* is often down-regulated or deleted in advanced PCa [5, 6]. In our previous studies, loss of KLF5 induced EMT and obvious apoptotic signaling, and dramatically inhibited sphere formation and tumor growth in two AR-null prostate

cancer cell lines PC-3 and DU 145. Through RNA-seq and ChIP-seq analyses, we have identified two prospective genes that may be under direct KLF5 regulation: placental alkaline phosphatase (*ALPP*) and family with sequence similarity 83 member A (*FAM83A*).

*FAM83A* is upregulated in several cancers, including prostate, pancreatic, and lung [7]. Previous studies have indicated that the FAM83 proteins may be involved in MAPK signaling and EMT in cancer cells, therefore serving as potential novel therapeutic targets for therapy resistant cancers [8]. In both pancreatic and breast cancer cells, silencing or down-regulation of FAM83A lead to decreased cell proliferation and increased sensitivity to chemotherapy and EGFR therapy respectively [9, 10].

A member of the ALP family of genes, ALPP is produced in the placenta and plays a role in fetal development [11]. Interestingly, the gene is also found to be overexpressed in some cancers, including prostate cancer. A previous study has shown that knock-down of ALPP in an androgen-independent mutated PCa cell line led to significant decrease in cell proliferation [12].

Our efforts are aimed towards examining the relationship between KLF5 and ALPP and the role of KLF5/ALPP axis AR-null prostate cancer cells. We examined protein-DNA interaction as well as trends in mRNA and protein level changes to ALPP in order to determine a direct KLF5 transcriptional regulation of *ALPP*. We also evaluated the effects of KLF5, TGF-β, and ALPP on cell proliferation, colony formation and chemosensitivity. Our results showed that knockdown of ALPP significantly stunts cell growth and contributes to chemoresistance. It also suggests that ALPP expression is important for cell proliferation and colony formation and may also increase chemosensitivity of AR-null prostate cancer cells.

## Results

### Identification of candidate genes under KLF5 regulation

KLF5 has been shown to regulate a wide variety of genes involved in many processes including cell proliferation, development, epithelial-mesenchymal transition (EMT), and apoptosis [13]. In order to identify potential genes that are under transcriptional activation by KLF5, we performed RNA sequencing (RNA seq.) and ChIP sequencing (ChIP seq.). We collected samples from PC-3 Parental, endogenous KLF5 knockout (P), and KLF5 overexpressing (K) groups. Due to low endogenous levels of KLF5 in PC-3 cells, ChIP assay is not viable for PC-3 samples. RNA sequencing was performed in duplicates on PC-3 samples and analyzed for significance with FDR adjusted p-value $< 0.01$, comparing parental to P and P to K. ChIP samples were obtained from DU 145 P and K groups with KLF5 antibody. Both RNA seq. and ChIP seq. were performed on DU 145 samples with only one replicate, and data was analyzed with p-value $< 0.05$. 8 genes showed significant differences in expression level when comparing KLF5 manipulated groups in all experiments. They are *ALPP, ELF3, FAM83A, LINC01133, PI3, PLEKHA7, SLPI*, and *KLF5* itself. Results of ChIP sequencing also showed KLF5 binding along the genome in DU 145 cells, one region being the promoter region of *ALPP* (Fig.1c).

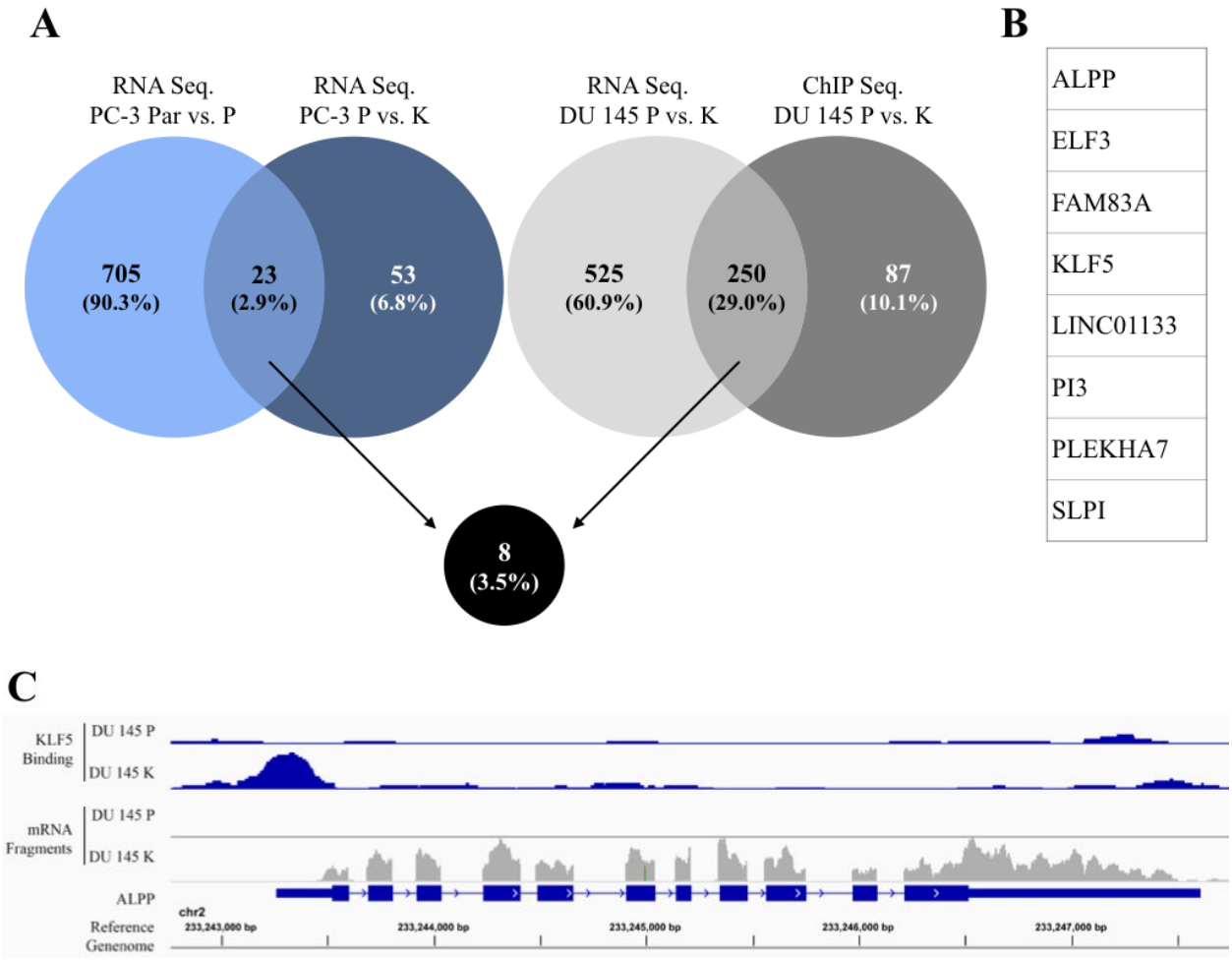



**Figure 1. ChIP & RNA Sequencing Results.** Panel A summarizes the results of RNA and ChIP seq performed on PC-3 Parental, P and K and DU 145 P and K groups. 8 genes show significant changes in expression level under all 4 conditions and they are listed in panel B. ChIP seq. with KLF5 antibody shows KLF5 binding in the promoter region of ALPP in DU 145 cells when KLF5 is expressed, and no binding when no KLF5 is expressed.

**Quantification of expression levels of 7 candidate genes**

We utilized Real-Time PCR (RT-PCR) to further narrow the pool of candidate genes. We conducted PCR on RNA samples from PC-3 and DU 145 Parental, P, and K groups for the 8 genes listed previously and GAPDH as an endogenous control. We ran the samples in triplicates and compared the fold changes of RNA expression level of each gene between Parental and P groups or P and K groups. ALPP showed among the highest fold changes for all experimental

conditions and FAM83A showed high fold changes in PC-3 groups. Both genes were downregulated in P groups and expression recovered in K groups (Fig. 2e-f). Consequently, we selected *ALPP* and *FAM83A* for the focus of further experiments.

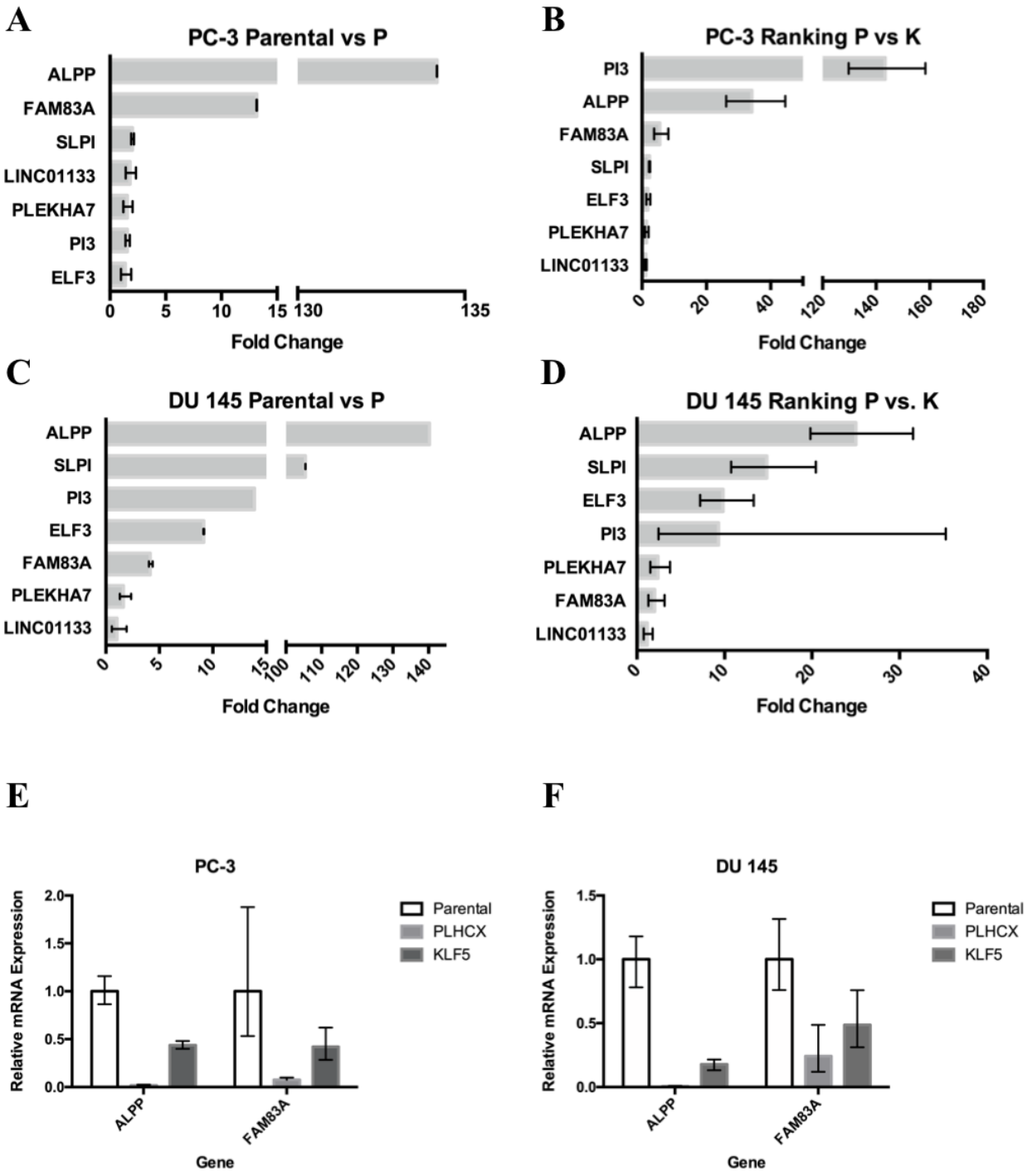


**Figure 2. RT-PCR Results.** RT-PCR was conducted using samples collected from PC-3 and DU 145 Parental, P, and K groups run in triplicates with only one experimental repeat. GAPDH was used as an endogenous control. **(A, C)** Fold changes of 7 candidate genes were compared between cells expressing endogenous KLF5 (Parental) and KLF5 knockout cells (P). ALPP shows the greatest fold change in both PC-3 and DU 145 groups. FAM83A and SLPI show high fold change in PC-3 and DU 145 groups respectively. **(B, D)** Fold changes were compared between P group and exogenous KLF5 overexpressing group (K). ALPP and FAM83A also show high fold changes in these groups. **(E, F)** mRNA expression level of ALPP and FAM83A in Parental, P and K groups in PC-3 and DU 145 are shown. The general trend shows that both ALPP and FAM83A have lower mRNA expression levels when endogenous KLF5 is knocked out and both genes' expression levels increase when exogenous KLF5 is expressed.

**KLF5 may bind to *ALPP* gene region and upregulate expression.**

Per PCR results, we expect that ALPP and FAM83A protein levels will increase with increased KLF5 expression. To test this hypothesis, we conducted western blotting and analyzed samples for KLF5, ALPP, FAM83A, and β-Actin, which served as a loading control. We first used anti-KLF5 antibody to validate the KLF5 constructs in Parental, P and K groups. Exogenous KLF5 protein has a slightly higher molecular weight than endogenous KLF5 due to an added flag tag, which can be seen in figure 3a. The faint protein band seen in PC-3 PLHCX group is of the same molecular weight as exogenous KLF5, possibly suggesting that a small amount of contamination from the PC-3 KLF5 group may have occurred during cell passaging.

Western blotting with anti-FAM83A was not successful, with high background noise and ambiguity surrounding the correct molecular weight the protein band was expected to be observed at. Though the known molecular weight of FAM83A protein is ~47 kDa, the antibody manufacturer indicated an expected band above ~55 kDa and other sources showed FAM83A protein bands at ~28 kDa and ~65 kDa. We therefore were not able to ascertain what size to expect visualization of a FAM83A protein band and no strong bands were produced by the western blot. We also probed PC-3 and DU 145 samples for ALPP protein expression. In PC-3 cells, there is low protein expression of ALPP in Parental cells, slightly higher expression in P

cells, and highest expression in the K group. It may be possible that the low expression of ALPP seen in P group can be attributed to the low expression of exogenous KLF5 as seen in figure 3a. In DU 145, low levels of ALPP expression are shown on the western blot, while the K group showed decreased expression. However previous western blots by a lab member showed a strong protein band for DU 145 K cells. We hypothesize that the discrepancy may be a result of high passage number of my cells, resulting in a compensation effect on pathways involving KLF5.

In order to assess KLF5 binding to *ALPP* or *FAM83A* genomic regions, we used ChIP PCR. A lab member designed ALPP and FAM83A primers using Primer Premiere (listed in Table 1) and assessed the efficiency of each primer using whole genome ChIP samples. ALPP primer #2 and FAM83A primer #5 were chosen to use for subsequent experiments. We pulled down DNA sequences from DU 145 P and K samples with anti-KLF5 antibody and performed two rounds of ChIP PCR in triplicates. Our results show a trend of slightly higher fold enrichment of ALPP in the K group than P group, which would indicate that KLF5 does bind to ALPP gene region. The fold enrichment for FAM83A is about equal, if not slightly higher in the K group. However, unpaired two-tailed student t-test with a 95% confidence interval showed no statistical significance in KLF5 binding for either ALPP or FAM83A genomic regions between P and K groups. High error may confound statistical analysis.

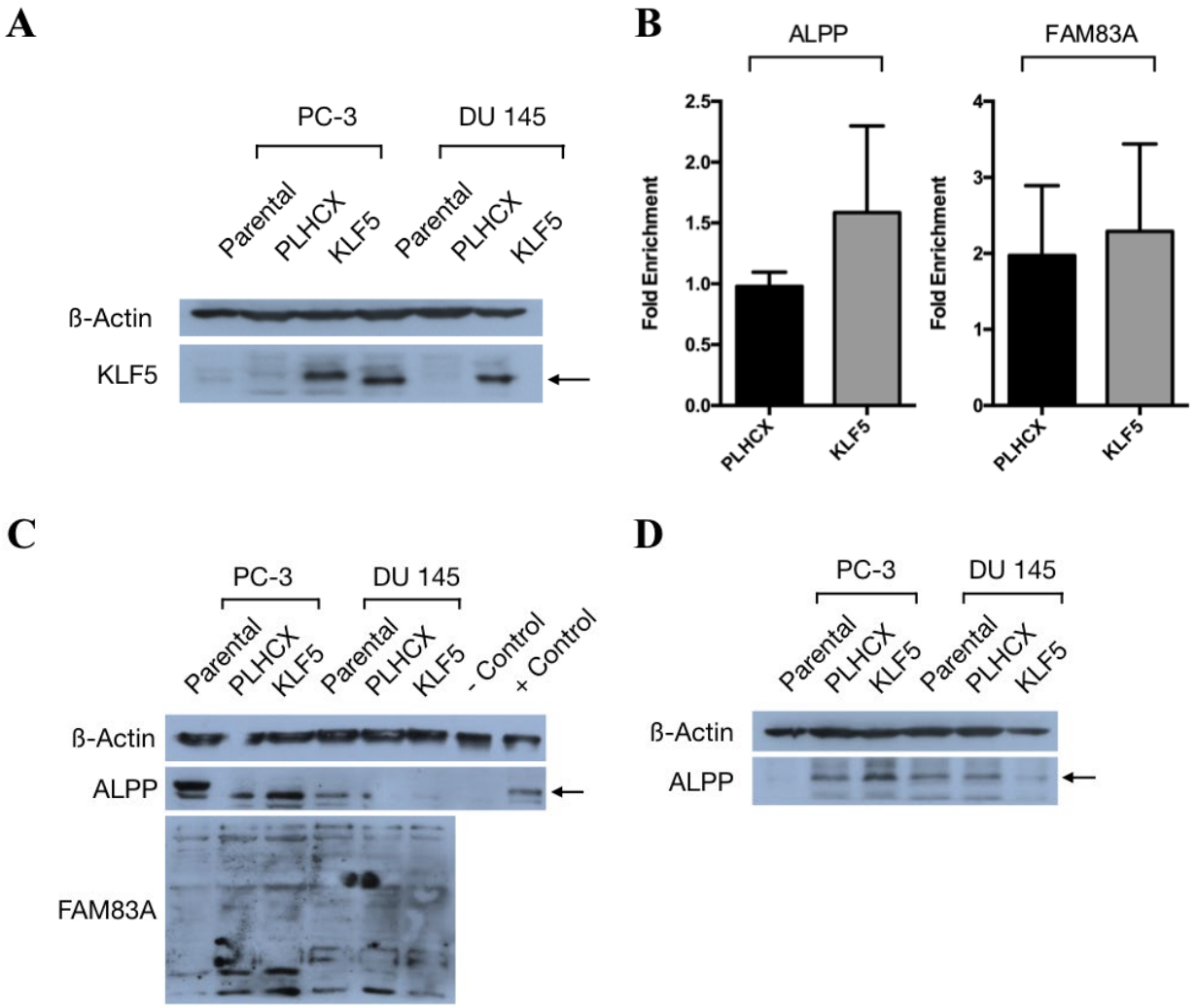



**Figure 3. KLF5 regulation of ALPP.** Western blotting was performed using protein samples obtained through direct lysis with Laemmli buffer from PC-3 and DU 145 Parental, P, and K samples. **(A)** KLF5 construct validation in PC-3 and DU 145 Parental P and K cells. **(B)** DU 145 P and K samples were pulled down with anti-KLF5 antibody and probed for ALPP and FAM83A sequences. ChIP PCR showed no significant difference in KLF5 binding at ALPP or FAM83A genomic regions between KLF5 knockout and KLF5 overexpressing groups (p=0.49 and p=0.84 respectively). Low levels of endogenous KLF5 in PC-3 samples make it inviable for ChIP assays. **(C)** Western blotting with anti-FAM83A probe. B-actin served as a loading control but was burned because of too high signal during film development. Bubbles in lanes 2 and 5 resulted in incomplete bands. **(C, D)** ALPP shows increase in expression in PC-3 K group when compared to either Parental or P group. However, in DU 145, ALPP decreased in expression level in the K group when compared to both Parental and P groups.

**KLF5 promotes cell proliferation and contributes to maintenance of epithelial status**

Previous studies have shown that KLF5 is responsible for increased cell proliferation and knockout of KLF5 significantly reduced sphere growth and promoted EMT [14]. In order to investigate KLF5's effect on sphere growth and cell proliferation we utilized colony formation assay. Knockout of endogenous KLF5 expression (P) cells showed dramatic changes to cell morphology in PC-3 cells but not in DU 145. PC-3 Parental cells form very rounded and compact colonies. The colonies formed by PC-3 P cells are fewer in number and more spread out than those formed by Parental cells. Individual PC-3 P cells show increasingly mesenchymal phenotypes, with cells visibly elongated and migrating outwards from spheres. PC-3 K cells form significantly larger spheres than both Parental and P and cells are closer in morphology to the parental group than the P group. However, some individual KLF5 cells are still seen with elongated phenotypes. We hypothesize that this may be attributed to the loss of KLF5 expression in some cells despite retention of Hygromycin B selection marker.

We did not observe such dramatic morphological difference between groups in DU 145 cells. Cells from all groups are generally rounded in morphology, though some DU 145 PLHCX cells did show a slightly more elongated morphology. Similar to PC-3 K cells, DU 145 K cells also grew significantly larger colonies than the P group. However, no significant differences were seen in colony number and size between parental and K groups. The DU 145 P group however, formed less colonies overall than parental cells. Results from PC-3 and DU 145 suggest that KLF5 contributes to the maintenance of an epithelial phenotype and promotes cell proliferation while knocking out KLF5 expression stunts cell proliferation and colony formation as well as facilitates EMT.

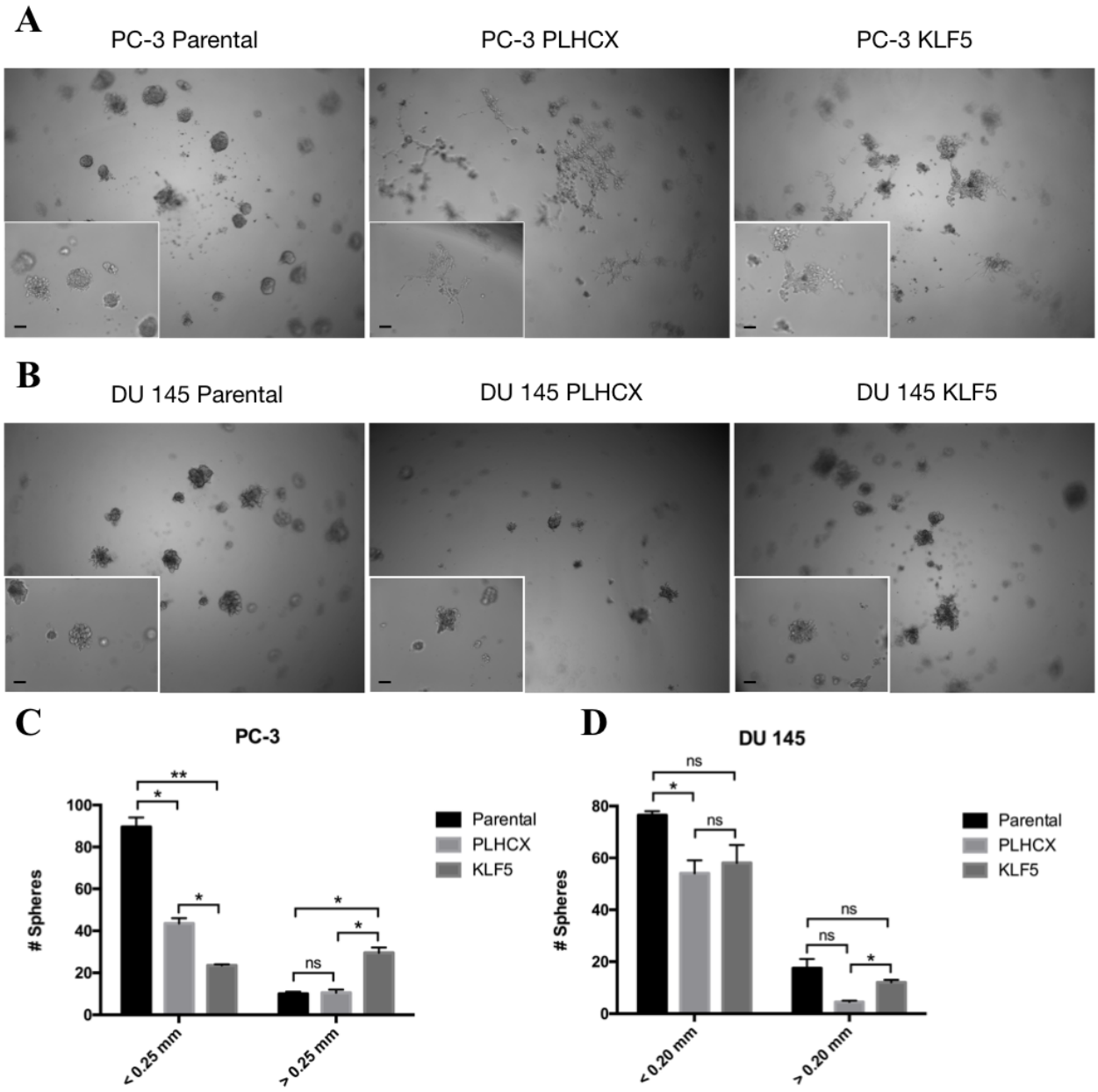



**Figure 4. Effect of KLF5 on cell proliferation and colony formation**. PC-3 and DU 145 Parental, P and K cells were plated in matrigel coated 96-well plates at a density of 300 cells per well. PC-3 groups were imaged at 4x and 10x magnification and counted at day 8 and DU 145 groups, which grow slower, were assessed at day 16. Scale bar represents 100 μm on both large and small images. Colony size was measured using a hemocytometer. Diameter thresholds used for counting colonies were 0.25 mm for PC-3 group and 0.20 mm for DU 145 group. *$p < 0.05$, **$p < 0.01$, ns = no significance.

**ALPP is necessary for cell proliferation and colony formation**

To investigate ALPP's effects on cell proliferation and colony formation, we conducted another colony formation assay using transient ALPP manipulated cells. Because of heavy workload and time constraints, we decided to move forward with functional assays using only one cell line. We chose PC-3 cells because they showed a greater difference in KLF5-related ALPP expression via PCR and western blotting, as well as more contrasting results from the colony formation assay than seen with DU 145. We transfected PC-3 Parental and K cells, which express KLF5, with a retroviral vector containing ALPP targeted shRNA (shALPP), and PC-3 P cells, KLF5 knockout cells, with a retroviral vector containing an ALPP gene insert (ALPP). Transfection with PLKO.1 empty vector served as a control.

In comparison to Parental control cells, control cells with knockout of KLF5 resulted in the formation of slightly fewer number of colonies. After overexpressing ALPP in these KLF5 knockout cells, cell proliferation and colony formation increased dramatically. Comparison between KLF5 knockout control cells and KLF5 overexpressing control cells revealed a slight increase in number of colonies formed as wells colony size in the latter group. Addition of shALPP to the KLF5 overexpressing group reverted the pro-proliferative effects of ALPP, causing little to no colony formation and very reduced cell growth. These results indicate that colony formation promoted by KLF5 expression was suppressed after knockdown of ALPP. On the other hand, KLF5 knockdown lead to a decrease in sphere formation and cell proliferation, a phenotype that was rescued by ALPP overexpression.

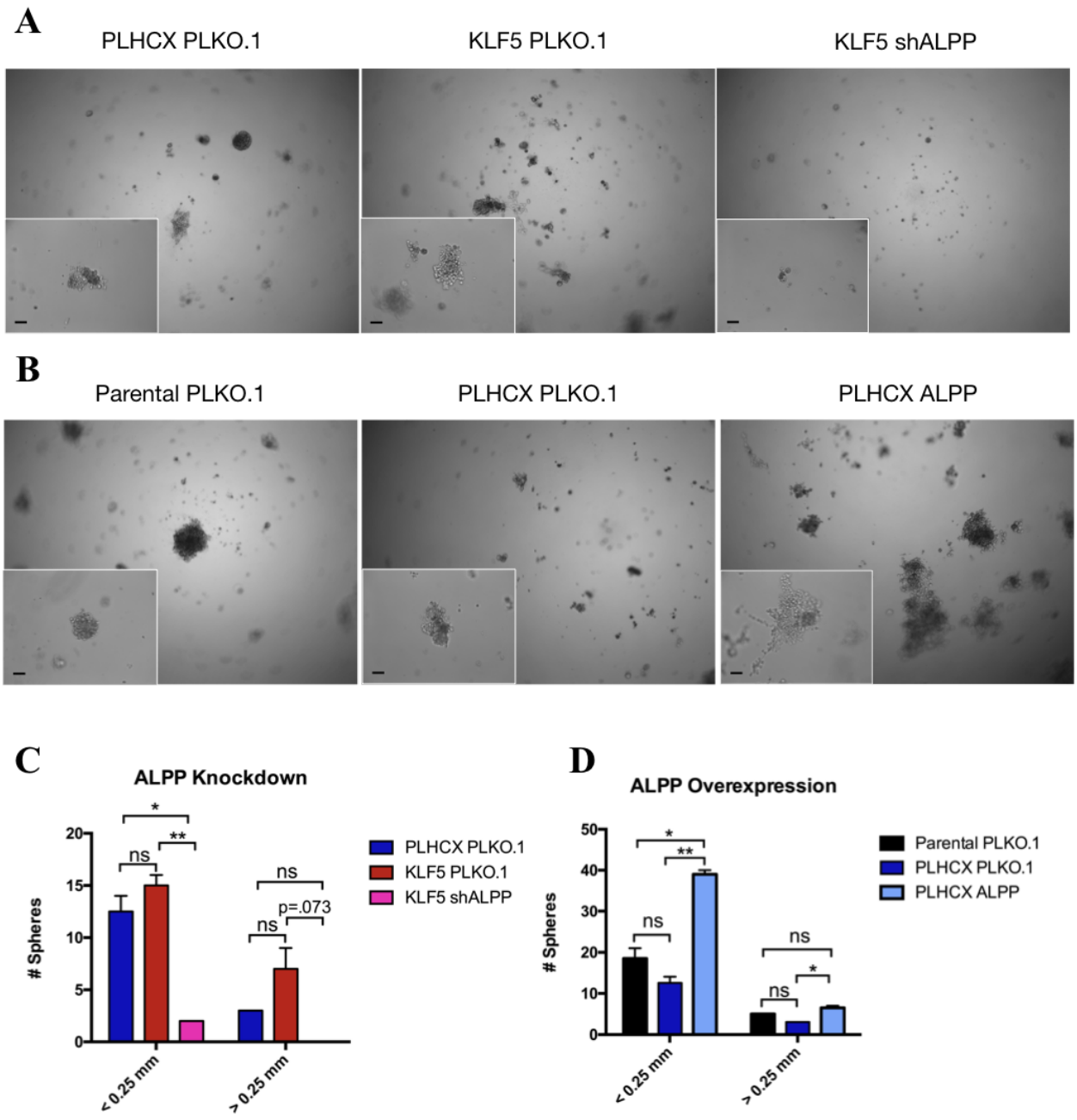



**Figure 5. Effect of ALPP on cell proliferation and colony formation**. PC-3 Parental and K cells transiently expressing ALPP-targeted shRNA and P cells transiently overexpressing ALPP were plated with the same conditions as above. PLKO.1 empty vector served as the control. Cells were imaged at day 18 under 4x and 10x magnification. Scale bar represents 100 μm. Colony size was measured using a hemocytometer. *$p < 0.05$, **$p < 0.01$, ns = no significance.

**KLF5 alone does not induce docetaxel resistance**

Previous studies have shown that KLF5 can have both pro- and anti-proliferative effects, and that its function may be determined by presence of TGF-β [6]. Studies in our lab have also indicated that TGF-β treatment induces chemoresistance mediated by KLF5 in prostate cancer cell models. In order to confirm the relationship between KLF5 and TGF-β on chemoresistance and cell survival, we conducted cytotoxicity assay using Cell Counting Kit-8 (CCK-8). We plated PC-3 and DU 145 P and K cells at a density of 2000 cells/well in triplicates, using either each cell group's respective cell culture media or cell culture media containing 10 ng/μl TGF-β1. 24-hours after plating, we treated cells with docetaxel. After 3 days of growth, CCK-8 solution was added, and cell viability was quantified using OD 450 nm. We used unpaired student's t-tests to show statistical significance. Statistical analysis was conducted on OD data at 0 docetaxel concentration to show any significant differences in cell proliferation between groups. Data was then normalized relative to the OD readout at 0 docetaxel concentration within each group, and statistical analysis was performed on the remaining data points to show chemoresistance between groups.

Our results show that in PC-3 cells, differential KLF5 expression did not cause any significant difference in cell proliferation after 3 days of growth. After normalization of data, PC-3 K cells show a significantly higher percent survival than other groups at high concentrations of Docetaxel treatment. However, at lower concentrations, no significant difference between cell viability was seen between Parental, P and K groups. Among DU 145 cell groups, there was a significantly higher cell density in the K group than either Parental or P. This may have been caused by the pro-proliferative effects of KLF5. Comparison of normalized data indicates that there is no significant difference in cell survival between P and K groups at

any docetaxel concentration. These results are in line with previous findings in our lab that KLF5 has no significant effect by itself on chemoresistance.

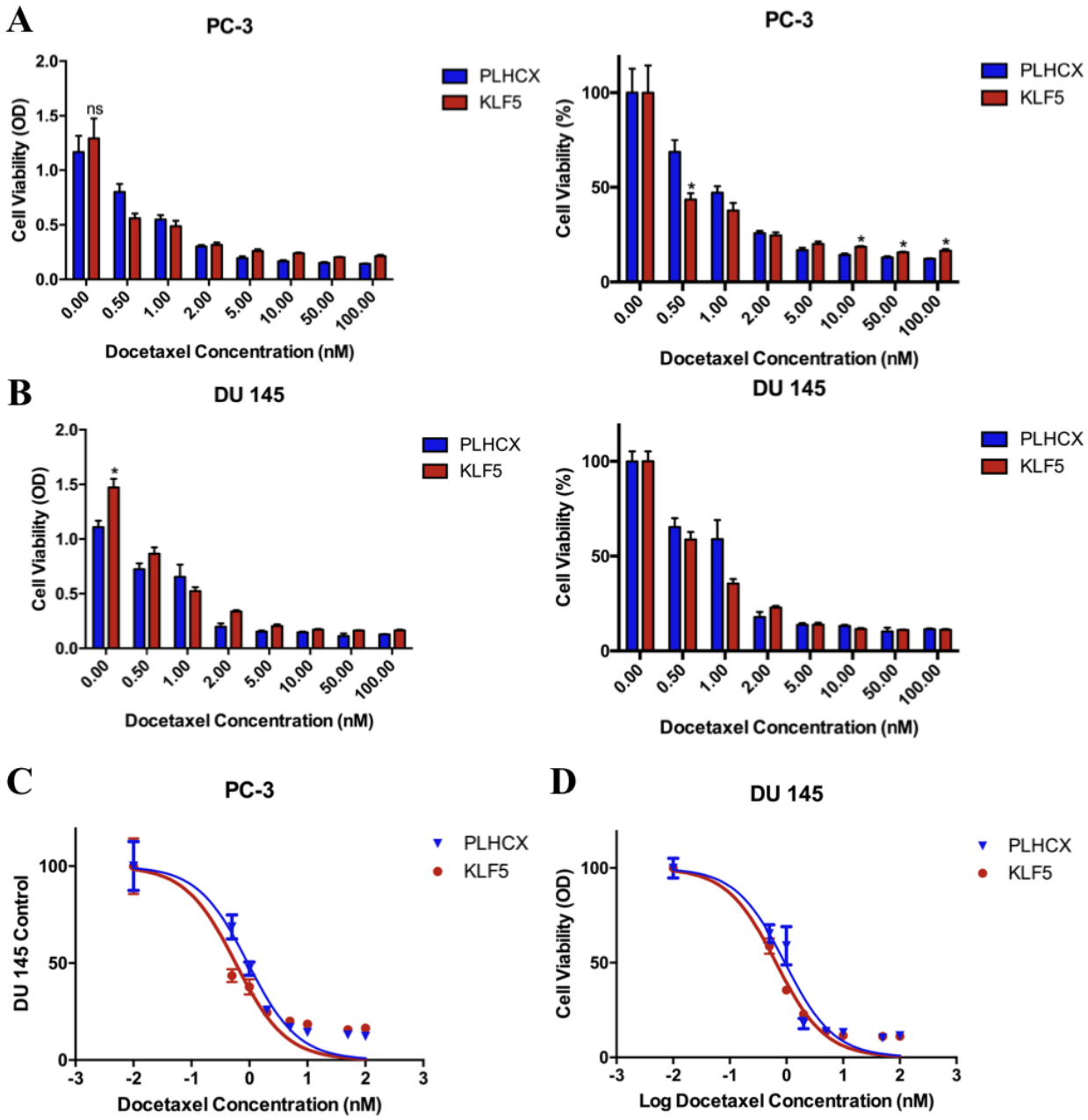



**Figure 6**. **Effect of KLF5 on chemoresistance.** Cells were plated at a density of 2000 cells/well in 96-well plates. Docetaxel treatment was administered 24 hours following plating at concentrations of 0, 0.5, 1, 2, 5, 10, 50, and 100 nM. CCK-8 solution was administered 3 days after docetaxel treatment. Plate readout was performed at OD 450 nm and OD 650 nm was used to control for background. Raw data points were analyzed for significance only at 0 docetaxel concentration, to analyze differences in cell proliferation between groups. Remaining data points

are analyzed after normalizing. (a) PC-3 P and K cells (b) DU 145 P and K cells (c, d) Non-linear regression line represents IC50 for normalized PC-3 and DU 145 data respectively. *$p<0.05$, ns = no significance.

**TGF-β induces docetaxel resistance mediated by KLF5 and reduces cell proliferation**

TGF-β signaling has been shown to play a part in inducing chemoresistance in several cancers [15, 16]. To investigate TGF-β's effects on docetaxel resistance in AR-null prostate cancer cell models, we first performed a cytotoxicity assay with PC-3 P and K cells plated in either normal cell culture medium or media containing 10 ng/μl TGF-β1 using the same conditions as previously mentioned. In both P and K groups, treatment with TGF-β resulted in significantly lower cell density at 0 docetaxel concentration. This may possibly be attributed to the anti-proliferative or toxic effects of TGF-β treatment. After normalizing data, both KLF5 knockout group (P) and KLF5 overexpressing group (K) showed increased cell viability when treated with TGF-β over untreated groups. This indicates that TGF-β elicits an increased resistance to docetaxel treatment. Though both statistically significant, the TGF-β-treated K group shows a much greater increase in cell survival versus the untreated group than the TGF-β P group shows versus its respective untreated group. These results indicate that the docetaxel resistance caused by TGF-β treatment in prostate cancer cells is mediated by KFL5.

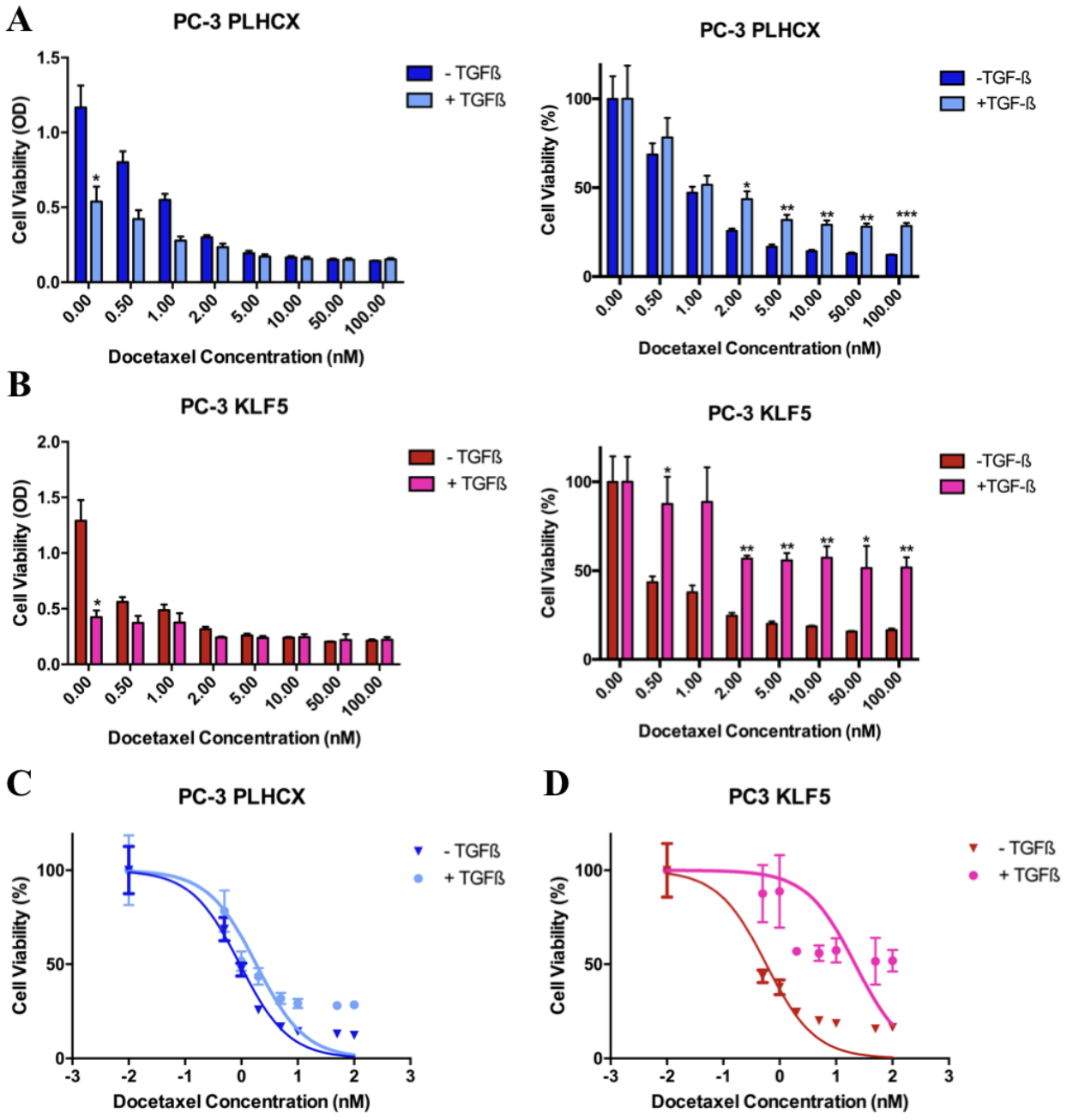
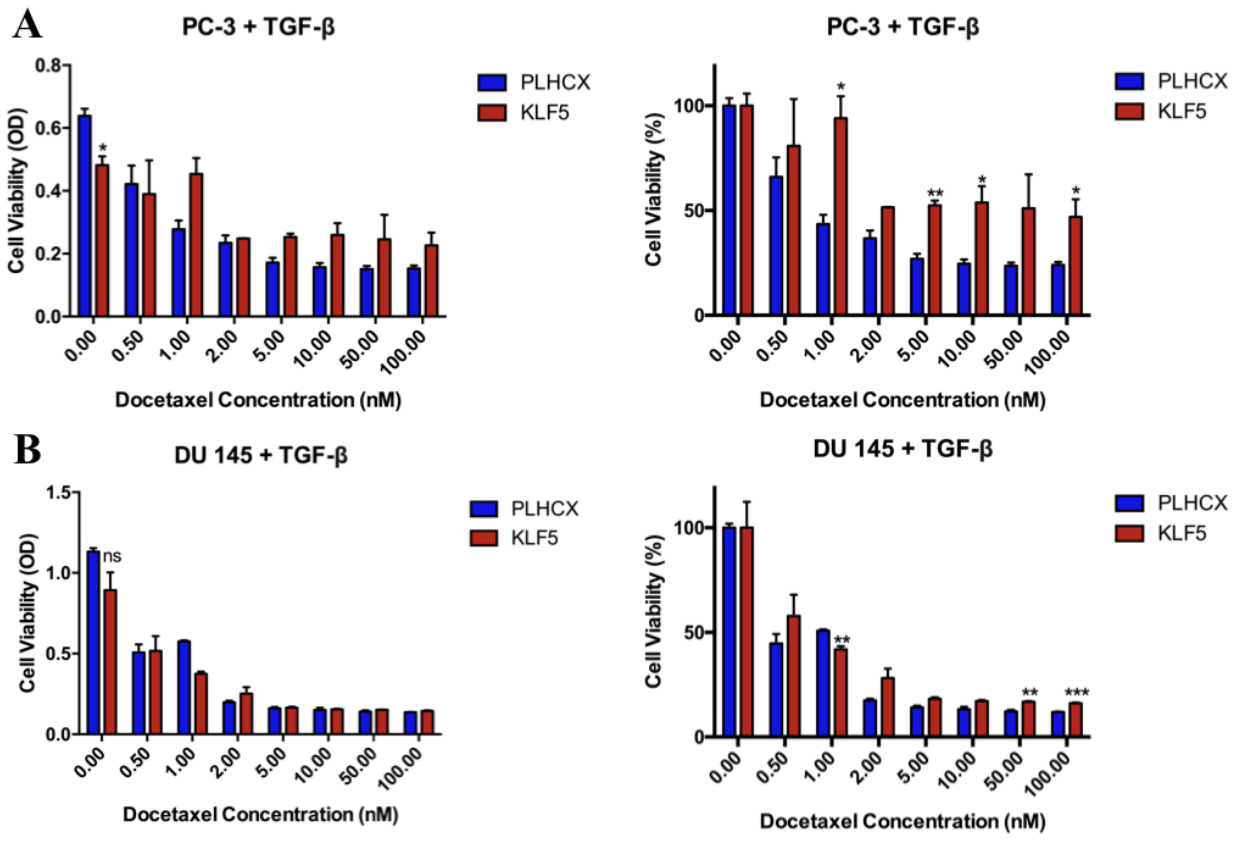



**Figure 7. Effect of TGF-β on chemoresistance.** (a) Cytotoxicity assay results, raw and normalized, for PC-3 KLF5 knockout cells (PLHCX) with or without TGF-β treatment. (b) PC-3 KLF5 overexpressing cells with or without TGF-β treatment. (c, d) non-linear regression line fit for normalized PC-3 and DU 145 cell viability data respectively. *p<0.05, **p<0.01, ***p<0.001, ns = no significance. Results show that TGF-β induces a small increase in chemoresistance to docetaxel in P group (a, c), but the effect is magnified by KLF5 overexpression (b, d).

**KLF5 mediates TGF-β-induced chemoresistance.**

We next analyzed the effects of KLF5 in conjunction with TGF-β. PC-3 and DU 145 P and K cells were grown in either normal cell culture media or TGF-β-containing media and plated for cytotoxicity assay as previously described. Results showed that in PC-3 cells, KLF5 overexpressing cells with TGF-β treatment showed markedly increased cell survival over PC-3 P cells treated with TGF-β, consistent with our hypothesis that KLF5 mediates chemoresistance in the presence of TGF-β treatment. Increased chemoresistance was less pronounced between TGF-β-treated DU 145 P and K groups, however at higher docetaxel concentrations, significantly higher cell viability in the K group over the P group was seen.



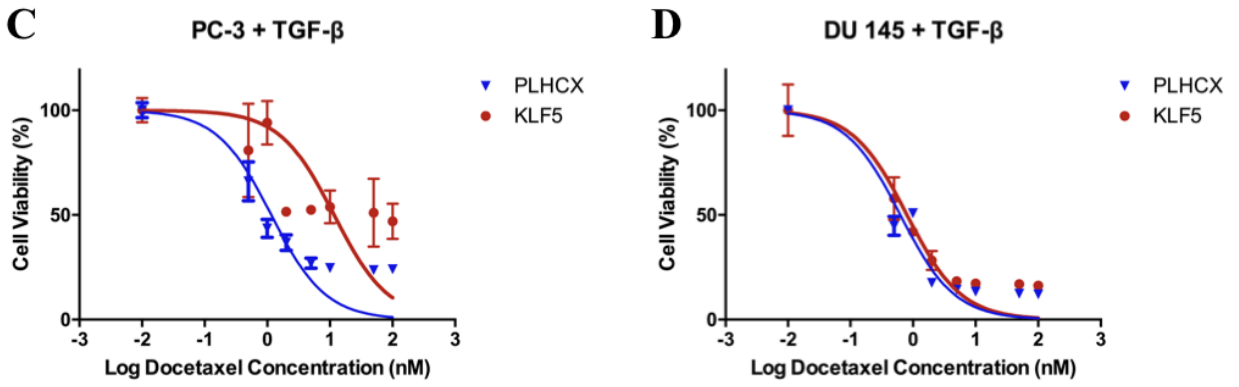


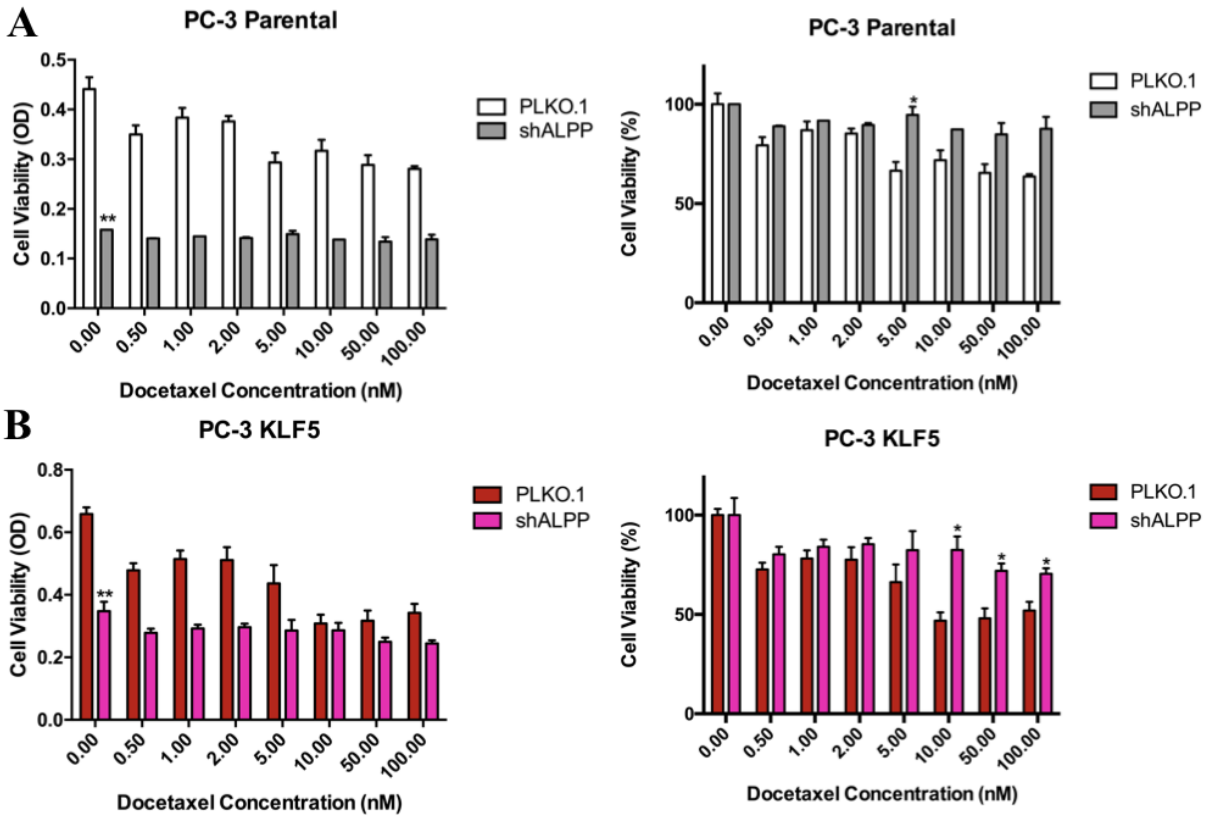

**Figure 8. Effects of KLF5 & TGF-β on Chemoresistance.** (a) Raw and normalized cytotoxicity assay results for PC-3 P and K cells with 10 ng/μL TGF-β treatment administered at time of plating. PC-3 K group shows improved chemoresistance over P group. (b) DU 145 P and K cells with 10 ng/μL TGF-β treatment. (c, d) non-linear regression line fit for normalized PC-3 and DU 145 cell viability data respectively. *p<0.05, **p<0.01, ***p<0.001, ns = no significance.

**ALPP is involved in cell proliferation and may sensitize cells to chemoresistance**

Following this evidence that KLF5 in conjunction with TGF-β increases chemoresistance, we aim to investigate ALPP's effect on this mechanism. As we know KLF5 upregulates ALPP expression at both the mRNA and protein level, we hypothesized that KLF5 mediates TGF- induced chemoresistance through upregulation of ALPP. We first focused on determining ALPP's effect on chemoresistance. We used ALPP manipulated cells for cytotoxicity assays following the same procedure as previously stated. PC-3 Parental and K cells were transfected with ALPP overexpression while PC-3 P cells were transfected with ALPP targeted shRNA.

Our results show that knockdown of ALPP in both Parental and KLF5 cells reduces cell proliferation, significantly decreasing cell density at 0 docetaxel concentration. After normalizing data, results show that shALPP groups show increase cell survival at higher concentrations of docetaxel than control groups. This would suggest that knockdown of ALPP

expression on its own may contribute to increased chemoresistance. ALPP overexpressing P cells showed considerable increase in cell proliferation compared to the control group. After normalizing, ALPP overexpressing cells showed a significantly greater response to docetaxel treatment than control group, indicating that ALPP overexpression may sensitize cells to chemotherapy. These results together show that ALPP plays an important role in cell proliferation, and knockdown of ALPP reduces cell proliferation significantly. Preliminary data also indicates that ALPP expression may act to sensitize cells to chemotherapy and knockdown of ALPP contributes to increased chemoresistance. This trend may go against our initial hypothesis that KLF5 mediates TGF-β induced chemoresistance by upregulating ALPP.



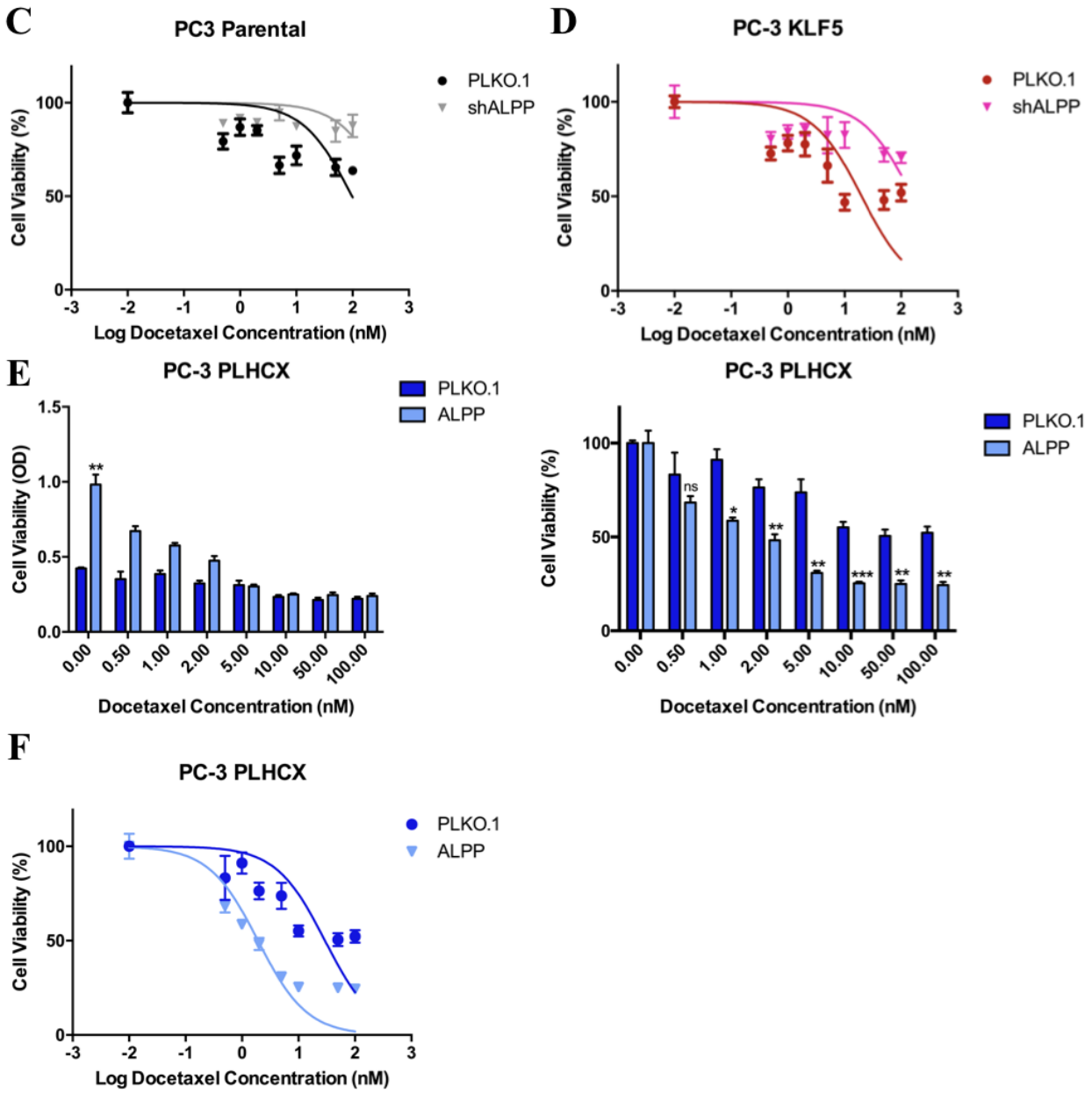



**Figure 9. Effect of ALPP on chemoresistance.** Cytotoxicity assay with ALPP manipulated cells. Retroviral vector containing ALPP targeted shRNA (shALPP) was administered to PC-3 Parental (a, c) and K cells (b, d). Retroviral vector containing ALPP gene insert was administered to PC-3 P cells (e-f). *p<0.05, **p<0.01, ***p<0.001, ns = no significance.

## Discussion

In this study, we aim to identify a gene transcriptionally activated by KLF5 that mediates KLF5's effects on cell proliferation and chemoresistance in AR-null prostate cancer cell models. We used the two cell lines PC-3 and DU 145, which both express low endogenous levels of KLF5, as well as cells with stable KLF5 knock-out and overexpression. We first analyzed ChIP sequencing and RNA sequencing to identify candidate genes whose expression differs significantly when KLF5 is manipulated. Of the 7 candidate genes identified by this method, we selected two to focus on based on results of RT-PCR: *ALPP* and *FAM83A*.

Next, we conducted western blotting to show KLF5 regulation of these genes at the protein level and ChIP PCR to show binding of KLF5 to these two gene regions. We were unable to produce successful western blotting or significant ChIP PCR results for FAM83A. However western blotting for ALPP showed that in KLF5 overexpressing PC-3 cells, ALPP expression is upregulated. In DU 145 cells, my western blot showed an unexpected decrease in ALPP expression in KLF5 overexpressing cells, contradictory to results produced previously by a lab member showing a strong upregulation of ALPP expression in DU 145 K cells. We hypothesize that this discrepancy may be due to a compensation effect in my cells, which had a high passage number by the time protein samples were collected. After long periods of passaging, the DU 145 K cell group may have adapted to compensate for KLF5 overexpression through other pathways, resulting in the lessening of effects caused by KLF5 overexpression. To test this hypothesis, we plan to perform western blotting for ALPP with protein samples collected from cells frozen at a much earlier passage number from the same batch of cells used in this experiment. ChIP PCR on DU 145 samples, although statistically insignificant, showed a trend for increased KLF5 binding

to ALPP gene region in KLF5 overexpressing cells. These results suggest that KLF5 may directly upregulate transcription of ALPP.

We next investigated the effects of KLF5 on cell proliferation and colony formation using colony formation assays. Results showed a stark contrast in cell morphology between Parental PC-3 cells and KLF5 knockout cells. Both Parental PC-3 and DU 145 cells formed a large number of small spheres that were rounded with clear boundaries. Knockout of KLF5 induced elongated, mesenchymal characteristics in individual cells in PC-3 strongly and DU 145 slightly. KLF5 overexpressing cells showed rounded morphology, more similar to Parental cells than knockout cells. Some cells in the K group were still seen with elongated cell shapes. We hypothesize that this could be due to the fact that not every cell may have acquired KLF5 overexpression through transfection, even if they do retain Hygromycin B marker.

In both PC-3 and DU 145, KLF5 knockout also resulted in the formation of significantly fewer colonies than Parental groups, the majority of which remained small in size. Significantly more large sized colonies formed in cells overexpressing KLF5 than in Parental or P groups. These results together indicate that KLF5 expression is needed for maintenance of an epithelial cell morphology as well as induces increased cell proliferation. These conclusions are in accordance with previous findings [17].

ALPP was also shown to have a significant effect on cell proliferation and colony formation through the colony formation assay. Using PC-3 Parental and K cells transiently expressing ALPP targeted shRNA (shALPP), we observed a drastic decrease in colony formation as well as cell proliferation when ALPP was knocked down. In PC-3 P cells transiently overexpressing ALPP (ALPP), we observed a significant increase in number of colonies formed as well as colony size. This shows that ALPP induces cell proliferation and may also contribute

to stemness, a trait that defines colony-formation ability, while knockdown of ALPP will heavily reduce cell proliferation rate and colony formation. ALPP knockdown or overexpression did not seem to have any significant effect on cell morphology. RT-PCR still remains to be performed in order to validate the knockdown and overexpression of ALPP in these cell groups.

Results of cytotoxicity assay confirmed previous studies in our lab showing that KLF5 contributes to chemoresistance only in the presence of TGF-β treatment. To further investigate how KLF5 regulation of ALPP may affect this mechanism, we first evaluated ALPP's effect on chemoresistance and cell proliferation. Our results reaffirmed the huge effects of ALPP knockdown on reduced cell proliferation as well as increased cell proliferation when ALPP is overexpressed. A discrepancy in the data is seen within the control PLKO.1 empty vector group. It seems the addition of the empty vector did change cell behavior from the expected norm, with less cell death occurring than usual in response to docetaxel treatment. This may indicate that the addition of the vector has a significant effect on cell behavior. However, comparing strictly between PLKO.1 control groups and experimental ALPP or shALPP groups can still give insight into ALPP's effect on chemoresistance and cell proliferation. Contrary to our original hypothesis however, the data would suggest that ALPP overexpression sensitizes PC-3 cells to chemotherapy while knockdown of ALPP increases chemoresistance. We would like to further investigate TGF-β's interaction with ALPP as well as KLF5's role in regulating ALPP's functional effects. We did execute cytotoxicity assay with ALPP manipulated cell groups and TGF-β, however the combination of anti-proliferative effects from TGF-β and shALPP produced very low cell densities, so we were unable to include these results. Future plans would include repeating this experiment with higher initial cell densities plated as well as including more groups in the transfection of ALPP or shALPP. These experiments will provide more insight on

the relationship between TGF-β and ALPP as well as KLF5 and ALPP in terms of mediating chemoresistance.

In summary, we showed that KLF5 upregulates ALPP expression at the mRNA and protein level likely through direct binding to the gene region. We also show that KLF5 induced pro-proliferative effects can be reverted by knockdown of ALPP. Similarly, reduced cell proliferation and colony formation due to KLF5 knockout can be rescued by ALPP overexpression. Finally, our preliminary data shows evidence that ALPP expression may cause sensitization of AR-null prostate cancer to docetaxel treatment.

## Methods

### Author Contributions

Baotong Zhang (B.Z.), Yixiang Li (Y.L.), Kathy Pei Li (K.P.L), and Jin-Tang Dong (J.T.D) conceived the project. Y.L., K.P.L., B.Z., and J.T.D. designed the experiments and performed data analysis. B.Z. and Y.L. performed ChIP Seq. and RNA Seq. K.P.L. performed remaining experiments. K.P.L., Y.L., and B.Z., performed data analysis. Y.L., B.Z., J.T.D., Gregg Orloff, and Kevin Karnes contributed to editing of thesis, written by K.P.L.

### Cell lines and other materials.

Cells used were the human prostate cancer cell lines PC-3 and DU 145 obtained from American Type Culture Collection (ATCC) (Manassas, VA) and were propagated per manufacturer's instructions. Both cell lines are androgen receptor pathway-independent and express low levels of endogenous KLF5. Endogenous KLF5 was knocked out in parental PC-3 and DU 145 cells

using CRISPR-Cas9 and successful single clones were selected from each group to create cultures (2-10H and 2-13M respectively). These cells were transfected with either empty pLHCX retroviral vector or KLF5 overexpressed in pLHCX with Hygromycin B selection marker to create stable manipulated cell lines. Plasmids were previously cloned by a lab member. 293-T cells were obtained from ATCC. TGF-β (240-B-002) was obtained from R&D Systems and docetaxel (Y0001133) from Sigma Aldrich. Lentiviral plasmid with ALPP gene insert (45581) was purchased from Addgene and ALPP knock-down plasmid from Sigma Aldrich.

**RNA & Chromatin Immunoprecipitation (ChIP) Sequencing**

RNA sequencing was performed on PC-3 par, P and K groups in duplicates. Data was analyzed to identify genes whose expression levels in both endogenous (parental) and exogenous (K) KLF5 expressing groups differed significantly from KLF5 knock-out group (P). Both RNA and ChIP sequencing were performed on DU 145 P and K groups with only one replicate. Pull-down assay was performed by Baotong Zhang on DU 145 samples with anti-KLF5 antibody (AF3758, R&D Systems).

**Real-Time PCR (RT-PCR)**

RNA was isolated from parental, P, and K groups of PC-3 and DU 145 cell lines using TRIzol Reagent method (Invitrogen). cDNA was synthesized for all 7 prospective genes and GAPDH, which served as an RNA-level control. The mRNA levels of each gene were assessed using ROX Dye in Takara SYBR green and their respective sequence-specific primers. The $2^{-\Delta\Delta Ct}$ method was utilized for quantification of mRNA levels. The primers used are:

FAM83A: 5'-CTCGGACTGGAGATTTGTCC-3' and 5'- GGAACTCCTCGTCAAACAGC-3', PI3: 5'- GCAGCTTCTTGATCGTGGTG-3' and 5'- GCCGTGGGCATCCTGAATGGG-3', SLPI: 5'-GAGATGTTGTCCTGACACTTGTG-3' and 5'- AGGCTTCCTCCTTGTTGGGT-3', ALPP: 5'-GTGAACCGCAACTGGTACTC-3' and 5'-GAGCTGCGTAGCGATGTCC-3', ELF3: 5'-GGCCGATGACTTGGTACTGAC-3' and 5'- GCTTGCGTCGTACTTGTTCTTC-3', PLEKHA7: 5'- GACGGGACAGTTTTCTCCAG-3' and 5'- TTGGTTTCTGTCTTGCTTCG-3', and LINC01133: 5'- GGAGCGAGATCCCTCCAAAAT-3' and 5'-GGCTGTTGTCATACTTCTCATGG-3'.

**Transient Viral Infection**

A lentiviral vector expressing ALPP was purchased from Addgene (#45581) and 2 lentiviral vectors expressing ALPP-targeted short hairpin RNAs (shRNA) were purchased from Sigma Aldrich. Empty PLKO.1 vector was used as a control. To produce virus, 293-T cells were cultured and transfected with empty PLKO.1 or ALPP expressing or shRNA expressing vectors. After a 48-hour incubation period, virus-containing media was collected. PC-3 and DU 145 were double transfected with virus containing media at 0 and 24 hours with polybrene. 48 hours after initial transfection, cells were re-plated into 96-well plates for cytotoxicity assay, colony formation assay, or harvested for RNA.

**Western Blotting**

Cells were rinsed with PBS and protein extracted via direct lysis with 2x Laemmli Buffer from Bio-Rad. Protein was separated by SDS-PAGE and western blotting was used to analyze expression levels of ALPP (~70 kDa), FAM83A (~47 kDa), KLF5 (~57 kDa), and ß-Actin (~42

kDa). The antibodies used were anti-FAM83A (SAB1103067, Sigma), anti-ALPP (3E5C7, CST), and anti-β-Actin (A2066, Sigma). Anti-KLF5 was produced as described in a previous study [18].

**ChIP PCR**

ChIP primers were designed by Yixiang Li using Primer Premiere and all are listed in Table 1. The same ChIP samples prepared by Baotong Zhang were used. IgV and Hg19 version of reference genome was used for data analysis.

**Cytotoxicity Assay**

Cells were plated in 96-well plates at a density of 2000 cells/well in duplicates or triplicates with either regular culture media or TGF-β containing media. The following day, the cells were treated with Docetaxel treatment at concentrations of 0, 0.5, 1, 2, 5, 10, 50 and 100 nM. After 3 days of growth, cells were incubated with Dojindo Cell Counting Kit-8 (CCK-8) solution and quantified at OD 450 nm, using OD 650 nm as background control.

**Colony Formation Assay**

Cells were plated in 96-well plates at a density of 300 cells/well atop 50 ul matrigel in 3-D Sphere media. Sphere media is comprised of prostate epithelial cell basal medium with added insulin, B27, EGF, and FGF. PC-3 cells were observed at 8 days and DU 145 cells were observed at 15 days for sphere growth and cell morphology. Colony numbers above and below a diameter threshold were recorded.

**Statistical Analysis**

Statistical analysis of data was conducted using Graphpad Prism. We utilized the unpaired, two-tailed Student's t-test to compare results between groups, with p-value $< 0.05$ signifying statistical significance.

**Supplemental Figures**

**Table 1. ChIP Primers Sequences**

| | Forward Sequence | Reverse Sequence |
|---|---|---|
| ALPP # 1 | ACTCAATACCGCACCCTTCC | CCCAGCCTCTTGACCTGAA |
| ALPP # 2 | AGGGTCAAGGTGGCAACG | TGGGCATGGAGTATGAGC |
| ALPP # 3 | CACAGTGAGCAGGGGTTG | AGTACAGATGCCATTTGGGT |
| FAM83A # 1 | CTCACTCCAGCGCCAACA | GACCGCAGAGGTTCCAGTT |
| FAM83A # 2 | TTGGCCTGAGAACTGGAAC | TGTGAGGAGGGAGCTGGA |
| FAM83A # 3 | AGGGAGGGGTGGGCACTT | TTCCCATGCTGTTTCAAAGATA |
| FAM83A # 4 | CCCACAGGGAAATTCTTAT | GCCTTTGTTACCAAGCAG |
| FAM83A # 5 | GAGGCTCCCTGCTTGGTA | GGGCTGGCTCAGACACCT |
| FAM83A # 6 | TTGCGAAACCCAGCAGGAA | TGCGGGATTGGCTTATCT |

**References**


1. Siegel, R.L., K.D. Miller, and A. Jemal, *Cancer statistics, 2019.* CA Cancer J Clin, 2019. **69**(1): p. 7-34.

2. Saraon, P., et al., *Mechanisms of Androgen-Independent Prostate Cancer.* Ejifcc, 2014. **25**(1): p. 42-54.

3. Devlin, H.L. and M. Mudryj, *Progression of prostate cancer: multiple pathways to androgen independence.* Cancer Lett, 2009. **274**(2): p. 177-86.

4. Bluemn, E.G., et al., *Androgen Receptor Pathway-Independent Prostate Cancer Is Sustained through FGF Signaling.* Cancer Cell, 2017. **32**(4): p. 474-489.e6.

5. Chen, C., et al., *KLF5 is frequently deleted and down-regulated but rarely mutated in prostate cancer.* Prostate, 2003. **55**(2): p. 81-8.

6. Li, X., et al., *Interruption of KLF5 acetylation converts its function from tumor suppressor to tumor promoter in prostate cancer cells.* Int J Cancer, 2015. **136**(3): p. 536-46.

7. Bartel, C.A., et al., *FAM83 proteins: Fostering new interactions to drive oncogenic signaling and therapeutic resistance.* Oncotarget, 2016. **7**(32): p. 52597-52612.

8. Cipriano, R., et al., *Conserved oncogenic behavior of the FAM83 family regulates MAPK signaling in human cancer.* Mol Cancer Res, 2014. **12**(8): p. 1156-65.

9. Chen, S., et al., *FAM83A is amplified and promotes cancer stem cell-like traits and chemoresistance in pancreatic cancer.* Oncogenesis, 2017. **6**(3): p. e300.

10. Bartel, C.A. and M.W. Jackson, *HER2-positive breast cancer cells expressing elevated FAM83A are sensitive to FAM83A loss.* PLoS One, 2017. **12**(5): p. e0176778.

11. She, Q.B., et al., *Growth factor-like effects of placental alkaline phosphatase in human fetus and mouse embryo fibroblasts.* FEBS Lett, 2000. **469**(2-3): p. 163-7.

12. Lange, P.H., et al., *Placental alkaline phosphatase as a tumor marker for seminoma.* Cancer Res, 1982. **42**(8): p. 3244-7.

13. Dong, J.T. and C. Chen, *Essential role of KLF5 transcription factor in cell proliferation and differentiation and its implications for human diseases.* Cell Mol Life Sci, 2009. **66**(16): p. 2691-706.

14. Xing, C., et al., *Different expression patterns and functions of acetylated and unacetylated Klf5 in the proliferation and differentiation of prostatic epithelial cells.* PLoS One, 2013. **8**(6): p. e65538.

15. Brown, J.A., et al., *TGF-β-Induced Quiescence Mediates Chemoresistance of Tumor-Propagating Cells in Squamous Cell Carcinoma.* Cell Stem Cell, 2017. **21**(5): p. 650-664.e8.

16. Cai, J., et al., *Simultaneous overactivation of Wnt/beta-catenin and TGFbeta signalling by miR-128-3p confers chemoresistance-associated metastasis in NSCLC.* Nat Commun, 2017. **8**: p. 15870.

17. Zhang, B., et al., *KLF5 activates microRNA 200 transcription to maintain epithelial characteristics and prevent induced epithelial-mesenchymal transition in epithelial cells.* Mol Cell Biol, 2013. **33**(24): p. 4919-35.

18. Chen, C., et al., *Ubiquitin-proteasome degradation of KLF5 transcription factor in cancer and untransformed epithelial cells.* Oncogene, 2005. **24**(20): p. 3319-27.