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13. SUPPLEMENTARY NOTES

14. ABSTRACT: During the first year of funding, we identified a specific set of genes elevated in PCa whose expression is predicted to be determined by the transcription factor CREB1 or ATF1. Using qRT-PCR and immunoblotting, we validated that some of these are elevated in tumor cells and not expressed in normal cells. We further demonstrated that activated CREB is specifically bound to the CREB binding elements in the promoters of GATA2 and TWIST1 in tumor cells. We generated Tet-inducible shRNA lines to knock down CREB1 and ATF1 in the tumor cells and found that CREB1 and ATF1 have opposite functions. CREB1 suppresses terminal luminal cell differentiation in tumor cells and its removal restores luminal properties. However, because CREB1 is required for luminal cell survival, they eventually die. In contrast, ATF1 is required for terminal luminal cell differentiation, and its removal from tumor cells does not restore terminal differentiation. We determined that these differences in function are related to their ability to suppress and enhance the expression of the chromatin binding protein, ING4, which is required for luminal cell differentiation and lost in tumor cells.

15. SUBJECT TERMS

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INTRODUCTION: Being able to determine which prostate cancer patient has indolent disease versus those who will die, unless aggressively treated, remains a major challenge. Despite recent studies that identified several possible PCa subtypes, there was relatively little difference in these subtypes between primary cancer and metastatic cancers. However, six of the PCa subclasses identified in those studies were notable in that they are defined by transcription factors; molecules that can control key genetic programs. These same transcription factors have been linked to differentiation and cell fate determination. Oncogenic events associated with PCa, including Myc overexpression or Pten loss, have also been linked to altered differentiation; however, how these oncogenes relate to the transcriptional programs involved in differentiation and cell fate determination remain unresolved. We propose there will be a specific combination of oncogenic events and specific differentiation alterations that produce an aggressive subtype of PCa. If this signature can be identified, then the ability to distinguish indolent from lethal disease can be achieved. We identified a gene signature that is a target of the transcription factor CREB, which is elevated in tumorigenic cells when PTEN is lost. Of the known oncogenes associated with PCa, PTEN loss is most closely associated with poor outcomes in patients. Virtually nothing is known about the function or nature of this CREB/PTEN signature in PCa.

KEYWORDS: CREB1, ATF1, GATA2, TWIST1, PTEN, ING4, differentiation, oncogenesis, gene expression, biomarker, chromatin, luminal cells, prostate cancer.

ACCOMPLISHMENTS:

What were the major goals of the project?

The **hypothesis** we are testing is that aberrant temporal CREB1/ATF1 activation, due to PTEN loss, prior to full luminal cell commitment, promotes a prostate cancer oncogenic program that is associated with poor outcomes in patients.

Our **Aims/Goals** are:	Percent completed
1) Determine the role of CREB1/ATF1 in differentiation and oncogenesis	
Milestones:	
1) Identification of CREB1 vs ATF1 specific gene targets (12 months)	50%
2) Manuscript – CREB1 vs ATF1 role in luminal cell differentiation (16 months)	90%
3) Manuscript – CREB1/ATF1 role in tumorigenesis (12 months)	10%
2) Identify the CREB1/ATF1 targets necessary for prostate cancer oncogenesis	
Milestones:	
1) Identification of PTEN/CREB-dependent targets (15 months)	10%
2) Manuscript – GATA2/Twist1 suppression of differentiation (32 months)	0%
3) CREB targets required for oncogenesis identified (24 months)	0%
4) Manuscript – CREB/Pten mechanisms of PCa oncogenesis (24 months)	0%
3) Define the CREB1/ATF1 signature in clinical human prostate cancer samples	
Milestones:	
1) TMAs received and ready for processing (28 months)	0%
2) Manuscript – CREB/Pten Signature and PCa outcomes (36 months)	0%

What was accomplished under these goals?

Aim 1: Determine the role of CREB1/ATF1 in differentiation and oncogenesis

Task 1 – Subtask 1: Generate EMP cells lines in which AFT1 or CREB1 are deleted by shRNA

We generated both normal iPrEC and tumorigenic EMP cells in which ATF1 and CREB1 are conditionally knocked down by Tet-inducible shRNA (**Fig. 1A,B**). CREB1 and ATF1 are very stable proteins, and it takes 5 days to decrease their expression using 100ng/ml Doxycycline.

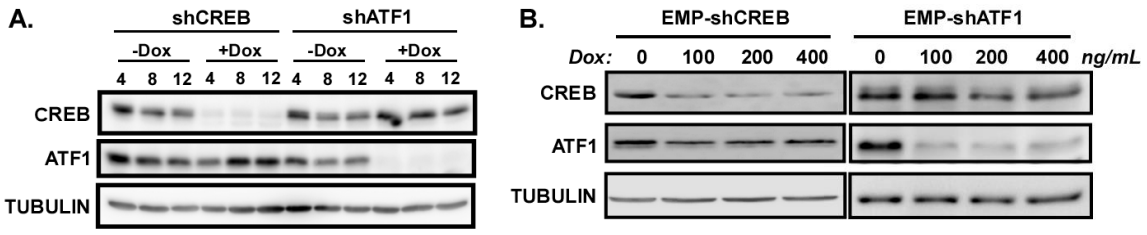


Fig. 1: Generation of shCREB and shATF1 cell lines. A) iPrECs and **B)** EMP cells were engineered to stably express Tet-inducible shRNAs targeted to CREB1 and ATF1 by lentiviral vector infection. Following 5 days of doxycycline treatment, knock-down was validated by immunoblotting.

Task 1 – Subtask 2 and 3: Quantify the expression of 529 predicted CREB1/ATF1 target genes and validate the targets by performing CREB1/ATF1 ChIP.

We have not yet assessed all 529 predicted CREB1/ATF1 target genes, but did validate one new CREB1 target gene, JFK. JFK is an E3-ligase and one of its targets is the chromatin reader protein, ING4 (cite). We previously demonstrated that transient induction of ING4 mRNA and protein is required for prostate luminal cell differentiation (cite), and postulated that JFK might be a feedback mechanism for turning off ING4 expression late in differentiation. Within our RNA-Seq data we found that JFK mRNA is induced late in luminal differentiation of normal iPrEC, but is not induced in tumorigenic EMP cells (**Fig. 2A**). This latter finding ruled out a role for elevated JFK in tumorigenic EMP cells as mechanism for ING4 loss in prostate cancer. We validated that JFK mRNA is induced during differentiation by qRT-PCR (**Fig. 2B**). JFK has a CREB1/ATF1 binding site in its promoter, and we were able to ChIP CREB1/ATF1 on this promoter (**Fig. 2C**). Unexpectedly, we also discovered that ING4 binds to the JFK promoter (**Fig. 2D**), suggesting that when ING4 is high, it may turn on its own repressor. Thus, we identified the ING4 E3-ligase, JFK, as a CREB1/ATF1 target that is responsible for inhibiting ING4 expression late in differentiation and ING4 may control the expression of its own inhibitor. These data are also consistent with the overall role of CREB1 in blocking ING4 expression (see data in **Fig. 4**). However, this does not fully explain all the mechanisms for how ING4 is repressed, as E3-ligase would only be responsible for its degradation, but not its decreased transcription.

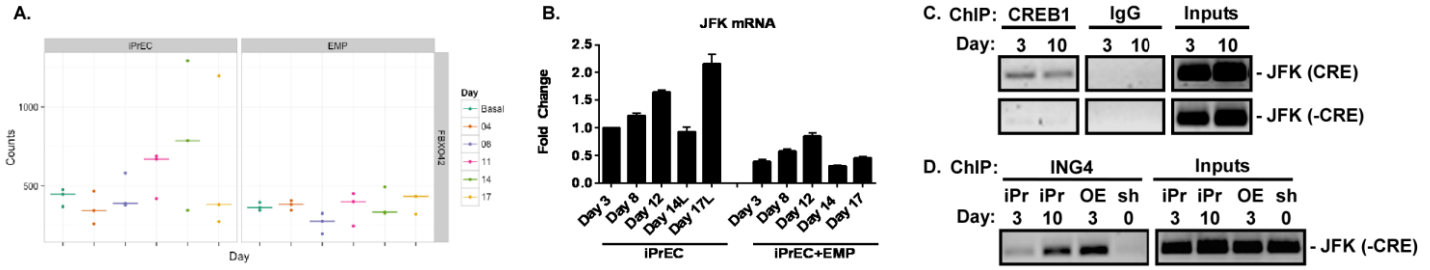


Fig. 2: CREB1/ATF1 controls the expression of JFK to set the timing of ING4 expression during luminal cell differentiation. A) RNA-Seq counts for JFK mRNA after Days 0, 4, 8, 11, 14, or 17 days of differentiation in normal iPrEC versus tumorigenic EMP cells. **B)** JFK mRNA expression measured by qRT-PCR in differentiating iPrEC and EMP cells. **C)** ChIP of CREB1 versus IgG control on the CRE element in the promoter of JFK assessed by PCR. A non-CRE containing region of JFK served as a control. **D)** ChIP of ING4 on the transcriptional start site of JFK – outside the CRE region in cells differentiated for 3 or 10 days, or in cells overexpressing (OE) ING4 or ING4 shRNA (sh).

In our preliminary data, we demonstrated that TWIST1 and GATA2, predicted CREB1/ATF1 targets in tumorigenic EMP cells, are elevated in EMP cells and absent in normal iPrEC (**Fig. 3A,B**). In EMP-shCREB1 cells, loss of CREB1 reduced the expression of GATA2, but did not affect Twist1 (**Fig. 3C**). To determine if these are direct transcriptional targets of CREB1 or ATF1, we performed ChIP using an antibody that specifically recognizes the active forms of both CREB1 and ATF1 – p-CREB/p-ATF1. We used histone 3 (H3) as our positive control, and probed regions outside of the predicted CREB-binding motifs as a negative control. We found that active p-CREB/p-ATF1 is bound to the promoters of both TWIST1 and GATA2 (**Fig. 3D**). Thus, TWIST1 and GATA2 are bona-fide p-CREB1/ATF1 targets in tumorigenic EMP cells; however, GATA2 maybe targeted by p-CREB1, while Twist1 may be targeted by p-ATF1.

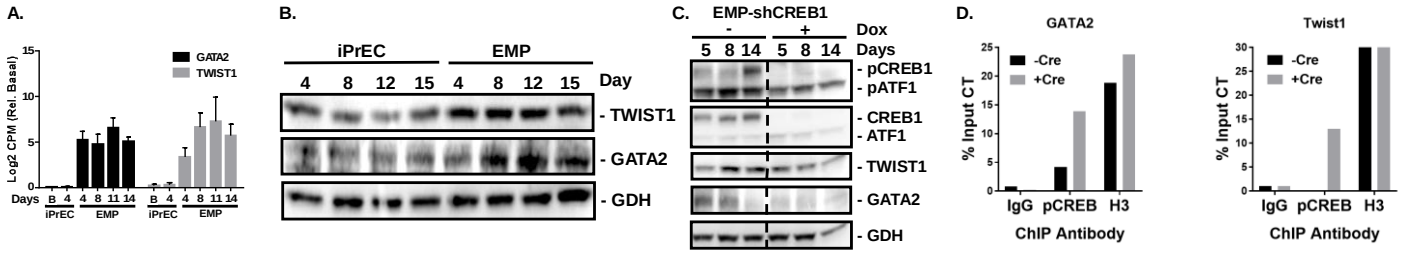


Fig. 3: Twist1 and GATA2 are direct targets of CREB1. A) Levels of Twist1 and GATA2 mRNA and **B)** protein in iPrEC and EMP cells differentiated for 0, 4, 8, 11/12, and 14/15 days measured by qRT-PCR and immunoblotting respectively. **C)** EMP-shCREB1 cells were differentiated for 5, 8, and 14 days with or without doxycycline to induce CREB1 shRNA and the levels of Twist1, GATA2, CREB, p-CREB, and GAPDH (GPD) assessed by immunoblotting. **D)** ChIP of p-CREB1/p-ATF1 on GATA2 and Twist1 Cre (+Cre) and not on non-Cre (-Cre) promoter regions in EMP cells. IgG and histone 3 (H3) served as negative and positive controls respectively.

Task 2 – Subtask 1: CREB1-dependent differentiation - determine if loss of CREB1 in the EMP cells will rescue differentiation.

We found that loss of CREB1 in normal iPrEC accelerates luminal cell differentiation (**Fig. 4A**); however, these newly differentiated luminal cells die based on increased Caspase 3 staining (**Fig. 4B**), indicating CREB1 is required for luminal cell survival. The tumorigenic EMP cells do not differentiate (**Fig. 4C**), but when we knock-down CREB1 upon doxycycline treatment, the same patches of differentiated luminal cells appear, as is seen in the normal iPrECs when CREB1 is knocked down (**Fig. 4D**). Like in iPrECs, these patches of luminal cells are also dying (**Fig. 4E**). Thus, loss of CREB1 in EMP restores the phenotype of normal iPrECs demonstrating the importance of CREB1 in prostate cancer tumorigenesis.

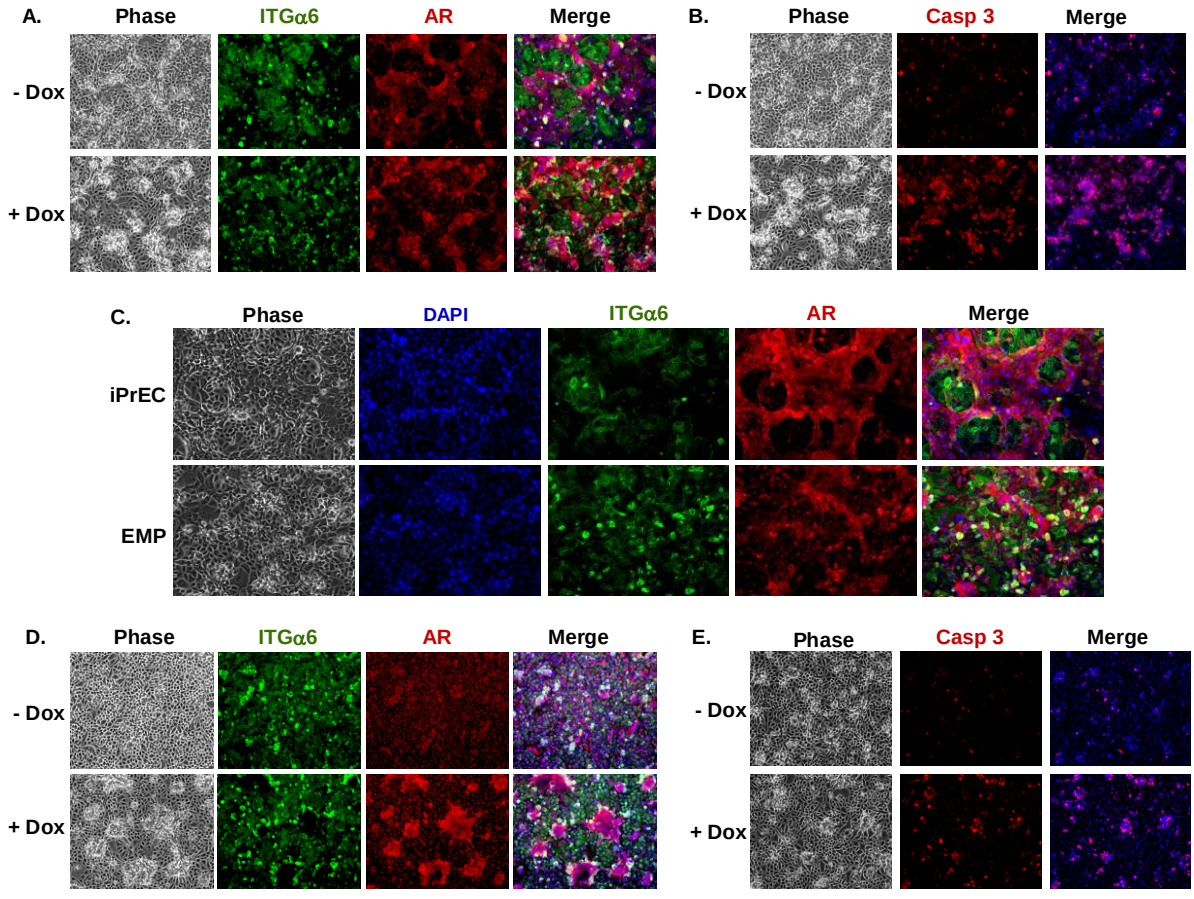


Fig. 4: CREB1 limits ING4 expression to prevent death of luminal cells and promotes tumor de-differentiation by preventing ING4 expression. A,B) iPrEC-shCREB1 were differentiated for 12 days in the presence or absence of doxycycline, to induce CREB1 shRNA, and levels of **A)** basal integrin α6 (ITGα6, green), luminal AR (red), and **B)** active caspase 3 (red) measured by immunostaining and nuclei counterstained (blue). **C)** iPrEC and EMP cells were differentiated for 12 days in culture and levels of basal integrin α6 (ITGα6, green) and luminal AR (red) measured by immunostaining and nuclei counterstained (blue). **D,E)** EMP-shCREB1 cells were differentiated for 12 days in the presence or absence of doxycycline, to induce CREB1 shRNA, and levels of **A)**

basal integrin α6 (ITGα6, green), luminal AR (red), and **B)** active caspase 3 (red) measured by immunostaining and nuclei counterstained (blue).

The acceleration of differentiation and induction of death of the differentiated luminal cells by CREB1 knockdown, is exactly what happens when we overexpress ING4 in iPrECs (cite). Transient induction of ING4 is required for luminal cell differentiation. Prolonged ING4 expression induces luminal cell death. Thus, we hypothesized that CREB1 might be the negative regulator of ING4 that turns it off during luminal cell terminal differentiation. In support of this, ING4 has a CRE binding domain in its promoter, and we were able to ChIP CREB1/ATF1 on that promoter element (**Fig. 5A**). Consistent with our hypothesis, we found that ING4 is elevated in CREB1 knockdown cells in both iPrECs and EMPs (**Fig. 5B,C**). We previously demonstrated that re-expressing ING4 in EMPs rescues differentiation (cite), but like in the iPrECs, the prolonged expression of ING4 induces death. Therefore, loss of CREB1 in EMP cells also restores differentiation by up-regulating ING4, but the differentiated cells die. We would argue that the death induced in the iPrEC and EMP cells upon CREB1 loss is due to prolonged ING4 expression, but we can't rule out the possibility that CREB1 has other targets that may promote survival as well.

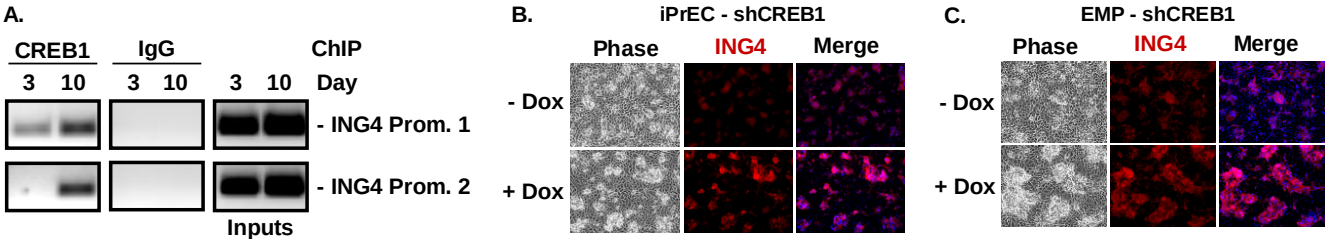


Fig. 5: CREB1 suppresses ING4 expression. A) ChIP of CREB1 on the two different CRE sites in the ING4 promoter after 3 and 10 days of differentiation compared to IgG control. **B,C)** ING4 expression measured in **B)** iPrEC-shCREB1 or **C)** EMP-shCREB1 cells were differentiated for 12 days in the presence or absence of doxycycline, to induce CREB1 shRNA, and ING4 levels assessed by immunostaining (red) and nuclear counterstain (blue).

Task 2 - subtask 2: ATF1-dependent differentiation – determine if loss of ATF1 in EMP cell will rescue differentiation

We found that loss of ATF1 in normal iPrECs prevents differentiation and makes the cells look like tumorigenic EMP cells, in that the cells co-express both basal (ITGα6) and luminal markers (AR, K18) (**Fig. 6A**) and so loss of ATF1 in tumorigenic EMP cells did not rescue differentiation (**Fig. 6B**). In contrast to CREB1, loss of ATF1 prevents the induction of ING4 (**Fig. 6C**). Thus, ATF1 is required for ING4 induction in luminal cells, while CREB1 acts to limit the duration of ING4 expression.

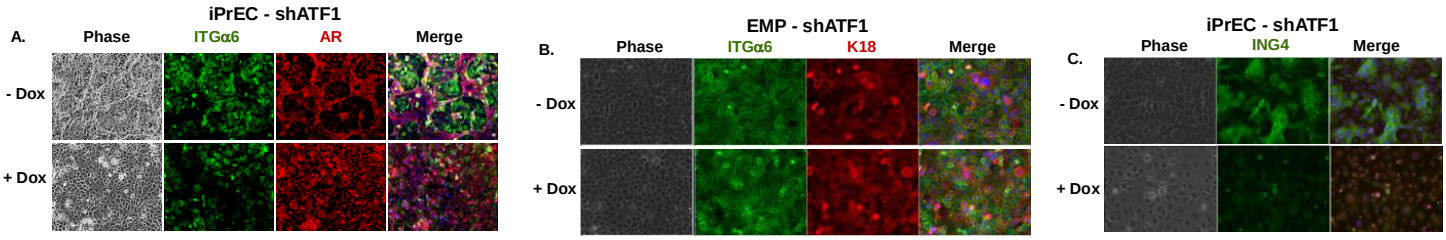
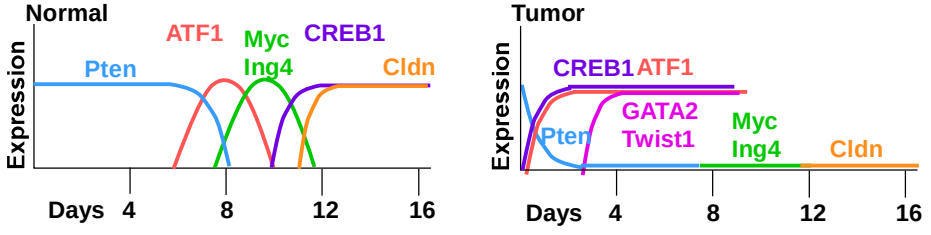


Fig. 6: ATF1 is required for luminal cell differentiation and its loss cannot rescue tumor cells because ING4 cannot be induced. iPrEC-shATF1 or EMP-shATF1 cells were differentiated for 12 days in the presence or absence of doxycycline to induce ATF1 shRNA and immuno-stained for **A)** ITGα6 and AR, **B)** ITGα6 and K18, and **C)** ING4.

All our findings to date, support our model that premature activation of CREB1, by loss of PTEN in the EMP cells, prevents ING4 from ever being induced during normal differentiation and promotes the tumorigenic phenotype (**Fig. 7**).

Fig. 7: Model for CREB1/ATF1 action in differentiation vs oncogenesis: In normal PrECs, Pten is elevated until around day 8, when ATF1 is transiently activated, followed by CREB1 activation. Transient ING4 peaks within this same time frame. Once CREB1 is activated, terminal differentiation genes, such



as claudin (Cldn), are turned on. In tumor cells in which Pten is lost, CREB1 and ATF1 are constitutively activated prior to ING4, and because CREB1 turns off ING4, ING4 cannot be induced. This leads to aberrant expression of transcriptional factors (i.e. GATA2 and Twist1).

Milestone: Manuscript – CREB1 vs ATF1 role in luminal cell differentiation

We have a preliminary draft of a manuscript describing the role of CREB1 and ATF1 in prostate luminal cell differentiation, based on the preliminary data from the grant application and these additional studies. We anticipate submitting it within the next month for publication.

Task 3 – Subtask 1: Local IACUC Approval

We obtained local IACUC approval to conduct the proposed animal experiments.

Task 3 – Subtask 2: ACURO Approval

We are awaiting ACURO approval of the protocol.

Aim 2: Identify the CREB1/ATF1 targets necessary for prostate cancer oncogenesis

Task 4 – Subtask 1: Perform RNA-Seq profiling to determine the transcriptional targets affected by PTEN loss.

We recently initiated these studies by first verifying that the tumorigenic EMP-shCREB1 cells we will be injecting to mice still overexpress Erg and Myc and have reduced PTEN (**Fig. 8A**). In addition, to using our tumorigenic EMP versus non-tumorigenic EM cells, we will also use another tumor cell line, 22Rv1, that has intact PTEN and compare it to a 22Rv1 line we generated by CRISPR that lacks PTEN (**Fig. 8B**). To validate the function of PTEN loss in 22Rv1ΔPTEN cells, we demonstrated that these cells are now more sensitive to death induced by a PI3K inhibitor (**Fig. 8C**). This approach will improve the rigor of the experiment and allow us to better focus in on the genes that are PTEN targets in both cell lines.

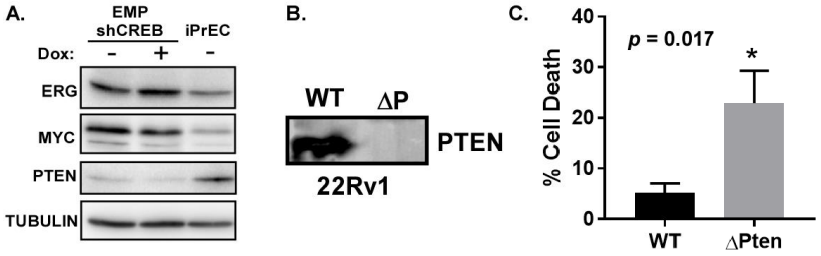


Fig. 8: Validation of tumor lines for in vivo mouse studies. A) Tumorigenic EMP-shCREB cells stimulated with or without 100ng/ml Doxycycline for 5 days were assessed for the levels of Erg, Myc, PTEN, and tubulin expression by immunoblotting. Normal iPrECs served as the control. B) PTEN was deleted in 22Rv1 cells using CRISPR and loss of PTEN (ΔP) validated by immunoblotting. **C)** Wildtype and PTEN-deleted 22Rv1 cells were treated for two days with 500nM PX866 and percent cell death measured by trypan exclusion. n=3; **p*=0.017

What opportunities for training and professional development has the project provided?

Nothing to Report

How were the results disseminated to communities of interest?

Nothing to Report

What do you plan to do during the next reporting period to accomplish the goals?

We are on track to finish all the Tasks in Aim 1 (Tasks 1-3), including our major milestones of generating two publications. We anticipate successful completion of 2 of the 3 Tasks in Aim 2 (Tasks 4-5). At this point we will have identified and validated several CREB1/ATF1 targets and can start on Aim 3 to identify antibodies and perform preliminary IHC staining on some tissues to prepare for the PCBN application required in Aim 3.

IMPACT

What was the impact on the development of the principal discipline(s) of the project?

We have identified new mechanisms that control normal luminal prostate differentiation and demonstrated how a specific oncogene, PTEN, through dysregulation of CREB1/ATF1 and disruption of

differentiation leads to the onset of prostate cancer. We've identified two regulatory mechanisms, i.e. CREB1 and JFK, around how an important differentiation regulatory protein, ING4, is induced and suppressed at the proper time of differentiation to commit the luminal cells to a terminally differentiated state. We further demonstrated how the dysregulation of CREB1/ATF1, due to PTEN loss, suppresses ING4 to allow for tumor cells to form. These findings fill in the question about why ING4 loss occurs in prostate cancer and how loss of PTEN contributes to ING4 loss.

These are the first studies to ever functionally link PTEN and ING4, to link CREB1/ATF1 with ING4, and to describe a new mechanism for how loss the tumor suppressor PTEN in prostate cancer promotes tumorigenesis. What makes these studies even more significant is that they were conducted in a unique human genetic model that mimics many properties of the human prostate gland.

Ultimately, by identifying the mechanisms by which PTEN leads to aggressive disease through CREB1/ATF1, we hope to determine which of these targets can serve as biomarkers for detecting potentially aggressive tumors much earlier.

What was the impact on other disciplines?

Epithelial differentiation occurs in multiple organs and some of the processes involved are likely to be conserved. ING4 and CREB1 expression are also high in liver and kidney, opening the possibility that a similar crosstalk may exist in those tissues.

The unique human prostate luminal epithelial differentiation model employed in these studies serve as a model for developing similar models in other organs. There is a large initiative in the research community to generate 'organs-on-a-chip' based on human tissue to better represent the genetics and physiology of human biology. The goal is to provide better model systems for testing and advancing therapeutic drugs to the clinic. The more we learn about the mechanisms operating in the model, the more we can contribute to defining the authenticity of the 'organs-on-a-chip' and their feasibility for therapy testing.

What was the impact on technology transfer?

Nothing to Report

CHANGES/PROBLEMS:

Changes in approach and reasons for change

Task 1 - Subtask 2: Quantify the level of mRNA expression of 529 predicted CREB1/ATF1 target genes using oligo bar-coded hybridization techniques such as NanoString in ATF1 or CREB1 shRNA knock-down cells.

We have been delayed on the analysis of the remaining CREB1/ATF1 targets, because the original nano-string technology we proposed to use is very expensive for the custom array we need. Therefore, we are now collaborating with Dr. Bruce Seligman to use his oligo-based hybridization TempO-Seq technology (cite). This approach will not only allow us to interrogate the specific CREB1/ATF1 targets already identified, but also the whole genome to identify other targets, for more than half the cost of nano-string. We are in the process of isolating the RNA from triplicate experiments for hybridization and sequencing.

Task 3 - Subtask 3 and 4: shCREB and shATF1 Xenografts – determine if loss of CREB1 or ATF1 in EMP cells will prevent tumorigenesis

We were originally going to use ultrasound as our method for monitoring tumor growth of orthotopically implanted Tet-inducible shCREB1 EMP cells. But the collaborator who was doing this for us left. We are now generating luciferase-expressing cells so that we can monitor tumor growth by IVIS. The same will be done with the Tet-inducible shATF1 EMP cells as well as the EMP control cells. The protocol submitted to ACURO reflect these changes.

Actual delays and actions to resolve them

I estimate we are 4-6 months behind our original scheduled timeline. The primary cause for this was a significant delay in getting a visa required to hire the personnel with the requisite experience for carrying out the ChIP and RNA-Seq experiments. Our original hire date was August 1, consummate with the funding, but the visa did not clear until late November. Then a couple months was required to get the new person familiar

with the models and techniques. A second delay was due to a need to find and train the personnel needed for the mouse studies and get the protocols approved. Part of this was caused by my move from VARI to University of Arizona, where the personnel who were originally slated to do the work moved on to other positions. Otherwise we are moving forward at the speed, and on task, as originally planned. We have not encountered any technical or feasibility problems.

PRODUCTS:

Journal publications

Nothing to Report

Books or other non-periodical, one-time publications.

Nothing to Report

Other publications, conference papers, and presentations.

Conference Presentations:

Four Abstracts are included in the Appendix

Oral Presentations

Watson MJ, Berger PL, Frank SB, and **Miranti, CK**. PTEN and CREB Cooperativity during Prostate Luminal Epithelial Differentiation is Disrupted in Prostate Cancer. **11th Prostate Cancer Symposium at Clark Atlanta University**, Atlanta, GA, September 26-28, 2016

Watson MJ, Berger PL, Frank SB, Basile AJ and **Miranti, CK**. Pten Loss Promotes Prostate Cancer Tumorigenesis through Disruption of a CREB-Dependent Differentiation Pathway. **University of Arizona Cancer Center Retreat**, Tucson, AZ, Apr 21, 2017.

Watson MJ, Burger PL, Frank SB, Winn M, and **Miranti, CK.** PTEN Loss Promotes Prostate Cancer Tumorigenesis through Disruption of a CREB-Dependent Differentiation Pathway. **SBUR Annual Meeting**: **Multiple Genetic and Epigenetic Mechanisms of Urologic Disease**, Tampa, FL, Nov 9-12, 2017.

Watson MJ, Burger PL, Frank SB, Winn M, and **Miranti, CK**. PTEN Loss Disrupts a CREB-Dependent Differentiation Pathway during Prostate Cancer Oncogenesis, **Ventana Medical Systems**, Oro Valley, AZ, Nov 13, 2017.

Watson MJ, Burger PL, Frank SB, Winn M, Tran J, Banerjee K, and **Miranti, CK**. How and Why Integrin α6β1 Drives Prostate Cancer. **Dana Farber Cancer Institute, Harvard Medical School**, Boston, MA Jun 12, 2018

Poster Presentations

Watson MJ, Burger P, Frank SB, Winn M, and Miranti CK. CREB1 and ATF1 Differentially Regulate Terminal Prostate Luminal Cell Differentiation by Controlling the Timing of ING4 Expression, while CREB1 Prevents ING4 Expression upon PTEN Loss in Prostate Cancer. **SBUR Annual Meeting**: **Multiple Genetic and Epigenetic Mechanisms of Urologic Disease**, Tampa, FL, Nov 9-12, 2017. Abstract Published: Am J Clin Exp Urol 2017;5(Suppl 1):1-92 Poster P51, p60. www.ajceu.us

Watson MJ, Burger P, Frank SB, Winn M, and Miranti CK. CREB1 and ATF1 Differentially Regulate Terminal Prostate Luminal Cell Differentiation by Controlling the Timing of ING4 Expression, while CREB1 Prevents ING4 Expression upon PTEN Loss in Prostate Cancer. **AACR Special Conference on Prostate Cancer**, Orlando, FL, Dec 2-5, 2017.

Website(s) or other Internet site(s)

Nothing to Report

Technologies or techniques

Nothing to Report

Inventions, patent applications, and/or licenses

Nothing to Report

Other Products

Nothing to Report

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Cindy Miranti, PhD.
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	0000-0003-0668-6106
Nearest person month worked:	1.2 months
Contribution to Project:	Supervisor
Funding Support:	University of Arizona

Name:	Sander Frank, PhD
Project Role:	Postdoctoral Fellow
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1 month
Contribution to Project:	ING4 studies,
Funding Support:	DOD W81XWH-14-1-0479

Name:	Kasturi Banerjee, PhD
Project Role:	Assistant Scientific Investigator
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	8 months
Contribution to Project:	ChIP and RNA-Seq, EMP differentiation, bioinformatics
Funding Support:	

Name:	Lin Tang, PhD
Project Role:	Assistant Scientific Investigator
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	2.4 months
Contribution to Project:	Mouse protocols and surgery
Funding Support:	

Name:	Jack Tran
Project Role:	Research Specialist
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	3.6 months
Contribution to Project:	iPrEC shCREB/ATF1 differentiation studies
Funding Support:	

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report

What other organizations were involved as partners?

Nothing to Report

SPECIAL REPORTING REQUIREMENTS

Not applicable

APPENDEX: Abstracts from oral and poster presentations.

Pten and CREB Cooperativity during Prostate Luminal Epithelial Differentiation is Disrupted in Prostate Cancer

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Myc is overexpressed in the majority of human prostate cancers (PCa), yet the mechanisms by Myc drives (PCa) are not fully understood. Using a novel *in vitro* differentiation model in which human basal prostate epithelial cells (PrECs) are induced to differentiate into luminal cells, we previously demonstrated that Myc is required for transient expression of the chromatin-binding protein ING4, whose transient induction and subsequent down regulation is required for normal luminal cell differentiation. ING4 expression is lost in >60% of primary prostate tumors, loss of ING4 prevented PrEC differentiation, and was necessary for Myc-dependent tumorigenesis *in vivo*. Furthermore, loss of Pten prevented ING4 induction and differentiation (Cancer Res 74:3357-68, 2014). Our objective is to identify the mechanism by which ING4 expression is controlled by Pten. Using RNA-Seq to interrogate genes induced during PrEC differentiation we determined that ~20% of the differentially regulated genes are targets of the transcription factor, CREB. Furthermore, CREB-regulated genes were highly expressed in PrECs transformed by overexpression of Erg, Myc, and shRNA to Pten (EMP cells). However, the CREB genes induced in normal PrECs were completely different from the genes induced in EMP cells, suggesting there is a major genetic switch surrounding CREB-mediated transcription in tumor cells vs normal cells. We found that during normal PrEC differentiation, Pten is elevated until around Day 8-10, then its expression drops and CREB is then activated around Day 12. Loss of Pten results in constitutive activation of CREB indicating that Pten acts to control the timing of CREB activation. ING4 is induced prior to CREB activation and decreases when CREB activity is high, and removal of CREB by shRNA results in constitutive induction of ING4. Thus, CREB is a negative regulator of ING4, downstream of Pten during normal PrEC differentiation. Thus, in the tumor cells when Pten is lost, CREB is constitutively activated and prevents ING4 induction. Furthermore, in this context CREB induces a different set of genes that promote tumorigenesis, rather than differentiation. Defining these differential targets may allow us to identify specific therapeutic targets or biomarkers.

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Pten Loss Promotes Prostate Cancer Tumorigenesis through Disruption of a CREB-Dependent Differentiation Pathway

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Background: Genetic drivers of prostate cancer (PCa) development, including Myc overexpression or Pten loss, are linked to prostate epithelial differentiation. But, how these oncogenes alter the differentiation program during PCa development remains unresolved. We previously showed, in normal human prostate epithelium, that Myc induces transient expression of a chromatin-binding protein, ING4, which is required for prostate luminal cell differentiation. However, ING4 expression is lost in >60% of primary tumors and ING4 loss prevents luminal cell differentiation. In fact, ING4 loss is necessary for Myc-overexpressing cells to produce tumors. We further found that Pten loss prevents luminal cell differentiation by blocking ING4 induction and 50% of primary tumors with ING4 loss also lack Pten. We sought to determine how Pten loss suppresses ING4 expression and differentiation.

Methods: We used RNA-Seq to identify gene signatures induced during human luminal cell differentiation and compared them to those induced in tumor cells with combined Myc overexpression and Pten loss (Myc/shPten). Gene signatures were validated by qRT-PCR and immunoblotting. Functional assessment of the role of specific genes on differentiation, ING4 expression, and tumorigenesis was carried out using shRNA knock-down and chromatin immunoprecipitation (ChIP).

Results: During luminal cell differentiation, ~30% of the differentially regulated genes are targets of the transcription factor CREB. We found that late in luminal cell differentiation, ATF1 activation precedes CREB1, at a time when Pten expression drops. CREB-target genes associated with luminal cell differentiation are induced at this time, including ING4 and its E3-ligase JFK. Accordingly, CREB1/ATF1 are bound to the promoters of both ING4 and JFK. Paradoxically, knock-down of CREB1 results in overexpression of ING4 and accelerated differentiation, while knock-down of ATF1 prevents ING4 induction and differentiation. Loss of CREB1 also eventually induces luminal cell death. Surprisingly, a large set of CREB-regulated genes are highly expressed in the Myc/shPten tumor cells; however, this set of genes is distinctly different from those induced in the normal cells. Pten loss, with or without Myc overexpression, is sufficient to constitutively activate CREB1 and ATF1 in normal prostate epithelium. Several CREB targets involved in progenitor cell maintenance, including GATA2 and Twist1, are elevated in the tumor cells. Knock-down of CREB1 in tumor cells restores ING4 expression and rescues differentiation.

Conclusions: Early in prostate luminal cell differentiation, elevated Pten prevents premature activation of ATF1 and CREB1. ATF1 is required for the transcriptional induction of ING4 and initiation of luminal differentiation, while CREB1 limits ING4 expression, possibly through induction of its E3-ligase, and is required for luminal cell survival. However, in the absence of Pten, CREB1 and ATF1 are activated prematurely, preventing ING4 induction and terminal differentiation, leading to maintenance of a progenitor cell-like proliferative population.

Impact: Determining which PCa patients are at risk for lethal disease remains a major challenge. We propose there will be a specific combination of oncogenic events and differentiation alterations that produce an aggressive subtype of PCa. If this signature can be identified, then the ability to distinguish indolent from lethal disease can be achieved.

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CREB1 and ATF1 differentially regulate terminal prostate luminal cell differentiation by controlling the timing of ING4 expression, while CREB1 prevents ING4 expression upon PTEN loss in prostate cancer

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We previously demonstrated that transient ING4 expression is required for luminal cell differentiation. Additionally, ING4 is downregulated in ~60% of primary prostate tumors and its loss is correlated with loss of PTEN. We further demonstrated in a primary prostate cancer model overexpressing ERG, MYC, and shPTEN (EMP) that loss of PTEN was responsible for ING4 downregulation, though the mechanism remained elusive. Utilizing RNA-seq and our *in vitro* differentiation model, we identified transcriptional nodes required for luminal differentiation. Of the ~600 differentially regulated genes during differentiation, the largest transcription factor signature (29% of genes) was CREB/ATF. A subset of these targets (Blimp1, Claudin1, Plk2, Chek1) were further validated by qRT-PCR and immunoblotting.

CREB/ATF bind constitutively to open chromatin CRE elements and can be activated by multiple kinases, including AKT. Both CREB1 and ATF1 are inducibly phosphorylated midway through luminal differentiation, with ATF1 preceding CREB1. Knockdown of CREB1 with shRNA increased ING4, accelerated differentiation, and induced premature luminal cell death. Conversely, knockdown of ATF1 blocked ING4 induction and prevented supra-basal layer formation. CREB1/ATF1 ChIP was enriched at the ING4 promoter at mid-differentiation, when ING4 expression peaks. Additionally, CREB1/ATF1 was constitutively bound to the promoter of JFK, an E3-ligase that targets ING4 and whose mRNA levels increase during differentiation. Thus, we propose that ATF1 is required to induce ING4 transcription while CREB1 suppresses ING4 by both transcriptional repression and induction of JFK. We compared the gene signature of differentiated cells to that of the tumorigenic EMPs. Surprisingly, 30% of the differentially expressed genes were CREB/ATF targets but there is less than 10% overlap, indicating CREB/ATF control distinct subsets of genes in differentiated luminal cells versus cancer cells. Some EMP-specific CREB/ATF targets (GATA2, TWIST1, Necdin, PPM1F) were further validated by qRT-PCR and immunoblotting. CREB1 and ATF1 were highly phosphorylated in EMP cells and knockdown of CREB1 restored ING4 expression and supra-basal formation.

Our working model is that AKT activation upon PTEN loss in transiently differentiating luminal cells results in premature and constitutive activation of CREB1/ATF1 bound to genes prior to induction of the ING4 chromatin switch. This prevents ING4 induction and the chromatin rearrangements required for terminal differentiation. In normal PrECs, CREB/ATF1 activation is tightly controlled by as of yet undetermined factors and is only permitted when the proper CRE binding sites are exposed. This model helps to explain how loss of PTEN disrupts luminal cell terminal differentiation to promote prostate cancer oncogenesis.

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Many genes aberrantly expressed in prostate cancer are involved in normal basal to luminal cell differentiation. We previously demonstrated that transient ING4 expression is required for luminal cell differentiation and is downregulated in ~60% of primary prostate tumors. We further demonstrated in a primary prostate cancer model overexpressing ERG, MYC, and shPTEN (EMP) that loss of PTEN was responsible for ING4 loss. Furthermore, half of the human tumor samples that lose ING4 have also lost PTEN. However, we did not know how PTEN loss inhibits ING4 expression. Utilizing our *in vitro* differentiation model, whereby prostate basal epithelial cells (iPrEC) treated with KGF and androgen induce a supra-basal layer of luminal-like cells, and RNA-seq we identified transcriptional nodes required for luminal differentiation. Differentially expressed genes were analyzed by GeneGo to identify enriched transcription-factor signatures. Of the ~600 differentially regulated genes during differentiation, the largest signature (29% of genes) was CREB/ATF targets. Induction of Blimp1, Claudin1, and Plk2 and inhibition of Chek1 were further validated by qRT-PCR and immunoblotting.

CREB/ATF bind constitutively to open chromatin CRE elements in the promoters of genes and are activated through signaling-induced phosphorylation at Ser133 by kinases, including AKT. We found that both CREB1 and ATF1 are inducibly phosphorylated midway through luminal differentiation, with ATF1 preceding CREB1. Knockdown of CREB1 with shRNA increased ING4, accelerated differentiation, and induced premature luminal cell death. Conversely, knockdown of ATF1 blocked ING4 induction and prevented supra-basal layer formation. CREB1/ATF1 ChIP was enriched at the ING4 promoter at mid-differentiation, when ING4 expression peaks. Additionally, CREB1/ATF1 was constitutively bound to the promoter of JFK, an E3-ligase that targets ING4 and whose mRNA levels increase during differentiation. Thus, we propose that ATF1 is required to induce ING4 transcription, while CREB1 suppresses ING4 and simultaneously activates its E3-ligase to tightly control the timing of ING4 expression. We compared the gene signature of luminal cells to that of the tumorigenic EMP cells and surprisingly found 30% of the differentially expressed genes were also CREB/ATF targets. However, there is less than 10% overlap in these targets, indicating CREB/ATF control distinct subsets of genes in differentiated luminal cells versus cancer cells. Some of EMP-specific CREB/ATF targets included GATA2, TWIST1, Necdin, and PPM1F, which were further validated by qRT-PCR and immunoblotting. CREB1 and ATF1 were highly phosphorylated in EMP cells and knockdown of CREB1 restored ING4 expression and supra-basal formation.

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