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14. ABSTRACT

The goal of this project is to identify specific differentiation events whose disruption by Myc and Pten leads to aggressive PCa. Our Aims are to 1) determine how ING4 controls prostate epithelial differentiation; 2) determine how loss of ING4 impacts tumorigenesis; and 3) determine how loss of ING4 in patients relates to tumor progression. We found the following: 1) Notch3 is a target of Myc required for differentiation. 2) CREB/ATF1 controls ING4 expression and differentiation, and the dynamics of CREB/ATF1 activation and its targets differ between normal and tumor cells. 3) Miz1 is an ING4 target required to suppress integrin α6 and β1 that is absent in tumor cells. 4) Erg negatively impacts differentiation, but only when expressed in the AR-positive cells. 5) Pten protein phosphatase activity sets the timing of differentiation, CREB/ATF1 activation, and induction of ING4. We identified targets of Myc, ING4, and CREB that can be used to screen human tissues proposed in Aim 3. We have met almost all of our first year objectives and extended into some of next year's objectives.

15. SUBJECT TERMS
Prostate epithelial differentiation, Myc, ING4, chromatin, integrins, Erg, Pten, Miz1, CREB, Notch, p38, prostate cancer oncogenesis, TMA, mouse model, human model.

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1. Introduction

Being able to determine which PCa patients have indolent disease and require minimal treatment, versus those who will die unless aggressively treated, remains a major challenge. The goal of this project is to test the **hypothesis** that Myc normally promotes prostate epithelial differentiation through chromatin remodeling mediated by ING4, such that loss of ING4 is required for Myc oncogenesis, which leads to aggressive disease through suppression of differentiation. Our **specific aims** are: Aim 1: Determine how ING4 controls prostate epithelial differentiation. We hypothesize that Myc normally promotes prostate epithelial differentiation through chromatin remodeling mediated by ING4. Aim 2: Determine how loss of ING4 impacts tumorigenesis. We hypothesize that loss of ING4 cooperates with specific oncogenes to disrupt terminal differentiation, which is required for aggressive tumorigenesis. Aim 3: Determine how loss of ING4 in patients relates to tumor progression. Our objectives are to 1) establish if there is a correlation between ING4 loss and over expression of Myc, Erg fusions, or Pten loss, and the relationship to disease recurrence in patients; and 2) determine how ING4 expression correlates with the expression of known differentiation markers in the tumors.

2. Keywords

Prostate epithelial differentiation, Myc, ING4, chromatin, integrins, Erg, Pten, Miz1, CREB, Notch, p38, prostate cancer oncogenesis, TMA, mouse model, human model

3. Accomplishments

The major goals of the project:
Underlined dates indicate completed or partially completed tasks.

Specific Aim 1: Determine how ING4 controls prostate epithelial differentiation

Major Task 1: ING4 and Myc Targets
- Subtask 1a: Determine how ING4 impacts expression of three Myc target genes
  - Months 1-2
- Subtask 1b: Carry out Myc ChIP analysis of three Myc target genes
  - Months 3-6

Major Task 2: ING4 and EZH1/2
- Subtask 2a: Determine how ING4 impacts EZH1/2 expression
  - Months 7-8
- Subtask 2b: Identify EZH1/2 targets controlled by ING4
  - Months 9-11
- Milestone #1: Prepare manuscript for publication
  - Months 11-12

Major Task 3: Global targets
- Subtask 3a: Initiate ChIP studies by optimizing and validating techniques
  - Months 1-6

Subtask 3b: Isolate mRNA for RNA-Seq studies
Months 5-6
Subtask 3c: Set up ChIP studies for sequencing
Months 6-20
Subtask 3d: Run RNA-Seq studies
Months 6-20
Subtask 3e: Analysis of ChIP-Seq and RNA-Seq data
Months 21-28
Subtask 3f: Validation of hits in model
Months 28-34
Milestone #2: Prepare manuscript for publication
Months 35-36

Specific Aim 2: Determine how loss of ING4 impacts tumorigenesis

Major Task 4: Oncogenic Suppression of ING4 and Tumorigenesis
Subtask 4a: IACUC animal protocol approval (80 mice).
Months 1-3
Subtask 4b: Generation of oncogenic iPrEC cell line combinations
Months 1-3
Subtask 4c: Testing of cell line combinations in vitro and in vivo
Months 4-8
Subtask 4d: Analysis of tumor tissues
Months 9-12
Subtask 4e: Analysis of tumor cell lines in 3D models
Months 13-15

Major Task 5: Oncogenic Control of ING4 Expression
Subtask 5a: Measure ING4 mRNA and protein in oncogenic lines
Months 9
Subtask 5b: Measure ING4 mRNA and protein stability in oncogenic lines
Months 10-11
Subtask 5c: Assess oncogenic effects on ING4 promotor
Months 10-15
Milestone #3: Prepare manuscript for publication
Months 16-17

Major Task 6: ING4 Loss and Myc Cooperation in Tumorigenesis
Subtask 6a: IACUC animal protocol approval (720 mice)
Months 1-3
Subtask 6b: Initiate in vitro fertilization to generate Pb-Myc and ING4 KO breeders
Months 4-6
Subtask 6c: Breeding to generate double mutant mice
Months 6-12
Subtask 6d: Monitoring and analysis of tumor development
Months 13-24

Subtask 6e: Assessment of tumor pathology and IHC
Months 25-30
Milestone #4: Prepare manuscript for publication
Months 31-32

Specific Aim 3: Determine how loss of ING4 in patients relates to tumor progression

Major Task 7: Regulatory processes
Subtask 7a: Apply for TMA samples from PCBN
Months 1-3
Subtask 7b: IRB paperwork and approval
Months 1-3

Major Task 8: Correlation of ING4 Loss with Outcome and Oncogenic Events
Subtask 8a: Optimization of IHC staining on VARI tester TMA
Months 1-6
Subtask 8b: IHC staining of PCBN arrays
Months 7-16
Subtask 8c: Statistical analysis of data
Months 17-18

Major Task 9: ING4 Loss and Tumor Differentiation
Subtask 9a: Optimization of IHC staining on VARI tester TMA
Months 19-24
Subtask 9b: IHC staining of PCBN and VARI arrays
Months 25-32
Subtask 9c: Statistical analysis of data
Months 32-34
Milestone #5: Prepare manuscript for publication
Months 35-36

What we accomplished under these goals:

Aim 1: Determine how ING4 controls prostate epithelial differentiation. We hypothesized that Myc normally promotes prostate epithelial differentiation through chromatin remodeling mediated by ING4. Thus, to figure out how Myc and ING4 cooperate to promote differentiation, we need to identify relevant targets of Myc and ING4. We took 2 approaches, interrogation of 'best guess' targets (**Tasks 1 and 2**), and a global approach using RNA-Seq and ChIP-Seq (**Task 3**).

In **Task 1** we looked at genes known to be regulated by Myc, specifically ODC, cyclin D1, and integrin α6. We found that ING4 suppresses ODC and cyclin D1 expression, and Myc does not ChIP on those targets in differentiating PrECs. We are currently working on integrin α6 as detailed further under Task 3.

In **Task 2** we interrogated 2 known chromatin modifying enzymes, EZH1/2 as possible targets of ING4. ING4 did not ChIP on these promoters, EZH1 expression was unchanged, and EZH2 expression went down upon ING4 induction. Thus, although EZH2 loss is likely

important for differentiation, it is not an ING4 target. Thus, our initial milestone of preparing a manuscript on these proposed Myc and ING4 targets was not achieved. However, we were much more successful at identifying both Myc and ING4 targets in Task 3 using RNA-Seq, which will allow us to reach a slightly delayed publication milestone.

For **Task 3**, we set up 3 major RNA-seq studies. The first was a time course of normal PrEC differentiation compared to tumorigenic PrECs (EMPs – Erg+Myc overexpression+Pten shRNA (See Aim 2)) taken at days 0, 4, 8, 11, 14, and 17 of differentiation. In the second experiment, to identify ING4-specific targets we compared normal PrECs differentiated for 10 days (time of peak ING4 expression), PrECs constitutively overexpressing ING4 differentiated for 3 days (these cells accelerate differentiation), PrECs expressing shING4 differentiated for 10 days, and undifferentiated PrECs. The third experiment was designed to identify both p38-MAPK and Myc targets. We previously determined that p38-MAPK is essential for initiating differentiation (Lamb *et al.*, 2010), and Myc is a target of p38. Therefore, we generated a Tet-inducible PrEC line to express constitutively active MMK6, the p38 upstream activator. A short pulse of doxycycline is sufficient to induce p38 phosphorylation and Myc induction 2-fold (equivalent to what we see in normal differentiating cells), peaking at 7 hours (**Fig. 1a**). Using an RNA-Seq technique which measures newly synthesized RNA, called Bru-Seq (Paulsen *et al.*, 2014), we compared untreated to 7 hour doxycycline-treated MKK6 cells.

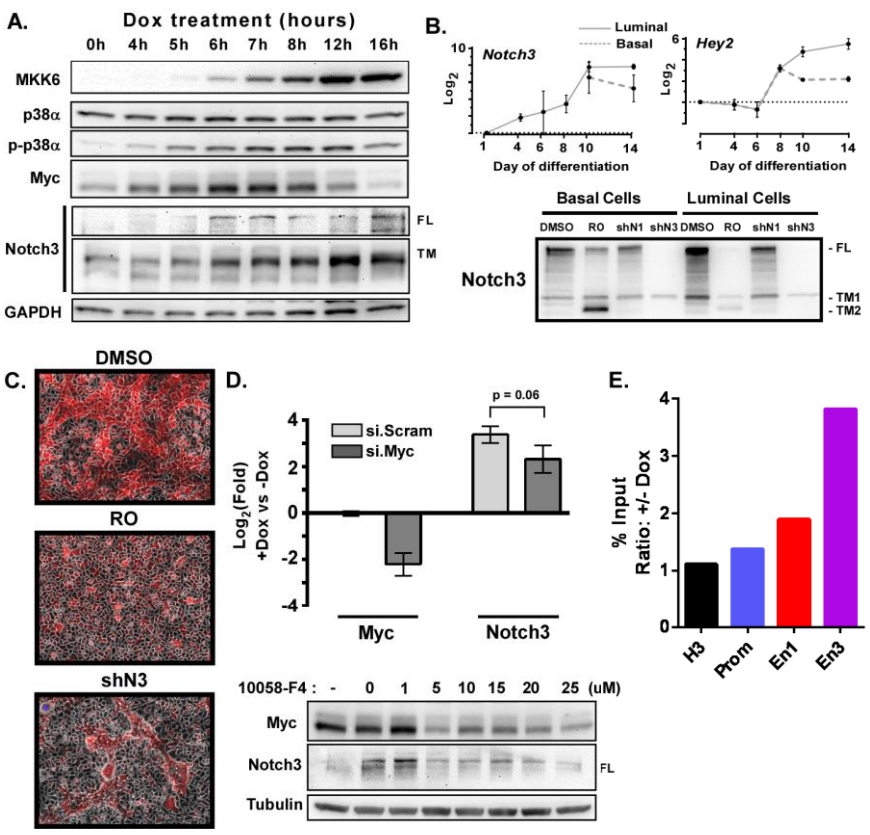


Figure 1: Notch3 induction requires Myc. A. MKK6 was induced in PrECs with a 4 hour doxycycline (Dox) pulse. Levels of MKK6, p38α, active p38α (p-p38α), Myc, Notch3 (full length (FL) or cleaved (TM), and GAPDH over time (hours (h)) measured by immunoblotting. **B.** Notch3 and Hey2 mRNA (graphs) measured by qRT-PCR at different times during differentiation. Top (secretory cells) and Bot (basal cells) were separated at days 8 and 10. Notch3 protein (full length (FL) or cleaved (TM1/TM2)) measured by immunoblotting in basal and luminal cells differentiated 16 days in the presence of vehicle (DMSO), γ-secretase inhibitor RO4929097 (RO) or Notch1 or Notch3 shRNA (shN1, shN3). **C.** PrECs treated as above and immunostained for E-cadherin (marks the luminal cells). **D.** MKK6 induced in PrECs for 7 hours after treatment with Myc (si.Myc) or scrambled (si.Scram) siRNA (graph) or Myc inhibitor (10068-F4) (blot). Levels of Myc or Notch3 mRNA (graph) or protein (blot) were measured in MKK6 cells induced with doxycycline (Dox). **E.** Myc ChIP on Notch3 promoter (Prom), enhancer 1 (En1) and enhancer 3 (En3) in MKK6 cells induced with doxycycline (+Dox) compared to uninduced cells (-).

Several sets of analyses were run on the RNA-Seq data sets. For the p38/Myc data we used GSEA to first subtract out stress-response genes known to be activated by p38-MAPK and to focus on those genes associated with differentiation, cytoskeleton, and cell adhesion (since differentiation is associated with the latter two events). The most highly induced gene in this list was Notch3. Correspondingly we observed induction of Notch3 full length and activated TM form 7-8 hours after MMK6 induction (**Fig. 1a**). Notch3 was also identified in the ING4 and time course RNA-Seq data. We then determined the relationship between Myc and Notch3 during differentiation. Some of our findings include 1) Notch3 mRNA and its target Hey2 is induced over 20-fold over the course of differentiation, and the majority of Notch3 protein ends up in the differentiated luminal cells (**Fig 1b**); 2) Blocking Notch3 with shRNA blocks differentiation (**Fig 1c**); 3) Inhibiting Myc reduces Notch3 induction (**Fig. 1d**), but Myc alone is not sufficient to induce Notch3; and 4) Myc ChIPs on two enhancers of the Notch3 gene (**Fig. 1e).** Thus, we identified at least one new Myc target. We are finishing the last stages of this study and preparing a manuscript to publish these findings.

In the second analysis, we took all the significant hits (those which changed by 2x or more in the time course and the ING4-manipulated cells) and passed them through Gene-Go to determine which transcription factors could be responsible for driving the expression of those genes. In addition to finding Myc and AR targets as expected, the factor with the most hits was CREB. What was even more striking, is that the set of CREB targets induced in normal PrECs during differentiation were completely different from the CREB targets induced in the tumorigenic EMP cells (**Fig. 2a**). To validate these findings, we monitored CREB and ATF1 (CREB binding partner) activation during differentiation and in the tumorigenic EMP cells. CREB activity is transiently induced during normal differentiation and peaks when ING4 is highest. ATF1 activation is also transient, but peaks a few days earlier when ING4 is induced (**Fig 2b**). In EMP cells, CREB and ATF1 were constitutively activated. We validated some of the CREB targets, demonstrating that BLIMP1 and CLDN1 are dramatically induced at the same time CREB is activated in normal PrECs, and are poorly induced in EMP cells (**Fig 2c**). We are currently validating EMP-specific CREB targets.

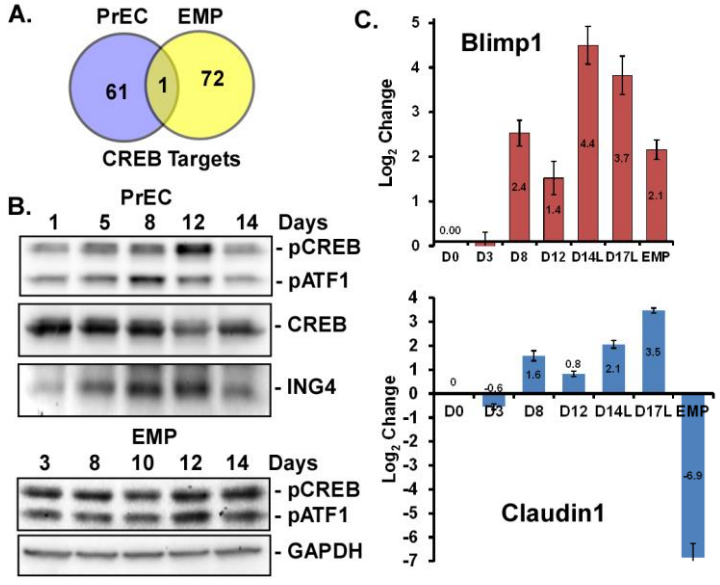
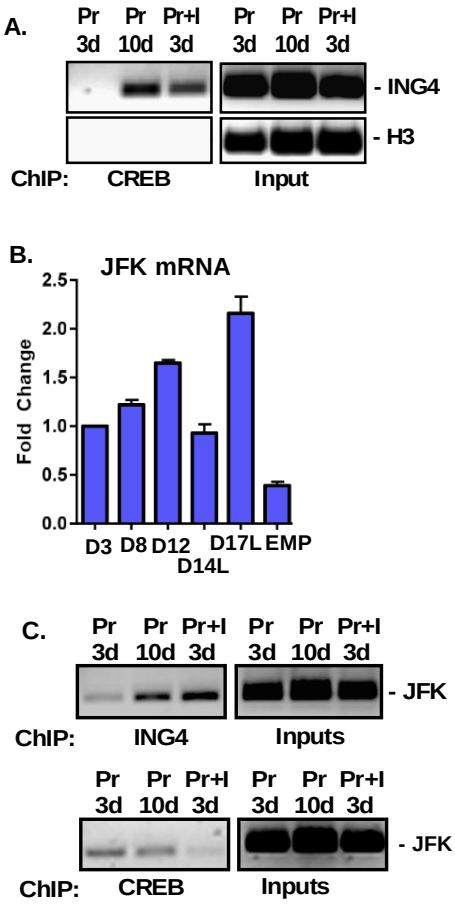


Figure 2: CREB Dynamics Differ Between Normal and Tumor Cells. A. The intersection of genes whose expression is increased in normal PrECs vs tumorigenic EMP cells that are transcriptional targets of CREB. **B.** CREB and ATF1 activation (pCREB, pATF1) as measured by phosph-immunoblotting various times (days) during PrEC and EMP cell differentiation compared to ING4 expression. **C.** CREB targets, Blimp1 and Claudin1 mRNA levels measured by qRT-PCR during PrEC differentiation and in EMP cells. D=days, L=luminal cells.

We used CREB/ATF1 and ING4 ChIP to begin teasing out the relationship between ING4 and CREB/ATF1 and to identify ING4 targets. We found CREB/ATF1 (antibody used for



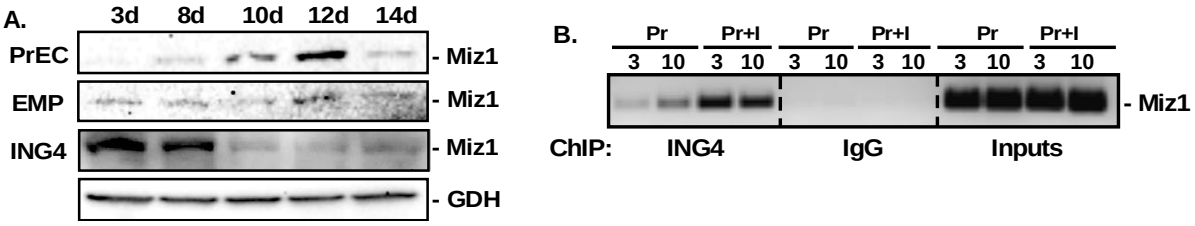
the ChIP does not distinguish CREB from ATF1) bound at the ING4 promoter (**Fig. 3a**) and ING4 overexpression enhanced this binding, suggesting ING4 influences CREB/ATF1 binding to its own promoter. We saw a 2-fold increase in expression of the ING4 E3 ligase (**Fig 3b**), JFK (Yan *et al.*, 2015). We found both ING4 and CREB/ATF1 bound to the promoter of JFK (**Fig. 3c**). However, in this case overexpression of ING4 resulted in increased ING4 binding as would be expected for a direct target, but ING4 over expression actually suppressed CREB/ATF1 binding at the JFK promoter. This suggests ING4 might limit the ability of CREB/ATF1 to induce JFK or change how JFK is induced. From these data we propose a working hypothesis whereby ING4 is initially induced by ATF1 (based on time course in Fig. 2b) and ING4 enhances CREB binding at the ING4 promoter (where CREB might limit ING4 expression), but at the same time induces JFK in a CREB/AFT1-independent manner to activate its own destruction later in differentiation, by activating its E3 ligase.

Figure 3: CREB binds ING4 promoter, and ING4 binds the promoter of its own E3 ligase, JFK. A. ChIP of CREB bound to ING4 promoter in normal PrECs (Pr) and PrECs overexpressing ING4 (Pr+I) at days 3 and 10 (3d, 10d) of differentiation. **B**. JKF mRNA levels in differentiating PrECs over time (D=days) and in tumorigenic EMP cells. L=luminal cells. **C.** ChIP of ING4 and CREB bound to JFK promoter in normal PrECs (Pr) and PrECs overexpressing ING4 (Pr+I) at days 3 and 10 (3d, 10d) of differentiation.

From the RNA-Seq data we also identified Miz1 as another ING4 target. Miz1 is a transcriptional repressor that suppresses Myc activity. In keratinocytes, the Miz1/Myc repressor binds integrin α6 and β1 promoters and is required to suppress their expression in suprabasal cells (Gebhardt *et al.*, 2006). We measured integrin mRNA during differentiation and found that integrin α3 and β4 were turned off 3 days before integrin α6 or β1. This supported our previous data based on immunostaining (Lamb *et al.*, 2010). Loss of integrin α3 and β4 coincided with Myc and Notch3 induction, while loss of integrin α6 and β1 coincided with ING4 expression. We found Miz1 expression is induced around the same time as ING4 during differentiation (**Fig. 4a**), and over expression of ING4 directly induces Miz1. Using ChIP we found increased ING4 binding to the Miz1 promoter 10 days after differentiation compared to 3 days. Overexpression of ING4 resulted in ING4 binding constitutively (at day 3) to the Miz1 promoter (**Fig 4b**). Thus, we propose ING4 is required to induce Miz1, which acts in concert with Myc to turn off integrin α6 and β1. Miz1 is not induced in the tumorigenic EMP cells (**Fig. 4a**), and these cells do not express ING4, and as a consequence integrin α6 and β1 expression are retained in these cells (Berger *et al.*, 2014).

Figure 4: Miz1 is an ING4 target. A. Miz1 expression measured by immunoblotting in normal PrECs, tumorigenic EMP, and PrECs overexpressing ING4 at different days during differentiation. **B.** ChIP of ING4 on the

Miz1 promoter in normal PrECs (Pr) and PrECs overexpressing ING4 (Pr+I) at days 3 and 10 (3d, 10d) of differentiation.



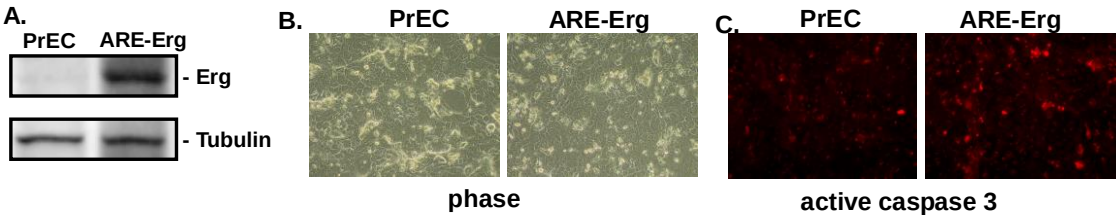
Thus, we made substantial progress, more than expected, successfully identifying both Myc and ING4 targets required for normal PrEC differentiation, and gained a better understanding of how ING4 controls differentiation. In addition, we identified other important markers of PrEC differentiation, Notch3, BLIMP1, Cldn1, CREB, ATF1, and JFK, and shown that some of these targets are disrupted in the tumorigenic EMP cells. We are well poised to begin to interrogate these markers in human tissues and relate them to patient outcomes as proposed in Aim 3. We also have additional potential Myc and ING4 targets within our RNA-Seq data to validate and study.

Aim 2: Determine how loss of ING4 impacts tumorigenesis. We hypothesize that loss of ING4 cooperates with specific oncogenes to disrupt terminal differentiation, which is required for aggressive tumorigenesis. Our published data indicate that ING4 is lost in 60% of primary prostate cancers. However, we do not know how ING4 is lost. We know that overexpression of Myc + Erg + Pten loss (EMP) generates tumorigenic cells that lose ING4 expression and fail to differentiate in vitro (Berger *et al.*, 2014). The goals of this aim are to determine which oncogenes are most important for ING4 loss in PCa development and progression using oncogenic manipulation of iPrECs in xenografts (**Task 4**), in vitro (**Task 5**) and development of an engineered mouse model (**Task 6**).

For **Task 4 and 5**, we want to identify which combination of oncogenes, i.e. Myc, Erg, or loss of Pten, is required to induce ING4 loss, prevent differentiation, and induce tumorigenesis. Thus far, we determined that overexpression of Myc, Erg, Myc+Erg, or loss of Pten alone, is not sufficient to induce tumors in orthotopic xenograft injections in vivo, is not sufficient to prevent differentiation in vitro, and do not suppress ING4 expression. We still need to test Pten in combination with either Erg or Myc alone. During these studies we became concerned that we were not detecting any impact of Erg overexpression on differentiation or tumorigenesis, and yet this should be an oncogene. Our original strategy was to overexpress the N-terminal truncated version of Erg found in tumors off a constitutive promoter such that it is expressed in both basal and luminal cells during differentiation. However, in human PCa this oncogene is only expressed in luminal-like cells in which AR is expressed. Thus, we re-engineered this gene to be expressed under the control of the PSA ARE enhancer and stably introduced it into PrECs. When we induce the differentiation of these cells, we saw specific induction of Erg (**Fig. 5a**). In these cells, it appeared that luminal cells would initially appear, but were not stable and appeared to be dying and would disappear (**Fig. 5b**). Cell death was confirmed by now see major alterations in their differentiation (**Fig 5c**). Thus, we propose there is something about Erg expression in luminal cells, and its absence in basal cells that is critical for its oncogenic properties. In the RNA-Seq data from the MMK6/p38/Myc induced differentiation model, one of the striking findings is the decrease in expression of Erg and almost all the ETV family members (all those found in PCa gene fusions), but not Ets genes. This suggests there is a

fundamental relationship between Erg/ETV loss in normal cells and its retention specifically in luminal cells that may drive PCa development. Thus, we will now use this modified Erg gene to repeat and extend these studies.

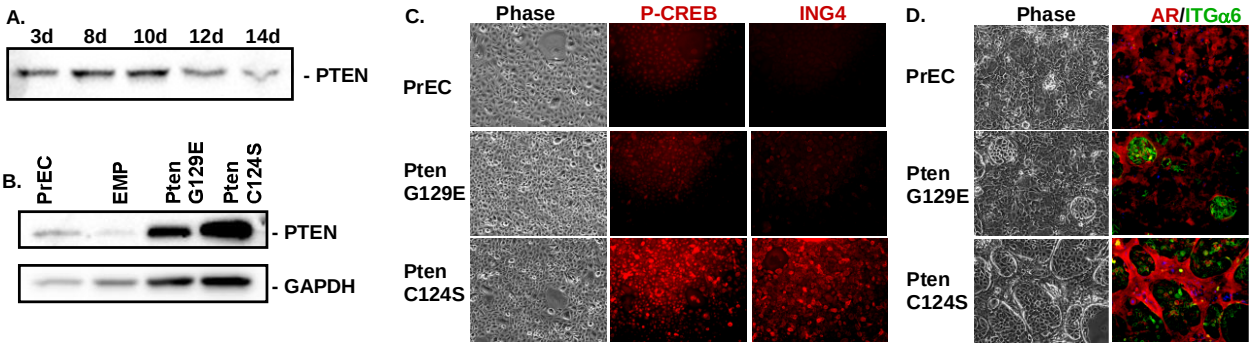
Figure 5: Erg under Control of Androgen Negatively Impacts Differentiation. A. Level of Erg and tubulin expression measured by immunoblotting in normal PrECs and PrECs expressing ARE-Erg after 21 days of differentiation. **B.** PrECs and PrECs with ARE-Erg differentiated for 16 days and viewed by phase contrast. Piles of luminal cells are appearing in the PrEC culture, but only single cells are appearing in ARE-Erg cells. **C.** PrECs and PrECs with ARE-Erg differentiated for 21 days and immunostained for cleaved/active caspase 3. More dead cells appear in the ARE-Erg cultures.



We know ING4 is lost in the EMP (Erg+Myc+shPten) cells, and not in the EM (Erg+Myc) cells (Berger *et al.*, 2014). Therefore, Pten contributes in some way to ING4 loss in tumors. During differentiation, Pten is elevated early (**Fig. 6a**), but decreases after ING4 is induced and CREB becomes active (after day 10). In EMP cells, CREB/ATF1 is constitutively activated (see Fig. 2b); therefore, we propose that Pten acts to suppress CREB/ATF1 activation in normal differentiation until the right time, and its loss leads to constitutive CREB/ATF1 activation. Loss of Pten results in elevated Akt activity due to loss of lipid phosphatase activity, and Akt is a potent activator of CREB (Caravatta *et al.*, 2008). However, it has also been reported that Pten itself can dephosphorylate and inactivate CREB, particularly when associated with differentiation (Gu *et al.*, 2011; Lyu *et al.*, 2015). We obtained 2 Pten dominant acting mutants, one which inactivates lipid phosphatase activity (G129E) and one that inactivates both the lipid and protein phosphatase (C124S) activity. We overexpressed these in PrECs (**Fig 6b**), and find that blocking only protein phosphatase activity (C124S), but not lipid phosphatase (G129E) results in constitutive CREB/ATF1 activation (**Fig. 6c**) as early as 4 days after differentiation, before it is detected in normal PrECs. We observed a corresponding increase in ING4 expression (Fig. 6c) and these cells still differentiate. In fact they differentiate more rapidly and do so in 4x less concentration of differentiation factors (**Fig. 6d**). This is consistent with our finding that loss of Pten alone doesn't suppress ING4 or differentiation and that CREB/ATF1 may induce ING4 (see Fig. 3a). Thus, there is a second event triggered by Myc or Erg, in addition to Pten loss that is required to suppress ING4.

Figure 6: Pten Protein Phosphatase Activity SePrts the Timing of Differentiation, CREB Activation and Induction of ING4. A. Pten expression was measured by immunoblotting of PrECs differentiated for 3, 8, 10, 12 or 14 days (d). **B.** Pten expression was measured by immunoblotting of normal PrECs, EMPs, or PrECs overexpressing Pten mutants G129E or C124S. **C.** Activated CREB/ATF1 (P-CREB) and ING4 levels were measured by immunostaining PrECs and Pten mutant cell lines differentiated for 4 days. This early in differentiation, normal PrECs and the lipid phosphatase mutant (G129E) have low levels of P-CREB, but no ING4, while the lipid/protein phosphatase mutant (C124S) has dramatically increased P-CREB and ING4 expression. **D.** Differentiation is accelerated in the lipid/protein phosphatase mutant (C124S) under suboptimal levels of differentiation factors. Differentiation was measured by immunostaining for AR in the luminal layer (red) and

integrin α6 (green) in the basal layer. Many more luminal cells with more robust AR staining was observed in the C124S mutant cells.



In **Task 6**, we proposed to cross ING4 KO mice to Pb-Myc mice to determine if loss of ING4 will accelerate the slow development of PCa seen in the Pb-Myc mice. We obtained and successfully rederived the ING4 mice into FVB/n mice. We have crossed the hets and are currently trying to identify the homozygotes. We were initially having some problems with the PCR screening of these mice. But after contacting the lab which generated these mice and following their protocol, we seem to be on track and have identified some homozygotes. We will be validating the KO by screening tissues for ING4 expression. We obtained and began breeding the Pb-Myc mice, which are on a FVB background. We are currently aging these mice for tumor studies and preparing to cross them to the ING4 KO mice.

We have completed almost all the Tasks we expected, but need to repeat a subset of the xenograft/in vitro studies with our new Erg construct.

Aim 3: Determine how loss of ING4 in patients relates to tumor progression. Our objectives for this aim are to 1) establish if there is a correlation between ING4 loss and over expression of Myc, Erg fusions, or Pten loss, and the relationship to disease recurrence in patients (**Task 8**); and 2) determine how ING4 expression correlates with the expression of known differentiation markers in the tumors (**Task 9**). We first had to get IRB and TMA paper work initiated (**Task 7**).

Task 7: We have successfully applied for and obtained IRB approval and TMAs from VARI for our test samples and final arrays. Validation of our antibodies on the tester samples is critical for obtaining final PCBN approval. We submitted an application to PCBN. We were asked to submit data on the specificity of our ING4 antibody and are currently working on this.

Task 8: In the mean time I directly contacted Dr. De Marzo at Johns Hopkins about potentially collaborating on this study since he is the expert on Pten and Myc IHC in prostate cancer. It turns out he has already conducted studies looking at Pten, Myc, and Erg expression in the samples we requested. Thus, he is willing to share that data with us for comparison and analysis of our ING4 staining when we receive the PCBN samples. He was extremely helpful in defining exactly what is needed to validate our antibody and to receive the PCBN samples.

Task 9: Now that we have identified Myc and ING4 targets that are important for PrEC differentiation (Aim 1), we are identifying and testing antibodies to Miz1, P-CREB, and Notch3 for their specificity and IHC staining on the VARI tester TMAs.

Thus, we have completed most of the tasks of this aim that we expected to have achieved at the end of year 1 and are track to continue making progress.

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." Describe how the results were disseminated to communities of interest.

Plans to accomplish the goals during the next reporting period:

Aim 1: Determine how ING4 controls prostate epithelial differentiation.
We will continue to identify, validate, and determine how our Myc and ING4 targets control PrEC differentiation. We will use this information to determine their role in tumorigenesis (Aim 2). We will follow up on the Myc/Notch3, CREB, Miz1, and JFK findings and publish them. We also determine the relationship between Notch3 and ING4. Since Myc is required for both Notch3 and ING4 induction, and based on the time course of induction, we suspect Notch3 is required for ING4 induction. We will begin setting up the ChIP-Seq experiments. We are scaling up and switching to an automated sonication system for generating our cell lysates. Information from these studies will be used to screen human TMAs in Aim3.

Aim 2: Determine how loss of ING4 impacts tumorigenesis.
We will move forward with our new ARE-Erg gene fusion to determine how it alters differentiation and may cooperate with loss of ING4 to promote tumorigenesis. We will continue to investigate the relationship between Myc, Pten, CREB, and ING4. Hits from Aim 1 will be interrogated and potentially introduced or inhibited into the tumor model to see how it influences ING4 expression and tumorigenesis. Once we have the xenografts repeated we will assess the tumor tissues for expression of our targets. Results from the tumor studies on different Myc and ING4 targets will be incorporated into the manuscripts describing those targets. Breeding of the ING4 x Pb-Myc mice will begin and we will be assessing tumor development and isolating the tumors at different time points.

Aim 3: Determine how loss of ING4 in patients relates to tumor progression.
Our priority will be to get the antibody specificity worked out for our ING4 antibody, so that we can receive the TMAs from PCBN and begin the outcomes analysis. We will continue to work on testing antibodies to our newly identified target and assessing them in the prostate cancer TMAs.

4. Impact

What was the impact on the development of the principal discipline(s) of the project?
The principal disciplines of our project are cancer biology, prostate cancer, oncogenesis, and differentiation. We are the only lab working with this prostate differentiation model and the first to show the involvement of ING4 in prostate epithelial differentiation – and indeed the first to show ING4 has anything to do with differentiation in any model. We were also the first to demonstrate ING4 is lost in PCa, and to define its relationship to Myc, a well-established oncogene in PCa. We've gone on to identify a potential link between Pten (a well-established tumor suppressor in PCa) and ING4 through CREB. Only a few in vitro studies have interrogated CREB in PCa cell lines; none have looked in human tissues or defined its targets.

Previous studies suggest there is a relationship between elevated cAMP/CREB signaling and aggressive disease and therapy resistance. Thus, defining how CREB normally functions in PrECs and how its dysregulation promotes aggressive PCa will be critical to defining indolent from lethal disease. We are the first to identify Notch3 as a crucial Myc target and driver of PrEC differentiation. Notch dysregulation is known to be associated with advanced PCa. It will be important to determine if Notch3 specifically is altered in tumors. We are the first to begin to decipher the exact mechanisms by which several known PCa onocgenes i.e. Myc, Erg, and loss of Pten, contribute to PCa development through dysregulation of differentiation. This work will contribute to defining the cell of origin in PCa. Moreover, because these studies use human cells, the mechanisms that are specifically important in human disease, as opposed to mice, will be better defined.

What was the impact on other disciplines?
Nothing to Report

What was the impact on technology transfer?
Nothing to Report

What was the impact on society beyond science and technology?
Nothing to Report.

5. Changes/Problems
Nothing to Report

6. Products

Publications, conference papers, and presentations
The following publication relevant to this project was published. A copy is in the appendix.

Berger PL, Frank SB, Schulz VV, Nollet EA, Edick MJ, Holly B, Chang TA, Hostetter G, Kim S and **Miranti CK**. 2014. Transient induction of ING4 by MYC drives prostate epithelial cell differentiation and its disruption drives prostate tumorigenesis. **Cancer Res** 74:3357-68.

Books or other non-periodical, one-time publications.
None

Other publications, conference papers, and presentations.
The following posters were presented. Underline indicates person presenting the poster. Copies are in the appendix.

Frank SB and Miranti CK. 2014. p38-MAPK Regulation of Notch via Myc is Required for Prostate Epithelial Cell Differentiation. Society for Basic Urologic Research. Dallas, TX, November 13-16.

Frank SB, Berger PL and Miranti CK. 2015. Myc governs a prostate epithelial differentiation program involving chromatin remodeling protein ING4 and Notch3: Disruption of which is necessary for human prostate cancer development. AACR: MYC: From Biology to Therapy. San Diego, CA, Jan 7-10.

The following abstracts have been submitted for presentation at up-coming meetings. The first will be an oral presentation. Still waiting to see if the second one will be accepted for an oral presentation.

Berger PL, Watson M, Winn ME and Miranti CK. 2015. Elucidating ING4 Targets Important in Prostate Epithelial Cell Differentiation and Examining CREB as a Key Regulator of ING4 Expression. Society for Basic Urologic Research. Fort Lauderdale, FL, Nov 12-15.

Berger PL, Watson M, Winn ME and Miranti CK. 2015. Key Intermediate Progenitor in Luminal Prostate Epithelial Differentiation Dictates Susceptibility to Myc Overexpression and Pten Loss in Prostate Cancer Cell of Origin. AACR: Developmental Biology and Cancer. Boston, MA, Nov 30-Dec 3.

Other Products

HUMAN In Vitro Differentiation Model: This model utilizes primary or immortalized basal epithelial cells isolated from patients. Cells are grown to confluency in defined medium and then treated with DHT and KGF. Over a period of 14-20 days, a subset of basal cells differentiate into functional secretory luminal cells (Lamb *et al.*, 2010). This model was initially developed using primary cells by a graduate student, Dr. Laura Lamb, who received a DOD Predoctoral Award for this work. We have since generated 2 immortalized cell lines and obtained one from Dr. John Isaacs. All three lines behave similar to the primary cells. These lines have now been stably modified to express a host of different genes or shRNA, either constitutively or under control of Tet-R or ARE. These cell lines and model greatly expand the HUMAN repertoire of tools available for PCa research.

Mouse Model: We are crossing Pb-Myc-Hi mice to ING4 KO mice. If successful, we anticipate this will generate a more aggressive Myc model for PCa.

RNA-Seq Data: We generated 3 sets of RNA-Seq data. This data defines the mRNA transcriptional program of normal HUMAN prostate epithelial differentiation from basal cells into luminal cells, from luminal cells to tumor cells, and defines those genes targeted by ING4, Myc, and p38-MAPK during differentiation. These data sets will be submitted to databases at the time of publication.

7. Participants & Other Collaborating Organizations

What individuals have worked on the project?

Van Andel Research Institute

Name: Cindy Miranti
Project Role: Principal Investigator
Research Identifier:
Nearest Person Month Worked: 2
Contribution to the project: Supervised and directed the project. Obtained necessary IACUC and IRB approvals. Managed, analyzed, and interpreted data. Submitted and presented abstracts at meetings. Wrote this report.

Name: Penny Berger
Project Role: Research Technician
Research Identifier:
Nearest Person Month Worked: 10
Contribution to the project: Designed, executed, interpreted, and prepared data on ING4, Miz1, and JFK. Managed and initiated the ING4 and Pb-Myc mouse breeding and crosses. Did the Xenograft studies. Worked on validating the ING4, Miz1, and P-CREB antibody for future IHC/TMA tissue studies.

Name: Sander Frank
Project Role: Graduate Student (MSU)
Research Identifier:
Nearest Person Month Worked: 6
Contribution to the project: Designed, executed, interpreted, and prepared data on p38-MAPK, Myc, and Notch3. Assisted with molecular biology and development of reagents on many aspects of the project.

Name: McLane Watson
Project Role: Assistant Research Technician
Researcher Identifier:
Nearest Person Month Worked: 4
Contribution to the project: Worked with Bioinformatics Core to analyze RNA-seq data. Validated and conducted CREB and Pten experiments.
Funding Support: Internal funds from VARI were used for salary support.

Translational Genomics Research Institute
Name: Suwon Kim
Project Role: Sub-contract PI
Research Identifier (e.g. ORCID ID):
Nearest Person Month Worked: 1
Contribution to the project: Ensured the progress and completion of RNA sequencing by working with the Collaborative Sequencing Center at TGen and communicated the data to the PI
Funding Support: The salary support was from the University of Arizona funds allocated for faculty salary.

Name: Madeline Keenen
Project Role: Technician
Research Identifier (e.g. ORCID ID):

Nearest Person Month Worked: 1
Contribution to the project: Performed QC of the RNA samples and initial steps of library preparation for the sequencing.

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

8. Special Reporting Requirements
None

9. Appendices

1. Frank SB and Miranti CK. 2014. p38-MAPK Regulation of Notch via Myc is Required for Prostate Epithelial Cell Differentiation. Society for Basic Urologic Research. Dallas, TX,
   November 13-16.

2. Frank SB, Berger PL and Miranti CK. 2015. Myc governs a prostate epithelial differentiation program involving chromatin remodeling protein ING4 and Notch3: Disruption of which is necessary for human prostate cancer development. AACR: MYC: From Biology to Therapy. San Diego, CA, Jan 7-10.

3. Berger PL, Frank SB, Schulz VV, Nollet EA, Edick MJ, Holly B, Chang TA, Hostetter G, Kim S and **Miranti CK**. 2014. Transient induction of ING4 by MYC drives prostate epithelial cell differentiation and its disruption drives prostate tumorigenesis. **Cancer Res** 74:3357-68.

Citations

Berger, P.L., Frank, S.B., Schulz, V.V., Nollet, E.A., Edick, M.J., Holly, B., Chang, T.T., Hostetter, G., Kim, S., and Miranti, C.K. (2014). Transient induction of ING4 by Myc drives prostate epithelial cell differentiation and its disruption drives prostate tumorigenesis. Cancer Res *74*, 3357-3368.

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p38-MAPK Regulation of Notch via Myc is Required for Prostate Epithelial Cell Differentiation

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Background: Researchers have made progress identifying major drivers of aggressive prostate cancer (e.g. AR, Myc, Erg) but mechanistic understanding of the early events of tumorigenesis remain poorly understood. The epithelium of the prostate is composed of basal and luminal cells that can be identified by distinct protein markers which are often co-expressed in prostate tumors. Moreover, many of the signaling pathways that are misregulated in prostate tumors have also been broadly implicated in epithelial differentiation (e.g. Myc, p38-MAPK, Notch). Thus, we propose that better understanding of differentiation pathways in the prostate will provide insight into understanding tumorigenesis. Specifically, we hypothesize that p38-MAPK regulation of Notch3 via Myc is required for prostate epithelial differentiation.

Methods: To test our hypothesis we utilized an *in vitro* differentiation model in which primary human basal prostate epithelial cells (PrECs) are cultured and induced to differentiate into luminal cells. RNAi techniques were used to knockdown expression of various genes in the context of PrEC differentiation. Alternately, tet-inducible expression of constitutively active MKK6 was used to temporally activate p38-MAPK signaling. Differentiation was monitored by phase-contrast microscopy and changes in gene expression were measured via quantitative real-time PCR and immunoblot. Promoter sequences were cloned from BACs into luciferase reporters to test Notch regulatory regions.

Results: Pharmacological inhibition of p38α with SB202190 or shRNA knockdown demonstrated that p38α was required for differentiation. Likewise, inhibition of Notch signaling with a γ-secretase inhibitor (RO4929097) prevented proper differentiation. RNA and protein from differentiation timecourse samples showed upregulation of the Notch1 and, to an even greater extent, Notch3 receptors. shRNA knockdown of Notch1 blocked differentiation and knockdown of Notch3 is currently underway. Constitutive p38-MAPK signaling had minimal effect on Notch1 but led to increased Notch3 mRNA and protein expression which was greatly abrogated upon knockdown of Myc. Notch3 mRNA upregulation was partially explained by increased mRNA stability but likely relies primarily on transcriptional upregulation independent of the classic promoter region.

Conclusions: This work is the first to define specific roles for the p38-MAPK and Notch pathways in human prostate epithelial differentiation. Moreover, we provide evidence for Myc as a novel link between p38-MAPK and Notch pathways. Additionally, we provide evidence of a distinct role for Notch3 in prostate differentiation. We expect that further understanding of these differentiation pathways will provide new insight into how oncogenic transformation in a transient differentiating prostate epithelial cell may give rise to cancer.

Myc governs a prostate epithelial differentiation program involving chromatin remodeling protein ING4 and Notch3: Disruption of which is necessary for human prostate cancer development

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Myc is overexpressed in the majority of human prostate cancers and is a known determinant of cell fate, yet the cell of origin from which prostate cancers arise is controversial. Furthermore, the mechanisms by which oncogenes such as Myc disrupt prostate epithelial cell fate are poorly understood. Using a novel human *in vitro* differentiation model in which prostate basal epithelial cells are induced to differentiate into lumenal cells, we previously demonstrated that Myc-driven prostate cancer develops in an intermediate progenitor cell population whose full differentiation is derailed upon oncogenic transformation (Berger et al, Cancer Res 74:3357-68, 2014). In basal prostate epithelial cells, Myc is required for transient expression of the chromatin-binding protein ING4, which is required for lumenal cell differentiation. In human tissues, ING4 expression is lost in >60% of primary prostate tumors. Loss of ING4 prevented differentiation and was necessary for Myc-dependent tumorigenesis *in vivo*. ING4 loss generated Myc-dependent tumor cells co-expressing basal and lumenal markers, indicating Myc-dependent oncogenesis disrupted an intermediate step in the prostate epithelial differentiation program.

Our objective for this study was to further elucidate the mechanisms by which Myc controls prostate epithelial cell fate. Myc is a known downstream target of Notch1, and several studies suggest Notch signaling is aberrant in prostate cancer; although the mechanistic details are vague. We found that Notch3 is required for lumenal cell differentiation and hypothesized that Notch3 expression is directly controlled by Myc. Inhibition of total Notch signaling with a γ-secretase inhibitor (RO4929097) prevented differentiation. Total Notch1 mRNA and protein levels change very little during differentiation; whereas both Notch3 mRNA and protein increase dramatically. Knock-down of Notch3 by shRNA blocked differentiation, while over expression of active Notch3 (NCID3) induced spontaneous differentiation. Less than 15% of the increase in Notch3 mRNA was attributable to increased mRNA stability, and required new protein synthesis. Temporally, Myc mRNA and protein levels increase prior to Notch3. Blocking Myc expression prevented Notch3 induction. The 2kb proximal promoter region of Notch3 lacked the elements that promote Notch3 induction. We identified a Notch3 enhancer element with Myc binding motifs that support differentiation-induced luciferase reporter activity. We further determined that p38α-MAPK is required for Myc and Notch3 induction. We are currently determining how Myc-dependent regulation of Notch3 influences Myc-dependent regulation of ING4. Thus, our studies demonstrate that at least 2 targets of Myc, ING4 and Notch3, control prostate epithelial cell fate, and that disruption of at least one of them is required for Myc-driven human prostate cancer development. In depth understanding of Myc-driven differentiation pathways will provide new insights into how oncogenic transformation by Myc in intermediate progenitor prostate epithelial cells gives rise to prostate cancer.

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Cancer Research

Transient Induction of ING4 by Myc Drives Prostate Epithelial Cell Differentiation and Its Disruption Drives Prostate Tumorigenesis

Penny L. Berger, Sander B. Frank, Veronique V. Schulz, et al.

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Transient Induction of ING4 by Myc Drives Prostate Epithelial Cell Differentiation and Its Disruption Drives Prostate Tumorigenesis

Penny L. Berger[1], Sander B. Frank[1,5], Veronique V. Schulz[1], Eric A. Nollet[1,4], Mathew J. Edick[1], Brittany Holly[2], Ting-Tung A. Chang[2], Galen Hostetter[3], Suwon Kim[6], and Cindy K. Miranti[1]

Abstract

The mechanisms by which Myc overexpression or Pten loss promotes prostate cancer development are poorly understood. We identified the chromatin remodeling protein, ING4, as a crucial switch downstream of Myc and Pten that is required for human prostate epithelial differentiation. Myc-induced transient expression of ING4 is required for the differentiation of basal epithelial cells into luminal cells, while sustained ING4 expression induces apoptosis. ING4 expression is lost in >60% of human primary prostate tumors. ING4 or Pten loss prevents epithelial cell differentiation, which was necessary for tumorigenesis. Pten loss prevents differentiation by blocking ING4 expression, which is rescued by ING4 re-expression. Pten or ING4 loss generates tumor cells that co-express basal and luminal markers, indicating prostate oncogenesis occurs through disruption of an intermediate step in the prostate epithelial differentiation program. Thus, we identified a new epithelial cell differentiation switch involving Myc, Pten, and ING4, which when disrupted leads to prostate tumorigenesis. Myc overexpression and Pten loss are common genetic abnormalities in prostate cancer, whereas loss of the tumor suppressor ING4 has not been reported. This is the first demonstration that transient ING4 expression is absolutely required for epithelial differentiation, its expression is dependent on Myc and Pten, and it is lost in the majority of human prostate cancers. This is the first demonstration that loss of ING4, either directly or indirectly through loss of Pten, promotes Myc-driven oncogenesis by deregulating differentiation. The clinical implication is that Pten/ING4 negative and ING4-only negative tumors may reflect two distinct subtypes of prostate cancer. *Cancer Res; 74(12); 3357–68. ©2014 AACR.*

Introduction

Normal prostate glands contain prostatic ducts composed of two distinct layers of epithelial cells: luminal cells that express androgen receptor (AR) and secrete prostate-specific antigen (PSA) and basal cells that express nuclear p63. It is thought that the stem or progenitor cells within or in proximity of the basal layer differentiate and give rise to the luminal cells (1, 2). Prostate tumors are often devoid of the cell layer distinction and express both luminal and basal cell markers, suggesting deregulated cell differentiation. That prostate cancer arises from deregulated differentiation is also supported by mouse models. The most notable example is loss of Nkx3.1, a known prostate-specific differentiation gene, which predisposes mice to develop prostate cancer in the context of additional oncogenic events (3). Two other well characterized oncogenic events linked with prostate cancer are loss of Pten or overexpression of Myc (4, 5). Both of which lead to downregulation of Nkx3.1 expression, but are also sufficient to induce prostate cancer in mice (6, 7). The prostate-specific oncogene, TMPRSS2-Erg, when overexpressed in mouse prostates leads to prostate intraepithelial neoplasia (PIN), with a corresponding change in differentiation, where progenitor cell markers Sca1 and integrin α6 are increased, whereas basal cell keratin is diminished and AR is expressed (8, 9). In addition, overexpression of Erg upregulates Myc expression and produces an expression profile consistent with a change in differentiation (10). A recent mouse study where Pten was deleted in either basal or luminal cells, demonstrated the appearance of $K5^+/K8^+$ intermediate tumor cells, further supporting the idea that deregulated differentiation is a hallmark of prostate cancer (11). However, the mechanism by which differentiation is deregulated is unknown.

We recently reported on an *in vitro* differentiation model in which AR-negative human basal prostate epithelial cells can be differentiated into AR-positive and androgen-responsive

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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postmitotic secretory cells (12). Based on known prostate and epithelial differentiation markers, and the demonstration that PSA can be secreted into the medium from the differentiated cells, this model recapitulates the biology and physiology of the human prostate gland *in vivo*. A major step in the differentiation process is the loss of integrin expression and cell–matrix adhesion, which is crucial to generate stable AR-expressing cells. This is accompanied by a dramatic shift in survival signaling pathways, whereby basal cells, which survive primarily through integrin-mediated activation of the Erk signaling pathway, give rise to secretory cells that depend on E-cadherin based cell–cell adhesion and activation of Akt for survival.

The separation of AR and integrin functions in the two different epithelial populations is wholly consistent with what is observed *in vivo*; that integrin expression is limited to the basal cells and AR is only in the secretory cells (13, 14). In prostate cancer this distinction is lost, whereby AR and integrin α6β1 are coexpressed in the tumors, where integrin α6β1 cooperates with AR to promote the survival of prostate cancer cells (15). Other markers typically associated with basal or intermediate cells, such as receptor tyrosine kinases EGFR and Met, bcl-2, and coexpression of basal and secretory keratins K5 and K8, are also found in tumor cells that express AR-dependent differentiation genes (14, 16, 17). Thus, the majority of the primary tumor population in prostate cancer resembles a potential differentiation intermediate. In addition, the unexplained loss of basal cells in prostate cancer points to altered differentiation as a major factor in prostate cancer (18).

Myc is overexpressed in up to 90% of primary prostate tumors, presenting itself as a major driver in prostate cancer (4). Recent studies have unraveled the function of Myc in reprogramming of somatic cells into pluripotent stem cells and the maintenance of self-renewal in stem cells (19), and is consistent with the idea that deregulated Myc prevents full differentiation of prostate epithelial cells, leading to prostate cancer when given additional molecular lesions. ING4 is a tumor suppressor whose expression is lost in several cancers; but whose role in prostate cancer is unknown (20). ING4 is a plant homeodomain–containing transcriptional regulator, which binds trimethylated histone H3 and recruits the HBO1 acetyltransferase to increase histone acetylation (21). ING4 was shown to block Myc-induced anchorage-independence and mammary hyperplasia in a mouse model of breast cancer, suggesting ING4 may function to suppress Myc (22, 23). We hypothesized there would be an interplay between Myc and ING4 in prostate epithelial cell differentiation that would be disrupted in prostate tumorigenesis. In this study, we determine how Myc, Pten, and ING4 are involved in normal prostate epithelial differentiation and demonstrate the importance of ING4 loss in promoting prostate oncogenesis through suppressing differentiation.

Materials and Methods

Cell lines

Primary basal prostate epithelial cells were isolated from clinical prostectomies as previously described (24, 25). Cultures were validated to be Mycoplasma-free and express only basal epithelial cell markers (12, 25). Cells were immortalized with retroviruses expressing HPV E6/E7 and hTert, selected in 150 μg/mL neomycin for 3 days, and the resulting population pooled. Cells retain all the basal markers of primary cells. Immortalized cells (iPrEC) were transformed by retroviruses expressing Erg and Myc (EM), and lentivirus expressing Pten shRNA (EMP) or ING4 shRNA (EMI), then selected and maintained in 0.35 μg/mL puromycin. All lines were maintained and passaged as previously described (24, 25).

Differentiation protocol

Differentiation and layer separation protocols were detailed previously (12). Briefly, iPrECs at confluency were treated in complete growth medium with 10 ng/mL keratinocyte growth factor (KGF; Cell Sciences) and 5 nmol/L R1881 (PerkinElmer) every other day for up to 21 days. For biochemical analysis, the suprabasal differentiated layer was separated from the basal layer as previously described (12).

Constructs

The wild-type retroviral pBabe-Myc construct was obtained from Dr. B. Knudsen. pLPCX-Erg was generated by subcloning the *ERG* cDNA *Not*I/*Spe*I fragment from pMax Dest ΔN-Erg (9), supplied by Dr. Vasioukhin, into *Not*I/*Xho*I of pLPCX. The wild-type (pMIG-ING4) and C-terminal deletion mutant (pMIG-ING4-ΔC1) of ING4 were described previously (23). The ING4 shRNA construct was generated by subcloning the oligo 5'-CCGGGCTAGGTGTGATCAACACTTTCTCGAGAAAGTGTT-GATCACACCTAGCTTTTTTG-3', complementary to the 3'-UTR of ING4, into a lentiviral vector to generate pLKO.1-shING4. The pLKO vector containing Pten shRNA was generated by first creating a pCR8-GW-TOPO-shLEGO shuttle vector. A 344bp PCR product containing a multicloning site, *Eco*RV/*Xba*I/*Sal*I/*Pst*I, the pLKO U6 promoter, an *Age*I site, a *Hin*dIII site, followed by a reverse multicloning site, *Pst*I/*Sal*I/*Xba*I/*Eco*RV, and an *Eco*RI site was TA cloned into pCR8-GW-TOPO. Oligo shPten2, 5′-CCGGTCCACAGCTAGAACTTAT-CAAACTCGAGTTTGATAAGTTCTAGCTGTGGTTTTTA-3′, was cloned into the *Age*I/*Hin*dIII site of the pCR8-GW-TOPO-shLEGO shuttle vector. The *Age*I/*Eco*RI fragment was subcloned into pLKO to generate pLKO-shPten2.

Virus generation and infection

Lentivirus shRNAs were generated by transfecting a packaging cell line, harvesting virus 3 days later and immediately infecting iPrECs. Cells were selected and pools maintained in 0.35 μg/mL puromycin. Retroviruses expressing ING4 or Myc were generated by transfecting Phoenix cells (National Gene Vector Biorepository), harvesting 2 days later and immediately infecting iPrECs. Myc expressing cells were selected and maintained in 0.35 μg/mL puromycin. ING4 construct has no selectable marker and cells were generated *de novo* as needed.

siRNA transfection

A pool of siRNAs against Myc and a nontargeting sequence were purchased from Origene. ON-Targetplus SMARTpool targeted to Bnip3, came from Dharmacon. Differentiated cultures were serially transfected every 2 days with Myc or

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control siRNA using siLentFect lipid reagent (Bio-Rad) following manufacturer's directions. Cells were placed in differentiation medium 18 hours after transfection.

Antibodies

Immunofluorescence. AR (C-19), Nkx3.1 (H-50), and TMPRSS2 (H-50) were purchased from Santa Cruz. ITGα6 (GoH3) was purchased from BD Pharmingen, and PSA (18127) from R&D Systems. Keratin 8 (M20) came from Abcam and Keratin 5 (AF-138) came from Covance. ING4 monoclonal antibody was generated as previously described (26) and a polyclonal antibody was obtained from Proteintech. Cleaved caspase-3 (Asp175)(5A1E) was purchased from Cell Signaling.

Immunoblotting. Myc (o6–340) was purchased from Millipore, Erg (C-20) from Santa Cruz, Pten (138G6) and p27 (Kip1) from Cell Signaling, and ING4 (EP3804) from GeneTex. Tubulin antibody (DM1A) was purchased from Sigma and GAPDH (6CS) from Millipore. Polyclonal integrin α6 (AA6A) antibody was a gift from Dr. A. Cress (University of Arizona; ref. 27).

Immunostaining and microscopy

Differentiated cultures were fixed in 4% paraformaldehyde and permeabilized with 0.2% Triton-X 100. After washing, cells were blocked with 2% normal goat serum for 2 hours. Primary antibodies, diluted in 1% BSA/PBS, were applied to samples overnight at 4°C. After washing, secondary conjugated antibodies diluted in 1% BSA/PBS were incubated for 1 to 2 hours. Nuclei were stained with Hoechst 33258 (Sigma) for 10 minutes at room temperature. Coverslips were mounted using Fluoromount-G (SouthernBiotech). Epifluorescent images were acquired on a Nikon Eclipse TE300 fluorescence microscope using OpenLab v5.5.0 image analysis software (Improvision). Confocal images were acquired by sequential detection on an Olympus FluoView 1000 LSM using FluoView software v5.0.

Immunoblotting

Total cell lysates were prepared for immunoblotting as previously described (24). Briefly, cells were lysed in RIPA buffer, 30 to 50 μg of total cell lysates were run on SDS polyacrylamide gels and transferred to PVDF membranes. Membranes were blocked in 5% BSA in TBST overnight at 4°C then probed with primary antibody, and HRP-conjugated secondary antibodies (Bio-Rad) in TBST + 5% BSA. Signals were visualized by chemiluminescence reagent with a CCD camera in a Bio-Rad Chemi-Doc Imaging System using Quantity One software v4.5.2 (Bio-Rad).

RT-PCR

Total RNA was isolated using Qiagen's RNeasy Kit. RNA was purified with RNase-free DNase and RNeasy Mini Kits (Qiagen). For qRT-PCR, 0.5 μg RNA was reversed transcribed using a reverse transcription system (Promega). Synthesized cDNA was amplified for qRT-PCR using SYBR Green Master Mix (Roche) with gene-specific primers and an ABI 7500 RT-PCR system (Applied Biosystems). Gene expression was normalized to 18s rRNA by the 2-ΔΔCt method (28). Primers for ING4 and Myc were as follows: ING4: 5′-TCGGAAGTTGCTTTGTTTTGC-3′, Myc: 5′-TTCGGGTAGTGGAAAACCAG-3′.

Mouse tumorigenesis

Half a million iPrEC, EM, EMP, EMI, or EM-vector cells were injected orthotopically into the prostates of 8-week nude mice. Mice were monitored by ultrasound between 8 and 18 weeks for the development of tumors. Mice were sacrificed between 16 and 18 weeks and prostate glands analyzed histologically for tumors. In one cohort of EMPs, 5 mice with tumors were castrated 16 weeks postorthotopic transplantation and measured by ultrasound for regression of tumors. All animal work was carried out following Institutional Animal Care and Use Committee approval at an Association for Assessment and Accreditation of Laboratory Animal Care-accredited facility.

Histology

Prostates isolated from mice were formalin-fixed and paraffin-embedded. Sections were analyzed following hematoxylin and eosin or immunohistochemical (IHC) staining. Human-specific MHC class I was purchased from Abcam, polyclonal ING4 was purchased from ProteinTech, and AR (N-20) was purchased from Santa Cruz. IHC was performed using automated immunostaining (Ventana Discovery XT). A human prostate tumor survey tissue microarray (TMA) was constructed as previously described (29). The prostate survey TMA contained 52 de-identified unique prostate carcinomas ranging from Gleason 6 to 9 and 23 control cores, including 14 cases of benign prostatic hypertrophy (BPH). TMA sectioned at 5 μm thicknesses was stained using standard DAB. IHC was performed with ING4 antibody as previously described (26, 30). For validation, sections were also stained with a commercial ING4 antibody (ProteinTech). Negative control was nonimmune rabbit antiserum without primary antibody. TMA staining was scored manually with IHC assigned to each core as composite scores of 0, 1, 2, or 3 with 0 to 1 representing complete to major loss of protein, and 2 to 3 near normal to wild-type levels.

Results

Myc and ING4 are transiently expressed during differentiation

When grown to confluency and treated with KGF plus androgen, primary cultures of basal prostate epithelial cells (PrEC) undergo differentiation such that a second suprabasal layer forms on top of the basal layer in about 2 weeks (12). An immortalized primary prostate epithelial cell line (iPrEC) was established by expressing the E6/E7 viral oncogenes and hTert. Treatment of confluent iPrEC cultures with 10 ng/mL KGF and 5 nmol/L R1881, a synthetic AR agonist, for 18 days resulted in a distinct top layer of cells that no longer expressed integrin α6, K14, or p63 but expressed AR and AR-dependent targets, such as TMPRSS2 and Nkx3.1 (Fig. 1A and Supplementary Figs. S1 and S2). These data indicate iPrECs retain the ability to differentiate.

ING4 expression was low to undetectable in untreated iPrECs, but by as early as 8 days of differentiation, distinct nuclear staining was detected in the newly forming suprabasal layer of differentiated cells (Fig. 1B). The initial increase in

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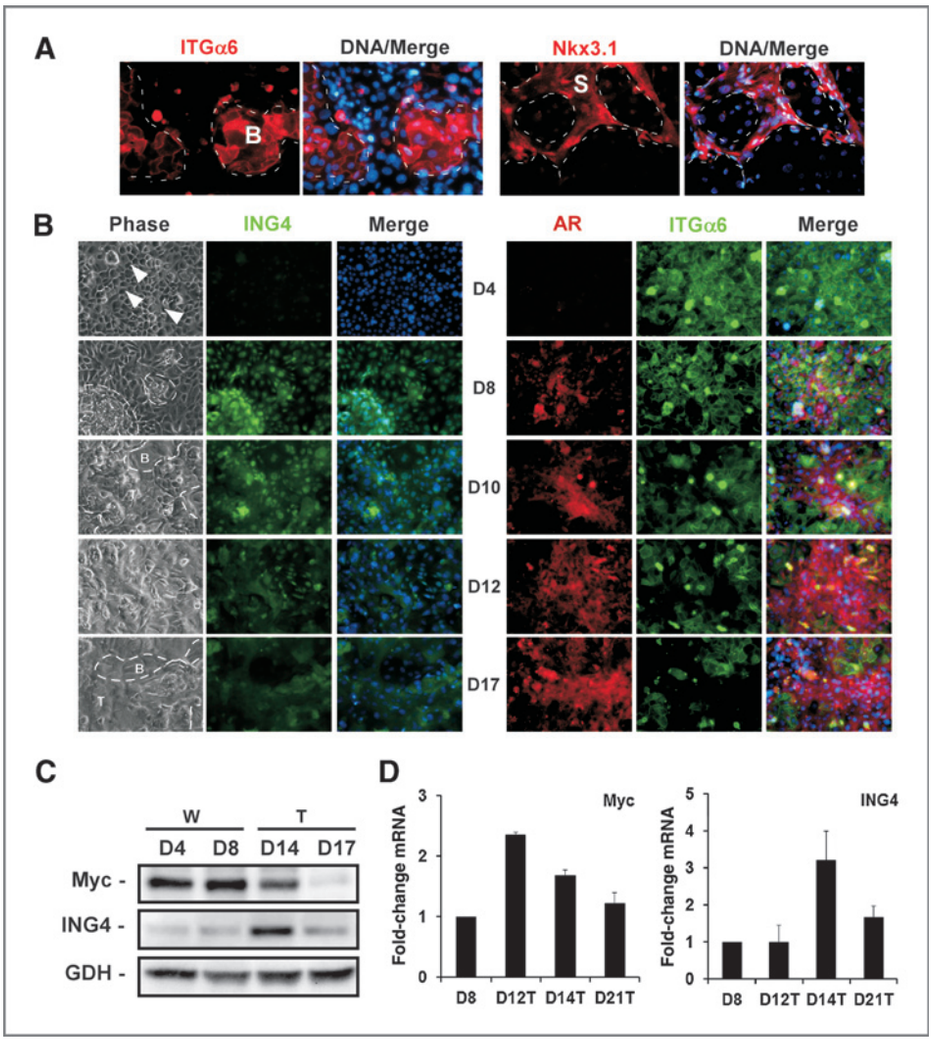

Figure 1. ING4 is transiently expressed in differentiated immortalized prostate epithelial cells. Confluent immortalized prostate epithelial cells (iPrEC) were induced to differentiate with 10 ng/mL KGF and 5 nmol/L R1881 for 4 to 21 days. A, terminally differentiated iPrECs were immunostained (red) and counterstained with Hoechst (blue), then imaged by confocal microscopy. Suprabasal cells (S) express NKX3.1, whereas only basal cells (B) express integrin α6. Dashed lines demarcate suprabasal and basal layer cells. B, iPrECs were immunostained 4 to 17 days after differentiation to detect ING4 (green), AR (red), or integrin α6 (green). C and D, protein or RNA was isolated from whole (W) differentiated cultures at days 4 to 8 or only the suprabasal (T, top) cells at days 12 to 21. C, ING4, Myc, and GAPDH (GDH) were detected by immunoblotting. D, ING4 and Myc mRNA were measured by qRT-PCR. Data are normalized to 18S rRNA and are mean ± SD.

ING4 expression was coincident with the increase in AR expression and the loss of integrin α6 expression, two hallmarks of differentiation (Fig. 1B and Supplementary Fig. S1B). At no time point were we able to dissociate ING4 expression from changes in AR or integrin α6 expression; nor were we able to separate loss of basal keratin K14 from integrin α6 loss (Supplementary Fig. S2), suggesting ING4 controls a major differentiation switch. Although AR persisted in the differentiated layer, ING4 expression was transient and no longer nuclear at later time points.

Once a sufficient number of cells have differentiated, typically between day 12 and 14, it is possible to separate the top layer of differentiated cells from the bottom layer (12). Immunoblot analysis of whole cultures from days 4 and 8, and the top layers from days 14 and 17 indicated a transient increase in ING4 protein expression at day 14, which returned to basal level expression by day 17 (Fig. 1C). ING4 mRNA expression also peaked at day 14 (Fig. 1D). The apparent lag in ING4 expression seen biochemically, compared with the immunostaining data, is most likely because of the low number of differentiated cells within the culture relative to the basal cells at early time points.

Over the same time course, Myc protein and mRNA expression were also transiently elevated (Fig. 1C and D). Myc expression preceded that of ING4 expression, suggesting a concerted temporal regulation of Myc and ING4 during iPrEC differentiation.

Myc-induced ING4 expression is required for differentiation

Cells were engineered to overexpress ING4, Myc, and/or ING4 shRNA (Fig. 2A). Although ING4 expression levels did not affect Myc expression, most notable was the increase in ING4 expression in the Myc overexpressing cells (Fig. 2A and B). These results suggest that Myc is responsible for the increase in ING4 expression during iPrEC differentiation.

Overexpression of ING4 or Myc accelerated the emergence of differentiated cells compared with the control iPrECs. The appearance of suprabasal layer cells, loss of integrin α6, and gain in Nkx3.1 expression was more robust between days 8 and

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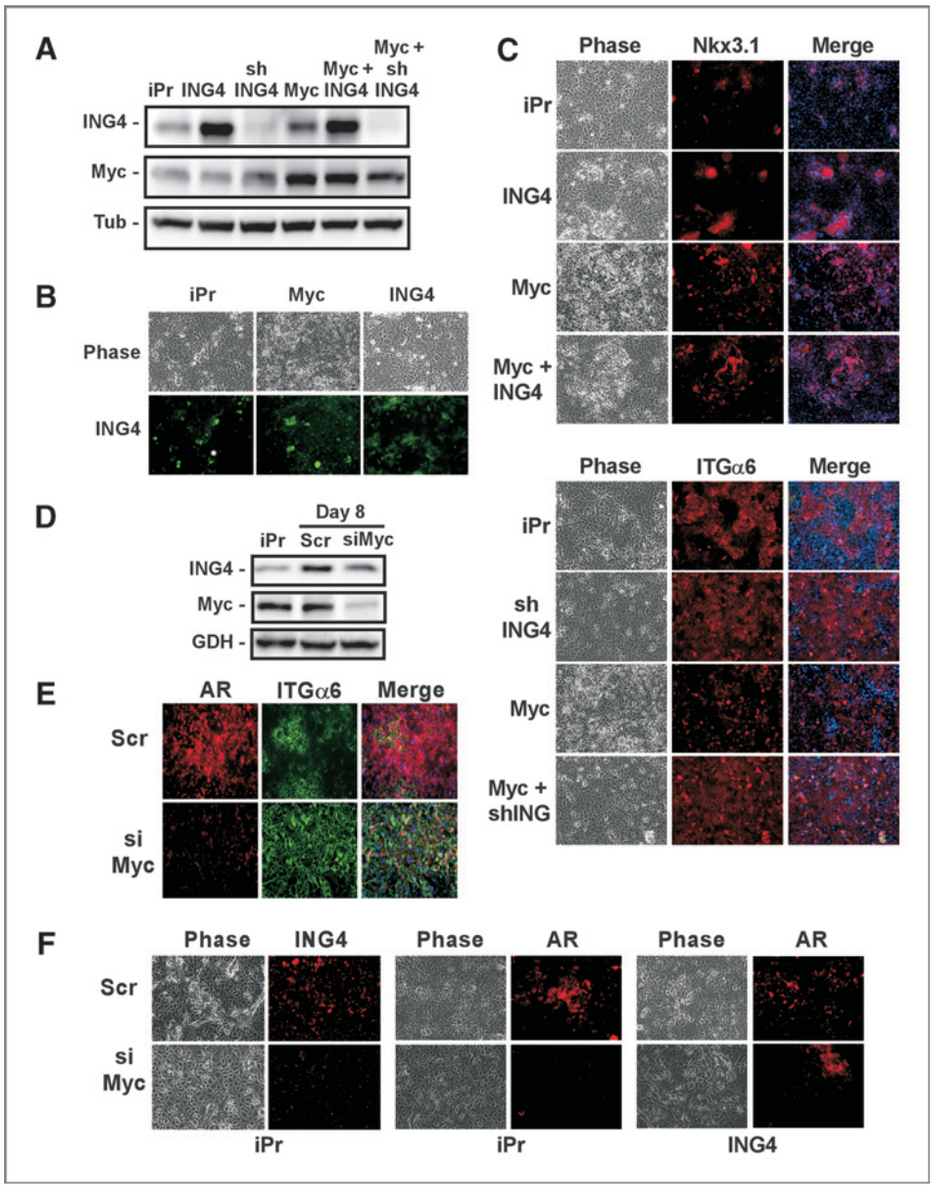

Figure 2. Myc-induced ING4 expression is required for differentiation. iPrECs were engineered to overexpress ING4, Myc, ING4 and Myc, ING4 shRNA (shING4), or Myc with shING4. A, ING4, Myc, and tubulin (Tub) expression in basal cells were measured by immunoblotting. B, cells differentiated for 8 days were immunostained for ING4 (green) and imaged by fluorescent microscopy. C, cells differentiated for 12 days were immunostained (red) to detect NKX3.1 or ITGα6, counterstained with Hoechst (blue), and imaged by fluorescent microscopy. D–F, one day after inducing differentiation, iPrECs (iPr) or iPrECs overexpressing ING4 (ING4) were serially transfected with Myc siRNA (siMyc) or a scrambled sequence (Scr) on days 2, 4, and 6. D, ING4 and Myc expression were detected in undifferentiated (iPr) or in siRNA-treated differentiated cells on day 8 by immunoblotting. E and F, differentiated iPrECs (iPr) or ING4-overexpressing cells were immunostained for ING4 (red), AR (red), or ITGα6 (green) on day 9.

12 in the ING4 or Myc overexpressing cells, whereas the control iPrECs do not robustly express the same set of differentiation markers until days 14 to 16 (day 12 shown in Fig. 2C). Combined overexpression of Myc and ING4 did not exert an additive effect on accelerating differentiation compared with cells overexpressing either Myc or ING4 alone (Fig. 2C). However, it should be noted that the higher levels of ING4 expression in the Myc+ING4 cell line (Fig. 2A) was not always observed; most likely it is not tolerated because of enhanced cell death (see Fig. 3). Thus, it is possible we did not achieve levels of ING4 overexpression required for an additive effect. Downregulation of ING4 expression by shRNA (shING4) severely retarded the emergence of differentiated cells (Fig. 2C). Reduced ING4 expression prevented cells from appearing in the suprabasal layer, and the concomitant loss of integrin α6 (Fig. 2C) and gain of AR, indicating an absolute necessity for ING4 to suppress integrin α6 and permit AR expression.

The ability of Myc to accelerate differentiation was blocked by shING4 (Fig. 2C), indicating ING4 functions downstream of Myc during differentiation. This epistatic relationship is further supported by the fact that transient inhibition of Myc expression between days 2 to 6 failed to induce ING4 expression (Fig. 2D and F) and completely blocked differentiation (Fig. 2E and F). Furthermore, ING4 overexpression rescued the differentiation blocked by siMyc (Fig. 2F). Taken together, our results indicate that a temporal peak in Myc expression is required for the subsequent induction and transient expression of ING4 during iPrEC differentiation.

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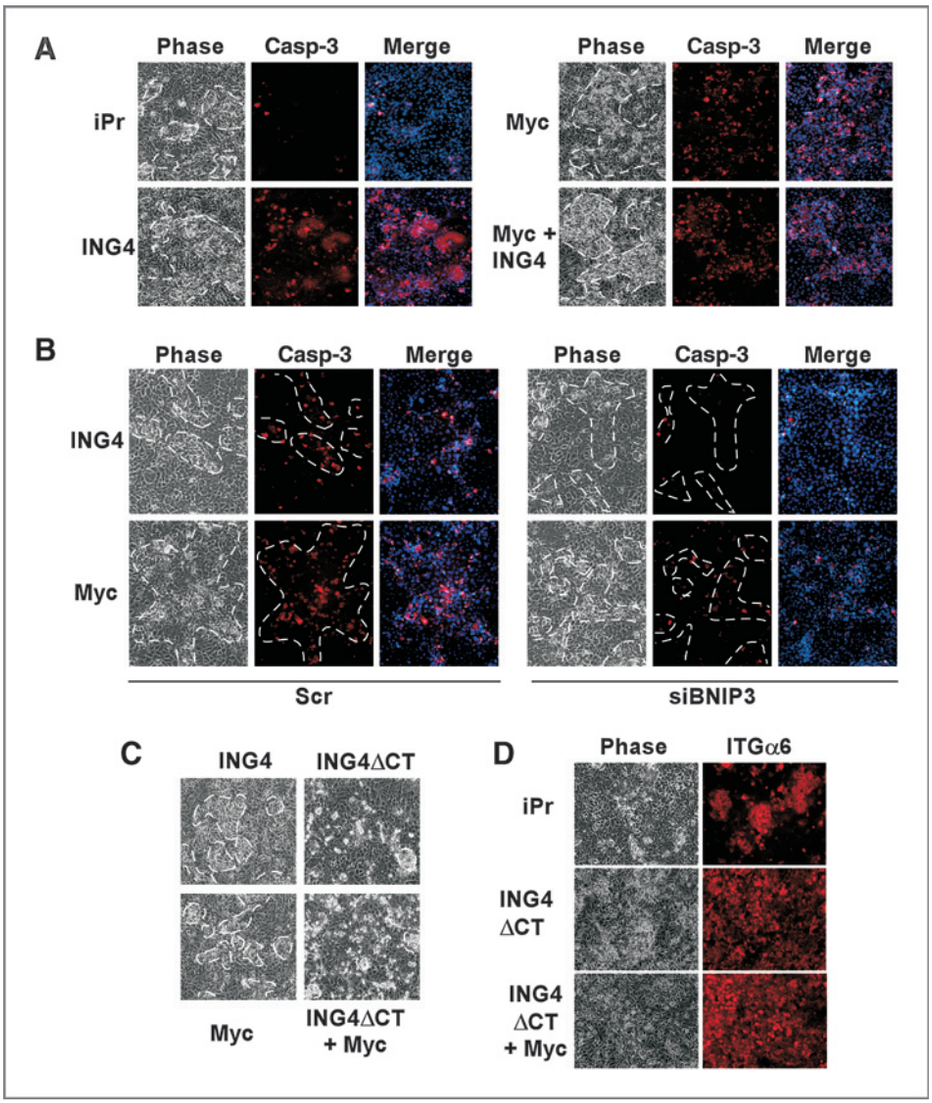

Figure 3. Constitutive Myc and ING4 expression leads to cell death. A, cell death was measured in cells differentiated for 12 days by immunostaining for caspase-3 activity (red). Nuclei were counterstained with Hoechst (blue). B, after 6 days of differentiation, Myc or ING4 overexpressing cells were transfected with Bnip3 siRNA (siBnip3) or a scrambled sequence (Scr) and immunostained 72 hours later for caspase-3 activity (red) and counterstained with Hoechst (blue). White dashes demarcate top layer. C, the C-terminal truncation mutant of ING4 was overexpressed in iPrECs (ING4ΔCT) or in Myc overexpressing cells and compared 8 days after differentiation by phase contrast to cells overexpressing wild-type ING4. D, after 12 days of differentiation, iPrECs (iPr), ING4ΔCT, and Myc plus ING4ΔCT expressing cells were immunostained for integrin α6 (red) and imaged by fluorescent microscopy.

Constitutive Myc and ING4 expression leads to cell death of the differentiated cells via Bnip3

Although Myc or ING4 overexpression initially accelerated iPrEC differentiation, the differentiated cells eventually became disorganized and dissociated from the basal cells, resulting in the loss of the differentiated cell layer (not shown). Differentiated cells from the control iPrEC cultures remained healthy and viable. At day 12, many more apoptotic cells were detected in the differentiating Myc or ING overexpressing cultures as evidenced by increased cleaved caspase-3 (Fig. 3A) and TUNEL staining (not shown) specifically in the suprabasal layer. The basal cell layer remained intact and displayed no evidence of cell death. Thus, sustained overexpression of Myc or ING4, specifically in the differentiated cells, ultimately causes their death.

A qRT-PCR screen for cell death effectors identified elevated expression of Bnip3 (not shown), which encodes a BH3-only proapoptotic protein. Inhibiting Bnip3 expression with siRNA blocked the death induced by ING4 or Myc overexpression, as measured by a reduction in caspase-3–positive cells (Fig. 3B). Blocking Bnip3 expression did not inhibit differentiation (Supplementary Fig. S3), indicating death occurs after differentiation. Thus, the death induced by Myc and ING4 overexpression in differentiated cells is mediated by elevated Bnip3 expression, leading to apoptosis.

The C-terminal domain of ING4 is required for iPrEC differentiation

Myc promotes the trimethylation of H3 at K4 (H3K4me3; ref. 31). ING4 functions in chromatin remodeling complexes by binding to histone H3K4me3 sites via its C-terminal PHD motif and recruiting the HBO1 acetyltransferase via the N-terminal domain (21, 32). Deletion of the PHD motif generates a dominant inhibitory mutant (23). The ability of ING4 to accelerate differentiation was abrogated when the C-terminal domain of ING4 (ING4ΔCT) was deleted (Fig. 3C). This is

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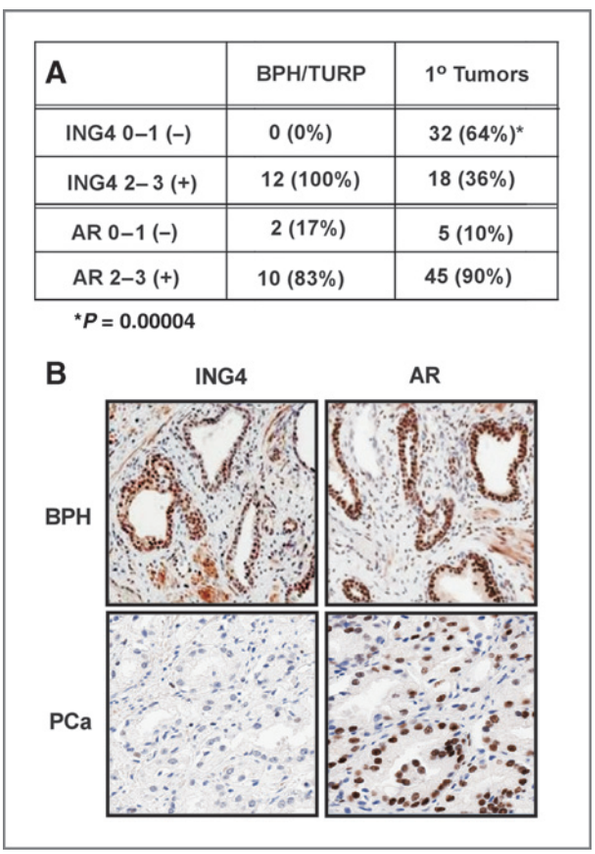
further evidenced by the failure to suppress integrin α6 expression (Fig. 3D) in the cells expressing ING4ΔCT. Furthermore, ING4ΔCT blocked the ability of Myc to induce differentiation. Cells that did appear in the suprabasal layer were dying as determined by caspase-3 immunostaining (not shown). Thus, the C-terminus of ING4 containing the PHD domain is required for iPrEC differentiation and survival of the emerging cells, suggesting that the Myc-ING4 differentiation program depends on ING4-dependent chromatin remodeling.

ING4 expression is lost in patient with prostate cancer tumors

To determine whether ING4 expression is altered in prostate cancer, a tissue microarray containing 50 malignant prostate tumors and 12 noncancerous prostates was surveyed for ING4 and AR expression (Fig. 4A). ING4 expression was detected in the nuclei of the luminal cell population of noncancerous samples (Fig. 4B). ING4 expression levels were scored on a scale ranging from 0 to 3; 0 for no detectable expression and 3 for distinct nuclear expression in accordance with a previous study (30). Although 100% of control (BPH or TURP) samples were positive for ING4, only 36% of tumor samples (18/50) were positive for nuclear ING4 expression (Fig. 4A). In contrast, 83% benign lesion sample (10/12) and 90% of the tumors were positive for AR (Fig. 4A). These results demonstrate that more than 60% of prostate tumors downregulate ING4 expression and this loss occurs in AR-positive cancer, indicating that ING4 loss may be a main event in prostate tumorigenesis.



A	BPH/TURP	1° Tumors
ING4 0–1 (–)	0 (0%)	32 (64%)*
ING4 2–3 (+)	12 (100%)	18 (36%)
AR 0–1 (–)	2 (17%)	5 (10%)
AR 2–3 (+)	10 (83%)	45 (90%)

*$P = 0.00004$

Figure 4. ING4 expression is lost in patients with prostate cancer. A tissue microarray of 50 cancerous and 12 noncancerous human prostate samples was immunostained for ING4 or AR. A, table of ING4 and AR histologic grading (scale 0–3; 3 being highest expression) comparing benign prostate hyperplasia/transurethral resection (BPH/TURP) and primary tumors (1°). *, $P = 0.0004$; $n = 50$. B, IHC staining of ING4 and AR in benign hyperplastic prostate tissue (BPH) and prostate cancer (PCa) tissue.

Loss of ING4 expression cooperates with Myc/Erg in prostate tumorigenesis

As reported previously, Myc overexpression alone in human iPrECs was not sufficient to generate a cell line that is tumorigenic in mice (33). Combined overexpression of Myc and the prostate-specific oncogene, Erg (10), was also not sufficient to generate human tumors. To test whether loss of ING4 is also required, we orthotopically injected iPrECs overexpressing Erg and Myc (EM) with or without shING4 (EMI), or a nontargeting shRNA (EMshCV) into prostates of nude mice. Cells overexpressing the two oncogenes Myc and Erg (EM) or in conjunction with a nontargeting shRNA (EMshCV) did not produce tumors in the mice 18 weeks following orthotopic injection. However, EMI cells produced tumors in 60% of the mice (Fig. 5A). Ultrasound imaging of tumors in mice 18 weeks following orthotopic injection is shown in Fig. 5B. Tumors were positive for AR, but negative for ING4 expression when compared with adjacent normal tissue (Fig. 5C and D). Thus, loss of ING4 is required in human cells to cooperate with Myc and Erg to produce prostate tumors.

Pten loss prevents ING4 expression

To further develop prostate cancer models, Pten expression was silenced by overexpressing Pten shRNA in the EM cells (EMP). Overexpression of Myc and Erg and knockdown of Pten was verified in EMP cells by immunoblotting (Fig. 6A). In EMP cells, the expression of integrin α6 was increased whereas the expression of the p27 cell-cycle inhibitor was reduced (Fig. 6A), consistent with changes observed in prostate cancer (13, 34).

Orthotopic injection of EMP, but not iPrECs, into the prostates of nude mice produced tumors that were detectable by ultrasound imaging as early as 8 weeks after injection. At 16 weeks, the tumors averaged 2.85 mm in diameter, ranging from 2.11 to 3.68 mm (Fig. 6B). The tumor penetrance was 60%, as 17 of 30 injections resulted in prostate tumor formation (Fig. 6C). IHC with human-specific MHC class I antibody revealed the presence of human cells demarcating the tumorigenic foci. The EMP tumors stained positive for AR (Fig. 6D) and castrating the mice 16 weeks after the tumors were established resulted in complete tumor regression, indicating a dependence on androgen for tumor maintenance (Fig. 6C).

When subjected to the differentiation protocol, EM cells were completely competent at differentiating as evidenced by the formation of distinct layers, loss of integrin α6, and gain of AR in the suprabasal layer (Fig. 7A). In contrast, the EMI cells failed to differentiate as evidenced by reduced numbers of suprabasal cells, poor AR expression, and retention of integrin α6 in all the cells. EMP cells also failed to differentiate, as evidenced by the lack of a suprabasal layer, and failure to lose

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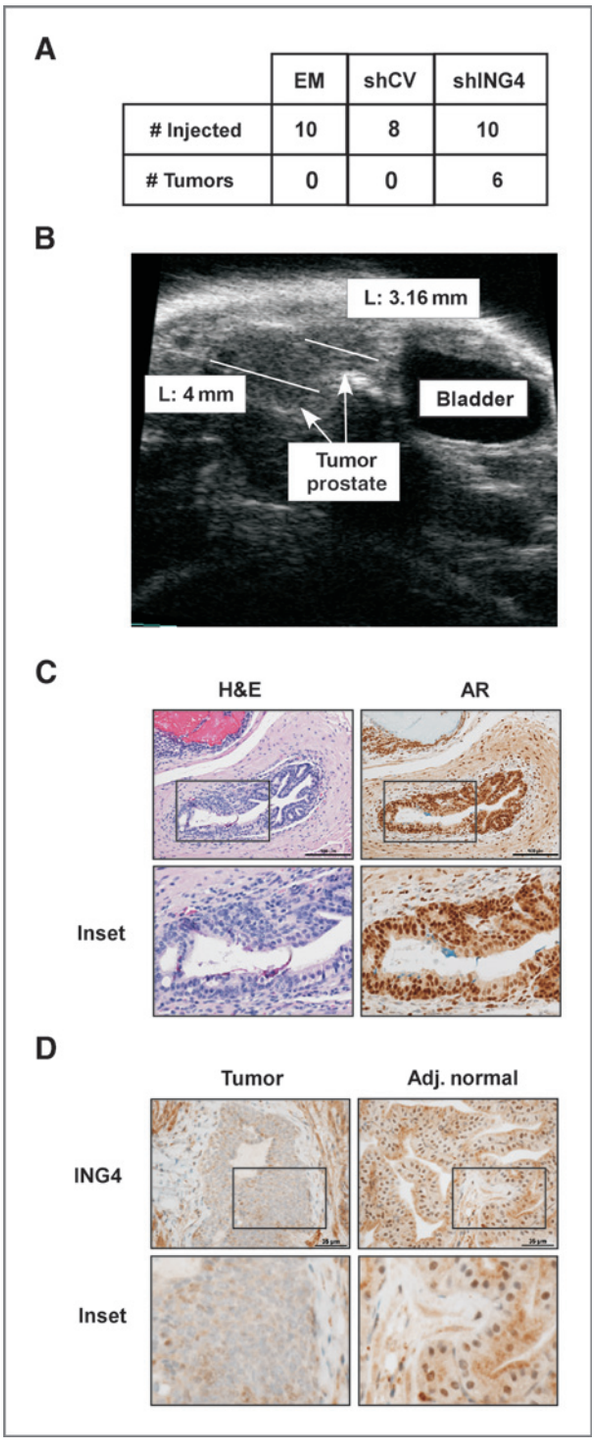

Figure 5. Loss of ING4 expression is required for tumorigenesis. A, iPrECs were engineered to stably overexpress Myc and Erg (EM) with or without shING4 (EMshING4) or a nontargeting shRNA (shCV). Number of mice in which tumors formed following orthotopic injection of EMshING4 compared with control EM and EMshCV cells 18 weeks postinjection. B, tumor measured by ultrasound imaging 18 weeks after orthotopic injection of EMshING4 into the prostates of nude mice. C, hematoxylin and eosin (H&E) and IHC staining of a tumor sample with AR. D, IHC staining of ING4 in normal mouse prostate and tumor sample.

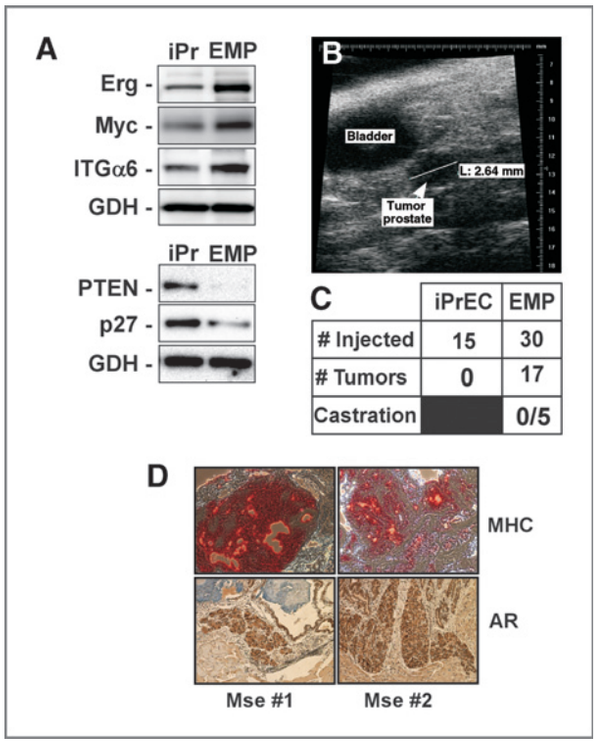

Figure 6. Pten loss promotes tumorigenesis. A, iPrECs (iPr) were engineered to stably overexpress Myc and Erg, along with Pten shRNA (EMP). Immunoblotting confirmed overexpression of Myc, Erg, integrin α6 (ITGα6), and loss of Pten and p27Kip. B, tumor measured by ultrasound imaging 16 weeks after orthotopic injection of EMP cells into the prostates of nude mice. C, number of mice in which tumors formed following orthotopic injection of EMP cells compared with control iPrECs 16 weeks postinjection. Sixteen weeks postinjection, 5 mice harboring EMP tumors were castrated and 11 weeks later the number of tumors that regressed was recorded. D, IHC staining of different tumor samples with human-specific MHC class I or AR.

integrin expression (Fig. 7A). However, in contrast to EMI cells, the EMP cells induced high AR and integrin α6 expression in the basal layer (Fig. 7A). Elevated integrin α6 expression in EMP cells was also observed by immunoblotting (Fig. 6A). This resulted in a population of cells coexpressing AR and integrin α6; reproducing the histopathology observed in clinical samples (13). The inability of EMI and EMP cells to differentiate, correlated with a failure of Myc to induce ING4 expression (Fig. 7A and B). The small clusters of AR-positive cells in the EMI culture are cells in which shING4 was poorly expressed, as evidenced by ING4 positivity in those clusters. Analysis of the keratin subtypes further revealed that EMP cells coexpress both basal keratin K5 and secretory keratin K8 (16) compared with normal iPrECs, where each keratin was distinctively expressed in their respective cell types (Fig. 7C). Thus, EMP cells have a dysfunctional differentiation program that prevents ING4 expression in the presence of Myc, resulting in tumorigenic cells with an intermediate differentiation phenotype. Re-expression of ING4 in EMP cells completely rescued the differentiation defect, restoring the suprabasal layer, AR expression, loss of integrin (Fig. 7B), and separation of the K5

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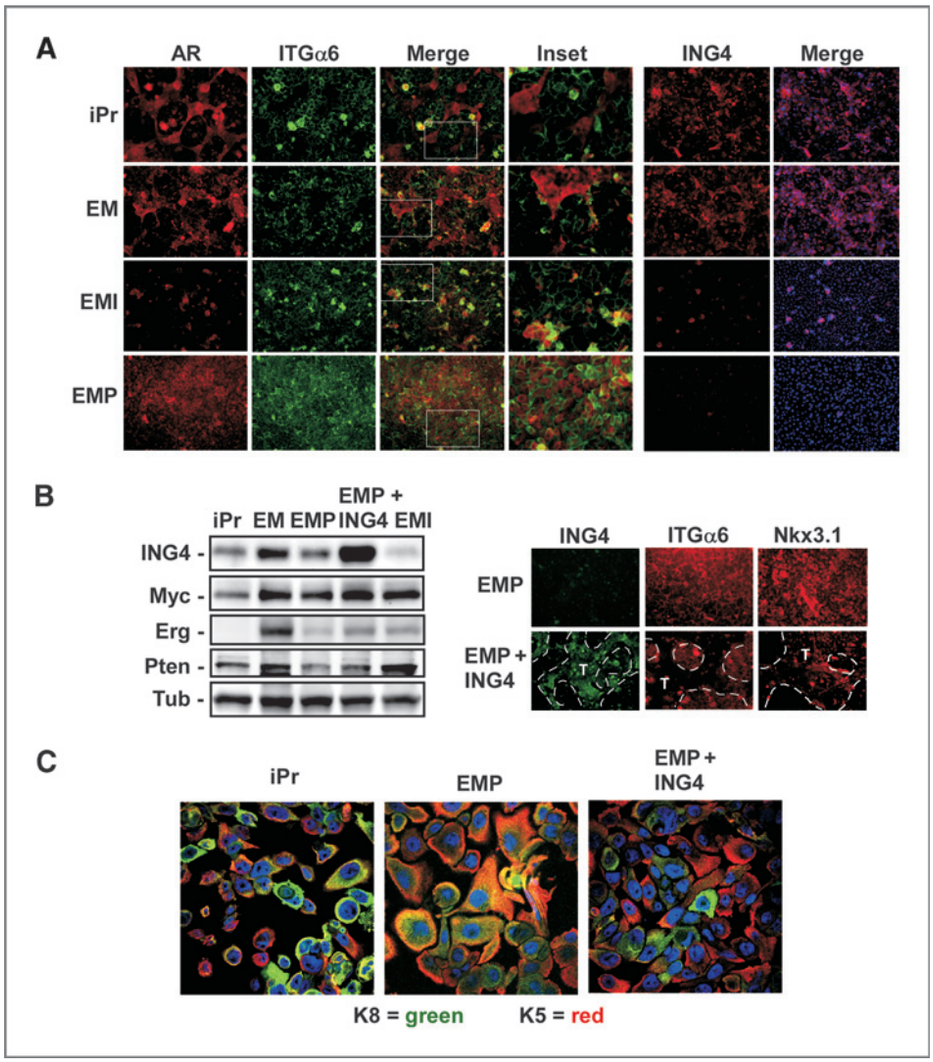

Figure 7. Pten-mediated loss of ING4 and altered differentiation in tumorigenic cells. A, iPrECs (iPr), EM, EMI, or EMP cells differentiated for 12 to 14 days were immunostained for AR (red), ITGα6 (green), or ING4 (red), counterstained with Hoechst (blue), and imaged by fluorescent microscopy. B, ING4, Myc, Erg, and Pten expression in undifferentiated iPrECs (iPr), EM, EMP, EMP+ING4, or EMI cells detected by immunoblotting. EMP or EMP-ING4 cells differentiated for 12 to 14 days were immunostained for ING4 (green), ITGα6 (red), or Nkx3.1 (red). Dashed lines demarcate supra (T) and basal cell layers. C, cultures of iPrECs, EMP, or EMP-ING4 cells were differentiated for 17 days, immunostained for keratins K8 (green) and K5 (red), counterstained with Hoechst (blue), and imaged by confocal microscopy.

and K8 populations (Fig. 7C). Expression of the ING4ΔCT mutant in EMP cells did not rescue the differentiation defect (not shown). Thus, the Myc-ING4 differentiation relay is no longer functional in the oncogenic EMP cells and Pten loss is responsible. Together our results support the conclusion that ING4 is required for differentiation of iPrECs and suggest that one of the major oncogenic events in prostate cancer is the uncoupling of the Myc-ING4 differentiation program.

Discussion

In immortalized human prostate epithelial cells with the capacity to differentiate *in vitro*, transient ING4 expression, dependent on Myc, is required for prostate epithelial differentiation. ING4 expression coincides with loss of matrix-based adhesion, downregulation of integrin, and acquisition of AR; blocking ING4 prevents the initiation of these processes. In normal differentiating iPrECs, the acquisition of AR expression and androgen responsiveness is observed only in cells in which integrin expression is lost (12). We found that neither AR nor androgen is required for ING4 expression (not shown), nor were we able to demonstrate any influence of ING4 on AR expression or its ability to activate its transcriptional targets in cells expressing AR. Thus, the role of ING4 in prostate epithelial differentiation lies at least in part within its capacity to target integrins. This is consistent with the observations in the Myc breast cancer mouse model, where overexpression of the C-terminal deletion mutant of ING4 (ING4ΔCT) restored integrin expression in the tumors (unpublished results; ref. 23). This is also consistent with the established role for Myc in directly suppressing integrin α6 and β1 transcription during differentiation (35). Our data indicate that ING4 is an essential component of the Myc-dependent effect on integrin expression, because removal of ING4 prevents Myc from suppressing integrin expression.

Myc or ING4 overexpression in basal cells is sufficient to accelerate differentiation toward luminal cells; however, improper prolonged expression of Myc or ING4 leads to cell death. Thus, the temporal, that is, Myc expression preceding ING4, and transient nature of Myc and ING4 expression is

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crucial for normal epithelial cell differentiation. The molecular mechanism of this concerted transcriptional relay is currently unclear. Previous ChIP analysis identified Myc bound to the ING4 promoter, suggesting ING4 is a direct target of Myc (36). However, we failed to detect an increase in ING4 mRNA in undifferentiated basal cells overexpressing Myc (not shown). Similarly, Myc overexpression in breast epithelial cells also did not increase ING4 expression (unpublished results). Thus, our results point to an indirect action of Myc in ING4 induction, or requiring additional factor(s) during the course of differentiation.

Differentiation is dependent on the ING4 C-terminal domain containing the PHD motif required for H3K4me3 binding (21). ING4 overexpression alters chromatin modifications (not shown), suggesting ING4 association with chromatin is required for differentiation. To our knowledge, this is the first time that the chromatin remodeling properties of ING4 have been linked to differentiation. Once bound, ING4 recruits the HBO1 acetyltransferase (21, 37), facilitates histone H3/H4 acetylation, and activates gene transcription (21, 38). Like ING4, Myc is extensively involved in chromatin remodeling (39, 40). In addition, recent studies have brought to light the chromatin remodeling activity of Myc in the maintenance of pluripotent stem cells (19, 41). Taken together, the relay from Myc to ING4 is likely to install epigenetic changes that govern differential transcription and ultimately prostate epithelial cell differentiation.

Myc overexpression alone often fails to transform normal human cells because of induction of cell death (33, 42). Myc or ING4 overexpression specifically induces death of the differentiated cells, but not the underlying basal cells. This supports the current paradigm that Myc activity manifests in a context-dependent manner such that Myc induces cell death in more differentiated cells, but maintains the proliferative and self-renewal capacity of less differentiated stem or progenitor cells. The death phenotypes induced by Myc overexpression are mediated in part by p53 and ING4 enhances p53 function (43). However, the death induced by Myc or ING4 overexpression in iPrECs is likely p53-independent, because the iPrECs express E6 that blocks p53 function. In iPrECs, Bnip3 is responsible for the observed cell death. Although p53 is reported to regulate Bnip3 (44, 45), our results describe an alternate mechanism of Bnip3 activation that is p53 independent. Nonetheless, ING4 may be part of the mechanism by which p53 regulates Bnip3. In prostate cancer, p53 loss is rare and associated with a small subset of late stage disease (46). Thus, loss of ING4 may be a mechanism by which prostate cancer cells escape the tumor suppressive effects of p53 when Myc is overexpressed. This idea is further supported when contrasting the prostate cancer tissue data, which demonstrate a 60% loss of ING4, with that of breast cancer where p53 loss is more highly prevalent and only 34% of the samples lack ING4.

ING4 expression is lost in more than 60% of prostate tumors, suggesting for the first time a significant contribution of ING4 loss to prostate tumorigenesis. The high prevalence of Myc overexpression in prostate cancer and its tendency to induce cell death suggests loss of ING4 is necessary for Myc-dependent prostate oncogenesis. Indeed, only Myc-overexpressing cells without ING4 are capable of generating tumors in mice. Moreover, loss of ING4 blocked tumor cell differentiation generating cells coexpressing both basal and luminal markers, a phenotype often seen in prostate cancer. The mechanism by which ING4 is lost in prostate cancer needs more investigation, but LOH at 12p13, the genomic region that contains the ING4 gene, has been reported in 10% to 20% of primary and up to 45% of metastatic prostate tumors (47, 48). Our data demonstrate loss of Pten is another mechanism that leads to ING4 loss. The molecular mechanism of Pten in the regulation of ING4 expression is presently unknown and likely to be indirect.

We have established a genetic link between Myc and ING4 in prostate epithelial differentiation and prostate cancer. Our data demonstrate that a Myc-ING4 temporal relay is required for normal prostate cell differentiation and when this relay is missing, it leads to prostate cancer. Whether the Myc-ING4 relay also governs cell differentiation in other cell types, including breast epithelia, needs to be addressed. We propose that ING4 dictates the downstream program driven by Myc toward differentiation, and in its absence Myc is directed toward targets that promote tumorigenesis. Pten loss resulting in the loss of ING4 expression, disruption of the Myc-ING4 relay, a block in differentiation, and susceptibility to tumorigenesis, reinforces the idea that ING4 plays a pivotal role in determining prostate epithelial cell fate.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S.B. Frank, B. Holly, T.-T.A. Chang, G. Hostetter, S. Kim, C.K. Miranti

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): P.L. Berger, B. Holly, T.-T.A. Chang, C.K. Miranti

Writing, review, and/or revision of the manuscript: P.L. Berger, B. Holly, T.-T.A. Chang, G. Hostetter, S. Kim, C.K. Miranti

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): P.L. Berger, V.V. Schulz, C.K. Miranti

Study supervision: T.-T.A. Chang, C.K. Miranti

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