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Identification of Androgen Receptor and Beta-Catenin Target Genes in Prostate and Prostate Cancer

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14. ABSTRACT

The biological role of β-catenin in prostate cancer is largely not known. The overall objective of this proposal is to determine the biological significance of androgen receptor (AR) and β-catenin crosstalk in prostate, prostate cancer, and castration resistant prostate cancer cells by identifying their transcriptional targets. These studies demonstrate that β-catenin can promote proliferation, survival and adhesion in prostate cancer cells, and this dependence on β-catenin may increase with prostate cancer progression. The β-catenin inhibitor iCRT-3 was a potent inhibitor of β-catenin, the AR-β-catenin complex, proliferation, survival and adhesion in prostate cancer cells. Wnt, androgen, or treatment with other growth factors could only partially rescue a subset of these phenotypes. β-catenin can interact with other steroid receptors, such as ERα, in cervical cancer cells. iCRT-3 can also inhibit cell proliferation and survival in cervical cancer cells and can partially inhibit the β-catenin-ERα complex, but Wnt treatment can fully rescue. Lastly, Split DamID constructs have been made and conditions optimized to determine the β-catenin, AR, and AR-β-catenin complex targets in prostate and prostate cancer cells. A new β-catenin response element in the proximal PSA/KLK3 promoter was discovered. Split DamID data suggests that both AR and β-catenin are binding this new response element. This will be confirmed and the biological significance established in future studies.

15. SUBJECT TERMS
cell biology, molecular biology, androgen receptor (AR), beta-catenin, proliferation, iCRT-3, Split DamID

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INTRODUCTION

Prostate cancer (PCa) is the most frequently diagnosed cancer in American men and second leading cause of cancer death in men. Androgen signaling via its receptor, androgen receptor (AR), plays an important role in PCa progression. While androgen ablation therapy has been the major therapeutic for metastatic PCa the last sixty years, it is not curative and patients ultimately relapse with castration resistant prostate cancer (CRPC). Surprisingly, inhibition of AR expression or its DNA binding activity in CRPC cells inhibits their proliferation and leads to cell death, suggesting that CRPC cells are still dependent on AR. Aberrant Wnt and β-catenin signaling is prevalent in many cancers, most notably colorectal cancer; however their role in prostate cancer is becoming increasingly appreciated. **My hypothesis is that AR targets different genomic targets in PCa progression through an interaction with β-catenin** (see **Fig. 1**). This hypothesis is founded on two key findings: 1) β-catenin acts as a transcriptional co-activator for AR and 2) β-catenin allows AR to respond to ligands other than androgen, possibly one of the mechanisms enabling AR activation in CRPC. The overall objective of this proposal is to determine the biological significance of androgen receptor (AR) and β-catenin crosstalk in prostate, prostate cancer, and castration resistant prostate cancer cells by identifying their transcriptional targets using a new technology termed Split DamID. By understanding the transcriptional targets of AR, β-catenin, and the AR-β-catenin complex in PCa progression, new therapeutic targets or biomarkers for PCa and CRPC may be identified. This grant also serves to train me to be a better prostate cancer research scientist through supporting various research training experiences and travel.

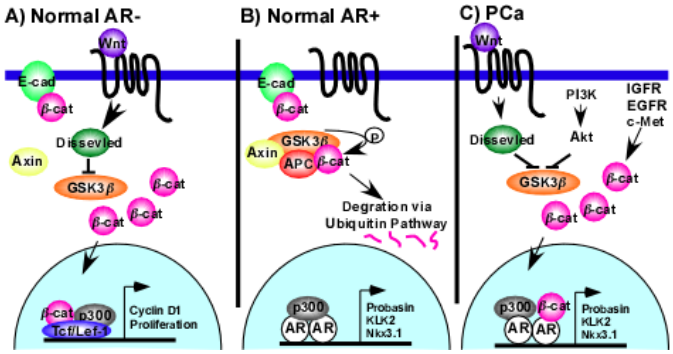

Figure 1. Hypothesized AR and β-catenin signaling in normal AR- and AR+ prostate cells and prostate cancer cells. A) β-catenin targets proliferation genes in normal, AR- prostate cells. **B)** Normal, non-proliferative AR+ prostate cells have little to no β-catenin activity due to increased E-cadherin expression, which sequesters β-catenin. **C)** In PCa, β-catenin preferentially pairs with AR instead of TCF. This shifts transcriptional targets of β-catenin to those also bound by AR, and makes AR active in the absence of physiological levels of DHT, playing a key role in PCa progression.

BODY

TRAINING PLAN UPDATE

I have been working hard to extend my skill set, expertise and knowledge by following the outlined training plan. I have been attending all the listed seminars, journal clubs, and research groups and have been presenting at them on an annual basis, thus improving my communication and presentations skills, as well as getting constructive feedback on my project. I attended the 2012 AACR Prostate Cancer Research conference, allowing me to broaden my knowledge on current research and remaining questions in prostate cancer. I also was able to network with other prostate cancer researchers. In 2013, I attended the AACR Translational Research in Cancer Research. I felt that this was especially impactful on my future research as this training really forced me to evaluate what my goals should be for meaningful laboratory studies that can really impact cancer patients' lives and treatments. Furthermore, I followed urology oncologists at the Dana Farber Cancer Institute in Boston, MA. I observed them as they

met with prostate cancer patients, evaluated the patients, and then determined the next steps in their treatment (or not). I talked with them privately on what their top concerns and challenges were in the clinic. I also spoke to prostate cancer patients and to get a better understanding of what it meant to live with the disease, go through the different therapies and diagnoses, and what they hoped would come out of future research. This was probably one of the most impactful training experiences I have had in all my training so I thank the DOD for this experience. Lastly, in the last year I have become involved in the Transdisciplinary Research in Energetics and Cancer (TREC) Center at Washington University. This U54 grant, lead by world-renowned Cancer Epidemiologist Graham Colditz, strives to encourage large collaborative projects in cancer research by exposing TREC postdocs to the essence of transdisciplinary science via interactive study/training. This includes participation in journal clubs and research-in-progress meetings that spans across numerous disciplines, including basic and clinical science, social sciences (anthropology, psychology, sociology, etc.), as well as economics and statistics. This has trained me in how to do "team science", and learn how to work with and communicate with experts from a range of disciplines. This is an invaluable skill since this is the direction that most research is going and it can be difficult to establish connections outside of your field. Furthermore, this also stresses the big picture in developing successful treatments for diseases; that challenges exist not only in developing interventions (which may or may not be a drug), but also in executing those interventions within in the target population and within a community. Lastly, this year I have also been working with Dr. Matt Haas to strengthen my molecular biology skills.

Overall, this year has been very transformative to my research philosophy in that I have started to develop skill sets that will allow me to do more transformation and translational research in the future. This upcoming year I hope to expand even further on these skills.

RESEARCH PLAN UPDATE

The overall objective of this proposal is to determine the biological significance of androgen receptor (AR) and β-catenin crosstalk in prostate, prostate cancer, and castration resistant prostate cancer cells by identifying their transcriptional targets. To achieve this objective, the following tasks outlined in the Statement of Work have been identified.

Summary of Aim 1:

The goal of Aim 1 was to determine if β-catenin signaling is required for CRPC cell proliferation. My *working hypothesis* was that β-catenin plays a pro-proliferative role in prostate cancer cells (**Fig. 1**). **Task 1** was to determine the level of β-catenin and β-catenin activity in paired prostate cancer androgen sensitive and CRPC lines. **Task 2** was to determine if loss of β-catenin is sufficient to induce loss of proliferation in paired prostate cancer androgen sensitive and CRPC lines. **Task 3** is to determine if β-catenin can interact with other steroid receptors in other cancers and if this was important to their proliferation. As such, this Aim is mostly complete.

Task 1 was to determine the level of β-catenin expression and activity in paired prostate cancer androgen sensitive and CRPC cell lines. I was able to acquire LNCaP, C4-2, and LAPC-4 cells, but after contacting the Dr. Charles Sawyers lab at MD Anderson Cancer Center and the Dr. Robert Reiter lab at UCLA, I was informed that neither lab still had the LAPC-3 cells (private communication) and these cells are not available through ATCC. As such, I focused on the androgen sensitive LNCaP cells and their CRPC derivative, C4-2 cells. LNCaP and C4-2 cells have approximately the same

levels of AR, but C4-2 cells had slightly more β-catenin by western blot (**Fig. 2A**). LNCaP cells were more responsive than C4-2 cells to androgen in androgen receptor reporter assays with the exception of the 4X-ARE-Luc reporter (**Fig. 2B**). However, LNCaP cells had little β-catenin activity in response to Wnt conditioned media by the β-catenin/TCF reporter, TOP-FLASH (**Fig. 2C**). In contrast, C4-2 cells exhibited a 5-fold increase in TOP-FLASH activity when stimulated with Wnt. Up-regulation of the Wnt/β-catenin pathway in CRPC has also been recently validated by next-generation sequencing in clinical patient samples (5).

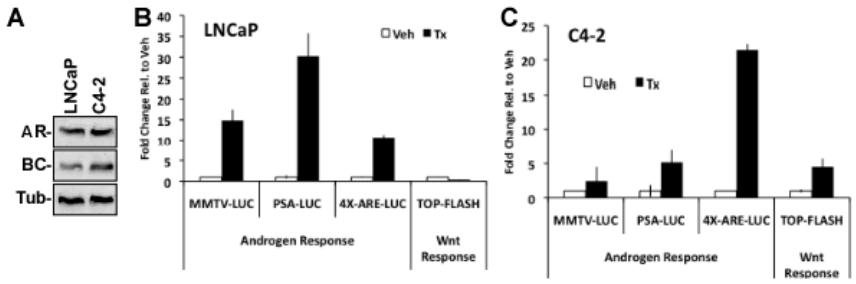

Figure. 2. β-catenin (BC) levels and expression in prostate cancer cell lines. A) AR and BC levels were monitored by immunoblotting in LNCaP and C4-2 cells. LNCaP **(B)** and C4-2 **(C)** cells treated (Tx)with reporter assays for AR (MMTV-Luc, PSA-Luc, 4X-ARE-LUC) or BC (TOP-FLASH) and stimulated with androgen or Wnt respectively. Data is standardized to vector only control and expressed as Fold Change. Error bars on all graphs represent standard deviations.

To determine if loss of β-catenin was sufficient to induce loss of proliferation in prostate cancer cell lines (**Task 2**), LNCaP and C4-2 cells were transfected with either shRNA or siRNA against β-catenin or AR. Non-targeting shRNA (NT, shEmpty, shScram) or shRNA targeting luciferase (shLuc) or GFP (shGFP) were used as controls for shRNA. Non-specific siRNA (Scram) was used as a control for siRNA. Alamar Blue was used as a readout for cell number as metabolically active cells reduce the reagent to make a fluorescent compound. Incubation time with reagent was optimized for LNCaP and C4-2 cells at 4h (**Fig. 3A**, C4-2 data not shown). Of the shRNA constructs tested, shBC2 and 5 gave the best knockdown of β-catenin mRNA to approximately ~25% of controls (**Fig. 3B**). For AR knockdown, shAR2 and 5 gave the best knockdown of AR at approximately 20% and 5% of controls respectively (**Fig. 3C**). Knockdown was about the same in both cell lines. Interestingly, shRNA against β-catenin also resulted in some loss of AR mRNA (**Fig. 3C**). Knockdown of AR had no effect on β-catenin mRNA levels (data not shown). Knockdown of AR by shRNA resulted in less LNCaP, but not C4-2, cells (**Fig. 3D**). Loss of β-catenin resulted in less C4-2, but not LNCaP, cells (**Fig. 3D**). Given that shRNA against β-catenin had some effect on AR mRNA levels as well, siRNA was also used as an alternative approach. siRNA against AR or β-catenin knocked down corresponding mRNA levels to approximately 20-30% (**Fig. 3E-F)** and was specific to each gene (data not shown). Knockdown of β-catenin also resulted in an equivalent knockdown of the β-catenin transcriptional target, Cyclin D1 (**Fig. 3F**). Knockdown of β-catenin in both LNCaP and C4-2 cells impaired their proliferation (**Fig. 3 G-H**). C4-2 cells grew faster than LNCaP cells and loss of β-catenin resulted in a larger reduction of cell growth than LNCaP cells. Together, this data is inconclusive if β-catenin can inhibit cell growth in LNCaP cells since the shRNA and siRNA knockdowns gave different results. However, β-catenin does play a role in promoting cell proliferation in C4-2 cells since knockdown by shRNA or siRNA resulted in reduced cell growth compared to controls.

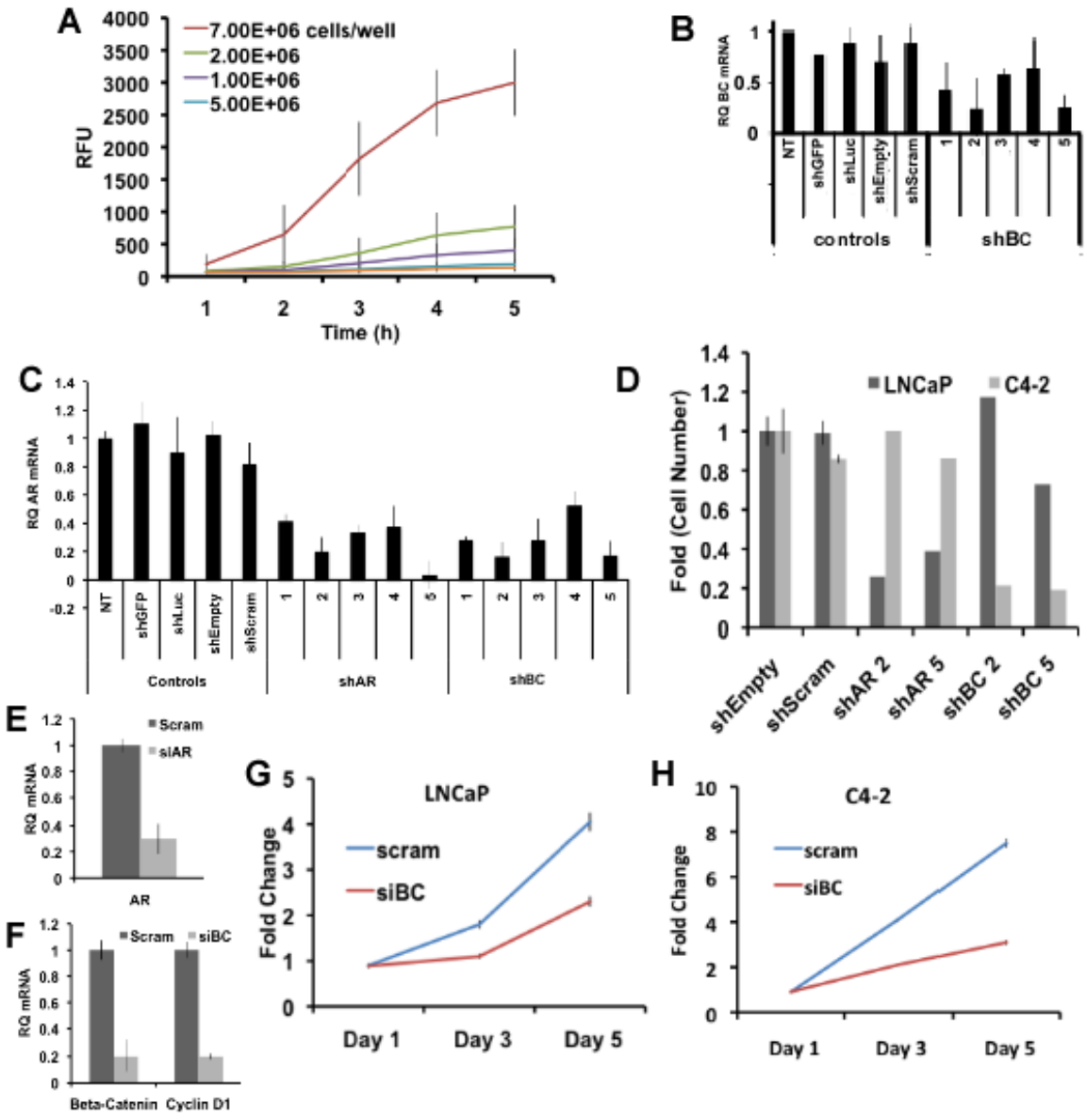

Figure 3. Effect of β-catenin (BC) knockdown on cell proliferation. A) Correlation of cell number and Alamar Blue incubation time with 590 nm fluorescence (RFU) in LNCaP cells. **B-C,E-F)** LNCaP cells were treated with shRNA or siRNA plasmids against BC (shBC or siBC), AR (shAR or siAR), or non-targeting control plasmids (NT, shGFP, shLuc, shEmpty, shScram, or scram siRNA) and mRNA expression of BC, AR or Cyclin D1 was monitored using qPCR. **F)** Cell proliferation of LNCaP and C4-2 cells treated with shRNA against AR, BC, or with control plasmids for 5 days and cell growth was measured and compared to shEmpty controls. G-H**)** Fold change in cell proliferation assays in LNCaP and C4-2 cells treated with siRNA pool against BC or non-specific sequence (scram). Error bars on all graphs represent standard deviations; n=2-4.

To further examine the role of β-catenin in prostate cancer cells, various pharmacological inhibitors and activators of the Wnt/β-catenin pathway were examined (summarized in **Table 1**). First, these compounds were tested for their ability to either inhibit or activate β-catenin using the TOP-FLASH reporter assay. BIO (Tocris), LiCl (Sigma), or Wnt conditioned media (Wnt) were able to activate TOP-FLASH reporter (**Fig. 4**). Inhibitors IWR-1 (Sigma), iCRT-3 (Sigma), iCRT-14 (Sigma), ICG-001(Selleckchem), or IQ-1 (Millipore) had no effect on their own (data not shown), but were able to largely reduce the activation Wnt induced to various degrees (**Fig. 4**). The synthetic androgen, R1881, had no effect on its own and could not inhibit Wnt stimulation of the reporter as expected (**Fig. 4**).

Table 1. β-catenin inhibitors

Compound	Target	Conc.
iCRT-3, 14	β-catenin:TCF interaction	25 uM
ICG001	β-catenin:CBP interaction	10 uM
IQ-1	β-catenin: p300 interaction	10 uM
IWR-1	Tankyrase/Axin	10 uM
BIO, LiCl	GSK (TSH/CREB)	5 nM, 20 mM

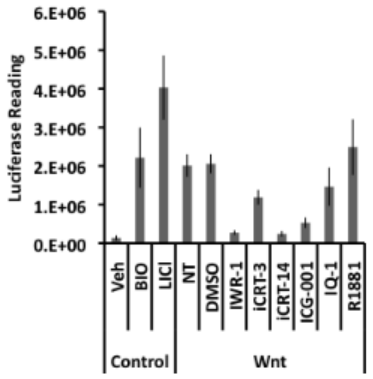

Figure 4. Effect of Wnt/b-catenin inhibitors and activators on TOP-FLASH reporter assay in STF-293 cells. STF-293 cells that stably express the TOP-FLASH reporter were treated with control or Wnt-conditioned media (Wnt), then treated with indicated inhibitors and activators of the Wnt/b-catenin pathway. Results were normalized to control reporter, FLOP-FLASH. Error bars represent standard deviations; n=3.

Prostate cancer cells were then treated with the β-catenin inhibitors in a blinded study. Both iCRT-3 and iCRT-14, which target the β-catenin and TCF interaction, inhibited cell proliferation in both LNCaP and C4-2 cells, with iCRT-3 having the greatest inhibition (**Fig. 5**). This was surprising since iCRT-14 had a great reduction in Wnt-induced TOPFLASH reporter activity than iCRT-3 (**Fig. 4**). Co-treatment with androgen (R1881) or Wnt could partially rescue, but did not bring proliferation back to normal levels (**Fig. 5 B-C, F-G**). IWR-1 treatment (tankyrase inhibitor; promotes β-catenin degredation) also resulted in a smaller inhibition on cell growth (**Fig. 5 A, E**). These were validated to be significant ($P<0.05$) by statistical analysis. Another tankyrase inhibitor, XAV939, has been recently shown to inhibit proliferation in a CRPC LNCaP cell line, but have only a minor inhibition in LNCaPs themselves (5). I also observed that inhibition was more pronounced in C4-2 cells compared to LNCaPs, which is also a reflection that C4-2 cells grow at a faster rate than LNCaP cells. This was true for the iCRT-3 and iCRT-14 inhibitors as well. In addition to using a different inhibitor, the authors of this study used a different CRPC LNCaP subline (LNCaP-AI) and used BrdU incorporation as a readout of proliferation, which may have influenced the degree of inhibition they observed (~80%) as compared to our studies (~30%). I am planning on testing XAV939 in future studied. Furthermore, treatment of either cell line with ICG-001, IQ-1, or the combination of both had no effect on cell proliferation in either cell line although this would inhibit the interaction of β-catenin with both p300 and CBP (**Fig. 5 D, H**). Together, this data suggests that inhibition of β-catenin with TCF (or other proteins that may interact with β-catenin using the same β-catenin interface as TCF) can prevent prostate cell proliferation.

Inhibition in cell proliferation can be a result of inability to progress in cell cycle or increase in cell death. To determine the effects of β-catenin on cell survival, cells were first starved 48h to remove any endogenous growth factors including steroids. Phenol red-free media was also used as phenol red has been shown to act as a steroid mimic (6, 7). I focused on iCRT-3 since iCRT-3 had the greatest inhibition of cell proliferation

(**Fig. 5**). Treatment of LNCaP or C4-2 cells with iCRT-3, but not vehicle (DMSO), R1881, or Wnt, resulted in a dramatic loss of adhesion in as few as six hours (**Fig. 6A**). Treatment with R1881, Wnt, or both in combination could not rescue this effect. There was a greater loss of adhesion in LNCaP than in C4-2 cells. Cell adhesion to matrix is required for cell survival of most epithelial cells, including in prostate and prostate cancer cells (8, 9). To determine if the cells were dying, both adherent and non-adherent cells were collected and stained with Trypan Blue. Trypan Blue is excluded from viable cells. Interestingly, ~80-95% of C4-2 cells were Trypan Blue positive when treated with iCRT-3, even in the presence of R1881, Wnt, or both (**Fig. 6B**). Only ~20% of LNCaP cells were Trypan Blue positive when treated with iCRT-3, and this could be rescued by Wnt but not R1881 (**Fig. 6B**). Treatment of cells with other growth factors also could not rescue the effect of iCRT-3 treatment (data not shown).

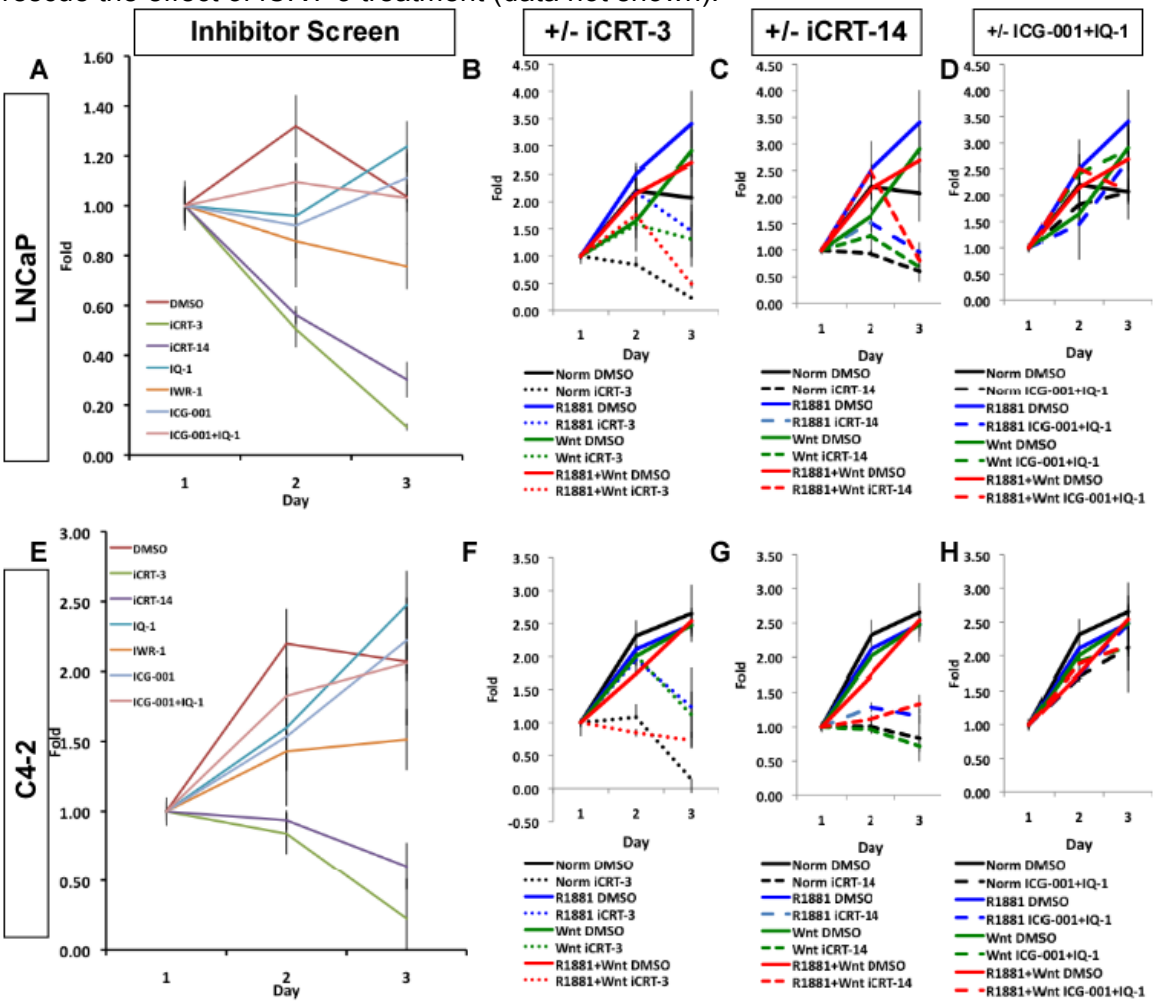

Figure. 5. iCRT-3 and i-CRT-14 are potent inhibitors of cell proliferation. LNCaP **(A-D)** or C4-2 **(E-H)** cells were treated with indicated inhibitors of Wnt/b-catenin and cell growth was monitored over time by Alamar Blue and compared to day 1. **B-D, F-H)** Cells treated with inhibitors in the presence of Wnt, R1881, or both. Error bars represent standard deviations; n=3.

To see if this was specific to iCRT-3, the panel of other β-catenin inhibitors (**Table 1**) was also tested. iCRT-14 treatment resulted in ~40% cell death in C4-2 cells, and about ~20% cell death in LNCaP cells. Other inhibitors had a minor effect on cell survival in both cell lines.

AR and β-catenin have been reported to interact (10-14). iCRT-3 was the most potent inhibitor in the cell proliferation and cell death assays (**Fig. 5+6**). iCRT-3 is thought to inhibit the interaction between β-catenin and TCF (15) but it is possible that it may prevent the interaction of other proteins with β-catenin that also use the same interface of β-catenin as TCF. Both AR (13, 14) and TCF (16) interact with β-catenin armadillo repeat 6, suggesting that iCRT-3 may also inhibit the ability of AR and β–catenin to interact. To test this, LNCaP cells were treated with vehicle control, iCRT-3, R1881 (androgen), or Wnt alone or in combination and either AR or β-catenin was immunoprecipitated. The β-catenin and AR interaction was most favored when Wnt was present in the conditions tested (**Fig. 7**). However, treatment with iCRT-3 could inhibit the interaction, even in the presence of Wnt. Therefore, iCRT-3 inhibits the interaction of AR and β-catenin in prostate cancer cells. These results have recently been replicated by another lab (17).

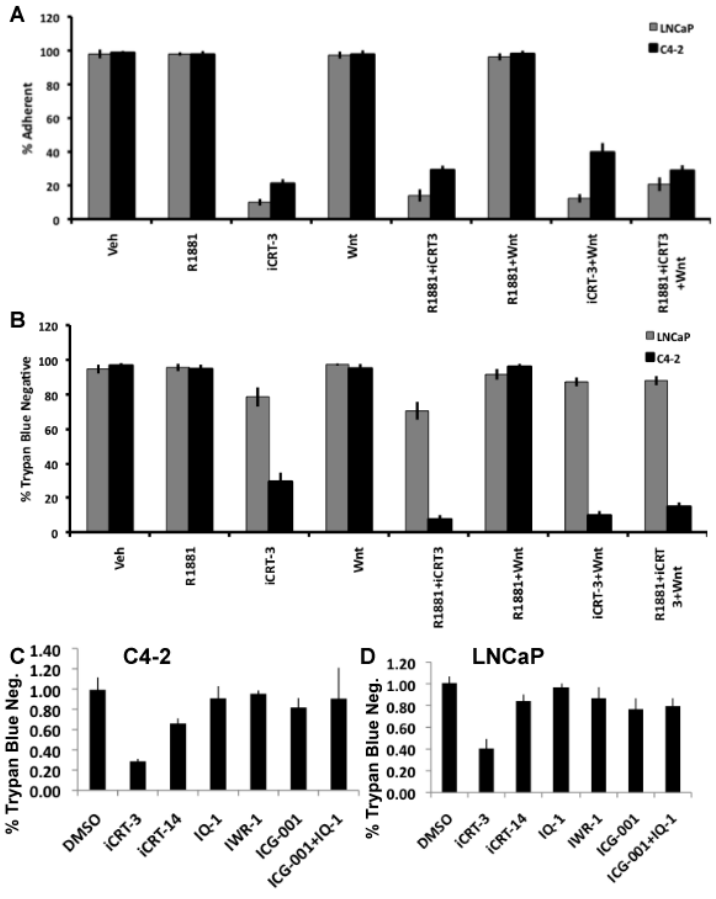

Figure 6. iCRT-3 induces loss of adhesion and cell death. A-B) LNCaP or C4-2 cells were treated with the indicated drugs or growth factors and the number of cells plated that became adherent **(A)** or percent viability as measured by Trypan Blue **(B)** was measured at 8 or 24 h. **C-D)** C4-2 **(C)** or LNCaP **(D)** cells were treated with indicated inhibitors and viability as measured by Trypan Blue staining was measured at 24h post-treatment. Error bars represent standard deviations; n=3.

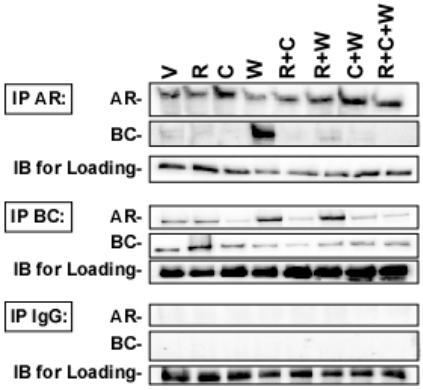

Figure 7. Androgen and Wnt regulate AR and β-catenin binding. LNCaP cells were treated with vehicle (V), 10 nM R1881 (R), 20 uM iCRT-3 (C), or 25% Wnt conditioned media (W), or in combination for 24h. Cells were lysed and AR, BC, or control IgG were immunoprecipitated (IP) and AR and BC were immunoblotted for.

Since AR and β-catenin have been demonstrated to interact, and AR and β-catenin have been demonstrated to both contribute to prostate cancer progression, **Task 3** sought to determine if the interaction between β-catenin and a hormone receptor was unique to prostate cancer or if this also played a role in other cancers. I screened other cancers that are thought to be hormone sensitive by doing pathway analysis (GeneGo software) on existing microarray datasets (18) and looking for cancers with Wnt/APC/β-catenin and AR in the top ten pathways or transcription factors affected (data not shown). Surprisingly, the top hit for the screen using these criteria was cervical cancer (Wnt/APC/β-catenin was top pathway effected, AR was ranked fifth). Estrogen receptor signaling was also a top pathway altered during cervical carcinogenesis, but this was always a lower hit than androgen signaling in all the datasets tested (ranked seventh). However, AR appears to be lost in cervical cancer progression (19) and was not detectable by western blotting in various cervical cancer cells lines (data not shown). In breast and neuronal cells, estrogen increases β-catenin expression, and activated estrogen receptor α (ERα) and β-catenin directly interact (20-23). Also, a drug that can reduce estrogen signaling, Indole-3-carbinol (I3C), prevents cervical cancer in HPV16-infected mice and was highly effective in inducing precancerous lesion regression in women (24-26). These effects may be due to the fact that I3C can also degrade β-catenin in prostate cancer cells (27). Given these facts, I hypothesized that HPV infection leads to increased activity of β-catenin, which contributes to the development of cervical cancer by up-regulating ERα targets. Furthermore, if β-catenin and ERα interact in cervical cancer cells, iCRT-3 could inhibit this interaction. ME180 cells are a cervical cancer cell line that expresses low but endogenous levels of ERα. Preliminary studies show that ERα and β-catenin co-immunoprecipitate with each other and Wnt may increase this interaction, though this may be because Wnt increases the total levels of β-catenin available (**Fig. 8A,B**). iCRT-3 can partially inhibit this interaction, although this may be due to decreased β-catenin and ERα expression. To determine if estrogen signaling was important for cell proliferation, cells were plated, starved and treated with either estrogen (E2) or estrogen inhibitor (ICI 182,780; ICI) for 48h as indicated in **Fig. 8C**. E2 resulted in increased number of metabolically active cells, and although there was a decrease in cells with the estrogen inhibitor, it was not significantly different (**Fig. 8D**). This may be because the drug was on the cells for only 48h and we have since learned that the ICI drug can take 48-72h to start becoming effective. Future studies will take this into consideration. To see if Wnt/β-catenin was important in cell proliferation, in a panel of cervical cancer cell lines the pathway was either activated with Wnt, inhibited with iCRT-3, or treated with both. In general, treatment with Wnt increased cell number although this was not always significant (**Fig. 8E**). However, treatment with iCRT-3 universally resulted in a dramatic decrease in metabolically active cells, suggesting cell death or senescence (**Fig. 8E**). This will be determined in future studies although cell morphology was rounded cells with a lot of extracellular debris, suggestive of cell death (data not shown). In contrast with the prostate cancer cells, the cells were still adherent to the plate and addition of Wnt resulted in a full rescue of cell number (**Fig. 8E**). This maybe through a non-canonical Wnt pathway and remains to be determined. In summary, in ME180 cervical cancer cells, there is basal levels of ERα and β-catenin interaction which may be increased in the presence of Wnt. iCRT-3 can inhibit. Estrogen and Wnt may be pro-proliferative in cervical cancer cells and Wnt can rescue cell death induced by iCRT-3. These studies have lead to a funded Center for Women's Infectious Disease Research Pilot grant.

In **summary** of Aim 1, I have demonstrated that β-catenin is important for cell proliferation of prostate cancer cells by shRNA, siRNA, and pharmological inhibitors.

C4-2 cells, a CRPC model, is more sensitive to β-catenin inhibition than the androgen sensitive LNCaP cells. This is probably due to the higher basal activity of β-catenin in C4-2 cells compared to LNCaP cells. iCRT-3 is a potent inhibitor of cell proliferation, survival and adhesion of C4-2 cells, and to a lesser degree LNCaP cells. iCRT-3 can also inhibit the interaction between AR and β-catenin. Francis et. al. have demonstrated that loss of β-catenin does not inhibit *in vivo* prostate cancer cell growth, but stabilized β-catenin can promote prostate cancer growth in a *Pten* loss genetic mouse model of prostate cancer (28). Lastly, hormone receptor crosstalk with β-catenin may be important in other cancers, such as ERα and β-catenin in cervical cancer.

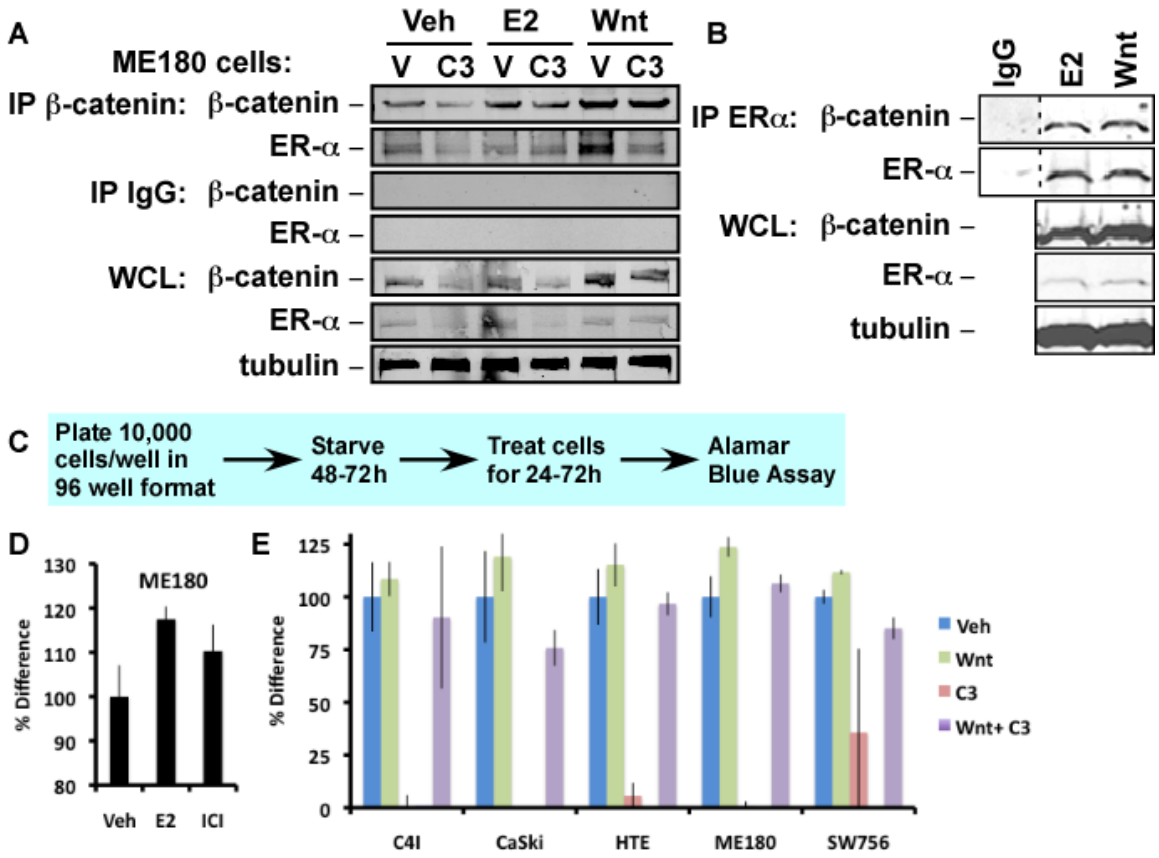

Figure 8. ERα and β-catenin in cervical cancer cells. A-B) ME180 cells were treated with vehicle (V), 1 nM estrogen (E2), 20 uM iCRT-3 (C), or 25% Wnt conditioned media (W), or in combination for 24h. Cells were lysed and ERα, BC, or control IgG were immunoprecipitated (IP). ERα and BC were immunoblotted for. Whole cell lysates (WCL) were also examined and tubulin was used as a loading control. **C)** Cell proliferation assay set-up. **D)** ME180 cells treated with estrogen (E2) or ER inhibitor (ICI) for 48 h. **E)** Various cervical cancer cell lines treated with Wnt or iCRT-3 (C3) for 48h. Error bars represent standard deviations; n=2.

Summary of Aim 2:

The goals of Aim 2 were to determine the genetic targets of β-catenin, AR, and β-catenin-AR in prostate and prostrate cancer cells. My *working hypothesis* was that β-catenin targets different genomic targets in normal prostate cells compared to PCa (**Fig. 1**). Specifically, β-catenin targets proliferation genes in normal, AR- prostate cells. Normal, non-proliferative AR+ prostate cells have little to no β-catenin activity due to increased E-cadherin expression (29), which sequesters β-catenin. In PCa, β-catenin preferentially pairs with AR instead of TCF. This shifts transcriptional targets of β-catenin to those also bound by AR, and makes AR active in the absence of physiological

levels of DHT, playing a key role in PCa progression. Mutations in the AF2 region of AR may drive the β-catenin-AR interaction in CRPC. In order to achieve the objective off identifying genetic targets of β-catenin, AR and the β-catenin-AR complex in prostate and prostate cancer cells, I had to optimize the novel technology, Split DamID, for these assays (Split DamID technology is summarized in **Fig. 9**).

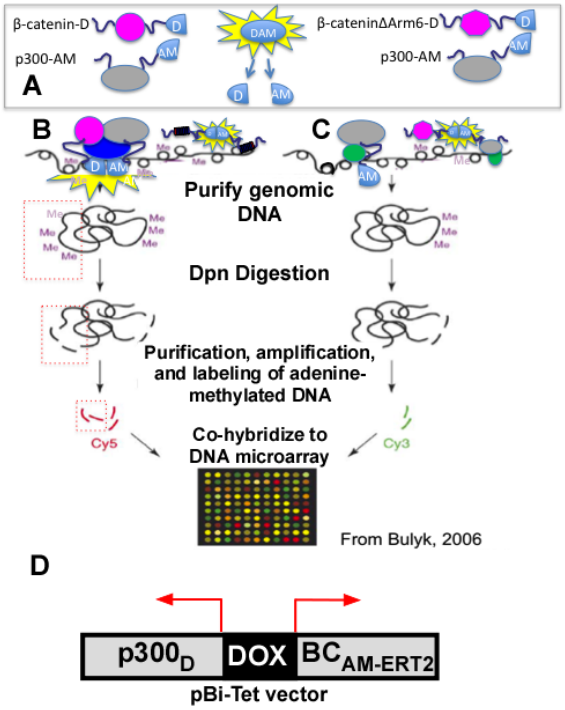

Figure 9. Overview of Split DamID. A) The *E. Coli* DNA Adenine Methyltransferase (DAM) was split into N-terminal (D) and C-terminal (AM) complementary halves. Halves were then fused to interacting transcription factors, such as p300 and β-catenin. **B)** When the two transcription factors interact with each other on DNA, the D and AM fragments reconstitute DAM activity. This enriches adenine methylation (A^m) on GA^mTC sequences near the binding sites of the two transcription factors of interest. DNA is purified, the methylated sites are digested with DpnII, adaptors are ligated to the fragments and amplified by adaptor-mediated PCR. Dpn fragments are identified by deep sequencing or co-hybridized with a **(C)** control library to a DNA microarray. Split DamID can identify transient interactions, operates at low levels of fusion protein expression, has improved sensitivity over ChIP, and does not require co-immunoprecipitation with an antibody (1-4). Furthermore, only a small amount of cellular material is required and one can probe the DNA bound by only by complexes containing two factors, like AR and β-catenin. **D)** Signal-to-noise ratio is minimized by putting the AM domain under the regulation of the ERT2 (activated by OHT. To control temporal and relative expression levels, proteins of interest are cloned into the doxycycline (DOX) responsive bi-directional expression vector pBi-Tet (Clontech). This allows co-expression of transcription factors under the control of DOX in cells of interest.

For **Task 1**, I had to develop the pBi-Tet vectors to be used in this assay. Cloning, mutating, and especially sequencing of vectors containing AR proved to be difficult since AR contains several polyglutamine tracts with extensive GC content (30). Despite this, all the vectors have been completed and sequence verified with the exception of the double AR and β-catenin mutant negative controls for AR and β-catenin DNA binding (**Table 2**). These are currently being tested to confirm mutations by complete sequencing of the vector. Furthermore, both the K5-rtTA and Probasin-rtTA mouse are now available from Jackson Labs, meaning the constructs and corresponding mice no longer are required to be generated (see **Table 2** for details). As such, **Task 1** is almost complete.

For **Task 2**, the genetic targets of β-catenin, AR and the β-catenin-AR complex in prostate and prostate cancer cells was to be elucidated using Split DamID. There were significant challenges in adapting the Split DamID technology to prostate cancer cells. At first, there was little to no amplification of the adenine methylated DNA ligation mediated PCR products (LMPs) (**Fig. 10A**). This was the consequence of the larger size of the Split DamID constructs generated for this project whereas the original Split DamID constructs made to investigate Notch signaling were smaller in size. Expression of the smaller Notch Split DamID constructs in prostate cells were able to generate detectable levels of LMPs (data not shown). To address this, the Split DamID constructs had to be transfected at higher expression levels in the prostate cancer cells. Several different reagents and protocols had to be tested given that prostate cancer cells are significantly more difficult to transfect compared to other cell lines. Optimizing the

Table 2. Split DamID Construct Progress	
Construct (purpose)	**Status**
pBI-Tet-p300-AM-ERT-2+β-catenin-V5-D (positive control for β-catenin alone)	Generated and Tested
pBI-Tet-p300-AM-ERT-2+AR-V5-D (positive control for AR alone)	Generated and Tested
pBI-Tet-SRC3-AM-ERT-2+AR-V5-D (positive control for AR with coactivator)	Generated and Tested
pBI-Tet-AR-AM-ERT-2+β-catenin-V5-D	Generated and Tested
pBI-Tet-p300-AM-ERT-2+β-cateninDArm6-V5-D (negative control for β-catenin)	Generated and Tested
pBI-Tet-p300-AM-ERT-2+ARDLBD-V5-D (negative control for AR)	Generated and Tested
pBI-Tet-p300-AM-ERT-2+TCF4-V5-D (positive control for TCF4 alone)	Generated and Tested
pBI-Tet-TCF4-AM-ERT-2+β-catenin-V5-D (test for TCF4-β-catenin complex)	Generated and Tested
pBI-Tet-β-catenin-AM-ERT-2+TCF4-V5-D (test for TCF4-β-catenin complex)	Generated and Tested
pBI-Tet-TCF4-AM-ERT-2+AR-V5-D (test for TCF4-AR complex)	Generated and Tested
pBI-Tet-ARDLBD-AM-ERT-2+β-cateninDArm6-V5-D (negative control for AR and β-catenin)	In Progress
pBI-Tet-ARDLBD;V581F-AM-ERT-2+β-catenin MT10DArm6-V5-D (negative control for AR and β-catenin)	In Progress
K5-rtTA	Mouse available from Jackson Labs Cat. No. 017519
Probasin-rtTA	Mouse available from Jackson Labs Cat. No. 021187

transfection protocol now allowed the Split DamID constructs to be expressed at sufficient levels for to generate detectable levels of LMPs. Stimulation of the transcription factors of interest (i.e. androgen for AR and Wnt for β-catenin) also improved generation of LMPs (data not shown). Another protocol that had to be optimized were the conditions under which the DpnII digest was done, in that while there was a reduced amount of LMPs generated in the absence of DpnII enzyme, there was still LMPs generated when there should have been none (**Fig. 10B**). It is important to note that control constructs ran in parallel to the Split DamID constructs gave either digested products or non-digested products with or without DpnII treatment respectively, showing that the original conditions tested were sufficient for simple DNA digests (data not shown). I purchased new Dam- bacterial cells to generate the DNA for these assays to ensure that the DNA being transfected into the cells was not a source of DNA methylation contamination, although we suspect this was not the cause since the same lot of bacterial cells were used to generate Split DamID constructs for Notch without these effects. Optimization of the ligation buffer mix (NEB Cut Smart Buffer) resolved this issue. Another problem that arose was the production of LMPs in the absence of DOX or OHT, possibly due to the higher levels of expression of the Split DamID constructs. This was resolved in the following ways: 1) cells were treated with phenol-red free media, as phenol-red could potentially be an OHT-mimic (6, 7); 2) the Tet-ON vector was stably transfected into prostate cancer cells; 3) induction of the Split DamID

constructs by OHT and DOX was limited to less than 8h. These significantly improved the dependence on DOX and OHT for Split DamID especially in C4-2 cells (**Fig. 10C**, right panel). Occasionally, loss of dependence on DOX and OHT still occurs in LNCaP cells although this is not common and I still seek to minimize (**Fig. 10C**, left panel). The last aspect of this assay that I have been optimizing is the production of clean and specific PCR bands for DNA sequence specific DNA. In some areas of the genome, DpnII and DpnI can cut as often as ever 150-200bp. DNA specific PCR products must

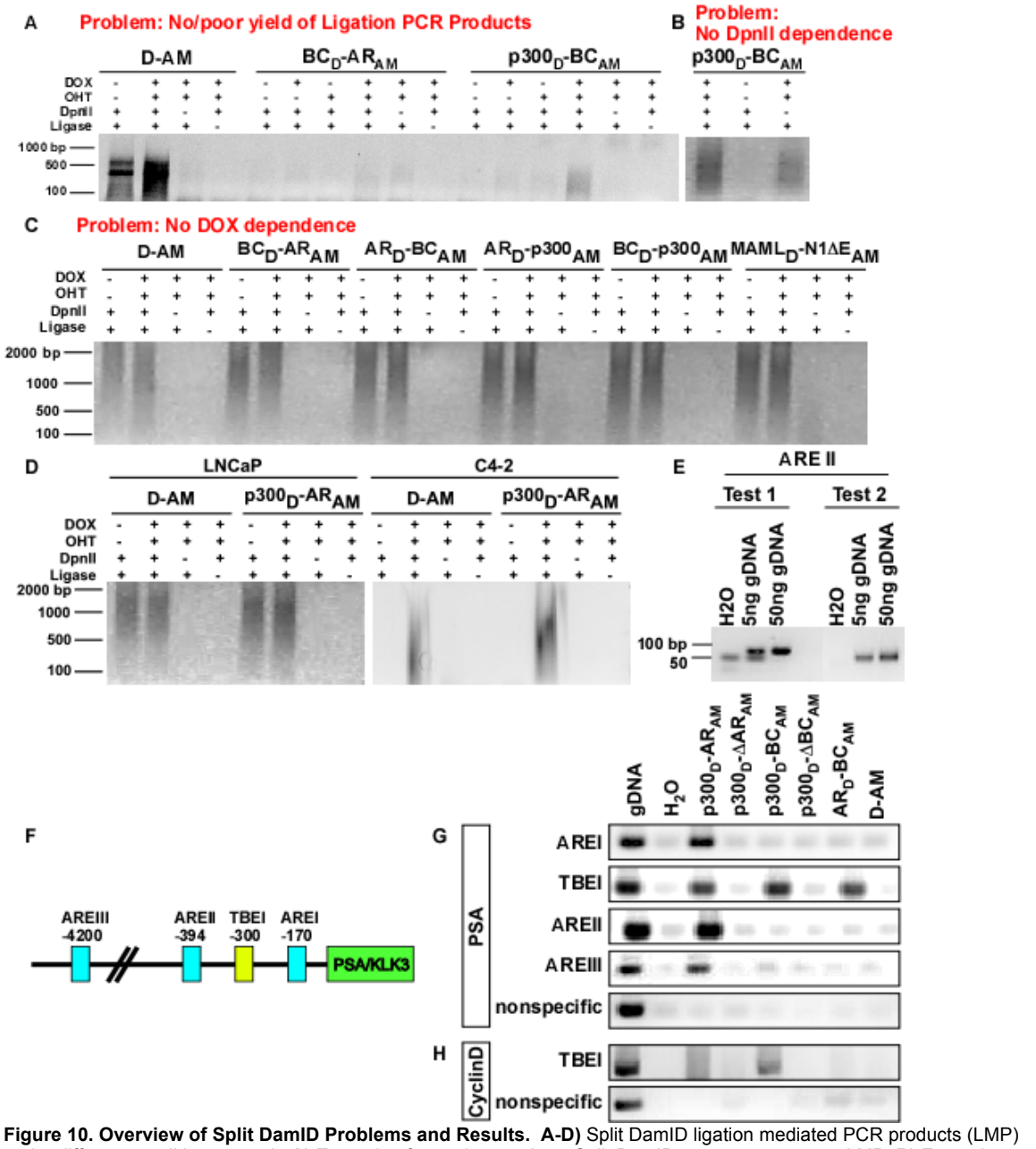

Figure 10. Overview of Split DamID Problems and Results. A-D) Split DamID ligation mediated PCR products (LMP) under different conditions tested. **A)** Example of experiment where Split DamID constructs gave no LMP. **B)** Example where there is LMP in absence of DpnII digestion. **C)** Example where LMP has lacks DOX dependence. **D)** Example of LMP in LNCaP and C4-2 cells. **E)** Target specific PCR for androgen response element (ARE) II in PSA promoter under two different test conditions, where test condition 2 gives no background bands. **F)** Schematic of human PSA promoter and or β-catenin binding sites (TBE). **G-H)** Target specific PCR for PSA **(G)** or Cyclin D1 **(H)** promoter. gDNA and H2O are positive and negative controls for PCR respectively. D-AM gives background levels of random DNA methylation. Nonspecific is DNA in the promoter that lacks an ARE or TBE. N=3-5.

be designed to run smaller than the Dpn fragment size, with some being about 100bp in size. This can run close to bands that may be present from dimer-primer pairs or the dye mobility front from Bromophenol Blue or Cresol Red under the conditions tested. As a result, a smaller non-specific band was sometimes detected in sequence specific PCRs (**Fig. 10E**, left panel "Test 1"). To correct this: 1) a DNA loading buffer with Orange G and Xylene cyanol (have dye migrations at 50 and 4000bp under the conditions tested, i.e. not where the PCR bands will be) were used; 2) three sets of PCR primers for each target were tested for the absence of dimer-primer pairs with the best one being selected; and 3) PCR cycling conditions were reduced by 4 cycles. This improved the ability to detect DNA sequence specific PCR products (**Fig. 10E**, right panel "Test 2").

I have validated that the Split DamID constructs work as expected on known AR and β-catenin targets in prostate cancer cell lines, including PSA/KLK3 for androgen receptor and Cyclin D1 for β-catenin amongst numerous others (**Fig. 10F-H,** data not shown). The PSA/KLK3 promoter is a known AR-target in prostate cancer and has two androgen responsive elements in the proximal promoter (AREI and II) and one in the enhancer (AREIII) (**Fig. 10F**). Transcriptional analysis of the promoter also revealed a putative TCF response element (TBEI) in between AREI and AREII, suggesting that this may be a sequence that β-catenin could interact with. Split DamID was used to determine if AR, β-catenin, or the AR-β-catenin complex was binding to any of these elements. A non-specific sequence (approximately located at -1kb) that did not contain any AREs or TBEs was used as a negative control for the Split DamID constructs. Genomic DNA (gDNA) and water (H_2O) were as positive and negative controls for the PCR reaction respectively. As expected, the AR (**Fig. 10G** as indicated by the $p300_D$-AR_{AM} construct, lane 3), but not the AR DNA binding mutant (**Fig. 10G** as indicated by the $p300_D$-ΔAR_{AM} construct, lane 4) bound to the AREI, AREII, and AREIII sequences as determined by PCR. β-catenin and the AR-β-catenin complex did not bind these sites and D-AM expressed by itself gave little detectable background, indicating that this is a specific PCR product to AR (**Fig. 10G,** lanes 5-8). Interestingly, AR, β-catenin and the AR-β-catenin complex all bound to the TBEI (**Fig. 10G,** lanes 3, 5, and 7). This was specific since mutation of AR or β-catenin or D-AM by itself did not produce a PCR product above background levels (**Fig. 10G,** lanes 4, 6, and 8). This is the first evidence that β-catenin and AR can bind to a promoter as a complex. Further studies will try to confirm this by ChIP and the biological significance of this site will be determined through reporter assays. For the β-catenin target Cyclin D1, only the wild type β-catenin construct binds the promoter (**Fig. 10H,** lane 5; please note that there is no specific band in lane 3 but rather a general and non-specific DNA smear). Pending the completion of the pBI-Tet-ARDLBD-AM-ERT-2+β-cateninDArm6-V5-D and pBI-Tet-ARDLBD;V581F-AM-ERT-2+β-catenin MT10DArm6-V5-D constructs (both negative controls), this task in on-target for completing a major screen of AR, β-catenin, and AR-β-catenin targets within the second year of this award.

Task 3 of Aim 2 is to develop transgenic mice for performing Split DamID analysis *in vivo.* Two of these mice, the probasin-rtTa and K5-rtTA, are already available through Jackson Labs and do not need to be generated (see **Table 2**). Production of the transgenic mice expressing the Split DamID constructs will commence once I validate that the constructs work for the *in vitro* major screen in **Task 2**.

In **summary** of Aim 2, despite numerous challenges of implementing Split DamID in prostate cancer cells, these obstacles have been overcome. A novel β-catenin response element that putatively binds β-catenin and AR in a complex has been identified in the proximal promoter for PSA/KLK3. This result is indicative that the Split

DamID constructs will work as expected and will discover and validate AR, β-catenin, and AR-β-catenin complex targets.

KEY RESEARCH ACCOMPLISHMENTS

1. Determined that β-catenin loss could inhibit cell proliferation of LNCaP and C4-2 prostate cancer cells.
2. Demonstrated that the β-catenin inhibitor iCRT-3 was a potent inhibitor of prostate cancer cell proliferation, adhesion, and survival.
3. Demonstrated that iCRT-3 can inhibit the interaction of β-catenin and AR in prostate cancer cells.
4. Determined that ERα and β-catenin interact in cervical cancer cells; Wnt can enhance this interaction and iCRT-3 only minimally inhibits.
5. Determined that cervical cancer cells have a small increase in proliferation upon estrogen treatment and in some cases Wnt treatment. iCRT-3 induces cell death or senescence, but Wnt can rescue.
6. Generated LNCaP and C4-2 cells with stable expression of the androgen receptor reporters MMTV-Luc, PSA-Luc, and ARE-4X-Luc and the control vector pGL3-empty-Luc; the β-catenin reporter TOP-FLASH and the control vector FLOP-FLASH; and the pBi-Tet vector for Split DamID studies.
7. Generated Split DamID constructs to determine the DNA targeted by AR, β-catenin, AR-β-catenin, TCF4, TCF4-β-catenin, and TCF4-AR.
8. Optimized Split DamID for prostate cancer *in vitro* studies.
9. Discovered a TCF/β-catenin response elemement in the PSA/KLK2 promoter that is putatively bound by a β-catenin/AR complex.

REPORTABLE OUTCOMES

The following items have been generated due to the research carried out in the last year.

1. Developed the prostate cancer cell lines indicated in the Key Research Accomplishments #6 listed above.
2. Applied for and received a Center for Women's Infectious Disease Pilot Grant for developing the Split DamID technology to look at β-catenin crosstalk with another hormone receptor, ERα, in cervical cancer cells.
3. Applied for and was accepted to the AACR Translational Cancer Research for Basic Scientists Workshop, with an emphasis on prostate cancer.
4. Applied to and was accepted into the Transdisciplinary Research in Energenics and Cancer (TREC) program at Washington University in St. Louis. This will provide addition funding for this project for reagents when needed.
5. This data has been presented at the Women's Health Research Seminar Series and the Developmental Biology Seminar Series at Washington University in St. Louis.

CONCLUSIONS

Prior to these studies, the biological role of β-catenin in prostate cancer was largely not known. These studies demonstrate that β-catenin can promote proliferation,

survival and adhesion in prostate cancer cells, and this dependence on β-catenin may increase with prostate cancer progression as inhibition of β-catenin in the CRPC cell line C4-2 had a larger effect than in the androgen sensitive LNCaP cells. Notably, the β-catenin inhibitor iCRT-3 was a potent inhibitor of β-catenin, the AR-β-catenin complex, proliferation, survival and adhesion in prostate cancer cells. Wnt, androgen, or treatment with other growth factors could only partially rescue a subset of these phenotypes. β-catenin can interact with other steroid receptors, such as ERα, in cervical cancer cells. iCRT-3 can also inhibit cell proliferation and survival in cervical cancer cells and can partially inhibit the β-catenin-ERα complex, but Wnt treatment can fully rescue. Lastly, Split DamID constructs have been made and conditions optimized to determine the β-catenin, AR, and AR-β-catenin complex targets in prostate and prostate cancer cells. A new β-catenin response element in the proximal PSA/KLK3 promoter was discovered. Split DamID data suggests that both AR and β-catenin are binding this new response element. This will be confirmed and the biological significance established in future studies.

So what: CRPC has no curative treatments and new therapeutic targets for this disease needs to be identified. This data suggests that β-catenin may be misregulated during prostate cancer progression and may be a possible target, probably in combination with other therapies since inhibition of individual pathways has largely proven to be ineffective. Targeting the Wnt/β-catenin pathway is equally problematic because no compounds targeting Wnt/β-catenin signaling have been approved for clinical use. As such, iCRT-3, a β-catenin inhibitor, had the most dramatic effects and could be tested as a potential therapeutic in animal models in future studies. Lastly, Split DamID has the potential to identify new therapeutic targets or biomarkers. Given the importance of this pathway in highly proliferative tissues such as the gut, there is caution about the toxicity of targeting β-catenin directly (31). Therefore, identification of downstream transcriptional targets of these two pathways may enable development of better and more targeted therapeutics for prostate cancer.

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APPENDIX

REVISED STATEMENT OF WORK (SOW)

Training Plan:

1. To receive project feedback and develop critical thinking skills, I will meet with my mentor Dr. Kelle Moley weekly.
2. In order to learn about new cancer and prostate cancer studies, cutting edge technologies, develop presentation skills, and establish collaborations, I will attend multidisciplinary seminars, journal clubs, rounds, and conferences at Washington University in Saint Louis relevant to prostate cancer including:
    a. Cell Cycle and Signaling Research In Progress, *weekly*
    b. Basic Science Seminar Series, Siteman Cancer Center, *monthly*
    c. Schwartz Center Rounds, Siteman Cancer Center, *monthly*
    d. Genitourinary (GU) Research Group, *monthly*
    e. GU Cancer Conference, *annually*
    f. GU Urology Lab Meeting, *monthly*
    g. Transdisciplinary Research in Epigenetics and Cancer Journal Clubs and Transdisciplinary Science Meetings, *biweekly and monthly*
3. To gain expertise generating animal models and Split DamID technology, as well as strengthen my molecular biology skills, I will gain training from other members of the Kopan laboratory, especially Dr. Matt Hass, as well as training seminars offered by Washington University Support cores that offer these services.
4. To continue to learn about new basic, preclinical, and clinical studies, I will attend at least one conference or training opportunity a year including:
    a. 2012 AACR Advances in Prostate Cancer Research Conference
    b. 2013 Meeting on Translational Research
    c. 2014 Meeting or Training Opportunity on Cancer

Research Plan:

The overall objective of this proposal is to determine the biological significance of androgen receptor (AR) and β-catenin crosstalk in androgen sensitive and castration resistant prostate cancer cells by identifying their transcriptional targets. To achieve this objective I will carry out the tasks outlined below.

AIM 1. Determine if β-catenin signaling is required for CRPC cell proliferation.

Task 1. Determine the level of β-catenin and β-catenin activity in paired prostate cancer androgen sensitive and CRPC lines (e.g. LNCaP and C4-2 cell respectively) by western blotting and qPCR. (Month 1).

1a. The LNCaP cell line will be purchased from ATCC. C4-2 cells will be acquired from MD Anderson Cancer Center or Dr. Robert Reiter at UCLA (Acquired).

Task 2. Determine if loss of β-catenin is sufficient to induce loss of proliferation in paired prostate cancer androgen sensitive and CRPC lines (LNCaP for androgen sensitive, C4-2 as castration independent).

2.a. Cells will be transfected with shRNA constructs against β-catenin to induce β-catenin loss. A scrambled shRNA sequence that is non-specific for any targets, as well as a shRNA targeting Green Fluorescent Protein (GFP) will be used as negative controls (reagents already in lab and being validated). Western blotting and qPCR will be used to validate target

knockdown. Cells will be plated at equal densities in 96 well plates with three wells acting as technical replicates per shRNA construct. The total number of viable cells will be measured by Alamar Blue staining (recently optimized for LNCaP cells in our hands). The number of viable cells for each shRNA will be measured every other day over a two week period. In addition, pharmological inhibitors targeting β-catenin will also be used (Month 1-2).

Task 3. Determine if β-catenin can interact with other steroid receptors in other cancers and if this is important to their proliferation.

1a. Cervical cancer cell lines will be purchased from ATCC. Cells will be immunoprecipitated for the steroid receptors AR, ERα, or β-catenin and immunoblotted for the same proteins to see if there is an interaction between any of the steroid receptors and β-catenin. If an interaction is found, the dependence on β-catenin for cell proliferation will be determined as outlines in Task 2 above (Months 11-12).

Outcomes: It will be determined if β-catenin is important for cancer cell proliferation.

AIM 2. Determine the genetic targets of β-catenin, AR, and β-catenin-AR in prostrate and prostate cancer cells.

Task 1. Develop constructs where β-catenin, AR and p300 are fused to complementary halves of DAM. The signal-to-noise ratio is minimized by putting the AM domain under the regulation of the ERT2. To control temporal and relative expression levels, proteins of interest will be cloned into the doxycycline (DOX) responsive bi-directional expression vector pBI-Tet (Clontech). This will allow co-expression of transcription factors under the control of DOX in cells of interest (In progress, Months 1-12).

1a. The following constructs will be made by cloning:

pBI-Tet-p300-AM-ERT-2+β-catenin-V5-D (positive control for β-catenin alone)

pBI-Tet-p300-AM-ERT-2+AR-V5-D (positive control for AR alone)

pBI-Tet-SRC3-AM-ERT-2+AR-V5-D (positive control for AR with coactivator)

pBI-Tet-AR-AM-ERT-2+β-catenin-V5-D

pBI-Tet-p300-AM-ERT-2+β-cateninΔArm6-V5-D (negative control for β-catenin)

pBI-Tet-p300-AM-ERT-2+ARΔLBD-V5-D (negative control for AR)

pBI-Tet-ARΔLBD-AM-ERT-2+β-cateninΔArm6-V5-D (negative control for AR and β-catenin)

pBI-Tet-ARΔLBD;V581F-AM-ERT-2+β-catenin MT10ΔArm6-V5-D (negative control for AR and β-catenin)

pBI-Tet-p300-AM-ERT-2+TCF4-V5-D (positive control for TCF4 alone)

pBI-Tet-TCF4-AM-ERT-2+β-catenin-V5-D (test for TCF4-β-catenin complex)

pBI-Tet-β-catenin-AM-ERT-2+TCF4-V5-D (test for TCF4-β-catenin complex)

pBI-Tet-TCF4-AM-ERT-2+AR-V5-D (test for TCF4-AR complex)

Task 2. Determine genetic targets of β-catenin, AR, β-catenin-AR, TCF4, TCF4-β-catenin, and AR- β-catenin *in vitro* in primary prostate epithelial cells, prostate epithelial cells induced to differentiate and express AR, and in the prostate cancer androgen sensitive and castration resistance cell lines. (Months 4-22).

2a. Transfect LNCaP (androgen sensitive) and C4-2 (castration resistant) cells with constructs in Task 1A. Perform Split DamID and deep bar sequencing of bar-coded DpnI fragment ends. (Months 4-9).

2b. Analyze results with help of Biostatistics Core, GTAC Sequencing Core in Department of Genetics at WUSTL and Dr. Matt Haas (expert in Split Dam-ID). Our lab was fortunate to receive American Recovery and Reinvestment Act (ARRA) funding (RC1 NS068277-01) that has been used to develop peak calling algorithms that will weigh in the DpnI digest in considering signal, enumerate the fragment ends, and filter out PCR artifacts. This has been done successfully for Notch1 and p300 targets. Since this could be potentially hundreds or thousands of DNA sequences bound by AR and β-catenin complexes, putative targets will be prioritized accordingly. 1) Analysis will be preformed on cells receiving DOX and tamoxifen and those which did not. 2) Only targets that transcribed within the experimental time frame will be analyzed for direct binding. 3) Negative controls (see 1a) will be subtracted from all results to remove background. The top five targets will be verified with ChIP (Months 10-16).

2c. Repeat 3a and 3b with primary prostate epithelial cells (obtained from ATCC) (Months 11-17).

2d. Repeat 3a and 3b with prostate epithelial cells induced to differentiate with KGF/androgen. (Months 12-18).

2e. Compare and analyze results from 2b, 2c, and 2d to find DNA sequences targeted by AR or β-catenin alone, or AR and β-catenin in complex only in prostate cancer and castrate resistant cells (Months 19-22).

Task 3. Develop transgenic mice expressing constructs where β-catenin, AR and p300 are fused to complementary halves of DAM under the control of prostate specific and DOX and tamoxifen dependent promoters using constructs developed in Task 1. (Months 10-24).

3a. Generate transgenic mice expressing the following vectors individually: (Months 10-18)

pBI-Tet-p300-AM-ERT-2+β-catenin-V5-D
pBI-Tet-p300-AM-ERT-2+AR-V5-D
pBI-Tet-AR-AM-ERT-2+β-catenin-V5-D.

3b. Generate bi-transgenic mice expressing either probasin-rtTA or K5-rtTA (already on-site) with the mice generated in Task 3a. The long term goals are to identify the AR and β-catenin in complex genetic targets *in vivo*.

Outcomes: The transcriptional targets of AR, β-catenin, TCF4 alone, or AR and β-catenin, TCF4 and β-catenin, or AR and β-catenin in complex will be identified in normal prostate AR- and AR+ cells, as well as prostate cancer androgen dependent and castration resistant cells *in vitro*. Differences between these targets with respect to stage in prostate cancer progression will be identified. Significant progress will be made towards determining these targets *in vivo* with the generation of bi-transgenic mice.

Rationale for Revised SOW:

The goal of **Aim 1** was to determine if β-catenin signaling is required for CRPC cell proliferation. My *working hypothesis* was that β-catenin plays a pro-proliferative role in prostate cancer (PCa) cells.

In **Aim 1, Task 3**, I was to determine if loss of β-catenin is sufficient to inhibit tumor growth *in vitro*. Before I could begin our studies, Francis et. al. published the results of this study (28). They showed that β-catenin is required for normal prostate development by controlling the number of progenitor cells in the epithelial buds of the prostate, but was not required for adult prostate homeostasis. c-Myc and Nkx3.1 are also regulated by β-catenin in normal prostate cells. They used the *Pten* deletion mouse model for prostate cancer, which is more clinically relevant than the transgenic adenocarcinoma mouse prostate (TRAMP, *PB-Tag*) PCa mouse model I proposed to use since PTEN is lost in ~30% of primary prostate cancer and ~60-70% metastatic prostate cancers (32); large T antigen, the transgene expressed in the TRAMP model, is not associated with human prostate cancer (33, 34). Loss of β-catenin did not prevent prostate cancer in the *Pten* deletion mouse model (28). However, expression of a stabilized β-catenin cooperated with *Pten* loss to drive prostate cancer progression, tumor size and proliferation but not invasion. In agreement with previous studies, expression of stabilized β-catenin alone resulted in development of non-invasive prostatic intraepithelial neoplasia (PIN) (35). Francis et. al. speculate in their discussion that the effects of stabilized β-catenin to promote cancer progression in cooperation with *Pten* loss are independent of AR, although they show no data looking at AR or its targets or examining the effects of castration on this model. Instead they propose that the effects may be through activation of the PI-3K/Akt pathway. While this may have biological significance, it is difficult to conclusively determine since this is in the context of *Pten* loss, and Pten is a major negative regulator of the PI-3K/Akt pathway. Given the considerable overlap in this published paper and our proposed study for **Aim 1, Task 3**, and the significant time and cost of mouse studies, it is recommended that **Aim 1, Task 3** should no longer be pursued.

To determine if these results could be applied to other cancers (i.e. can hormone receptors including androgen receptor or estrogen receptor interact with β-catenin? Is β-catenin important for their proliferation? If so, does this alter the transcription targets of the involved transcription factors), we screened other cancers that are thought to be hormone sensitive by doing pathway analysis on existing microarray datasets and looking for cancers with Wnt/β-catenin and androgen signaling in the top ten pathways affected. Surprisingly, the top hit for the screen using these criteria was cervical cancer. Estrogen receptor signaling was also a top pathway altered during cervical carcinogenesis, but this was always a lower hit than androgen signaling in all the datasets tested. We have added cervical cancer studies as the **new Aim 1, Task 3** to see if the results of our prostate cancer studies can be applicable to other cancers as well.

The goals of **Aim 2** were to determine the genetic targets of β-catenin, AR, and β-catenin-AR in prostate and prostrate cancer cells. My *working hypothesis* was that β-catenin targets different genomic targets in normal prostate cells compared to PCa.

Task 3 of Aim 2 is to develop transgenic mice for performing Split DamID analysis *in vivo.* Two of these mice, the probasin-rtTa and K5-rtTA, are already available through Jackson Labs and do not need to be generated.

Interestingly, the interaction of β-catenin with AR inhibits β-catenin-TCF mediated transcription with respect to Cyclin D1 and the TCF reporter TOPFLASH (36, 37). This is dependent on androgen and can be blocked with AR agonists. It is thought that this is due to a direct competition for free β-catenin between AR and TCF. The fate of classical, TCF-dependent β-catenin targets, and the impact their loss may have on PCa, remains to be investigated. I propose to address this by adding to **Aim 2 Tasks 1 and 2**, by generation of the following TCF4 Split DamID constructs: pBI-Tet-p300-AM-ERT-2+TCF4-V5-D (positive control for TCF4 alone), pBI-Tet-TCF4-AM-ERT-2+β-catenin-V5-D (test for TCF4-β-catenin complex), pBI-Tet-β-catenin-AM-ERT-2+TCF4-V5-D (test for TCF4-β-catenin complex), and pBI-Tet-TCF4-AM-ERT-2+AR-V5-D (test for TCF4-AR complex) and expressing them in the prostate cancer cell lines.

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