AD_____

Á

Award Number: W81XWH-10-1-0709

TITLE: Tracking Origins of Prostate Cancer - An Innovative In Vivo Modeling

PRINCIPAL INVESTIGATOR: Kethandapatti ÁÒÈBalaji, M.D.

CONTRACTING ORGANIZATION: Wake Forest University
                                                    Winston-Salem, NC 27157

REPORT DATE: September 201H

TYPE OF REPORT: Annual

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

DISTRIBUTION STATEMENT: Approved for Public Release;
                                              Distribution Unlimited
Á

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
September 201H	Annual	1 September 201G– 31 August 201H

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Tracking Origins of Prostate Cancer - An Innovative In Vivo Modeling	**5b. GRANT NUMBER** W81XWH-10-1-0709
	5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)	5d. PROJECT NUMBER
Kethandapatti Balaji Xiaolan Fang, Kennyth Gyabaah, Sandy Sink	**5e. TASK NUMBER**
E-Mail: kbalaji@wfubmc.edu	**5f. WORK UNIT NUMBER**

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)	8. PERFORMING ORGANIZATION REPORT NUMBER
Wake Forest University Winston-Salem, NC 27157	

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012	
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

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

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Heterogeneity, variable and often unpredictable clinical course are fundamental challenges in management of patients with prostate cancer. To make rapid advances in understanding of disease mechanism that can be translated to clinical care in short order, there is an immediate need for innovative in vivo disease models that accurately recapitulate human disease at cellular level. We propose to develop an innovative and hitherto not attempted in vivo prostate cancer model that will delineate the exact cell of origin through different stages of prostate cancer development and progression. We propose to study possible cell(s) of origin for prostate cancer by combinatorial expression of florescent magenta, cyan and yellow primary color proteins in prostate at development in mice. The study includes (1) Construction of "Prorainbow" plasmid with fluorescent proteins (XFPs) under control by prostate epithelial and basal cell-specific promoters. (2)Establish mouse line with the resulting "Prorainbow" construct and generation of transgenic mice by crossing with Cre mice. (3) Study the transgenic Prorainbow mice under normal and oncogenic conditions. The study is expected to produce in vivo animal of prostate cancer with unique capabilities.

15. SUBJECT TERMS
Prostate cancer, in vivo model, Prorainbow, tumor development

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

Annual Report

Title: Tracking Origins of Prostate Cancer – an Innovative *in vivo* Modeling

Table of Contents

Introduction

Prostate cancer is the most frequently diagnosed cancer of men and the most common cancer overall (1). The cancer cells may metastasize from the prostate to other parts of the body, particularly the bones and lymph nodes. About 20% of patients undergoing radical prostatectomy develop metastasis beyond 5 years, suggesting metastasis is an early event and removal of primary tumor does not significantly decrease the rate of metastasis. Thus, understanding the role of the genetic changes leading to origination and development of primary tumor and metastasis would provide for a targeting strategy to clinical therapy. The goal of this project is to study the origin of cancer cells within the prostate. Since development of human prostate cancer proceeds through a serious of defined states, we would utilize a newly developed fluorescent protein labeling technique, Brainbow, which has been used to study the nervous system development in Brain (2). Similar to the 'Brainbow' concept we propose 'Prorainbow' modeling to track prostate cell proliferation and differentiation by labeling individual early prostate precursor cell a unique color. In case of a tumor or metastasis, we can track down the ancestor normal cell by matching to the tumor cell color. We can then track these color distributions and pattern changes with time course, which will build up a dynamic vision of prostate cancer progression. Also, we want to examine functions of Protein Kinase D1 (PKD1) and Phosphatase and Tensin homolog (Pten) in conditional knockout mice in the development of cancer formation and metastasis in the prostate. Successful development of florescent labeled *in vivo* animal model will be unique in the field of prostate cancer research and provide much needed advance to understand progression of prostate cancer.

We proposed to testify the stated hypothesis with following aims:
1) Construction of 'Prorainbow' plasmid with fluorescent proteins (XFPs) under control by prostate epithelial and basal cell-specific promoters.
2) Establish mouse line with the resulting 'Prorainbow' construct and generation of transgenic mice by crossing with Cre mice.
3) Study the transgenic Prorainbow mice under normal and oncogenic conditions.

Body

Aim (1) Construction of 'Prorainbow' plasmid with fluorescent proteins (XFPs) under control by prostate epithelial and basal cell-specific promoters.

Task I. Generation of *Probasin* promoter controlled XFP.
This part is done and was reported in prior annual report.

Task II. Generation of *Cytokeratin 5* promoter controlled XFP.
This part is done and was reported in prior annual report.

Aim (2) Establish mouse line with the resulting 'Prorainbow' construct and generation of transgenic mice by crossing with Cre mice.

Task I. Obtain institutional approval for animal study.

This part is done and was reported in prior annual report.

Task II. Generation of Prorainbow construct expressing transgenic mice (n=3~5).
We've generated nine PB-XFP transgenic mouse founders, and eight CRK5-XFP founders as described in prior annual report.

Task III. Cross-breeding of Cre mice with Prorainbow transgenic mice.
Three different breeding schemes were planned to validate the Prorainbow system and study normal prostate development.
1) ROSA-CreER X PB-XFP
2) PB-Cre4 x CRK5-XFP

3) PB-Cre4 x CMV-XFP

After the founder mice (nine PB-XFP animals and eight CRK5-XFP animals) were transferred to Wake Forest University and passed the quarantine requirements, they were bred with Cre-mice (ROSA-Cre or PB-Cre) to test the expression of fluorescent proteins (XFP) in prostate.

For the first breeding method, ROSA-CreER mice were ordered from JaxMice (stock 008463, B6.129-Gt(ROSA)26 Sor tm1(cre/ERT2)Tyj/J). For PB-XFP founders, six out of nine lines (511, 512, 513, 517, 518 and 520) were kept for testing. Line 514 was lost due to low transgene inheritance (the female founder gave birth to three litters and all the pups screened were wild type). Line 515 was lost due to health concerns (the female founder had severe malocclusion, was found dead a few weeks after arrival). Line 516 was lost due to fighting (the male founder was severely wounded by the breeding female mouse and the injury did not heal well necessitating euthanasia). The remaining lines were bred with ROSA-Cre animals using specific schemes (Fig.1A, 1D), and we got male pups with correct genotypes for five lines (Fig.2A, line 511 as an example).

A ROSA-CreER $^{lox/lox}$ X PB-XFP $^{lox/+}$

25%

♂ ROSA-CreER $^{lox/+}$; PB-XFP $^{lox/+}$

B ♀ CRK5-XFP $^{lox/+}$ X ♂ PB-Cre4

12.5%

♂ PB-Cre4; CRK5-XFP $^{lox/+}$

C ROSA-CreER $^{lox/lox}$ X CRK5-XFP $^{lox/+}$

25%

♂ ROSA-CreER $^{lox/+}$; CRK5-XFP $^{lox/+}$

D ROSA-Cre X PB-XFP

Founder	Total pups screened	ROSA-Cre; PB-XFP males
511M	27	5
512M	22	3
513M	10	4
514F	18	0
515F	0	0
516M	0	0
517M	16	5
518F	21	1
520F	17	0

E ROSA-Cre X CRK5-XFP

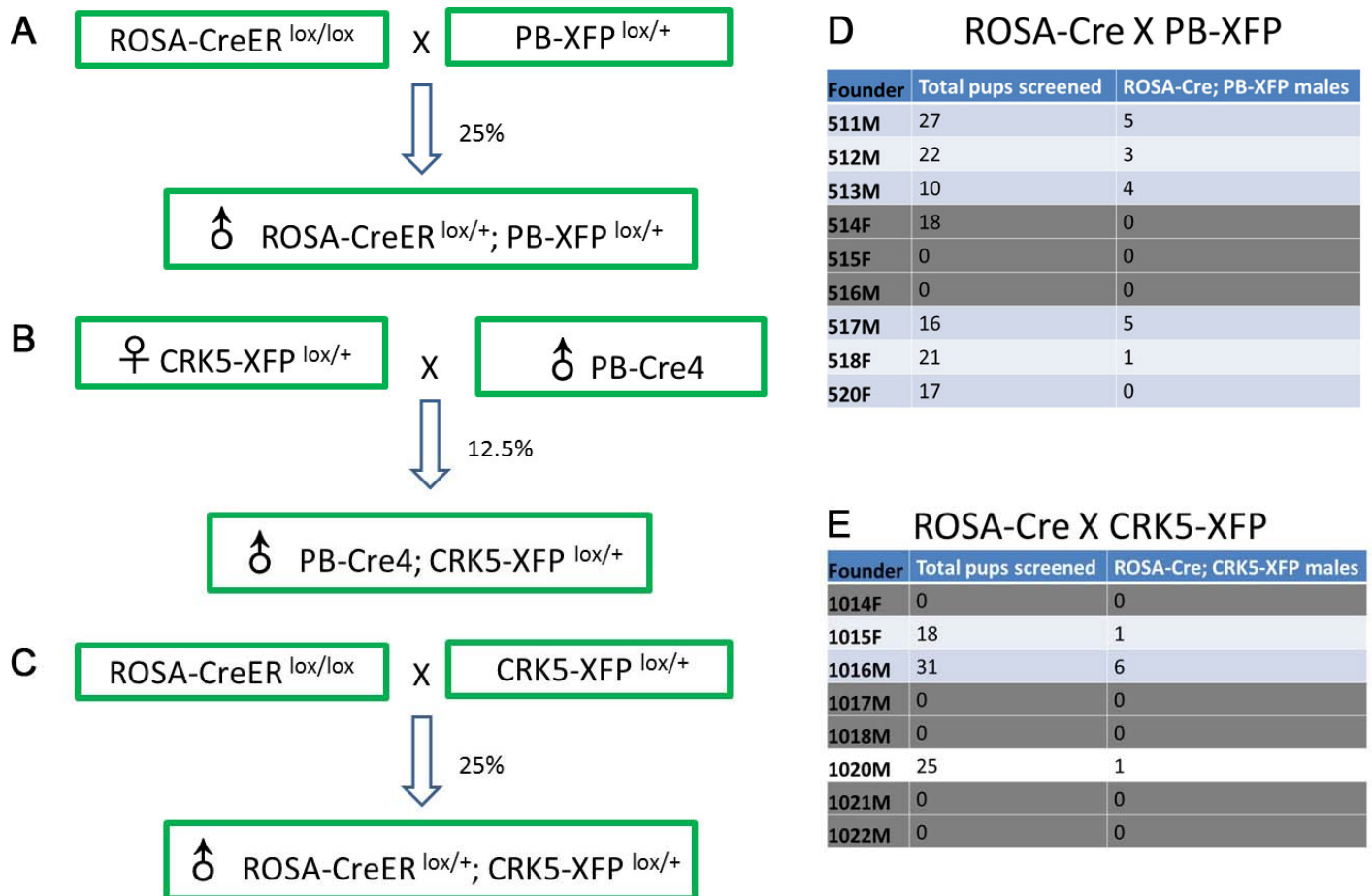| Founder	Total pups screened	ROSA-Cre; CRK5-XFP males
1014F	0	0
1015F	18	1
1016M	31	6
1017M	0	0
1018M	0	0
1020M	25	1
1021M	0	0
1022M	0	0

Figure 1 Breeding schemes for transgenic animals carrying PB-XFP and CRK5-XFP. A, breeding between ROSA-CreER and PB-XFP animals. B, breeding between PB-Cre4 males and CRK5-XFP females. C, breeding between ROSA-CreER and CRK5 animals. D, summary of screening results of ROSA-CreER; PB-XFP animals. E, summary of screening results of ROSA-CreER; CRK5-XFP animals.
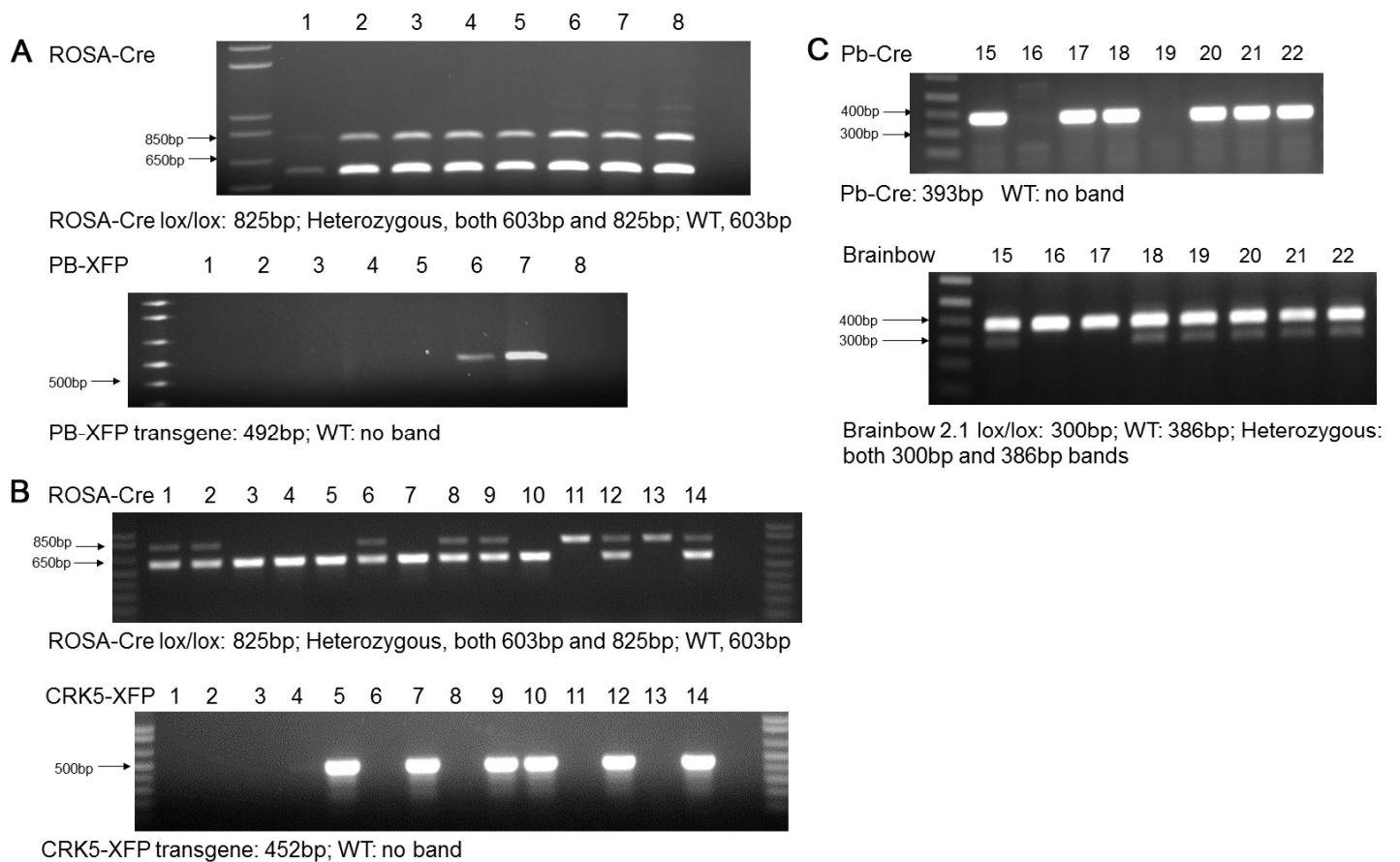
Figure 2 Screening for transgenic pups. A, Genotyping results for ROSA-Cre; PB-XFP pups. #6 and #7 are positive candidates for line 511. B, genotyping results for ROSA-Cre; CRK5-XFP pups. #9, 12 and 14 are positive candidates for line 1016 and 1020. C, genotyping results for PB-Cre4; Brainbow2.1 [lox/+] pups.

For the second breeding method, PB-Cre males were obtained through breeding in our laboratory. For CRK5-XFP founders, three out of eight lines (1015, 1016 and 1020) were kept for testing. Line 1014 was lost due to health concerns (the female founder had severe lesion on the back of neck and shoulder and had to be euthanized before any pup was born). Line 1017, 1018, 1021 and 1022 were lost due to infertility (no pup was born in all four breeding pairs, even though we keep female breeders young and fertile). Due to the difficulty with breeding with PB-Cre animals (Fig.1B), we switched to ROSA-Cre breeding (Fig.1C). This time the method worked out well for the three remaining transgenic lines (Fig. 1E), and we got male pups of correct genotypes (ROSA-Cre; CRK5-XFP) from two of them (Fig.2B, lines 1016 and 1020).

For the third breeding method, ROSA-Brainbow2.1 mice were ordered from JaxMice (stock 013731, Gt(ROSA)26Sortm1(CAG-Brainbow2.1)Cle/J). We crossed the mice with PB-Cre4 animals, and got PB-Cre4; CMV-XFP (Brainbow 2.1) pups (Fig.2C).

Task IV. Cross-breeding of Prorainbow transgenic mice for cancer research.

In the prior report, we showed that we got PKD1 knock-out animals and PKD1-PTEN double knock-out animals. After several rounds of long and complicated breeding, we got knock-out animals (both PKD1 knock-out and PKD1-PTEN double knock-out) with CMV-XFP (Brainbow 2.1) (Fig. 3).
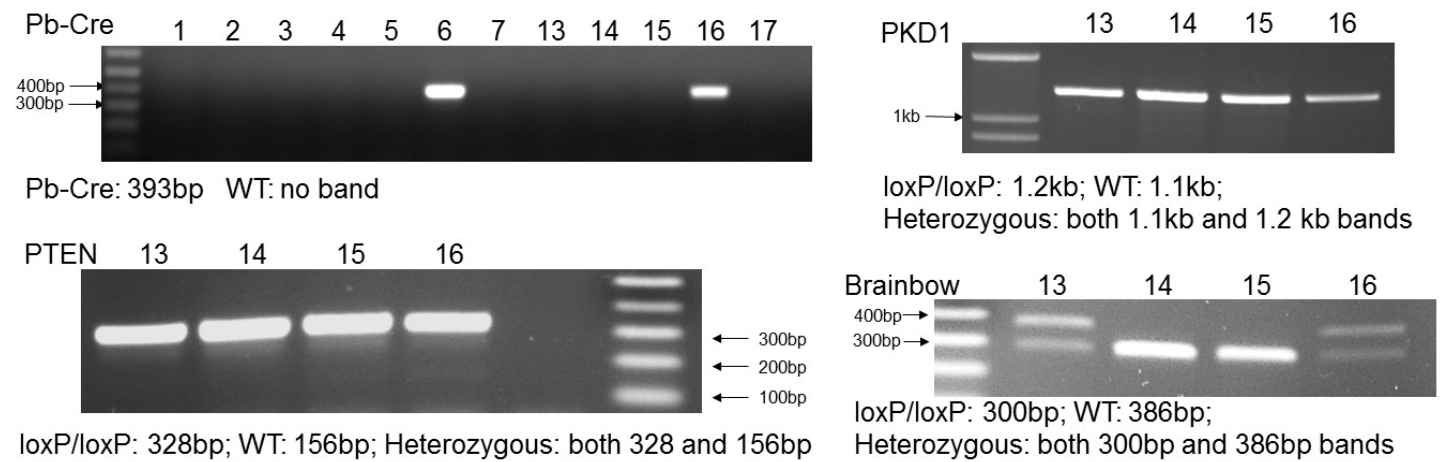
Pb-Cre 1 2 3 4 5 6 7 13 14 15 16 17
400bp→ 300bp→
Pb-Cre: 393bp   WT: no band

PKD1   13   14   15   16
1kb→
loxP/loxP: 1.2kb; WT: 1.1kb;
Heterozygous: both 1.1kb and 1.2 kb bands

PTEN   13   14   15   16
←300bp
←200bp
←100bp
loxP/loxP: 328bp; WT: 156bp; Heterozygous: both 328 and 156bp

Brainbow   13   14   15   16
400bp→ 300bp→
loxP/loxP: 300bp; WT: 386bp;
Heterozygous: both 300bp and 386bp bands

Figure 3 Screening for PKD1 PTEN double knock-out animal with CMV-XFP (Brainbow 2.1). Mouse#16 is a positive candidate.

Aim (3) Study the transgenic Prorainbow mice under normal and oncogenic conditions.

Task I. Evaluate combinatorial expression of XFP in prostate of Prorainbow mice.

For ROSA-Cre X PB-XFP breeding, among the six remaining founder lines, we got five lines with ROSA-Cre; PB-XFP males. We induced ROSA-Cre expression in three adult males with Tamoxifen injection (10mg/ml in corn oil, 100µl/adult/day, for five consecutive days). We checked prostate for all of them, and none of them showed epithelial expression of fluorescent proteins (Fig. 4). We did observe some fluorescent signals in stromal cells. To check whether those signals are background noise, we harvested prostate from non-induced control animal (litter mate with same genotype) and did similar check (Fig.5). We also checked signals in kidney of induced animal, as well as that of non-induced control (Fig.5). No fluorescent signal was detected in epithelial cells in either prostate or other organs (Fig. 6, 7). However, we did see weak signals from neighboring stromal cells in the prostate tissue and cells in kidney in induced animals (Fig6, 7). To rule out the possibility of injection problems, we plan to detect Cre protein expression in the prostate. We are still waiting for the last two lines to produce male pups with correct genotype.

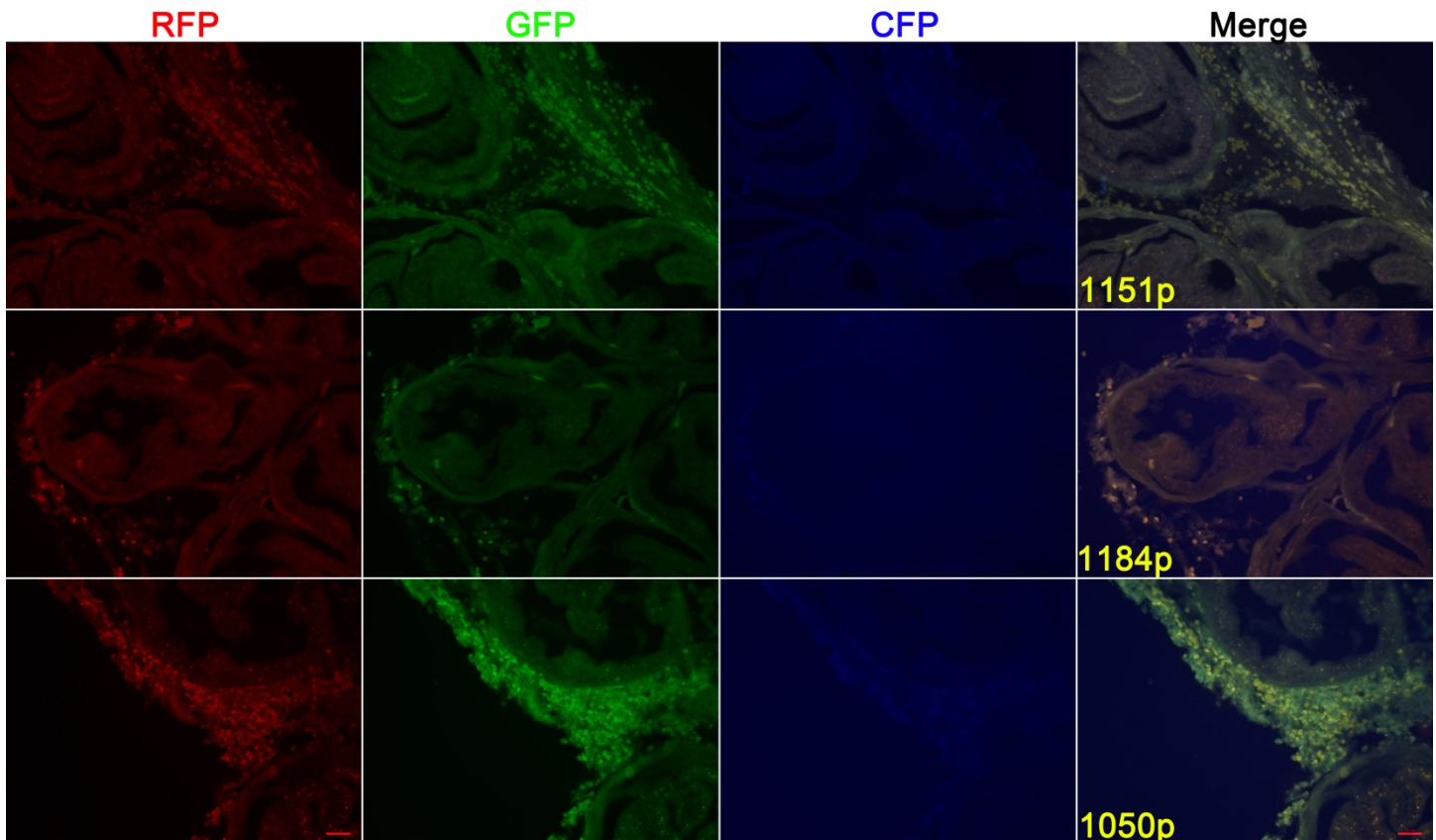RFP   GFP   CFP   Merge
1151p
1184p
1050p

Figure 4 No epithelial XFP expression was observed in prostate tissue of ROSA-CreER; PB-XFP males induced with Tamoxifen. Paraffinized prostate sample were observed for endogenous fluorescent protein expression. #1151, #1184 and #1050 are male animals from line 511, 512 and 513, respectively. p stands for prostate tissue. Scale bar is 20µm.
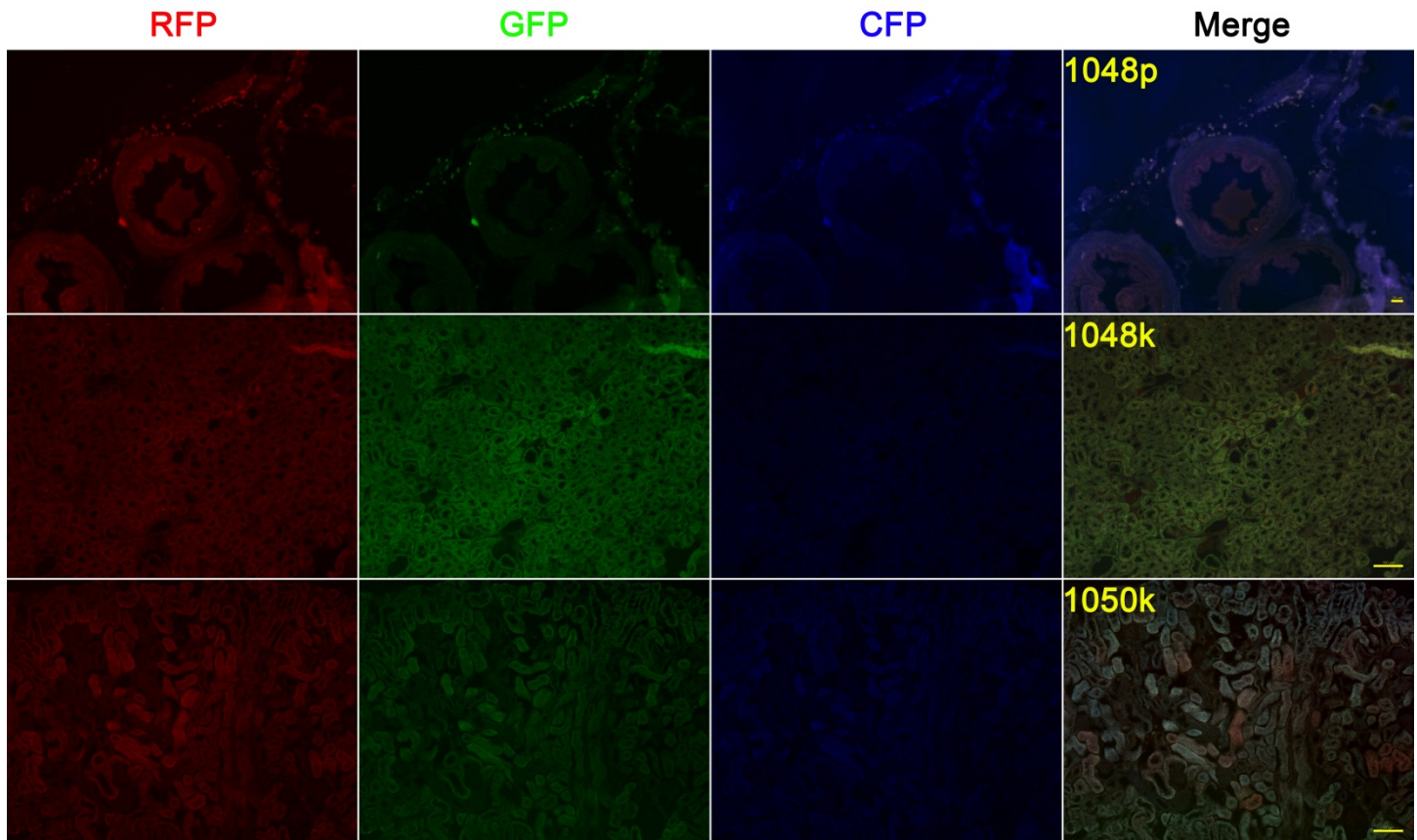

Figure 5 No epithelial XFP expression was observed in prostate or kidney tissue of ROSA-CreER; PB-XFP males that is not induced. Paraffinized prostate sample were observed for endogenous fluorescent protein expression. #1048 and #1050 are male animals from founder line 513. #1048 was non-induced, while #1050 was induced with Tamoxifen. p stands for prostate tissue. k stands for kidney tissue. Scale bar is 20µm.

For PB-Cre4 X CRK5-XFP breeding, there were fertility issues with PB-Cre; CRK5-XFP animals. Based on the difficulty with breeding, we switched to ROSA-Cre, and we got three lines with ROSA-Cre; CRK5-XFP (Fig. 1E, 2B). This breeding scheme worked out well. The induction is ongoing, and we will try the PB-Cre4 breeding as soon as we get young pups with CRK5-XFP transgenes.

For PB-Cre4 x CMV-XFP breeding, we got several mice with PB-Cre; Brainbow 2.1 [lox/+] mice.
We observed strong and distinct fluorescent signals in epithelial cells using both normal and confocal fluorescent microscopes (Fig.6, 7)
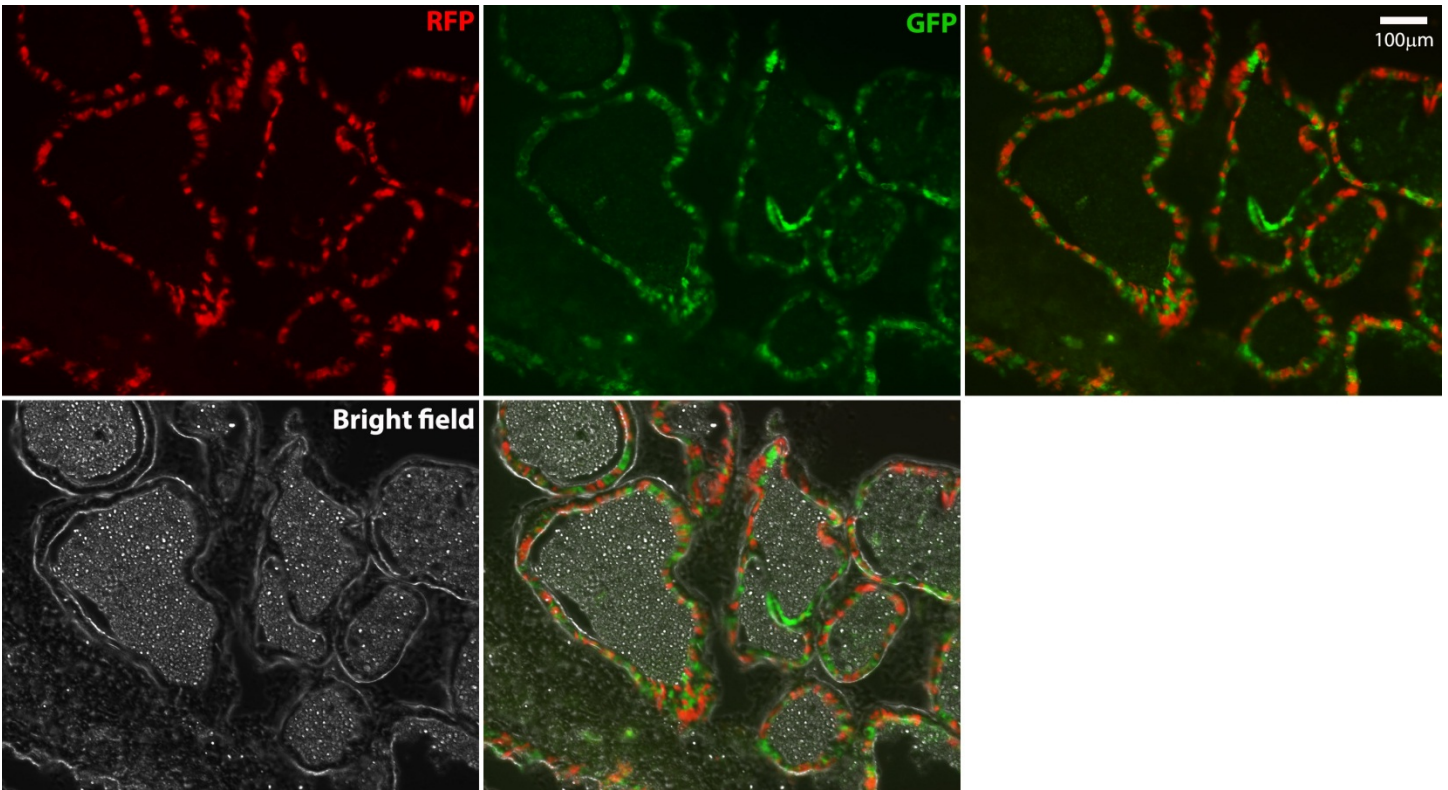
Figure 6 Fluorescent protein expression in frozen section of PB-Cre; Brainbow 2.1 lox/+ mouse prostate with normal fluorescent microscopy. Scale bar, 100μm.
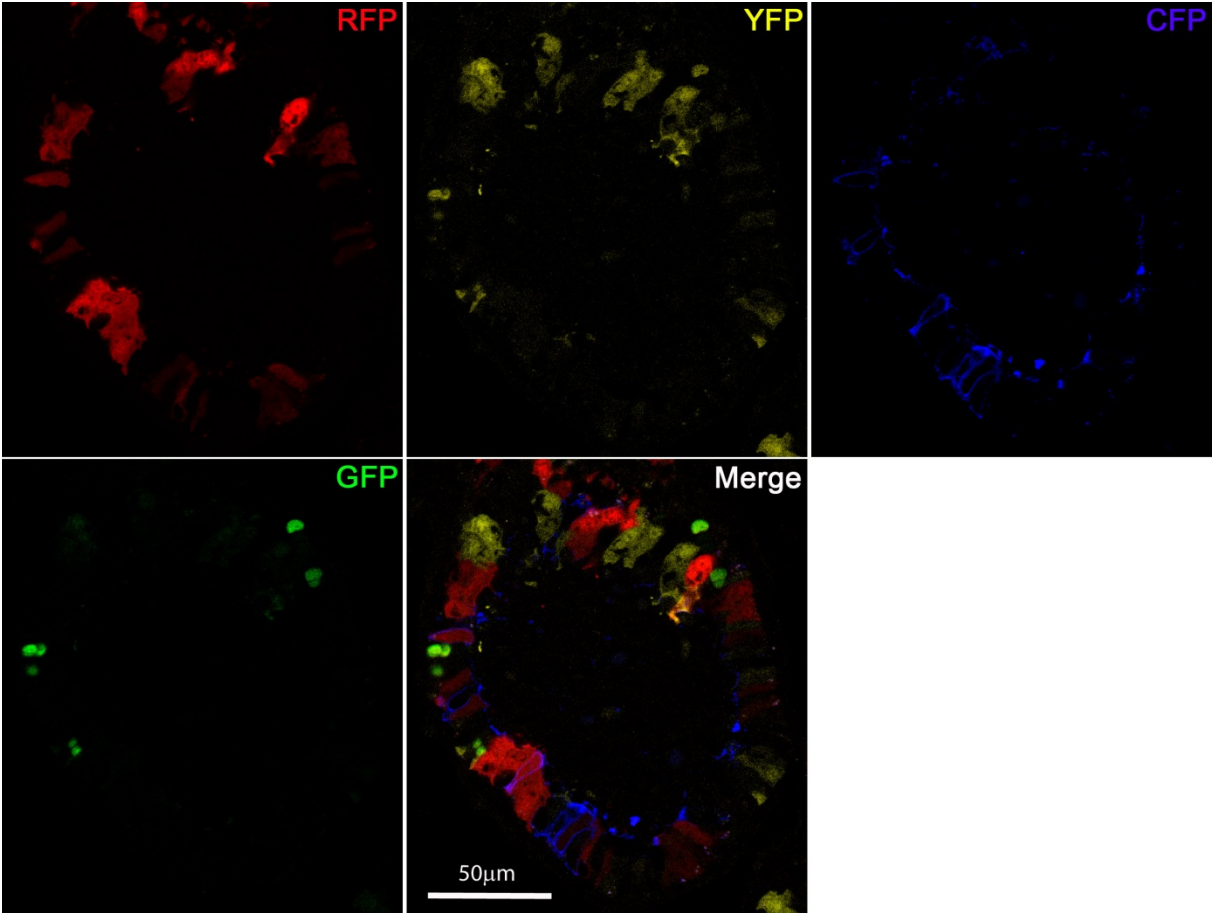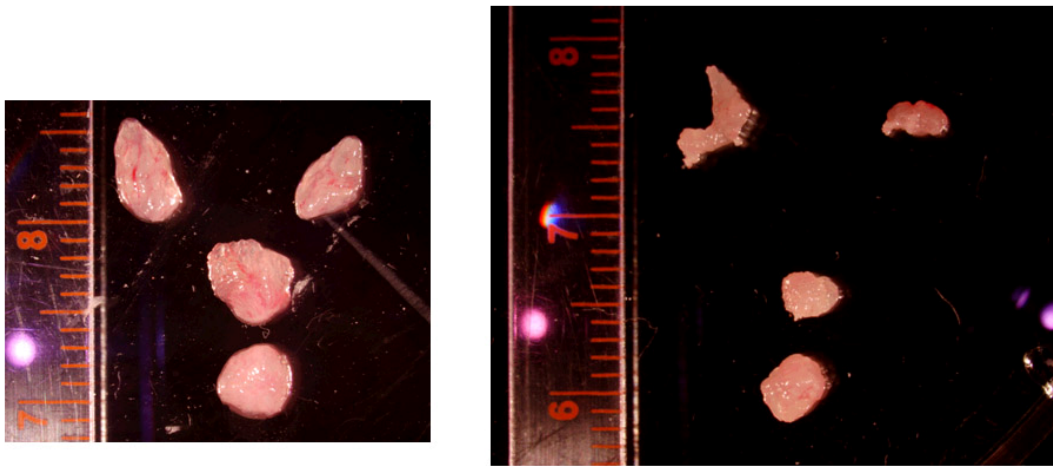

Figure 7 Fluorescent protein expression in frozen section of PB-Cre; Brainbow 2.1 lox/+ mouse prostate with confocal fluorescent microscopy. As expected, RFP and YFP localize in cytoplasm, CFP on cell membrane, and GFP in nucleus. Scale bar, 50μm.

We also harvested other organs (kidney and liver) and no XFP signals were detected in those organs. The results were confirmed with both IHC and endogenous protein check in frozen sections. We confirmed that the expression is strong and specific to prostate.

Task II. Study of Prorainbow and PTEN or PKD1 knock-out mice hybrids.

We harvested prostate, kidney and lung from PB-Cre4; PKD1 lox/lox; CMV-XFP/+ animals (PKD1 knock-out with Brainbow 2.1 expression) and from PB-Cre4; PKD1 lox/lox; PTEN lox/lox; CMV-XFP/+ animals (PKD1 PTEN double knock-out with Brainbow 2.1 expression). In PKD1 knock-out animals, we didn't observe obvious size difference between prostate tissues from knock-out animal and those from control littermate (Fig. 8). The prostate tissue was labeled with different fluorescent colors in PKD1 knock-out prostate (Fig. 9).

PKD1 KO (195 days)   Control littermate (195 days)

Figure 8 Prostate tissues harvested from PKD1 knock-out animal and control litter mate. No obvious size difference was observed. Top, anterior lobes; middle, dorsal-lateral lobes; bottom, ventral lobe.
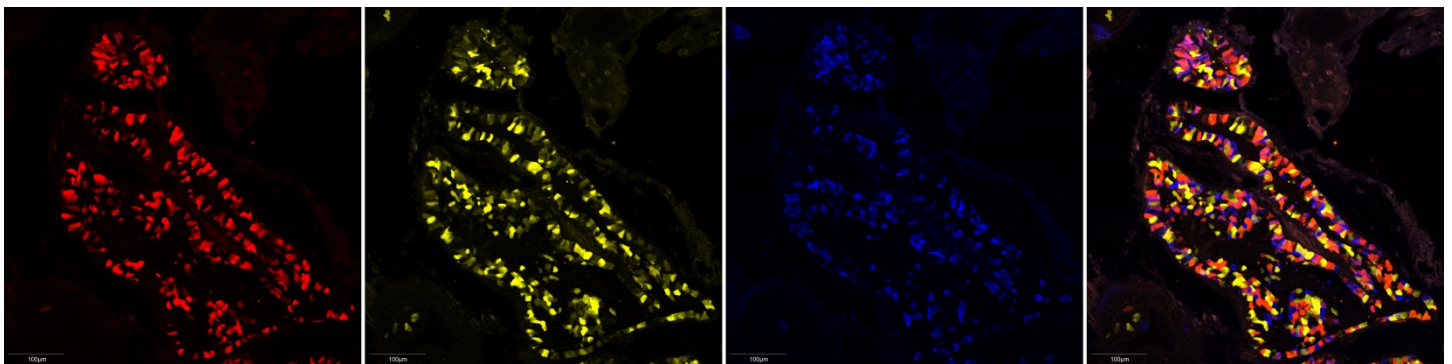

Figure 9 Fluorescent protein expression in frozen section of PB-Cre; PKD1 $^{lox/lox}$; Brainbow 2.1 $^{lox/+}$ mouse prostate with confocal fluorescent microscopy. Scale bar, 100µm.

In PKD1 PTEN double knock-out animals, however, we observed obvious prostate enlargement and cancer progression in prostate organ, while the other organs (kidney, liver and lung) didn't show any sign of tumor formation (Fig. 10). For the double knock-out animals, we did histological check with H&E staining to confirm the tumor formation (Fig. 11), and also checked other organs (kidney, liver and lung), but didn't find any metastatic lesion at 8 months (222 days old) (Fig. 12). XFP expression in prostate epithelial cells was strong and specific in double knock-out animal (Fig. 13).
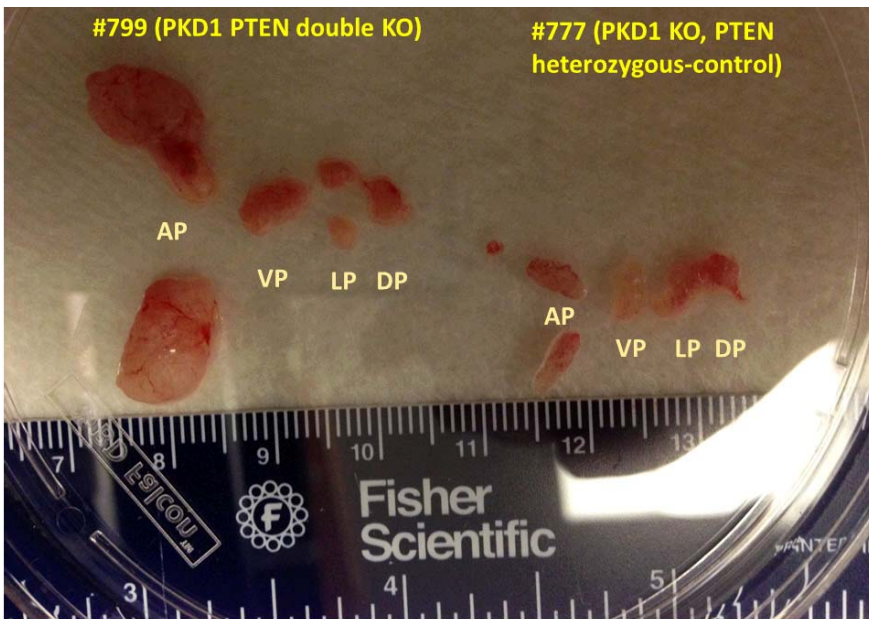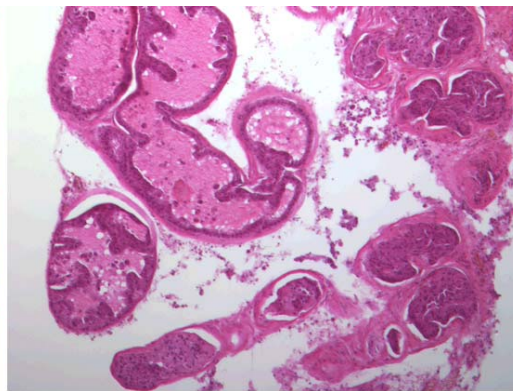
10

Figure 10 Prostate tissues harvested from PKD1 PTEN double knock-out animal and control litter mate. Prostate tissue from double knock-out animal was significantly increased. AP, anterior prostate; VP, ventral prostate; LP, lateral prostate; DP, dorsal prostate. Genotype of animal #799 is PB-Cre4; PKD1$^{lox/lox}$; PTEN$^{lox/lox}$; Brainbow 2.1$^{lox/+}$. Genotype of animal#777 is PB-Cre4; PKD1$^{lox/lox}$; PTEN$^{lox/+}$; Brainbow 2.1$^{lox/+}$. Animals were eight months old when prostate tissues were harvested.
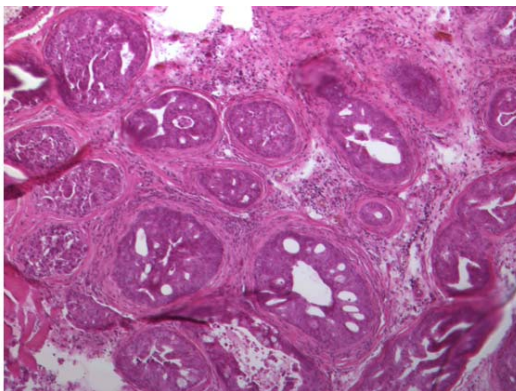
#799 (PKD1 PTEN double KO)   #777 (control)

Figure 11 Histological analyses of prostate tissues from PKD1 PTEN double knock-out animal and control litter mate. Tissues were paraffinized and stained with H&E. Tumor progression and invasion were observed in double knock-out animals. Genotype of animal #799 is PB-Cre4; PKD1$^{lox/lox}$; PTEN$^{lox/lox}$; Brainbow 2.1$^{lox/+}$. Genotype of animal#777 is PB-Cre4; PKD1$^{lox/lox}$; PTEN$^{lox/+}$; Brainbow 2.1$^{lox/+}$. Animals were eight months old when prostate tissues were harvested.
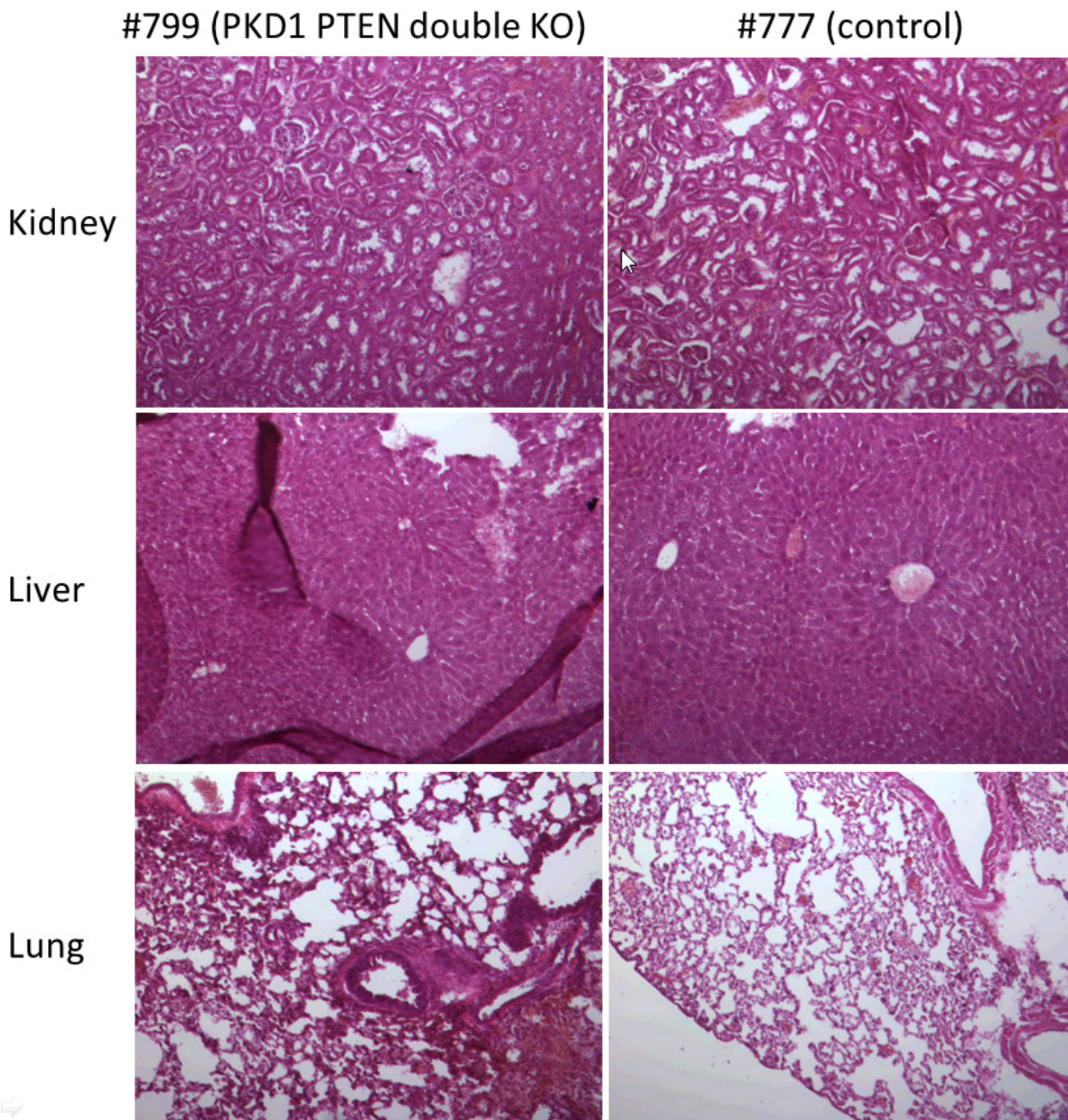
Figure 12 Histological analysis of lung tissues from PKD1 PTEN double knock-out animal and control litter mate. Tissues were paraffinized and stained with H&E. Tumor progression and invasion were observed in double knock-out animals. Genotype of animal #799 is PB-Cre4; PKD1$^{lox/lox}$; PTEN$^{lox/lox}$; Brainbow 2.1$^{lox/+}$. Genotype of animal#777 is PB-Cre4; PKD1$^{lox/lox}$; PTEN$^{lox/+}$; Brainbow 2.1$^{lox/+}$. Animals were eight months old when prostate tissues were harvested.
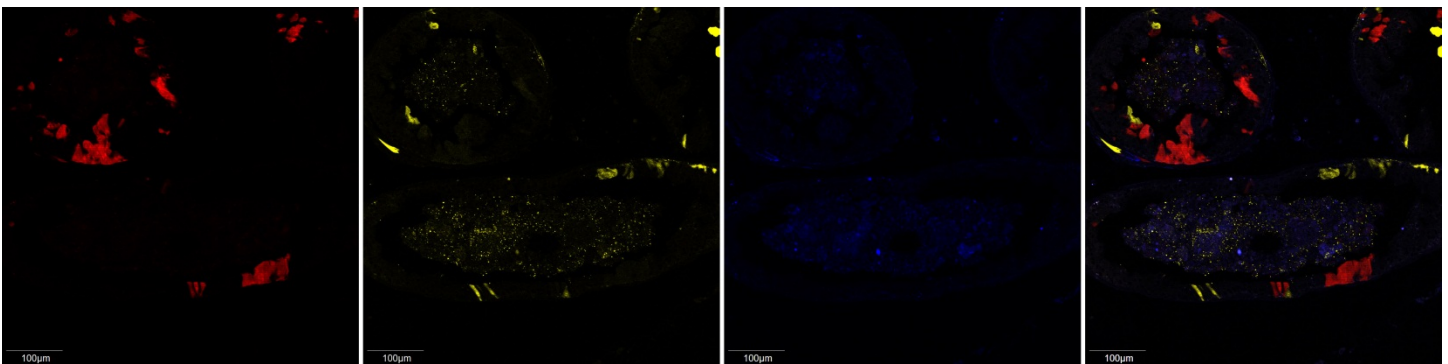

Figure 13 Fluorescent protein expression in frozen section of PB-Cre; PKD1$^{lox/lox}$; PTEN$^{lox/lox}$; Brainbow 2.1$^{lox/+}$ mouse prostate with confocal fluorescent microscopy. Scale bar, 100µm.

Interestingly, quite a few cells lost XFP in prostate tissue in double knock-out animals (Fig. 13). In order to check whether loss of XFP expression is due to cell proliferation we carried out immunostaining with Ki67 protein (a cellular marker for proliferation), and observed co-expression of Ki67 signal and endogenous XFP signals in tumor cells (Fig. 14). The results suggest that loss of XFP expression was neither due to cell proliferation nor is specific to proliferating cells. We continue to breed the mice and monitor for metastasis. If metastatic cells are found, we will establish whether these cells have the same color spectrum (monoclonal) or diverse color spectra (polyclonal). We can also track back the prostate progenitors of the metastatic cells by matching the color spectra.
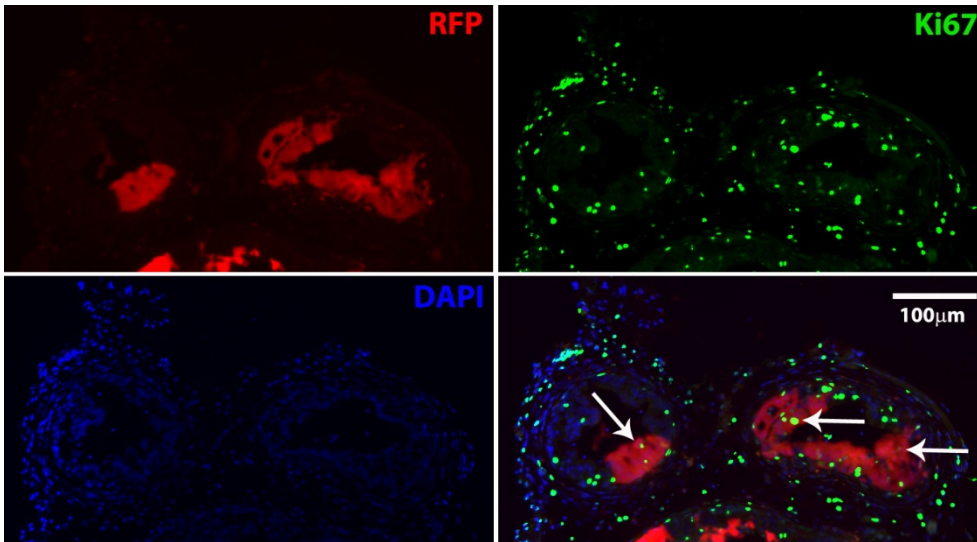

Figure 14 Fluorescent protein could label prostate tumor cells in double knock-out animal (genotype: PB-Cre; PKD1 [lox/lox]; PTEN [lox/lox]; Brainbow 2.1 [lox/+]). Frozen section was stained with anti-Ki67 antibody and DAPI. Arrows indicate proliferating epithelial cells (cancer cells in prostate) that are labeled with fluorescent proteins. Scale bar, 100µm.

We plan to finish this part in the following 6-12 months.

Key research accomplishments
We successfully produced prostate specific PKD1 knock-out mouse and PKD1 PTEN double knock-out mouse with CMV-XFP (Brainbow 2.1) fluorescent signal in prostate. We confirmed that XFP signals could express specifically in prostate under PB-Cre4 control, and could label both tumor cells and normal cells. We didn't observe any sign of tumor progression in PKD1 knock-out animals at eight months, but we did see an obvious enlargement of prostate tissue in PKD1 PTEN double knock-out animal. Tumor progression was confirmed by H&E staining. We tested the PB-XFP and CRK5-XFP transgene expression in majority of transgenic animals, yet no transgenic line was approved to be expressing fluorescent proteins.

Reportable outcomes
None.

Conclusion
We could use CMV-XFP to specifically label normal cells and tumor cells in mouse prostate, and use the same color pattern to track back its cellular origin.

Reference

1.   Siegel R, Naishadham D, Jemal A. Cancer statistics, 2013. CA: A Cancer Journal for Clinicians. 2013;63(1):11-30.

2.   Livet J, Weissman TA, Kang H, Draft RW, Lu J, Bennis RA, et al. Transgenic strategies for combinatorial expression of fluorescent proteins in the nervous system. Nature. 2007;450(7166):56-62.