INVESTIGATION OF RAS DEPENDENT SIGNALING PATHWAYS IN PROMOTING EXPERIMENTAL PROSTATE CANCER METASTASIS

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ABSTRACT

INVESTIGATION OF RAS DEPENDENT SIGNALING PATHWAYS IN PROMOTING EXPERIMENTAL PROSTATE CANCER METASTASIS

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Even with advances in the detection and treatment of localized prostate cancers the spread of the disease to distal sites is correlated with having a poor prognosis.[1] A subset of advanced human prostate cancers with metastatic capability have a combined loss of the tumor suppressors *PTEN* and *P53*.[2] However modeling the conditional loss of both genes in the prostates of mice only results in organ-confined disease.[3] Identifying genetic events and additional pathways that better model the phenotype and behavior of advanced cancers will create more useful preclinical models for studying the mechanisms that are important for metastasis and also for testing combinatorial therapies to help patients control their disease. The work described in this dissertation builds on and extends the current understanding of the pathways that are important for advanced prostate cancers to metastasize to the lungs, lymph nodes, bone and brain. Here we characterize the histologic phenotype and metastatic properties resulting from the overexpression of oncogenic $KRAS^{G12V}$ in the Clone 2 cell line, which was established from the *PB-Cre4(+)*$Pten^{(fl/fl)}TP53^{(fl/fl)}$ mouse model of prostate cancer. Adenocarcinoma that was CK8+, CK5- and P63- was the major phenotype that was observed for Clone 2-$Kras^{G12V}$ orthotopic tumors. Metastasis to the lung and lymph nodes occurred from Clone 2-$Kras^{G12V}$ orthotopic tumors. Brain metastasis occurred when Clone 2-$Kras^{G12V}$ cells were injected via an intracardiac route. Previous work with the *Pten;Trp53* knockout mouse model, primary tumor spheres derived from the model

and the Clone 2 cell line have shown that both *Pten* and *Trp53* regulate aspects of cellular differentiation in murine prostate epithelial cells, which may also directly or indirectly affect the frequency of metastasis.[3, 4] While retaining the ability to respond to TGFβ1 Clone 2-Kras^{G12V} CK8+ adenocarcinoma cells did not undergo an *in vivo* TGFβ1-mediated epithelial-to-mesenchymal transition (EMT) unlike Clone 2 CK8+, CK5+/-, P63- adenocarcinoma cells. When TGFβ-SMAD dependent signaling was impaired Clone 2-Kras^{G12V} cells became bi-phenotypic for both luminal and basal lineage markers (CK8+, CK5+, P63+) and formed lung and lymph node metastases following orthotopic implantation. The bi-potential tumor cells formed both bone and brain metastases following intracardiac injection. Additional experiments suppressing IKBα-p65-mediated NFκB showed a known relationship whereby Clone 2-Kras^{G12V} cells required NFκB for both invasion and metastatic colonization. Together these data show that 1) the expression of oncogenic Kras^{G12V} influenced the *in vivo* phenotype of Clone 2 cells, 2) resulted in metastasis and 3) identified the TGFβ and NFκB pathways as being important for aspects of the metastatic phenotype of Ras dependent tumor cells as well as the ability to remain fully committed as luminal epithelial cells.

This thesis is dedicated to my family, friends and clinical and research colleagues who have provided me with both support and guidance during my early career as a veterinary anatomic pathologist.

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KEY TO ABBREVIATIONS

PB, probasin

Cre, cre-recombinase

RAS, resistance to audiogenic seizures

KRAS, Kirsten rat sarcoma viral oncogene

HRAS, Harvey rat sarcoma virus oncogene

NRAS, Neural rat sarcoma virus oncogene

RRAS, related RAS viral (r-ras) oncogene

GEF, guanine nucleotide exchange factor

GAP, GTPase-activating protein

Ral, v-ral simian leukemia viral oncogene A

Braf, Braf transforming gene

Ezh, enhancer of zeste 2 polycomb repressive complex 2 subunit

Rb, retinoblastoma 1

PSA, prostate specific antigen

PIN, prostatic intraepithelial neoplasia

AMACR, Alpha-methylacyl-CoA racemase

Nkx3.1, NK3 homeobox 1

Myc, myelocytomatosis oncogene

TP53, tumor protein p53

Trp53, transformation related protein p53

PTEN/Pten, phosphatase and tensin homolog

PI3K, phosphatidylinositol 3-kinase

Akt, v-akt murine thymoma viral oncogene homolog 1

MAPK, mitogen activated kinase-like protein

Mek, MAP kinse-ERK kinase

Erk, extracellular regulated MAP kinase

P63, tumor protein p63

VIM, vimentin

CDH1, Ecadherin, cadherin 1

SLUG, *Snai2*, snail family zinc finger 2

CDH2, Ncadherin, cadherin 2

Tgfβ1, transforming growth factor beta 1

Tgfβ2, transforming growth factor beta 2

Tgfβ3, transforming growth factor beta 3

TGFβRI, transforming growth factor beta receptor I

ALK5, aurora-like kinase 5

TGFβRII, transforming growth factor beta receptor II

SMAD 2, SMAD family member 2

SMAD 3, SMAD family member 3

SMAD 4, SMAD family member 4

dnRII, dominant-negative TGFβRII

NFκB, nuclear factor of kappa light polypeptide gene enhancer in B-cells

TNFα, tumor necrosis factor alpha

TRAF6, TNF receptor-associated factor 6

Rel A/P65 v-rel avian reticuloendotheliosis viral oncogene homolog A

Rel B/P50, avian reticuloendotheliosis viral (v-rel) oncogene related B

Nfkb1, Nfkb1 nuclear factor of kappa light polypeptide gene enhancer in B cells 1, p105

Nfkb2, nuclear factor of kappa light polypeptide gene enhancer in B cells 2, p49/p100

IKBα, NFKB inhibitor alpha

Iκκα, inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase alpha

Iκκβ, inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta

IκκY/NEMO, IκB Kinase Complex subunit gamma

SR, IKBα super repressor

NICD, Notch 1 intra-cellular domain

CHAPTER ONE: Introduction to Human Prostate Cancers and Experimental Prostate Cancer Models

1.1 A general overview of human prostate cancers in the United States population

Prostate cancer is the most common cancer in the American male population, and it is the fifth leading cause of cancer related deaths in the United States. The number of deaths was 21.4 per 100,000 men per year based on 2008-2012 data from the National Institutes of Health Surveillance, Epidemiology, and End Results Program (SEER). In 2015, 220,800 of prostate cancer were diagnosed and 27,540 patient succumb to the disease.[1] Metastatic disease is usually the cause of death in patients with advanced cancers. The most common site for prostate cancers to colonize include the bone microenvironment (84%) followed by distant lymph nodes (10.6%), the liver(10.2%), and within the thorax (9.1%). Interestingly of patients that go on to have bone metastases, approximately 12.4% will also have detectable disease in the brain (12.4%).[5] However statistical data from SEER studies and other published reports show that prostate cancer is not often a deadly disease. The standard diagnostic test for prostate cancer, serum prostate-specific antigen (PSA) has led to an increase in the detection of early prostate cancers. However, because PSA is not a biomarker for disease progression there is currently a dilemma whereby patients may be either over treated or undertreated for their cancers.[6] Thus the development and characterization of new experimental models to study aggressive prostate cancers with lethal metastatic potential will be important for determining the genetic features and signaling pathways that are essential for an aggressive disease phenotype. Additionally the creation of new

experimental models is important for testing new combinatorial therapies that may benefit patients with advanced disease.

1.2 The histologic assessment of prostate cancers

While several histologic classification systems for prostate cancer exist, the Gleason scoring system is commonly used and is well researched regarding its prognostic significance for prostate tumors.[7, 8] The majority of prostate tumors have a histologic phenotype that is classified as prostatic acinar adenocarcinoma. This tumor type is positive for the luminal marker cytokeratin 8/18 (CK8/18) and negative for basal markers cytokeratin 5/6 (CK5/6) and P63.[9] Other histologic variants of prostate cancer that have been reported include: prostatic ductal adenenocarcinomas and adenocarcinomas with mucinous, signet ring, adenosquamous, basaloid, sarcomatoid, lymphoepithelioma-like, small cell (neuroendocrine), urothelial (transitional cell), undifferentiated, pseudohyperplastic, xanthomatous, or atrophic phenotypes.[10] Some of these phenotypes have been correlated with having a more adverse outcome.[10-12] The Gleason system[13] was designed to provide prognostic information only for prostatic acinar adenocarcinomas, but it also includes information for grading other prostate cancer subtypes with features of glandular differentiation.

The putative precursor lesion for prostate cancer, prostatic intra-epithelial neoplasia (PIN),[14] is divided into a low grade and high grade designation and is not graded according to the Gleason system. Unfortunately using histology alone to stratify prostate cancers into low risk and high risk groups has not always been a consistent predictor for biological behavior of a tumor, and sampling bias may be to blame for some of the inaccurate assessments of malignancy in a subset of tumors. [15] While it is well documented in the literature that prostate cancers with ≥ 8 Gleason scores[16] are at risk for aggressive disease it is unclear whether patients with intermediate scores,

such as Gleason grades 6 or 7, will have indolent tumors or aggressive diseases that require treatment or placement under active surveillance. [17]

In addition to an incomplete classification scheme for prostate cancers there is currently a limited translatability of published *in vitro* and *in vivo* genetic and functional studies, which could be used as the basis for a universal subtyping scheme for stratifying patients into risk groups and treatment protocols. Without the ability to stratify prostate cancers at a phenotypic or genetic level in a robust way, the lack of a clinically relevant classification system has led to costly and unnecessary treatments of less aggressive diseases and sometimes the under treatment of a more malignant tumor.[6] To this end, large scale and comprehensive genomic studies have been undertaken to define common and important genetic lesions that can be used to provide a molecular subtyping scheme for diagnosis, treatment and the prediction of patient outcome.

1.3 The genetic basis of prostate cancer tumorigenesis and progression

Prostate cancer is unusual from other epithelial cancers regarding the genetic changes that have been characterized as necessary for a tumor to development. Both *in vitro* and *in vivo* genetic studies point to chromosomal rearrangements events[18], copy number alterations[2, 19, 20] resulting in loss of tumor suppressors and activation of the transcription factor *c-myc*[21] as key drivers of tumorigenesis and early progression rather than instances of oncogene addiction.[22, 23] The key genetic events common to the development of the putative precursor lesion, prostatic intraepithelial neoplasia (PIN), and adenocarcinomas include an increase in the expression of Alpha-methylacyl-CoA racemase (AMACR) in PIN and carcinoma lesions and loss of function of tumor suppressors, such as *NKX3.1,[24, 25] PTEN,[26]* and *P53.*[27] This inherent genomic instability and uncontrolled proliferation at the onset of tumorigenesis is thought to give rise to additional oncogenic stresses that are needed to overcome cellular induced senescence, which lead to both driver and by-stander genetic effects. Such effects may include the acquisition of activating point mutations in oncogenes like Ras proteins and Rb family members, [2, 28] the accumulation of epigenetic events, such as the overexpression of the polycomb group protein EZH, and the acquisition of stem and progenitor cell characteristics which promote down-regulation of E-cadherin leading to epithelial to mesenchymal transitions.[29] A number of tissue based analyses and genetic *in vitro* and *in vivo* model systems are being created for the purpose of generating molecular signatures from these experimental systems to stratify indolent versus aggressive disease. [20, 30-32]

The advantage of using these genome based approaches to generate molecular signatures for both a generalized and personalized subtyping approaches for prostate cancers are that genetic events that are found can be translated back to *in vitro* and *in vivo* systems, such as xenograft, allograft and mouse preclinical models, to better understand the specific contributions of single and combined genetic events. An important consideration for these studies is that the kinetics and selective pressures leading to lesion development in the man versus the mouse and in *in vitro* models are different. While it is difficult to determine driver versus passenger events in tumors and the correct combinations of events that need to occur to promote particular tumor cell behaviors, such as invasion and metastasis, this combined bench-to-bedside approach and back can be effective for studying this group of cancers. [33-37]

1.4 Ras dysregulation and prostate cancer

Ras dysregulation is a common event that is required for tumor initiation in many solid epithelial cancers such as colon, lung, and pancreas. In these cancers activating mutations in Kirsten-Ras (KRAS) are more commonly encountered. [38] In addition to direct changes to Ras biochemistry, chromosomal rearrangements resulting in amplification of Ras genes, mutations in upstream receptor tyrosine kinase inhibitors, like EGFR, or altered functions to negative and positive regulators of Ras signaling can result in aberrant activation leading dysregulation of wild-type Ras signals.[39] Genitourinary malignancies, specifically bladder cancers, frequently have mutations in Harvey-Ras (HRAS)[40] Interestingly, unlike in bladder or renal cancers, which share a similar embryonic origin to the prostate, genetic events involving HRAS are not documented to occur in prostate cancers. Instead a subset of prostate cancers have activating point mutations involving two isoforms of Ras, specifically *KRAS,* and less often *Neural-RAS* (NRAS). These mutations are rare and documented to be at a 5% frequency of all of the cancers that are sampled (http://cancer.sanger.ac.uk/cosmic, COSMIC, Catalog of Somatic Mutations in Cancer). Interestingly often prostate cancers with Ras mutations occur within specific populations, such as patients of Asian ancestry.[41] This population has a greater risk for developing aggressive lethal disease compared with men of European descent.[42]

Downstream effector proteins in the Ras pathway, such as MAPK, are frequently upregulated in genetic and genomic studies, which most commonly include Caucasian men with aggressive primary tumors, biochemical recurrence and/or metastatic disease.[2] Together these findings highlight that signatures constituting perturbations

of the Ras signaling axis, including the identification of specific markers and pathways that are linked uniquely to having Ras dysfunction, could also be useful for stratifying many prostate cancers into low and high-risk disease groups for certain patient populations.

1.5 Experimental prostate cancer models of Ras dysregulation

RAS proteins are found to be constitutively activated through somatic mutations, fusions, and gene copy number variations in approximately 20-30% of human tumors.[43] Better defining and understanding the RAS signaling network and points where there is crosstalk with other pathways that are implicated in metastatic disease will be useful. Experimental studies have generated a number of effective preclinical models of Ras dysregulation to better define not only the functional biology of Ras regarding prostate cancer, but also to aid with the identification of Ras dependent druggable targets. Figure 1 shows the Ras signal transduction cascade and some of its better defined alterations in prostate cancer with regards to experimental model systems.

Several models of Ras dysregulation using both human cell lines and genetically engineered mouse models have been described for prostate. Many of the published models often rely on the constitutive or inducible introduction of a point mutation in a RAS isoform, often KRAS or HRAS, for understanding the role of Ras dysregulation regarding tumorigenesis and metastasis. Studies conducted using human prostate cancer cell lines and genetically engineered mouse models of prostate cancers have largely validated the genomic data, which indicates that Ras dysregulation and the upregulation of MAPK signaling are important features for advanced cancers having metastatic potential.[2]

The DU145 and PC3 human prostate cancer cell lines, two androgen receptor (AR) negative cell lines, have been most commonly used to study the tissue specific role of oncogenic Ras in prostate cancer. DU145 prostate cancer cells were derived

from a patient brain metastasis over 30 years ago, are heterozygous for PTEN and have metastatic potential by different injection methods.[44] The PC3 cell line was derived from a patient bone metastasis, has an epithelial phenotype and metastatic potential.[45] Early studies using the DU145 and PC3 model systems tested four different HRas effector mutants in these cell lines.[46] Each effector mutant had biologic properties leading to the upregulation of specific signaling pathways downstream of Ras including the MAPK/ERK, PI3K/AKT, and Ral-GEF pathways. Interestingly the *HRas*^{V12G37} mutant increased signaling through the RalA pathway leading to more bone metastases. To improve the metastatic potential cells were re-derived from a bone metastasis formed by DU145^{V12G37} cells in order to establish the DU145/RasB1 cell line as a unique preclinical model for studying bone and brain metastatic disease.[47] This model has been extensively used to study Ras dependent signaling pathways, such as TGFβ and NFκB.[48] [49] Together these experimental systems have established the importance for examining Ras dysregulation in androgen independent prostate cancers with lethal metastatic potential.

Kras mutations are infrequently reported for prostate cancers. However, several mouse studies have used activating mutations in order to model aspects of Ras dysregulation increased MAPK signaling that is observed in genomic analyses of patient tumors. Two conditional mouse models of Ras dysregulation using a Kras^{G12D} mutant have been characterized. In the first study *Pten* loss synergized with a Kras^{G12D} mutation resulting in an aggressive primary disease and metastatic potential to the lungs and lymph nodes.[50] Interestingly the expression of oncogenic Ras increased the stem and progenitor properties of the transformed cells leading to an *in vivo*

epithelial-to-mesenchymal transition (EMT) that correlated with *in vivo* metastatic potential. Further experimental support for the role for Ras in increasing the metastatic potential of prostate cancer cells comes from a second mouse model. This model combined the loss of the tumor suppressor *Nkx3.1* with activation of PI3-Kinase/AKT through *Pten* deletion and used a $Kras^{G12D}$ mutation to dysregulate Ras signaling. The three genetic events synergized to promote metastatic disease through increased transcription of *Etv4* and its downstream targets, including *Ezh*. [51] This study used a lineage tracing approach to show that tumor cells colonized distal sites before overt metastatic disease was detectable by *in vivo* imaging. This observation suggested that Ras dysregulation was important for the ability to seed novel microenvironments, such as the lungs. However, bone metastasis was not observed indicating that additional events were required to fully recapitulate lethal disease observed in the human disease. In contrast to the previous study, primary and metastatic cells remained committed to the luminal lineage and did not show phenotypes that would indicate tumor cells had acquired lineage plasticity. This difference in EMT capability by Ras dependent tumor cells shows that a number of different signals besides Ras dysregulation are important for the acquisition of stem and progenitor cell characteristics that may confer cellular plasticity.

Several unique experimental models have been described that do not rely on the introduction of an activating Ras mutation to model dysregulation in this signaling axis. One study used the DU145 and RWPE cell lines and showed that the Kras gene could be amplified through a chromosomal rearrangement event involving *UBE2L3*, an E3 ubiquitin ligase and *KRAS.[52]* Using an algorithm based approach the study identified

a number of gene rearrangements leading to Kras gene amplification. Another model used a $BRAF^{V600E}$ mutation combined with *Pten* loss to upregulate the MAPK pathway and drive lethal metastatic disease.[53] Finally, one experimental system modeled the combined loss of function of *Pten* and Sprouty genes *Spry1* and *Spry2*, which are negative regulators Ras. These events upregulated MAPK signaling resulting in an aggressive and metastatic disease.[54]

Together the findings from these experimental systems compliment the genomic evidence showing a role for Ras dysregulation in the acquisition of invasive and metastatic phenotypes.[2] The studies also provide a strong rational for understanding the Ras status of prostate tumors, which may represent a distinct molecular subgroup of prostate cancers with metastatic potential. However, Ras biology is complex and currently evolving as the field defines and characterizes sometimes very different biologic outcomes resulting from direct alterations to Ras isoforms and the positive and negative cellular regulators of the signaling axis. For cancers that commonly have Ras dysregulation the most important challenge to date is that all of the Ras isoforms are currently considered to be undruggable targets. This problem highlights the need for better models to study and identify effective and less toxic molecular targets that are druggable and can reduce or elimiate these types of cancers.

1.6 Ras signaling overview

The Ras protein isoforms (Harvey-ras, Kirsten-ras, Neural-ras, R-ras) are signal transducing guanosine triphosphatases (i.e small GTP binding proteins) that together play central roles in the regulation of cell proliferation and motility. Much of Ras signaling biology regarding its oncogenic properties has been characterized from early studies using the NIH 3T3 cell line.[55] Ras proteins are positively regulated by guanine nucleotide exchange factors (GEFs) and negatively regulated by GTPase-activating proteins (GAPS) that may be stimulated by upstream receptor tyrosine kinases or G-protein coupled receptors. The stabilization of Ras at the plasma membrane occurs by post-translational farnesylation by GEFs, although other compensatory post-translational modifications may occur when this activity is blocked. Farnesylation of the Ras protein leads to signaling activity, which is then regulated by the proportion of bound GDP to GTP. In the GTP bound state Ras is active and may bind to downstream target enzymes to affect dependent signaling pathways including Raf/MEK/ERK, PI3K/Akt and RalGEF/Ral and specific target genes including NFκB and c-Myc. GTP is hydrolyzed by the intrinsic GTPase activity of the Ras protein to GDP leading to a reduced or inactive signaling state. [56, 57]

While signaling is increased through Ras dependent downstream effector pathways like MEK and PI3K, epithelial cancers with Ras deregulation also require TGFβ and NFκB.[58] Several studies in prostate have shown that both pathways have defined roles in the progression of prostate cancers, especially regarding metastasis. In the clinical setting it is clear that targeting Ras dependent pathways like MEK and AKT alone are not enough to stop progression because of upstream feedback loops and

additional genetic events that also regulate these tertiary pathways. A better strategy has been the development of combinatorial therapies targeting multiple key points of cross talk of several pathways at primary, secondary, and tertiary points of intersection. Because Ras is not currently a druggable target and has variable biology based on the events leading to its dysregulation the creation of models for studying TGFβ and NFκB in the context of Ras dysregulation is important. The biology regarding TGFβ and NFκB is equally complex to Ras signaling as research has defined unique and tissue specific effects when either of these pathways are altered downstream of specific genetic events.

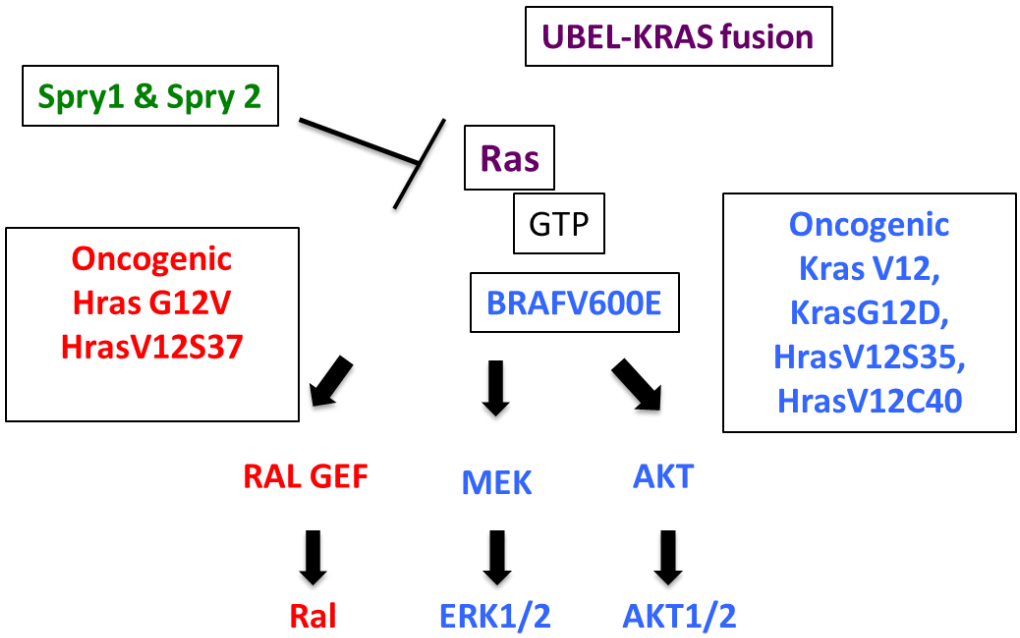

Figure 1. The Ras signaling pathway and alterations described in experimental prostate cancer model systems.

1.7 TGFβ signaling overview

TGFβ and members forming the SMAD dependent signal transduction cascade are regulated by cytokine binding of TGFβ1, TGFβ2, or TGFβ3 ligands. TGFβ dependent signals regulate aspects of cell fate determination, growth, and cellular movement. Therefore almost all cell types both secrete TGFβ ligands and express TGFβ receptors. The targeted deletion of either *tgfβR1, or tgfβR2* is embryonic lethal while deletion of the *tgfb2* gene is perinatal lethal.[59-61] Because TGFβ affects many areas of cellular homeostasis it is an important pathway to study and to target in cancer cells. Figure 2 shows the TGFβ SMAD dependent signal transduction cascade. TGFβ ligands activate the canonical signal transduction cascade by binding to serine/threonine receptor kinases known as type I (TβR-I or ALK5) and type II (TβR-II) receptors. TβRII receptors form heterotetramers resulting in receptor-mediated auto-phosphorylation of SMADs 2 and 3 on specific regions of the linker portion of each protein. Following phosphorylation the activated receptor-SMADs 2 and 3 form a complex with co-SMAD4 leading to nuclear translocation and TGFβ-mediated gene transcription. The receptor SMADs continuously shuttle between the cytoplasmic and nuclear compartments of the cell to affect transcription [62, 63]. However, DNA binding cannot take place without phosphorylation of the linker regions of each molecule and in conjunction with the SMAD4 molecule. TGFβ SMAD dependent regulation may be affected by a number of pathways, including MAPK, which can also phosphorylate linker regions to exert a point of negative regulation in SMAD dependent signaling. [62]

TGFβ SMAD signaling in prostate tumors is associated with the acquisition of a bone metastatic phenotype, partly due to the observation that TGFβ driven gene profiles

are upregulated in bone metastases[64] as well as a number of functional studies showing that TGFβ receptors or the SMAD signaling factors appear to mediate aspects of invasion and colonization in this microenvironment. Because both Ras and TGFβ share several points of crosstalk re-examination and development of new experimental models continue to be useful for determining the biologic effects resulting from the perturbation of both of these major signaling pathways.

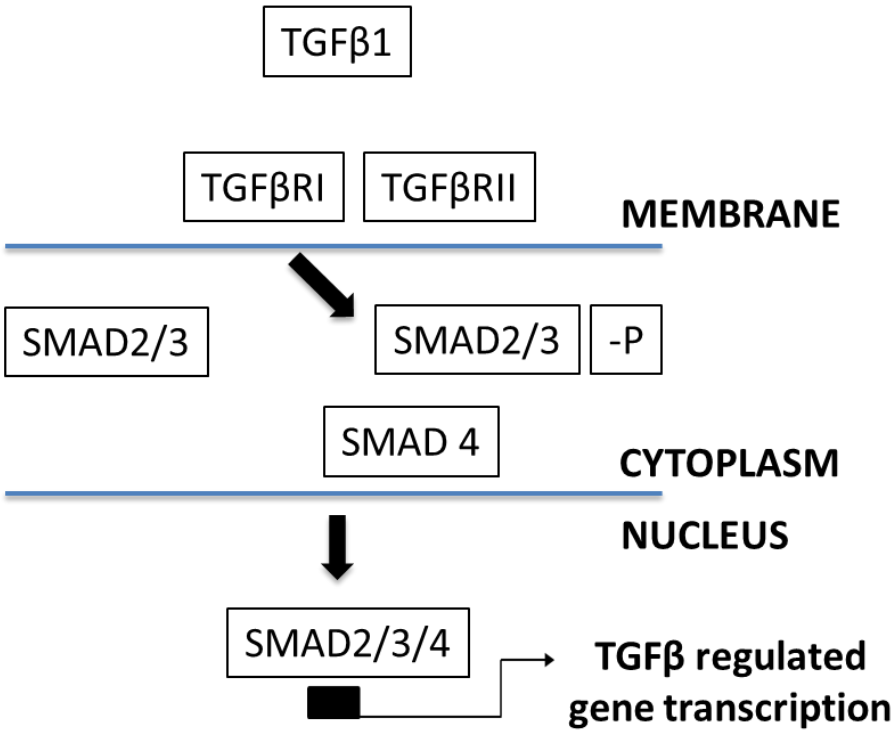

Figure 2. Schematic of the Canonical TGFβ SMAD dependent pathway.

1.8 NFκB signaling overview

Nuclear factor κB was first described in the context of the innate immune system as a nuclear protein that binds the kappa immunoglobulin-light chain enhancer.[65] Studies have shown that NFκB has diverse roles in inflammatory responses, apoptosis and cell proliferation, which implicate it as one of the central pathways that often becomes dysregulated across a broad spectrum of cancers. NFκB is constitutively active in hematopoietic malignancies [66] as well as a variety of epithelial cancers such as breast, liver, lung, and in a subset of prostate cancers with high Gleason grades.[67] The present section provides an overview of the canonical NFκB pathway and then focuses on prostate cancer models used to study this pathway.

NFκB regulation is complex and involves combinations of protein/protein interactions, phosphorylation events, and feedback loops in order to coordinate cellular functions. The signaling factor p65 (RelA), which is part of the canonical arm, is at the tertiary layer of this signaling axis where it is activated by the I-kappa B kinase (IKK) complex. Canonical NFκB activation requires the IKK complex to regulate the NFκB inhibitory protein IκB to which p65 is bound in order to allow the nuclear translocation of p65 [68]. A number of events must take place for the nuclear translocation of p65.

-NFkB regulation by Iκκ and IKB proteins and ubiquitination

The inhibitory of kB inhibitory proteins (IKB) are one level of regulation within the NFκB pathway. IKB proteins mask the nuclear localization sequence located on each p65 subunit. Regulation of the IKBa/p65/p50 complex is the best-studied example of

NFκB signal transcution and is designated as the “canonical” arm of the pathway (Figure 3). In this arm of the pathway a typical stimulus, often a TNF-alpha ligand family member, like TNFα, binds the TRAF6 receptor to activate a number of MAP3Ks. Signals then converge on the IkappaB Kinase (Iκκ) multimeric protein complex containing two IKB-specific kinases Iκκα and Iκκβ and a scaffold protein IκκΥ/NEMO. Activation of the secondary layer of the pathway results in the phosphorylation of the IKB proteins on two serine resides (32 and 36 for canonical IKBα). Phosphorylated IKB is then targeted for ubiquitination and proteasome-mediated degradation. NFκB signaling factor p65 is then able to bind other p50 (RelB) or p65 factors and translocate to the nucleus to activate target genes.[69-72]

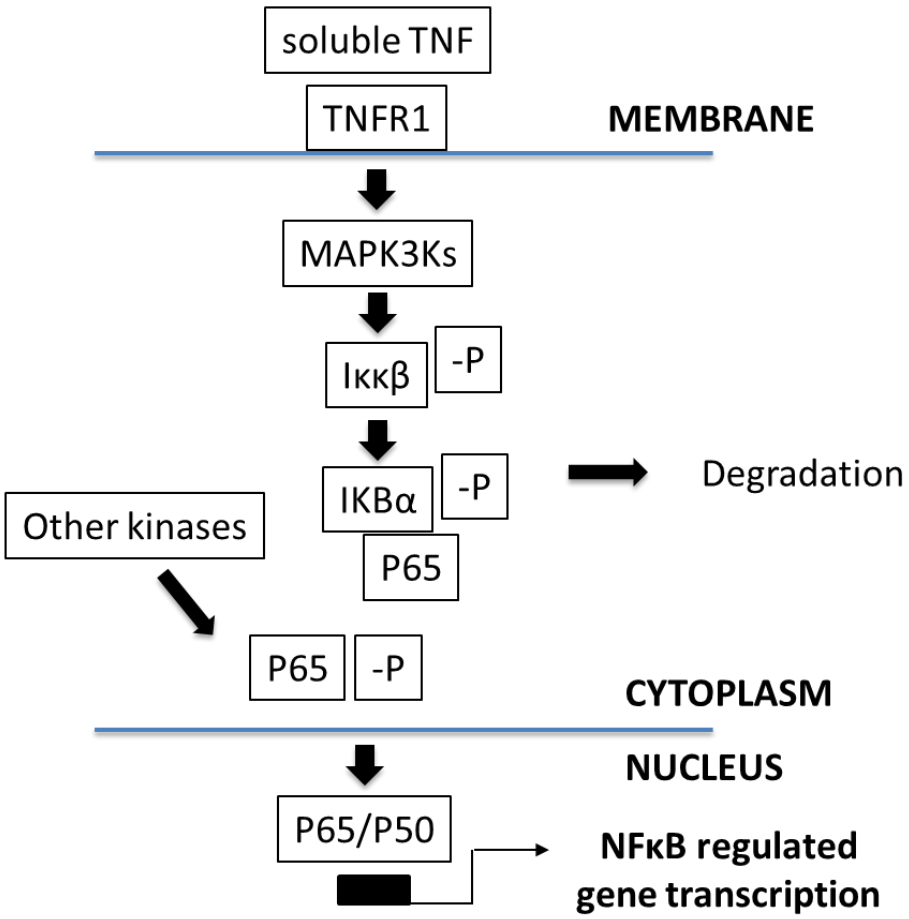

Figure 3. Schematic of the canonical NFκB pathway.

-NFκB regulation by post-translational modifications of p65

Another level of regulation exists in posttranslational events. Liberated NFκB p50-p65, p50-50, or p65-65 dimers are subject to post-translational regulation through phosphorylation and acetylation events. Kinases, such as PI3K and TANK binding kinase 1 (TBK1), can enhance NFκB translocation via phosphorylation of serine 536 on p65 in order to effect gene transcription [72]. The phosphorylation of p65 at serine 536 greatly enhances DNA binding and transcription while phosphorylation at serine 276 acts as negative regulator for this part of the pathway. The upregulation of phospho-$p65^{ser536}$ expression in prostate cancer cells has been correlated with the acquisition of invasion potential.[73]

-NFκB regulation by proteolytic cleavage

Another level of NFκB regulation rests on the proteolytic cleavage of NFκB1/p105 and NFκB2/p100 to p50 and p52, respectively. NFκB1/p100/p50 regulates canonical NFκB signals while NFκB2/p100/p50 regulates a non-canonical signal typified by NIK-mediated p52 translocation. For the canonical signal NFκB1/p100 cleavage to p50 can promote p50 dimerization with itself or liberated p65, leading to nuclear translocation and active signal transduction. Often proteolytic cleavage occurs as a positive feedback mechanism to the IKB-p65 mediated pathway regulation downstream of specific stimuli indicating that both the canonical and non-canonical arms of the pathway regulate each another.

-Experimental methods used to suppress NFκB

Because NFκB activation augments many aspects of human disease methods have been developed to understand individual components and the functional contribution of each molecule to the different arms of the pathway.

-Genetic repression through an NFκB super-repressor

Repression of NFκB by IKB proteins provides a direct approach to target the main arm of the pathway. Mutations of IKBα serines 32 and 36 inhibit Iκκ-mediated phosphorylation and degradation of IKB inhibitory proteins that affect the p65 mediated arm of NFκB. The reduction in canonical signaling can also feed into the non-canonical signaling arms with continued suppression reducing overall signal transduction.

-Pharmacologic inhibition

NFκB pathway inhibitors such as curcumin or sanguinarine are derived from natural products and affect the p65-mediated arm of the pathway, which can then exert regulation on non-canonical p52-mediated pathway signals. Synthetic inhibitors (BAY-11-7082) are created for their ability to target specific pathway components such as the Iκκ-complex. Additionally other pharmacologic inhibitors target pathway Iκκ-mediated regulation or alter ligands associated with inflammation (i.e. NSAIDs and TNFα inhibitors). IKB-mediated regulation of NFκB requires an intact proteasome.

Proteosomal inhibitors, like Bortezomib, can suppress NFκB activation through the stabilization of phosphorylated IKB complexes and have been used in the clinical setting for the treatment of hematopoietic malignancies that have a requirement for NFκB [74]. Unfortunately because of the pleotrophic nature of NFκB signaling and the low specificity of the targeted drugs the therapeutic use of NFκB and proteosomal inhibitors remains very limited.

-NFκB and prostate cancer

The development of distal metastatic disease, especially to bone, is one of the most important clinical concerns associated with treatment of advanced prostate cancers. The identification of signaling pathways that regulate the growth of prostate cancer cells in the bone microenvironment is of great therapeutic interest. NFκB is upregulated in advanced prostate cancers and metastatic lesions.[49, 67, 75] Additionally NFκB is a key pathway that can affect both bone and brain metastasis in experimental models of advanced prostate cancers.[49] Experiments conducted with the DU145 cells that were transfected with KrasG37 showed that the bone and brain metastatic phenotype correlated with the upregulation of NFκB downstream of the TWEAK-FN14 receptor signaling axis. Likewise the overexpression of ETS transcription factors ESE1 and ELF3 can sustain p65-mediated NFκB signaling in LNCaP, 22RV1, and DU145 human cell and drive aggressive disease phenotypes [76]. Another third model found that DAB2IP functions as a signaling scaffold between NFκB and Ras to prevent metastasis as tested in $Hras^{V12}$ transformed PrEC cells and a clone of the PC-3 human cell line [77]. DAB2IP silencing by EZH resulted in constitutive Ras

expression leading to an upregulation of NFκB signaling and the ability for cells to undergo an EMT, which correlated with increased invasive and metastatic abilities. Proof of principle experiments with an IKBα-superrepressor impaired NFκB in prostate cancer cells, reversed the EMT phenotype and reduced metastasis.

Few mouse models of NFκB dysregulation have been created and described. One mouse model showed that either heterozygous or homozygous deletion of IKBα in combination with the Hi-MYC mouse model resulted in an aggressive primary disease phenotype [78]. Interestingly NFκB deregulation alone did not induce high grade disease. Also, metastasis was not observed in the combined NFκB and MYC model system suggesting that specific oncogenic drivers, such as Ras, may be important for NFκB-dependent metastasis in mouse models. An NFκB recurrence signature termed NARP21 was generated from the model and found to be useful for predicting metastasis free survival in human prostate cancer databases. Together these published studies highlight the important role NFκB in advanced prostate cancers and the need to develop additional models to study this pathway.

1.9 A review of epithelial-mesenchymal transition

Epithelial-mesenchymal transition (EMT) is a biological program that allows for epithelial cells to undergo a series of genetic and epigenetic changes that enable them to assume the lineage profiles and behavioral characteristics of mesenchymal cells.[79] Because the acquisition of mesenchymal cell characteristics often corresponds with an increase in invasiveness as well as resistance to cell death signals, this phenotype is considered to be an important trait for cancer cells to acquire in order to metastasize or become resistant to therapies. A number of mouse models and human and animal cell lines, including prostate cancer models, have shown that carcinoma cells can acquire a mesenchymal phenotype.[50, 80] EMT phenotypes that are observed in tumors are frequently characterized by having either dual staining profiles for both the intermediate filament proteins vimentin and pan-cytokeratin, which is consistent with a partial EMT phenotype. Alternatively tumor cells can have a complete loss of cytokeratin and E-cadherin expression resulting in tumor cells that become singly positive for vimentin, N-cadherin, or fibronectin. The complete loss of epithelial cell markers and the expression of mesenchymal cell markers is consistent with a complete EMT phenotype.[81] Tumor cells that are anatomically located at the invasive edges of primary tumors or tumor emboli in circulation and at distal tissue sites most often have either morphologic or immunohistochemical features associated with either a partial or complete EMT phenotype. Therefore it is thought that these populations of cells with acquired plasticity can more efficiently complete the entire invasion-metastasis cascade rather than other tumor cell populations composing the bulk of a neoplasm.[81] [82] However often metastases do not contain evidence of an EMT, which makes the role for EMT in cancer

metastasis still controversial.[83] Therefore it is hypothesized that cancer cells having this type of phenotypic plasticity are in a state of “partial” EMT that is reversed in the process called a mesenchymal-to-epithelial transition (MET). It is the MET that is hypothesized to facilitate more efficient metastatic colonization of new microenvironments. The EMT phenotypes that are described in experimental models are mediated by a number of signaling pathways that are also implicated in cancer progression including Wnt, Notch, and TGFβ.[84] Therefore characterizing the type of EMT that is occurring within experimental models is important for understanding the biological properties of these systems and also for constructing more accurate models of the human disease that model metastasis.

1.10 The *PB-Cre4+Pten$^{fl/fl}$Trp53$^{fl/fl}$* prostate cancer mouse model is useful for studying advanced human prostate cancers.

The combined loss of PTEN and loss of TP53 are common genetic aberrations occurring in approximately 21% of human prostate cancers.[2] Characterization of the *PB-Cre4+Pten$^{fl/fl}$Trp53$^{fl/fl}$* model of prostate cancer has shown that the tumors produced in the mouse as well as transplanted primary tumor cells have luminal progenitor cell properties that confer unique histologies and different metastatic potentials to mimic many features of aggressive human prostate cancers.[85] [3] Additionally subpopulations of *Pten;Trp53* deficient tumor cells have multi-lineage potential to undergo an EMT that is mediated by a TGFβ1-Slug dependent signaling cascade.[80, 86] The ability to undergo an EMT is often correlated with having an increase in metastatic potential. However, recent studies with pancreatic cancer models show that this is not always the case even though EMT cells have other features of progression, such as drug resistance.[87] The EMT that is observed to occur by subpopulations of *Pten;Trp53* deficient tumor cells is an irreversible process that results in a complete EMT tumor phenotype and tumor cells that have acquired little to no metastatic abilities.[3] These data show that *Pten;Trp53* deficient tumor cells need to acquire additional genetic or epigenetic events for metastasis to occur efficiently. Thus, the *Pten;Trp53* knockout mouse model is useful for asking which pathways must be altered for metastasis to occur. Because the model system also has multi-lineage plasticity, it is also useful for asking questions about pathways that regulate lineage relationships in prostate cancers and how these pathways and phenotypes may affect metastasis.

1.11 Thesis goals and outline

A role for Ras in prostate cancer metastasis is well established and is a reasonable starting point for the generation and characterization of an advanced prostate cancer model system that may be used for basic research purposes and in preclinical studies. The experiments that are described in this dissertation use the poorly metastatic Clone 2 murine adenocarcinoma cell line to create the Clone 2-Kras^{G12V} cell line, a model of advanced, lethal prostate cancer. The Clone 2-Kras^{G12V} model incorporates several features of advanced prostate cancers including: having Ras dysregulation that leads to the upregulation of MAPK signaling, *Pten* and *Trp53* combined loss, a luminal adenocarcinoma phenotype (CK8+, TP63-) and the ability to metastasize to the lungs, regional lymph nodes and brain. We show that the Clone 2-Kras^{G12V} model also has intact TGFβ and NFκB signal transduction cascades, which make it useful for asking questions about if and how these pathway are important for lineage commitment choices and metastatic potential. The studies that are presented here contain the *in vitro* examination of the following: tumor cell lineage commitment, the state of the TGFβ and NFκB signal transduction pathways in a basal state and following stimulation with ligands that are known to induce positive signal, as well as the tumorigenic and invasive potential of the genetically modified lines as tested by soft agar and chemotactic directed invasion assays, respectively. More importantly for each question that is asked there are pathology analyses from *in vivo* experiments that assess the ability of each genetically modified line that was created to form orthotopic tumors in immunocompromised mice, assess the differences between *in vivo* tumor cell lineage commitment following changes to the pathways of interest and, finally, the effect

on the incidence of metastasis when pathways are altered or when tumor cells are introduced into mice by different injection methods. The hypothesis underlying this dissertation is that oncogenic $Kras^{G12V}$ expression in the context of the combined loss of both *Pten* and *Trp53* tumor suppressors modifies 1) TGFβ-mediated SLUG-driven epithelial-mesenchymal signaling pathways to prevent an irreversible sarcomatoid transition and 2) increase metastatic potential through TGFβ and NFκB.

The three specific aims for Chapters 3 through 6 that are designed to test this central hypothesis include:

1) *To characterize the effect on in vivo lineage commitment, invasive potential and metastatic outcome by constitutively expressing oncogenic $Kras^{G12V}$ in Clone 2 Pten;Trp53 deficient prostate cancer cells.*
2) *To characterize the effect on in vivo lineage commitment and metastatic outcome by constitutively expressing an inducible dnRII into both the Clone 2-$Kras^{G12V}$ and Clone 2 Pten;Trp53 deficient prostate cancer cell lines.*
3) *To characterize the effect on in vivo lineage commitment and metastatic outcome by constitutively expressing an inducible IκBα super repressor (SR) into both the Clone 2-$Kras^{G12V}$ and Clone 2 Pten;Trp53 deficient prostate cancer cell lines.*

Establishing a model of advanced prostate cancer that has both phenotypic and biologic behaviors that are comparable to lethal, metastatic cancers provides a unique resource to investigate questions regarding cell fate decisions and pathways that are

important for metastasis. These models may also be useful in the preclinical setting to test promising combinatorial therapies.

In Chapter 2 the general methodologies and the reagents used for the experiments in Chapters 3 through 6 are discussed.

Chapter 3 evaluates the synergy between oncogenic $Kras^{G12V}$ and the combined loss of both *Pten* and *Trp53* for increasing the metastatic potential of Clone 2 cells. In these studies the *in vitro* effects on epithelial cell lineage commitment, MAPK signaling, colony formation and the ability for directed chemotactic invasion are initially examined. The *in vivo* studies show that $KRAS^{G12V}$ expression allows adenocarcinoma cells to maintain their commitment as luminal epithelial cells and acquire invasive potential in order to metastasize to distal sites following either orthotopic implantation or intracardiac injection. Together these experiments show that the Clone 2-$Kras^{G12V}$ line is a more faithful model of advanced human prostate cancers. The *in vivo* experiments in this chapter establish the basis from which to evaluate the effects on cell lineage commitment and metastasis when important signaling pathways like TGFβ and NFκB are perturbed.

Chapter 4 explores the functional role of TGFβ in the Clone 2-$Kras^{G12V}$ model system regarding *in vivo* lineage commitment, invasive ability and the frequency of metastatic colonization of distal sites as assessed by histology and/or bioluminescent imaging. In these studies having an intact TGFβ-SMAD signaling cascade is important for luminal lineage commitment and retention of full invasive potential that is observed in Clone 2-$Kras^{G12V}$ and as described in Chapter 3. Uniquely these studies show that while TGFβ is important for full invasive potential this pathway is dispensable for

metastasis, especially to the bone and brain, as tested in Clone 2 and Clone 2-Kras^{G12V} cells.

Chapter 5 examines whether NFκB is important for Clone 2-Kras^{G12V} cells to retain full invasive and metastatic capabilities. In many Ras dependent prostate cancer models and prostate clinical specimens signaling through NFκB has been shown to be important for invasion and metastasis. While the suppression of the canonical NFκB pathway in Clone 2-Kras^{G12V} cells did not affect the growth of tumor cells following orthotopic implantation, the suppression of NFκB selected for cell populations that expressed basal epithelial cell markers. The phenotypic change correlated with a reduction in both invasion and metastasis by Clone 2-Kras^{G12V} cells in a subset of mice. Therefore these studies indicate that the role for NFκB is in the Clone 2-Kras^{G12V} model is for luminal lineage commitment and the retention of full *in vivo* invasive and metastatic potential.

Because both TGFβ and NFκB are shown to be important for the *in vivo* luminal adenocarcinoma phenotype of Clone 2-Kras^{G12V} cells Chapter 6 provides data from additional *in vitro* experiments that confirm both TGFβ and NFκB select for tumor cells with a luminal epithelial cell phenotype. We briefly examined whether TGFβ could signal through a Notch-mediated mechanism to affect aspects of luminal lineage commitment. However experiments examining the relationship between TGFβ and Notch in the Clone 2-Kras^{G12V} model were inconclusive and require further study to draw meaningful conclusions.

In Chapter 7 the conclusions from Chapters 3 through 6 are summarized to show that the Clone 2-Kras^{G12V} and parental Clone 2 models are useful systems for studying

questions about lineage commitment and metastasis. The experimental data discussed in Chapters 3 through 6 indicate that Clone 2-Kras^{G12V} cells retain at least the bipotential lineage plasticity of the parent Clone 2 line but do not spontaneously undergo EMT *in vivo* and have a robust metastatic phenotype in the *in vivo* setting. Additionally TGFβ and NFκB are important for both lineage commitment and metastasis in the Clone 2 and Clone 2-Kras^{G12V} models. Therefore the data in Chapters 3-6 show that each model, as well as the genetically modified lines developed from each, can be used to test questions that are centered on lineage, metastasis and/or combinatorial therapies that are designed to target lethal, metastatic prostate tumors.

CHAPTER TWO: General Materials and Methodology for Dissertation Research

This chapter describes the methods that are used for the studies described in chapters three through six.

Cell culture reagents and constructs: Clone 2 cells were derived from a *Pten;Trp53*KO prostate cancer mouse model and previously characterized. [3] Murine clone 2 and clone 2-Kras^{G12V} cells were plated in 100-cm^2 culture dishes and grown to 90% confluence in the appropriate media, Prostate basal epithelial growth media (PrEBM) with supplements (Lonza). The following growth factor used to stimulate the TGFβ SMAD dependent pathway was human recombinant TGF-β1 (R&D Systems) at a concentration of 2ng/mL. The growth factor used to stimulate the NFκB pathway was mouse recombinant TNFα (R&D Systems) at a concentration of 30ng/mL. The mPol2p-Hs.KRASG12V, TRE3Gp-Mm.TGFβRIIDN-IRES-mCherry (dnRII), and TRE3Gp-Hs.IKBαSR-IRES-mCherry (SR) or the FerH-Hs.IKKβ^{S177E,S181E} lentiviral vectors were from the Protein Expression Laboratory, NCI-Frederick, Frederick, MD. The NCID vector was a kind gift from Dr. Li Xin of Baylor College of Medicine.

Lentivirus production and establishment of genetically modified lines: A semi-confluent 60-mm plate of HEK293T was transfected with 4μg of VSVg plasmid and a mixture of the packaging–encoding vectors PsPAX (6 μg) and the lentiviral vector of interest (8 μg). Virus-containing media was collected 48 hours post-transfection and filtered with a 0.45μM filter; the virus supernatant, polybrene, and PrEBM media + supplements and without antibiotics was placed on the cell lines of interest for 72 hours

before removal and expansion of the infected line. After vector infection, stable selection of infected cells is performed using antibiotic selection. Serial FACS analysis and cell sorting for vectors expressing fluorescent tags were performed. Cells with high expression of mCherry were used for all experiments. After stable selection the confirmation of vector expression and function was carried out by western blot analysis for markers of vector expression (mCherry) and pathway specific markers (tp65, tIκκβ and pSMAD2/3).

TGFβ1 and TNFα stimulations: To test pathways cells were treated with regular PrEBM media or a combination of 2 ng/mL human recombinant TGFβ1 + PrEGM and supplements (Lonza) or 30 ng/mL mouse recombinant TNFα + PrEGM and supplements. Cells were harvested after 0, 15, 30 or 60 minutes for either total RNA or protein. Negative controls consisted of untreated cells or cells expressing genetic repressors for pathways of interest. Experiments were performed in triplicate with cell lines from different passages with at least two technical replicates per time point.

Pharmacologic inhibitors: To test the role of specific pathways and kinases cells were treated with regular PrEBM media and supplements (Lonza) also containing a vehicle control (DMSO) or pathway inhibitors diluted in DMSO at varying concentrations dependent upon the IC50 and MTS assays. Inhibitors were diluted to a concentration of 10μM in DMSO. The following inhibitors were used: 1) BAY 11-7082 (Selleckchem), an NF-κB inhibitor, which inhibits TNFα-induced IκBα phosphorylation with IC50 of 10 μM, 2) GW788388 (Selleckchem), an ALK5 inhibitor that also inhibits TGF-β type II receptor

and activin type II receptor activities, but does not inhibit BMP type II receptor, and 3) the TGFβR1 inhibitor (SB431542) was from Sigma (St Louis, MO, USA)

Protein isolation: Total protein was collected from cell extracts by washing and scraping cells in 1X RIPA lysis buffer (Boston BioProducts) containing appropriate phosphatase and protease inhibitors (Roche). Supernatant was collected and total protein concentrations are determined by the bichoncinic acid (BCA) method. Nuclear and cytoplasmic fractionation of cell proteins were performed using the NE-PER Kit (Thermo Scientific, Rockford, IL) according to the manufacturer's protocol, and protein concentrations were determined by the BCA method. Western blot analysis was done with the BioRad Semi-Dry Rapid Western blotting system according to manufacturer's protocol. Detailed antibody information is provided in supplement Table 1.

Western blot analysis: Proteins were subjected to SDS-PAGE electrophoresis on 4-20%Tris-glycine acrylamide gels, followed by transfer onto PDVF membranes (BioRad). Primary and secondary antibodies were diluted based on previously optimized laboratory protocols. Bands were visualized with the HRP-conjugated secondary antibodies (1:2000) and ECL chemiluminescent substrate. Gels were evaluated qualitatively. Samples will be considered positive when there is an immunoreactive band of the appropriate size that appears to be the same as reported by the manufacturer's antibody data sheet and scientific literature. Either α-tubulin or LaminB1 were used as protein loading controls for the whole cell lysates and cytoplasmic fractions or nuclear fractions, respectively. Western blot antibody information and

conditions can be found in Table 2. Western blot analyses shown in the paper are representative of experiments performed in triplicate with at least two technical replicates per antibody tested.

FACS analysis and cell sorting: A volume of 7-aminoactinomycin D (Sigma Aldrich) (100 µg/ml) was added prior to analysis for mCherry expression. Fluorescence-activated cytometry (FAC) analysis and cell sorting using FACSDiva software for mCherry (TRE3Gp-Mm.TGFβRIIDN-IRES-mCherry and TRE3Gp-Hs.IKBαSR-IRES-mCherry). Controls consisted of non-mCherry expressing murine cells labeled with 7'-AAD.

Matrigel invasion assay: Boyden chamber assays were used to provide quantitative analysis of different migratory responses of the un-induced and induced vector expressing prostate cancer cells. Initial migration to regular culture media, culture media with 5% FBS, and culture media with the treatments were assessed. The newly studied cells were allowed to migrate for 24 hours at 37◦C. The membranes were fixed with Diff-Quix fixative. The migrated cells were microscopically counted. Experiments were performed in triplicate. Differences between experimental groups were evaluated using a unpaired t-test with Welch's correction. Assays were performed with biologic and technical triplicates.

Soft agar assay: 5000 cells were plated per well in a 0.36% suspension of agar on top of a bottom agar at a 0.6% concentration. Media was refreshed every 3-4 days.

Experiments were conducted over a 7 week period. At the end of the experiment cells were stained with Nitrotetrazolium blue chloride and allowed to sit at 37C overnight before being quantified using a BioCount Instrument. Triplicate wells were set up for each cell line or condition that was tested. Results represent 3 independent experiments.

Immunocytochemistry: For immunocytochemistry cultured cells were plated onto either 8-well glass or 8-well glass chamber slides at a density of 5,000 cells/well and allowed to adhere for 48 hours. Adherent cells in control and experimental conditions were fixed in 4% paraformaldehyde in PBS for 10 minutes, followed by permeabilization with 0.5% Triton X-100 in PBS for 5 minutes. Non-specific sites are blocked by incubation in 2% BSA/20% goat serum in PBS for 60 minutes. Cells are then incubated overnight at 4°C with the specified primary antibodies in 2% BSA/PBS. Cells were washed three times with PBS containing 0.1% Tween-20, incubated with Alexa Fluor 488 conjugated goat anti-mouse IgG and Alexa Fluor 586 conjugated goat anti-rabbit (1:200) in 2% BSA for 30 minutes at room temperature, and finally washed and mounted using the anti-fade reagent Fluoro-gel II with DAPI (Electron Microscopy Sciences). Fluorescent images are captured using an upright fluorescent Zeiss Axioplan microscope (Zeiss, Thornwood, NY). To estimate the number of cells expressing CK8, CK5 and P63 five micrographs are taken at ×200 for each combination of markers, the percentages of positive cells are counted, and data is reported as the mean average of the five fields using ImageJ software. Experiments were performed in duplicate with mean and standard error mean calculated.

***In vivo* metastasis assays:** 5-6 week old male athymic nude mice (Ncr $^{nu/nu}$) were obtained from NCI, Frederick Animal Care Facility. Care and experimental procedures were carried out in accordance with the procedures outlined in the *Guide for the Care and Use of Laboratory Animals* and the laboratory's animal study protocols as approved by an Institutional Animal Care and Use Committee. To assess lineage commitment and full metastatic capacity, $1X10^6$ tumor cells in 20µl PBS were placed in the anterior prostate lobe. To assess metastatic capability to brain and bone, $1X10^5$ tumor cells in 100µl PBS were inoculated into the left cardiac ventricle of the mice as previously described.[46] All cell lines tested were derived from the Clone 2 *Pten*$^{-/-}$*Trp53*$^{-/-}$ murine prostate cancer cells contained a GFP/luciferase fusion protein. To induce the expression of either SR or dnRII vector mice were fed a doxycycline chow diet (BioRad) *ad libitum* 24 hours before the initiation of a study continuing until its end. Control mice injected with un-induced, vector expressing cells were fed a normal chow diet supplied by the animal facility (Purina, St. Louis, Mo). To monitor the development of metastasis, mice were imaged weekly using the Xenogen IVIS Spectrum and analyzed using the Living Image ver 4.4 software program (PerkinElmer). Mice were euthanized following weight loss of greater than 10% body weight or after demonstrating signs of paralysis. Four long bones (two front limbs with scapulae and two hind limbs), spine and brain were collected and fixed in 4% paraformaldehyde for radiologic and histological analysis (Histoserv Inc., Germantown, MD). Animal experiments were performed three times using two separate biologic replicates.

Histology: Orthotopic tumors were harvested and fixed with 4% paraformaldehyde for 24 hours, rinsed well in PBS, and transferred to 70% ethanol before standard histological processing, sectioning, and staining (Histoserv, Germantown, MD). For the purpose of histopathological analysis for progression, for each mouse, two H&E sections separated by 100 μm were initially analyzed. For the purposes of imunophenotyping the lesions, sequential serial sections were used for immunohistochemical staining where possible. Two serial sections (separated by 100 μm) of the liver, kidneys, spleen, brain, and decalcified longitudinal sections of the lumbar spine were analyzed from each animal to assay for metastasis. Bright field images were taken using an upright Zeiss Axioplan microscope (Zeiss, Oberkochen, Germany), Nikon Ecipse Ni, or Leica/Aperio slide scanner and ImageScope software.

Immunohistochemistry: Unstained slides were deparaffinized, and antigen retrieval was performed in a citrate buffer (Dako targeted antigen retrieval solution; Carpinteria, CA) in a steamer at 100°C for 15 minutes, or autoclave at 121°C for 20 minutes, followed by a 15-minute incubation at room temperature. Blocking was performed with Cyto Q Background Buster reagent (Innovex Biosciences, Richmond, CA) for 60 minutes at room temperature for mouse and rabbit primary antibodies. Primary antibody incubation was performed overnight at 4°C, followed by secondary antibody incubation at room temperature for 30 minutes. Secondary goat anti-rabbit biotinylated IgG (E0432, DAKO), and goat anti-mouse biotinylated IgG (E0433, Dako), used at 1:200 dilution. The ABC peroxidase kit (Vector Laboratories, Burlingame, CA) was used, followed by DAB (Dako) for chromagen visualization. All slides were counterstained with

hematoxylin (Dako). Primary antibodies and the concentrations are noted in Table 2. Positive immunohistochemical controls consisted of murine wild-type prostate luminal and basal epithelium (CK8, CK5, p63, AR) or sarcomatoid carcinomas (SLUG), colonic epithelium (SMAD2/3/4). For negative controls, the primary antibodies were omitted and tissues where staining was expected to be absent were evaluated. Antibody information and conditions can be found in Table 1.

Immunofluorescence: Double immunofluorescence was performed on tissue sections using the same protocol as used for IHC with the following exceptions: The secondary antibodies were Alexa Fluor 488 conjugated goat anti-mouse IgG (A11001) and Alexa Fluor 586 conjugated goat anti-rabbit IgG (A11011), 1:200, (Invitrogen, Carlsbad, CA). Slides were mounted with Vectashield hard mount with DAPI (Vector Laboratories, Burlingame, CA). The same primary antibodies at the concentrations indicated are used as for IHC and are listed in Table 3 where applicable for IF.

Statistical methods: *In vivo* animal results and TMA analysis were expressed as plots showing the median and box boundaries extending between 25th to 75th percentiles, with whiskers down to the minimum and up to the maximum value. All *in vitro* data were expressed as mean ± SD. All data were analyzed using Prism software (GraphPad Software, Inc.) and differences between individual groups were determined by the appropriate statistical tests noted for each assay when discussed in the individual chapters.

Table 1. Antibodies used for western blot analysis.

All primary antibody incubations for western blotting were performed overnight at 4°C.

Antibody	Information	Incubation condition for primary antibody
RAS	Rabbit Polyclonal Antibody, Cell Signaling, #3965	1:1000
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	Rabbit monoclonal Antibody, Cell Signaling, 4370	1:1000
Total ERK1/2	Mouse monoclonal Antibody, Cell Signaling, 9107	1:1000
Total P65	Rabbit Polyclonal Antibody, Santa Cruz, #3702	1:1000
LAMINB1	Rabbit Polyclonal Antibody, Abcam	1:1000
α-TUBULIN	Monoclonal Antibody, Sigma	1:2500
IκBα	Rabbit Polyclonal Antibody, Santa Cruz	1:1000
IKKβ	Monoclonal Antibody, Millipore	1:1000
phospho-SMAD2/3 (D27F4)	Rabbit Monoclonal Antibody, Cell Signaling, #8828	1:500
E-cadherin	Rabbit monoclonal, Cell signaling, #3195	1:1000
N-Cadherin	Rabbit, Cell signaling, #4061	1:1000
Vimentin	Mouse Monoclonal (V9), Santa Cruz, sc-6260	1:1000

Table 2. Antibodies used for immunohistochemical and immunofluorescence analyses of tissue sections and immunocytochemistry on glass 8 well chamber slides.

Antibody	Antigen retrieval method	Blocking Condition	Incubation condition for primary antibody	Detection
phospho-44/42 ERK1/2 Cell Signaling Rabbit polyclonal	Steam cook citrate buffer pH 6.0	Cyto Q Background buster (Innovex BioSciences)	Room temperature 1 hour 1:200	ABC kit (Vector Laboratories)
Total p65 Santa Cruz SC372 Rabbit polyclonal	Steam cook citrate buffer pH 6.0	Cyto Q Background buster (Innovex BioSciences)	4°C Over night 1:500	ABC kit (Vector Laboratories)
Phospho-p65ser536 Abcam Rabbit polyclonal	Microwave citrate buffer pH 9	5% goat serum/2% BSA in 1XTBST	4°C Over night 1:100	ABC kit (Vector Laboratories)
Phospho-SMAD2/3 Cell Signaling Rabbit polyclona	Microwave citrate buffer pH 9	5% goat serum/2% BSA in 1XTBST	4°C Over night 1:100	ABC kit (Vector Laboratories)
Pan-p63 Millipore, NAB-4315	Steam cook citrate buffer pH 6.0	Cyto Q Background buster (Innovex BioSciences)	4°C Over night 1:750	ABC kit (Vector Laboratories)
Ly6G Rabbit	Steam cook citrate buffer pH 6.0	Cyto Q Background buster (Innovex BioSciences)	4°C Over night 1:500	ABC kit (Vector Laboratories)
F4/80 Mouse monoclonal	Steam cook citrate buffer pH 6.0	Cyto Q Background buster (Innovex BioSciences)	4°C Over night 1:500	ABC kit (Vector Laboratories)
Cytokeratin 8 Covance Mouse monoclonal	Steam cook citrate buffer pH 6.0	Cyto Q Background buster (Innovex BioSciences)	4°C Over night 1:750	Alexa 488, Green (Invitrogen)
Cytokeratin 5 Covance Rabbit polyclonal	Steam cook citrate buffer pH 6.0	Cyto Q Background buster (Innovex BioSciences)	4°C Over night 1:750	Alexa 586, red (Invitrogen)
Vimentin Santa Cruz Rabbit polyclonal	Steam cook citrate buffer pH 6.0	Cyto Q Background buster (Innovex BioSciences)	4°C Over night 1:200	Alexa 586, red (Invitrogen)
Ecadherin Cell Signaling Rabbit polyclonal	Steam cook citrate buffer pH 6.0	Cyto Q Background buster (Innovex BioSciences)	4°C Over night 1:200	Alexa 488, green (Invitrogen)

CHAPTER THREE: Generation and characterization of the Clone 2-Kras^{G12V} murine prostate adenocarcinoma cell line

3.1 Introduction

Human prostate adenocarcinoma has a luminal phenotype characterized by both cytokeratin 8 (CK8) and nuclear androgen receptor (AR) labeling and the loss of TP63 expression.[9] There are few experimental models of advanced prostate cancer that mimic the phenotype of human tumors and are also highly metastatic. Therefore the creation of a model that more closely mimics the histologic phenotype and biological behavior of metastatic prostate cancers will be a useful research tool to answer basic questions about aggressive cancers and for testing promising combinatorial therapies.

Integrative genomic studies have identified dysregulation of components within the Ras signaling axis leading to constitutive activation of the downstream MAPK pathway as key events for prostate cancers to acquire metastatic potential.[1] Experimental model systems have been created to determine the different mechanisms through which Ras dysregulation may increase the frequency of prostate cancer metastasis. To date these models have used *HRAS* mutants or the *Kras^{G12D}* mutant in the context of either heterozygous or homozygous *Pten* loss or combined *Nkx3.1* and *Pten* loss[51] to substantiate the human genetic studies.[46] [47] The present study expands on these published findings and examines Ras dysregulation in the context of *Pten* and *Trp53* combined loss, which are two genetic events that define a subset of aggressive, metastatic prostate cancers.[2] In this study we used the Clone 2 cell line as model for investigating two risk factors for having advanced prostate cancer with metastatic potential: 1) combined *Pten* and *Trp53* loss and 2) Ras dysregulation leading

to the upregulation of MAPK signaling. In these studies we assessed the suitability of the newly generated Clone 2-Kras^{G12V} cell line as a model for studying metastasis in the setting of aggressive prostate cancer. We found that Ras activation impaired sarcomatoid differentiation in Clone 2 and increased the frequency of experimental metastasis to the lungs, lymph nodes, and brain. Thus, the Clone 2-Kras^{G12V} model system will be useful for the identification and study of Ras dependent pathways that regulate cellular differentiation and the metastatic potential of prostate cancer cells.

3.2 Results

3.2.a Overexpression of Kras^{G12V} in Clone 2 cells increases MAPK signaling and increases *in vitro* invasion.

Human *KRASG12V* cDNA was stably infected into the poorly metastatic Clone 2 adenocarcinoma cell line using a constitutive lentiviral vector and with the goal of evaluating the effect of oncogenic KRAS^{G12V} on tumor phenotype and metastatic ability. Western blot analyses confirmed that there was overexpression of KRAS in the Clone 2-Kras^{G12V} cell line after infection. (Figure 4A) Clone 2-Kras^{G12V} cells had activation of the downstream MEK/ERK1/2 pathway as measured by an increase in both total and phospho-ERK1/2 expression levels. (Figure 4A) There was not a significant difference in the ability of Clone 2 and Clone 2-Kras^{G12V} cells to form colonies in soft agar, which suggested that there would not be a difference in the ability of tumor cells to grow at an orthotopic site. (Figure 4B) Interestingly Clone 2-Kras^{G12V} cells had a significant increase in chemotactic directed invasion through a Matrigel membrane. (Figure 4C)

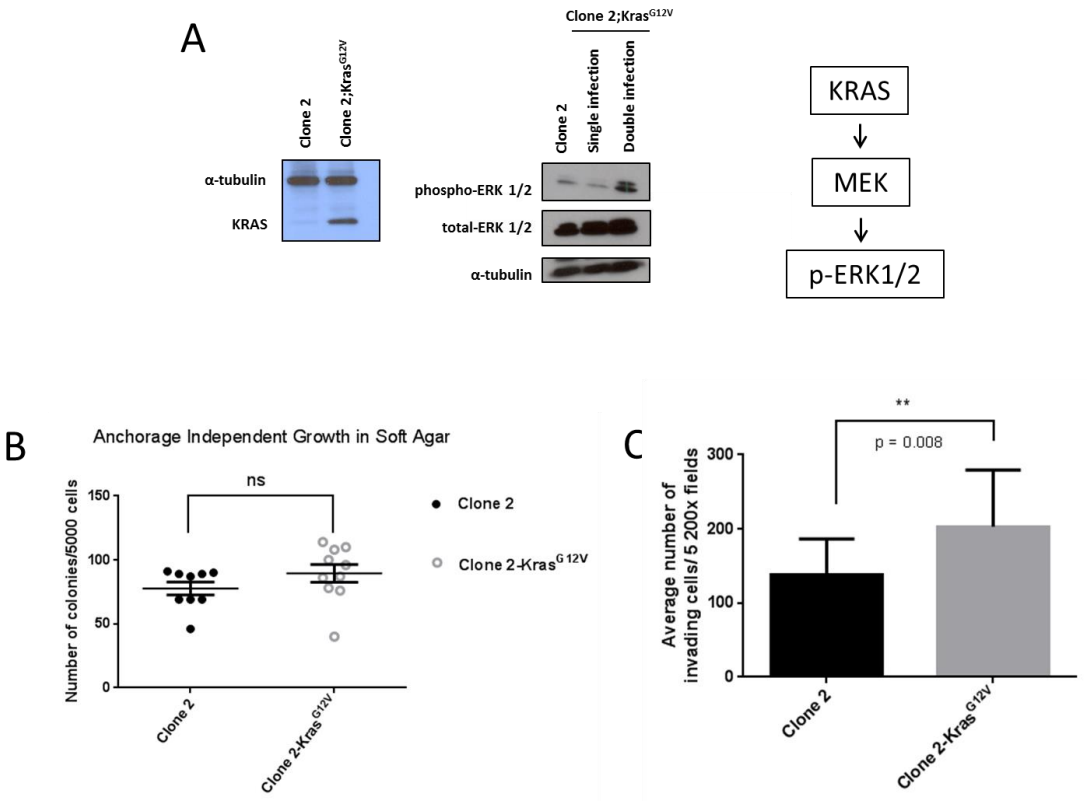

Figure 4. Ectopic Kras^{G12V} expression increases both MAPK signaling and *in vitro* invasive potential of Clone 2 cells.

A. Western blot analysis of whole cell lysates show that KRAS is overexpressed in Clone 2-Kras^{G12V} cells. Overexpression of KRAS correlates with an increase in both pERK1/2 and tEKR1/2 expression, which is downstream of having Ras activation. Alpha tubulin serves as a loading control for both sets of western blots.

B. Anchorage independent colony formation remains unchanged for Clone 2 cells following ectopic Kras^{G12V} expression. Results represent 9 replicates for Clone 2 and 10 replicates for Clone 2-Kras^{G12V} that were tested in 3 independent experiments. Significance was determined by a Mann-Whitney test.

C. *In vitro* chemotactic directed invasion through a Matrigel membrane is significantly increased following the constitutive expression of Kras^{G12V} in Clone 2

cells. The results represent 3 independent experiments with 18 replicates/condition tested. Significance was determined using an unpaired Student's T-test.

3.2.b Clone 2-Kras^{G12V} tumor cells metastasize from orthotopic sites and after intracardiac injection.

We employed both orthotopic prostate implantation and intracardiac injection techniques to initially address whether having constitutive expression of oncogenic KRAS^{G12V} would increase the frequency of metastasis of Clone 2 cells. The results for the orthotopic studies are listed in Table 3. The results from the intracardiac studies are listed as part of Table 11. Figure 5 shows representative histologies and the immunophenotype of orthotopic tumors that formed from Clone 2 or Clone 2-Kras^{G12V} cells placed into BALB/c$^{nu/nu}$ mice. Clone 2 cells formed sarcomatoid carcinomas with the bulk of the tumor being composed of pleomorphic spindle cells that were often interspersed with foci of osseous and cartilaginous differentiation, regions of glandular differentiation, and areas of stratified squamous epithelia without keratinization. The establishment of micro- and macro-metastases in the lung, lymph node, liver, brain, or bone was not observed in mice injected with Clone 2 cells, except for 1 mouse that formed lung metastases. This observation was consistent with previously published findings.[3] Thus, because Clone 2 tumors showed multi-lineage potential they were broadly determined to be sarcomatoid carcinomas that were locally invasive but poorly metastatic.

On gross examination of the mice that were injected with Clone 2-Kras^{G12V} cells all animal had evidence of peritoneal carcinomatosis, which frequently correlated with

having mild to marked abdominal ascites. Histologically Clone 2-Kras^{G12V} cells formed adenocarcinomas that were composed of haphazardly arranged glands lined by simple, one-layered epithelium as well as regions where the tumor cells formed solid nests that lacked glandular differentiation. Clone 2-Kras^{G12V} cells were locally invasive, and tumor emboli were frequently found in both the lymphatic and blood vasculature. Metastatic colonization of either or both the lungs and regional lymph nodes were observed in all of the mice in this cohort.

Prostate cancers that acquire the ability to metastasize to bone are lethal. An intracardiac injection technique was used to test whether Clone 2-Kras^{G12V} cells could metastasize to either the brain or the bone. Clone 2 cells did not form metastases (0/6). However in some mice there was evidence of Clone 2 tumor cell growth within the left ventricle of the heart, which indicated that the cells that were injected were viable. Second, this result indicated that Clone 2 cells could not readily colonize distal sites even when placed directly into circulation. Clone 2-Kras^{G12V} cells colonized the brain (6/9), but not the bone, suggesting that additional pathways beyond oncogenic Kras are required for a bone metastasis phenotype. The results are presented in Table 11 of Chapter 5.

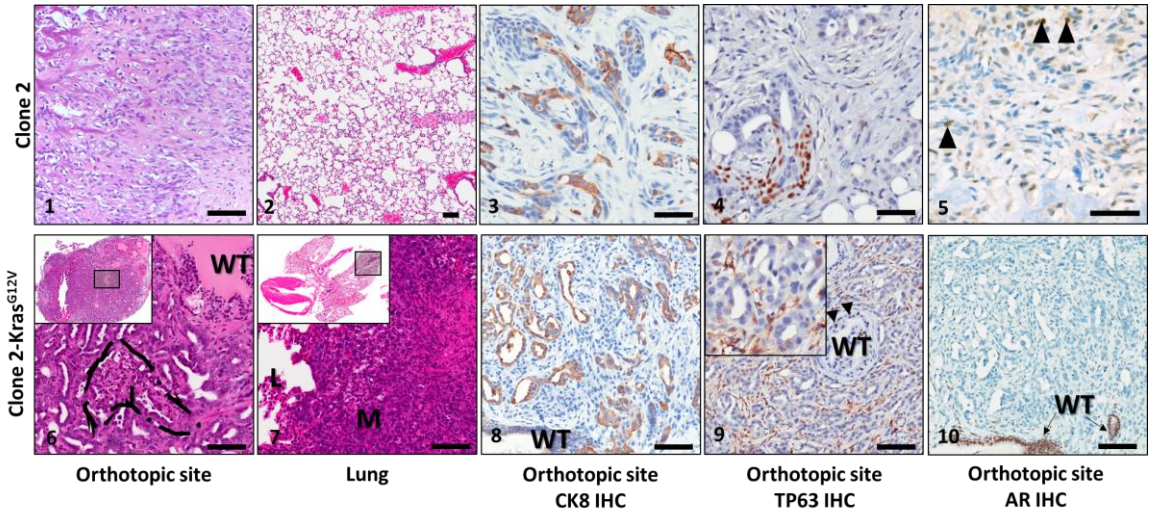

Figure 5. Pathology findings for Clone 2 and Clone 2-Kras^{G12V} expressing orthotopic tumors.

1. Representative histology of a Clone 2 sarcomatoid carcinoma, 200X, H&E.
2. Normal lung from a nude mouse injected with Clone 2 cells. Metastatic disease from an orthotopic site is not commonly observed for Clone 2 cells. Chapter 3. 2X, H&E.
3. Clone 2 cells with glandular differentiation express CK8, 200X, anti-CK8 antibody with hematoxylin counterstain.
4. Subpopulations of Clone 2 cells have morphologic features of complex epithelia and express nuclear P63, 200X, anti-P63 antibody with hematoxylin counterstain.
5. Subpopulations of Clone 2 cells express nuclear AR (black arrowheads), but expression in other cell morphologies and throughout the bulk of orthotopic tumors is largely absent, 200X, anti-AR antibody with hematoxylin counterstain.
6. Representative histology from a Clone 2-Kras^{G12V} orthotopic tumor. The area circled shows tumor associated inflammation and necrosis. 200X, H&E; Insert 1X, H&E.

7. Representative histology from a metastatic lesion in a nude mouse injected with Clone 2-Kras^{G12V} cells at an orthotopic site. 200X, H&E; Insert 1X, H&E.
8. Clone 2-Kras^{G12V} cells form glandular structures and solid nests that are positive for CK8, 100X, anti-CK8 antibody with hematoxylin counterstain.
9. Glandular structures formed by Clone 2-Kras^{G12V} cells are uniformly P63-, 100X anti-P63 antibody with hematoxylin counterstain; Insert 200X.
10. Clone 2-Kras^{G12V} cells do not have detectable AR expression in either the nucleus or the cytoplasm by IHC. A wild-type prostate gland from nude mouse prostate is an internal positive control for AR labeling. 100X anti-AR antibody with hematoxylin counterstain; Insert 200X. Scale bar represents 50µM. I = inflammation, WT = wild-type, L = lung, M = metastasis.

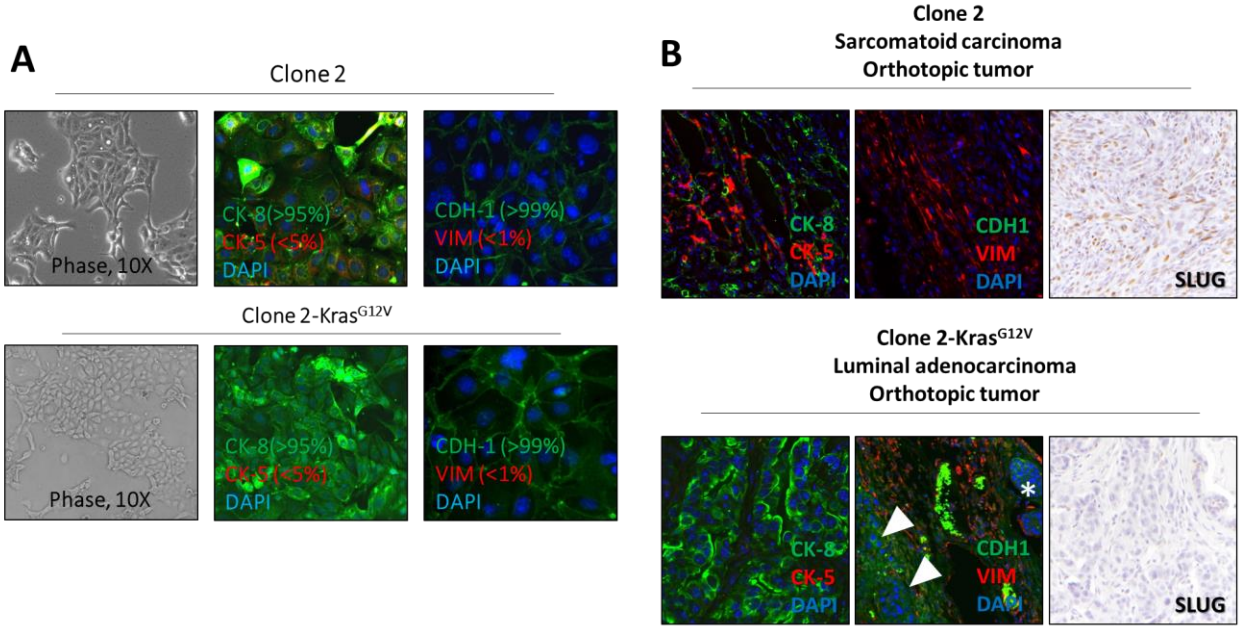

Figure 6. Comparison of *in vitro* and *in vivo* lineage marker expression differences in Clone 2 and Clone 2-Kras^{G12V} cell lines and orthotopic tumors

A. *In vitro* assessment of prostate cell lineage (CK8, CK5) and EMT markers (VIM, CDH1) in Clone 2 and Clone 2-Kras^{G12V} cells. Representative phase contrast images show that Clone 2 tumor cells have an epitheliod morphology and primarily express CK8 with subpopulations of CK5+ and dual CK8/5+ cells. Tumor cells express CDH1 and have low or absent VIM expression. Together these features are consistent with having a luminal adenocarcinoma phenotype. Clone 2-Kras^{G12V} tumor cells also have an epitheliod cellular morphology and express only CK8 and CDH1, which is consistent with having a luminal adenocarcinoma phenotype.

B. *In vivo* assessment of prostate cell lineage and EMT markers in Clone 2 (n=3) and Clone 2-Kras^{G12V} orthotopic tumors (n=4). Clone 2 orthotopic tumors have subpopulations of cells that are CK8+, CK5+, or dual CK8/5+ in areas where epithelial morphologies are retained. Clone 2 tumors are VIM+, CDH1- and SLUG+ in areas of sarcomatoid differentiation. Clone 2-Kras^{G12V} orthotopic tumors are CK8+, CK5-, CDH1+, VIM- and SLUG-. (*) Asterisk represents a CHD1+ Clone 2-Kras^{G12V} tumor embolus in vessels. The white arrowheads represent CDH1+ primary tumor cells at the invasive edge of the mass.

3.2.c Clone 2-Kras^{G12V} cells form adenocarcinomas with a luminal phenotype at orthotopic sites.

Next we immunophenotyped Clone 2 and Clone 2-Kras^{G12V} cell lines and orthotopic tumors for prostate specific lineage markers (Figures 5 and 6) and markers of an epithelial-to-mesenchymal transition (EMT) (Figure 6). *In vitro* both cell lines expressed CK8. However, Clone 2 cells also had subpopulations of tumor cells that

were dual CK8+/CK5+ or CK5+ alone. Clone 2 cells formed sarcomatoid carcinomas that expressed Vimentin (VIM) but not E-cadherin (CDH1). Clone 2 tumor cells with luminal and squamous histologies expressing combinations of CK8 and CK5 and P63, respectively. Previous studies conducted using *Pten;Trp53* deficient AC1, AC2 and SC1 cells have shown that the sarcomatoid carcinoma phenotype arises from tumor cells that express SLUG. Furthermore the complete EMT phenotype that is observed in the model is mediated by TGFβ, which acts as one of the main transcriptional regulators of the mesenchymal determinant factor, *Snai2* (SLUG). Therefore SLUG is both a key molecular and phenotypic marker for transition towards having a spindle-cell morphology and a complete EMT tumor phenotype in the *Pten;Trp53* prostate cancer model [80, 86] Figure 6B shows that Clone 2 sarcomatoid carcinomas expressed nuclear SLUG, and this expression is paralleled by a complete EMT tumor phenotype in the *in vivo* setting.

Nude mice injected with Clone2;Kras^{G12V} cells developed CK8+, P63-, CK5- , CDH1+, VIM-, SLUG- adenocarcinomas. The immunophenotype for Kras^{G12V}-expressing tumor cells was also comparable to both the histology and immunophenotype of human prostate adenocarcinomas. [9] Therefore the loss of SLUG expression by Clone 2-Kras^{G12V} tumor cells suggested that there was a loss of lineage plasticity in Clone 2 cells when oncogenic KRAS^{G12V} was introduced. Because work with the AC3, AC1 and SC1 cell lines have all shown that *Snai2*/SLUG levels are regulated by TGFβ[80] the inhibition of the sarcomatoid carcinoma phenotype also suggested that Ras dependence may have affected TGFβ signaling.

3.2.d Clone 2 and Clone 2-Kras^{G12V} orthotopic tumors have different tumor microenvironments

We observed that there was notable differences between the tumor microenvironments of Clone 2 and Clone 2-Kras^{GV12} orthotopic tumors. These differences included a significant increase the areas of necrosis and an increased presence of polymorphonuclear and mononuclear immune cell infiltrates in all of the Clone 2-Kras^{G12V} orthotopic tumors (Figure 7A). The changes to the tumor microenvironment that were observed for the Clone 2-Kras^{G12V} orthotopic tumors and metastases are also reported as histologic features for other cancer models with Ras dysregulation. It is suggested that having a microenvironment with inflammation and necrosis drives aspects of Ras-mediated tumorigenesis and progression through cytokine and chemokine pathways that converge on NFκB.[88, 89] Clone 2 (n=15) and Clone 2-Kras^{G12V} (n=25) orthotopic tumors were examined and designated as unremarkable or having areas of necrosis and cellular inflammation. (Table 3) A subset of Clone 2 and Clone 2-Kras^{G12V} orthotopic tumors were analyzed for necrosis with areas of necrosis being annotated in multiple cross-sections in an H&E slide using Aperio ImageScope software. All of the Clone 2-Kras^{G12V} tumors had a statistically significant increase in the percentage of area of the tumor undergoing morphologic features of necrosis, which included a loss of cellular architectural detail, cells with apoptotic body formation, accumulations of acellular eosinophilic material and large numbers of viable and degenerate infiltrating immune cells. (Figure 7B) The presence of tumor associated necrosis correlated with an increase in immune cell infiltration, so we also broadly assessed the kinds of cellular infiltrates that were present in the Clone 2-Kras^{G12V} tumor microenvironments. We were limited to only examining markers for

innate immune cells as the mice used for the orthotopic assays were deficient for both B and many T lymphocyte populations. Immune cell infiltrates consisted of histiocytes that expressed F4/80, a common tissue histiocyte antigen. Cellular infiltrates with a segmented nuclear morphology expressed Ly6G, a polymorphonuclear leukocyte marker for both neutrophils and eosinophils that is also transiently expressed in immature monocyte populations, respectively (Figure 7C).

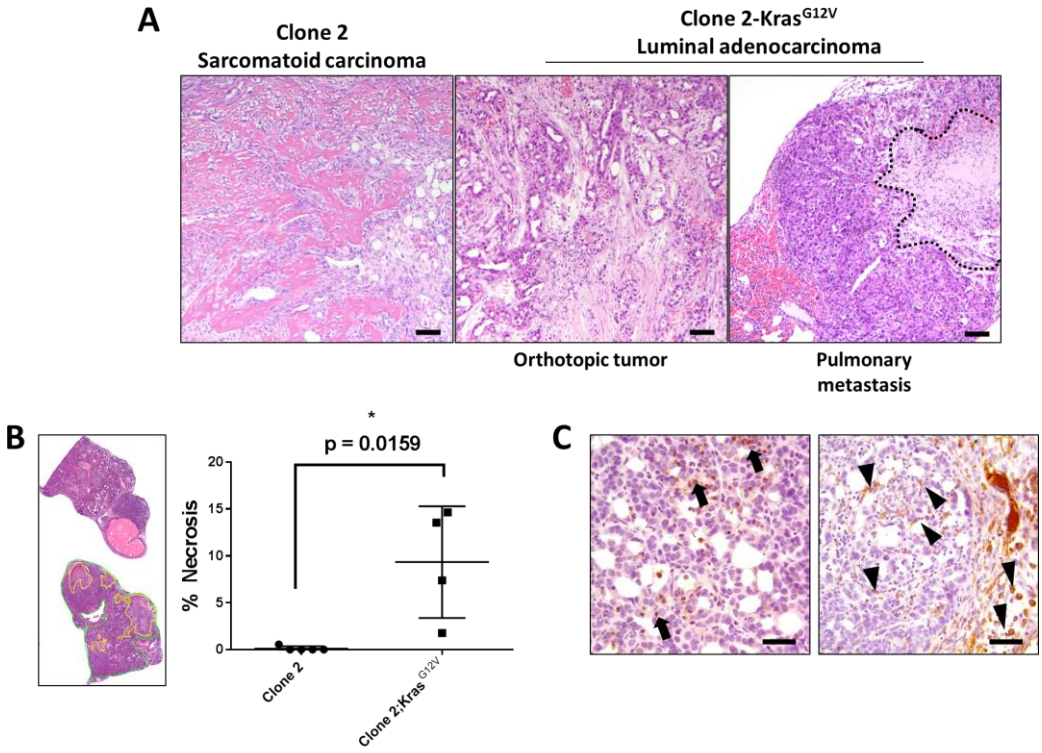

Figure 7. Characterization of the differences between the Clone 2 and Clone 2-Kras^{G12V} orthotopic tumor microenvironments

A. Representative image of a Clone 2 sarcomatoid carcinoma with osseous differentiation showing that the tumor microenvironment does not have areas of necrosis or large numbers of infiltrating immune cells. 100X, H&E. The representative sections of a Clone 2-Kras^{G12V} orthotopic tumor (100X, H&E) and

a metastatic lung tumor (100X, H&E) both show that tumors in this group had large areas of necrosis and a notable increase in the numbers of infiltrating immune cells. The black dashed line in the third image highlights an area in a metastatic lesion designated as being necrotic.

B. Example image of whole slide annotation used to quantify the percentage of necrosis/tumor in a subset of Clone 2 (n=5) and Clone 2-Kras^{G12V} orthotopic tumors (n=4) using Aperio Slide Scanner and the ImageScope software package. Significance was determined by a Mann-Whitney test.

C. Representative images of IHC labeled sections highlight that immune cell infiltrates were characterized as being either F4/80 macrophages (black arrows) or Ly6G+ polymorphonuclear leukocytes (black arrowheads). N= 5 Clone 2-Kras^{G12V} orthotopic tumors/stain were assessed.

Scale bar represents 50µM

Table 3. Histopathologic analyses of Clone 2 and Clone 2-Kras^{G12V} tumors following orthotopic implantation.
Animals that were reviewed by histology were included in this table. Results are from three biologic replicates.

Table 3. Histopathologic analyses of Clone 2 and Clone 2-Kras^{G12V} tumors following orthotopic implantation			
Cell line	**Tumor phenotype**	**Metastasis**	**Necrosis**
Clone 2	Sarcomatoid carcinoma > luminal adenocarcinoma > basosquamous carcinoma	1/15 (6.67%)	1/15 (6.67%)
Clone 2-Kras^{GV12}	Luminal adenocarcinoma	26/26 (100%)	26/26 (100%)

Table 4. Immunophenotypic analyses of Clone 2 and Clone 2-Kras^{G12V} tumors following orthotopic implantation

Table 4. Immunophenotypic analyses of Clone 2 and Clone 2-Kras^{G12V} tumors following orthotopic implantation				
	Clone 2			Clone 2-Kras^{G12V}
IHC Marker	**Adenocarcinoma**	**Basosquamous**	**Sarcomatoid**	**Adenocarcinoma**
CK8	(++++)	(-)	(-)	(++++)
CK5	(+/-)	(++/-)	(-)	(-)
P63	(-)	(++/-)	(-)	(-)
AR	(+/-)	(+/-)	(+/-)	(-)
VIM	(-)	(-)	(++++)	(-)
CDH1	(++++)	N/A	(-)	(+)
SLUG	(+/-)	(+/-)	(++++)	(-)
Nuclear P-ERK1/2	(+/-)	(+/-)	(+/-)	(++)

Data represents IHC analyses from 5 tumors/antibody/group

++++ Marker labeled >76% of cells

+++ Marker labeled 51-75% of cells

++ Marker labeled 26-50% of cells

+ Marker labeled 25% or less of cells

- Absence of detectable labeling by IHC

3.3 Discussion

The combined loss of *PTEN* and *TP53* as well as dysregulation of the RAS signaling axis are all common genetic aberrations in advanced human prostate cancers with metastatic potential.[2] The most significant finding for these studies is that Clone 2-Kras^{G12V} cells formed luminal adenocarcinomas (CK8+,CK5-,TP63-) that are highly metastatic. The development of a murine model of prostate cancer metastasis that not only has the genetic changes that are correlated with advanced disease but also the histologic phenotype and comparable biological behaviors has translational importance. Published studies using the *PB-Cre4*$^{+}$; *Pten*$^{fl/fl}$*;TP53*$^{fl/fl}$ murine prostate cancer model as well as the work presented here all show that the combined deletion of *Pten* and *Trp53* in murine prostate epithelial cells is sufficient for both *in vitro* and *in vivo* invasion with the limited ability of tumor cells to colonize distal sites.[3] We observed this problem directly as metastases was rarely observed in mice injected with Clone 2 cells by either orthotopic implantation or intracardiac inoculation. Additionally we observed that the prostate cancer epithelial cells that have the potential for multi-lineage plasticity leading to a complete EMT tumor phenotype and without robust metastasis are best marked by the nuclear expression of mesenchymal marker SLUG.[80] Clone 2-Kras^{G12V} tumor cells do not have evidence for multi-lineage plasticity as they do no express SLUG by IHC. Therefore several statements can be made from previous studies with this model and the data described in Chapter 3: 1) the sarcomatoid phenotype is marked by SLUG expression, and 2) the complete EMT phenotype that results in sarcomatoid differentiation greatly reduces the metastatic ability of *Pten;Trp*53 deficient prostate cancer cells.

Often the ability to undergo an EMT correlates with *in vivo* metastasis in various experimental metastasis models.[90] However, many of the cancer models described as having an EMT tumor phenotype also retain the ability to undergo a mesenchymal to epithelial (MET) transition for full metastatic potential. At least one study using a pancreatic cancer model has shown that an experimentally induced EMT reduces metastasis.[87] This study shows additional experimental evidence outside of studies conducted with the *PB-Cre4*$^{+}$; *Pten*$^{fl/fl}$*;TP53*$^{fl/fl}$ murine prostate cancer model that an EMT impairs metastatic potential.

While an irreversible EMT can affect the metastatic potential of experimental model systems, the inhibition of the sarcomatoid carcinoma phenotype in *Pten;Trp53* deficient prostate cancer cells does not completely explain why Clone2-Kras^{G12V} cells are highly metastatic. This concept is reinforced by the demonstration that depletion of *Slug* in AC3 cells also impairs the spindle cell phenotype that is observed both *in vitro* and *in vivo*. However *Slug* depletion and restoration of an epithelial cell phenotype does not correlate with *in vivo* metastatic capability following subcutaneous, orthotopic or intracardiac injections of these tumor cells.[80] Evidence from clinical samples shows that Ras dysregulation and the upregulation of MAPK signaling is present in the setting of advanced metastatic disease.[2] Other mouse models of lethal prostate cancer also confirm that oncogenic Ras/MAPK upregulation can synergize with either *Pten* loss or *Nkx3.1* and *Pten* combined loss to drive aggressive primary disease with a high metastatic rate. [50, 51] In one studying having oncogenic Ras activation correlated with a high metastatic rate even when an EMT tumor phenotype was observed.[50] Another conclusion that can be made from the data presented in this

chapter is that additional genetic or epigenetic changes are altogether necessary for *Pten;Trp53* deficient prostate cancer cells to metastasize efficiently.

Another significant finding was that Clone 2-Kras^{G12V} orthotopic tumors have a different tumor microenvironment from Clone 2 orthotopic tumors. Inflammation contributes to the development of many epithelial cancers that also have dysregulation in Ras signaling. Microenvironmental inflammation is also correlated with having a more aggressive disease.[91] Patients that have chronic prostatic inflammation have an increased risk of death from prostate cancer.[86] Several prostate studies also have shown that an inflammatory microenvironment can promote tumorigenesis and enhance the biological aggressiveness of Ras dependent tumors by signaling through the TGFβ and NFκB pathways.[92-94] Additionally having chronic inflammation can influence how prostate cancer cells use aspects of TGFβ and NFκB for lineage commitment choices.[95] Thus the Clone 2-Kras^{G12V} line provides an alternative way to study these types of experimental questions.

The published studies and the findings that are described in Chapter 3 from the characterization of the Clone 2-Kras^{G12V} model all show that this system can be useful for studying questions that are important for understanding the phenotype and biology of lethal, metastatic prostate cancers. Interestingly, Clone 2-Kras^{G12V} cells did not have detectable nuclear AR by IHC when compared with Clone 2 orthotopic tumors, which already had low to moderate, nuclear AR expression. Therefore this model may also be useful for studying the role of AR-independent signaling regarding metastatic potential. Therefore a metastatic prostate adenocarcinoma cell line with genetic alterations and pathway changes that are found in advanced tumors, such as the Clone 2-Kras^{G12V}

model, can serve as an important tool for determining molecular mechanisms driving cellular plasticity, metastasis and drug resistance in aggressive cancers.

CHAPTER FOUR: Generation and characterization of the Clone 2;dnRII and Clone 2-Kras^{G12V};dnRII murine prostate cancer cell lines.

4.1 Introduction

Experiments conducted with the Clone 2-Kras^{G12V} cell line in Chapter 3 showed that oncogenic Kras^{G12V} expression impaired TGFβ-mediated sarcomatoid differentiation. This phenotypic change correlated with a significant increase in experimental metastasis. Thus it was suspected that Ras dependence altered TGFβ. Evidence from other experimental cancer models, such as the PC3 cell line, implicate TGFβ directed pathways in bone metastasis.[64] However, the varied roles for the TGFβ SMAD dependent pathway regarding prostate cancer metastasis is still not well understood. The deletion of *Smad 4* in the *Pten;Trp53* knockout mouse allows tumors cells with a sarcomatoid carcinoma phenotype to grow into and colonize the bone microenvironment, suggesting that this pathway is dispensable for metastatic capability.[96] Clearly there are conflicting conclusions about the role for TGFβ regarding the metastatic capabilities of aggressive prostate cancers. Therefore the Clone 2-Kras^{G12V} cell line provides another way to study if and how this pathway may be important for metastasis. In this chapter we investigate whether the TGFβ pathway is important for Ras dependent metastasis. These studies show that Clone 2-Kras^{G12V} cells do not need the TGFβ SMAD dependent pathway for metastatic ability. In fact, retention of signaling either through TGFβRII or the SMADs may be protective again the development of lethal bone and brain metastases.

In the mouse TGFβ is one pathway that is important for commitment to a luminal epithelial cell lineage.[97] Clone 2-Kras^{G12V} cells expressing an inducible dominant negative TGFβRII (dnRII) showed time dependent increases in the expression of basal lineage markers following the short or long term inhibition of TGFβRII-SMAD dependent signaling. The acquisition of a basal epithelial cell phenotype by dnRII expressing tumor cells correlated with these cells also having metastatic potential to both the bone and the brain. Therefore these studies also show that the Clone 2-Kras^{G12V} model can be used to identify and study specific lineages of cells within tumors that have the potential to seed novel microenvironments and form lethal metastases.

4. 2 Results

4.2.a Clone 2-Kras^{G12V} tumor cells have an intact TGFβ signal transduction cascade

We first investigated the possibility that the expression of oncogenic Kras^{G12V} may have altered TGFβ signal transduction. Previous work with the *Pten;Trp53* deficient mouse model showed that an intact TGFβ1-TGFβRII pathway is important for the complete EMT tumor phenotype of some *Pten;Trp53* deficient cell lines.[80] While not all *Pten;Trp53* deficient cell lines can display multi-lineage plasticity when placed back into an orthotopic site the *C*lone 2 line readily undergoes a TGFβ-mediated EMT and has a sarcomatoid carcinoma phenotype that is comparable to the tumor histologies that are observed in the *Pten;Trp53*KO mouse model and the AC1, AC3 and SC1 cell lines.[3] Because Clone 2-Kras^{G12V} cells did not undergo an *in vivo* TGβ-dependent EMT we hypothesized that Ras dysregulation altered the *in vivo* response to TGFβ1, and this change was important for metastasis.

We first assayed whether the TGFβ SMAD dependent signal transduction cascade remained intact for Clone 2-Kras^{G12V} cells by IHC and western blot analyses. Both Clone 2 and Clone 2-Kras^{G12V} cell lines expressed pSMAD2/3 following stimulation with exogenous TGFβ1 as assessed by western blot analysis. (Figure 8A) IHC analyses of Clone 2 and Clone 2-Kras^{G12V} orthotopic tumors also showed pSMAD2/3 expression was detectable in both the nucleus and cytoplasm, which indicated that active signal transduction was probably occurring in the *in vivo* setting. (Figure 8B) Next we used a semi-quantitative approach to evaluate whether there were differences in pSMAD2/3 expression between Clone 2 and Clone 2-Kras^{G12V} orthotopic tumors.

(Figure 8C) The intensity scoring method for evaluating pSMAD2/3 expression between the groups was: 0, none; 1, weak; 2, moderate/intermediate; 3, strong; and 4, over-stained. The scores for the percentage of positive cells were: 0, none; 1, up to 10%; 2, up to 25%; 3, up to 50%; 4, up to 75%; and 5, up to 100%. This scheme resulted in a 20-point combined staining score. We did not find a significant difference in combined scores when examining either nuclear or cytoplasmic expression of pSMAD2/3 in the two groups. Clone 2 tumor cells had the following combined scores for nuclear labeling: 12 (3/6 tumors) and 16 (3/6 tumors). The following combined scores were calculated for Clone 2 pSMAD2/3 cytoplasmic labeling: 4(6/6 tumors). Clone 2-Kras^{G12V} tumor cells had the following combined scores for nuclear labeling: 6 (1/7 tumors), 8 (4/7 tumors), 16 (2/7 tumors) and for cytoplasmic labeling: 3 (5/7 tumors) and 4 (2/7).

Because TGFβ signaling was occurring *in vivo* we reasoned that changes to the TGFβ-SMAD dependent pathway could not entirely account for the altered phenotype and behavior of Clone 2 cells following oncogenic Ras expression. (Chapter 3) Interestingly, the presence of nuclear pSMAD2/3 in Clone 2-Kras^{G12V} lung metastases suggested that the pathway may have a pro-metastatic role in this model, which was consistent with other published reports using Ras transformed DU145 human prostate cancer cells.[98] Because TGFβ and its downstream dependent pathways are widely implicated in prostate cancer metastasis and we observed that signaling was not apparently different between Clone 2 and Clone 2-KrasG12V cells in either the *in vitro* or *in vivo* settings, we then asked whether this pathway was necessary for *in vivo* metastasis.

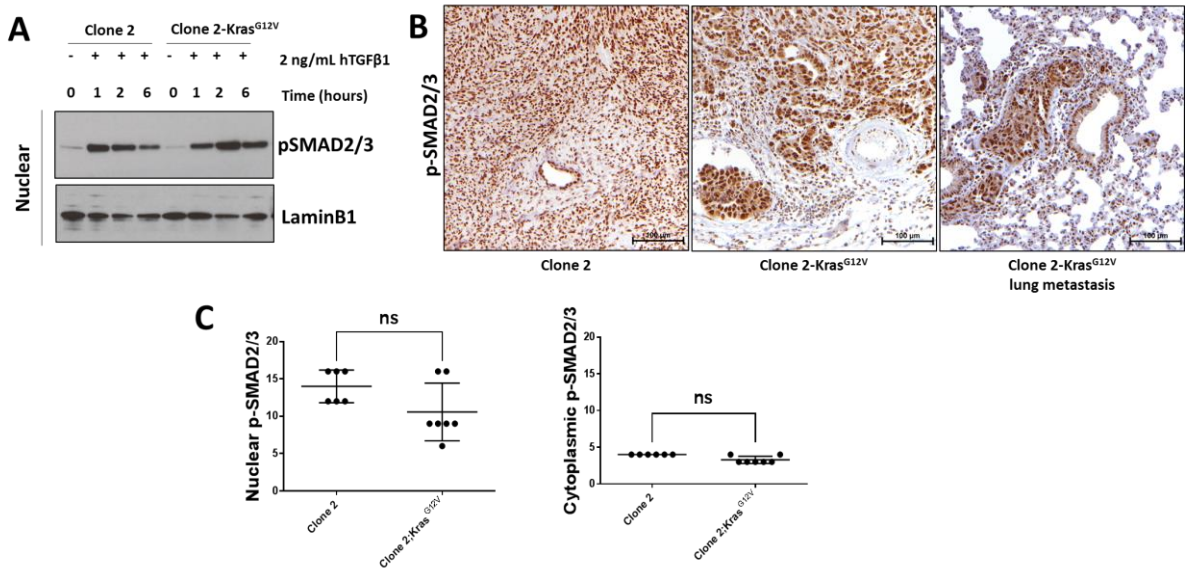

Figure 8. *In vitro* and *in vivo* analyses of TGFβ SMAD dependent signaling in Clone 2 and Clone2-Kras^{G12V} cell lines and orthotopic tumors and Clone2-Kras^{G12V} metastases.

A. Western blot analyses of nuclear lysates from Clone 2 and Clone 2-Kras^{G12V} cells shows that the TGFβ pathway is not constitutively active but is responsive to TGFβ1 stimulation as measured by pSMAD2/3.

B. Nuclear pSMAD2/3 expression is present in both Clone 2 tumors and Kras^{G12V} expressing orthotopic tumors and metastases.

C. Nuclear and cytoplasmic pSMAD2/3 expression levels by IHC expressed as a combined score as evaluated in Clone 2 (n=6) and Clone 2-Kras^{G12V} (n=7) orthotopic tumors. The central line represents the mean. Error bars are ± SEM. Significance was measured using a Mann-Whitney test.

4.2.b Clone 2-Kras^{G12V};dnRII cells form both basosqumaous and adenosquamous carcinomas that can metastasize following orthotopic implantation

Because the TGFβ signaling cascade was intact and there was evidence of active transduction in the *in vivo* setting we suppressed the pathway to test whether it was a requirement for Clone 2-Kras^{G12V} metastasis. Previous studies in a mouse model of combined loss of *Pten* and *Smad4* showed that there is an increase in the metastatic colonization of regional lymph nodes by *Pten;Smad4* deficient tumor cells.[99] In a similar mouse model with combined loss of *Pten, Trp53 and Smad 4* locally aggressive prostatic sarcomatoid carcinomas acquired the ability to invade into and efface portions of the adjacent lumbar spine.[96] However, this study did not provide definitive evidence as to whether these tumor cells could also fulfill the six steps of the invasion-metastasis cascade in order to colonize the bone microenvironment as would be expected in the human disease setting. Thus our orthotopic model would be more robust test for asking whether TGFβ-SMAD dependent signaling was important metastasis. To suppress the pathway we expressed a tetracycline responsive dominant negative TGFβRII (dnRII) in both the Clone 2-Kras^{G12V} and Clone 2 cell lines. The experimental strategy is outlined in Figure 9A. Following the induction of the genetic repressor by doxycycline MCHERRY fluorescent protein was expressed from an internal ribosomal entry site that allowed for sorting of pure populations for the downstream experiments. After confirming that our vector reduced p-SMAD2/3 expression in whole cell lysates (Figure 9B) we observed that the *in vitro* invasive potential of both Clone 2;dnRII and Clone 2-Kras^{G12V};dnRII cells was reduced when compared with the invasive potentials of the parental cell lines. (Figure 9C) Based on these assays we hypothesized that tumor cells

may not be able to efficiently invade or colonize distal sites following orthotopic implantation.

Next we orthotopically implanted dnRII expressing tumor cells into the prostates of nude mice. All of the mice injected with Clone 2-Kras^{G12V};dnRII cells (25/25) formed tumors. Clone 2-Kras^{G12V};dnRII orthotopic tumors were comprised of mixtures of both adenocarcinoma and basosquamous phenotypes (4/25), which were termed adenosquamous carcinomas, or had a complete basosquamous phenotype (21/25). Tumors having a complete basosquamous carcinoma phenotype were derived from Clone 2-Kras^{G12V};dnRII cells that have been continuously cultured in doxycycline for greater than two months time following the selection and expansion of mCHERRY high and intermediate expressing tumor cell populations. The data indicated that the suppression of TGFβ was more selective for the growth and expansion of tumor cell populations that were committed to a basal lineage. (Figure 9D and Table 5)

Next we assessed the immunophenotypes of the histologies observed for the orthotopic tumors. Tumor areas having both basaloid and squamous morphologies were positive for P63. A subset of tumor cells having a squamous morphology and features of keratohyaline granules and keratinization did not express P63. However, prior studies with *Pten;Trp53* deficient mouse model system showed that separating out CD49f^{hi} tumor cell populations selected for *in vivo* morphologies were either adenosquamous or basosquamous in histology. The tumor cells with these morphologies often expressed combinations of P63 and CK5, and that the expression of these lineage markers in propagated tumors correlated with tumor cells being a fully and terminally differentiated as basal epithelial cells.[85] Adenocarcinoma

morphologies were strongly positive for CK8 and did not express either P63 or CK5. Neither the basaloid nor squamous morphologies were positive for CK8. Phospho-SMAD2/3 expression was analyzed in a set of 4 tumors harvested 3-5 weeks post-implantation. Tumor cells at the orthotopic site had either low or undetectable levels of phospho-SMAD2/3 in the nucleus and low expression in the cytoplasm, which was consistent with having a reduction in TGFβ signaling in the *in vivo* setting. (Figure 9D)

We observed that induction of the dnRII in Clone 2-Kras^{G12V} cells had negative effects on both tumor cell invasion in the *in vivo* setting (Figure 9E) as measured by a significant reduction in the number of foci of lymphovascular invasion at the orthotopic site. However while the number of pulmonary metastases per mouse was lower, this different was not found to be significant. (Figure 9F) Additionally nude mouse survival was also not statistically significant between the two groups. (Figure 9G) Thus we concluded that this pathway is not fully required for Clone 2-Kras^{G12V} dependent invasion and metastasis.

In breast cancer models a reduction in either TGFβRII or TGFβ-SMAD dependent signaling can facilitate metastasis to microenvironments that are high in endogenous TGFβ1, including lymph nodes and bone.[100] These prior studies led us to hypothesize that Clone 2-Kras^{G12V};dnRII expressing cells may be able to readily colonize sites with high TGFβ1 levels, including the lymph node and bone marrow microenvironments. We analyzed whether there was an increase in the incidence of lymph node metastasis in mice injected with Clone 2-Kras^{G12V};dnRII cells but did not observe any differences. (Table 6 and Figure 10A) Interestingly disseminated tumor cells could be observed in the bone marrow of mice injected with Clone 2-

Kras^{G12V};dnRII tumor cells. (Figure 10A) Because of the short survival times following the orthotopic implantation of cells we next used an intracardiac model to test whether metastatic colonization of the bone microenvironment would occur.

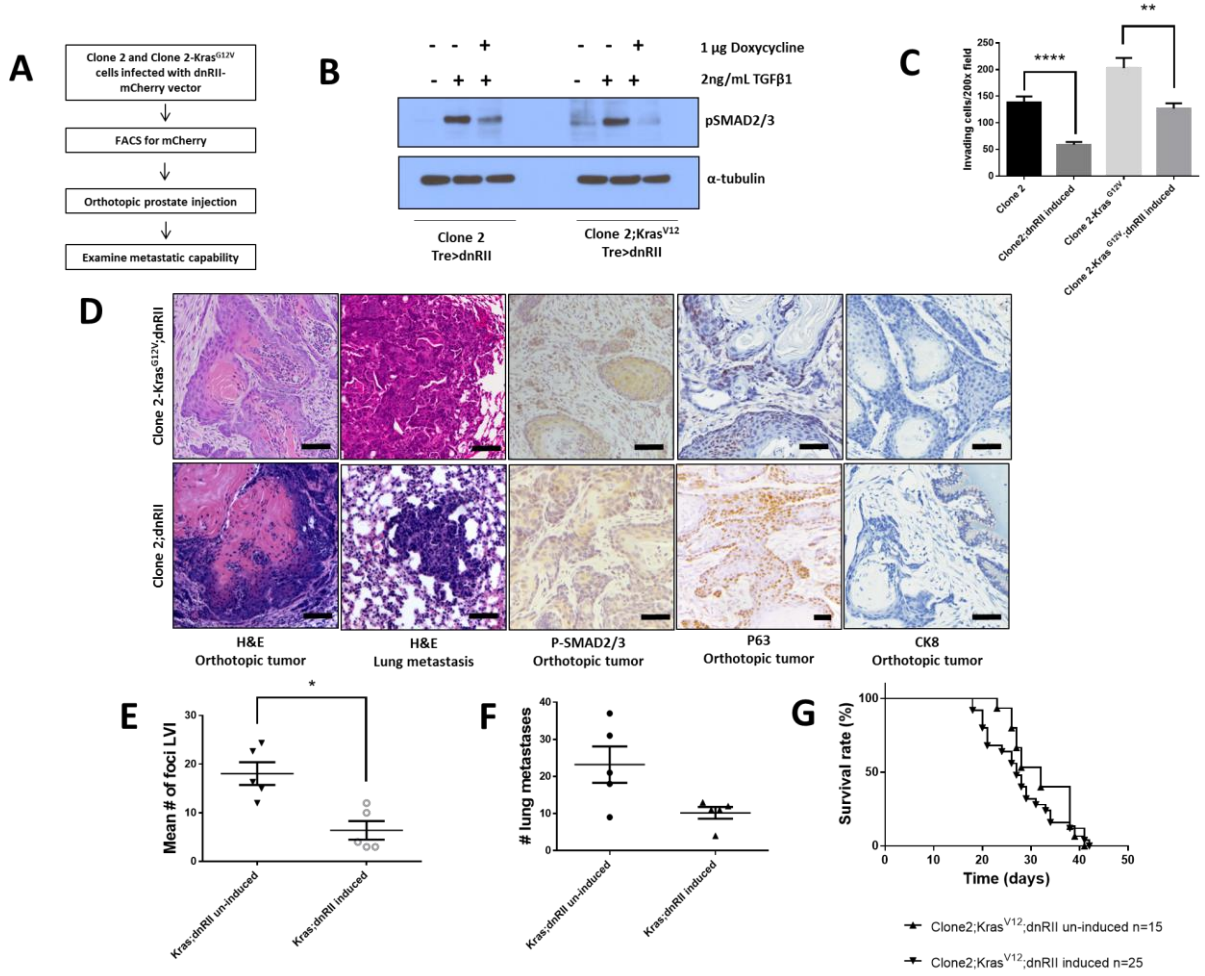

Figure 9*. In vitro* and *in vivo* findings for the Clone 2-Kras^{G12V};dnRII and Clone 2;dnRII orthotopic studies

A. Schematic of the experimental design for the Clone 2-Kras^{G12V};dnRII orthotopic studies.
B. Expression of phospho-SMAD2/3 in response to doxycycline treatment and induction of the dnRII vector. Clone 2>TRE dnRII and Clone 2;Kras^{G12V}>TRE

dnRII cells were treated with 1 μg/ml Dox, stimulated with 2ng/mL of human TGFβ1 and assayed for expression of pSMAD2/3 in whole cell lysates by Western blot.

C. Chemotactic directed invasive of Clone 2 and Clone 2-Kras^{G12V} through a Matrigel membrane is reduced in cells expressing an induced dnRII. ** = < 0.0019 using an unpaired T test with Welch's correction.

D. Representative histologies of Clone 2-Kras^{G12V};dnRII induced and Clone 2;dnRII induced orthotopic tumors. Mice injected with tumor cells from either group formed tumors with adenosquamous or fully basosquamous cellular morphologies that had metastatic potential. Orthotopic tumor cells did not express nuclear pSMAD2/3, which was consistent with impaired SMAD dependent signaling. P63 is expressed in areas of basal and squamous differentiation while CK8 expression is not detectable in cells having these morphologies. Scale bar represents 50μM.

E. There is a decrease in the mean number of foci of lymphovascular invasion at the edges of orthotopic tumors formed by Clone 2-Kras^{G12V};dnRII tumor cells. * = < 0.0159 using a Mann-Whitney test.

F. The number of lung metastases per nude mice injected with Clone 2-Kras^{G12V};dnRII induced tumor cells is not significantly different from mice injected with Clone 2-Kras^{G12V};dnRII uninduced tumor cells.

G. There are no significant differences in nude mouse survival between the un-induced and induced Clone 2-Kras^{G12V};dnRII study groups.

4.2.c Clone 2;dnRII cells form basosquamous carcinomas that can metastasize following orthotopic implantation

Because Clone 2 tumor cells have multi-lineage potential and an *in vivo* EMT phenotype we hypothesized that the suppression of TGFβ SMAD dependent signaling would inhibit sarcomatoid differentiation and increase metastasis. After confirming that the vector would reduce pSMAD2/3 levels (Figure 9B) we also showed that Clone 2;dnRII cells also had a reduction in directed invasion when compared with the parental cell line. (Figure 9C) Again, this data suggested that TGFβ was important for full invasive potential. The majority of the mice injected with Clone 2;dnRII cells (11/16) formed tumors. Of the mice that formed tumors all had evidence of distal spread of tumor cells (11/11). (Table 5) Unfortunately a large number of the control mice failed to develop orthotopic tumors following implantation (1/15). Additionally metastatic colonization of a lymph node was observed in the one mouse that was injected with the un-induced cells. Therefore additional *in vivo* studies are needed to better understand whether there are differences between the metastatic potentials of Clone 2 and Clone 2;dnRII induced cells.

Clone 2;dnRII orthotopic tumors that formed at orthotopic sites were basosquamous carcinomas with 1 mouse having a tumor with an adenosquamous phenotype. (Figure 9D) Tumor cells with basaloid and squamous morphologies were positive for P63. Subsets of cells having a squamous cell morphology did not express P63. The expression of CK8 was either low or completely absent in the cells with basaloid and squamous morphologies. The one tumor with an adenosquamous morphology had areas with a glandular morphology that were strongly positive for CK8

and did not express P63. Four tumors were analyzed for pSMAD2/3 expression. Tumor cells had either low or undetectable levels of phospho-SMAD2/3 in the nucleus and low expression in the cytoplasm, which was consistent with having an inhibition of TGFβ signaling in the *in vivo* setting. As anticipated the un-induced Clone 2;dnRII tumor cells formed a sarcomatoid carcinoma that was devoid of osseous or cartilaginous metaplasia and was CK8- and P63-.

4.2.d Clone 2;dnRII and Clone 2-Kras^{G12V};dnRII cells form brain and bone metastases following intracardiac injection

We used an intracardiac injection model to overcome the limits of the orthotopic technique in order to test whether TGFβ insensitivity could result in a bone metastasis phenotype. (Figure 10B) Un-induced vector containing and vector-induced cells were stably infected with a lentiviral vector encoding for luciferase/enhanced green fluorescent protein reporter gene to follow *in vivo* progression by bioluminescent imaging (BLI). (Figure 10C) The appearance of signal by BLI became evident in the brain and long bones as early as 2 weeks post-intracardiac injection for both the Clone 2;dnRII and Clone 2-Kras^{G12V};dnRII expressing groups. All of the mice remained on study for a period of 3-6 weeks post injection. Of the mice that were injected with Clone 2-Kras^{G12V};dnRII cells 5/10 had signals in the long bones and 13/15 in the brain by BLI. 1/5 mice injected with Clone-Kras^{G12V};dnRII un-induced cells had a positive signal at the level of the metatarsal bone that most likely represented a soft tissue lesion. Therefore it was not counted as a metastatic lesion. This conclusion was in line with prior intracardiac studies using the Clone 2-Kras^{G12V} cells, whereby injection did not result in metastatic bone lesions in any mice (0/9, Chapter 5, Table 11). Brain signals were

observed in 2/5 mice injected with Clone 2-Kras^{G12V};dnRII un-induced cells. This observation was also comparable with the historical controls, which showed that 6/9 mice injected with Clone 2-Kras^{G12V} parental cells had evidence of metastatic disease in the brain. (Chapter 5, Table 11) Of the 10 mice that were injected with Clone 2-dnRII induced cells 7/10 had a luciferase positive signal in the brain. None of the five mice injected with Clone 2;dnRII un-induced cells developed evidence of metastatic disease by BLI. This result was also similar to the first intracardiac experiments with Clone 2 parental cells that also showed all six mice in the cohort did not develop evidence of metastatic disease. (Chapter 5, Table 11) The findings from these intracardiac studies are summarized in Table 7 of this Chapter.

The bone metastases that formed from Clone 2-Kras^{G12V};dnRII cells had a basosquamous morphology and were osteolytic type lesions as assessed by histology and staining with hematoxylin and eosin with Orange G (H&E, Orange G). Brain metastases that formed in mice injected with either Clone 2;dnRII or Clone 2-Kras^{G12V};dnRII expressing cells had basosquamous morphologies. The tumor cells having the combinations of basaloid and squamous cell types were positive for TP63 and CK5, respectively. CK8 was expressed in rare subpopulations of cells (<5%) at the edges of areas where there was basosquamous differentiation or was entirely absent from the metastatic lesions. Nuclear phospho-SMAD2/3 was not detected in brain metastases, indicating that the TGFβ SMAD signal transduction was reduced/absent within the metastatic lesions. (Figure 10E) Thus, these studies provide evidence that bi-potential P63+ CK5+ CK8+/- tumor cells can colonize both the bone and brain. More importantly, TGFβ SMAD dependent signaling is not a requirement for either bone or

brain metastasis as assessed by the results from the Clone 2-Kras^{G12V} ;dnRII and Clone 2;dnRII intracardiac experiments.

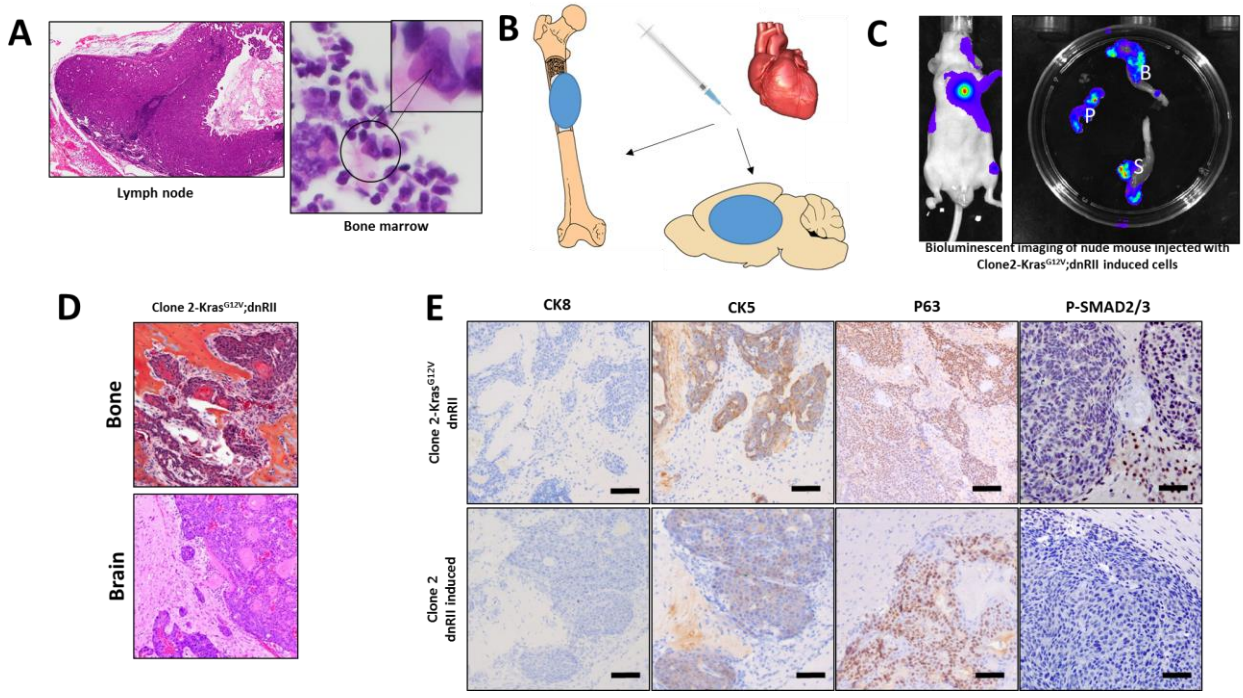

Figure 10. Experimental design and pathology findings for the Clone 2;dnRII and Clone 2-Kras^{G12V};dnRII intracardiac studies.

A. Examples of a Clone 2-Kras^{G12V};dnRII lymph node metastasis and a circulating tumor cell found within the bone marrow cavity from the orthotopic studies.
B. Schematic of the experimental design for intracardiac metastasis assays.
C. Representative bioluminescent imaging confirms the presence of tumors in the brain and long bone in a nude mouse injected with Clone 2-Kras^{G12V};dnRII cells. The *ex-vivo* imaging of limbs shows there is a true signal at the level of the bone as well as in the adjacent soft tissue structures. P = prostate, B = bone, S = soft tissue.

D. Representative histologies of the bone (200X,H&E with orange G) and brain (200X, H&E only) metastases formed following intracardiac injection of Clone 2-Kras^{G12V};dnRII cells. Metastatic tumor cells had a basosquamous phenotype.

E. Immunophenotype of Clone 2-Kras^{G12V};dnRII induced and Clone 2;dnRII brain metastases in mice following intracardiac injection. Brain metastases either have low expression of CK8 or a complete absence of this marker. Tumor cells with a squamous morphology are CK5 positive while tumor cells with both basaloid and squamous morphologies are P63 positive. All of the tumor cells are negative for nuclear P-SMAD2/3.

Table 5. Histopathology findings from the Clone 2;dnRII and Clone 2-Kras^{G12V};dnRII orthotopic implantation studies

Animals that were reviewed by histology were included in this table. Results are from two biologic replicates.
** 1 out 15 Clone 2;dnRII uninduced tumors formed following orthotopic implantation*

Table 5. Histopathology findings from the Clone 2;dnRII and Clone 2-Kras^{G12V};dnRII orthotopic implantation studies		
Cell line	**Primary tumor phenotype**	**Metastasis**
Clone 2;dnRII Un-induced	Sarcomatoid carcinoma > luminal adenocarcinoma > basosquamous carcinoma	1/11
Clone 2dnRII Induced	Basosquamous or Adenosquamous carcinoma	11/11
Clone 2-Kras^{G12V};dnRII Un-induced	Adenosquamous or Basosquamous carcinoma	15/15
Clone 2-Kras^{G12V};dnRII Induced	Basosquamous or Adenosquamous adenocarcinoma	25/25

Table 6. Comparison of the frequency of lymph node metastasis from mice with and without an intact TGFβ signaling cascade.

Table 6. Comparison of the frequency of lymph node metastasis from mice with and without an intact TGFβ signaling cascade	
No. of mice injected with induced cells with LN mets	11/13
No. of mice injected with un-induced cells with LN mets	10/13

Table 7. Sites of metastasis observed for the Clone 2;dnRII and Clone 2-Kras^{G12V};dnRII intracardiac studies as assessed by bioluminescent imaging.

Table 7. Pathology findings from the Clone 2;dnRII and Clone 2-Kras^{G12V};dnRII intracardiac studies		
Cell line	**Brain metatasis**	**Bone metastasis**
Clone 2;dnRII Uninduced	0/5	0/5
Clone 2;dnRII Induced	7/10	0/10
Clone 2-Kras^{G12V};dnRII Uninduced	2/5	0/5
Clone 2-Kras^{G12V};dnRII Induced	13/15	5/10

Table 8. Immunophenotypic analyses of Clone 2;dnRII and Clone 2-Kras^{G12V};dnRII orthotopic tumors

Table 8. Immunophenotypic analyses of Clone 2;dnRII and Clone 2-Kras^{G12V};dnRII tumors					
	Clone 2			**Clone 2-Kras^{G12V}**	
IHC Marker	**Adenocarcinoma**	**Basosquamous**	**Sarcomatoid**	**Adenocarcinoma**	**Basosquamous**
CK8	(++++)	(+/-)	(-)	(++++)	(+/-)
CK5	(+/-)	(+++/-)	(-)	(-)	(+++/-)
P63	(-)	(++/-)	(-)	(-)	(++/-)

Data represents IHC analyses from 5 tumors/antibody/group

++++ Marker labeled >76% of cells

+++ Marker labeled 51-75% of cells

++ Marker labeled 26-50% of cells

+ Marker labeled 25% or less of cells

- Absence of detectable labeling by IHC

4.3 Discussion

We investigated whether TGFβ was important for the metastatic potential of Clone 2-Kras^{G12V} cells. The functional *in vivo* studies show that 1) TGFβ is an important signal for full invasive capabilities of prostate tumor cells, and 2) this pathway is essential for the differentiation and continued selection for tumor cells that are committed to a luminal epithelial lineage. More importantly, our studies support the idea that the inactivation of TGFβ receptor mediated SMAD signaling by genetic repression enables Clone 2-Kras^{G12V} cells to more effectively colonize the bone microenvironment as tested by intracardiac injection.

Genomic studies of human clinical samples have shown that the complete inactivation of TGFβ signaling by either the deletion or mutation of one of the canonical TGFβ signaling pathway components, either at the level of the receptors or Smad molecules, is not documented in prostate cancer primary or metastatic tumors that have been examined to date.[2] However previous experimental studies have documented that the loss of TGFβ does occur and that it is potentially important for the early stages leading to the development of PIN lesions and some human prostate cancers.[101] Other studies implicate TGFβ in progenitor cell homeostasis and loss of SMAD dependent signaling as being an important event for driving intratumoral heterogeneity and the selection for tumor cell populations with bi-lineage potential.[99, 102] The findings in Chapter 4 also show that the loss of TGFβ SMAD signaling in Clone 2-Kras^{G12V} cells selects for cells populations with bi-lineage potential that express two key markers of basal epithelial cell differentiation.

Consistent with previous work in other epithelial cancers, we observed that the constitutive activation of oncogenic $Kras^{G12V}$ in the *Pten;Trp53* deficient Clone 2 model enabled metastasis by cells having a luminal epithelial histology and immunophenotype; however, Clone 2-$Kras^{G12V}$ tumor cells did not exhibit a key feature of lethal cancers, mainly the ability to spread to the bone. We demonstrated that in these cells with robust metastatic potential TGFβ signaling remained intact, and that when this pathway was suppressed invasion was negatively affected. These data suggest that TGFβ is important for invasion and, therefore, can indirectly affect metastasis. Our data also suggests that ongoing signaling through this pathway appears to impeded both bone and brain metastasis. In this regard, other experimental prostate cancer models have also shown a similar change in the biological behavior of tumor cells when receptor II mediated signaling is inhibited.[103] Furthermore there is clinical evidence that correlates the loss of the TGF-β type II receptor (TβRII) in tumor cells with those cancers being classified as a higher Gleason grade.[101] These data generated in another experimental model of aggressive prostate cancer and also in the clinical setting independently support our current findings. Therefore we conclude that TGFβ SMAD dependent signaling is not entirely essential for prostate cancer progression, provided tumor cells possess other genetic changes associated with having advanced disease.

Analyses of well-characterized prostate cancer cell lines, such as PC3 and DU145, often show that TGFβ signaling factors and TGFβ driven metastasis gene profiles are upregulated in aggressive adenocarcinomas and bone metastases.[64] However, the applicability of these findings have not been completely validated in tissue

samples. There are also questions about the representative nature of these two models, especially regarding selective pressures and genetic drift over time as well as the current genetic and biologic features of advanced prostate cancers that been treated with newer androgen deprivation therapies or immunotherapies. Therefore, having a number of alternative experimental models of lethal metastatic disease will be useful for either proving or disproving the role for TGFβ regarding prostate cancer metastasis.

In summary these studies have established that the loss of TGFβ SMAD dependent signaling through the expression of a dnRII enables the rapid progression of disease in both the Clone 2-Kras^{G12V} and Clone 2 models. At the same time, we established that tumor cells expressing oncogenic Kras^{G12V} could acquire both a bone and brain metastatic phenotype when placed directly into circulation. Thus, we conclude that having a period of inactivation of TGFβ SMAD dependent signaling is one potential mechanism for the development of more advanced disease phenotypes including bone and brain metastasis. Therefore the Clone 2-Kras^{G12V} and Clone 2 lines offer additional tools for studying lethal metastatic disease and validating findings from other model systems and the clinic.

CHAPTER FIVE: Generation and characterization of the Clone 2;SR and Clone 2-Kras^{G12V};SR murine prostate cancer cell lines

5.1 Introduction

Previous work has shown that oncogenic Kras^{G12V} leading to the upregulation of MAPK signaling is important for experimental prostate cancer metastasis as tested in *Pten;Trp53* deficient Clone 2 murine prostate cancer cells. Evidence from clinical specimens and other experimental cancer models harboring Ras mutations all indicate that the Ras signaling axis uses the canonical NFκB pathway for aspects of tumorigenesis and metastasis. However, the role for canonical NFκB for prostate cancer metastasis in the *Pten;Trp*53 knockout mouse prostate cancer model has not been previously demonstrated. Furthermore the role for canonical NFκB in human prostate cancers and metastatic disease is not well understood and should be explored as it represents a pathway with a number of druggable targets.

In this chapter we investigate whether the NFκB pathway is important for the metastatic capability of Clone 2-Kras^{G12V} cells. In the mouse NFκB is one pathway that is important for luminal epithelial lineage commitment of prostate cells. It is also important for the growth of Kras dependent tumors of the lungs. [90][92][58] To directly test whether these observations were also true in the Clone 2-Kras^{G12V} model we stably express an induced IKBα-super repressor (SR) into the line to stop canonical signaling and show that a reduction in NFκB signal transduction selects for basal epithelial cells that had poor invasive and metastatic potentials. We also show that the canonical NFκB pathway is not required for metastasis as Clone 2;SR expressing cells can

colonize the lungs. Therefore these studies highlight that NFκB has critical roles for both lineage commitment of prostate cancer cells as well as invasion and metastasis as tested in the Clone 2-Kras^{G12V} model.

5.2 Results

5.2.a The NFkB signaling factor p65 is upregulated in a subset of human prostate cancers and metastases.

NFκB has been implicated in prostate cancer progression.[96] To determine whether NFκB was important for human prostate cancer metastasis we stained and analyzed two tissue microarrays (TMA) for total P65 (Figure 11). The TMAs contained both primary prostate cancers (UWTMA48) and metastases (UWTMA22). Both TMAs were acquired from the University of Washington. In other published reports clinical samples often have a low overall frequency of nuclear P65 staining, which is attributed to specimen handling and fixation methods.[97] Because there were a large number of tissue cores containing low percentages of prostate cancer cells with nuclear p65 staining, an expanded scale was used for scoring: 0, none; 1, up to 10%; 2, up to 25%; 3, up to 50%; 4, up to 75%; and 5, up to 100%. The intensity scoring method for p65 was: 0, none; 1, weak; 2, moderate/intermediate; 3, strong; and 4, strong and obscuring details. This system yielded a 20-point combined staining score. For nuclear p65, combined scores were 0, negative; 1–3, weak; 4–6, intermediate; and 8–20, strong. Cores were marked as negative if less than 1% of the prostate epithelial cells were positive for nuclear NFκB p65 staining. A subset of primary prostate tumors and metastases had a statistically significant increase in the combined values of intensity and percentage positivity compared with normal prostate or BPH samples. Interestingly, a higher percentage of metastases had intense staining compared with primary tumors, suggesting a potential role for p65 mediated NFκB for human prostate cancer cell invasion and metastasis.

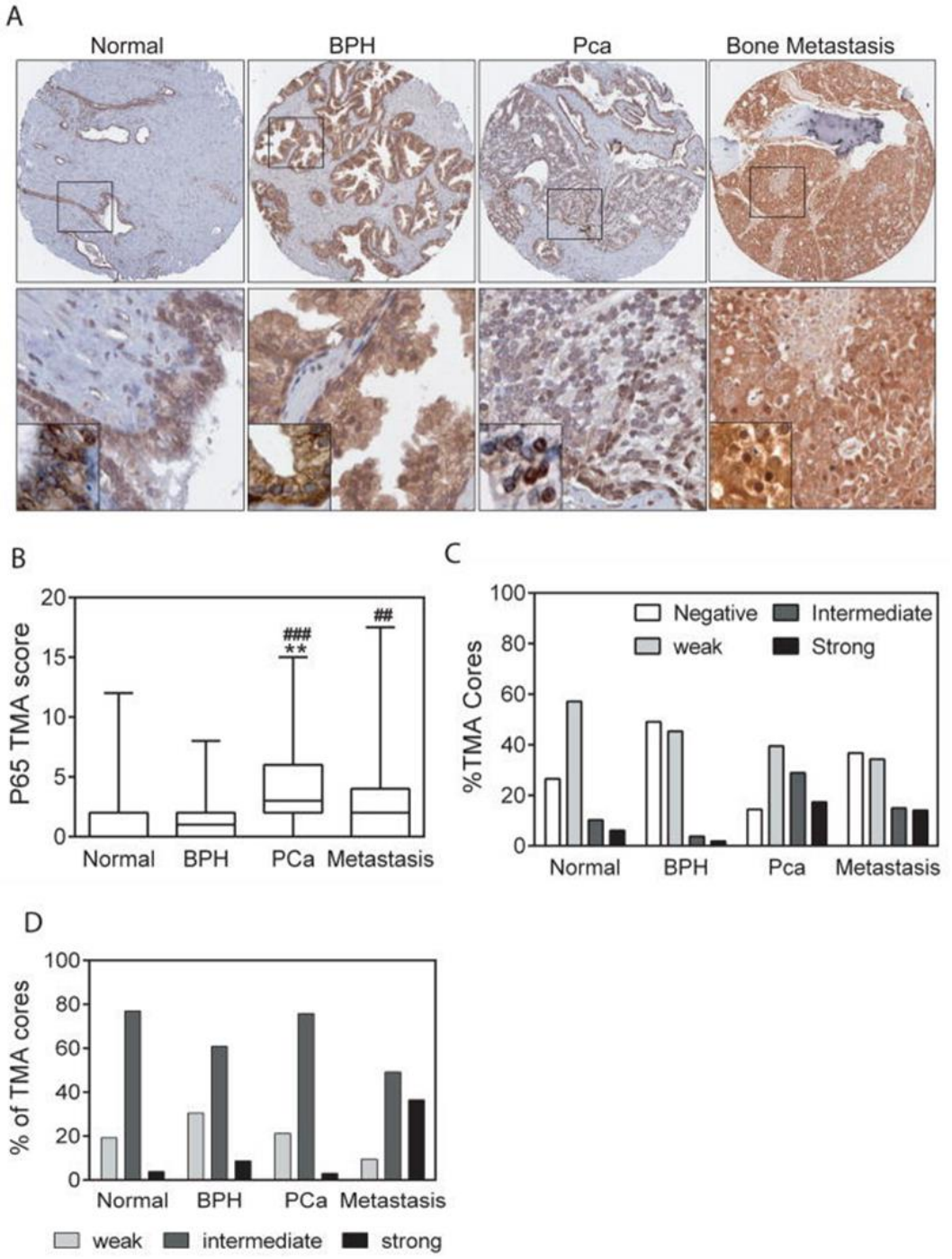

Figure 11. NFκB signaling factor p65 is upregulated in a subset of human prostate cancers and metastases.

A. Representative images and scores of tissue cores, including normal prostate tissue (0), BPH (0), primary prostate cancer (4) (UWTMA 48), and bone

metastasis (3) (UWTMA22) stained for p65. In the bone metastasis core, the cytoplasmic staining is strong, while nuclear staining is <10%.

B. Plots summarizing the p65 scores in different groups of patient samples. The numbers of patient tissue samples in each group are: Normal, BPH, primary prostate cancer (55), and metastasis (115). **p<0.01 vs Normal, $^{\#\#}$ p<0.01, $^{\#\#\#}$ p<0.001 vs BPH.

C. Percentage of TMA tissue samples with negative, weak (1–3), intermediate (4–6) or high (>6) nuclear p65 staining from B.

D. Further analysis for staining intensity in tissue samples from B that had a range of 1–10% of cells being positive for nuclear p65 positive. Shown is the distribution of the selected samples for staining intensity with weak (1), intermediate/moderate (2) and strong (3) intensity.

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5.2.b Clone 2-Kras^{G12V} tumor cells with invasive and metastatic potential express p-P65^{ser536}

Published studies and our own examination of total P65 expression in clinical samples suggested that the canonical arm of the pathway is constitutively activated in a subset of prostate cancer cells with metastatic potential. There is also evidence in the literature that the nuclear localization of p65 is correlated with having signaling cascade that is active in the *in vivo* setting.[98] Because we established the relevance for NFκB for the human disease we wanted to next test whether NFκB was important for the

metastatic phenotype we observed for the Clone 2-Kras^{G12V} *in vivo* studies. (Chapter 3) We assayed phospho-P65^{ser536} expression by immunohistochemistry. This phosphoisoform of p65 is upregulated in invasive prostate cancer cells by both canonical and non-canonical kinases (Figure 12A).[68][104] We observed that p-P65^{ser536} was expressed in both the cytoplasmic and nuclear compartments of Clone 2 sarcomatoid carcinoma cells and Clone 2-Kras^{G12V} adenocarcinoma cells. (Figure 12B) Interestingly, Clone 2-Kras^{G12V} cells, especially tumor cells with invasive and metastatic potential, had increased percentages of cells with more intense labeling by the anti-p-P65^{ser536} antibody.

Because there was an apparent difference in the IHC labeling between the two groups we used a semi-quantitative approach to evaluate both the percentage of positive tumor cells and the intensity of labeling by the anti-p-P65^{ser536} antibody in 10 high power (40X) fields of each tumor. This method was comparable to the scoring scheme for the TMA analyses that was described in section 5.2.a. The intensity scoring method for p65 was: 0, none; 1, weak; 2, moderate/intermediate; 3, strong; and 4, overstained. The scores for the percentage of positive cells were: 0, none; 1, up to 10%; 2, up to 25%; 3, up to 50%; 4, up to 75%; and 5, up to 100%. This scheme resulted in a 20-point combined staining score. For either the cytoplasmic or nuclear expression of p-p65^{ser536}, combined scores were placed into the following categories: 0, negative; 1–3, weak; 4–6, intermediate; and 8–20, strong. Clone 2-Kras^{G12V} tumor cells had intermediate to strong cytoplasmic expression of p-P65^{ser536} by IHC and the following combined scores: 8 (4/5 tumors) and 6 (1/5 tumors). Clone 2-Kras^{G12V} tumor cells had the following combined scores for nuclear labeling: 4 (4/5 tumors) and 2 (1/5

tumors). Clone 2 tumor cells had weak to intermediate cytoplasmic expression of p-P65^{ser536} by IHC and the following combined scores: 6 (1/5 tumors), 4 (3/5 tumors) and 2 (1/5 tumors). Clone 2 tumor cells had the following combined scores for nuclear labeling: 4 (1/5 tumors) and 0 (4/5 tumors). The one Clone 2 tumor with nuclear labeling in the intermediate category also had the largest percentage of tumor cells with an epithelial cell morphology. Clone 2-Kras^{G12V} tumor cells had a significant increase in both the cytoplasmic and nuclear expression of p-p65^{ser536} by IHC when compared with the combined scores for Clone 2 tumor cells. The results from the IHC findings and the semi-quantitative analyses of Clone 2 and Clone 2-Kras^{G12V} orthotopic tumors show that there is a difference in NFκB signaling between the two models and that Clone 2-Kras^{G12V} cells may require this pathway for aspects of invasion and metastasis.

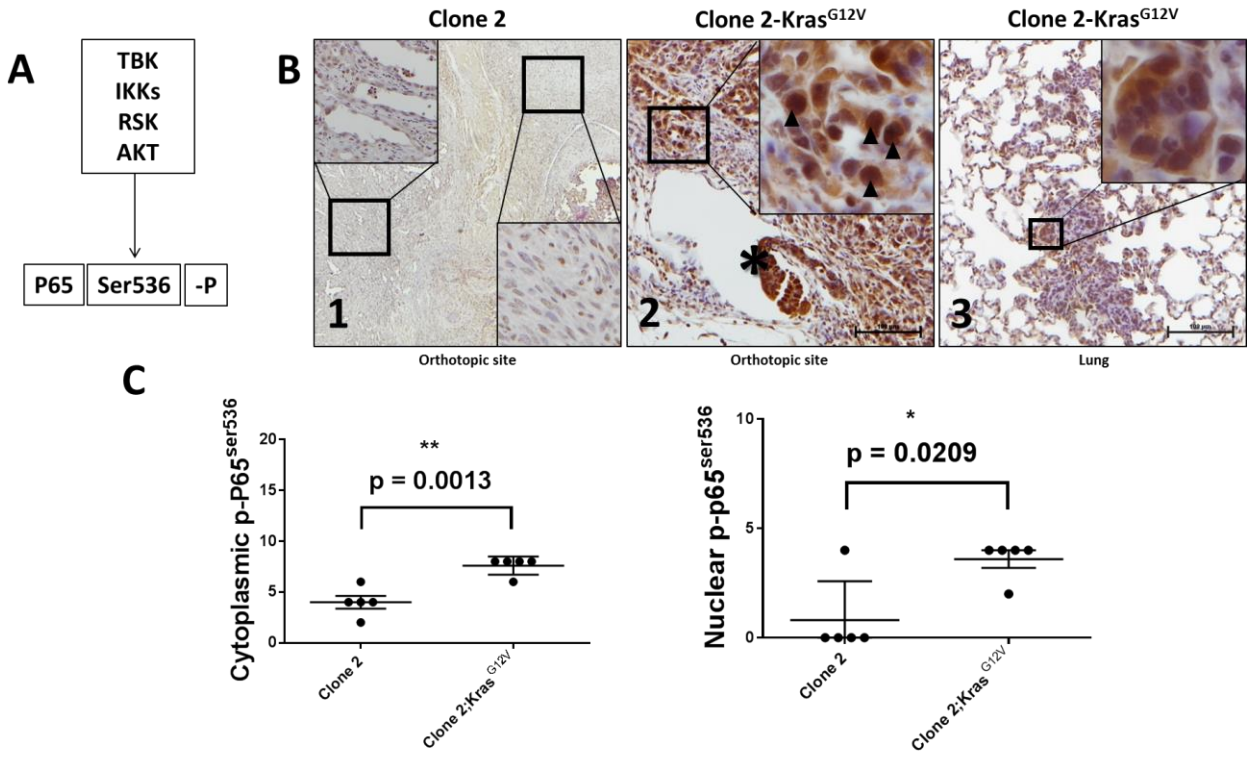

Figure 12. Clone 2-Kras^{G12V} tumor cells with invasive and metastatic potential express phospho-P65^{ser536}

A. Schematic of some kinases that may phosphorylate p65 at serine 536.

B. Immunohistochemical labeling for active NFκB signal transduction. Clone 2 (1) and Clone 2-Kras^{G12V} tumor cells (2) both express the marker at the orthotopic sites. However p-P65 is more highly expressed in cells invading vessels (*) and metastatic Clone 2-Kras^{G12V} cells (3). Image 1, 2X magnification image with 2 inserts taken at 600X. Images 2 and 3, 200x magnification with inserts taken at 600X.

C. Nuclear and cytoplasmic p-P65^{ser536} expression levels assessed by semi-quantitative analyses of Clone 2 (n=5) and Clone 2-Kras^{G12V} (n=5) orthotopic tumors labeled with anti-phospho-P65^{ser536}. The central line represents the mean.

Error bars are ± SEM. Significance was measured using an unpaired T test with Welch's correction.

5.2.c Clone 2-Kras^{G12V} cells require NFκB for *in vitro* colony formation and invasion

To test whether inhibiting the NFκB pathway would affect Ras dependent metastasis, we expressed an inducible IκBα super repressor (SR) into Clone 2-Kras^{G12V} cells. The schematic of the experimental design is shown in Figure 13A. Following doxycycline induction of the genetic repressor mCHERRY fluorescent protein is expressed from an internal ribosomal entry site (IRES). The expression of mCHERRY was used to sort for pure populations for all of the downstream experiments. We analyzed the expression of mCHERRY in both un-induced and induced Clone 2-Kras^{G12V} cells harboring the SR to determine whether there was tight regulation of the inducible system. The fluorescence-activated cell sorting (FACS) analyses showed that MCHERRY expression was only present when the vector was induced with doxycycline in both cell lines (Figure 13B). In western blot analysis of cytoplasmic extracts from both un-induced and induced Clone 2-Kras^{G12V} and Clone 2 cells, IκBα-super repressor infected cells showed an increase in total IκBα expression compared with un-induced cells and in the presence of positive pathway stimulation indicating that the vector was functioning as expected (Figure 13C).

Constitutive NFκB activation is reportedly required for the proliferation and growth of Ras dependent cancers. [54] Because other Ras dependent cancers have a requirement for NFκB we wanted to know whether the growth of Clone 2-Kras^{G12V} cells also would be negatively affected. We observed that there was a decrease in the ability of Clone 2-Kras^{G12V};SR expressing cells to form colonies in soft agar. These

experiments suggested that *in vivo* growth at an orthotopic site could be reduced or absent because there was a potential requirement for constitutive NFκB signaling for cell growth and proliferation by the tumor cells.

Based on the IHC results (Section 5.2.b) we expected that Clone 2-Kras^{G12V} cells would have either a partial or full requirement for NFκB for invasion and metastatic colonization. We observed that Clone 2-Kras^{G12V};SR cells had reduced *in vitro* invasion towards a chemoattractant. Because there were negative effects on tumor cell growth that were observed in the soft agar assay experiments it was difficult to determine whether the reduction in invasion was a direct or an indirect effect from NFκB inhibition. Interesting Clone 2;IKBα-SR induced cells did not have a significant increase in invasive capability nor a reduced ability to form colonies in soft agar suggesting that there was not an *in vitro* requirement for constitutive NFκB signaling, at least in this cell line. We then asked whether there would be similar effects on *in vivo* tumor cell growth and invasive and metastatic potentials of Clone 2-Kras^{G12V} tumor cells that were orthotopically implanted into nude mice.

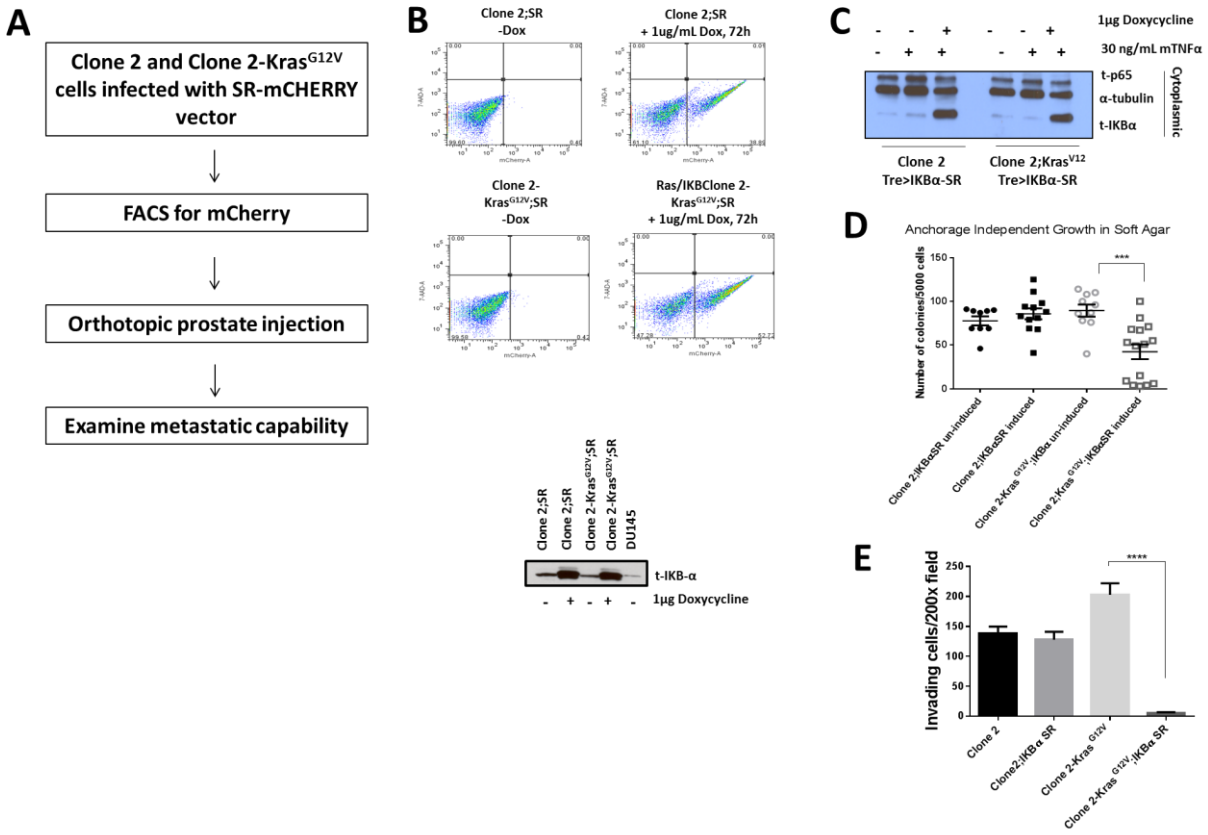

Figure 13. ***In vitro*** **evaluation of NFκB signal transduction, colony forming abilities and invasion following induction of the IKBα super repressor in both Clone 2-Kras^{G12V} and Clone 2 cells**

A. Schematic of the experimental design for testing whether NFκB is important for Clone 2-Kras^{G12V} metastasis.

B. FACS plots showing that there is tight regulation of the inducible system in both Clone 2;SR and Clone 2-Kras^{G12V};SR lines. Western blot analyses of cell lines used for FACS show that induction of the vector increases total IKBα levels.

C. Clone 2>TRE IκBα SR and Clone 2;Kras^{G12V}>TRE IκBα SR cells were treated with 1 µg/ml Dox, stimulated with 30ng/mL of mouse TNFα and assayed for expression of total P65 and IκBα in the cytosol by Western blot. The analyses

show that SR induced cells have increases in total IKBα following the induction of the IκBα super repressor and in the presence of positive pathway stimulation.

D. Clone 2-Kras^{G12V};SR expressing cells have a significant reduction in the number of colonies formed in soft agar compared with Clone 2-Kras^{G12V};SR un-induced cells. *** = < 0.001 using an unpaired T test with Welch's correction.

E. Clone 2-Kras^{G12V};SR induced cells have a reduction in chemotactic directed *in vitro* invasion as tested by Matrigel assay. **** = < 0.0001 using an unpaired T test with Welch's correction.

5.2.d Histopathologic analysis of Clone 2-Kras^{G12V};SR orthotopic tumors

Several lines of evidence suggest that NFκB is both required for Ras driven epithelial tumors to form and for prostate cancer cells to invade and colonize distal sites such as the bone and brain.[49, 58] Our approach to test whether NFκB was important for aspects of growth and invasion and metastasis was to implant SR expressing cells into the prostates of nude mice to see whether tumors would form and if distal metastasis could occur.

NFκB is important for prostate cells to become committed to a luminal epithelial lineage.[97] Clone 2-Kras^{G12V};SR cells formed bi-phenotypic orthotopic tumors that were mixtures of both basal epithelial and luminal epithelial phenotypes. The four highly metastatic Clone 2-Kras^{G12V};SR orthotopic tumors that were harvested at 4-5 weeks following orthotopic implantation were comprised of mixtures of cell types with glandular (adenocarcinoma) or basaloid and squamous (basosquamous) morphologies, and were termed adenosquamous carcinomas. The mice injected with SR induced tumor cells

that were harvested between 4 and 7 weeks were classified as basosquamous carcinomas with poor metastatic potential. The histologies from the tumors in the induced cohort suggested that there was a selective pressure *in vivo* for epithelial cells with a basal cell phenotype to survival and continue to contribute to the bulk of the tumor mass. Furthermore, the basal epithelial cells that were selected had poor metastatic potential. We assayed CK8, CK5 and P63 lineage marker expression in 5 orthotopic tumors. Cells with glandular differentiation were strongly positive for CK8 and did not express either P63 or CK5 by IHC. Tumor areas having both basaloid morphologies were positive for P63 while areas having a squamous morphology were CK5+, P63+/- and CK8-. Tumor cells having a squamous morphology and being immediate adjacent to areas of keratinization did not always label with P63 but did label with CK5. Phospho-P65^{ser536} expression was analyzed in 5 tumors taken between 4-8 weeks and having a basosquamous morphology. We observed that >95% of tumor cells at the orthotopic site had low or undetectable expression of phospho-P65^{ser536} in the nucleus by IHC. Low to moderate expression of phospho-P65^{ser536} was observed in the cytoplasm of the tumor cells, which was consistent with an inhibition of NFκB signaling in the *in vivo* setting. (Figure 14A) As expected the mice that were injected with un-induced cells formed metastatic luminal adenocarcinomas (5/5).

One question was whether the inhibition of NFκB had negatively affected tumor cell growth, which could also have effects on invasion and metastasis. The majority of mice implanted with Clone 2-Kras^{G12V};SR cells formed tumors (15/21). Ki-67 labeling of orthotopic tumors harvested in a 4-7 week time period identified regions of tumor cell proliferation consistent with growth when the super repressor was continuously induced

(d0) or induced 14 days (d14) following implantation. (Figure 14B) Thus, we also concluded that NFκB was not entirely required for *in vivo* tumor formation.

In the tumors that formed we observed a decrease in lymphovascular invasion and the metastatic colonization of the lungs in a subset of mice that correlated with a significant increase in nude mouse survival from the *in vivo* studies. (Figure 14C) Four out of the 21 mice that were taken approximately 4 and 5 weeks post implantation developed lung metastases that were grossly visible at the time of necropsy. The majority of tumors (17/21 mice) had no evidence of distal disease or had small numbers of tumor cell emboli trapped within the pulmonary capillaries, which did not represent the ability to fully metastasize and grow within a novel microenvironment. Therefore we concluded that NFκB was important for aspects of Ras dependent metastasis but it was not entirely necessary.

We also observed that metastatic potential was inversely correlated with the phenotype of the orthotopic tumors. The four mice with metastases formed orthotopic tumors that had an adenocarcinoma morphology. These findings suggested three possibilities: 1) the tumor cells no longer stably expressed the vector, 2) NFκB signal transduction was not sufficiently suppressed in the *in vivo* setting or 3) the *in vivo* selection for cells with a basal lineage simultaneous selected for cells with a reduced potential to metastasize from an orthotopic site. Thus our findings show that Clone 2-Kras^{G12V} cells have certain requirements NFκB for both luminal lineage commitment and for efficient invasion and metastasis from an orthotopic site.

5.2.e Histopathologic analysis of Clone 2;SR orthotopic tumors

We also tested whether induction of the SR repressor would affect the growth, lineage characteristics and metastatic potential of Clone 2 cells. Clone 2;SR cells were able to form tumors following orthotopic implantation (11/15) as suggested by the soft agar assay results (Figure 13D). The orthotopic tumors formed from the vector induced cells had basosquamous and adenocarcinoma histologies that were comparable to the Clone 2-Kras^{G12V};SR tumors indicating that induced cells retained at least the bi-lineage potential of the Clone 2 parental cell line. A subset of mice (5/11) formed tumors that only had a basosquamous morphology. Again we assayed CK8, CK5 and P63 lineage marker expression in three orthotopic tumors. Areas of the tumors having a basaloid morphology were positive for P63, while tumor cells having a squamous phenotype variably expressed P63 but were strongly CK5+. Adenocarcinoma morphologies, where present, were positive for CK8 only. (Figure 14A) Phospho-P65^{ser536} expression was analyzed in 4 tumors taken between 4-8 weeks and having a basosquamous morphology. Tumor cells had either low or undetectable expression of phospho-P65^{ser536} in the nucleus with low or moderate expression in the cytoplasm. (Figure14A) These data again indicated that the inhibition of NFκB by inducing the SR resulted in the selective pressure for the survival and continued proliferation basal epithelial cells in the *in vivo* setting.

We observed metastasis in 8 out of 11 mice. (Table 9) Metastatic tumor cells also had basaloid and squamous morphologies that were observed within the corresponding orthotopic tumors. The presence of metastatic basal cell populations in the lungs suggested that the inhibition of NFκB likely co-selected for a population basal

cells with metastatic potential. Three out of 9 Clone 2;IKBα-SR un-induced tumors were produced following implantation. All tumors of the tumors in the un-induced group had mixed histologic phenotypes that were commonly observed for Clone 2 tumors including areas with luminal, basosquamous, and sarcomatoid differentiation with osseous and cartilaginous metaplasia. Two out of three of the mice in the un-induced group had lymphovascular invasion and formed metastatic lesions in regional lymph nodes. Thus, these data show that NFκB inhibition in Clone 2 cells leads to the selective survival of basal cells with metastatic potential. Because it appeared that NFκB was not always a requirement for metastasis from an orthotopic site, we questioned whether having constitutive NFκB signal transduction in Clone 2 cells could cause metastasis.

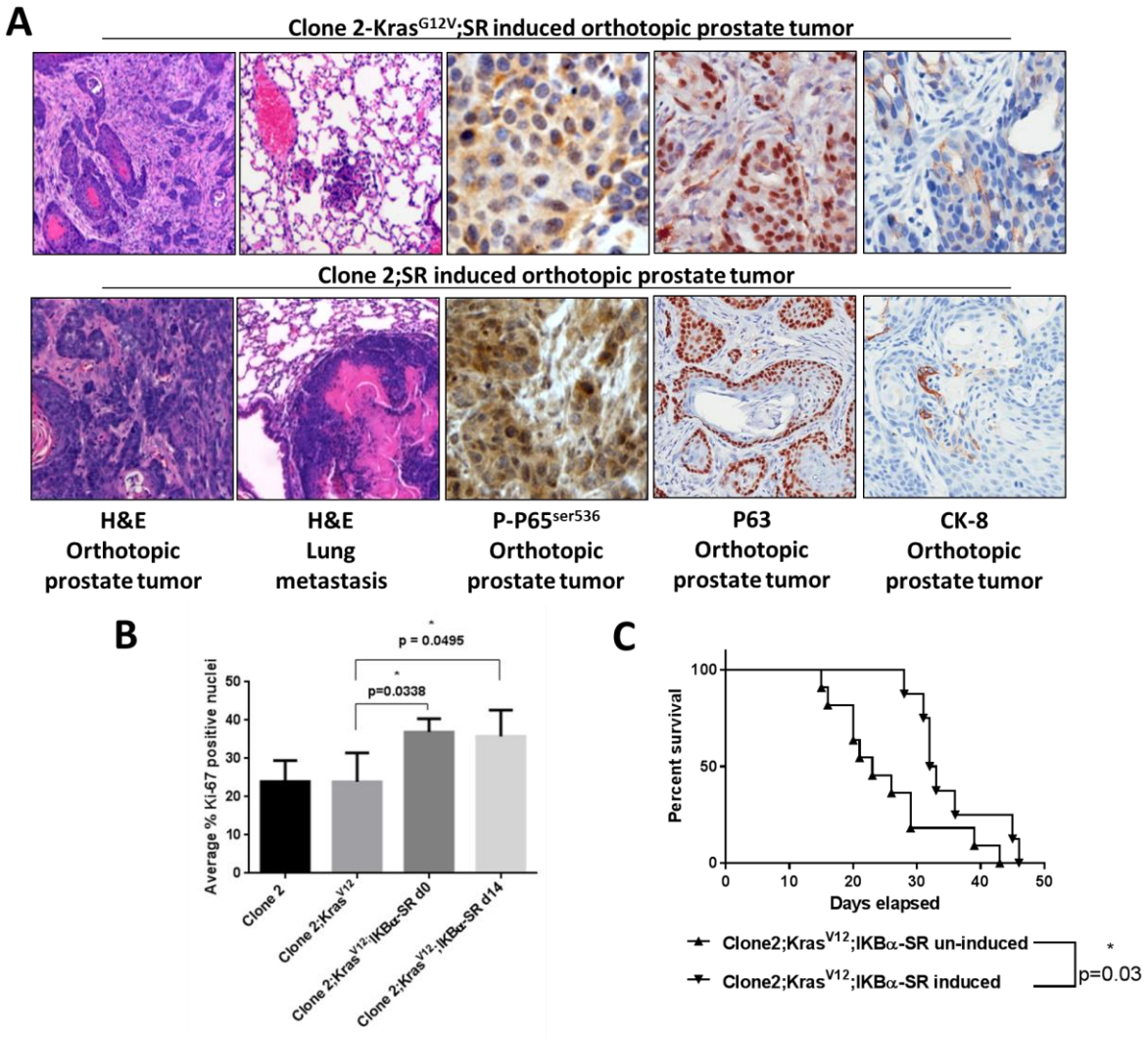

Figure 14. Pathology analyses from Clone 2-Kras^{G12V};SR and Clone 2;SR *in vivo* orthotopic studies

A. Representative histologies of the orthotopic tumors and metastatic lesions following the induction of the SR in Clone 2-Kras^{G12V} and Clone 2 cells. Both Clone 2-Kras^{G12V};SR and Clone 2;SR orthotopic tumor cells express p-P65^{ser536} in the cytoplasm by IHC. The orthotopic tumors have a basosquamous phenotype that is characterized by the expression of P63 in basaloid cells and some cells having a squamous morphology. There is low or absent CK8 expression in basosquamous tumors.

B. Induction of the super-repressor in Clone 2-Kras^{G12V} cells at d0 or d14 following orthotopic implantation does not impair tumor cell growth as measured by the percentage of Ki-67 positive tumor cells. Error bars represent the mean ±SD for n=4-5 mice per experimental condition. Significance determined by an unpaired t-test with Welch's correction.

C. NFκB suppression significantly increases athymic nude mouse survival when the SR is induced in Clone 2-Kras^{G12V} cells. Significance is determined by a Mantel-Cox test.

Table 9. Pathologic analyses for Clone 2;SR and Clone 2-Kras^{G12V};SR orthotopic implantation studies

Animals that were reviewed by histology were included in this table. Results are from two biologic replicates.

Table 9. Pathologic analyses for Clone 2;SR and Clone 2-Kras^{G12V};SR orthotopic implantation studies		
Cell line tested	**Histologic phenotype**	**Metastasis**
Clone 2;IKBα-SR Uninduced	Sarcomatoid carcinoma > luminal adenocarcinoma > basosquamous carcinoma	2/3
Clone 2 IKBα-SR Induced	Adenosquamous or Basosquamous carcinoma	8/11
Clone 2-Kras^{G12V};IKBα-SR Uninduced	Luminal adenocarcinoma	5/5
Clone 2-Kras^{G12V}; IKBα-SR Induced	Adenosquamous or Basosquamous carcinoma	4/21

Table 10. Immunophenotypic analyses of Clone 2;SR and Clone 2-Kras^{G12V};SR tumors

Table 10. Immunophenotypic analyses of Clone 2;SR and Clone 2-Kras^{G12V};SR tumors					
	Clone 2			Clone 2-Kras^{G12V}	
IHC Marker	Adenocarcinoma	Basosquamous	Sarcomatoid	Adenocarcinoma	Basosquamous
CK8	(++++)	(+/-)	(-)	(++++)	(+/-)
CK5	(+/-)	(+++/-)	(-)	(-)	(+++/-)
P63	(-)	(++/-)	(-)	(-)	(++/-)

Data for Clone 2;SR tumors represents 3 tumors/antibody

Data for Clone 2-Kras ^{G12V} ;SR tumors represents5 tumors/antibody

5.2.f Constitutive NFκB expression does not increase Clone 2 metastasis.

Because we observed different results following the suppression of NFκB in our two models we next asked whether activation of this pathway was necessary or merely sufficient for metastasis. We constitutively expressed a mutant Ikkβ^{177E,S181E} vector in Clone 2 cells as the most direct experiment. The Clone 2;Ikkβ line had both increased cytoplasmic IKKβ and nuclear p65 expression by western blot analysis indicating that the vector was functional. (Figure 15A)

Ikkα-mediated NFκB signal transduction can induce breast cancer cells to undergo an EMT that results in metastasis.[101] We observed that the constitutive expression of Ikkβ increased CDH-2 (N-cadherin) expression suggesting that *in vivo* sarcomatoid differentiation would occur. Next we tested the tumor-forming and

metastatic abilities of Clone 2;IκκβSS^{177E,S181E} cells by intracardiac, orthotopic or subcutaneous injection into nude mice. (Table 11) When tested by intracardiac injection (n=4) indications of tumor cell growth were not detected in the heart, lungs or brain. In another set of *in vivo* experiments tumors did not develop following orthotopic implantation into nude mice (n=8). However, when the cells were injected using a subcutaneous route 3/6 nude mice formed tumors. The subcutaneous tumors demonstrated multi-lineage potential that had been observed in previous *in vivo* studies using the Clone 2 line and had the following morphologies: glands lined by a single layer of simple epithelium, complex and stratified squamous epithelium and sarcomatoid differentiation without evidence of osseous or cartilaginous metaplasia. Neither lymphovascular invasion nor metastasis was observed in the lungs in mice following introduction into nude mice by three separate injection methods. Thus we concluded that 1) the constitutive expression of the mutant IκκβSS^{177E,S181E} vector in Clone 2 negatively affected *in vivo* tumor cell growth, 2) did not alter the multi-lineage potential of Clone 2 cells and 3) was insufficient for metastasis.

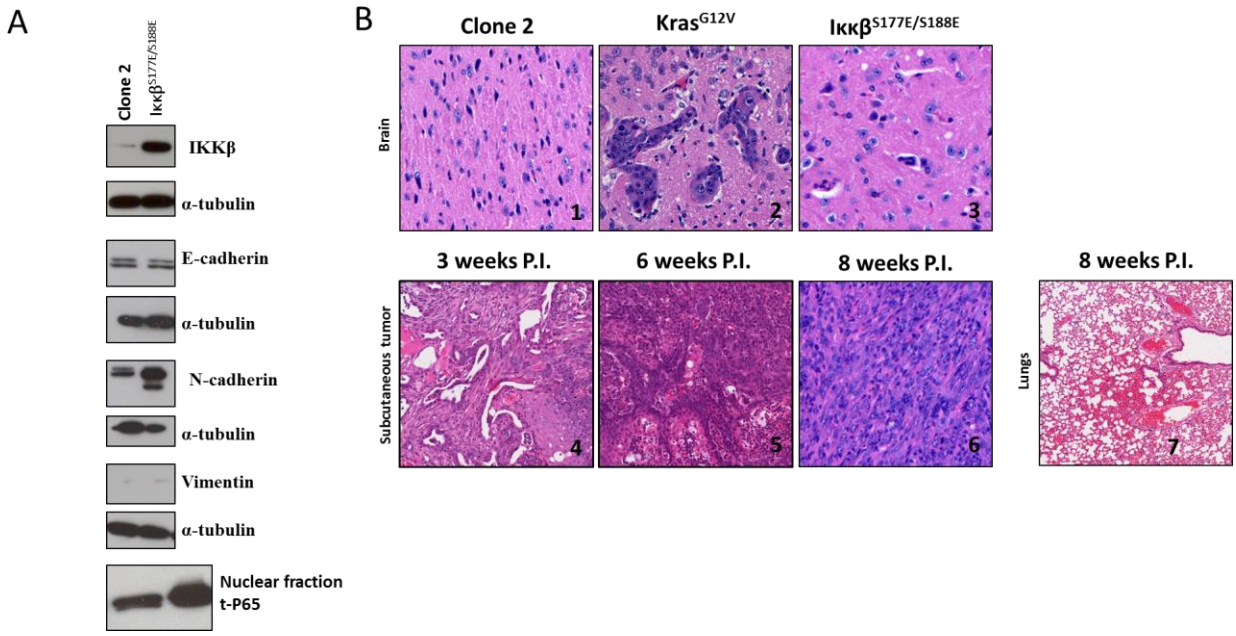

Figure 15. Constitutive NFκB activation does not impair sarcomatoid differentiation nor does it increase the frequency of metastasis by Clone 2 cells

A. Western blot analyses of nuclear and cytoplasmic fractions from Clone 2 and Clone 2-IκκβS177E/S188E cells. Vector expressing cells have an increase in total Iκκβ in the cytoplasmic fraction. This increase is paralleled by another increase in CDH2 (N-cadherin) in the cytoplasm, which is consistent with having an EMT phenotype. Nuclear total-P65 expression is increased in Clone 2-IκκβS177E/S188E cells.

B. Representative images from the intracardiac study (1-3) and the subcutaneous injection study shows that NFκB activation does not increase the frequency of brain metastasis by intracardiac injection. Representative images from the subcutaneous study (4-6) show that NFκB activation results in the formation of subcutaneous tumors with multi-lineage plasticity and poor metastatic potential (7).

Table 11. Pathologic analyses of Clone 2;IκκβS177E,S181E *in vivo* studies

Table 11. Pathologic analyses of Clone 2;IκκβS177E,S181E *in vivo* studies		
Cell line	**Metastasis**	**Injection Method**
Clone 2	0/6	Intracardiac
Clone2;KrasG12V	6/9	Intracardiac
Clone 2;IκκβS177E,S181E	0/4	Intracardiac
Clone 2;IκκβS177E,S181E	0/9	Orthotopic
Clone 2;IκκβS177E,S181E	0/6	Subcutaneous

5.3 Discussion

Investigation of NFκB in the Clone 2-Kras^{G12V} model shows that this pathway is dispensable for *in vivo* tumor growth, but it is critical for invasion and metastasis. However little is known about the participation of NFκB in invasion and metastasis using other experimental prostate cancer models that do not rely on the introduction of Ras activating mutations. Thus, the data generated from the Clone 2 cell line provides a unique opportunity for investigating the additional roles for NFκB regarding metastasis. Since NFκB is known to be important for the survival and continued proliferation of luminal epithelial cells in the mouse prostate,[97] the basal cell immunophenotype that was documented in Clone 2-Kras^{G12V} and Clone 2 orthotopic tumors following induction of the SR is consistent with these previously published studies.

Work performed with a Ras-driven lung cancer model show a requirement for NFκB for tumors to develop in mice.[58] We also observed that there was a growth requirement that could be measured as a reduction in the formation of colonies by Clone 2-Kras^{G12V};SR induced cells when placed into inert soft agar. However, most Clone 2-Kras^{G12V};SR induced tumors formed at orthotopic sites, indicating that this pathway is not entirely required in the *in vivo* setting. Additionally we showed that Clone 2-Kras^{G12V} ;SR orthotopic tumors have an increase in the expression of Ki-67, a marker of cellular proliferation. Previously published work using the *PB-Cre4+; Pten$^{fl/fl}$;Trp53$^{fl/fl}$* model, tumor organoids and serially propagated primary tumor cell populations all show that the locally aggressive tumors that arise from the combined loss of *Pten* and *Trp53* in mouse prostate epithelial cell populations are attributed to two possibilities: 1) the

decreased senescence of terminally differentiated populations forming the bulk of the neoplasm and also 2) from the amplification of and continued renewal of the bulky tumor mass over time by cells having a stem and progenitor cell phenotype.[3, 4, 85] Therefore, given the advanced genetic changes that are already present in Clone 2 cells these events probably select for a growth advantage in the *in vivo* setting, so it is unlikely that suppressing the canonical arm of NFκB in either cell line would have entirely prevented the formation of tumors at an orthotopic site.

We also investigated whether Clone 2-Kras^{G12V} cells required NFκB for metastasis. The results from the Matrigel invasion assays suggested that Clone 2-Kras^{G12V} tumor cells would have reduced *in vivo* invasive potential. At first this reduction in invasion was hypothesized to be a result of the negative effects on growth following NFκB inhibition by the super repressor and as measured by the soft agar assays. However, when Clone 2-Kras^{G12V} ;SR orthotopic tumors formed and the frequency of lung metastasis was greatly reduced, these findings implicated NFκB as having a more direct role regarding the full invasive and metastatic potential of Ras dependent Clone 2 cells. More broadly we also asked whether NFκB was necessary or merely sufficient for *Pten;Trp53* deficient cells to metastasize. The *in vivo* data from the control Clone 2;SR line and the Clone 2-Ikkβ studies showed that this pathway is not entirely necessary for metastasis. These data also highlight that Clone 2-Kras^{G12V} cells probably have a unique requirement for NFκB regarding aspects of tumor cell invasion and metastasis that is similar to the findings from models of Ras-driven prostate cancer.[49]

As discussed in Chapters 3 and 4 the complete EMT tumor phenotype that is observed in both the *Pten;Trp53* mouse model and from tumors formed following the orthotopic implantation of the clonally derived murine cell lines is not correlated with having robust metastatic potential. However the introduction of a strong oncogenic driver such as $Kras^{G12V}$ increases the frequency of metastasis by Clone 2 cells, and this increased frequency of metastasis correlated well with the loss of sarcomatoid differentiation, which marks a complete EMT phenotype. (Chapter 3) Again, we observed the same correlation for the Clone 2;SR experiments. Clone 2;SR cells formed either adenosquamous or basosquamous carcinomas and had lung metastases following orthotopic implantation without clear evidence of a complete EMT phenotype. Additional experiments using the Clone 2-Ikkβ cell line also showed that having constitutive activation of NFκB selects for multi-lineage tumors with a complete EMT phenotype and poor metastatic potential. Again, multi-lineage plasticity *in vivo* does not correlate with the development of *in vivo* metastasis as tested in *Pten;Trp53* deficient murine prostate cancer cells. While these *in vivo* studies are limited in their sample size together the data presented in Chapter 5 show the complexities of NFκB signaling. The data also show that there are several epithelial cell populations that are under selective pressure following the activation or inhibition of NFκB signaling, and each of these populations have different metastatic abilities. Currently from this data we can conclude that 1) NFκB is one pathway that is important for the complete EMT tumor phenotype in the *Pten;Trp53*KO mouse model and 2) intact NFκB signaling can be protective again the development of metastatic disease in advanced tumors that do not have Ras dysregulation.

In summary these studies have established a role for NFκB regarding the invasive and metastatic potential of Clone 2-Kras^{G12V} cells, provided additional rationale for studying NFκB in other experimental prostate model systems, and confirmed that NFκB is important for the selection of *Pten;Trp53* deficient cells defined as having a luminal lineage commitment. These studies show initial experimental evidence that less toxic NFκB inhibitors may benefit some prostate cancer patients, especially those patients that have cancers containing a Kras mutation. The studies with the Clone 2;SR line also show that the inhibition of NFκB can lead to experimental metastasis. Therefore additional studies should be carefully undertaken to first understand the specific role for NFκB in different models of prostate cancer in order to define when it is most appropriate to target this pathway in human prostate tumors. Therefore both the Clone 2-Kras^{G12V} and Clone 2 lines represent important resources for studying questions centered around NFκB and prostate cancers.

CHAPTER SIX: Lineage changes in Clone2-Kras^{G12V} cells following the suppression of TGFβ and NFκB signal transduction

6.1 Introduction

The studies that are described in Chapters 4 and 5 using the Clone 2-Kras^{G12V} and Clone 2 models have revealed that both the TGFβ and NFκB pathways select for epithelial cell populations that are committed to the luminal lineage. Evidence from other experimental prostate cancer models also shows that both pathways work in a hierarchical way through Notch to affect cell fate choices, specifically luminal differentiation.[97, 105]

In this chapter we more directly investigate whether TGFβ or NFκB have a role in luminal epithelial cell differentiation in the Clone 2-Kras^{G12V} model. These studies indicate that both TGFβ and NFκB are important for Clone 2-Kras^{G12V} cells to become fully committed to a luminal epithelial cell lineage. Additionally we also show that Notch is important for Clone 2-Kras^{G12V} cells to remain fully committed as luminal cells. However a direct relationship between TGFβ and Notch is not observed in this model. Thus these studies show that all three pathways are important for luminal lineage commitment as tested in the Clone 2-Kras^{G12V} model and serve as a foundation for future studies of each pathway regarding cell fate decisions.

6.2 Results

6.2.a Pharmacologic inhibition of TGFβ in Clone 2 and Clone 2-Kras^{G12V} cells increases the expression of basal lineage markers.

Studies conducted in Clone 2 and Clone 2-Kras^{G12V} cells showed that TGFβ was important for tumor cells to maintain a luminal lineage commitment. (Chapter 4) To test whether TGFβ was important for remaining committed as a luminal cell we treated both Clone 2 and Clone 2-Kras^{G12V} cells with an ALK5 inhibitor at a dose of 180nM (ALK5i). This ALK5 inhibitor was chosen because it was reported to be more selective for a TGFβRII mediated response. The absolute numbers of cells expressing CK8, CK5 and P63 or with co-expression of these lineage markers were determined by counting at least 200 cells. The pharmacologic inhibition of ALK5 mediated signaling induced a bi-lineage phenotype with the following lineage marker expression patterns: CK8+ or P63+ only cells with subpopulations of cells co-expressing combinations of CK8 and P63. (Figure 16A) These data showed by a second method that TGFβ is important for luminal lineage commitment in the tested lines.

One question that remained to be determined was whether P63+ only cells would revert to a CK8+ only immunophenotype when treatment stopped. In this experiment Clone 2-Kras^{G12V} cells were first treated with the ALK5i for 2 weeks. Treatment ceased after this time point, and the cells were then continuously cultured into PrEBM media + supplements for an additional 2 weeks. Again the absolute numbers of cells expressing CK8, CK5 and P63 or with co-expression of these lineage markers were determined by counting at least 200 cells. We observed that even after the cessation of the treatment there were still cell populations that singly expressed either P63 or CK5, as well as bi-

potential populations that co-expressed these markers with CK8. There were also cell populations that only expressed CK8. In the *Pten;Trp53* knockout mouse model system the acquisition of CK5 expression by serially propagated tumor cells is correlated with having an irreversible basal epithelial cell phenotype.[85] While it was unclear whether these bi-potential populations could become recommitted as luminal cells the presence of CK5 positive cells indicated that there were populations of tumor cells that could not re-acquire a luminal phenotype. Therefore these data indicated that the basal lineage commitment by tumor cells after short term TGFβ inhibition was not an entirely reversible phenotype.

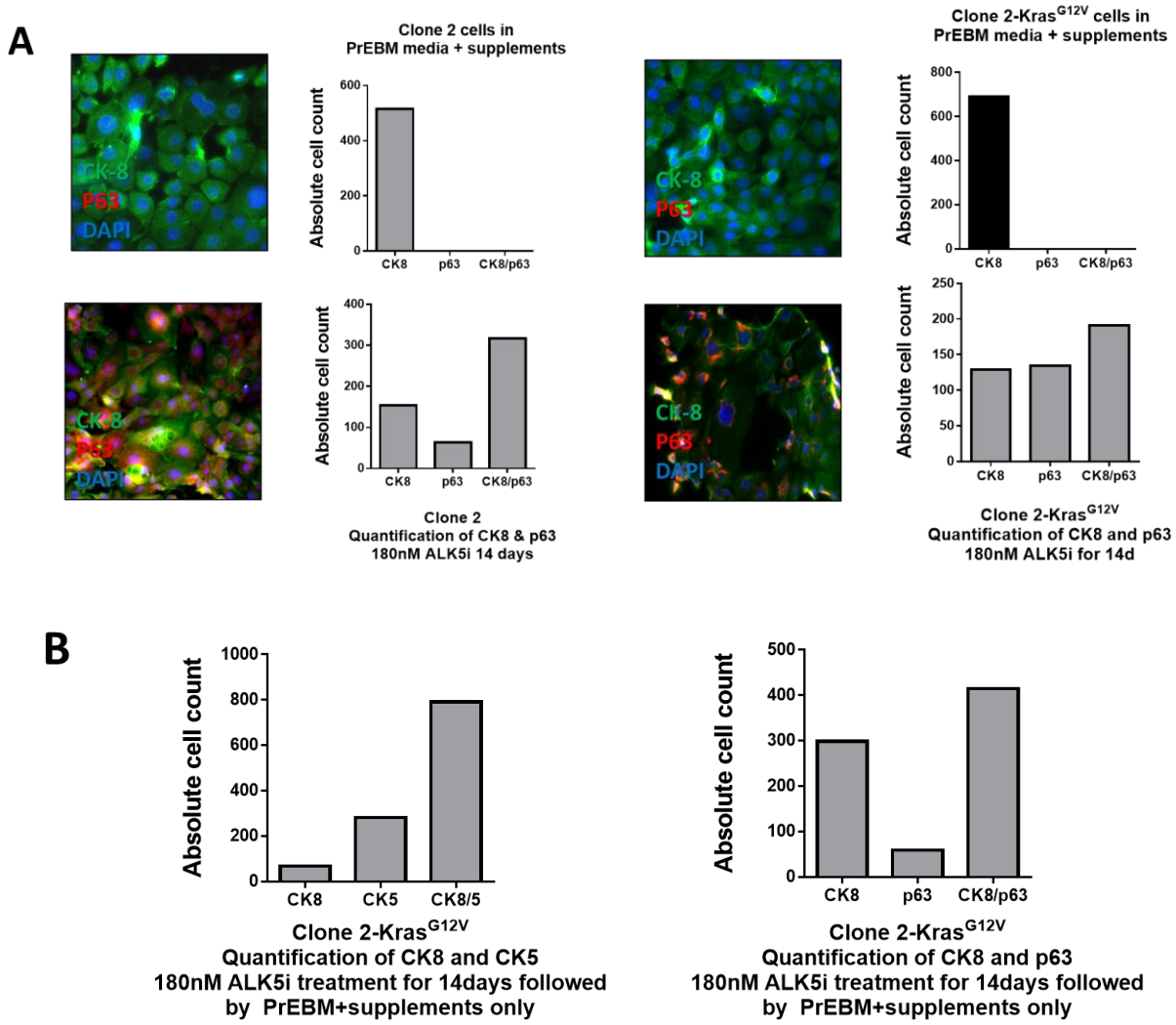

Figure 16. Pharmacologic inhibition of TGFβ signaling in Clone 2 and Clone 2-Kras^{G12V} cells increase the expression of basal lineage markers.

Representative immunofluorescence images and graphs showing the quantification of chamber slides stained with prostate lineage markers.

C. Quantification of CK8 and P63 expression by Clone 2 and Clone 2-Kras^{G12V} cells in basal prostate media and following the addition of an ALK5 inhibitor in the culture media.

D. Quantification of CK8, P6 and CK5 expression by Clone 2-Kras^{G12V} cells 14 days after stopping the ALK5 inhibitor treatment shows the expression of basal cell markers by sub-cultured cells is still observed.

6.2.b Pharmacologic inhibition of NFκB in Clone 2 and Clone 2-Kras^{G12V} cells increases the expression of basal lineage markers

As described in Chapter 5 we observed that Clone 2 and Clone 2-Kras^{G12V} cells also had a requirement for NFκB in order to be remain committed as luminal epithelial cells. These data are supported by other studies, which show that in the mouse NFκB is one pathway that is important for the survival and proliferation of luminal epithelial cells.[97] Again we used a pharmacologic inhibitor of this pathway as a second method for testing whether NFκB was essential for luminal lineage commitment. Both Clone 2 and Clone 2-Kras^{G12V} cells were treated with an Ikkβ inhibitor at a dose of 180nM (IKBαi). This Ikkβ inhibitor was chosen because it was reported to be more selective for an IKBα-mediated signal. The absolute numbers of cells expressing CK8 and P63 or having co-expression of these lineage markers were determined by counting at least 200 cells. The pharmacologic inhibition of Ikkβ mediated signaling induced basal lineage marker expression that was characterized by combinations of CK8+ and P63+ only cells with subpopulations of cells co-expressing the markers. (Figure 17A) These data confirmed that NFκB was important for cells to retain full luminal lineage commitment.

We also assayed whether cells that had an immunophenotype that was characteristic for a basal epithelial cell would be able to differentiate back towards a luminal lineage after the treatment was stopped. In a similar manner to the method

described in 6.2.a, Clone 2-Kras^{G12V} cells were first treated with the Iκκβ inhibitor for 2 weeks and then continuously cultured into PrEBM media + supplements for an additional two weeks. Again the absolute numbers of cells expressing combinations of CK8, CK5 and P63 were determined by counting at least 200 cells. We observed that even after the cessation of the treatment in the two-week period to follow there were still cell populations that expressed either P63 or CK5 alone, as well as bi-potential populations that co-expressed these markers with CK8. Therefore these data indicated that the commitment to the basal lineage was not an entirely reversible phenotype. We observed a similar result following genetic repression of NFκB. (Figure 18) In this one example, induction of the super-repressor to sort for mCHERRY+ cells resulted in a subpopulation of tumor cells that expressed basal lineage makers, which had not been previously detected in either the parental or vector un-induced lines. Thus these results show that NFκB is a second pathway regulating basal and luminal lineage commitment choices for *Pten;Trp53* deficient prostate cancer cells. Because both TGFβ and NFκB suppression had similar phenotypic effects on both Clone 2 and Clone 2-Kras^{G12V} cells we conducted further experiments to determine whether these pathways acted in a hierarchical or parallel manner.

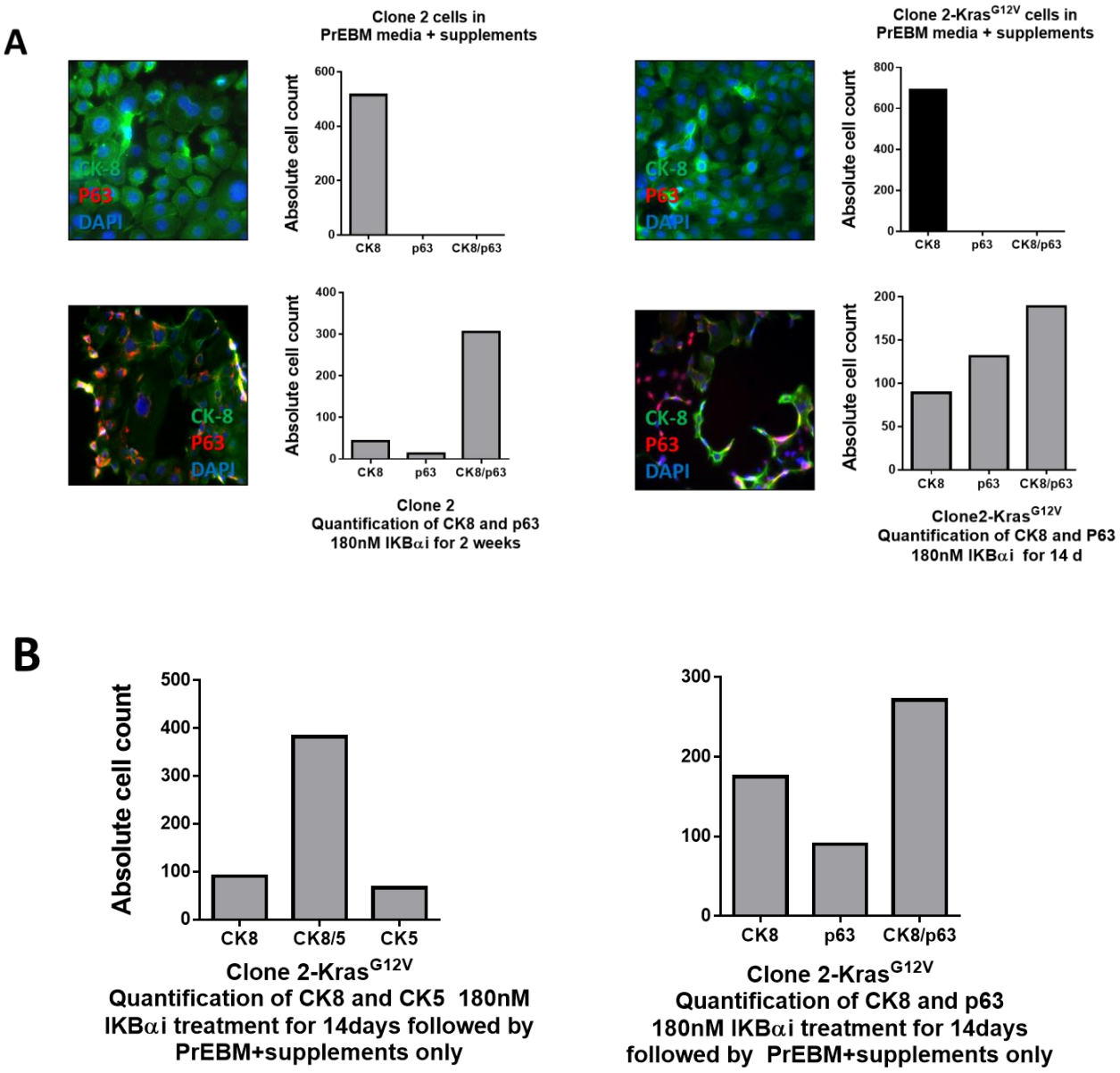

Figure 17. Pharmacologic inhibition of NFκB in Clone 2 and Clone 2-Kras^{G12V} cells increases the expression of basal lineage markers.

Representative immunofluorescence images from the quantification of tumor cells plated onto chamber slides shows that pharmacologic repression of this pathway results in an increase in P63 expression as assayed by IF labeling.

A. Quantification of CK8 and P63 expression by Clone 2 and Clone 2-Kras^{G12V} cells in basal prostate media and following the addition of an Ικκβ inhibitor (noted as IKBαi in the figure) in the culture media. Quantification of CK8 and P63 expression by Clone 2 and Clone 2-Kras^{G12V} cells following treatment with an Ικκβ inhibitor in basal prostate media for 14 days.

B. Quantification of CK8, P6 and CK5 expression by Clone 2-Kras^{G12V} cells 14 days after stopping the Ικκβ inhibitor treatment shows that the expression of basal cell markers still persist in sub-cultured lines.

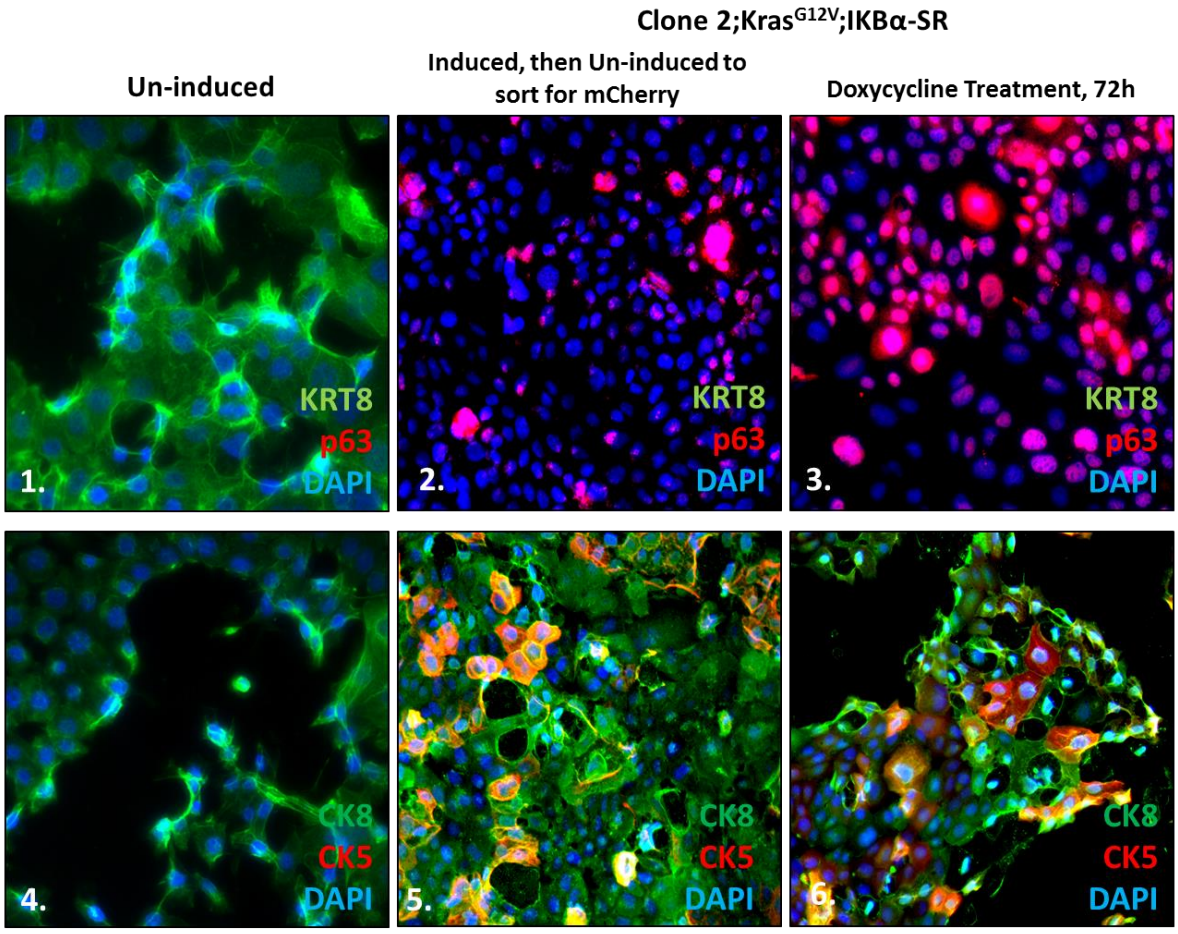

Figure 18. Genetic suppression of NFκB signaling in Clone 2-Kras^{G12V} cells increases the expression of basal lineage markers.

Representative immunofluorescence images from Clone 2-Kras^{G12V} cells containing the IKBα-SR that was un-induced (1, 4), induced for mCHERRY sorting only (2, 5) and then induced for 72 hours in 1μg of doxycycline (3, 6). Images show that genetic repression of NFκB for short periods of time increase basal lineage marker expression. This finding is comparable to the results following pharmacologic suppression of the pathway and the *in vivo* studies with the cell lines containing the induced genetic repressor.

6.2.c Nuclear translocation of p65 occurs following TGFβ1 stimulation in Clone 2 cells.

Because TGFβ or NFκB inhibition both resulted in the expression of basal lineage markers by Clone 2 and Clone 2-Kras^{G12V} cells we asked whether there was a hierarchical relationship between the two pathways. TGFβ1 stimulation resulted in the nuclear translocation of total P65 in the Clone 2 line but not in Clone 2-Kras^{G12V} cells (Figure 19). This finding indicated that TGFβ and NFκB did not have a hierarchical relationship in Clone 2-Kras^{G12V} cells. Because there was not a direct relationship between TGFβ and NFκB we asked whether there was another point of cross talk between the two pathways that could explain why suppression of both pathways both resulted in a basal epithelial cell phenotype. One pathway that represents a point of cross talk between TGFβ and NFκB is Notch.

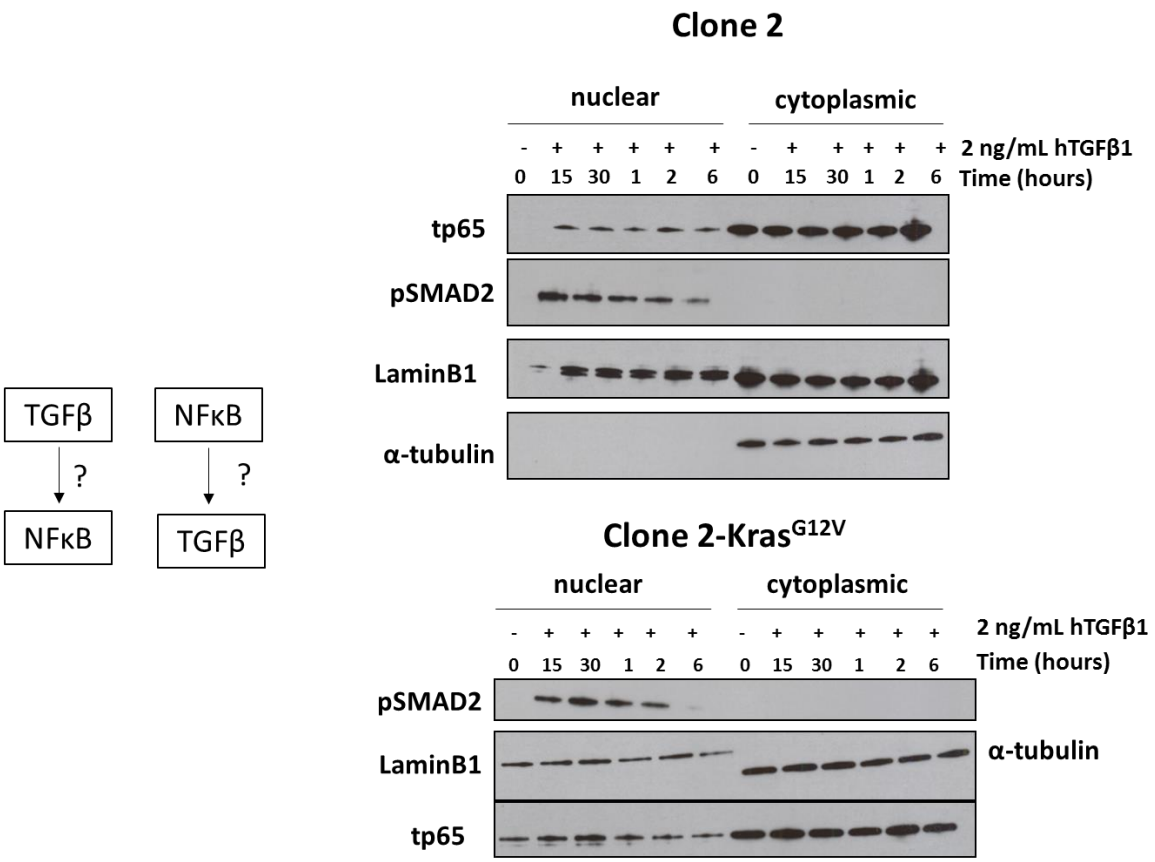

Figure 19. NFκB p65 translocation is TGFβ1 dependent for Clone 2 cells.

Western blot analyses show that TGFβ1 stimulation induces nuclear translocation of total-P65 in Clone 2 cells. In contrast Clone 2-Kras^{G12V} cells have persistent nuclear expression of total-P65 that remains the same even in the presence of TGFβ1 stimulation. These data show that the relationship between TGFβ and NFκB regarding lineage commitment is not linear in Clone 2-Kras^{G12V} cells. Both Clone 2 and Clone 2-Kras^{G12V} cells have nuclear translocation of pSMAD2/3 following TGFβ1 stimulation.

6.2.d Pharmacologic inhibition of Notch in Clone 2 and Clone 2-Kras^{G12V} cells increases the expression of basal lineage markers

TGFβ-mediated Notch signaling is necessary for luminal differentiation and the continued proliferation and survival of luminal committed cells in the mouse prostate.[97] We first treated Clone 2 and Clone 2-Kras^{G12V} cells with DAPT, a ϒ-secretase inhibitor to determine whether pan-Notch inhibition would induce the expression of basal lineage markers. The DAPT inhibitor impairs Notch signaling by all four Notch receptor mediated pathways, so it does not directly show whether there is a specific contribution by one particular arm of the Notch signaling cascade.[106] Treatment at 1μM resulted in Clone 2 and Clone 2-Kras^{G12V} cells that had a bi-potential phenotype that was characterized by combinations of CK8, CK5 and P63 expression (Figure 20A and B). Together these results showed that Notch was a third pathway that can affect luminal lineage commitment as tested in Clone 2 and Clone 2-Kras^{G12V} cells. Often Notch 1 and Notch2 signals are important for epithelial lineage commitment programs in the mouse prostate.[97] Next we tested whether Notch was regulated by TGFβ in the Clone 2-Kras^{G12V} model. We examined this possibility by directly assaying lineage commitment markers CK8 and P63 by IF labeling in Clone 2-Kras^{G12V} cells expressing a vector for constitutive Notch1 and also receiving a TGFβ inhibitor in the cell culture media.

6.2.e Pharmacologic inhibition of TGFβ in the presence of constitutive Notch expression does not rescue the luminal phenotype of Clone 2-Kras^{G12V} cells.

To ascertain whether TGFβ may signal through Notch to affect luminal lineage commitment we stably expressed a constitutive lentiviral vector expressing the Notch1

intracellular domain (NICD) into Clone-Kras^{G12V} cells. We hypothesized that if Notch was a direct downstream target for a TGFβ-mediated program then its constitutive activation would result in Clone 2-Kras^{G12V} cells maintaining a luminal immunophenotype even when the TGFβ pathway was impaired. After two serial FACS experiments Clone-Kras^{G12V} ;NICD expressing cells were plated on 8 well chamber slides and treated with the TGFβ inhibitor SB431542,which suppresses TGFβRI signaling. The expression of luminal and basal markers were examined at either 1, 3, 7 or 14 days of treatment. The continued treatment resulted in a growing number of tumor cells expressing basal cell lineage markers P63+ or CK5+ cells over a period of 14 days (Figure 20C and D). The results from this pilot experiment suggested that TGFβ may not signal through Notch 1 to maintain a luminal epithelial phenotype. Thus, because an effect was not observed further experiments were not pursued.

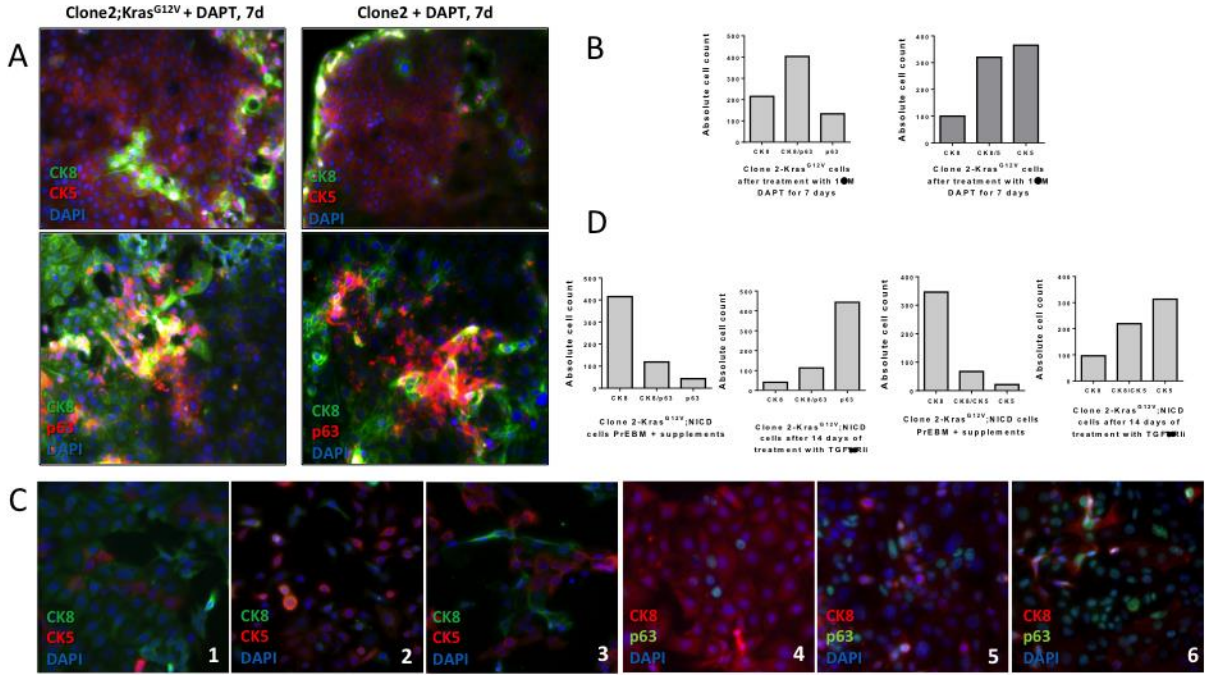

Figure 20. Assessment of lineage marker expression following experimental manipulations to the Notch and TGFβ pathways

A. Representative IF images of Clone 2 and Clone 2-Kras^{G12V} cells treated with DAPT for 7 days shows that a large number of tumor cell express either CK5 or P63.

B. Quantification of chamber slides for CK8, CK5 and P63 expression by Clone 2-Kras^{G12V} cells following treatment with DAPT for 7 days shows that there is an increase in the expression of basal lineage markers over time.

C. Representative images of Clone 2-Kras^{G12V};NICD expressing cells labeled for CK8, CK5 and P63 before treatment (1, 4) following treatment for 1 day (2, 5) or after 7 days (3, 6) of being placed in culture with a TGFβRI inhibitor.

D. Quantification of chamber slides at the day 14 time point for CK8, CK5 and P63, which show that loss of TGFβ correlates with an increase in basal markers even when Notch1 is present.

6.3 Discussion

The majority of prostate tumors (>95%) are overwhelmingly CK8+ luminal adenocarcinomas and do not contain P63+ CK5+ basal cells, although rare P63+ subtypes do exist.[107] Because there is paucity in the diversity in tumor phenotypes that are observed in primary cancers that arise within the prostate, there are many open questions regarding the cell of origin of prostate tumors. There are also more basic questions that are centered on the identification of different classes of stem and progenitor cells that give rise to the unique cellular populations composing the normal prostate glands of the mouse and the anatomic zones in man. Many studies conducted in mice and by propagating cells taken from either mouse or human prostate tumors have suggested that luminal epithelial cells may not possess the same progenitor cell activities unlike basal epithelial cells, thus making basal cells the putative cell of origin for tumors [108, 109]. However other reports have suggested the opposite and that it is the increased susceptibility to anoikois by luminal cells that makes them difficult to culture and study with regards to their self-renewal properties.[97] A recent study using FACS sorted primary tumor cells taken from *Pten;Trp53* deficient mouse adenocarcinomas also shows that difference classes of luminal progenitor cells exist within the mouse prostate and can be studied *in vitro* and *in vivo*.[85]

Because we observed that Clone 2-Kras^{G12V} tumor cells could transdifferentiate between luminal and basal epithelial cell lineages we asked whether TGFβ or NFκB were acting in either a parallel or hierarchical manner to affect this phenotypic switch. Other mouse models have shown that TGFβ can signal through both Notch and NFκB in order for cells to become committed to a luminal lineage.[97, 105] However,

this same relationship could not be determined for the Clone 2-Kras^{G12V} model. Thus, the data in this chapter only shows that TGFβ, NFκB and Notch are important for aspects of luminal lineage commitment in the tested models.

While no tissue-specific markers for luminal progenitor cells have been identified, expression of keratins 8 and 18 are most characteristic of these cells. In contrast the expression of keratins 5 and 6 characterize cells composing complex stratified epithelia and also label prostatic epithelial cells that are reported to have a transit-amplifying phenotype as well as terminally committed basal cells as assessed both in the prostates of mice, man and experimental prostate cancer models derived from these two species.[110-112] *Trp63,* a transcription factor that is related to the tumor suppressor *Trp53*, is reported to represent an earlier stage epithelial progenitor cell that can give rise to both basal and luminal committed cells in the mouse.[113] The studies discussed in Chapter 3 initially demonstrated that Clone 2-Kras^{G12V} tumor cells were terminally committed as a luminal epithelial cells because the orthotopic tumors initially lacked any clear evidence for multi-lineage or bi-lineage differentiation, as they did not have detectable basal cell populations or a complete EMT tumor phenotype either *in vitro* or in the *in vivo* setting. Furthermore this phenotype strongly correlated with having a robust metastatic potential. However, the suppression of either TGFβ or NFκB signaling in Clone–Kras^{G12V} cells by two different methods, genetic repression or pharmacologic inhibition, both show that this cell line also contains progenitor cell populations, like Clone 2, that have bi-lineage differentiation potential. Additionally each of the lineages that emerge following the inhibition of either TGFβ or NFκB have unique metastatic potentials.

The differences between the *in vivo* metastatic outcomes as described in the dnRII and SR studies with Clone 2-Kras^{G12V} cells suggest that there are several different kinds of progenitor epithelial populations that cannot be effectively separated by CK8, P63 or CK5 expression, and each of these populations have dramatically different biological behaviors. Therefore it is reasonable to hypothesize that the basal cells that resulted from the suppression of NFκB in Clone 2-Kras^{G12V} tumors may represent a different class of basal cells that cannot effectively metastasize and can be targeted to reduce bulky tumor growth at the primary site. Likewise identifying and then targeting basal cells with metastatic potential that are selected for following inhibition of TGFβ receptor and/or SMAD signaling in Clone 2-Kras^{G12V} tumors may be an effective strategy for preventing or reducing metastatic disease. Therefore the findings from these studies will require validation in other aggressive prostate cancer models and from data generated using patient specimens. In all, the data in Chapters 4-6 raises a more central question of whether there are pathway specific markers that can be used to identify the unique basal and luminal progenitor populations that are present within Clone 2-Kras^{G12V} cells to show which cells are more likely to colonize distal sites or remain localized to the prostate. By knowing this information, one can then ask whether these markers may also represent safely druggable targets. Therefore both the Clone 2 and Clone 2-Kras^{G12V} cell lines not only serve as useful tools for asking questions about the pathways that regulate the orderly progression of the epithelial lineages composing the mouse prostate but also how cells selected downstream of specific pathway signals have biological behaviors that may be predictable and can be exploited in order to reduce either the burden of disease or cure the patient.

CHAPTER SEVEN: Conclusions

Many studies of metastatic prostate cancers rely on data that is derived from three human prostate cancer cell lines, LNCaP, DU145 and PC3. While these cell lines are the most readily available models for *in vitro* and *in vivo* studies, they are not entirely representative of the biology of the tumor cells that are selected for when cancers are diagnosed earlier and treated with second generation androgen deprivation therapies or immunomodulatory drugs. Because the diagnosis of metastatic prostate cancer is correlated with a poor patient outcome it is important to continually generate and validate new experimental systems that can be used to investigate how and why these changes in tumor biology arise. Thus the studies using the Clone 2-Kras^{G12V} and Clone 2 cell lines that are reported in this thesis show that these two models can be used to study and answer questions regarding basic prostate tumor biology and serve as good preclinical models for testing combinatorial therapies.

In summary, these studies have characterized novel models of lethal, metastatic prostate cancers and have shown that each genetically modified cell line is suitable for asking questions about the lineage commitment of cancer cells that have a stem and progenitor phenotype and the specific roles for pathways regarding metastasis in the *in vivo* setting. First, this work has established a role for oncogenic Kras for increasing the frequency of metastasis by *Pten;Trp*53 deficient murine prostate cancer cells as tested in the Clone 2 cell line. Second, these studies determined that TGFβ is not entirely required for metastasis as tested in the Clone 2-Kras^{G12V} and Clone 2 models. Thirdly, work with Clone 2-Kras^{G12V} cells shows that NFκB is important for Ras-mediated invasion and metastasis. Finally the work in this dissertation shows that TGFβ, NFκB

and Notch are important for luminal lineage commitment choices as tested in Clone 2 and Clone 2-Kras^{G12V} cells. A graphical abstract summarizing several key findings from Chapters 3 through 6 is provided in Figure 21. While these models can be used to study the TGFβ, NFκB and Notch pathways, which all have shared importance regarding both cell fate decisions and in the acquisition of metastatic potential, the cell lines may also aid in the identification of new pathways or pathway relationships that result in lethal disease phenotypes. Thus, both the Clone 2 and Clone 2-Kras^{G12V} lines represent important resources for the prostate cancer research community.

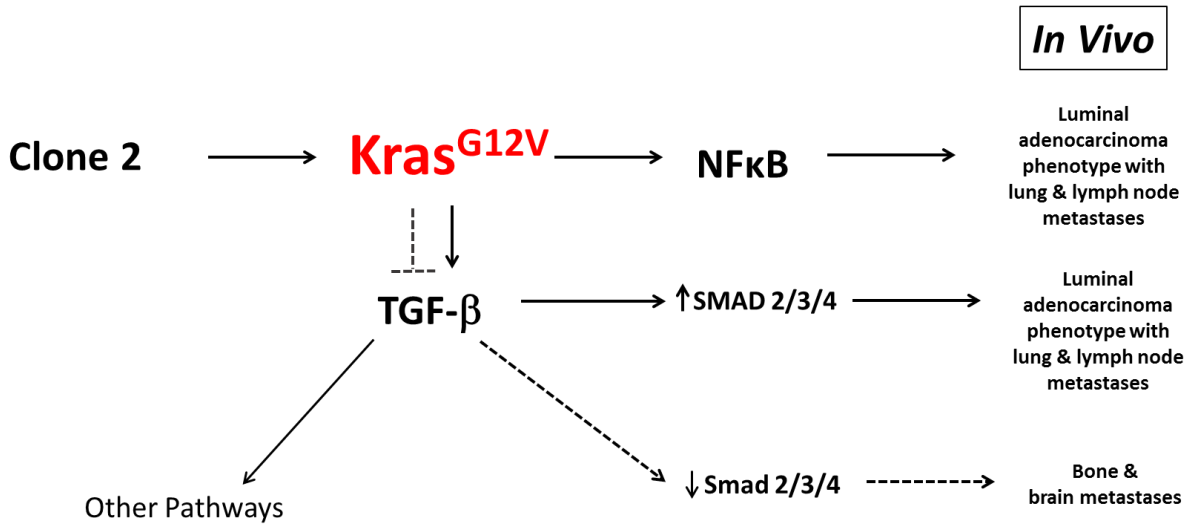

Figure 21. Working model for the role of TGFβ and NFκB for lineage commitment and metastatic potential of Clone 2-Kras^{G12V} as examined in the *in vivo* setting

BIBLIOGRAPHY

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1. Howlader N, N.A., Krapcho M, Garshell J, Miller D, Altekruse SF, Kosary CL, Yu M, Ruhl J, Tatalovich Z,Mariotto A, Lewis DR, Chen HS, Feuer EJ, Cronin KA (eds), *SEER Cancer Statistics Review, 1975-2012, National Cancer Institute. Bethesda, MD, http://seer.cancer.gov/csr/1975_2012/, based on November 2014 SEER data submission, posted to the SEER web site, April 2015.*

2. Taylor, B.S., et al., *Integrative Genomic Profiling of Human Prostate Cancer.* Cancer Cell, 2010. **18**(1): p. 11-22.

3. Martin, P., et al., *Prostate Epithelial Pten/TP53 Loss Leads to Transformation of Multipotential Progenitors and Epithelial to Mesenchymal Transition.* The American Journal of Pathology, 2011. **179**(1): p. 422-435.

4. Abou-Kheir, W., et al., *Self-Renewing Pten(-/-)TP53(-/-) Protospheres Produce Metastatic Adenocarcinoma Cell Lines with Multipotent Progenitor Activity.* PLoS ONE, 2011. **6**(10): p. e26112.

5. Gandaglia, G., et al., *Distribution of metastatic sites in patients with prostate cancer: A population-based analysis.* The Prostate, 2014. **74**(2): p. 210-216.

6. Loeb, S., et al., *Overdiagnosis and Overtreatment of Prostate Cancer.* European urology, 2014. **65**(6): p. 1046-1055.

7. Humphrey, P.A., *Gleason grading and prognostic factors in carcinoma of the prostate.* Mod Pathol, 2004. **17**(3): p. 292-306.

8. Epstein JI, A.W., Jr., Amin MB, Egevad LL., *The 2005 International Society of Urological Pathology (ISUP) Consensus Conference on Gleason Grading of Prostatic Carcinoma.* Am J Surg Pathol, 2005. **29**(9): p. 1228-42.

9. Hameed, O. and P.A. Humphrey, *Immunohistochemistry in diagnostic surgical pathology of the prostate.* Semin Diagn Pathol, 2005. **22**(1): p. 88-104.

10. Grignon, D.J., *Unusual subtypes of prostate cancer.* Mod Pathol, 2004. **17**(3): p. 316-327.

11. Abrahamsson, P.-A., *Neuroendocrine differentiation in prostatic carcinoma.* The Prostate, 1999. **39**(2): p. 135-148.

12. Mazzucchelli, R., et al., *Rare and unusual histological variants of prostatic carcinoma: clinical significance.* BJU International, 2008. **102**(10): p. 1369-1374.

13. Gleason, D.F. and G.T. Mellinger, *Prediction of prognosis for prostatic adenocarcinoma by combined histological grading and clinical staging.* J Urol, 1974. **111**(1): p. 58-64.

14. Häggman MJ, M.J., Wojno KJ, Oesterling JE., *The relationship between prostatic intraepithelial neoplasia and prostate cancer: critical issues.* J Urol, 1997. **158**(1): p. 12-22.

15. Presti, J.C., *Prostate Biopsy: Current Status and Limitations.* Reviews in Urology, 2007. **9**(3): p. 93-98.

16. Anast, J.W., et al., *Relating biopsy and clinical variables to radical prostatectomy findings: Can insignificant and advanced prostate cancer be predicted in a screening population?* Urology, 2004. **64**(3): p. 544-550.

17. Lucia, M.S., et al., *Pathologic Characteristics of Cancers Detected in the Prostate Cancer Prevention Trial: Implications for Prostate Cancer Detection and Chemoprevention.* Cancer Prevention Research, 2008. **1**(3): p. 167-173.

18. Tomlins, S.A., et al., *Recurrent Fusion of TMPRSS2 and ETS Transcription Factor Genes in Prostate Cancer.* Science, 2005. **310**(5748): p. 644-648.

19. Hieronymus, H., et al., *Copy number alteration burden predicts prostate cancer relapse.* Proceedings of the National Academy of Sciences, 2014. **111**(30): p. 11139-11144.

20. Grasso, C.S., et al., *The mutational landscape of lethal castration-resistant prostate cancer.* Nature, 2012. **487**(7406): p. 239-243.

21. Koh, C.M., et al., *MYC and Prostate Cancer.* Genes & Cancer, 2010. **1**(6): p. 617-628.

22. Sharma, S.V. and J. Settleman, *Oncogene addiction: setting the stage for molecularly targeted cancer therapy.* Genes & Development, 2007. **21**(24): p. 3214-3231.

23. Pagliarini, R., W. Shao, and W.R. Sellers, *Oncogene addiction: Pathways of therapeutic response, resistance, and road maps toward a cure.* EMBO Reports, 2015. **16**(3): p. 280-296.

24. Kim, M.J., et al., *Nkx3.1 Mutant Mice Recapitulate Early Stages of Prostate Carcinogenesis.* Cancer Research, 2002. **62**(11): p. 2999-3004.

25. Bhatia-Gaur, R., et al., *Roles for Nkx3.1 in prostate development and cancer.* Genes & Development, 1999. **13**(8): p. 966-977.

26. Trotman, L.C., et al., *Pten Dose Dictates Cancer Progression in the Prostate.* PLoS Biol, 2003. **1**(3): p. e59.

27. Qian, J., et al., *Loss of p53 and c-myc Overrepresentation in Stage T2-3N1-3M0 Prostate Cancer are Potential Markers for Cancer Progression.* Mod Pathol, 0000. **15**(1): p. 35-44.

28. Collado, M., et al., *Tumour biology: Senescence in premalignant tumours.* Nature, 2005. **436**(7051): p. 642-642.

29. Xu, K., et al., *EZH2 Oncogenic Activity in Castration-Resistant Prostate Cancer Cells Is Polycomb-Independent.* Science, 2012. **338**(6113): p. 1465-1469.

30. Irshad, S., et al., *A Molecular Signature Predictive of Indolent Prostate Cancer.* Science Translational Medicine, 2013. **5**(202): p. 202ra122.

31. Markert, E.K., et al., *Molecular classification of prostate cancer using curated expression signatures.* Proceedings of the National Academy of Sciences, 2011. **108**(52): p. 21276-21281.

32. Tomlins, S.A., et al., *Urine TMPRSS2:ERG Fusion Transcript Stratifies Prostate Cancer Risk in Men with Elevated Serum PSA.* Science Translational Medicine, 2011. **3**(94): p. 94ra72.

33. Alkan, C., S. Sajjadian, and E.E. Eichler, *Limitations of next-generation genome sequence assembly.* Nat Meth, 2011. **8**(1): p. 61-65.

34. *Points to consider in the clinical application of genomic sequencing.* Genet Med, 2012. **14**(8): p. 759-761.

35. Fairfield, H., et al., *Mutation discovery in mice by whole exome sequencing.* Genome Biol, 2011. **12**(9): p. R86.

36. Gilissen, C., et al., *Unlocking Mendelian disease using exome sequencing.* Genome Biology, 2011. **12**(9): p. 228.

37. Cheon, D.-J. and S. Orsulic, *Mouse Models of Cancer.* Annual Review of Pathology: Mechanisms of Disease, 2011. **6**(1): p. 95-119.

38. Downward, J., *Targeting RAS signalling pathways in cancer therapy.* Nat Rev Cancer, 2003. **3**(1): p. 11-22.

39. Sharma, S.V., et al., *Epidermal growth factor receptor mutations in lung cancer.* Nat Rev Cancer, 2007. **7**(3): p. 169-181.

40. Castillo-Martin, M., et al., *Molecular pathways of urothelial development and bladder tumorigenesis.* Urologic Oncology: Seminars and Original Investigations, 2010. **28**(4): p. 401-408.

41. Sahu, A., M.K. Iyer, and A.M. Chinnaiyan, *Insights into Chinese prostate cancer with RNA-seq.* Cell Res, 2012. **22**(5): p. 786-788.

42. Ito, K., *Prostate cancer in Asian men.* Nat Rev Urol, 2014. **11**(4): p. 197-212.

43. Pylayeva-Gupta, Y., E. Grabocka, and D. Bar-Sagi, *RAS oncogenes: weaving a tumorigenic web.* Nat Rev Cancer, 2011. **11**(11): p. 761-774.

44. Stone KR, M.D., Wunderli H, Mickey GH, Paulson DF., *Isolation of a human prostate carcinoma cell line (DU 145).* Int J Cancer, 1978. **15**(3): p. 274-81.

45. Kaighn ME, N.K., Ohnuki Y, Lechner JF, Jones LW., *Establishment and characterization of a human prostatic carcinoma cell line (PC-3).* Invest Urol, 1979. **17**(1): p. 16-23.

46. Yin, J., et al., *Activation of the RalGEF/Ral Pathway Promotes Prostate Cancer Metastasis to Bone.* Molecular and Cellular Biology, 2007. **27**(21): p. 7538-7550.

47. JuanYin, J., et al., *Cediranib/AZD2171 Inhibits Bone and Brain Metastasis in a Preclinical Model of Advanced Prostate Cancer.* Cancer research, 2010. **70**(21): p. 8662-8673.

48. Siu, M.K., et al., *Transforming growth factor-[beta] promotes prostate bone metastasis through induction of microRNA-96 and activation of the mTOR pathway.* Oncogene, 2015. **34**(36): p. 4767-4776.

49. Yin, J., et al., *AR-Regulated TWEAK-FN14 Pathway Promotes Prostate Cancer Bone Metastasis.* Cancer Research, 2014. **74**(16): p. 4306-4317.

50. Mulholland, D.J., et al., *Pten Loss and RAS/MAPK Activation Cooperate to Promote EMT and Metastasis Initiated from Prostate Cancer Stem/Progenitor Cells.* Cancer Research, 2012. **72**(7): p. 1878-1889.

51. Aytes, A., et al., *ETV4 promotes metastasis in response to activation of PI3-kinase and Ras signaling in a mouse model of advanced prostate cancer.* Proceedings of the National Academy of Sciences, 2013. **110**(37): p. E3506-E3515.

52. Edgren, H., S. Kangaspeska, and O. Kallioniemi, *KRAS Oncogene Rearrangements and Gene Fusions: Unexpected Rare Encounters in Late-Stage Prostate Cancers.* Cancer Discovery, 2011. **1**(1): p. 12-13.

53. Wang, J., et al., *B-Raf Activation Cooperates with PTEN Loss to Drive c-Myc Expression in Advanced Prostate Cancer.* Cancer Research, 2012. **72**(18): p. 4765-4776.

54. Schutzman, J.L. and G.R. Martin, *Sprouty genes function in suppression of prostate tumorigenesis.* Proceedings of the National Academy of Sciences, 2012. **109**(49): p. 20023-20028.

55. Malumbres, M. and M. Barbacid, *RAS oncogenes: The first 30 years.* Nature Reviews Cancer, 2003. **3**(6): p. 459-465.

56. Stephen, Andrew G., et al., *Dragging Ras Back in the Ring.* Cancer Cell, 2014. **25**(3): p. 272-281.

57. Takai, Y., T. Sasaki, and T. Matozaki, *Small GTP-Binding Proteins*. Vol. 81. 2001. 153-208.

58. Meylan, E., et al., *Requirement for NF-[kgr]B signalling in a mouse model of lung adenocarcinoma.* Nature, 2009. **462**(7269): p. 104-107.

59. Dickson, M.C., et al., *Defective haematopoiesis and vasculogenesis in transforming growth factor-beta 1 knock out mice.* Development, 1995. **121**(6): p. 1845-1854.

60. Oshima, M., H. Oshima, and M.M. Taketo, *TGF-beta receptor type II deficiency results in defects of yolk sac hematopoiesis and vasculogenesis.* Developmental Biology, 1996. **179**(1): p. 297-302.

61. Sanford, L.P., et al., *TGFbeta2 knockout mice have multiple developmental defects that are non-overlapping with other TGFbeta knockout phenotypes.* Development, 1997. **124**(13): p. 2659-2670.

62. Massagué, J. and R.R. Gomis, *The logic of TGFβ signaling.* FEBS Letters, 2006. **580**(12): p. 2811-2820.

63. Shi, Y. and J. Massagué, *Mechanisms of TGF-β Signaling from Cell Membrane to the Nucleus.* Cell, 2003. **113**(6): p. 685-700.

64. Fournier, Pierrick G.J., et al., *The TGF-β Signaling Regulator PMEPA1 Suppresses Prostate Cancer Metastases to Bone.* Cancer Cell, 2015. **27**(6): p. 809-821.

65. Baltimore, D., *Discovering NF-κB.* Cold Spring Harbor Perspectives in Biology, 2009. **1**(1).

66. Staudt, L.M., *Oncogenic Activation of NF-κB.* Cold Spring Harbor Perspectives in Biology, 2010. **2**(6).

67. Lessard, L., et al., *NF-κB nuclear localization and its prognostic significance in prostate cancer.* BJU International, 2003. **91**(4): p. 417-420.

68. Oeckinghaus, A. and S. Ghosh, *The NF-κB Family of Transcription Factors and Its Regulation.* Cold Spring Harbor Perspectives in Biology, 2009. **1**(4).

69. Hayden, M.S. and S. Ghosh, *Signaling to NF-κB.* Genes & Development, 2004. **18**(18): p. 2195-2224.

70. Israël, A., *The IKK Complex, a Central Regulator of NF-κB Activation.* Cold Spring Harbor Perspectives in Biology, 2010. **2**(3).

71. Wertz, I.E. and V.M. Dixit, *Signaling to NF-κB: Regulation by Ubiquitination.* Cold Spring Harbor Perspectives in Biology, 2010. **2**(3).

72. Wan, F. and M.J. Lenardo, *Specification of DNA Binding Activity of NF-κB Proteins.* Cold Spring Harbor Perspectives in Biology, 2009. **1**(4).

73. Zhang, L., et al., *Function of phosphorylation of NF-kB p65 ser536 in prostate cancer oncogenesis*. 2015. 2015.

74. Kanarek, N., et al., *Ubiquitination and Degradation of the Inhibitors of NF-κB.* Cold Spring Harbor Perspectives in Biology, 2010. **2**(2).

75. Gannon, P.O., et al., *Large-scale independent validation of the nuclear factor-kappa B p65 prognostic biomarker in prostate cancer.* European Journal of Cancer, 2013. **49**(10): p. 2441-2448.

76. Longoni, N., et al., *ETS Transcription Factor ESE1/ELF3 Orchestrates a Positive Feedback Loop That Constitutively Activates NF-κB and Drives Prostate Cancer Progression.* Cancer Research, 2013. **73**(14): p. 4533-4547.

77. Wu, K., et al., *The role of DAB2IP in androgen receptor activation during prostate cancer progression.* Oncogene, 2014. **33**(15): p. 1954-1963.

78. Jin, R., et al., *NF-kappaB gene signature predicts prostate cancer progression.* Cancer Res, 2014. **74**(10): p. 2763-72.

79. Kalluri, R. and R.A. Weinberg, *The basics of epithelial-mesenchymal transition.* The Journal of Clinical Investigation, 2009. **119**(6): p. 1420-1428.

80. Liu, Y.-N., et al., *Critical and Reciprocal Regulation of KLF4 and SLUG in Transforming Growth Factor β-Initiated Prostate Cancer Epithelial-Mesenchymal Transition.* Molecular and Cellular Biology, 2012. **32**(5): p. 941-953.

81. Cardiff, R.D., *The Pathology of EMT in Mouse Mammary Tumorigenesis.* Journal of Mammary Gland Biology and Neoplasia, 2010. **15**(2): p. 225-233.

82. Ruscetti, M., et al., *Tracking and Functional Characterization of Epithelial–Mesenchymal Transition and Mesenchymal Tumor Cells during Prostate Cancer Metastasis.* Cancer Research, 2015. **75**(13): p. 2749-2759.

83. Jadaan, D.Y., M.M. Jadaan, and J.P. McCabe, *Cellular Plasticity in Prostate Cancer Bone Metastasis.* Prostate Cancer, 2015. **2015**: p. 651580.

84. Moustakas, A. and C.-H. Heldin, *Signaling networks guiding epithelial–mesenchymal transitions during embryogenesis and cancer progression.* Cancer Science, 2007. **98**(10): p. 1512-1520.

85. Agarwal, S., et al., *Identification of Different Classes of Luminal Progenitor Cells within Prostate Tumors.* Cell Reports, 2015. **13**(10): p. 2147-2158.

86. Liu, Y.N., et al., *MiR-1 and miR-200 inhibit EMT via Slug-dependent and tumorigenesis via Slug-independent mechanisms.* Oncogene, 2013. **32**(3): p. 296-306.

87. Zheng, X., et al., *Epithelial-to-mesenchymal transition is dispensable for metastasis but induces chemoresistance in pancreatic cancer.* Nature, 2015. **advance online publication**.

88. Pylayeva-Gupta, Y., et al., *Oncogenic Kras-Induced GM-CSF Production Promotes the Development of Pancreatic Neoplasia.* Cancer Cell, 2012. **21**(6): p. 836-847.

89. Sparmann, A. and D. Bar-Sagi, *Ras-induced interleukin-8 expression plays a critical role in tumor growth and angiogenesis.* Cancer Cell, 2004. **6**(5): p. 447-458.

90. Yang, J., et al., *Twist, a Master Regulator of Morphogenesis, Plays an Essential Role in Tumor Metastasis.* Cell, 2004. **117**(7): p. 927-939.

91. Sfanos, K.S. and A.M. De Marzo, *Prostate cancer and inflammation: the evidence.* Histopathology, 2012. **60**(1): p. 199-215.

92. Elkahwaji, J.E., *The role of inflammatory mediators in the development of prostatic hyperplasia and prostate cancer.* Research and Reports in Urology, 2013. **5**: p. 1-10.

93. Pickup, M., S. Novitskiy, and H.L. Moses, *The roles of TGFβ in the tumour microenvironment.* Nature reviews. Cancer, 2013. **13**(11): p. 788-799.

94. Quail, D.F. and J.A. Joyce, *Microenvironmental regulation of tumor progression and metastasis.* Nature medicine, 2013. **19**(11): p. 1423-1437.

95. Kwon, O.-J., et al., *Increased Notch signalling inhibits anoikis and stimulates proliferation of prostate luminal epithelial cells.* Nat Commun, 2014. **5**.

96. Ding, Z., et al., *Telomerase Reactivation following Telomere Dysfunction Yields Murine Prostate Tumors with Bone Metastases.* Cell, 2012. **148**(5): p. 896-907.

97. Kwon, O.-J., et al., *Increased Notch signaling inhibits anoikis and stimulates proliferation of prostate luminal epithelial cells.* Nature communications, 2014. **5**: p. 4416-4416.

98. Siu, M.K., et al., *Transforming growth factor-[beta] promotes prostate bone metastasis through induction of microRNA-96 and activation of the mTOR pathway.* Oncogene, 2014.

99. Ding, Z., et al., *SMAD4-dependent barrier constrains prostate cancer growth and metastatic progression.* Nature, 2011. **470**(7333): p. 269-273.

100. Padua, D. and J. Massague, *Roles of TGF[beta] in metastasis.* Cell Res, 2009. **19**(1): p. 89-102.

101. Kim, I.Y., et al., *Loss of expression of transforming growth factor-beta receptors is associated with poor prognosis in prostate cancer patients.* Clinical Cancer Research, 1998. **4**(7): p. 1625-1630.

102. Salm, S.N., et al., *TGF-β maintains dormancy of prostatic stem cells in the proximal region of ducts.* The Journal of Cell Biology, 2005. **170**(1): p. 81-90.

103. Tu, W.H., et al., *The Loss of TGF-β Signaling Promotes Prostate Cancer Metastasis.* Neoplasia (New York, N.Y.), 2003. **5**(3): p. 267-277.

104. Sasaki, C.Y., et al., *Phosphorylation of RelA/p65 on Serine 536 Defines an IκBα-independent NF-κB Pathway.* Journal of Biological Chemistry, 2005. **280**(41): p. 34538-34547.

105. Valdez, Joseph M., et al., *Notch and TGFβ Form a Reciprocal Positive Regulatory Loop that Suppresses Murine Prostate Basal Stem/Progenitor Cell Activity.* Cell Stem Cell, 2012. **11**(5): p. 676-688.

106. Olsauskas-Kuprys, R., A. Zlobin, and C. Osipo, *Gamma secretase inhibitors of Notch signaling.* OncoTargets and therapy, 2013. **6**: p. 943-955.

107. Tan, H.-L., et al., *Prostate Adenocarcinomas Aberrantly Expressing p63 Are Molecularly Distinct from Usual-Type Prostatic Adenocarcinomas.* Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc, 2015. **28**(3): p. 446-456.

108. Goldstein, A.S., et al., *Trop2 identifies a subpopulation of murine and human prostate basal cells with stem cell characteristics.* Proceedings of the National Academy of Sciences, 2008. **105**(52): p. 20882-20887.

109. Garraway, I.P., et al., *Human Prostate Sphere-Forming Cells Represent a Subset of Basal Epithelial Cells Capable of Glandular Regeneration in Vivo.* The Prostate, 2010. **70**(5): p. 491-501.

110. van Leenders, G.J.L.H., et al., *Expression of basal cell keratins in human prostate cancer metastases and cell lines.* The Journal of Pathology, 2001. **195**(5): p. 563-570.

111. Moll, R., M. Divo, and L. Langbein, *The human keratins: biology and pathology.* Histochemistry and Cell Biology, 2008. **129**(6): p. 705-733.

112. Wang, Y., et al., *Cell differentiation lineage in the prostate.* Differentiation, 2001. **68**(4-5): p. 270-9.

113. Huang, Y., et al., *Prostate Sphere-forming Stem Cells Are Derived from the P63-expressing Basal Compartment.* Journal of Biological Chemistry, 2015. **290**(29): p. 17745-17752.