PBK PROMOTES AGGRESSIVE BEHAVIOR OF PROSTATE CANCER CELLS THROUGH UPREGULATION OF A B-CATENIN/TCF/LEF- AND MYC-DRIVEN PRO-METASTASIS GENE EXPRESSION PROGRAM

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Abstract

A key question in prostate cancer research is how to distinguish aggressive prostate cancer from indolent neoplasms. Early treatment of aggressive cancers is essential to maximize therapeutic potential but treatment carries significant health risks and the majority of men diagnosed with prostate cancer will not die from the disease. Predictive nomograms have been developed to assess the risk of malignant progression but they are currently not robust enough to accurately recommend patients for therapy or surveillance. Biomarkers of aggressive prostate cancer are therefore urgently sought. This project aimed to evaluate the role of PDZ domain-binding kinase/T-LAK cell-originated protein kinase (PBK/TOPK) in prostate cancer and to determine if PBK expression contributes to aggressive behavior of prostate cancer cells. Ectopic overexpression of PBK up-regulates the invasive ability of prostate cancer cells. Production of matrix metalloproteinases-2 and -9, which are key players in metastatic invasion, is up-regulated, and the promoters of these genes are transcriptionally activated by PBK via increased \( \beta \)-catenin-TCF/LEF signaling. Genetic knockdown or pharmacological inhibition of PBK function in aggressive prostate cancer cells caused reduced invasiveness and down-regulation of metalloproteinase production. We also demonstrated that PBK increases expression and transcription of the metastasis-promoting gene RANKL. This effect is mediated through PBK activity-dependent
stabilization and β-catenin/TCF/LEF-induced transcription of the oncogenic transcription factor c-Myc. Analysis of human prostate cancer bone metastasis samples reveals significantly higher co-expression of PBK, MYC and RANKL, compared to non-tumor prostate and localized cancer patient samples. Finally, analysis of a large set of human prostate cancer samples and multiple, independent prostate cancer datasets revealed that PBK levels and nuclear localization are significantly associated with shorter recurrence-free survival, stage, grade and distant metastasis. Our in vitro and in situ data are in agreement that PBK could be a prognostic biomarker for prostate cancer that would discriminate aggressive prostate cancer from indolent disease, and also a potential target for the therapeutic intervention of aggressive prostate cancer in men.
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Finally, I dedicate this thesis to my mother, whom we lost to that beast, cancer, too soon. You shared with me your love of the world and your ever-present eagerness to learn about it and help those in it. We miss you, your laugh and your joie de vivre every day.
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List of Abbreviations

AR – androgen receptor
APC – adenomatous polyposis complex
AML – acute myeloid leukemia
BPH – benign prostate hyperplasia
CHX - cycloheximide
CKI – casein kinase I
DRE – digital rectal exam
EMT – epithelial-mesenchymal transition
ECM – extracellular matrix
ERK – extracellular-regulated kinase
ESC – embryonic stem cell
GEMM – genetically engineered mouse model
GSK3 – glycogen synthase kinase 3
JNK – c-Jun N-terminal kinase
MAPKK – mitogen-activated protein kinase kinase
MMP – matrix metalloproteinase
NF-κB – nuclear factor of nuclear factor kappa-light-chain-enhancer of activated B cells
OPG - osteoprotegerin
PBK – PDZ domain-binding kinase
PBS – phosphate-buffered saline
PCSM – prostate cancer-specific mortality

PDX – patient-derived xenograft

PSA – prostate-specific antigen

RANKL – receptor activator of NF-κB ligand

TRAF – TNFR-associated factor

USPSTF – US Preventative Services Task Force
Chapter I: Introduction
1.1 Prostate Development and Anatomy

The adult human prostate is a golf ball-sized tubulo-alveolar gland at the neck of the bladder, encircling the urethra (Figure 1A). Its function is to produce and secrete an alkaline fluid that makes up a large fraction of the volume of the ejaculate and protects the sperm in the acidic vagina, as well as other compounds that may assist fertilization. Development of the prostate begins at about the tenth week of gestation, and is reliant on the production of testosterone, which begins about two weeks earlier. 5α-dihydrotestosterone-dependent androgen receptor (AR) signaling triggers outgrowth of prostatic buds from the urogenital sinus and their invasion into the surrounding mesenchyme. These buds are solid cords of epithelial cells that then lengthen and branch during fetal growth. Androgens in the first postnatal year induce lumens to develop, which are lined with multiple levels of immature cells that express cytokeratins of stratified or simple epithelium. The organ remains the same until puberty, at which point the increase in circulating androgens causes the epithelial cells to differentiate into a bilayer, consisting of peripheral cuboidal basal cells and luminal, columnar, secretory cells. The luminal epithelial cells are the predominant cell type in the prostate; they are terminally differentiated, AR-positive cells that require a continual supply of androgens for survival and secrete the components, notably prostate-specific antigen (PSA) and prostatic acid phosphatase, of the prostatic fluid into the acini lumen. The basal cells that line the basement membrane are less differentiated and do not possess secretory activity. They have lower or undetectable levels of AR and do not need androgens for survival. Finally, a significant population of AR-negative neuroendocrine cells is interspersed with the basal epithelial cells.
Historically, different models have been used to describe the anatomy of the adult prostate; more recently, McNeal developed a widely accepted model based on zones, reflecting clear histological differences (Figure 1B)\textsuperscript{4,5}. The ventral or anterior side of the prostate is almost entirely fibromuscular, while the posterior side is glandular, and the glands surround the ejaculatory ducts as they proceed into the urethra. The central zone constitutes \textasciitilde25\% of the prostate and is a vertical wedge of glandular tissue with its base above the gland capsule. Its ducts lie close to the ejaculatory duct orifices and follow them proximally. A thin band of stroma lies between the central and the peripheral zone. The peripheral zone accounts for about 70\% of the adult prostate and is a disc of tissue that surrounds the central zone and extends caudally along the urethra. Notably, almost all carcinomas arise in this zone, as well as most cases of prostatitis. The transition zone is a small wedge of tissue normally less than 5\% of the mass of the adult prostate, excepting cases of benign prostate hyperplasia (BPH). It lies immediately lateral to the distal end of the sphincter and is interspersed with fibers of the outer half of the sphincter. Additionally, it is glandular nodules in this zone that are the site of origin of BPH, which McNeal suggested should rather be termed benign nodular hyperplasia. Finally, he considered the anterior fibromuscular stroma, a thick sheath of non-glandular tissue that forms the entire anterior surface of the prostate, as playing a role in the development of the prostate but not important in its function or pathology\textsuperscript{6,7}.
Figure 1: Anatomy of the adult prostate (A) A diagram of the frontal and sagittal sections of the male urogenital complex. McNeal’s model of prostatic zones (1983) are indicated: central zone (CZ), peripheral zone (PZ), anterior fibromuscular stroma (AFS), and transition zone (TZ). (B) A larger representation of McNeal’s model: peripheral zone (yellow), central zone (red), transition zone (blue), anterior fibromuscular stroma (green).

Source: Timms BG, Differentiation (2008), 76(6), 565-77.
1.2 Prostate Cancer

1.2.1 Incidence and Risk Factors

Prostate cancer is the most commonly diagnosed non-skin cancer in American men. In 2015, 220,800 men were diagnosed with the disease and 27,540 were estimated to die from it, a number of cancer deaths that is surpassed only by lung cancer. The greatest risk factor for prostate cancer is age: 87% of prostate cancer cases were diagnosed in men aged 60 or over. Following that, a number of other, less significant risk factors have been identified, including race, genetic predisposition, body fat and diet. African American men are 1.7 times as likely as non-Hispanic white men to be diagnosed with prostate cancer, and 2.4 times as likely to die from the disease. This is thought to be mostly due to inequalities in access to high-quality health care and differences in prevalence of risk factors. It is worth noting though, for breast cancer at least, black women were more likely to die from a breast cancer diagnosis despite uniform treatment, even after controlling for socioeconomic status, stage of disease, tumor characteristics, and follow up, indicating that there may be an underlying race/ethnic biological contribution, in addition to well-noted differences in health care access and socioeconomic-associated risk factors.

A family history increases the risk of developing prostate cancer, and a number of genetic polymorphisms have been identified that predispose individuals to develop the disease, namely BRCA1/2, MSH1/2, and HOXB13, among others. Additionally, abdominal obesity does increase the risk of developing high-grade prostate cancer, which may be due to increases in circulating insulin-like
growth factor. Finally, there is weak and often contradictory evidence that some dietary interventions or habits may modify risk of aggressive or any prostate cancer, for foods ranging from milk (promotes or delays progression) and coffee (decreases risk) to more specific compounds like β-carotene (increases risk or has no effect) and selenium (higher levels associated epidemiologically with reduced risk but supplementation had no effect or increased risk).

1.2.2 Diagnosis of Prostate Cancer

Two diagnostic tools are commonly used to screen men for prostate cancer: the PSA blood test and the digital rectal exam (DRE). Although the use of the PSA test and the age at which to begin screening is controversial, the American Cancer Society guidelines recommend that a non-African American man with no family history of prostate cancer, who has been informed of the current uncertainties in screening and diagnosis, should begin screening at age 50.

The blood test to detect circulating levels of PSA (the formal gene name is kallikrein-related peptidase 3, or KLK3) has become a commonly used screening tool since it was reported that use of the test, in conjunction with the DRE, enhanced early detection of prostate cancer. The kallikrein-related peptidases are a family of 15 homologous serine endoproteases. Like the S1 family of proteases, KLKs are synthesized as a pre-pro-enzyme. The signal pre-domain targets the KLK to the endoplasmic reticulum and Golgi apparatus, where the domain is cleaved, and the remaining peptide glycosylated and secreted into the extracellular space. A trypsin-like protease (which can be other KLKs, allowing for an activation cascade upon transcription of the KLK gene) then cleaves off the regulatory pro-domain,
completing its activation\textsuperscript{26}. The broadly accepted physiological role for KLK3 is to liquefy semen\textsuperscript{27}, but its proteolytic activity may also facilitate signaling events in the extracellular space, i.e., generation or inactivation of peptide agonists/hormones, release of extra-cellular matrix (ECM)- or membrane-bound growth factors or signaling adhesive molecules such as integrins\textsuperscript{28}. Its presence in the circulation may therefore be a simple reflection of its production as a gene regulated by the AR\textsuperscript{29}, which drives prostate cancer development and progression, and the increased porosity of the altered cancer microenvironment or the ability of KLK3 itself to degrade ECM proteins separating prostate acini from the circulation, but a large body of evidence has implicated KLK3 as facilitating almost every step of cancer development and progression\textsuperscript{26}. It must be noted that, in the context of KLK3 use as a cancer biomarker, that its expression is also regulated by numerous other factors, including progestin\textsuperscript{30}, corticosteroid\textsuperscript{31}, thyroid hormone\textsuperscript{32}, among others\textsuperscript{29}.

The DRE aids diagnosis of prostate cancer as well, by allowing the physician to manually palpitate and feel the rigidity associated with cancer development in the normally pliable prostate. However, it must be noted that not all prostate cancers are rigid and rough to the touch; it was the additional, increased sensitivity of the PSA blood test\textsuperscript{25} that led to its widespread adoption.

The vast majority of prostate malignancies are acinar adenocarcinomas, with other subtypes, including mucinous, ductal, neuroendocrine and adenosquamous, comprising less than 1\% of diagnosed prostate cancer cases\textsuperscript{33}. Additionally, although neuroendocrine cancers rarely arise de novo, androgen depletion
treatment can cause an acinar adenocarcinoma to gain an increasing proportion or characteristics of neuroendocrine tumors\textsuperscript{34}.

Similar to other solid cancers, prostate cancer is broadly classified according to degree of invasion of surrounding tissue and spread to more distant sites, termed stage (figure 2), and the degree to which the neoplasm resembles differentiated prostate epithelial tissue, using a grading scheme known as the Gleason score. Stage I tumors are detectable only by needle biopsy, and are not palpable or visible to imaging. Stage II tumors are still confined to the prostate and may be present in one or both sides (in relation to the urethra) of the prostate. Stage III cancers are those that have escaped the prostate, perhaps reaching the seminal vesicles, while stage IV cancers have metastasized to further tissues, including the urethral sphincter, rectum, bladder, or pelvic wall, with bone, followed by lung, liver, pleura and adrenals, being the most common sites of metastasis\textsuperscript{35,36}.
Figure 2: Stages of prostate cancer  Prostate cancer is staged according to the degree of its spread, both within and out of the prostate.

Source: National Cancer Institute
1.2.2.1 The Gleason Score in Diagnosis and Prognosis of Prostate Cancer

The Gleason score is the pathologist’s judgment of how closely the neoplasm resembles well-formed, differentiated prostate tissue. In contrast to the stage of the disease, the Gleason score is a measure of the cellular status of a tumor. As such, it possesses significant prognostic information: the Gleason score is significantly associated with metastasis and prostate-cancer specific mortality (PCSM)\textsuperscript{37,38} and is the most powerful prognostic factor in the diagnostic biopsy\textsuperscript{39}.

Given intra-tumor heterogeneity, multiple regions of varying differentiation are common. From a radical prostatectomy, the pathologist reports the two most prevalent patterns, on a scale of one to five (with less differentiation given a higher number, figure 3). From a needle biopsy, the pathologist determines the most prevalent and the highest-grade pattern. This is because it was found that there was a poor correlation of the two most prevalent patterns from needle biopsy with cancer outcome and significant disagreement among pathologists on the actual Gleason score\textsuperscript{40}. The two patterns reported by the pathologist are then summed to obtain the Gleason score.

Interpretation and analysis of the Gleason score of a prostate cancer has become increasingly sophisticated and informative since the original association of the histological grading with PCSM\textsuperscript{37}. Prostate cancers with a Gleason score of 3 + 3 have been reported to have five-year recurrence-free survival rates of 95-100\%\textsuperscript{41,42} and, in a large study of 14,000 such cancers, none were found to have metastasized to the lymph nodes\textsuperscript{43}. Thus, it has been suggested to term 3 + 3 Gleason score
prostate cancers as “indolent lesions of epithelial origin,” to minimize patients fears associated with a cancer diagnosis and subsequent, unnecessary overtreatment\textsuperscript{44}.

Additionally, the relative proportions of the two reported patterns possess significant prognostic information as well. Patients with Gleason score 3 + 4 prostate cancer have a significantly better five-year, biochemical recurrence-free survival compared to patients with a Gleason score 4 + 3 prostate cancer (88% vs. 63%)\textsuperscript{45}. Even more specifically, prostate cancers with minimal (< 5%) pattern 4 may be suitable for active surveillance, based on other patient factors, while cancers with extensive pattern 4 (40-50%) warrant treatment\textsuperscript{44}. These reports show that Gleason score is a powerful tool in prognosis of prostate cancer, and sophistication in its use can enhance its utility. Moreover, as discussed in the next section, combining it with other clinical factors results in models that have significantly improved prognostic and predictive power.
Figure 3: A schematic illustrating the different Gleason scores As prostate tumors display increasingly less well-differentiated prostate gland structure, the pathologist assigns an increasingly high Gleason score to them. Note that Gleason pattern 5 is assigned to cancers that resemble an undifferentiated sheet of cells or possess markedly abnormal glandular structure.

1.2.2.2 Differentiation of Indolent from Aggressive Prostate Cancers

Historically, prostate cancer patients would typically only seek medical attention, and receive their diagnosis of prostate cancer, after they had already developed aggressive, metastatic cancer, due to the paucity of screening tools and lack of symptoms of early stage prostate cancer\textsuperscript{46}. With the rise of screening and the PSA blood test, more prostate cancers are being diagnosed and at earlier stages\textsuperscript{25,47}. Earlier detection of cancer is beneficial, as it allows treatment to begin when it is most efficacious. In this regard, the PSA test has been effective, reducing the age at diagnosis by five to eight years\textsuperscript{48}. However, the overdiagnosis of prostate cancer, or the amount of diagnosed cases that would have remained asymptomatic and undiagnosed in the absence of the PSA test, was estimated at \(\sim 40\%\)\textsuperscript{48}. It is for this reason that the US Preventative Services Task Force (USPSTF) recently recommended against the use of the PSA test for men of all ages. Reviewing the data, they found that to save one life from prostate cancer, 1,000 men would have to be screened, and that the associated subsequent biopsies would result in one serious, biopsy-associated complication per those 1,000 patients. Additionally, even though 82\% of men diagnosed with prostate cancer will die from causes \textit{unrelated} to the cancer\textsuperscript{49}, 90\% of diagnosed men do elect for treatment. Therefore, the USPSTF estimated that, due to the increase in treatment, there would be two serious cardiovascular events, one deep vein thrombosis or pulmonary embolus, 18 men with urinary incontinence and 29 men with erectile dysfunction. They judged that the harm associated with PSA screening, particularly overtreatment (treatment of
men diagnosed with prostate cancer but whose neoplasm would never progress to a malignant, life-threatening stage), outweighed the benefit\textsuperscript{50}.

This judgment is due at least in part to the lack of our ability to accurately distinguish indolent neoplasms that will have no adverse health effects in the life span of the patient to aggressive cancers that will metastasize and directly contribute to a significant worsening of patient quality of life or death. Prognostic nomograms have been developed that utilize a combination of factors, notably stage, grade, and PSA levels, among others, to calculate an expected prognosis (summarized in table 1). The predictive value of these schemes ranges from 73\% to 90\%; therefore, a significant proportion of patients will be incorrectly advised a course of action. Additionally, these nomograms have been developed to make predictions about cancer outcome based on pathologic criteria following prostatectomy. Critically, no instrument has been developed specifically to predict outcomes of men under active surveillance, resulting in considerable confusion and anxiety among men diagnosed with prostate cancer and predisposing patients and physicians alike towards treatment\textsuperscript{51}.

Therefore, it seems likely that to further improve predictive and prognostic efforts, novel markers are needed. In this regard, prostate cancer has a paucity of routinely used molecular markers. These could take the form of plasma proteins that contribute to progression of the tumor phenotype, such as RANKL\textsuperscript{52} or MIC1\textsuperscript{53}, or tumor intrinsic biomarkers, examples of both of which have been shown to possess independent, significant prognostic value\textsuperscript{54,55}.
<table>
<thead>
<tr>
<th>Nomogram</th>
<th>Variables utilized</th>
<th>Robustness</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>D’Amico\textsuperscript{56}</td>
<td>Stage, grade, PSA level</td>
<td></td>
<td>Not designed to suggest treatment</td>
</tr>
<tr>
<td>Epstein\textsuperscript{57}</td>
<td>PSA density, Gleason score, number and extent cancer-positive cores</td>
<td>95% positive predictive value, 66% negative predictive value, and accuracy of 73%</td>
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<tr>
<td>Kattan\textsuperscript{58}</td>
<td>PSA level, clinical stage, Gleason score, prostate volume, length tissue affected, tissue cancer-free in cores</td>
<td>Area under the curve of (~0.79); \textit{c}-index 0.68 - 0.78</td>
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</tr>
<tr>
<td>Nakanishi\textsuperscript{59}</td>
<td>Age, PSA density, length tumor</td>
<td>Accuracy 73%</td>
<td></td>
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<tr>
<td>Chun\textsuperscript{60}</td>
<td>PSA, clinical stage, Gleason score, length cancer and percentage positive cores</td>
<td>90% predictive value</td>
<td></td>
</tr>
<tr>
<td>Capra\textsuperscript{61}</td>
<td>Age and PSA at diagnosis, Gleason score of the biopsy, clinical stage, percent of cancer-positive biopsies</td>
<td>\textit{c}-index 0.68 – 0.81;</td>
<td></td>
</tr>
</tbody>
</table>

\textit{Table 1: Commonly used and recently developed prostate cancer nomograms}
1.3 Kinome

1.3.1 Role of the Kinome in Cancer

The human kinome, encoding about 518 protein kinases, plays a pivotal role in normal cell physiology\textsuperscript{62,63}. By virtue of their diverse target utilization, the kinases influence growth and differentiation by altering an expansive array of molecular switches\textsuperscript{64,65}. Kinases are critical in what has come to be termed cell signaling, i.e., the integration of multiple signals, whether factors from outside the cell or related to the status of the cell, to produce a coherent output: what actions the cell performs, such as proliferation, movement, or apoptosis. In this manner, the dysregulated expression or function of these kinases leads to pathological conditions and a significant fraction of them are responsible for uncontrolled growth stimulation and malignant development\textsuperscript{66-69}. Indeed, the first oncogene identified, vSrc\textsuperscript{70}, is a kinase, and the second, Ras\textsuperscript{71}, directly regulates kinase signaling.

In a list of \textasciitilde{}120 genes that are commonly mutated and are considered ‘driver’ mutations in cancer, kinases account for 19 members\textsuperscript{72}. This significant fraction is in addition to those kinases for which aberrant expression is sufficient to ‘drive’ the tumor phenotype\textsuperscript{73-75}. It is difficult to estimate the size of the latter number, but one recent review notes that compounds that target 42 different kinases are currently under development\textsuperscript{76}, in addition to another 24 non-overlapping kinases currently targeted by the current clinically approved kinase inhibitors\textsuperscript{77}. Notably, almost all of the kinase inhibitors already approved or under development have oncological indications, underlining how mutation or aberrant
expression or activation of kinases is a profound motivating force in the development and progression of cancer. Additionally, plenty of work remains to be done, as more than 100 kinases have completely unknown function and about 50% of all kinases have relatively little known about them. These proteins should not be discounted; one screen of kinases in a variety of primary and cancer cell lines found considerable variation in kinases necessary for growth between primary cells from different tissues and even different cancer cell lines arising from the same tissue, and the authors noted that there was no bias towards well-characterized kinases in this screen\textsuperscript{78}. This suggests that potentially clinically useful kinases are still waiting to be explored.

1.3.2 PDZ Domain-Binding Kinase

PDZ-binding kinase (PBK) or T-LAK cell-originated protein kinase (TOPK) is a dual specificity serine/threonine kinase and a member of the MAP kinase kinase (MAPKK) family of proteins. It was initially characterized in the same year by two different groups, one of who found it in a screen of hDLG-binding partners\textsuperscript{79} and the other in a cDNA subtraction assay to find genes highly expressed in activated killer T cells\textsuperscript{80}. PBK plays a positive regulatory role in proper chromosomal separation and cytokinesis through phosphorylation of various targets\textsuperscript{81}, including LGN/GPSM2\textsuperscript{82} and histone H3\textsuperscript{83}. Additionally, during mitosis, PBK phosphorylates the zinc finger motif, the most common motif present in transcription factor proteins, of hundreds of proteins. This reduces their affinity for DNA and ensures the chromatin is free of C2H2 zinc finger transcription factors, assisting proper chromosomal condensation and segregation\textsuperscript{84}. Therefore, although the physiological role of PBK has received
little attention, it does seem to facilitate proper chromosomal segregation in mitosis through multiple mechanisms.

While PBK is not detectable in most human normal adult tissues, it is expressed to a significant level in certain proliferating normal cells, such as the testicular and placental tissues\(^79\). Analysis of PBK expression during spermatogenesis suggested that PBK is expressed at the outer cell layer of seminiferous tubules, which are populated with spermatogonia and primary spermatocytes, but not in the mature spermatozoa\(^{85}\). Additionally, it is frequently up-regulated in human cancers of diverse tissue origins. The statistically significant associations of PBK expression with various clinicopathological features have been summarized in table 2; and high, aberrant expression of PBK compared to matching, non-tumor tissue has been found less rigorously in breast\(^{86}\), liver\(^{87}\), and skin\(^{88}\), although little is known about the functional consequences of the constitutive, higher levels of PBK seen in these various cancers.
<table>
<thead>
<tr>
<th>Cancer tissue/type</th>
<th>Association with PBK expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
<td>Survival(^9^); lymph node metastasis, histological type, TNM stage, survival(^9^); overall and disease-free survival(^9^)</td>
</tr>
<tr>
<td>Cervix</td>
<td>Histological type, differentiation, lymph node metastasis, vaginal and cervical invasion, TNM stage, tumor size(^9^)</td>
</tr>
<tr>
<td>Urinary bladder transitional carcinoma</td>
<td>Cancer, muscle invasion, stage(^9^)</td>
</tr>
<tr>
<td>Hemalogic neoplasms</td>
<td>Associated with acute myeloid leukemia, acute lymphoblastic leukemia(^9^); Burkitt’s lymphoma, acute lymphoblastic leukemia and relapsed myeloma(^9^)</td>
</tr>
<tr>
<td>Glioblastoma</td>
<td>As a panel of nine genes, with all subtypes of GBM and overall survival(^9^)</td>
</tr>
<tr>
<td>Colorectal</td>
<td>Survival, advanced pT stage and poor outcome of anti-EGFR therapy(^9^)</td>
</tr>
<tr>
<td>Prostate</td>
<td>Gleason score(^9^)</td>
</tr>
</tbody>
</table>

Table 2: PBK is associated with dismal outcomes in a variety of cancers
1.3.2.1 Regulation of PBK

The physiological distribution of PBK has been reported to be absent from most adult tissues and limited to the testis, placenta, activated lymphoid cells and neural progenitor cells. PBK mRNA levels vary dramatically with the phase of the cell cycle, with levels highest during G2/M phase, so it is unclear if the role of PBK in mitosis is restricted to the above tissues, which have relatively high levels of proliferating cells, or if the lack of detection in other tissues is due to lower levels of proliferating cells.

At the molecular level, the oncogenic transcription factor MYC was found to indirectly induce transcription of PBK through induction of E2F1 expression, which binds directly to the PBK promoter and promotes its transcription in lymphoma cells. The cell-cycle transcription factor CREB1 also binds directly to the PBK promoter and stimulates its transcription. Additionally, the EWS-FLI1 fusion protein, which is found in over 90% of Ewing sarcoma tumors, can bind directly to the first intron of the PBK gene and induce its expression.

I performed bioinformatic analysis of the PBK promoter with the TFSEARCH program and found high-confidence binding sites for the SOX and RUNX families of transcription factors. These results suggest additional scope for exploring the role of the oncologically important transcription factors SOX and RUNX in regulating PBK expression and their impact on cancer development or progression.

Post-translationally, the CDK1/cyclin B1 complex phosphorylates PBK, fully activating it. Conversely, CHFR, a mitotic checkpoint E3 ubiquitin ligase that suspends entry into mitosis in reaction to cellular stress, ubiquitylates PBK and

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induces its proteasomal degradation\textsuperscript{105}. These post-translational modifications provide another mechanism to link PBK activity to the mitotic cycle.

The multiple levels of regulatory mechanisms to tie PBK level and activity to the mitotic cycle suggest its importance in mitosis. Additionally, as many of these upstream activators are overexpressed in cancer\textsuperscript{106-109}, it provides an explanation for the aberrant, constitutive expression of PBK frequently reported in various cancers.

1.3.2.2 PBK Signaling Pathways

PBK has high homology to the MAP kinase kinase family of proteins and it was initially characterized as a direct activator of p38\textsuperscript{80}. However, subsequent reports disagreed on the exact MAPK that PBK phosphorylates, with extracellular-regulated kinase 2 (ERK2)\textsuperscript{110} and c-Jun N-terminal kinase (JNK)\textsuperscript{111} also being identified as activated by PBK. Additionally, some authors stated that PBK was specifically able to induce p38, but not ERK or JNK, phosphorylation\textsuperscript{80}, or that PBK had no effect on ERK or p38 phosphorylation status\textsuperscript{112}. Notably, all of these experiments were performed in cancer cells of various origins, suggesting that there is considerable tissue specificity in the consequences of PBK expression.

In addition to regulating MAPK signaling, PBK also phosphorylates the tumor suppressor PTEN\textsuperscript{105}. This results in activation of AKT, increasing the invasive ability of the lung cancer cells studied\textsuperscript{113}. PBK also directly interacts with the major tumor suppressor p53, and down-regulates its transcriptional activity (it is unclear if phosphorylation is required for this effect)\textsuperscript{114}, attenuating the DNA damage response and promoting entry into the cell cycle. Finally, PBK phosphorylates IκBα,
a tumor suppressor that regulates NF-κB (nuclear factor of nuclear factor kappa-light-chain-enhancer of activated B cells) signaling\textsuperscript{115}, and increases chemoresistance of HeLa cervical cancer cells\textsuperscript{116}.

Although the role of PBK in activation of MAPK signaling is controversial despite its high identity to MAPKK protein family members, it does potentiate important cancer signaling pathways through inhibition of various tumor suppressors. It is unknown whether this is part of PBK’s physiological role, deactivating tumor suppressor proteins to facilitate a smooth progression through mitosis, or a reflection of the constitutively higher levels expressed in various cancer types and cell lines, promiscuously phosphorylating non-physiological targets. In either case, it is becoming more and more clear that PBK contributes to diverse aspects of the tumor phenotype through an array of molecular targets.

### 1.4 β-Catenin Signaling

The WNT/β-catenin signaling pathway is highly evolutionarily conserved in metazoa. The name WNT derives from a contraction of the first identified member of this protein family in \textit{Drosophila} and the proto-oncogene \textit{INT-1}, which was first cloned in a mouse mammary tumor. There are nine WNT family members in \textit{Drosophila} and 19 in both mice and humans. While some of these are redundant, deletion of others is embryonic lethal, demonstrating that some have unique and non-overlapping roles in development. Indeed, there is now abundant evidence that WNT signaling controls many developmental processes, from gastrulation and early pattern formation to organogenesis. In adult humans as well, WNT signaling continues to serve key functions in processes ranging from stem cell maintenance in
regenerating tissues such as hair follicles and intestinal epithelia to wound repair and bone homeostasis\textsuperscript{117}.

The WNT signaling pathway is centered on the stability of its effector molecule, β-catenin. In the absence of WNT signaling, β-catenin is either sequestered by E-cadherin at the plasma membrane, where it forms a chain linking E-cadherin to α-catenin and the actin cytoskeleton, or it is bound by the destruction complex, composed of casein kinase I (CKI), adenomatous polyposis complex (APC), axin and glycogen synthase kinase 3 (GSK3). CKI phosphorylates β-catenin, triggering GSK3 phosphorylation of β-catenin as well. The E3 ubiquitin ligase β-TrCP can then bind to β-catenin phosphorylated in this manner, ubiquitylating it and inducing its proteasomal degradation. Therefore, beta-catenin not sequestered by E-cadherin in non-WNT-stimulated cells has a half-life of approximately 30 minutes\textsuperscript{117}.

In the canonical WNT pathway, the WNT proteins are translated and subjected to glycosylation and palmitoylation before secretion into the extracellular space. There, they bind to the FZS/LRP5-6 receptors. Ligand binding induces receptor dimerization and Dishevelled-dependent CKI and GSK3 phosphorylation of the intracellular domain of the dimerized receptors. This phosphorylation recruits Axin, completing the disruption of the destruction complex. Cytoplasmic β-catenin levels are stabilized and rise. β-catenin is then free to translocate through the nuclear pore complex into the nucleus, where it interacts with and switches the TCF/LEF family of transcription factors from a repressive to an activating mode, inducing expression of target genes (figure 4)\textsuperscript{118}.
Figure 4: Schematic diagram of canonical WNT/β-catenin signaling (a) In non-WNT-stimulated cells, β-catenin is degraded, and TCF/LEF are in a repressive mode. (b) Upon WNT binding to the FZD/LRP receptors, the destruction complex is disrupted and β-catenin accumulates. It enters the nucleus, binds to TCF/LEF-family transcription factors and switches them to a transcriptionally activating mode.

1.4.1 B-catenin Signaling in Prostate Cancer

B-catenin was first characterized as playing a key role in cancer through its interaction with APC, a tumor suppressor that is frequently mutated in colorectal cancer\textsuperscript{119}. Since then, a large body of evidence, collected in vitro, animal models and human cancer, has emerged showing that β-catenin signaling plays a key role in prostate cancer. As is typical of most cellular signaling pathways, β-catenin is regulated at multiple levels by a plethora of different molecules, and a large majority of these have been implicated in prostate cancer.

As the key signaling molecule of the WNT pathway, β-catenin has been repeatedly found to be present at high nuclear levels in a number of reports. Chen et al. reported that 43\% of prostate tumors with a Gleason score below seven, and 78\% of prostate tumors with a Gleason score above 7 had high β-catenin levels, with increased staining particularly in lymph node and bone metastases\textsuperscript{120}. Two other groups found \textasciitilde15-20\% of low Gleason score prostate adenocarcinomas and \textasciitilde40\% of high Gleason score adenocarcinomas had aberrantly elevated β-catenin levels\textsuperscript{121,122}, indicating that β-catenin seems likely to play an important role in a large subset of human prostate cancers.

At the genetic level, stabilizing mutations have been found in \textasciitilde5\% of human prostate cancers\textsuperscript{123,124} and mutations in APC are much rarer in prostate cancer compared to colorectal cancer\textsuperscript{125}. The discrepancy with the total β-catenin levels is most likely due to the fact that prostate cancer has been characterized as having a low mutational load relative to other cancers\textsuperscript{126}. Rather, hypermethylation of CpG islands in the promoter region of a large subset of genes is a much more common
event in prostate cancer development and progression. Indeed, hypermethylation of the APC promoter and other negative regulators of β-catenin is a frequently observed event in prostate cancer.

In terms of positive activators of the WNT signaling pathway, WNT1, together with β-catenin, was present at high levels in approximately half of primary prostate cancer specimens and ~80% of distant metastases, suggesting a role in development and progression of prostate cancer. WNT5A is likewise present at high levels in prostate cancer skeletal metastases, and WNT7B was also found at increasingly high levels as prostate cancers progressed to higher stages. Fatty acid synthase (FAS), the protein responsible for palmitoylation of WNTs prior to their secretion, was significantly associated with prostate cancer and stabilization of β-catenin in a large set of prostate cancer patients. Thus, there is a significant body of evidence suggesting a role for the β-catenin signaling pathway in development and progression of human prostate cancer, through activation or expression of these pathways and others.

How does WNT signaling contribute to the prostate cancer phenotype? A large part of the answer is reflected in many of the β-catenin/TCF/LEF-target genes, such as MYC, MMP7, and VEGF, which play key roles in cancer development and progression. Additionally, β-catenin/TCF/LEF signaling can stimulate TWIST1/2 and SNAI1/2 expression, repressing E-cadherin gene expression and inducing an epithelial-mesenchymal transition (EMT). EMT is thought to be a key event in cancer cell acquisition of invasive and drug-resistant properties. Finally, a complex relationship exists between β-catenin and the AR, that principal driver of
prostate cancer. β-catenin and the AR do indeed form a complex in prostate cancer cells\textsuperscript{136}. This interaction increases AR activity at the expense of β-catenin/TCF/LEF transcriptional activity, suggesting that the AR and TCF/LEF transcription factors compete for rate-limiting β-catenin. Additionally, activity of this complex is promoted by androgens, which increases β-catenin mRNA levels\textsuperscript{137} and protein nuclear translocation\textsuperscript{138}. β-catenin overexpression can also directly increase AR mRNA transcription. However, β-catenin can also inhibit AR activity indirectly through activation of MDM2-dependent AR proteasomal degradation\textsuperscript{139}. In a marked contrast to all these β-catenin-overexpression-dependent results, silencing of β-catenin increased, rather than decreased, AR transcriptional activity\textsuperscript{140}, possibly through GSK3-depedent activation of the AR\textsuperscript{141}. Finally and interestingly, an in vivo prostate cancer xenograft model found reactivation of β-catenin and AR signaling simultaneously in castrate, but not non-castrate, mice, suggesting a mechanism for clinical anti-androgen resistance\textsuperscript{142}. All of these reports support the observations of elevated β-catenin levels in human prostate cancer by providing various, disparate mechanisms wherein β-catenin contributes to the tumor phenotype.

1.5 MYC

The MYC gene is interesting because its history is closely intertwined with that of cancer biology. MYC is a member of the MYC family of transcription factors, of which there are three genes (\textit{MYC, MYCL}, and \textit{MYCN}), each with multiple polypeptide products. Discovery of MYC was indirect and took place during the era when it was increasingly thought that cancers had a viral initiation, as the v-Myc
oncogene was isolated first as a causative agent in retrovirus-induced fulminating chicken tumors\textsuperscript{143-145}, and later identified as having a cellular origin. The cellular version was labeled c-Myc\textsuperscript{146} (the official HUGO gene name being MYC).

The commonly held view of MYC function has evolved over the years as well. The MYC protein translated from the canonical AUG start site has an N-terminal transcriptional activation domain and nuclear localization signal, and a C-terminal basic DNA-binding motif linked to a helix-loop-helix-leucine zipper dimerization motif. It is through this dimerization motif that MYC interacts with MAX, which is necessary for MYC to perform many of its functions\textsuperscript{147-149}. The MYC-MAX dimer binds to the canonical E-box motif, the most frequently occurring DNA-binding motif in the human genome\textsuperscript{150}, and activates transcription, seemingly through multiple mechanisms. MYC can recruit various histone-modifying enzymes to the chromatin to remodel the chromatin and promote transcription in this manner\textsuperscript{151-153}. Indeed, cells with high MYC activity have high levels of euchromatic and hyperacetylated chromatin\textsuperscript{154}. MYC also interacts directly with cyclin T1, which is part of the positive transcription elongation factor b complex. This complex phosphorylates the C-terminal domain of RNA polymerase, allowing it to continue productive transcription after binding the DNA promoter and beginning transcription of a short (\textless{}60 nucleotides) sequence. Enabling this transcriptional pause release does seem to be a major function of MYC\textsuperscript{155}. Seemingly unrelated to its transcription-inducing function, MYC also recruits DNA replication licensing factors to initiate DNA replication\textsuperscript{156} and plays an important role in mRNA cap methylation, polysome loading and rate of translation\textsuperscript{157}. 

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1.5.1 Role of MYC in Cancer

MYC is one of the most frequently expressed and amplified oncogenes in human cancer\textsuperscript{158}, including aggressive prostate cancer\textsuperscript{159}. Confirming this, early in vitro studies established that MYC can transform normal embryonic fibroblasts\textsuperscript{160} and drive tumorigenesis in a number of murine tissues\textsuperscript{161-163}. However, it must be noted that there is a significant time delay between activation of MYC and tumorigenesis, demonstrating that cells require further oncogenic alterations to transform\textsuperscript{164}. Additionally, knockdown of MYC in cancer cell lines\textsuperscript{165-167} and in transgenic mouse models\textsuperscript{168} is sufficient to inhibit growth or other aspects of the tumor phenotype, highlighting that MYC is important for the development and maintenance of cancer.

The ability of MYC to bind MAX and promote transcription does seem to be a necessary feature of its oncogenic ability\textsuperscript{149,169} and the exact genes induced are responsible for the different effects MYC may have on the cancer phenotype\textsuperscript{165,168}. In the exact subset of genes MYC induces, however, there is considerable disagreement. MYC has been found at almost all the promoters in open chromatin in several cell types\textsuperscript{170} and the presence of epigenetic marks suggestive of open chromatin correlate highly with MYC promoter binding\textsuperscript{171}. In an interesting study in resting B cells, upon stimulation of the cells, MYC acted as a non-linear amplifier of gene expression, not as a simple on-off switch, acting universally at transcriptionally active genes\textsuperscript{172}. Therefore, in addition to its ability to globally regulate transcriptional unpausing, this model postulates that MYC acts as a general transcriptional amplifier and that MYC-dependent gene repression is actually an
artifact of the normalization process. However, a pair of subsequent studies disagreed, arguing that, increases in MYC-promoter binding doesn’t universally produce increased expression of that gene\textsuperscript{173} or that the ratio of MYC to MIZ1 bound at each promoter dictates the degree and direction of change in gene expression\textsuperscript{174} (however, the data is open to interpretation\textsuperscript{175}). In any case, interestingly, Walz et al. did find that increases in MYC levels increased MYC promoter occupancy and transcription of genes that promote migration, angiogenesis and metastasis\textsuperscript{174}. This suggests a mechanism how increasing levels of MYC contribute to cancer development and progression: increased MYC levels drive proliferation, as well as the expression of genes that support this process, e.g., cell-cycle, general transcription and translation, and energy metabolism genes\textsuperscript{176}, and increasingly higher levels of MYC induces expression of genes associated with more aggressive aspects of the tumor phenotype.

1.5.1.1 N-Myc in Resistance to Hormone Therapy in Clinical Human Prostate Cancer

N-Myc (the official HUGO gene name of which is MYCN) was first characterized in 1983 in two separate reports of a subset of neuroblastoma cell lines that harbor genomic amplification and subsequent high expression of a MYC family member\textsuperscript{177,178}. Similar to MYC, N-Myc is a basic helix-loop-helix-leucine zipper transcription factor that promotes transcription of genes involved in several key cellular processes, including proliferation, growth, energy metabolism, apoptosis and differentiation\textsuperscript{179}. While MYC is ubiquitous and continues to be highly expressed in dividing adult cells, N-Myc is not expressed in adult tissues but is important in embryonic development of B-cells, kidney, intestine and particularly
the nervous system. N-Myc and MYC can compensate for each other in embryonic stem cells, and N-Myc can replace MYC in normal growth and development but MYC cannot replace N-Myc in proper embryonic development of the nervous system, suggesting that the two proteins share a common set of target genes but also induce key, distinct transcriptional programs.

N-Myc is amplified in ~20% of neuroblastomas and its levels correlate with advanced stage, poor outcome and unfavorable clinical features. This association was significant enough that neuroblastoma patients are now stratified by N-Myc levels, with those possessing elevated N-Myc selected for more aggressive treatment. In human prostate cancer, N-Myc is amplified in 5% of adenocarcinoma and ~40% of neuroendocrine prostate cancers.

Despite the low incidence of neuroendocrine prostate cancer upon initial diagnosis (less than 1%), it is associated with ~25% of PCSM. Long-term treatment with androgen receptor antagonists, such as enzalutamide or bicalutamide, is the standard clinical treatment for advanced stage prostate cancers or prostate cancers that recur. However, induction of a neuroendocrine phenotype in prostate cancer cells mediates cell growth in the presence of these agents both in vitro and is associated with hormone therapy resistance in clinical human prostate cancer, as well as development of bone metastases. Notably, N-Myc, in cooperation with Aurora kinase A, is the driving force in induction of a neuroendocrine phenotype in prostate cancer cells. These reports show that, similar to its family member MYC, N-Myc contributes to a poorer outcome for human prostate cancer patients, although through a different mechanism:
acquisition of a neuroendocrine phenotype and subsequent castration resistance and aggressive behavior.

1.5.2 Regulation of MYC

The key role of MYC in regulating numerous cellular processes and transformation is highlighted by the numerous mechanisms regulating its expression and stability. As expected, MYC gene expression is increased by a large number of the mitogenic signaling pathways and transcription factors, including TCF, CBF, SMAD, and NF-κB, and AP1/JUN\textsuperscript{194}. Cis-regulatory modules as far away as 450,000 bases have been found to be biologically and clinically significant\textsuperscript{195}. The comprehensive expression of MYC therefore may be due to plethora of factors rather than one single strong driver; loss of MYC in a specific cell population due to loss of one tissue-specific transcription factor has been demonstrated\textsuperscript{196}. Additionally, the MYC mRNA is regulated by several miRNAs: let-7\textsuperscript{197}, miR-34\textsuperscript{198}, and miR-145\textsuperscript{199}.

The MYC protein has a short half-life in the cell, approximately 30 minutes\textsuperscript{200}, and various post-translational modifications can stabilize or destabilize the protein further or impact on its function in other ways. One recurring motif is that of the phosphodegron: phosphorylation at a particular site regulates the ability of a particular ubiquitin E3 ligase to bind and ubiquitylate MYC, inducing its proteasomal degradation. A number of kinases can phosphorylate MYC at serine 62, including ERK, JNK, CDK1 and DYRK2. This permits GSK3 to phosphorylate MYC at serine 58, recruiting the E3 ligase FBXW7 and inducing assembly of (standard degradation-associated) lysine 48-linked polyubiquitin chains and proteasomal
degradation of MYC\textsuperscript{201}. Conversely, the PLK1 kinase phosphorylates MYC around amino acids 260, enhancing β-TrCP E3 ubiquitin ligase binding, assembly of atypical, heterotypic polyubiquitin chains on MYC and its stabilization\textsuperscript{202}. This regulation itself can have multiple layers, as PAK phosphorylation of MYC enhances subsequent PLK1-dependent MYC phosphorylation\textsuperscript{203}. Post-translational modifications of MYC and their effects are summarized in table 3. Numerous mechanisms exist to regulate MYC activity, ensuring that it is able to perform its task of facilitating and supporting cell growth while minimizing the risk of MYC-driven tumorigenesis.
<table>
<thead>
<tr>
<th>Catalytic protein</th>
<th>Modification</th>
<th>Effect on MYC</th>
</tr>
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<tbody>
<tr>
<td>FBXO32</td>
<td>K48-linked polyubiquitylation</td>
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</tr>
<tr>
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<td>CHIP</td>
<td>K48-linked polyubiquitylation</td>
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<td>CUL4</td>
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<td>FBXW7</td>
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<td>B-TrCP</td>
<td>Atypical, heterotypic polyubiquitylation</td>
<td>Stabilization</td>
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<td>PLK1</td>
<td>Phosphorylation</td>
<td>Stabilization by recruitment of β-TrCP</td>
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<td>PAK</td>
<td>Phosphorylation</td>
<td>Facilitates PLK1 phosphorylation of MYC, stabilizing it</td>
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<td>HECT</td>
<td>Lysine 63-linked polyubiquitylation</td>
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<td>SKP2</td>
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<td>GCN5 and TIP60</td>
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<td></td>
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**Table 3: Post-translational modifiers of MYC and the effect of these modifications**
1.6 Receptor Activator of NF-κB Ligand (RANKL)

The receptor activator of NF-κB ligand (RANKL, the official gene name of which is TNFSF11) is a key osteoclastogenic signaling protein and member of the pleiotropic tumor necrosis factor (TNF) family. It was discovered and characterized in the 1990’s as a result of multiple, independent commercial and academic research efforts. The osteoprotective decoy receptor osteoprotegerin (OPG) had been previously cloned and found to strikingly increase bone deposition in mice through reducing osteoclast number\textsuperscript{211}, but its ligand was unknown. A screen of cell surface molecules that bound OPG identified what was termed OPG ligand, a 317-amino acid protein that induced rapid hypercalcemia in mice and osteoclast differentiation of bone marrow precursor cells, in combination with colony-stimulating factor 1\textsuperscript{212}. Meanwhile, a tagged form of the extracellular form of RANK was used in a screen to find a ligand, isolating RANKL, which was discovered to be identical to OPG ligand\textsuperscript{213}. Mouse genetic studies confirmed that both RANKL and RANK are necessary for osteoclast development, as mice missing either develop osteopetrosis and are resistant to the effects of calcitropic hormones and have defects in lymph node formation and B- and T-cell differentiation\textsuperscript{214-216}. More recent work has shown that RANKL-RANK signaling is the major effector that drives the proliferative expansion of mammary epithelial cells and expansion of mammary stem cells in response to progesterone during pregnancy\textsuperscript{217-219}.

RANKL is a type II transmembrane protein with a small N-terminal intracellular domain, a transmembrane region and a C-terminal extracellular region that trimerizes and binds its receptor, a characteristic typical of TNF family
members. After translation, RANKL is inserted into the membrane, at which point matrix metalloproteinase (MMP) 14 and ADAM10 (a distintegrin and metalloproteinase domain-containing protein) cleave off the extracellular portion. Both the membrane-bound and soluble forms are biologically active\textsuperscript{220,221}.

The osteoclast is a multinucleated, differentiated cell of the monocyte lineage that resides at the tissue/bone interface and resorbs bone, therefore playing a key role in serum calcium and bone homeostasis. Its differentiation is induced when RANKL binds to RANK on the immature hemateopoietic precursor cell. The RANKL-RANK binding induces oligomerization of the receptor RANK, recruiting TNFR-associated factor (TRAF)\textsuperscript{222}. This activates at least six distinct signaling pathways that are important for osteoclast differentiation: NF-κB, p38, ERK, JNK, AKT and Src. All of these result in changes to the cell cytoskeleton, motility and gene expression (notably, expression of the osteoclast proteins cathepsin K, TRAP, β3-integrin, the calcitonin receptor and NFATC1), resulting in the differentiation of the bone marrow precursor cell into a mature, active osteoclast\textsuperscript{223}. Together, osteoblasts, osteocytes and bone marrow cells adjust the relative levels of OPG and RANKL in response to a variety of endocrine, paracrine, cytokine and mechanical stress signals, to achieve bones that, under physiological conditions, are the minimum mass needed to support their quotidian function and to maintain serum calcium homeostasis\textsuperscript{224}(figure 5).
Figure 5: RANKL in differentiation of osteoclast cells A simplified version of the OPG-RANKL-RANK pathway. RANKL, either on the membrane of stromal or osteoblast cells or as a soluble factor, may be sequestered by OPG or may trimerize and activate its receptor, RANK. Following RANK oligomerization, TRAF is recruited and multiple signaling pathways activated, leading to induction of osteoclast differentiation.

1.6.1 Role of RANKL in Development of Bone Metastases

RANKL-RANK signaling is critical for development and establishment of distant bone metastases. Prostate cancer bone metastases are primarily osteoblastic but also demonstrate aberrant osteoclastic activity. This leads to local bone structures with pathological outgrowths but are structurally weak. These cause considerable bone pain and are prone to fracture, in addition to hypercalcemia and spinal cord and nerve compression syndromes, resulting in a large negative impact on patient quality of life\textsuperscript{225}.

RANKL contributes to development of metastases through multiple mechanisms. An increase in osteoclast activity liberates various growth factors and calcium on the bone, driving further tumor growth and malignant activity. This cooperative interaction between cancer cells and the bone microenvironment is termed the vicious cycle (figure 5). Additionally, RANKL can act directly on RANK-positive cancer cells, activating the same signaling pathways as in osteoclasts cells but which can intensify the tumor phenotype in the context of tumor cells. OPG treatment of prostate cancer cells reduced their migratory and invasive abilities\textsuperscript{226}. In a mouse model of spontaneous mammary cancer, treatment of the mice with soluble RANK did not decrease the median time to mammary tumor development, but significantly decreased the number of tumors and spontaneous lung metastases\textsuperscript{227}. The observation that RANKL inhibition decreased soft tissue metastases is noteworthy, as it suggests that RANKL has an osteoclast-independent promoting effect on cancer cell metastasis. Silencing of RANK in prostate cancer LNCaP or ARCAP\textsubscript{M} cells abrogated their tendency to form skeletal metastases\textsuperscript{228}. 
suggesting that RANKL promotes acquisition of aggressive behavior in prostate cancer cells through an autocrine-signaling mechanism. Conversely, RANKL treatment of mice orthotopically engrafted with breast cancer MT2 cells increased the number and occurrence of pulmonary metastases\textsuperscript{229} and RANKL overexpression in prostate cancer cells increased metastases not only to bone but also to lung, lymph node and the adrenal gland, as well as increasing the metastatic ability of control cells co-injected with the RANKL-overexpressing cells\textsuperscript{228}. Concordantly, human metastatic prostate cancers have higher levels of both RANK and RANKL, compared to localized tumors\textsuperscript{230}. Additionally, exposing the RANK-positive prostate cancer cell line PC-3 to RANKL increased its migratory and invasive abilities\textsuperscript{231} and expression of the pro-metastatic molecules MMP9, IL-6, IL-8, VEGF and several CXCL family members\textsuperscript{232} and RANKL treatment of prostate cancer DU145 cells significantly increased their invasive behavior\textsuperscript{233}.

The ability of RANKL to promote cancer cell metastasis has been confirmed in multiple clinical trials. Denosumab is a humanized monoclonal antibody that binds RANKL and prevents it from activating RANK\textsuperscript{234}. Denosumab treatment of metastasis-free prostate cancer patients did increase bone metastasis-free survival, but unfortunately not overall survival\textsuperscript{235,236}, and overall survival in lung cancer patients\textsuperscript{237} (clinical trials are under way in high-risk, early-stage breast cancer patients to determine if denosumab increases progression-free or overall survival for patients of that cancer type). Therefore, a significant body of preclinical and clinical evidence shows that RANKL is an important signaling molecule in development of aggressive cancer behavior.
**Figure 6: The vicious cycle of bone metastases** Increased RANKL levels induced by cancer cells leads to differentiation and activation of osteoclast cells. The increased osteolytic activity liberates growth factors that are embedded in the bone matrix, further driving the proliferation and malignant behavior of the metastasized cancer cells.

**Source:** Mundy GR, Nature Reviews Cancer (2002), 2: 584-93.
Chapter II: Hypothesis and Specific Aims
2.1 Hypothesis

Aberrant expression of PBK contributes to prostate cancer invasion and metastasis, via regulation of a cancer-associated signaling pathway that is involved in the metastatic process.

2.2 Specific Aims

1. Elucidate the role of PBK in promoting metastatic ability of prostate cancer cells
   A. Evaluate the impact of PBK-gain or loss of function on the invasive ability of prostate cancer cells
   B. Determine the mechanism by which PBK effects its pro-metastatic changes on cell behavior.

2. Establish the clinical significance of PBK expression in human prostate cancer
   A. Examine a cohort of prostate non-tumor and tumor samples to determine if PBK levels are significantly associated with any clinicopathological variables
Chapter III: Materials and Methods
3.1 Cell Lines, Plasmids and Reagents

LNCaP, VCaP, 22Rv1, PC-3M and HT1080 (American Type Culture Collection, Manassas, VA) and BPH-1 (cells were obtained from Dr. Simon Hayward, Vanderbilt University) cells were cultured in phenol red-free Improved Minimum Essential Medium (IMEM; Life Technologies, Grand Island, NY) containing 10% FBS (Atlanta Biologicals, Flowery Branch, GA), 2 mmol/L glutamine, 100 U/mL penicillin G sodium, and 100 μg/mL streptomycin sulfate (Sigma-Aldrich, St. Louis, MO), at 37°C and 5% CO2. Primary prostate epithelial cells (PrEC) were obtained from Lonza (Walkersville, MD) and were cultured in K-SFM with L-Glutamine, EGF and BPE (Invitrogen). All cell lines used were tested and authenticated at the Tissue Culture Shared Resource at Georgetown University by DNA fingerprinting short-tandem repeats.

Two different PBK shRNA, and one scrambled shRNA, constructs (Origene, Rockville, MD) were transfected into PC-3M cells to create stable PBK knockdown and scrambled vector transfected control cells, respectively. To overexpress PBK, pLenti-suCMV-RFP-2A-Puro (GenTarget Inc., San Diego, CA) was infected into VCaP and LNCaP cells to create stable cell lines and pLenti-suCMV-null-RFP-puro (GenTarget) was used to create control cells. Forty-eight hours after transfection or infection, cells were selected with puromycin, individually cloned, and screened for loss or gain of PBK expression.

HI-TOPK-032 was obtained from Tocris Bioscience (Bristol, UK). OTS514 was a generous gift from Oncotherapy Sciences (Tokyo, Japan).
3.2 Western Blot Analysis

Cells were harvested to extract total protein, which was resolved using 8-15% SDS-PAGE and electro-transferred to nitrocellulose membrane (Amersham Biosciences, Pittsburg, PA). The membranes were blocked with a 5% non-fat dried milk solution dissolved in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) and incubated with the indicated antibodies overnight with rocking at 4°C. The membranes were then washed with TBS-T, incubated for an hour with horseradish peroxidase-conjugated secondary antibodies (1:5,000x diluted; Santa Cruz Biotechnology, Dallas, TX) and visualized using chemiluminescent reagent (Santa Cruz Biotechnology). The LAS-1000 Imager (Fuji, Tokyo, Japan) was used to take pictures. Antibodies against PBK (#2286-1 Epitomics, Burlingame, CA), MMP9, MYC, β-catenin (#3852, 9562, 5625, respectively, Cell Signaling, Danvers, MA), actin, MMP2, RANKL, ubiquitin (#47772, 10736, 9628, 9133, respectively; Santa Cruz Biotechnology, Dallas, TX), active β-catenin, RANKL (#05-665 and MABD183, respectively, Millipore, Billerica, MA) FLAG and tubulin (Sigma Aldrich #F3165 and T5168, respectively) were used at the concentration recommended by the respective manufacturer.

3.3 MMP-2 and -9 Promoter Activity Assays

VCaP or PC-3M cells were transiently transfected with MMP-2 and MMP-9 promoter luciferase constructs238, TCF/LEF-Luciferase (Promega Corp., Madison WI), RANKL promoter luciferase constructs228239 or pGL4 empty vector (Promega)
constructs, as well as a control *Renilla* luciferase reporter plasmid (Promega). Forty-eight hours after transfection, luciferase activity was measured in cell lysates using the Dual Luciferase Assay kit (Promega), following the manufacturer's protocol, and normalized to *Renilla* luciferase activity.

### 3.4 Gelatin Zymography

Equal numbers of cells were plated in 10 cm plates. Once the cells had attached, the media was replaced with half the normal volume of serum-free media. The cells were incubated with the media for 16-18 hours and the media was collected. LNCaP- and VCaP-conditioned media required concentration using Amicon Ultra-0.5 Centrifugal Filter Unit with Ultracel-10 membrane (#UFC5010BK, Millipore) and 10 μl of concentrated, conditioned media were loaded into the gelatin zymography. For PC3M-conditioned media, 25 μl of non-concentrated media were loaded directly into the gelatin zymography. For HT1080-conditioned media, 10 μl of non-concentrated media were loaded directly into the gelatin zymography. Conditioned media was resolved on a 10% acrylamide, 1% gelatin SDS-PAGE. The gels were then incubated with renaturing buffer (2.5% Triton X-100) for 30 minutes, washed twice with distilled water, incubated for 30 minutes with developing buffer (10x stock: 0.5 M Tris-HCl, pH 7.8, 2 M NaCl, 0.05 CaCl$_2$, and 0.2% Brij 35), which was then replaced with fresh developing buffer, and incubated overnight at 37$^\circ$C. The following day, the gel was stained with Coomassie Blue for one hour and images taken.
3.5 Reverse Transcriptase-PCR

RNA was extracted from cells using Trizol (Invitrogen) and converted to cDNA and measured via quantitative PCR using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA), according to manufacturer’s instructions. RNA derived from samples of human prostate tissue was obtained from Georgetown University Tissue Core with approved IRB protocol. Semi-quantitative, one-step reverse transcriptase PCR was performed using the Verso One-Step RT-PCR kit (Thermo Scientific, Waltham, MA), according to the manufacturer’s instructions.

3.6 Immunofluorescent Staining

Cells were grown on ECL-coated chamber slides (Upstate Biotechnology Inc, Lake Placid, NY, USA) in phenol red-free IMEM. After the indicated treatments, cells were fixed using methanol, air dried, and rehydrated with phosphate-buffered saline (PBS). 0.2% bovine serum albumin (BSA) in PBS was used to block the cells, after which the cells were incubated overnight with primary antibody at 4°C. The slides were washed three times with PBS incubated with 4 μg/ml Alexa Fluor 488- or 594-conjugated secondary antibody (Invitrogen) for one hour. Slides were washed thrice with PBS and incubated with DAPI for 5 minutes. Slides were washed again with PBS and visualized using a fluorescent microscope (Olympus BX, Olympus Corp, Tokyo, Japan). Images were imported into Adobe Photoshop.

Active β-catenin antibody was purchased from Millipore (cat. # 05-665).
3.7 Immunohistochemistry

Immunohistochemistry (IHC) was carried out on prostate cancer tissue microarrays purchased from US Biomax (cat. # PR956b and 8011, US Biomax, Rockville, MD), or on human prostate non-cancer and cancer samples obtained from GUMC/LCCC Histopathology & Tissue Shared Resource (figure 19; https://lombardi.georgetown.edu/research/sharedresources/htsr). Patient samples and data were received de-identified to protect participant confidentiality and had Georgetown University IRB-approval and consent under protocols 1992-048 and 2007-345. The Histopathology & Tissue Shared resource is partially supported by NIH/NCI grant P30-CA051008 (Cancer Center Support Grant to Lombardi Comprehensive Cancer Center). Hematoxylin and eosin (H&E) stained slides from archival FFPE blocks were reviewed by the HTSR’s board certified pathologist for the identification of prostate cancer foci as well as adjacent normal tissue.

Tissue sections were deparaffinized in xylene, rehydrated in a graded alcohol series, subjected to antigen retrieval via heating at 95° C in citrate buffer, washed with PBS, blocked with 2% BSA in PBS, treated with 3% H2O2 to block endogenous peroxidase activity, and incubated with primary antibody overnight at 4°C. Antibodies against PBK, MYC (cat. #4942, 5605, Cell Signaling) or RANKL (cat. #MABD183, Millipore) were used at 1:100 dilution. The slides were then stained with the Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA), according to the manufacturer’s instructions, and 3,3′-diaminobenzidine, and counterstained
with hematoxylin or propidium iodide. Slides were photographed using a camera-equipped microscope (Zeiss AxioPlan2 Imaging System).

The stained slides were then analyzed with a pathologist, a Gleason score determined for the available portion of the biopsy and a cytoplasmic staining score was obtained by summing two separate scores for distribution (negative = 0; focal or < 10% positive = 1; regional or 11-50% positive = 2; diffuse or > 50% positive = 3) and intensity (negative = 0; weak = 1; moderate = 2; intense = 3). A median value for PBK expression was found (3) was found and any samples possessing at least that degree of PBK expression were considered positive for PBK expression. Additionally, if any nuclear PBK was observed, that sample was considered nuclear positive.

3.8 Cell Invasion and Migration Assays

For LNCaP, VCaP and 22Rv1 cells, a 0.1 ml suspension of 20,000 cells (for PC-3M cells, only 10,000 cells were used) was subjected to the Boyden chamber invasion assay (BD Biosciences, San Jose, CA), according to the manufacturer’s directions. Cells were treated with 5 μM of HI-TOPK-032 for 12 hours prior to addition of cells to the modified Boyden chamber and HI-TOPK-032 was present for the duration of the assay as well. PC-3M cells were allowed to invade for 20 hours before fixation, while 22Rv1 and VCaP cells were allowed to invade for 24 and 72 hours, respectively. All cells were treated with mitomycin (10 ng/ml) while in the Boyden chamber to control for any differences in proliferation between control and treated cells. After crystal violet staining, pictures were taken of four representative fields of view and invaded cells were counted.
Stably genetically modified cells were grown in 24-well plates. Once cells had reached 90% confluency, a scratch was made in a representative region using a sterile 200μl pipette tip. The plates were washed two times with sterile PBS and 500 μl of complete media further added to the plate. The plates were left for 16 hours and photographs were taken under inverted microscope.

3.9 MTT Assay

Equal numbers of cells were plated in 96-well plates and treatments were carried out for the indicated periods of time as described in the individual experiments. MTT reagent (10 μl) was added to each well and cells were incubated at 37° C for four hours. Isopropanol:HCl solution (100:1) as added to each well (100 μl) and mixed thoroughly. Optical absorbance was then measured at 570 nm.

3.10 Immunoprecipitation

Immunoprecipitation of poly-ubiquitylated MYC was carried out according to a published protocol. Cells were lysed in 2% SDS, 150 mM NaCl, 10 mM Tris-HCl, ph 8.0, and boiled for ten minutes to denature deubiquitinases. The lysate was then diluted ten times in 10 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% Triton, clarified by centrifugation, and 1 mg of lysate incubated overnight with anti-MYC antibody (#sc-40, Santa Cruz Biotech). The mixture was then rocked with protein A/G-agarose beads for four hours and the beads washed three times with 10 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% NP-40. The beads were then boiled with 50 μl 2x Laemmli buffer and the eluate analyzed via western blotting.
3.11 Statistical Analysis

All data were derived from at least three independent experiments and statistical analysis was conducted using the Prism 3 GraphPad software. Values were presented as means ± SEM. Significance level was calculated using Student’s t-test or ANOVA, as appropriate, for the in vitro experiments, while the Kruskal-Wallis test was used to determine if the groups in the immunohistochemistry experiments were significantly different. A p value <0.05 was considered significant. Once significance was established for the results of the immunohistochemistry experiments, the Mann-Whitney test was used to establish which groups differed significantly from the others.

3.12 Primers

<table>
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<tr>
<th>Target gene</th>
<th>Sequence</th>
<th>Conditions</th>
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<td>Tm 56° C</td>
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<tr>
<td>MMP2-qRev</td>
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<td>Sequence</td>
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<td>Semi-quantitative PCR primers</td>
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<td>PBK-Rev</td>
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<tr>
<td>FLAG-MYC-Fw</td>
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<tr>
<td>FLAG-MYC-Rev</td>
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Table 4: Primers used for semi-quantitative and quantitative PCR
Chapter IV: Results
4.1 PBK Expression is Commensurate with the Invasive Properties of Prostate Cancer Cells

Given the aberrant expression of PBK in a variety of cancer types, we decided to investigate the expression of PBK in a panel of prostate cell lines. PBK expression correlates with aggressiveness and invasive ability in a panel of prostate cell lines (figure 7A and B), including the hormone responsive LNCaP and VCaP, hormone-refractory 22Rv1 and highly aggressively metastatic derivative PC-3M\textsuperscript{241}. BPH-1 (a SV40-immortalized hyperplastic prostate epithelial cell line) and primary prostate epithelial cells (PrEC) were included in the study as non-tumorigenic cells. PBK expression was not detectable in PrEC or BPH-1 cells, whereas LNCaP and VCaP express low levels of PBK, and 22Rv1 and PC-3M possess increasingly higher levels of PBK (figure 7A). PBK gene expression was also analyzed via quantitative PCR and mRNA levels among the cell lines in the panel were found to be similar to the observed protein levels (figure 7C).

To establish if this observation was relevant to clinical prostate cancer, PBK expression was analyzed in normal and prostate cancer samples of human origin. Normal (n=4), benign prostate hyperplasia (n=4) and prostate cancer (n=8) samples were lysed and PBK protein levels were determined using Western blot analyses (figure 7D). Similar to the prostate cells (PrEC, BPH-1 and cancer cells), normal prostate and benign prostate hyperplasia displayed no detectable PBK, while six of the eight prostate cancer samples were positive for PBK. Interestingly, the two tissue samples that lacked PBK were stage I and II prostate cancers while four of the six tissue samples with detectable PBK levels were stage IV prostate cancer. PBK
expression was also analyzed via semi-quantitative RT-PCR and this data closely matched PBK protein levels (figure 7E), thereby confirming that PBK is expressed exclusively in tumor samples and not in normal prostatic tissue. These data show that PBK expression correlates well with the clinical phenotype, in addition to its correlation with in vitro invasiveness observed in prostate cancer cell lines.
**Sample # | Clinical Stage | Distant Metastases**
--- | --- | ---
1-4 | Non-tumor | No
5 | T2a | No
6 | T1c | No
7 | T3b | Invaded Seminal vesicles
8 | T1c | No
9-12 | BPH | No
13-16 | Stage 4 | Yes
Figure 7: Levels of PBK in prostate cancer cell lines and tissue samples correlate with aggressive behavior. (A) Western immunoblot assay showing PBK levels in primary normal prostate epithelial cells (PrEC), non-tumorigenic benign prostatic hyperplasia cell line (BPH-1), hormone-sensitive prostate cancer cell lines (LNCaP, VCaP), hormone-refractory prostate cancer cell line (22Rv1), and androgen receptor-negative prostate cancer cell line (PC-3M). Tubulin level was utilized as an internal control for equal protein loading. (B) Quantification of Matrigel invasion assay with the above cell lines showing number of cells invaded per field of view through the Matrigel matrix. The vertical axis is discontinuous for accurate presentation of high and low invasion. Four fields of view were counted and averaged for each cell line. (C) Quantitative PCR analysis of PBK expression in a panel of prostate cell lines. (D) Western immunoblot assay showing PBK levels in human normal (non-tumorigenic adjacent to tumorigenic tissue) prostate tissue (lane 1-4), benign prostatic hyperplasia tissue (lane 9-12) and prostate cancer tissue samples (lane 5-8 and 13-16). The clinical stage for the samples is shown in the table below. (E) One microgram of RNA from patient non-tumor (lanes 1-4) and tumor samples (lanes 5-8) was subjected to a one-step, semi-quantitative RT-PCR to analyze relative PBK expression. \( n \geq 3 \).

4.2 Invasive Properties of Prostate Cancer Cells are Modulated by Ectopic Expression of PBK or Knockdown of PBK Expression

The observation that PBK expression level is commensurate with the invasive properties of prostate cancer cells prompted the question of if prostate cancer cells with low endogenous PBK show increased invasiveness upon ectopic expression of PBK. To this end, VCaP and LNCaP cells, which express low levels of PBK, were infected with a PBK expression vector and stable cell lines overexpressing PBK were isolated (figure 8A). PBK overexpression resulted in increased invasiveness of the transfected clones, compared to parental cells and empty vector-infected controls (figures 8C and D). Interestingly, upon overexpression of PBK, VCaP cells acquired a spindle-shaped morphology, a characteristic of more aggressive cell type (figure 8I).

Conversely, PC-3M cells, which express significantly higher levels of PBK and are highly invasive compared to VCaP and LNCaP cells, were transfected with a silencing PBK shRNA vector, causing abrogation of PBK expression (figure 8B). In contrast to VCaP cells overexpressing PBK, silencing of PBK in PC-3M cells changed their previous spindle-like epithelial morphology to a more flattened one with increased cytoplasm (figure 8J). These cells had significantly decreased invasive ability, compared to parental cells or scrambled shRNA-transfected cells (figures 8E, F).

Figures 8G and H show the results of an in vitro wound-healing assay. Narrowing gaps between dotted lines demonstrate cell migration, which is increased upon ectopic PBK expression in VCaP cells while knockdown of PBK
expression in PC-3M cells decreased cell migration. Together, these data indicate that PBK causally regulates the migratory and invasive ability of prostate cancer cells.
Figure 8: PBK causally modulates the invasive and migratory potential of prostate cancer cells. (A) Ectopic expression of PBK in hormone-sensitive LNCaP and VCaP cells is measured by Western blot analysis. Non-transfected and empty vector-infected cells were used as controls. (B) Western blot showing knockdown of PBK in PC-3M cells. Non-transfected and scrambled shRNA-transfected cells were used as controls. Representative images of (C) LNCaP or VCaP cells, either appropriate controls or stably overexpressing PBK, stained with crystal violet after being subjected to a modified Boyden chamber invasion assay, in addition to (E) PC-3M cells, with PBK expression stably knocked down and appropriate controls. (D and F) Quantification of cells that had invaded from three different experiments. Invaded cells were counted in four fields of view from each experiment. Quantitative data are represented as SEM ± SE. ** represents a p-value <0.01 and *** a p-value <0.001. Representative images of cell migration 16h after creation of a wound in (G) VCaP-PBK and (H) PC-3M-shPBK stably modified cells compared to their respective controls (empty vector or scrambled-shRNA stable cells, respectively). The black dotted line indicates the boundary of the area covered by cells. (I) VCaP cells stably transfected with empty vector or PBK expression vector. VCaP PBK-overexpressing cells show more projections at the periphery of the colony compared to VCaP empty vector cells. (J) PC-3M cells stably expressing a scramble shRNA-GFP or PBK-shRNA-GFP construct were visualized using fluorescent microscopy. PBK knockdown PC-3M cells are more flat and broad compared to scrambled shRNA-GFP transfected cells. Scale bars are 200 μm. n ≥ 3.

4.3 Metalloproteinases MMP-2 and MMP-9 Levels are Modulated by PBK

To identify effectors of the PBK-dependent induction of invasive potential, a microarray was utilized to identify changes in gene expression between control PC-3M cells and PC-3M cells with PBK expression stably knocked down (full data available on request – it’s a 124 page-long table). Ingenuity Pathway Analysis software was used to pick out key genes, pathways and biological processes altered (table 5).

<table>
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<tr>
<th>Pathways</th>
<th>p-value</th>
<th>Selected Key Genes</th>
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<tbody>
<tr>
<td>↓Prostate Cancer</td>
<td>3.40E-05</td>
<td>↑RARRES1, mir-Let7D, VCAN, TFP12, OPRM1, AHRDGI8, BTG3</td>
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<tr>
<td>↓Metastasis</td>
<td>2.20E-05</td>
<td>↑SERPINE1, MIRLET7D ↓S100A4, PTGS2, MMP1,2,3,9, 10,13, TNFSF11</td>
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<tr>
<td>↓Invasion</td>
<td>1.09E-05</td>
<td>↑MIR133B ↓S100A4, DNMT3B, ELF3, S100P</td>
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<tr>
<td>↓Proliferation of Prostate Cancer Cell Lines</td>
<td>6.16E-04</td>
<td>↓CTNNB1, FGFP1, LCP1</td>
</tr>
</tbody>
</table>

Table 5: A selection of pathways and key genes differentially expressed in the PC3M-shPBK microarray

Ingenuity Pathway Analysis was used to analyze the data and detect significantly regulated pathways and genes. A small selection of the analysis is shown here, along with some key genes to illustrate the point.

Interestingly, several members of the MMP family were significantly down-regulated with loss of PBK expression. MMP-2 and MMP-9 are implicated in tissue invasion in metastatic malignancies\textsuperscript{242}. Consequently, the effect of PBK expression on MMP-2 and -9 levels was measured in the level of gelatinolytic activity in cell-
conditioned media and by immunoblot assays. Figures 9A-D show that PBK expression upregulates MMP-2 and -9 gelatinolytic activity of conditioned media and their protein levels as well.

To confirm that the alterations in MMP protein levels are due to similar changes coordinated by PBK at the transcript level, quantitative PCR was performed. Figures 9E and F show that the accumulation of MMP-2 and -9 mRNAs is significantly increased in the presence of PBK expression, as indicated by quantitative PCR analyses, whereas, MMP-2 and -9 mRNAs are significantly decreased after PBK knockdown as compared to appropriate controls. To determine if this is due to increased transcriptional activation at the promoters of these genes, VCaP and PC-3M cells were transfected with MMP-2 and -9 promoter luciferase reporter constructs. Transcription from the MMP-2 and -9 promoters is indeed positively regulated by PBK expression (figure 9G). Conversely, transcription from the MMP-2 and -9 promoters is decreased by knockdown of PBK (figure 9H). Taken together, these data show that PBK modulates MMP-2 and -9 levels in conditioned media through transcriptional upregulation of the genes.
A

Vector - + - Pos. Con.
PBK - - + MMP9 - +
MMP2 - MMP9 PC3M

B

Vector - + - Pos. Con.
shPBK - - + MMP9 - +
MMP2 - MMP9 VCaP

C

Vector - + - Pos. Con.
PBK MMP2 72 kDa 63 kDa
MMP9 1 1.2 1.9
MMP9 Tubulin 84 kDa
MMP9 1 0.9 3.5
MMP2 50 kDa
VCaP 1 1.1 1.2

D

Vector - + - Pos. Con.
shPBK - - + MMP2 72 kDa 63 kDa
MMP9 1 1.3 0.3
MMP9 84 kDa
Tubulin 50 kDa
PC3M 1 1.5

E

Relative Expression
Vector PBK Exp. Vector
MMP2 400
MMP9 300

F

Relative Expression
Scramble shRNA PBK shRNA
MMP2 1.4
MMP9 1.2

G

Fold Change in Normalized Luciferase Activity
Non-transfected Empty Vector PBK Exp. Vector
MMP2 8
MMP9 6

H

Fold Change in Normalized Luciferase Activity
Non-transfected Scramble shRNA PBK shRNA
MMP2 2
MMP9 1.5

Figure 9: PBK modulates expression of metalloproteinases MMP-2 and MMP-9. Gelatin zymographies of cell-conditioned media from (A) VCaP cells stably overexpressing PBK and (B) PC-3M cells with PBK stably knocked down, with appropriate controls. Conditioned media from HT1080 fibrosarcoma cells was used as a positive control (Pos Con) for MMP-2 and –9. Western immunoblots for MMP-2 and MMP-9 in cell extracts from (C) VCaP and (D) PC-3M. qPCR analyses comparing MMP-2 and MMP-9 mRNA levels in (E) PBK-overexpressing VCaP and (F) PBK knockdown PC-3M cells, along with their respective controls. Luciferase activities of MMP-2 and MMP-9 promoters in (G) PBK-overexpressing VCaP and (H) PBK-knocked down PC-3M cells were compared with appropriate controls. * represents a p-value < 0.05, and ** a p-value < 0.01. n ≥ 3.

4.4 PBK Enhances Cell Invasiveness via β-Catenin-TCF/LEF Signaling

Since PBK is a protein kinase and has not been reported to have any ability to directly regulate gene expression, it seemed likely that it was effecting its transcriptional changes through regulation of a signaling pathway. To this end, the effect of PBK overexpression on the activation status of multiple signaling pathways was investigated (figure 10A). Among the three pathways that PBK overexpression activated, the β-catenin-TCF/LEF signaling pathway was most associated with cancer cell invasion and metastasis and MMP production. For this reason, β-catenin signaling was selected for further study.

VCaP, PC-3M and their stably transfected derivatives were transiently transfected with TCF/LEF reporter constructs. Overexpression of PBK in VCaP cells increased TCF/LEF transcriptional activity (figure 10B) and loss of PBK in PC-3M cells decreased TCF/LEF transcriptional activity (figure 10C). These results were corroborated using another TCF/LEF reporter construct, TOP-FLASH (figure 10D and E). To confirm that canonical β-catenin signaling was indeed active, we examined levels of active (non-phosphorylated) β-catenin in control and stably modified VCaP and PC3M cells. Indeed, overexpression of PBK in VCaP cells resulted in a robust nuclear accumulation of active β-catenin (figure 10F), while loss of PBK in PC-3M cells caused a loss of both nuclear and total active β-catenin, as expected (figure 10G).

To demonstrate that PBK-dependent invasion is mediated through β-catenin signaling, we performed rescue experiments, wherein VCaP cells stably overexpressing PBK were treated with a pharmacological inhibitor (iCRT3), which
inhibits the interaction between β-catenin and its transcriptional coactivators, TCF/LEF, and then subjected the cells’ conditioned media to gelatin zymography or the cells to a Matrigel invasion assay (figures 10H-J). Efficacy of this inhibitor (iCRT3) was determined by measuring its effect on TOP-FLASH luciferase activity, and a downstream target gene, cyclin D1, by Western blot analysis (figures 10K and L). Notably, inhibition of β-catenin signaling reduced gelatinolytic activity of conditioned media and invasive ability of PBK-overexpressing VCaP cells to almost control levels, showing that β-catenin signaling mediates the upregulation of PBK-dependent invasion in prostate cancer cells.
**Figure 10: β-catenin/TCF/LEF signaling mediates PBK-dependent invasion in prostate cancer cells.** (A) Promega Minimal Promoter Reporter luciferase constructs, as well as Renilla luciferase, were transiently transfected into VCaP control and PBK-overexpressing cells. There days later, cells were lysed and luciferase activity measured. TCF/LEF-luciferase reporter activities in (B) VCaP cells stably overexpressing PBK and (C) PC-3M with stably knocked down PBK in PC-3M cells, with appropriate controls, and confirmed with TOP-FLASH, normalized to FOP-FLASH, transient transfection of stably modified (D) VCaP and (E) PC-3M cells. Immunofluorescence of stably modified (F) VCaP and (G) PC-3M cells showing localization of active (non-phosphorylated) β-catenin. Scale bars are 100 μm. (H) Conditioned media was collected after overnight incubation with HT1080 cells (positive control – Pos Con), or VCaP vector-transfected, PBK-overexpressing or PBK overexpressing cells treated for the same time with 50 μM iCRT3 and analyzed via gelatin zymography. (I) A modified Boyden chamber invasion assay of VCaP vector-transfected, PBK-overexpressing or PBK overexpressing cells treated for the duration of the assay with 50 μM iCRT3. Cells were allowed to invade for 24 hours, then fixed, and stained. (J) Quantification of cells that invaded through Matrigel was counted from four representative view fields of three independent experiments. Efficacy of iCRT3 was determined by (K) TOP-FLASH luciferase and (L) protein levels of a β-catenin/TCF/LEF-target gene, cyclin D1, in cell lysate measured via Western blotting, after 24 hours of treatment at a dose of 50 μM, compared to vehicle-treated control. * represents a p-value < 0.05, ** a p-value < 0.01, and *** a p-value < 0.001. n ≥ 3.

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4.5 Pharmacological Inhibition of PBK Mimics Knockdown Expression and Modulates Downstream Signaling

An ATP-competitive inhibitor of PBK, HI-TOPK-032, has been reported to reduce the growth of colon cancer cells\textsuperscript{244}. Consequently, HI-TOPK-032 was used to determine if the downstream effects of PBK knockdown expression could be replicated by treatment with this inhibitor. Additionally, as the inhibitor is a small molecule that competes for the ATP-binding site, it will not have a large impact on protein-protein binding and therefore should be suggestive if PBK’s kinase activity is required to mediate these invasive behaviors.

To this end, PC-3M and 22Rv1 prostate cancer cells, both of which express high levels of PBK and have high invasive ability, were treated with the inhibitor. Interestingly, the PBK inhibitor was found to be efficacious in causing more than a five-fold inhibition of invasion in both PC-3M and 22Rv1 cells (figures 11A and B). Use of 22Rv1 cells along with PC-3M cells demonstrates that in both cell lines inhibition of PBK decreases invasive potential and this effect is not cell line specific. Similarly, MMP-2 and -9 levels, as measured in the conditioned media, were significantly reduced in the presence of HI-TOPK-032, in both prostate cancer cell lines (figures 11C and D). Conditioned media from a fibrosarcoma cell line, HT1080, was used as a positive control for MMP-2 and -9. TCF/LEF luciferase reporter activity was also reduced dose-dependently in both PC3M and 22Rv1 cells after HI-TOPK-032 treatment (figure 11E). Similar results were also observed when MMP-9 promoter activity was monitored in the presence of PBK inhibitor (figure 11F). It is also interesting to note that PBK inhibitor was likewise able to inhibit gelatinase.
activity in a fibrosarcoma cell line, HT1080, which expresses very high levels of MMP-2 and -9 (figure 11G). Taken together, these data suggest that, similar to genetic knockdown, pharmacological inhibition of PBK strongly reduces invasive ability of cancer cells through a reduction in its phosphorylation-dependent signaling, via down-regulation of β-catenin-mediated production of MMP-2 and -9.
Figure 11: Pharmacological inhibition of PBK mimics the knockdown effect seen in prostate cancer cells (A) PC-3M or 22RV1 cells were treated with 5 μM of HI-TOPK-032 or DMSO (as control) for 12 hours and then subjected to a modified Boyden chamber invasion assay for the period of time as described in Materials and Methods and subsequently stained with crystal violet. (B) Quantification of invaded cells from four representative fields of view from three independent experiments. Gelatin zymographies using conditioned media of HI-TOPK-032-treated or DMSO-treated (control) (C) PC-3M or (D) 22Rv1 cells, respectively. (E) Similar to genetic knockdown, pharmacological inhibition of PBK decreases β-catenin-TCF/LEF luciferase activity, in both PC-3M and 22Rv1 cells, as well as (F) MMP-9 promoter luciferase activity. Cells were transfected with TCF/LEF or MMP-9 promoter luciferase reporter constructs, respectively, for 48 hours and then treated with 5 μM for 24 hours, prior to being lysed and luciferase activity measured. (G) In situ DQ gelatin staining of HT1080 cells treated with vehicle or 5 μM HI-TOPK-032. HT1080 cells were pre-treated with DMSO (control) or HI-TOPK-032 for 24 hours, then DQ gelatin was added to HT1080 cells in culture for an hour. MMPs enzymatically cleave DQ-gelatin, yielding highly fluorescent fragments. Representative pictures were taken under Olympus fluorescent microscope (upper panels) and bright field images (lower panels) at same magnification. * represents a p-value < 0.05, ** a p-value < 0.01, *** a p-value < 0.001, and **** a p-value <0.0001. n ≥ 3.

4.6 PBK Modulates the Proliferative Activity of Prostate Cancer Cells

To determine if PBK activity is regulating prostate cancer cell proliferation, in addition to its role in promoting acquisition of invasive behavior, cell growth was examined in the presence or absence of PBK (figure 12A). Overexpression of PBK in VCaP cells increased cell proliferation significantly, while the loss of PBK in PC3M cells significantly reduced cell proliferation, as determined by MTT assay. The effect of the pharmacological inhibitor of PBK on cell growth was also examined. Non-toxic concentrations of HI-TOPK-032 inhibited growth of VCaP, 22Rv1 and PC-3M (figure 12B). These data suggest that PBK is able to promote multiple aspects of the prostate cancer phenotype, namely, invasive behavior and proliferative activity.
**Figure 12: PBK modulates growth of prostate cancer cell lines.** MTT assays were performed 72 hours after plating stably genetically modified VCaP and PC-3M cells. Normalized MTT values showing that (A) PBK overexpression in VCaP cells significantly increases growth compared to empty vector stable cells, whereas PBK knockdown slows growth in PC-3M cells compared to scrambled shRNA transfected cells. (B) MTT assays were also performed using various concentrations of HI-TOPK-032, with DMSO as control, in three different prostate cancer cell lines. Normalized MTT value showing growth inhibition of VCaP, 22Rv1 and PC-3M cells after treatment with HI-TOPK-032 for 72 hours. Data are mean ± SE. * indicates a p-value <0.05; ** p-value <0.01; *** p-value <0.001. n = 4.

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4.7 Immunohistochemical Analyses of Prostate Cancer Tissue Microarrays Show Abundance of PBK in High-Grade Carcinoma and Distant Metastasis

The clinical relevance of this study of the role of PBK expression in prostate cancer was examined by analyzing a large patient cohort (n=120). Before staining the tissue microarrays, the anti-PBK antibody was first validated for immunohistochemical use in rat testes sections. Spermatogonial germ cells are a physiological source of PBK protein and mRNA expression, so testes sections were used to validate the antibody. Indeed, the spermatogonial germ cells, but not the mature spermatids, possessed high levels of PBK, while the rest of the section had no detectable staining (figure 13A), validating the antibody for immunohistochemical analysis.

Examination of tumor-adjacent normal tissues and prostate cancer sections with low Gleason score, as assessed locally in conjunction with a Board-certified pathologist, showed they were frequently negative or possessed weak PBK protein expression (figures 13B: a, b). Tissue sections from tumors with higher Gleason score and, hence, more aggressive disease had much stronger PBK levels (figure 13B, c-i). Higher PBK level was significantly associated with intermediate and high Gleason scores, as well as being significantly higher in any stage cancer sample, compared to tumor adjacent non-tumor tissue (figures 13, C-D). Nuclear PBK localization also strongly correlated with Gleason score and cancer stage (figures 13, E-F). Most interestingly, metastatic lesions of prostate cancer in abdominal wall, lymph node and bone (figures 13B: g-i) show robust PBK levels and nuclear localization. Intriguingly, there was also some positive immunostaining for PBK in
the basal layer of epithelial cells in non-tumor tissue sections adjacent to cancer tissue, suggesting a possible role for PBK in this cell type (figure 13B, a).

To confirm the PBK localization further, prostate cancer cells lines, along with non-tumorigenic BPH-1 and PrEC, were analyzed (figure 13H). In concordance with this finding in human prostate cancer tissue samples, non-tumorigenic primary prostate epithelial (PrEC) and BPH-1 cells had no detectable PBK protein expression, whereas in the tumorigenic cell lines VCaP, 22Rv1 and PC-3M, PBK protein was localized predominantly in the nucleus, but also displayed some cytoplasmic staining (figure 13H), similar to what we observed in the human prostate cancer tissue samples.

Together, these data provide strong evidence that PBK is present in prostate cancer cells and not in normal non-tumorigenic cells and its level increases with distant metastasis and invasive ability.
Figure 13: PBK protein levels and localization in human prostate cancer tissue samples and in distant metastasis. (A) The anti-PBK antibody was validated for immunohistochemical use in rat testes sections. (B) Human prostate cancer samples were immunostained with PBK-specific antibody and counterstained with hematoxylin: (a) Normal prostate tissue (non-tumorigenic adjacent to tumor tissue), (b) low- (Gleason score < 7), (c) intermediate- (Gleason score 7) and (d-f) high grade prostate cancer (Gleason score > 7), as well as prostate cancer metastases to the (g) abdominal wall, (h) lymph node and (i) bone. Scale bars are 100 μm. The correlation between the (C) cytoplasmic staining score and Gleason score (n=120), as well as the (D) correlation between cytoplasmic staining score and prostate cancer stage was determined. (E and F) represent quantification of percentage of PBK-positive nuclei in various grades and stages, respectively. (G) Showing the total percentages of PBK positivity among tumor samples in the tissue array. (H) Immunofluorescence staining of PBK in normal primary prostate epithelial cells (PrEC), BPH-1 (non-tumorigenic cell line), and prostate cancer cell lines (VCaP, 22Rv1 and PC-3M). Note nuclear localization of PBK in prostate cancer cells along with some cytoplasmic staining. * represents a p-value < 0.05, and ** a p-value < 0.01.

4.8 Nuclear Exclusion of PBK Does Not Diminish its Induction of MMP Production

The intriguing observation that nuclear levels of PBK correlated particularly well with prostate cancer stage and grade, while being completely absent in non-tumor prostate tissue, prompted the examination of the effect of nuclear exclusion of PBK on its observed effects. To this end, a nuclear export signal (NES), followed by two ‘hinge’ glycine residues, as was previously shown to be efficacious in prostate cancer cells\textsuperscript{245}, was cloned into the N-terminus of the PBK insert in the expression vector used to stably overexpress PBK (NES-PBK). VCaP cells were transiently transfected with either a wild-type PBK or NES-PBK expression vector. Wild-type PBK expression vector localized PBK predominantly in the nucleus with some staining in the cytoplasm, whereas NES-PBK was localized mainly in the cytoplasm (data not shown). However, there was no apparent difference between nuclear PBK vs. cytoplasmic PBK expression on MMP-2 and MMP-9 mRNA expression or secretion into conditioned media (figure 14A and B). Both wild-type and NES-PBK are equally potent at inducing MMP production and both MMPs are biologically active, as shown by gelatin zymography. These data suggest that cytoplasmic vs. nuclear localization of PBK has no bearing on its ability to activate β-catenin signaling.
A

MMP Gene Expression in VCaP cells

- Control
- PBK
- NES-PBK

B

<table>
<thead>
<tr>
<th>Condition</th>
<th>MMP-2</th>
<th>MMP-9</th>
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<td>Vector</td>
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<td>PBK Exp. Vector</td>
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<td>NES-PBK Exp Vector</td>
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MMP-9  
MMP-2  
VCaP
**Figure 14:** Nuclear vs. cytoplasmic PBK does not affect PBK-dependent induction of MMP-2 and -9 gene expression or protein levels. VCaP cells were transiently transfected with expression vectors for either wild-type PBK or PBK with an N-terminal nuclear export signal (NES). (A) After 72 hours, RNA was isolated and expression of the indicated genes examined via qPCR and normalized to GAPDH. (B) 72 hours after transfection, cell media was replaced with serum-free media and the cells were incubated overnight. The following day, the media was concentrated and used to perform a gelatin zymography. $n = 3$. 


4.9 PBK Expression Correlates with RANKL Expression in Human Prostate Cancer

During the course of this investigation, several findings prompted further research. Samples of human prostate cancer bone metastases possessed strikingly high levels of PBK, as compared to non-tumor prostate tissue or even primary tumors. This led us to revisit the microarray data. In the genes that were significantly affected by loss of PBK expression in the aggressive PC-3M prostate cancer cell line, levels of RANKL were significantly diminished. Since increasing evidence suggests that the rate-limiting steps of macrometastasis development are survival and proliferation of cancer cells in a foreign microenvironment\textsuperscript{246,247}, steps which are likely to be aided by RANKL-dependent disruption of local bone homeostasis\textsuperscript{248}, we sought to determine if PBK regulated expression of RANKL in prostate cancer cells.

Accordingly, we examined levels of PBK and RANKL in a panel of prostate cancer cell lines. Indeed, the relative protein levels of both RANKL and PBK in the panel were highly similar (figure 15A), suggesting a possible link.

Subsequently, we analyzed PBK and RANKL mRNA and protein levels in a number of prostate non-tumor and tumor samples at varying stages. Surprisingly, benign prostate hyperplasia (BPH) did possess a low level of RANKL, but no detectable PBK. However, the stage 4 prostate cancer samples (which had spread to form distant metastases) had considerably higher levels of RANKL, as well as high levels of PBK (figure 15B). Additionally, non-tumor prostate tissue had low levels of PBK and RANKL expression, as assessed by qPCR, and again there seems to be a trend of co-expression of PBK and RANKL, particularly in the stage 3 prostate cancer
sample (figure 15C). Altogether, these data suggest that PBK and RANKL expression are up-regulated as human prostate cancer progresses to higher stages.
**Figure 15:** Levels of PBK in prostate cancer cell lines and tissue samples correlate with levels of RANKL. (A) Western blot showing PBK and RANKL levels in primary normal prostate epithelial cells (PrEC), hormone-sensitive prostate cancer cell lines (LNCaP, VCaP), hormone-refractory prostate cancer cell line (22Rv1), and androgen receptor-negative prostate cancer cell line (PC-3M). Tubulin level was utilized as an internal control for equal protein loading. (B) Western immunoblot assay showing PBK and RANKL levels in human benign prostatic hyperplasia and stage IV prostate cancer tissue samples. (C) Quantitative PCR analysis of PBK and RANKL expression in a panel of prostate non-tumor and tumor tissue samples.
4.10 PBK Promotes Production of RANKL in Prostate Cancer Cells

Based on the finding that PBK and RANKL are often co-expressed in prostate cancer samples and cell lines, we sought to determine if gain or loss of PBK could causally regulate levels of RANKL in prostate cancer cells. Indeed, treatment of PC-3M cells with PBK siRNA did result in a significant decline of RANKL secreted into cell-conditioned media, compared to PC-3M cells treated with non-targeting siRNA (Figure 16A). Conversely, gain of PBK expression in VCaP cells resulted in a large increase in RANKL levels, compared to control cells (figure 16B).

*RANKL* mRNA levels were then assessed by qPCR in response to changes in PBK expression. Loss of PBK expression in PC-3M cells decreased *RANKL* mRNA (figure 16C), while gain of PBK expression in VCaP cells significantly increased levels of *RANKL* mRNA (figure 16D). To establish if these changes were due to changes in transcription or another post-transcriptional effect, PC-3M and VCaP cells were transfected with RANKL promoter-luciferase constructs and luciferase activity was measured in response to changes in PBK expression. Loss of PBK expression in PC-3M cells did significantly reduce transcriptional activity at the RANKL promoter (figure 16E), while gain of PBK expression in VCaP cells significantly increased RANKL promoter transcriptional activity (figure 16F). Taken together, these data demonstrate that PBK causally increases levels of RANKL in prostate cancer cells through an increase in rates of *RANKL* transcription.
Figure 16: PBK causally increases transcription and production of RANKL

Western blot showing that (A) loss of PBK in PC-3M cells decreases levels of RANKL secreted into conditioned media, while (B) gain of PBK expression in VCaP cells increased RANKL levels in cell-conditioned media. Quantitative PCR measurement of RANKL mRNA after (C) PBK knockdown with siRNA in PC-3M cells or (D) PBK overexpression in VCaP cells, as compared to appropriate, respective controls. Columns, means of three independent experiments with triplicate samples; bars, SEM. (E) PC-3M transiently transfected with non-targeting siRNA or PBK siRNA for 24 hours or (F) VCaP cells stably transfected with empty vector or PBK-expression vector were transfected with RANKL promoter luciferase reporter construct and Renilla luciferase plasmids. Promoter activity was measured three days later and normalized to Renilla luciferase activity. Significance was calculated with a two-tailed student’s t-test. n = 3. Columns, means of three independent experiments with quadruplicate samples; bars, SEM. * represents a p-value < 0.05, ** a p-value < 0.01, and *** a p-value < 0.001.
4.11 MYC Mediates PBK-Dependent RANKL Production

Since PBK is a kinase and has never been reported to have any direct effects on transcription, we hypothesized that PBK was regulating activity of a transcription factor that induces transcription of the RANKL gene. We investigated whether the loss of any of several transcription factors that have been reported to bind directly to the RANKL promoter impacts PBK-dependent RANKL transcription. The transcription factor that had the most significant and reproducible effects on PBK-dependent RANKL production was the oncogenic protein MYC. To determine if MYC mediates production of RANKL in the aggressive PC-3M prostate cancer cells, which produce high levels of both RANKL and PBK, we knocked down MYC and observed that RANKL production was almost completely abolished (figure 17A). To confirm that MYC was responsible for PBK-dependent induction of RANKL expression, PC-3 cells overexpressing PBK were treated with MYC siRNA. As before, ectopic expression of PBK in PC-3 cells significantly increased RANKL levels, and MYC knockdown almost entirely suppressed RANKL production (figure 17B), suggesting that MYC is the transcription factor that mediates PBK-dependent generation of RANKL.

To determine if MYC was directly responsible for the PBK-dependent induction of RANKL transcription, the genomic data directly upstream of the RANKL transcription start site was analyzed for the presence of an E-box, the canonical DNA element to which the active MYC/MAX heterodimer binds. There is an E-box (CACATG) at 1.3 kilobases upstream of the transcription start site. Therefore, luciferase reporter constructs with varying lengths of the RANKL promoter were
transfected into PC-3M cells in the presence or absence of PBK siRNA. Knockdown of PBK expression did significantly reduce transcriptional activity of a 2.5 or 2 kilobase RANKL promoter luciferase construct, but loss of PBK had no effect on luciferase levels in cells transfected with a RANKL promoter construct of 1.2 kilobases (figure 17C). To determine more directly if that canonical E-box is responsible for PBK-dependent RANKL transcription, a 2.5 kilobase RANKL promoter-luciferase construct with the E-box deleted was transfected into PC-3M cells in the presence or absence of PBK siRNA. Loss of PBK expression had no effect on transcriptional activity at this mutant RANKL promoter (figure 17C), suggesting that the E-box, and therefore direct MYC binding, is essential for PBK-dependent induction of RANKL transcription. All these data together demonstrate that PBK works to induce RANKL expression through the oncogenic transcription factor MYC.
**Figure 17:** PBK-dependent RANKL production requires the transcription factor MYC. PC-3M cells (A) were transfected with non-targeting or MYC siRNA. 48 hours later, the cell media was replaced with serum-free media. Cells were incubated overnight and the following day, protein levels of RANKL in the concentrated, conditioned media and of MYC and actin in the cellular lysate were examined via Western blotting. (B) Protein levels of RANKL, PBK, MYC, and actin were measured in the cell-conditioned media or cellular lysate, respectively, of PC-3 cells stably overexpressing PBK transfected with non-targeting or MYC siRNA, as well as parental PC-3 cells. (C) PC-3M transiently transfected with non-targeting siRNA or PBK siRNA for 24 hours were transiently transfected with RANKL promoter luciferase reporter construct possessing the indicated lengths of genomic RANKL promoter and Renilla luciferase plasmids. Promoter activity was measured three days later and normalized to Renilla luciferase activity. Significance was calculated with a two-tailed student’s t-test. n = 3. Bars, means of three independent experiments with quadruplicate samples; lines with caps, SEM. * represents a p-value < 0.05, ** a p-value < 0.01, and *** a p-value < 0.001.
4.12 PBK Posttranslationally Stabilizes MYC Levels

Since we showed previously that PBK positively regulates β-catenin/TCF/LEF signaling" and as MYC is a well-characterized target gene of the β-catenin/TCF/LEF signaling pathway, I hypothesized that PBK was inducing production of RANKL indirectly through an increase in β-catenin-mediated transcription of MYC. Therefore, I aimed to rescue RANKL levels in PC-3M cells treated with PBK siRNA by transfecting in exogenous MYC. However, loss of PBK in PC-3M cells consistently had a striking effect on levels of exogenous MYC (figure 18A, B), significantly reducing the levels of FLAG-tagged MYC. Since expression of this exogenous MYC is under the control of the exogenous cytomegalovirus (CMV) promoter, PBK could not be regulating its expression through modulation of β-catenin/TCF/LEF signaling, and indeed, levels of exogenous FLAG-MYC mRNA did not change with loss of PBK expression (figure 18C).

To confirm that PBK is regulating levels of MYC, MYC was assessed via Western blotting with gain or loss of PBK expression. As expected, loss of PBK in PC-3M cells significantly decreased MYC levels, compared to appropriate controls (figure 18D); conversely, gain of PBK significantly increased MYC levels in VCaP cells (figure 18E). Similarly, pharmacological inhibition of PBK using a recently developed competitive inhibitor, OTS514250, significantly reduced MYC levels within two hours of treatment (figure 18F).

To investigate further how PBK was effecting the reduction in MYC protein levels, MYC mRNA levels were quantified using qPCR. As expected, there was a significant decrease in MYC mRNA at three days after PBK siRNA treatment of PC
3M cells (figure 18G). Additionally, endogenous MYC mRNA levels were significantly decreased after two hours of OTS514 treatment (figure 18H). To determine if this was due to PBK-dependent modulation of β-catenin/TCF/LEF signaling, cellular levels of active (i.e., non-phosphorylated) β-catenin were measured after OTS514 treatment. Indeed, active, but not total, β-catenin decreased within one hour of PBK inhibition and the decline in MYC levels preceded or paralleled this decline (figure 18I, J).

Based on the earlier findings that PBK reduced levels of exogenous MYC, the ability of PBK to regulate the posttranslational stability of MYC was investigated. Treatment of PC-3M cells with the peptide synthesis inhibitor cycloheximide in conjunction with OTS514 decreased MYC levels more rapidly than treatment of the cells with cycloheximide alone (figure 18K, L). Inhibition of the proteasome via MG132 treatment rescued the OTS514-induced decline in MYC levels, suggesting that inhibition of PBK increases proteasomal degradation of MYC (figure 18M). Since polyubiquitination of MYC is generally associated with its degradation\textsuperscript{251,252}, the ubiquitination status of MYC was examined. One hour of OTS514 treatment was sufficient to induce a large increase in the amount of polyubiquinated MYC (figure 18N).

Expression levels of other MYC target genes were assessed to confirm that there was a global loss of functional MYC upon disruption of PBK expression or activity. As expected, expression levels of several other MYC target genes, \textit{CDK4}, \textit{ODC1} and \textit{LDHA}, were also significantly decreased with loss of PBK (figure 18O), demonstrating that the effect of the PBK-dependent increase in MYC levels was not
restricted solely to RANKL production. All these data, taken together, show that PBK significantly alters gene expression by up-regulating both β-catenin/TCF/LEF-induced transcription and posttranslational stabilization of MYC.

Inhibition of PBK actually decreased phosphorylation at the well-studied phosphodegron phosphorylation site Thr58 on MYC (figure 18P). This change would normally be predicted to stabilize MYC; however, we consistently observed the opposite. This suggests that PBK regulates MYC stability through a distinct mechanism.
Figure 18: PBK increases MYC levels through transcriptional and post-translational means. Schematic (A) of the experiment performed in (B). HEK293T cells were transfected with FLAG-MYC and one day later, split equally into two plates. One plate was then transfected with scramble shRNA and the other with PBK shRNA. Two days later, the cells were lysed and (B) protein levels analyzed via western blotting or (C) RNA levels via semi-quantitative RT-PCR. (D) PC-3M cells were transfected with non-targeting siRNA or PBK siRNA and lysed three days later. Protein levels analyzed with western blotting or (G) gene expression via qPCR. (E) VCaP cells stably expressing empty vector or PBK expression vector, as well as parental VCaP cells, were lysed and protein levels measured with western blotting. PC-3M cells were treated with 100 nM of OTS514 for the indicated time points and their (H) gene expression was analyzed with qPCR or (F,J) their protein levels with western blotting or (J) immunofluorescence, compared to untreated cells. Significance was calculated with a two-tailed student’s t-test. Bars, means of three independent experiments with quadruplicate samples; lines with caps, SEM. * represents a p-value < 0.05, ** a p-value < 0.01, and *** a p-value < 0.001. n = 3.
**Figure 18:** PBK increases MYC levels through transcriptional and post-translational means continued

PC-3M cells were treated with 100 nM of OTS514 for the indicated time points and protein levels were analyzed via (J) immunofluorescence, compared to untreated cells. (K) PC-3M cells were treated with either cycloheximide (CHX) alone or CHX plus OTS514 for the indicated time points. The cells were then lysed and their protein levels measured with western blotting. (L) Relative protein levels of MYC were quantified and graphed. Significance was calculated with a two-tailed student’s t-test. Bars, means of three independent experiments with quadruplicate samples; lines with caps, SEM. * represents a p-value < 0.05, ** a p-value < 0.01, and *** a p-value < 0.001. n = 3.
Figure 18: PBK increases MYC levels through transcriptional and post-translational means continued (M) PC-3M cells were treated with OTS514 and/or MG132 for two hours, lysed and their protein levels measured via western blotting, compared to untreated cells. (N) PC-3M cells were treated with MG132, and one plate of cells with OTS514, for one hour. The cells were then lysed and subjected to immunoprecipitation with an anti-MYC antibody (Santa Cruz). Protein levels in the eluate were measured with western blotting, (O) Expression of MYC target genes was measured with qPCR in PC-3M cells three days after transfection with non-targeting or PBK siRNA. (P) PC-3M cells were treated with 100 nM OTS514 for one hour, lysed and their protein levels measured via western blotting, compared to DMSO-treated or untreated cells. Significance was calculated with a two-tailed student's t-test. Bars, means of three independent experiments with quadruplicate samples; lines with caps, SEM. * represents a p-value < 0.05, ** a p-value < 0.01, and *** a p-value < 0.001. n = 3.
4.13 PBK is Associated with Distant Metastases, Shorter Recurrence-Free Survival And Prostate Cancer

To determine if the above finding of a causal link between PBK, MYC and RANKL is clinically relevant, we sought to establish if the three proteins were significantly likely to be found together in human samples of metastatic prostate cancer. However, acquiring biopsies of metastatic prostate cancer is non-trivial, as prostate cancer is rarely biopsied at this stage. Therefore, $n$ is only two for these experiments. In non-tumor prostate samples, PBK expression was limited to basal epithelial cells, as previously shown (figure 13B and 19A). PBK levels were higher in the localized prostate cancer sections and the bone metastasis samples had strikingly high levels of both cytoplasmic and nuclear PBK (figure 19A). MYC staining was even more limited: MYC was detectable in the nuclei of a few basal and luminal epithelial cells. Similar to PBK, MYC levels were significantly higher in localized prostate and even greater in both bone metastasis samples. RANKL was surprisingly present in the stroma and on the luminal edge of the epithelial cells, as well as in the cytoplasm of localized prostate cancer. However, again, RANKL staining was much more intense in both bone metastasis samples.

The PBK, MYC and RANKL immunohistochemistry data were highly suggestive of a clinical association between PBK, MYC and RANKL. To determine if PBK is significantly associated with any clinical outcomes, we interrogated publicly available databases of gene expression in patient samples. The large TCGA prostate cancer dataset ($n = 550$) showed a significant association between higher PBK expression and shorter recurrence-free survival (figure 19B), as well as between
PBK expression and prostate cancer stage and grade (data not shown). Despite the large sample size, there were only eight deaths due to prostate cancer, so it was not possible to determine if there was a statistically significant correlation between PBK expression and overall survival. Searching the Oncomine database revealed that PBK expression is significantly elevated in prostate carcinoma, compared to non-tumor prostate tissue, in the Grasso dataset\textsuperscript{253} (figure 19C) and that PBK expression is significantly higher in metastatic prostate cancer, compared to localized cancer, in the independent Varambally dataset\textsuperscript{254} (figure 19D). Taken together, all these clinical data support our hypothesis that there is a causal link between PBK, MYC and RANKL in human prostate cancer and PBK contributes to malignant progression of prostate cancer.
Prostate cancer
Varambally dataset

P < 0.001; 4th percentile-ranked gene

PBK RNA expression

Localized (n = 26) Metastatic (n = 58)
**Figure 19: PBK is associated with distant metastasis, shorter recurrence-free survival and prostate cancer.** (A) Human prostate non-tumor, localized prostate cancer and bone metastasis samples were immunostained with PBK-, MYC- and RANKL-specific antibodies and counterstained with hematoxylin. Bone sections are marked with ‘b’. Scale bars are 50 μm. (B) Prostate cancer patients in the TCGA dataset were stratified into low and high PBK-expressing groups based on a median value of PBK expression. The recurrence-free survival versus time in days was then plotted and significance determined with the log-rank test. The Oncomine website software was used to analyze and plot PBK expression and calculate significance in (C) prostate gland versus carcinoma in the Grasso dataset\(^ {253}\) and in (D) localized versus metastatic prostate cancer in the Varambally dataset\(^ {254}\). The y-axis represents the log\(_2\) median-centered RNA expression intensity of PBK while the x-axis represents binned sample groups.
Chapter V: Discussion
Development of detectable macrometastases is a defining step in progression of prostate cancer. It simultaneously necessitates the use of systemic therapies, reduces patient quality of life and drastically lowers the five-year survival rate. Screening and identifying early stage prostate cancers is essential, to allow treatment to begin when it would be most efficacious and curative. The current challenge is to distinguish aggressive neoplasms from indolent ones, so as to minimize the substantial health hazards associated with overtreatment. Prognostic nomograms have been devised to determine the likely course of the disease; however, they still possess a fair degree of uncertainty in predicting the risk of progression\textsuperscript{255}. Therefore, the identification of markers of aggressive behavior is still a pressing research question, and prostate cancer is particularly underrepresented in the use of molecular markers in the clinic. It is with this deficiency in mind that the investigation of the role of PBK in aggressive prostate cancer was commenced.

As an easily assayable measure of cancer cells’ tendency to metastasize, the ability of the cells to invade through a Matrigel matrix was measured in relation to PBK levels. Indeed, total PBK protein expression correlated well with the invasive ability of a panel of the prostate cancer cell lines used in this thesis. Preliminary investigation of human tumor-adjacent normal prostate, BPH and tumor samples showed a striking disparity in PBK levels: tumor-adjacent normal prostate and BPH samples had no detectable PBK, as did lower stage prostate cancer. PBK levels increased with the degree of the spread of the cancer cells, with the stage IV prostate cancer samples all expressing a large amount of PBK. Confirmation of the
correlation of PBK with cancer stage was then sought in a larger set of tumor-adjacent normal prostate and tumor samples (n =120). Here a significant association between PBK and prostate cancer Gleason score was also observed. Moreover, our data show a very high percentage (59%) of prostate cancers with distant metastases expresses high levels of PBK. These results are in good agreement with two earlier studies of lung cancer where either 59%89 or 54%91 of stage III-IV lung cancer samples were shown to have PBK expression. Our results are also in agreement with the finding of Shiraishi et al.98 that PBK expression was significantly increased in prostate cancer, compared to normal prostate. Additionally, while non-tumor samples completely lacked any nuclear PBK, there was a very strong correlation of nuclear PBK with cancer grade and stage and distant metastases. The association of nuclear PBK with cancer stage and grade in our study is a novel observation and it seems that nuclear PBK may possess more prognostic information than PBK levels alone. Nuclear exclusion of PBK via addition of an N-terminal nuclear export signal peptide sequence had no effect on PBK-dependent MMP production. Therefore, it is most likely that PBK activates β-catenin signaling through a cytoplasmic mechanism. Our study was unable to define a role for nuclear PBK. However, nuclear localization of PBK may mediate acquisition of aggressive behavior in advanced prostate cancer and this topic should be explored in the future. The other possibility is that nuclear PBK could be indicative of dysregulated nuclear transport, which is a common feature of cancer progression256,257.
The presence of PBK in basal epithelial cells in non-tumor tissue sections was also a novel and unexpected observation. Although there are conflicting reports\textsuperscript{258,259}, basal cells in the prostatic acini are believed to be a stem cell-like population capable of recreating both prostate epithelial cell layers\textsuperscript{260}. PBK is also present in spermatogonial germ cells\textsuperscript{85}, neural progenitor cells\textsuperscript{100} and placenta\textsuperscript{79}, but absent in adult epithelial cell types\textsuperscript{80}, suggesting its association with a stem cell type. It is also interesting to note that there are several high-confidence binding sites in the \textit{PBK} promoter of the SOX transcription factor, which is critical in initiation and maintenance of embryonic stem cells (ESC). All of these findings together suggest a possible role for PBK in a stem/progenitor cell type.

Pathologically, cancer stem cells are crucial for the recurrence and metastasis of any cancer. Stem-like cancer cells have been identified in several cancers, including blood\textsuperscript{261}, brain\textsuperscript{262}, breast\textsuperscript{263}, pancreas\textsuperscript{264} and prostate\textsuperscript{265} cancers. Although they are frequently found to make up only a small percentage of a tumor, there is accumulating evidence that they play a key role in metastatic spread and drug therapy resistance, owing to their higher motility and expression of survival factors and slower cycling\textsuperscript{3}. Interestingly, a comprehensive, systems examination of genes important in glioblastoma stem cells identified PBK as important in this cell type\textsuperscript{96}. Genetically or pharmacologically targeting PBK expression or function in glioblastoma-initiating cells significantly decreased their growth, survival, sphere formation and \textit{in vivo} tumor-initiating ability\textsuperscript{266}. In a large patient cohort (n=550) analyzed in the Cancer Genome Atlas Project, high PBK expression was associated with shorter recurrence-free survival and we demonstrated that PBK facilitates
invasion and metastasis in human prostate cancer cells. These are all clinical events that are attributable to cancer stem cells\textsuperscript{267}. Furthermore, the Wnt/β-catenin signaling pathway is important in development of castration-resistant prostate cancer\textsuperscript{268-270} and in prostate cancer stem cells\textsuperscript{271-273} (as well as in normal embryonic development of the prostate\textsuperscript{274}). PBK mediates its acquisition of aggressive behavior in prostate cancer cells through activation of β-catenin signaling\textsuperscript{249}. Finally, we observed that PBK up-regulated expression of the prostate stem cell marker CD44 (data not shown). All of these disparate findings in the literature and our observations suggest that PBK may play a key role in initiation or maintenance of cancer stem cells. Prostate cancer stem cells are resistant to chemotherapy and radiation and hormone therapy, so elucidation of the role of druggable proteins in cancer stem cells could have significant clinical implications.

In addition to PBK’s association with cancer stem cell features, we did establish that PBK does causally mediate invasive behavior of prostate cancer cells. Our investigation in both AR-positive (22Rv1) and AR-negative (PC-3M) cells showed that inhibition of PBK decreased their invasive ability by approximately 90%, whereas overexpression PBK in VCaP or LNCaP cells increased their invasive ability more than ten-fold. These results suggest that the presence of PBK is necessary for a large fraction of the invasive behavior of prostate cancer cells. Pharmacological inhibition of PBK in PC-3M cells and of β-catenin/TCF/LEF interaction in VCaP cells stably overexpressing PBK did show that PBK-dependent invasion requires β-catenin transcriptional activity, suggesting that PBK and β-catenin work together to establish a pro-invasion gene expression program. Our
data are in good agreement with previous reports that $\beta$-catenin plays a key role in facilitating invasiveness and metastasis in prostate cancer cell lines both in vitro$^{275}$ and in vivo$^{276}$.

To further investigate how PBK orchestrates invasive ability, downstream effectors were sought in a microarray of gene expression changes following PBK knockdown in a prostate cancer cell line. The matrix metalloproteinases MMP-2 and MMP-9 play a key role in metastatic development in human cancer patients$^{277,278}$. Using the Ingenuity Pathway Analysis software to analyze the microarray data identified MMP-2 and MMP-9 as significantly down-regulated genes following PBK knockdown. On the other hand, over-expression of PBK up-regulates expression of MMP-2 and MMP-9. Moreover, expression from the MMP-2 and MMP-9 gene promoters is also modulated by PBK, indicating that transcriptional control of these genes is positively regulated by PBK. These matrix metalloproteinases have been demonstrated to be involved in the penetration and invasion through the basement membrane and the endothelial barriers in vivo, allowing invading cells to appear in circulation$^{279}$. Notably, $\beta$-catenin/TCF/LEF inhibition of VCaP cells overexpressing PBK abolished PBK-induced MMP-2 and MMP-9 production, suggesting that PBK induces aggressive behavior through a $\beta$-catenin/TCF/LEF-mediated pro-invasion gene expression program.

In addition to induction of MMP-2 and MMP-9 expression, manipulation of PBK had a profound effect on cellular morphology. PBK knockdown in PC-3M led to the cells losing their elongated, spindle-like morphology and becoming wider and more polygonal, while the reverse morphological change happened upon stable expression of exogenous PBK in VCaP cells, which have low endogenous expression.
of PBK. We initially investigated EMT-related markers upon observation of this shift and there was a significant increase in levels of N-cadherin, SNAIL1, TWIST and ZEB1 and 2, with a concomitant loss of E-cadherin, in VCaP cells overexpressing PBK but we did not observe the converse happening in PC-3M cells with reduced expression of PBK. So, EMT may be important in the morphological shift VCAP cells undergo upon PBK overexpression, and changes in EMT factors in PC-3M may require longer silencing of PBK or other redundant genes may regulate EMT in these cells.

Although we have not ascertained the mechanism by which PBK activates β-catenin signaling, PBK inhibition did rapidly (within 30-60 minutes) decrease active β-catenin levels. This time scale suggests a PBK-dependent post-translational modification, and not changes in gene expression, that directly impinge on β-catenin. β-catenin stability is regulated by a large number of molecules; interestingly, a number of them possess PDZ domains, for which PBK has been shown to possess significant affinity79. This includes DVL, APC and a number of other proteins280, suggesting signaling nodes where PBK might interact with this pathway to effect its pro-invasion gene expression program.

Local invasion is an easily measurable aspect of aggressive behavior of cancer cells, but accumulating evidence suggests that, while the tissue architecture of the epithelium does serve as a barrier and suppressor of tumor invasiveness281,282, escape from the primary tumor and intravasation into the circulation is actually an early event in progression of at least a subset of cancer patients283,284. The rate-limiting step in metastatic development seems to be survival and growth in the novel microenvironments of the circulation and the distant organ
site\textsuperscript{285-287}, particularly in prostate cancer, where the appearance of macrometastases can occur more than a decade after resection of the primary tumor\textsuperscript{288}. Additionally, we also noticed in the large set of prostate cancer samples that distant metastases to the bones and other sites had strikingly high levels of PBK. With this in mind, we reexamined the microarray data to seek potential mediators of these later steps of the metastatic cascade. We discovered RANKL as a probable lead, given its importance in development of bone metastases in prostate cancer.

Levels of PBK and RANKL were analyzed in a panel of prostate cancer cell lines. As there was a striking correlation of the levels of the two proteins, further assessment was carried out in a panel of samples of non-tumor human prostate, benign prostatic hyperplasia and prostate tumors of increasing invasiveness and spread. In these human patient samples, there was a strong correlation between levels of either mRNA or protein of PBK and RANKL. These results suggested that RANKL could be a downstream effector of PBK-dependent metastasis.

Genetic manipulation of PBK expression did indeed causally regulate \textit{RANKL} expression via an increase in transcriptional activity at the promoter. Beyond regulating bone resorption, pre-clinical evidence demonstrates that RANKL can promote the aggressive behavior of cancer cells\textsuperscript{233,289} and induce changes in the microenvironment that further stimulate tumor growth and malignant behavior\textsuperscript{290}. These reports are borne out by clinical data showing that blocking RANKL specifically via a neutralizing, monoclonal antibody increases time to recurrence in prostate cancer\textsuperscript{236} and overall survival in lung cancer\textsuperscript{237}. Induction of RANKL expression, as well as of other pro-osteoclastogenesis factors such as parathyroid
hormone, IL-6 and TGF-β (data not shown), is therefore a potential mechanism by which PBK could contribute to progression through the later, rate-limiting steps of cancer metastasis.

A search of the RANKL promoter revealed a small number of transcription factors that regulate its expression, including c-Myc. Further investigation showed that MYC was responsible for PBK-dependent RANKL expression. MYC is a potent oncogene and a large body of evidence links it to almost every aspect of the tumor phenotype, including malignant spread\textsuperscript{291}. MYC has been previously shown to induce RANKL expression in prostate cancer cells\textsuperscript{228}, confirming its potential as a link between PBK and RANKL.

Once we discovered that PBK expression increased total MYC levels, while loss of PBK decreased total MYC, we assumed that it was a consequence of PBK-dependent activation of β-catenin/TCF/LEF signaling, as MYC is a well-characterized target gene of this pathway. However, while trying to show that exogenous MYC rescued the loss of RANKL observed upon PBK knockdown or inhibition, we noticed that FLAG-tagged MYC levels were consistently lower in PBK siRNA-treated samples. This could not be due to regulation of β-catenin, since the exogenous MYC was under control of an exogenous CMV promoter. This surprising finding led us to investigate if PBK regulates MYC stability. Pharmacological inhibition of PBK did lead to a significant decrease in MYC protein stability and also rapidly increased its ubiquitylation. This result suggests that PBK regulates the ubiquitylation status of MYC, and that inhibition of PBK leads to its rapid polyubiquitylation and degradation. Whether PBK phosphorylates MYC or another
factor has not been established yet and further experiments are necessary in this area. Numerous kinases and E3 ligases regulate MYC stability; moreover, PBK has been found in a complex with the deubiquitinase USP5^{292}, raising the possibility that PBK could regulate the ubiquitylation machinery. Although the mechanism is uncertain, our data indicate that PBK regulates MYC levels through transcriptional as well as posttranslational mechanisms.

MYC has also been reported to induce $PBK$ expression indirectly through the cell-cycle transcription factor E2F1^{101}. We confirmed the ability of MYC to regulate $PBK$ expression in prostate cancer cells as well (data not shown). Therefore, PBK and MYC are involved in a cycle of positive feedback (figure 20). This positive feedback loop would explain our observation that inhibition of PBK with any of three pharmacological inhibitors consistently decreases levels of both PBK and MYC. Under physiological conditions this feedback mechanism is likely to facilitate progression through mitosis, but the constitutively higher levels of MYC and PBK in cancer could contribute to the development and aggressiveness of prostate and many other cancers^{89,158,293}. Therefore, it seems likely that both PBK and MYC could mediate some of the malignant behavior associated with each other’s expression in cancer.
Figure 20: Schematic diagram of PBK activity We showed in this work that PBK activates β-catenin signaling and this results in increased MMP-2 and -9 production, resulting in increased cancer cell invasion. PBK also induces transcription and post-translational stabilization of MYC, which directly increases RANKL transcription. MYC also regulates PBK transcription, showing the existence of a positive feedback loop between the two proteins.
Altogether, our data show that PBK facilitates multiple steps in the metastatic cascade. This raises the possibility that PBK could serve as a prognostic marker of aggressive behavior in prostate cancer. Differentiating indolent from aggressive prostate cancer remains a clinical challenge and appropriate biomarkers of aggressive neoplasms of the prostate are necessary to increase prognostic accuracy and survival of patients with metastatic prostate cancer. While PBK has not been examined in the context of providing information during initial prognosis, as part of a panel of 31 genes, it accurately staged prostate cancer patients\(^\text{294}\) and accurately predicted biochemical recurrence and time to death in patients who had radical prostectomy\(^\text{295}\). This agrees with our finding that PBK is significantly associated with a shorter time to recurrence in the TCGA prostate cancer data set. However, due to the low number of mortalities from prostate cancer (8 out of 550 patients in a 12-year span) in this data set, it was not possible to establish if PBK is a significant, independent predictor of cancer mortality. Further validation of PBK as a prognostic marker would require, as for any potential biomarker, a large, prospective trial carried out for at least several years to reach a statistically significant conclusion. Until that study can be done, retrospective analyses of existing data sets can contribute or refine parameters but prospective trials are necessary to eliminate any potential sources of bias and definitively establish a biomarker-assisted model to identify high-risk prostate cancer.

While basic understanding about the role of PBK in cancer is unfolding, efforts to develop the potential clinical utility of PBK as a therapeutic target are already underway. An academic and industry collaboration developed two novel
inhibitors of PBK, OTS514 and OTS964, that induced complete regression of lung tumor xenografts in mice\(^{250}\). OTS514 potently inhibited growth of PBK-positive cancer cell lines and, while it did show some hematopoietic toxicity in mice, this off-target effect was completely alleviated by a non-targeted liposomal formulation. OTS514 was also shown to effectively inhibit growth of acute myeloid leukemia (AML) cells and increase survival of AML-engrafted mice\(^{112}\). A different, rationally designed inhibitor of PBK, known as HI-TOPK-032, potently inhibited growth of colorectal cancer xenografts in mice\(^{244}\) and inhibited growth and sphere-forming ability of glioblastoma-initiating cells\(^{266}\), as discussed above. The effectiveness of pharmacological inhibition of PBK in these disparate cancer types, combined with the reports of PBK mediating growth\(^{296,297}\) or tumorigenic ability\(^{110,298}\) and being associated with advanced cancers of various tissue origins\(^{92,93,299}\), show that PBK could be a viable therapeutic target in a variety of different cancers. This growth-inhibitory effect is consistent with the physiological role of PBK mediating successful mitosis and its cancer-associated role of tumor suppressor inhibition. The caveat, as for all targeted therapies, is that PBK-negative cell lines are much less sensitive to PBK inhibition\(^{250}\) and patients would have to be confirmed to possess PBK-positive tumors before treatment. The work presented here aims to demonstrate that PBK has an oncogenic role beyond promoting and mediating growth, i.e., that it also enables invasive and metastatic behavior. This is in agreement with a recent report showing that genetic knockdown of PBK in circulating prostate cancer cells greatly reduced their metastatic ability\(^{300}\). While development of agents that specifically target the metastatic process has lagged
behind those that target cell division or survival, this may be due to a lack of understanding when metastases are seeded. Cancers such as those of the breast or prostate can have a long latency between detection and removal of the primary tumor and development of macrometastases, while lung and pancreatic adenocarcinomas progress rapidly to macrometases upon reaching a distant organ\textsuperscript{301}. It seems that escape from the primary site is an early event in at least a subset of prostate cancers\textsuperscript{283,284}. Therefore, the ability of PBK to mediate multiple steps in the metastatic cascade, in addition to cancer cell growth and survival, highlights it as a potential therapeutic target.

Taken together, our findings demonstrate that PBK activates both $\beta$-catenin/TCF/LEF, MYC and RANKL signaling to promote the acquisition of invasive and metastatic abilities in prostate cancer cells and that it is associated with high-grade prostate cancer and distant metastases. Both $\beta$-catenin and MYC are well-studied molecules that have been definitively tied to cancer development and progression but which have proven difficult to target directly with drugs. PBK has a limited expression profile in adults but high aberrant expression in cancer and it mediates multiple aspects of the tumor phenotype. Therefore, it merits further consideration as both a prognostic biomarker of aggressive behavior in prostate cancer and as a direct therapeutic target to treat metastatic disease.
Chapter VI: Future Directions
The work presented in this thesis generates more questions than it answers and suggests many more directions for further investigation. The molecular mechanisms through which PBK activates β-catenin signaling and posttranslationally stabilizes MYC are still unknown. Inhibition of PBK decreases β-catenin protein levels within one hour, suggesting that PBK directly regulates some component of Wnt signaling pathway and isn’t mediating this effect indirectly through a change in gene expression. The β-catenin core pathway is well established, and these proteins could be assayed for changes in phosphorylation status using Phos-tag, a reagent that resolves differentially phosphorylated species of a target protein on a Western blot. Beyond the core pathway members, there are a lot of proteins that impinge on β-catenin stability\textsuperscript{280}, so identifying the mediating factor becomes more difficult. A stringent screen of PBK-binding partners using mass spectrometry could be useful in identifying the mechanistic connection.

Similarly, we were unable to ascertain how PBK stabilizes MYC protein levels. As discussed above, there are several kinases, E3 ubiquitin ligases and acetyltransferases that have been demonstrated to regulate MYC protein stability. There is also the possibility that PBK directly phosphorylates MYC at a less well-studied or novel phosphorylation site. Analyzing MYC phosphorylation was technically difficult: the protein is very unstable and, for unknown reasons, does not resolve well on a Phos-tag Western blot. Analysis of the stability of discrete regions of MYC in the context of PBK presence or absence may yield further clues.

The lack of an identified substrate hampered our mechanistic research. We were unable to replicate any of the reported PBK-mediated phosphorylation events.
This may be due to differences in biological context; there is only one other publication on PBK in prostate cancer cells. Oh et al. reported that PBK activates JNK1 signaling through phosphorylation of the JIP1 scaffold protein in melanoma cells\textsuperscript{111}. The presence or absence of other scaffold proteins in different cell types may determine the mechanism and effect of PBK activity. In any case, identification of a substrate of PBK would greatly assist investigation of the molecular biology of PBK and potential future drug design efforts. As mentioned above, a stringent, mass spectrometry-based screen of PBK-interaction partners could facilitate this process.

Murine in vivo experiments would serve as a useful pre-clinical model to confirm that PBK contributes to metastatic progression of prostate cancer. Intracardiac injection of prostate cancer cells places them into systemic circulation and measures progression through the later, rate-limiting steps of the metastatic cascade, while orthotopic implantation of prostate cancer cells requires more expertise but assays the whole metastatic process. Unfortunately, there is not a genetic mouse model of prostate cancer that accurately recapitulates the metastatic process of human prostate cancer\textsuperscript{302}. Patient-derived xenografts of prostate cancers have been reported to more accurately mimic human prostate cancer and metastasis\textsuperscript{303}, so this may be the most faithful (and expensive) method to study the role of PBK in malignant progression of human prostate cancer.

Physiologically, we repeatedly observed PBK immunohistochemical staining in prostate basal epithelial cells, a novel observation in a putative stem cell population. These cells are enriched for stem/progenitor cells that can, from a single cell, generate an entire prostate epithelial organoid\textsuperscript{260} and may also be the cell of
origin for at least a subset of human prostate cancers. Investigation of the role of PBK in this cell type may illuminate signaling networks in both physiological and cancer stem cells, a troublesome cell type that may contribute to cancer drug resistance, recurrence and metastasis.

The findings in this thesis, in conjunction with published reports, show that PBK may have prognostic or therapeutic utility in a range of cancer types. Elucidation of the potential of PBK in either role would require prospective trials. Retrospective studies already showed that PBK, as part of a panel of 31 genes, accurately predicts stage at initial diagnosis or recurrence after radical prostatectomy or radiation therapy. A large prospective trial would thus be necessary to determine if this panel could be part of a biomarker-assisted model to recommend patients for active surveillance or different treatments.

Finally, as PBK seems to mediate cancer cell growth, survival, invasion and metastasis, preclinical evidence suggests that pharmacological inhibition of PBK could have therapeutic potential. Indeed, Nakamura, who led the group that developed two novel inhibitors of PBK, stated that he is planning to begin clinical trials of one. The findings in this thesis suggest that PBK may be a therapeutic target in advanced prostate cancer. Pharmacology studies need to be performed to evaluate the most efficacious potential inhibitor and further pre-clinical studies would be beneficial to establish this claim further, e.g., if PBK inhibition causes regression of patient-derived prostate cancer xenografts in mice and if particular subtypes of prostate cancer are more vulnerable. These data would be essential in
bringing a PBK inhibitor into the clinic and increasing the range of treatment options for patients with aggressive prostate cancer.
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