Defining and modeling a unique molecular subclass of prostate cancer

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1. Gutachter: Prof. Dr. Mark A. Rubin

2. Gutachter: Prof. Dr. Rupert Mutzel

Disputation am 31.01.2018

I want to thank Prof. Dr. Mark Rubin and Prof. Dr. Rupert Mutzel for taking the time to read and review my dissertation. And I want to thank the member of my committee to be part of my defense and evaluate the work and research I have conducted.

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"I came to New York as a student and left as a married woman, mom of a wonderful daughter and with the strong foundation for a successful career as a scientist."

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1. Focus and question to answer for this thesis

Mutations in *SPOP*, which encodes for the substrate recognition subunit of the Cull3 ubiquitin ligase complex, have been reported in first whole genome sequencing reports of prostate cancer patients. The goal of this PhD thesis is to provide knowledge about the role of mutated *SPOP* in prostate cancer. This includes answering first in class questions with increasing levels of complexity:

- **Further characterization of the mutations, such as position in the gene, allelic frequency and predicted impact on protein functionality**
- **What is the mutation frequency of *SPOP* across multiple cohorts and ethnicity? Which includes the development of a high throughput screening assay**

Create a mouse model, which will help us to answer following questions:

- **Does mutation in *SPOP* cause prostate cancer initiation? If so, possible mechanism?**
- **Does mutated *SPOP* have a driving role in prostate cancer progression?**

2. Introduction

2.1 Anatomy and histology of the prostate

The prostate is an accessory gland of the male reproduction system the size of a walnut and located below the bladder and in front of the rectum [1]. The main function of the prostate is to produce the fluid component of the semen [1].

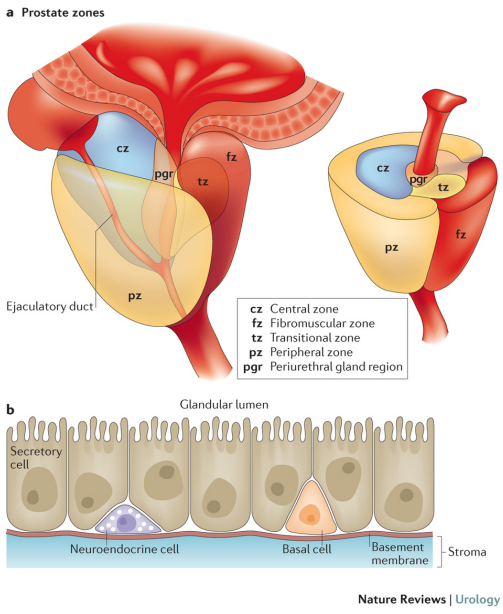


Figure 1: Gross and microscopic anatomy of the prostate gland (license number: 4217601510627)

The prostate gland is a two lobed organ wrapped around the urethra and can be divided into 4 major zones as shown in figure 1 (Illustration adapted from Verze et al., Nature Reviews, 2016) [2–4].

- Central zone (cz)
- Fibromuscular Zone (fz)
- Transitional zone (tz)
- Peripheral Zone (pz)

On a histological level the prostate is structured into a large number of branched glands which all lead through ducts into the prostatic urethra. An

individual prostate gland is built of three differentiated epithelial cell types [5]. The most prominent cell type is the secretory epithelial cell (luminal cells) which shows high level of androgen receptor (AR) therefore expresses prostate-specific antigen (PSA) and characteristic markers such as cytokeratins 8 and 18[6]. Basal cells can be found in-between the columnar shaped luminal cells and are thought to harbor a population of stem cells and therefore are important for the regeneration of the epithelium. They express cytokeratins 5 and 14 and it is important to note that AR is below detection level and not required for growth[7]. The third and least frequent cell type are the neuroendocrine cells which are thought to provide a paracrine function in the gland[8]. They express distinct markers such as chromogranin A and synaptophysin and like the basal cells they do not express AR[8]. The inner layer built by the luminal, basal, and neuroendocrine cells is surrounded by a basement membrane, which then all together forms a gland. The connective tissue consisting of smooth muscle, nerves and lymphatics form the fibromuscular stroma which accounts for about 70% of the prostate mass [1].

2.2 The pathology of the prostate/ Prostatic diseases

There is a wide spectrum of prostate pathology ranging from prostatitis over benign prostatic hyperplasia (BPH) to indolent and aggressive prostate cancer. It is interesting that the prevalence for each pathological state is different between lobes. As an example, the vast majority of BPH can be found in central zone whereas 75% of all prostate cancer cases seem to have their origin in the peripheral zone[9]. More research needs to be done to fully understand the biology behind the connection of malignancy and the specific zones. *During my time in Dr. Rubin´s lab I generated a new mouse model characterizing a specific molecular subclass of prostate cancer (PCa) and therefore I will only focus on PCa from this point on.*

Acinar adenocarcinoma accounts for 95% of all hormone naïve clinically localized prostate cancers[10]. The origin of this type of adenocarcinoma lies in the cells forming the gland. The remaining 5% of prostate cancer starts from cells that either line the ducts (ductal adenocarcinoma), cells covering the glands (Squamous cell cancer), cells which are part of the neuroendocrine system (carcinoid tumors) including the very aggressive small cell carcinoma or it starts from cells which are capable to develop into connective tissue, blood vessels or muscle cells (sarcomatoid cancer)[11].

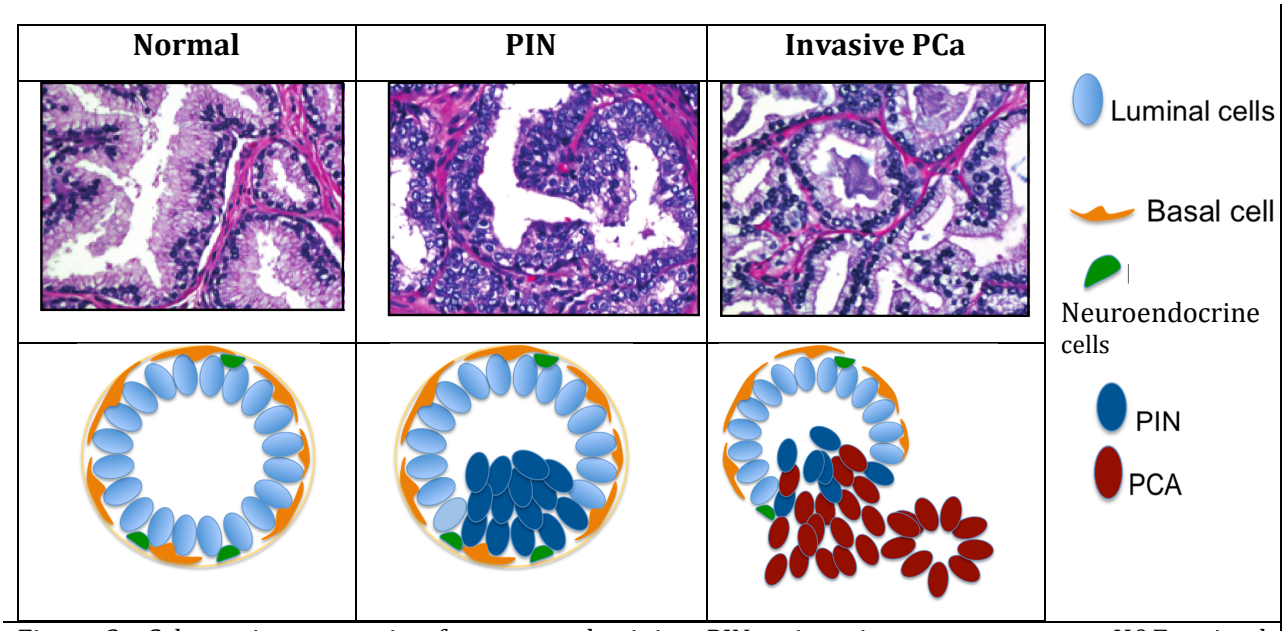


Figure 2: Schematic progression from normal acini to PIN to invasive prostate cancer. H&E stained microphotographs taken by Dr. Verena Sailer.

Prostatic Intraepithelial Neoplasia (PIN) is widely accepted to be the first step of prostate carcinogenesis[12]. Normal prostatic glands turn into PIN when cells start to proliferate unconfined without invading the basement membrane [13] (Figure 2). There are two grades of PIN; the low grade and the high grade PIN. But the high variability between pathologist in calling low-grade PIN limits its clinical usage and therefore will not be reported at all[12]. The increasing frequency of PIN as well as PCa with increasing age and the strong correlation between high-grade PIN and subsequent development of invasive adenocarcinoma led to the widely accepted consensus in the field of high-grade PIN being the precursor of PCa. Additionally over the past years scientists confirmed the similarity of genetic features between high-grade PIN and adjacent PCa such as loss of 8q12-21, mutation in *SPOP* or *TMPRSS-ERG* fusion[12,14–18]. In the early 1900s Virchow and Broders first formulated the relationship between increasing grade of neoplasm with increasing malignancy[19,20]. In the 1960s Dr. Donald F Gleason, a pathologist in Minnesota, developed a prostate cancer grading system. The Gleason score is the most widely accepted grading system, which is entirely based on histological growth patterns of carcinoma cells in H&E-stained prostatic tissue sections. A drawing by Dr. Gleason (Figure 3, Adapted from Peter A Humphrey, Modern Pathology, 2017) shows how he consolidated 9 different growth patterns into 5 grades[19]. Every pathological review of prostate cancer consists of a histological score from 2-10, which results from adding the primary and the second most dominant grade together. The minor grade has to have a prevalence of at least 3% otherwise the most dominant grade is multiplied by two[19]. As an important side note, because prostate cancer is a multifocal and heterogeneous disease multiple

studies have reported that on an average one patient has about 2.7 Gleason grade patterns ranging from 1-5 independent nodules[21–23].

With nearly 400,000 new diagnosed cases each year in Europe alone and 307,000 deaths per year worldwide makes prostate cancer the fifth most common cause of death due to cancer in males - after lung, liver, stomach and bowel[24,25]. The majority of diagnosed low (Gleason 6) or intermediate (Gleason 7) Gleason score prostate adenocarcinomas remain localized and therefore indolent. Only a subset show an aggressive phenotype and have a high potential to end lethal [26–28]. To date, scientists were not able to find a reliable biomarker to distinguish between indolent and aggressive PCa and therefore many patient go through unnecessary treatment. On the other hand by waiting and observing one might miss the opportunity to treat a yet indolent lesion turning into an aggressive tumor. A recently identified gene signature might potentially be able to help to better characterize low grade tumors while under surveillance[26]. The signature is linked to aging and senescence and the future will show how much impact it can have improving clinical diagnostic and patient outcome.

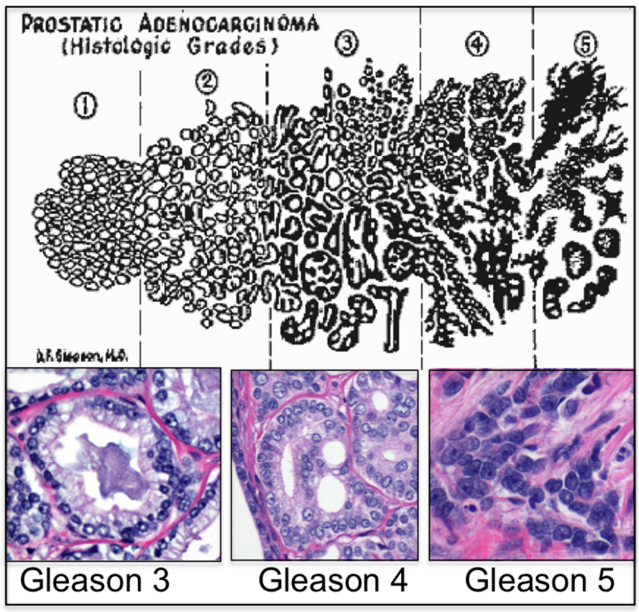


Figure 3: This illustration shows Dr. Gleason's own simplified drawing of the five Gleason grades of prostate cancer. Grade 1 appears on the far left and grade 5 on the far right. (license number: 4217610880836)

2.2.1 The role of AR in prostate development and tumorigenesis

Androgen receptor (AR) is a member of the nuclear receptor superfamily and has a critical role in prostate development and maintenance[29,30]. In benign prostate glands androgen does not promote cell proliferation rather induces the differentiation of basal cells into luminal cells as well as the production and secretion of prostatic differentiation marker[31–33]. Without a doubt luminal cells express the highest amount of AR but recent research shows that there are traces of AR in a subset of basal cells as well as stromal cells[34–38]. Basal cells are characterized by a higher proliferation rate, and are known to be able to self-renew. Lu *et al.* and Planz *et al.* both reported in 1999 a reciprocal feedback loop between stromal cells and the epithelial compartment[35,37]. They suggest that androgens stimulate the stromal cells to secret peptide growth factors called “andromedins” which

then stimulate the growth of basal cells, stimulates differentiation and repress apoptosis pathway[31,39]. Such suggested factors include fibroblast growth factor 7 and 10 [37], keratinocytes growth factors [35] as well as insulin growth factors [40]. This has been shown in multiple studies for mouse and rat cells, and mainly during fetal development, but compelling human data is still missing. Without the presence of androgens AR is localized exclusively in the cytoplasm bound to heat shock proteins (HSP). The primary and well characterized androgen is testosterone. The vast majority of testosterone is produced by the gonads. Modifications by the 5α-reductase isoenzymes converts testosterone to its more potent dihydrotestosterone (DHT). DHT binds with a high affinity to AR inducing conformational changes leading to the disassociation of AR and HSPs, dimerization and migration to the nucleus[41]. Once in the nucleus and after further modification such as phosphorylation and SUMOlyation, AR acts as a transcription factor. Bound AR can have an enhancing as well as repressing effect on transcription in a complex role with other co-factors, such as SRC3 as well as pioneer factors. The "AR cistrome" which includes all DNA regions, mainly promoter and enhancer regions, indicating AR affinity, is very specific for a cell type and developmental stage leading to an androgen specific gene signature.

The crucial role of activated AR in prostate development and functionality as well as the high AR addiction of almost every prostate tumor cell brought AR to the center of attention for research and drug development for the last decades. Understanding the function of AR on all levels including normal prostate development - prostate cancer initiation - prostate cancer progression and metastasis as well as castration resistant prostate cancer (CRPC) is required for most precise and efficient patient care.

Undeniably AR holds a central role in prostate cancer. Even at the stage of CRPC the cancer cells retain AR expression and often dependency on AR signaling. But surprisingly little is known of the involvement of AR and androgens itself on tumor initiation. Studies in mice have shown that overexpression of AR is not enough to induce a tumor but the precursor HG-PIN. A very interesting question still remains unanswered: At which step and how do AR binding sites switch from the focus of differentiation related genes to proliferation related genes. Zhou at al. called this step the `malignancy switch´[33]. Despite the central role of AR in prostate cancer neither high or low androgen levels have been conveniently shown to correlate with prostate cancer initiation. The only widely accepted correlated "bio-factor" to this point is age. Some studies suggest that chronic low level of androgens, which potentially leads to increased level of AR might increase the risk of cancer incidence. This could provide a partial explanation of the correlation with age, which comes with decreased level of androgens and cancer incidence. On the other hand it is worth mentioning than two preventative trials have shown reduced risk of prostate cancer in men treated with 5a-reductase inhibitor over a period of 4-7 years[42,43]. A very recent study investigates the role of

oxidative stress leading to DNA damage and higher chance to create DNA rearrangement such as the TMPRSS-ERG fusion[44]. Further, the study suggests that AR itself does not play a direct role in the process of gene fusion. But the presence of AR causes an increased transcription rate which then makes the DNA more accessible for DNA damage causing molecules[44]. Other groups identified AR as a direct cause for two genes to rearrange through an AR induced DNA looping bringing two genes into close proximity which then allows rearrangements more likely to happen[45–47]. The concept of inflammation in the prostate being causative to cancer has been discussed previously[48–50]. Tam et al reported in 2003 that castration leads to inflammation in the prostate of rat[51]. Another theory says that prolonged inflammation increased the rate of highly proliferative "cells with stem like features" which some people consider the cell of origin for cancer (reviewed in [52]). None of the described models gives a complete explanation what causes the `malignancy switch´.

2.2.2 Non AR pathway alterations in prostate cancer

Despite the important role of AR in prostate cancer initiation and progression, multiple other transcriptional regulators and pathways have been described to have a key role in prostate cancer. Such pathways include PI3K/mTOR , MYC, Ras/MAPK/ERK, Jack/STAT, Wnt, NF-kB and SPINK[53–55].

Androgen receptor signaling and phosphoinositide 3-kinase (PI3K) signaling are two major pathways important for cell survival and growth, and that contribute to malignancy. Alterations in these two pathways, using PTEN alteration as a major representative of the PI3K pathway, can be seen in around 30% of localized PCa, and the percentage increases with disease progression underlining their important role in tumorigenesis (Figure 4).

PI3K molecules can be grouped into three classes (I; II; III). Class I molecules and in particular class IA are clearly involved in oncogenic processes and they harbor a catalytic (p110) as well as regulatory (p85/p55) subunit. Further, class I molecules can be divided into the two subgroups, IA and IB. Both catalytic as well as the regulatory subunits are each encoded by three genes (α, β and γ)[56]. Their catalytic function can be seen as messenger between activated transmembrane receptors and key cell pathways such as proliferation, survival and differentiation. The activation of class I PI3K molecules sets off a cascade of phosphorylations of inositol-containing lipids leading to phosphorylated PIP2 and then PIP3. PIP3 in turn then has the ability to recruit PH-domain-containing proteins to the cell membrane[56]. One such target is the oncoprotein AKT. Through phosphorylation AKT regulates the activity of many proteins involved in growth, cell cycle and apoptosis such as mTOR, MDM2 and NF-kB[56].

Typically, AR and the PI3K/mTOR pathways balance each other out; meaning the activation of one keeps the other in check and prevents cells from growing out of control. This has been demonstrated in multiple studies in the past few years and can be summarized as a reciprocal feedback between PI3K/mTOR and the AR pathway. The exact mechanism is yet to be determined[57,58].

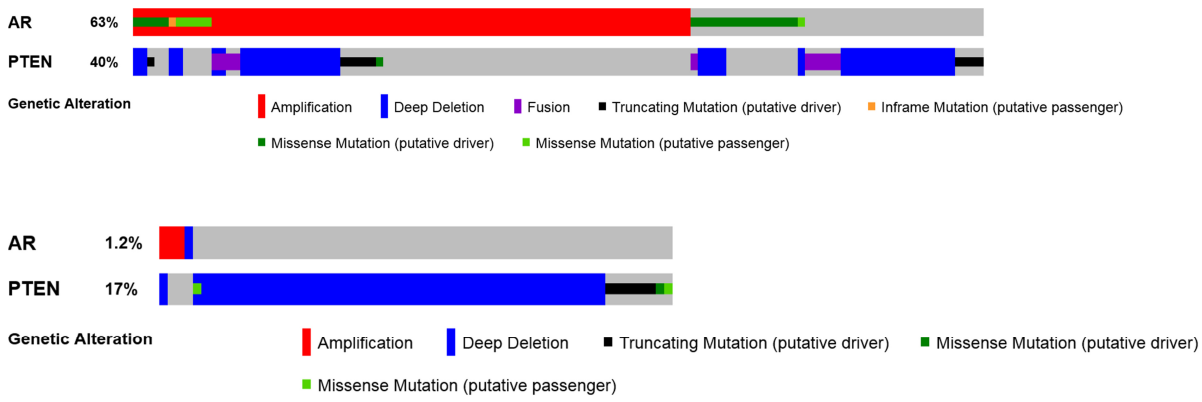


Figure 4:
Top: Genetic alterations in AR and Pten in localized prostate cancer (top – Cohort:Localized Prostate cancer; Prostate Adenocarcinoma (TCGA, Cell 2015)) and advanced prostate cancer (bottom – cohort:Metastatic Prostate Cnacer, SU2C/PCF Dream Team (Robinson et al., Cell 2015)).Figure generated using cbioportal.

2.2.3 Treatment of prostate cancer

If at the point of diagnosis the cancer is still localized, with no sign of spread to distant organs, by removing the whole prostate, radical prostatectomy, the patient has a very high chance to become and stay cancer free. Recent studies have also suggested that radiation therapy and active surveillance are equally beneficial for the care of clinically localized disease. Once the cancer spreads outside of the prostate treatment other than surgery will be needed. Because of its central role in tumor development and progression the most efficient treatment of disseminated prostate cancer involves the inhibition of androgen receptor by either surgical or chemical castration. Chemical castration currently happens on multiple levels in the AR activating pathway either in mono or combinational therapy.

Blocking androgen production by giving **gonadotropin-releasing hormone** (GnRH) **agonist** which will initially lead to an increase of leutinizing hormones (LH) and consequentially androgens but shortly after will lead to an down regulation of blood testosterone levels.

Non-steroidal antiandrogens (NSAA) with the functionality of blocking the interaction of androgens and AR itself. Giving NSAA in combination with GnRH agonist will provide an additional AR inhibition mechanism especially during the time the agonist will lead to an increase

of androgens. Once both blocking agents are fully effective the actual level of circulating testosterone in the blood drops below 0.2 ng/dl which is comparable to men who underwent surgical castration[59].

Unfortunately, for the majority of patients the tumor growth will relapse, and at this stage will be adapted to the chemical castration. This type of prostate cancer is called: Castration resistant prostate cancer (CRPC). Despite the lack of circulating testosterone the tumor remains AR sensitive. After failing first line androgen depravation therapy (ADT) patients with biochemical recurrence of elevated PSA level have a limited amount of treatment options.

Second generation of anti-androgens (e.g., enzalutamide) have shown to have a life prolonging effect for a couple of months when given to patients with CRPC. **CYP17 inhibitor**, such as abiraterone acetate, is another level of interfering in the activation of AR activity. CYP17 is a key enzyme involved in androgen production. Adrenal glands as well as the tumor cells themselves have been shown to be able to produce low levels of androgens, which are thought to be enough to keep promoting cancer growth.

Once AR targeting therapies fail, a less tumor specific treating approach can be administered. Doxetaxel and Cabazitaxel, members of compound group taxanes, are FDA approved chemotherapeutic agents for CRPC[60]. Taxanes target the dynamic ongoing microtubule assembling and disassembling process which makes it unable for the cells to go through an efficient cell division process ultimately leading to catastrophe in the tumor cells.

A small percentage of men will progress into advanced prostate cancer with metastases. Majority of those patients will show metastasis located in the bone and unfortunately, to this date a treatment leading to cure is not available[61]. After failing androgen depravation, then called metastatic castration resistant prostate cancer (mCRPC), two main strategies fighting the metastasized cancer are to either target the bone homeostasis or to administer radioactive substances conjugated to calcium molecules, radiopharmaceuticals[62].

Research has been done to get a clear picture on how prostate cancer and their metastases can overcome ADT and still remain AR sensitive. Some of those mechanisms include amplified AR level, mutations in AR leading to decreased ligand specificity and changes in the expression of AR co-regulators[41,63].

2.2.4 Molecular characterization of prostate cancer

As previously discussed many external factors can play a role in prostate cancer initiation. Next to age, inflammation, diet and an overall healthy life style, genetic alterations have the strongest impact on cancer initiation, progression but also treatment success[48,50,64,65].

Uncharacteristically for solid tumors, prostate cancer is a cancer of gene fusions and chromosomal rearrangements and is on the lower end of the spectrum for frequency of somatic mutations, with around 1 mutation per Mb, compared to other tumor types (Figure 5, adapted from Lawrence at al., Nature, 2016) [66,67].

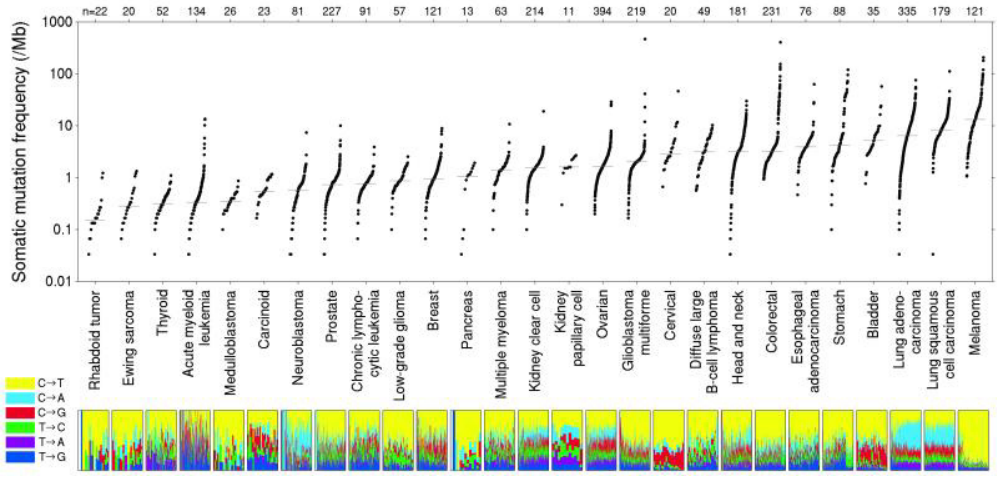


Figure 5: Mutational burden across different tumor entities (license number: 4207680051437)

Around 50% of of all prostate cancer show at least one gene fusion[66].

In 2005 Tomlins et al described the first recurrent and most prevalent gene fusion, *TMPRSS2-ERG*[68]. The vast majority of gene fusions seen in prostate cancer involve an AR responsive promoter element as the 5´ partners as well as a growth regulating oncogene as the 3´ fusion partner. ERG is a member of the ETS (erythroblast transformation-specific) family, which encode for oncogenic transcription factors. Other known *ETS* fusion partners are *ETV4*, *ETV1*, *ETV5* and *FLI1*[69]. Till this date TMPRSS2-ERG positive tumors mark the largest subclass in prostate cancer seen in about 50% off all patients[70]. Over many years fusion positive tumors were thought to be characteristically similar and were combined into one subclass. Multi institutional efforts such as The Cancer Genome Atlas (TCGA) made it possible to reveal that despite involving genes from the same family every gene fusion leads to its own distinct tumor profile, including gene signature and methylation profile (Figure 6, adapted from Abeshouse at al.; Cell,2015) [70]. About one third of hormone naïve localized prostate cancers do not show a known fusion but rather have a specific mutation. The most common mutation seen in about 10% up to 15% of all patients occurs in the *SPOP* gene[17,70]. Other recurrent mutated genes are *P53*, *FOXA1* as well as *MED12*[17,70] (Figure 6).

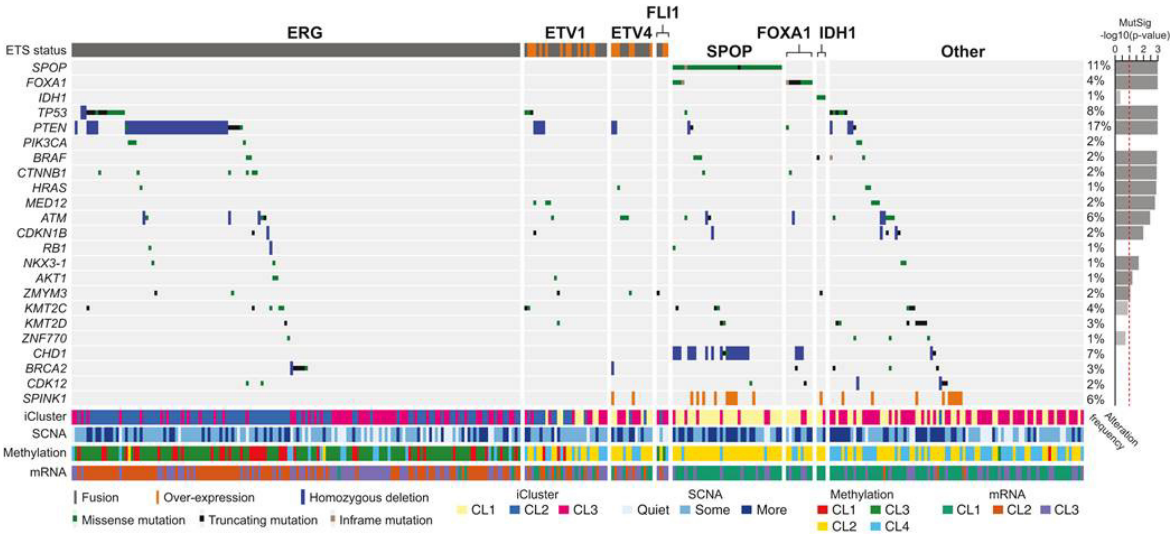


Figure 6: Recurrent Alterations in Primary Prostate Cancer (The cancer genome atlas) (license number: 4217601156379)

The molecular makeup of a tumor includes some or all of a list of changes such as specific mutations, chromosomal rearrangement, gene fusions, and a transcriptional as well as methylation profile. Further characteristics could include overall mutation load and rearrangement burden, copy number variations or abnormalities in functional subgroups of genes such as those involved in DNA damage repair or cell cycle check points. Ultimately, understanding the difference between subgroups will not only help determine new biomarkers and more predictable disease progression but will lead to specific and hopefully more successful treatment plans. Till this point and despite large efforts of analyzing multiple cohorts and their molecular features the Gleason score is still the most reliable marker to predict decease progression[71].

Figure 7 reflects a timeline of when and how characteristic alterations in prostate cancer have been first described.

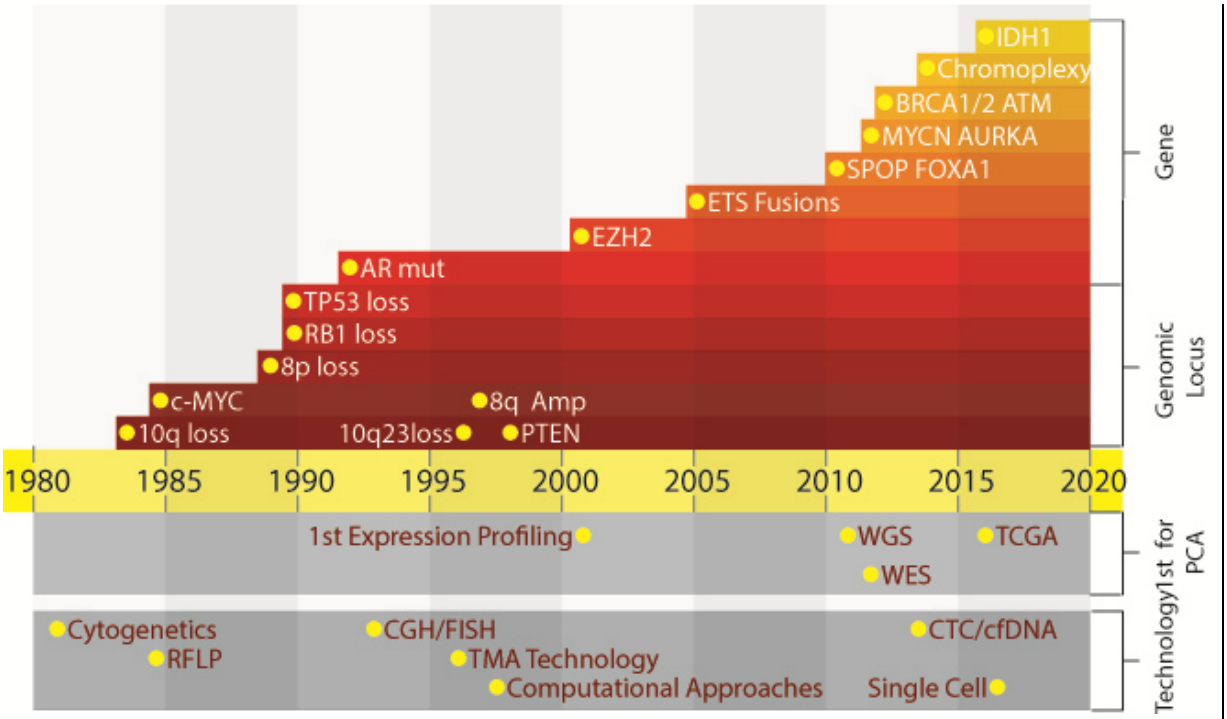


Figure 7: Rubin and Demichelis, The Genomics of Prostate Cancer in Cold Spring Harbor Library, Prostate Cancer, Shen M and Rubin M (2017, in press)

2.2.5 Molecular features of advanced prostate cancer

In a multi-institutional effort (largely funded by Stand up 2 Cancer (SU2C)-PCF) scientists (including Dr. Rubin) sequenced 150 metastatic castration resistant prostate cancer (mCRPC) and analyzed the genomics of advanced prostate cancer on context of disease progression and clinical outcome. Comparing the prevalence of recurrent aberrations in localized primary disease versus advanced prostate cancer further indicated the central role of AR in disease progression but also functional loss of TP53, further activation of PI3K pathway or alteration causing DNA repair deficiency[72] (Figure 8, adapted from Robinson et al., Cell, 2015). Knowing and further identifying pathways which looking at the genomics seem to be crucial in disease progression and helped shifting the research into new areas such as Poly(ADP-ribose) polymerase (PARP) inhibition in advanced prostate cancer showing signs of DNA repair deficiency[73,74] .

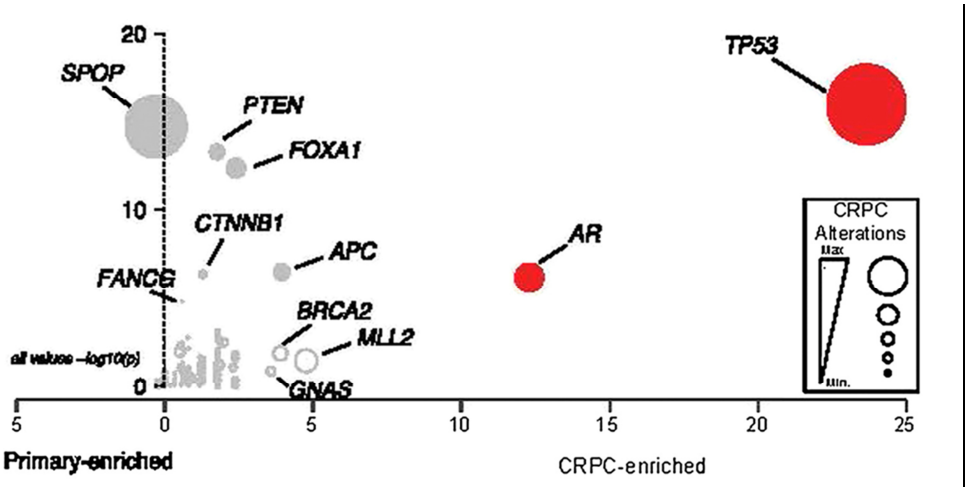


Figure 8: Mutations enriched in mCRPC relative to hormone naïve primary prostate cancer (license number: 4218180165066)

2.2.6 Models in prostate cancer

Great efforts has been made in characterizing cohorts and correlating molecular features to either prospective or retrospective clinical data such as prostate cancer stage, overall survival or time till biochemical relapse[71,75,76]. One major downside of trying to understand prostate cancer by looking at a cohort is the molecular complexity of a single patient. In 1971 Alfred G Knudson formulated the two-hit hypothesis[77]. It says, that the evolution of cancer will require at least two mutations[77]. Most patients accumulate multiple such genetic and epigenetic changes over the course of disease progression. This leads to the struggle of distinguishing between alterations driving a specific cancer profile and ultimately disease progression, and alterations with no obvious significance, referred to as passenger mutations or alterations[78,79]. In order to fully explore the significance of a single alteration it is ideal to study the change in a genetically normal background.

Unfortunately, only handful human prostate cancer cell lines exist. The cell line closest to benign prostate cells (RWPE) shows only minimal AR activation post DHT stimulation and all other cell lines harbor multiple alterations ranging from *P53* mutation and *TMPRSS2-ERG* fusion (VCaP) over mutated *AR* (22RV) and *PTEN* deletion with *ETV1* overexpression (LNCaP)[80]. Further well established prostate cancer cell lines include the AR negative PC3 and DU145 and the LAPC4 which express AR but mutation in *P53*[81]. To answer a specific scientific question the best suitable cell line has to be selected carefully.

The use of mouse models allows research to be done in a fairly regulated setting. The Cre-loxP recombination system, which was published in 1984 by Hoess and Abremski, made it possible to generate conditional genomic alterations[82]. This system can be used to generate conditional

knockouts, knock-ins or transgenic expression. By flanking a region of interest with loxP sites specific gene expression can be interrupted in the presence of Cre providing a conditional gene deletion tool. On the flip side using a loxP-Stop-LoxP-transgene construct will allow the transgenic expression only in the presents of Cre. Further, linking Cre expression to a tissue specific promoter will subsequently lead to tissue specific conditional knock-out/ knock-in.

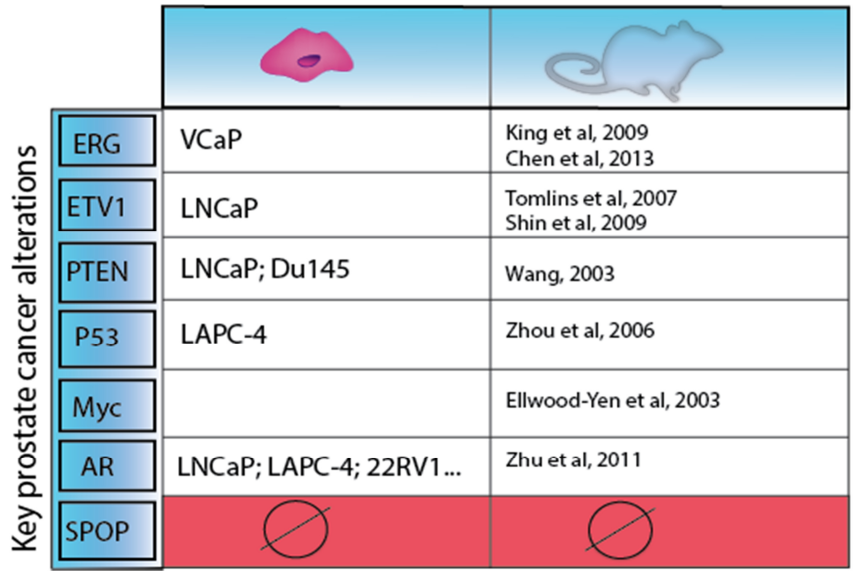


Figure 9: Overview over available and most used cell and mouse lines for prostate cancer research

Prostate specific promoters include PSA, TMPRSS2 and NKX3.1 but the most prominent and widely used promoter to regulate Cre expression remains the probasin (PB) promoter, PB-Cre4. The third generation of this promoter describes a ~500bp long sequence from the rat probasin promoter leading to high level transgene expression across all mouse prostate lobes and high androgen specificity[83,84]. Contrary to the human prostate the mouse prostate contains multiple lobes; the ventral, dorsal, lateral and anterior lobe with every lobe having a unique histological architecture and gene expression profile. There is still no consensus of which of the lobes recapitulate the human prostate and in particular prostate cancer the best but gene expression profiling efforts show the highest similarity between the dorsolateral lobe and the peripheral zone of the human prostate[6,85,86]. Combining PB-Cre4 with a R26 reporter construct has revealed the highest Cre expression in the dorsolateral lobe followed by the ventral lobe and the lowest expression in the anterior lobe[83].

One such mouse model, which helped tremendously gaining better understanding of biggest subclass the ERG rearrangement positive prostate cancer was published in 2013 by Dr. Chen and colleague. Switching to a different transgenic model, away from directly probasin driven overexpression, as well as further manipulation of the genetic background finally led to a prostate cancer causing mouse model recapitulating many features of human prostate cancer. With this model several new functions of TMPRSS2-ERG protein were investigated such as ERG

overexpression causing rather invasive and not proliferative features, leading to AR cistrome changes, ERG being a pioneer factor of AR and ERG overexpression restoring AR signature in setting of PTEN loss. All the new findings were proven to be relevant in human ERG rearranged prostate cancers as well[87]. Understanding core functionalities and features of every PCa subclass is the most critical step in developing more targeted and efficient therapies.

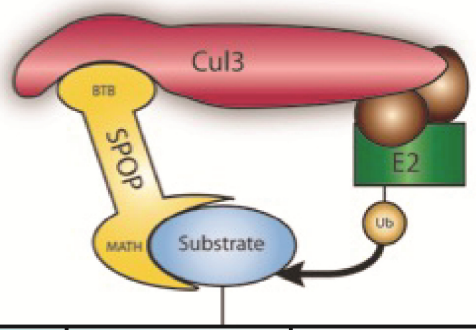
As summarized in figure 9, to date no stable cell line expressing a recurrent mutation in *SPOP* has been established and nor has a mouse model been created to explore the role of mutated SPOP in prostate cancer initiation and progression. Multiple reviews provide extensive summaries of well-described mouse models and their phenotypes[88–90].

2.3 Speckle type POZ/BTB protein (SPOP) and its role as a substrate recognition subunit

Speckle type POZ/BTB protein (SPOP) acts as the substrate-recognition component of a Cullin3-based E3-ubiquitin Ligase. The ubiquitination of a protein serves as a mechanism to tag proteins for cellular events. Such events include change of location, histone regulation[91], DNA repair[92], transcriptional regulation[93] or the well-studied event of proteolytic degradation via the 26S proeasome[94]. In a nutshell protein ubiquitination is a three step process: Ubiquitin (Ub) activating enzyme E1 uses ATP to generate the highly active Ub thiolester. Activated Ub is subsequentially transferred to the Ub carrier/conjugating enzyme E2s. E3s which are the Ub-protein ligases are required to facilitate the transfer of the activated Ub-E2 complex onto a lysine of the targeted protein[9595,96]. The array of ubiquitination substrates reflects the diversity within the family of E3-ubiquitin ligases (>1000)[96]. E3-ubiquitin ligases can be grouped into four protein families: Homologous to E6AP Carboxy Terminus (HECT), Really Interesting New Gene (RING), UFD2 homology (U-box) and the culling-RING ligases[95,97]. Whereas the HECT, RING and the U-box E3 ligases are a single polypeptide ligase the culling-RING ligases form a complex multi-subunit complex. Core compartments of this complex are a cullin, the scaffold protein, a RING-box which interacts with E2 as well as with the C terminus of cullin, and a substrate recognition protein which interacts with the substrate and the N terminus of cullin. Culling-RING E3 ligases include the well described Skip, Cul1, F-box (SCF) complex as well as the largest E3 ligase subfamily, the cullin3-based E3-ubiquitin ligases[98]. Most cullin3-based ligases recruit a POZ/BTB (from here on only BTB) domain containing substrate recognition subunit[98–100]. The BTB domain is structurally very similar to the cullin binding region of Skp1 and interacts with cullin3[101]. For substrate recognition majority of the BTB-subunit show a second protein interacting domain such as MATH or Kelch[101]. One such BTB-MATH containing substrate recognition subunit is Speckle type POZ/BTB protein (SPOP) (Figure 10)[99]. First described in 1997, SPOP was found in the serum of a scleroderma patient and got its name based on the subnuclear localization pattern[102].

Soon after, a fundamental role of SPOP in modulating the Hedghog pathway through degradation of transcription factor Ci and Gli was indetified[103]. Most of the early research has been carried out in *Drosophila*. The same group then further first described the S/T-rich SPOP binding sites (SBS) and the homodimerization of two SPOP molecules through their BTB domain, which has been confirmed in subsequent studies and is of high relevance for SPOP mutation in prostate cancer[104]. The group around Wesley Errington and others put in a lot of effort to further describe the structural complexity of the Cull3-SPOP complex. Through their work we learned that SPOP molecules do not only form a dimeric structure but rather assemble in large oligomers[105,106]. In addition to the important role of SPOP in normal development, SPOP was found to degrade multiple proteins involved in chromatin remodeling and X-chromosome silencing[107–109].

Over the years a steadily growing list of substrates have been described for SPOP but not until 2011 has the focus shifted from a developmental role to modulating transcription especially in prostate cancer[103,107–115] (Figure 10).



Substrate	Functionality	Pathway	Identified	Authorship
Ci	Hedgehog	Development	2006	Zhang
Gli2/3	Hedgehog	Development	2006	Zhang
Puc	JNK; Apopotosis	Development	2009	Liu
PIP4K2B	Kinase	Growth	2008	Bunce
DAXX	Regulator	Apoptosis	2006	Kwon
	Chaperone	Chromatin regulator		
macroH2A	Nucleosome	Chromatin regulator	2002	Takahashi
Dek	DNA binding	Chromatin regulator	2014	Theurillat
BMI1	Polycomb complex	Chromatin regulator	2005	H-Munoz
Pdx-1/IPF1	Insulin pathway	Transcription regulator	2004	Liu
SRC3	NR activator	Transcription regulator	2011	Li
AR	Nuclear receptor	Transcription regulator	2014	An
TRIM24	AR activator	Transcription regulator	2014	Theurillat

Figure 10: Known SPOP substrates their functional role and year in which interaction was first described.

2.3.1 SPOP as putative oncogene in prostate cancer

The role of *SPOP* as a tumor suppressor was first described by Li et al. in 2011. They identified steroid receptor co-activatior-3 (SRC-3) as a SPOP substrate and revealed that a high percentage of breast cancer patients show loss of the *SPOP* locus[114]. Controversially in a large sequencing effort led by the cancer genome atlas - TCGA and published in 2015 SPOP showed to be amplified in 8% of invasive breast cancer giving *SPOP* a rather oncogenic role[116]. Around this time the Rubin and Garraway labs in collaboration made the first observation that SPOP is a potentially recurrent mutated gene with 2 or 7 cases demonstrating mutation[117]. This was later confirmed in a much larger study led by Rubin and Garraway labs when our group first identified recurrent mutations in *SPOP,* and in particular recurrent mutations in the MATH domain, the substrate recognition domain[17]

Over the course of prostate cancer development multiple genetic alterations accumulate in one cell till a point at which the cell growth turns into developing malignancy. Even in established cancer, cells still acquire further alterations and new clones start to arrive. Prostate cancer is known to be a very heterogeneous disease and 80% of patients show two or more tumor nodules[118]. Additionally, a single tumor nodule can contain multiple clones[119]. New sequencing analysis tools have been developed to place a given alteration on a scale from clonal (all cancer cells show alteration) to very subclonal[119]. We have been able to show that mutation in *SPOP* can be found in HG-PIN adjacent mutant *SPOP* positive prostate cancer suggesting it occurs early in the natural history of prostate cancer[17]. Later this finding has been supported by deep sequencing results showing high clonality for *SPOP* mutation[120].

3. List of publications and contributions

Summary of publications of work in which I had a leading role or a significant contribution to are shown in table 1. Because of the especially strong impact on my PhD work I will focus on three main publications in my cumulative thesis for further discussions:

- Exome sequencing identifies recurrent SPOP, FOXA1 and MED12 mutations in prostate cancer, Barbieri et al., Nature Medicine, 2012
- SPOP mutations in prostate cancer across demographically diverse patient cohorts, Blattner et al., Neoplasia, 2014
- SPOP mutation drives prostate tumorigenesis in vivo through coordinate regulation of PI3K/mTOR and AR signaling, Blattner et al., Cancer Cell, 2017

Year	Journal	Title	Author	Contribution
2017	Nature Medicine	Prostate cancer–associated SPOP mutations confer resistance to BET inhibitors through stabilization of BRD4	14th	• Generating stable mouse cell lines expressing SPOPmut • Helping to conduct drug screen on SPOP organoid model
2017	Nature Communications	Inherited determinants of early recurrent somatic mutations in prostate cancer	4th	• Screening cohort for SPOP mutation status and helping interpret results • Help with the design of SNP validation and screen of larger cohorts
2017	Cancer Cell	SPOP mutation drives prostate tumorigenesis in vivo through coordinate regulation of PI3K/mTOR and AR signaling	**First**	• Generating GEM model • Phenotype observation and description • Identification of oncological changes • Translate and confirm results in patient data
2016	Nature, Scientific Reports	Image-based computational quantification and visualization of genetic alterations and tumor heterogeneity	14th	• Screening cohort for SPOP mutation status and helping interpret results
2016	PLoS One	Serum Autoantibodies in Chronic Prostate Inflammation in Prostate Cancer Patients	5th	• Screening cohort for SPOP mutation status and helping interpret results
2015	Elife	SPOP mutation leads to genomic instability in prostate cancer"	4th	• Generating stable mouse cell lines expressing SPOPmut • Validation of genomic instability in SPOPmut mouse cell lines
2014	Neoplasia	SPOP mutations in prostate cancer across demographically diverse patient cohorts	**First**	• Screening multiple cohorts for SPOP mutation status • Correlation of mutation status with clinical outcome and available patient data
2014	Science	Prostate cancer. Ubiquitylome analysis identifies dysregulation of effector substrates in SPOP-mutant prostate cancer	8th	• Screening cohort for SPOP mutation status and helping interpret results
2014	Clinc Cancer Research	Evidence for molecular differences in prostate cancer between African American and Caucasian men	4th	• Screening cohort for SPOP mutation status and helping interpret results
2013	Proc Natl Acad Sci	Prostate cancer-associated mutations in speckle-type POZ protein (SPOP) regulate steroid receptor coactivator 3 protein turnover	11th	• Screening cohort for SPOP mutation status and helping interpret results
2012	Nature Genetics	Exome sequencing identifies recurrent SPOP, FOXA1 and MED12 mutations in prostate cancer	5th	• Validation of SPOP mutation via sanger sequencing • Detecting SPOP mutation in HG-Pin as well as matching Tumor • Showing individuality of ERG rearrangement positive and SPOPmut cancer in collision cases

Table 1: List of publications I was involved in either as first or as contributing author during my time in Dr. Mark A. Rubin´s Lab from 01.2011 until 12.2016.

4. Summary in English and German

4.1.1 Summary in English

Back in 2011, when recurrent mutations in *SPOP* were first described in prostate cancer, nothing about the specific role of SPOP in prostate was known. Only one year later our group was able to investigate a few key characteristics of this newly emerged prostate cancer subgroup which still hold up till this day: 1) SPOP mutations occur in about 10-15% of primary prostate cancer 2) Mutations in SPOP cluster in the substrate-binding-cleft and the MATH domain 3) Striking mutual exclusivity between two common events, ERG rearrangement and SPOP mutations 4) Heterozygous nature of the mutations 5) Strong genomic instability of the SPOP mutant subclass.

Speckle-type POZ protein, SPOP, acts as a substrate recognition and binding protein, serving as a bridge between the substrate and ubiquitin ligase Cullin-3 ultimately leading to ubiquitination and degradation of the substrate. Despite the hurdles we experienced generating prostate cell lines stably expressing mutant SPOP we were able to show *SPOP* mutation causes some oncogenic features such as increased invasion capability. I characterized the *SPOP* status in 720 prostate cancer samples from six international cohorts spanning Caucasian, African American, and Asian patients. This large scale screening effort of samples confirmed our initial number of SPOP mutant affecting about 10-15% prostate cancer patients and led to my first publication as first author in Neoplasia, 2014. We were able to affirm the statistically significant relationship between *SPOP* mutation and loss of *CHD1*, and the mutually exclusivity between *ERG*-rearrangement and *SPOP* mutation. Still struggling with the lack of any models recapitulating this subgroup we decided to generate a mouse model. In my second first author publication published in Cancer Cell, 2017, we describe the effect of mutant SPOP on multiple pathways in murine prostates as well as prostate cancer cell lines. Using a prostate specific probasin driven Cre system we generated mice expressing SPOP-F133V exclusively in the luminal cells of the prostate. Analyzing this model carefully we first described that mutant SPOP had a cancer initiating function in the setting of homozygous loss of *Pten*. In the background of heterozygous loss of *Pten*, mutant *SPOP* expressing prostate tissue develops high grade prostatic intraepithelial neoplasia. Comparing the *SPOP* mutant-specific transcriptional signature we retrieved from *SPOP-F133V* mouse prostate cells to a large human patient cohort (TCGA) confirmed the relevance and comparability of our murine model to human samples on molecular level. We also for the first time described an activation of the PI3K pathway in mutant *SPOP* cell lines and mouse tissue. Previous work has published a negative reciprocal feedback between PI3K pathway activation and AR activity. We were excited to take a further step towards a better understand of this new subclass when we first described that in murine prostate cell and tissue *SPOP* mutation causes simultaneous activation of two major prostate cancer pathways, AR and PI3K.

4.1.2. Zusammenfassung auf Deutsch

Im Jahr 2011, als erstmals wiederkehrende Mutationen in *SPOP* im Prostatakrebs beschrieben wurden, war nichts über die spezifische Rolle von SPOP in der Prostata bekannt. Nur ein Jahr später gelang es unserer Gruppe, einige Schlüsselmerkmale dieser neu definierten Prostatakrebs-Untergruppe zu formulieren: 1. *SPOP* Mutationen treten bei etwa 10-15% lokalen Prostatakarzinomen auf. 2. Mutationen in *SPOP* fallen in die Substrat-bindende MATH-Domäne. 3. Auffallendes gegenseitiges Ausschließen zwischen zwei häufigen genomischen Alteratione: *ERG*-Neuanordnung und *SPOP*-Mutationen. 4. Heterozygote Erscheinung der Mutationen. 5. Starke genomische Instabilität in der *SPOP* mutierten Unterklasse.

Speckle-Typ POZ-Protein, SPOP, wirkt als Substraterkennungs- und Bindungsprotein und dient als Brücke zwischen dem Substrat und der Ubiquitin-Ligase Cullin-3. Dies führt letztendlich zur Ubiquitinierung und zum Abbau des Substrats. Trotz der Schwierigkeiten, die wir bei der Generierung von dauerhaft exprimierenden SPOP mutanten Prostatazelllinien erfuhren, konnten wir zeigen, dass Mutationen in *SPOP* einige onkogene Merkmale, wie eine erhöhte Invasionsfähigkeit, hervorrufen. Ich habe den *SPOP* Status von 720 Prostatatumorproben aus sechs internationalen Kohorten charakterisiert. Diese Kohorten umfassen Patienten von europäischer, afroamerikanischer und asiatischer Abstammung. Diese groß angelegte Charakterisierung bestätigte unsere anfängliche berichtete Prozentzahl von etwa 10-15% *SPOP* mutierten Tumoren und führte zu meiner ersten Veröffentlichung als Erstautor bei Neoplasia im Jahre 2014. Des Weiteren konnten wir die statistisch signifikante Beziehung zwischen *SPOP*-Mutation und dem Verlust von *CHD1* und das gegenseitige Ausschließen von *ERG*-Neuanordnung und *SPOP*-Mutation bestätigen. Da wir immer noch mit dem Fehlen jeglicher Modelle, spezifisch für diese Untergruppe, zu kämpfen hatten, entschieden wir uns selber ein Mausmodell zu erstellen. In meiner zweiten Erstautoren-Publikation, die in Cancer Cell im Jahre 2017 veröffentlicht wurde, beschrieben wir die Auswirkung von mutiertem SPOP auf vielfältige Signalwege in der Prostata von Mäusen sowie in Prostatakrebszelllinien. Unter Verwendung eines prostataspezifischen Cre-Systems erzeugten wir Mäuse, welche SPOP-F133V ausschließlich in den Luminalzellen der Prostata exprimieren. Durch sorgfältige Analyse dieses Modells konnten wir zum ersten Mal beschreiben, dass mutiertes SPOP eine krebsauslösende Funktion in Kombination mit homozygotem Pten-Verlust hat. In Kombination mit einem heterozygoten Verlust an Pten entwickelt SPOP mutiertes Prostatagewebe hochgradige prostatische intraepitheliale Neoplasie. Ein Vergleich der SPOP-Mutanten spezifischen Transkriptionssignatur, die wir von *SPOP-F133V* exprimierenden Mausprostatazellen erhielten, zu einer großen humanen Patientengruppe (TCGA) bestätigte die Relevanz und Vergleichbarkeit unseres Mausmodels auf molekularer Ebene. Auch beschrieben wir zum ersten Mal eine Aktivierung des PI3K-Signalweges in mutierten SPOP-Zelllinien und Mausgewebe. Frühere

Arbeiten wiesen auf eine negative reziproke Rückkopplung zwischen PI3K-Signalwegaktivierung und AR-Aktivität hin. Ein weiterer Schritt auf dem Weg zu einem besseren Verständnis dieser neuen Unterklasse gelang uns, als wir erstmals beschreiben konnten, dass Mutationen in *SPOP* in murinen Prostatazellen und -geweben gleichzeitig die Aktivierung von zwei bekannten Prostatakrebs relevanten Signalwegen, AR und PI3K, bewirken.

5. Discussion

Despite the enormous amount of new knowledge and better understanding we gained for the recently defined *SPOP* mutant prostate cancer subgroup, there are still several unanswered questions and discussion points. These include the comparison between mutant SPOP in prostate cancer and endometrial cancer, the mutual exclusivity between SPOP and ERG rearrangement, the possible loss of function versus gain of function or the difference in mutation frequency between cohorts and stage of disease. In this last part of my thesis I want to discuss some of those still unanswered questions and bring them into the perspective of future research and patient relevance.

Having screened hundreds of prostate cancer samples, tumor heterogeneity and methodology account for the biggest **discrepancy of mutation frequency** in *SPOP* across cohorts. This starts with consistency in pathological review including staging of the disease and estimating tumor cell content, choice of preservation procedure, which can have a major impact on DNA quality, and mostly the sensitivity of the detection method. A true biological difference is the much lower frequency of *SPOP* mutation in a cohort of patients with African heritage[121,122]. This low frequency of close to 5% was confirmed with next generation sequencing and therefore sensitivity concerns can be taken out of equation. The same cohort had a notable higher frequency of overexpression of a gene called *SPINK,* which similar to *SPOP* mutation, shows a mutually exclusive nature with ERG-rearrangement[121]. Raising the possibility of SPINK1 mutation as well as SPINK1 overexpression and SPOP mutation together to combine a molecular similar subgroup. Research published this year and carried out in collaboration with us suggests that there might be a germline predisposition component to the likelihood to gain a mutation in SPOP[123].

Another notable and very interesting **discrepancy in SPOP mutation frequency is between localized PCa (~10-15%) and metastatic CRPC (~ 6-8%)**[70,72]**.** Taking what we learned analyzing our mouse model, that SPOP stabilizes the AR signature in PCa, one possible explanation could be a higher sensitivity to androgen deprivation therapy. As we have shown in a separate publication, Boysen et al., Elife, 2015, *SPOP* mutation leads to a shift from homologous recombination to the more error prone non-homologous recombination. There is a good rational behind the chance that

this shift sensitizes patients harboring a *SPOP* mutation to additional DNA damage caused by radiation therapy. Targeting the tumor with a combination of AR depravation and radiation therapy might lead to a greater response rate, and therefore a drop of *SPOP* mutation in advanced prostate cancer. In order to possibly answer this question the medical history has to be carefully reviewed and compared and further subgroup comparing in-vivo drug screen should be carried out. The question if *SPOP* mutant PCa might not be as aggressive as other subtypes might rather be a question of currently better fitting treatment than clinical manifestation. Despite the considerable amount of patients, n= 720 with clinical follow up data, we screened in a study conducted in 2014 we did not gain enough power to correlate SPOP mutation with biochemical recurrence (BCR)[122]. Additionally we did not find a significant difference in Gleason grade or in age at time of initial diagnosis[122]. Assuming an overall mutation frequency of 10% across different cohorts a 15% rate of BCR within 5 years[124] and a required 80% power to detect a significant difference in BCR would a require a sample size of several thousand screened patients.
We were able to show that the mutation in *SPOP* happens very early during disease initiation progress and the high clonality has been confirmed in a subsequent publication by Sylvan Baca and colleagues in 2013[17,120]. Mutation in *SPOP* might be thought of as a "**gate keeper**" event which then allows cells to accumulate further abnormalities without going into a crises and ultimately leading to cancer. A similar scenario has been discussed for ERG rearrangement. If this is the case one should consider the possibility that mutant SPOP might not be a necessary alteration anymore after progression and further accumulation of new and maybe more potent driver events. New subclones and metastases might arise, mutant AR or loss of TP53, and over time and various treatments, SPOP mutation might get lost due to lack of selection pressure or through weak competition with other genetically independent clones.

SPOP protein is ubiquitously expressed in all tissues but recurrent mutations in *SPOP* are only found to play a role in prostate and endometrial cancer, interestingly both hormone driven organs. Notably the set of mutations found in **endometrial cancer** map just like those in prostate cancer into the substrate binding cleft and the MATH domain, but interestingly the affected amino acid residues represent a **separate set from those found by us and others in PCA**[125]. In a recent publication, prostate cancer cells expressing endometrial specific *SPOP* mutations have been used as a negative similar to wt SPOP control. This again indicates the functional specificity and precision of the mutations unique to prostate and vice versa[112].
Mutated SPOP in endometrial cancer, similar to prostate cancer, stabilizes ER respectively AR[125]. We were able to demonstrate in our latest publication that not only AR itself is stabilized by mutant SPOP, but rather a whole set of AR-centric proteins including cofactors and pioneers. In the scenario of AR we hypothesize that careful upregulating of multiple components of a largely joint

network might lead to a more robust oncogenic event than the strong upregulation of one key protein. It would be interesting to see if the same holds up for ER in *SPOP* mutant endometrial cell lines. Data published in 2017 shows opposing effects on the abundance of BRD2/3/4 dependent on SPOP mutation position and cell linage[126]. It might be of interest to further explore the mechanistic similarities and differences between these two independent but yet similar sets of mutations. Further research and retrospective analysis of material and clinical outcome of both tumor entities might help identify new possibilities for targeted treatment.

In endometrial cancer as well as prostate cancer mutation of SPOP is thought to lead to loss of function rather than gain of function, more precisely **partial loss of function**. Several clues throughout my PhD taught me the importance of this differentiation. One of the first things we noticed was that to my knowledge no patient with a deletion, heterozygous or homozygous, of SPOP has been reported or analyzed by our group and others. Mutations leading to a complete loss of function of the protein are usually, even if rarely, accompanied by some deleterious events in other patients. Taking the precise positioning of the mutation in prostate as well as endometrium indicates a more precise effect than a global loss of function. In our initial publication in 2012 we demonstrated a striking stringent normal expression of SPOP[17]. In our hands it was not possible to generate stable cell lines overexpressing SPOP, wt or mutant. Looping back to our latest publication a slight downregulation of endogenous SPOP can be noted upon the ectopic expression of SPOP-F133V.

In the complex with Cul3 two SPOP molecules form a dimer[105]. With immunoprecipitation experiments we showed that mutant SPOP binds to WT SPOP in a one to one ratio[127]**.** This indicates that the mutant protein has a dominant negative effect over the wt protein. Partial wild type functionality of SPOP despite mutation in the substrate binding site seems to be crucial to the prostate cells and serves as explanation why we have never observed loss of heterozygosity and homozygous mutation. To this point we cannot exclude the possibility to a partial gain of function but analyzing our proteomics data published in 2017 in Cancer Cell proteins upregulated in the setting of mutant SPOP show an negative enrichment of SPOP binding consensus (SBC) making likely to be a more of an indirect effect[128].

The **AR malignancy switch** can be seen as the point at which the focus of AR targets switch from differentiation towards proliferation, and it is a crucial step in disease initiation. As shown in our recent publication SPOP mutation does not solely affect AR, it changes the abundance of multiple AR associated proteins such as Trim24, Hoxb13 and Ep300[128]. The dysregulation of AR itself and AR cofactors and pioneers might kick off the mentioned AR malignancy switch without driving the cell into suicide. To further explore this hypothesis our inducible mouse model will be used for AR ChIP sequencing. The comparison of changes in the AR binding site between 1) normal SPOPwt

expressing cells with 2) still normal but SPOP mutant expressing cells and 3)SPOP mutant tumor cells will provide crucial data to further understand the role of AR in disease initiation and progression in the SPOP mutant setting. High enrichment of lost CHD1 as well as a very distinct methylation pattern are observed in the SPOP mutant subclass of prostate cancer. Large research effort is currently going on to understand the beneficial co-occurrence of lost CHD1 and *SPOP* mutation during tumor development[70]. CHD1 being a chromatin remodeler together with the observed changes in multiple AR associated factors indicates a central role of epigenetic alterations feeding into the AR cistrome changes. One could hypothesize that the deletion of CHD1 leads to changes in the accessibility of genes for AR or AR related factors to efficiently bind. All these changes would not be reached by simply overexpressing AR. A full understanding of the disease initiation process would give the possibility of new cancer preventative treatment options.
A high degree of rearrangements and genomic breaks, indicating genomic instability, is a dominant feature of the *SPOP* mutant prostate cancer subclass[17,127]. In a study published 2015 we showed less efficient DNA repair machinery in SPOP mutant prostate cancer cells[127]. Mutant SPOP pushing the DNA repair machinery from homologous to the more error prone **non-homologs repair machinery** in combination with the mentioned AR malignancy switch might create cellular preconditions for high cancer receptiveness. Having generated a model developing prostate cancer we now are able to compare the level of genomic instability in our model to human prostate cancer samples. Currently ongoing research and clinical trials focuses on possible of increased susceptibility of further DNA repair impairing treatment such as PARP inhibition for patients with *SPOP* mutant PCa. Our mouse model will be of great resource to the community to study the effect of a variety of treatment options and combinations in-vivo on a highly comparable level.

It was suggested that **mutated SPOP causes the upregulation of ERG**[129,130], and therefore mutant *SPOP* could serve as a mechanism to phenocopy to oncogenic outcome of ERG rearrangement in prostate cancer. In a manuscript, currently in press, in the Journal of Clinical Investigation, we showed that we do not see any increase of ERG expression in the presence of mutant SPOP. As mentioned in the introduction, a lot of effort has been undertaken to precisely define subclasses of prostate cancer[17,70,120]. *SPOP* mutant prostate cancer shows a very distinct gene expression profile, a high rate of genomic instability harboring many rearrangements and copy number alterations, such as the loss of Chromosome 5q21 and 6q21, a unique methylation pattern[17,70,120,127]. Taking the striking discrepancy of SPOP mutant prostate cancer and ERG-rearranged prostate cancer in respect to above mentioned parameters clearly points towards two totally independent types of prostate cancer. There are still about 20-30% of patients whose driving event or subclass has not yet been identified and who fall into the category of "others"[70]. Off those tumors about 30% show

very similar features to those of *SPOP* mutant cases and it will be interesting to see which yet unknown mechanism might "phenocopy" mutations in *SPOP*.

When we first decided to generate a mouse model we had to make a decision on which specific mutation we want to model. Across diverse cohorts we and others noticed that about 50% of all SPOP mutation causes an amino acid exchange on position F133, the most prominent being F133V. With the little data we had available at this time we did not notice a big difference between the reported mutations. A few publications later we do see differences in the strength of alterations, changes in the protein abundance of a given substrate, the mutations can cause but to my knowledge there has not been a reported opposite effect between mutations at this point[70,112,127]. In our recent Cancer Cell paper we used the transcriptional data we got from SPOP-F133V stably expressing murine prostate cells to generate a SPOP-mutant signature by using the most altered genes compared to SPOP wt cells. Based on this gene set we generated a hierarchical clustering and heatmap of the TCGA prostate cancer cohort. A highly significant clustering of human *SPOP* mutant prostate cancer, including but not solely F133 alterations, as well as other co-occurring genomic alterations, such as 6q15 deletion, did not only show the high value of our model to recapitulate human prostate cancer but also that this model can be used to model multiple *SPOP* mutations.

Clinically localized prostate cancer shows a mutual exclusivity with genomic alterations in the PI3K pathway across multiple cohorts[128]. This mutual exclusivity no longer holds up in patients with advanced disease. In mice mutant SPOP caused prostate cancer in the background of homozygously deleted Pten and high-grade prostatic intraepthelial neoplasia (HG-PIN) with similar histological features in mice with heterozygous deleted Pten. Traces of similar histology, strong nuclear atypia, can be found in mice expressing mutant SPOP with intact PTEN and were absent in mice with different Pten status and intact SPOP. Therefore we hypothesize that our model can be used as a **disease progression model**. Tracking little changes caused by mutant SPOP using Pten wt mice, the beginning of tumorigenesis in the background of heterozygous loss of Pten and advanced disease in combination with full loss of function of Pten. This model has the potential to be used not only to define better and more precise treatments, but also to understand and eventually block the transition from HG-PIN to advanced disease. A yet unanswered question is how mutated SPOP is able to overcome the reported **negative reciprocal feedback regulation** between activation of AR and PI3K[57,58]. D. Mulholland suggest in his publication in 2011 that deletion of AR leads to transcriptional downregulation of Fkbp5 which serves as a scaffold protein between PHLPP and AKT and therefore low abundant FKBP5 ultimately reduces PHLPP mediated AKT inhibition[58]. Murine prostate expressing mutant SPOP in setting of Pten deletion showed to have higher transcript level of FKBP5 than prostate tissue from age matched mice expressing normal SPOP in setting of Pten deletion. Those preliminary experiments suggest that PHLPP should still be able to more efficiently inhibit the phosphorylation of AKT, yet we see very high

pAKT levels in the same tissue. Further experiments have to be planned and carried out to determine the functionality of PHLPP in cells with mutated SPOP. One possibility could be that mutated SPOP overcomes the inhibitory effect of PHLPP by activating AKT with a stronger force from a different angle. We noticed that *SPOP* mutant tissue has a strong phosphorylation effect on the residue S473 and less on T308. The Amino acid T308 is targeted only by PI-3kinase signaling whereas phosphorylation on the S473 site is initiated mainly by mTORC2[131]. This point to a particular role of mTOR in *SPOP* mutated cells. This question could potentially be answered by treating mutant SPOP expressing cells with inhibitor targeting either PI-3kinases or mTOR either in combination with AR inhibiting agents or in the setting of active AR. One question I still don't fully understand is: if *SPOP* mutation in combination with loss of Pten in mice can cause tumor formation why is this very rarely seen in localized prostate cancer? One aspect, as I mentioned above, might be that even though PI3K and mTOR has a big overlapping spectrum of their pathway, in the early stages cells might be pushed too hard by overstimulated PI3K pathway with losing PTEN. In our model we artificially force the luminal cells to express mutant SPOP in the background of deleted PTEN, which ultimately lead to cancer. But there still might be the possibility that this process caused a high rate of apoptotic cells, which in a regular setting would end the oncogenic transformation for this particular cell. Staining tissue slides for apoptotic marker of the prostate of young mice expressing both alterations and comparing those to other combinational hits, such as overexpression of mTOR, would give us a better insight into this question.

We yet have to nominate all the proteins dysregulated by mutated SPOP and their individual role in tumorigenesis. However, the increased number of substrates affected by the mutations indicates that mutant SPOP induced tumorigenesis is caused via dysregulation of multiple signaling pathways and networks at the same time and not a single target event. During tumorigenesis the yet normal cell has to accumulate abnormalities without pushing the cell into suicide which I believe mutated SPOP is doing by dysregulating multiple pathways such as AR, PI3K and DNA damage response at the same time but all on a gentle level.

6. Impact of this work

During his life time about one out of seven men will be diagnosed with prostate cancer[132]. Prostate cancer is a highly heterogeneous disease but for many years oncologists had no molecular stratification mechanism. A big step towards defining subgroups and biomarkers was taken when Dr. Tomlins and Dr. Rubin together with colleagues were able to show that about 50% off all prostate cancer harbored a rearrangement involving a prostate specific promoter element, commonly TMPRSS2, and an ETS transcription factor such as ERG which heavily influencing proliferation and differentiation[133]. When I started my work in Dr. Rubin´s lab in 2011 his team just had received the sequencing data of the first few whole genomes and whole exome sequenced prostate cancer samples and recognized a common mutation hot-spot region in SPOP, speckle-type POZ protein. Nothing was known about the particular function of those mutations in prostate cancer. Since then we have learned a lot through work I have been fortunate enough to be part of but also conducted myself. We investigated that *SPOP* mutation occurs early, and that *SPOP* mutation has a dominant negative affect and leads to a partial loss of function. We learned that across different ethnicities mutation in *SPOP* occur in about 4-15% off patients, is mutually exclusive to *ERG* rearrangements, and that this frequency does not increase in patients with advanced disease. We painfully had to learn that prostate cells are very restricted to a normal expression of SPOP and that sometimes it is better to generate a new antibody than trying to optimize available products. I was able to generate a mouse model recapitulating human *SPOP* mutated prostate cancer. With the help of this model we were able to first describe that mutant SPOP has a tumor initiation function by activating concordantly two major prostate cancer related pathways, AR and PI3K; making mutation in SPOP a new biomarker for about 10-15% of all prostate cancer patients. The generated model is a major resource for the research community who will now be able to define better treatment options for patients harboring mutation in *SPOP* in an in-vivo system. These researchers will now be able to explore to response to a combinational treatment of AR and PI3K inhibition in combination with or without radiation therapy. All the knowledge we gained during the time for a newly defined subclass of prostate cancer helps to further explore the potential of precision medicine and ultimately has the potential to increase the survival rate of a subset of newly diagnosed prostate cancer patients.

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8. Appendix

8.1. Curriculum Vitae

Due to privacy protection reasons the curriculum vitae will not be part in this for the public accessible version.

8.2. Abstract of publications and key results relevant for this work

8.2.1. Manuscript 1

Exome sequencing identifies recurrent SPOP, FOXA1 and MED12 mutations in prostate cancer

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8.2.1.1. Abstract

Prostate cancer is the second most common cancer in men worldwide and causes over 250,000 deaths each year. Overtreatment of indolent disease also results in significant morbidity. Common genetic alterations in prostate cancer include losses of NKX3.1 (8p21) and PTEN (10q23), gains of AR (the androgen receptor gene) and fusion of ETS family transcription factor genes with androgen-responsive promoters. Recurrent somatic base-pair substitutions are believed to be less contributory in prostate tumorigenesis but have not been systematically analyzed in large cohorts. Here, we sequenced the exomes of 112 prostate tumor and normal tissue pairs. New recurrent mutations were identified in multiple genes, including MED12 and FOXA1. SPOP was the most frequently mutated gene, with mutations involving the SPOP substrate-binding cleft in 6–15% of tumors across multiple independent cohorts. Prostate cancers with mutant SPOP lacked ETS family gene rearrangements and showed a distinct pattern of genomic alterations. Thus, SPOP mutations may define a new molecular subtype of prostate cancer.

8.2.1.2. Key results

(i) Mutations identified in SPOP cluster exclusively in the substrate-binding cleft, the MATH domain

(ii) SPOP mutations occur solely as heterozygous missense mutations

(iii) Mutation in SPOP occur similar to ERG rearrangement as an early and clonal event in the natural history of prostate cancer

(iv) Proven mutually exclusivity between SPOP mutation and ERG rearrangement within same tumor nodule

8.2.2. Manuscript 2

SPOP mutations in prostate cancer across demographically diverse patient cohorts

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8.2.2.1. Abstract

Recurrent mutations in the Speckle-Type POZ Protein (SPOP) gene occur in up to 15% of prostate cancers. However, the frequency and features of cancers with these mutations across different populations is unknown. To investigate SPOP mutations across diverse cohorts we validated a series of assays employing high-resolution melting (HRM) analysis and Sanger sequencing for mutational analysis of formalin-fixed paraffin-embedded material. Prostate cancer samples (n=720) from six international cohorts spanning Caucasian, African American, and Asian patients, including both prostate-specific antigen-screened and unscreened populations, were screened for their SPOP mutation status. Status of SPOP was correlated to molecular features (ERG rearrangement, PTEN deletion, and CHD1 deletion) as well as clinical and pathologic features.

8.2.2.2. Key results

(i) Developed assay can be used to screen for mutations in *SPOP* from fresh and fixed material as well as low input amount of DNA

(ii) Mutation in SPOP can be found across various ethnicity

(iii) Mutation rate ranges from 5% to 14% in localized prostate cancer patients

(iv) Reinforcement of mutually exclusivity between SPOP mutation and ERG rearrangement across cohorts and hundreds of samples

(v) We did not find significant association between SPOP mutations and clinical or pathologic parameters but high correlation of SPOP mutation and CHD1 deletions

8.2.3. Manuscript 3

SPOP mutation drives prostate tumorigenesis in vivo through coordinate regulation of PI3K/mTOR and AR signaling

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8.2.3.1. Abstract

Recurrent point mutations in SPOP occur early in 10% of prostate cancers defining a distinct molecular subclass. Here, we report development of a conditional mouse model to define the role of SPOP mutation as a driver of prostate tumorigenesis in vivo. Our findings show that SPOP mutation drives prostate neoplasia in vivo through coordinate deregulation of both PI3K/mTOR and AR pathways. The discovery that SPOP mutation can activate two of the major pathways in prostate cancer exposes not only the biology of the SPOP mutant subclass, but the central importance of these pathways and their context across the spectrum of prostate cancer. These findings provide insight to both the unique and common features of molecular subtypes of human prostate cancer, and highlight potential opportunities for precision therapy.

8.2.3.2. Key results

(i) SPOP mutation has tumor initiation capability
(ii) Selective loss of function towards the remaining wild-type allele
(iii) SPOP mutation leads to activation of the PI3K pathway
(iv) SPOP mutated cells show active AR despite an activated PI3K pathway
(v) SPOP mutation affects simultaneously multiple AR centric proteins

8.3. Print of publications

Aus urheberrechtlichen Gründen werden die drei in Punkt 8.2.1 bis 8.2.3 erwähnten Publikationen in diesem der Öffentlichkeit zugängigen Format nicht dargestellt.