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ONCOGENIC GRPR OVEREXPRESSION IN PROSTATE CARCINOMAS HARBORING ETS REARRANGEMENTS: UNCOVERING DOWNSTREAM TARGETS AND THERAPEUTIC POTENTIAL

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PUBLICATIONS

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“Be the change you want to see in the world”

Mahatma Gandhi

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SUMMARY

Prostate carcinoma (PCa) is the most incident neoplasia in men and the second leading cancer-related cause of death. PCa is a heterogeneous disease and current therapeutic strategies are dependent on TNM staging, Gleason scoring, PSA levels and overall health status. Primary treatment consists mainly of radical prostatectomy and/or radiation therapy, which may be supplemented with androgen ablation. Although many patients are identified with local, surgically curable, disease, there is a subset of patients that progress or show metastatic prostate cancer, where the gold standard therapy is androgen ablation. Moreover, recurrence is frequent, and many patients develop metastatic disease, for which chemotherapy is only moderately effective. A better understanding of the genetics and molecular pathways involved in prostate carcinogenesis should contribute to the current challenge of identifying promising molecular targets involved in PCa progression.

Genomic rearrangements involving members of the ETS family of transcription factors are recurrently found in PCa, with *ERG* and *ETV1* being reported in 50% and 10% of the cases, respectively. These aberrant alterations are also present in precursor lesions of PCa, suggesting a role in the early events that ultimately led to a prostate carcinoma. ETS members have generally been associated with the regulation of cell growth, proliferation, differentiation, and apoptosis, through activation or repression of target genes. Therapeutic targeting of ETS and other transcription factors has been challenging due to their nuclear localization and molecular embedding in DNA–protein and protein–protein complexes. Consequently, it has been of utmost importance to identify and to characterize the downstream molecular targets of ETS rearrangements, as some of them could potentially be more amenable to targeted therapy.

In a previous work, using a genome-wide scale and exon-level expression microarray platform on a clinical series of PCa enriched for *ERG* and *ETV1* rearrangements, we have shown that *ERG* and *ETV1* regulate both specific and shared target genes in PCa. Our group reported a list of 27 target genes shared by *ERG* and *ETV1* rearrangements. Our results, using VCaP and LNCaP knockdown cell line models, clearly validate *KCNH8*, *TMEM45B* and *GRPR* as downstream targets of both *ERG* and *ETV1*, as also indicated by our demonstration of direct binding of ERG to the promoter of these genes using ERG-immunoprecipitated chromatin from VCaP cells. *GRPR* encodes the gastrin-releasing peptide receptor and has been described as overexpressed in several cancer types, including PCa. Considering the overexpression of *GRPR* in a high proportion of PCa that harbor either *ERG* or *ETV1* rearrangements, the cellular

localization of its protein product, the relevance of its function, the availability of blocking agents, and the several previous reports on its oncogenic activity, makes it a promising therapeutic target for these particular molecular subtypes of PCa**.**

In **Paper I**, we have shown that effective knockdown of *GRPR* in LNCaP and VCaP cells attenuates their malignant phenotype by decreasing proliferation, invasion and anchorage-independent growth, while increasing apoptosis. Besides validating *GRPR* as a potential target gene of both *ERG* and *ETV1* transcription factors, our data revealed the activation of different intermediate players of GRPR/ETS in *in vitro*-mediated proliferation/apoptosis and invasion/ anchorage-independent growth, associated with disease aggressiveness. Considering what is known concerning the activity of these intermediates and the data shown here, we propose a model for GRPR signaling under an ETS-rearrangement cellular context, where TYK2, MST1 and *p*-Akt may constitute promising therapeutic targets that should be explored in combination with GRPR inhibitors for treating this particular subtype of prostate cancer.

TYK2 overexpression has been observed in several malignancies including breast cancer cell lines, prostate cancer and squamous cervical carcinomas (Übel *et al.* 2013) and some studies have described the involvement of this tyrosine kinase in mediating prostate cancer invasion. Considering the oncogenic role of *TYK2* in prostate carcinogenesis and its overexpression in tumors harboring ETS rearrangements, a combined therapy of a GRPR antagonist and a TYK2 inhibitor in prostate carcinomas with ETS rearrangements was evaluated in **Paper II**. Our data provides further evidence to sustain a therapeutic effect of dual GRPR/TYK2 blockade in prostate carcinogenesis, since *in vitro* treatment with GRPR antagonist in combination with a tyrosine kinase inhibitor targeting TYK2, drastically reduces the tumorigenic behavior of LNCaP and VCaP cell lines. Additional *in vitro* and *in vivo* ongoing studies will be crucial to further test the possible therapeutic potential of dual GRPR/TYK2 blockade in the molecular PCa subtype characterized by rearrangements of the ETS transcription factors.

RESUMO

Os carcinomas da próstata (PCa) são uma das neoplasias mais incidentes em homens e a segunda causa de morte relacionada com cancro. Os tumores da próstata são bastante heterogéneos e a terapia utilizada actualmente depende do estadio TMN, do score de Gleason, dos níveis de PSA no sangue e do estado de saúde em geral. O tratamento primário neste tipo de neoplasias é a prostatectomia radical e/ou radioterapia, eventualmente suplementada com ablação androgénica.

A maioria dos pacientes apresentam doença localizada potencialmente curável através de cirurgia, mas existe um subgrupo de pacientes cuja doença progride ou que apresentam metastização. Adicionalmente, a recorrência de doenca é frequente e muitos doentes desenvolvem doença metastática, para a qual o tratamento por quimioterapia é apenas moderadamente eficaz. O estudo e compreensão da genética e das vias moleculares envolvidas na carcinógenese da prostata poderá contribuir para a identificação de alvos moleculares involvidos na progressão do PCa.

Rearranjos genómicos envolvendo membros da família dos factores de transcrição ETS são frequentemente observados em PCa, com maior frequência para os genes *ERG* e *ETV1* (50% e 10% dos casos, respectivamente). Estas alterações foram também identificadas em lesões precursoras de PCa, sugerindo um papel como eventos iniciais da carcinógenese prostática. Os membros da família ETS têm vindo a ser associados com a regulação do crescimento celular, da proliferação, da diferenciação e da apoptose através da activação e repressão de genes alvo. Contudo, a terapia dirigida aos ETS e a outros factores de transcrição tem vindo a ser um desafio devido à sua localização nuclear e aos complexos DNA-proteína e proteína-proteína. Consequentemente, é de extrema importância identificar e caracterizar alvos moleculares dos rearranjos ETS que poderão ser alvos de terapia dirigida.

Num estudo prévio, utilizando uma plataforma de *microarrays* de expressão génica e exónica global numa série clínica de PCa enriquecida para casos com presença de rearranjos *ERG* e *ETV1*, demonstramos que os genes *ERG* e *ETV1* regulam genes alvo quer específicos, quer comuns. O nosso grupo descreveu uma lista de 27 genes-alvo partilhados em casos de rearranjos *ERG* ou *ETV1*. Os nossos resultados utilizando linhas celulares VCaP and LNCaP silenciadas para os ETS em questão validaram os genes *KCNH8*, *TMEM45B* e *GRPR* como alvos do *ERG* e *ETV1*, tal como demonstrado pela ligação directa do ERG ao promotor desses genes utilizando immunoprecipitação da cromatina para o *ERG* em células VCaP. O gene *GRPR* codifica o receptor do péptido libertador de gastrina e tem sido descrito como sobrexpresso em diversos tumores, entre

os quais PCa. A sobrexpressão de *GRPR* numa grande proporção de PCa com rearranjos do *ERG* ou *ETV1*, a localização celular do seu produto protéico, a relevância da sua função, a disponibilidade de fármacos antagonistas e diversos trabalhos demonstrando a sua actividade oncogénica, torna este receptor um promissor alvo terapêutico neste subgrupo particular de PCa.

Na **publicaçao I**, demonstrámos que o silenciamento do gene *GRPR* nas linhas celulares LNCaP e VCaP atenua o seu fenótipo maligno através da diminuição da proliferação, invasão e crescimento independente de ancoragem, e do aumento dos níveis de apoptose. Para além de validarmos o gene *GRPR* como potencial alvo terapêutico de ambos os factores de transcriçao *ERG* e *ETV1*, os nosos resultados revelaram a activação de diferentes intermediários da proliferação/apoptose e invasão/ crescimento independente de ancoragem mediado pela sinalização GRPR/ETS *in vitro.* Considerando aquilo que estava descrito na literatura relativamente à actividade destes intermediários, propomos um modelo de sinalização celular desencadeado pelo GRPR no contexto ETS, onde TYK2, MST1 e *p*-Akt poderão constituir promissores alvos terapêuticos que deverão ser explorados em combinação com inibidores GRPR para o tratamento deste subgrupo particular de PCa.

A sobreexpressão de TYK2 tem vindo a ser observada em diversas neoplasias, incluindo linhas celulares de cancro da mama, PCa e carcinomas cervicais escamosos, e alguns estudos descrevem o seu envolvimento na mediação da invasão de PCa. Deste modo, e considerando o papel oncogénico de TYK2 na carcinógenese prostática e a sua sobrexpressão em tumores com rearranjos ETS, foi avaliado na **publicação II** uma terapia combinada de um antagonista GRPR e de um inibidor TYK2 em PCa com rearranjos ETS. Os nossos dados revelaram o efeito terapêutico do duplo bloqueio GRPR/TYK2 na carcinógenese prostática, onde o tratamento com um antagonista do GRPR em combinação com um inibidor do TYK2 levou à diminuição drástica das características tumorigénicas das linhas celulares LNCaP e VCaP. Estudos adicionais *in vitro* e *in vivo* em curso serão cruciais para melhor avaliar o potencial terapêutico da dupla inibição GRPR/TYK2 no subtipo molecular de PCa caracterizado por rearranjos dos factores de transcrição ETS.

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LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
AFMS	Anterior fibromuscular stroma
AJCC	American Joint Committee on Cancer
AMACR	α-methylacel-coA racemase
ASAP	Atypical small acinar proliferation
ASOs	Antisense oligonucleotides
BPH	Benign prostatic hyperplasia
BsMol	Bispecific molecules
cDNA	complementary DNA
CGH	Comparative genomic hybridization
COPA	Cancer outlier profile analysis
CpG	Cytosine - phosphate - guanine
CRPC	Castration-resistant prostate cancer
DNA	Deoxyribonucleic acid
DRE	Digital rectal examination
EBRT	External-beam radiotherapy
ETS	E26 transformation-specific
FISH	Fluorescence in situ hybridization
GI	gastrointestinal
GPCR	G-protein coupled receptor
GRP	Gastrin-releasing peptide
GRPR	Gastrin-releasing peptide receptor
GS	Gleason score
H&E	Hematoxylin and eosin staining
HGPIN	High-grade prostatic intraepithelial neoplasia
IC50	half maximal inhibitory concentration
LHRH	Luteinizing hormone-releasing hormone
LOH	Loss-of-heterozygosity
mAbs	Monoclonal antibodies
mCRPC	Metastatic castration-resistant prostate cancer
miRNA	Micro RNA
mRNA	Messenger RNA
NPT	morphologically normal prostate tissues

NTT	N-terminal truncated
PCa	Prostate cancer
PIA	Proliferative inflammatory atrophy
PIN	Prostatic intraepithelial neoplasia
PNT	Pointed domain
PSA	Prostate-specific antigen
PYNATYP	ASAP associated with HGPIN
RACE	Rapid amplification of cDNA ends
RNA	Ribonucleic acid
RP	Radical prostatectomy
RT-PCR	Reverse transcriptase-polymerase chain reaction
shRNA	Silenced RNA
TNM	Tumor Node Metastasis
TRUS	Transrectal ultrasonography
TURP	Transurethral resection of the prostate
UICC	International Union Against Cancer
WT	Wild type

Chapter 1

INTRODUCTION

1. ANATOMY OF THE PROSTATE GLAND

The prostate gland is an exocrine walnut-shaped organ of the male reproductive system, whose main function is to secrete a thin, slightly alkaline fluid that forms a portion of the seminal fluid (DeVita *et al.* 2008, Shen and Abate-Shen 2010). The prostate is enclosed by a capsule composed of collagen, elastin and large amounts of smooth muscle and it is located in the pelvic region, posterior to the lower portion of the symphysis pubis superior to the perineal membrane, anterior to the rectum, inferior to the urinary bladder and surrounding the urethra (DeVita *et al.* 2008, Bhavsar and Verma 2014). In normal adult it reaches approximately 25 cm^3 and weights about 20g (DeVita *et al.* 2008, Hricak and Scardino 2009).

In 1969, McNeal proposed that the prostate gland was composed by four zones: peripheral, central, transition and the anterior fibromuscular stroma (AFMS) (Figure 1) (McNeal 1981, Hricak and Scardino 2009, Shen and Abate-Shen 2010).

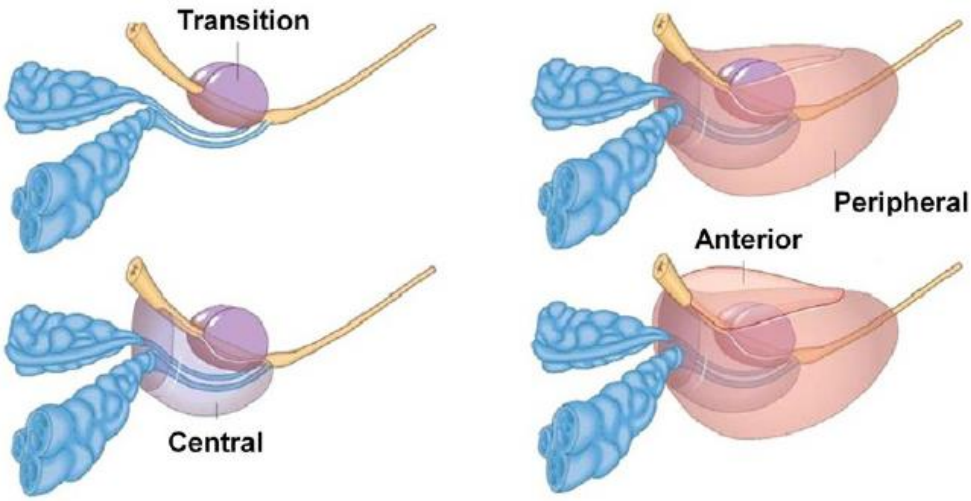


Figure 1. Zonal anatomy of the normal prostate as described by McNeal (McNeal 1988).

The peripheral zone is the one closest to rectum, comprises 70% of the organ, has a mesodermal origin and consists of the area palpable by digital rectal examination (DRE). About 70% of adenocarcinomas occur in this zone and chronic prostatitis and postinflammatory atrophy are also more common than in the other zones. The central zone is a cone shaped region that surrounds the ejaculatory ducts, comprises about 25% of the glandular tissue and only 1-5% of prostate carcinomas occur in this region. The transition zone has an endodermal origin that forms only 5-10% of the glandular tissue, and consists of two equal lobules that surround the urethra. Age-related benign prostatic hyperplasia (BPH) is frequently limited to this specific region and only 15% of prostate cancer arises from this zone. The AFMS is an anterior band composed mostly by striated muscle, contiguous to the bladder smooth muscle and external sphincter (DeVita *et al.* 2008, Hricak and Scardino 2009).

Histologically, the architecture of the prostate looks like a branched duct gland, each containing two cell layers, a luminal secretory columnar and an underlying basal cell layer. Additionally, rare neuroendocrine cells are observable in normal epithelium of prostate glands (DeVita *et al.* 2008, Hricak and Scardino 2009).

2. PROSTATE GLAND PATHOLOGY

Prostate disorders are very diverse and include benign, pre-malignant and malignant conditions. These disorders have become a huge health concern in an aging world population, as they are commonly associated with increased age.

2.1 BENIGN PROSTATIC HYPERPLASIA

Benign prostatic hyperplasia (BPH) is one of the most common urological diseases in aging men, affecting around 50% of men over 50 years old (Alcaraz *et al.* 2009). Nearly 90% of 80-year-old men will have histologic BPH (Bushman 2009) and about half of these men will develop prostatic enlargement, although this lesion is not considered to be a precursor of prostate cancer (Kristal 2010). BPH is characterized by a hyperplastic growth of epithelial and stromal cells in the prostate and occurs more frequently at the transition region (Hricak and Scardino 2009, Timms and Hofkamp 2011).

2.2 PRE-MALIGNANT LESIONS

Individuals with pre-malignant lesions present an increased risk to developed cancer when compared to the general population (Berney and Warren 2013). Prostatic intraepithelial neoplasia (PIN) is a neoplastic proliferation of the prostatic acinar cells, confined by the basement membrane and without invasion of the stroma (Epstein 2009, Berney and Warren 2013). Histologically, PIN is divided in low- and high-grade PIN (HGPIN), but only the last one is considered a precursor of invasive carcinomas (Epstein 2009). HGPIN lesions present higher levels of cellular proliferation markers and prominent nucleoli, which it is related with its ability to progress towards a prostate carcinoma. HGPIN and prostate carcinomas are both multifocal and heterogeneous, and there are several other features in common between both lesions: firstly, the epidemiologic evidence, since prostate cancer and HGPIN undergo parallel increase with age; topographic proximity of both lesions has been observed in about 70% of the cases; morphologic similarities have been described in both lesions (progressive loss of basal cells, atypia with increased size and nuclear irregularity) and also some genetic alterations

(gain or loss of specific chromosomes and expression of α-methylacel-CoA racemase (AMACR)) (Ramon and Denis 2007, Tewari 2013). The earliest evidence of carcinoma is typically an early stromal invasion, and this step occurs at sites of acinar outpouching and basal cell disruption in acini with PIN. This microinvasion is present in about 2% of high-power microscopic fields of PIN, with equal distribution between all architectural patterns. Several studies have evaluated the predictive value of HGPIN for prostate cancer, concluding that HGPIN provides a higher significant risk ratio. In the era of sextant-biopy, has been reported a 30.2% risk of cancer in the following biopsy after one or two cores diagnosed with HGPIN, 40% with three cores and 75% with more than three cores (Kronz *et al.* 2001). However, in an initial 11-13 core biopsy scheme, the presence of HGPIN by itself is no longer an indication to repeat biopsy, as the risk of cancer in the following biopsy is reduced to 22% (Karakiewicz *et al.* 2005).

Proliferative inflammatory atrophy (PIA) is morphologically characterized by glandular atrophy associated with glandular proliferation and chronic inflammatory cells, whose epithelial cells are intermediate between the basal and secretory cell phenotype (Tewari 2013). Recent studies have described that PIA is heterogeneous and subsets are histologically definable (DeVita *et al.* 2008). Proliferative inflammatory atrophy (PIA) has been suggested as a precursor of HGPIN, due to identification of the same genetic and morphologic changes among them, although its potential premalignant role is controversial and has yet not been fully defined (Ramon and Denis 2007). Contrarily, PIA is common throughout the entire prostate gland and is also present in young adults, whereas HGPIN and prostate carcinomas develop predominantly in the peripheral zones in older age groups (Tewari 2013).

Atypical small acinar proliferation (ASAP) is an area of glands that fulfill some of the cytological and architectural features of a carcinoma, although not all. This clinical finding typically occurs in just 5% of biopsies. Due to their similarities with prostate carcinomas, these lesions have been called as “borderline for malignancy”. Several studies have shown that the diagnosis of ASAP increases the risk for the later diagnosis of malignancy in about 35-45% (Tewari 2013, Dorin *et al.* 2015). Occasionally, prostate biopsies show both HGPIN and ASAPs, and these can happen in two distinct ways. First, the ASAP focus and the HGPIN are unrelated spatially, being detected in different biopsies or different parts of the same biopsy. Second, and the most studied entity, ASAP may be associated with HGPIN, frequently called PINATYP or PIN/ASAP. There are a few studies on PINATYP prognosis, all pointing to a higher risk of a subsequent diagnosis of malignant lesion, equivalent or even higher than that observed when HGPIN or ASAP are identified alone (Kronz *et al.* 2001).

2.3 MALIGNANT LESIONS

Prostate adenocarcinoma comprises about 95% of prostatic malignant conditions. Other prostate malignant lesions include intralobular acinar carcinomas, ductal carcinomas, small cell or scirrhous pattern tumors, a rare clear cell variant resembling renal cell carcinomas, and mucinous carcinoma (DeVita *et al.* 2008).

Prostate adenocarcinomas arise in acinar and proximal ductal epithelium. The peripheral zone of the prostate is affected in about 75% of cases, although transitional and central zone tumors occur as well in about 15% and 10% of patients, respectively (DeVita *et al.* 2008). The histopathologic diagnosis of adenocarcinoma is made on the basis of a combination of several histologic features (Tabela 1), grouped into primary (architecture of the glands) and secondary criteria (cytologic). Tertiary criteria are helpful and supportive of the diagnosis (Tewari 2013).

Table 1 - Criteria for diagnosis of prostate cancer

Primary criteria
Architectural – diagnostic
1. Small glands (microacini)
2. Crowded glands
3. Haphazardly arranged glands, not in lobules
4. Fused glands
5. Infiltrative pattern
6. Small glands around/between benign glands
7. Perineural invasion
8. Mucinous fibroplasia
9. Glomerulations
Secondary criteria
Cytologic – diagnostic
7. Absence of basal cells
8. Large nucleoli
9. Large hyperchromatic nuclei, with an increased nucleus-cytoplasm ratio
Terciary criteria
Cytoplasmic/luminal – supportive
1. Luminal blue mucin
2. Luminal pink amorphous secretions
3. Crystalloids
4. Sharp/rigid luminal borders
5. Amphophilic or foamy cytoplasm

Adenocarcinomas range from clinically indolent to extremely aggressive neoplasms (Hricak and Scardino 2009), and the high degree of PCa heterogeneity is potentially relevant to understand these different states. Although PCa is described as a disease of older men, studies of prostate specimens from healthy men with ages of 20 to 40 years, frequently showed presence of histologic foci of prostate cancer. This data suggests that cancer initiation has already taken place at a relatively early age (Shen and Abate-Shen 2010).

Multifocal adenocarcinoma of the prostate is present in more than 85% of the cases, which supports the idea that prostate gland can present multiple neoplastic transformation events, many of these giving rise to latent and clinically undetectable disease (Shen and Abate-Shen 2010). The basal cell layer is lost progressively from BPH, to HGPIN and finally PCa, suggesting a relationship between these lesions (Figure 2) (Chrisofos *et al.* 2007, Shen and Abate-Shen 2010). Therefore, there is still a lot to be understood on how prostate cancer evolves and if this potential relationship presents a real chain of events.

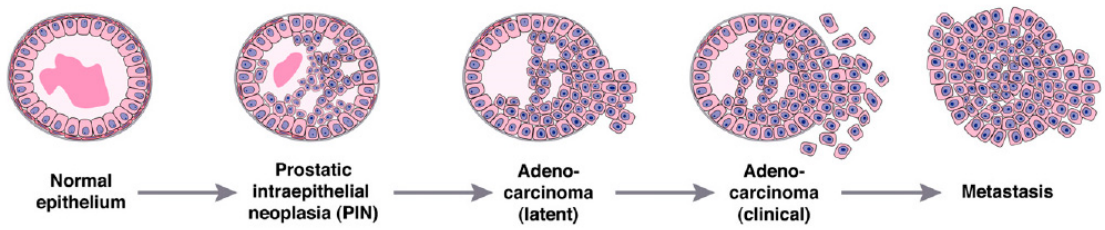


Figure 2. Progression pathway in prostate cancer (adapted from (Shen and Abate-Shen 2010).

3. PROSTATE CANCER

3.1 EPIDEMIOLOGY

The incidence and mortality rate of prostate cancer varies worldwide. PCa is the thirst most common neoplasia among men, with 1,094,916 cases in the year 2012. This represents 14.8% of all cancer cases in men (68% in developed countries and 32% in developing countries) (Ferlay J 2012). Incidence rates vary by more than 25-fold worldwide, with the highest rates found in the developed countries of Oceania, Europe and North America (Jemal *et al.* 2011). Incidence variance could be explained by differences in age-structure between populations of different regions, since PCa is associated with advancing age. Other reasons for this difference are genetic susceptibility, exposure to unknown external risk factor or differences in the detection techniques implemented and screening programs, such as the prostate-specific antigen (PSA) test (Crawford 2003, Ramon and Denis 2007). PCa is a less prominent cause of death from cancer, counting 307,481 deaths in 2012 (6.6% of cancer deaths in men) (Ferlay J 2012). Associated with the high incidence rate and relatively low mortality rate, PCa became the most prevalent form of cancer in men. Likewise, prostate cancer mortality varies worldwide, with highest rates in Caribbean and Scandinavia and the lowest in China, Japan and countries of the former Soviet Union. Again, this difference probably occurs due to different age-structure in populations from these countries or genetic susceptibility (Jemal *et al.* 2011) (Figure 3).

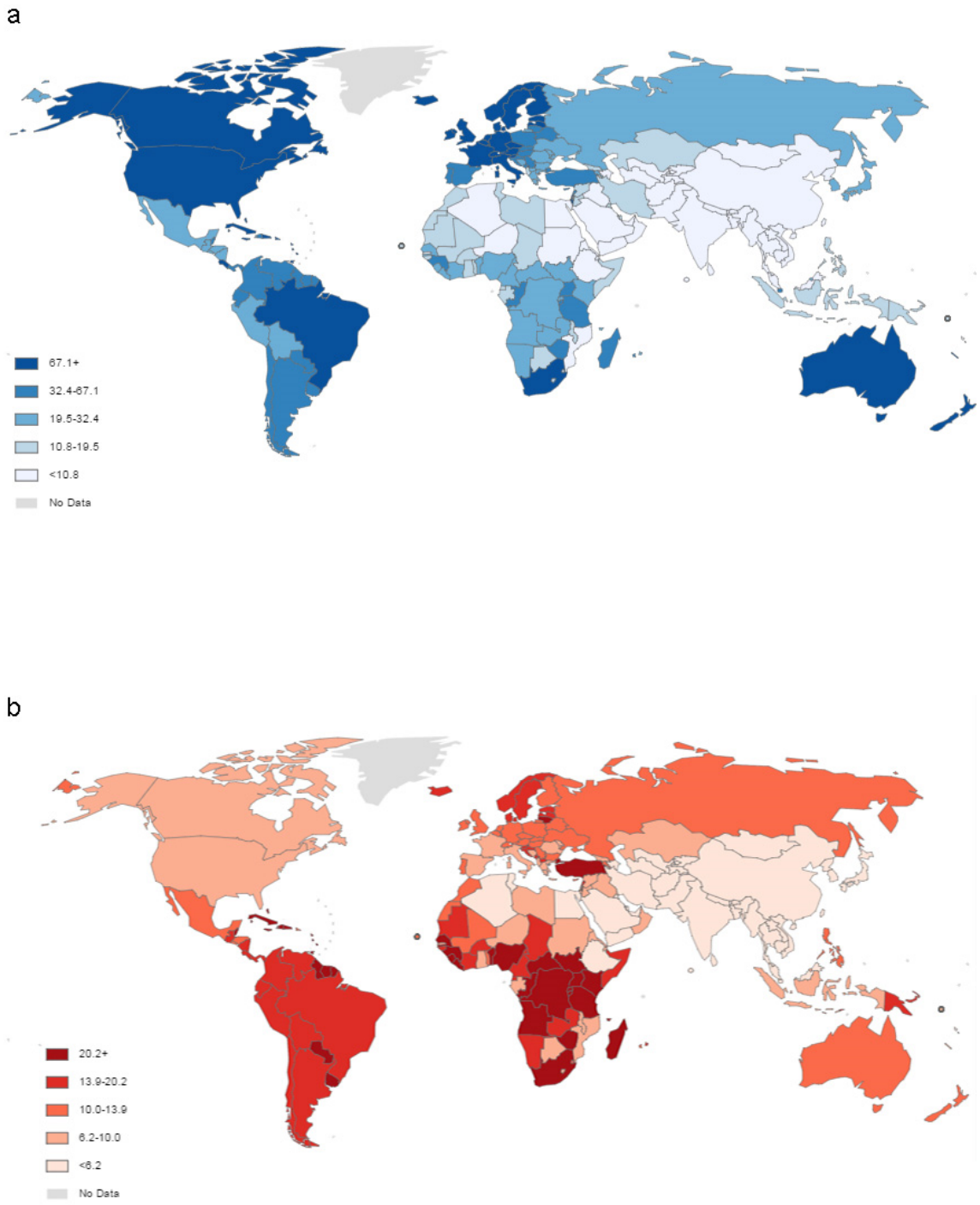


Figure 3. International variation in age-standardized prostate cancer incidence (a) and mortality (b) rates by world area (adapted from GLOBOCAN 2012). Rate per 100000.

Among European men, PCa is the second most incident neoplasm and is the fourth leading cause of cancer-related death. In Portugal, 6,622 new PCa patients were diagnosed in 2012, being the second most frequent neoplasia detected among men (23.3% of total) and ranking fourth in cancer mortality (11.1% of total cancer cases) (Figure 4)(Ferlay J 2012).

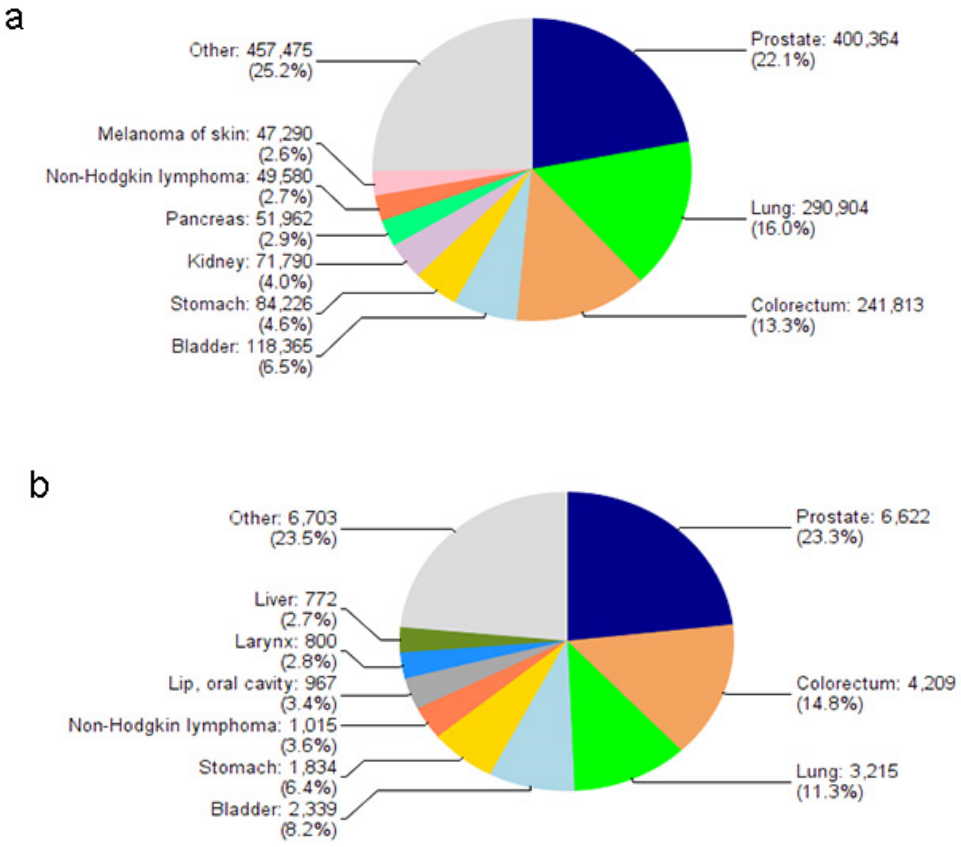


Figure 4. Cancer incidence in Europe and Portugal in males (adapted from Globocan 2012).

3.2 RISK FACTORS

PCa risk has been related to several factors including diet, environmental exposures and obesity. However, there are only three well-established risk factors for this particular cancer: family history, ethnicity and age.

Family history of PCa increases risk of developing the disease. Indeed, having a first degree relative affected by PCa more than doubles the risk of developing this malignant disease, and the risk is much higher for men with several affected relatives (Brawley 2012, Albright *et al.* 2015). This could be explained not only by genetic predisposition, but also by the exposure to the same environmental factor within a family (Brawley 2012). Other well-established risk factor is race, as it has been observed an unbalanced incidence between ethnic groups. When compared with Caucasians, African American men have higher incidence and, besides being diagnosed earlier, have higher tumor burdens within each stage category, a 1.5-fold higher frequency of metastatic disease at presentation, and lower survival rates (Chornokur *et al.* 2011, Siegel *et al.* 2015). Asians have lower prostate cancer incidence rates both in the United States and in Asia (DeVita *et al.* 2008). It is uncertain why some populations have higher incidence than others, although genetic susceptibility, exposure to environmental factors, culture and socioeconomic differences may play a role in this observation (Brawley 2012).

Age is the most important risk factor for PCa incidence and mortality. It is well established that PCa is age-related. Indeed, PCa rarely occurs until the age of 49, but the incidence rate increases thereafter. Men with up 49 years of age have a probability of developing PCa of 0.3% (1 in 304), but this probability increases to 2.3% (1 in 44) for men with 50 to 59, 6.3% (1 in 16) for men with 60 to 69 and 10.9% (1 in 9) for men who are 70 years old or older (Siegel *et al.* 2015).

4. DIAGNOSIS OF PROSTATE CANCER

PCa is normally asymptomatic until invasion occurs, at which point curative therapy is unusual, demanding the need for implementation of screening methods on a population basis. Several studies have concluded that there is no evidence for introducing widespread screening for PCa (Ilic *et al.* 2013). However, early detection should be offered to well-informed men (Heidenreich *et al.* 2014). Diagnostic tools to diagnose PCa include digital rectal examination (DRE), serum concentration of PSA, and transrectal ultrasound (TRUS)–guided biopsies.

4.1 DIGITAL RECTAL EXAMINATION

DRE is critical to establish the clinical stage of PCa. In fact, this exam provides information about location, size, and extent of the primary cancer and is essential for prognostic and treatment planning. The sensitivity of DRE for PCa diagnosis depend both on the stage of the tumor and the experience of the examiner. While DRE is of limited value in detecting tumors confined to the prostate gland, it remains a useful adjunct to PSA testing to identify higher-risk cancer when PSA levels are low (Tewari 2013). In fact, twenty percent (20%) of tumors detected by DRE when the PSA is less than 2 ng/ml are not organ confined (Okotie *et al.* 2007).

4.2 PROSTATE-SPECIFIC ANTIGEN

PSA screening test was first implemented in 1987 and in subsequent years incidence rates raised significantly in the United States (Barry 2001). PSA is a glycoprotein with protease activity produced by epithelial cells surrounding prostate acini and ducts. This protein is secreted in the lumina of these ducts and is normally present in low concentration in the plasma but in some prostate disorders, such as inflammation, HBP or cancer, its concentration increases (Ramon and Denis 2007). PSA expression is prostate specific rather than prostate cancer specific, which makes it a test with reduced accuracy (Crawford 2003). Nevertheless, the PSA test has been an outstanding help for

PCa management by clinicians, although its nonspecificity has led to overdiagnosis and overtreatment of indolent tumors (Duffy 2011).

Conventionally, a patient with PSA above 4 ng/ml is purposed to have a prostate biopsy, since approximately 70% of cancers will be detected using this cutoff (Catalona *et al.* 1994). About 30% of men with PSA in the range of 4–10 ng/ml will have PCa on biopsy (Gann *et al.* 1995), but it has been reported a high prevalence of PCa among men with a PSA ≤4 ng/ml and that at these PSA levels several men can harbor clinically significant disease. Indeed, in men with PSA test between 3.1-4.0 ng/ml, 26.9% had PCa and of these 25% were high-grade tumors (Thompson *et al.* 2004).

To improve the specificity of PSA in early detection of PCa, several modifications of serum PSA value have been described, including PSA density, PSA density of the transition zone, age-specific reference ranges, and PSA molecular forms (Heidenreich *et al.* 2014).

Screening for prostate cancer is primarily performed using DRE and PSA test, yet the specificity and sensitivity of both of these modalities are not ideal (Holmström *et al.* 2009). However, DRE and PSA together as early detection tools have allowed diminishing stage at detection in recent years, as 70-80% of PCa now diagnosed are organ confined and with increased chances of cure (DeVita *et al.* 2008). According to international guidance, screening must start at age of 50 and it must be regular throughout the years until the seventh decade of life (Wolf *et al.* 2010).

4.3 TRANSRECTAL ULTRASONOGRAPHY

The transrectal ultrasound probe was introduced by Watanabe and colleagues in 1968 and the first report of the diagnostic procedure were led by Holm and Gammelgard in 1981 (Tewari 2013). Transrectal ultrasonography (TRUS) has become the standard imaging tool used by urologists to assess the prostate and assist guidance of needles for directed tissue biopsies to obtain material for histopathologic examination. Patients with high levels of PSA and/or an inconclusive and suspicious DRE are submitted to TRUS, to determine presence of PCa. The number of collected cores must be adapted depending on patient's age, PSA level and prostate volume. European guidelines suggest that in a 30-40 mg organ, eight cores must be obtained at least (Heidenreich *et al.* 2014). Patient

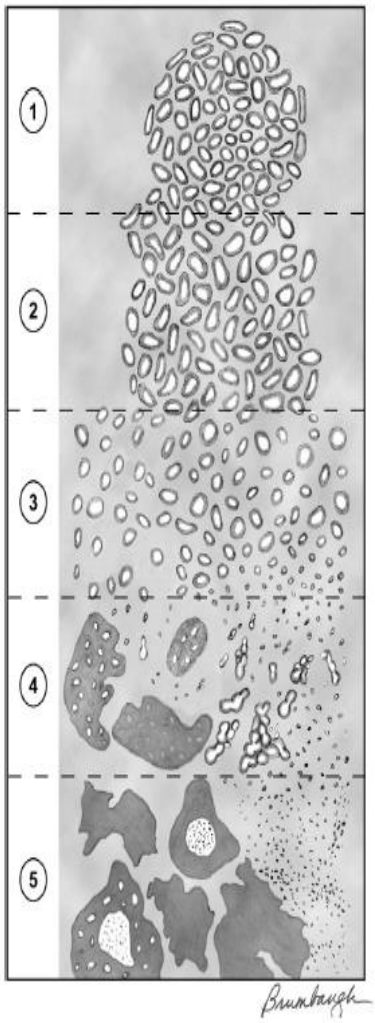
age, potential comorbidities, and the therapeutic consequences should also be considered (Heidenreich *et al.* 2014).

TRUS is also useful in assessing prostate volume and calculating PSA density. Limitations of this imaging modality include the difficulty to characterize the integrity of prostatic capsule and to visualize early extra capsular extension or seminal vesicle involvement (DeVita *et al.* 2008).

5. GRADING AND STAGING OF PROSTATE ADENOCARCINOMAS

5.1 HISTOLOGICAL GRADING: GLEASON SCORE

Histological grading has been proven to be one of the main predictors of prostate adenocarcinomas outcome. Gleason grading was firstly developed in 1966 by Donald Gleason and his colleagues, and has recently been updated to meet the needs of daily diagnostic practice (Lotan and Epstein 2010). Gleason grading score has become the strongest prognostic factor for clinical behavior and treatment response, including recurrence risk after radical prostatectomy or radiotherapy. The Gleason grading system is based entirely on the histological pattern evaluated at low magnification in H&E-stained prostatic tissue sections. This grading system is based on the degree of differentiation of the two most common patterns (Figure 5). The grade of the most common pattern is added to the grade of the second most common pattern (both ranging from 1 to 5) with pattern 1 being the most differentiated and 5 the most poorly differentiated. The sum of the primary and secondary patterns is called Gleason score (GS), ranging from 2 to 10 (Ramon and Denis 2007, DeVita *et al.* 2008). The Gleason score is reported with both primary and secondary patterns. The 2005 ISUP consensus update also demands that if a tertiary pattern 5 is observed on needle biopsy, it should be reported in place of the secondary pattern, to reflect the prognostic impact of Gleason pattern 5 adenocarcinoma and the uncertainty about possible sampling error. An example, a positive core with Gleason patterns 4 + 3 and tertiary pattern 5 on needle biopsy, should be diagnosed as GS 9 (4 + 5). However, in prostatectomies, when the entire carcinoma is available, the consensus is to grade such tumor as Gleason score 7 (4 + 3) and include additionally the tertiary pattern 5 (Epstein 2010).



2005 ISUP Modified Gleason System

Gleason pattern 1

- Completely circumscribed nodule of tightly packed but separate, uniform, rounded to oval, medium-sized acini

Gleason pattern 2

- Circumscribed nodule of small acini, with some variation in size, which are less tightly packed than in pattern 1 and may show minimal peripheral invasion into stroma but never into benign lobules

Gleason pattern 3

- Discrete glandular units, frequently with irregular contours
- Infiltrates in and amongst non-neoplastic prostate acini
- Marked variation in size and shape.
- Smoothy circumscribe small cribiform nodules of tumor

Gleason pattern 4

- Fused microacinar glands
- Ill-defined glands with poorly formed glandular lumina
- Cribriform glands with na irregular border
- Hypernephromatoid

Gleason pattern 5

- Glandular architecture is completely lost, composed of single cells, cords or solid sheets
- Comedocarcinoma with central necrosis surrounded by papillary, cribriform or solid masses

Figure 5. Modified Gleason score system for histological grading of PCa (adapted from (Epstein *et al.* 2005).

5.2 TUMOR STAGING

An accurate staging is critical for prognosis assessment and treatment planning for PCa. In the earlier 1950s, staging systems for solid tumors began to consider the tumor, lymph node and metastasis to categorize prognosis (Hricak and Scardino 2009). A unified TNM (tumor, lymph node, metastasis) staging system for prostatic carcinoma was first introduced only in 1992, by the American Joint Committee on Cancer (AJCC) and the International Union Against Cancer (UICC) (Cheng *et al.* 2012). Since them, several revisions have been undertaken to optimize the prognostic accuracy. At this time, the 2010 TNM staging system revision of the AJCC/UICC is the most widely used.

The TNM staging system consists on the evaluation of the extension of the primary tumor (T), presence and extension of involved lymph nodes (N) and distant metastases (M). Within each category of TNM, there are different sublevels (Table 2) based on tumor volume or extent (T1–T4), amount and/or size of lymph node metastases (N0–N1), and distant metastases (M0–M1) (Cheng *et al.* 2012). This system comprises two types of staging considering timing of data collection, namely clinical and pathological staging. Clinical staging reports to any information on cancer extension before definitive treatment and is established by DRE examination, TRUS, magnetic resonance imaging (MRI) and also serum PSA level (Falzarano and Magi-Galluzzi 2011). Pathologic staging is obtained after radical prostatectomy (RP) and is centered in macro and microscopic examination of the surgical specimen and dissected regional lymph nodes (Falzarano and Magi-Galluzzi 2011, Cheng *et al.* 2012). Considering extent of disease outside the prostate, N-staging is assessed by pelvic lymphadenectomy, while M-staging is obtained by bone scan (Edge and Compton 2010). Agreement between clinical and pathological stages would simplify assessment of patient prognosis and guide appropriate therapy. However, clinical staging is an inaccurate tool to predict the final pathological stage (Cheng *et al.* 2012). Furthermore, over 80% of PCa are multifocal, which makes accurate clinical staging even more difficult and uncertain (Andreoiu and Cheng 2010). Clinical tumor understaging has consistently been shown to range from 40% to 60% (Campbell *et al.* 2001, Heidenreich *et al.* 2014). This is evidently associated to the multifocal and histologically heterogeneous nature of prostatic carcinomas.

Table 2. Clinical and pathologic classification of PCa by the The American Joint Committee on Cancer (adapted from (Cheng *et al.* 2012)).

Primary Tumor – Clinical (T)		
TX		Primary tumor cannot be assessed
T0		No evidence of primary tumor
T1		Clinically inapparent tumor neither palpable nor visible by imaging
	T1a	Tumor incidental histologic finding in 5% or less of tissue resected
	T1b	Tumor incidental histologic finding in more than 5% of tissue resected
	T1c	Tumor identified by needle biopsy (e.g., because of elevated PSA)
T2		Tumor confined within prostate*
	T2a	Tumor involves one-half of one lobe or less
	T2b	Tumor involves more than one-half of one lobe but not both lobes
	T2c	Tumor involves both lobes
T3		Tumor extends through the prostate capsule
	T3a	Extracapsular extension (unilateral or bilateral)
	T3b	Tumor invades seminal vesicle(s)
T4		Tumor is fixed or invades adjacent structures other than seminal vesicles such as external sphincter, rectum, bladder, levator muscles, and/or pelvic wall
Primary Tumor - Pathologic (pT)		
pT2		Organ confined
	pT2a	Unilateral, one-half of one side or less
	pT2b	Unilateral, involving more than one-half of side but not both sides
	pT2c	Bilateral disease
pT3		Extraprostatic extension
	pT3a	Extraprostatic extension or microscopic invasion of bladder neck
	pT3b	Seminal vesicle invasion
pT4		Invasion of rectum, levator muscles, and /or pelvic wall
Regional Limph Nodes - Clinical (N)		
NX		Regional lymph nodes were not assessed
N0		No regional lymph node metastasis
N1		Metastasis in regional lymph node(s)
Regional Limph Nodes - Pathologic (pN)		
pNX		Regional nodes not sampled
pN0		No positive regional nodes
pN1		Metastases in regional node(s)
Distant metastasis - Clinical (M)		
M0		No distant metastasis
M1		Distant metastasis
	M1a	Nonregional lymph node(s)
	M1b	Bone(s)
	M1c	Other site(s) with or without bone disease
Distant metastasis - Pathologic (pM)		
pM0		No distant metastasis
pM1		Distant metastasis
	pM1a	Non-regional lymph node(s)
	pM1b	Bone(s)
	pM1c	Other site(s) with or without bone disease. When more than one site of metastasis is present, the most advanced category is used. pM1c is most advanced

6. TREATMENT OF PROSTATE CANCER

Considering the complexity and heterogeneity of PCa, it is difficult to determine a standard treatment option for all patients. Selecting the optimal therapeutic approach from an array of choices requires individualization of treatment plans based on several features: age, life expectancy, life quality, TNM classification, GS and preoperative serum PSA level (Ramon and Denis 2007). For organ-confined PCa, the therapeutic options include active surveillance, radical prostatectomy (RP), and radiation therapy. For invasive or metastatic tumors, the options available include hormone and/or radiation therapy and chemotherapy. The present challenge is to distinguish men with aggressive local disease for whom treatment might be useful (DeVita *et al.* 2008). Concerning PCa therapeutic strategies, there is an urgent need to develop new and better therapeutic options. Thus, it is crucial to attend this goal by increasing knowledge on the biology of PCa carcinogenesis.

6.1 ACTIVE SURVEILLANCE AND WATCHFUL WAITING

Active surveillance is a strategy adopted for low-risk patients that includes active prostate cancer monitoring with scheduled attention to both local and PSA progression. With this option patients are not treated but kept under surveillance and treated only when risk of progression increases during follow-up (Heidenreich *et al.* 2014). This approach is a strategy to avoid overtreatment. Criteria to select PCa patients to this procedure are: clinically confined PCa, GS equal or less than 6 and low PSA serum level (less than 10ng/mL) (Heidenreich *et al.* 2014). The follow-up of these patients consists in frequent PSA evaluation and prostatic biopsies at 2, 5 and 10 years (Ramon and Denis 2007). On the other hand, the watchful waiting is a less intensive type of follow-up that means fewer tests and relay more on changes in a man's symtoms to decide if treatment is needed (Heidenreich *et al.* 2014)

6.2 RADICAL PROSTATECTOMY

Radical prostatectomy (RP) is a surgical procedure that removes the entire prostate gland and both seminal vesicles and is frequently accompanied by bilateral pelvic lymph node dissection (Heidenreich and Pfister 2014). This treatment is offered to men with localized PCa (cT1a–T2b, Gleason score 2–7 and PSA inferior to 20 ng/mL), life expectancy of 10 or more years and its intent is to totally eradicate disease (Ramon and Denis 2007, Heidenreich and Pfister 2014).

6.3 RADIOTHERAPY

Radiation therapy has played a significant role in management of PCa over the years. There are two main types of radiation therapy: external-beam radiotherapy and brachytherapy. Regarding efficiency in patient survival, radiation therapy has achieved rates of disease-free survival similar to those obtained by RP (Jani and Hellman 2003). External-beam radiotherapy (EBRT) is the procedure with lower morbidity rates, which enables its application to broad range of PCa patients, whom do not tolerate RP or brachytherapy (Jani and Hellman 2003). Brachytherapy consists in the placement of a small radioactive source into or near the tumor, allowing a more localized distribution of radiation and resulting in fewer side effects than external-beam radiotherapy (Jani and Hellman 2003). Brachytherapy is typically administrated in patients harboring localized PCa, with low-volume and low-grade (Jani and Hellman 2003). Moreover combined use of external-beam therapy and brachytherapy is frequent in patients with intermediate to high-risk disease, leading to good survival rates (Ramon and Denis 2007).

6.4 HORMONOTHERAPY

The benefits of surgical castration and estrogen treatment on prostate size and symptoms in metastatic PCa were firstly reported in 1941 by Huggins and Hodges. These effects showed that PCa were androgen dependent (Hammerer and Madersbacher 2012), indeed, androgens are essential for the growth and perpetuation of tumor cells (Walsh 1975). Prostate is a hormone-responsive gland and androgen deprivation can be

achieved by suppressing the secretion of testicular androgens by surgical (orchiectomy) or chemical castration (luteinizing hormone-releasing hormone (LHRH) agonists or antagonists) or by inhibiting its action using competing compounds at the level of their receptor (anti-androgens) (Hammerer and Madersbacher 2012). Thus, androgen ablation has been the first therapeutic approach for patients with advanced PCa. It has previously been demonstrated that hormonal therapy combined with external beam-radiotherapy administered to local PCa presents high cure rates (Jani and Hellman 2003). These therapeutic approaches achieve a symptom relief in 70-80% of patients, although virtually all the patients will develop castration-resistant PCa (CRPC) after 18 to 24 months of hormone treatment (Felici *et al.* 2012).

6.5 CYTOTOXIC CHEMOTHERAPY

Since 1990's, the field of cytotoxic chemotherapy has significantly improved survival and life quality in patients with metastatic castration-resistant prostate cancer (mCRPC) and has become a standard of care in this disease (Sonpavde *et al.* 2014). In 1999, the combination of mitoxantrone and prednisone became the first line therapy to show clinical benefit in mCRPC (Tannock *et al.* 1996). Interestingly, it was approved based on palliative benefits, despite no improvement in overall survival. In 2004, a phase III trial showed that docetaxel, a taxane microtubule inhibitor, conferred a survival benefit in mCRPC and was approved for the first-line treatment of these PCa. Since 2010, multiple cytotoxic agents have been included to the chemotherapy resource, such as sipuleucel-T, cabazitaxel, abiraterone acetate plus prednisone and radium-223 (Kantoff *et al.* 2010, De Bono *et al.* 2011, Cabot *et al.* 2012, Fizazi *et al.* 2012, Parker *et al.* 2013). There is no accepted standard sequence of cytotoxic agents and the choice is dependent of several patient characteristics, such as presence of symptoms, sites of metastasis, previous docetaxel exposure, comorbidities, patient preference, and the cost and availability of different treatments (Sonpavde *et al.* 2014).

7. GENETIC CHANGES IN PROSTATE CANCER

Prostate carcinoma is a multifactorial disease influenced by both environmental and genetic factors. Besides advancing age and ethnic background, the strongest epidemiological risk factor for PCa is a positive family history. Over the past 20 years, much has been done in the field of gene abnormalities associated with prostate cancer risk, including familial aggregation studies, twin studies, family-based linkage studies, mutation screening and molecular epidemiological studies (Alberti 2010). The increasing knowledge in understanding the molecular basis of PCa will be crucial to establish efficient biomarkers for early detection, distinguish between indolent and aggressive PCa, and also to develop new and more effective therapies.

7.1 GERMLINE MUTATIONS

PCa can be divided into three groups: sporadic (up to 85%), hereditary (5%) and familial (10%) (Kral *et al.* 2011). Hereditary PCa has been defined as families that meet at least one of the following three criteria: three or more first-degree relatives affected with PCa in any nuclear family; occurrence of PCa in each of three successive generations in either of the proband's paternal or maternal lineages; or at least two relatives, both affected with PCa diagnosed before age 55 (Carter *et al.* 1993). Cases that do not fulfill the reported criteria but have at least two affected relatives are defined as familial forms. The clinicopathologic characteristics and tumor progression features are similar between hereditary and sporadic PCa, except in the age at diagnosis that commonly arises 6 years earlier in hereditary PCa. Recent studies suggest that hereditary PCa is a complex disease, involving multiple susceptibility genes with variable phenotypic expression. Family-based studies have identified strong candidate susceptibility genes involved in the hereditary form of prostate cancer, including *RNaseL*, *ElaC2*, *MSR1* and *HOXB13* and additional weak candidate susceptibility loci have been suggested to be involved in hereditary PCa (Table 3).

Table 3. Genes involved in prostate cancer development

Gene localization	Candidate gene/locus	Gene Function
Strong candidates for susceptibility genes		
1q25.3	*RNaseL/HPC1*	Antiviral and pro-apoptotic
17p11	*ELaC2/HPC2*	Induces tRNA maturation
8p22-23	*MSR1*	Involved in arterial wall deposition of cholesterol and in endocytosis of low density lipoproteins
17q21-23	*HOXB13*	Transcription factor
Weak candidates for susceptibility genes (low-risk alleles)		
Xq27-28	*HPCX*	
20q13	*HPC20*	
17q21	*BRCA1*	Regulation of cell cycle progression and DNA repair
13q12-13	*BRCA2*	DNA recombination and repair
1q42-43	*PCAP*	

In 1996, the first prostate cancer susceptibility locus was identified. Termed *HPC1* locus and located in chromosome 1q24–25, it was subsequently identified as the RNaseL gene. *RNaseL* codifies an important enzyme in immune response to viral infection, induction of apoptosis and cell cycle, and cell differentiation regulation. Germ-line mutations in *RNaseL* gene have been reported in up to 13 % of all familial prostate cancer cases (Casey *et al.* 2002).

Other strong candidate for susceptibility in PCa is the *ELAC2* gene. This gene is located on 17p11 and codifies an essential enzyme for tRNA biosynthesis. Mutations in this gene increase 2%–5% the risk of PCa. However, germ-line *HPC2*/*ELaC2* mutations are rare in hereditary prostate cancer, indicating that this gene plays a limited role in genetic susceptibility to this disease (Wang *et al.* 2001).

The *MSR1* gene has also been implicated as a candidate gene to hereditary prostate cancer. This gene is located at the 8p22 chromosome region and encodes the macrophage scavenger receptor type A, involved in several cell functions such as the

modulation of interaction between foreign cells and macrophages, cell adhesion and phagocytosis. Several studies have described MSR1 mutations linked to prostate cancer and, in fact, a specific region was characterized as linked to an increased risk of prostate cancer in Caucasian population (Xu *et al.* 2002, Beuten *et al.* 2010).

Finally, *HOXB13* encodes for an important transcription factor in prostate development. Recently, Ewing and collaborators identified a recurrent germ-line mutation (G84E) in *HOXB13*, in a previously recognized region of linkage at 17q21–22, as harboring an increased risk for familial prostate cancer (Ewing *et al.* 2012). Importantly, despite being a rare mutation in prostate cancer families (about 5%), *HOXB13* G84E mutation is significantly associated with predisposition to PCa (Xu *et al.* 2013).

7.2 EPIGENETIC CHANGES

Epigenetics is defined as the study of mechanisms that initiate and maintain patterns of gene function and regulation in a heritable manner without affecting DNA sequence. Epigenetic alterations include: DNA methylation, histone modifications and non-coding RNAs, specially microRNAs (Sandoval and Esteller 2012). These alterations arise early and have been associated also with PCa progression. In addition, there are many studies describing the application of epigenetic alterations for biomarker development and also the developing of inhibitors to block epigenetic mechanisms of carcinogenesis.

DNA methylation occurs by addition of a methyl group at the 5' position of a cytosine ring inside CpG dinucleotides. Gene promoter regions are frequently enriched with CpG dinucleotides, and these stretches are commonly known as CpG islands (Jones 2012). *GSTP1* is one of the best-known genes hypermethylated in PCa. *GSTP1* encodes an enzyme involved in oxidative damage response and protect cells from DNA damage and cancer initiation. Promoter hypermethylation of *GSTP1*, and subsequently loss of expression, was frequently found in PCa (over 90%), as well as in HGPIN. These observations support that promoter hypermethylation is an early event in tumorigenesis. Since it can be detected in tumor tissues and body fluids, extensive work has been done in an attempt to make methylation of *GSTP1* a useful biomarker to PCa (Jerónimo *et al.* 2001, Nakayama *et al.* 2004).

DNA hypomethylation has also been observed in PCa. This aberrant DNA methylation was the first cancer-related epigenetic alteration reported. A well-studied example is the hypomethylation of *LINE1* in around 50% of all PCa samples. Several other genes were observed as upregulated due to promoter hypomethylation in PCa, including *CAGE*, *CYP1B1*, *HPDSE*, *PLAU*, *CRIP1*, *S100P* and *WNT5A* (Jerónimo *et al.* 2011).

Histone modifications are changes of basic amino acid residues (such as lysine, arginine and serine) on histone tails, including acetylation, phosphorylation, methylation, ubiquitylation, sumoylation, citrullination, and ADP ribosylation (Bannister and Kouzarides 2011). These modifications disturb the affinity to DNA, change the chromatin structure, and subsequently influence gene expression. The study of histone modifications in PCa progression presents a huge challenge due to the complexities of this field. Several histone-modifying enzymes have been described as affected in PCa, including HDACs, HMTs and HDMs (Bannister and Kouzarides 2011). Among these, the best studied is the enhancer of zeste homolog 2 (*EZH2*). This histone methyltransferase is responsible for the trimethylation of H3K27 (H3K27me3) and subsequently gene silencing (Cao *et al.* 2002). Physically it interacts with DNMTs and helps to their binding at *EZH2* target promoters (Viré *et al.* 2006). Overexpression of *EZH2* has been associated with high proliferation rate and tumor aggressiveness in PCa (Bachmann *et al.* 2006).

MicroRNAs (miRNA/miR) are small noncoding RNAs (18–25 nucleotides in length) that can regulate the expression of multiple genes by binding with complementary mRNA sequences and altering their expression through a RNA-induced silencing complex (Catto *et al.* 2011). Each miRNA frequently regulates multiple mRNAs, and each mRNA can be targeted by multiple miRNAs (Catto *et al.* 2011). MicroRNA expression is often altered in cancer and can act as oncogenes (when overexpressed) or tumor suppressors (via downregulation), depending on their expression status and specific targets. Several reports have implicated the deregulation of miRNA in PCa with alterations in important pathways of cell growth and disease development (Table 4) (Catto *et al.* 2011). In fact, miRNA deregulation in PCa affects epigenetic reprogramming, blockade of apoptosis, promotion of cell cycle, migration, and invasion, and is an alternative mechanism sustaining androgen-independent growth (Coppola *et al.* 2010). Therefore, miRNAs, as modulators of gene expression frequently altered in PCa, might potentially be used as biomarkers or novel therapeutic targets.

Table 4. Summary of miRNAs with altered expression in PCa, including their targeted messenger RNAs and pathways (adapted from (Catto *et al.* 2011).

miRNA	Expression	miRNA target	Pathway
miR-20a	*up*	E2F1-3	Apoptosis
miR-21	*up*	PTEN, AKT, androgen pathway	Apoptosis, mTOR, androgen independence
miR-24	*up*	FAF1	Apoptosis
miR-32	*up*	BCL2L11 (Bim)	Apoptosis
miR-106b	*up*	P21, E2F1	Cell cycle control/apoptosis and proliferation
miR-125b	*up*	P53, BBC3 (Puma), BAK1	Apoptosis
miR148a	*up*	CAND1	Cell cycle control
miR-221	*up*	P27(kip1)	Cell cycle control and androgen independence
miR-222	*up*	P27(kip1)	Cell cycle control and androgen independence
miR-521	*up*	Cockayne syndrome protein A	DNA repair
miR-1	*down*	Exportin-6, tyrosine kinase 9	Gene expression
miR-7	*down*	ERBB-2 (EGFR, HER2)	Signal transduction
miR-15a-16 cluster	*down*	CCND1 and WNT3a	Cell cycle regulation, apoptosis and proliferation
miR-34a	*down*	HuR/Bcl2/SIRT1	Apoptosis and drug resistance
miR-34c	*down*	E2F3, bcl2	Apoptosis and proliferation
miR-101	*down*	EZH2	Gene expression
miR-107	*down*	Granulin	Proliferation
miR-143	*down*	MYO6, ERK5	Cell migration, proliferation
miR-145	*down*	MYO6, BNIP3L, CCNA2, TNFSF10	Cell migration, apoptosis, cell cycle control
miR-146a	*down*	ROCK1	-
miR-148a	*down*	MSK1	Proliferation, stress response and drug resistance
miR-205	*down*	IL-24 and IL-32, Cepsilon	Cell growth and invasion, EMT
miR-331-3P	*down*	ERBB-2, CDCA5, KIF23	Signal transduction, cell cycle control
miR-449a	*down*	HDAC-1	Gene expression
miR-1296	*down*	MCM family	DNA replication
Let-7a	*down*	E2F2 and CCND2	Cell cycle control and proliferation

7.3 COPY NUMBER ALTERATIONS

Extensive genomic analyses of prostate cancer have identified copy number alterations and chromosomal rearrangements associated with prostate carcinogenesis. Important somatic alterations have been identified by comparative genomic hybridization (CGH), such as gains or losses of chromosomal regions (Boyd *et al.* 2012). Global analyses of copy number profiles of primary PCa tumors and metastases have identified recurrent aberrations, including gains at 1q, 3q, 7q and 8q and losses at 1p, 6q, 8p, 9p, 13q, and 16p (Boyd *et al.* 2012). Importantly, numerous of these genetic alterations have also been identified in PIN and PIA lesions, which have further suggested these lesions as precursors to PCa. Focal amplifications, containing key regulatory genes, have been frequently observed in PCa, including androgen receptor at Xq12 and *MYC* at 8q24 ((Demichelis *et al.* 2009, Taylor *et al.* 2010, Lonigro *et al.* 2011, Barbieri *et al.* 2013).

Several studies have demonstrated that the number of copy number alterations is correlated with features of PCa progression, such as Gleason grade, advanced tumor stage, and other poor prognostic features. Indeed, a genetic model of prostate carcinogenesis based on genomic imbalances detected by CGH has been proposed (Figure 6). This model shows that losses of 8p and 13q are independent initiating events, followed by intermediate events (8q gain and 6q, 16q and 18q losses) and finally a cluster of events that includes both gains and losses of chromosomal regions (Ribeiro *et al.* 2006).

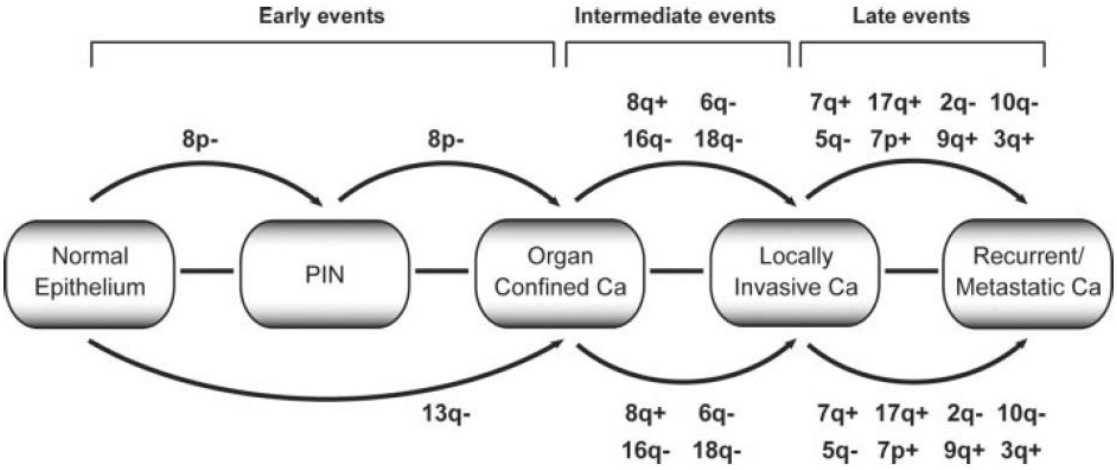


Figure 6. Genetic model of PCa progression based on genomic imbalances detected by comparative genomic hybridization (adapted from (Ribeiro *et al.* 2006).

7.4 GENES COMMONLY ALTERED

There are several molecular events that are believed to occur frequently in PCa. Even though each event has been associated with a possible role in cancer initiation or progression, it is unclear if there is a temporal sequence associated with these events, or whether there is a causal relationship between them.

Loss-of-heterozygosity (LOH) at 8p21.2 is present in up to 85% of high-grade PIN lesions and adenocarcinomas (Swalwell *et al.* 2002, Bethel *et al.* 2006). This region includes *NKX3.1*, a homeobox gene that has been observed down-regulated in PCa. Such alteration is an initial event of the prostate carcinogenesis and is involved in multiple important pathways (Abate-Shen *et al.* 2008). Although LOH of 8p21 progressively increases in frequency with cancer grade, *NKX3.1* mRNA expression did not correlate with copy-number loss, suggesting the possibility of alternative tumor suppressors in this region (Taylor *et al.* 2010).

MYC (v-myc myelocytomatosis viral oncogene homolog) at chromosome 8q24 is frequently amplified in prostate cancer (Taylor *et al.* 2010); however, this often involves amplification of the entire arm of chromosome 8, leading to the possibility of other oncogenes in the region. *MYC* encodes a transcription factor (c-Myc) with multiple downstream target genes, leading to cell cycle progression, cell survival, and tumorigenesis. Recent studies have suggested a role for *MYC* overexpression in cancer initiation, as nuclear MYC protein is up-regulated in many PIN lesions and the majority of carcinomas in the absence of gene amplification (Gurel *et al.* 2008).

The androgen receptor (*AR*) gene that codifies a ligand-dependent nuclear transcription factor is located on Xq11–12. One of the principal functions of this protein is to activate the expression of target genes (Dehm and Tindall 2006). AR is essential for growth and differentiation of the normal prostate and is also responsible for treatment failure in castration-resistant metastatic disease (Chen *et al.* 2004, Tran *et al.* 2009). In PCa, AR has been observed with increased activation by multiple alterations, including gene amplification, point mutations and alterations in splicing leading to constitutively active variants. Recent studies point these alterations as more frequent in metastatic CRPC. Indeed, AR amplification was present in about 40% and mutation in 10% of metastatic PCa, but completely absent in primary tumors (Taylor *et al.* 2010). Alterations in the *AR* gene itself do not play a role in the pathogenesis of prostate cancer, but instead

emerge during treatment as a mechanism of resistance to therapies targeting the androgen axis.

The phosphatase and tensin homologue gene (*PTEN*) is located on chromosomal region 10q23. *PTEN* is an essential protein in the regulation of PI3K/Akt signaling pathway, as its main negative regulator. This protein is also involved is several important cell processes including apoptosis, cell cycle progression, cell proliferation, angiogenesis, aging and DNA damage response (Govender and Chetty 2012). It was originally identified as a tumor suppressor gene, frequently mutated or deleted in many neoplasms, including those of the prostate (Salmena *et al.* 2008). Deletions at the *PTEN* locus occur in approximately 40% of primary PCa and 70-80% in advanced PCa (Squire *et al.* 2011). Loss of PTEN caused by inactivating point mutations is less frequent, about 5-10% of primary PCa (Grasso *et al.* 2012, Weischenfeldt *et al.* 2013). Recent studies have demonstrated that *PTEN* undergoes copy number loss as an early event in prostate carcinogenesis and is correlated with progression to aggressive, castration-resistant disease (Verhagen *et al.* 2006, Schmitz *et al.* 2007, Sircar *et al.* 2009, Taylor *et al.* 2010). Alteration of *PTEN* expression in human prostate cancer cell lines or targeted deletion of *PTEN* in PCa mouse models is sufficient for the development of castration resistance (Lin *et al.* 2004, Bertram *et al.* 2006, Wu *et al.* 2006). Whereas this could reflect the ability of *PTEN* to interact directly with *AR*, the mechanistic details by which PTEN loss promotes castration resistance remains to be resolved.

7.5 SIGNALING PATHWAYS IN PROSTATE CANCER

The PI3K/Akt/mTOR pathway is a key oncogenic signaling pathway that has been linked to tumorigenesis and resistance to both conventional and targeted anticancer therapies in a wide variety of tumor types (McCubrey *et al.* 2011). This signaling pathway has been involved in numerous essential cell functions, including the regulation of cellular survival, differentiation and stem cell-like properties, growth, proliferation, metabolism, migration and angiogenesis (Bitting and Armstrong 2013). In prostate cancer, activation of the PI3K/Akt/mTOR pathway has been strongly implicated in tumor progression (Taylor et al. 2010). Activation of the PI3K/Akt/mTOR pathway by mutation, altered expression, or copy number alterations, has been reported in 42% of primary prostate tumors and 100% of metastatic tumors leading to prostate cancer progression (Taylor *et al.* 2010). Several studies have showed that the functional consequences of Pi3K/Akt/mTOR pathway activation are mostly relevant for castration-resistant prostate cancer (Majumder *et al.* 2004, Uzgare and Isaacs 2004, Gao *et al.* 2006, Xin *et al.* 2006). Therefore, the PI3K/Akt/mTOR pathway appears to be fundamental to the metastatic potential of PCa, offering a strong rationale for targeting the PI3K/Akt/mTOR pathway in disease treatment.

Since the discovery that castration of men with advanced disease eventually ends with cancer relapse, much have been done in the field of androgen-signaling in PCa. Several reports have described *AR* gene amplification, point mutations, and alteration in splicing leading to increased activity in PCa (Taylor *et al.* 2010). Furthermore, AR pathway interacts with other oncogenic signaling pathways, including PI3K/Akt signaling. Based on the whole genome of PCa, an interesting role for androgen signaling in driving prostate carcinogenesis has been proposed: as rearrangement breakpoints are more common near androgen receptor-binding sites, AR-mediated transcription could predispose to genomic rearrangements through transcriptional stress (Berger *et al.* 2011). Thus, androgen stimulation can potentially bring together the TMPRSS2 and ERG loci, ultimately promoting gene fusion of both genes (Haffner *et al.* 2010). Androgen-mediated transcriptional activity could act as an initial driver of many genomic rearrangements in PCa, and emphasize androgen signaling as a critical signaling pathway in both primary and advanced prostate cancer.

The mitogen-activated protein kinase (MAPK) pathway has a critical role in many human neoplasias (including lung, ovary, melanoma, pancreas and GI tract); however, its role in PCa is not well established. Up-regulation of MAPK pathway components and

upstream intermediates are common and enriched in prostate cancer metastases, although mutations in these components are rare (Taylor *et al.* 2010).

The Wnt proteins are secreted cysteine-rich proteins that have important roles in the developing embryo and in tissue homeostasis in adults (Clevers 2006). The Wnt/β-catenin signaling pathway is implicated in the maintenance of stem and progenitor cells in several adult tissues, including blood, intestine and skin (Wend *et al.* 2010). The deregulation of this pathway can occur in many types of cancer, such as colon, liver, skin, and prostate cancer (Giles *et al.* 2003, MacDonald *et al.* 2009, Kypta and Waxman 2012). An important feature of Wnt/β-catenin signaling is the stabilization of the transcriptional co-activator β-catenin, which regulates the expression of many genes implicated in cancer and is also an essential component of cadherin cell adhesion complexes. The last feature has special importance for the formation and function of the prostate gland (Heuberger and Birchmeier 2010). The activation of the Wnt/β-catenin pathway has effects on prostate cell proliferation, differentiation and the epithelial–mesenchymal transition, which is thought to regulate the invasive behavior of tumor cells. Studies focused in the development of inhibitors for Wnt/β-catenin signalling have recently emerged, as their action might reduce the self-renewal of prostate cancer stem or progenitor cells, which could be of potential therapeutic benefit (Kypta and Waxman 2012).

Hedgehog (Hh) signaling pathway genes are essential components in cell proliferation, differentiation and tissue polarity during embryonic development (Ingham 1998). During prostate development, the Hedgehog signaling is required for ductal morphogenesis and proliferation, and its activity is relatively low in adult normal prostate gland (Berman *et al.* 2004). This pathway has been reported as deregulated in several human cancers, including basal cell carcinomas, medulloblastomas, small cell lung cancer and GI cancers (Rubin and de Sauvage 2006). Emerging studies have related the Hh signaling to the development and progression of PCa to a more aggressive and to therapy-resistant disease states (Karhadkar *et al.* 2004, Sheng *et al.* 2004, Kim *et al.* 2011); however there is still a lot of controversy about how this deregulation occurs.

Finally, the deregulated expression of oncogenic tyrosine kinases, such as Src or Her2/Neu, has been studied in prostate neoplasm (Mellinghoff *et al.* 2004, Fizazi 2007). In fact, SRC tyrosine kinases have been implicated in aggressive PCa, progression to metastasis and castration resistance (Fizazi 2007). Several studies have demonstrated that Src is highly expressed in PCa cell lines, as well as in the majority of prostate cancer specimens. Additionally, AR signaling leads to activation of Src signaling in PCa, which can lead to castration resistance and cellular proliferation and invasiveness (Migliaccio *et*

al. 2000, Agoulnik *et al.* 2005, Kraus *et al.* 2006). A number of experiments have proved that Src inhibitors decrease the oncogenic potential of prostate cancer cell lines *in vitro* (Recchia *et al.* 2003, Lombardo *et al.* 2004, Nam *et al.* 2005), and also reduce prostate cancer growth and metastasis in mouse xenograft studies (Park *et al.* 2008, Saad and Lipton 2010). However, further research regarding the optimal use of these agents in the clinic still needs to be performed.

7.6 ETS REARRANGEMENTS IN PROSTATE CANCER

Recurrent chromosomal rearrangements in prostate carcinomas were firstly reported by Tomlins and collaborators (Tomlins *et al.* 2005), through an unconventional bioinformatics approach termed as the "Cancer Outlier Profile Analysis" (COPA) algorithm, used to analyze DNA microarray studies. This algorithm has allowed the identification of high expression genes on DNA microarrays data, and using this strategy in prostate cancer profiling studies two members of the ETS transcription factor family gene, *ERG* and *ETV1*, were identified. Then using 5'-RNA ligase-mediated rapid amplification of cDNA ends (5'RACE), the authors found recurrent chromosomal rearrangements of the 5' ends of the ETS family member (*ERG* or *ETV1*) with sequences from the 5' untranslated region of the androgen-regulated gene *TMPRSS2* (Tomlins *et al.* 2005). The gene fusion of *TMPRSS2* with *ERG* or *ETV1* only occurred in cases with overexpression of the respective ETS gene, and fusions were not detectable in benign prostate tissues. Using fluorescence in situ hybridization (FISH), more than 50% of a prostate-specific antigen (PSA)–screened cohort of prostatectomy samples had ETS rearrangements, confirming their existence at the chromosomal level. Analysis of *TMPRSS2-ERG* positive and *TMPRSS2-ERG* negative PCa cell lines showed that the *TMPRSS2-ERG* fusion resulted in androgen-regulated expression of *ERG*. In fact, upon androgen stimulation of VCaP cell line (harboring *TMPRSS2-ERG*) an increase of *ERG* expression occured, whereas no effect was observed in LNCaP cell line (*TMPRSS2-ETS* negative). Thus, the androgen-responsive elements that normally restrict the expression of *TMPRSS2* to the prostate drove the aberrant overexpression of the truncated ETS oncogenes (Tomlins *et al.* 2005). Many subsequent studies have validated the ocurrence of ETS gene fusions in about 47% of 10,779 prostate cancer cases (Pettersson *et al.* 2012). Furthermore, the identification of ETS rearrangements in about 20% of HGPIN and none of HBP or normal prostate tissue suggests that ETS gene fusions could be an early

event in prostate carcinogenesis (Cerveira *et al.* 2006, Perner *et al.* 2007, Clark *et al.* 2008, Park *et al.* 2008, van Leenders *et al.* 2011).

7.6.1ETS TRANSCRIPTION FACTOR FAMILY

The E26 transformation-specific (ETS) transcription factor family is one of the largest families of transcription regulators, composed of 28 members (Figure 7). All of them have an evolutionary conserved DNA-binding domain, named ETS domain, at the C-terminal part of the protein (Seth and Watson 2005, Hollenhorst *et al.* 2011). The human ETS factors are classified into 12 subgroups based on the ETS domain sequence homology (Seth and Watson 2005, Watson *et al.* 2010, Hollenhorst *et al.* 2011). The ETS domain is a sequence of 85 amino acids forming a helix-turn-helix DNA-binding structure that recognizes a GGAA/T core consensus sequence (ETS binding site) in regulatory regions of target genes (Wei *et al.* 2010, Hollenhorst *et al.* 2011). A second conserved domain present in a subset of ETS factors is the pointed domain (PNT). This domain, presented in 11 of the 28 ETS members, codifies a 65 to 85 amino acid helix-loop-helix domain important in protein-protein interactions and oligomerization (Seth and Watson 2005). Besides ETS and PNT domains, ETS proteins may also present activation and repression domains and, rarely, B-box and OST domains (Hollenhorst *et al.* 2011).

7.6.2 ETS REARRANGEMENTS DIVERSITY

Although gene fusions between *TMPRSS2* and *ERG* represent the most common subtype of fusions found in PCa, rearrangements involving other ETS family members have been described (Tomlins *et al.* 2009). In fact, *ETV1* fusion products are present in approximately 10% of PCa, and this gene can be rearranged with the 5' untranslated region of several other genes besides *TMPRSS2* (*TMRSS2-ETV1* is present in only 1% of PCa). Thereafter, the screening of microarrays datasets for ETS gene outlier expression led to the identification of fusion genes involving the ETS variant 4 and 5 genes, *ETV4* and *ETV5* (Tomlins *et al.* 2006, Helgeson *et al.* 2008). The ongoing effort to screen PCa patients for gene fusions, in combination with the recent technological advances, has resulted in a comprehensive gene fusion landscape. Several other novel 5′ promoter or other upstream sequences of androgen-inducible genes (*HERV_K22q11.23*, *SLC45A3*,

C15orf21, *HNRPA2B1*, *KLK2*, *CANT1*) have been identified (Tomlins *et al.* 2008) (Table 5). ETS gene fusions in prostate cancer seem mutually exclusive, but in multifocal disease more than one fusion event can be found (Mehra *et al.* 2007, Furusato *et al.* 2008, Paulo *et al.* 2012).

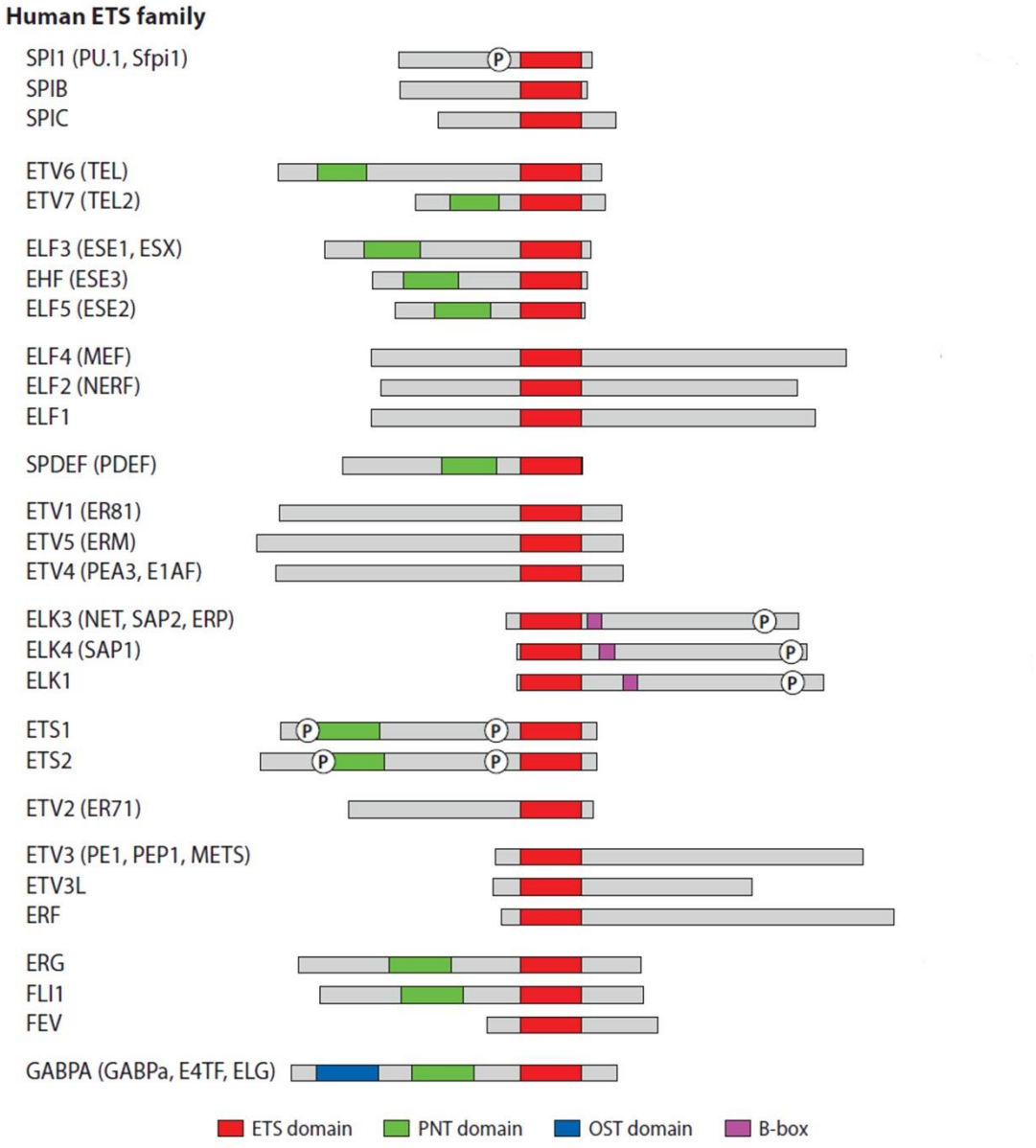


Figure 7. Structural and functional domains of the ETS family of transcription factors. Nomenclature (HUGO) and domain organization of the 28 human ETS proteins. Boxes identify the DNA-binding ETS domain (red), PNT domain (green), OST domain (blue), and B-box (magenta) of the ETS factors. (adapted from Hollenhorst *et al.* 2011).

Table 5. Known ETS genes and 5' partners involved in gene fusions.

Translocation partner	Locus	Androgen response	Structure of encoded protein	Gene function
ERG				
TMPRSS2	21q22	Up-regulated	NTT; Fusion (5aa); WT	Transmembrane serine protease
SLC45A3	1q32	Up-regulated	NS	Solute carrier protein
HERPUD1	16q13	Up-regulated	Fusion (49aa)	Multipass membrane protein – Ubiquitin-dependent degradation
NDRG1	8q24.3	Up-regulated		Alfa/beta hydrolase
ETV1				
TMPRSS2	21q22	Up-regulated	NTT; Fusion (5aa)	Transmembrane serine protease
SLC45A3	1q32	Up-regulated	NTT	Solute carrier protein
ACSL3	2q36.1	Up-regulated	NTT	Acetyl-CoA synthetase
HERV-K22	22q11.23	Up-regulated	NTT	Endogenous retrovirus
HERV-K17	17q13.1	Up-regulated	NTT	Endogenous retrovirus
FOXP1	3p13	Up-regulated	Fusion (19aa)	Transcriptional repressor
EST14	14q21.1	Up-regulated	NTT; Fusion (5aa)	Non-coding RNA
Chromosome 14	14q13.3-21.1	Up-regulated	WT	NA
C15orf21	15q21.1	Down-regulated	NTT	Unidentified open reading frame
HNRPA2B1	7p15	No effect	Fusion (2aa)	Ribonuclear protein – pre-mRNA processing
ETV4				
TMPRSS2	21q22	Up-regulated	NTT; Fusion (5aa)	Transmembrane serine protease
KLK2	19q13.33	Up-regulated	NTT	Kallikrein peptidase - serine protease
CANT1	17q25.3	Up-regulated	NTT	calcium activated nucleotidase
DDX5	17q24.1	No effect	Fusion (102aa)	RNA helicase
ETV5				
TMPRSS2	21q22	Up-regulated	Fusion (84aa); WT	Transmembrane serine protease
SLC45A3	1q32	Up-regulated	NTT	Solute carrier protein

NTT, N-terminal truncated; WT, wild type; NS, not specified; NA, not applicable

Besides the diversity of fusion partners described, an additional level of complexity is introduced by both rearrangements mechanism and the multiple fusion-transcript isoforms. Both *TMPRSS2* and *ERG* are located less than 3 megabases (Mb) apart on chromosome 21, and the gene fusion *TMPRSS2-ERG* can occur either through interchromosomal insertion (Teixeira 2008) or through deletion of the intervening region on chromosome 21. Moreover, the most common *TMPRSS2-ERG* fusion transcript isoform involves the exon 1 of *TMPRSS2* and exon 4 of *ERG*, although more than 20 *TMPRSS2-ERG* different transcripts have been described. The majority of *TMPRSS2-ERG* rearrangements linked exon 1 of *TMPRSS2* with different exons of *ERG*, resulting in overexpression of N-terminal truncated (NTT) ERG proteins (Wang *et al.* 2006, Clark *et al.* 2007, Liu *et al.* 2007, Hu *et al.* 2008). This is also observed for other ETS transcription factors involved in rearrangements, in which the majority of the 5' partners contribute to the fusion transcripts with untranslated exons. Whereas *ETV1* and *ETV4* have frequently lost part of the 5' translated region, *ETV5* has its wildtype (WT) protein overexpressed (Tomlins *et al.* 2007).

7.6.3 Prognostic value of ETS rearrangements

Owing to the high frequency of PCa harboring *ETS* rearrangements, several studies have investigated the effect of *TMPRSS2-ERG* rearrangement on prognosis of PCa patients. However, the conclusions are contradictory, *ERG* rearrangements have been reported as associated with both more aggressive and more indolent disease, due to several variables such as heterogeneity of study cohorts and management, the impact of sampling, multifocality and intraprostate molecular heterogeneity, and the variability of measured outcomes (Clark and Cooper 2009). Population-based studies focused on non-PSA screened populations with PCa diagnosed by transurethral resection of the prostate (TURP) and conservatively management (watchful waiting) have shown a significant association between ETS fusions and poor prognosis (Demichelis *et al.* 2007, Attard *et al.* 2010), while retrospective radical prostatectomy series studies have produced inconsistent results regarding aggressiveness and prognosis of ETS fusion-positive PCa (Clark and Cooper 2009). With the lower frequency of *ETV1* rearrangements, it is more difficult to evaluate an association of *ETV1* rearrangement and clinicopathological variables. Attard and collaborators have analyzed 22 PCa samples harboring *ETV1* rearrangement and did not show an association with survival (Attard *et al.* 2008). More recently, Baena and colaborators have associated ETV1-regulated pathways with higher

Gleason score and metastasis (Baena *et al.* 2013), supporting previous reports (Shin *et al.* 2009).

7.6.4 ETS TARGET GENES

Cancer results from a multi-step sequence of genetic and epigenetic changes that lead to crucial alterations in cell physiology, such as loss of growth controls and normal apoptotic response, as well as sustained angiogenesis, invasion, and metastasis (Hanahan and Weinberg 2011). The ETS transcription factors are able to act as positive or negative regulators of gene expression of crucial elements that are involved in those important biological pathways. Consequently, ETS have been associated to cellular proliferation, differentiation, apoptosis, tissue remodeling, angiogenesis, metastasis and transformation. In the last years, several studies have made significant efforts in identification of ETS target genes. Among ETS target genes validated so far are genes associated with cell proliferation control (cyclins and cdks), motility (HGF), invasion (uPA, PAI, MMPs, TIMPs), extravasation (MMPS, integrins), micro-metastasis (Osteopontin; BSP and Osteonectin), and establishment and maintenance of distant site metastasis and angiogenesis (integrin b3, VEGF, Flt-1/KDR, Tie2) (Sementchenko and Watson 2000, Hsu *et al.* 2004). As stated above, a significant proportion of PCa carry ETS rearrangements leading to overexpression of the ETS transcription factor involved. *In vitro* studies have revealed that *ERG* activates plasminogen and Wnt pathways to promote degradation of the extracellular matrix and decrease cell adhesion, but very few genes have been validated as direct *ERG* targets (Tomlins *et al.* 2008, Gupta *et al.* 2010, Mohamed *et al.* 2011). Regarding *ETV1* rearrangements, there are some *in vitro* and *in vivo* models linking overexpression of *ETV1* with the invasion potential of cancer cells by activation of matrix metalloproteinase *MMP1* and *ITGB3* integrin (Cai *et al.* 2007, Tomlins *et al.* 2007, Hermans *et al.* 2008).

Therapeutic targeting of ETS and other transcription factors has been challenging due to their nuclear localization and molecular embedding in DNA–protein and protein–protein complexes (Konstantinopoulos and Papavassiliou 2011, Findlay *et al.* 2013). Consequently, it has been of utmost importance to identify and to characterize the downstream molecular targets of ETS rearrangements, as potentially some of them could be more amenable to targeted therapy.

In our previous work, and using a genome-wide scale and exon-level expression microarray platform on a clinical series of PCa enriched for *ERG* and *ETV1* rearrangements, we have shown that *ERG* and *ETV1* regulate both specific and shared target genes in PCa (Paulo *et al.* 2012). Our group reported a list of 57 *ERG*-associated genes in primary PCa, which 8 were also deregulated in VCaP cells with the *TMPRSS2-ERG* fusion (*SH3RF1, TMBIM1, PLA1A, CACNA1D, ATP8A2, HLA-DMB, PDE3B,* and *TDRD1*). Of the 15 genes highly associated with tumors harboring *ETV1* rearrangements, only 2 genes were shown to have the expected overexpression in the LNCaP cell line harboring an *ETV1* rearrangement (*FKBP10* and *GLYATL2*). We also reported a list of 27 target genes shared by *ERG* and *ETV1* rearrangements. Our results, using the VCaP and LNCaP knockdown cell line models, clearly validate *KCNH8*, *TMEM45B* and *GRPR* as downstream targets of both *ERG* and *ETV1*, as also indicated by our demonstration of direct binding of ERG to the promoter of these genes using ERG-immunoprecipitated chromatin from VCaP cells (Paulo *et al.* 2012). *KCNH8* have been previously associated with tumors harboring ERG rearrangements (Glinsky *et al.* 2004, Jhavar *et al.* 2008, Jhavar *et al.* 2009). *TMEM45B* encodes a putative membrane protein with unknown function, so its role in prostate carcinogenesis might be worth exploring. Finally, *GRPR* encodes the gastrin-releasing peptide receptor and has been described as overexpressed in several cancer types, including PCa (Cornelio *et al.* 2007, Beer *et al.* 2012). Considering the overexpression of *GRPR* in a high proportion of PCa that harbor either *ERG* or *ETV1* rearrangements, the cellular membrane localization of its protein product, the relevance of its function, the availability of blocking agents, and the several previous reports on its oncogenic activity, makes it a promising therapeutic target for these particular molecular subtypes of PCa.

8. GASTRIN-RELEASING PROTEIN RECEPTOR

The Gastrin-releasing protein receptor (GRPR) is a member of the G-protein coupled receptor superfamily (GPCR). GPCR superfamily is the largest family of cell-surface molecules involved in signal transmission that regulate many cell functions, including cell proliferation, survival and motility, and have lately arisen as crucial players in tumor growth, angiogenesis and metastasis. The aberrant overexpression of GPCR members and their activation by specific agonists represents the most frequent form used by cancer cells to stimulate GPCRs signaling networks (Dorsam and Gutkind 2007).

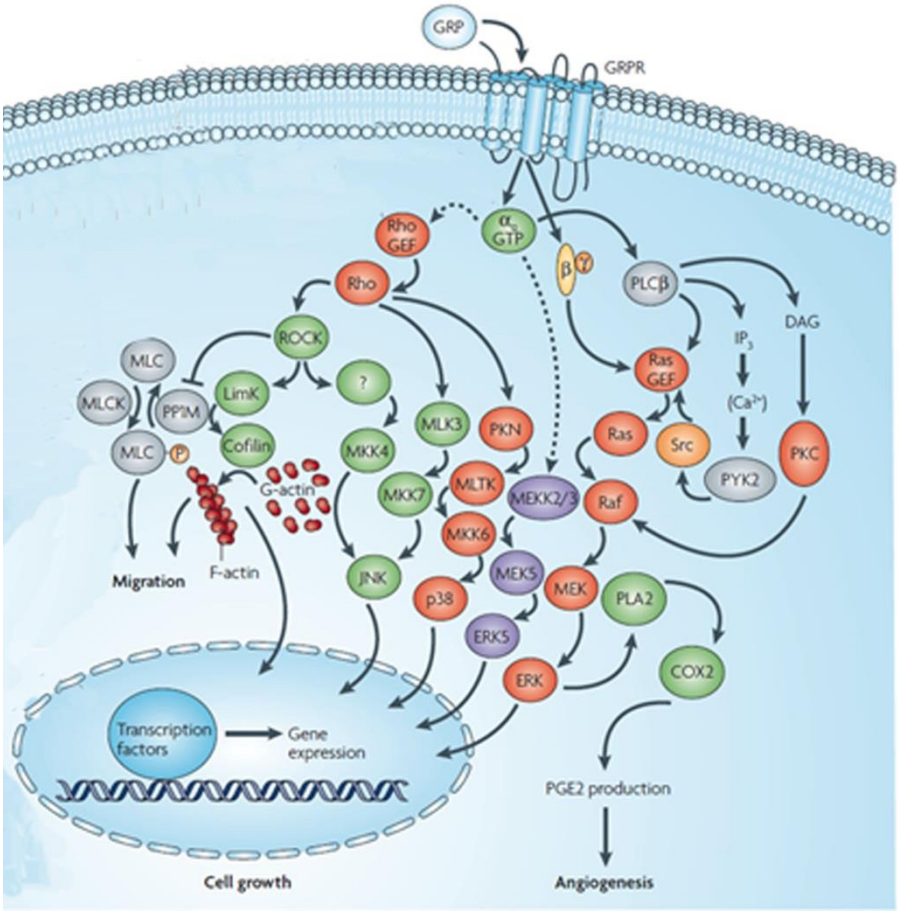


Figure 8. Some oncogenic signaling pathways associated to gastrin-releasing peptide receptor activation (adapted from (Dorsam and Gutkind 2007).

The expression of GRPR in normal tissues is restricted to gastric, respiratory, endocrine, muscle and nervous systems (Xiao *et al.* 2001). The activation of this receptor is mediated by its specific ligand GRP (gastrin-releasing peptide). GRP belongs to the regulatory peptide family, involved in several modulatory roles in distinct regions of the human body, including brain, gastrointestinal tract, vascular and endocrine systems. Several signaling mechanisms are activated through the binding of GRPR, including phospholipases (A_2, B_1, B_3 and D), cAMP, PKC, cyclooxygenase, and protein kinase cascades (among them the Raf/MEK/ERK kinase cascade) (Figure 8) (Hohla and Schally 2010). Generally, regulatory peptides and their receptors are present at low concentrations in physiologically organs, although the receptors are often highly expressed in human cancers. In fact, *GRPR* is known to be overexpressed in several human malignancies, including neuroblastoma, lung, breast, pancreatic, colorectal, gastric, esophageal, and prostatic cancer (Cornelio *et al.* 2007). *GRPR* activation by GRP ligation lead to the regulation of many functions of the gastrointestinal and central nervous systems, including the release of gastrointestinal hormones, contraction of smooth musculature in the gastrointestinal and urogenital tract, hormone release from the pancreas, stomach and other endocrine organs, and proliferation of epithelial cells (Mansi *et al.* 2013).

GRPR overexpression in prostate tumors has been identified in radical prostatectomy at both mRNA and protein level (Bartholdi *et al.* 1998, Markwalder and Reubi 1999, Sun *et al.* 2000, Weber 2009). Markwalder and Reubi detected that primary prostatic carcinomas express *GRPR* at much higher levels than non-neoplastic prostate glands, and this protein was also detected at higher levels in high-grade prostatic intraepithelial neoplasia (HGPIN), usually considered a tumor precursor lesion (Markwalder and Reubi 1999). The discovery of *GRPR* overexpression in several cancer cells led to approaches to inhibit the autocrine growth effect of GRP peptides on tumor growth, including receptor antagonists, monoclonal antibodies (mAbs) against GRP, antisense oligonucleotides (ASOs), and bispecific molecules (BsMol). Additionally, GRP analogues for imaging were also developed (Hohla and Schally 2010).

The three main GRPR antagonists' options are the nonradioactive bombesin analogues for long-term antiproliferation treatment, the radioactive bombesin analogues for targeted radiotherapy, and the cytotoxic bombesin analogues for targeted cytotoxic therapy (Mansi *et al.* 2013). Concerning GRPR nonradioactive analogues, a large number of compounds have been synthesized and several studies have reported the antiproliferative effect of GRPR antagonists *in vitro* and *in vivo* in distinct tumor models (Schally *et al.* 2001, Hohla and Schally 2010). Although the mechanisms involved in the

tumor growth inhibition by GRPR antagonists are not completely understood, it is already known that it includes reduction of epidermal growth factor receptor levels (Bajo *et al.* 2002), inhibition of neovascularization, and the decrease of oncogene expression (Schally *et al.* 2001, Jensen *et al.* 2008). Among GRPR antagonists, the compound RC-3095 [D-Tpi^{6},Leu^{13}Ψ(CH_2NH) Leu^{14}]bombesin(6-14) showed strong inhibitory effect on several experimental cancers *in vitro* and *in vivo* (Hohla and Schally 2010). In fact, several reports have described the effect of selective GRPR antagonists on inhibition of tumor growth in numerous models, including prostate cancer cell lines (PC-3, DU-145, MDA-PCa-2b) (Stangelberger *et al.* 2005a, Stangelberger *et al.* 2005b, Stangelberger *et al.* 2005c), although the associated mechanisms are not yet fully understood. The tumor-inhibitory mechanism of GRP antagonists appears to be more complex than a simple competitive action on the receptor, although the main mechanism of tumor inhibitory action of RC-3095 appears to involve the reduction in levels of key members of oncogenic signaling (Mansi *et al.* 2013). Considering the impressive preclinical antitumor activity of RC-3095, a phase I clinical trial was conducted in which RC-3095 was administered to 25 patients with different advanced solid malignancies, including six with PCa. According to this initial study, no side effects were observed, but the tumor-reducing effects were not convincing. However, maximal doses could not be reached by the methods used, despite dose escalation (Schwartsmann *et al.* 2006). Our recent study concerning the connection between *GRPR* overexpression and *ERG* and *ETV1* rearrangements (Paulo *et al.* 2012) may help understand how the expression of this molecule is regulated and the potential use of *GRPR* as a therapeutic target for this particular subset of prostate carcinomas harboring ETS rearrangements.

Chapter 2

AIMS

The aims of this thesis were:

1. To perform technical and biological validation of the differential RNA expression of the gastrin-releasing peptide receptor (*GRPR*) between non-malignant prostate samples and prostate carcinomas with and without the *ETS* rearrangements;

2. To study the phenotypic impact of *in vitro* silencing of *GRPR* using prostate cancer cell lines that show the same pattern of RNA and protein expression as those identified in primary tumors;

3. To characterize the downstream pathway effects of *GRPR* silencing and to identify the most promising therapeutic targets;

4. To evaluate the potential of an anti-oncogenic therapy targeting *GRPR* and its downstream targets using prostate cancer cell line models with ETS rearrangements.

Chapter 3

ORIGINAL PUBLICATION #1

Uncovering potential downstream targets of oncogenic GRPR overexpression in prostate carcinomas harboring ETS rearrangements

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ABSTRACT

Gastrin-releasing peptide receptor (GRPR) is known to be overexpressed in several human malignancies, including prostate cancer, and has been implicated in multiple important neoplastic signaling pathways. We recently have shown that GRPR is an *ERG* and *ETV1* target gene in prostate cancer, using a genome-wide scale and exon-level expression microarray platform. Due to its cellular localization, the relevance of its function and the availability of blocking agents, GRPR seems to be a promising candidate as therapeutic target. Our present work shows that effective knockdown of GRPR in LNCaP and VCaP cells attenuates their malignant phenotype by decreasing proliferation, invasion and anchorage-independent growth, while increasing apoptosis. Using an antibody microarray we were able to validate known and identify new targets of GRPR pathway, namely AKT1, PKCε, TYK2 and MST1. Finally, we show that overexpression of these GRPR targets is restricted to prostate carcinomas harboring *ERG* and/or *ETV1* rearrangements, establishing their potential as therapeutic targets for these particular molecular subsets of the disease.

INTRODUCTION

Prostate carcinoma (PCa) is the most incident neoplasia in men and the second leading cancer-related cause of death [1]. PCa is a heterogeneous disease and current therapeutic strategies are dependent on TNM staging, Gleason scoring, PSA levels and overall health status. Primary treatment consists mainly of radical prostatectomy and/or radiation therapy, which may be supplemented with androgen ablation [2]. Although many patients are identified with locally, surgically curable, disease, there is a subset of patients that progress or show metastatic prostate cancer, where the gold standard therapy is androgen ablation. Moreover, recurrence is frequent, and many patients develop metastatic disease, for which chemotherapy is only moderately effective [3]. Thus, novel therapeutic approaches to metastatic prostate cancer are needed.

A better understanding of the genetics and molecular pathways involved in prostate carcinogenesis should contribute to the current challenge of identifying promising molecular targets involved in PCa progression. Genomic rearrangements involving members of the ETS family of transcription factors are recurrently found in PCa, with *ERG* and *ETV1* being reported in 50% and 10% of the cases, respectively [4, 5]. ETS members have generally been associated with the regulation of cell growth, proliferation, differentiation, and apoptosis,

through activation or repression of target genes [6]. Therapeutic targeting of ETS and other transcription factors has been challenging due to their nuclear localization and molecular embedding in DNA–protein and protein–protein complexes [7, 8]. Therefore, it is important to characterize the downstream molecular targets of these aberrant transcription factors, as some of them may be more amenable to targeted therapy. Using a genome-wide scale and exon-level expression microarray platform, we have shown that *ERG* and *ETV1* regulate both specific and shared target genes in PCa [9]. The most overexpressed gene of our list of shared *ERG* and *ETV1* targets was *GRPR*, which encodes for a membrane-bound gastrin-releasing peptide receptor. GRPR, a member of the G-protein coupled receptor superfamily, is expressed in gastric, respiratory, endocrine, muscle and nervous systems [10]. Both GRPR and its specific ligand GRP (gastrin-releasing peptide), are known to be overexpressed in several human malignancies, including neuroblastoma, lung, breast, pancreatic, colorectal, gastric, esophageal and prostatic cancer [11]. In the prostate, GRPR expression was also detected at high levels in the tumor precursor lesion high-grade prostatic intraepithelial neoplasia (HGPIN) [12].

The discovery of GRPR overexpression in cancer cells led to the test of specific GRP analogues for imaging or targeted therapy [13]. In fact, several reports have described the effect of selective GRPR antagonists on inhibition of tumor growth in numerous models, including prostate cancer cell lines (PC-3, DU-145, MDA-PCa-2b) [14-16], although the associated mechanisms are not yet completely understood. In this context, further knowledge of GRPR biology is of major importance. The link we observed between GRPR overexpression and *ERG* and *ETV1* rearrangements may help understand how the expression of this protein is regulated and, especially, clarify the potential use of GRPR as a therapeutic target for the entire subset of PCa harboring ETS rearrangements. In this study, we aimed to characterize the oncogenic role of GRPR in prostate cancer in an ETS context and to identify specific players involved in the GRPR pathway with potential to be used as therapeutic targets for this particular subset of prostate cancers.

RESULTS

GRPR is overexpressed in prostate tumors and cell lines harboring *ERG* and *ETV1* rearrangements

To validate previous findings showing GRPR overexpression in tumors harboring ETS rearrangements [9], the mRNA expression of *GRPR* was evaluated in a partially-independent series of 160 PCa and 15 morphologically normal prostate tissues (NPT) by real time RT-PCR. We confirmed a statistically significant *GRPR* overexpression in both *ERG* and *ETV1* rearrangement-positive PCa comparing with NPT samples ($p<0.001$) and ETS-negative PCa ($p<0.001$) (Fig.1A). Expression of *GRPR*, both at mRNA and protein levels, was detected in *ERG* and *ETV1* rearrangement-positive prostate cancer cell lines VCaP and LNCaP, respectively (Fig. 1B). For each cell line, two independent silenced populations (shGRPR#1 and shGRPR#2) and a non-targeting control (Scramble) were established. As observed by both real-time RT-PCR and western blot, successful silencing was achieved in both cell lines, allowing a decrease in *GRPR* expression of 60-70% in LNCaP cells and of about 50% in VCaP cells (Fig. 1C).

Stable knockdown of *GRPR* expression impairs proliferation and promotes apoptosis

To evaluate the impact of *GRPR* silencing in the acquisition of early-stage characteristics of prostate cancer cells in the context of *ERG* and *ETV1* rearrangements, proliferation and apoptosis were assessed. *GRPR* silenced cell populations (shGRPR) of both cell line models displayed significantly reduced cell viability (Fig. 2A) and increased apoptosis (Fig. 2B), comparing to the corresponding scramble controls. In fact, at 96h in culture, *GRPR* silencing led to a 30% decrease ($p<0.05$) in the number of viable cells in both cell lines, and to a 2 and 1.5-fold increase ($p<0.05$) in apoptosis levels, in LNCaP and VCaP cells, respectively.

GRPR is involved in the activation of invasion and anchorage independent properties *in vitro*

To evaluate whether GRPR could be involved in the phenotypic characteristics of advanced prostate cancer cells, we evaluated the impact of *GRPR* silencing in invasion potential and in the capacity to grow without attachment. Using the *in vitro* Matrigel invasion assay, and comparing to scrambled cells, shGRPR cell populations from both cell lines showed a significant reduction of their invasion ability (around 50% decrease, $p<0.05$) (Fig. 3A). Similarly, looking at the capacity of cells to grow without attachment, we found that cell populations with stable *GRPR* silencing developed about 50% fewer colonies than scrambled controls ($p<0.05$) (Fig. 3B).

Identification of potential *in vitro* GRPR downstream targets by antibody microarray

To discover potential downstream targets of GRPR, we used KAM-850, an antibody microarray that features 850 specific antibodies (Supplementary Table 1). This platform was used to compare the differential protein

expression pattern between scrambled and shGRPR populations in both LNCaP and VCaP cell line models. In order to find potential oncogenes regulated by GRPR that could be interesting for targeted therapy of PCa with ETS rearrangements, we focused on down-regulated targets shared by both cell lines (Fig. 4A, Supplementary Table 2). Through this analysis we found a list of nine proteins with decreased expression levels in both cell lines and, based on their cell pathways association, we focused our attention in five of them (Fig. 4B): PLK2, TYK2, MST1, *p*-AKT1 (Ser473) and *p*-PKCε (Ser729). We also included in the remaining analysis pan-AKT1 and pan-PKCε in order to detected total AKT and PKCε protein. To validate these results, western blot analysis was performed using RIPA protein extracts (Fig. 4C). We confirmed that *GRPR* silencing leads to a decreased expression of TYK2, PLK2, MST1 and *p*-AKT1 in both LNCaP and VCaP cells. Total PKCε and *p*-PKCε showed a small decrease in expression only in shGRPR-VCaP cells, and no changes were observed in the expression of total AKT1 in both cell line models.

In vivo validation of *in vitro* GRPR downstream targets

To evaluate whether the *in vitro* association between the expression of GRPR and of these potential targets under an ETS-rearrangement context would be observed *in vivo*, protein extracts of six NPT and 18 PCa (six of each ETS subgroup), randomly selected, were analyzed by western blot (Fig. 4D). This approach showed that, overall, the expression of AKT1 was higher in PCa samples when compared with NPT. Interestingly, tumors with *ETV1* rearrangement showed consistently higher expression of TYK2, MST1 and *p*-AKT1, when compared with both NPT and other PCa subgroups, in which the expression pattern of those proteins showed to be highly heterogeneous. Regarding PKCε and *p*-PKCε expression, both *ETV1* and *ERG* rearrangement-positive PCa samples showed consistently higher expression when compared with NPT, although high protein levels were also detected in some ETS-negative PCa. We were unable to detect PLK2 expression in prostate tissues using two different

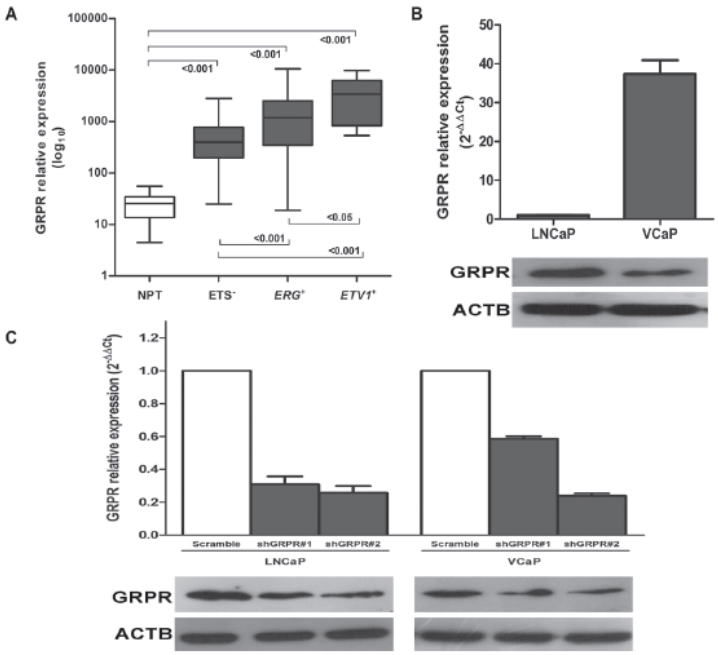

Figure 1: GRPR expression in prostate carcinomas and cell line models of *ERG* and *ETV1* rearrangements. (A) Validation of *GRPR* overexpression in a partially-independent series of 160 prostatectomy tumors, including 79 samples with *ERG* rearrangement, 16 samples with *ETV1* rearrangement, and 65 samples without known ETS rearrangements, and 15 morphologically normal prostate tissues (NPT) by Real Time RT-PCR. ETV1$^+$ and ERG$^+$ represent PCa with rearrangements involving *ETV1* and *ERG*, respectively, and ETS$^-$ represents PCa negative for known ETS rearrangements. *p*-values of two-group comparisons (MW) are shown. (B) Real Time RT-PCR (top) and immunoblotting (bottom) of *GRPR* expression in the cell line models of *ETV1* and *ERG* rearrangements, LNCaP and VCaP, respectively. (C) Real Time RT-PCR (top) and immunoblotting (bottom) of *GRPR* expression after stable silencing in LNCaP and VCaP cell lines. For each cell line, a negative control (scramble) and two independently silenced cell populations (shGRPR#1 and shGRPR#2) were established.

primary antibodies. Considering the stronger association between the expression of some of the *in vitro* identified GRPR candidate target genes and the presence of *ETV1* rearrangements in PCa samples, we thought to investigate that association using our previously established model of *ETV1* silencing in LNCaP cells. We observed that *ETV1* silencing leads to a decrease in GRPR protein levels (as expected [9]), but also to a decrease in the expression of TYK2, MST1, *p*-AKT1 and *p*-PKCε (Fig. 4C). In agreement with the data observed for cell populations with GRPR silencing, immunoblotting of *ETV1* silenced LNCaP cells did not show differences in AKT1 and PKCε expression. Contrarily, higher PLK2 expression was observed in shETV1-LNCaP cells.

DISCUSSION

Following our previous work that provided the identification of potential target genes regulated by both

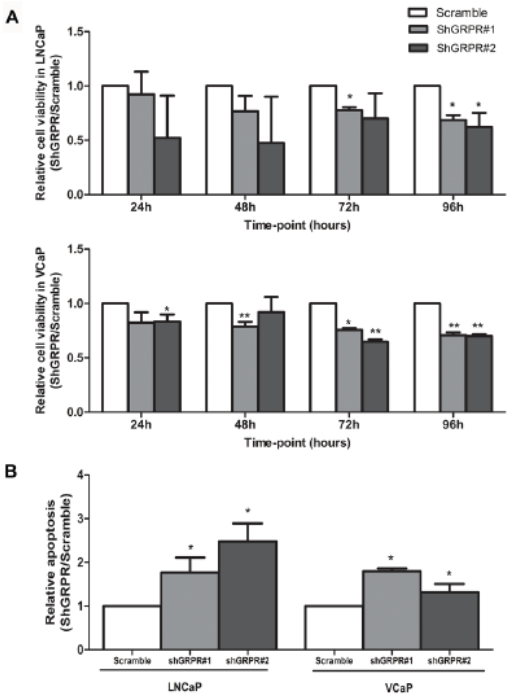

Figure 2: Impact of *GRPR* silencing in LNCaP and VCaP cell lines in cell viability and apoptosis. (A) Quantitative analysis of metabolically active cells by the MTT assay, at four time-points. (B) Quantification analysis of apoptotic levels at 96h in culture. For both assays, results are shown for each silenced cell population relative to the scramble cells, from three independent experiments. Statistically significant *p* values are showed by an asterisk (*$p<0.05$; **$p<0.01$).

ERG and *ETV1* transcription factors in PCa, we focused our attention in *GRPR*, the top-most differentially expressed gene of a list of 27 ETS candidate targets [9]. Due to its cellular localization, the relevance of its function and the availability of blocking agents, GRPR seems to be a promising candidate for targeted therapy. Several emergent studies point to the potential of GRPR as a therapeutic target, supporting its role as an important player of signaling pathways in cancer cells, namely cell proliferation, metastasis and angiogenesis [17]. After validating a higher expression of *GRPR* in PCa samples harboring either *ERG* or *ETV1* rearrangements, we decided to evaluate the phenotypic impact of this receptor *in vitro* by knocking down its expression in either *ERG*- or *ETV1*-rearranged prostate cell lines (VCaP and LNCaP, respectively). Upon successful and stable *GRPR* silencing in both cell lines, we observed a decline of malignant cells' phenotype through reduction of cell proliferation, invasion and ability to growth in the absence of cell attachment, and by an increment of apoptosis. Although GRPR and its specific peptide have been associated with an oncogenic role in different tissues and models, the present work is the first report ascertaining the malignant impact of this receptor in prostate carcinogenesis. The observed phenotypic effects and the lack of proved efficacy of GRPR antagonists as therapeutic approaches [18], prompted us to look for potential GRPR target proteins using an antibody microarray, focusing on relevant cellular pathways frequently deregulated in tumorigenesis. Multiple molecular pathways are involved in the proliferation and survival of prostate cancer cells during tumor progression. Among these survival-signaling pathways, up-regulation of the PI3K/Akt pathway is particularly important, considering its role in survival enhancement and apoptosis inhibition [19]. Other authors reported that GRP can induce Akt phosphorylation at Serine 473 in a non-small cell lung carcinoma cell line, and that this activation occurred through transactivation of the epidermal growth factor receptor (EGFR), a known Akt activator [20]. In this work, we observed a significant increase in apoptosis levels and a reduction of cell viability after GRPR knockdown, eventually as a result of disturbing PI3K/Akt pathway via down-regulation of *p*-AKT1 (Ser473). Considering the increased levels of *p*-AKT1 (Ser473) observed in tumors harboring ETS rearrangements, these observations support the hypothesis that ETS overexpression up-regulates the expression of GRPR and subsequently leads to up-regulation of *p*-AKT1 (Ser473), placing ETS transcription factors as upstream regulators of GRPR overexpression in PCa. Interestingly, PKCε, a protein kinase described to be overexpressed in most solid tumors (including those of the prostate) and to have crucial roles in several aspects of tumor development, namely cell transformation, proliferation, cancer cell survival, EMT, migration and invasion [21, 22], was also found overexpressed in our ETS-positive tumors. In our

cell line models, PKCε seemed to be more dependent of the ETS context than of the GRPR overexpression, as no significant effect was observed on PKCε/*p*-PKCε expression upon silencing of *GRPR* in both cell line models (VCaP and LNCaP) but a significant decrease of *p*-PKCε was observed in LNCaP cells upon silencing of *ETV1*. In fact, *p*-PKCε was identified as the active kinase that phosphorylates AKT1 at serine 473 leading to full AKT activation [23]. We therefore suggest a link between ETS overexpression and increased PKCε/*p*-PKCε expression, as a GRPR alternative mediator of *p*-AKT1 (Ser473) activation. These findings are in agreement with studies proposing that high levels of ETS protein collaborate with constitutively activated AKT kinase, leading to the development of more aggressive PCa [24].

Additionally, our work revealed that GRPR plays an important role in anchorage-independent growth and invasion in the human prostate cancer cell lines tested, as GRPR silencing led to a significantly decrease in the invasive capacity of both LNCaP and VCaP cell lines. This effect could be the result of down-regulation of TYK2 and MST1 expression, as observed by immunoblotting of GRPR silenced populations from both cell lines. In fact, overexpression of TYK2 (a member of the Janus family of non-receptor tyrosine kinases, JAKs) has been described in several malignancies, such as PCa and squamous cervical carcinomas, as well as in breast cancer cell lines [25], with some studies showing its involvement in enhancing prostate cancer invasion [26, 27]. Similarly, the signaling initiated by the binding of MST1 to its receptor (MST1R) is an important pathway for invasive growth in different neoplasias [28]. However, we were only able to detect strong expression of both TYK2 and MST1 proteins in *ETV1*-positive PCa, and silencing of *ETV1* in LNCaP cells (which also leads to a drastic impair of both cell invasion and anchorage-independent growth [29]) only showed a significant effect in the expression of TYK2, but not in MST1. These observations may indicate that both GRPR and ETV1 may regulate the expression of TYK2 and MST1, which potentially act cumulatively when overexpression of both is present. Taken together, these data support the hypothesis that targeting TYK2 and/or MST1 with specific inhibitors could be a useful approach in the blockage of prostate cancer progression in *ETV1*-positive PCa.

A decrease in PLK2 expression was also observed in our *GRPR* silenced cell populations (with higher impact in LNCaP cells), however the opposite effect was observed in response to *ETV1* silencing in the LNCaP cell line. This suggests that PLK2 expression levels would be the result of a balance between the two factors, with ETV1/ETS transcription factors acting as repressors and GRPR as an activator. Nevertheless, no information was obtained from our series of prostate tissues, since PLK2 expression was not detected in any of the samples analyzed using two different antibodies. This observation, however, is in

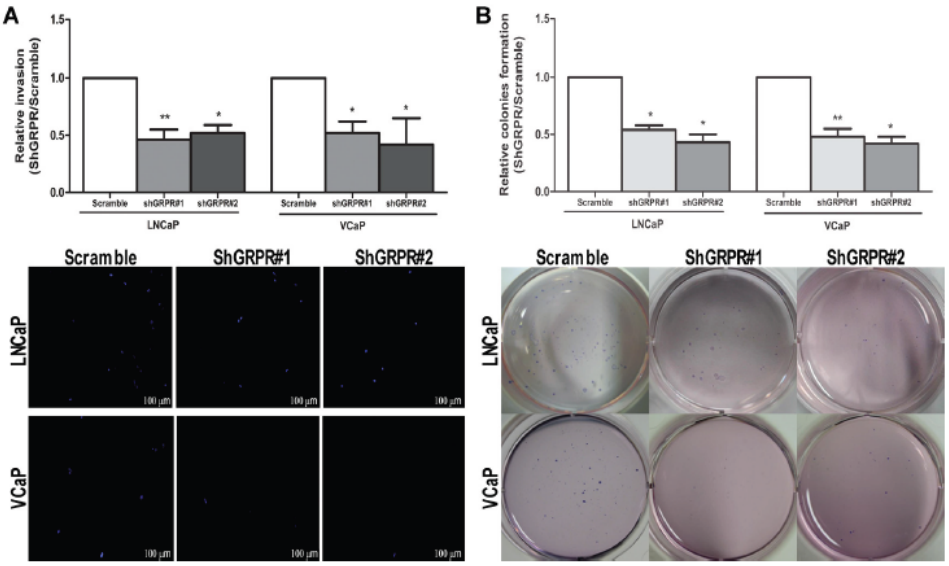

Figure 3: Impact of *GRPR* silencing in LNCaP and VCaP cell lines in invasion and anchorage-independent growth. (A) Quantitative analysis (top) and qualitative visualization (bottom) of cell invasion using Matrigel Invasion Chambers. (B) Quantitative analysis (top) and qualitative visualization (bottom) of anchorage-independent growth by the Soft agar colony formation assay. Results are shown for each silenced cell population relative to the scramble cells from three independent experiments. Statistically significant *p* values are showed by an asterisk (**p*<0.05; ***p*<0.01).

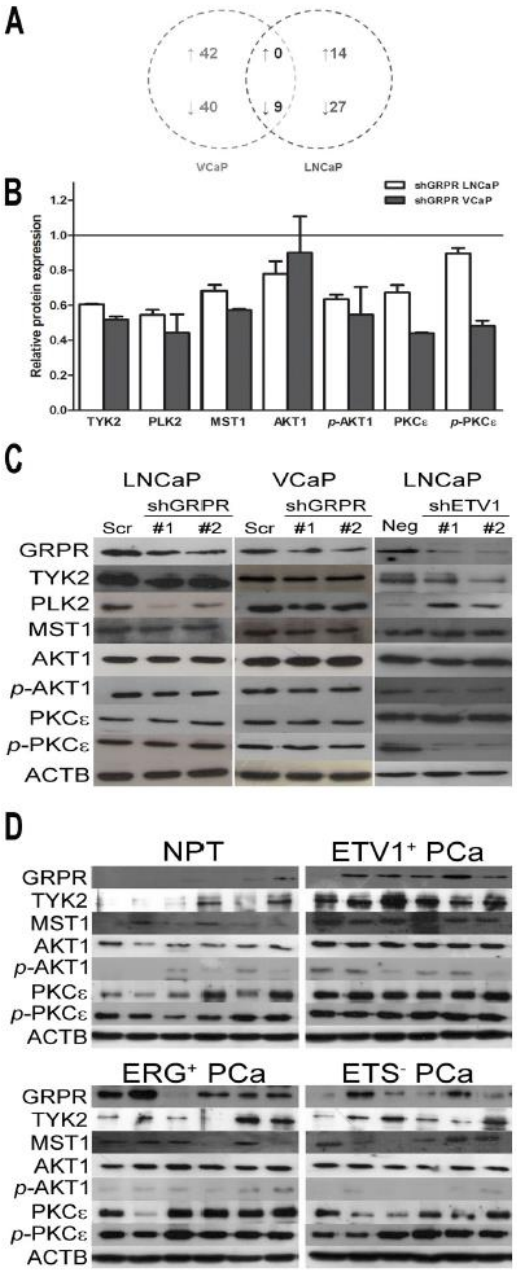

Figure 4: Dissection of potential GRPR downstream targets. (A) Venn-diagram of the number of significantly deregulated proteins (at least 1.2-fold) in GRPR silenced cell populations from LNCaP and VCaP cell lines, showing specific and shared deregulated candidate targets (Supplementary Table 2). (B) Relative protein expression of potential targets of GRPR in LNCaP and VCaP cell lines, revealed by KAM-850 antibody microarray. Globally normalized protein expression was compared between shGRPR and scramble (relative protein expression: 1.0) for each cell line, and shared targets of silenced GRPR cell lines were selected after Z score calculation. (C) Immunoblotting validation of previously selected down-regulated targets of GRPR in LNCaP and VCaP cell line models of GRPR silencing and in the LNCaP model of ETV1 silencing. (Scr – scramble; Neg - negative). (D) Immunoblotting analysis of previously selected down-regulated targets of GRPR in protein extracts of ETS-subtyped prostate tumors. $ETV1^+$ and ERG^+ represent PCa with rearrangements involving *ETV1* and *ERG*, respectively, ETS^- represents PCa negative for known ETS rearrangements and NTP represents morphologically normal prostate tissues.

accordance to the low PLK2 expression levels described for normal and tumorous prostate tissues (Supplementary Figure 1), suggesting that PLK2 expression levels in cell lines may result from adaptation to *in vitro* conditions, and further reflect the relevance of looking into tumor samples to validate *in vitro* associations.

In this study, we report the oncogenic role of GRPR in different biological processes of prostate cancer progression through activation of specific targets involved in cancer-associated signaling pathways (including PI3K/Akt and JAK-STAT). Besides validating GRPR as a potential target gene of both *ERG* and *ETV1* transcription factors, our data reveal the activation of different intermediate players of GRPR/ETS *in vitro*-mediated proliferation/apoptosis and invasion/anchorage-independent growth, associated with disease aggressiveness. Considering what is known concerning the activity of these intermediates and the data shown here, we propose a model for GRPR signaling under an ETS-rearrangement cellular context (Fig. 5), where TYK2, MST1 and *p*-Akt may constitute promising therapeutic targets that should be explored in combination with GRPR inhibitors for treating these particular subtypes of prostate cancer.

MATERIAL AND METHODS

Tissue samples

We used a series of prostate carcinomas previously characterized for ETS rearrangements [30] and selected 160 samples to represent the various molecular subtypes of PCa, including 79 samples with *ERG* rearrangement, 16 samples with *ETV1* rearrangement, and 65 samples without known ETS rearrangements. As control samples, 15 morphologically normal prostate tissues (NPT) were used (collected from peripheral zones of non-cancerous prostate from bladder cancer patients submitted to cystoprostatectomy) [9]. The groups of *ETV1*-positive PCa and NPT samples included the 13 and nine samples, respectively, which we had previously analyzed by expression microarrays [9]. This study was approved by the institutional review board.

Cell lines and reagents

The human prostate cell lines used in this study were LNCaP and VCaP. Both cell lines were maintained

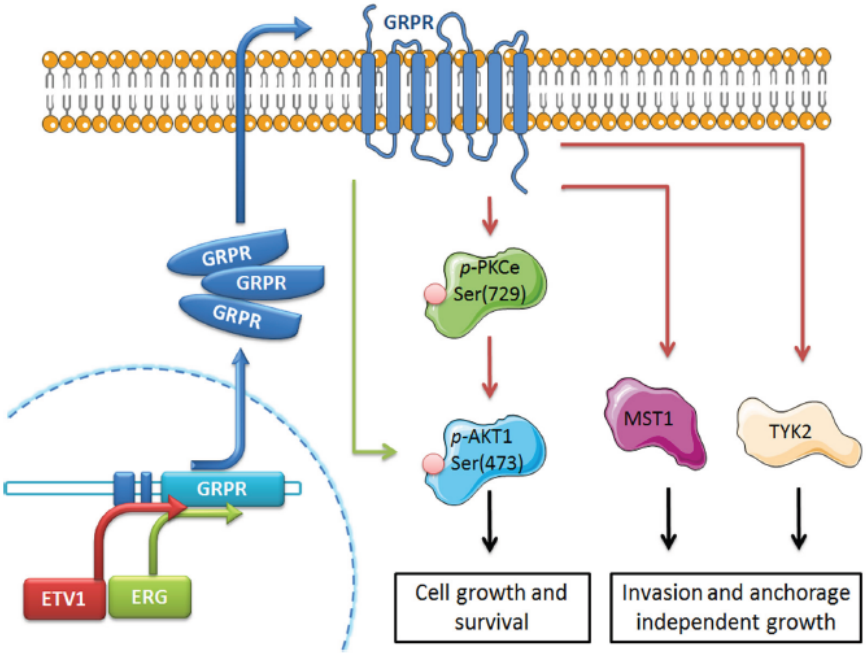

Figure 5: Proposed model for GRPR involvement in the acquisition of oncogenic properties of prostate cancer cells harboring ETS rearrangements. Overexpression of ETV1 or ERG in prostate cancer cells (as those harboring *ETV1* or *ERG* rearrangements) increases the transcription of the GRPR gene and consequently leads to overexpression of the GRPR protein. As a G-protein coupled receptor, overexpressed GRPR leads to an increased expression/activation of targets known to be involved in particular cancer pathways, namely AKT1, PKCε, MST1 and TYK2. These deregulated proteins thus constitute promising therapeutic targets for these particular cancer subsets. Green and red arrows represent activation of targets associated with ERG and ETV1, respectively.

in standard growth medium, supplemented with 10% fetal bovine serum (Gibco by Life Technologies, Carlsbad, CA, USA) and 1% penicillin/streptomycin solution (Gibco) in a humidified chamber (37°C, 5% CO_2). LNCaP cells were acquired from the German Resource Centre for Biological Material (DSMZ, Braunschweig, Germany) and VCaP cells from the European Collection of Cell Cultures (Sigma-Aldrich, St Louis, MO). For validation purposes, both prostate cell lines were karyotyped by G banding and probed for *ERG* and *ETV1* rearrangements by FISH analysis. Cultures were considered *Mycoplasma*-free by routine testing for *Mycoplasma spp.* contamination (PCR Mycoplasma Detection Set; Clontech Laboratories Inc., Mountain View, CA, USA).

RNA isolation, cDNA synthesis and real-time RT-PCR

Total RNA extraction from tissue samples with TRIzol (Invitrogen by Life Technologies) was previously described [30]. For cDNA synthesis, 200 ng of RNA and the TransPlex Whole Transcriptome Amplification Kit (Sigma-Aldrich) were used, following the manufacturer's instructions. For cell lines, total RNA was extracted using the Illustra TriplePrep Kit (GE Healthcare Bio-science Corporation, NJ, USA), and cDNA was obtained from 1µg of RNA using oligo-dT primers and the H-minus RevertAid cDNA synthesis kit (Fermentas, Ontario, Canada), according to the manufacturer's instructions. Real-time RT-PCR was performed using pre-developed TaqMan® Gene Expression assays (Applied Biosystems, Foster City, CA, USA). Amplification reactions were carried out in triplicates on a 7500 Sequence Detection System (Applied Biosystems), with *GUSB* used as a reference gene. Relative expression was obtained using the comparative Ct method [31].

GRPR and *ETV1* stable silencing

The expression of GRPR was stably silenced in the prostate cancer cell lines (LNCaP and VCaP) by specific short-hairpin RNAs (GRPR shRNA; sc-106924-V) and the shRNA Lentiviral Particles Transdution System, both from Santa Cruz Biotechnology Inc. (CA, USA). A negative scrambled shRNA lentiviral particle (sc-108080) was used to generate a biological control. Cells were plated in a 12-well plate to reach 50%-70% confluence on the day of infection. The lentiviral particles were used to infect the prostate cancer cell lines after addition of polybrene (4.0 µg/ml, Sigma-Aldrich). Effectively transfected cells grew under selective pressure by Puromycin dihydrochloride (cat. 631306, Clontech Laboratories Inc.) at 2.5 µg/ml. The LNCaP cell line model with stable *ETV1* silencing (LNCaP-shETV1 and LNCaP-shNeg populations) was previously established [9].

Protein extraction and Western blotting

Protein was extracted from sub-confluent cell lines using RIPA lysis buffer (sc-24948, Santa Cruz Biotechnology Inc.) and from tissue samples using the organic fractions obtained after RNA separation with TRIzol (Invitrogen), according to the manufacturer's instructions. Protein concentration was measured using the bicinchoninic acid (BCA) protein assay kit from Thermo Scientific (Waltham, MA, USA) and 40 µg or 10ug (cell lines or tissues, respectively) of total protein were loaded in 10% (w/v) Bis-Tris–containing polyacrylamide gels under reducing conditions for SDS-PAGE. After proteins transfer to a nitrocellulose membrane (Merck Millipore, Billerica, MA, USA), blots were blocked with 5% fat-free milk (Bio-Rad Laboratories, Hercules, CA, USA) in TBS-T (50 mM Tris, 150 mM NaCl, 0.1% Tween-20, pH 7.4) and incubated with primary antibodies at 4°C overnight. Blots were then incubated with a horseradish peroxidase–conjugated secondary antibody for 1h at room temperature, and developed with the enhanced chemiluminescence Western blotting detection system Immun-Star™ WesternC™ Kit (Bio-Rad Laboratories), according to the manufacturer's indications. The primary antibodies used were: rabbit anti-GRPR (ab39883, 1:500, Abcam, Cambridge, UK); rabbit anti-phospho-AKT (Ser473) (bs-0876R, 1:10000, Bioss Inc., MA, USA); rabbit anti-phospho-PKCε (Ser729) (06-821-I, 1:2000, Merck Millipore); and rabbit anti-PKCε (sc-214, 1:2000), goat anti-MST1 (N-19) (sc-6213, 1:100), goat anti-PLK2 (C-18) (sc-9577, 1:100), rabbit anti-TYK2 (sc-169, 1:2000) and mouse anti-AKT1 (B-1) (sc-5298, 1:10000), all purchased from Santa Cruz Biotechnology Inc. A mouse anti-β-actin monoclonal antibody (A1978, 1:8000, Sigma-Aldrich) was used as loading control.

Cell proliferation assay

The MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay was used for cell viability measurement. LNCaP (1.0×10^4) and VCaP cells (2.5×10^4) were seeded in 96-well plates (Sarstedt AG & Co, Nümbrecht, Germany) in 200 µL of complete growth medium and incubated in a humidified chamber (37°C and 5% CO_2). Cells were allowed to adhere and then viability assay was performed at different time-points (24h – 96h). At each time-point, growth medium was replaced by medium containing MTT at 1.0 mg/mL (Sigma-Aldrich) and cells incubated for 1 hour in a humidified chamber. MTT-containing medium was removed and formazan crystals were dissolved using DMSO (Sigma-Aldrich). Finally, plates were shaken for 15 minutes for complete homogenization and absorbance levels were measured at 540 nm with background correction at 630 nm using a microplate reader (Fluostar Omega, BMG Labtech,

Offenburg, Germany). For each time-point, an average value of measurements from nine replicate wells was obtained. Cell viability was estimated by correcting and normalizing the average absorbance values obtained in each time-point (Tn) to the average absorbance values of the time zero (T0) by the following formula: (Tn-T0)/T0. Relative cell viability was obtained by normalizing values of each silenced cell population to its respective control. Three independent assays were performed.

Apoptosis assay

Apoptosis assay was performed according to the manufacturer's instructions (Biocolor, Newtownabbey, Northern Ireland). The APOPercentage assay is a dye-uptake assay which stains only apoptotic cells with a red dye, whereas normal and necrotic cells remain unlabeled. Cells were seeded in 96-well plates at a density of 1.0 x 10^4 cells (LNCaP) or 2.5 x 10^4 cells (VCaP) for 96h. Cells were then stained with APOPercentage dye for 1 hour and washed twice with PBS to remove non-cell bound dye. Dye Release Reagent was added to each well and the plate was shaken for 10 minutes. Absorbance levels were measured at 550 nm with background correction at 620 nm using a microplate reader (Fluostar Omega). An average value of measures from nine replicate wells was obtained for each cell population. Relative apoptosis was obtained by normalizing values of each silenced cell population to its respective control. Three independent assays were performed.

Invasion assay

Cell invasion through a three-dimensional extracellular matrix was evaluated by a Matrigel invasion assay using BD Matrigel Invasion Chambers with 8.0 µm pore (BD Biocoat, Bedford, MA, USA). Briefly, the matrigel-coated transwell chambers were rehydrated and 2.5 x 10^4 LNCaP cells or 5.0 x 10^4 VCaP cells in 500 µL of serum-free medium were plated in the upper chamber, in triplicate wells. Complete growth medium was added to the lower chamber. After 48 or 72 hours (LNCaP and VCaP, respectively), the cells on the upper surface were removed with cotton swabs, and invaded cells at the lower surface were fixed with methanol, stained with DAPI and counted under a microscope. Relative cell invasion was obtained by normalizing values of each silenced cell population to its respective control. Three independent assays were performed.

Soft agar colony formation assay

LNCaP and VCaP cells (1.0 x 10^4 or 5.0 x 10^4, respectively) were resuspended in 0.2% low melting agarose in complete growth medium and plated on top of 1 ml underlayer of 0.6% low melting agarose in the same medium in 6-well cell culture plates. After 4 weeks of incubation in a humidified chamber, colonies were stained with 0.05% crystal violet, photographed and counted. Relative aggregation was obtained by normalizing values of each silenced cell population to its respective control. Three independent assays were performed.

Kinex™ antibody microarray (KAM-850)

To perform the Kinex™ analyses (Kinexus Bioinformatics Corporation, Vancouver, Canada), 50 µg of total protein lysate from each sample were covalently labeled with a proprietary fluorescent dye and incubated on the chip array. The six protein extracts analyzed, from the *GRPR* silenced cell line models of LNCaP and VCaP cells (GRPR sh#1, GRPR sh#2 and scramble control), were obtained using the kinexus protein lysis buffer (Kinexus Bioinformatics Corporation). The KAM-850 antibody microarray contains over 850 antibodies among pan- and phospho site-specific with wide coverage of cell signaling proteins and pathways. Each array produces a pair of 16-bit images, which were captured with a ScanArray Reader laser array scanner (Perkin-Elmer, Waltham, MA, USA). Image capture, signal quantification, background correction and Z score transformation [32] were performed by Kinexus Bioinformatics Corporation. A Z ratio of ±1.2 was inferred as significant.

Statistical analysis

All *in vitro* data were obtained from three independent experiments, each including triplicate wells per condition. Statistical analyses were conducted using SPSS software version 21.0 (IBM-SPSS Inc., Chicago, IL, USA) and graphs were built using GraphPad Prism 5.0 software (GraphPad Software Inc., La Jolla, CA, USA). *In vitro* studies data were analyzed by paired Student's *t* test. Kruskal-Wallis test (KW) and Mann-Whitney U test (MW) were used for multi-group comparisons, as appropriate. All p values were based on two-sided hypothesis testing and a $p<0.05$ was considered statistically significant.

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Regional do Norte (MT). PP is a Posdoc fellow from FCT (PEst-OE/SAU/UI0776/2014).

CONFLICT OF INTEREST

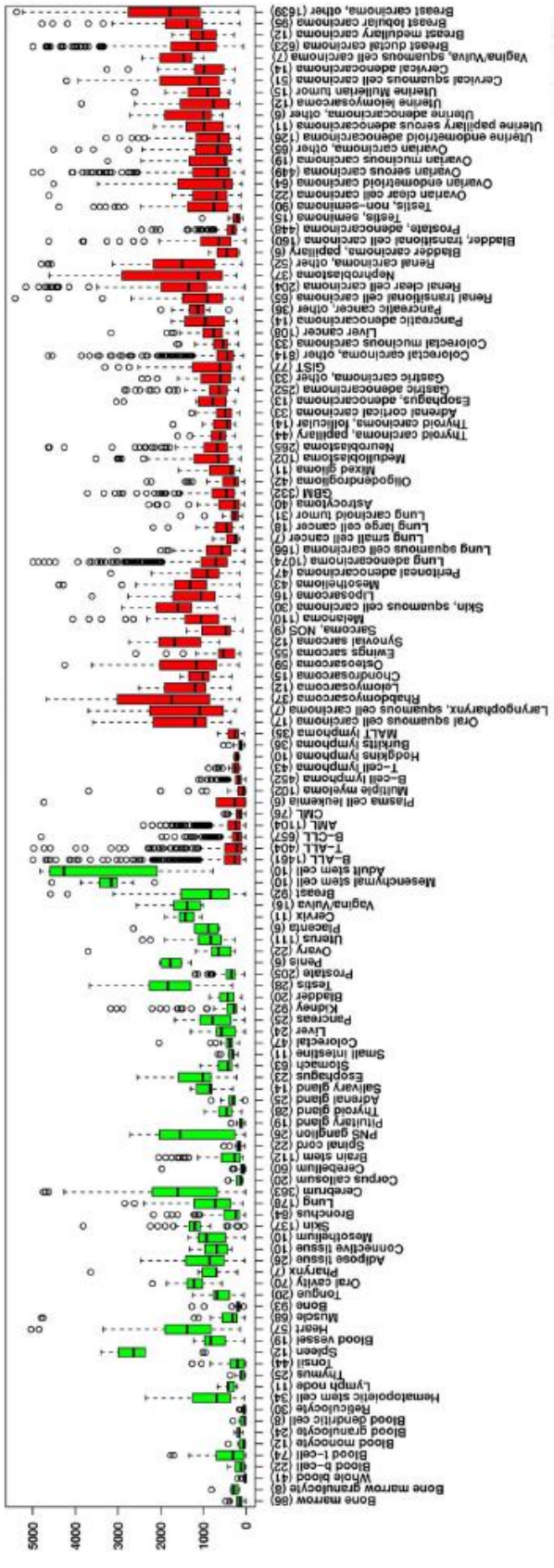
There is no conflict of interest

REFERENCES

1. Siegel R, Ma J, Zou Z and Jemal A. Cancer statistics, 2014. CA: a cancer journal for clinicians. 2014; 64(1):9-29.
2. Ramon J and Denis L. Prostate Cancer. Springer, Berlim. 2007.
3. Feldman BJ and Feldman D. The development of androgen-independent prostate cancer. Nature reviews Cancer. 2001; 1(1):34-45.
4. Clark JP and Cooper CS. ETS gene fusions in prostate cancer. Nature reviews Urology. 2009; 6(8):429-439.
5. Kumar-Sinha C, Tomlins SA and Chinnaiyan AM. Recurrent gene fusions in prostate cancer. Nature reviews Cancer. 2008; 8(7):497-511.
6. Oikawa T and Yamada T. Molecular biology of the Ets family of transcription factors. Gene. 2003; 303:11-34.
7. Konstantinopoulos PA and Papavassiliou AG. Seeing the future of cancer-associated transcription factor drug targets. Jama. 2011; 305(22):2349-2350.
8. Findlay VJ, LaRue AC, Turner DP, Watson PM and Watson DK. Understanding the role of ETS-mediated gene regulation in complex biological processes. Advances in cancer research. 2013; 119:1-61.
9. Paulo P, Ribeiro FR, Santos J, Mesquita D, Almeida M, Barros-Silva JD, Itkonen H, Henrique R, Jeronimo C, Sveen A, Mills IG, Skotheim RI, Lothe RA, et al. Molecular subtyping of primary prostate cancer reveals specific and shared target genes of different ETS rearrangements. Neoplasia. 2012; 14(7):600-611.
10. Xiao D, Wang J, Hampton LL and Weber HC. The human gastrin-releasing peptide receptor gene structure, its tissue expression and promoter. Gene. 2001; 264(1):95-103.
11. Cornelio DB, Roesler R and Schwartsmann G. Gastrin-releasing peptide receptor as a molecular target in experimental anticancer therapy. Annals of oncology: official journal of the European Society for Medical Oncology / ESMO. 2007; 18(9):1457-1466.
12. Markwalder R and Reubi JC. Gastrin-releasing peptide receptors in the human prostate: relation to neoplastic transformation. Cancer research. 1999; 59(5):1152-1159.
13. Hohla F and Schally AV. Targeting gastrin releasing peptide receptors: New options for the therapy and diagnosis of cancer. Cell cycle. 2010; 9(9):1738-1741.
14. Stangelberger A, Schally AV, Varga JL, Zarandi M, Szepeshazi K, Armatis P and Halmos G. Inhibitory effect of antagonists of bombesin and growth hormone-releasing hormone on orthotopic and intraosseous growth and invasiveness of PC-3 human prostate cancer in nude mice. Clinical cancer research : an official journal of the American Association for Cancer Research. 2005; 11(1):49-57.
15. Stangelberger A, Schally AV, Varga JL, Hammann BD, Groot K, Halmos G, Cai RZ and Zarandi M. Antagonists of growth hormone releasing hormone (GHRH) and of bombesin/gastrin releasing peptide (BN/GRP) suppress the expression of VEGF, bFGF, and receptors of the EGF/HER family in PC-3 and DU-145 human androgen-independent prostate cancers. The Prostate. 2005; 64(3):303-315.
16. Stangelberger A, Schally AV, Varga JL, Zarandi M, Cai RZ, Baker B, Hammann BD, Armatis P and Kanashiro CA. Inhibition of human androgen-independent PC-3 and DU-145 prostate cancers by antagonists of bombesin and growth hormone releasing hormone is linked to PKC, MAPK and c-jun intracellular signalling. European journal of cancer. 2005; 41(17):2735-2744.
17. Jensen RT, Battey JF, Spindel ER and Benya RV. International Union of Pharmacology. LXVIII. Mammalian bombesin receptors: nomenclature, distribution, pharmacology, signaling, and functions in normal and disease states. Pharmacological reviews. 2008; 60(1):1-42.
18. Mansi R, Fleischmann A, Macke HR and Reubi JC. Targeting GRPR in urological cancers--from basic research to clinical application. Nature reviews Urology. 2013; 10(4):235-244.
19. Morgan TM, Koreckij TD and Corey E. Targeted therapy for advanced prostate cancer: inhibition of the PI3K/Akt/mTOR pathway. Current cancer drug targets. 2009; 9(2):237-249.
20. Liu X, Carlisle DL, Swick MC, Gaither-Davis A, Grandis JR and Siegfried JM. Gastrin-releasing peptide activates Akt through the epidermal growth factor receptor pathway and abrogates the effect of gefitinib. Experimental cell research. 2007; 313(7):1361-1372.
21. Basu A and Sivaprasad U. Protein kinase Cepsilon makes the life and death decision. Cellular signalling. 2007; 19(8):1633-1642.
22. Gorin MA and Pan Q. Protein kinase C epsilon: an oncogene and emerging tumor biomarker. Molecular cancer. 2009; 8:9.
23. Zhang J, Baines CP, Zong C, Cardwell EM, Wang G, Vondriska TM and Ping P. Functional proteomic analysis of a three-tier PKCepsilon-Akt-eNOS signaling module in cardiac protection. American journal of physiology Heart and circulatory physiology. 2005; 288(2):H954-961.
24. Zong Y, Xin L, Goldstein AS, Lawson DA, Teitell MA and Witte ON. ETS family transcription factors collaborate with alternative signaling pathways to induce carcinoma from adult murine prostate cells. Proceedings of the National Academy of Sciences of the United States of America. 2009; 106(30):12465-12470.
25. Ubel C, Mousset S, Trufa D, Sirbu H and Finotto S.

Establishing the role of tyrosine kinase 2 in cancer. Oncoimmunology. 2013; 2(1):e22840.

26. Ide H, Nakagawa T, Terado Y, Kamiyama Y, Muto S and Horie S. Tyk2 expression and its signaling enhances the invasiveness of prostate cancer cells. Biochemical and biophysical research communications. 2008; 369(2):292-296.
27. Schuster C, Muller M, Freissmuth M, Sexl V and Stoiber D. Commentary on H. Ide et al., "Tyk2 expression and its signaling enhances the invasiveness of prostate cancer cells". Biochemical and biophysical research communications. 2008; 366(4):869-870.
28. Yao HP, Zhou YQ, Zhang R and Wang MH. MSP-RON signalling in cancer: pathogenesis and therapeutic potential. Nature reviews Cancer. 2013; 13(7):466-481.
29. Mesquita D, Barros-Silva JD, Santos J, Skotheim R, Lothe RA, Paulo P and Teixeira MR. Specific and redundant activities of ETV1 and ETV4 in prostate cancer aggressiveness revealed by co-overexpression cellular contexts. Oncotarget. (in press)
30. Paulo P, Barros-Silva JD, Ribeiro FR, Ramalho-Carvalho J, Jeronimo C, Henrique R, Lind GE, Skotheim RI, Lothe RA and Teixeira MR. FLI1 is a novel ETS transcription factor involved in gene fusions in prostate cancer. Genes, chromosomes & cancer. 2012; 51(3):240-249.
31. Schmittgen TD and Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. Nature protocols. 2008; 3(6):1101-1108.
32. Cheadle C, Vawter MP, Freed WJ and Becker KG. Analysis of microarray data using Z score transformation. The Journal of molecular diagnostics : JMD. 2003; 5(2):73-81.



Supplementary Figure 1: Box-whisker plot of the PLK2 gene's expression (ENSG00000145632) in healthy and cancer tissues in IST (in silico transcriptomics) online. Green boxes indicate healthy tissues, red boxes indicate cancers.

Supplementary Table 1: List of the 850 antibodies among pan- and phospho site-specific included in the antibody microarray KAM-850 Kinexus.

No.	Antibody	Target	Phosp	Full Target Protein Name	Refseq	Uniprot Link
1	NN001	14-3-3 z	Pan-specific	14-3-3 protein zeta (cross-reacts with other	NP_003397	P63104
2	NN166	4E-BP1	Pan-specific	Eukaryotic translation initiation factor 4E binding	NP_004086	Q13541
3	PN001	4E-BP1	S65	Eukaryotic translation initiation factor 4E binding	NP_004086	Q13541
4	PN114	4E-BP1	T45	Eukaryotic translation initiation factor 4E binding	NP_004086	Q13541
5	PN128	4E-BP1	T70	Eukaryotic translation initiation factor 4E binding	NP_004086	Q13541
6	NK001	Abl	Pan-specific	Abelson proto-oncogene-encoded protein-	NP_005148	P00519
7	PN002	AcCoA	S80	Acetyl coenzyme A carboxylase	NP_000655	Q13085
8	NN135-1	Acetylated	Pan-specific	Acetylated Lysine	NA	NA
9	NN135-2	Acetylated	Pan-specific	Acetylated Lysine	NA	NA
10	NK002	ACK1	Pan-specific	Activated p21cdc42Hs protein-serine kinase	NP_005772	Q07912
11	PN003-	Adducin a	S726	Adducin alpha (ADD1)	NP_058432	P35611
12	NN002	AIF	Pan-specific	Apoptosis inducing factor (programed cell death	NP_004199	O95831
13	NN003	AK2	Pan-specific	Adenylate kinase 2	NP_001616	P54819
14	NK003	ALK	Pan-specific	Anaplastic lymphoma kinase	NP_004295.2	Q9UM73
15	NK004	ALS2CR7	Pan-specific	Amyotrophic lateral sclerosis 2 chromosomal	NP_631897	Q96Q40
16	PK002	AMPKa1/2	T183	5'-AMP-activated protein kinase subunit alpha	NP_006242	Q13131
17	NK006	ANKRD3	Pan-specific	Ankyrin repeat domain protein-serine kinase 3	NP_065690	P57078
18	NN004	APG1	Pan-specific	Hsp 70-related heat shock protein 1 (osmotic	NP_055093	O95757
19	NN122	APG2	Pan-specific	Hsp 70-related heat shock protein 4 (HSP70RY)	NP_002145.3	P34932
20	PN189	APP	T668	Amyloid beta A4 protein	NP_000475.1	P05067
21	NN121	Arrestin b1	Pan-specific	Arrestin beta 1	NP_004032	P49407
22	PN133	Arrestin b1	S412	Arrestin beta 1	NP_004032	P49407
23	NK007	ASK1	Pan-specific	Apoptosis signal regulating protein-serine kinase	NP_005914	Q99683
24	NK007-2	ASK1	Pan-specific	Apoptosis signal regulating protein-serine kinase	NP_005914	Q99683
25	PK143	ASK1	S966	Apoptosis signal regulating protein-serine kinase	NP_005914	Q99683
26	NN160	ATF2	Pan-specific	Activating transcription factor 2 (CRE-BP1)	NP_001871	P15336
27	PN006-1	ATF2	T69 + T71	Activating transcription factor 2 (CRE-BP1)	NP_001871	P15336
28	PN115	ATF2	S112	Activating transcription factor 2 (CRE-BP1)	NP_001871	P15336
29	NK008-2	Aurora A	Pan-specific	Aurora Kinase A (serine/threonine protein	NP_940835	O14965
30	NK193	Aurora B	Pan-specific	Aurora Kinase B (serine/threonine protein	NP_004208	Q96GD4
31	NK009	Aurora C	Pan-specific	Aurora Kinase C (serine/threonine-protein	NP_003151	Q9UQB9
32	NK010	Axl	Pan-specific	Axl proto-oncogene-encoded protein-tyrosine	NP_001690	P30530
33	PN008	B23 (NPM)	T199	B23 (nucleophosmin, numatrin, nucleolar protein	NP_002511	P06748
34	PN008-2	B23 (NPM)	T199	B23 (nucleophosmin, numatrin, nucleolar protein	NP_002511	P06748
35	PN009	B23 (NPM)	T234/T237	B23 (nucleophosmin, numatrin, nucleolar protein	NP_002511	P06748
36	PN012-1	Bad	S99	Bcl2-antagonist of cell death protein	NP_004313	Q92934
37	NN000	Bak	Pan-specific	Bcl2 homologous antagonist/killer (BCK2L7)	NP_001179	Q16611
38	NN005	Bax	Pan-specific	Apoptosis regulator Bcl2-associated X protein	NP_620116	Q07812
39	NN006	Bcl2	Pan-specific	B-cell lymphoma protein 2 alpha	NP_000624	P10415
40	NN006-1	Bcl2	Pan-specific	B-cell lymphoma protein 2 alpha	NP_000624	P10415
41	NN007	Bcl-xL	Pan-specific	Bcl2-like protein 1	NP_612815	Q07817
42	NN008	Bcl-xS/L	Pan-specific	Bcl2-like protein 1	NP_612815	Q07817
43	PK164	Bcr	Y177	Breakpoint cluster region protein	NP_004318.3	P11274
44	NN009	Bid	Pan-specific	BH3 interacting domain death agonist	NP_001187	P55957
45	NK011	BLK	Pan-specific	B lymphoid tyrosine kinase	NP_001706	P51451
46	PN013	BLNK	Y84	B-cell linker protein	NP_037446	O75498
47	NK012	BMX (Etk)	Pan-specific	Bone marrow X protein-tyrosine kinase	NP_001712	P51813
48	PK003	BMX (Etk)	Y40	Bone marrow X protein-tyrosine kinase	NP_001712	P51813
49	PN014	BRCA1	S1497	Breast cancer type 1 susceptibility protein	NP_009225	P38398
50	PN116	BRCA1	S1423	Breast cancer type 1 susceptibility protein	NP_009225	P38398
51	NK013	BRD2	Pan-specific	Bromodomain-containing protein-serine kinase 2	NP_005095	P25440
52	NK014	Btk	Pan-specific	Bruton's agammaglobulinemia tyrosine kinase	NP_000052	Q06187
53	PK004	Btk	Y223	Bruton's agammaglobulinemia tyrosine kinase	NP_000052	Q06187
54	NK015	BUB1A	Pan-specific	BUB1 mitotic checkpoint protein-serine kinase	NP_004327	O43683
55	NN174	CA9	Pan-specific	Carbonic anhydrase 9	NP_001207.2	Q16790
56	PN015	Caldesmo	S789	Caldesmon	NP_004333	Q05682
57	NN136-2	Calnexin	Pan-specific	Calnexin	NP_001019820.1.	P27824
58	NN137-1	Calreticulin	Pan-specific	Calreticulin	NP_004334.1.	P27797
59	NK211	CAMK1a	Pan-specific	Calcium/calmodulin-dependent protein-serine	NP_003647.1	Q14012
60	NK016-1	CaMK1d	Pan-specific	Calcium/calmodulin-dependent protein-serine	NP_003647	Q8IU85
61	NK016-2	CaMK1d	Pan-specific	Calcium/calmodulin-dependent protein-serine	NP_003647	Q8IU85
62	PK005-1	CaMK2a	T286	Calcium/calmodulin-dependent protein-serine	NP_741960	Q9UQM7
63	NK018-2	CAMK2b	Pan-specific	Calcium/calmodulin-dependent protein-serine	NP_742081	Q13554
64	NK019-2	CAMK2d	Pan-specific	Calcium/calmodulin-dependent protein-serine	NP_742126	Q13557
65	NK021	CaMK4	Pan-specific	Calcium/calmodulin-dependent protein-serine	NP_001735	Q16566
66	NK021-2	CaMK4	Pan-specific	Calcium/calmodulin-dependent protein-serine	NP_001735	Q16566
67	NK021-3	CaMK4	Pan-specific	Calcium/calmodulin-dependent protein-serine	NP_001735	Q16566
68	NK022	CaMKK	Pan-specific	Calcium/calmodulin-dependent protein-serine	NP_006540	Q8N5S9
69	NN010	CAS	Pan-specific	Cellular apoptosis susceptibility protein (CSE1L)	NP_001307	P55060
70	NK023	CASK/Lin2	Pan-specific	Calcium/calmodulin-dependent protein-serine	NP_001119526.1	O14936
71	NN011	CASP1	Pan-specific	Caspase 1 (Interleukin-1 beta convertase)	NP_001214	P29466
72	NN011-2	CASP1	Pan-specific	Caspase 1 (Interleukin-1 beta convertase)	NP_001214	P29466
73	NN013	CASP3	Pan-specific	Caspase 3 (apopain, cysteine protease CPP32)	NP_004337	P42574
74	NN013-3	CASP3	Pan-specific	Caspase 3 (apopain, cysteine protease CPP32)	NP_004337	P42574
75	NN016	CASP6	Pan-specific	Caspase 6 (apoptotic protease Mch2)	NP_001217	P55212
76	NN017	CASP7	Pan-specific	Caspase 7 (ICE-like apoptotic protease 3 (ICE-	NP_01218	P55210
77	NN017-2	CASP7	Pan-specific	Caspase 7 (ICE-like apoptotic protease 3 (ICE-	NP_01218	P55210
78	NN019-2	CASP9	Pan-specific	Caspase 9 (ICE-like apoptotic protease 6 (ICE-	NP_033938	P55211
79	PN162	Catenin a	S641	Catenin (cadherin-associated protein) alpha	NP_001894.2	P35221

80	PN166	Catenin b	S33	Catenin (cadherin-associated protein) beta 1	NP_001895	P35222
81	PN167	Catenin b	Y333	Catenin (cadherin-associated protein) beta 1	NP_001895	P35222
82	NN021	Catenin b1	Pan-specific	Catenin (cadherin-associated protein) beta 1	NP_001895	P35222
83	NN021-1	Catenin b1	Pan-specific	Catenin (cadherin-associated protein) beta 1	NP_001895	P35222
84	NN167	Caveolin 1	Pan-specific	Caveolin 1	NP_001744.2	Q03135
85	PN147	Caveolin 1	Y14	Caveolin 1	NP_001744.2	Q03135
86	NN022-1	Caveolin 2	Pan-specific	Caveolin 2	NP_001224	P51636
87	PN018	Caveolin 2	S36	Caveolin 2	NP_001224	P51636
88	PN171	Cbl	Y700	Signal transduction protein CBL	NP_005179.2	P22681
89	NP001	CD45	Pan-specific	Leukocyte common antigen CD45 receptor-	NP_002829	P08575
90	NP002	Cdc25B	Pan-specific	Cell division cycle 25B phosphatase	NP_004349	P30305
91	NP003	Cdc25C	Pan-specific	Cell division cycle 25C phosphatase	NP_001781	P30307
92	PP005	Cdc25C	S216	Cell division cycle 25C phosphatase	NP_002882.3	P30307
93	NK024	CDC2L5	Pan-specific	Cell division cycle 2-like protein-serine kinase 5	NP_003709	Q14004
94	NN023	Cdc34	Pan-specific	Cell division cycle 34 (ubiquitin-conjugating	NP_004350	P49427
95	NN024	Cdc42	Pan-specific	Cell division control protein 42 homolog	NP_001782	P60953
96	NK025-1	CDK1	Pan-specific	Cyclin-dependent protein-serine kinase 1	NP_001777	P06493
97	NK025-2	CDK1	Pan-specific	Cyclin-dependent protein-serine kinase 1	NP_001777	P06493
98	NK025-3	CDK1	Pan-specific	Cyclin-dependent protein-serine kinase 1	NP_001777	P06493
99	NK025-4	CDK1	Pan-specific	Cyclin-dependent protein-serine kinase 1	NP_001777	P06493
100	NK025-5	CDK1	Pan-specific	Cyclin-dependent protein-serine kinase 1	NP_001777	P06493
101	NK025-6	CDK1	Pan-specific	Cyclin-dependent protein-serine kinase 1	NP_001777	P06493
102	PK006	CDK1/2	T14+Y15	Cyclin-dependent protein-serine kinase 1/2	NP_001777	P06493
103	PK007-1	CDK1/2	Y15	Cyclin-dependent protein-serine kinase 1/2	NP_001777	P06493
104	PK007-2	CDK1/2	Y15	Cyclin-dependent protein-serine kinase 1/2	NP_001777	P06493
105	PK007-3	CDK1/2	Y15	Cyclin-dependent protein-serine kinase 1/2	NP_001777	P06493
106	PK008	CDK1/2	T161	Cyclin-dependent protein-serine kinase 1/2	NP_001777	P06493
107	NK033	CDK10	Pan-specific	Cyclin-dependent protein-serine kinase 10	NP_003665	Q15131
108	NK026-2	CDK2	Pan-specific	Cyclin-dependent protein-serine kinase 2	NP_001789	P24941
109	NK026-3	CDK2	Pan-specific	Cyclin-dependent protein-serine kinase 2	NP_001789	P24941
110	NK026-4	CDK2	Pan-specific	Cyclin-dependent protein-serine kinase 2	NP_001789	P24941
111	NK026-5	CDK2	Pan-specific	Cyclin-dependent protein-serine kinase 2	NP_001789	P24941
112	NK026-6	CDK2	Pan-specific	Cyclin-dependent protein-serine kinase 2	NP_001789	P24941
113	NK026-7	CDK2	Pan-specific	Cyclin-dependent protein-serine kinase 2	NP_001789	P24941
114	NK027	CDK4	Pan-specific	Cyclin-dependent protein-serine kinase 4	NP_000066	P11802
115	NK027-2	CDK4	Pan-specific	Cyclin-dependent protein-serine kinase 4	NP_000066	P11802
116	NK028-1	CDK5	Pan-specific	Cyclin-dependent protein-serine kinase 5	NP_004926	Q00535
117	NK028-2	CDK5	Pan-specific	Cyclin-dependent protein-serine kinase 5	NP_004926	Q00535
118	NK028-4	CDK5	Pan-specific	Cyclin-dependent protein-serine kinase 5	NP_004926	Q00535
119	NK028-5	CDK5	Pan-specific	Cyclin-dependent protein-serine kinase 5	NP_004926	Q00535
120	NK029	CDK6	Pan-specific	Cyclin-dependent protein-serine kinase 6	NP_001250	Q00534
121	NK029-2	CDK6	Pan-specific	Cyclin-dependent protein-serine kinase 6	NP_001250	Q00534
122	NK029-3	CDK6	Pan-specific	Cyclin-dependent protein-serine kinase 6	NP_001250	Q00534
123	PK165	CDK6	Y13	Cyclin-dependent protein-serine kinase 6	NP_001250	Q00534
124	NK030-2	CDK7	Pan-specific	Cyclin-dependent protein-serine kinase 7	NP_001790	P50613
125	NK030-3	CDK7	Pan-specific	Cyclin-dependent protein-serine kinase 7	NP_001790	P50613
126	NK031-2	CDK8	Pan-specific	Cyclin-dependent protein-serine kinase 8	NP_001252	P49336
127	NK031-4	CDK8	Pan-specific	Cyclin-dependent protein-serine kinase 8	NP_001252	P49336
128	NK031-5	CDK8	Pan-specific	Cyclin-dependent protein-serine kinase 8	NP_001252	P49336
129	NK032	CDK9	Pan-specific	Cyclin-dependent protein-serine kinase 9	NP_001252	P50750
130	NK199	CDKL1	Pan-specific	Cyclin-dependent kinase-like 1	NP_004187.2	Q00532
131	NK034	Chk1	Pan-specific	Checkpoint protein-serine kinase 1	NP_001265	O14757
132	NK034-2	Chk1	Pan-specific	Checkpoint protein-serine kinase 1	NP_001265	O14757
133	PK162	Chk1	S280	Checkpoint protein-serine kinase 1	NP_001265	O14757
134	NK035	Chk2	Pan-specific	Checkpoint protein-serine kinase 2	NP_009125	O96017
135	PK119	Chk2	T68	Checkpoint protein-serine kinase 2	NP_009125	O96017
136	NN025	c-IAP1	Pan-specific	Cellular inhibitor of apoptosis protein 1	NP_001156	Q13490
137	NK036	CK1d	Pan-specific	Casein protein-serine kinase 1 delta	NP_001884	P48730
138	NK037-1	CK1e	Pan-specific	Casein protein-serine kinase 1 epsilon	NP_001885	P49674
139	NK198	CK1g	Pan-specific	Casein kinase I gamma 1 isoform	NP_071331	Q9HCP0
140	NK040	CK1g2	Pan-specific	Casein protein-serine kinase 1 gamma 2	NP_001310	P78368
141	NK041	CK2a	Pan-specific	Casein protein-serine kinase 2 alpha/ alpha	NP_001887	P68400
142	PK167	CK2a	T360/S362	Casein protein-serine kinase 2 alpha/ alpha	NP_001887	P68400
143	PN130	c-Myc	T58/S62	Myc proto-oncogene protein	NP_002458.2	P01106
144	NN026	Cofilin 1	Pan-specific	Cofilin 1	NP_005498	P23528
145	PN019	Cofilin 1	S3	Cofilin 1	NP_005498	P23528
146	PN020	Cofilin 2	S3	Cofilin 2	NP_068733	Q9Y281
147	PN148	Connexin	S368	Gap junction alpha-1 protein	NP_000156.1	P17302
148	PN022-2	Cortactin	Y466	Cortactin (amplaxin) (mouse)	NP_031829	Q14247
149	NK042	COT	Pan-specific	Osaka thyroid oncogene protein-serine kinase	NP_005195	P41279
150	NK042-2	COT	Pan-specific	Osaka thyroid oncogene protein-serine kinase	NP_005195	P41279
151	NN027	COX2	Pan-specific	Cyclo-oxygenase 2 (prostaglandin G/H synthase	NP_000954	P35354
152	NK043	CPG16/Ca	Pan-specific	Serine/threonine-protein kinase DCAMKL1	NP_004725	O15075
153	PN023	CREB1	S129+S133	cAMP response element binding protein 1	NP_004370	P16220
154	PN024	CREB1	S133	cAMP response element binding protein 1	NP_004370	P16220
155	PN024-2	CREB1	S133	cAMP response element binding protein 1	NP_004370	P16220
156	NN149-1	Crystallin	Pan-specific	Crystallin alpha B (heat-shock 20 kDa like-	NP_001876	P02511
157	NN149-2	Crystallin	Pan-specific	Crystallin alpha B (heat-shock 20 kDa like-	NP_001876	P02511
158	NN149-3	Crystallin	Pan-specific	Crystallin alpha B (heat-shock 20 kDa like-	NP_001876	P02511
159	PN025	Crystallin	S19	Crystallin alpha B (heat-shock 20 kDa like-	NP_001876	P02511

160	PN110	Crystallin	S45	Crystallin alpha B (heat-shock 20 kDa like-	NP_001876	P02511
161	NK044	Csk	Pan-specific	C-terminus of Src tyrosine kinase	NP_004374	P41240
162	NK044-2	Csk	Pan-specific	C-terminus of Src tyrosine kinase	NP_004374	P41240
163	NN028	Cyclin A	Pan-specific	Cyclin A1	NP_003905	P78396
164	NN029	Cyclin B1	Pan-specific	Cyclin B1	NP_114172	P14635
165	PN190	Cyclin B1	S147	Cyclin B1	NP_114172	P14635
166	NN030-1	Cyclin D1	Pan-specific	Cyclin D1 (PRAD1)	NP_444284	P24385
167	NN030-2	Cyclin D1	Pan-specific	Cyclin D1 (PRAD1)	NP_444284	P24385
168	NN031	Cyclin E	Pan-specific	Cyclin E1	NP_001229	P24864
169	PN191	Cyclin E	T395	Cyclin E1	NP_001229	P24864
170	NN032	Cyclin G1	Pan-specific	Cyclin G1	NP_004051	P51959
171	NN033	CytoC	Pan-specific	Cytochrome C	NP_061820	P99999
172	PN026	Dab1	Y198	Disabled homolog 1	NP_066566	O75553
173	NK210	DAPK1	Pan-specific	Death-associated protein kinase 1	NP_004929	P53355
174	NK046	DAPK2	Pan-specific	Death-associated protein kinase 2	NP_055141	O9UIK4
175	NN034	DAXX	Pan-specific	Death-associated protein 6 (BING2)	NP_001341	O9UER7
176	NN163	DDIT3(CH	Pan-specific	DNA damage-inducible transcript 3 protein	NP_001181986.1	P35639
177	NN035-	DFF35	Pan-specific	DNA fragmentation factor alpha (ICAD) 35-kDa	NP_004392	O00273
178	NK219	DGKz	Pan-specific	Diacylglycerol kinase zeta	NP_963290	O13574
179	NK048	DNAPK	Pan-specific	DNA-activated protein-serine kinase	NP_008835	P78527
180	NK048-2	DNAPK	Pan-specific	DNA-activated protein-serine kinase	NP_008835	P78527
181	PN027	Dok2	Y139	Docking protein 2	NP_034201	O60496
182	PN027-2	Dok2	Y139	Docking protein 2	NP_034201	O60496
183	NK050	DRAK2	Pan-specific	DAP kinase-related apoptosis-inducing protein-	NP_004217	O94768
184	NK051	eEF2K	Pan-specific	Elongation factor-2 protein-serine kinase	NP 037434	O00418
185	NN175	EFNA5	Pan-specific	Ephrin-A5	NP_001953.1	P52803
186	PN173	EFNB2	Y316	EPH-related receptor tyrosine kinase ligand 5	NP_004084.1	P52799
187	NK052-1	EGFR	Pan-specific	Epidermal growth factor receptor-tyrosine kinase	NP_005219	P00533
188	PK010	EGFR	Y1172	Epidermal growth factor receptor-tyrosine kinase	NP_005219	P00533
189	PK010-2	EGFR	Y1172	Epidermal growth factor receptor-tyrosine kinase	NP_005219	P00533
190	PK011-1	EGFR	Y1197	Epidermal growth factor receptor-tyrosine kinase	NP_005219	P00533
191	PK121	EGFR	T693	Epidermal growth factor receptor-tyrosine kinase	NP_005219	P00533
192	PK122-1	EGFR	Y1092	Epidermal growth factor receptor-tyrosine kinase	NP_005219	P00533
193	PK123	EGFR	Y1110	Epidermal growth factor receptor-tyrosine kinase	NP_005219	P00533
194	NN038-1	eIF2a	Pan-specific	Eukaryotic translation initiation factor 2 alpha	NP_004085	P05198
195	PN028-1	eIF2a	S52	Eukaryotic translation initiation factor 2 alpha	NP_004085	P05198
196	PN028-2	eIF2a	S52	Eukaryotic translation initiation factor 2 alpha	NP_004085	P05198
197	PN172	eIF4B	S422	Eukaryotic translation initiation factor 4B	NP_001408.2	P23588
198	NN039-1	eIF4E	Pan-specific	Eukaryotic translation initiation factor 4 (mRNA	NP_001959	P06730
199	PN030-1	eIF4E	S209	Eukaryotic translation initiation factor 4 (mRNA	NP_001959	P06730
200	PN030-2	eIF4E	S209	Eukaryotic translation initiation factor 4 (mRNA	NP_001959	P06730
201	PN031	eIF4G	S1107	Eukaryotic translation initiation factor 4 gamma	NP_004944	O04637
202	PN193	eIF4G	S1232	Eukaryotic translation initiation factor 4 gamma	NP_004944	O04637
203	NN168	Elk-1	Pan-specific	ETS domain-containing protein Elk-1	NP_001107595.1	P19419
204	PN149	Elk-1	S383	ETS domain-containing protein Elk-1	NP_001107595.1	P19419
205	PN170	Elk-1	S389	ETS domain-containing protein Elk-1	NP_001107595.1	P19419
206	NN173	Epcam	Pan-specific	Epithelial cell adhesion molecule	NP_002345.2	P16422
207	NK053	EphA1	Pan-specific	Ephrin type-A receptor 1 protein-tyrosine kinase	NP_005223	P21709
208	PN198	ER-alpha	S104	estrogen receptor alpha	NP_000116.2	P03372
209	NK054-1	ErbB2	Pan-specific	ErbB2 (Neu) receptor-tyrosine kinase	NP_004439	P04626
210	NK054-2	ErbB2	Pan-specific	ErbB2 (Neu) receptor-tyrosine kinase	NP_004439	P04626
211	PK013-1	ErbB2	Y1248	ErbB2 (Neu) receptor-tyrosine kinase	NP_004439	P04626
212	PK013-2	ErbB2	Y1248	ErbB2 (Neu) receptor-tyrosine kinase	NP_004439	P04626
213	PK134	ErbB2	T686	ErbB2 (Neu) receptor-tyrosine kinase	NP_004439	P04626
214	PK163	ErbB3	Y1328	Tyrosine kinase-type cell surface receptor HER3	NP_001005915.1	P21860
215	NK055-	Erk1	Pan-specific	Extracellular regulated protein-serine kinase 1	AAA36142.1,	P27361
216	NK055-	Erk1	Pan-specific	Extracellular regulated protein-serine kinase 1	AAA36142.1,	P27361
217	NK055-	Erk1	Pan-specific	Extracellular regulated protein-serine kinase 1	AAA36142.1,	P27361
218	NK055-	Erk1	Pan-specific	Extracellular regulated protein-serine kinase 1	AAA36142.1,	P27361
219	NK055-	Erk1	Pan-specific	Extracellular regulated protein-serine kinase 1	AAA36142.1,	P27361
220	NK055-	Erk1	Pan-specific	Extracellular regulated protein-serine kinase 1	AAA36142.1,	P27361
221	PK168-	Erk1	Y204	Extracellular regulated protein-serine kinase 1	AAA36142.1,	P27361
222	PK170-	Erk1	T202	Extracellular regulated protein-serine kinase 1	AAA36142.1,	P27361
223	NK056	Erk2	Pan-specific	Extracellular regulated protein-serine kinase 2	NP_002736	P28482
224	NK057-2	Erk3	Pan-specific	Extracellular regulated protein-serine kinase 3	NP_002739	Q16659
225	NK058	Erk4	Pan-specific	Extracellular regulated protein-serine kinase 4	NP_002738	P31152
226	NK206-1	Erk5	Pan-specific	Extracellular regulated protein-serine kinase 5	NP_620602	Q13164
227	NK206-2	Erk5	Pan-specific	Extracellular regulated protein-serine kinase 5	NP_620602	Q13164
228	NK206-3	Erk5	Pan-specific	Extracellular regulated protein-serine kinase 5	NP_620602	Q13164
229	PK016	Erk5	T218+Y220	Extracellular regulated protein-serine kinase 5	NP_620602	Q13164
230	PK016-3	Erk5	T218+Y220	Extracellular regulated protein-serine kinase 5	NP_620602	Q13164
231	NN040	ERP57	Pan-specific	ER protein 57 kDa (protein disulfide isomerase-	NP_005304	P30101
232	NN041	ERP72	Pan-specific	ER protein 72 kDa (protein disulfide isomerase-	NP_004902	P13667
233	PN174	Ezrin	T567	cytovillin 2	NP_001104547.1	P15311
234	PN175	Ezrin	Y353	cytovillin 2	NP_001104547.1	P15311
235	NK060	FAK	Pan-specific	Focal adhesion protein-tyrosine kinase	NP_005598	Q05397
236	PK017	FAK	Y397	Focal adhesion protein-tyrosine kinase	NP_005598	Q05397
237	PK017-1	FAK	Y397	Focal adhesion protein-tyrosine kinase	NP_005598	Q05397
238	PK018-3	FAK	Y576	Focal adhesion protein-tyrosine kinase	NP_005598	Q05397
239	PK020	FAK	S722	Focal adhesion protein-tyrosine kinase	NP_005598	Q05397

240	PK020-3	FAK	S722	Focal adhesion protein-tyrosine kinase	NP_005598	Q05397
241	PK021	FAK	S732	Focal adhesion protein-tyrosine kinase	NP_005598	Q05397
242	PK022-2	FAK	S843	Focal adhesion protein-tyrosine kinase	NP_005598	Q05397
243	PK024	FAK	S910	Focal adhesion protein-tyrosine kinase	NP_005598	Q05397
244	PK151	FAK	Y576/Y577	Focal adhesion protein-tyrosine kinase	NP_005598	Q05397
245	NN042	FAS	Pan-specific	Tumor necrosis factor superfamily member 6	NP_003789	P25445
246	NN043	FasL	Pan-specific	Tumor necrosis factor ligand, member 6	NP_000630	P48023
247	NK061	Fes	Pan-specific	Fes/Fps protein-tyrosine kinase	NP_001996	P07332
248	NN172	FHL2	Pan-specific	Four and a half LIM domains protein 2	NP_001034581.1	Q14192
249	NN127	FKBP52	Pan-specific	FK506-binding protein 4	NP_002005	Q02790
250	PN194	FKHR	S256	Forkhead box protein O1	NP_002006.2	Q12778
251	PN195	FKHR	S319	Forkhead box protein O1	NP_002006.2	Q12778
252	PN145-	FKHRL1	T32	Forkhead-like transcription factor 1 (FOXO3A)	NP_001446	Q43524
253	PN145-	FKHRL1	T32	Forkhead-like transcription factor 1 (FOXO3A)	NP_001446	Q43524
254	NN044	Fos	Pan-specific	Fos-c FBJ murine osteosarcoma oncoprotein-	NP_005243	P01100
255	PN033	Fos	T232	Fos-c FBJ murine osteosarcoma oncoprotein-	NP_005243	P01100
256	PN146	FRS2	Y348	Fibroblast growth factor receptor substrate 2	NP_001036020.1	Q8WU20
257	NK065	Fyn	Pan-specific	Fyn proto-oncogene-encoded protein-tyrosine	NP_002028	P06241
258	PN192	Gab1	Y627	GRB2-associated binder 1	NP_002030.2	Q13480
259	PN098	GAP-43	S41	Growth associated protein 43 (Neuromodulin)	NP_002036	P17677
260	PN196	GATA1	S142	Erythroid transcription factor	NP_002040.1	P15976
261	NK066	GCK	Pan-specific	Germinal centre protein-serine kinase	NP_004570	Q12851
262	PN034	GFAP	S8	Glial fibrillary acidic protein	NP_002046	P14136
263	PN178	GluR1	S849	Glutamate receptor 1	NP_000818.2	P42261
264	NN045	GNB2L1	Pan-specific	Guanine nucleotide-binding protein beta	NP_006089	P63244
265	NK067	GRK2	Pan-specific	G protein-coupled receptor-serine kinase 2	NP_001610	P25098
266	PK025	GRK2	S670	G protein-coupled receptor-serine kinase 2	NP_001610	P25098
267	NK068	GRK3	Pan-specific	G protein-coupled receptor-serine kinase 3	NP_005151	P35626
268	NN046	GroEL	Pan-specific	GroEL homolog (may correspond to Hsp60)	NP_002147	P10809
269	NN047	Grp75	Pan-specific	Glucose regulated protein 75	NP_004125	P38646
270	NN048	Grp78	Pan-specific	Glucose regulated protein 78	NP_005338	P11021
271	NN048-2	Grp78	Pan-specific	Glucose regulated protein 78	NP_005338	P11021
272	NN049	Grp94	Pan-specific	Glucose regulated protein 94 (endoplasmin)	NP_003290	P14625
273	NK069-	GSK3a	Pan-specific	Glycogen synthase-serine kinase 3 alpha	NP_063937	P49840
274	NK069-	GSK3a	Pan-specific	Glycogen synthase-serine kinase 3 alpha	NP_063937	P49840
275	PK026-	GSK3a	S21	Glycogen synthase-serine kinase 3 alpha	NP_063937	P49840
276	PK028-	GSK3a	Y279	Glycogen synthase-serine kinase 3 alpha	NP_063937	P49840
277	NK070	GSK3b	Pan-specific	Glycogen synthase-serine kinase 3 beta	NP_002084	P49841
278	NK070-1	GSK3b	Pan-specific	Glycogen synthase-serine kinase 3 beta	NP_002084	P49841
279	NK071	Haspin	Pan-specific	Haploid germ cell-specific nuclear protein-serine	NP_114171	Q8TF76
280	NN169	HDAC4	Pan-specific	Histone deacetylase 4	NP_006028.2	P56524
281	PN179-	HDAC4	S246	Histone deacetylase 4	NP_006028.2	P56524
282	PN188	HDAC5	S498	Histone deacetylase 5	NP_001015053.1	Q9UQL6
283	NN050	hHR23B	Pan-specific	UV excison repair protein RAD23 homolog B	NP_002865	P54727
284	NN051	Hip	Pan-specific	Hsp70/Hsc70 interacting protein (ST13)	NP_003923	P50502
285	PN035	Histone H1	phospho CDK1 sites	Histone H1 phosphorylated	NP_005316	Q02539
286	PN036	Histone	S140	Histone H2A variant X	NP_002096	P16104
287	PN037	Histone	S15	Histone H2B	NP_778225	P33778
288	PN038	Histone H3	S11	Histone H3.3	NP_003521	P84243
289	PN039	Histone H3	S29	Histone H3.3	NP_003521	P84243
290	PN100	Histone H3	T12	Histone H3.3	NP_003521	P84243
291	PN101	Histone H3	T4	Histone H3.3	NP_003521	P84243
292	PN101-2	Histone H3	T4	Histone H3.3	NP_003521	P84243
293	NN052	HO1	Pan-specific	Heme oxygenase 1	NP_002124	P09601
294	NN053	HO2	Pan-specific	Heme oxygenase 2	NP_002125	P30519
295	NK072	Hpk1	Pan-specific	Hematopoetic progenitor protein-serine kinase 1	NP_009112	O92918
296	NN054	Hsc70	Pan-specific	Heat shock 70 kDa protein 8	NP_006588	P11142
297	NN054-2	Hsc70	Pan-specific	Heat shock 70 kDa protein 8	NP_006588	P11142
298	NN055	HSF4	Pan-specific	Heat shock transcription factor 4	NP_001529	O9ULV5
299	NN062	Hsp105	Pan-specific	Heat shock 105 kDa protein	NP_006635	O92598
300	NN152-1	Hsp27	Pan-specific	Heat shock 27 kDa protein beta 1 (HspB1)	NP_001531	P04792
301	PN040-1	Hsp27	S15	Heat shock 27 kDa protein beta 1 (HspB1)	NP_001531	P04792
302	PN040-2	Hsp27	S15	Heat shock 27 kDa protein beta 1 (HspB1)	NP_001531	P04792
303	PN041	Hsp27	S78	Heat shock 27 kDa protein beta 1 (HspB1)	NP_001531	P04792
304	PN042-1	Hsp27	S82	Heat shock 27 kDa protein beta 1 (HspB1)	NP_001531	P04792
305	PN042-2	Hsp27	S82	Heat shock 27 kDa protein beta 1 (HspB1)	NP_001531	P04792
306	PN042-3	Hsp27	S82	Heat shock 27 kDa protein beta 1 (HspB1)	NP_001531	P04792
307	NN057	Hsp40	Pan-specific	DnaJ homolog, subfamily B member 1	NP_006136	P25685
308	NN057-2	Hsp40	Pan-specific	DnaJ homolog, subfamily B member 1	NP_006136	P25685
309	NN057-3	Hsp40	Pan-specific	DnaJ homolog, subfamily B member 1	NP_006136	P25685
310	NN058	Hsp47	Pan-specific	Heat shock 47 kDa protein (collagen-binding	NP_001226	P29043
311	NN059-1	Hsp60	Pan-specific	Heat shock 60 kDa protein 1 (chaperonin,	NP_002147	P10809
312	NN059-2	Hsp60	Pan-specific	Heat shock 60 kDa protein 1 (chaperonin,	NP_002147	P10809
313	NN059-3	Hsp60	Pan-specific	Heat shock 60 kDa protein 1 (chaperonin,	NP_002147	P10809
314	NN060	Hsp70	Pan-specific	Heat shock 70 kDa protein 1	NP_005336	P08107
315	NN060-2	Hsp70	Pan-specific	Heat shock 70 kDa protein 1	NP_005336	P08107
316	NN060-3	Hsp70	Pan-specific	Heat shock 70 kDa protein 1	NP_005336	P08107
317	NN164	Hsp90a	Pan-specific	Heat shock 90 kDa protein alpha	NP_005339	P07900
318	NN061	Hsp90a/b	Pan-specific	Heat shock 90 kDa protein alpha/beta	NP_005339	P07900
319	NN061-16	Hsp90a/b	Pan-specific	Heat shock 90 kDa protein alpha/beta	NP_005339	P07900

320	NN061-2	Hsp90a/b	Pan-specific	Heat shock 90 kDa protein alpha/beta	NP_005339	P07900
321	NN061-3	Hsp90a/b	Pan-specific	Heat shock 90 kDa protein alpha/beta	NP_005339	P07900
322	NN061-4	Hsp90a/b	Pan-specific	Heat shock 90 kDa protein alpha/beta	NP_005339	P07900
323	NN165	Hsp90b	Pan-specific	Heat shock 90 kDa protein beta	NP_031381	P08238
324	NN165-1	Hsp90b	Pan-specific	Heat shock 90 kDa protein beta	NP_031381	P08238
325	PN176	Hsp90b	S255	Heat shock 90 kDa protein beta	NP_031381	P08238
326	NN063	HspBP1	Pan-specific	Hsp70 binding protein 1	NP_036399	Q9NZL4
327	PN103	Huntingtin	S421	Huntington's disease protein	NP_002102	P42858
328	NN130	I1PP2A	Pan-specific	Acidic leucine-rich nuclear phosphoprotein 32	NP_006296	P39687
329	NN131	I2PP2A	Pan-specific	Protein SET	NP_003002	Q01105
330	NK073	ICK	Pan-specific	Intestinal cell protein-serine kinase (MAK-related	NP_057597	Q9UPZ9
331	NK074	IGF1R	Pan-specific	Insulin-like growth factor 1 receptor protein-	NP_000866	P08069
332	PK152	IGF1R	Y1280	Insulin-like growth factor 1 receptor protein-	NP_000866	P08069
333	PK153	IGF1R	Y1165/Y1166	Insulin-like growth factor 1 receptor protein-	NP_000866	P08069
334	PK139	IGF1Rb/IR	Y1161/Y1185	Insulin-like growth factor 1 /Insulin	NP_000866	P08069
335	NN064	IkBa	Pan-specific	Inhibitor of NF-kappa-B alpha (MAD3)	NP_065390	P25963
336	NN064-2	IkBa	Pan-specific	Inhibitor of NF-kappa-B alpha (MAD3)	NP_065390	P25963
337	NN064-4	IkBa	Pan-specific	Inhibitor of NF-kappa-B alpha (MAD3)	NP_065390	P25963
338	PN164	IkBa	Y42	Inhibitor of NF-kappa-B alpha (MAD3)	NP_065390	P25963
339	NN065	IkBb	Pan-specific	Inhibitor of NF-kappa-B beta (thyroid receptor	NP_002494	Q15653
340	PN168	IkBe	S22	NF-kappa-B inhibitor epsilon	NP_004547.2	O00221
341	NK075-1	IKKa	Pan-specific	Inhibitor of NF-kappa-B protein-serine kinase	NP_001269	O15111
342	NK075-2	IKKa	Pan-specific	Inhibitor of NF-kappa-B protein-serine kinase	NP_001269	O15111
343	NK075-3	IKKa	Pan-specific	Inhibitor of NF-kappa-B protein-serine kinase	NP_001269	O15111
344	NK075-4	IKKa	Pan-specific	Inhibitor of NF-kappa-B protein-serine kinase	NP_001269	O15111
345	NK075-5	IKKa	Pan-specific	Inhibitor of NF-kappa-B protein-serine kinase	NP_001269	O15111
346	NK075-6	IKKa	Pan-specific	Inhibitor of NF-kappa-B protein-serine kinase	NP_001269	O15111
347	PK030-	IKKa	S180	Inhibitor of NF-kappa-B protein-serine kinase	NP_001269	O15111
348	PK154	IKKa	T23	Inhibitor of NF-kappa-B protein-serine kinase	NP_001269	O15111
349	NK076-1	IKKb	Pan-specific	Inhibitor of NF-kappa-B protein-serine kinase	NP_001547	O14920
350	NK076-2	IKKb	Pan-specific	Inhibitor of NF-kappa-B protein-serine kinase	NP_001547	O14920
351	NK076-3	IKKb	Pan-specific	Inhibitor of NF-kappa-B protein-serine kinase	NP_001547	O14920
352	NK076-4	IKKb	Pan-specific	Inhibitor of NF-kappa-B protein-serine kinase	NP_001547	O14920
353	NN161	IKKg	Pan-specific	I-kappa-B kinase gamma/NF-kappa-B essential	NP_003630	Q9Y6K9
354	NK078-2	ILK1	Pan-specific	Integrin-linked protein-serine kinase 1	NP_034692	Q13418
355	NK078-3	ILK1	Pan-specific	Integrin-linked protein-serine kinase 1	NP_034692	Q13418
356	PN043	Integrin a4	S1027	Integrin alpha 4 (VLA4)	NP_000876	P13612
357	PN044	Integrin b1	S785	Integrin beta 1 (fibronectin receptor beta	NP_002202	P05556
358	NK079	IR (INSR)	Pan-specific	Insulin receptor beta chain	NP_000199	P06213
359	PK032-1	IR (INSR)	Y999	Insulin receptor	NP_000199	P06213
360	PK033	IR/IGF1R	Y1189/Y1190	Insulin receptor / Insulin-like growth factor 1	NP_000866	P06213
361	NK080	IRAK1	Pan-specific	Interleukin 1 receptor-associated kinase 1	NP_001560	P51617
362	NK080-2	IRAK1	Pan-specific	Interleukin 1 receptor-associated kinase 1	NP_001560	P51617
363	NK081	IRAK2	Pan-specific	Interleukin 1 receptor-associated kinase 2	NP_001561	O43187
364	NK081-2	IRAK2	Pan-specific	Interleukin 1 receptor-associated kinase 2	NP_001561	O43187
365	NK082	IRAK3	Pan-specific	Interleukin 1 receptor-associated kinase 3	NP_009130	Q9Y616
366	NK083-1	IRAK4	Pan-specific	Interleukin 1 receptor-associated kinase 4	NP_057207	Q9NWZ3
367	NK083-2	IRAK4	Pan-specific	Interleukin 1 receptor-associated kinase 4	NP_057207	Q9NWZ3
368	PN045	IRS1	Y612	Insulin receptor substrate 1	NP_005535	P35568
369	PN046-2	IRS1	Y1179	Insulin receptor substrate 1	NP_005535	P35568
370	PN117	IRS1	S312	Insulin receptor substrate 1	NP_005535	P35568
371	PN118	IRS1	S639	Insulin receptor substrate 1	NP_005535	P35568
372	NK084-1	JAK1	Pan-specific	Janus protein-tyrosine kinase 1	NP_002218	P23458
373	NK084-2	JAK1	Pan-specific	Janus protein-tyrosine kinase 1	NP_002218	P23458
374	PK126	JAK1	Y1034	Janus protein-tyrosine kinase 1	NP_002218	P23458
375	NK085	JAK2	Pan-specific	Janus protein-tyrosine kinase 2	NP_004963	O60674
376	PK034-1	JAK2	Y1007+Y1008	Janus protein-tyrosine kinase 2	NP_004963	O60674
377	PK034-2	JAK2	Y1007+Y1008	Janus protein-tyrosine kinase 2	NP_004963	O60674
378	NK086	JAK3	Pan-specific	Janus protein-tyrosine kinase 3	NP_000206	P52333
379	NK087	TAO3 (JIK)	Pan-specific	STE20-like protein-serine kinase	NP_057365	Q9H2K8
380	NK217	JNK1	Pan-specific	Jun N-terminus protein-serine kinase (stress-	NP_620637.1	P45983
381	NK088-1	JNK1/2/3	Pan-specific	Jun N-terminus protein-serine kinase (stress-	NP_002741	P45983
382	NK088-2	JNK1/2/3	Pan-specific	Jun N-terminus protein-serine kinase (stress-	NP_002741	P45983
383	NK088-3	JNK1/2/3	Pan-specific	Jun N-terminus protein-serine kinase (stress-	NP_002741	P45983
384	PK035-1	JNK1/2/3	T183 + Y185	Jun N-terminus protein-serine kinase (stress-	NP_002741	P45983
385	PK035-2	JNK1/2/3	T183 + Y185	Jun N-terminus protein-serine kinase (stress-	NP_002741	P45983
386	PK035-4	JNK1/2/3	T183 + Y185	Jun N-terminus protein-serine kinase (stress-	NP_002741	P45983
387	NK189	JNK2	Pan-specific	Jun N-terminus protein-serine kinase (stress-	NP_002744	P45984
388	NK196	JNK2/3	Pan-specific	Jun N-terminus protein-serine kinase (stress-	NP_002743.3	P45984
389	NK197	JNK3	Pan-specific	Jun N-terminus protein-serine kinase (stress-	NP_002744.1	P53779
390	NN162	Jun	Pan-specific	Jun proto-oncogene-encoded AP1 transcription	NP_002219	P05412
391	PN047	Jun	S63	Jun proto-oncogene-encoded AP1 transcription	NP_002219	P05412
392	PN048-1	Jun	S73	Jun proto-oncogene-encoded AP1 transcription	NP_002219	P05412
393	PN048-2	Jun	S73	Jun proto-oncogene-encoded AP1 transcription	NP_002219	P05412
394	PN154	Jun	S243	Jun proto-oncogene-encoded AP1 transcription	NP_002219	P05412
395	PN155	Jun	Y170	Jun proto-oncogene-encoded AP1 transcription	NP_002219	P05412
396	PN163	Jun	T91	Jun proto-oncogene-encoded AP1 transcription	NP_002219	P05412
397	NP004	KAP	Pan-specific	Cyclin-dependent kinase associated	NP_005183	Q16667
398	NN153	KDEL	Pan-specific	ER lumen protein retaining receptor 1	NP_006792.1	P24390
399	NK089	KHS	Pan-specific	Kinase homologous to SPS1/STE20	NP_006566	Q9Y4K4

400	PK036	Kit	Y703	Kit/Steel factor receptor-tyrosine kinase	NP_006566	P10721
401	PK037	Kit	Y730	Kit/Steel factor receptor-tyrosine kinase	NP_006566	P10721
402	PK038	Kit	Y936	Kit/Steel factor receptor-tyrosine kinase	NP_006566	P10721
403	PK150	Kit	Y721	Kit/Steel factor receptor-tyrosine kinase	NP_006566	P10721
404	NK090	Ksr1	Pan-specific	Protein-serine kinase suppressor of Ras 1	NP_055053.1	O8IVT5
405	NK090-2	Ksr1	Pan-specific	Protein-serine kinase suppressor of Ras 1	NP_055053.1	O8IVT5
406	NP005	LAR	Pan-specific	LCA antigen-related (LAR) receptor tyrosine	NP_002831	P10586
407	NK091	LATS1	Pan-specific	Large tumor suppressor 1 protein-serine kinase	NP_004681	O95835
408	NK092-2	Lck	Pan-specific	Lymphocyte-specific protein-tyrosine kinase	NP_005347	P06239
409	NK092-3	Lck	Pan-specific	Lymphocyte-specific protein-tyrosine kinase	NP_005347	P06239
410	PK039	Lck	S158	Lymphocyte-specific protein-tyrosine kinase	NP_005347	P06239
411	PK040	Lck	Y192	Lymphocyte-specific protein-tyrosine kinase	NP_005347	P06239
412	PK041	Lck	Y505	Lymphocyte-specific protein-tyrosine kinase	NP_005347	P06239
413	PK149	Lck	Y394	Lymphocyte-specific protein-tyrosine kinase	NP_005347	P06239
414	NK093	LIMK1	Pan-specific	LIM domain kinase 1	NP_002305	P53667
415	PK042-	LIMK1	Y507+T508	LIM domain kinase 1	NP_002305	P53667
416	NK095	Lyn	Pan-specific	Yes-related protein-tyrosine kinase	NP_002341	P07948
417	PK043	Lyn	Y508	Yes-related protein-tyrosine kinase	NP_002341	P07948
418	NK096	MAK	Pan-specific	Male germ cell-associated protein-serine kinase	NP_005897	P20794
419	NK097	MAPKAPK	Pan-specific	Mitogen-activated protein kinase-activated	NP_116584	P49137
420	PK044	MAPKAPK	T222	Mitogen-activated protein kinase-activated	NP_004750	P49137
421	PN049-	MAPKAPK	T334	Mitogen-activated protein kinase-activated	NP_004750	P49137
422	PN049-	MAPKAPK	T334	Mitogen-activated protein kinase-activated	NP_004750	P49137
423	PN050-1	MARCKS	S159+S163	Myristoylated alanine-rich protein kinase C	NP_002347	P29966
424	NK098	MARK	Pan-specific	MAP/microtubule affinity-regulating protein-	NP_061120	O9P0L2
425	NN067	Mcl1	Pan-specific	Myeloid cell leukemia differentiation protein 1	NP_068779	Q07820
426	PN169	MDM2	S166	double minute 2	NP_002383.2	Q00987
427	NN155	MEF-2	Pan-specific	Myelin expression factor 2 (MYEF2)	NP_057216.2	O9P2K5
428	NK099-1	MEK1	Pan-specific	MAPK/ERK protein-serine kinase 1 (MKK1)	NP_002746	Q02750
429	NK099-2	MEK1	Pan-specific	MAPK/ERK protein-serine kinase 1 (MKK1)	NP_002746	Q02750
430	NK099-3	MEK1	Pan-specific	MAPK/ERK protein-serine kinase 1 (MKK1)	NP_002746	Q02750
431	NK099-4	MEK1	Pan-specific	MAPK/ERK protein-serine kinase 1 (MKK1)	NP_002746	Q02750
432	NK099-5	MEK1	Pan-specific	MAPK/ERK protein-serine kinase 1 (MKK1)	NP_002746	Q02750
433	PK046-1	MEK1	T292	MAPK/ERK protein-serine kinase 1 (MKK1)	NP_002746	Q02750
434	PK046-2	MEK1	T292	MAPK/ERK protein-serine kinase 1 (MKK1)	NP_002746	Q02750
435	PK046-3	MEK1	T292	MAPK/ERK protein-serine kinase 1 (MKK1)	NP_002746	Q02750
436	PK047-2	MEK1	S298	MAPK/ERK protein-serine kinase 1 (MKK1)	NP_002746	Q02750
437	PK048-1	MEK1	T386	MAPK/ERK protein-serine kinase 1 (MKK1)	NP_002746	Q02750
438	PK048-2	MEK1	T386	MAPK/ERK protein-serine kinase 1 (MKK1)	NP_002746	Q02750
439	PK048-3	MEK1	T386	MAPK/ERK protein-serine kinase 1 (MKK1)	NP_002746	Q02750
440	PK045-	MEK1/2	S218+S222	MAPK/ERK protein-serine kinase 1/2 (MKK1/2)	NP_002746	Q02750
441	NK221	MEK1/2	Pan-specific	MAPK/ERK protein-serine kinase 1/2 (MKK1/2)	NP_002746	Q02750
442	NK100-1	MEK2	Pan-specific	MAPK/ERK protein-serine kinase 2 (MKK2)	AAH00471.1	P36507
443	NK100-2	MEK2	Pan-specific	MAPK/ERK protein-serine kinase 2 (MKK2)	AAH00471.1	P36507
444	NK100-3	MEK2	Pan-specific	MAPK/ERK protein-serine kinase 2 (MKK2)	AAH00471.1	P36507
445	PK049	MEK2	T394	MAPK/ERK protein-serine kinase 2 (MKK2)	AAH00471.1	P36507
446	PK049-2	MEK2	T394	MAPK/ERK protein-serine kinase 2 (MKK2)	AAH00471.1	P36507
447	PK050	MEK2	T394	MAPK/ERK protein-serine kinase 2 (MKK2)	NP_075627	P36507
448	NK101	MEK3	Pan-specific	MAPK/ERK protein-serine kinase 3 (MKK3)	NP_659732	P46734
449	NK101-3	MEK3	Pan-specific	MAPK/ERK protein-serine kinase 3 (MKK3)	NP_659732	P46734
450	PK127	MEK3	S218	MAPK/ERK protein-serine kinase 3 (MKK3)	NP_659732	P46734
451	PK051	MEK3/6	S218/S207	MAPK/ERK protein-serine kinase 3/6 (MKK3/6)	NP_002747	P46734
452	PK051-2	MEK3/6	S218/S207	MAPK/ERK protein-serine kinase 3/6 (MKK3/6)	NP_002747	P46734
453	PK051-3	MEK3/6	S218/S207	MAPK/ERK protein-serine kinase 3/6 (MKK3/6)	NP_002747	P46734
454	NK102	MEK3b	Pan-specific	MAPK/ERK protein-serine kinase 3 beta isoform	NP_659731	P46734
455	NK103	MEK4	Pan-specific	MAPK/ERK protein-serine kinase 4 (MKK4)	NP_003001	P45985
456	NK103-2	MEK4	Pan-specific	MAPK/ERK protein-serine kinase 4 (MKK4)	NP_003001	P45985
457	NK103-3	MEK4	Pan-specific	MAPK/ERK protein-serine kinase 4 (MKK4)	NP_003001	P45985
458	PK052	MEK4	S257+T261	MAPK/ERK protein-serine kinase 4 (MKK4)	NP_003001	P45985
459	PK155	MEK5	S311+T315	MAPK/ERK protein-serine kinase 5 (MKK5)	NP_660143	Q13163
460	NK104	MEK5	Pan-specific	MAPK/ERK protein-serine kinase 5 (MKK5)	NP_660143	Q13163
461	NK104-2	MEK5	Pan-specific	MAPK/ERK protein-serine kinase 5 (MKK5)	NP_660143	Q13163
462	NK105-1	MEK6	Pan-specific	MAPK/ERK protein-serine kinase 6 (MKK6)	NP_002749	P52564
463	PK129	MEK6	S207	MAPK/ERK protein-serine kinase 6 (MKK6)	NP_002749	P52564
464	NK106-2	MEK7	Pan-specific	MAPK/ERK protein-serine kinase 7 (MKK7)	NP_005034	O14733
465	NK107	MEKK1	Pan-specific	MAPK/ERK kinase kinase 1	NP_005912.1	Q13233
466	NK107-2	MEKK1	Pan-specific	MAPK/ERK kinase kinase 1	NP_005912.1	Q13233
467	NK107-3	MEKK1	Pan-specific	MAPK/ERK kinase kinase 1	NP_005912.1	Q13233
468	NK107-4	MEKK1	Pan-specific	MAPK/ERK kinase kinase 1	NP_005912.1	Q13233
469	NK108	MEKK2	Pan-specific	MAPK/ERK kinase kinase 2	NP_006600.3	O9Y2U5
470	NK108-2	MEKK2	Pan-specific	MAPK/ERK kinase kinase 2	NP_006600.3	O9Y2U5
471	NK109	MEKK4	Pan-specific	MAPK/ERK kinase kinase 4	NP_005913	O9Y6R4
472	PK055-1	Met	Y1230+Y1234+Y1235	Hepatocyte growth factor (HGF) receptor-	NP_000236.	P08581
473	NP006	MKP1	Pan-specific	MAP kinase phosphatase 1 (VH1, DUSP1)	NP_004408	P28562
474	NP007	MKP2	Pan-specific	MAP kinase phosphatase 2 (VH2)	NP_001385	Q13115
475	PN051-1	MLC(MLR	S19	Myosin regulatory light chain 2, smooth muscle	NP_291024	P19105
476	PN051-2	MLC(MLR	S19	Myosin regulatory light chain 2, smooth muscle	NP_291024	P19105
477	NK208	MLK3	Pan-specific	Mixed-lineage protein-serine kinase 3	NP_002410	Q16584
478	PK056	MLK3	T277+S281	Mixed-lineage protein-serine kinase 3	NP_002410	Q16584
479	NN132	mMOB1	Pan-specific	Preimplantation protein 3	NP_056202	O9Y3A3

480	PK057	Mnk1	T250+T255	MAP kinase-interacting protein-serine kinase 1	NP_003675	Q9BUB5
481	NK111	Mnk2	Pan-specific	MAP kinase-interacting protein-serine kinase 2	NP_060042	Q9HBH9
482	NN069	MSH2	Pan-specific	DNA mismatch repair protein mutS homolog2,	NP_000242	P43246
483	PK058	Msk1	S376	Mitogen & stress-activated protein-serine kinase	NP_004746	O75582
484	NK113-1	MST1	Pan-specific	Mammalian STE20-like protein-serine kinase 1	NP_006273	Q13043
485	NK113-2	MST1	Pan-specific	Mammalian STE20-like protein-serine kinase 1	NP_006273	Q13043
486	NK113-3	MST1	Pan-specific	Mammalian STE20-like protein-serine kinase 1	NP_006273	Q13043
487	NK113-4	MST1	Pan-specific	Mammalian STE20-like protein-serine kinase 1	NP_006273	Q13043
488	NK114	MST2	Pan-specific	Mammalian STE20-like protein-serine kinase 2	NP_006272	Q13188
489	NK115	MST3	Pan-specific	Mammalian STE20-like protein-serine kinase 3	NP_003567	Q9Y6E0
490	PK116	mTOR	S2448	Mammalian target of rapamycin (FRAP)	NP_004949	P42345
491	PN186	Myc	S373	Myc proto-oncogene protein	NP_002458.2	P01106
492	PN199	Myc	T58	Myc proto-oncogene protein	NP_002458.2	P01106
493	PN182	MyoD	S200	Myoblast determination protein 1	NP_002469.2	P15172
494	PN052	MYPT1	T696	Myosin phosphatase target 1	NP_446342	O14974
495	PN187	NBS1	S343	Nijmegen breakage syndrome protein 1	NP_002476.2	O60934
496	NK117-1	Nek2	Pan-specific	NIMA (never-in-mitosis)-related protein-serine	NP_002488	P51955
497	NK117-2	Nek2	Pan-specific	NIMA (never-in-mitosis)-related protein-serine	NP_002488	P51955
498	NK117-3	Nek2	Pan-specific	NIMA (never-in-mitosis)-related protein-serine	NP_002488	P51955
499	NK117-4	Nek2	Pan-specific	NIMA (never-in-mitosis)-related protein-serine	NP_002488	P51955
500	NK117-5	Nek2	Pan-specific	NIMA (never-in-mitosis)-related protein-serine	NP_002488	P51955
501	NK118	Nek4	Pan-specific	NIMA (never-in-mitosis)-related protein-serine	NP_003148	P51957
502	NK119	Nek7	Pan-specific	NIMA (never-in-mitosis)-related protein-serine	NP_598001	Q8TDX7
503	NN070	NFkappaB	Pan-specific	NF-kappa-B p50 nuclear transcription factor	NP_003989	P19838
504	NN071	NFkappaB	Pan-specific	NF-kappa-B p65 nuclear transcription factor	NP_003989	Q04206
505	PN053-1	NFkappaB	S276	NF-kappa-B p65 nuclear transcription factor	NP_003989	Q04206
506	PN156	NFkappaB	S529	NF-kappa-B p65 nuclear transcription factor	NP_003989	Q04206
507	PN157	NFkappaB	S536	NF-kappa-B p65 nuclear transcription factor	NP_003989	Q04206
508	NK207	NIK	Pan-specific	NF-kappa beta-inducing kinase	NP_003945.2	Q99558
509	NK212	NLK	Pan-specific	Serine/threonine protein kinase NLK	NP_057315.3	Q9UBE8
510	PN054	NMDAR2B	Y1474	N-methyl-D-aspartate (NMDA) glutamate	NP_000825	Q13224
511	NN074	NME7	Pan-specific	Nucleotide diphosphate kinase 7 (nm23-H7)	NP_037462	Q9Y5B8
512	PN055-1	NR1	S896	N-methyl-D-aspartate (NMDA) glutamate	NP_000823	Q05586
513	NN075	NT5E	Pan-specific	Ecto-5'-nucleotidase (CD73 antigen)	NP_002517	P21589
514	NN083	p107	Pan-specific	Retinoblastoma (Rb) protein-related p107	NP_002886.2	P28749
515	NN077	p18 INK4c	Pan-specific	p18 INK4c cyclin-dependent kinase inhibitor	NP_523240	P42773
516	NN078	p21 CDKI1	Pan-specific	cyclin-dependent kinase inhibitor 1 (MDA6)	NP_000380	P38936
517	NN081-	p25	Pan-specific	CDK5 regulatory subunit p25	NP_003876.1	Q15078
518	NN080	p27 Kip1	Pan-specific	p27 cyclin-dependent kinase inhibitor 1B	NP_004055	P46527
519	PN056	p27 Kip1	T187	p27 cyclin-dependent kinase inhibitor 1B	NP_004055	P46527
520	PK060-1	p38a	T180+Y182	Mitogen-activated protein-serine kinase p38	NP_001306	Q16539
521	PK060-2	p38a	T180+Y182	Mitogen-activated protein-serine kinase p38	NP_001306	Q16539
522	PK060-3	p38a	T180+Y182	Mitogen-activated protein-serine kinase p38	NP_001306	Q16539
523	NK120-1	p38a	Pan-specific	Mitogen-activated protein-serine kinase p38	NP_001306	Q16539
524	NK120-10	p38a	Pan-specific	Mitogen-activated protein-serine kinase p38	NP_001306	Q16539
525	NK120-2	p38a	Pan-specific	Mitogen-activated protein-serine kinase p38	NP_001306	Q16539
526	NK120-3	p38a	Pan-specific	Mitogen-activated protein-serine kinase p38	NP_001306	Q16539
527	NK120-4	p38a	Pan-specific	Mitogen-activated protein-serine kinase p38	NP_001306	Q16539
528	NK120-5	p38a	Pan-specific	Mitogen-activated protein-serine kinase p38	NP_001306	Q16539
529	NK120-7	p38a	Pan-specific	Mitogen-activated protein-serine kinase p38	NP_001306	Q16539
530	NK059-1	p38g	Pan-specific	Mitogen-activated protein-serine kinase p38	NP_002960	P53778
531	NK059-2	p38g	Pan-specific	Mitogen-activated protein-serine kinase p38	NP_002960	P53778
532	NN082	p53	Pan-specific	Tumor suppressor protein p53	NP_000537	P04637
533	PN057-1	p53	S392	Tumor suppressor protein p53	NP_000537	P04637
534	PN057-2	p53	S392	Tumor suppressor protein p53	NP_000537	P04637
535	PN057-3	p53	S392	Tumor suppressor protein p53	NP_000537	P04637
536	PN158	p53	S33	Tumor suppressor protein p53	NP_000537	P04637
537	PN159	p53	S37	Tumor suppressor protein p53	NP_000537	P04637
538	PN160	p53	S6	Tumor suppressor protein p53	NP_000537	P04637
539	NN123	p73	Pan-specific	Tumor suppressor protein p73	NP_005418	O15350
540	NP008	PAC1	Pan-specific	Dual specificity MAP kinase protein	NP_004409	Q05923
541	NN084	PACSIN1	Pan-specific	Protein kinase C + casein kinase substrate in	NP_065855	Q9BY11
542	NK122	PAK1	Pan-specific	p21-activated kinase 1 (alpha)	NP_002567	Q13153
543	NK122-2	PAK1	Pan-specific	p21-activated kinase 1 (alpha)	NP_002567	Q13153
544	NK122-4	PAK1	Pan-specific	p21-activated kinase 1 (alpha)	NP_002567	Q13153
545	PK130	PAK1	T212	p21-activated kinase 1 (alpha)	NP_002567	Q13153
546	NK224	PAK1/2/3	Pan-specific	p21-activated kinase 1/2/3	NP_002567	Q13153
547	PK061	PAK1/2/3	S144/S141/S154	p21-activated kinase 1/2/3	NP_002567	Q13153
548	NK200	PAK2	Pan-specific	p21-activated kinase 2 (gamma)	NP_002568.2	Q13177
549	NK200-2	PAK2	Pan-specific	p21-activated kinase 2 (gamma)	NP_002568.2	Q13177
550	NK123	PAK3	Pan-specific	p21-activated kinase 3 (beta) (serine/threonine-	NP_002569	O75914
551	NN085-1	PARP1	Pan-specific	Poly [ADP-ribose] polymerase 1 (ADPRT)	NP_001609	P09874
552	NN085-2	PARP1	Pan-specific	Poly [ADP-ribose] polymerase 1 (ADPRT)	NP_001609	P09874
553	PN058	Pax2	S394	Paired box protein 2	NP_003978	Q02962
554	NN086	Paxillin 1	Pan-specific	Paxillin 1	NP_002850	P49023
555	PN059	Paxillin 1	Y31	Paxillin 1	NP_002850	P49023
556	PN060-1	Paxillin 1	Y118	Paxillin 1	NP_002850	P49023
557	PN060-3	Paxillin 1	Y118	Paxillin 1	NP_002850	P49023
558	NN087	PCNA	Pan-specific	Proliferating cell nuclear antigen	NP_002583	P12004
559	NK125	PCTK1	Pan-specific	PCTAIRE-1 protein-serine kinase	NP_148978	O00536

560	PK063	PDGFRa	Y754	Platelet-derived growth factor receptor kinase	NP_006197	P16234
561	PK065	PDGFRb	Y716	Platelet-derived growth factor receptor kinase	NP_032835	P09619
562	NN141-1	PDI	Pan-specific	Protein disulfide-isomerase	NP_000909.2	P07237
563	NK126-1	PDK1	Pan-specific	3-phosphoinositide-dependent protein-serine	NP_002604	O15530
564	NK126-2	PDK1	Pan-specific	3-phosphoinositide-dependent protein-serine	NP_002604	O15530
565	PK066	PDK1	S241	3-Phosphoinositide-dependent protein-serine	NP_002604	O15530
566	PN061	PED15	S116	Phosphoprotein-enriched in diabetes/astrocytes	NP_003759	O15121
567	NN088	PERP	Pan-specific	p53-induced protein PIGPC1	NP_071404	Q9H230
568	NN089	PI3K	Pan-specific	Phosphatidylinositol 3-kinase regulatory subunit	NP_852664	P27986
569	PN127	PI3K	Y467/Y199	Phosphatidylinositol 3-kinase regulatory subunit	NP_852664	P27986
570	NN114	PI3KR4	Pan-specific	Phosphoinositide-3-kinase, regulatory subunit 4	NP_055417	Q99570
571	NK192	PI4KCB	Pan-specific	phosphatidylinositol 4-kinase, catalytic, beta	NP_002642	Q5VWC1
572	NK209	PIP5K2a	Pan-specific	Phosphatidylinositol 4-phosphatase 5-kinase	NP_005019.2	P48426
573	NK213	PITSLRE	Pan-specific	PITSLRE serine/threonine-protein kinase	NP_277021.1	P21127
574	NK127-1	PKA Ca/b	Pan-specific	cAMP-dependent protein-serine kinase catalytic	NP_002721	P17612
575	PK067	PKA Ca/b	T198	cAMP-dependent protein-serine kinase catalytic	NP_002721	P17612
576	PK068	PKA Cb	S339	cAMP-dependent protein-serine kinase catalytic	NP_002722	P22694
577	NN116	PKA R1a	Pan-specific	cAMP-dependent protein-serine kinase type I	NP_002725	P10644
578	NK128	PKA R2a	Pan-specific	cAMP-dependent protein-serine kinase	NP_004148	P13861
579	PK069	PKA R2a	S99	cAMP-dependent protein-serine kinase	NP_523671	P13861
580	NK129	PKBa	Pan-specific	Protein-serine kinase B alpha	NP_005154	P31749
581	NK129-2	PKBa	Pan-specific	Protein-serine kinase B alpha	NP_005154	P31749
582	PK071-2	PKBa	T308	Protein-serine kinase B alpha	NP_005154	P31749
583	PK072-1	PKBa	S473	Protein-serine kinase B alpha	NP_005154	P31749
584	PK072-3	PKBa	S473	Protein-serine kinase B alpha	NP_005154	P31749
585	PK072-5	PKBa	S473	Protein-serine kinase B alpha	NP_005154	P31749
586	PK148	PKBa	Y474	Protein-serine kinase B alpha	NP_005154	P31749
587	NK130-1	PKBb	Pan-specific	Protein-serine kinase B beta	NP_001617	P31751
588	NK130-2	PKBb	Pan-specific	Protein-serine kinase B beta	NP_001617	P31751
589	NK130-3	PKBb	Pan-specific	Protein-serine kinase B beta	NP_001617	P31751
590	NK130-5	PKBb	Pan-specific	Protein-serine kinase B beta	NP_001617	P31751
591	NK130-6	PKBb	Pan-specific	Protein-serine kinase B beta	NP_001617	P31751
592	NK130-7	PKBb	Pan-specific	Protein-serine kinase B beta	NP_001617	P31751
593	NK131-1	PKBg	Pan-specific	Protein-serine kinase B gamma	NP_005456	O9Y243
594	NK131-2	PKBg	Pan-specific	Protein-serine kinase B gamma	NP_005456	O9Y243
595	NK201	PKC	Pan-specific	Protein-serine kinase C alpha	NP_002728	P17252
596	NK218	PKCh	Pan-specific	Protein kinase C eta type	NP_006246.2	P24723
597	NK132	PKCa	Pan-specific	Protein-serine kinase C alpha	NP_002728	P17252
598	PK073	PKCa	S657	Protein-serine kinase C alpha	NP_002728	P17252
599	PK074	PKCa/b2	T638/T641	Protein-serine kinase C alpha/beta 2	NP_002728	P17252
600	NK133	PKCb1	Pan-specific	Protein-serine kinase C beta 1	NP_002729	P05771
601	NK133-2	PKCb1	Pan-specific	Protein-serine kinase C beta 1	NP_002729	P05771
602	PK075	PKCb1/2	T500	Protein-serine kinase C beta 1/2	NP_997700	P05771
603	PK075-2	PKCb1/2	T500	Protein-serine kinase C beta 1/2	NP_997700	P05771
604	NK134-2	PKCb2	Pan-specific	Protein-serine kinase C beta 2	AAA60095	P05771-2
605	PK076-2	PKCb2	T642	Protein-serine kinase C beta 2	NP_002729	P05771
606	NK135	PKCd	Pan-specific	Protein-serine kinase C delta	NP_006245	Q05655
607	PK077-1	PKCd	Y313	Protein-serine kinase C delta	NP_006245	Q05655
608	PK077-2	PKCd	Y313	Protein-serine kinase C delta	NP_006245	Q05655
609	PK078	PKCd	T507	Protein-serine kinase C delta	NP_006245	Q05655
610	PK079-1	PKCd	S645	Protein-serine kinase C delta	NP_006245	Q05655
611	PK080	PKCd	S664	Protein-serine kinase C delta	NP_006245	Q05655
612	NK136	PKCe	Pan-specific	Protein-serine kinase C epsilon	NP_005391	Q02156
613	NK136-2	PKCe	Pan-specific	Protein-serine kinase C epsilon	NP_005391	Q02156
614	PK081-1	PKCe	S729	Protein-serine kinase C epsilon	NP_005391	Q02156
615	NK137	PKCg	Pan-specific	Protein-serine kinase C gamma	NP_002730	P05129
616	PK082-1	PKCg	T514	Protein-serine kinase C gamma	NP_002730	P05129
617	PK082-2	PKCg	T514	Protein-serine kinase C gamma	NP_002730	P05129
618	PK083	PKCg	T655	Protein-serine kinase C gamma	NP_002730	P05129
619	PK084	PKCg	T674	Protein-serine kinase C gamma	NP_002730.1	P05129
620	PK085	PKCh	T655	Protein-serine kinase C eta	NP_006246	P24723
621	NK138-1	PKCl/i	Pan-specific	Protein-serine kinase C lambda/iota	NP_002731	P41743
622	PK087	PKCl/i	T564	Protein-serine kinase C lambda/iota	NP_002731	P41743
623	NK142	PKCm	Pan-specific	Protein-serine kinase C mu (Protein kinase D)	NP_002733	Q15139
624	PK092	PKCm	S738+S742	Protein-serine kinase C mu (Protein kinase D)	NP_002733	Q15139
625	PK093-1	PKCm	S910	Protein-serine kinase C mu (Protein kinase D)	NP_002733	Q15139
626	PK093-2	PKCm	S910	Protein-serine kinase C mu (Protein kinase D)	NP_002733	Q15139
627	NK140	PKCq	Pan-specific	Protein-serine kinase C theta	NP_006248	Q04759
628	PK088	PKCq	T538	Protein-serine kinase C theta	NP_006248	Q04759
629	PK089-1	PKCq	S676	Protein-serine kinase C theta	NP_006248	Q04759
630	PK090-1	PKCq	S695	Protein-serine kinase C theta	NP_006248	Q04759
631	NK141	PKCz	Pan-specific	Protein-serine kinase C zeta	NP_002735	Q05513
632	PK091	PKCz/l	T410/T412	Protein-serine kinase C zeta/lambda	NP_002735	Q05513
633	NK143	PKG1	Pan-specific	Protein-serine kinase G1 (cGMP-dependent	NP_006249	Q13976
634	NK202	PKG1a	Pan-specific	cGMP-dependent protein kinase 1, alpha	NP_006249	Q13976
635	NK203	PKG1b	Pan-specific	cGMP-dependent protein kinase 1, beta	NP_006249.1	P14619
636	NN115	PKM2	Pan-specific	Pyruvate kinase, isozymes M1/M2	NP_872270	P14618
637	NK144-1	PKR1	Pan-specific	Double-stranded RNA-dependent protein-serine	NP_002750	P19525
638	PK132	PKR1	T446	Double-stranded RNA-dependent protein-serine	NP_002750	P19525
639	NN156	PLC	Pan-specific	1-phosphatidylinositol-4,5-bisphosphate	NP_002652.2	P16885

640	PN144	PLCg1	Y783	1-phosphatidylinositol-4,5-bisphosphate	NP_877963.1	P19174
641	PN165	PLCg1	Y771	1-phosphatidylinositol-4,5-bisphosphate	NP_877963.1	P19174
642	PN143	PLC	Y753	1-phosphatidylinositol-4,5-bisphosphate	NP_002652.2	P16885
643	NK145	Plk1	Pan-specific	Polo-like protein-serine kinase 1	NP_005021	P53350
644	PK117	Plk1	T210	Polo-like protein-serine kinase 1	NP_005021	P53350
645	NK146	Plk2	Pan-specific	Polo-like protein kinase 2 (SNK)	NP_006613	Q9NYY3
646	NK146-2	Plk2	Pan-specific	Polo-like protein kinase 2 (SNK)	NP_006613	Q9NYY3
647	NK147	Plk3	Pan-specific	Polo-like protein kinase 3 (CNK)	NP_004064	Q9H4B4
648	NP009	PP1/Ca	Pan-specific	Protein-serine phosphatase 1 alpha	NP_002699	P62136
649	NP009-2	PP1/Ca	Pan-specific	Protein-serine phosphatase 1 alpha	NP_002699	P62136
650	PP001	PP1/Ca	T320	Protein-serine phosphatase 1 aphfa	NP_002699	P62136
651	NP010	PP1/Cb	Pan-specific	Protein-serine phosphatase 1 beta	NP_002700	P62140
652	NP010-2	PP1/Cb	Pan-specific	Protein-serine phosphatase 1 beta	NP_002700	P62140
653	NP011	PP1/Cg	Pan-specific	Protein-serine phosphatase 1 gama	NP_002701	P36873
654	NP033	PP2A B'	Pan-specific	Protein-serine phosphatase 2A subunit - B56	NP_001186685.1	Q15172
655	NP012	PP2A/Aa/b	Pan-specific	Protein-serine phosphatase 2A - subunit - alpha	NP_002707	P30153
656	NP035	PP2A/Bb	Pan-specific	Protein-serine phosphatase 2A - B regulatory	NP_001120853.1	Q00005
657	NP032	PP2A/Bg2	Pan-specific	Protein-serine phosphatase 2A - B regulatory	NP_001193923.1	Q9Y2T4
658	NP013-	PP2A/Ca	Pan-specific	Protein-serine phosphatase 2A alpha isoform	NP_002706	P67775
659	NP015	PP2B/Aa	Pan-specific	Protein-serine phosphatase 2B alpha isoform	NP_000935	Q08209
660	NP016-	PP2Ca	Pan-specific	Protein-serine phosphatase 2C alpha	NP_066283	P35813
661	NP012-2	PP2A/Aa/b	Pan-specific	Protein-serine phosphatase 2A alpha and beta	NP_002707	P30153
662	NP018	PP2Cd	Pan-specific	Protein-serine phosphatase 2C delta isoform	NP_110395	O15297
663	NP019	PP4/A'2	Pan-specific	Protein-serine phosphatase 4 subunit (PPX/A'2)	NP_005125	Q8TF05
664	NP020	PP4C	Pan-specific	Protein-serine phosphatase X - catalytic subunit	NP_002711	P60510
665	NP020-2	PP4C	Pan-specific	Protein-serine phosphatase X - catalytic subunit	NP_002711	P60510
666	NP021	PP5C	Pan-specific	Protein-serine phosphatase 5 - catalytic subunit	NP_006238	P53041
667	NP021-2	PP5C	Pan-specific	Protein-serine phosphatase 5 - catalytic subunit	NP_006238	P53041
668	NP022	PP6C	Pan-specific	Protein-serine phosphatase 6 - catalytic subunit	NP_002712	O00743
669	PN062	PRAS40	T246	Proline-rich Akt substrate 40 kDa (Akt1S1)	NP_115751	Q96B36
670	NK148	PRK1	Pan-specific	Protein kinase C-related protein-serine kinase 1	NP_002732	Q16512
671	PK095-	PRK1	T774	Protein kinase C-related protein-serine kinase 1	NP_002732	Q16512
672	NK149	PRK2	Pan-specific	Protein kinase C-related protein-serine kinase 2	NP_006247	Q16513
673	NK149-2	PRK2	Pan-specific	Protein kinase C-related protein-serine kinase 2	NP_006247	Q16513
674	NK150	PRKAB1	Pan-specific	5'-AMP-activated protein kinase (AMPK), beta-1	NP_006244	Q9Y478
675	NK151	WNK4	Pan-specific	Putative protein-serine kinase WNK4	NP_115763	Q96J92
676	PN104	Progestero	S294	Progesterone receptor	NP_000917	P06401
677	NK152	PRP4K	Pan-specific	Protein-serine kinase PRP4 homolog	NP_003904	Q13523
678	NN142	PSD-95	Pan-specific	Disks large homolog 4	NP_001356.1	P78352
679	NK204	PSTAIRE	Pan-specific	PSTAIRE	NA	NA
680	NP023	PTEN	Pan-specific	Phosphatidylinositol-3,4,5-trisphosphate	NP_000305	P60484
681	NP023-2	PTEN	Pan-specific	Phosphatidylinositol-3,4,5-trisphosphate	NP_000305	P60484
682	PP003	PTEN	S380+T382+S385	Phosphatidylinositol-3,4,5-trisphosphate	NP_000305	P60484
683	PP006	PTEN	S380+T382+T383	Phosphatidylinositol-3,4,5-trisphosphate	NP_000305	P60484
684	PP006-1	PTEN	S380+T382+T383	Phosphatidylinositol-3,4,5-trisphosphate	NP_000305	P60484
685	NP024	PTP1B	Pan-specific	Protein-tyrosine phosphatase 1B (PTPN1)	NP_002818	P18031
686	NP025	PTP1C	Pan-specific	Protein-tyrosine phosphatase 1C	NP_002822	P29350
687	NP026	PTP1D	Pan-specific	Protein-tyrosine phosphatase 1D	NP_002825	Q06124
688	NP026-2	PTP1D	Pan-specific	Protein-tyrosine phosphatase 1D	NP_002825	Q06124
689	PP004	PTP1D	S580	Protein-tyrosine phosphatase 1D	NP_002825	Q06124
690	NP036	PTPD1	Pan-specific	Protein-tyrosine phosphatase non-receptor type	NP_008970.2	Q16825
691	NP027	PTP-PEST	Pan-specific	Protein tyrosine phosphatase, non-receptor type	NP_001124480.1	Q05209
692	NK153	PyDK2	Pan-specific	Pyruvate dehydrogenase kinase isoform 2	NP_002602	Q15119
693	NK154	Pyk2	Pan-specific	Protein-tyrosine kinase 2	NP_004094	Q14289
694	PK097-3	Pyk2	Y579	Protein-tyrosine kinase 2	NP_004094	Q14289
695	NN150	Rab5	Pan-specific	Ras-related protein Rab-5A	NP_004153.2.	P20339
696	NN092-1	Rac1	Pan-specific	Ras-related C3 botulinum toxin substrate 1	NP_001782	P63000
697	PN063	Rac1	S71	Ras-related C3 botulinum toxin substrate 1	NP_008839	P63000
698	PN064	Rad17	S656	Rad17 homolog	NP_579921	O75943
699	NK155-2	Raf1	Pan-specific	Raf1 proto-oncogene-encoded protein-serine	NP_002871	P04049
700	NK155-3	Raf1	Pan-specific	Raf1 proto-oncogene-encoded protein-serine	NP_002871	P04049
701	NK155-4	Raf1	Pan-specific	Raf1 proto-oncogene-encoded protein-serine	NP_002871	P04049
702	PK098	Raf1	S259	Raf1 proto-oncogene-encoded protein-serine	NP_002871	P04049
703	NK205	RafA (Araf)	Pan-specific	A-Raf proto-oncogene serine/threonine-protein	NP_001645.1	P10398
704	NK205-2	RafA (Araf)	Pan-specific	A-Raf proto-oncogene serine/threonine-protein	NP_001645.1	P10398
705	NK156	RafB (Braf)	Pan-specific	RafB proto-oncogene-encoded protein-serine	NP_004324	P15056
706	NK156-2	RafB (Braf)	Pan-specific	RafB proto-oncogene-encoded protein-serine	NP_004324	P15056
707	NN093	Rb	Pan-specific	Retinoblastoma-associated protein 1	NP_000312	P06400
708	PN065	Rb	T356	Retinoblastoma-associated protein 1	NP_000312	P06400
709	PN066	Rb	S612	Retinoblastoma-associated protein 1	NP_000312	P06400
710	PN067	Rb	S780	Retinoblastoma-associated protein 1	NP_000312	P06400
711	PN068	Rb	S807	Retinoblastoma-associated protein 1	NP_000312	P06400
712	PN069	Rb	S807+S811	Retinoblastoma-associated protein 1	NP_000312	P06400
713	PN070	Rb	T821	Retinoblastoma-associated protein 1	NP_000312	P06400
714	PN071	Rb	T826	Retinoblastoma-associated protein 1	NP_000312	P06400
715	PN113	Rb	S608	Retinoblastoma-associated protein 1	NP_000312	P06400
716	PN131-1	Rb	S795	Retinoblastoma-associated protein 1	NP_000312	P06400
717	NN170	RelB	Pan-specific	Transcription factor RelB	NP_006500.2	Q01201
718	PN151	RelB	S573	Transcription factor RelB	NP_006500.2	Q01201
719	NK157	RIP2/RICK	Pan-specific	Receptor-interacting serine/threonine-protein	NP_003812	O43353

720	NK158	RIPK1	Pan-specific	Receptor-interacting protein-serine kinase 1	NP_003795	Q13546
721	NK159-1	ROKa	Pan-specific	RhoA protein-serine kinase alpha	NP_004841	O75116
722	NK159-2	ROKa	Pan-specific	RhoA protein-serine kinase alpha	NP_004841	O75116
723	NK160	ROKb	Pan-specific	RhoA protein-serine kinase beta	NP_005397	Q13464
724	NK161	RONa	Pan-specific	Macrophage-stimulating protein receptor alpha	NP_002438	O04912
725	NK162	ROR2	Pan-specific	ROR2 neurotrophic receptor-tyrosine kinase	NP_004551	O01974
726	NK163	ROS	Pan-specific	Orosomucoid 1 receptor-tyrosine kinase	NP_002935	P08922
727	NK164	RSK1	Pan-specific	Ribosomal S6 protein-serine kinase 1	NP_002944	O15418
728	NK164-2	RSK1	Pan-specific	Ribosomal S6 protein-serine kinase 1	NP_002944	O15418
729	PK157	RSK1	S363	Ribosomal S6 protein-serine kinase 1	NP_002944	O15418
730	PK158	RSK1	T348	Ribosomal S6 protein-serine kinase 1	NP_002944	O15418
731	PK099	RSK1/2	S221/S227	Ribosomal S6 protein-serine kinase 1/2	NP_002944	O15418
732	PK100	RSK1/2	S363/S369	Ribosomal S6 protein-serine kinase 1/2	NP_002944	O15418
733	PK100-2	RSK1/2	S363/S369	Ribosomal S6 protein-serine kinase 1/2	NP_002944	O15418
734	PK101-1	RSK1/2	S380/S386	Ribosomal S6 protein-serine kinase 1/2	NP_002944	O15418
735	PK101-2	RSK1/2	S380/S386	Ribosomal S6 protein-serine kinase 1/2	NP_002944	O15418
736	PK102	RSK1/2/3	T573/T577/T570	Ribosomal S6 protein-serine kinase 1/2/3	NP_002944	O15418
737	PK103	RSK1/3	T359+S363/T356+S360	Ribosomal S6 protein-serine kinase 1/3	NP_002944	O15418
738	NK165	RSK2	Pan-specific	Ribosomal S6 protein-serine kinase 2	NP_004577	P51812
739	NK167	RYK	Pan-specific	RYK tyrosine-protein kinase	NP_001005861.1	P34925
740	PN073	S6	S235	40S ribosomal protein S6	NP_001001	P62753
741	PK156	S6K	S424	p70 ribosomal protein-serine S6 kinase	NP_003152	P23443
742	PK166	S6K	S411	p70 ribosomal protein-serine S6 kinase	NP_003152	P23443
743	NK223	S6Kb1	Pan-specific	Ribosomal protein-serine S6 kinase beta 1	NP_003152	P23443
744	NK223-1	S6Kb1	Pan-specific	Ribosomal protein-serine S6 kinase beta 1	NP_003152	P23443
745	NK223-2	S6Kb1	Pan-specific	Ribosomal protein-serine S6 kinase beta 1	NP_003152	P23443
746	PK145	S6Kb1	T252	Ribosomal protein serine S6 kinase beta 1	NP_003152	P23443
747	PK146	S6Kb1	T444+S447	Ribosomal protein serine S6 kinase beta 1	NP_003152	P23443
748	PK147	S6Kb1	T412	Ribosomal protein serine S6 kinase beta 1	NP_003152	P23443
749	NK222	S6Kb2	Pan-specific	Ribosomal protein-serine S6 kinase beta 2	NP_003943	O9UBS0
750	NN133	SG2NA	Pan-specific	Striatin-3	NP_001077362.1	Q13033
751	PN074	Shc1	Y349+Y350	SH2 domain-containing transforming protein 1	NP_003020	P29353
752	PN161	Shc1	Y349	SH2 domain-containing transforming protein 1	NP_003020	P29353
753	NN095	Smac/DIA	Pan-specific	2nd mitochondria-derived activator of caspase	NP_620308	O9NR28
754	PN183	Smad1	S465	Mothers against decapentaplegic homolog 1	NP_005891	O15797
755	PN075	Smad1/5/8	S463+S465/S463+S465/S465+	Mothers against decapentaplegic homologs	NP_005891	O15797
756	PN184	Smad2	S467	Mothers against decapentaplegic homolog 2	NP_005891	O15796
757	PN185	Smad2	T200	Mothers against decapentaplegic homolog 2	NP_005891	O15796
758	NN096	Smad2/3	Pan-specific	SMA- and mothers against decapentaplegic	NP_005892	O15796
759	PN125	SMC1	S957	Structural maintenance of chromosomes protein	NP_006297.2.	O14683
760	PN177	SNCA	Y136	Alpha-synuclein	NP_000336.1	P37840
761	PN197	SNCA	S129	Alpha-synuclein	NP_000336.1	P37840
762	NN145	SOCS2	Pan-specific	Suppressor of cytokine signaling 2	NP_003868.1.	O14508
763	NN097	SOCS4	Pan-specific	Suppressor of cytokine signalling 4 (SOCS7)	NP_543143	Q8WXH5
764	NN098	SOD	Pan-specific	Superoxide dismutase 1	NP_000445	P00441
765	NN068	SOD (Mn)	Pan-specific	Superoxide dismutase [Mn]	NP_000627	P04179
766	NN068-1	SOD (Mn)	Pan-specific	Superoxide dismutase [Mn]	NP_000627	P04179
767	NN099	SODD	Pan-specific	Silencer of death domains (Bcl2 associated	NP_004865	O95429
768	PN077	SOX9	S181	SRY (sex determining region Y)-box 9	NP_000337	P48436
769	NN100	SPHK1	Pan-specific	Sphingosine kinase 1	NP_892010	O9NYA1
770	NN101	SPHK2	Pan-specific	Sphingosine kinase 2	NP_064511	O9NRA0
771	NK172	Src	Pan-specific	Src proto-oncogene-encoded protein-tyrosine	NP_005408	P12931
772	NK172-2	Src	Pan-specific	Src proto-oncogene-encoded protein-tyrosine	NP_005408	P12931
773	NK172-3	Src	Pan-specific	Src proto-oncogene-encoded protein-tyrosine	NP_005408	P12931
774	NK172-4	Src	Pan-specific	Src proto-oncogene-encoded protein-tyrosine	NP_005408	P12931
775	PK107	Src	Y419	Src proto-oncogene-encoded protein-tyrosine	NP_005408	P12931
776	PK108	Src	Y530	Src proto-oncogene-encoded protein-tyrosine	NP_005408	P12931
777	NN102-	STAT1a	Pan-specific	Signal transducer and activator of transcription 1	NP_009330	P42224
778	NN102-	STAT1a	Pan-specific	Signal transducer and activator of transcription 1	NP_009330	P42224
779	NN139	STAT1a	Pan-specific	Signal transducer and activator of transcription 1	NP_009330	P42224
780	PN078-	STAT1a	S727	Signal transducer and activator of transcription 1	NP_009330	P42224
781	PN079-	STAT1a	Y701	Signal transducer and activator of transcription 1	NP_009330	P42224
782	NN103	STAT2	Pan-specific	Signal transducer and activator of transcription 2	NP_005410	P52630
783	PN080	STAT2	Y690	Signal transducer and activator of transcription 2	NP_005410	P52630
784	NN104	STAT3	Pan-specific	Signal transducer and activator of transcription 3	NP_003141	P40763
785	NN104-2	STAT3	Pan-specific	Signal transducer and activator of transcription 3	NP_003141	P40763
786	PN081-1	STAT3	S727	Signal transducer and activator of transcription 3	NP_003141	P40763
787	PN082	STAT3	Y705	Signal transducer and activator of transcription 3	NP_003141	P40763
788	PN082-1	STAT3	Y705	Signal transducer and activator of transcription 3	NP_003141	P40763
789	NN117	STAT4	Pan-specific	Signal transducer and activator of transcription 4	NP_003142	Q14765
790	NN105	STAT5A	Pan-specific	Signal transducer and activator of transcription	NP_003143	P42229
791	PN083	STAT5A	Y694	Signal transducer and activator of transcription	NP_003143	P42229
792	PN083-1	STAT5A	Y694	Signal transducer and activator of transcription	NP_003143	P42229
793	PN119	STAT5A	S780	Signal transducer and activator of transcription	NP_003143	P42229
794	NN106	STAT5B	Pan-specific	Signal transducer and activator of transcription	NP_036580	P51692
795	NN107	STAT6	Pan-specific	Signal transducer and activator of transcription 6	NP_003144	P42226
796	NN108	STI1	Pan-specific	Stress induced phosphoprotein 1	NP_006810	P31948
797	NK173	STK33	Pan-specific	FLJ35932 protein-serine kinase	NP_112168	O8NEF5
798	NN134	Striatin	Pan-specific	Striatin	NP_003153.2.	O43815
799	NK174	Syk	Pan-specific	Spleen protein-tyrosine kinase	NP_003168	P43405

800	PK159	Syk	Y323	Spleen protein-tyrosine kinase	NP_003168	P43405
801	NN171	Synapsin 1	Pan-specific	Synapsin 1 isoform Ia	NP_008881	P17600
802	PN084	Synapsin 1	S9	Synapsin 1 isoform Ia	NP_008881	P17600
803	NK175-2	TAK1	Pan-specific	TGF-beta-activated protein-serine kinase 1	NP_663306	O43318
804	NK175-3	TAK1	Pan-specific	TGF-beta-activated protein-serine kinase 1	NP_663306	O43318
805	NK175-4	TAK1	Pan-specific	TGF-beta-activated protein-serine kinase 1	NP_663306	O43318
806	NK175-5	TAK1	Pan-specific	TGF-beta-activated protein-serine kinase 1	NP_663306	O43318
807	PN085	Tau	S516	Microtubule-associated protein tau	NP_005901	P10636
808	PN086	Tau	S516+S519	Microtubule-associated protein tau	NP_005901	P10636
809	PN090	Tau	S713	Microtubule-associated protein tau	NP_005901	P10636
810	PN090-2	Tau	S713	Microtubule-associated protein tau	NP_005901	P10636
811	PN091	Tau	S717	Microtubule-associated protein tau	NP_005901	P10636
812	PN092	Tau	S721	Microtubule-associated protein tau	NP_005901	P10636
813	PN106	Tau	S519	Microtubule-associated protein tau	NP_005901	P10636
814	PN107	Tau	S739	Microtubule-associated protein tau	NP_005901	P10636
815	PN121	Tau	T522	Microtubule-associated protein tau	NP_005901	P10636
816	PN122	Tau	T548	Microtubule-associated protein tau	NP_005901	P10636
817	NK220-1	TBK1	Pan-specific	Serine/threonine-protein kinase TBK1	NP_037386	Q9UHD2
818	NK220-2	TBK1	Pan-specific	Serine/threonine-protein kinase TBK1	NP_037386	Q9UHD2
819	NK176	TEK (TIE2)	Pan-specific	Angiopoietin-1 receptor-tyrosine kinase	NP_444515	Q02763
820	NK177	Tlk1	Pan-specific	Tousled-like protein-serine kinase 1	NP_036422	Q9UKI8
821	NP034	TPIPb	Pan-specific	trisphosphatehosphatase TPTE2	NP_001135440.1	Q6XPS3
822	NN110	TRADD	Pan-specific	Tumor necrosis factor receptor type 1	NP_003789	Q15628
823	NN111	Trail	Pan-specific	Tumor necrosis factor-related apoptosis-	NP_003801	P50591
824	NK178	TrkA	Pan-specific	Nerve growth factor (NGF) receptor-tyrosine	NP_002520	P04629
825	NK179	TrkB	Pan-specific	BNDF/NT3/4/5 receptor- tyrosine kinase	NP_006171	Q16620
826	PK160	TrkB	Y706	BNDF/NT3/4/5 receptor- tyrosine kinase	NP_006171	Q16620
827	NK180	TTK	Pan-specific	Dual specificity protein kinase	AAA61239.1	P33981
828	NK181	Tyk2	Pan-specific	Protein-tyrosine kinase 2 (Jak-related)	NP_003322	P29597
829	NK181-2	Tyk2	Pan-specific	Protein-tyrosine kinase 2 (Jak-related)	NP_003322	P29597
830	NK183-1	Tyro10	Pan-specific	Neurotrophic receptor-tyrosine kinase	NP_006173	Q16832
831	NK183-2	Tyro10	Pan-specific	Neurotrophic receptor-tyrosine kinase	NP_006173	Q16832
832	PN093-1	Tyrosine	S70	Tyrosine hydroxylase isoform a	NP_954986	P07101
833	PN109	Tyrosine	S18	Tyrosine hydroxylase isoform a	NP_954986	P07101
834	NN176	VEGF-C	Pan-specific	Vascular endothelial growth factor C	NP_005420.1	P49767
835	PK161	VEGFR2	Y1059	Vascular endothelial growth factor receptor-	NP_002244	P35968
836	PK133	VEGFR2	Y1214	Vascular endothelial growth factor receptor-	NP_002244	P35968
837	NP030	VHR	Pan-specific	Dual specificity protein phosphatase 3	NP_004081	P51452
838	PN094	Vimentin	S34	Vimentin	NP_003371	P08670
839	NK184	Vrk1	Pan-specific	Vaccinia related protein-serine kinase 1	NP_003375	O99986
840	NK185	Wee1	Pan-specific	Wee1 protein-tyrosine kinase	NP_003381	P30291
841	NP037	WIP1	Pan-specific	Protein phosphatase 1D	NP_003611.1.	O15297
842	NK186	Yes	Pan-specific	Yamaguchi sarcoma proto-oncogene-encoded	NP_005424	P07947
843	NK186-2	Yes	Pan-specific	Yamaguchi sarcoma proto-oncogene-encoded	NP_005424	P07947
844	NK214	YSK1	Pan-specific	Serine/threonine-protein kinase 25	NP_006365.2	O00506
845	NK187	ZAP70	Pan-specific	Zeta-chain (TCR) associated protein-tyrosine	NP_003168	P43403
846	NK187-2	ZAP70	Pan-specific	Zeta-chain (TCR) associated protein-tyrosine	NP_003168	P43403
847	PK109	ZAP70/Syk	Y319/Y352	Zeta-chain (TCR) associated protein-tyrosine	NP_001070	P43403
848	CN003	pThr(MmA	pThr	pThr(MmAb)	NA	NA
849	NK188-1	ZIPK	Pan-specific	ZIP kinase (death associated protein-serine	NP_001339	O43293
850	NK188-2	ZIPK	Pan-specific	ZIP kinase (death associated protein-serine	NP_001339	O43293
851	CN001	Actin	Pan-specific	Actin	NP_001092.1	P60709
852	CN002	Tubulin	Pan-specific	Tubulin	NP_006000.2	Q71U36
853	CN004	pThr(RpAb	pThr	pThr(RpAb)	NA	NA
854	CN005	4G10	pTyr	4G10	NA	NA

Supplementary Table 2A - Antibody list of proteins with significant differential expression between shGRPR and scrambled in LNCaP.

No.	Antibody Codes	Target Protein Name	Phospho Site (Human)	Full Target Protein Name	Uniprot Link	Average Z-ratio
89	NP001	CD45	Pan-specific	Leukocyte common antigen CD45 receptor-tyrosine phosphatase (LCA, T200)	NA	-2,12
666	NP021	PP5C	Pan-specific	Protein-serine phosphatase 5 - catalytic subunit (PPT)	Q99683	-2,10
87	PN018	Caveolin 2	S36	Caveolin 2	P00519	-1,92
408	NK092-2	Lck	Pan-specific	Lymphocyte-specific protein-tyrosine kinase	Q07912	-1,83
73	NN013	CASP3	Pan-specific	Caspase 3 (apopain, cysteine protease CPP32)	P63104	-1,67
76	NN017	CASP7	Pan-specific	Caspase 7 (ICE-like apoptotic protease 3 (ICE-LAP3), Mch3)	Q13541	-1,58
646	NK146-2	Plk2	Pan-specific	Polo-like protein kinase 2 (serum -inducible kinase (SNK))	P49407	-1,57
844	NK214	YSK1	Pan-specific	Serine/threonine-protein kinase 25	P15336	-1,53
442	NK100-1	MEK2 (MAP2K2)	Pan-specific	MAPK/ERK protein-serine kinase 2 (MKK2)	Q96Q40	-1,48
419	NK097	MAPKAPK2	Pan-specific	Mitogen-activated protein kinase-activated protein kinase 2	O95831	-1,40
422	PN049-PN112-2	MAPKAPK2a	T334	Mitogen-activated protein kinase-activated protein kinase 2 alpha	P54819	-1,39
81	PN167	Catenin b	Y333	Catenin (cadherin-associated protein) beta 1	Q13541	-1,38
398	NN153	KDEL receptor 1	Pan-specific	ER lumen protein retaining receptor 1	NA	-1,35
88	PN171	Cbl	Y700	Signal transduction protein CBL	Q13085	-1,35
658	NP013-NP014	PP2A/Ca	Pan-specific	Protein-serine phosphatase 2A - catalytic subunit - alpha isoform	Q99683	-1,35
639	NN156	PLC R(PLCg2)	Pan-specific	1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase gamma-2	P49407	-1,33
828	NK181	Tyk2	Pan-specific	Protein-tyrosine kinase 2 (Jak-related)	Q99683	-1,32
83	NN021-1	Catenin b1	Pan-specific	Catenin (cadherin-associated protein) beta 1	Q13541	-1,32
464	NK106-2	MEK7 (MAP2K7)	Pan-specific	MAPK/ERK protein-serine kinase 7 (MKK7)	Q13131	-1,31
615	NK137	PKCg	Pan-specific	Protein-serine kinase C gamma	P05067	-1,27
428	NK099-1	MEK1 (MAP2K1)	Pan-specific	MAPK/ERK protein-serine kinase 1 (MKK1)	Q9UM73	-1,25
412	PK041	Lck	Y505	Lymphocyte-specific protein-tyrosine kinase	P35611	-1,22
84	NN167	Caveolin 1	Pan-specific	Caveolin 1	Q13541	-1,21
846	NK187-2	ZAP70	Pan-specific	Zeta-chain (TCR) associated protein-tyrosine kinase, 70 kDa	P15336	-1,21
584	PK072-3	PKBa (Akt1)	S473	Protein-serine kinase B alpha	O95757	-1,20
612	NK136	PKCe	Pan-specific	Protein-serine kinase C epsilon	P34932	-1,20

486	NK113-3	MST1	Pan-specific	Mammalian STE20-like protein-serine kinase 1 (KRS2)	Q13043	-1,20
541	NN084	PACSIN1	Pan-specific	Protein kinase C + casein kinase substrate in neurons protein 1	Q9BY11	1,22
8	NN135-1	Acetylated Lysine	Pan-specific	Acetylated Lysine	NA	1,42
513	NN075	NT5E	Pan-specific	Ecto-5'-nucleotidase (CD73 antigen)	P21589	1,56
672	NK149	PRK2 (PKN2)	Pan-specific	Protein kinase C-related protein-serine kinase 2	Q16513	1,58
504	NN071	NFkappaB p65	Pan-specific	NF-kappa-B p65 nuclear transcription factor	Q04206	1,59
508	NK207	NIK (MAP3K14)	Pan-specific	NF-kappa beta-inducing kinase	Q99558	1,71
512	PN055-1	NR1	S896	N-methyl-D-aspartate (NMDA) glutamate receptor 1 subunit zeta	Q05586	1,73
527	NK120-4	p38a MAPK	Pan-specific	Mitogen-activated protein-serine kinase p38 alpha	Q16539	1,94
509	NK212	NLK	Pan-specific	Serine/threonine protein kinase NLK	Q9UBE8	2,02
530	NK059-1	p38g MAPK (Erk6)	Pan-specific	Mitogen-activated protein-serine kinase p38 gamma (MAPK12)	P53778	2,20
506	PN156	NFkappaB p65	S529	NF-kappa-B p65 nuclear transcription factor	Q04206	2,65
499	NK117-4	Nek2	Pan-specific	NIMA (never-in-mitosis)-related protein-serine kinase 2	P51955	2,79
495	PN187	NBS1	S343	Nijmegen breakage syndrome protein 1	O60934	2,96
498	NK117-3	Nek2	Pan-specific	NIMA (never-in-mitosis)-related protein-serine kinase 2	P51955	3,75

Supplementary Table 2B - Antibody list of proteins with significant differential expression between shGRPR and scrambled in VCaP.

No.	Antibody Codes	Target Protein Name	Phospho Site (Human)	Full Target Protein Name	Uniprot Link	Average Z-ratio
288	PN038	Histone H3	S11	Histone H3.3	P84243	-2,29
111	NK026-5	CDK2	Pan-specific	Cyclin-dependent protein-serine kinase 2	P24941	-2,16
408	NK092-2	Lck	Pan-specific	Lymphocyte-specific protein-tyrosine kinase	P06239	-1,95
630	PK090-1	PKCq	S695	Protein-serine kinase C theta	Q04759	-1,90
646	NK146-2	Plk2	Pan-specific	Polo-like protein kinase 2 (serum -inducible kinase (SNK))	Q9NYY3	-1,90
615	NK137	PKCg	Pan-specific	Protein-serine kinase C gamma	P05129	-1,85
612	NK136	PKCe	Pan-specific	Protein-serine kinase C epsilon	Q02156	-1,81
613	NK136-2	PKCe	Pan-specific	Protein-serine kinase C epsilon	Q02156	-1,79
257	NK065	Fyn	Pan-specific	Fyn proto-oncogene-encoded protein-tyrosine kinase	P06241	-1,65
614	PK081-1	PKCe	S729	Protein-serine kinase C epsilon	Q02156	-1,64
606	NK135	PKCd	Pan-specific	Protein-serine kinase C delta	Q05655	-1,63
119	NK028-5	CDK5	Pan-specific	Cyclin-dependent protein-serine kinase 5	Q00535	-1,62
315	NN060-2	Hsp70	Pan-specific	Heat shock 70 kDa protein 1	P08107	-1,61
631	NK141	PKCz	Pan-specific	Protein-serine kinase C zeta	Q05513	-1,56
633	NK143	PKG1	Pan-specific	Protein-serine kinase G1 (cGMP-dependent protein kinase)	Q13976	-1,56
607	PK077-1	PKCd	Y313	Protein-serine kinase C delta	Q05655	-1,55
608	PK077-2	PKCd	Y313	Protein-serine kinase C delta	Q05655	-1,53
221	PK168-PK169	Erk1 (MAPK3)+ Erk2 (MAPK1)	Y204	Extracellular regulated protein-serine kinase 1 (p44 MAP kinase)+Extracellular regulated protein-serine kinase 2 (p42 MAP kinase)	P27361	-1,53
314	NN060	Hsp70	Pan-specific	Heat shock 70 kDa protein 1	P08107	-1,52
604	NK134-2	PKCb2	Pan-specific	Protein-serine kinase C beta 2	P05771-2	-1,51
228	NK206-3	Erk5 (MAPK7)	Pan-specific	Extracellular regulated protein-serine kinase 5 (Big MAP kinase 1 (BMK1))	Q13164	-1,48
470	NK108-2	MEKK2 (MAP3K2)	Pan-specific	MAPK/ERK kinase kinase 2	Q9Y2U5	-1,45
176	NN163	DDIT3(CHOP)	Pan-specific	DNA damage-inducible transcript 3 protein	P35639	-1,44
478	PK056	MLK3	T277+S281	Mixed-lineage protein-serine kinase 3	Q16584	-1,43
828	NK181	Tyk2	Pan-specific	Protein-tyrosine kinase 2 (Jak-related)	P29597	-1,41
616	PK082-1	PKCg	T514	Protein-serine kinase C gamma	P05129	-1,38
584	PK072-3	PKBa (Akt1)	S473	Protein-serine kinase B alpha	P31749	-1,34
397	NP004	KAP	Pan-specific	Cyclin-dependent kinase associated phosphatase (CDK inhibitor 3, CIP2)	Q16667	-1,33
113	NK026-7	CDK2	Pan-specific	Cyclin-dependent protein-serine kinase 2	P24941	-1,33

393	PN048-2	Jun	S73	Jun proto-oncogene-encoded AP1 transcription factor	P05412	-1,30
396	PN163	Jun	T91	Jun proto-oncogene-encoded AP1 transcription factor	P05412	-1,29
638	PK132	PKR1	T446	Double-stranded RNA-dependent protein-serine kinase (EIF2AK2)	P19525	-1,29
625	PK093-1	PKCm (PKD)	S910	Protein-serine kinase C mu (Protein kinase D)	Q15139	-1,29
464	NK106-2	MEK7 (MAP2K7)	Pan-specific	MAPK/ERK protein-serine kinase 7 (MKK7)	O14733	-1,28
842	NK186	Yes	Pan-specific	Yamaguchi sarcoma proto-oncogene-encoded tyrosine kinase	P07947	-1,27
398	NN153	KDEL receptor 1	Pan-specific	ER lumen protein retaining receptor 1	P24390	-1,27
579	PK069	PKA R2a	S99	cAMP-dependent protein-serine kinase regulatory type 2 subunit alpha	P13861	-1,24
211	PK013-1	ErbB2 (HER2)	Y1248	ErbB2 (Neu) receptor-tyrosine kinase	P04626	-1,21
76	NN017	CASP7	Pan-specific	Caspase 7 (ICE-like apoptotic protease 3 (ICE-LAP3), Mch3)	P55210	-1,20
486	NK113-3	MST1	Pan-specific	Mammalian STE20-like protein-serine kinase 1 (KRS2)	Q13043	-1,20
696	NN092-1	Rac1	Pan-specific	Ras-related C3 botulinum toxin substrate 1	P63000	1,20
562	NN141-1	PDI	Pan-specific	Protein disulfide-isomerase	P07237	1,22
707	NN093	Rb	Pan-specific	Retinoblastoma-associated protein 1	P06400	1,23
144	NN026	Cofilin 1	Pan-specific	Cofilin 1	P23528	1,24
153	PN023	CREB1	S129+S133	cAMP response element binding protein 1	P16220	1,25
47	NK012	BMX (Etk)	Pan-specific	Bone marrow X protein-tyrosine kinase	P51813	1,26
41	NN007	Bcl-xL	Pan-specific	Bcl2-like protein 1	Q07817	1,27
700	NK155-3	Raf1	Pan-specific	Raf1 proto-oncogene-encoded protein-serine kinase	P04049	1,27
795	NN107	STAT6	Pan-specific	Signal transducer and activator of transcription 6	P42226	1,28
21	NN121	Arrestin b1	Pan-specific	Arrestin beta 1	P49407	1,28
811	PN091	Tau	S717	Microtubule-associated protein tau	P10636	1,31
780	PN078-PN135	STAT1a	S727	Signal transducer and activator of transcription 1 alpha	P42224	1,35
166	NN030-1	Cyclin D1	Pan-specific	Cyclin D1 (PRAD1)	P24385	1,36
766	NN068-1	SOD (Mn)	Pan-specific	Superoxide dismutase [Mn]	P04179	1,43
733	PK100-2	RSK1/2	S363/S369	Ribosomal S6 protein-serine kinase 1/2	Q15418	1,43
796	NN108	STI1	Pan-specific	Stress induced phosphoprotein 1 (Hsc70/Hsp90 organizing protein (Hop))	P31948	1,43
270	NN048	Grp78	Pan-specific	Glucose regulated protein 78	P11021	1,47
823	NN111	Trail	Pan-specific	Tumor necrosis factor-related apoptosis-inducing ligand	P50591	1,48
2	NN166	4E-BP1	Pan-specific	Eukaryotic translation initiation factor 4E binding protein 1 (PHAS1)	Q13541	1,51
682	PP003	PTEN	S380+T382+S385	Phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase	P60484	1,51

				and protein phosphatase and tensin homolog deleted on chromosome 10		
57	NN136-2	Calnexin	Pan-specific	Calnexin	P27824	1,52
156	NN149-1	Crystallin aB	Pan-specific	Crystallin alpha B (heat-shock 20 kDa like-protein)	P02511	1,56
58	NN137-1	Calreticulin	Pan-specific	Calreticulin	P27797	1,58
783	PN080	STAT2	Y690	Signal transducer and activator of transcription 2	P52630	1,62
776	PK108	Src	Y530	Src proto-oncogene-encoded protein-tyrosine kinase	P12931	1,67
159	PN025	Crystallin aB	S19	Crystallin alpha B (heat-shock 20 kDa like-protein)	P02511	1,74
356	PN043	Integrin a4	S1027	Integrin alpha 4 (VLA4)	P13612	1,76
686	NP025	PTP1C	Pan-specific	Protein-tyrosine phosphatase 1C (SHP1, SHPTP1, PTPN6)	P29350	1,81
138	NK037-1	CK1e	Pan-specific	Casein protein-serine kinase 1 epsilon	P49674	1,83
685	NP024	PTP1B	Pan-specific	Protein-tyrosine phosphatase 1B (PTPN1)	P18031	1,84
732	PK100	RSK1/2	S363/S369	Ribosomal S6 protein-serine kinase 1/2	Q15418	1,86
666	NP021	PP5C	Pan-specific	Protein-serine phosphatase 5 - catalytic subunit (PPT)	P53041	1,87
269	NN047	Grp75	Pan-specific	Glucose regulated protein 75	P38646	1,89
390	NN162	Jun	Pan-specific	Jun proto-oncogene-encoded AP1 transcription factor	P05412	2,01
788	PN082-1	STAT3	Y705	Signal transducer and activator of transcription 3	P40763	2,02
163	NN028	Cyclin A	Pan-specific	Cyclin A1	P78396	2,02
721	NK159-1	ROCK2	Pan-specific	RhoA protein-serine kinase alpha	O75116	2,05
662	NP018	PP2Cd	Pan-specific	Protein-serine phosphatase 2C - catalytic subunit - delta isoform	O15297	2,08
798	NN134	Striatin	Pan-specific	Striatin	O43815	2,33
777	NN102-NN124	STAT1a	Pan-specific	Signal transducer and activator of transcription 1 alpha	P42224	2,75
720	NK158	RIPK1	Pan-specific	Receptor-interacting protein-serine kinase 1	Q13546	3,07
782	NN103	STAT2	Pan-specific	Signal transducer and activator of transcription 2	P52630	3,29

Supplementary Table 2C - Antibody list of proteins down-regulated between shGRPR and scrambled in both LNCaP and VCaP.

No.	Antibody Codes	Target Protein Name	Phospho Site (Human)	Full Target Protein Name	Uniprot Link	Average Z-ratio
408	NK092-2	Lck	Pan-specific	Lymphocyte-specific protein-tyrosine kinase	Q07912	-1,95
646	NK146-2	Plk2	Pan-specific	Polo-like protein kinase 2 (serum -inducible kinase (SNK))	P49407	-1,90
612	NK136	PKCe	Pan-specific	Protein-serine kinase C epsilon	P34932	-1,81
828	NK181	Tyk2	Pan-specific	Protein-tyrosine kinase 2 (Jak-related)	Q99683	-1,41
584	PK072-3	PKBa (Akt1)	S473	Protein-serine kinase B alpha	O95757	-1,34
464	NK106-2	MEK7 (MAP2K7)	Pan-specific	MAPK/ERK protein-serine kinase 7 (MKK7)	Q13131	-1,28
398	NN153	KDEL receptor 1	Pan-specific	ER lumen protein retaining receptor 1	NA	-1,27
76	NN017	CASP7	Pan-specific	Caspase 7 (ICE-like apoptotic protease 3 (ICE-LAP3), Mch3)	Q13541	-1,20
486	NK113-2	MST1	Pan-specific	Mammalian STE20-like protein-serine kinase 1 (KRS2)	Q13043	-1,20

Chapter 4

ORIGINAL PUBLICATION #2

Therapeutic potential of dual GRPR and TYK2 inhibition in prostate cancer harboring ETS rearrangements

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Therapeutic potential of dual GRPR and TYK2 inhibition in prostate cancer harboring ETS rearrangements

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Keywords: prostate cancer, ETS-positive tumors, *GRPR*, TYK2, therapeutic potential

Running title: GRPR/TYK2 chemical blockade in ETS-positive prostate carcinomas

Abstract

Gastrin-releasing peptide receptor (GRPR) is known to be overexpressed in several human malignancies, including prostate cancer (PCa), and has been implicated in multiple important neoplastic signaling pathways. Recently, we have identified GRPR as a target of overexpressed ETS transcription factors in PCa and showed that effective knockdown of GRPR in LNCaP and VCaP cells attenuates their malignant phenotype. Additionally, we identified TYK2, an important element for the enhancement of PCa invasiveness, as a downstream target of GRPR overexpression. In this work we aimed to evaluate the anti-oncogenic potential of a therapy targeting GRPR and TYK2 signaling using *in vitro* models of PCa with ETS rearrangements. Our present work shows that a combination treatment targeting both GRPR and TYK2 with the specific inhibitors RC-3095 and Tyrphostin 1, respectively, was able to impair the malignant phenotype of LNCaP and VCaP cells, supporting a therapeutic potential of GRPR/TYK2 blockade for PCa harboring ETS rearrangements.

Introduction

Prostate cancer (PCa) is a highly heterogeneous disease and radical prostatectomy is the only available treatment with curative intent. A significant proportion of the PCa are detected in advanced stage, where the treatment options are limited (1). Genomic rearrangements involving members of the ETS family of transcription factors are frequently found in PCa, with *ERG* and *ETV1* being rearranged in around 50% and 10% of all PCa cases, respectively (2, 3). These aberrant alterations are also present in the HGPIN precursor lesions, suggesting a role in early events of prostate carcinogenesis (4-8). Owing to the high frequency of PCa harboring *ERG* rearrangements, several studies have investigated their association with prognosis, but data are contradictory (2). In contrast, *ETV1* rearrangements have been consistently associated with more aggressive PCa. In 2013, Baena and collaborators associated ETV1-regulated pathways with higher Gleason score and metastasis (9), supporting previous reports (10, 11). Considering the complexity of transcription factors in general, several groups have attempted to ascertain which genes are deregulated upon *ERG* and *ETV1* overexpression, aiming to identify alternative downstream targets with therapeutic potential (12).

In previously studies from our group, we found Gastrin-releasing peptide receptor (*GRPR*) as overexpressed in patients harboring *ERG* and *ETV1* rearrangements (13) and further demonstrated the malignant role of *GRPR* in prostate carcinogenesis (14). GRPR is a member of the G-protein coupled receptor superfamily that is overexpressed in several human malignancies, being implicated in multiple neoplastic signaling pathways (15). In prostate cancer, GRPR overexpression has been identified in radical prostatectomy specimens at both mRNA and protein levels (16-19). Additionally, higher levels of this protein were also detected in high-grade prostatic intraepithelial neoplasias (HGPIN), considered precursor lesions of PCa (17). The discovery of *GRPR*

overexpression in several cancer types led to approaches to inhibit the autocrine growth effect of GRP on tumor growth, including receptor antagonists, and several studies have reported the antiproliferative effect of GRPR antagonists both *in vitro* and *in vivo* in distinct tumor models (20, 21).

Moreover, we demonstrated that TYK2 (tyrosine kinase 2), a member of the Janus family of non-receptor tyrosine kinases (JAKs), is a target of the GRPR oncogenic pathway, being equally deregulated in ETS-positive PCa (14). Members of the JAK family play important roles in cellular growth, development, differentiation, survival and apoptosis of various cell types (22), and some reports have demonstrated that JAKs are expressed in prostate cancer tissues and cell lines (23, 24). The particular role of TYK2 in cancer has been extensively studied, with overexpression being described in several breast cancer cell lines, prostate cancers and squamous cervical carcinomas (25-27). Ide and collaborators further showed that blockade of TYK2 signaling by a small interfering RNA or by the JAK inhibitor tyrphostin A1 impaired the invasion capacity in human prostate cancer cells (25). The mechanism of how TYK2 contributes to the invasiveness of malignant cells is not completely understood, although reports have emerged implicating Jak/Stat signaling on tissue infiltration (28-30) and on regulation of metalloproteinases expression, important elements of metastasis (31-33). Considering the oncogenic role of TYK2 in prostate carcinogenesis and its overexpression in tumors harboring ETS rearrangements, a combined therapy of a GRPR antagonist and a TYK2 inhibitor may be a promising therapeutic approach for prostate carcinomas with ETS rearrangements. In this work, we aimed to evaluate the potential of an anti-oncogenic therapy targeting GRPR and TYK2 signaling using *in vitro* models of PCa with ETS rearrangements.

Methods

Cell lines and reagents

The human prostate cell lines used in this study were LNCaP and VCaP, which were maintained in standard growth conditions, as previously described (14). Cultures were considered *Mycoplasma*-free by routine testing for *Mycoplasma spp.* contamination (PCR Mycoplasma Detection Set; Clontech Laboratories Inc., Mountain View, CA, USA).

The GRPR antagonist RC-3095 (R9653; Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 0.1% dimethyl sulphoxide (DMSO; Sigma-Aldrich) in PBS and the TYK2 antagonist Tyrphostin 1 (T7040-25MG; Sigma-Aldrich) was dissolved in dimethyl sulphoxide (DMSO; Sigma-Aldrich) to a concentration of 50 mM.

IC50 assay

To establish the dose-response curve of Tyrphostin 1, the cell viability of VCaP and LNCaP cell lines was assessed using the MTT assay in the presence of different concentrations of Tyrphostin 1. The IC50 value was determined by the concentration of Tyrphostin 1 (Tyr1) that was required for 50% of cell inhibition at 24 hours of treatment (Figure 1). For the anti-GRPR treatment, cells were exposed to concentrations of RC-3095 previously describe (1 and 10 µM) (34-36).

Cell proliferation and apoptosis assays

The MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay and the apoptosis assay (Biocolor, Newtownabbey, Northern Ireland) were performed as previously described (14). LNCaP ($1.0x10^4$) and VCaP cells ($2.5x10^4$) were seeded in 96-well plates (Sarstedt AG & Co, Nümbrecht, Germany) in 200 µL of serum-containing medium and incubated in normal growth conditions until cell adherence. For the anti-GRPR treatment, cells were exposed to two concentrations of RC-3095 (1 and 10 µM) (34-36). For the anti-TYK2 treatment, cells were exposed to 300 nM of Tyrphostin 1,

following to the concentration determined by the IC50 assay for both cell lines. For the combined treatments, cells were exposed to two different combinations of RC-3095 (1 and 10 µM) with 300 nM of Tyrphostin 1. Cells exposed to DMSO at 0.1% were used as control for both RC-3095 and/or Tyrphostin 1 treatments. At 24 hours after treatment the viability and apoptosis assays were performed. For viability measurement, treatment medium was replaced by medium containing MTT at 1mg/mL (Sigma-Aldrich, Schnelldorf, Germany) and incubated for 1 hour in regular growth conditions. The MTT solution was removed and formazan crystals were dissolved using DMSO (Sigma-Aldrich) in constant stirring for 15 minutes. Absorbance levels were measured using a microplate reader (Fluostar Omega, BMG Labtech, Offenburg, Germany) at a wavelength of 540 nm with background deduction at 630 nm. For quantification of apoptosis levels, cells were stained with APOPercentage dye for 1 hour and washed twice with PBS to remove non-cell bound dye. Dye Release Reagent was added to each well and the plate was shaked for 10 minutes. The absorbance levels were measured using the microplate reader (Fluostar Omega) at a wavelength of 550nm.

Invasion assay

Cell invasion through a three-dimensional extracellular matrix was evaluated by a Matrigel invasion assay using BD Matrigel Invasion Chambers (BD Biocoat, Bedford, MA, USA) with 8.0 µm pore, as previously described (14). Briefly, the matrigel-coated transwell chambers were rehydrated and $2.5x10^4$ LNCaP cells and $5.0x10^4$ VCaP cells, resuspended in 500 µL of serum-free medium, were plated in triplicate. For the anti-GRPR and anti-TYK2 treatments, cells were exposed to either 10 µM of RC-3095 or 300 nM of Tyrphostin 1, respectively. Cells exposed to DMSO at 0.1% were used as control for both treatments. After 48 or 72 hours (LNCaP and VCaP, respectively), cells in the upper surface of the transwell chambers were washed, and the invaded cells at the lower surface were fixed, stained with DAPI and counted under the microscope.

Statistical analysis

All *in vitro* data were carried out in triplicate and three independent experiments were performed. Statistical analyses were conducted using SPSS software (IBM-SPSS Inc., Chicago, IL, USA) and data analyzed by paired Student's *t* test. Graphs were built using GraphPad Prism 5.0 software (GraphPad Software Inc., La Jolla, CA, USA). All *p* values are based on two-sided hypothesis testing and $p<0.05$ was considered statistically significant.

Results

Combinatory inhibition of GRPR and TYK2 signaling impairs cell viability and induces apoptosis

To evaluate the impact of inhibition of GRPR and TYK2 signaling in the early-stage characteristics of prostate cancer cells in the context of *ERG* and *ETV1* rearrangements, proliferation and apoptosis were assessed. GRPR signaling inhibition by the specific antagonist RC-3095 at 10 µM was only effective in LNCaP cells, which displayed a significantly reduced cell viability of 17% comparing with control (Fig. 2A). Likewise, the TYK2 signaling inhibition by Tyrphostin 1 at 300 nM was only effective in the LNCaP cell line, leading to a significant 28% decrease of cell viability (Fig. 2A). When both drug dosages were used in combination, a significant decrease of cell viability was observed in both LNCaP and VCaP cell lines, showing 50% and 80% decrease, respectively ($p<0.01$; Fig. 2A). The lower dosage of RC-3095 (1 µM) in combination with 300 nM of Tyrphostin 1 showed an equally significant decrease of cell viability in LNCaP cells, reaching around 46% viability decrease ($p<0.01$), but had no significant impact in VCaP cells. Only the blockade of both GRPR and TYK2 in combination was significantly effective in inducing apoptosis. In fact, both dose combinations of RC-3095 (1 and 10 µM) with Tyrphostin 1 led to significant increase in apoptosis levels in both prostate cell lines, reaching 3.0- and

5.8-fold increase for the higher RC-3095 dosage combination in LNCaP and VCaP, respectively (Fig. 2B).

RC-3095 and Tyrphostin 1 combination treatment decreases the invasion capacity of prostate cancer cells

To evaluate whether the GRPR antagonist and the TYK2 inhibitor were efficient in inhibiting the phenotypic characteristics of advanced prostate carcinomas, we evaluated the impact of RC-3095 and Tyrphostin1 treatment, respectively, in the invasion potential. Comparing with control cells, *in vitro* treatment with RC-3095 at 10 µM in combination with 300 nM of Tyrphostin 1 significantly decreased the invasion ability of both LNCaP and VCaP prostate cell lines (of around 62% and 30%, respectively; $p<0.05$) (Fig. 3). On the other hand, isolated treatment with RC-3095 or Tyrphostin 1 was ineffective on cell invasion inhibition in both cell lines (Fig. 3).

Discussion

Considering the relevance of ETS rearrangements in a significant proportion of prostate carcinomas, the identification and characterization of the downstream targets is crucial to define the signaling pathways that are deregulated by ETS overexpression and to define new therapeutic target options to this particular subset of PCa. Several studies have described GRPR as a therapeutic target and support the relevance of this receptor as an important activator of oncogenic signaling pathways in PCa cells. In our recent work, we reported for the first time the oncogenic role of GRPR in different biological processes of prostate cancer progression in an ETS-positive context, and identified TYK2 as a GRPR target potentially involved in cancer-associated signaling pathways, namely invasion (14). Given the observed deregulation of these two oncogenic players in prostate cancers (14), we decided to test their potential as therapeutic targets by evaluating the phenotypic impact of GRPR and TYK2 blockade *in vitro*. Our present work shows that a

combined treatment targeting both GRPR and TYK2 with specific inhibitors is efficient in inhibiting both early-stage and more advanced phenotypic characteristics of prostate carcinomas *in vitro*, in the context of *ERG* and *ETV1* rearrangements. In fact, the drug combination with 10 µM RC-3095 and 300 nM Tyrphostin 1 was able to decrease both proliferation and invasion, while increasing apoptosis.

Several studies have demonstrated the strong inhibitory effect of GRPR antagonists on distinct tumors models, including prostate cancer cell lines (PC-3, DU-145, MDA-PCa-2b) (20, 37-39). The RC-3095 is a small molecule that binds to the extracellular domain of the receptor, leading to an inhibition of tumor growth in several experimental models, through a number of different mechanisms that are not completely understood (40). Nevertheless, and considering the preclinical antitumor activity of RC-3095, a phase I clinical trial was conducted in which RC-3095 was administered to 25 patients with different advanced solid malignancies, including six cases with PCa. In this initial study, no side effects were observed but tumor-reducing effects were also not convincing (41). Despite the inclusion of PCa individuals in this study cohort, no information was available regarding ETS status. According to our data, the pilot study could have benefited from a more accurate selection of the PCa patients based on the ETS rearrangement status of the prostate carcinomas, a molecular subtyping that is nowadays possible.

TYK2 overexpression has been observed in several malignancies, such as breast cancer cell lines, prostate cancer and squamous cervical carcinomas (22), and some studies have described the involvement of this tyrosine kinase in enhancing prostate cancer invasion (25, 42). Ide and collaborators have demonstrated that TYK2 signaling blockade by the JAK inhibitor tyrphostin 1 at 100 µM impaired the invasion capacity in the DU-145 human prostate cancer cells (25), although the same drug dosage was not efficient to impair DU-145 cell proliferation. In the present study, the reported drug dosage was not able on its own to affect the phenotypic characteristics of any of the cell lines used (LNCaP and VCaP) (data not shown).

Considering the worst prognosis described for ETV1-positive PCa (Attard *et al.* 2008, Shin *et al.* 2009, Baena *et al.* 2013) and our previous observations of a higher GRPR and TYK2 protein expression in prostate carcinomas harboring ETV1 rearrangements (14), together with the fact that single treatments with RC-3095 or Tyrphostin 1 were only efficient in the LNCaP cell line that harbors an ETV1-rearrangement, a combined therapy of RC3095 antagonist and a tyrosine kinase inhibitor targeting for TYK2 seems to be a promising and more effective approach to drive antitumor activity in prostate carcinomas with ETV1 rearrangements. Nevertheless, the elucidation of the application of the combined treatment approach in other prostate human cell lines, as well as in other human cancer cell line models, requires further investigation. Moreover, *in vivo* studies are also crucial to test the possible therapeutic application of this combined treatment in PCa. Furthermore, our data show that the dual GRPR/TYK2 inhibition may be effective also in inducing apoptosis in PCa characterized by the common *ERG* rearrangements in addition to those involving *ETV1*, being a therapeutic strategy potentially applicable to about 60% of PCa.

In conclusion, our data provides further evidence to support a therapeutic effect of GRPR/TYK2 blockade in PCa harboring ETS rearrangements by showing that combined treatment of RC-3095 and Tyrphostin 1 drastically impairs the *in vitro* tumorigenic behavior of PCa cell lines with these rearrangements.

References

1. Ramon J, Denis L. Prostate Cancer. Berlim: Springer 2007.
2. Clark JP, Cooper CS. ETS gene fusions in prostate cancer. Nature Reviews Urology. 2009;6(8):429-39.
3. Kumar-Sinha C, Tomlins SA, Chinnaiyan AM. Recurrent gene fusions in prostate cancer. Nature Reviews Cancer. 2008;8(7):497-511.
4. Cerveira N, Ribeiro FR, Peixoto A, Costa V, Henrique R, Jerönimo C, et al. TMPRSS2-ERG Gene Fusion Causing ERG Overexpression Precedes Chromosome Copy Number Changes in Prostate Carcinomas, Paired HGPIN Lesions. Neoplasia. 2006;8(10):826-32.
5. Clark J, Attard G, Jhavar S, Flohr P, Reid A, De-Bono J, et al. Complex patterns of ETS gene alteration arise during cancer development in the human prostate. Oncogene. 2008;27(14):1993-2003.
6. Park K, Tomlins SA, Mudaliar KM, Chiu Y-L, Esgueva R, Mehra R, et al. Antibody-based detection of ERG rearrangement-positive prostate cancer. Neoplasia. 2010;12(7):590-8.
7. Perner S, Mosquera J-M, Demichelis F, Hofer MD, Paris PL, Simko J, et al. TMPRSS2-ERG fusion prostate cancer: an early molecular event associated with invasion. The American journal of surgical pathology. 2007;31(6):882-8.
8. van Leenders GJ, Boormans JL, Vissers CJ, Hoogland AM, Bressers AA, Furusato B, et al. Antibody EPR3864 is specific for ERG genomic fusions in prostate cancer: implications for pathological practice. Modern Pathology. 2011;24(8):1128-38.
9. Baena E, Shao Z, Linn DE, Glass K, Hamblen MJ, Fujiwara Y, et al. ETV1 directs androgen metabolism and confers aggressive prostate cancer in targeted mice and patients. Genes & development. 2013;27(6):683-98.
10. Attard G, Clark J, Ambroisine L, Mills I, Fisher G, Flohr P, et al. Heterogeneity and clinical significance of ETV1 translocations in human prostate cancer. British journal of cancer. 2008;99(2):314-20.
11. Shin S, Kim T-D, Jin F, van Deursen JM, Dehm SM, Tindall DJ, et al. Induction of prostatic intraepithelial neoplasia and modulation of androgen receptor by ETS variant 1/ETS-related protein 81. Cancer research. 2009;69(20):8102-10.
12. Turner DP, Watson DK. ETS transcription factors: oncogenes and tumor suppressor genes as therapeutic targets for prostate cancer. Expert Rev Anticancer Ther. 2008;8(1):33-42.

13. Paulo P, Ribeiro FR, Santos J, Mesquita D, Almeida M, Barros-Silva JD, et al. Molecular subtyping of primary prostate cancer reveals specific and shared target genes of different ETS rearrangements. Neoplasia. 2012;14(7):600-11.
14. Santos J, Mesquita D, Barros-Silva JD, Jerónimo C, Henrique R, Morais A, et al. Uncovering potential downstream targets of oncogenic GRPR overexpression in prostate carcinomas harboring ETS rearrangements. Oncoscience. 2015;in print.
15. Cornelio D, Roesler R, Schwartsmann G. Gastrin-releasing peptide receptor as a molecular target in experimental anticancer therapy. Annals of Oncology. 2007;18:1457-66.
16. Bartholdi MF, Wu JM, Pu H, Troncoso P, Eden PA, Feldman RI. In situ hybridization for gastrin-releasing peptide receptor (GRP receptor) expression in prostatic carcinoma. International journal of cancer. 1998;79(1):82-90.
17. Markwalder R, Reubi JC. Gastrin-releasing peptide receptors in the human prostate relation to neoplastic transformation. Cancer research. 1999;59(5):1152-9.
18. Sun B, Halmos G, Schally AV, Wang X, Martinez M. Presence of receptors for bombesin/gastrin-releasing peptide and mRNA for three receptor subtypes in human prostate cancers. The Prostate. 2000;42(4):295-303.
19. Weber HC. Regulation and signaling of human bombesin receptors and their biological effects. Current Opinion in Endocrinology, Diabetes and Obesity. 2009;16(1):66-71.
20. Hohla F, Schally AV. Targeting gastrin releasing peptide receptors: New options for the therapy and diagnosis of cancer. Cell cycle. 2010;9(9):1738-41.
21. Schally AV, Comaru-Schally AM, Nagy A, Kovacs M, Szepeshazi K, Plonowski A, et al. Hypothalamic hormones and cancer. Frontiers in neuroendocrinology. 2001;22(4):248-91.
22. Übel C, Mousset S, Trufa D, Sirbu H, Finotto S. Establishing the role of tyrosine kinase 2 in cancer. Oncoimmunology. 2013;2(1): e22840.
23. Chang CY-M, Kung H-J, Evans CP. Nonreceptor Tyrosine Kinases in Prostate. Neoplasia. 2007;9(2):90-100.
24. Drake JM, Graham NA, Stoyanova T, Sedghi A, Goldstein AS, Cai H, et al. Oncogene-specific activation of tyrosine kinase networks during prostate cancer progression. Proceedings of the National Academy of Sciences. 2012;109(5):1643-8.
25. Ide H, Nakagawa T, Terado Y, Kamiyama Y, Muto S, Horie S. Tyk2 expression and its signaling enhances the invasiveness of prostate cancer cells. Biochemical and biophysical research communications. 2008;369(2):292-6.

26. Song XC, Fu G, Yang X, Jiang Z, Wang Y, Zhou GW. Protein expression profiling of breast cancer cells by dissociable antibody microarray (DAMA) staining. Molecular & cellular proteomics. 2008;7(1):163-9.
27. Zhu X, Lv J, Yu L, Zhu X, Wu J, Zou S, et al. Proteomic identification of differentially-expressed proteins in squamous cervical cancer. Gynecologic oncology. 2009;112(1):248-56.
28. Mellado M, Rodríguez-Frade JM, Mañes S, Martínez-A C. Chemokine signaling and functional responses: the role of receptor dimerization and TK pathway activation. Annual review of immunology. 2001;19(1):397-421.
29. Müller A, Homey B, Soto H, Ge N, Catron D, Buchanan ME, et al. Involvement of chemokine receptors in breast cancer metastasis. Nature. 2001;410(6824):50-6.
30. Wiley HE, Gonzalez EB, Maki W, Wu M-t, Hwang ST. Expression of CC chemokine receptor-7 and regional lymph node metastasis of B16 murine melanoma. Journal of the National Cancer Institute. 2001;93(21):1638-43.
31. Itoh M, Murata T, Suzuki T, Shindoh M, Nakajima K, Imai K, et al. Requirement of STAT3 activation for maximal collagenase-1 (MMP-1) induction by epidermal growth factor and malignant characteristics in T24 bladder cancer cells. Oncogene. 2006;25(8):1195-204.
32. Li WQ, Dehnade F, Zafarullah M. Oncostatin M-induced matrix metalloproteinase and tissue inhibitor of metalloproteinase-3 genes expression in chondrocytes requires Janus kinase/STAT signaling pathway. The Journal of Immunology. 2001;166(5):3491-8.
33. Tsareva SA, Moriggl R, Corvinus FM, Wiederanders B, Schutz A, Kovacic B, et al. Signal Transducer and Activator of Transcription 3 Activation Promotes Invasive Growth of Colon Carcinomas through Matrix Metal loproteinase Induction. Neoplasia. 2007;9(4):279-91.
34. Qin Y, Ertl T, Cai R-Z, Halmos G, Schally AV. Inhibitory effect of bombesin receptor antagonist RC-3095 on the growth of human pancreatic cancer cells in vivo and in vitro. Cancer research. 1994;54(4):1035-41.
35. Sotomayor S, Munoz-Moreno L, Carmena MJ, Schally AV, Sanchez-Chapado M, Prieto JC, et al. Regulation of HER expression and transactivation in human prostate cancer cells by a targeted cytotoxic bombesin analog (AN-215) and a bombesin antagonist (RC-3095). International journal of cancer Journal international du cancer. 2010;127(8):1813-22.
36. Rick FG, Abi-Chaker A, Szalontay L, Perez R, Jaszberenyi M, Jayakumar AR, et al. Shrinkage of experimental benign prostatic hyperplasia and reduction of prostatic

cell volume by a gastrin-releasing peptide antagonist. Proceedings of the National Academy of Sciences of the United States of America. 2013;110(7):2617-22.

37. Stangelberger A, Schally AV, Varga JL, Hammann BD, Groot K, Halmos G, et al. Antagonists of growth hormone releasing hormone (GHRH) and of bombesin/gastrin releasing peptide (BN/GRP) suppress the expression of VEGF, bFGF, and receptors of the EGF/HER family in PC-3 and DU-145 human androgen-independent prostate cancers. The Prostate. 2005a;64(3):303-15.
38. Stangelberger A, Schally AV, Varga JL, Zarandi M, Cai R-Z, Baker B, et al. Inhibition of human androgen-independent PC-3 and DU-145 prostate cancers by antagonists of bombesin and growth hormone releasing hormone is linked to PKC, MAPK and c-jun intracellular signalling. European Journal of Cancer. 2005b;41(17):2735-44.
39. Stangelberger A, Schally AV, Varga JL, Zarandi M, Szepeshazi K, Armatis P, et al. Inhibitory effect of antagonists of bombesin and growth hormone-releasing hormone on orthotopic and intraosseous growth and invasiveness of PC-3 human prostate cancer in nude mice. Clinical Cancer Research. 2005c;11(1):49-57.
40. Mansi R, Fleischmann A, Mäcke HR, Reubi JC. Targeting GRPR in urological cancers—from basic research to clinical application. Nature Reviews Urology. 2013;10(4):235-44.
41. Schwartsmann G, DiLeone L, Horowitz M, Schunemann D, Cancella A, Pereira A, et al. A phase I trial of the bombesin/gastrin-releasing peptide (BN/GRP) antagonist RC3095 in patients with advanced solid malignancies. Investigational new drugs. 2006;24(5):403-12.
42. Schuster C, Müller M, Freissmuth M, Sexl V, Stoiber D. Commentary on H. Ide et al.,“Tyk2 expression and its signaling enhances the invasiveness of prostate cancer cells”. Biochemical and biophysical research communications. 2008;366(4):869-70.

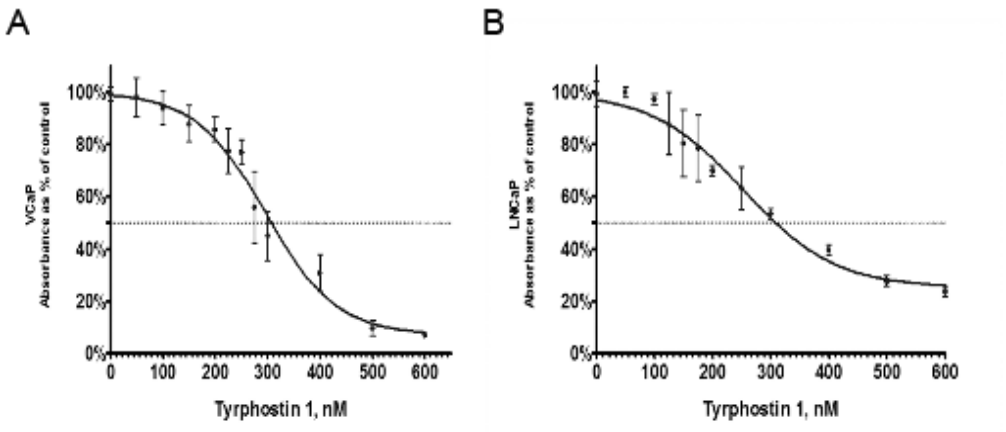

Figure 1: Dose-response curve of Tyrphostin 1 in VCaP (*left*) and LNCaP (*right*) cell lines, assessed with the MTT assay.

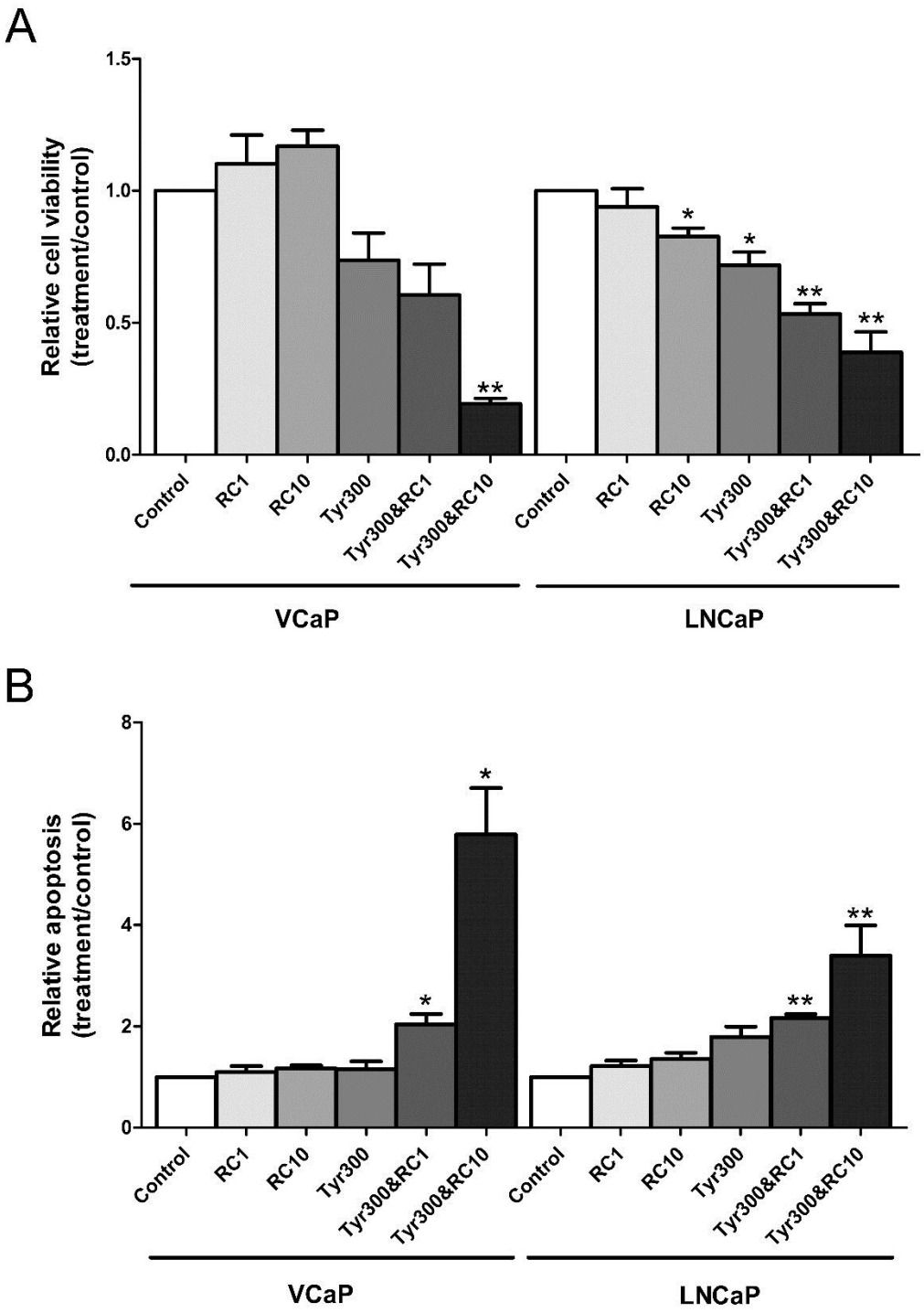

Figure 2: Impact of GRPR and/or TYK2 inhibition in the cell viability and apoptosis of the LNCaP and VCaP cells at 24 hours after treatment. **(A)** Quantitative analysis of metabolically active cells by the MTT assay. **(B)** Quantitative analysis of the apoptotic levels. For both assays, results are shown for treated cell populations relative to the control cells, from three independent experiments. Statistically significant *p* values are showed by an asterisk (*p<0.05; **p<0.01). RC1, RC-3095 at 1 µM; RC10, RC-3095 at 10 µM; Tyr300, Tyrphostin 1 at 300 nM.

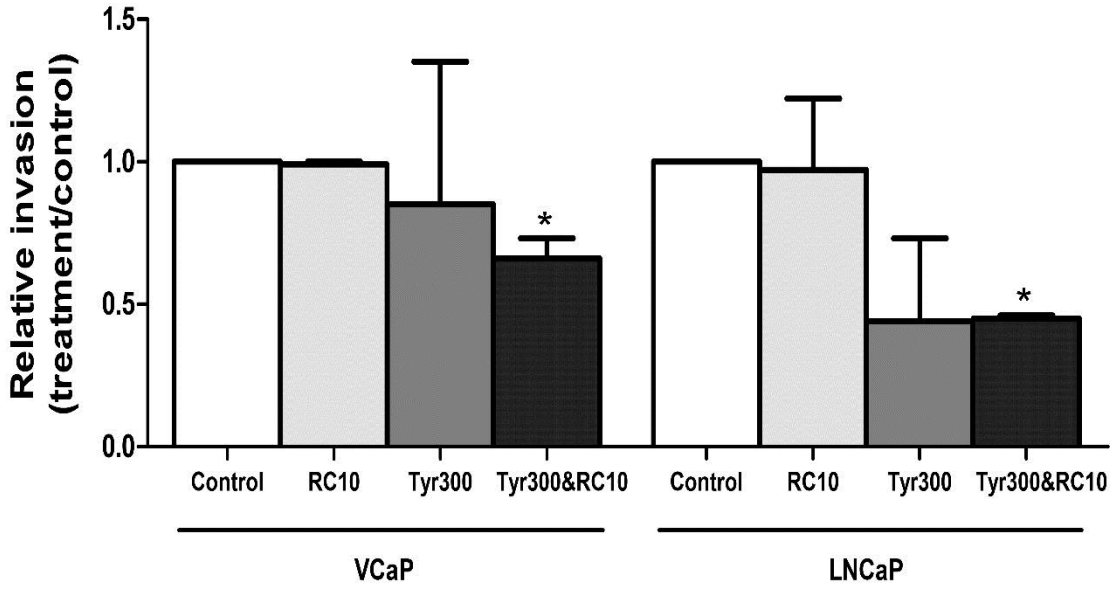

Figure 3: Evaluation of the impact of GRPR and/or TYK2 inhibition in the invasive ability of LNCaP and VCaP cells, using Matrigel Invasion Chambers. Results are shown for treated cell population relative to the control cells, from three independent experiments. Statistically significant *p* values are showed by an asterisk (*$p<0.05$). RC10, RC-3095 at 10 μM; Tyr300, Tyrphostin 1 at 300 nM.

Chapter 5

GENERAL DISCUSSION

PCa is still a major health concern worldwide, mainly due to insufficient biological knowledge about its onset and progression (Shen and Abate-Shen 2010). Considering the complexity and heterogeneity of PCa, there is no standard treatment for all patients. The selection of the optimal therapeutic approach is based on several individual features, and the therapeutic options available range from active surveillance, radical prostatectomy and radiation therapy to hormone and/or radiation therapy and chemotherapy. Nowadays, the challenge is to distinguish men with aggressive local disease for whom treatment might be useful from those with indolent disease for whom treatment would bring a decrease in life quality (DeVita *et al.* 2008). Concerning PCa therapeutic strategies, there is an urgent need to develop new and better therapeutic options. Increasing the knowledge on the molecular biology of PCa carcinogenesis is therefore crucial to identify efficient biomarkers for early detection, distinguish between indolent and aggressive PCa, and to develop new and more effective therapies.

Genomic rearrangements involving members of the ETS family of transcription factors are frequently found in PCa, with *ERG* and *ETV1* being rearranged in about 50% and 10% of all PCa cases, respectively (Kumar-Sinha *et al.* 2008, Clark and Cooper 2009)(Cerveira *et al.* 2006, Perner *et al.* 2007, Clark *et al.* 2008, Park *et al.* 2010, van Leenders *et al.* 2011). Owing to the high frequency of PCa patients harboring *ETS* rearrangements, several studies have explored their association with prognosis, although the relevance of these rearrangements in prostate carcinogenesis is still poorly understood (Clark and Cooper 2009). Only *ETV1* rearrangements have been regularly associated with a more aggressive PCa. However, therapeutic targeting of ETS and other transcription factors has been challenging due to their nuclear localization and molecular embedding in DNA–protein and protein–protein complexes. Consequently, it has been of utmost importance to identify and to characterize the downstream molecular targets of ETS rearrangements, as this knowledge might bring further insight on current understanding of prostate carcinogenesis, identify potential biomarkers, and ascertain potential targeted therapies.

In a previous work in our group key players of the overexpressed ETS transcription factors in PCa were described using a genome-scale and gene-level expression microarray platform (Paulo *et al.* 2012). Specific and/or shared target genes were identified, which helped to clarify the signaling pathways deregulated in each molecular subtype of PCa. Because *ERG* and *ETV1* belong to the same family of transcription factors, the existence of shared target genes was expected. In fact, in the study above mentioned, a list of 27 target genes shared by *ERG* and *ETV1* rearrangements were reported, seven of which were validated by cell line models. Additionally, direct binding of

ERG to the promoter of some of these genes were also demonstrated by ERG-immunoprecipitated chromatin from VCaP cells (Paulo *et al.* 2012). Among them the top most interesting gene was *GRPR*, which encodes for the gastrin-releasing peptide receptor and has been described as overexpressed in several cancer types, including PCa (Cornelio *et al.* 2007, Beer *et al.* 2012). Considering the overexpression of *GRPR* in a high proportion of PCa that harbor either *ERG* or *ETV1* rearrangements, the cellular localization of its protein product, the relevance of its function, the availability of blocking agents and the previous reports of *GRPR* overexpression in several neoplasms, including PCa, make this an interesting downstream target.

1. Overexpression of *GRPR* in prostate tumors and cell lines harboring *ERG* and *ETV1* rearrangements

As a first step, validation of the differential expression of *GRPR* between non-malignant prostate samples and PCa with and without ETS rearrangements was performed in a partially independent series of 160 PCa and 15 morphologically normal prostate tissues (NPT) by real time RT-PCR (**Paper I**). Although *GRPR* overexpression was observed in all subtypes of PCa comparing with NPT samples, stratification of the samples by their ETS status showed that *GRPR* overexpression was particularly elevated in both *ERG* and *ETV1* rearrangement positive PCa comparing with NPT samples and also with ETS-negative PCa. Additionally, the expression of *GRPR*, both at mRNA and protein levels, was detected in the *ERG* and *ETV1* rearrangement positive prostate cancer cell lines VCaP and LNCaP, respectively.

2. Stable knockdown of *GRPR* in LNCaP and VCaP cells attenuates their malignant phenotype

To evaluate the oncogenic role of *GRPR,* two independent silenced populations (shGRPR#1 and shGRPR#2) were successfully established for each cell line model using the shRNA Lentiviral Particles Transdution System with specific short-hairpin RNAs and scrambled shRNA lentiviral particle as biological negative control.

Although GRPR and its specific peptide have been associated with an oncogenic role in different tissues and models, **Paper 1** is the first report ascertaining the malignant impact of this receptor in prostate carcinogenesis. In this work, the impact of *GRPR* silencing in the acquisition of early-stage characteristics, as well as in the phenotypic

characteristics of advanced prostate cancer cells, in the context of *ERG* and *ETV1* rearrangements, was clearly demonstrated. In fact, *GRPR* silenced cell populations (shGRPR) of both cell line models displayed a decline of early malignant features of PCa phenotype through reduction of cell viability and increment of apoptosis. Furthermore, the impact of *GRPR* silencing was evaluated in the phenotypic characteristics of advanced prostate cancer cells, namely invasion potential and in the capacity to grow without attachment. Using the *in vitro* Matrigel invasion assay, we were able to demonstrate that GRPR silencing in both cell lines significant reduces about 50% of their invasion ability. Similarly, looking at the capacity of cells to grow without attachment, cell populations with stable *GRPR* silencing developed about 50% fewer colonies than scrambled controls.

3. Downstream pathway targets of *GRPR*

The observed phenotypic effects of *GRPR* silencing prompted us to look for potential *GRPR* target proteins using the KAM-850 antibody microarray, which contains over 850 antibodies pan- and phospho site-specific, with wide coverage of cell signaling proteins and pathways frequently deregulated in tumorigenesis. This platform was used to compare the differential protein expression patterns between scrambled and shGRPR populations in both LNCaP and VCaP cell line models. In order to find potential oncogenes regulated by *GRPR* that could be interesting for targeted therapy of PCa with ETS rearrangements, focus was directed to down-regulated targets shared by both cell lines. Through this analysis, a list of nine proteins with decreased expression levels in both cell lines was found and, based on their cell pathway association, we concentrated our attention in five of them: PLK2, TYK2, MST1, *p*-AKT1 (Ser473) and *p*-PKCε (Ser729).

We next evaluated by western blotting whether the *in vitro* association between the expression of *GRPR* and these potential targets, under an ETS-rearrangement context, was observed also *in vivo*. This approach showed that, overall, the expression of AKT1 was higher in PCa samples when compared with NPT. Interestingly, tumors with *ETV1* rearrangement showed consistently higher expression of TYK2, MST1 and *p*-AKT1, when compared with both NPT and other PCa subgroups, in which the expression pattern of those proteins was shown to be highly heterogeneous. Regarding PKCε and *p*-PKCε expression, both *ETV1* and *ERG* rearrangement-positive PCa samples showed consistently higher expression when compared with NPT, although high protein levels were also detected in some ETS-negative PCa.

Multiple molecular pathways are involved in proliferation and survival of prostate cancer cells during tumor progression. Among these survival signaling pathways, up-regulation of the PI3K/Akt/mTOR pathway is particularly important, as it has been strongly implicated in tumor progression, mostly considering its role in survival enhancement and apoptosis inhibition (Morgan *et al.* 2009). Thus, the observed significant increase in apoptosis levels and the reduction of cell viability after *GRPR* knockdown could be related to a disturbance of the PI3K/Akt pathway via down-regulation of *p*-AKT1 (Ser473). Considering the increased levels of *p*-AKT1 (Ser473) observed in tumors harboring ETS rearrangements, these observations support the hypothesis that ETS overexpression up-regulates the expression of *GRPR* and subsequently leads to up-regulation of *p*-AKT1 (Ser473), placing ETS transcription factors as upstream regulators of *GRPR* overexpression in PCa. Concerning PKCε, a protein kinase described to be overexpressed in several solid tumors (including PCa), we also found its overexpression in ETS-positive tumors. However, PKCε seemed to be more dependent on the ETS context than on the *GRPR* overexpression, as no significant effect was observed on PKCε/*p*-PKCε expression upon silencing of *GRPR* in both cell line models (VCaP and LNCaP) and a significant decrease of *p*-PKCε was observed in LNCaP cells upon silencing of *ETV1*. In fact, *p*-PKCε was identified as the active kinase that phosphorylates AKT1 at serine 473, leading to full AKT activation (Zhang *et al.* 2005). We therefore suggest a link between ETS overexpression and increased PKCε/*p*-PKCε expression, as a GRPR alternative mediator of *p*-AKT1 (Ser473) activation. These findings are in agreement with studies proposing that high levels of ETS protein collaborate with constitutively activated AKT kinase, leading to the development of more aggressive PCa (Zong *et al.* 2009).

Moreover, we have demonstrated that *GRPR* plays an important role in anchorage-independent growth and invasion in prostate cancer, as GRPR silencing led to a significantly decrease in the invasive capacity of both LNCaP and VCaP cell lines. This observation could be a consequence of down-regulation of TYK2 and MST1 expression, as observed by immunoblotting of *GRPR* silenced populations from both cell lines. In fact, overexpression of TYK2 (a member of the Janus family of non-receptor tyrosine kinases, JAKs) has been described in several malignancies, such as breast cancer cell lines, prostate cancers and squamous cervical carcinomas (Ide *et al.* 2008, Song *et al.* 2008, Zhu *et al.* 2009), with some studies showing its involvement in enhancing prostate cancer invasion (Ide *et al.* 2008). Likewise, the signaling initiated by the binding of MST1 to its receptor (MST1R) is an important pathway for invasive growth in different neoplasias (Yao *et al.* 2013). However, we were only able to detect strong expression of both TYK2 and

MST1 proteins in *ETV1*-positive PCa, and silencing of *ETV1* in LNCaP cells only showed a significant effect in the expression of TYK2, but not in MST1. These observations may indicate that both *GRPR* and *ETV1* regulate the expression of TYK2 and MST1, which potentially act cumulatively when overexpression of both is present.

A decrease in PLK2 expression was also observed in *GRPR* silenced cell populations, with higher impact in LNCaP cells, however the opposite effect was observed in response to *ETV1* silencing in the LNCaP cell line. This suggests that PLK2 expression levels would be the result of a balance between the two factors, with ETV1/ETS transcription factors acting as repressors and GRPR as an activator. Nevertheless, no information was obtained from our series of prostate tissues, since PLK2 expression was not detected in any of the samples analyzed using two different antibodies. This observation, however, is in accordance to the low PLK2 expression levels described for normal and tumorous prostate tissues, suggesting that PLK2 expression levels in cell lines may result from adaptation to *in vitro* conditions, further illustrating the importance of looking into tumor samples to validate *in vitro* associations.

4. Therapeutic potential of dual GRPR and TYK2 inhibition in prostate cancer harboring ETS rearrangements

Several studies have described GRPR as a therapeutic target and support the relevance of this receptor as an important activator of oncogenic signaling pathways in PCa cells. In **Paper I**, we reported for the first time the oncogenic role of GRPR in different biological processes of prostate cancer progression in an ETS-positive context, and identified TYK2 as a GRPR target potentially involved in cancer-associated signaling pathways, namely invasion. Given the observed deregulation of these two oncogenic players in prostate cancers, we aimed to evaluate the anti-oncogenic potential of a therapy targeting GRPR and TYK2 signaling using *in vitro* models of PCa with ETS rearrangements (**Paper II**).

To evaluate the impact of inhibition of GRPR and TYK2 signaling in the early-stage characteristics of prostate cancer cells in the context of *ERG* and *ETV1* rearrangements, proliferation and apoptosis were assessed. GRPR signaling inhibition by RC-3095 or TYK2 signaling inhibition by Tyrphostin 1 were only effective in LNCaP cells. When both drugs were used in combination, a significant decrease of cell viability was observed in both LNCaP and VCaP cell lines, showing 50% and 80% decrease, respectively.

Concerning increasing apoptosis levels, only the dual blockade of GRPR and TYK2 was significantly effective. Additionally, to evaluate whether the GRPR antagonist and the TYK2 inhibitor were efficient in inhibiting the phenotypic characteristics of advanced prostate carcinomas, we evaluated the impact of RC-3095 and Tyrphostin1 treatment, respectively, in the invasion potential. Once again, the isolated treatment with RC-3095 or Tyrphostin 1 was ineffective on cell invasion inhibition in both cell lines, but the combined treatment with RC-3095 and Tyrphostin 1 significantly decreased the invasion ability of both LNCaP and VCaP prostate cell lines (of around 62% and 30%, respectively). We have therefore revealed that a combined treatment targeting both GRPR and TYK2 with specific inhibitors is efficient in inhibiting both early-stage and more advanced phenotypic characteristics of prostate carcinomas *in vitro*, in the context of *ERG* and *ETV1* rearrangements. In fact, the drug combination with 10 µM RC-3095 and 300 nM Tyrphostin 1 was able to decrease both proliferation and invasion, while increasing apoptosis.

Several studies have demonstrated the strong inhibitory effect of GRPR antagonists on distinct tumors models, including prostate cancer cell lines (PC-3, DU-145, MDA-PCa-2b) (Stangelberger *et al.* 2005a, Stangelberger *et al.* 2005b, Stangelberger *et al.* 2005c, Hohla and Schally 2010). Considering the preclinical antitumor activity of RC-3095, a phase I clinical trial was conducted in which RC-3095 was administered to 25 patients with different advanced solid malignancies, including six cases with PCa, in whom no side effects were observed but tumor-reducing effects were also not convincing (Schwartsmann *et al.* 2006). According to our present data, the pilot study could have benefited from a more accurate selection of the PCa patients based on the ETS rearrangement status of the prostate carcinomas, a molecular subtyping that is nowadays possible.

Several studies have investigated the association between ETS rearrangements with prognosis. Concerning ERG rearrangements the data obtained is contradictory. In contrast, *ETV1* rearrangements have been consistently associated with more aggressive PCa. ETV1-regulated pathways were effectively associated to higher Gleason score and metastasis (Baena *et al.* 2013). Considering the worst prognosis described for ETV1-positive PCa and our previous observations of a higher GRPR and TYK2 protein expression in prostate carcinomas harboring ETV1 rearrangements (**Paper I**), together with the fact that single treatments with RC-3095 or Tyrphostin 1 were only efficient in the LNCaP cell line that harbors an ETV1-rearrangement, a combined therapy of GRPR antagonist and a tyrosine kinase inhibitor targeting TYK2 seems to be a promising and more effective approach to drive antitumor activity in prostate carcinomas with ETV1

rearrangements. Furthermore, our data show that the dual GRPR/TYK2 inhibition may be effective also in inducing apoptosis in PCa characterized by the common *ERG* rearrangements in addition to those involving *ETV1*, being a therapeutic strategy potentially applicable to about 60% of PCa.

Chapter 6

CONCLUSIONS

The aims of this thesis were addressed in the included publications and the following conclusions can be presented:

- The differential expression of *GRPR* between non-malignant prostate samples and PCa with and without ETS rearrangements was evaluated and this work has confirmed the striking *GRPR* overexpression in both *ERG* and *ETV1* rearrangement-positive PCa comparing with NPT samples and also with ETS-negative PCa;

- The oncogenic role of GRPR in different biological processes of prostate cancer progression was demonstrated through evaluation the phenotypic impact of *GRPR* silencing in prostate cancer cells in the context of *ERG* and *ETV1* rearrangements;

- Downstream players of GRPR/ETS involved in cancer-associated signaling pathways, namely PLK2, TYK2, MST1, *p*-AKT1 (Ser473), and *p*-PKCε (Ser729), were identified;

- A combination treatment targeting both GRPR and TYK2 with the specific inhibitors RC-3095 and Tyrphostin 1, respectively, was able to impair the malignant phenotype of LNCaP and VCaP cells, supporting a therapeutic potential of dual GRPR/TYK2 blockade for PCa harboring ETS rearrangements.

Chapter 7

FUTURE PERSPECTIVES

The combination treatment targeting both GRPR and TYK2 with the specific inhibitors was able to impair the malignant phenotype of LNCaP and VCaP cells, supporting a therapeutic potential of dual GRPR/TYK2 blockade for PCa harboring ETS rearrangements. However, further *in vitro* and *in vivo* studies are crucial to test the possible therapeutic application of this combined treatment in PCa. There are already two ongoing studies on this issue:

I. To ascertain the impact of inhibition of GRPR and TYK2 signaling in the early-stage and advanced characteristics of prostate carcinomas in the context of *de novo* expression of *ERG* and *ETV1* in the PNT2 benign prostate cell line, proliferation, apoptosis and invasion potential will be evaluated in the presence of specific inhibitors targeting GRPR and TYK2.

II. To evaluate the impact of a therapy targeting GRPR and TYK2 signaling in *in vivo* models, subcutaneous mouse xenographs will be developed. Subcutaneous injection of LNCaP and VCaP cells will promote the development of a tumor xenograph, and the effect of the combination treatment targeting GRPR and TYK2 with the specific inhibitors will be assessed.

REFERENCES

Abate-Shen, C., Shen, M. M. and Gelmann, E. (2008). Integrating differentiation and cancer: the Nkx3. 1 homeobox gene in prostate organogenesis and carcinogenesis. Differentiation 76(6): 717-727.

Agoulnik, I. U., Vaid, A., Bingman, W. E., Erdeme, H., Frolov, A., Smith, C. L., Ayala, G., Ittmann, M. M.*, et al.* (2005). Role of SRC-1 in the promotion of prostate cancer cell growth and tumor progression. Cancer research 65(17): 7959-7967.

Alberti, C. (2010). Hereditary/familial versus sporadic prostate cancer: few indisputable genetic differences and many similar clinicopathological features. Eur Rev Med Pharmacol Sci 14(1): 31-41.

Albright, F., Stephenson, R. A., Agarwal, N., Teerlink, C. C., Lowrance, W. T., Farnham, J. M. and Albright, L. A. C. (2015). Prostate cancer risk prediction based on complete prostate cancer family history. The Prostate 75(4): 390-398.

Alcaraz, A., Hammerer, P., Tubaro, A., Schröder, F. H. and Castro, R. (2009). Is there evidence of a relationship between benign prostatic hyperplasia and prostate cancer? Findings of a literature review. European urology 55(4): 864-875.

Andreoiu, M. and Cheng, L. (2010). Multifocal prostate cancer: biologic, prognostic, and therapeutic implications. Human pathology 41(6): 781-793.

Attard, G., Clark, J., Ambroisine, L., Mills, I., Fisher, G., Flohr, P., Reid, A., Edwards, S.*, et al.* (2008). Heterogeneity and clinical significance of ETV1 translocations in human prostate cancer. British journal of cancer 99(2): 314-320.

Attard, G., de Bono, J. S., Clark, J. and Cooper, C. S. (2010). Studies of TMPRSS2-ERG gene fusions in diagnostic trans-rectal prostate biopsies. Clinical Cancer Research: 1078-0432. CCR-1009-2253.

Bachmann, I. M., Halvorsen, O. J., Collett, K., Stefansson, I. M., Straume, O., Haukaas, S. A., Salvesen, H. B., Otte, A. P.*, et al.* (2006). EZH2 expression is associated with high proliferation rate and aggressive tumor subgroups in cutaneous melanoma and cancers of the endometrium, prostate, and breast. Journal of Clinical Oncology 24(2): 268-273.

Baena, E., Shao, Z., Linn, D. E., Glass, K., Hamblen, M. J., Fujiwara, Y., Kim, J., Nguyen, M.*, et al.* (2013). ETV1 directs androgen metabolism and confers aggressive prostate cancer in targeted mice and patients. Genes & development 27(6): 683-698.

Bajo, A. M., Schally, A. V., Krupa, M., Hebert, F., Groot, K. and Szepeshazi, K. (2002). Bombesin antagonists inhibit growth of MDA-MB-435 estrogen-independent breast cancers and decrease the expression of the ErbB-2/HER-2 oncoprotein and c-jun

and c-fos oncogenes. Proceedings of the National Academy of Sciences 99(6): 3836-3841.

Bannister, A. J. and Kouzarides, T. (2011). Regulation of chromatin by histone modifications. Cell research 21(3): 381-395.

Barbieri, C. E., Bangma, C. H., Bjartell, A., Catto, J. W., Culig, Z., Grönberg, H., Luo, J., Visakorpi, T.*, et al.* (2013). The mutational landscape of prostate cancer. European urology 64(4): 567-576.

Barry, M. J. (2001). Prostate-specific–antigen testing for early diagnosis of prostate cancer. New England Journal of Medicine 344(18): 1373-1377.

Bartholdi, M. F., Wu, J. M., Pu, H., Troncoso, P., Eden, P. A. and Feldman, R. I. (1998). In situ hybridization for gastrin-releasing peptide receptor (GRP receptor) expression in prostatic carcinoma. International journal of cancer 79(1): 82-90.

Beer, M., Montani, M., Gerhardt, J., Wild, P. J., Hany, T. F., Hermanns, T., Müntener, M. and Kristiansen, G. (2012). Profiling gastrin-releasing peptide receptor in prostate tissues: Clinical implications and molecular correlates. The Prostate 72(3): 318-325.

Berger, M. F., Lawrence, M. S., Demichelis, F., Drier, Y., Cibulskis, K., Sivachenko, A. Y., Sboner, A., Esgueva, R.*, et al.* (2011). The genomic complexity of primary human prostate cancer. Nature 470(7333): 214-220.

Berman, D. M., Desai, N., Wang, X., Karhadkar, S. S., Reynon, M., Abate-Shen, C., Beachy, P. A. and Shen, M. M. (2004). Roles for Hedgehog signaling in androgen production and prostate ductal morphogenesis. Developmental biology 267(2): 387-398.

Berney, D. M. and Warren, A. Y. (2013). Premalignant Lesions of the Prostate. Prostate Cancer: A Comprehensive Perspective, Springer**:** 215-233.

Bertram, J., Peacock, J. W., Fazli, L., Mui, A. L. F., Chung, S. W., Cox, M. E., Monia, B., Gleave, M. E.*, et al.* (2006). Loss of PTEN is associated with progression to androgen independence. The Prostate 66(9): 895-902.

Bethel, C. R., Faith, D., Li, X., Guan, B., Hicks, J. L., Lan, F., Jenkins, R. B., Bieberich, C. J.*, et al.* (2006). Decreased NKX3. 1 protein expression in focal prostatic atrophy, prostatic intraepithelial neoplasia, and adenocarcinoma: association with gleason score and chromosome 8p deletion. Cancer research 66(22): 10683-10690.

Beuten, J., Gelfond, J. A., Franke, J. L., Shook, S., Johnson-Pais, T. L., Thompson, I. M. and Leach, R. J. (2010). Single and multivariate associations of MSR1, ELAC2, and RNASEL with prostate cancer in an ethnic diverse cohort of men. Cancer Epidemiology Biomarkers & Prevention 19(2): 588-599.

Bhavsar, A. and Verma, S. (2014). Anatomic imaging of the prostate. Biomed Res Int 2014: 728539.

Bitting, R. L. and Armstrong, A. J. (2013). Targeting the PI3K/Akt/mTOR pathway in castration-resistant prostate cancer. Endocrine-related cancer 20(3): R83-R99.

Boyd, L. K., Mao, X. and Lu, Y.-J. (2012). The complexity of prostate cancer: genomic alterations and heterogeneity. Nature Reviews Urology 9(11): 652-664.

Brawley, O. W. (2012). Prostate cancer epidemiology in the United States. World journal of urology 30(2): 195-200.

Bushman, W. (2009). Etiology, epidemiology, and natural history. Urologic Clinics of North America 36(4): 403-415.

Cabot, R. C., Harris, N. L., Rosenberg, E. S., Shepard, J.-A. O., Cort, A. M., Ebeling, S. H., McDonald, E. K., Scher, H. I.*, et al.* (2012). Increased survival with enzalutamide in prostate cancer after chemotherapy. New England Journal of Medicine 367(13): 1187-1197.

Cai, C., Hsieh, C.-L., Omwancha, J., Zheng, Z., Chen, S.-Y., Baert, J.-L. and Shemshedini, L. (2007). ETV1 is a novel androgen receptor-regulated gene that mediates prostate cancer cell invasion. Molecular endocrinology 21(8): 1835-1846.

Campbell, T., Blasko, J., Crawford, E. D., Forman, J., Hanks, G., Kuban, D., Montie, J., Moul, J.*, et al.* (2001). Clinical staging of prostate cancer: reproducibility and clarification of issues. International journal of cancer 96(3): 198-209.

Cao, R., Wang, L., Wang, H., Xia, L., Erdjument-Bromage, H., Tempst, P., Jones, R. S. and Zhang, Y. (2002). Role of histone H3 lysine 27 methylation in Polycomb-group silencing. Science 298(5595): 1039-1043.

Carter, B., Bova, G., Beaty, T., Steinberg, G., Childs, B., Isaacs, W. and Walsh, P. (1993). Hereditary prostate cancer: epidemiologic and clinical features. The Journal of urology 150(3): 797-802.

Casey, G., Neville, P. J., Plummer, S. J., Xiang, Y., Krumroy, L. M., Klein, E. A., Catalona, W. J., Nupponen, N.*, et al.* (2002). RNASEL Arg462Gln variant is implicated in up to 13% of prostate cancer cases. Nature genetics 32(4): 581-583.

Catalona, W., Hudson, M., Scardino, P., Richie, J., Ahmann, F., Flanigan, R., Dekernion, J., Ratliff, T.*, et al.* (1994). Selection of optimal prostate specific antigen cutoffs for early detection of prostate cancer: receiver operating characteristic curves. The Journal of urology 152(6 Pt 1): 2037-2042.

Catto, J. W., Alcaraz, A., Bjartell, A. S., White, R. D. V., Evans, C. P., Fussel, S., Hamdy, F. C., Kallioniemi, O.*, et al.* (2011). MicroRNA in prostate, bladder, and kidney cancer: a systematic review. European urology 59(5): 671-681.

Cerveira, N., Ribeiro, F. R., Peixoto, A., Costa, V., Henrique, R., Jerönimo, C. and Teixeira, M. R. (2006). TMPRSS2-ERG Gene Fusion Causing ERG Overexpression

Precedes Chromosome Copy Number Changes in Prostate Carcinomas, Paired HGPIN Lesions. Neoplasia 8(10): 826-832.

Chen, C. D., Welsbie, D. S., Tran, C., Baek, S. H., Chen, R., Vessella, R., Rosenfeld, M. G. and Sawyers, C. L. (2004). Molecular determinants of resistance to antiandrogen therapy. Nature medicine 10(1): 33-39.

Cheng, L., Montironi, R., Bostwick, D. G., Lopez-Beltran, A. and Berney, D. M. (2012). Staging of prostate cancer. Histopathology 60(1): 87-117.

Chornokur, G., Dalton, K., Borysova, M. E. and Kumar, N. B. (2011). Disparities at presentation, diagnosis, treatment, and survival in African American men, affected by prostate cancer. The Prostate 71(9): 985-997.

Chrisofos, M., Papatsoris, A., Lazaris, A. and Deliveliotis, C. (2007). Precursor lesions of prostate cancer. Critical reviews in clinical laboratory sciences 44(3): 243-270.

Clark, J., Attard, G., Jhavar, S., Flohr, P., Reid, A., De-Bono, J., Eeles, R., Scardino, P.*, et al.* (2008). Complex patterns of ETS gene alteration arise during cancer development in the human prostate. Oncogene 27(14): 1993-2003.

Clark, J., Merson, S., Jhavar, S., Flohr, P., Edwards, S., Foster, C., Eeles, R., Martin, F. L.*, et al.* (2007). Diversity of TMPRSS2-ERG fusion transcripts in the human prostate. Oncogene 26(18): 2667-2673.

Clark, J. P. and Cooper, C. S. (2009). ETS gene fusions in prostate cancer. Nature Reviews Urology 6(8): 429-439.

Clevers, H. (2006). Wnt/β-catenin signaling in development and disease. Cell 127(3): 469-480.

Coppola, V., De Maria, R. and Bonci, D. (2010). MicroRNAs and prostate cancer. Endocrine-related cancer 17(1): F1-F17.

Cornelio, D., Roesler, R. and Schwartsmann, G. (2007). Gastrin-releasing peptide receptor as a molecular target in experimental anticancer therapy. Annals of Oncology 18: 1457-1466.

Crawford, E. D. (2003). Epidemiology of prostate cancer. Urology 62(6): 3-12.

De Bono, J. S., Logothetis, C. J., Molina, A., Fizazi, K., North, S., Chu, L., Chi, K. N., Jones, R. J.*, et al.* (2011). Abiraterone and increased survival in metastatic prostate cancer. New England Journal of Medicine 364(21): 1995-2005.

Dehm, S. M. and Tindall, D. J. (2006). Ligand-independent androgen receptor activity is activation function-2-independent and resistant to antiandrogens in androgen refractory prostate cancer cells. Journal of Biological Chemistry 281(38): 27882-27893.

Demichelis, F., Fall, K., Perner, S., Andrén, O., Schmidt, F., Setlur, S., Hoshida, Y., Mosquera, J.*, et al.* (2007). TMPRSS2: ERG gene fusion associated with lethal prostate cancer in a watchful waiting cohort. Oncogene 26(31): 4596-4599.

Demichelis, F., Setlur, S. R., Beroukhim, R., Perner, S., Korbel, J. O., LaFargue, C. J., Pflueger, D., Pina, C.*, et al.* (2009). Distinct genomic aberrations associated with ERG rearranged prostate cancer. Genes, Chromosomes and Cancer 48(4): 366-380.

DeVita, V., Theodore, T. and SA, R. (2008). Cancer: Principles & Practice of Oncology, Wolters Kluwer/Lippincott Williams & Wilkins.

Dorin, R. P., Wiener, S., Harris, C. D. and Wagner, J. R. (2015). Prostate atypia: Does repeat biopsy detect clinically significant prostate cancer? The Prostate.

Dorsam, R. T. and Gutkind, J. S. (2007). G-protein-coupled receptors and cancer. Nature Reviews Cancer 7(2): 79-94.

Duffy, M. J. (2011). Prostate-specific antigen: does the current evidence support its use in prostate cancer screening? Annals of clinical biochemistry 48(4): 310-316.

Edge, S. B. and Compton, C. C. (2010). The American Joint Committee on Cancer: the 7th edition of the AJCC cancer staging manual and the future of TNM. Annals of surgical oncology 17(6): 1471-1474.

Epstein, J. I. (2009). Precursor lesions to prostatic adenocarcinoma. Virchows Archiv 454(1): 1-16.

Epstein, J. I. (2010). An update of the Gleason grading system. The Journal of urology 183(2): 433-440.

Epstein, J. I., Allsbrook Jr, W. C., Amin, M. B., Egevad, L. L. and Committee, I. G. (2005). The 2005 International Society of Urological Pathology (ISUP) consensus conference on Gleason grading of prostatic carcinoma. The American journal of surgical pathology 29(9): 1228-1242.

Ewing, C. M., Ray, A. M., Lange, E. M., Zuhlke, K. A., Robbins, C. M., Tembe, W. D., Wiley, K. E., Isaacs, S. D.*, et al.* (2012). Germline mutations in HOXB13 and prostate-cancer risk. New England Journal of Medicine 366(2): 141-149.

Falzarano, S. M. and Magi-Galluzzi, C. (2011). Prostate cancer staging and grading at radical prostatectomy over time. Advances in anatomic pathology 18(2): 159-164.

Felici, A., Pino, M. S. and Carlini, P. (2012). A changing landscape in castration-resistant prostate cancer treatment. Frontiers in endocrinology 3.

Ferlay J, S. I., Ervik M, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray, F (2012). GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11

Findlay, V. J., LaRue, A. C. and Turner, D. P. (2013). Understanding the role of ETS-Mediated Gene Regulation in Complex Biological Processes. Advances in cancer research 119**:** 1-61.

Fizazi, K. (2007). The role of Src in prostate cancer. Annals of oncology 18(11): 1765-1773.

Fizazi, K., Scher, H. I., Molina, A., Logothetis, C. J., Chi, K. N., Jones, R. J., Staffurth, J. N., North, S., *et al.* (2012). Abiraterone acetate for treatment of metastatic castration-resistant prostate cancer: final overall survival analysis of the COU-AA-301 randomised, double-blind, placebo-controlled phase 3 study. The lancet oncology 13(10): 983-992.

Furusato, B., Gao, C.-L., Ravindranath, L., Chen, Y., Cullen, J., McLeod, D. G., Dobi, A., Srivastava, S., *et al.* (2008). Mapping of TMPRSS2–ERG fusions in the context of multi-focal prostate cancer. Modern Pathology 21(2): 67-75.

Gann, P. H., Hennekens, C. H. and Stampfer, M. J. (1995). A prospective evaluation of plasma prostate-specific antigen for detection of prostatic cancer. Jama 273(4): 289-294.

Gao, H., Ouyang, X., Banach-Petrosky, W. A., Gerald, W. L., Shen, M. M. and Abate-Shen, C. (2006). Combinatorial activities of Akt and B-Raf/Erk signaling in a mouse model of androgen-independent prostate cancer. Proceedings of the National Academy of Sciences 103(39): 14477-14482.

Giles, R. H., van Es, J. H. and Clevers, H. (2003). Caught up in a Wnt storm: Wnt signaling in cancer. Biochimica et Biophysica Acta (BBA)-Reviews on Cancer 1653(1): 1-24.

Glinsky, G. V., Glinskii, A. B., Stephenson, A. J., Hoffman, R. M. and Gerald, W. L. (2004). Gene expression profiling predicts clinical outcome of prostate cancer. Journal of Clinical Investigation 113(6): 913.

Govender, D. and Chetty, R. (2012). Gene of the month: PTEN. Journal of clinical pathology 65(7): 601-603.

Grasso, C. S., Wu, Y.-M., Robinson, D. R., Cao, X., Dhanasekaran, S. M., Khan, A. P., Quist, M. J., Jing, X., *et al.* (2012). The mutational landscape of lethal castration-resistant prostate cancer. Nature 487(7406): 239-243.

Gupta, S., Iljin, K., Sara, H., Mpindi, J. P., Mirtti, T., Vainio, P., Rantala, J., Alanen, K., *et al.* (2010). FZD4 as a mediator of ERG oncogene–induced WNT signaling and epithelial-to-mesenchymal transition in human prostate cancer cells. Cancer research 70(17): 6735-6745.

Gurel, B., Iwata, T., Koh, C. M., Jenkins, R. B., Lan, F., Van Dang, C., Hicks, J. L., Morgan, J., *et al.* (2008). Nuclear MYC protein overexpression is an early alteration in human prostate carcinogenesis. Modern pathology 21(9): 1156-1167.

Haffner, M. C., Aryee, M. J., Toubaji, A., Esopi, D. M., Albadine, R., Gurel, B., Isaacs, W. B., Bova, G. S., *et al.* (2010). Androgen-induced TOP2B-mediated double-strand breaks and prostate cancer gene rearrangements. Nature genetics 42(8): 668-675.

Hammerer, P. and Madersbacher, S. (2012). Landmarks in hormonal therapy for prostate cancer. BJU international 110(s1): 23-29.

Hanahan, D. and Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. cell 144(5): 646-674.

Heidenreich, A., Bastian, P. J., Bellmunt, J., Bolla, M., Joniau, S., van der Kwast, T., Mason, M., Matveev, V.*, et al.* (2014). EAU guidelines on prostate cancer. Part 1: screening, diagnosis, and local treatment with curative intent—update 2013. European urology 65(1): 124-137.

Heidenreich, A. and Pfister, D. (2014). Pelvic lymphadenectomy in clinically localised prostate cancer: counting lymph nodes or dissecting primary landing zones of the prostate? European urology 66(3): 447-449.

Helgeson, B. E., Tomlins, S. A., Shah, N., Laxman, B., Cao, Q., Prensner, J. R., Cao, X., Singla, N.*, et al.* (2008). Characterization of TMPRSS2: ETV5 and SLC45A3: ETV5 gene fusions in prostate cancer. Cancer research 68(1): 73-80.

Hermans, K. G., van der Korput, H. A., van Marion, R., van de Wijngaart, D. J., Ziel-van der Made, A., Dits, N. F., Boormans, J. L., van der Kwast, T. H.*, et al.* (2008). Truncated ETV1, fused to novel tissue-specific genes, and full-length ETV1 in prostate cancer. Cancer research 68(18): 7541-7549.

Heuberger, J. and Birchmeier, W. (2010). Interplay of cadherin-mediated cell adhesion and canonical Wnt signaling. Cold Spring Harbor perspectives in biology 2(2): a002915.

Hohla, F. and Schally, A. V. (2010). Targeting gastrin releasing peptide receptors: New options for the therapy and diagnosis of cancer. Cell Cycle 9(9): 1738-1741.

Hollenhorst, P. C., McIntosh, L. P. and Graves, B. J. (2011). Genomic and biochemical insights into the specificity of ETS transcription factors. Annual review of biochemistry 80: 437-471.

Holmström, B., Johansson, M., Bergh, A., Stenman, U.-H., Hallmans, G. and Stattin, P. (2009). Prostate specific antigen for early detection of prostate cancer: longitudinal study. BMJ 339: b3537.

Hricak, H. and Scardino, P. (2009). Contemporary Issues in Cancer Imaging: Prostate Cancer, Cambridge Univ Press.

Hsu, T., Trojanowska, M. and Watson, D. K. (2004). Ets proteins in biological control and cancer. Journal of cellular biochemistry 91(5): 896-903.

Hu, Y., Dobi, A., Sreenath, T., Cook, C., Tadase, A. Y., Ravindranath, L., Cullen, J., Furusato, B.*, et al.* (2008). Delineation of TMPRSS2-ERG splice variants in prostate cancer. Clinical Cancer Research 14(15): 4719-4725.

Ide, H., Nakagawa, T., Terado, Y., Kamiyama, Y., Muto, S. and Horie, S. (2008). Tyk2 expression and its signaling enhances the invasiveness of prostate cancer cells. Biochemical and biophysical research communications 369(2): 292-296.

Ilic, D., Neuberger, M. M., Djulbegovic, M. and Dahm, P. (2013). Screening for prostate cancer. The Cochrane Library.

Ingham, P. (1998). Transducing Hedgehog: the story so far. The EMBO Journal 17(13): 3505-3511.

Jani, A. B. and Hellman, S. (2003). Early prostate cancer: clinical decision-making. The Lancet 361(9362): 1045-1053.

Jemal, A., Bray, F., Center, M. M., Ferlay, J., Ward, E. and Forman, D. (2011). Global cancer statistics. CA: a cancer journal for clinicians 61(2): 69-90.

Jensen, R., Battey, J., Spindel, E. and Benya, R. (2008). International Union of Pharmacology. LXVIII. Mammalian bombesin receptors: nomenclature, distribution, pharmacology, signaling, and functions in normal and disease states. Pharmacological reviews 60(1): 1-42.

Jerónimo, C., Bastian, P. J., Bjartell, A., Carbone, G. M., Catto, J. W., Clark, S. J., Henrique, R., Nelson, W. G.*, et al.* (2011). Epigenetics in prostate cancer: biologic and clinical relevance. European urology 60(4): 753-766.

Jerónimo, C., Usadel, H., Henrique, R., Oliveira, J., Lopes, C., Nelson, W. G. and Sidransky, D. (2001). Quantitation of GSTP1 methylation in non-neoplastic prostatic tissue and organ-confined prostate adenocarcinoma. Journal of the National Cancer Institute 93(22): 1747-1752.

Jhavar, S., Brewer, D., Edwards, S., Kote-Jarai, Z., Attard, G., Clark, J., Flohr, P., Christmas, T.*, et al.* (2009). Integration of ERG gene mapping and gene-expression profiling identifies distinct categories of human prostate cancer. BJU international 103(9): 1256-1269.

Jhavar, S., Reid, A., Clark, J., Kote-Jarai, Z., Christmas, T., Thompson, A., Woodhouse, C., Ogden, C.*, et al.* (2008). Detection of TMPRSS2-ERG translocations in human prostate cancer by expression profiling using GeneChip Human Exon 1.0 ST arrays. The Journal of Molecular Diagnostics 10(1): 50-57.

Jones, P. A. (2012). Functions of DNA methylation: islands, start sites, gene bodies and beyond. Nature Reviews Genetics 13(7): 484-492.

Kantoff, P. W., Higano, C. S., Shore, N. D., Berger, E. R., Small, E. J., Penson, D. F., Redfern, C. H., Ferrari, A. C.*, et al.* (2010). Sipuleucel-T immunotherapy for castration-resistant prostate cancer. New England Journal of Medicine 363(5): 411-422.

Karakiewicz, P., Perrotte, P., McCormack, M., Peloquin, F., Perreault, J., Arjane, P., Widmer, H. and Saad, F. (2005). Early detection of prostate cancer with ultrasound-guided systematic needle biopsy. The Canadian journal of urology 12: 5-8.

Karhadkar, S. S., Bova, G. S., Abdallah, N., Dhara, S., Gardner, D., Maitra, A., Isaacs, J. T., Berman, D. M.*, et al.* (2004). Hedgehog signalling in prostate regeneration, neoplasia and metastasis. Nature 431(7009): 707-712.

Kim, T. J., Lee, J. Y., Hwang, T. K., Kang, C. S. and Choi, Y. J. (2011). Hedgehog signaling protein expression and its association with prognostic parameters in prostate cancer: a retrospective study from the view point of new 2010 anatomic stage/prognostic groups. Journal of surgical oncology 104(5): 472-479.

Konstantinopoulos, P. A. and Papavassiliou, A. G. (2011). Seeing the future of cancer-associated transcription factor drug targets. JAMA 305(22): 2349-2350.

Kral, M., Rosinska, V., Student, V., Grepl, M., Hrabec, M. and Bouchal, J. (2011). Genetic determinants of prostate cancer: a review. Biomedical Papers 155(1): 3-9.

Kraus, S., Gioeli, D., Vomastek, T., Gordon, V. and Weber, M. J. (2006). Receptor for activated C kinase 1 (RACK1) and Src regulate the tyrosine phosphorylation and function of the androgen receptor. Cancer research 66(22): 11047-11054.

Kronz, J. D., Allan, C. H., Shaikh, A. A. and Epstein, J. I. (2001). Predicting cancer following a diagnosis of high-grade prostatic intraepithelial neoplasia on needle biopsy: data on men with more than one follow-up biopsy. The American journal of surgical pathology 25(8): 1079-1085.

Kumar-Sinha, C., Tomlins, S. A. and Chinnaiyan, A. M. (2008). Recurrent gene fusions in prostate cancer. Nature Reviews Cancer 8(7): 497-511.

Kypta, R. M. and Waxman, J. (2012). Wnt/β-catenin signalling in prostate cancer. Nature Reviews Urology 9(8): 418-428.

Lin, H.-K., Hu, Y.-C., Lee, D. K. and Chang, C. (2004). Regulation of androgen receptor signaling by PTEN (phosphatase and tensin homolog deleted on chromosome 10) tumor suppressor through distinct mechanisms in prostate cancer cells. Molecular Endocrinology 18(10): 2409-2423.

Liu, W., Ewing, C. M., Chang, B. L., Li, T., Sun, J., Turner, A. R., Dimitrov, L., Zhu, Y.*, et al.* (2007). Multiple genomic alterations on 21q22 predict various TMPRSS2/ERG fusion transcripts in human prostate cancers. Genes, Chromosomes and Cancer 46(11): 972-980.

Lombardo, L. J., Lee, F. Y., Chen, P., Norris, D., Barrish, J. C., Behnia, K., Castaneda, S., Cornelius, L. A.*, et al.* (2004). Discovery of N-(2-chloro-6-methyl-phenyl)-2-(6-(4-(2-hydroxyethyl)-piperazin-1-yl)-2-methylpyrimidin-4-ylamino)thiazole-5-

carboxamide (BMS-354825), a dual Src/Abl kinase inhibitor with potent antitumor activity in preclinical assays. Journal of medicinal chemistry 47(27): 6658-6661.

Lonigro, R. J., Grasso, C. S., Robinson, D. R., Jing, X., Wu, Y.-M., Cao, X., Quist, M. J., Tomlins, S. A.*, et al.* (2011). Detection of somatic copy number alterations in cancer using targeted exome capture sequencing. Neoplasia 13(11): 1019-1025.

Lotan, T. L. and Epstein, J. I. (2010). Clinical implications of changing definitions within the Gleason grading system. Nature Reviews Urology 7(3): 136-142.

MacDonald, B. T., Tamai, K. and He, X. (2009). Wnt/β-catenin signaling: components, mechanisms, and diseases. Developmental cell 17(1): 9-26.

Majumder, P. K., Febbo, P. G., Bikoff, R., Berger, R., Xue, Q., McMahon, L. M., Manola, J., Brugarolas, J.*, et al.* (2004). mTOR inhibition reverses Akt-dependent prostate intraepithelial neoplasia through regulation of apoptotic and HIF-1-dependent pathways. Nature medicine 10(6): 594-601.

Mansi, R., Fleischmann, A., Mäcke, H. R. and Reubi, J. C. (2013). Targeting GRPR in urological cancers—from basic research to clinical application. Nature Reviews Urology 10(4): 235-244.

Markwalder, R. and Reubi, J. C. (1999). Gastrin-releasing peptide receptors in the human prostate relation to neoplastic transformation. Cancer Research 59(5): 1152-1159.

McCubrey, J. A., Steelman, L. S., Kempf, C. R., Chappell, W. H., Abrams, S. L., Stivala, F., Malaponte, G., Nicoletti, F.*, et al.* (2011). Therapeutic resistance resulting from mutations in Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR signaling pathways. Journal of cellular physiology 226(11): 2762-2781.

McNeal, J. E. (1981). The zonal anatomy of the prostate. The prostate 2(1): 35-49.

McNeal, J. E. (1988). Normal histology of the prostate. The American journal of surgical pathology 12(8): 619-633.

Mehra, R., Han, B., Tomlins, S. A., Wang, L., Menon, A., Wasco, M. J., Shen, R., Montie, J. E.*, et al.* (2007). Heterogeneity of TMPRSS2 gene rearrangements in multifocal prostate adenocarcinoma: molecular evidence for an independent group of diseases. Cancer research 67(17): 7991-7995.

Mellinghoff, I. K., Vivanco, I., Kwon, A., Tran, C., Wongvipat, J. and Sawyers, C. L. (2004). HER2/neu kinase-dependent modulation of androgen receptor function through effects on DNA binding and stability. Cancer cell 6(5): 517-527.

Migliaccio, A., Castoria, G., Domenico, M. D., de Falco, A., Bilancio, A., Lombardi, M., Barone, M. V., Ametrano, D.*, et al.* (2000). Steroid-induced androgen receptor–oestradiol receptor β–Src complex triggers prostate cancer cell proliferation. The EMBO journal 19(20): 5406-5417.

Mohamed, A. A., Tan, S.-H., Sun, C., Shaheduzzaman, S., Hu, Y., Petrovics, G., Chen, Y., Sesterhenn, I. A.*, et al.* (2011). ERG oncogene modulates prostaglandin signaling in prostate cancer cells. Cancer biology & therapy 11(4): 410-417.

Morgan, T. M., Koreckij, T. D. and Corey, E. (2009). Targeted therapy for advanced prostate cancer: inhibition of the PI3K/Akt/mTOR pathway. Current cancer drug targets 9(2): 237.

Nakayama, M., Gonzalgo, M. L., Yegnasubramanian, S., Lin, X., De Marzo, A. M. and Nelson, W. G. (2004). GSTP1 CpG island hypermethylation as a molecular biomarker for prostate cancer. Journal of cellular biochemistry 91(3): 540-552.

Nam, S., Kim, D., Cheng, J. Q., Zhang, S., Lee, J.-H., Buettner, R., Mirosevich, J., Lee, F. Y.*, et al.* (2005). Action of the Src family kinase inhibitor, dasatinib (BMS-354825), on human prostate cancer cells. Cancer research 65(20): 9185-9189.

Okotie, O. T., Roehl, K. A., Han, M., Loeb, S., Gashti, S. N. and Catalona, W. J. (2007). Characteristics of prostate cancer detected by digital rectal examination only. Urology 70(6): 1117-1120.

Park, K., Tomlins, S. A., Mudaliar, K. M., Chiu, Y.-L., Esgueva, R., Mehra, R., Suleman, K., Varambally, S.*, et al.* (2010). Antibody-based detection of ERG rearrangement-positive prostate cancer. Neoplasia 12(7): 590-598.

Park, S. I., Zhang, J., Phillips, K. A., Araujo, J. C., Najjar, A. M., Volgin, A. Y., Gelovani, J. G., Kim, S.-J.*, et al.* (2008). Targeting SRC family kinases inhibits growth and lymph node metastases of prostate cancer in an orthotopic nude mouse model. Cancer research 68(9): 3323-3333.

Parker, C., Nilsson, S., Heinrich, D., Helle, S., O'Sullivan, J., Fosså, S., Chodacki, A., Wiechno, P.*, et al.* (2013). Alpha emitter radium-223 and survival in metastatic prostate cancer. New England Journal of Medicine 369(3): 213-223.

Paulo, P., Barros-Silva, J. D., Ribeiro, F. R., Ramalho-Carvalho, J., Jerónimo, C., Henrique, R., Lind, G. E., Skotheim, R. I.*, et al.* (2012). FLI1 is a novel ETS transcription factor involved in gene fusions in prostate cancer. Genes, Chromosomes and Cancer 51(3): 240-249.

Paulo, P., Ribeiro, F. R., Santos, J., Mesquita, D., Almeida, M., Barros-Silva, J. D., Itkonen, H., Henrique, R.*, et al.* (2012). Molecular subtyping of primary prostate cancer reveals specific and shared target genes of different ETS rearrangements. Neoplasia 14(7): 600-611.

Perner, S., Mosquera, J.-M., Demichelis, F., Hofer, M. D., Paris, P. L., Simko, J., Collins, C., Bismar, T. A.*, et al.* (2007). TMPRSS2-ERG fusion prostate cancer: an early molecular event associated with invasion. The American journal of surgical pathology 31(6): 882-888.

Pettersson, A., Graff, R. E., Bauer, S. R., Pitt, M. J., Lis, R. T., Stack, E. C., Martin, N. E., Kunz, L.*, et al.* (2012). The TMPRSS2: ERG rearrangement, ERG expression, and prostate cancer outcomes: a cohort study and meta-analysis. Cancer Epidemiology Biomarkers & Prevention 21(9): 1497-1509.

Ramon, J. and Denis, L. (2007). Prostate Cancer. Berlim, Springer

Recchia, I., Rucci, N., Festuccia, C., Bologna, M., MacKay, A., Migliaccio, S., Longo, M., Šuša, M.*, et al.* (2003). Pyrrolopyrimidine c-Src inhibitors reduce growth, adhesion, motility and invasion of prostate cancer cells in vitro. European Journal of Cancer 39(13): 1927-1935.

Ribeiro, F. R., Diep, C. B., Jerónimo, C., Henrique, R., Lopes, C., Eknæs, M., Lingjærde, O. C., Lothe, R. A.*, et al.* (2006). Statistical dissection of genetic pathways involved in prostate carcinogenesis. Genes, Chromosomes and Cancer 45(2): 154-163.

Rubin, L. L. and de Sauvage, F. J. (2006). Targeting the Hedgehog pathway in cancer. Nature reviews Drug discovery 5(12): 1026-1033.

Saad, F. and Lipton, A. (2010). SRC kinase inhibition: targeting bone metastases and tumor growth in prostate and breast cancer. Cancer treatment reviews 36(2): 177-184.

Salmena, L., Carracedo, A. and Pandolfi, P. P. (2008). Tenets of PTEN tumor suppression. Cell 133(3): 403-414.

Sandoval, J. and Esteller, M. (2012). Cancer epigenomics: beyond genomics. Current opinion in genetics & development 22(1): 50-55.

Schally, A. V., Comaru-Schally, A. M., Nagy, A., Kovacs, M., Szepeshazi, K., Plonowski, A., Varga, J. L. and Halmos, G. (2001). Hypothalamic hormones and cancer. Frontiers in neuroendocrinology 22(4): 248-291.

Schmitz, M., Grignard, G., Margue, C., Dippel, W., Capesius, C., Mossong, J., Nathan, M., Giacchi, S.*, et al.* (2007). Complete loss of PTEN expression as a possible early prognostic marker for prostate cancer metastasis. International journal of cancer 120(6): 1284-1292.

Schwartsmann, G., DiLeone, L., Horowitz, M., Schunemann, D., Cancella, A., Pereira, A., Richter, M., Souza, F.*, et al.* (2006). A phase I trial of the bombesin/gastrin-releasing peptide (BN/GRP) antagonist RC3095 in patients with advanced solid malignancies. Investigational new drugs 24(5): 403-412.

Sementchenko, V. I. and Watson, D. K. (2000). Ets target genes: past, present and future. Oncogene 19(55): 6533-6548.

Seth, A. and Watson, D. K. (2005). ETS transcription factors and their emerging roles in human cancer. European Journal of Cancer 41(16): 2462-2478.

Shen, M. and Abate-Shen, C. (2010). Molecular genetics of prostate cancer: new prospects for old challenges. Genes Dev 24(18): 1967-2000.

Sheng, T., Li, C., Zhang, X., Chi, S., He, N., Chen, K., McCormick, F., Gatalica, Z.*, et al.* (2004). Activation of the hedgehog pathway in advanced prostate cancer. Molecular cancer 3(1): 29.

Shin, S., Kim, T.-D., Jin, F., van Deursen, J. M., Dehm, S. M., Tindall, D. J., Grande, J. P., Munz, J.-M.*, et al.* (2009). Induction of prostatic intraepithelial neoplasia and modulation of androgen receptor by ETS variant 1/ETS-related protein 81. Cancer research 69(20): 8102-8110.

Siegel, R. L., Miller, K. D. and Jemal, A. (2015). Cancer statistics, 2015. CA: a cancer journal for clinicians 65(1): 5-29.

Sircar, K., Yoshimoto, M., Monzon, F. A., Koumakpayi, I. H., Katz, R. L., Khanna, A., Alvarez, K., Chen, G.*, et al.* (2009). PTEN genomic deletion is associated with p-Akt and AR signalling in poorer outcome, hormone refractory prostate cancer. The Journal of pathology 218(4): 505-513.

Song, X. C., Fu, G., Yang, X., Jiang, Z., Wang, Y. and Zhou, G. W. (2008). Protein expression profiling of breast cancer cells by dissociable antibody microarray (DAMA) staining. Molecular & cellular proteomics 7(1): 163-169.

Sonpavde, G., Wang, C. G., Galsky, M. D., Oh, W. K. and Armstrong, A. J. (2014). Cytotoxic chemotherapy in the contemporary management of metastatic castration-resistant prostate cancer (mCRPC). BJU international.

Squire, J. A., Park, P. C., Yoshimoto, M., Alami, J., Williams, J. L., Evans, A. and Joshua, A. M. (2011). Prostate cancer as a model system for genetic diversity in tumors. Adv Cancer Res 112: 183-216.

Stangelberger, A., Schally, A. V., Varga, J. L., Hammann, B. D., Groot, K., Halmos, G., Cai, R. Z. and Zarandi, M. (2005a). Antagonists of growth hormone releasing hormone (GHRH) and of bombesin/gastrin releasing peptide (BN/GRP) suppress the expression of VEGF, bFGF, and receptors of the EGF/HER family in PC-3 and DU-145 human androgen-independent prostate cancers. Prostate 64(3): 303-315.

Stangelberger, A., Schally, A. V., Varga, J. L., Zarandi, M., Cai, R.-Z., Baker, B., Hammann, B. D., Armatis, P.*, et al.* (2005b). Inhibition of human androgen-independent PC-3 and DU-145 prostate cancers by antagonists of bombesin and growth hormone releasing hormone is linked to PKC, MAPK and c-jun intracellular signalling. European Journal of Cancer 41(17): 2735-2744.

Stangelberger, A., Schally, A. V., Varga, J. L., Zarandi, M., Szepeshazi, K., Armatis, P. and Halmos, G. (2005c). Inhibitory effect of antagonists of bombesin and growth

hormone-releasing hormone on orthotopic and intraosseous growth and invasiveness of PC-3 human prostate cancer in nude mice. Clinical Cancer Research 11(1): 49-57.

Sun, B., Halmos, G., Schally, A. V., Wang, X. and Martinez, M. (2000). Presence of receptors for bombesin/gastrin-releasing peptide and mRNA for three receptor subtypes in human prostate cancers. The Prostate 42(4): 295-303.

Swalwell, J. I., Vocke, C. D., Yang, Y., Walker, J. R., Grouse, L., Myers, S. H., Gillespie, J. W., Bostwick, D. G.*, et al.* (2002). Determination of a minimal deletion interval on chromosome band 8p21 in sporadic prostate cancer†. Genes, Chromosomes and Cancer 33(2): 201-205.

Tannock, I. F., Osoba, D., Stockler, M. R., Ernst, D. S., Neville, A. J., Moore, M. J., Armitage, G. R., Wilson, J. J.*, et al.* (1996). Chemotherapy with mitoxantrone plus prednisone or prednisone alone for symptomatic hormone-resistant prostate cancer: a Canadian randomized trial with palliative end points. Journal of Clinical Oncology 14(6): 1756-1764.

Taylor, B. S., Schultz, N., Hieronymus, H., Gopalan, A., Xiao, Y., Carver, B. S., Arora, V. K., Kaushik, P.*, et al.* (2010). Integrative genomic profiling of human prostate cancer. Cancer cell 18(1): 11-22.

Teixeira, M. R. (2008). Chromosome mechanisms giving rise to the TMPRSS2-ERG fusion oncogene in prostate cancer and HGPIN lesions. The American journal of surgical pathology 32(4): 640-644.

Tewari, A. (2013). Prostate Cancer: A Comprehensive Perspective. London, Springer.

Thompson, I. M., Pauler, D. K., Goodman, P. J., Tangen, C. M., Lucia, M. S., Parnes, H. L., Minasian, L. M., Ford, L. G.*, et al.* (2004). Prevalence of prostate cancer among men with a prostate-specific antigen level≤ 4.0 ng per milliliter. New England Journal of Medicine 350(22): 2239-2246.

Timms, B. G. and Hofkamp, L. E. (2011). Prostate development and growth in benign prostatic hyperplasia. Differentiation 82(4): 173-183.

Tomlins, S. A., Bjartell, A., Chinnaiyan, A. M., Jenster, G., Nam, R. K., Rubin, M. A. and Schalken, J. A. (2009). ETS gene fusions in prostate cancer: from discovery to daily clinical practice. European urology 56(2): 275-286.

Tomlins, S. A., Laxman, B., Dhanasekaran, S. M., Helgeson, B. E., Cao, X., Morris, D. S., Menon, A., Jing, X.*, et al.* (2007). Distinct classes of chromosomal rearrangements create oncogenic ETS gene fusions in prostate cancer. Nature 448(7153): 595-599.

Tomlins, S. A., Laxman, B., Varambally, S., Cao, X., Yu, J., Helgeson, B. E., Cao, Q., Prensner, J. R.*, et al.* (2008). Role of the TMPRSS2-ERG gene fusion in prostate cancer. Neoplasia 10(2): 177-188.

Tomlins, S. A., Mehra, R., Rhodes, D. R., Smith, L. R., Roulston, D., Helgeson, B. E., Cao, X., Wei, J. T.*, et al.* (2006). TMPRSS2: ETV4 gene fusions define a third molecular subtype of prostate cancer. Cancer research 66(7): 3396-3400.

Tomlins, S. A., Rhodes, D. R., Perner, S., Dhanasekaran, S. M., Mehra, R., Sun, X.-W., Varambally, S., Cao, X.*, et al.* (2005). Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. Science 310(5748): 644-648.

Tran, C., Ouk, S., Clegg, N. J., Chen, Y., Watson, P. A., Arora, V., Wongvipat, J., Smith-Jones, P. M.*, et al.* (2009). Development of a second-generation antiandrogen for treatment of advanced prostate cancer. Science 324(5928): 787-790.

Übel, C., Mousset, S., Trufa, D., Sirbu, H. and Finotto, S. (2013). Establishing the role of tyrosine kinase 2 in cancer. Oncoimmunology 2(1): e22840.

Uzgare, A. R. and Isaacs, J. T. (2004). Enhanced redundancy in Akt and mitogen-activated protein kinase-induced survival of malignant versus normal prostate epithelial cells. Cancer research 64(17): 6190-6199.

van Leenders, G. J., Boormans, J. L., Vissers, C. J., Hoogland, A. M., Bressers, A. A., Furusato, B. and Trapman, J. (2011). Antibody EPR3864 is specific for ERG genomic fusions in prostate cancer: implications for pathological practice. Modern Pathology 24(8): 1128-1138.

Verhagen, P., Van Duijn, P., Hermans, K., Looijenga, L., van Gurp, R., Stoop, H., Van der Kwast, T. and Trapman, J. (2006). The PTEN gene in locally progressive prostate cancer is preferentially inactivated by bi-allelic gene deletion. The Journal of pathology 208(5): 699-707.

Viré, E., Brenner, C., Deplus, R., Blanchon, L., Fraga, M., Didelot, C., Morey, L., Van Eynde, A.*, et al.* (2006). The Polycomb group protein EZH2 directly controls DNA methylation. Nature 439(7078): 871-874.

Walsh, P. (1975). Physiologic basis for hormonal theapy in carcinoma of the prostate. The Urologic clinics of North America 2(1): 125-140.

Wang, J., Cai, Y., Ren, C. and Ittmann, M. (2006). Expression of variant TMPRSS2/ERG fusion messenger RNAs is associated with aggressive prostate cancer. Cancer research 66(17): 8347-8351.

Wang, L., McDonnell, S. K., Elkins, D. A., Slager, S. L., Christensen, E., Marks, A. F., Cunningham, J. M., Peterson, B. J.*, et al.* (2001). Role of HPC2/ELAC2 in hereditary prostate cancer. Cancer research 61(17): 6494-6499.

Watson, D. K., Turner, D. P., Scheiber, M. N., Findlay, V. J. and Watson, P. M. (2010). ETS transcription factor expression and conversion during prostate and breast cancer progression. Open Cancer J 3: 24-39.

Weber, H. C. (2009). Regulation and signaling of human bombesin receptors and their biological effects. Current Opinion in Endocrinology, Diabetes and Obesity 16(1): 66-71.

Wei, G. H., Badis, G., Berger, M. F., Kivioja, T., Palin, K., Enge, M., Bonke, M., Jolma, A.*, et al.* (2010). Genome-wide analysis of ETS-family DNA-binding in vitro and in vivo. The EMBO journal 29(13): 2147-2160.

Weischenfeldt, J., Simon, R., Feuerbach, L., Schlangen, K., Weichenhan, D., Minner, S., Wuttig, D., Warnatz, H.-J.*, et al.* (2013). Integrative genomic analyses reveal an androgen-driven somatic alteration landscape in early-onset prostate cancer. Cancer cell 23(2): 159-170.

Wend, P., Holland, J. D., Ziebold, U. and Birchmeier, W. (2010). Wnt signaling in stem and cancer stem cells. Seminars in cell & developmental biology, Elsevier.

Wolf, A., Wender, R. C., Etzioni, R. B., Thompson, I. M., D'Amico, A. V., Volk, R. J., Brooks, D. D., Dash, C.*, et al.* (2010). American Cancer Society guideline for the early detection of prostate cancer: update 2010. CA: a cancer journal for clinicians 60(2): 70-98.

Wu, Z., Conaway, M., Gioeli, D., Weber, M. and Theodorescu, D. (2006). Conditional expression of PTEN alters the androgen responsiveness of prostate cancer cells. The Prostate 66(10): 1114-1123.

Xiao, D., Wang, J., Hampton, L. L. and Weber, H. C. (2001). The human gastrin-releasing peptide receptor gene structure, its tissue expression and promoter. Gene 264(1): 95-103.

Xin, L., Teitell, M. A., Lawson, D. A., Kwon, A., Mellinghoff, I. K. and Witte, O. N. (2006). Progression of prostate cancer by synergy of AKT with genotropic and nongenotropic actions of the androgen receptor. Proceedings of the National Academy of Sciences 103(20): 7789-7794.

Xu, J., Lange, E. M., Lu, L., Zheng, S. L., Wang, Z., Thibodeau, S. N., Cannon-Albright, L. A., Teerlink, C. C.*, et al.* (2013). HOXB13 is a susceptibility gene for prostate cancer: results from the International Consortium for Prostate Cancer Genetics (ICPCG). Human genetics 132(1): 5-14.

Xu, J., Zheng, S. L., Komiya, A., Mychaleckyj, J. C., Isaacs, S. D., Hu, J. J., Sterling, D., Lange, E. M.*, et al.* (2002). Germline mutations and sequence variants of the macrophage scavenger receptor 1 gene are associated with prostate cancer risk. Nature genetics 32(2): 321-325.

Yao, H. P., Zhou, Y. Q., Zhang, R. and Wang, M. H. (2013). MSP-RON signalling in cancer: pathogenesis and therapeutic potential. Nat Rev Cancer 13(7): 466-481.

Zhang, J., Baines, C. P., Zong, C., Cardwell, E. M., Wang, G., Vondriska, T. M. and Ping, P. (2005). Functional proteomic analysis of a three-tier PKCepsilon-Akt-eNOS signaling module in cardiac protection. Am J Physiol Heart Circ Physiol 288(2): H954-961.

Zhu, X., Lv, J., Yu, L., Zhu, X., Wu, J., Zou, S. and Jiang, S. (2009). Proteomic identification of differentially-expressed proteins in squamous cervical cancer. Gynecologic oncology 112(1): 248-256.

Zong, Y., Xin, L., Goldstein, A. S., Lawson, D. A., Teitell, M. A. and Witte, O. N. (2009). ETS family transcription factors collaborate with alternative signaling pathways to induce carcinoma from adult murine prostate cells. Proc Natl Acad Sci U S A 106(30): 12465-12470.