U.PORTO

MESTRADO EM ONCOLOGIA

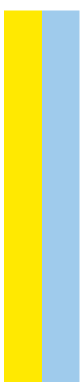
ESPECIALIZAÇÃO EM ONCOLOGIA LABORATORIAL

Clarifying the role of GRPR and ETV1 overexpression in EGFR-mediated JAK/STAT signaling - putative therapeutic targets for prostate carcinomas with *ETV1* rearrangements

Ana Catarina Pinto Azeredo

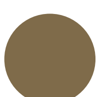
M

2021



U.PORTO

Ana Catarina Pinto Azeredo. Clarifying the role of GRPR and ETV*1* overexpression in EGFR-mediated JAK/STAT signaling - putative therapeutic targets for prostate carcinomas with *ETV1* rearrangements

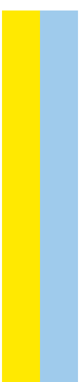


M.ICBAS **2021**

Clarifying the role of GRPR and ETV1 overexpression in EGFR-mediated JAK/STAT signaling - putative therapeutic targets for prostate carcinomas with *ETV1* rearrangements

Ana Catarina Pinto Azeredo

INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR



ANA CATARINA PINTO AZEREDO

CLARIFYING THE ROLE OF GRPR AND ETV1 OVEREXPRESSION IN EGFR-MEDIATED JAK/STAT SIGNALING – PUTATIVE THERAPEUTIC TARGETS FOR PROSTATE CARCINOMAS WITH *ETV1* REARRANGEMENTS

Dissertação de Candidatura ao Grau de Mestre em Oncologia – Especialização em Oncologia Laboratorial – submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto.

Orientador:

Doutora Paula Cristina Martins dos Santos Paulo
Investigadora Auxiliar no Grupo de Oncogenética do Centro de Investigação do Instituto Português de Oncologia do Porto.

Co-orientador:

Professor Doutor Manuel Rodrigues Teixeira
Professor Catedrático Convidado
Departamento de Patologia e Imunologia Molecular Instituto de Ciência Biomédicas de Abel Salazar da Universidade do Porto.
Diretor do Departamento de Genética e Coordenador do Grupo de Oncogenética do Centro de Investigação do Instituto Português de Oncologia do Porto.

ACKNOWLEDGMENTS

Em primeiro lugar quero expressar a minha gratidão ao Professor Manuel Teixeira, por me ter possibilitado integrar o seu grupo de investigação e me ter co-orientado durante esta Tese de mestrado. Muito obrigada pelo seu apoio, orientação e disponibilidade.

Quero dirigir também um agradecimento muito especial à minha orientadora, a Doutora Paula Paulo, por toda a dedicação e empenho neste projeto. Obrigada pelo conhecimento que partilhou comigo, pelas muitas horas despendidas para que este trabalho fosse possível, pela paciência e, sobretudo, por acreditar sempre em mim e nas minhas capacidades, encorajando-me sempre para novos desafios. Estarei eternamente grata por todo o companheirismo que teve comigo.

A todo o grupo de Oncogenética por me ter acolhido. Em particular à Marta, Luísa, Andreia e Maria por toda a ajuda, pelos conselhos partilhados, por ouvirem as minhas frustrações e por partilharem comigo bons momentos.

O meu maior agradecimento é dirigido à minha família.

Aos meus pais por me terem transmitido valores que me acompanham ao longo da vida, por acreditarem comigo nos meus sonhos e me ajudarem a realizá-los, e por me levantarem em todas as minhas quedas.

Ao Cláudio por ser a melhor pessoa que podia ter escolhido para estar ao meu lado. Obrigada por rires e chorares comigo, por acreditares em mim quando eu mesma já não acredito e por estares presente em todos os momentos da minha vida. Obrigada pelo teu amor incondicional e por me fazeres feliz. Sem ti nada disto seria possível.

À minha irmã por me fazer ver a vida de uma forma mais leve, por me cultivar o instinto de proteção e de exemplo, por todos os desabafos compartilhados e declarações de orgulho que aquecem o coração.

Aos meus avós por me ajudarem a crescer, por acreditarem nos meus sonhos e me terem encorajado sempre a lutar por eles, vivendo um dia de cada vez. Obrigada, sobretudo, por me fazerem sentir “em casa”.

Dedico esta dissertação à minha mãe, que embora não fisicamente presente, sei que esteve num lugar especial sempre a dar-me força e a ajudar-me a levantar em todos os meus percalços. Pelo exemplo de força, dedicação e persistência que é para mim. Por ter sido a melhor mãe que uma filha pode desejar ter na vida. Por sempre realçar o orgulho que sentia por mim. E porque sei que vai estar sempre comigo a celebrar todas as minhas vitórias.

"*Quero voltar para os braços da minha mãe*"

INDEX

ACKNOWLEDGMENTS III
INDEX VII
FIGURE INDEX XIII
TABLE INDEX XVII
RELEVANT ABREVIATIONS XXI
ABSTRACT XXV
RESUMO XXIX
INTRODUCTION 33
1. Prostate 35
1.1. Anatomy and histology 35
1.2. Prostatic lesions 36
2. Prostate Cancer 37
2.1. Epidemiology 37
2.2. Etiology 38
2.3. Diagnosis 39
2.3.1. Prostate Specific Antigen 40
2.3.2. Digital Rectal Examination 40
2.3.3. Biopsy and Gleason Score 40
2.4. Treatment 41
2.4.1. Options for localized prostate cancer 41
2.4.2. Options for metastatic castration-resistant prostate cancer 42
3. Genetic alterations 42
3.1. Germline variants 42
3.2. Somatic variants 43
4. The ETS family of transcription factors 44
4.1. ETS subfamilies 44
4.2. ETS rearrangements 45
4.2.1. Oncogenic functions 46
4.2.2. Prognostic value of ETS rearrangements 46
4.3. ETS as therapeutic targets 47
5. GRPR 48
5.1. GRPR and prostate cancer 48
5.2. GRPR as therapeutic target 49

5.3. GRPR and EGFR transactivation 50
6. EGFR 51
6.1. Gene, protein, and receptor 51
6.2. Signaling and signaling pathways 52
6.3. Mutations and oncogenic functions 53
6.4. EGFR inhibitors and mechanisms of resistance 53
7. JAK/STAT signaling pathway 54
7.1. JAKs overview 55
7.1.1. TYK2 56
7.2. STATs overview 56
7.2.1. Oncogenic functions 58
7.3. JAK/STAT Inhibitors 59
AIMS 61
MATERIALS AND METHODS 65
1. Cell lines and cell culture 67
1.1. Validation of the cell models 68
1.1.1. RNA extraction 69
1.1.2. cDNA synthesis 69
1.1.3. qRT-PCR 69
2. Assessment of proteins expression 71
2.1. Cellular stimulation 71
2.2. Protein extraction and quantification 71
2.3. Western Blot 72
2.3.1. Densitometry analysis 74
3. Identification of direct ETV1-regulated genes 74
RESULTS 77
1. ETV1 modulates the expression of GRPR 79
2. ETV1 induces the expression and activation of EGFR 80
3. GRPR positively regulates the activation of EGFR under ETV1 overexpression 82
4. EGFR also regulates the expression of ETV1 82
5. STATs are effectors of the ETV1-GRPR-EGFR signaling cascade 83
5.1. TYK2 83
5.2. STAT5A / P-STAT5A 84
5.3. STAT3 / P-STAT3 85

6. ERG negatively regulates the identified EGFR-STATs cascade 87
7. ETV1 binds to the promoter region of *GRPR*, *EGFR*, and *STAT3* genes 88
DISCUSSION 89
1. Establishing a link between ETV1 and EGFR 91
2. GRPR positively regulates the activation of EGFR 94
3. Downstream effectors of the ETV1-EGFR 94
4. Implication of the discovered signaling cascade for prostate cancer treatment 98
CONCLUSION 101
FUTURE PRESPECTIVES 105
REFERENCES 109
APPENDIX 125
Appendix 1 127
Appendix 2 131

FIGURE INDEX

Figure 1. Cellular organization of the prostate tissue. 35

Figure 2. Anatomic division of the prostate in three glandular zones (central, transitional and peripheral) and respective fraction of diagnosed prostate carcinomas 36

Figure 3. Simplified model of prostate cancer development and progression 37

Figure 4. Estimates for incidence and mortality of male cancers in 2020, worldwide. 37

Figure 5. Estimates for incidence and mortality of male cancers in 2020, in Portugal. 38

Figure 6. Guidelines for Prostate cancer early detection – NCCN Clinical Practice Guidelines in Oncology, version 1.2021. 39

Figure 7. Hierarchical clustering of the ETS family members according to the homology of the DNA binding sequence. 44

Figure 8. Mode of action of GRPR agonists and antagonists. 49

Figure 9. Conformational structure model of the EGFR domains and changes upon EGF binding. 51

Figure 10. Main signaling pathways activated by EGFR. 52

Figure 11. JAK/STAT signaling pathway. 55

Figure 12. STATs structural domains. 57

Figure 13. Mode of activation of a TaqMan® probe. 70

Figure 14. ETV1 overexpression leads to increased GRPR expression. 79

Figure 15. ETV1 silencing leads to decreased GRPR expression. 80

Figure 16. ETV1 overexpression leads to increased EGFR expression and activation in PNT2 cells upon EGF stimulus. 81

Figure 17. ETV1 silencing leads to decreased EGFR expression and activation in LNCaP cells. 81

Figure 18. EGFR activation is differentially regulated by GRPR in LNCaP and VCaP cells 82

Figure 19. EGF stimulus increases the expression of ETV1. 83

Figure 20. Total TYK2 expression is not regulated by ETV1. 84

Figure 21. TYK2 total expression is not regulated by GRPR. 84

Figure 22. ETV1 overexpression and EGF stimulus induce increased STAT5A activation. 85

Figure 23. GRPR silencing results in increased STAT5A phosphorylation. 85

Figure 24. ETV1 overexpression and EGF stimulus induce increased STAT3 expression and activation... 86

Figure 25. GRPR silencing results in decreased STAT3 activation in LNCaP cells. 86

Figure 26. ERG overexpression negatively regulates the expression and activation of EGFR, STAT3, and STAT5A... 87

Figure 27. ETV1 binds to the promoter regions of *EGFR* and *STAT3* genes. 88

Figure 28. Proposed model for ETV1-mediated and GRPR-dependent EGFR-STATs oncogenic pathway. 97

TABLE INDEX

Table 1. Grade group and correspondent Gleason score 41

Table 2. STAT activation in the different solid tumor types 58

Table 3. Main characteristics of the prostatic cell lines used 67

Table 4. Cell line models used in this study 68

Table 5. Optimized blotting conditions for the detection of specific proteins and phosphorylated isoforms 73

RELEVANT ABREVIATIONS

ADT	Androgen Deprivation Therapy
AR	Androgen Receptor
ATP	Adenosine triphosphate
BPH	Benign Prostatic Hyperplasia
CRPC	Castration-Resistant Prostate Cancer
ChIP	Chromatin Immunoprecipitation
DNA	Deoxyribonucleic acid
DRE	Digital Rectal Examination
EGF	Epidermal growth factor
EGFR	Epidermal Growth Factor Receptor
EMA	European Medicines Agency
EMT	Epithelial to mesenchymal transition
ERG	v-ets avian erythroblastosis virus E26 oncogene homolog
ERK	Extracellular signal-regulated kinases
ESMO	European Society for Medical Oncology
ETS	E-26 transformation-specific
ETV1	ETS variant 1
ETV4	ETS variant 4
ETV5	ETS variant 5
FDA	Food and Drug Administration
FLI1	Friend leukaemia virus integration 1
GG	Gleason Grade
GRP	Gastrin Releasing Peptide
GRPR	Gastrin Releasing Peptide Receptor
GS	Gleason Score
GUSB	Beta glucuronidase
HBOC	Hereditary Breast and Ovarian Cancer Syndrome
HER	Human Epidermal Receptor
HGPIN	High-Grade Prostatic Intraepithelial Neoplasia

HNSCC	Head and neck squamous cell carcinomas
IL	Interleukin
JAKs	Janus Kinases
mAbs	Monoclonal antibodies
MAPK	Mitogenic-activated protein kinase
mCRPC	Metastatic Castration-Resistant Prostate Cancer
MMR	Mismatch Repair
mRNA	Messenger RNA
NCBI	National Center for Biotechnology Information
NCCN	National Comprehensive Cancer Network
NSCLC	Non-small cell lung cancer
PCa	Prostate cancer
P-EGFR	Phospho-EGFR
PIAS	Protein Inhibitors of Activated STAT
PIN	Prostatic Intraepithelial Neoplasia
PSA	Prostate Specific Antigen
P-STAT3	Phospho-STAT3
P-STAT5A	Phospho-STAT5A
qRT-PCR	Quantitative reverse transcription PCR
RP	Radical Prostatectomy
SOCS	Suppressors of Cytokine Signaling
STAT	Signal Transducers and Activators of Transcription
TCGA	The Cancer Genome Atlas
TGF-α	Transforming Growth Factor-alpha
TKIs	Tyrosine kinase inhibitors
TYK2	Tyrosine Kinase 2
Tyr	Tyrosine
TMPRSS2	Transmembrane Protease Serine 2

ABSTRACT

Abstract

Prostate cancer (PCa) is the second most incident neoplasia and the fifth leading cause of death from cancer in men, worldwide. Therefore, the search for new targeted therapies focusing on genetic alterations and new signaling pathways involved in disease progression is one of the main topics in PCa research.

The genomic rearrangements involving the ETS transcription factor family are one of the most frequent genetic alterations in PCa. Although ERG rearrangements are present in 50-60% of the prostate carcinomas, their prognostic value is controversial. Conversely, rearrangements/overexpression of ETV1, present in 10-15% of the carcinomas, have been consistently associated with higher tumor aggressiveness and poor prognosis.

Previous studies in our group have identified GRPR, the human gastrin-releasing peptide receptor, as a target shared by ERG and ETV1, and TYK2, a tyrosine kinase of the JAK family, as potential specific effector of ETV1 overexpression. Other studies reported that the GRPR transactivates the epidermal growth factor receptor (EGFR) in prostate, lung, and head and neck carcinomas, and that EGFR can activate the JAK/STATs oncogenic pathway. Therefore, the main aim of this study was to understand if EGFR activity is involved in the interplay between GRPR and TYK2, and if there is ETV1-specificity in a GRPR-EGFR-JAK/STAT signaling pathway, opening horizons for new targeted therapeutics.

To evaluate the association between ETS or GRPR overexpression and both EGFR and JAK/STATs activation, we used prostate-derived cellular models with modulation of ETV1/ERG or GRPR expression, previously established. Using western blot, we evaluated changes in the expression/activation of GRPR, EGFR, TYK2, STAT3, and STAT5A, both in cells stimulated and unstimulated with EGF.

Our results show that ETV1 and ERG overexpression differentially regulate the expression/activation of EGFR, STAT3, and STAT5A, in prostate-derived cell populations with *de novo* ETS overexpression, with ETV1 promoting and ERG repressing the EGFR-STATs pathway. Additionally, we observed that EGFR-mediated activation of STAT3 is determined by GRPR overexpression and specific of the ETV1 background. Both in ERG and ETV1 overexpressing cells, GRPR silencing leads to EGFR-mediated STAT5A activation.

We conclude that ETV1 overexpression is sufficient to induce the activation of the EGFR pathway, that STATs are downstream effectors of the ETV1-EGFR oncogenic cascade, and that GRPR modulates the ETV1-EGFR STAT effector. On the other hand, the observation of an inverse regulatory mechanism by ERG overexpression, reinforces that activation of the EGFR-STATs cascade may be specific to the ETV1 context, eventually

explaining the higher clinicopathological aggressiveness described for ETV1-positive tumors. Although the role of TYK2 in the new ETV1-regulated and GRPR-mediated oncogenic pathway is not yet clarified, this work describes for the first time an ETV1 oncogenic pathway involving EGFR activation, thus, supporting the therapeutic potential of EGFR targeted therapies in prostate carcinomas with ETV1 overexpression.

RESUMO

Resumo

O cancro da próstata é a segunda neoplasia mais incidente e a quinta principal causa de morte por cancro nos homens a nível mundial. Por isso, a pesquisa de novas terapias tendo por base alterações genéticas e vias de sinalização envolvidas na progressão da doença tem sido um dos principais tópicos na investigação em cancro da próstata.

Entre as alterações genéticas mais frequentes em cancro da próstata destacam-se os rearranjos genómicos da família de fatores de transcrição ETS. Embora rearranjos envolvendo o ERG estejam presentes em 50-60% dos carcinomas da próstata, o seu valor prognóstico é controverso. Por outro lado, os rearranjos/sobre-expressão do ETV1, presentes em 10-15% dos carcinomas, têm sido consistentemente associados a elevada agressividade tumoral e a pior prognóstico.

Estudos anteriores do nosso grupo identificaram o GRPR (recetor do péptido libertador de gastrina) como alvo comum da sobre-expressão de ERG e ETV1, e a TYK2 (cinase de tirosina da família JAK) como potencial efetor específico da sobre-expressão de ETV1. Outros estudos mostraram que o GRPR transativa o recetor do fator de crescimento epidérmico (EGFR) em carcinomas da próstata, pulmão e cabeça e pescoço e que o EGFR pode ativar a via oncogénica JAK/STAT. Assim, o principal objetivo deste estudo foi perceber se o EGFR está por trás da relação entre o GRPR e a TYK2 e se a via de sinalização GRPR-EGFR-JAK/STAT é especifica para o ETV1, abrindo horizontes para novos alvos terapêuticos.

Para avaliar a associação entre a sobre-expressão dos ETS ou do GRPR e a ativação do EGFR e das JAK/STATs recorremos a modelos celulares derivados de próstata com modelação da expressão de ETV1/ERG ou de GRPR, previamente estabelecidos. Através da técnica de *western blot*, avaliamos alterações na expressão/ativação de GRPR, EGFR, TYK2 e STAT3 e STAT5A, em células estimuladas e não estimuladas com EGF.

Os nossos resultados mostram que, em populações celulares derivadas de próstata com sobre-expressão *de novo* de ETS, a sobre-expressão de ETV1 e de ERG regula de forma distinta a expressão/ativação do GRPR, EGFR, STAT3 e STAT5A, com o ETV1 a promover e o ERG a reprimir a via EGFR-STATs. Para além disso, observamos que a ativação da STAT3 mediada pelo EGFR é determinada pela sobre-expressão de GRPR e específica para o contexto ETV1. Por fim, verificamos que, tanto em células com sobre-expressão de ERG como de ETV1, o silenciamento do GRPR levou à ativação da STAT5A, mediada pelo EGFR.

Deste modo, concluímos que a sobre-expressão de ETV1 é suficiente para induzir a ativação da via do EGFR, que as STATs são efetoras *downstream* da cascata oncogénica

ETV1-EGFR e que o GRPR modula a STAT efetora da via ETV1-EGFR. Por outro lado, a observação de um mecanismo de regulação inversa para a sobre-expressão de ERG, reforça que a ativação da cascata EGFR-STATs possa ser específica do contexto ETV1, explicando, eventualmente, a elevada agressividade clinicopatológica descrita para os carcinomas positivos para ETV1. Embora ainda não tenha sido esclarecido o papel da TYK2 na nova via oncogénica regulada pelo ETV1 e mediada pelo GRPR, este trabalho descreve, pela primeira vez uma via oncogénica associada ao ETV1 que envolve a ativação do EGFR, suportando, assim, o potencial terapêutico de terapias dirigidas ao EGFR em carcinomas da próstata com sobre-expressão de ETV1.

INTRODUCTION

1. Prostate

1.1. Anatomy and histology

The prostate is the main accessory gland that belongs to the male reproductive system (Verze *et al*, 2016). It has the shape of an almond with a normal volume of 20 to 30 grams (Langan, 2019), and is located in the subperitoneal compartment, posterior to the symphysis pubis, anterior to the rectum, and inferior to the urinary bladder (Lee *et al*, 2011). It is responsible for producing the prostatic fluid, which has a relevant role in male fertility since it is associated with sperm activation and capacitation (Verze *et al*, 2016).

In histological terms, the prostate is organized in ducts and glands with an inner layer of epithelium surrounded by stroma (Figure 1). The main function of the stroma is to offer an adequate microenvironment for the epithelial part. The epithelium performs the major glandular function (Verze *et al*, 2016), and is composed of three types of epithelial cells: secretory or luminal, basal, and neuroendocrine (Shen & Abate-Shen, 2010; Verze *et al*, 2016; Wang *et al*, 2018).

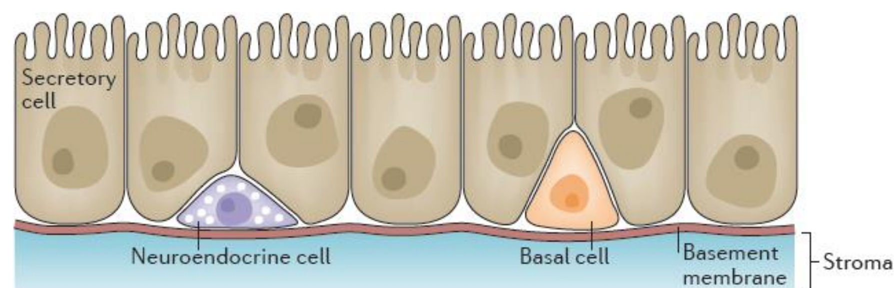


Figure 1. Cellular organization of the prostate tissue [adapted from (Verze *et al*, 2016)].

A model of the prostatic anatomic division was established in 1981 by McNeal and it continues to be accepted nowadays. The scheme proposed by McNeal divides the prostate in a fibromuscular zone and in three glandular zones: central, transition, and peripheral (McNeal, 1981; Lee *et al*, 2011; Verze *et al*, 2016; Sathianathen *et al*, 2018). The central zone surrounds the ejaculatory ducts and projects under the urinary bladder, the transition zone surrounds the urethra proximal to the ejaculatory ducts, and the peripheral zone surrounds both structures (Figure 2) (Verze *et al*, 2016). McNeal found that while benign prostatic hyperplasia often occurs in the transition zone, about 75% of the prostate carcinomas develop in the peripheral zone (McNeal, 1981; Lee *et al*, 2011; Sathianathen *et al*, 2018)

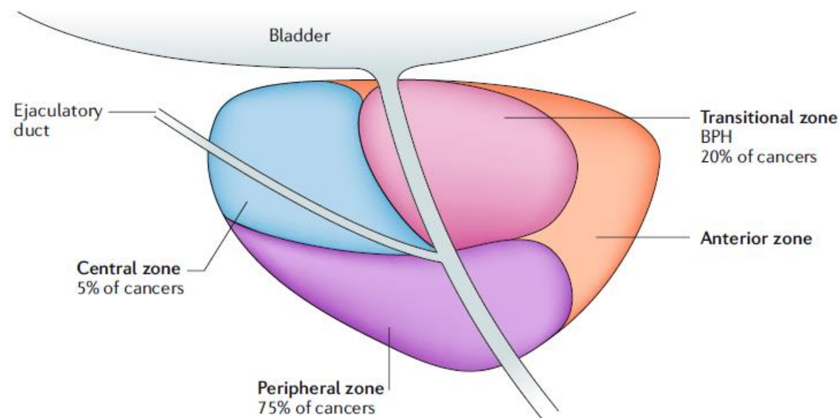


Figure 2. Anatomic division of the prostate in three glandular zones (central, transitional and peripheral) and respective fraction of diagnosed prostate carcinomas [adapted from (Sathianathen *et al*, 2018)].

1.2. Prostatic lesions

Benign prostatic hyperplasia (BPH) is one of the most frequently diagnosed prostatic lesions. It occurs mainly in the transitional zone and is characterized by the proliferation of prostatic cells with a change in the prostate volume (Skinder *et al*, 2016). This increased prostate state leads to urethral obstruction and lower urinary tract symptoms, resulting in recurrent urinary infections and discomfort during urination (Skinder *et al*, 2016; Langan, 2019). The risk of being diagnosed with BPH increases with age, with the highest prevalence being observed in men between 70 to 79 years old (Skinder *et al*, 2016).

Prostate malignant transformation is a multistage process, eventually developing from a prostatic intraepithelial neoplasia (PIN) lesion (Wang *et al*, 2018) (Figure 3). High-Grade PIN (HGPIN) is characterized by the abnormal proliferation of epithelial cells in the prostate ducts and glands, being accepted as a transition phase between the benign lesions and PCa, and hence, as a precursor lesion of prostate carcinoma (De Marzo *et al*, 2016; Zhou, 2018). As well as BPH, the diagnosis of HGPIN increases with age, being found in 5% of the prostate biopsies and in a large percentage of the radical prostatectomies. The identification of HGPIN lesions in a biopsy is associated with a ~25% risk of a prostate carcinoma diagnosis in a subsequent biopsy (Zhou, 2018).

The next steps in malignant transformation are localized PCa, followed by local invasion and, lastly, metastatic disease (Figure 3) (Wang *et al*, 2018). Prostate carcinoma usually metastasizes to adjacent lymph nodes, following spread to the liver, lungs, pleura, and/or bones. Bone metastases occur as osteoblastic lesions and are often painful, causing hypercalcemia and fractures (Shen & Abate-Shen, 2010; Wang *et al*, 2018).

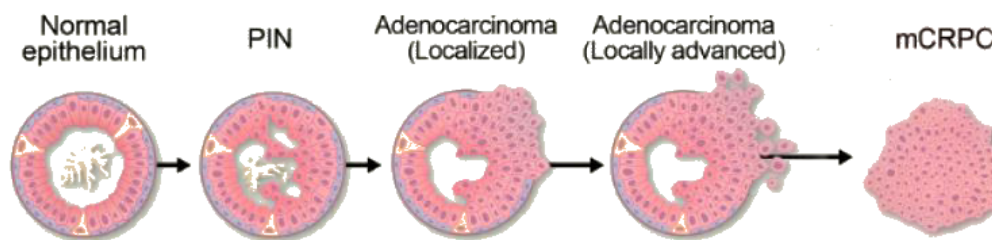


Figure 3. Simplified model of prostate cancer development and progression – Malignant transformation presumably starts with a PIN lesion, which evolves to localized adenocarcinoma. Disease continuum may lead to advanced adenocarcinoma, ultimately progressing to metastatic castration resistant prostate carcinoma (mCRPC) [adapted from (Wang *et al*, 2018)].

2. Prostate Cancer

2.1. Epidemiology

Prostate cancer (PCa) is the second most incident neoplasia in men and the fifth leading cause of cancer mortality, worldwide. In 2020, estimates point to 1,414,259 new diagnoses, corresponding to 14.1% of all cancers in men, and 375,304 deaths, representing 6.8% of all male cancer deaths (Figure 4) (Ferlay *et al*, 2021; Sung *et al*, 2021). According to GLOBOCAN projections, in 2040 there will be 2,235,568 new PCa cases and 720,661 PCa deaths, corresponding to an increase of 58.1% and 92.0%, respectively, comparing to 2020 (Culp *et al*, 2020; Ferlay *et al*, 2021; Sung *et al*, 2021).

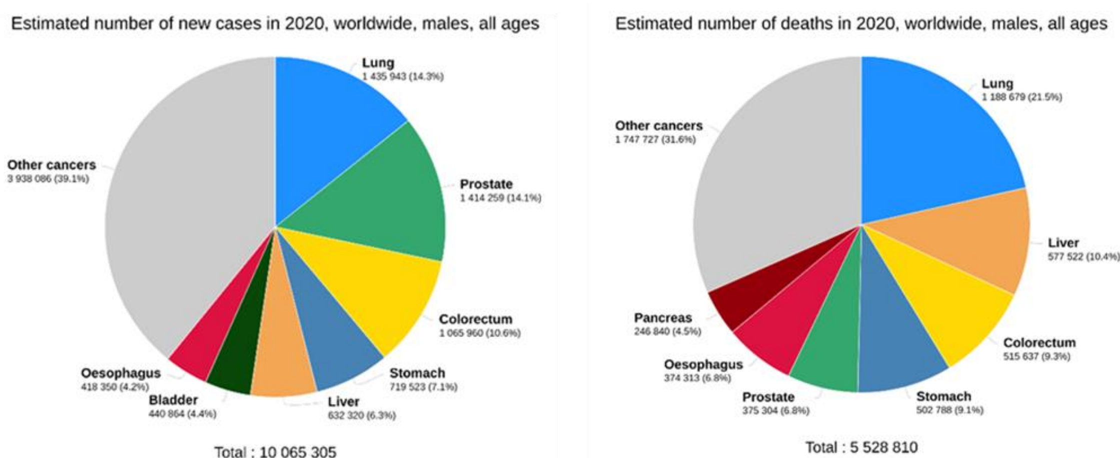


Figure 4. Estimates for incidence and mortality of male cancers in 2020, worldwide [adapted from GLOBOCAN 2020, available at https://gco.iarc.fr/].

In Portugal, in 2020, PCa was the most incident cancer, with 6,759 new diagnoses cases, representing 20% of all cancers in men, and the third leading cause of cancer mortality, with 1,917 deaths from PCa, which correspond to 10.5% of all cancer deaths in men (Figure 5). Projections for 2040 point to 8,216 new cases and 2,844 deaths of PCa, with an increase of 21.6% and 48.4%, respectively (Sung *et al*, 2021).

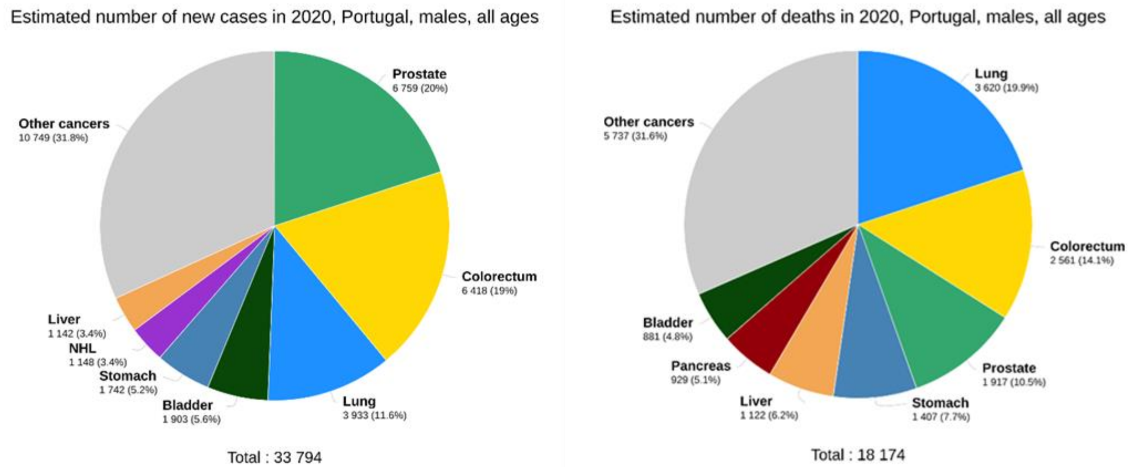


Figure 5. Estimates for incidence and mortality of male cancers in 2020, in Portugal [adapted from GLOBOCAN 2020, available at https://gco.iarc.fr/].

2.2. Etiology

As previously referred, PCa is a very frequent disease, however, the knowledge about its etiology is scarce. Risk factors as aging, ethnicity, family history, genetic variations (namely in the *BRCA1* and *BRCA2* genes), and cancer predisposing syndromes (as the Hereditary Breast and Ovarian Cancer Syndrome (HBOC) and Lynch Syndrome) are well established (Perdana *et al*, 2016; Brandão *et al*, 2020; Culp *et al*, 2020; Sung *et al*, 2021).

The age associated with an increased risk of developing this disease is also related to the patient’s ethnicity. For Caucasian men, the risk significantly increases after the age of 50, while African men above 40 years old are already at increased risk. Contrarily, Asian men have the lowest incident rates of PCa. Additionally, older men (above age 70) have higher likelihood to be diagnosed with PCa in an advanced stage and exhibit lower overall survival (Perdana *et al*, 2016).

Regarding family history, epidemiological studies have shown that a man with a first degree relative who suffered from PCa has 2 to 3 times higher probability of having the disease, increasing to 10 times if three immediate family members are affected, when compared with a man without family PCa history (Daniyal *et al*, 2014; Perdana *et al*, 2016).

Besides these well-established risk factors, there are a few lifestyle and environmental factors that have been associated with PCa development, such as smoking, alcohol consumption, diet, obesity, sunlight exposure, and chemical exposure, among others (Perdana *et al*, 2016; Pernar *et al*, 2018; Culp *et al*, 2020; Sung *et al*, 2021).

The risk associated with genetic variations will be described in Chapter 3.

2.3. Diagnosis

According to the National Comprehensive Cancer Network (NCCN) and the European Society for Medical Oncology (ESMO) guidelines, PCa diagnosis begins with the baseline evaluation of family cancer history, ethnicity, familial or personal history of high-risk germline variants, history of prostate disease, and prior analyses and/or exams. After that, Prostate Specific Antigen (PSA) levels and findings on digital rectal examination (DRE) are considered. If the PSA levels are higher than 3 ng/mL and/or there are suspected alterations on DRE, men are assigned to biopsy or *follow up* according to the risk factors previously assessed and considering complementary exams, namely, second PSA analysis, Magnetic Resonance Imaging (MRI) examination, or additional biomarkers to improve screening specificity (Figure 6) (Parker *et al*, 2020; National Comprehensive Cancer Network, 2021).

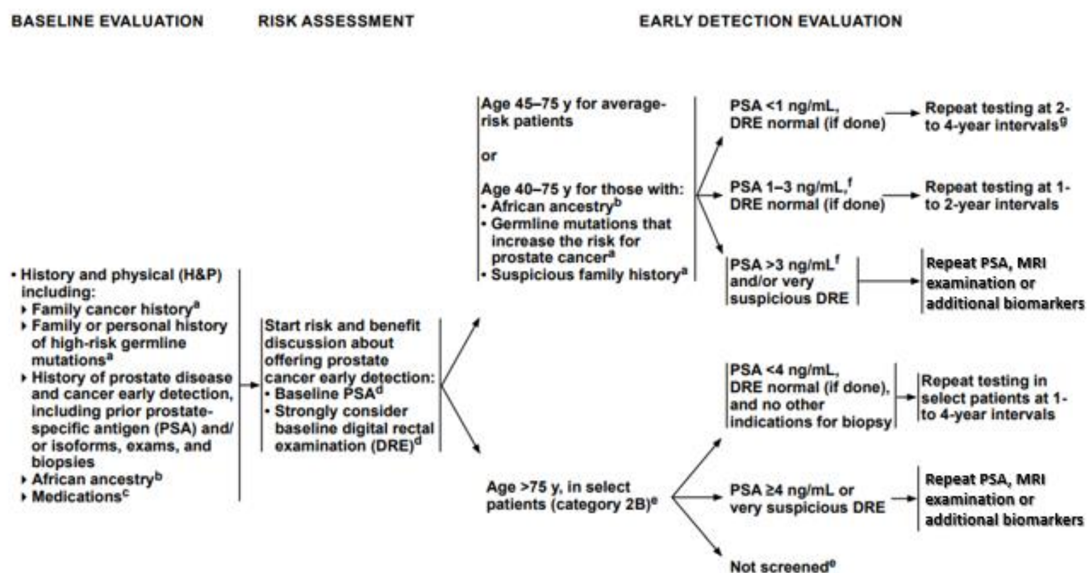


Figure 6. Guidelines for Prostate cancer early detection – NCCN Clinical Practice Guidelines in Oncology, version 1.2021 [adapted from (National Comprehensive Cancer Network, 2021)].

Different types of prostate carcinoma can be diagnosed, namely neuroendocrine carcinoma, basal cell carcinoma, squamous cell carcinoma, urothelial carcinoma, and adenocarcinoma, being the latter the most frequent (Inamura, 2018).

2.3.1. Prostate Specific Antigen

PSA is a protease secreted by prostatic epithelial cells that lyses the clotted ejaculate to enhance sperm motility. Although levels may be elevated in PCa, it is not a cancer-specific marker since it is also increased in other prostate alterations such as BPH or prostatic infections (Daniyal *et al*, 2014; Barry & Simmons, 2017).

2.3.2. Digital Rectal Examination

Digital Rectal Examination (DRE) has been supporting PCa diagnosis for many years, with abnormal results increasing PCa likelihood. It is an inexpensive, easy to perform, and relatively non-invasive technic, however, its effectiveness depends on examiner's experience and skills. Thus, DRE results should be analyzed in combination with other diagnostics tools (Ragsdale *et al*, 2014; Barry & Simmons, 2017).

2.3.3. Biopsy and Gleason Score

Prostate biopsy is the gold standard for PCa diagnosis, and is performed using transrectal ultrasound to obtain 10 to 12 tissue samples. The histological appearance and architecture of the prostate cells are then evaluated by microscopy, according to a specific scoring system – the Gleason Score (GS) (Litwin & Tan, 2017; Sathianathen *et al*, 2018). This system consists in the sum of the two most predominant grades of histological differentiation, both on a scale of 1 (the most differentiated tumor) to 5 (the least differentiated tumor) (Lee *et al*, 2011; Litwin & Tan, 2017).

In 2014, the Gleason Score was revised, and the different sums were organized into five grades (Table 1) (Epstein *et al*, 2016; Litwin & Tan, 2017; Sehn, 2018). This classification is important because it enables the differentiation of the Gleason score 7 in two groups with different prognostic value: 3 + 4 (grade 2) and 4 + 3 (grade 3), with grade 3 carcinomas showing worse prognosis compared to grade 2 carcinomas. Following this classification, patients with Gleason Grade (GG) carcinoma = 1 have a good prognosis, so this alteration also aims to prevent overtreatment in a diagnosis of a grade 1 carcinoma (Epstein *et al*, 2016; Sehn, 2018).

Table 1. Grade group and correspondent Gleason score
[adapted from (Sehn, 2018)].

Grade group	Gleason score
1	≤6 (usually 3+3)
2	7 (3+4)
3	7 (4+3)
4	8 (4+4, 3+5, 5+3)
5	9, 10 (4+5, 5+4, 5+5)

2.4. Treatment

Before a treatment option is applied, the patient should be informed about the benefits and side effects of the treatment. Prostate cancer treatment may cause dysfunction, infertility, and urinary problems. Therefore, the different options must be discussed between the person and both the urologist and the oncologist (Parker *et al*, 2020).

2.4.1. Options for localized prostate cancer

According to NCCN, localized disease can be stratified into five risk groups: very low, low, intermediate, high, and very high, being the intermediate risk divided in "favourable" and "unfavourable" (National Comprehensive Cancer Network, 2021).

For patients with "very low", "low" or "favourable intermediate" risk, the therapeutic options are active surveillance, external beam radiotherapy (EBRT) or brachytherapy (BT), and radical prostatectomy (RP) (Parker *et al*, 2020; National Comprehensive Cancer Network, 2021). Active surveillance is a strategy of disease monitoring, which involves PSA testing, physical examinations, and repeated biopsies (Litwin & Tan, 2017; Parker *et al*, 2020). For patients with "unfavourable intermediate", "high" or "very high" risk, combined therapies are suggested, namely, EBRT with androgen deprivation therapy (ADT), EBRT with BT and ADT, or RP alone (Parker *et al*, 2020; National Comprehensive Cancer Network, 2021).

Prostate cancer is a hormone-responsive cancer and ADT usually leads to tumor regression, using agents that block the androgen pathway. However, some carcinomas can become unresponsive to ADT resulting in castration-resistant prostate cancer (CRPC) (Wang *et al*, 2018). CRPC is characterized by disease progression during ADT treatment, with or without metastatic lesions (Hotte & Saad, 2010; Wang *et al*, 2018).

2.4.2. Options for metastatic castration-resistant prostate cancer

For patients suffering from metastatic castration-resistant prostate cancer (mCRPC), the preferred treatment options are the combination of ADT with Apalutamide, Abiraterone, or Enzalutamide (second generation androgen pathway inhibitors), or ADT with Docetaxel (chemotherapy). In certain circumstances, an immunologic vaccine (Sipuleucel-T), Radium – 223 (usually for symptomatic bone metastases), or secondary hormone therapy can be recommended (Litwin & Tan, 2017; Parker *et al*, 2020; National Comprehensive Cancer Network, 2021).

Despite the available treatment options, metastatic disease continues to be the leading cause of mortality from PCa, with about 70% of men with advanced disease showing metastatic progression (Hotte & Saad, 2010; Wang *et al*, 2018). Thus, it is essential to develop targeted therapies to patients with metastatic PCa to reduce these elevated rates. In this manner, it is important to understand the genetic changes and signaling pathways that are involved in disease progression. Recently, PARP inhibitors were recently approved for the treatment of mCRPC for carcinomas with deleterious variants (germline or somatic) in genes of the homologous recombination repair pathway (Jang *et al*, 2020; National Comprehensive Cancer Network, 2021).

3. Genetic alterations

3.1. Germline variants

Germline variants are alterations that are present in the DNA of germ cells (egg and sperm), therefore, inherited by direct biological line, being found in every cell of the body. Thereby, inherited variants may predispose to hereditary cancer (Cheng *et al*, 2019).

In hereditary PCa, the most common germline variants known, so far, are those associated with DNA repair genes, such as *BRCA1*, *BRCA2*, *ATM*, *CHEK2*, *RAD51D*, *PALB2*, *MSH2*, *MSH6*, *MLH1* e *PMS2* (Cheng *et al*, 2019; Brandão *et al*, 2020; Raghallaigh & Eeles, 2021).

BRCA1 and *BRCA2* are the most well-stablished PCa predisposing genes, involved in homologous recombination repair (Brandão *et al*, 2020; Raghallaigh & Eeles, 2021). In 2019, a meta-analysis showed that carriers of *BRCA* deleterious variants had a 1.90-fold increased risk of developing PCa, and that the risk was higher for *BRCA2* carriers (2.64-fold) than for *BRCA1* carriers (1.35-fold). Moreover, patients with *BRCA2* variants were also associated with an increased risk of PCa death (Oh *et al*, 2019). Castro et al. had found similar results, concluding that men with *BRCA1/2* deleterious variants had worse survival

outcomes and a higher probability of developing regional and distant metastases (Castro *et al*, 2013).

Variants in *BRCA2* are more frequent than in other DNA repair genes. In 2016, a multicenter study recruited 692 men with metastatic PCa and identified DNA repair gene mutations in 11.8% of the cases. Variants in *BRCA2* were present in 5.3% of men, *ATM* in 1.6%, *CHEK2* in 1.9%, *BRCA1* in 0.9%, and *RAD51D* and *PALB2* in 0.4% (Pritchard *et al*, 2016). The germline variants in *BRCA1*, *BRCA2*, *ATM*, *CHEK2*, and *PALB2* genes are associated with predisposition to breast and/or ovarian cancer, which is often associated with increased risk of PCa development (Petrucelli *et al*, 1998; Brandão *et al*, 2020).

The *MSH2*, *MSH6*, *MLH1*, and *PMS2* genes are responsible for mismatch repair (MMR). Germline variants in these genes are associated with Lynch syndrome (LS), a hereditary condition that increases the risk of developing colorectal cancers, as well as other malignancies (Medina-Arana *et al*, 2012; Cheng *et al*, 2019). Several studies have found a relation between LS and the risk for PCa. A meta-analysis collected data from molecular and risk studies of patients with PCa and deleterious variants in MMR genes and analyzed the association of both conditions, concluding that carriers had 2 to 3 times higher risk of PCa, and that *MSH2* was the most frequently mutated gene (Ryan *et al*, 2014), which was consistent with previous risk studies. Another study followed 188 men diagnosed with LS and registered the PCa development up to 14 years after LS diagnosis. Of the 188 men studied, 11 were diagnosed with PCa, seven of which carrying deleterious variants in *MSH2*, two in *MSH6*, one in *MLH1*, and one in *PMS2*, which represent a 5-fold increased risk of developing PCa (Haraldsdottir *et al*, 2014).

3.2. Somatic variants

Somatic variants are non-inherited alterations that can occur in the DNA of any cell except in the germ cells, and that may be acquired over the entire life (Karki *et al*, 2015).

The Cancer Genome Atlas (TCGA) Network research group characterized 333 primary prostate cancers and found that 74% of the carcinomas could be subtyped according to the profile of somatic variations, with variants in DNA repair genes being found in only 19% of the carcinomas. Among the somatic changes, 53% had genomic fusions involving the *ETS* gene family (*ERG*, *ETV1*, *ETV4* and *FLI1*), 11% were positive for variants in *SPOP*, 3% in *FOXA1*, and 1% in *IDH1*, defining the seven PCa molecular subtypes (The Cancer Genome Atlas Research Network, 2015).

The relevance of ETS rearrangements will be detailed in Chapter 4.

Variants in *SPOP* were found to be mutually exclusive of ETS alterations (The Cancer Genome Atlas Research Network, 2015). Previously, *SPOP* variations have been

considered early molecular events in prostate carcinogenesis since they were found in HGPIN precursor lesions (Barbieri *et al*, 2012). However, their influence on PCa prognosis has been controversial. A study showed that *SPOP* variations do not correlate with survival, disease relapse, or mortality in men with PCa (Blattner *et al*, 2014), whereas another study observed poor prognosis in patients with these genetic changes (García-Flores *et al*, 2014).

FOXA1 is a chromatin remodeler whose inactivating mutations leads to the loss of its capacity to repress cell motility. Variants in *FOXA1* have been found in localized and metastatic PCa and are mutually exclusive of ETS and *SPOP* mutations (Arora & Barbieri, 2018).

Although *IDH1* variants are less common in PCa than in other cancer types such as leukemia and gliomas, they are associated with early age of PCa developing (The Cancer Genome Atlas Research Network, 2015; Arora & Barbieri, 2018).

4. The ETS family of transcription factors

4.1. ETS subfamilies

The ETS are a family of transcription factors composed of 28 genes. These genes are divided into 11 subfamilies of up to 3 members each, according to their similarity in the DNA binding domain (Figure 7). The two most important subfamilies in PCa are the ERG and the PEA3 subfamily. The ERG subfamily comprises *ERG*, *FLI1*, and *FEV*, while *ETV1*, *ETV4*, and *ETV5* belong to the PEA3 subfamily. Inside a subfamily, the homology of the entire protein between members is high, however, between subclasses, the different ETS members only share homology in the DNA binding domain (Tandefelt *et al*, 2014; Fry *et al*, 2018; Nicholas *et al*, 2019).

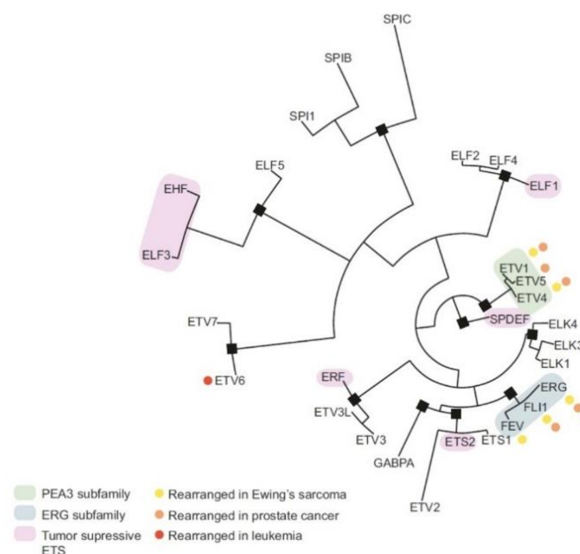


Figure 7. Hierarchical clustering of the ETS family members according to the homology of the DNA binding sequence. Common rearrangements in cancer are marked in coloured dots. The green blot represents

the PEA3 subfamily, the blue the ERG subfamily, and the pink the tumor suppressive ETS. Squares correspond to different subfamilies [adapted from (Nicholas *et al*, 2019)].

The DNA binding domain is composed of 84 to 90 amino acids which bind to DNA as a monomer. This domain has a structure of a winged helix-turn-helix (wHTH), with 3 alpha-helices and 4 beta-strands, and recognizes the 5'- GGA(A/T) - 3' core consensus sequence, binding with high-affinity in the regulatory region of target genes. After DNA binding, ETS can act as activators or repressors of the transcription (Tandefelt *et al*, 2014; Fry *et al*, 2018; Nicholas *et al*, 2019).

4.2. ETS rearrangements

Members of ERG and PEA3 subfamilies are often mutated in Ewing's sarcoma, myeloid leukaemia, and PCa, by genomic rearrangements (Sizemore *et al*, 2017). In PCa, ETS rearrangements usually involve the fusion of the 3' end of the ETS, which includes the DNA binding domain, with the 5' end of the partner gene, frequently an androgen-regulated promotor, leading to overexpression of the ETS transcription factor (Nicholas *et al*, 2019).

ERG rearrangements are found in 40 to 50% of prostate carcinomas, *ETV1* rearrangements/overexpression in 8 to 10%, and *ETV4*, *ETV5*, and *FLI1* in 2 to 5% of the cases, being mutually exclusive (The Cancer Genome Atlas Research Network, 2015; Nicholas *et al*, 2019).

The most common 5' partner is the *TMPRSS2* gene, which encodes a transmembrane serine protease regulated by androgens. The *TMPRSS2-ERG* fusion is found in a large percentage of the prostate cancers, frequently caused by interstitial deletion of the genomic region between both genes, at 21q22 (Tomlins *et al*, 2005; Tandefelt *et al*, 2014). *TMPRSS2* can also be found rearranged with *ETV1*, *ETV4*, and *ETV5*, by inter-chromosomal rearrangement, although at much lower frequencies (Tandefelt *et al*, 2014; Nicholas *et al*, 2019).

Rearrangements between *ERG* and other fusion partners, such as *SLC45A3*, *NDRG1*, and *HERPUD1*, are rare, while for *ETV1*, more than ten 5' fusion partners have been described, those being *SLC45A3*, *HERV-K*, *HERVK17*, *C15orf21*, *HNRPA2B1*, *OR51E2*, *EST14*, *FLJ35294*, *FOXP1*, and *ACSLS*, with the most common alteration involving full-length ETV1 translocation to a specific region on chromosome 14, which also contains the non-coding EST14 gene (Tomlins *et al*, 2007a; Clark & Cooper, 2009; Barros-Silva *et al*, 2013; Tandefelt *et al*, 2014; Nicholas *et al*, 2019).

4.2.1. Oncogenic functions

As previously mentioned, when overexpressed, the ETS activate or repress target genes, having important roles in the regulation of cell growth, proliferation, differentiation, apoptosis, and invasion (Tomlins *et al*, 2007b; Clark & Cooper, 2009; Mesquita *et al*, 2015).

It has been consensual that *ERG*, *ETV1*, and *ETV4* are oncogenes, since they are frequently overexpressed in PCa cells and are not expressed in normal prostate tissue (Hollenhorst *et al*, 2004; Nicholas *et al*, 2019). Although *ETV5* and *FLI1* are also not expressed in normal prostate cells (Hollenhorst *et al*, 2004), they are rearranged in a lower percentage of prostate tumors (Paulo *et al*, 2012a), so their oncogenic function is not so clear (Nicholas *et al*, 2019). Some studies found that *ETV5* fusions are also oncogenic, but the role of *FLI1* rearrangements is still unclear (Helgeson *et al*, 2008; Kedage *et al*, 2016).

ETS rearrangements are considered early molecular events in prostate carcinogenesis since they are detected in HGPIN precursor lesions, but not in BPH (Cerveira *et al*, 2006). In these lesions, the ETS rearrangements have been pointed not as the initial event of prostate tumorigenesis, but as mediators of PIN progression to prostate carcinoma. Moreover, these rearrangements are also found in advanced PCa states, including in metastatic cancer at similar frequencies (Carver *et al*, 2009; Taris *et al*, 2014; Nicholas *et al*, 2019).

4.2.2. Prognostic value of ETS rearrangements

Although *ERG* rearrangements are detected in almost 50% of primary prostate tumors (The Cancer Genome Atlas Research Network, 2015), this alteration has been controversial as a prognostic marker (Arora & Barbieri, 2018). Initially, Petrovics et al. showed that *ERG* overexpression in prostate tumor cells was a prognostic indicator of pathologic state and disease-free survival after radical prostatectomy (RP) (Petrovics *et al*, 2005). Later, other studies suggested a correlation between *TMPRSS2-ERG* fusion and tumor stage, namely, Wang and colleagues described that different isoforms of this fusion were associated with aggressive disease, seminal vesicle invasion, and poor outcome following RP (Wang *et al*, 2006), and Rajput et al. found that *TMPRSS2-ERG* fusion was increased in less differentiated PCa, suggesting ERG rearrangement as a diagnostic marker (Rajput *et al*, 2007). The most recent studies contradicted the association of ERG overexpression as a prognostic marker, showing that *TMPRSS2-ERG* rearrangement is not correlated with outcome, disease aggressiveness, recurrence, or mortality, for men treated with RP (Gopalan *et al*, 2009; Pettersson *et al*, 2012). In CRPC, a single study found ERG overexpression associated with longer overall survival (Taris *et al*, 2014).

On the other hand, despite their smaller frequency (in ~10% of the PCa), two studies were able to show that ETV1 overexpression has a negative impact on PCa patients. In 2013, Baena et al. found that ETV1 expression was associated with higher disease aggressiveness and poor outcome (Baena *et al*, 2013), an observation later confirmed by Segalés and colleagues who affirmed that ETV1 overexpression has a "non-negligible role" in PCa (Segalés *et al*, 2019). Previous studies from our group reported that ETV1 was associated with increased cellular invasion in PCa cells (Mesquita *et al*, 2015).

4.3. ETS as therapeutic targets

Although *ERG*, *ETV1*, *ETV4*, and *ETV5* have oncogenic functions in PCa, they are also necessary for vital functions in other organs. ERG is also expressed in hematopoietic stem cells, helping in self-renewal, and in endothelial cells, allowing migration. ETVs are important for the development of some organs such as lung, kidney, salivary and mammary glands. In normal cells, as in PCa cells, these transcription factors alter the expression of genes related to cell development, proliferation, differentiation, migration, and apoptosis (Clark & Cooper, 2009; Tandefelt *et al*, 2014; Fry *et al*, 2018; Nicholas *et al*, 2019).

Moreover, some ETS as *SPDEF*, *EHF*, *ETS2*, *ELF3*, *ELF1*, and *ERF* have tumor suppressive functions, and their main action is to compete with oncogenic ETS for the DNA binding site (Gu *et al*, 2007; Albino *et al*, 2012; Longoni *et al*, 2013; Linn *et al*, 2016; Bose *et al*, 2017; Budka *et al*, 2018; Nicholas *et al*, 2019).

As ETS perform important physiological functions in normal cells, a major problem with ETS targeting drugs is tumor specificity, once they should not interfere with other normal ETS functions (Nicholas *et al*, 2019). In addition, the use of transcription factors as therapeutic targets is challenging because of their localization in the nucleus and activity in multiprotein complexes (Konstantinopoulos & Papavassiliou, 2011). Still, a couple of compounds have been reported to inhibit ETS transcription factors with different specificities (Hsing *et al*, 2020).

Due to the difficulty of using ETS as therapeutic targets and considering that ETS overexpression is relevant for PCa progression, our research group has focused on the identification of ETS-downstream targets with potential therapeutic utility. In 2012, Paulo et al. found that prostate carcinomas with *ERG* or *ETV1* rearrangements have overexpression of GRPR, showing direct regulation of ERG by binding to the GRPR promoter region (Paulo *et al*, 2012b). Later, Santos et al. reported TYK2 as a possible effector of the GRPR pathway in *ETV1*-positive tumors, further observing a decrease in TYK2 expression after *GRPR* silencing *in vitro*. In addition, a decrease in both GRPR and TYK2 protein levels were observed after *ETV1* silencing, thus, establishing a link between ETV1, GRPR, and TYK2,

and sustaining the potential of GRPR and TYK2 as therapeutic targets in prostate carcinomas with *ETV1* overexpression (Santos *et al*, 2015).

5. GRPR

The Gastrin Releasing Peptide (GRP) is a regulatory peptide that belongs to the family of bombesin-related peptides. Bombesin is a short peptide that was first isolated from the skin of frogs, and its mammalian version is GRP, first discovered in the porcine stomach (Hohla & Schally, 2010; Mansi *et al*, 2013; Ischia *et al*, 2014).

GRP has diverse functions in gastrointestinal and central nervous systems, such as the release of gastrointestinal hormones from the stomach, pancreas, and other endocrine organs, the proliferation of epithelial cells, and the stimulation of the contraction of smooth muscles (Hohla & Schally, 2010; Mansi *et al*, 2013).

The bombesin-related peptides' functions are performed by G-protein coupled receptors, which bind to G-protein by their intracellular domain. These receptors are divided into three subtypes: GRP Receptor (GRPR), Neuromedin B receptor (NMBR), and Bombesin Receptor Subtype 3 (BRS-3) (Hohla & Schally, 2010; Mansi *et al*, 2013; Ischia *et al*, 2014).

The *GRPR* gene is located in chromosome X, and the transmembrane receptor, as other G-protein coupled receptors, contains an extracellular N-domain, seven transmembrane domains, three extracellular and three intracellular loops, and an intracellular C-tail (Xiao *et al*, 2001; Liapakis *et al*, 2017).

GRPR is found overexpressed in several cancers, namely, lung, breast, colorectal, pancreatic, gastric, head and neck, renal, brain, and prostatic carcinomas (Hohla & Schally, 2010; Mansi *et al*, 2013).

5.1. GRPR and prostate cancer

GRPR has been demonstrated to have an important role in PCa development. An initial study of Markwalder and Reubi using tumors obtained from radical prostatectomies concluded that GRPR expression was higher in primary prostatic carcinomas than in non-neoplastic prostatic tissues. In addition, they also observed GRPR overexpression in HGPIN, thus, being an early molecular event in prostate carcinogenesis (Markwalder & Reubi, 1999). In 2015, Santos et al. found that silencing of GRPR leads to the attenuation of the malignant phenotype of PCa cell lines, decreasing proliferation and invasion, and increasing apoptosis (Santos *et al*, 2015).

The tumor progression induced by GRPR seems to be associated with the interaction between GRP and the androgen receptor (AR). A preclinical study showed that,

in LNCaP cell lines with GRP overexpression, there was activation of the AR in the absence of androgen stimulus, which increased the cellular growth and motility. Moreover, orthotopic implantation of these cellular models in castrated nude mice led to the development of aggressive tumors. These results suggest that GRP activates AR signaling even in androgen-depleted conditions, being an important marker for CRPC (Yang *et al*, 2009). Later, Qiao al. showed that the activation of GRPR increases the activity of the NF-kB transcription factor, which, in turn, increases the expression of an AR variant depleted of ligand-binding domain, which is activated in the absence of ligand, contributing to progression to CRPC (Qiao *et al*, 2016)

5.2. GRPR as therapeutic target

Due to the reports of GRPR overexpression in numerous cancers, including PCa, different approaches have been developed to inhibit GRPR activity. These strategies are receptor antagonists, monoclonal antibodies, a vaccine, antisense oligonucleotides, and bispecific molecules (Hohla & Schally, 2010). GRPR antagonists can be nonradioactive, radioactive or cytotoxic, and can be useful in both cancer treatment and diagnosis. The main difference between agonists and antagonist are the mode of action; while agonists bind to the receptor and are internalized along with the receptor, exerting cell destruction from the inside of the cells, the antagonists bind to the receptor and block its internalization, impairing cell signaling (Figure 8). By accumulating at higher levels in the cells, antagonists show higher efficacy (Mansi *et al*, 2013).

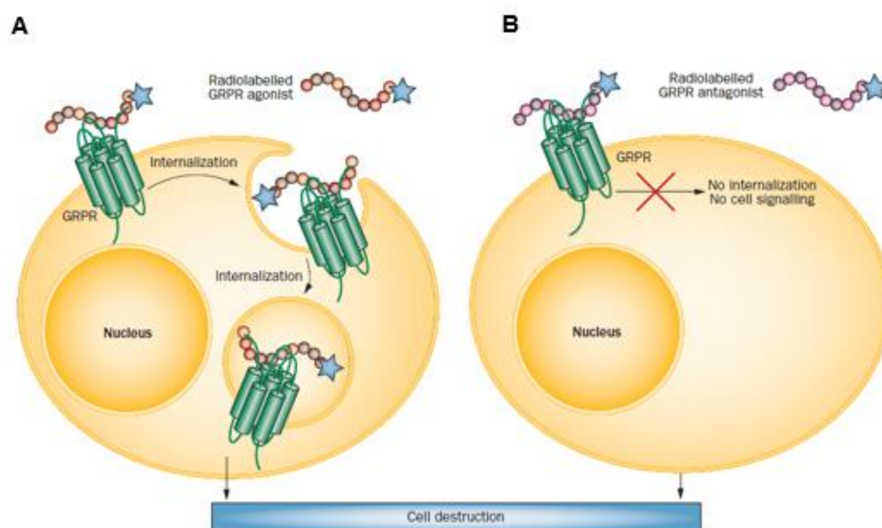


Figure 8. Mode of action of GRPR agonists and antagonists. The agonist binds to the receptor and is internalized, exerting its destructive action within the cell (**A**). The antagonist binds to the receptor blocking its internalization (**B**) [adapted from (Mansi et al, 2013)].

As previously described, GRP can induce AR activation, thus, blocking GRPR in CRPC seems a promising therapeutic opportunity (Roesler & Schwartsmann, 2013). However, in a study of 530 prostate carcinomas, GRPR overexpression was inversely correlated with increased Gleason score, PSA value, and tumor size (Beer *et al*, 2012), potentially limiting the use of GRPR antagonists in the treatment of patients with high-grade PCa (Roesler & Schwartsmann, 2013).

Moreover, considering the high biological complexity of prostate tumors, targeting only a receptor or a signaling pathway by GRPR antagonists as a monotherapy is not expected to have a substantial effect on PCa progression (Roesler & Schwartsmann, 2013). In a study using cell lines and a mice model of glioma, it was shown that combined therapy of a GRPR antagonist with chemotherapy improved the impact in cell proliferation and tumor growth when compared with the GRPR antagonist as monotherapy (de Oliveira *et al*, 2009). Recently, Case et al. observed that combined therapy of a GRPR antagonist with ADT impaired castration-resistant proliferation *in vitro* and tumor growth *in vivo*, suggesting that combined therapy is sufficient to inhibit CRPC (Case *et al*, 2021).

5.3. GRPR and EGFR transactivation

GRPR is associated with the induction of tyrosine kinases activation, such as ERK and members of the Src family, and also leads to the transactivation of the Epidermal Growth Factor Receptor (EGFR) (Hohla & Schally, 2010).

EGFR activating mutations and EGFR amplification are found in approximately 30% of the patients with non-small cell lung cancer (NSCLC) (Zhang *et al*, 2016), and 21% of the head and neck squamous cell carcinomas (HNSCC) (Cancer Genome Atlas Network, 2015), respectively. In cell lines of these two types of cancer, it was shown that GRP, the GRPR ligand, induces EGFR phosphorylation upon GRPR activation, consequently, activating the MAPK pathway and promoting cellular proliferation (Lui *et al*, 2003; Thomas *et al*, 2005). The same pathway was observed to be active in PCa cells (Xiao *et al*, 2003).

Due to the cross-talk between GRPR and EGFR in NSCLC and HNSCC, some research groups explored the potential of combined therapies with a GRPR antagonist and a EGFR inhibitor. In NSCLC cells it was shown that combined therapy with PD176252 (a GRPR antagonist) and gefitinib (a EGFR inhibitor) leads to enhanced cell death and decreased cellular proliferation when compared with each therapy alone (Thomas *et al*, 2005). In HNSCC cell lines it was seen that the combination of PD176252 and erlotinib (an EGFR inhibitor) increases the percentage of apoptotic cells and decreases cell invasion and colony formation when compared with either treatment alone (Zhang *et al*, 2007). So far, we found no reports on the potential of these combined therapies for PCa treatment.

6. EGFR

6.1. Gene, protein, and receptor

EGFR, also recognized as HER1 or ERBB1, belongs to the human epidermal receptor (HER) family, along with HER2, HER3, and HER4 (Quesnelle *et al*, 2007; Sabbah *et al*, 2020).

The EGFR gene is located at the short arm of chromosome 7 and is composed of 31 exons, which encode for a transmembrane glycoprotein, member of the protein kinases superfamily (Sabbah *et al*, 2020).

As other HER members, EGFR protein consists of an extracellular domain, a hydrophobic transmembrane region, an intracellular tyrosine kinase domain, and a C-terminal domain (Figure 9). The HER members differ in the extracellular and C-terminal domains and are similar in the tyrosine kinase domain (Sigismund *et al*, 2018; Sabbah *et al*, 2020).

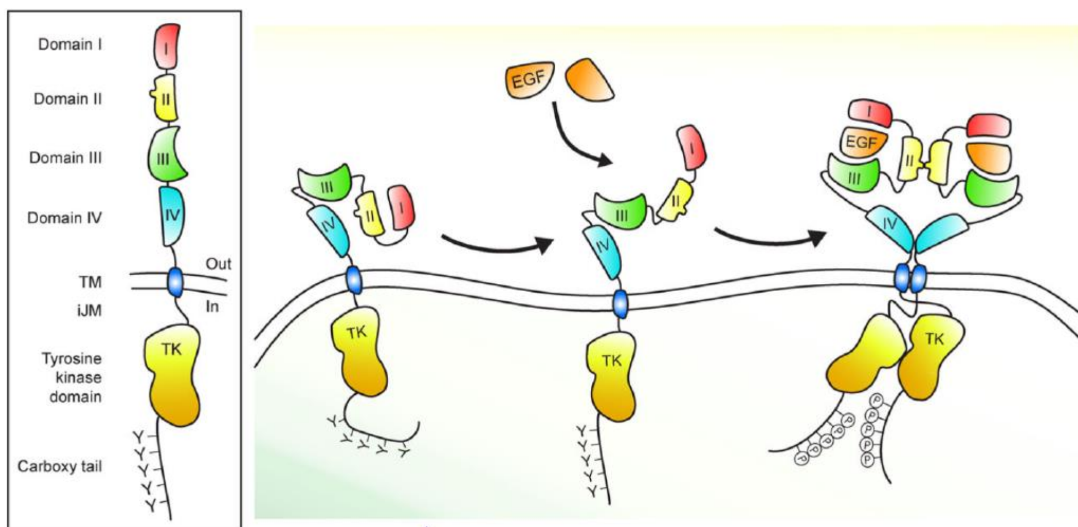


Figure 9. Conformational structure model of the EGFR domains and changes upon EGF binding. EGFR is composed of a 4-domain extracellular region, a hydrophobic transmembrane region, an intracellular tyrosine kinase domain, and a C-terminal carboxy tail. The ligand binds to the extracellular domain of the receptor and causes conformation changes allowing the receptor dimerization. The intracellular tyrosine kinase domain contains structural features as the activation loop, the catalytic domain, the hinge region, and ATP tyrosine kinase domain, which will trigger intracellular signal transduction [adapted from (Sigismund *et al*, 2018)].

6.2. Signaling and signaling pathways

Several ligands can bind to EGFR, namely, the amphiregulin, betacellulin, epidermal growth factor (EGF), heparin-binding EGF-like growth factor, and transforming growth factor-alpha (TGF-α). Ligand binding to EGFR causes conformation changes in the extracellular domain allowing dimerization, either with other EGFR molecule (homodimerization) or with other HER family members (heterodimerization). After dimer formation, the receptor becomes active by autophosphorylation of the tyrosine residues (Figure 9). Once active, EGFR activates phosphorylation reactions inducing multiple downstream signaling cascades, namely RAS/RAF/MAPK, PI3K/AKT, PLC/PKC, and JAK/STAT (Figure 10) (Quesnelle *et al*, 2007; Huang & Fu, 2015; Sigismund *et al*, 2018; Sabbah *et al*, 2020).

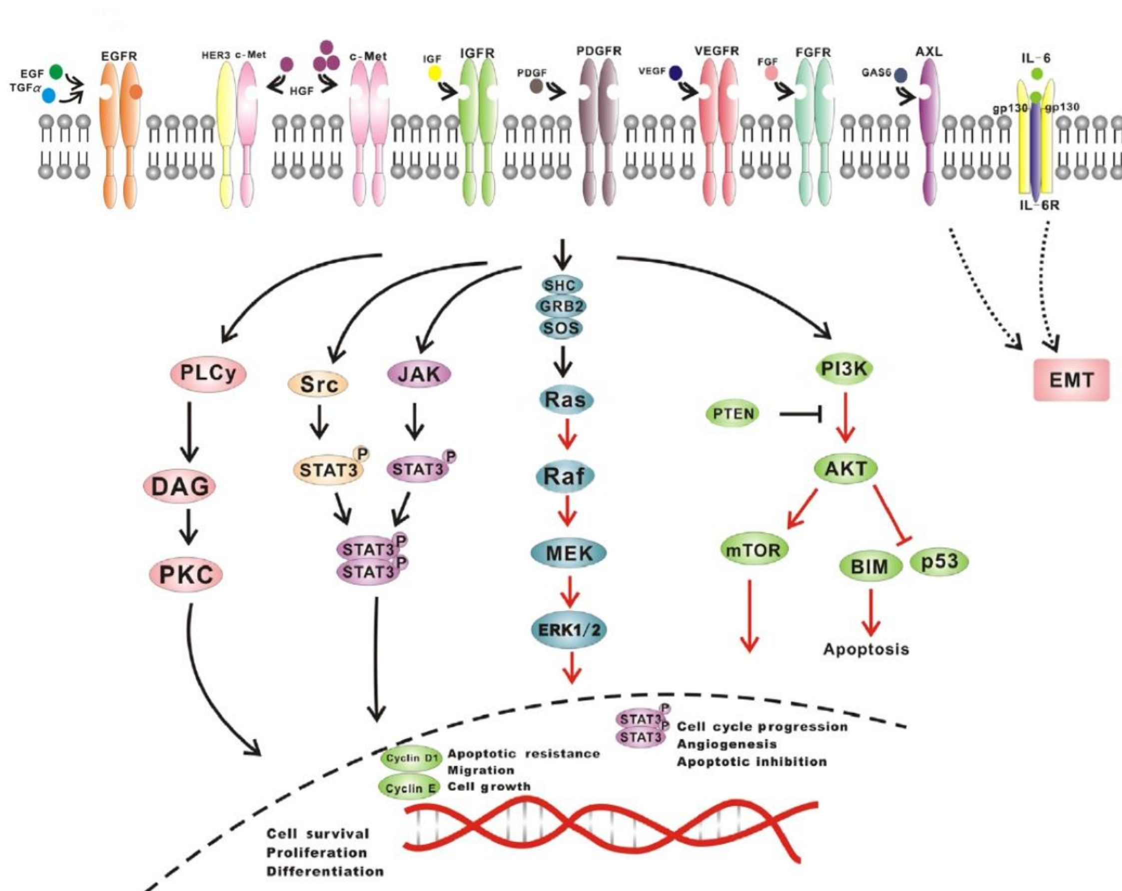


Figure 10. Main signaling pathways activated by EGFR. Activated EGFR can induce activation of several oncogenic pathways, as the RAS/RAF/MAPK, PI3K/AKT, PLC/PKC, and JAK/STAT pathways. STATs migrate to the nucleus and regulate the transcription of target genes involved in cell cycle progression, angiogenesis, and apoptotic inhibition. Other receptors may activate the same pathways, underlying the cause for resistance to EGFR target therapies [adapted from (Huang & Fu, 2015)].

6.3. Mutations and oncogenic functions

The activation of the different signaling pathways by EGFR allows signaling from the membrane to the nucleus, leading to the activation of proteins involved in cell proliferation, division, mitosis, survival, and differentiation (Figure 10). As previously mentioned, in cancer, EGFR is found frequently altered by mutations, gene amplification, and/or protein overexpression. Oncogenic mutations are more frequently found in exons 19 to 21, affecting the catalytic domain of the receptor (Sigismund *et al*, 2018; Sabbah *et al*, 2020). These alterations lead to increased EGFR signaling which contributes to cancer development and progression. EGFR alterations are present in diverse types of cancer namely, in breast, head and neck, renal, ovarian, colon, and NSCLC, which are characterized by increased aggressiveness, growth, and metastization (Quesnelle *et al*, 2007; Sabbah *et al*, 2020). This knowledge led to the development of several EGFR inhibitors, currently used in clinical practice.

6.4. EGFR inhibitors and mechanisms of resistance

There are two strategies for EGFR inhibition: the monoclonal antibodies (mAbs) and the small molecular weight tyrosine kinase inhibitors (TKIs). The antibodies target the extracellular domain of the receptor, blocking the interaction between the ligand and the receptor. On the other hand, TKIs displace ATP in the tyrosine kinase domain, interfering with the autophosphorylation of the receptor. Either way, both compounds lead to the inhibition of EGFR activation, interrupting the downstream signaling cascades (Quesnelle *et al*, 2007; Sigismund *et al*, 2018; Sabbah *et al*, 2020).

Some mAbs are approved by Food and Drug Administration (FDA) and European Medicines Agency (EMA), namely cetuximab, panitumumab, and necitumumab. Cetuximab is approved for the treatment of metastatic HNSCC and metastatic colorectal cancer (Licitra *et al*, 2013), panitumumab is approved for the treatment of metastatic colorectal cancer (Yau, 2019), and necitumumab for metastatic NSCLC (Cai *et al*, 2020).

Regarding TKIs, erlotinib, gefitinib, afatinib, and osimertinib are approved by FDA and EMA for the treatment of NSCLC (Solassol *et al*, 2019).

Despite the good results obtained with these inhibitors, patients develop drug resistance. The main mechanisms of resistance to EGFR inhibitors are secondary EGFR mutations, activating mutations in downstream effectors (KRAS, BRAF or PI3K), and activation of alternative pathways by other transmembrane receptors (as HER3, c-Met, IGFR, PDGFR, VGEFR, FGFR, AXL, and IL-6). Among these, IL-6 and AXL are particularly interesting since they are associated with epithelial to mesenchymal transition (EMT),

mediated by STAT3 and SLUG, respectively (Figure 10) (Huang & Fu, 2015; Picon & Guddati, 2020).

The importance of the STAT3 signaling in the resistance to target therapies has been highlighted by several research groups (Lee *et al*, 2014), who showed the benefits of STAT3 inhibition upon acquired resistance to EGFR-target in different cancer models (Sen *et al*, 2012; Chen *et al*, 2017; Yang *et al*, 2019; Zheng *et al*, 2021).

7. JAK/STAT signaling pathway

JAK/STAT activation can be mediated by receptors of cytokines as interleukins (ILs), interferons, or hormones, by receptors of growth factors as the EGFR, by G-protein coupled receptors, or by non-receptor tyrosine kinases as Src (Figure 11). Among cytokines, the most important activator of STAT3 is IL-6. IL-6 binds to its receptor and leads to the dimerization of the gp130 receptor subunits, which in turn activates JAKs, responsible for STAT3 activation (Quesnelle *et al*, 2007; Gu *et al*, 2020; Verhoeven *et al*, 2020).

The JAK/STAT pathway is initiated by the binding of the ligand (interleukins, interferons, hormones, or growth factors) to their respective receptors. After ligand binding, the receptor dimerizes and activates the associated JAK proteins. Once active, JAKs auto-phosphorylate and trans-phosphorylate tyrosine residues in the receptor. Phosphorylated tyrosines in the receptor, at the cytoplasmatic domain, allow the coupling of the STAT monomers by the STAT SH2 domain. When the link between the receptor and the STAT is established, the JAKs can phosphorylate the STAT in their specific tyrosine residues. One monomer of STAT is phosphorylated in each part of the receptor, and, after the phosphorylation, STATs bind to each other by the SH2 domain, forming dimers, and becoming activated. Dimers are then transported to the nucleus via importins, where they bind to specific DNA regulatory elements, regulating the transcription of the target genes, either promoting or repressing transcription (Figure 11) (Bharadwaj *et al*, 2020; Verhoeven *et al*, 2020).

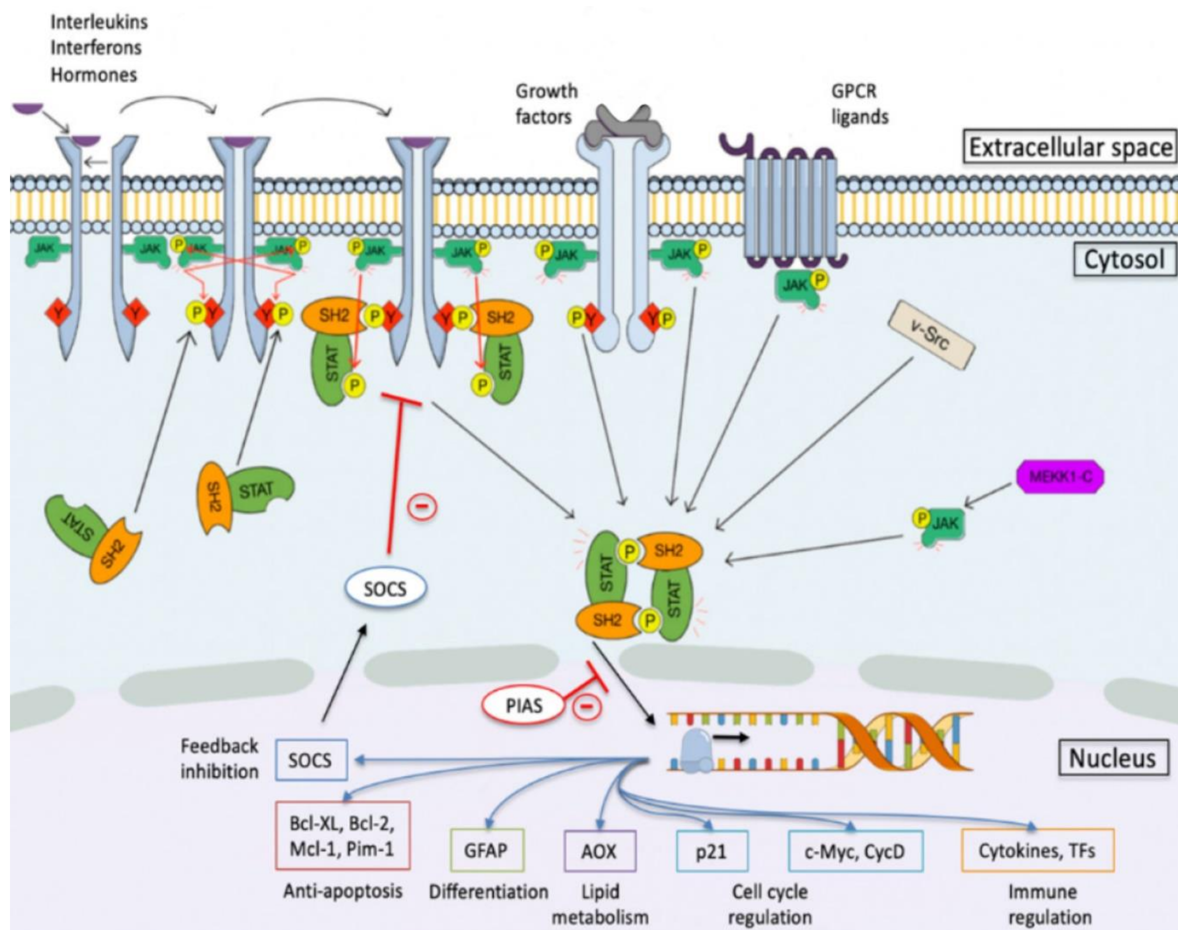


Figure 11. JAK/STAT signaling pathway. This signaling pathway can be activated by different types of receptors, non-receptor tyrosine kinases, as Src, and other proteins kinases, as MAPKs. Receptor phosphorylation by JAKs allows STAT binding, being subsequently phosphorylated and activated by JAKs. MAPKs recruit JAKs to the proximity of STATs for activation. Once in dimers, STATs migrate to the nucleus where they regulate the transcription of target genes. STATs are inhibited by Suppressors of Cytokine Signaling (SOCS) and Protein Inhibitors of Activated STAT (PIAS) [adapted from (Verhoeven *et al*, 2020)].

7.1. JAKs overview

The Janus Kinases (JAKs) family is composed of four elements: JAK1, JAK2, JAK3, and TYK2 (Tyrosine Kinase 2), that act as intermediates between the transmembrane receptors and cytoplasmic STATs (Bharadwaj *et al*, 2020).

Structurally, this family of proteins is constituted by four structural domains. The FERM domain mediates the stable association between the JAK and the intracellular tails of the receptor, and the SH2 domain is involved in the binding to the receptor. The pseudokinase domain is responsible for the negative regulation of the kinase activity, being a target of mutations. The kinase domain has the catalytic activity, where phosphorylation takes place at tyrosine residues (Leitner *et al*, 2017; Bharadwaj *et al*, 2020; Borcherding *et al*, 2021).

As the major function of JAKs is the activation of STATs, they are also regulated by the Suppressors of Cytokine Signaling (SOCS). Moreover, JAKs can also be inhibited through dephosphorylation by protein tyrosine phosphatases (Leitner *et al*, 2017; Borcherding *et al*, 2021).

JAKs can contribute to tumorigenesis by aberrant expression, increasing the expression or activation of upstream receptors, disruption of the negative regulators, as SOCS, or mutations in JAKs domains. Increased activation of JAKs enhances STAT activation, which, in turn, changes the expression of genes, involved in cellular proliferation, angiogenesis and apoptosis (Wöss *et al*, 2019; Bharadwaj *et al*, 2020). For the purpose of this thesis, only TYK2 will be further detailed.

7.1.1. TYK2

TYK2 gene is located in chromosome 19, and the protein has a molecular weight of approximately 134 kDa (Leitner *et al*, 2017; Borcherding *et al*, 2021).

TYK2 can be activated by many cytokine receptors, and its activation is related to the phosphorylation of the tyrosine residues 1054/1055, mediating the signaling of the receptors of Interferon type I (IFN-I), IL-10, IL-12, and IL-13. Moreover, TYK2 also participates in the IL-6 signaling, associated with the gp130 receptor. Since TYK2 can be activated by different receptors, it has been associated with both tumor suppressor and oncogenic functions. The tumor suppressor functions are mediated mainly by IFN-I and IL-12 signaling (Leitner *et al*, 2017; Wöss *et al*, 2019; Borcherding *et al*, 2021), while oncogenic functions are mainly associated with activation of the oncogenic STATs, namely, STAT3 and STAT5A/B. In fact, TYK2 aberrant expression levels lead to cellular transformation associated with STAT3 phosphorylation, stimulating cellular proliferation and blocking apoptosis. Additionally, TYK2 has also been described to promote migration, invasion, and metastasis in multiple types of cancer (Wöss *et al*, 2019; Borcherding *et al*, 2021).

In PCa, increased expression of TYK2 and its signaling was associated with enhancement of cellular invasion (Ide *et al*, 2008; Schuster *et al*, 2008), and metastization (Santos *et al*, 2015). Additionally, TYK2 and STAT3 were found activated in mCRPC (Drake *et al*, 2013).

7.2. STATs overview

The family of Signal Transducers and Activators of Transcription (STATs) is composed of seven proteins: STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6. The genes encoding STATs are located in different chromosomes, with STAT1 and

STAT4 at 2q32.2-q32.3, STAT2 and STAT6 at 12q13.3, and STAT3, STAT5A, and STAT5B at 17q21.2 (Gu *et al*, 2020; Verhoeven *et al*, 2020).

Regarding the structure of these proteins, they are all constituted by an N-terminal domain (ND), a coiled-coil domain (CCD), a DNA-binding domain (DBD), an Src-homology 2 (SH2) domain, and a transactivation domain (TAD) (Figure 12) (Gu *et al*, 2020; Verhoeven *et al*, 2020). The CCD domain is involved in nuclear localization, promoting protein-protein interactions, and DBD determines the DNA association. The SH2 domain, together with the ND, mediates the dimerization of the monomers of the STAT during their activation, being also important for the interaction of the STAT monomers with the upstream kinases. The TAD contains serine and tyrosine residues that are targets of phosphorylation, leading to protein activation (Figure 12). The TAD is also responsible for the recruitment of additional transcriptional activators, increasing the transcriptional activity of the STAT proteins (Quesnelle *et al*, 2007; Gu *et al*, 2020; Verhoeven *et al*, 2020).

Thus, STATs have the capability to transduce signals from the cell membrane into the nucleus, being activated in response to extracellular signaling proteins and playing essential roles in the regulation of physiologic processes by altering the transcription of the genes associated with cellular proliferation, differentiation, apoptosis, and angiogenesis (Verhoeven *et al*, 2020).

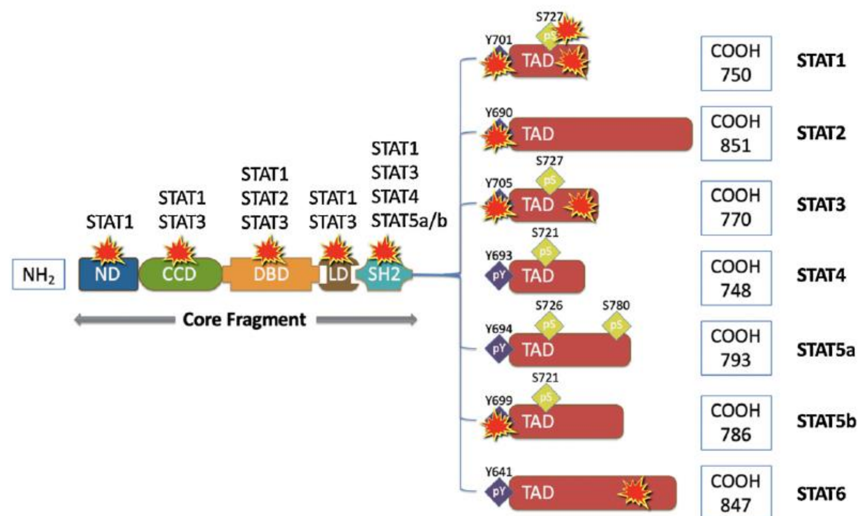


Figure 12. STATs structural domains. The STAT proteins are constituted by an N-terminal domain (ND), a coiled-coil domain (CCD), a DNA-binding domain (DBD), an Src-homology 2 (SH2) domain, and a transactivation domain (TAD). TAD contains the serine and tyrosine residues that are targets of phosphorylation, differing between STATs [adapted from (Verhoeven *et al*, 2020)].

7.2.1. Oncogenic functions

Due to the physiological functions of STATs in normal cells, the dysregulation of the signaling mediated by these proteins has important roles in the development and progression of cancer (Verhoeven *et al*, 2020).

Among the seven STATs described, STAT1, STAT3, STAT5A, and STAT5B have been demonstrated to play a role in cancer, and have been found in diverse tumors and cancer cell lines, both in hematologic malignancies and solid tumors (Quesnelle *et al*, 2007; Verhoeven *et al*, 2020). The main solid tumors showing activation of STATs are represented in Table 2.

Table 2. STAT activation in the different solid tumor types
[adapted from (Quesnelle *et al*, 2007).

Solid tumor type	STAT activation
Breast	Stat1, Stat3, Stat5
Head and neck	Stat1, Stat3, Stat6
Lung	Stat3, Stat5
Prostate	Stat3, Stat5
Colon	Stat3
Glioma	Stat3
Melanoma	Stat3
Ovarian	Stat3
Pancreatic	Stat3
Renal	Stat3
Liver	Stat3

STAT1 has been associated with activation of genes involved in cell cycle arrest and apoptosis, thus performing tumor suppressor functions (Hosui *et al*, 2012; Zhang *et al*, 2014). On the other hand, STAT3 and STAT5 have demonstrated to have oncogenic activity. STAT3 has been related to tumor invasion, metastasis, and poor prognosis (Kusaba *et al*, 2006; Chen *et al*, 2013). Specifically in PCa, it was observed that STAT3 promotes cellular migration, being involved in the metastatic behavior (Abdulghani *et al*, 2008). Furthermore, STAT5 was shown to contribute to the metastatic behavior of PCa, increasing migration and cellular invasion (Gu *et al*, 2010). Moreover, STAT5 activation was associated with poor prognosis in PCa (Li *et al*, 2005).

STAT3 and STAT5 perform their oncogenic functions by the regulation of the expression of genes related to cell proliferation, such as Cyclin D1 and c-Myc, angiogenesis, as VEGF, and apoptosis, as Survivin, Mcl-1, Bcl-2, and Bcl-XL (Quesnelle *et al*, 2007; Gu *et al*, 2020; Verhoeven *et al*, 2020).

7.3. JAK/STAT Inhibitors

Selective inhibitors for JAKs were initially developed for the treatment of autoimmune diseases, and organ transplantations. The first generation of TYK2 inhibitors targets the kinase domain, however, JAKs show high homology in this domain, thus, selectivity is a problem in this type of inhibitors. The next generation of TYK2 inhibitors is focused on the pseudokinase domain, with one already having passed a phase III clinical trials for the treatment of psoriasis (Wöss *et al*, 2019; Borcherding *et al*, 2021).

STAT signaling activation is naturally regulated by protein tyrosine phosphatases that can act in the membrane, blocking the interaction between the STAT and the receptor, or in the nucleus by the dephosphorylation of activated STAT. Additionally, SOCS can bind and inactivate STATs. Another mechanism of negative regulation is Protein Inhibitors of Activated STAT (PIAS), which bind to phosphorylated STATs preventing DNA recognition (Quesnelle *et al*, 2007; Verhoeven *et al*, 2020).

However, when STAT expression and activation are changed, as in cancer cells, these mechanisms of regulation are not enough, so a lot of inhibitors, mainly for STAT3, have already been developed. There are two main categories of STAT3 inhibitors: direct and indirect inhibitors. Indirect inhibitors interfere with the downstream cascade, blocking and inhibiting the cytokines, receptors, or kinases that phosphorylate STAT3. Moreover, they can activate negative regulators of STAT, blocking STAT shuttling between the nucleus and the cytoplasm. Direct inhibitors inhibit STAT mRNAs or a specific domain of the protein structure, as the ND, DBD, or SH2 domain (Verhoeven *et al*, 2020). Some studies with direct inhibitors of STAT3 have demonstrated a reduction in the malignant phenotype associated with this protein (Xiao *et al*, 2015; Zuo *et al*, 2015; Zhang *et al*, 2017), supporting the therapeutic potential of STAT3 targeted therapies in PCa.

AIMS

Aims

Previous observations have suggested TYK2 as an effector of the GRPR pathway in prostate carcinomas with ETV1 overexpression (Santos *et al*, 2015). However, the signaling pathway underlying GRPR and TYK2 interaction has not been described. Considering the described interplay between GRPR and EGFR and between EGFR and the JAK/STAT pathway, the main aims of this master thesis project were to understand if EGFR activity could be involved in the signaling pathway linking GRPR and TYK2, and whether ETV1 overexpression could contribute to this interaction, opening prospects for new therapeutic targets in ETV1-positive prostate carcinomas.

To achieve these aims it was necessary to clarify:

1. how ETV1 and GRPR modulate the expression and activation of EGFR;
2. if ETV1 and GRPR are involved in the activation of the JAK/STAT pathway;
3. whether TYK2 involvement in STATs pathway has ETS-specificity;
4. if ETV1 regulates directly the transcription of EGFR and JAKs/STATs.

MATERIALS AND METHODS

1. Cell lines and cell culture

We used the non-tumorigenic prostate-derived cell line, PNT2, and two established cell populations derived from PCa metastases with underlying ETS rearrangements: the LNCaP cell line harboring an ETV1 rearrangement, and the VCaP cell line carrying an ERG rearrangement. LNCaP and VCaP cells were previously obtained from the German Resource Centre for Biological Material (DSMZ, Braunschweig, Germany) and PNT2 cells were obtained from the European Collection of Cell Cultures (Sigma-Aldrich, St. Louis, MO, USA). The main characteristics of these prostatic cell lines are described in Table 3.

Table 3. Main characteristics of the prostatic cell lines used

Cell line	Cell type	Origin	ETS rearrangements
LNCaP	Carcinoma; Epithelial cells; Adherent, single cells, and loosely attached clusters.	Isolated from metastasis in the left supraclavicular lymph node of a 50-year-old Caucasian male.	Insertion of ETV1 into an intronic sequence at 14q13.3-14q21.1 (Tomlins *et al*, 2007a).
VCaP	Epithelial cells; Adherent.	Established from vertebral bone metastasis of a 59-year-old Caucasian male with hormone-refractory PCa.	TMPRSS2-ERG (Nicholas *et al*, 2019).
PNT2	Epithelial cells; Adherent.	Primary culture obtained from a normal prostate of a 33-year-old male at post mortem (Berthon *et al*, 1995).	None

[adapted from the ATCC website: www.atcc.org]

In the present study, LNCaP cell populations with GRPR or ETV1 silencing, VCaP cell populations with GRPR silencing, and PNT2 cell populations with *de novo* expression of ETV1 or ERG (Paulo *et al*, 2012b; Santos *et al*, 2015) were used. The different models are summarized in Table 4.

All cell lines were kept growing in a monolayer in a humidified chamber, at 37ºC and with 5% of CO_2. LNCaP- and PNT2-derived cell lines were grown in RPMI-1640 medium (Gibco, Invitrogen™, Carlsbad, CA, USA) and VCaP-derived cells were grown in DMEM medium (PAN-Biotech GmbH, Aidenbach, Germany). All the media were supplemented with 10% of Fetal Bovine Serum (FBS) and 1% of Penicillin/Streptomycin (both from Gibco). The cell lines were regularly tested for contaminations with *Mycoplasma spp.*, using a PCR Mycoplasma Detection Set from Clontech Laboratories Inc. (Mountain View, CA, USA).

Table 4. Cell line models used in this study

Cell line	**Model**	**Description**
LNCaP	LNCaP ShNeg	Control LNCaP cells obtained after transduction with a non-shRNA sequence (retroviral mediated).
	LNCaP ShETV1 C2	ETV1 silenced clones obtained after LNCaP transduction with an ETV1-targeting shRNA sequence (retroviral mediated).
	LNCaP ShETV1 C3	
	LNCaP Sc	Control LNCaP cells obtained after transduction with a *scrambled*, non-targeting, shRNA sequence (lentiviral mediated).
	LNCaP ShGRPR 10	GRPR silenced populations obtained after LNCaP transduction with GRPR-targeting shRNAs (lentiviral mediated).
	LNCaP ShGRPR 15	
VCaP	VCaP Sc	Control VCaP cells obtained after transduction with a *scrambled*, non-targeting, shRNA sequence (lentiviral mediated).
	VCaP ShGRPR 15	GRPR silenced populations obtained after VCaP transduction with GRPR-targeting shRNAs (lentiviral mediated).
	VCaP ShGRPR 20	
PNT2	PNT2 Neo	Control PNT2 cells obtained after transfection with a pMSCV-Neo empty vector (carrying the Neomycin resistance gene only.)
	PNT2 ETV1 C1	ETV1 overexpressing clone obtained after transfection of PNT2 cells with a pMSCV-Neo vector carrying full-length ETV1 (mimicking the most common ETV1 rearrangement, present in LNCaP cells).
	PNT2 ΔERG C3	ERG overexpressing clone obtained after transfection of PNT2 cells with a pMSCV-Neo vector carrying a 5' truncated ERG sequence (mimicking the most common TMPRSS2-ERG rearragment, present in VCaP cells).

1.1. Validation of the cell models

To proceed with our study, it was necessary to confirm silencing of ETV1 in LNCaP cells, silencing of GRPR in LNCaP and VCaP cells, and ETS overexpression in PNT2 cells. For this purpose, RNA was extracted from the cell lines, converted into complementary DNA (cDNA) and, finally, the expression of GRPR, ETV1, and ERG was analyzed with quantitative reverse transcription PCR (qRT-PCR).

1.1.1. RNA extraction

Sub-confluent cells were washed using Phosphate Buffered Saline (PBS) (GRiSP, Porto, Portugal) and trypsinized with TrypLE™ Express Enzyme (Gibco). Cells were harvested and centrifuged at 300 × *g* for 5 minutes to form a pellet. The pellet was washed twice using PBS before RNA extraction using the RNeasy® Mini Kit (QIAGEN, Frederick, MD, USA), following manufacturer's instructions.

Briefly, about 1×10^7 cells were lysed with 600 μL of Buffer RLT and a 20-gauge needle. Then, 600 μL of 70% ethanol was added to the homogenized lysate and the total volume of lysate was transferred to a RNeasy spin column. After centrifugation, column-bound nuclei-acids were washed once with 700 μL of Buffer RW1 and twice with 500 μL of Buffer RPE. RNA was eluted with 50 μL of RNAse-Free Water and concentration was measured using the Qubit™ 4 Fluorometer (Invitrogen™).

1.1.2. cDNA synthesis

RNA obtained from LNCaP, VCaP, and PNT2 cell populations was converted into cDNA using the RevertAid H Minus First Strand cDNA Synthesis Kit (ThermoFisher Scientific™, Waltham, MA, USA) according to the manufacturer instructions. Briefly, 1 μg of RNA was converted into cDNA using 1 μL of oligo $(dT)_{18}$ primers in a 20 μL buffered reaction containing 20 U of RiboLock RNase Inhibitor, 1 mM of dNTP Mix, and 200 U of RevertAid H Minus M-MuLV Reverse Transcriptase, in a two-step reaction. Briefly, initial sample denaturation and primer binding occurred at 65ºC for 5 minutes, followed by 2 minutes incubation on ice. After the adding of the remaining reaction components, cDNA synthesis took place at 42ºC for 60 minutes, followed by enzyme inactivation at 70ºC for 5 minutes.

1.1.3. qRT-PCR

Pre-developed primer/probe assays specific to GRPR, ETV1, and ERG were used, along with an assay targeting Beta glucuronidase (GUSB), as endogenous control (ThermoFisher Scientific™). The probes used have the TaqMan technology with a FAM fluorophore covalently attached to the 5'-end and a quencher dye (NFQ) at the 3'-end. The principle of TaqMan® probes activity is represented in Figure 13.

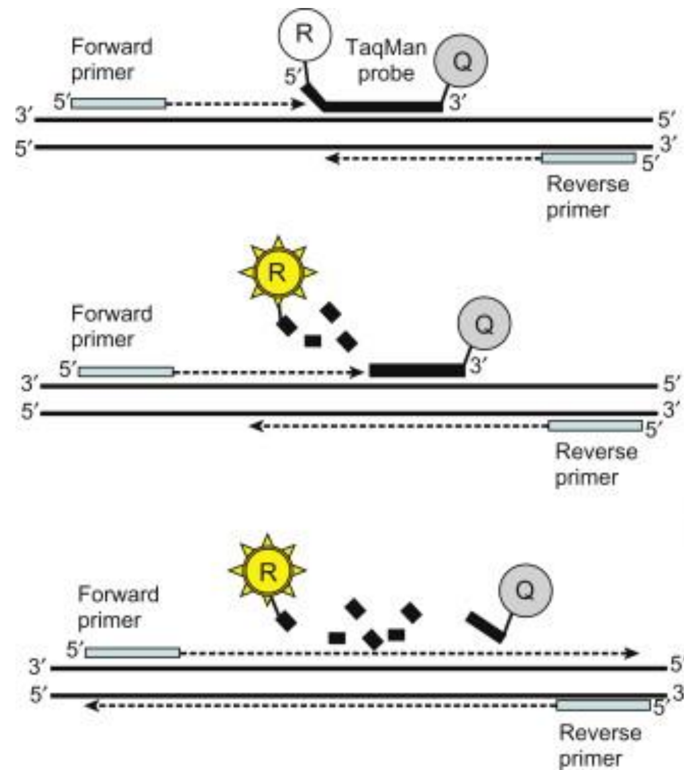


Figure 13. Mode of activation of a TaqMan® probe. When the probe is intact, the proximity between the reporter fluorescent and the quencher dyes allows that the quencher dye represses the fluorescence emitted by the reporter dye. After annealing the primers and the probe hybridized with the template, and during the extension, the probe is cleaved by the 5'-exonuclease activity of the Taq DNA polymerase, releasing the reported dye from the quencher and allowing the emission of the fluorescence by the reporter dye. The emitted signal is measured, being proportional to the amount of accumulated PCR product [adapted from (Butler, 2012)].

The reactions were prepared in 96-well plates and performed in duplicated, using for each reaction 2 µL of cDNA mixed with 1x TaqMan® assay, and 1x SensiFAST™Probe Lo-ROX mastermix (Bio-Rad, Hercules, CA, USA) in a final reaction volume of 25 µL. The reactions were performed on the Applied Biosystems 7500 Real-time PCR system (Applied Biosystems, ThermoFisher Scientific™) under the following conditions: 50ºC for 2 minutes, 95ºC for 10 minutes, and 45 cycles of 95ºC for 15 seconds and 60 ºC for 1 minute.

A standard curve was built using triplicates of four consecutive 10x dilutions of a positive control template to evaluate amplification efficiencies of the target genes and the endogenous reference gene. For GRPR and ERG, cDNA from VCaP cells was used, while for ETV1 we used cDNA from LNCaP cells.

For the analysis, GRPR, ETV1, and ERG expression levels were normalized to the expression of the GUSB housekeeping gene, to correct differences in RNA input, using the comparative threshold cycle (Ct) method. The Ct is inversely proportional to the initial copy number, and it represents the cycle at which the amplification plot crosses the threshold

value, where fluorescence is higher than the background. The ΔCt value is calculated by the difference between the Ct of the target gene and the Ct of the housekeeping gene.

For the representation of the results, the Relative Quantification (RQ) was used, which represents the gene expression in a specific cellular context relative to the respective control.

2. Assessment of proteins expression

In order to establish which proteins belong to the signaling pathway studied, all cell lines were first stimulated with EGF or IL-6 (as JAK/STAT positive control), and after that, all proteins were extracted and quantified. Using the *western blot* technique, total expression levels of ETV1, GRPR, EGFR, TYK2, STAT3, and STAT5A were analyzed. Additionally, the phosphorylated forms, which represent the active forms of EGFR, STAT3, and STAT5 were also evaluated.

2.1. Cellular stimulation

To induce activation of EGFR or IL-6R, cells were serum-starved for 24 hours and 1 µg of EGF (Gibco) or 500 ng of IL-6 (BioLegend®, San Diego, CA, USA) were added to T75 culture flasks containing 5-10×10^6 cells (French *et al*, 2003). After stimulation for 20 minutes, cells were washed twice with ice-cold PBS and used for direct protein extraction on ice.

2.2. Protein extraction and quantification

For each PBS-drained T75 flask, 200 µL of RIPA lysis buffer (Santa Cruz Biotechnology, Dallas, Texas, USA) were used to scrap cells monolayer. Lysates were collected and passed through a 20-gauge needle for lysis enhancement. The cell lysate was set on ice for 10 minutes, followed by centrifugation at 18,000 × *g* for 10 minutes at 4ºC to obtain the clean protein extract (supernatant).

For quantification of the protein concentration, the Qubit™ 4 Fluorometer and the respective kit for protein quantification (Qubit™ Protein BR Assay Kit, Invitrogen™) were used, following manufacturer's recommendations. Qubit™ 4 Fluorometer detects fluorescent dyes that are specific for one type of molecule (DNA, RNA, or protein) in the sample. These dyes only emit fluorescence when are bound to the target molecules. Protein detection is achieved with dyes that emit fluorescence when bound to the primary amines found in proteins.

2.3. Western Blot

Western blot is a technique of protein expression analysis that is based on the interaction between the antibody and the antigen, allowing the identification of specific proteins in a complex mixture. The main steps are protein separation by gel electrophoresis, according to their molecular weight, followed by immobilization into a membrane and immunodetection (Hnasko & Hnasko, 2015)

Initially, for protein separation, a 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed. For each sample to be analyzed, 30 to 60 µg of total protein extract were mixed with 1x loading buffer (0.1 M Tris-HCl – pH 6.8, 0.4% SDS, 2% glycerol, and 0.01% bromophenol blue), and denatured at 95ºC for 5 minutes. Denatured samples were loaded in the gel and proteins were separated according to their size, using a current of 120 V for 90 minutes.

After electrophoresis, the proteins were transferred to a 0.22 µm nitrocellulose membrane, using the Trans-Blot Turbo Transfer System (Bio-Rad) and the 1x Tris-Glycine buffer with 20% of methanol. The transference was performed for 15 minutes with a current of 25 V and 1.3 A.

To avoid unspecific bindings to the antibodies, the membranes were blocked in 5% non-fat-dry milk or BSA in TBS-0.1% Tween 20 (TBS-T), for 1 hour. Then, membranes were incubated with the primary antibodies overnight, at 4ºC, under agitation. After incubation with the primary antibody, the membranes were washed 3 times, for 10 minutes each, with TBS-T, and then incubated with the secondary antibody, diluted in the same blocking solution used in the primary antibody, for 1 hour, at room temperature, under agitation. After three washes with TBS-T, proteins were detected by chemiluminescence, using the Clarity^{TM} Western ECL Substrate (Bio-Rad), and the bands were visualized by autoradiography. Optimized conditions for each antibody are summarized in Table 5.

Table 5. Optimized blotting conditions for the detection of specific proteins and phosphorylated isoforms

Protein	Blocking Solution	Primary antibody	Dilution	Secondary antibody (HRP[#]-linked)	Dilution
TYK2	5% BSA	Cell Signaling[a] #14193	1:500	Goat anti-rabbit Santa Cruz Biotechnology	1:100,000
GRPR	5% non-fat-dry milk	Abcam[b] ab39883	1:250	Goat anti-rabbit Santa Cruz Biotechnology	1:100,000
EGFR	5% non-fat dry milk	Proteintech[c] 66455-1-Ig	1:10,000	Goat anti-mouse Bio-Rad	1:2,500
STAT3	5% non-fat dry milk	Abcam[b] ab119352	1:2,000	Goat anti-mouse Bio-Rad	1:2,500
STAT5A	5% non-fat dry milk	Abcam[b] ab32043	1:1,000	Goat anti-rabbit Santa Cruz Biotechnology	1:100,000
P-EGFR (Tyr1068)*	5% BSA	Abcam[b] ab40815	1:3,000	Goat anti-rabbit Santa Cruz Biotechnology	1:100,000
P-STAT3 (Tyr705)*	5% BSA	Cell Signaling[a] #9131	1:500	Goat anti-rabbit Santa Cruz Biotechnology	1:100,000
P-STAT5A (Tyr694)*	5% BSA	Abcam[b] ab32364	1:3,000	Goat anti-rabbit Santa Cruz Biotechnology	1:100,000
ETV1	5% non-fat dry milk	Sigma-Aldrich[d] SAB1403794	1:2,000	Goat anti-mouse Bio-Rad	1:2,500
β-actin	5% non-fat dry milk	Sigma-Aldrich[d] A1978	1:8,000	Goat anti-mouse Bio-Rad	1:2,500

a - Danvers, Massachusetts, USA; *b* - Cambridge, MA, USA; *c* - Manchester, UK; *d* - St. Louis, MO, USA; # - Horseradish Peroxidase (HRP); * - Phosphorylated Tyrosine residues.

2.3.1. Densitometry analysis

The relative quantification of the blots was made using ImageJ (1.8.0_172), available at the National Institutes of Health (USA), under the instructions provided at http://www.yorku.ca/yisheng/Internal/Protocols/ImageJ.pdf. Initially, the films were scanned and saved in TIFF format for software input. Images were converted into grayscale in ImageJ, and the regions of interest (ROI) were defined using the box tool. For each protein, the defined size of the ROI was maintained between the different conditions of the cell line. After defining the ROI, the pixel density in the bands was measured using the Mean Gray Value option. The same process was made for the β-actin, used as a loading control, and the respective backgrounds. The measurements were exported to Microsoft Excel, where the calculations were made. First, the pixel density for all data was inverted using the formula 255 – X, where X represents the Mean Gray Value obtained for the proteins and their background. Then, background values were subtracted to protein values and, finally, the densitometry analysis for each protein was normalized for its control by dividing the quantification of the test protein by the quantification of the loading control.

The values obtained with the densitometry analysis were transferred to the Prism – GraphPad Software for statistical analysis. The results from two independent experiments were analyzed using the unpaired two-tailed t-tested, and a *p-value* lower than 0.05 was considered statistically significant.

3. Identification of direct ETV1-regulated genes

To verify if ETV1 directly regulates the expression of GRPR, EGFR, TYK2, STAT3, and STAT5A, we searched the GEO DataSets database from the National Center for Biotechnology Information (NCBI) for Chromatin Immunoprecipitation (ChIP) data in PCa cells using an ETV1 antibody.

We obtained no results for ChIP experiments using a specific antibody for ETV1, however, two studies have used alternative approaches with the same purpose, namely, the study from Baena et al. (GSE39388) (Baena *et al*, 2013) and the study from Hollenhorst et al. (GSE29808) (Hollenhorst *et al*, 2011).

In the first study, the researchers have induced expression of an ETV1 protein linked to biotin, allowing ETV1-bound DNA to be caught by the affinity between biotin and streptavidin (bioChIP). The cell line used for bioChIP was LNCaP, characterized by ETV1 overexpression. Additionally, the conventional ChIP was also performed using an ERG antibody in VCaP cell lines, which have ERG overexpression. After the ChIP, target genes were identified using the Affymetrix Human Promoter 1.0R Array. The identified target genes were classified into three subsets, namely, ERG-ETV1 common targets, ERG-targets

only, and ETV1-targets only. Peak identification was calculated by MAT (Model-based Analysis of Tiling-array) scores, which describe the intensity of binding to each probe. Only the peaks associated with a *p-value* lower than 10^{-4} were considered. The genomic region defined as promotor region was in a distance of -8,000bp to 2,000bp of the transcription start site (TSS), and both genomic positions and nearby coding genes were reported according to NCBI Human Genome Assembly Build 36 (hg18) (Baena *et al*, 2013) (GSE39388). Results are publicly available as Supplementary Material to the main publication, in Excel spreadsheets. Therefore, we browsed the different published lists for the genes of interest.

In the second study, the researchers applied a similar approach for detection of either ETV1- or ERG-bound DNA. Briefly, they induced the expression of ETV1 or ERG protein linked to a FLAG epitope in RWPE-2 cells, allowing ETV1- or ERG-bound DNA to be caught by the binding to an Anti-FLAG antibody. After the ChIP, the target genes were identified through next generation sequencing (NGS), and ChIP-seq data was analyzed using the USeq software. *P-value* was calculated for significance, which was controlled by calculating the FDR (False Discovery Rate), obtained by the ratio between the false positive and the sum of the false positive with the true positives. Thus, only binding regions with a FDR inferior to 0.01 were considered, and genomic coordinates were outputted using the NCBI Human Genome Assembly Build 36 (hg18) (GSE29808) (Hollenhorst *et al*, 2011). Results are publicly available as Supplementary Material to the main article, in Excel spreadsheets. To look for an association with our genes of interest, genomic coordinates were imported into the UCSC Genome Browser website (https://genome.ucsc.edu/) as Custom Tracks, and the browser was used to look for a match between the ChIP-seq regions and the promoter regions of *GRPR*, *EGFR*, and the different JAKs and STATs genes.

RESULTS

We initiated our study by validating the silencing of ETV1 in LNCaP cells, the silencing of GRPR in LNCaP and VCaP cells, and the *de novo* ETS expression in PNT2 cells, with qRT-PCR (Figure A-1 to Figure A-3 – *Appendix* 1).

Additionally, as described in the literature, considering the IL-6 receptor as the cognate receptor involved in the activation of the JAK/STAT signaling pathway (Ge *et al*, 2012), we decided to validate, by western blot, the activation of this pathway in our cell models using IL-6 stimulus (Figure A-4).

After observing that the cell models maintain the ETS status and that control cell populations show activation of STAT3 and STAT5A upon IL-6 stimulus, we considered that the cell models were valid to achieve the proposed aims.

1. ETV1 modulates the expression of GRPR

To investigate whether the expression of ETV1 influences the expression of GRPR, we evaluated both the expression of GRPR at the mRNA level by qRT-PCR and at the protein level by western blotting, using two prostate-derived cell lines, namely PNT2 and LNCaP. In the non-tumorigenic PNT2 cell model, we studied the differences between the wild-type cells, represented as PNT2 Neo, and cells with *de novo* expression of ETV1, represented as PNT2 ETV1. As the LNCaP tumorigenic cell model already overexpresses ETV1, we studied the relevance of ETV1 silencing in two independent clonal cell populations, represented as LNCaP ShETV1 C2 and C3.

In the non-tumorigenic cell model, we observed that *de novo* expression of ETV1 associates with an increase in GRPR expression, both at the mRNA and protein level, comparing with the control (Figure 14 and Figure A-5). In the tumorigenic LNCaP model, we found a tendency to a decrease in the GRPR expression in the two independent populations with ETV1 silencing, when compared with the control (Figure 15 and Figure A-5). Both observations suggest that ETV1 positively regulates the expression of GRPR.

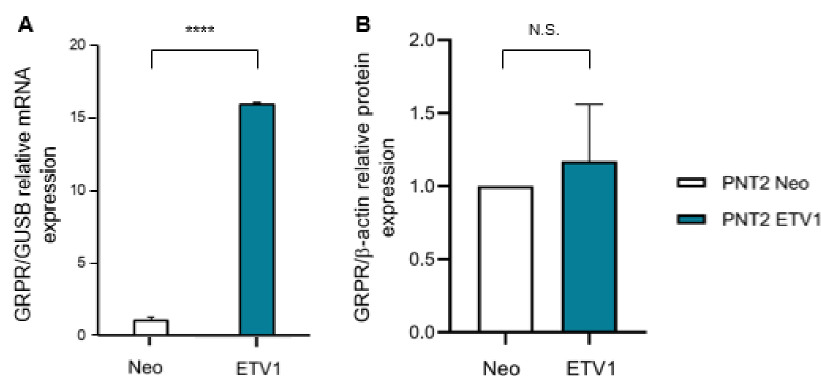


Figure 14. ETV1 overexpression leads to increased GRPR expression. (A) Relative expression of GRPR at mRNA level. GUSB was used as the internal control. Bars represent the standard deviation of the

mean. Unpaired two-tailed t-test was used for statistical analysis (**** P ≤ 0.001) **(B)** Quantification of the GRPR protein expression evaluated by western blot. β-actin was used as loading control. Bars represent the standard deviation of the mean from two independent experiments. Unpaired two-tailed t-test was used for statistical analysis (N.S. - not statistically significant, P > 0.05).

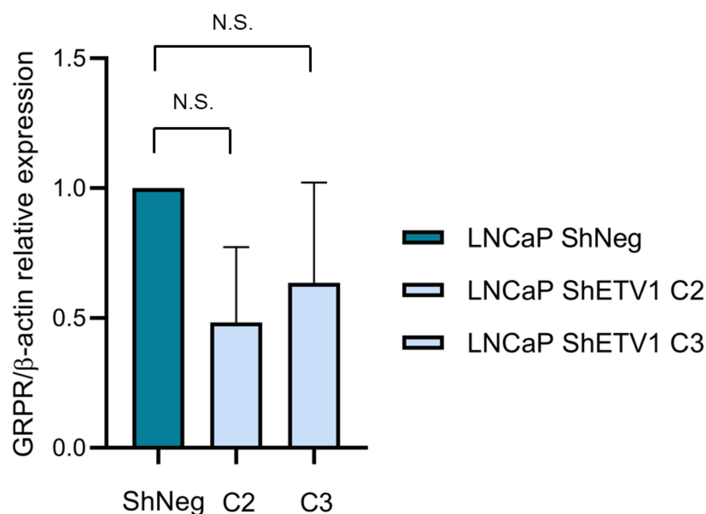


Figure 15. ETV1 silencing leads to decreased GRPR expression. Quantification of the GRPR expression at the protein level by western blot. β-actin was used as loading control. Bars represent the standard deviation of the mean from two independent experiments. Unpaired two-tailed t-test was used for statistical analysis (N.S. - not statistically significant, P > 0.05).

2. ETV1 induces the expression and activation of EGFR

To test if the expression of ETV1 affects total expression and activation of EGFR, we evaluated the expression of total EGFR and Phospho-EGFR (EGFR phosphorylated in 1068 tyrosine residue) by western blot in the two, EGF-stimulated, prostate-derived cell lines with modulation of ETV1 expression: PNT2 and LNCaP.

In the PNT2 cell model, we observed that *de novo* expression of ETV1 associates with an increase in total EGFR expression, when compared with the control (PNT2 Neo). This correlation demonstrated to be independent of whether the cells were untreated (UT) or treated with EGF (Figure 16A and Figure A-6). On the other hand, stimulus with EGF was needed for EGFR activation at tyrosine 1068, changing with *de novo* ETV1 expression (Figure 16B).

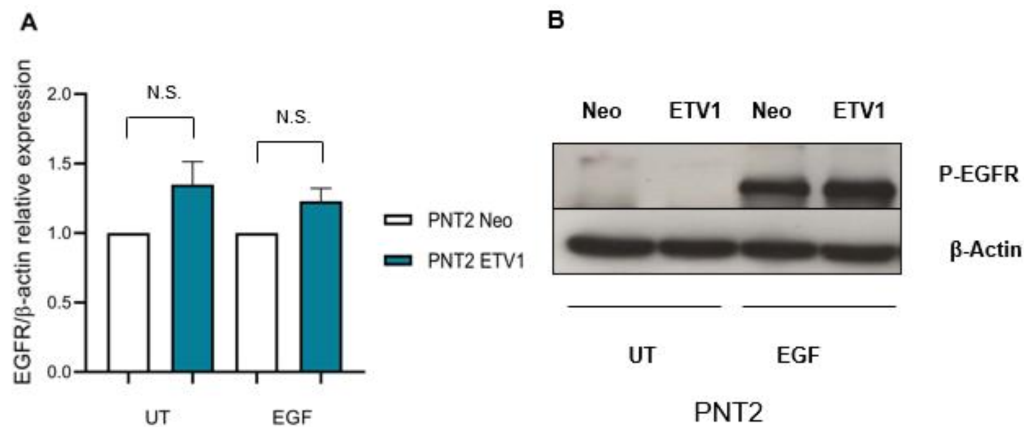


Figure 16. ETV1 overexpression leads to increased EGFR expression and activation in PNT2 cells upon EGF stimulus. (A) Quantification of total EGFR expression obtained by western blot. Bars represent the standard deviation of the mean from two independent experiments. Unpaired two-tailed t-test was used for statistical analysis (N.S. - not statistically significant, P > 0.05). **(B)** Protein blots of P-EGFR expression for the control cells (Neo) and PNT2 cells with ETV1 overexpression (ETV1) β-actin was used as loading control. UT represents the untreated cells and EGF the cells stimulated with EGF.

In the LNCaP cell model, we observed a significant decrease in total EGFR expression in the two independent populations with ETV1 silencing, independently of EGF stimulus (Figure 17A and Figure A-7). Again, we found that activation of EGFR at tyrosine 1068 is dependent on the stimulus with EGF and decreases with ETV1 silencing (Figure 17B).

Both cell models point to a positive regulation of the expression and activation of EGFR by ETV1.

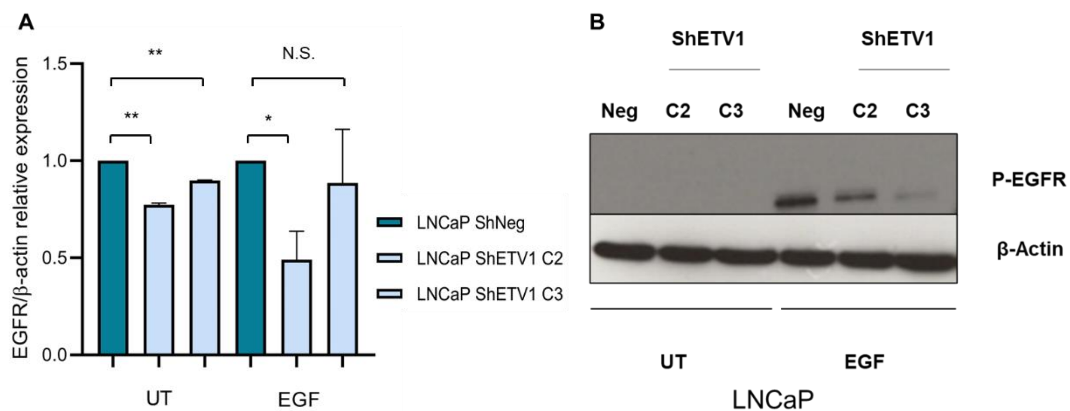


Figure 17. ETV1 silencing leads to decreased EGFR expression and activation in LNCaP cells. (A) Quantification of total EGFR expression obtained by western blot. Bars represent the standard deviation of the mean from two independent experiments. Unpaired two-tailed t-test was used for statistical analysis (** P < 0.01; * P < 0.05; N.S. - not statistically significant, P > 0.05). **(B)** Protein blots of P-EGFR expression for control (ShNeg) and silenced ETV1 clones (ShETV1). β-actin was used as loading control. UT represents the untreated cells and EGF the cells stimulated with EGF.

3. GRPR positively regulates the activation of EGFR under ETV1 overexpression

To investigate whether the expression of GRPR regulates the expression and activation of EGFR in a specific ETS cellular context, we evaluated the expression of total EGFR and P-EGFR in the two tumorigenic cell models of ETV1 and ERG rearrangements, LNCaP and VCaP cells, respectively. In both, we studied the effect of GRPR silencing in two independent cell populations, represented as LNCaP ShGRPR 10 and 15, and as VCaP ShGRPR 15 and 20, upon EGF stimulation.

Although total EGFR expression was not changed by GRPR silencing (Figure A-8), EGFR activation revealed variation. As previously observed in LNCaP cells with ETV1 silencing, the stimulus with EGF is needed for EGFR activation at tyrosine 1068, which decreases in the two independent populations with GRPR silencing (Figure 18). In the VCaP cell line, an inverse regulation was observed, with the EGFR activation at tyrosine 1068 increasing with GRPR silencing (Figure 18). This inverse regulation reinforces that the positive regulation of EGFR activation by the GRPR may be ETV1-specific.

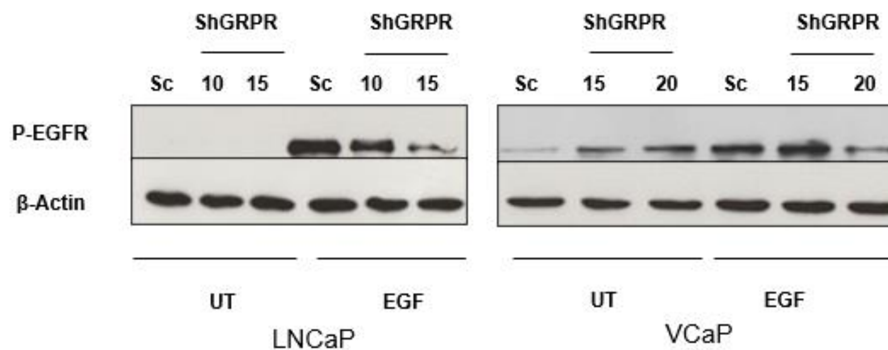


Figure 18. EGFR activation is differentially regulated by GRPR in LNCaP and VCaP cells. Protein blots of P-EGFR expression for control (Sc) and GRPR silenced (ShGRPR) cells upon EGF stimulus. β-actin was used as loading control. UT represents the untreated cells and EGF the cells stimulated with EGF.

4. EGFR also regulates the expression of ETV1

After we have observed that ETV1 modulates the expression of EGFR, we questioned whether EGFR also has the capacity to regulate the expression of ETV1. To achieve this purpose, we analyzed the expression of ETV1 by western blot in the LNCaP derived cells untreated (UT) and treated with EGF (EGF).

Observing the blots, we found that, in the cell line without silencing of ETV1 (LNCaP ShNeg), the expression of ETV1 is significantly increased with EGF stimulus, comparing

with untreated cells (UT) (Figure 19 and Figure A-9), suggesting a mechanism of positive feedback, in which the EGFR also increases the expression of ETV1.

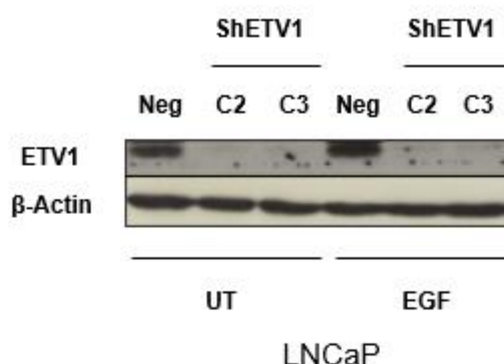


Figure 19. EGF stimulus increases the expression of ETV1. Protein blots of ETV1 expression of control cells (ShNeg) and the ETV1 silenced clones (ShETV1). β-actin was used as loading control. UT represents the untreated cells and EGF the cells stimulated with EGF.

5. STATs are effectors of the ETV1-GRPR-EGFR signaling cascade

After observing that ETV1 positively regulates the expression of GRPR and EGFR, we questioned if the JAK/STAT pathway could be downstream of the EGFR or GRPR signaling cascade mediated by ETV1. Considering previous findings placing TYK2 downstream of GRPR (Santos *et al*, 2015), we analyzed the expression of TYK2, and the expression and activation of STAT5A and STAT3 by western blot in the different cellular models. To analyse the influence of ETV1 in these JAK/STAT effectors, we used two cell models with modulation of ETV1 expression: PNT2 and LNCaP. To explore whether these effectors were regulated by GRPR, we studied the two cell models with GRPR silencing: LNCaP and VCaP. Additionally, to establish the role of EGFR in the signaling pathway, we compared EGF unstimulated with stimulated cells.

5.1. TYK2

Regarding total expression of TYK2, we observed that both in the cellular models with modulation of ETV1 and in those with modulation of GRPR, there are no changes in the global expression of TYK2 (Figure 20 and 21). Thus, neither ETV1 nor GRPR regulate total TYK2 expression. Changes in the phosphorylation of TYK2 have, not yet, been accessed.

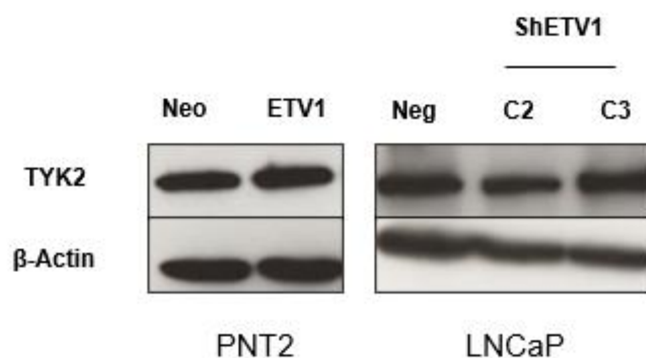


Figure 20. Total TYK2 expression is not regulated by ETV1. Protein blots of TYK2 expression in control cells (PNT2 Neo and LNCaP ShNeg) and cells with modulation of ETV1 expression (PNT2 ETV1 and LNCaP ShETV1 C2/C3). β-actin was used as loading control.

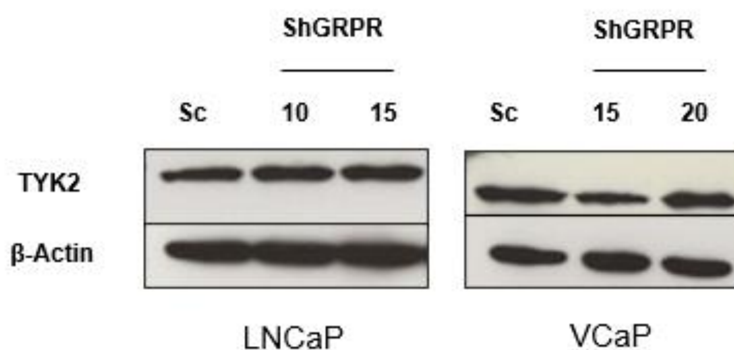


Figure 21. TYK2 total expression is not regulated by GRPR. Protein blots of TYK2 expression in control (Sc) and GRPR silenced (ShGRPR) cells. β-actin was used as loading control.

5.2. STAT5A / P-STAT5A

While for total STAT5A levels, we have not observed a pattern consistent with either an ETV1 or GRPR regulation, showing also no differences between EGF untreated and treated cells (Figure A-10), the STAT5A activated form (P-STAT5A, Tyr694) revealed variation in the several cellular models.

In the non-tumorigenic PNT2 cell model, overexpression of ETV1 induced an increase in the activation of STAT5A at tyrosine residue 694, comparing to the control, which is enhanced by EGF stimulus. Concordantly, in the tumorigenic LNCaP cell model, we observed that ETV1 silencing (ShETV1) led to the abrogation of STAT5A activation (Figure 22). These observations place ETV1 and EGFR as positive regulators of STAT5A activation. Interestingly, an hyperphosphorylated form of STAT5A was observed upon EGF stimulus and directly associated with ETV1 expression in both PNT2 and LNCaP cell models.

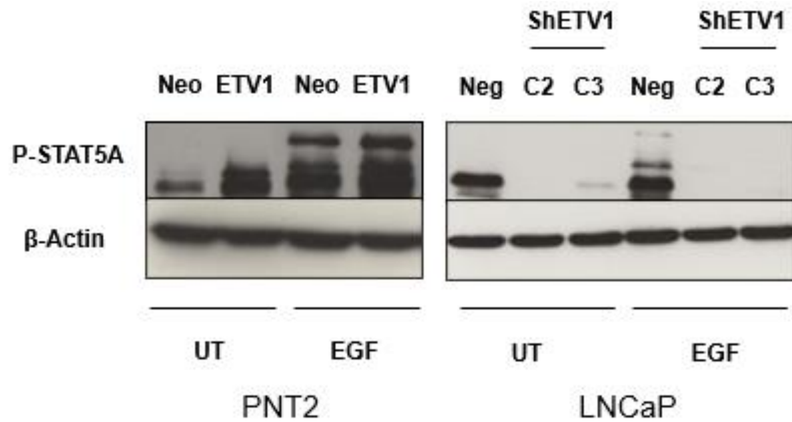


Figure 22. ETV1 overexpression and EGF stimulus induce increased STAT5A activation. Protein blots of P-STAT5A expression in control cells (PNT2 Neo and LNCaP ShNeg) and cell populations with modulation of ETV1 expression (PNT2 ETV1 and LNCaP ShETV1 C2/C3). β-actin was used as loading control. UT represents the untreated cells and EGF the cells stimulated with EGF.

On the other hand, in both LNCaP and VCaP malignant cell lines, we observed higher P-STAT5A levels in the two independent populations with GRPR silencing comparing with the control populations (Figure 23), placing GRPR as negative regulator of STAT5A activation, apparently, without ETS specificity.

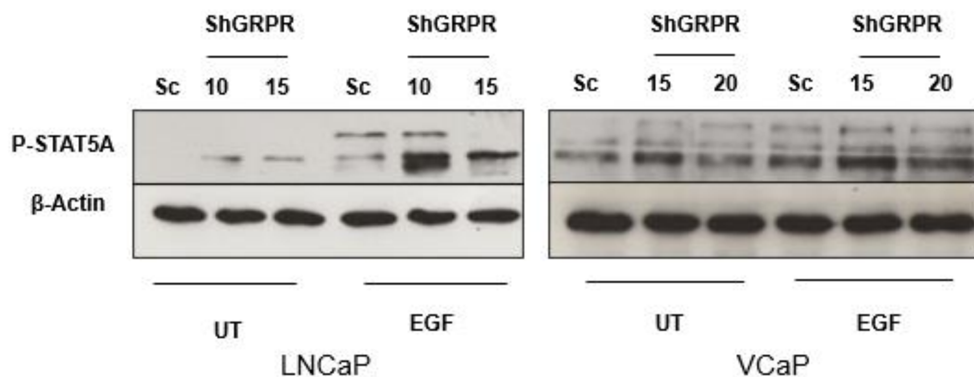


Figure 23. GRPR silencing results in increased STAT5A phosphorylation. Protein blots of TYK2 expression in control (Sc) and GRPR silenced (ShGRPR) cell populations. β-actin was used as loading control. UT represents the untreated cells and EGF the cells stimulated with EGF.

5.3. STAT3 / P-STAT3

In the PNT2 cell model, we observed that overexpression of ETV1 (PNT2 ETV1) increases the expression of STAT3, comparing with the control, independently of EGF stimulus. On the other hand, the stimulus with EGF enhances STAT3 activation at the tyrosine residue 705, which, in stimulated cells is higher for cells with ETV1 expression (Figure 24). In the LNCaP cell model, we observed that EGF stimulus is necessary for STAT3 activation and that this activation decreases with ETV1 silencing (ShETV1), when

compared with ShNeg control (Figure 24). These results support a role for ETV1 in EGFR-mediated STAT3 activation and that ETV1 expression is sufficient to increase STAT3 expression.

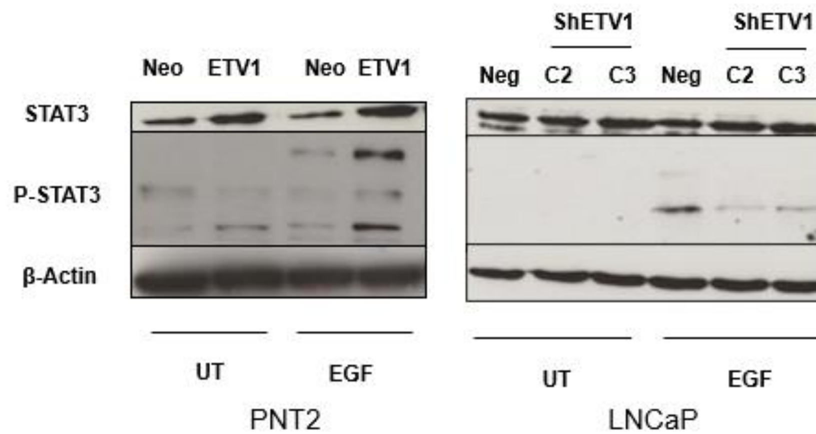


Figure 24. ETV1 overexpression and EGF stimulus induce increased STAT3 expression and activation. Protein blots of STAT3 and P-STAT3 expression in control cells (PNT2 Neo and LNCaP ShNeg) and the cell populations with modulation of ETV1 expression (PNT2 ETV1 and LNCaP ShETV1 C2/C3). β-actin was used as loading control. UT represents the untreated cells and EGF the cells stimulated with EGF.

In the LNCaP cell model with modulation of GRPR expression, we found that activation of STAT3 decreases with GRPR silencing in the two independent populations. The stimulus with EGF leads to hyperphosphorylation of STAT3, which also decreases with GRPR depletion, comparing with the Sc control (Figure 25). In VCaP cells we observed no activation of STAT3, with or without EGF stimulus (Figure 25). These results show that GRPR positively regulates the activation of STAT3, in LNCaP cells, suggesting that different ETS backgrounds may underline different GRPR-mediated signaling.

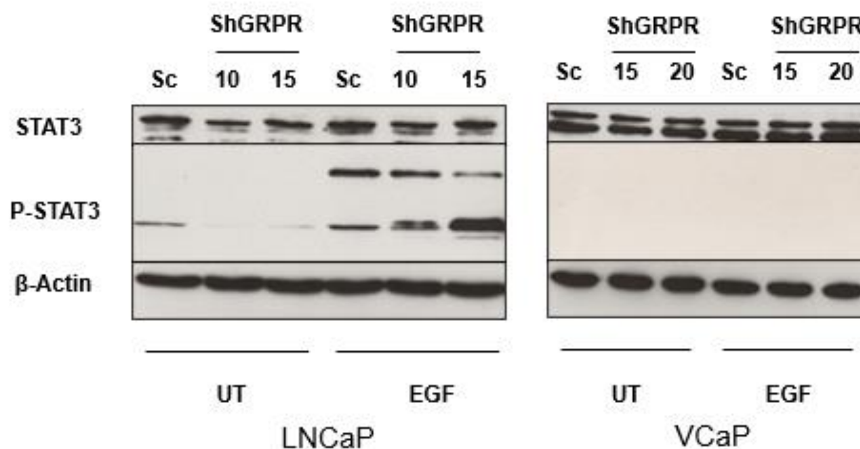


Figure 25. GRPR silencing results in decreased STAT3 activation in LNCaP cells. Protein blots of STAT3 and P-STAT3 expression in control (Sc) and GRPR silenced (ShGRPR) cells. β-actin was used as loading control. UT represents the untreated cells and EGF the cells stimulated with EGF.

6. ERG negatively regulates the identified EGFR-STATs cascade

To investigate if ERG also positively regulates the identified signaling cascade or whether this regulation is specific of ETV1 context, we evaluated the expression and activation of EGFR, STAT3, and STAT5A by western blotting in the PNT2 cell model with *de novo* ERG overexpression, and compared these levels with those previously obtained for PNT2-Neo and PNT2-ETV1 by densitometry analysis.

Interestingly, overexpression of ERG was found to regulate negatively the EGFR-STATs signaling pathway, independently of whether the cells were or not stimulated with EGF (Figure 26). In fact, we observed that ERG overexpression significantly reduces the activation of EGFR in stimulated cells, and in both untreated and treated cells, ERG significantly decreases the activation of STAT3.

These observations, not only validate the existence of ETV1 and ERG different signaling cascades, but also reinforce that the activation of the GRPR-EGFR-STAT3 pathway is specific of ETV1 overexpressing cells.

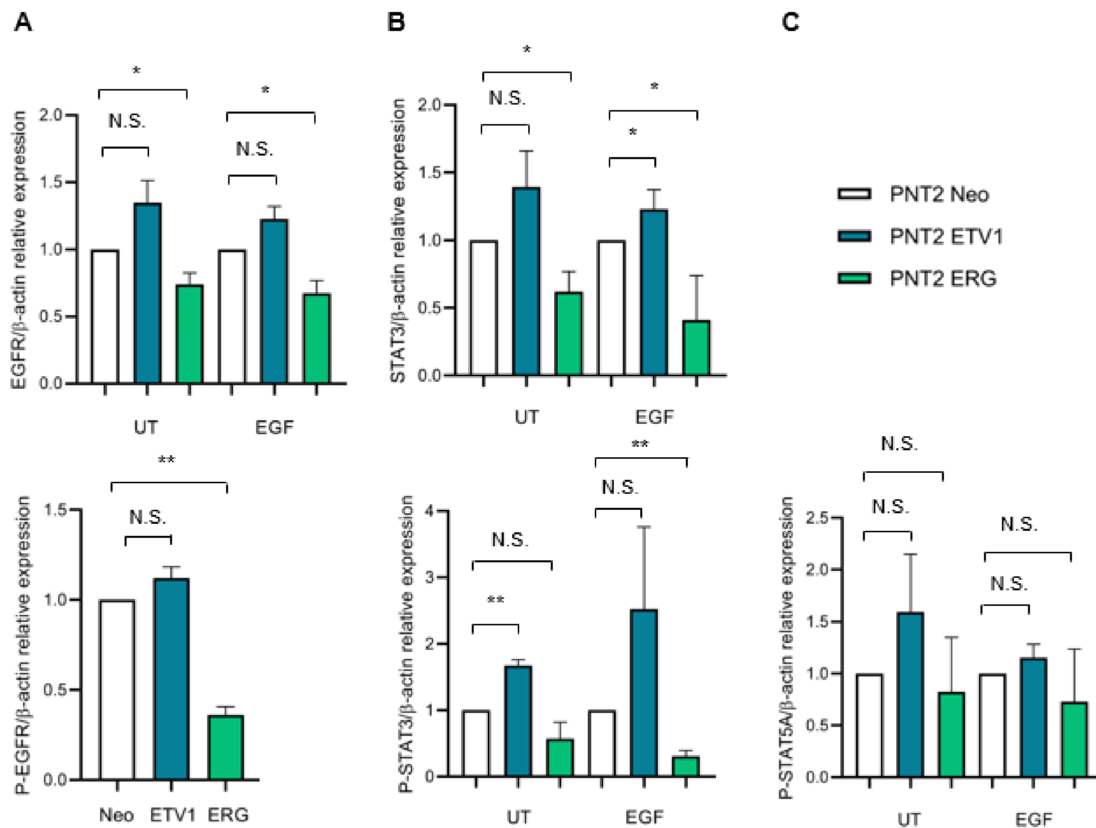


Figure 26. ERG overexpression negatively regulates the expression and activation of EGFR, STAT3, and STAT5A. Relative quantification of the expression levels of EGFR, P-EGFR, STAT3, P-STAT3, and P-STAT5A, obtained by western blot, normalized to β-actin. Bars represent the standard deviation of the mean from two independent experiments. Unpaired two-tailed t-test was used for statistical analysis (** $P < 0.01$; * $P < 0.05$; N.S. - not statistically significant, $P > 0.05$). UT represents the untreated cells and EGF the cells stimulated with EGF.

7. ETV1 binds to the promoter region of *GRPR*, *EGFR*, and *STAT3* genes

To analyze whether the ETV1 transcription factor directly regulates the expression of the *GRPR*, *EGFR*, and both JAKs and STATs genes by binding to their promoter region we used data from Baena et al. (GSE39388) and Hollenhorst et al. (GSE29808), obtained by Chromatin Immunoprecipitation (Hollenhorst *et al*, 2011; Baena *et al*, 2013).

Browsing the published list of ETV1 targets in data from Baena et al., *GRPR*, *EGFR*, and *STAT3* genes were identified among the significant bindings (*p-value* < 10^{-4}). However, while the genomic region identified near the *STAT3* gene is the promoter region, the regions identified near the *GRPR* and *EGFR* genes are outside these genes' promoters regions (>10,000 bp away from the TSS) (Figure 27). Additionally, in Baena et al. data, ETV1 was also bound to the promoter region of genes encoding other STATs, namely, *STAT1* and *STAT6*. Contrarily, none of these genes was found in the published list of ERG targets.

In the data obtained from Hollenhorst et al., analyzed in the UCSC Genome Browser website, we observed that ETV1, but not ERG, binds to the promoter region of *EGFR* and *STAT3* genes (Figure 27). These results support that the regulation of the transcription of *EGFR* and *STAT3* genes is ETV1-specific.

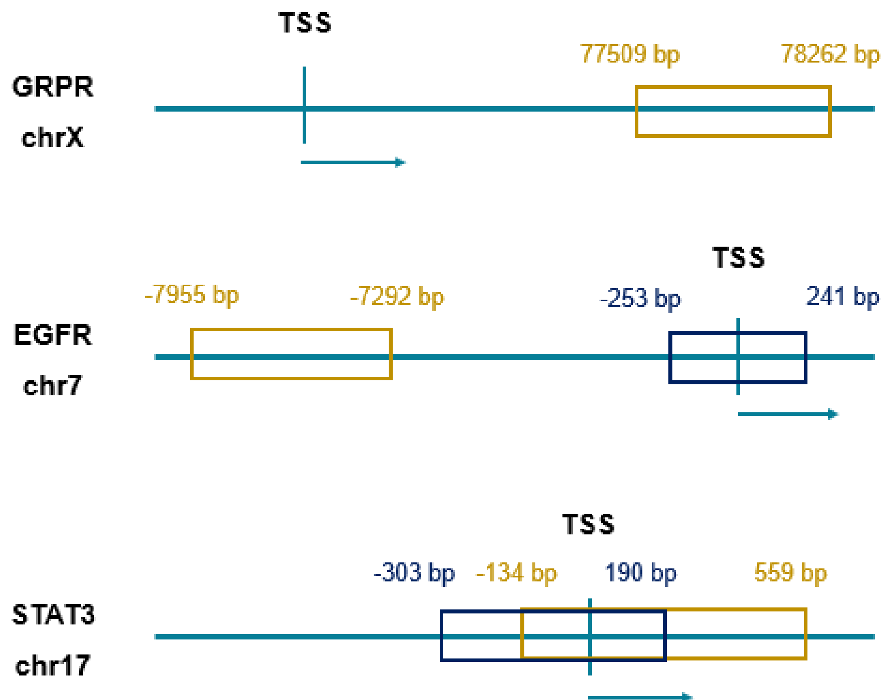


Figure 27. ETV1 binds to the promoter regions of *EGFR* and *STAT3* genes. Schematic representation of the analyzed results, where TSS represents the Transcription Start Site. Rectangles represent the identified ETV1-bound genomic regions, using data from GSE39388 (dark yellow) and GSE29808 (dark blue).

DISCUSSION

As previously mentioned, prostate cancer is the second most incident neoplasia and the fifth leading cause of death from cancer in men, worldwide (Sung *et al*, 2021). Despite advancements in molecular therapeutics, the currently available options for the treatment of, incurable, metastatic disease are reduced (Litwin & Tan, 2017; Parker *et al*, 2020). In fact, apart from the anti-androgenic treatment approaches, only very recently (2019) PARP inhibitors emerged as targeted therapeutics for mCRPC, despite being an option for a very small proportion of cases, specifically, carcinomas with deleterious variants in genes of the homologous recombination repair pathway (Jang *et al*, 2020). Therefore, the discovery of new targeted therapies focusing on new genetic alterations and/or signaling pathways involved in disease progression is one of the main topics in current prostate cancer research.

The rearrangements/overexpression of ETS transcription factors is one of the most frequent genetic alterations found in prostate carcinomas, with ERG and ETV1 defining two independent molecular subtypes (The Cancer Genome Atlas Research Network, 2015). Although ERG rearrangements are more frequent, the prognostic value of ERG subtyping is controversial (Abou-Ouf *et al*, 2016). Conversely, overexpression of ETV1, present in 10-15% of the prostate carcinomas, has been consistently associated with higher tumor aggressiveness and poor prognosis (Baena *et al*, 2013; Mesquita *et al*, 2015; Segalés *et al*, 2019).

Due to the difficulty of using ETS as therapeutic targets (Konstantinopoulos & Papavassiliou, 2011; Nicholas *et al*, 2019), our research group has been studying possible effectors of ETV1 overexpression, that can be used as alternative therapeutic targets. In that sense, GRPR and TYK2 were identified as potential effectors of ETV1 overexpression, both in prostate cancer cell lines and tumor samples (Paulo *et al*, 2012b; Santos *et al*, 2015). However, the mechanism underlying this association remains to be elucidated.

In light of the established association between GRPR and EGFR (Lui *et al*, 2003; Xiao *et al*, 2003; Thomas *et al*, 2005), which, in turn, is described to activate the JAK/STAT signaling pathway (Huang & Fu, 2015; Sigismund *et al*, 2018), we questioned whether EGFR could mediate the interaction between GRPR and TYK2 in ETV1 overexpressing cells, eventually opening horizons for a new therapeutic approach.

1. Establishing a link between ETV1 and EGFR

Due to the undeniable role of EGFR in activating oncogenic signaling pathways that contribute to proliferation, survival and differentiation (Sabbah *et al*, 2020), its association to a specific PCa molecular subtype may be an important step towards a new targeted PCa treatment.

Studying the possible link between ETV1 and EGFR, we found, for the first time, that ETV1 overexpression increases EGFR expression, both in the tumorigenic LNCaP cells and in the non-tumorigenic PNT2 cells. Our results are further supported by expression arrays of cell models with modulation of ETV1 or ERG, obtained from GEO DataSets database. As observed in our LNCaP cell model, data from GSE39388 also shows that LNCaP cells depleted of ETV1 have decreased EGFR expression, when compared with non-silenced ETV1 cells (Figure A-12 – *Appendix* 2). In RWPE cells (a non-tumorigenic model of prostatic cells, as PNT2), the expression of EGFR was shown to be higher after *de novo* ETV1 expression (GSE29438 and GSE39388, Figure A-13), which is also consistent with our data from PNT2 ETV1 cells. Additionally, in RWPE cells with *de novo* ERG expression, decreased EGFR expression is also observed (Figure A-14, GSE39388), again, in accordance with the results we obtained from PNT2 ERG cells. These observations support that increased EGFR expression could be ETV1-specific.

A similar regulation of the EGFR expression has been reported for another PEA3 subfamily member – ETV4. In colorectal cancer cells, silencing of ETV4 was associated with a decrease in EGFR expression, both at mRNA and protein level. Moreover, results from ChIP followed by qPCR, showed that ETV4 could bind to the promoter region of EGFR, increasing its transcription and, thus, positively regulating its expression (Leng *et al*, 2021). As ETV4 belongs to the same subfamily of ETV1, and considering the high homology in the DNA binding domain between members of the same subfamily (Nicholas *et al*, 2019), these observations suggest that direct regulation of EGFR expression may not be ETV1-specific, but PEA3-specific.

To clarify if ETV1 binds to the promoter region of *EGFR* gene, we had initially planned to perform ChIP analysis in our ETV1-overexpressing cell models (PNT2-ETV1 and LNCaP-shETV1), however, due to reports from different research groups regarding the nonexistence of a validated ETV1 antibody for ChIP, we decided to look for publicly available ChIP data at GEO DataSets Database (NCBI). We found ChIP Datasets from two studies where the authors have used alternative approaches to identify ETV1-regulated regions – GSE29438 and GSE39388 (Hollenhorst *et al*, 2011; Baena *et al*, 2013). Browsing ETV1- and ERG-bound genomic regions, obtained by Hollenhorst et al. using the RWPE cells with *de novo* ETV1 and ERG expression (Hollenhorst *et al*, 2011), the promoter region of the *EGFR* gene was among the ETV1-specific targets. Additionally, browsing data obtained by Baena et al. using LNCaP and VCaP cells for ChIP analysis of ETV1- and ERG-bound genomic regions (Baena *et al*, 2013), respectively, we identified a region of ETV1 binding whose nearest gene was *EGFR*, although far from the EGFR promoter. Despite being a largely unknown subject, it is possible that this region may act as a transcription enhancer, as enhancers are characterized to be distant from the transcription start site, but

in which the transcription factors can bind and regulate gene expression (Peng & Zhang, 2018). These two results support that ETV1 regulates the transcription of EGFR, leading to increased expression. As no ERG-bound genomic regions were associated with EGFR, and considering previous reports for ETV4 (Leng *et al*, 2021), it is likely that among the targets of ETS transcription factors, EGFR regulation in PCa is PEA3-specific.

Moreover, having in mind that oncogenic EGFR activity is driven by receptor phosphorylation, we also investigated whether ETV1 influences EGFR activation. First, we observed that EGF stimulus is necessary for activation of EGFR, both in the PNT2 benign and in the LNCaP tumorigenic cell lines. Concordantly, it was previously observed that, in the absence of EGF, both E1 (primary normal prostatic cells) and LNCaP cells, express very low levels of phosphorylated EGFR, comparing with androgen-independent prostate cancer cells, which exhibit high autocrine activation of EGFR (Sherwood *et al*, 1998). In fact, in VCaP cells (androgen-independent), we detected EGFR activation even in the absence of EGF stimulus, suggesting the presence/expression of other EGFR ligands that can activate this receptor (Sigismund *et al*, 2018) – autocrine regulation (Sherwood *et al*, 1998). Then, in stimulated cells, we observed increased EGFR activation in ETV1 overexpressing cells, comparing with cells without ETV1 expression. Considering that ETV1 increases the expression of EGFR, in ETV1-overexpressing cells there will be more EGFR at the cell membrane available to be activated in the presence of EGF stimulus, than in cells without ETV1 expression. A recent publication suggested that point mutations in ETV1 may stimulate EGFR signaling in lung cancer, contributing to resistance to EGFR inhibitors (Zhou *et al*, 2021), thus, supporting the existence of an oncogenic ETV1-EGFR-mediated signaling cascade. On the other hand, in PNT2 stimulated cells we observed that the EGFR activation is negatively regulated by ERG overexpression, again suggesting that increased activation of EGFR could be ETV1- or PEA3-specific.

Interestingly, in the LNCaP cell model, we also found that stimulus with EGF increases the expression of ETV1, favoring a mechanism of positive feedback in which EGFR and ETV1 cooperate in a *feedforward* loop, to enhance signaling and potentiate the malignant phenotype in prostate cancer cells. As there are no data in the literature supporting this hypothesis, further investigation is needed.

Altogether, our results reveal the existence of a positive interaction between ETV1 and EGFR, which seems to be specific for cells with overexpression of this ETS transcription factor or its sub-family.

2. GRPR positively regulates the activation of EGFR

In this study, we observed that total expression of EGFR was not affected by depletion of GRPR expression. On the other hand, both in LNCaP and VCaP cells, GRPR levels modulate EGFR activation: positively in LNCaP cells, and negatively in VCaP cells. In VCaP cells, this effect is observed even in the absence of EGF stimulus, which is explained by the fact that, in these cells, EGF stimulus is not necessary for EGFR activation, as previously described (Sherwood *et al*, 1998). Still, these opposite observations of GRPR involvement in EGFR activation suggest that GRPR may be necessary for EGFR activation in ETV1 overexpressing cells. GRPR silencing in the PNT2 cell models with *de novo* expression of ETV1 or ERG could clarify whether this GRPR-dependency is specific of ETV1-overexpressing cells or is shared with ETV1 or ERG overexpression.

The positive regulation of EGFR activation by GRPR is concordant with the literature. In fact, previous studies reported that GRPR transactivates EGFR, both in lung, head and neck squamous and prostate cancer cells, namely PC3 and DU145 (Lui *et al*, 2003; Xiao *et al*, 2003; Thomas *et al*, 2005). Considering that DU145 and PC3 cell lines overexpress ETV4 and ETV1 (Pellecchia *et al*, 2012; Mesquita *et al*, 2015), it is reasonable to hypothesize that the transactivation of EGFR by GRPR may be PEA3-specific.

Moreover, browsing data obtained by Baena et al. from ChIP analysis of ETV1- and ERG-bound genomic regions (Baena *et al*, 2013), we identified a region of ETV1 binding whose nearest gene was *GRPR,* although far from the GRPR promoter. Again, although there are no reports of GRPR enhancers, it is possible that this region may act as an enhancer region (Peng & Zhang, 2018).

3. Downstream effectors of the ETV1-EGFR

After having discovered that EGFR is regulated and activated by ETV1, and that its activation is also positively regulated by GRPR, which was previously associated with TYK2 expression (Santos *et al*, 2015), we questioned whether elements of the JAK/STAT pathway could be downstream effectors of the GRPR-EGFR signaling cascade mediated by ETV1.

Regarding TYK2, we found no differences in the total expression of this protein, neither in the cell models with modulation of ETV1 expression, nor in the cell models with modulation of GRPR expression. Considering previous observations of decreased TYK2 expression with either ETV1 or GRPR silencing (Santos *et al*, 2015), a possible explanation for the lack of reproducible results can be related with the use of different protein lysis buffers. In the present study, a buffer mainly composed of Tris and Nonidet P-40 detergent was used, while the lysis buffer used by Santos et al. has MOPS and Triton X-100 detergent

as main components. Triton X-100 is normally used for isolating protein complexes bound to the membrane, thus, it is possible that the use of a different detergent may compromise the extraction of the fraction of proteins attached to membrane receptors, in which case, TYK2, GRPR and also EGFR could be affected. Still, the analysis of the activation of TYK2 (phosphorylated TYK2 levels) is essential to clarify its role in this oncogenic cascade.

Apart from the, previously observed, relationship between the expression of TYK2 and ETV1 by our group, there are no additional studies reporting a regulation of TYK2 or other JAKs by any member of the ETS family. On the other hand, regarding the link between JAKs and GRPR, two studies have described that both GRPR and TYK2 are involved in the activation of the NF-kB signaling, further associated with progression from an androgen-dependent to a castrate-resistant prostate tumor (Yang *et al*, 2005; Qiao *et al*, 2016).

Additionally, a study using mice models to evaluate how IL-33/ST2 (IL-3 receptor) signaling is involved in chronic itch, suggested that IL-33 signaling contributes to chronic itch through activation of the JAK2-STAT3 cascade, which also regulates GRP/GRPR signaling-related itch response (Du *et al*, 2019).

Thus, in the event that TYK2 is confirmed as not being involved in the ETV1-GRPR-EGFR signaling, and attending to the suggested relationship between JAK2-STAT3 cascade activation and GRPR signaling, it would be interesting to evaluate if JAK2 is the JAK member acting downstream of this pathway, using our cell models with modulation of GRPR expression.

While studying STATs, we found that overexpression of ETV1 is sufficient to increase STAT3 expression in the non-tumorigenic PNT2 cells. This observation is complemented by ChIP data from both Hollenhorst et al., using RWPE cells with *de novo* ETV1 and ERG expression (Hollenhorst *et al*, 2011), and Baena et. al, using LNCaP and VCaP cells (Baena *et al*, 2013), in which ETV1 was found to bind to the STAT3 promoter. On the other hand, no ERG-bound genomic regions associated with *STAT3* were found, a result that contrasts with our observations in PNT2 with *de novo* expression of ERG, showing decreased STAT3 expression and activation. It is thus possible that ERG regulates STAT3 expression and activation indirectly, by regulating the expression of an unknown intermediate player. In fact, indirect regulation of STATs activity was recently observed in a study focusing in ETV4, in which ETV4 silencing was found to reduce STAT3 phosphorylation in colon carcinoma cells by decreasing the transcription of a described intermediate (Yao *et al*, 2021). Additionally, with ETV4 being a close member of ETV1 (same ETS subfamily), this observation also supports that STAT3 activation may be specific of the PEA3-subfamily, in accordance with the results obtained in our LNCaP cell models with ETV1 silencing and to the lack of STAT3 activation in VCaP cells (ERG positive).

Regarding ETV1 involvement in STAT5A expression and activation, careful analysis of the blots is needed. Observing the blots obtained for both STAT5A and P-STAT5A in LNCaP Neg cells (Figure A-10 and Figure 22), we see no expression with the antibody used to detect total STAT5A, while there is high expression of P-STAT5A. Discarding the possibility of unspecific labelling, due to the consistent abrogation of P-STAT5A in the two LNCaP clonal populations with ETV1 silencing, we hypothesize that the antibody targeting STAT5A only detects the unphosphorylated form. Based on this hypothesis we assessed total STATA5A levels considering the sum of the expression obtained with both antibodies, combining Y694-phosphorylated and -unphosphorylated forms of STAT5A. This analysis revealed a decrease in total STAT5A expression with ETV1 silencing in LNCaP cells (Figure A-11, *Appendix 1*). Although requiring validation with a different Anti-STAT5A antibody, this result, along with the higher levels of activated STAT5A observed in both PNT2 and LNCaP cell models with ETV1 overexpression, are in agreement with a positive regulation of STAT5A signaling by ETV1. Interestingly, while data obtained from expression arrays on ETV1 modulated cells (GSE29438 and GSE39388) does not show increased STAT5A expression (*not shown*), *de novo* ERG expression in RWPE cells induces decreased levels of STAT5A (Figure A-14, *Appendix 2*), an observation in line with the effect seen for activated STAT5A in our PNT2 cells with *de novo* ERG expression. To our knowledge, direct regulation of STAT5 expression by ETS transcription factors was only reported for ETV6 (another ETS), which was found to directly activate STAT5 in hematopoietic cells, driving the development of myeloproliferative neoplasms (Takeda *et al*, 2011). Collectively, these observations place ERG as a negative regulator of STAT5A expression and activation in prostate cancer cells, while a positive regulation can be ETV1-specific.

Exploring the link between EGFR and STATs activation, we observed that EGFR activation leads to hyperphosphorylation of both STAT3 and STAT5A in ETV1 overexpressing cells, validating the existence of an ETV1-mediated EGFR-STATs oncogenic cascade. In fact, while both the association between EGFR and STAT3 activation and their contribution to tumor development are well documented (Wu *et al*, 2014; Huang & Fu, 2015; Song *et al*, 2020), the relationship between EGFR and STAT5A phosphorylation has not been so explored. However, some studies have shown that STAT5 activation is mediated by EGFR and leads to cell proliferation, migration and invasion, by different EGF-like growth factors (Leong *et al*, 2002; Ospina-Prieto *et al*, 2015; Heo *et al*, 2018). STAT5 was also associated with therapeutic resistance in glioblastoma cells, being reported as a downstream effector of mutated EGFR and the underlying cause of resistance to EGFR inhibitors (Roos *et al*, 2018).

Searching for evidence supporting the involvement of GRPR in the ETV1-EGFR-STATs oncogenic cascade, we found that, in ETV1 overexpressing LNCaP cells, activation

of STAT3 is decreased after GRPR silencing, an effect not observed in the ERG-overexpressing VCaP cells. To validate that the positive regulation of STAT3 activation by GRPR is specific to an ETV1-overexpressing cell context we must study the effect of GRPR silencing in PNT2 ETV1 and PNT2 ERG cells. In the literature, GRP (ligand of GRPR) is described to increase the proliferation and migration of the vascular smooth muscle cells in rats through STAT3 activation (Park *et al*, 2017), which reinforces the contribution of STAT3 to angiogenesis (Hu *et al*, 2020) and the putative involvement of GRPR in the acquisition of advanced oncogenic features. Curiously, we observed that GRPR negatively regulates the activation of STAT5A, both in ETV1- and ERG-overexpressing cells, an interplay not previously reported in any cellular context.

Considering all our results, we established a signaling cascade occurring in prostate cells with overexpression of ETV1 (Figure 28). ETV1 increases the transcription of *GRPR*, *EGFR* and *STAT3* genes through binding to the promoter or enhancer regions, leading to increased protein expression. Overexpressed GRPR promotes EGFR activation, eventually by a yet unidentified intermediate, which results in STAT3 activation, possibly mediated by TYK2. Decreased GRPR will result in EGFR-mediated activation of STAT5A in an ETV1-dependent manner, eventually activated by another protein or receptor. Once active, STATs migrate to the nucleus, regulating the transcription of genes involved in angiogenesis, proliferation, and resistance to apoptosis (among other oncogenic pathways) (Verhoeven *et al*, 2020).

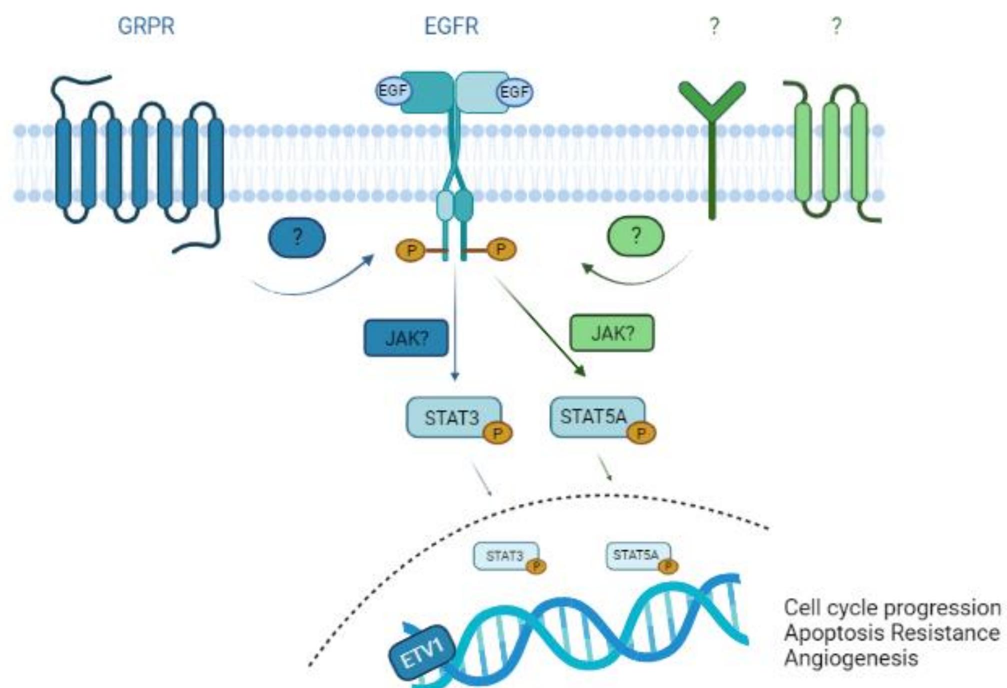


Figure 28. Proposed model for ETV1-mediated and GRPR-dependent EGFR-STATs oncogenic pathway.

4. Implication of the discovered signaling cascade for prostate cancer treatment

We consider that this work has significantly contributed to the future development of a new targeted prostate cancer treatment, since we have identified a novel oncogenic pathway driven by therapeutically targetable players, namely, GRPR, EGFR, and STAT3. As previously mentioned, there are several effective EGFR inhibitors already approved for the treatment of other carcinomas (Licitra *et al*, 2013; Solassol *et al*, 2019; Yau, 2019; Cai *et al*, 2020), and several molecules have been suggested as possible inhibitors of GRPR or STAT3 (Mansi *et al*, 2013; Xiao *et al*, 2015; Zuo *et al*, 2015; Zhang *et al*, 2017). Our observations support the potential of these inhibitors for the treatment of patients with prostate carcinomas harboring rearrangements/overexpression of ETV1, or PEA3 members (ETV1, ETV4 or ETV5) in general, since it has been reported that ETV4 also regulates EGFR expression and STAT3 activation (Leng *et al*, 2021; Yao *et al*, 2021). As these ETS rearrangements are present in a relatively low percentage of patients (Nicholas *et al*, 2019), its oncogenic role has not been well studied, however, the available evidence suggest that patients with ETV4 rearrangements could also benefit from a therapy targeting this pathway, thereby enlarging the number of eligible patients.

As we have observed that GRPR contributes to EGFR-mediated STAT3 activation, and that GRPR silencing leads to increased STAT5A activation, it is possible that by inhibiting GRPR alone *in vivo*, cells would activate STAT5A as an alternative STAT effector of ETV1-EGFR-mediated signaling. These results highlight the importance of combined therapies that act in different mediators of the oncogenic signaling cascade. In fact, in lung and head and neck cancer cells, the use of GRPR antagonists or EGFR inhibitors alone have limited effects in cell proliferation, invasion and apoptosis, when compared with the effect obtained by combination of both (Thomas *et al*, 2005; Zhang *et al*, 2007). Additionally, STAT3 has been reported to be activated by other receptors, underlying a resistance mechanism to EGFR inhibitors, and STAT3 inhibition has shown to restore cells' sensitivity to tyrosine kinase inhibitors (Yang *et al*, 2019; Zheng *et al*, 2021). In prostate cancer cells, Case et al. recently showed that combined inhibition of GRPR with androgen-deprivation was sufficient to control tumor growth and disease progression *in vivo* (Case *et al*, 2021). Interestingly, the cell line models used by Case et al., LNCaP and 22Rv1, have overexpression of ETV1 and ETV4, respectively (Mesquita *et al*, 2015). Although the ETS context was not addressed by the authors, in our understanding, this specific ETS background can be the underlying condition for the observed therapeutic efficacy.

Therefore, our results support the utility of a combined therapy targeting GRPR and either EGFR, STAT3, STAT5A or the, yet unveiled, JAK upstream effector, in prostate carcinomas with ETV1 (or PEA3) rearrangements, which deserves further investigation.

CONCLUSION

Conclusion

At the end of this work, the main findings can be summarized as follows:

- We found that ETV1 regulates positively the expression of GRPR, EGFR and STAT3, in prostate cells, by binding to the promoter or enhancer regions of the *GRPR*, *EGFR*, and *STAT3* genes;

- We discovered that oncogenic activation of EGFR is a feature of ETV1 overexpressing cells, a pathway repressed by ERG overexpression;

- We observed that ETV1-mediated EGFR signaling leads to the activation of both STAT3 and STAT5A;

- We revealed the existence of two GRPR-dependent EGFR-STAT pathways in ETV1 overexpressing cells: one mediated by GRPR and leading to activation of STAT3, and the other, repressed by GRPR and leading to STAT5A activation.

FUTURE PRESPECTIVES

Future Perspectives

Despite having achieved important milestones, the current project will benefit from complementary analyses, namely:

- To increase the consistency of the results and the statistical power of the most interesting observations, a third independent experiment will be performed. In fact, some alterations have not reached statistical significance presumably due to the small number of experiments;

- As the antibody used for total STAT5A expression did not reveal clarifying results, we intend to evaluate the impact of ETV1 and ERG in total STAT5A expression using a different antibody;

- We have, not yet, clarified the role of TYK2 in the new ETV1-GRPR-EGFR-STAT3 oncogenic cascade. To clarify this aim it is mandatory to evaluate the levels of phosphorylated TYK2 in the different cell models and growth conditions. If it turns out that there is no involvement of TYK2 in this oncogenic pathway, the involvement of JAK2 will be evaluated. Moreover, if we confirm that TYK2 is involved in the ETV1-GRPR-EGFR-STAT3 pathway, it will be important to evaluate, by co-immunoprecipitation, if TYK2 binds to EGFR;

- Our study supports the therapeutic potential of EGFR target therapies in prostate carcinomas with ETV1 overexpression. Thereby, in the near future, we intend to study the anti-oncogenic effect of anti-EGFR targeted therapies, alone or in combination with a GRPR antagonist, a JAK inhibitor or a STAT3/STAT5A inhibitor, in prostate cell lines with or without ETV1 overexpression.

REFERENCES

Abdulghani J, Gu L, Dagvadorj A, Lutz J, Leiby B, Bonuccelli G, Lisanti MP, Zellweger T, Alanen K, Mirtti T, Visakorpi T, Bubendorf L, Nevalainen MT (2008) Stat3 promotes metastatic progression of prostate cancer. *Am J Pathol*, 172 (6): 1717–1728.

Abou-Ouf H, Zhao L, Bismar TA (2016) ERG expression in prostate cancer: biological relevance and clinical implication. *J Cancer Res Clin Oncol*, 142 (8): 1781–1793.

Albino D, Longoni N, Curti L, Mello-Grand M, Pinton S, Civenni G, Thalmann G, D'Ambrosio G, Sarti M, Sessa F, Chiorino G, Catapano C V, Carbone GM (2012) ESE3/EHF controls epithelial cell differentiation and its loss leads to prostate tumors with mesenchymal and stem-like features. *Cancer Res*, 72 (11): 2889–2900.

Arora K, Barbieri CE (2018) Molecular Subtypes of Prostate Cancer. *Curr Oncol Rep*, 20 (8): 58–66.

Baena E, Shao Z, Linn DE, Glass K, Hamblen MJ, Fujiwara Y, Kim J, Nguyen M, Zhang X, Godinho FJ, Bronson RT, Mucci LA, Loda M, Yuan G-C, Orkin SH, Li Z (2013) ETV1 directs androgen metabolism and confers aggressive prostate cancer in targeted mice and patients. *Genes Dev*, 27 (6): 683–698.

Barbieri CE, Baca SC, Lawrence MS, Demichelis F, Blattner M, Theurillat J-P, White TA, Stojanov P, Van Allen E, Stransky N, Nickerson E, Chae S-S, Boysen G, Auclair D, Onofrio RC, Park K, Kitabayashi N, MacDonald TY, Sheikh K, Vuong T, Guiducci C, Cibulskis K, Sivachenko A, Carter SL, Saksena G, Voet D, Hussain WM, Ramos AH, Winckler W, Redman MC, Ardlie K, Tewari AK, Mosquera JM, Rupp N, Wild PJ, Moch H, Morrissey C, Nelson PS, Kantoff PW, Gabriel SB, Golub TR, Meyerson M, Lander ES, Getz G, Rubin MA, Garraway LA (2012) Exome sequencing identifies recurrent SPOP, FOXA1 and MED12 mutations in prostate cancer. *Nat Genet*, 44 (6): 685–689.

Barros-Silva JD, Paulo P, Bakken AC, Cerveira N, Løvf M, Henrique R, Jerónimo C, Lothe RA, Skotheim RI, Teixeira MR (2013) Novel 5' fusion partners of ETV1 and ETV4 in prostate cancer. *Neoplasia*, 15 (7): 720–726.

Barry MJ, Simmons LH (2017) Prevention of Prostate Cancer Morbidity and Mortality: Primary Prevention and Early Detection. *Med Clin North Am*, 101 (4): 787–806.

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

Berthon P, Cussenot O, Hopwood L, Leduc A, Maitland N (1995) Functional expression of sv40 in normal human prostatic epithelial and fibroblastic cells - differentiation pattern of

nontumorigenic cell-lines. *Int J Oncol*, 6 (2): 333–343.

Bharadwaj U, Kasembeli MM, Robinson P, Tweardy DJ (2020) Targeting Janus Kinases and Signal Transducer and Activator of Transcription 3 to Treat Inflammation, Fibrosis, and Cancer: Rationale, Progress, and Caution. *Pharmacol Rev*, 72 (2): 486–526.

Blattner M, Lee DJ, O'Reilly C, Park K, MacDonald TY, Khani F, Turner KR, Chiu Y-L, Wild PJ, Dolgalev I, Heguy A, Sboner A, Ramazangolu S, Hieronymus H, Sawyers C, Tewari AK, Moch H, Yoon GS, Known YC, Andrén O, Fall K, Demichelis F, Mosquera JM, Robinson BD, Barbieri CE, Rubin MA (2014) SPOP mutations in prostate cancer across demographically diverse patient cohorts. *Neoplasia*, 16 (1): 14–20.

Borcherding DC, He K, Amin N V, Hirbe AC (2021) TYK2 in Cancer Metastases: Genomic and Proteomic Discovery. *Cancers (Basel)*, 13 (16): 4171–4190.

Bose R, Karthaus WR, Armenia J, Abida W, Iaquinta PJ, Zhang Z, Wongvipat J, Wasmuth E V, Shah N, Sullivan PS, Doran MG, Wang P, Patruno A, Zhao Y, Zheng D, Schultz N, Sawyers CL (2017) ERF mutations reveal a balance of ETS factors controlling prostate oncogenesis. *Nature*, 546 (7660): 671–675.

Brandão A, Paulo P, Teixeira MR (2020) Hereditary Predisposition to Prostate Cancer: From Genetics to Clinical Implications. *Int J Mol Sci*, 21 (14): 5036–5059.

Budka JA, Ferris MW, Capone MJ, Hollenhorst PC (2018) Common ELF1 deletion in prostate cancer bolsters oncogenic ETS function, inhibits senescence and promotes docetaxel resistance. *Genes Cancer*, 9 (5–6): 198–214.

Butler JM (2012) DNA Quantitation. *Advanced Topics in Forensic DNA Typing* (*Elsevier)*, Chapter 3: 49–67.

Cai W-Q, Zeng L-S, Wang L-F, Wang Y-Y, Cheng J-T, Zhang Y, Han Z-W, Zhou Y, Huang S-L, Wang X-W, Peng X-C, Xiang Y, Ma Z, Cui S-Z, Xin H-W (2020) The Latest Battles Between EGFR Monoclonal Antibodies and Resistant Tumor Cells. *Front Oncol*, 10: 1249–1264.

Cancer Genome Atlas Network (2015) Comprehensive genomic characterization of head and neck squamous cell carcinomas. *Nature*, 517 (7536): 576–582.

Carver BS, Tran J, Chen Z, Carracedo-Perez A, Alimonti A, Nardella C, Gopalan A, Scardino PT, Cordon-Cardo C, Gerald W, Pandolfi PP (2009) ETS rearrangements and prostate cancer initiation. *Nature*, 457 (7231): E1; discussion E2-3.

Case TC, Merkel A, Ramirez-Solano M, Liu Q, Sterling JA, Jin R (2021) Blocking GRP/GRP-

R signaling decreases expression of androgen receptor splice variants and inhibits tumor growth in castration-resistant prostate cancer. *Transl Oncol*, 14 (11): 101213–101222.

Castro E, Goh C, Olmos D, Saunders E, Leongamornlert D, Tymrakiewicz M, Mahmud N, Dadaev T, Govindasami K, Guy M, Sawyer E, Wilkinson R, Ardern-Jones A, Ellis S, Frost D, Peock S, Evans DG, Tischkowitz M, Cole T, Davidson R, Eccles D, Brewer C, Douglas F, Porteous ME, Donaldson A, Dorkins H, Izatt L, Cook J, Hodgson S, Kennedy MJ, Side LE, Eason J, Murray A, Antoniou AC, Easton DF, Kote-Jarai Z, Eeles R (2013) Germline BRCA mutations are associated with higher risk of nodal involvement, distant metastasis, and poor survival outcomes in prostate cancer. *J Clin Oncol Off J Am Soc Clin Oncol*, 31 (14): 1748–1757.

Cerveira N, Ribeiro FR, Peixoto A, Costa V, Henrique R, Jerónimo C, Teixeira MR (2006) TMPRSS2-ERG gene fusion causing ERG overexpression precedes chromosome copy number changes in prostate carcinomas and paired HGPIN lesions. *Neoplasia*, 8 (10): 826–832.

Chen Y, Wang J, Wang X, Liu X, Li H, Lv Q, Zhu J, Wei B, Tang Y (2013) STAT3, a Poor Survival Predicator, Is Associated with Lymph Node Metastasis from Breast Cancer. *J Breast Cancer*, 16 (1): 40–49.

Chen Z, Wang T, Tu X, Xie W, He H, Wang M, Zhang J (2017) Antibody-based targeting of CD24 enhances antitumor effect of cetuximab via attenuating phosphorylation of Src/STAT3. *Biomed Pharmacother*, 90: 427–436.

Cheng HH, Sokolova AO, Schaeffer EM, Small EJ, Higano CS (2019) Germline and Somatic Mutations in Prostate Cancer for the Clinician. *J Natl Compr Canc Netw*, 17 (5): 515–521.

Clark JP, Cooper CS (2009) ETS gene fusions in prostate cancer. *Nat Rev Urol*, 6 (8): 429–439.

Culp MB, Soerjomataram I, Efstathiou JA, Bray F, Jemal A (2020) Recent Global Patterns in Prostate Cancer Incidence and Mortality Rates. *Eur Urol*, 77 (1): 38–52.

Daniyal M, Siddiqui ZA, Akram M, Asif HM, Sultana S, Khan A (2014) Epidemiology, Etiology, Diagnosis and Treatment of Prostate Cancer. *Asian Pacific J Cancer Prev*, 15 (22): 9575–9578.

Drake JM, Graham NA, Lee JK, Stoyanova T, Faltermeier CM, Sud S, Titz B, Huang J, Pienta KJ, Graeber TG, Witte ON (2013) Metastatic castration-resistant prostate cancer reveals intrapatient similarity and interpatient heterogeneity of therapeutic kinase targets.

Proc Natl Acad Sci U S A, 110 (49): E4762-9.

Du L, Hu X, Yang W, Yasheng H, Liu S, Zhang W, Zhou Y, Cui W, Zhu J, Qiao Z, Maoying Q, Chu Y, Zhou H, Wang Y, Mi W (2019) Spinal IL-33/ST2 signaling mediates chronic itch in mice through the astrocytic JAK2-STAT3 cascade. *Glia*, 67 (9): 1680–1693.

Epstein JI, Egevad L, Amin MB, Delahunt B, Srigley JR, Humphrey PA (2016) The 2014 International Society of Urological Pathology (ISUP) Consensus Conference on Gleason Grading of Prostatic Carcinoma: Definition of Grading Patterns and Proposal for a New Grading System. *Am J Surg Pathol*, 40 (2): 244–252.

Ferlay J, Colombet M, Soerjomataram I, Parkin DM, Piñeros M, Znaor A, Bray F (2021) Cancer statistics for the year 2020: An overview. *Int J Cancer*. 1–12.

French JD, Walters DK, Jelinek DF (2003) Transactivation of gp130 in Myeloma Cells. *J Immunol*, 170 (7): 3717–3723.

Fry EA, Mallakin A, Inoue K (2018) Translocations involving ETS family proteins in human cancer. *Integr Cancer Sci Ther*, 5 (4): 1–26.

García-Flores M, Casanova-Salas I, Rubio-Briones J, Calatrava A, Domínguez-Escrig J, Rubio L, Ramírez-Backhaus M, Fernández-Serra A, García-Casado Z, López-Guerrero JA (2014) Clinico-pathological significance of the molecular alterations of the SPOP gene in prostate cancer. *Eur J Cancer*, 50 (17): 2994–3002.

Ge D, Gao AC, Zhang Q, Liu S, Xue Y, You Z (2012) LNCaP prostate cancer cells with autocrine interleukin-6 expression are resistant to IL-6-induced neuroendocrine differentiation due to increased expression of suppressors of cytokine signaling. *Prostate*, 72 (12): 1306–1316.

Gopalan A, Leversha MA, Satagopan JM, Zhou Q, Al-Ahmadie HA, Fine SW, Eastham JA, Scardino PT, Scher HI, Tickoo SK, Reuter VE, Gerald WL (2009) TMPRSS2-ERG gene fusion is not associated with outcome in patients treated by prostatectomy. *Cancer Res*, 69 (4): 1400–1406.

Gu L, Vogiatzi P, Puhr M, Dagvadorj A, Lutz J, Ryder A, Addya S, Fortina P, Cooper C, Leiby B, Dasgupta A, Hyslop T, Bubendorf L, Alanen K, Mirtti T, Nevalainen MT (2010) Stat5 promotes metastatic behavior of human prostate cancer cells in vitro and in vivo. *Endocr Relat Cancer*, 17 (2): 481–493.

Gu X, Zerbini LF, Otu HH, Bhasin M, Yang Q, Joseph MG, Grall F, Onatunde T, Correa RG, Libermann TA (2007) Reduced PDEF expression increases invasion and expression of mesenchymal genes in prostate cancer cells. *Cancer Res*, 67 (9): 4219–4226.

Gu Y, Mohammad IS, Liu Z (2020) Overview of the STAT-3 signaling pathway in cancer and the development of specific inhibitors. *Oncol Lett*, 19 (4): 2585–2594.

Haraldsdottir S, Hampel H, Wei L, Wu C, Frankel W, Bekaii-Saab T, de la Chapelle A, Goldberg RM (2014) Prostate cancer incidence in males with Lynch syndrome. *Genet Med*, 16 (7): 553–557.

Helgeson BE, Tomlins SA, Shah N, Laxman B, Cao Q, Prensner JR, Cao X, Singla N, Montie JE, Varambally S, Mehra R, Chinnaiyan AM (2008) Characterization of TMPRSS2:ETV5 and SLC45A3:ETV5 gene fusions in prostate cancer. *Cancer Res*, 68 (1): 73–80.

Heo J, Kim JG, Kim S, Kang H (2018) Stat5 phosphorylation is responsible for the excessive potency of HB-EGF. *J Cell Biochem*, 119 (7): 5297–5307.

Hnasko TS, Hnasko RM (2015) The Western Blot. *Methods in Molecular Biology*. 1318: 87–96.

Hohla F, 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 PC, Ferris MW, Hull MA, Chae H, Kim S, Graves BJ (2011) Oncogenic ETS proteins mimic activated RAS/MAPK signaling in prostate cells. *Genes Dev*, 25 (20): 2147–2157.

Hollenhorst PC, Jones DA, Graves BJ (2004) Expression profiles frame the promoter specificity dilemma of the ETS family of transcription factors. *Nucleic Acids Res*, 32 (18): 5693–5702.

Hosui A, Klover P, Tatsumi T, Uemura A, Nagano H, Doki Y, Mori M, Hiramatsu N, Kanto T, Hennighausen L, Hayashi N, Takehara T (2012) Suppression of signal transducers and activators of transcription 1 in hepatocellular carcinoma is associated with tumor progression. *Int J cancer*, 131 (12): 2774–2784.

Hotte SJ, Saad F (2010) Current management of castrate-resistant prostate cancer. *Curr Oncol*, 17 (Suppl 2): S72-9.

Hsing M, Wang Y, Rennie PS, Cox ME, Cherkasov A (2020) ETS transcription factors as emerging drug targets in cancer. *Med Res Rev*, 40 (1): 413–430.

Hu R, Han Q, Zhang J (2020) STAT3: A key signaling molecule for converting cold to hot tumors. *Cancer Lett*, 489: 29–40.

Huang L, Fu L (2015) Mechanisms of resistance to EGFR tyrosine kinase inhibitors. *Acta*

Pharm Sin B, 5 (5): 390–401.

Ide H, Nakagawa T, Terado Y, Kamiyama Y, Muto S, Horie S (2008) Tyk2 expression and its signaling enhances the invasiveness of prostate cancer cells. *Biochem Biophys Res Commun*, 369 (2): 292–296.

Inamura K (2018) Prostatic cancers: understanding their molecular pathology and the 2016 WHO classification. *Oncotarget*, 9 (18): 14723–14737.

Ischia J, Patel O, Bolton D, Shulkes A, Baldwin GS (2014) Expression and function of gastrin-releasing peptide (GRP) in normal and cancerous urological tissues. *BJU Int*, 113, Suppl 2: 40–47.

Jang A, Sartor O, Barata PC, Paller CJ (2020) Therapeutic Potential of PARP Inhibitors in the Treatment of Metastatic Castration-Resistant Prostate Cancer. *Cancers (Basel)*, 12 (11): 3467–3780.

Karki R, Pandya D, Elston RC, Ferlini C (2015) Defining 'mutation' and 'polymorphism' in the era of personal genomics. *BMC Med Genomics*, 8: 37–43.

Kedage V, Selvaraj N, Nicholas TR, Budka JA, Plotnik JP, Jerde TJ, Hollenhorst PC (2016) An Interaction with Ewing's Sarcoma Breakpoint Protein EWS Defines a Specific Oncogenic Mechanism of ETS Factors Rearranged in Prostate Cancer. *Cell Rep*, 17 (5): 1289–1301.

Konstantinopoulos PA, Papavassiliou AG (2011) Seeing the future of cancer-associated transcription factor drug targets. *JAMA*, 305 (22): 2349–2350.

Kusaba T, Nakayama T, Yamazumi K, Yakata Y, Yoshizaki A, Inoue K, Nagayasu T, Sekine I (2006) Activation of STAT3 is a marker of poor prognosis in human colorectal cancer. *Oncol Rep*, 15 (6): 1445–1451.

Langan RC (2019) Benign Prostatic Hyperplasia. *Prim Care*, 46 (2): 223–232.

Lee CH, Akin-Olugbade O, Kirschenbaum A (2011) Overview of Prostate Anatomy, Histology, and Pathology. *Endocrinol Metab Clin North Am*, 40 (3): 565–575.

Lee H-J, Zhuang G, Cao Y, Du P, Kim H-J, Settleman J (2014) Drug Resistance via Feedback Activation of Stat3 in Oncogene-Addicted Cancer Cells. *Cancer Cell*, 26 (2): 207–221.

Leitner NR, Witalisz-Siepracka A, Strobl B, Müller M (2017) Tyrosine kinase 2 - Surveillant of tumours and bona fide oncogene. *Cytokine*, 89: 209–218.

Leng Y, Chen Z, Ding H, Zhao X, Qin L, Pan Y (2021) Overexpression of microRNA-29b inhibits epithelial-mesenchymal transition and angiogenesis of colorectal cancer through the ETV4/ERK/EGFR axis. *Cancer Cell Int*, 21 (1): 17–35.

Leong PL, Xi S, Drenning SD, Dyer KF, Wentzel AL, Lerner EC, Smithgall TE, Grandis JR (2002) Differential function of STAT5 isoforms in head and neck cancer growth control. *Oncogene*, 21 (18): 2846–2853.

Li H, Zhang Y, Glass A, Zellweger T, Gehan E, Bubendorf L, Gelmann EP, Nevalainen MT (2005) Activation of signal transducer and activator of transcription-5 in prostate cancer predicts early recurrence. *Clin cancer Res an Off J Am Assoc Cancer Res*, 11 (16): 5863–5868.

Liapakis G, Matsoukas M-T, Karageorgos V, Venihaki M, Mavromoustakos T (2017) Family B G Protein-coupled Receptors and their Ligands: From Structure to Function. *Curr Med Chem*, 24 (31): 3323–3355.

Licitra L, Störkel S, Kerr KM, Van Cutsem E, Pirker R, Hirsch FR, Vermorken JB, Von Heydebreck A, Esser R, Celik I, Ciardiello F (2013) Predictive value of epidermal growth factor receptor expression for first-line chemotherapy plus cetuximab in patients with head and neck and colorectal cancer: Analysis of data from the EXTREME and CRYSTAL studies. *Eur J Cancer*, 49 (6): 1161–1168.

Linn DE, Penney KL, Bronson RT, Mucci LA, Li Z (2016) Deletion of Interstitial Genes between TMPRSS2 and ERG Promotes Prostate Cancer Progression. *Cancer Res*, 76 (7): 1869–1881.

Litwin MS, Tan H-J (2017) The Diagnosis and Treatment of Prostate Cancer: A Review. *JAMA*, 317 (24): 2532–2542.

Longoni N, Sarti M, Albino D, Civenni G, Malek A, Ortelli E, Pinton S, Mello-Grand M, Ostano P, D'Ambrosio G, Sessa F, Garcia-Escudero R, Thalmann GN, Chiorino G, Catapano C V, Carbone GM (2013) ETS transcription factor ESE1/ELF3 orchestrates a positive feedback loop that constitutively activates NF-κB and drives prostate cancer progression. *Cancer Res*, 73 (14): 4533–4547.

Lui VWY, Thomas SM, Zhang Q, Wentzel AL, Siegfried JM, Li JY, Grandis JR (2003) Mitogenic effects of gastrin-releasing peptide in head and neck squamous cancer cells are mediated by activation of the epidermal growth factor receptor. *Oncogene*, 22 (40): 6183–6193.

Mansi R, Fleischmann A, Mäcke HR, Reubi JC (2013) Targeting GRPR in urological

cancers--from basic research to clinical application. *Nat Rev Urol*, 10 (4): 235–244.

Markwalder R, Reubi JC (1999) Gastrin-releasing peptide receptors in the human prostate: relation to neoplastic transformation. *Cancer Res*, 59 (5): 1152–1159.

De Marzo AM, Haffner MC, Lotan TL, Yegnasubramanian S, Nelson WG (2016) Premalignancy in Prostate Cancer: Rethinking What we Know. *Cancer Prev Res (Phila)*, 9 (8): 648–656.

McNeal JE (1981) The zonal anatomy of the prostate. *Prostate*, 2 (1): 35–49.

Medina-Arana V, Delgado L, Bravo A, Martín J, Fernández-Peralta AM, González-Aguilera JJ (2012) Tumor spectrum in lynch syndrome, DNA mismatch repair system and endogenous carcinogens. *J Surg Oncol*, 106 (1): 10–16.

Mesquita D, Barros-Silva JD, Santos J, Skotheim RI, Lothe RA, Paulo P, Teixeira MR (2015) Specific and redundant activities of ETV1 and ETV4 in prostate cancer aggressiveness revealed by co-overexpression cellular contexts. *Oncotarget*, 6 (7): 5217–5236.

National Comprehensive Cancer Network. (2021). NCCN Clinical Practice Guidelines in Oncology - Prostate Cancer Early Detection, Version 1.2021, available at https://www.nccn.org/professionals/physician_gls/pdf/prostate_detection.pdf

National Comprehensive Cancer Network. (2021). NCCN Clinical Practice Guidelines in Oncology - Prostate Cancer, Version 2.2021, available at https://www.nccn.org/professionals/physician_gls/pdf/prostate.pdf

Nicholas TR, Strittmatter BG, Hollenhorst PC (2019) Oncogenic ETS Factors in Prostate Cancer. *Adv Exp Med Biol*, 1210: 409–436.

Oh M, Alkhushaym N, Fallatah S, Althagafi A, Aljadeed R, Alsowaida Y, Jeter J, Martin JR, Babiker HM, McBride A, Abraham I (2019) The association of BRCA1 and BRCA2 mutations with prostate cancer risk, frequency, and mortality: A meta-analysis. *Prostate*, 79 (8): 880–895.

de Oliveira MS, Cechim G, Braganhol E, Santos DG, Meurer L, de Castro CGJ, Brunetto AL, Schwartsmann G, Battastini AMO, Lenz G, Roesler R (2009) Anti-proliferative effect of the gastrin-release peptide receptor antagonist RC-3095 plus temozolomide in experimental glioblastoma models. *J Neurooncol*, 93 (2): 191–201.

Ospina-Prieto S, Chaiwangyen W, Pastuschek J, Schleussner E, Markert UR, Morales-Prieto DM (2015) STAT5 is Activated by Epidermal Growth Factor and Induces Proliferation and Invasion in Trophoblastic Cells. *Reprod Sci*, 22 (11): 1358–1366.

Park H-J, Kim M-K, Kim Y, Bae SS, Kim HJ, Bae S-K, Bae M-K (2017) Gastrin-releasing peptide promotes the migration of vascular smooth muscle cells through upregulation of matrix metalloproteinase-2 and -9. *BMB Rep*, 50 (12): 628–633.

Parker C, Castro E, Fizazi K, Heidenreich A, Ost P, Procopio G, Tombal B, Gillessen S (2020) Prostate cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol Off J Eur Soc Med Oncol*, 31 (9): 1119–1134.

Paulo P, Barros-Silva JD, Ribeiro FR, Ramalho-Carvalho J, Jerónimo C, Henrique R, Lind GE, Skotheim RI, Lothe RA, Teixeira MR (2012a) FLI1 is a novel ETS transcription factor involved in gene fusions in prostate cancer. *Genes Chromosomes Cancer*, 51 (3): 240–249.

Paulo P, Ribeiro FR, Santos J, Mesquita D, Almeida M, Barros-Silva JD, Itkonen H, Henrique R, Jerónimo C, Sveen A, Mills IG, Skotheim RI, Lothe RA, Teixeira MR (2012b) Molecular subtyping of primary prostate cancer reveals specific and shared target genes of different ETS rearrangements. *Neoplasia*, 14 (7): 600–611.

Pellecchia A, Pescucci C, De Lorenzo E, Luceri C, Passaro N, Sica M, Notaro R, De Angioletti M (2012) Overexpression of ETV4 is oncogenic in prostate cells through promotion of both cell proliferation and epithelial to mesenchymal transition. *Oncogenesis*, 1 (7): e20-30.

Peng Y, Zhang Y (2018) Enhancer and super-enhancer: Positive regulators in gene transcription. *Anim Model Exp Med*, 1 (3): 169–179.

Perdana NR, Mochtar CA, Umbas R, Hamid ARA (2016) The Risk Factors of Prostate Cancer and Its Prevention: A Literature Review. *Acta Med Indones*, 48 (3): 228–238.

Pernar CH, Ebot EM, Wilson KM, Mucci LA (2018) The Epidemiology of Prostate Cancer. *Cold Spring Harb Perspect Med*, 8 (12): a030361-030379.

Petrovics G, Liu A, Shaheduzzaman S, Furusato B, Sun C, Chen Y, Nau M, Ravindranath L, Chen Y, Dobi A, Srikantan V, Sesterhenn IA, McLeod DG, Vahey M, Moul JW, Srivastava S (2005) Frequent overexpression of ETS-related gene-1 (ERG1) in prostate cancer transcriptome. *Oncogene*, 24 (23): 3847–3852.

Petrucelli N, Daly MB, Pal T (1998) BRCA1- and BRCA2-Associated Hereditary Breast and Ovarian Cancer. *GeneReviews® [Internet].*

Pettersson A, Graff RE, Bauer SR, Pitt MJ, Lis RT, Stack EC, Martin NE, Kunz L, Penney KL, Ligon AH, Suppan C, Flavin R, Sesso HD, Rider JR, Sweeney C, Stampfer MJ, Fiorentino M, Kantoff PW, Sanda MG, Giovannucci EL, Ding EL, Loda M, Mucci LA (2012)

The TMPRSS2:ERG rearrangement, ERG expression, and prostate cancer outcomes: a cohort study and meta-analysis. *Cancer Epidemiol biomarkers Prev a Publ Am Assoc Cancer Res cosponsored by Am Soc Prev Oncol*, 21 (9): 1497–1509.

Picon H, Guddati AK (2020) Mechanisms of resistance in head and neck cancer. *Am J Cancer Res*, 10 (9): 2742–2751.

Pritchard CC, Mateo J, Walsh MF, De Sarkar N, Abida W, Beltran H, Garofalo A, Gulati R, Carreira S, Eeles R, Elemento O, Rubin MA, Robinson D, Lonigro R, Hussain M, Chinnaiyan A, Vinson J, Filipenko J, Garraway L, Taplin M-E, AlDubayan S, Han GC, Beightol M, Morrissey C, Nghiem B, Cheng HH, Montgomery B, Walsh T, Casadei S, Berger M, Zhang L, Zehir A, Vijai J, Scher HI, Sawyers C, Schultz N, Kantoff PW, Solit D, Robson M, Van Allen EM, Offit K, de Bono J, Nelson PS (2016) Inherited DNA-Repair Gene Mutations in Men with Metastatic Prostate Cancer. *N Engl J Med*, 375 (5): 443–453.

Qiao J, Grabowska MM, Forestier-Roman IS, Mirosevich J, Case TC, Chung DH, Cates JMM, Matusik RJ, Manning HC, Jin R (2016) Activation of GRP/GRP-R signaling contributes to castration-resistant prostate cancer progression. *Oncotarget*, 7 (38): 61955–61969.

Quesnelle KM, Boehm AL, Grandis JR (2007) STAT-mediated EGFR signaling in cancer. *J Cell Biochem*, 102 (2): 311–319.

Raghallaigh HN, Eeles R (2021) Genetic predisposition to prostate cancer: an update. *Fam Cancer*.

Ragsdale JW 3rd, Halstater B, Martinez-Bianchi V (2014) Prostate cancer screening. *Prim Care*, 41 (2): 355–370.

Rajput AB, Miller MA, De Luca A, Boyd N, Leung S, Hurtado-Coll A, Fazli L, Jones EC, Palmer JB, Gleave ME, Cox ME, Huntsman DG (2007) Frequency of the TMPRSS2:ERG gene fusion is increased in moderate to poorly differentiated prostate cancers. *J Clin Pathol*, 60 (11): 1238–1243.

Roesler R, Schwartsmann G (2013) GRPR antagonists for prostate cancer--prospects and caveats. *Nat Rev Urol*, 10 (7): 424.

Roos A, Dhruv HD, Peng S, Inge LJ, Tuncali S, Pineda M, Millard N, Mayo Z, Eschbacher JM, Loftus JC, Winkles JA, Tran NL (2018) EGFRvIII-Stat5 Signaling Enhances Glioblastoma Cell Migration and Survival. *Mol Cancer Res*, 16 (7): 1185–1195.

Ryan S, Jenkins MA, Win AK (2014) Risk of prostate cancer in Lynch syndrome: a systematic review and meta-analysis. *Cancer Epidemiol biomarkers Prev a Publ Am Assoc*

Cancer Res cosponsored by Am Soc Prev Oncol, 23 (3): 437–449.

Sabbah DA, Hajjo R, Sweidan K (2020) Review on Epidermal Growth Factor Receptor (EGFR) Structure, Signaling Pathways, Interactions, and Recent Updates of EGFR Inhibitors. *Curr Top Med Chem*, 20 (10): 815–834.

Santos J, Mesquita D, Barros-Silva JD, Jerónimo C, Henrique R, Morais A, Paulo P, Teixeira MR (2015) Uncovering potential downstream targets of oncogenic GRPR overexpression in prostate carcinomas harboring ETS rearrangements. *Oncoscience*, 2 (5): 497–507.

Sathianathen NJ, Konety BR, Crook J, Saad F, Lawrentschuk N (2018) Landmarks in prostate cancer. *Nat Rev Urol*, 15 (10): 627–642.

Schuster C, Müller M, Freissmuth M, Sexl V, Stoiber D (2008) Commentary on H. Ide et al., 'Tyk2 expression and its signaling enhances the invasiveness of prostate cancer cells'. *Biochem Biophys Res Commun*, 366 (4): 869–870.

Segalés L, Juanpere N, Lorenzo M, Albero-González R, Fumadó L, Cecchini L, Bellmunt J, Lloreta-Trull J, Hernández-Llodrà S (2019) Strong cytoplasmic ETV1 expression has a negative impact on prostate cancer outcome. *Virchows Arch*, 475 (4): 457–466.

Sehn JK (2018) Prostate Cancer Pathology: Recent Updates and Controversies. *Mo Med*, 115 (2): 151–155.

Sen M, Joyce S, Panahandeh M, Li C, Thomas SM, Maxwell J, Wang L, Gooding WE, Johnson DE, Grandis JR (2012) Targeting Stat3 abrogates EGFR inhibitor resistance in cancer. *Clin cancer Res an Off J Am Assoc Cancer Res*, 18 (18): 4986–4996.

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

Sherwood ER, Van Dongen JL, Wood CG, Liao S, Kozlowski JM, Lee C (1998) Epidermal growth factor receptor activation in androgen-independent but not androgen-stimulated growth of human prostatic carcinoma cells. *Br J Cancer*, 77 (6): 855–861.

Sigismund S, Avanzato D, Lanzetti L (2018) Emerging functions of the EGFR in cancer. *Mol Oncol*, 12 (1): 3–20.

Sizemore GM, Pitarresi JR, Balakrishnan S, Ostrowski MC (2017) The ETS family of oncogenic transcription factors in solid tumours. *Nat Rev Cancer*, 17 (6): 337–351.

Skinder D, Zacharia I, Studin J, Covino J (2016) Benign prostatic hyperplasia: A clinical review. *JAAPA*, 29 (8): 19–23.

Solassol I, Pinguet F, Quantin X (2019) FDA- and EMA-Approved Tyrosine Kinase Inhibitors in Advanced EGFR-Mutated Non-Small Cell Lung Cancer: Safety, Tolerability, Plasma Concentration Monitoring, and Management. *Biomolecules*, 9 (11): 668–686.

Song X, Liu Z, Yu Z (2020) EGFR Promotes the Development of Triple Negative Breast Cancer Through JAK/STAT3 Signaling. *Cancer Manag Res*, 12: 703–717.

Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, Bray F (2021) Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA Cancer J Clin*, 71 (3): 209–249.

Takeda Y, Nakaseko C, Tanaka H, Takeuchi M, Yui M, Saraya A, Miyagi S, Wang C, Tanaka S, Ohwada C, Sakaida E, Yamaguchi N, Yokote K, Hennighausen L, Iwama A (2011) Direct activation of STAT5 by ETV6-LYN fusion protein promotes induction of myeloproliferative neoplasm with myelofibrosis. *Br J Haematol*, 153 (5): 589–598.

Tandefelt DG, Boormans J, Hermans K, Trapman J (2014) ETS fusion genes in prostate cancer. *Endocr Relat Cancer*, 21 (3): R143-52.

Taris M, Irani J, Blanchet P, Multigner L, Cathelineau X, Fromont G (2014) ERG expression in prostate cancer: the prognostic paradox. *Prostate*, 74 (15): 1481–1487.

The Cancer Genome Atlas Research Network (2015) The Molecular Taxonomy of Primary Prostate Cancer. *Cell*, 163 (4): 1011–1025.

Thomas SM, Grandis JR, Wentzel AL, Gooding WE, Lui VWY, Siegfried JM (2005) Gastrin-releasing peptide receptor mediates activation of the epidermal growth factor receptor in lung cancer cells. *Neoplasia*, 7 (4): 426–431.

Tomlins SA, Laxman B, Dhanasekaran SM, Helgeson BE, Cao X, Morris DS, Menon A, Jing X, Cao Q, Han B, Yu J, Wang L, Montie JE, Rubin MA, Pienta KJ, Roulston D, Shah RB, Varambally S, Mehra R, Chinnaiyan AM (2007a) Distinct classes of chromosomal rearrangements create oncogenic ETS gene fusions in prostate cancer. *Nature*, 448 (7153): 595–599.

Tomlins SA, Mehra R, Rhodes DR, Cao X, Wang L, Dhanasekaran SM, Kalyana-Sundaram S, Wei JT, Rubin MA, Pienta KJ, Shah RB, Chinnaiyan AM (2007b) Integrative molecular concept modeling of prostate cancer progression. *Nat Genet*, 39 (1): 41–51.

Tomlins SA, Rhodes DR, Perner S, Dhanasekaran SM, Mehra R, Sun X-W, Varambally S, Cao X, Tchinda J, Kuefer R, Lee C, Montie JE, Shah RB, Pienta KJ, Rubin MA, Chinnaiyan AM (2005) Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. *Science*, 310 (5748): 644–648.

Verhoeven Y, Tilborghs S, Jacobs J, De Waele J, Quatannens D, Deben C, Prenen H, Pauwels P, Trinh XB, Wouters A, Smits ELJ, Lardon F, van Dam PA (2020) The potential and controversy of targeting STAT family members in cancer. *Semin Cancer Biol*, 60: 41–56.

Verze P, Cai T, Lorenzetti S (2016) The role of the prostate in male fertility, health and disease. *Nat Rev Urol*, 13 (7): 379–386.

Wang G, Zhao D, Spring DJ, DePinho RA (2018) Genetics and biology of prostate cancer. *Genes Dev*, 32 (17–18): 1105–1140.

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

Wöss K, Simonović N, Strobl B, Macho-Maschler S, Müller M (2019) TYK2: An Upstream Kinase of STATs in Cancer. *Cancers (Basel)*, 11 (11): 1728-1745.

Wu J, Patmore DM, Jousma E, Eaves DW, Breving K, Patel A V, Schwartz EB, Fuchs JR, Cripe TP, Stemmer-Rachamimov AO, Ratner N (2014) EGFR-STAT3 signaling promotes formation of malignant peripheral nerve sheath tumors. *Oncogene*, 33 (2): 173–180.

Xiao D, Qu X, Weber HC (2003) Activation of extracellular signal-regulated kinase mediates bombesin-induced mitogenic responses in prostate cancer cells. *Cell Signal*, 15 (10): 945–953.

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

Xiao H, Bid HK, Jou D, Wu X, Yu W, Li C, Houghton PJ, Lin J (2015) A novel small molecular STAT3 inhibitor, LY5, inhibits cell viability, cell migration, and angiogenesis in medulloblastoma cells. *J Biol Chem*, 290 (6): 3418–3429.

Yang CH, Murti A, Valentine WJ, Du Z, Pfeffer LM (2005) Interferon alpha activates NF-kappaB in JAK1-deficient cells through a TYK2-dependent pathway. *J Biol Chem*, 280 (27): 25849–25853.

Yang JC, Ok J, Busby JE, Borowsky AD, Kung H-J, Evans CP (2009) Aberrant activation of androgen receptor in a new neuropeptide-autocrine model of androgen-insensitive prostate cancer. *Cancer Res*, 69 (1): 151–160.

Yang L, Lin S, Kang Y, Xiang Y, Xu L, Li J, Dai X, Liang G, Huang X, Zhao C (2019) Rhein sensitizes human pancreatic cancer cells to EGFR inhibitors by inhibiting STAT3 pathway.

J Exp Clin Cancer Res, 38 (1): 31-43.

Yao D, Bao Z, Qian X, Yang Y, Mao Z (2021) ETV4 transcriptionally activates HES1 and promotes Stat3 phosphorylation to promote malignant behaviors of colon adenocarcinoma. *Cell Biol Int*, 45 (10): 2129–2139.

Yau TO (2019) Precision treatment in colorectal cancer: Now and the future. *JGH open an open access J Gastroenterol Hepatol*, 3 (5): 361–369.

Zhang Q, Bhola NE, Lui VWY, Siwak DR, Thomas SM, Gubish CT, Siegfried JM, Mills GB, Shin D, Grandis JR (2007) Antitumor mechanisms of combined gastrin-releasing peptide receptor and epidermal growth factor receptor targeting in head and neck cancer. *Mol Cancer Ther*, 6 (4): 1414–1424.

Zhang T, Li J, Yin F, Lin B, Wang Z, Xu J, Wang H, Zuo D, Wang G, Hua Y, Cai Z (2017) Toosendanin demonstrates promising antitumor efficacy in osteosarcoma by targeting STAT3. *Oncogene*, 36 (47): 6627–6639.

Zhang Y-L, Yuan J-Q, Wang K-F, Fu X-H, Han X-R, Threapleton D, Yang Z-Y, Mao C, Tang J-L (2016) The prevalence of EGFR mutation in patients with non-small cell lung cancer: a systematic review and meta-analysis. *Oncotarget*, 7 (48): 78985–78993.

Zhang Y, Zhang Y, Yun H, Lai R, Su M (2014) Correlation of STAT1 with apoptosis and cell-cycle markers in esophageal squamous cell carcinoma. *PLoS One*, 9 (12): e113928-113941.

Zheng Q, Dong H, Mo J, Zhang Y, Huang J, Ouyang S, Shi S, Zhu K, Qu X, Hu W, Liu P, Wang Y, Zhang X (2021) A novel STAT3 inhibitor W2014-S regresses human non-small cell lung cancer xenografts and sensitizes EGFR-TKI acquired resistance. *Theranostics*, 11 (2): 824–840.

Zhou M (2018) High-grade prostatic intraepithelial neoplasia, PIN-like carcinoma, ductal carcinoma, and intraductal carcinoma of the prostate. *Mod Pathol*, 31 (S1): 71–79.

Zhou Y, Bai H, Xia J, Xu W-Y, Cheng L, Xiong L (2021) Novel ETV1 mutation in small cell lung cancer transformation resistant to EGFR tyrosine kinase inhibitors. *Ann Transl Med*, 9 (14): 1150-1166.

Zuo M, Li C, Lin J, Javle M (2015) LLL12, a novel small inhibitor targeting STAT3 for hepatocellular carcinoma therapy. *Oncotarget*, 6 (13): 10940–10949.

APPENDIX

Appendix 1

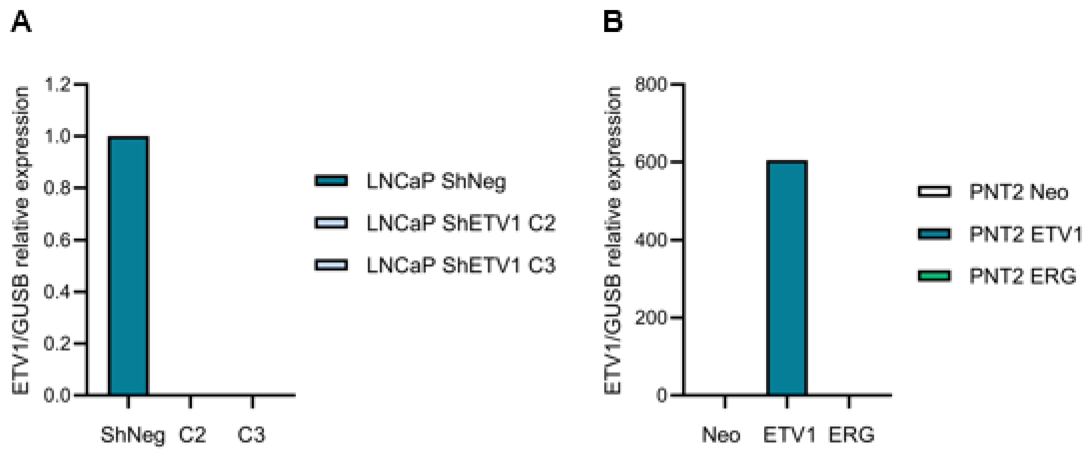


Figure A-1. Validation of the ETV1 cell models by qRT-PCR. (A) The two shETV1 clones derived from LNCaP cells show depletion of ETV1 expression and **(B)** The PNT2-ETV1 cell population shows *de novo* overexpression of ETV1.

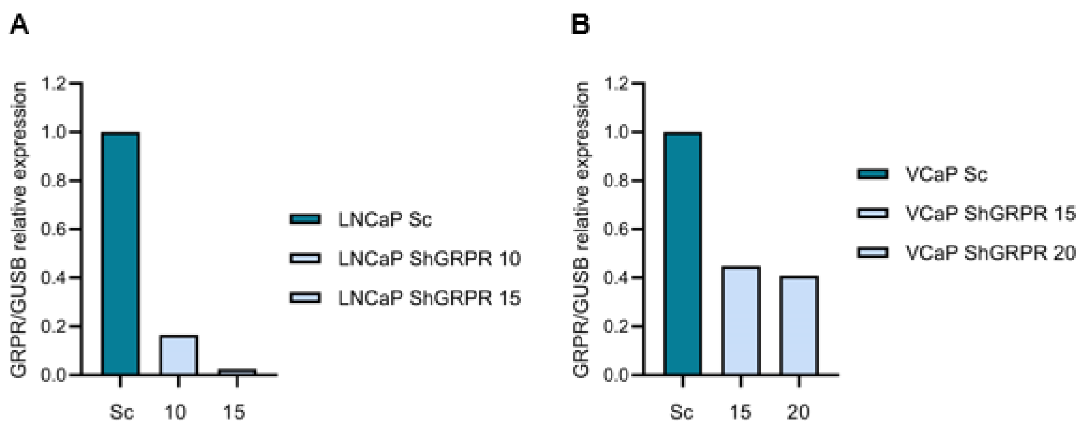


Figure A-2. Validation of GRPR silencing in LNCaP and VCaP cell models (A and **B).** The two shGRPR populations derived from LNCaP **(A)** or VCaP **(B)** cells show silencing of GRPR expression.

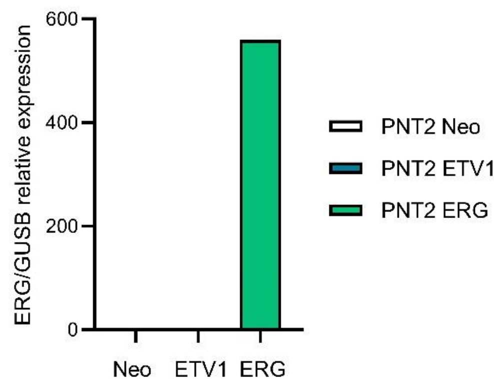


Figure A-3. Validation of *de novo* ERG expression of the PNT2-ERG cells.

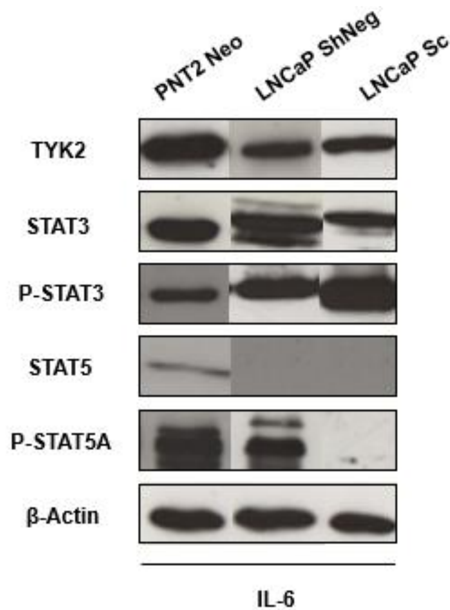


Figure A-4. Expression of different elements of the JAK/STAT pathway upon IL-6 stimulus. Protein blots for TYK2, STAT3, P-STAT3, STAT5A, and P-STAT5A are shown. β-actin was used as the loading control.

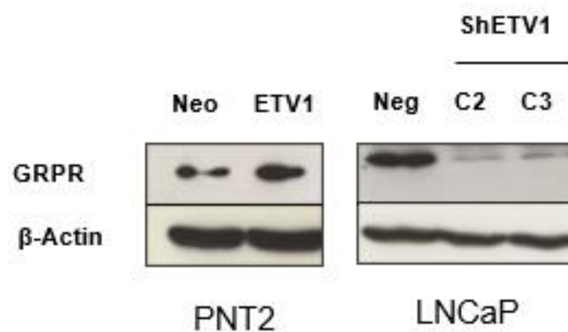


Figure A-5. ETV1 overexpression leads to increased GRPR expression. Protein blots of GRPR expression in control cells (Neo and ShNeg) and cells with modulation of ETV1 expression (PNT2 ETV1 and LNCaP ShETV1 C2/C3). β-actin was used as the loading control.

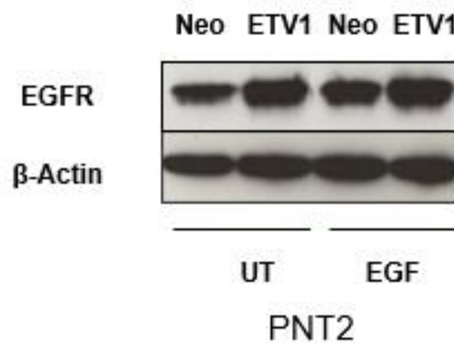


Figure A-6. ETV1 overexpression leads to increased EGFR expression in PNT2 cells. Protein blots of EGFR expression for the control cells (Neo) and PNT2 cells with ETV1 overexpression (ETV1). β-actin was used as loading control. UT represents the untreated cells and EGF the cells stimulated with EGF.

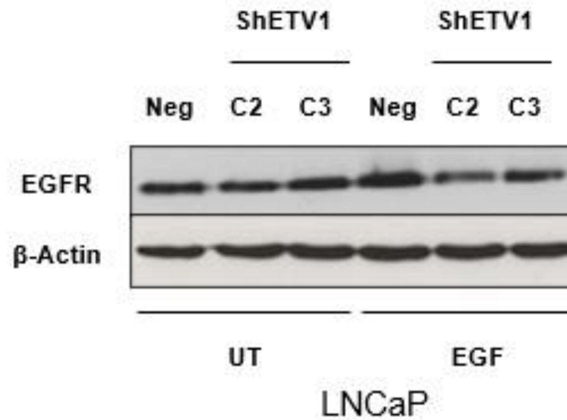


Figure A-7. ETV1 silencing leads to decreased EGFR expression in LNCaP cells independently of EGF stimulus. Protein blots of EGFR expression for control (ShNeg) and ETV1 silenced clones (ShETV1). β-actin was used as loading control. UT represents the untreated cells and EGF the cells stimulated with EGF.

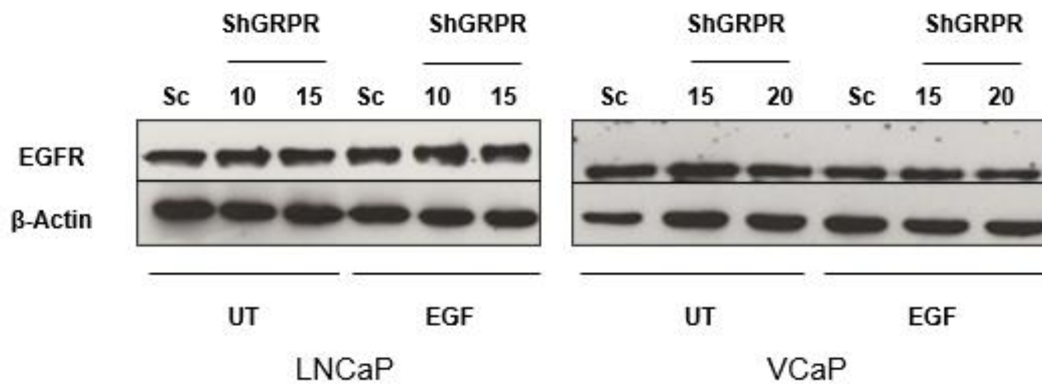


Figure A-8. Total EGFR expression was not changed by GRPR silencing. Protein blots of EGFR expression for control (Sc) and GRPR silenced (ShGRPR) cells. β-actin was used as the loading control. UT represents the untreated cells and EGF the cells stimulated with EGF.

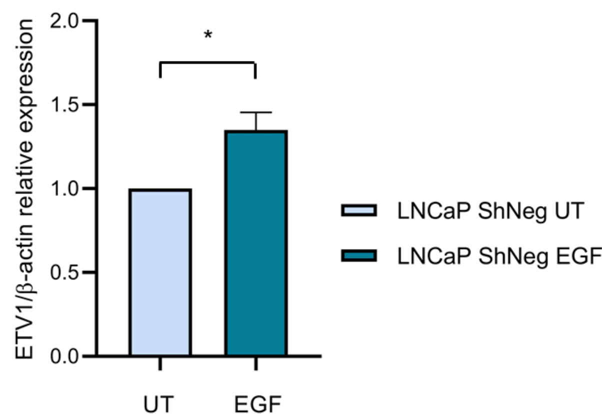


Figure A-9. EGF stimulus increases the expression of ETV1. Quantification of ETV1 expression obtained by western blot. β-actin was used as the loading control. Bars represent the standard deviation of the mean from two independent experiments. Unpaired two-tailed t-test was used for statistical analysis (* $P < 0.05$). UT represents the untreated cells and EGF the cells stimulated with EGF.

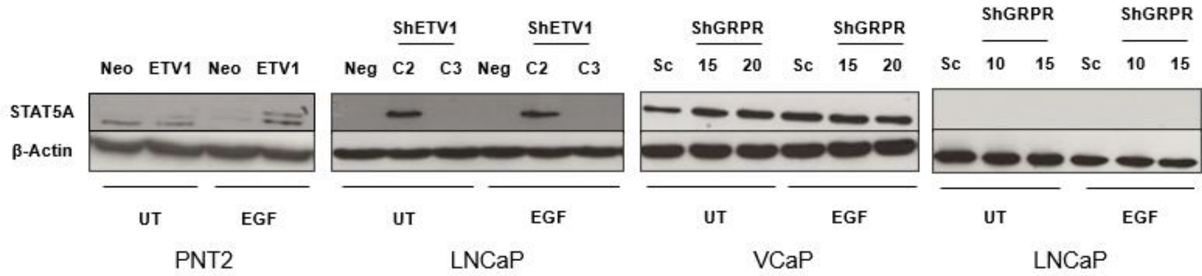


Figure A-10. STAT5A expression is independent of EGF stimulus and does not show a pattern consistent with regulation by either ETV1 or GRPR. Protein blots of STAT5A expression in control cells (PNT2 Neo, LNCaP ShNeg, VCaP Sc, and LNCaP Sc) and in cells with modulation of ETV1 (PNT2 ETV1 and LNCaP ShETV1 C2/C3) or GRPR (ShGRPR) expression. β-actin was used as loading control. UT represents the untreated cells and EGF the cells treated with EGF.

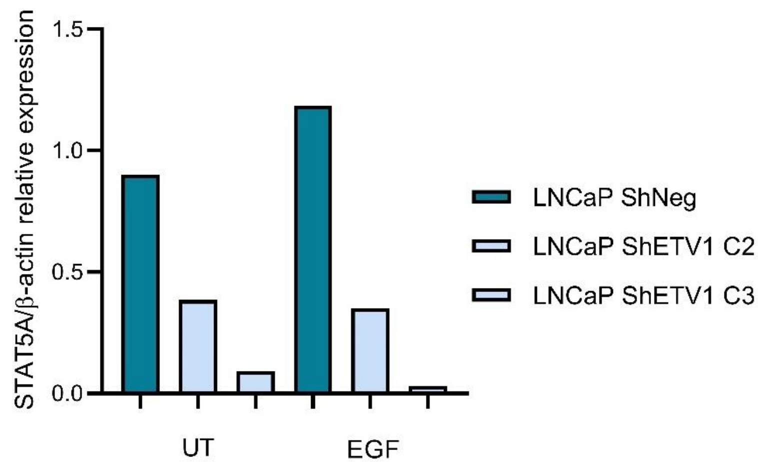


Figure A-11. STAT5A total expression decreases with ETV1 silencing in LNCaP cells. Total expression of STAT5A was obtained by the sum of the expression of Y694-phosphorylated and -unphosphorylated forms of STAT5A, previously showed in Figure 22 and Figure A-10. β-actin was used as loading control.

Appendix 2

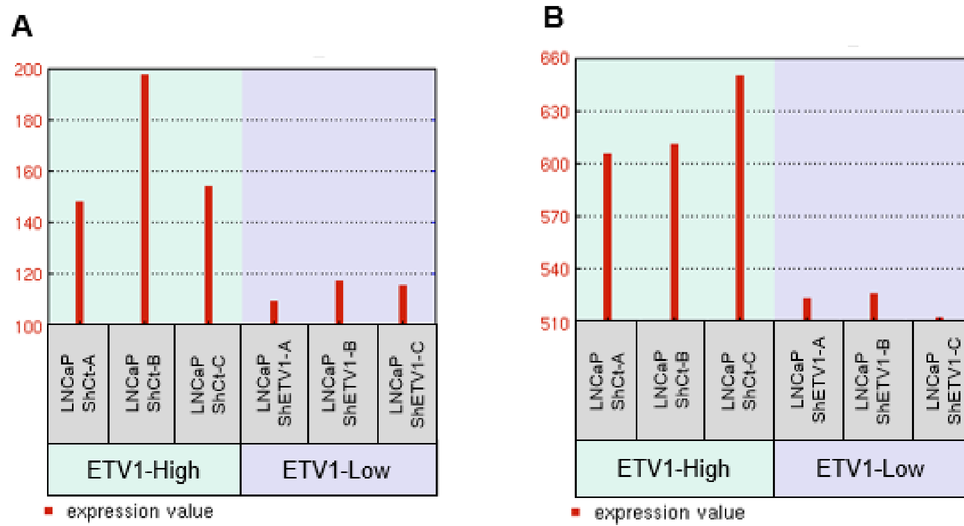


Figure A-12. The expression of both GRPR (A) and EGFR (B) decreases in LNCaP cells after ETV1 silencing. Data obtained from expression arrays available at GEO DataSets database GSE39388. LNCaP shCt A-C represents the different cellular replicates of control cells (ETV1-High) and LNCaP ShETV1 A-C represents the different cellular replicates with ETV1 silencing (ETV1-Low).

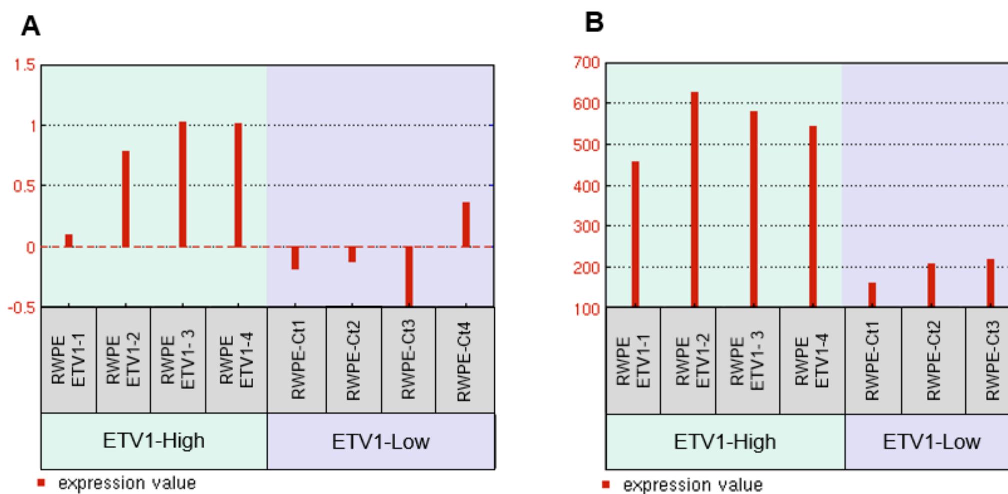


Figure A-13. The expression of EGFR increases with *de novo* ETV1 expression in RWPE cells. Data obtained from expression arrays available at the GEO DataSets database, specifically, **(A)** GSE29438 and **(B)** GSE39388. RWPE ETV1 1-4 represents the different cellular replicates with *de novo* ETV1 expression (ETV1-High) and RWPE Ct 1-4 represents the different cellular replicates of control cells (ETV1-Low).

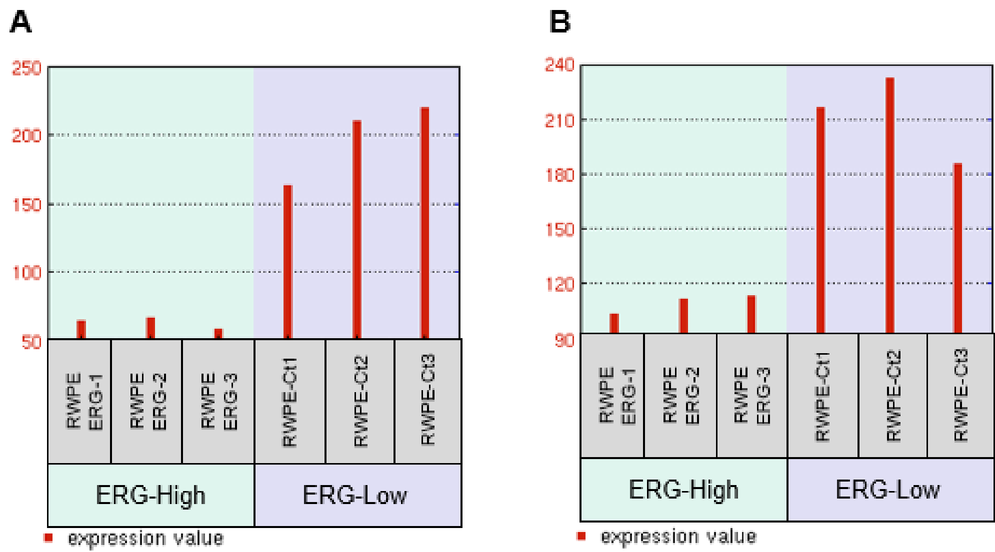


Figure A-14. The expression of both EGFR (A) and STAT5A (B) decreases with *de novo* ERG expression in RWPE cells. Data obtained from expression arrays available at GEO DataSets database (GSE39388). RWPE ERG 1-3 represents the different cellular replicates with *de novo* ERG expression (ERG-High) and RWPE Ct 1-3 represents the different cellular replicates of control cells (ERG-Low).