Universidade de Aveiro

Secção Autónoma de Ciências da Saúde

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LUÍS MIGUEL DOS SANTOS SOUSA

TCTEX1D4 E PPP1: A VIA DO TGFβ E O CANCRO DA PRÓSTATA

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biomedicina Molecular, realizada sob a orientação científica da Professora Doutora Margarida Sâncio da Cruz Fardilha, Professora Auxiliar Convidada da Secção Autónoma das Ciências da Saúde da Universidade de Aveiro

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palavras-chave

TCTEX1D4, PPP1, interação proteica, via do TGFβ, fosforilação proteica, cancro da próstata

resumo

T-complex testis expressed protein 1 domain containing 4 (TCTEX1D4) é uma cadeia leve de dineina que foi identificada como sendo uma proteína que interage com a PPP1. As funções específicas da TCTEX1D4 ainda permanecem desconhecidas mas a identificação dos seus interactores pode elucidar sobre as suas funções em sistemas biológicos. No interactoma da TCTEX1D4 merece particular destaque a presença de diversas proteínas associadas à via de sinalização do TGFβ e cuja desregulação se encontra associada ao cancro da próstata. Desta forma, foi objectivo deste trabalho avaliar a existência de TCTEX1D4 e do complexo TCTEX1D4-PPP1 em células de cancro da próstata, procurar desvendar o papel da TCTEX1D4 na via de sinalização do TGFβ e identificar eventuais alterações associadas à malginidade no cancro da próstata.

keywords

TCTEX1D4, PPP1, protein interaction, TGFβ pathway, protein phosphorylation, prostate cancer.

abstract

T-complex testis expressed protein 1 domain containing 4 (TCTEX1D4) is a dynein light chain that has been identified as interacting with PPP1. Whilst specific functions of TCTEX1D4 remain unclear, the identification of its interactors may help elucidating its biological function. Concerning to TCTEX1D4 interactome, the presence of several proteins of the TGFβ signaling pathway which deregulation is associated with prostate cancer is of particular interest. Thereof, it was purpose of this work to assess of existence of TCTEX1D4 and the TCTEX1D4-PPP1 complex in prostate cancer cells, clarify its role within the TGFβ signaling pathway and identify possible alterations during prostate cancer carcinogenesis.

Index

Abbreviations

ALK	Activin receptors-like kinases
AR	Androgen receptor
AFS	Anterior fibromuscular stroma
AMH	Anti-muellerian hormone
ASAP	Atypical small acinar proliferation
bFGF	Basic fibrolast growth factor
BPH	Benign prostatic hyperplasia
BCA	Bicinchoninic acid
BMP	Bone morphogenetic protein
BMPR	Bone morphogenetic protein receptor
BSA	Bovine serum albumin
BRCA1	Breast cancer 1, early onset
CZ	Central zone
CD	Cluster of Differentiation
Co-Smad	Common smad
cDNA	Complementary DNA
DNA	Deoxyribonucleic acid
DHT	Dihydrotestosterone
DLC	Dynein light chain
EGF	Epidermal growth factor
EGFR	Epidermal Growth Factor eeceptor
EST	Expression sequence tag
ECM	Extracellular matrix
FBS	Fetal bovine serum
FCS	Fetal calf serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GADD34	Growth arrest and DNA damage-inducible protein 34
GDF	Growth differentiation factor
HPRT	Hypoxanthine phosphoribosyltransferase
IP	Immunoprecipitation
I-Smad	Inhibitory smad
IGF	Insulin-like Growth Factor
KGF	Keratinocyte growth factor
LCMM	Lightcycler Master Mix
mRNA	Messanger RNA
MTOC	Microtubule organizing center
MAPK	Mitogen-activated protein kinase
NCBI	National Center for Biotechnology Information
NGF	Nerve growth factor
PZ	Peripheral zone
PTEN	Phosphatase and tensin homologue
PBS	Phosphate buffered saline

PI3K	Phosphatidylinositol 3-kinase
PPP1	Phosphoprotein phosphatase 1
PPP1C	Phosphoprotein phosphatase 1 catalytic subunit
PIP	Phosphoprotein phosphatase 1 Interacting Protein
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived Growth Factor Receptor
PCR	Polimerase chain reaction
PAGE	Polyacrylamide gel electrophoresis
PIA	Proliferative inflammatory atrophy
PCa	Prostate cancer
PSA	Prostate-specific antigen
PAP	Prostatic acid phosphatase
PIN	Prostatic intraepithelial neoplasia
PPs	Protein phosphatases
qPCR	Quantitative polymerase chain reaction
RIPA	Radioimmunoprecipitation assay
R-Smad	Regulatory smad
RNA	Ribonucleic acid
STPP	Serine/threonine protein phosphatase
SARA	Smad anchor for receptor activation protein
SBE	Smad-binding elements
SDS	Sodium dodecylsulfate
TCTEX1D4	T-complex testis expressed protein 1 domain containing 4
TGFα	Transforming Growth Factor α
TGFβ	Transforming Growth Factor β
TGFβR	Transforming Growth Factor β Receptor
TZ	Transition zone
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline-Tween 20
TBS-TT	Tris-buffered saline-Tween 20-Triton X-100
PTP	Tyrosine phosphatase
WR	Working reagent

I. Introduction

I. 1. Prostate gland

Prostate is the largest male accessory gland, located in the subperitoneal compartment between the pelvic diaphragm and the peritoneal cavity [5, 6]. The human prostate is composed of one non-glandular or stromal and three glandular compartments, tightly fused within a pseudocapsule [5, 7]. The three glandular elements are: central zone (CZ), transition zone (TZ) and peripheral zone (PZ), with the non-glandular zone being the anterior fibromuscular stroma (AFS) [5, 6]. The localization of the human prostate in the male reproductive tract and its division is depicted in Figure 1.

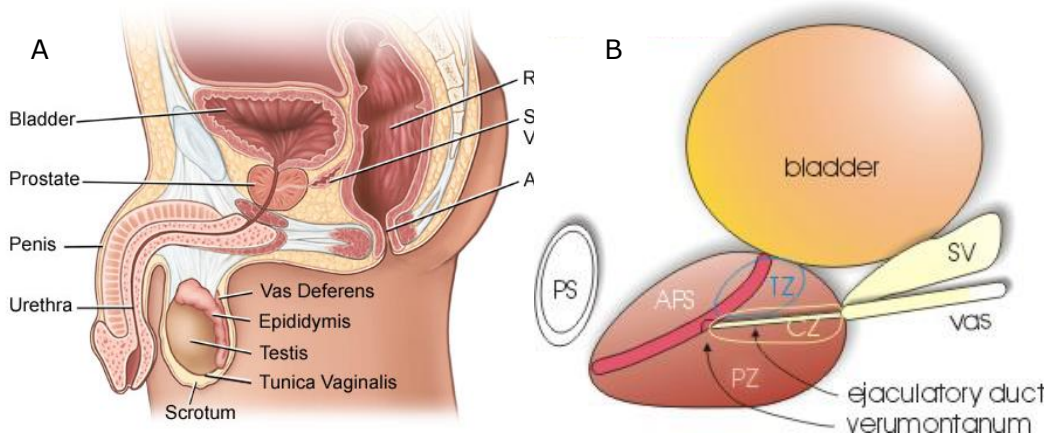

Figure 1 - A) Localization of the prostate gland in the male reproductive tract. B) Zonal division of the prostate. R – Rectum; SV - Seminal Vesicles; A – Anus; PS – Pubic Symphysis; AFS - Anterior Fibromuscular Stroma; TZ - Transition Zone; CZ - Central Zone; PZ - Peripheral Zone. Adapted from[8, 9].

These zones have different embryologic origins and can be distinguished by their histology, anatomic landmarks, biological functions and susceptibility to pathologic disorders, as summarized in Table 1 [5, 10].

Table 1 - Prostate gland is divided into three zones with different embryonic origins, histological and pathological findings. PCa – Prostate cancer; BPH – Benign Prostatic Hyperplasia.

Feature/Zone	Central Zone	Transition Zone	Peripheral Zone
Volume	25%	5%	70%
Embryonic Origin	Wolffian Duct	Urogenital Sinus	
Epithelium	Complex, large polygonal glands	Simple, small rounded glands	
Stroma	Compact	Fibromuscular	Loose
Origin of PCa	5%	25%	70%
Origin of BPH	-	100%	-

The histologic architecture of the prostate is that of a branched duct gland. Two cell layers, a luminal secretory columnar cell layer and an underlying basal cell layer, line each duct [6, 10]. The predominant secretory cells are columnar and characterized by expression of androgen receptor (AR), cytokeratins 8 and 18, CD57, prostatic acid phosphatase (PAP), human kallikrein-2 and prostate-specific antigen (PSA). Basal cells are cuboid and express cytokeratins 5, 10, 11, 14, 15 and 17, p63, CD44, and low levels of AR. Normal prostate also has neuroendocrine cells, which are androgen-independent and express chromogranin A and a variety of peptide hormones. The existence of epithelial stem cells in the prostate is well-accepted and associated with the expression of cytokeratins 5 and 14 [2, 11]. Prostate cells are in close contact with a complex stroma consisting of smooth muscle, blood vessels, blood borne cells, nerves, fibroblasts and extensive extracellular matrix (ECM). All these components work as a functional unit, with interactions between stroma and epithelium playing a pivotal role in normal prostate growth, development and function [12].

Prostate has several functions. As an exocrine gland, it produces the prostatic fluid, which comprises 30% of the volume of the ejaculate. The pr1ostatic fluid contains several constituents, such as: 1) zinc, with anti-bacterial properties; 2) PAP and other enzymes and nutrients that nurture sperm; 3) coagulase and other substances related to coagulation that facilitate movement and fertilization; 4) through its pH it increases sperm viability by reducing the acidity of the urethra. Prostate also participates in the control of urinary output and ejaculation and contributes to the metabolism of testosterone, converting it to the most potent androgen Dihydrotestosterone (DHT) [6, 13].

It has been established that androgens, testosterone and DHT, are the most potent and relevant mitogens of the normal prostate. In fact, continuous stimulation by androgens is crucial for normal prostatic development and function. AR is usually located in the cytoplasm. Upon presence of the ligand, it is translocated to the nucleus, where it exerts its transcription factor activity, binding to Androgen-Responsive Elements and promoting the transcription of target genes. Overall, it stimulates proliferation, differentiation and secretion. However, androgens actions are mainly indirect, through the stimulation of the production of diverse growth factors and its receptors. Among these, the more important ones are EGF, TGFα, KGF, IGF, NGF, PDGF and bFGF. In order to counteract the effects of these growth factors, both in stroma (bFGF) and epithelium (mainly TGFα and EGF), TGFβ has been identified as a key growth modulator in normal prostate, by inducing growth

inhibition and differentiation [6, 12, 14-16]. Commonly, these rely on intracellular pathways of signal transduction like JAK/STAT3, Ras/Raf/Mek/MAPK, Smads and PI3K/AKT/mTOR [17]. A more detailed description of the effects of androgens and other hormones and growth factors is presented in Table 2.

Table 2 - Overview of the effects of hormones and growth factors in the prostate gland. Based on [6, 12, 14-16].

Hormone/Growth Factor	Action
Androgens	Suppression of stromal growth; Increase in epithelial height and secretory activity; increases DNA synthesis, mRNA and Protein levels and enzymatic activity
Estrogens	Gonadotropin inhibition at hypothalamus or pituitary gland levels; Lowers epithelial height and secretion
Gestagens	Stimulates weight gain and secretion
Prolactin	Stimulates weight gain and secretion; Stimulates accumulation of androgens
Insulin	Stimulates weight gain and secretion; Prevents autophagy
bFGF	Mitogen, especially in stroma; Stimulates angiogenesis
EGF and TGFα	Mitogen, especially in epithelium
PDGF, NGF, IGF, IL6	Mitogens; prevention of apoptosis
TGFβ	Prevents epithelial cell growth; Stimulates stromal cell growth and angiogenesis

I. 2. Prostate cancer

Benign prostatic hyperplasia (BPH) and prostate cancer (PCa) are the two most common diseases in elderly men. Although they both occur in the same population, they are unrelated illnesses [5]. Worldwide, PCa is the 2nd most incident cancer in men, with 900 000 people diagnosed each year. In terms of cancer-related mortality, it ranks 6th worldwide (260 000 deaths each year). In Portugal, the situation is similar to other developed countries, with PCa being the most diagnosed cancer in men and 3rd cause of cancer-related death. Portuguese men have a lifetime risk of PCa diagnosis of 1/6 and 1 in every 35 portuguese men will die from PCa. Notably, worldwide significant differences (up to 25-fold) in PCa epidemiology

are evident: PCa is far more common in Europe, Australia and North America, with lower incidence in Asia and Africa [12, 18-20]. This may be due to the existence of several environmental and inherited risk factors. The main risk factor for the general population is ageing. Other risk factors or proposed risk factors are listed in Table 3.

Table 3 - Risk Factors associated with PCa. BRCA2 – Breast cancer 2, early onset; HPC1 - hereditary prostate cancer 1; HPC2 - hereditary prostate cancer 2; HSV – Herpes Simplex Virus; HPV – Human Papilloma Virus; HCV – Hepatitis C Virus. Based on [21].

Type of Risk Factor	Examples
Familial	Associated with *BRCA2, HPC1, HPC2* mutations
Ethnicity	African American
Pathological/Medical	Vasectomy, Prostatitis, STDs (HSV, HPV, HCV, Gonorrhea, syphilis and chlamydia infections)
Socioeconomic	Income and education, occupation (farmers, plumbers, mechanics, etc.)
Environmental/Behavioral	Exposure to cadmium, smoking, diet, obesity, lack of exercise, sexual behavior

I. 2. 1. Molecular basis of PCa

The term cancer encompasses a heterogenic group of disorders that share a profound growth deregulation as a result of an accumulation of several somatic mutations that determine the progressive acquisition of malignity. Carcinomas of the prostate are often clinically silent, particularly during their early stages [22]. Locally advanced PCa may produce signs and symptoms including local discomfort and evidence of lower urinary tract obstruction. Occasionally, the first evidence of PCa is due to metastasis either in bone (the most common site of metastasis, causing bone pain or fractures) or in the liver (causing liver enlargement) [23]. The cellular origin of PCa has been attributed either to the dedifferentiation and mortality acquisition of differentiated luminal cells or to the malignant transformation of prostate stem cells that reside among the basal cells. It has also been observed that PCa can arise from basal cells, although the aggressive potential of luminal and basal cells populations differs [24]. Proliferative inflammatory atrophy (PIA), prostatic intraepithelial neoplasia (PIN) and atypical small acinar proliferation (ASAP) are regarded as the main PCa precursors, with several molecular mechanisms being proposed as triggers for each stage of progression and the alterations in each well-defined (Figure 2) [2]. PIA cells may undergo transformation leading to PCa directly or indirectly via the development of

High-Grade PIN [3]. ASAP are the precancerous lesions more related to cancer [25]. Despite the fact that most malignant tumors are monoclonal in origin, by the time they become clinically evident their constituent cells are extremely heterogeneous. At the molecular level, tumor progression most likely results from multiple mutations that accumulate independently in different cells, generating subclones with different characteristics [22]. Due to its marked heterogeneity and multifocal nature, the definition of a general mechanism of acquisition of malignity in PCa is rather difficult [26]. Even though some characteristic chromosomal changes and mutations have been defined (Figure 2), no single alteration can be defined as the cause of PCa. Besides alterations in genetic level, PCa also includes inflammation [27], oxidative stress and DNA damage [28], telomere shortening and telomerase activity [29] and epigenetic modifications [30], all of which contribute in a cooperative manner to the acquisition of malignity. Nevertheless, it appears that alterations in hormonal systems and growth factors signaling may represent a key aspect of PCa development and sustainability. Initially, the majority of the tumors are androgen-dependent. However, they progress to an androgen-independent state [31]. Alterations in growth factors, its receptors and their signal transduction pathways are also described, namely in EGF, PDGF, TGFα and TGFβ [2, 26]. A closer insight into alterations of TGFβ signaling pathway in PCa will be presented below.

I. 2. 2. Prostate Cancer – a need for new targets and biomarkers

Most human prostatic carcinomas are initially responsive to androgen ablation therapy, surgical castration and radiotherapy. However, when prostatic carcinomas progress to androgen-independent tumors, radical prostatectomy is the only option for treatment [32]. Nonetheless, these therapeutic options present several side effects, including urinary incontinence and impotence [33]. After metastasis formation no curative treatment is currently available, being surgical or medical castration the standard palliative treatments, with androgen deprivation producing effects on 80% of patients [12]. Regarding PCa detection, digital rectal examination, PSA levels and transrectal ultrasonography are commonly used for screening although the utility of these (especially PSA) are under discussion. Nevertheless, biopsy is always necessary for a definitive diagnosis [34, 35]. Since PCa is usually a silent disease in the first stages, the establishment of a specific panel of biomarkers, in either tissue or body fluids, which might complement the routinely applied diagnosis techniques in order to achieve earlier and more accurate diagnosis is desirable [36].

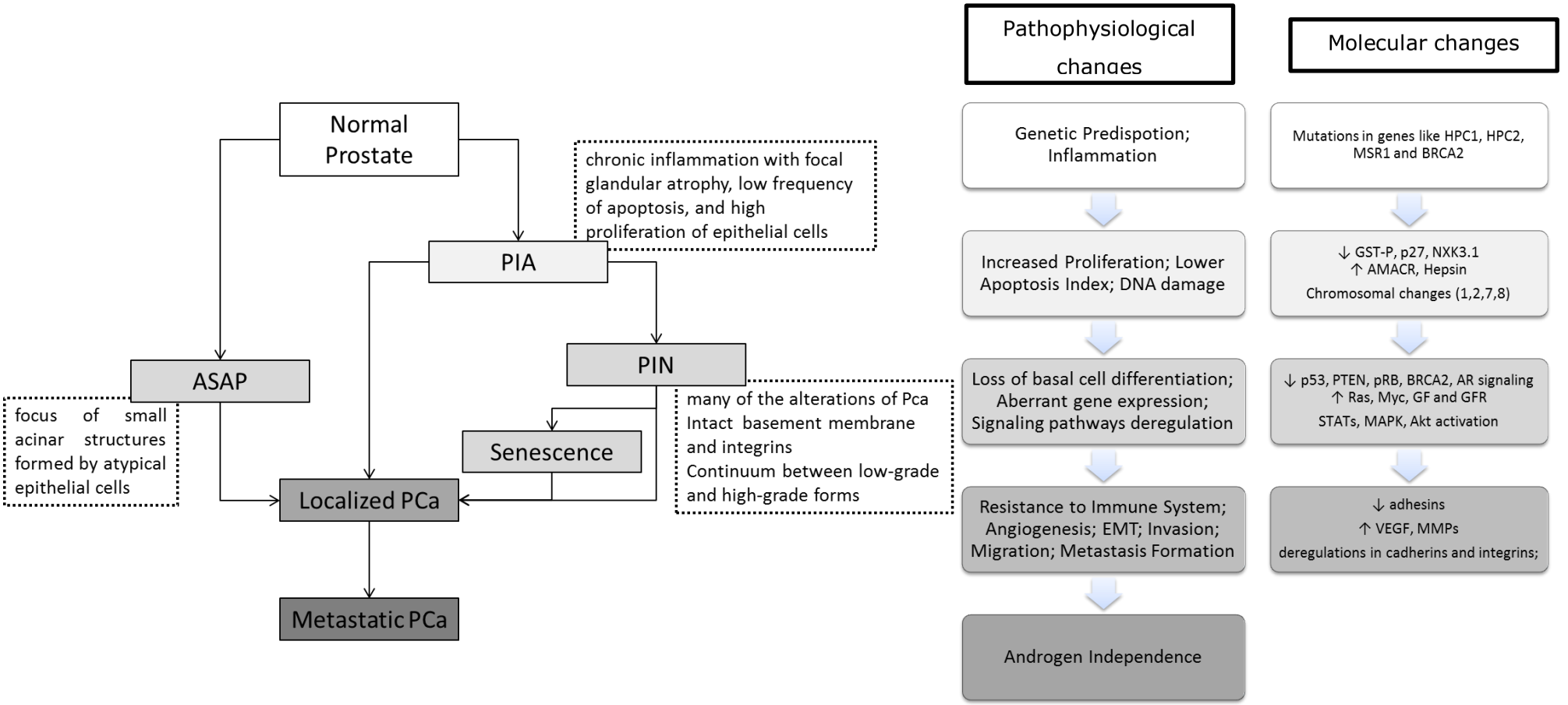

Figure 2 - PCa progression. Histoplatological changes associated with the precancerous lesions in dashed boxes. Pathophysiological and molecular changes during PCa progression are also displayed. EMT - Epithelial-mesenchymal transition; HPC1 - Hereditary Prostate Cancer 1; HPC2 - Hereditary Prostate Cancer 2; MSR1 - macrophage scavenger receptor 1; BRCA2 - breast cancer 2, early onset; GST-P - Glutathione-S-transferase; AMACAR - alpha-methylacyl-CoA racemase; p53 - protein 53; PTEN - phosphatase and tensin homologue; pRB - protein Retinoblastoma; AR - Androgen-Receptor; GF - Growth Factors; GFR - Growth Factor Receptors; STATs - signal transducer and activator of transcription; MAPK - Mitogen-Activated Protein Kinase; VEGF - Vascular endothelial growth factor; MMPs - Matrixmetello proteinases. Based on [1-4]

I. 2. TGFβ signaling

The transforming growth factor beta (TGFβ) superfamily comprises over than 42 members, all of which are generated from a pre-pro-peptide precursor. Besides TGFβ1, 2 and 3, this superfamily includes the bone morphogenetic proteins (BMPs), the activins, the growth differentiation factors (GDFs) and the anti-muellerian hormone (AMH), among others [37]. Virtually all types of cells produce and are sensitive to TGFβ superfamily members. These play fundamental roles in several cellular processes which may vary according to the ligand, the tissue and the conditions [38].

TGFβ is a cytokine with pleiotropic effects that is produced mainly by fibroblasts and epithelial cells [39]. In the epithelium, TGFβ inhibits cellular proliferation [40], whilst in the mesenchyme it promotes cellular proliferation [41, 42]. Other functions attributed to TGFβ are: synthesis of extracellular matrix [43], expression of integrins [44], modulation of immune response [45], angiogenesis [46] and wound healing [22]. BMPs display a broad range of effects distinct from those of TGFβ, even though sharing similar structure and signal transduction mechanisms. Among these, bone and cartilage formation and embryogenesis are the most relevant [47, 48]. Activins play crucial roles in the activation of FSH [49], erythropoiesis [50] and survival of neurons [51]. TGFβ family ligands dimerize, most commonly forming homodimers, and propagate the signal by interacting with membrane surface receptors presented in the target cell [52]. A total of 12 transmembrane Ser/Thr kinase receptors have been identified which are usually divided into two types: 5 constitutively active type II receptors (TGFβRII) and 7 non-constitutively active type I receptors (TGFβRI). Type III Receptors (or Co-receptors), which lack catalytic activity, have also been identified, namely endoglin (CD105) and betaglycan (TGFβRIII), which facilitate the interaction between the ligand and TGFβRII [52] (Figure 3). Ligands display more affinity to the type II receptors and the binding of the TGFβ to the type II receptor enables it to phosphorylate the GS domain of the type I receptor, activating its catalytic activity [38, 53, 54]. The type I receptors are denominated activin receptors-like kinases (ALKs) and once activated exert their catalytic activity by phosphorylating the C-terminal SxS domain in the main intracellular signal transducers of the pathway, the Smads [55]. Eight Smads have been identified in the human and mouse genomes: five regulatory Smads (R-Smads 1/2/3/5/8), one common Smad (Smad4, also known as Co-Smad) and two inhibitory Smads (I-Smads 6/7). The R-

Smads, after being phosphorylated by the type I receptors, form trimers with the Co-Smad [55]. Generally, BMPs, AMH and some GDFs (like GDF10) bind to ALKs 1/2/3/6 which propagate the signals via Smads 1/5/8, whereas TGFβ, Nodal, Activins and other GDFs (as GDF1) bind to ALKs 4/5/7 which propagate it through Smads 2/3 [38]. The fine dynamic equilibrium between these two opposing pathways often determines the ultimate outcome of the signal. After the formation of the complex, it is then translocated to the nucleus via microtubules and dyneins [35, 56].

Once in the nucleus, the trimers act as transregulatory elements to activate or repress the expression of genes such as *Sp1, Id1, Id2* and *Myc*. R-Smads/Co-Smad complex can also recruit transcription co-activators or co-repressors to modulate the amplitude of the activation/repression of the transcription [38]. Moreover, besides activating the canonical Smad-dependent signaling pathway, TGFβ can also activate other signaling pathways in a Smad-independent manner, such as MAPK, PI3K and small GTPases pathways [57, 58]. An overview of the TGFβ signaling pathways is depicted in Figure 3.

I. 2.1 TGFβ signaling alterations in PCa

During cell malignant transformation a number of alterations occur at molecular and cellular levels (genetic, epigenetic and somatic) and in the surrounding microenvironment contributing to an increased survivability and proliferative advantage [14, 59-61]. The traditional hallmarks of cancer were defined as: a) insensitivity to anti-growth signals; b) evasion of apoptosis; c) self-sufficiency in growth signals; d) sustained angiogenesis; e) limitless replicative potential; and f) tissue invasion and metastasis [62]. Moreover, two new emerging hallmarks, namely deregulation of the cellular energetics and avoidance of immune destruction have arisen. Additionally, two consequential characteristics of neoplasia facilitate the acquisition of the hallmarks previously defined, being these the genomic instability (and the consequent accumulation of mutations) and the tumor-promoting inflammation [63].

TGFβ, as a potent pleiotropic cytokine, has a defined yet complex role in mediating each of these hallmarks [14]. In normal tissues TGFβ functions as a formidable barrier to the development of cancer hallmarks [64]. It inhibits cellular proliferation [65], migration and invasion [66], and promotes apoptosis [67], cell adhesion [66] and cellular differentiation [68].

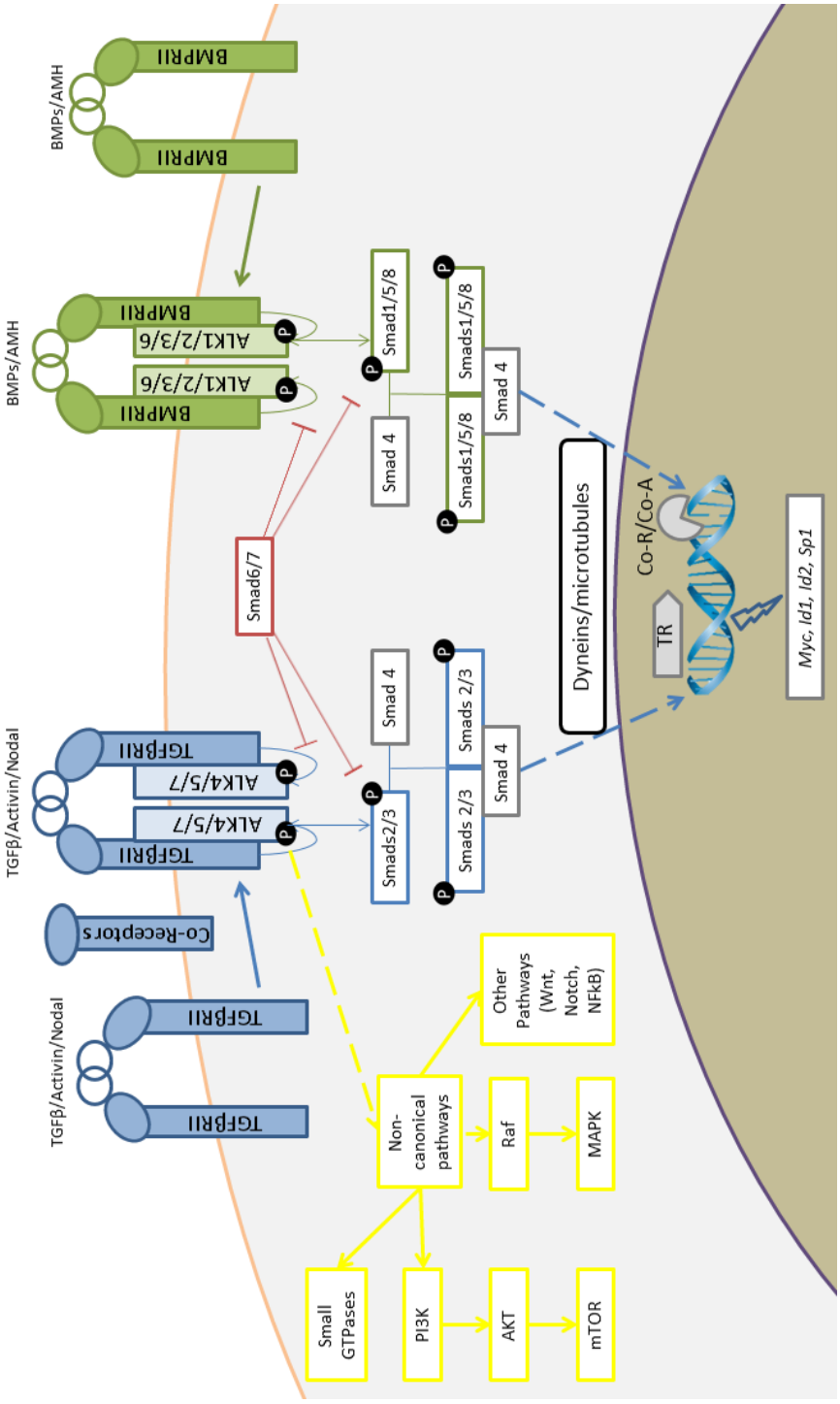

Figure 3 - TGFβ signaling pathway. Dimers of the TGFβ superfamily bind to type II TGFβR. TGFβ, Activin and Nodal bind to TGFβRII (or ActRII) which phosphorylates the ALKs 4/5/7 which propagates the signal via Smads2/3, in a phosphorylation-dependent way. Co-receptors like endoglin or Betaglycan facilitate binding to TGFβRs (Center, blue). On the opposite side, BMPs and AMH bind BMPRII, which phosphorylates ALKs 2/3/6, which, in turn, propagate this signal through phosphorylation of Smad1/5/8 (right, green). R-Smads form trimers which are translocated into the nucleus by dynenins and microtubules. In the nucleus they transregulate (TR) the expression of genes like Id1 and recruit co-repressors (Co-R) or co-activators (Co-A). I-Smads act inhibiting the signaling both at the receptor level and preventing Smad activation (Center, red). TGFβ also activates several non-canonical pathways (Left, Yellow).

However, TGFβ plays a dual role in cancer since in late-stage tumors the cellular machinery subverts the signaling pathway in order to promote the progression of the cancer [69]. In fact, alterations in TGFβ signaling in human cancers have been associated with the acquisition of all the cancer hallmarks, by losing of some of its normal functions (like growth inhibition) whilst retaining other effects that constitute a proliferative advantage (such as immunosuppression) as depicted in Figure 4.

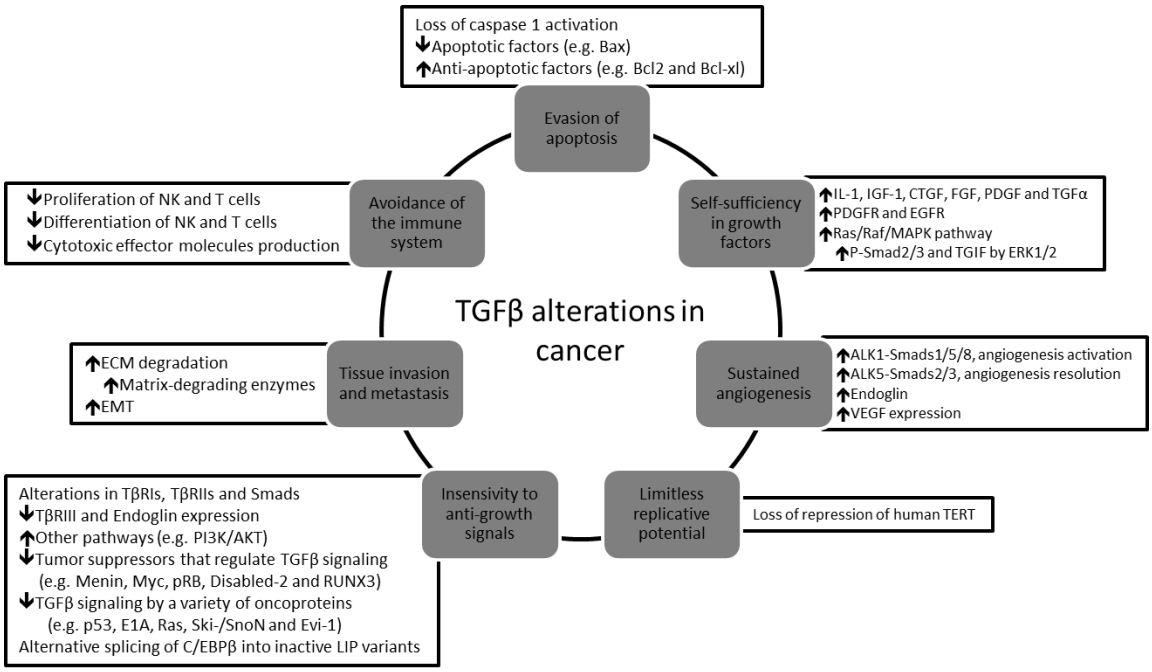

Figure 4 - All traditional cancer hallmarks are disrupted at variable extent, with insensivity to anti-growth signals comprising the most well described alterations. White boxes: TGFβ superfamily ligands, receptors, downstream effectors or in the responses exerted by TGFβ signaling pathway; Grey boxes: major cancer hallmark. TGFβ, transforming growth factor beta; IL-1, interleucin-1; IGF-1, insulin-like growth factor-1; CTGF, connective tissue growth factor; FGF, fibroblast growth factor; PDGF, platelet-derived growth factor; TGFα, transforming growth factor alpha; PDGFR, PDGF receptor; EGFR, epidermal growth factor receptor; MAPK, mitogen-activated protein kinase; TGIF, transforming growth-interacting factor; VEGF, vascular endothelial growth factor; TERT, telomerase reverse transcriptase; pRb, protein retinoblastoma; LIP, liver-enriched inhibitory protein; ECM, extracellular Matrix; EMT, epithelial–mesenchymal transition.

Concerning PCa, several alterations at different levels of the TGFβ signaling pathway have been defined.

Ligands

In PCa there is a dramatic increase in TGFβ1 mRNA and protein levels, which are correlated with high Gleason score, bone metastasis, angiogenesis and clinical outcome [14, 70]. In cancer, the TGFβ-induced growth inhibition is disrupted by the neoplasic surrounding environment [15]. It has also been shown that highly metastatic androgen-independent PCa cells have the ability to activate TGFβ [15] and that TGFβ can promote cancer growth, viability and aggressiveness [15, 71].

These effects were not due to a direct increase on cell proliferation, but rather by effects in the host, namely increases in angiogenesis and invasiveness, accompanied by a suppression of immune response and cell adhesion [15, 71].

Notably, TGFβ is its most potent inducer; however it causes auto-induction at high concentrations only in malignant cells [72]. Even though most studies are centered in TGFβ, other ligands of the TGFβ superfamily may also play pivotal roles in PCa (e.g. Activins, BMP6, GDF15 and Nodal/BMP16) [73, 74].

Receptors

Up to 30% of PCa cases have downregulation or absence of a TGFβ receptor [14]. Mutations of TGFβRII are common in lung and laryngeal cancers, but not in PCa [74]. Nevertheless, some PCa cells express a truncated TGFβRI mRNA transcript [75], lack a TGFβRII gene [76], have TGFβRs epigenetically downregulated [77, 78], or carry some sort of TGFβRII mutation [79]. The fact that TGFβRI and TGFβRII are decreased in metastasis vs. primary tumors may indicate an active role for this alteration in cancer progression [80]. The decrease of TGFβRs in prostate tumor cells appears to lead to growth inhibition resistance, thus resulting in clonal expansion [12, 81-84]. On the other hand, distinct BMPRs appear to elicit different responses, with a shift from BMPR1B towards BMPRIA expression being associated with cancer, as the latter is associated with prostate growth [85, 86].

Several studies have also reported the loss of TGFβRIII as the most common alteration during PCa progression being this alteration even more evident in metastasis [87]. Moreover, loss of TGFβRIII correlates with disease state, metastatic disease and PSA recurrence [87]. In a similar manner, endoglin (the other type III receptor) levels are lower in PCa cells vs. normal prostate cells and even lower in metastasis. Endoglin has been found to inhibit invasiveness, metastasis formation and motility while increasing cell adhesion, neovascularization and growth [88-91]. In contrast, its levels are higher in endothelial cells, being this associated with ongoing angiogenesis. Notably, endoglin may play a pivotal role regulating the fine equilibrium between Smad1/5/8 vs. Smad2/3 signaling [85, 89].

Effectors

Smad alterations in PCa are also found, although not as extensively described as alterations in TGFβ ligands and receptors. In the initial stages of prostate tumor development, ALK2-Smad1/5/8 signaling is promoted to increase the growth and neovascularization, whereas in late-stage tumors there is a shift towards ALK5-Smad2/3 signaling that leads to the acquisition of malignant capabilities, namely enhanced invasiveness, migration and metastasis formation [88, 90]. Smad4

promoter methylation has also been reported [92]. High levels of Ski, a co-repressor of Smad2/3, were detected only in PCa cells [73] (Figure 5).

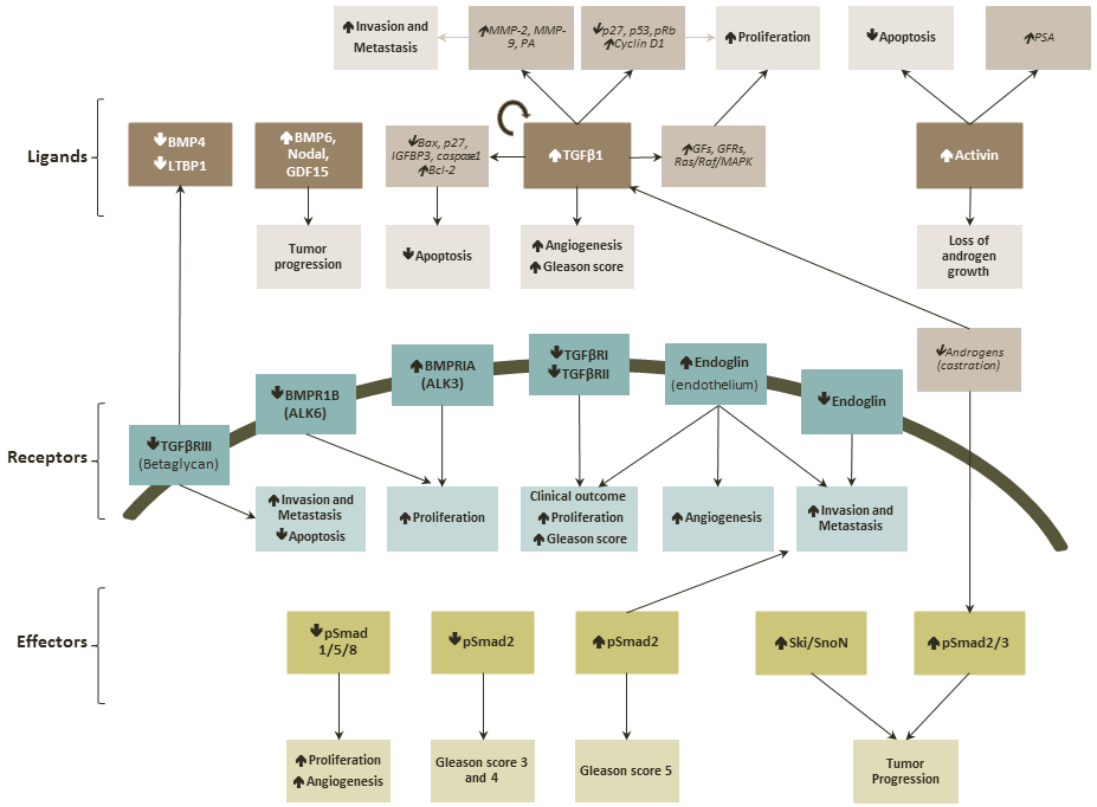

Figure 5 - Main effects or alterations related to the TGFβ signaling pathway that drive to prostate cancer hallmarks are depicted in the scheme. Dark grey boxes: ligands, receptors or downstream effectors of the TGFβ signaling pathway; Light grey boxes: alteration in other targets (italics) or alteration in cancer cell hallmarks or effects (bold); Black arrows inside boxes: increase/decrease or activation/inactivation; Black arrows: effect or alteration. PSA, prostate-specific androgen; MMP-2, matrix metalloproteinase 2; MMP-9, matrix metalloproteinase 9; PA, plasminogen activator; pRb, protein retinoblastoma; BMP, bone morphogenetic protein; LTBP1, latent TGFβ binding protein 1; GDF15, growth differentiation factor 15; IGFBP3, insulin-like growth factor binding protein 3; GF, growth factors; GFR, growth factor receptor; MAPK, mitogen-activated protein kinase; BMPR, bone morphogenetic protein receptor; TGFβR, TGFβ receptor; ALK, activin receptor-like kinase; EGFR, epidermal growth factor receptor.

I. 2. 2. TGFβ and androgens

The relationship between TGFβ and androgens is also relevant. After androgen withdrawal, TGFβ and TGFβRs mRNA levels are upregulated, at least transiently [12, 70, 93]. Androgens have also been found to downregulate Smads expression and activation. DHT bounds to androgen receptor and this complex binds to active Smad 3, inhibiting the association of Smad 3 with SBE and, therefore, blocking the signal. Also, DHT leads to the inactivation of *Sp1* suppressing its binding to TGFβRII promoter, thus decreasing TGFβRII levels [74, 94, 95]. It has been recognized that

the AR status determines the sensitivity of PCa cells to TGFβ-mediated apoptosis [96] and the ability to evade it is of paramount importance in the development of PCa [14].

I. 2. 3. TGFβ signaling pathway as a target in PCa

TGFβ signaling pathway components have been regarded as possible targets for PCa therapy for several years [97]. Preclinical studies on direct targeting of TGFβ (ligand) using antisense approaches and antibodies, and indirect inhibition of its membrane receptors, provide promise for the potential therapeutic value of targeting TGFβ signaling. There is evidence that inhibiting the TGFβ signaling is a way to control tumor progression in cancer. The inhibition of TGFβRI kinase is also under study [98]. Moreover, genistein, currently in phase II of clinical trials, acts through activation of Smad 1, thus suppressing PCa cell invasion, in an ALK2-dependent way [89]. Due to the prominent role of phosphorylation in TGFβ signaling and its deregulation in PCa, the targeting of phosphorylation systems of this pathway may represent a suitable way to address this issue.

I. 3. Phosphorylation

The phenomenon of protein phosphorylation was recognized more than 100 years ago; however, it was only in the 1950s that the first known example of "reversible protein phosphorylation" was identified, as regulating phosphorylase activation, thus concluding the research started 30 years before by Carl and Gerti Cori [99-102]. Phosphorylation is the most important and common mechanism of acute and reversible regulation of proteins [103]. From one third to up to 70% of all proteins are regulated by this mechanism [104, 105], comprising circa 100 000 phosphorylation sites [106]. In eukaryotic cells the majority of the phosphorylation events occur in three residues, namely serine, threonine and, lastly, tyrosine, in a 1000:100:1 ratio [107]. However, histidine, arginine and lysine may also undergo phosphorylation [108]. Reversible protein phosphorylation is a ubiquitous and important intracellular control mechanism, being involved in almost all signal transduction pathways, cellular and physiological processes [109], in a variety of very distinct cellular processes, from photosynthesis [110] to cell cycle control [111] (Table 4). Although the importance of this process is particularly relevant in eukaryotic cells, it is so ubiquitous that it plays an important role even in

prokaryotic cells [112-114] and viral activity [115], thus demonstrating its paramount importance and high prevalence in life systems. Alterations in this process were naturally correlated with the development of several diseases [116, 117], as listed in Table 4. Also, a number of naturally occurring toxins and pathogens also exert their effects by altering the phospho-state of proteins [117].

Table 4 - Phosphorylation is involved in a wide variety of molecular, cellular and physiological processes. Alterations in phosphorylation have been associated with the development of several pathologies. Mutations in kinases or phosphatases are the cause of several diseases or syndromes.

Molecular Process	Cellular Process	Physiological Process	Pathologies	Genetic Disorders
DNA replication	Differentiation	Immunity	Alzheimer's Disease	Hirschsprungis disease
Transcription	Meiosis	Embryogenesis	Parkinson's Disease	Ataxia-telangiectasia
Translation	Mitosis	Injury healing	Cancer	Chraniosynostosis
Enzymatic regulation	Necrosis	Hormonal and nervous control	Diabetes Mellitus	Li-fraumeni syndrome
Splicing	Apoptosis	Angiogenesis	Heart Failure	X-linked myotubular myopathy

Protein phosphorylation systems require four entities: a) a protein kinase; b) a protein phosphatase; c) a target protein and d) a phosphate (Figure 6). Protein kinases role is to catalyze the transfer of the gamma-phosphate of ATP to the target protein, which becomes phosphorylated and changes its properties accordingly. This process is reversed by protein phosphatases (PPs) [118-121].

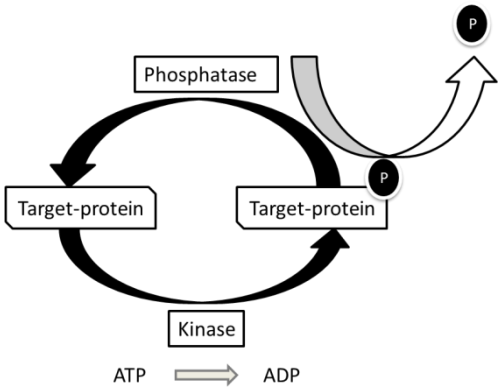

Figure 6 - The mechanism of reversible phosphorylation. P - Phosphate

At a biochemical level, protein activity, subcellular localization, stability, half-life and interactions can be controlled by phosphorylation [122, 123]. The fact that multisite phosphorylation can occur, enables several of such effects to operate in the same protein and can also determinate the extent and duration of a response, being the key to signal integration [124]. The critical feature of phosphoamino acids

in proteins is that they act as new chemical entities that do not resemble any natural amino acid, and thereby provide a means of diversifying the chemical nature of proteins, inducing conformational changes through allosteric mechanisms, creating binding sites for molecules, altering the properties of association/dissociation of proteins among other effects [125-127].

There are over than 500 kinases encoded in the human genome [128], comprising 2-3% of the human genome, thus representing one of the largest gene family [122, 129]. Of these, nearly two thirds are Ser/Thr kinases, with tyrosine kinases comprising the remaining 33% [130]. Unlike kinases in which all belong to the same gene family, PPs are divided in several unrelated families (Figure 7). Tyrosine phosphatases (PTPs) can be either cytosolic (9 subclasses) or transmembranar (5 subclasses) [131, 132]. All PTPs are monomeric and the domains that flank the catalytic domain control its activity and localization, with an unique catalytic mechanism [131]. Nowadays, circa 100 members of this family have been identified, thus making the number of PTPs similar to the number of tyrosine kinases [133]. Dual-specificity phosphatases are usually included in the PTPs family, in spite of their ability to act upon tyrosine, serine or threonine residues [131, 132]. Non-specific alkaline and acidic phosphatases can also be found, either in the intracellular level or in the extracellular milieu [134].

Ser/Thr PPs (STPPs) exert their effect by removing phosphate groups in serine or threonine residues. Initially, STPPs were divided according to 13 biochemical parameters [135]. This functional division was not reflected in the phylogenetic division. Three distinct gene families are now recognized: PPMs, FCPs and PPPs. PPMs (Mg^{2+} dependent) include PP2C, pyruvate dehydrogenase and relatives [136]. FCP family includes SCPs 1-3 and FCP1 phosphatases, which display specificity towards RNA polymerase II [137] (Figure 7). All members of the PPP superfamily have catalytic cores that share the same structural fold and catalytic mechanism, with the differences between these enzymes residing mainly in the solvent exposed loops that determine the shape and charge of the surface, and henceforth the affinity for ligands [105].

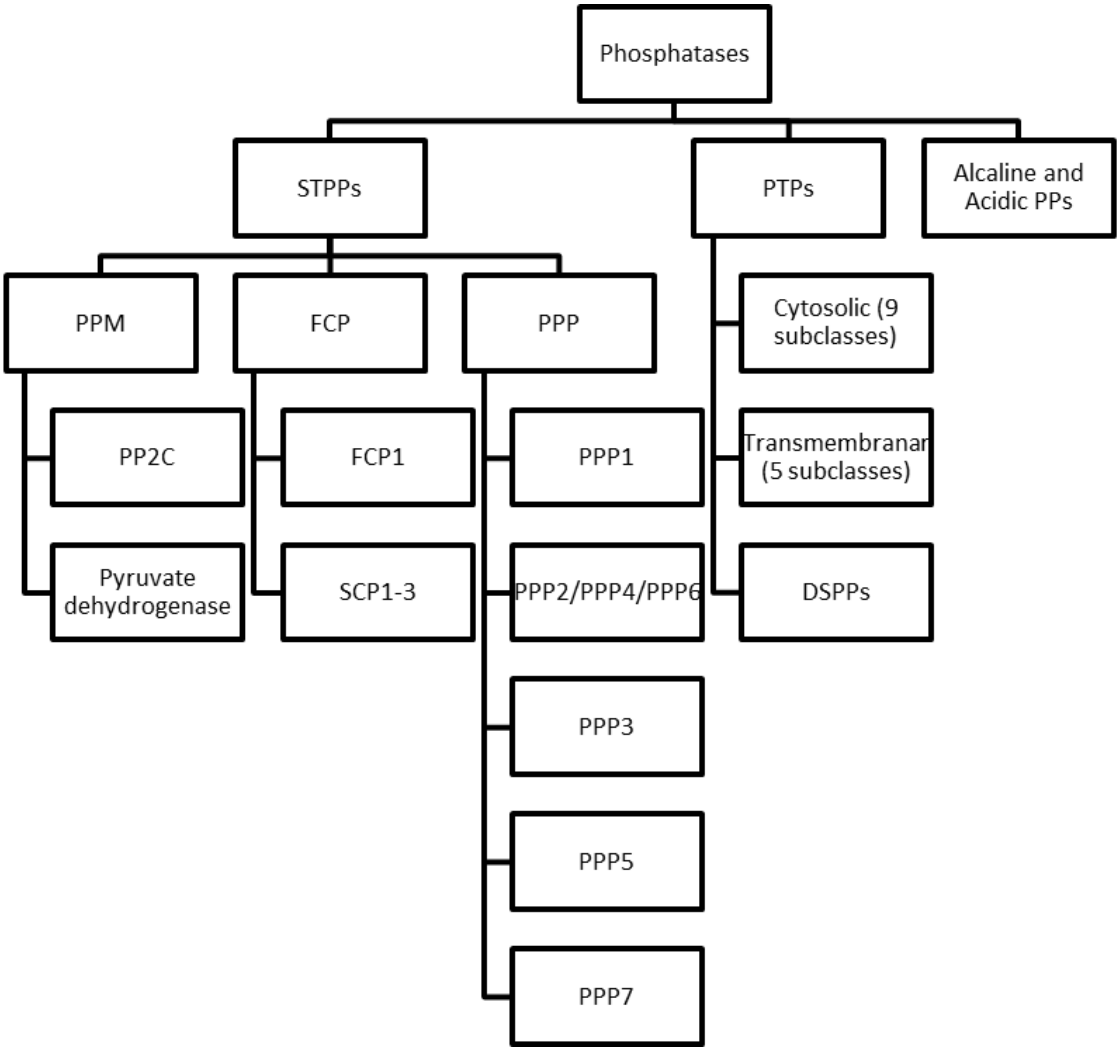

Figure 7 - Phylogenetic division of PPs.

Surprisingly, only 13 genes encode PPPs catalytic subunits, which have to counteract the effects of nearly 350 Ser/Thr kinases [138]. The discrepancy between the number of STPPs and kinases (circa 20 times fewer STPPs) is answered by the different strategies of achieving regulated specificity [139]. While in the case of Ser/Thr kinases diversity is achieved by gene duplication, in the case of STPPs it is achieved by their unparalleled ability to form stable protein complexes. This is due to the existence of a variety of different holoenzymes, each with its own substrates and mode of regulation [105, 138, 140].

I. 4. PPP1

PPP1 (phosphoprotein phosphatase 1) and PPP2 are major PPs that account for more than 90% of dephosphorylation events. In fact, 650 different PPP1 complexes and 70 PPP2 holoenzymes are taught to be contained in mammals, thus indicating that PPP1 catalyzes the majority of dephosphorylation events in eukaryotic cells [105, 138, 140]. Naturally, PPP1 has been associated with the regulation of a number of signal transduction pathways, cellular and physiological processes and its deregulation with several diseases, namely cancer, male infertility, neurodegenerative and cardiovascular diseases [116, 141, 142].

In mammals, the PPP1 catalytic subunit (PPP1C) is encoded by 3 highly related genes (*PPP1CA*, *PPP1CB* and *PPP1CC*). Also, alternative splicing of *PPP1CC* gene generates the ubiquitously expressed PPP1CC1 isoform and the testis-enriched and sperm specific PPP1CC2 isoform [140, 143]. As with other PPPs, PPP1 is very unspecific *in vitro* and usually cannot be differentiated by substrate specificity or ability to interact with regulatory subunits. Only minor differences in N- and C-terminus exist between the isoforms (PPP1CC1 and PPP1CC2 only differ at the C-terminus), with an overall identity of over 90% [130, 139]. However, *in vivo* it displays an exquisite specificity of targets and functions [139]. Several aspects contribute to this enhanced specificity *in vivo.* Although all isoforms (except for PPP1CC2) are ubiquitously expressed, some differences in the expression levels are found between the tissues (Table 5) and even within the same tissue [144]. Also, minor differences in terms of subcellular localization are also described (Table 5).

*T*able 5 - Subcellular localization and tissue distribution of the PPP1C isoforms

PPP1C isoform	Subcellular localization		Tissue distribution
	Nuclear	During Mitosis	
PPP1CA	Nuclear matrix	Centrosomes	Ubiquitous, abundant in brain
PPP1CB	Chromatin	Chromosomes	Ubiquitous, abundant in liver and kidney
PPP1CC	Nucleolus	Mitotic spindle	Ubiquitous, abundant in brain, small intestine and lung (PPP1CC2 is testis-enriched and sperm-specific)

Nonetheless, as with other PPPs, the specificity is mostly achieved by the interaction with regulatory subunits, or PIPs (PPP1 interacting proteins) [142]. Over than 200 PIPs have been identified [116], but many more remain to be found,

as well as multiple proteins that interact with PPP1 in an indirect manner. Preferential binding of PIPs to individual PPP1C isoforms has also been described [138, 139, 145]. These regulatory subunits have been classified into 4 classes (Table 6), regarding its effects in PPP1. Another level of regulation is achieved by post-translational modifications or allosteric regulation of both PPP1C and PIPs, which can induce conformational changes that modulate their activity or the interaction between PIPs, PPP1C and the substrate [138]. The exquisite specificity of PPP1 *in vivo* is also explained by the fact that some PIPs are expressed in a cell type-dependent manner, accounting for cell type-specific PPP1 activity [105].

Table 6 - Functions of PIPs and the effect exerted.

Function	Example	Effect
Substrate	BRCA1	Activation
	Aurora-A	Inactivation
	Ikaros	Stabilization
Targeting	GADD34	Targeting to ER
	URI	Targeting to Mitochondria
	NOM1	Nucleoli
Activity modulation	PPP1R2	Inhibition of PPP1
Unclassified	HOX11	Unkown

As with other PPPs catalytic subunits, PPP1C is highly abundant but does not exist as free monomers in eukaryotic cells, being always associated with one or two PIPs [139]. Therefore, rather than seeing PPP1C as a single pleiotropic or promiscuous phosphatase, it should be considered as a large family of biochemically diverse holoenzymes, with individual PPP1C-PIPs displaying high specificity, even though sharing a common catalytic subunit [138].

I. 4. 1. PPP1 binding motif

The analysis of known PIPs have led to the establishment of a PPP1-binding motif, the well-known RVxF, which is found in 70% of PIPs. This sequence is degenerate, with the redefined consensus motif being K/R V/I x F/W, where x is any residue other than Phe, Ile, Met, Tyr, Asp, or Pro. Mutations in the hydrophobic residues (V/I) or aromatic residues (F/W) are sufficient to weaken or disrupt binding of

regulatory subunits to PPP1C. The residues that are necessary for PIP-PPP1C binding, particularly 287-293, are invariant in all isoforms. Sequences flanking this motif may play a role in defining the affinity or specificity for a PPP1C isoform [105, 138, 139]. Binding to RVxF does not cause important conformational changes of the catalytic subunit and does not have a major impact effect on its activity. In fact, RVxF may act as an anchor for PPP1C, enabling regulatory subunits to make additional contacts with the phosphatase in an ordered and cooperative manner. The absence of one of these associations can have either have no effect, weaken or disrupt the association, depending on the strength of the remaining interactions [139]. As previously indicated, not all PIPs display the RVxF motif, with several other more uncommon PPP1C-binding motifs being identified throughout the years (Table 7).

Table 7 - PPP1 Binding Motifs. Note: SILK motif needs to be from 7 to 107 aa of distance from the RVxF motif;X(0,1) is any aa, present or absent; {P} is any aa except P.

Motif	Sequence	PIPs
RVxF	[RK]-X(0,1)-[VI]-{P}-[FW]	PPP1R8, PPP1R10
SILK	K-[GS]-I-L-[RK]	NOM-1, WBP
MyPhoNE	R-X-X-Q-[VIL]-[KR]-X-[YW]	PPP1R12A, PPP1R12B
PPP1R2 degenerate motif	R-[KR]-X-H-Y	PPP1R2
Apoptotic Motif	[RK]-X(0,1)-[VI-]X-F-X-X-[RK]-X-[RK]	Bad, Bcl-2
Other motifs	RARA RNYF YSNEDYDR	PPP1R15A iASPP sds22

I. 4. 2. Role of PPP1 and other phosphatases in TGFβ signaling

As previously stated, TGFβ-induced signaling relies in a series of phosphorylation events that are triggered by the binding of the ligand to TGFβRII. Since TGFβRI and TGFβRII are Ser/Thr Kinases, an increasing number of protein phosphatases, particularly of the STPP family, have been reported to regulate the TGFβ pathway through interactions with both receptors and Smad proteins [146]. It is well known that PPP2 inhibits TGFβRI, BMPRII and the R-Smads, either directly or via its regulatory subunits. The role of other phosphatases, including PPM1A, FCPs, SCPs

and Dual-Specificity Phosphatases, in TGFβ signaling regulation has also been described [146]. PPP1 acts as a negative regulator of TGFβRs through its binding to Smad anchor for receptor activation protein (SARA). SARA presents PPP1 to ALK5 receptor promoting its dephosphorylation and consequent signal attenuation [147]. This targeting involves the inhibitory Smad7, and another PIP, GADD34 (PPP1R15B). It has also been shown that Smad7 recruits PPP1C to ALK1, inhibiting Smad 1/5/8 dependent pathway [148, 149]. The PIP that recruits PPP1 to ALK1 still needs to be elucidated, however, a Smad anchor for BMP signaling called Endofin was recently discovered. In a similar way of what happens to SARA, PPP1 also binds to Endofin and GADD34 to dephosphorylate the ALK3 and ALK6 receptors but without any intervention of the inhibitory Smad7 [150].

I. 4. 3. Role of phosphorylation and PPP1 in PCa

Constitutive activation of oncogenic kinases is one of the hallmarks observed in cancer cells, driving uncontrolled cell proliferation, invasion and metastasis . Transmembrane kinases, such as epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR), and cytoplasmic kinases, such as Raf, MAPK and Akt are mutated or constitutively activated in several types of human cancer, including PCa. For example, an increased Akt signaling, a Ser/Thr kinase involved in the control of cell size/growth, proliferation and survival has been associated with poor clinical outcome in a variety of tumors [152, 153]. In PCa it is commonly observed the loss of the tumor suppressor gene that encodes PTEN, a lipid phosphatase that negatively regulates PI3K/Akt signaling by dephosphorylation of phosphatidylinositol 3,4,5 phosphate, either by mutation or by suppression via microRNA miR-153 [154]. PTEN loss enhances the activation of several signaling pathways which are normally upregulated in PCa, including PI3K/Akt and MAPK, resulting in the acquisition of malignant characteristics such as reduced apoptosis, increased proliferation and metastasis formation [155]. In PCa, PPP1 and PPP2 also act as tumor suppressors.

PPP1 has been associated to PCa either directly or via its PIPs. PPP1 directly dephosphorylates and attenuates the two major tumor suppressors, p53 and pRb, which deregulation has been associated with PCa [156-158]. PPP1 also dephosphorylates Akt, regulating its activity and also its downstream targets, promoting apoptosis (Xiao, et al., 2010). Several PIPs have also linked PPP1 to PCa, such as NIPP1 and AR. PPP1/NIPP1 complex has been recently described as a regulator of cancer cell migration, specifically in PCa cells [159]. Chen and colleagues have shown that AR and PPP1 interact, and that PPP1 inhibition enhanced AR degradation. On the other hand, PPP1 overexpression increased AR

expression and transcriptional activity in PCa cells [160]. In similar ways, many other PIPs or pathways regulated by PPP1 associated with PCa have been described like Apaf-1, Aurora kinases, BRCA1, Bcl-2, CDC25, and caspases [161-168]

I. 4. 4. PPP1 and PIPs as molecular targets

Protein phosphatases represent attractive targets for when a deregulation of the phosphorylation system is present, such as cancer. PPP1 inhibition is usually regarded as not viable due to PPP1 ubiquitous expression and pleiotropic effects, which leads to toxicity. For these reasons, it is rather more suitable to target PIPs instead of protein phosphatases directly as they may be more event, tissue and subcellular compartment specific [169]. Nowadays, two targeted PPP1-PIP complexes have been described and approved by FDA. The levels of PPP1-GADD34 complex are diminished in cells treated with salubrinal, a small molecule that protect the cell from ER-stress-induced apoptosis [170]. The other complex involves PPP1 and histone deacetylases (HDACs) and is an attractive target to antitumor drugs. Trichostatin A, for example, disrupts the interaction between PPP1 and HDAC6 in glioblastoma and PCa cells, ultimately leading to the suppression of the AKT signaling pathway, usually upregulated in PCa. The introduction of oligonucleotide antisense therapy may contribute to an even more specific targeting, thusly producing less side-effects [170].

I. 5. TCTEX1D4 - Dyneins and the T-complex

The trafficking of membranous vesicles and particles inside the cell is a process of great importance in cell physiology [171]. Two general movements are described, one towards the cell membrane or anterograde, in which kinesins are the motor proteins, and another towards the nucleus or retrograde, in which dyneins are the driving force [172]. Dyneins are massive molecular motor complexes of 1-2 MDa that generate force towards the minus-end of microtubules [173] and are divided into axonemal (associated with cilia and flagella) and cytoplasmic [174]. These complexes are composed of 4 subunits: a) heavy chains, an ATPase that binds to the microtubule and drives cargo movement; b) intermediate chains, which mediates cargo binding and regulates the motor activity; c) light intermediate chains, mediates cargo binding to the dynein; d) light chains (DLC), the unit for cargo binding and specificity. Cytoplasmic dyneins are involved in several motile dynamic processes such as meiosis, mitosis and maintenance of the Golgi apparatus. Dyneins have been found to be regulated by phosphorylation [173]. Regarding DLCs, 3 families have been defined, namely LC8, Roadblock/LC7 and DYNT1/TCTEX1. TCTEX1 and the related TCTEX2 were firstly identified in the mice t-complex [173]. The mouse t-complex is localized in chromosome 17 and corresponds to a naturally occurring variant [175]. The t-haplotypes result from a series of inversions and mutations that lock together a set of genes with effects in embryonic differentiation, male fertility and chromosome behavior [175, 176].

I. 5. 1. TCTEX1D4 Protein

T-complex testis expressed protein 1 domain containing 4 (TCTEX1D4), a DLC from the TCTEX1/DYNLT1 family, has been recently identified as a new PIP in testis library [177-180]. TCTEX1D4 has 221 amino acids, with a predicted molecular weight of 23 352 Da and is encoded by a 2-exon gene in 1p34.1 locus. It has two distinct domains: the first 95 amino acids account for the disordered domain, with the remaining 126 residues constituting the globular domain. The globular domain is similar to the globular domains of the DYNLT1 family, with 2 α-helixes and 4 β-strands. Using bioinformatics, many putative serine phosphorylation sites have been identified. Surprisingly, no threonine or tyrosine phospho-sites were identified, thus leading to the hypothesis that TCTEX1D4 function is mainly regulated by serine phosphorylation mechanisms. Moreover, a N-glycosylation site (205-200) and several binding domains were identified: APC/C D box (28-36), cyclins (161-

165) and MAPK (167-176). As with most PIPs, the RVxF motif (RVSF) is found in TCTEX1D4, in the N-terminus (90-93). It was found to interact with all PPP1 isoforms and it appears to be important to complex formation and function. Nevertheless, the mutation of RVSF to AAAA only partially decreased the binding ability (35%), probably because the proline-rich surrounding environment forces the structure to the RVSF motif, even when mutated. Regarding TCTEX1D4 tissue expression, the EST profile in various species has localized it in the female reproductive tract (ovary, oviduct, placenta, uterus and embryo), head (hypothalamus, brain, striatum, tongue) and lung. Concerning subcellular localization studies in testis and sperm, TCTEX1D4 is present in the cytoplasm (near cell-cell junctions, MTOC and microtubule-like structures) and in the cell nucleus. The interaction with PPP1 was found to occur in the nucleus and in the MTOC. [177, 179].

Regarding possible functions, TCTEX1D4 has been proposed to be a player in TGFβ signaling pathway, cell-to-cell junctions and microtubule dynamics. The existence of binding sites for APC/C, cyclins and MAPK has also raised the possibility of playing a role in proliferation, differentiation and cell cycle regulation. Regarding microtubule dynamics it is at least partially responsible for PPP1 targeting to MTOC. In terms of TGFβ signaling, TCTEX1D4 has been found to bind to two TGFβ receptors which downregulation has been associated with PCa, Endoglin and TβRII, inhibiting TGFβ 2/3 signaling. In terms of the interaction of TCTEX1D4 with Endoglin, it has been found to occur mostly in the cell membrane, cellular protrusions and only rarely in vesicles. Of particular interest, it has already been shown that specific Smad pathways may require specific DLC [181] which can play a pivotal role in defining the intracellular signal transduction pathway, of which, ultimately, opposing effects of TGFβ may arise. PPP1 is known to bind and dephosphorylate intermediate chains, so binding to TCTEX1D4 is most likely to facilitate the access to intermediate chains, thus regulating the dynein complex function. The flexible arm of the disordered N-terminus is thought to help the binding of TCTEX1D4 to diverse regulatory proteins and cargoes [178, 179]. The unraveling of TCTEX1D4 interactome may be of great importance in understanding its role in the cell.

With this work, it is intended to unveil the role of the recently identified PIP TCTEX1D4 in TGFβ signaling and in PCa. It was proposed that TCTEX1D4 might act as a DLC in this system. Ultimately, TCTEX1D4-PPP1 may represent a suitable target for PCa therapy, as the targeting of very specific PIPs represents a good strategy in cancer therapy and TGFβ signaling pathway is a desirable target in PCa.

II. Aims

The main objectives of the work were as it follows:

- To establish several cell lines representing normal, androgen-dependent malignant and androgen-independent malignant prostate cells
- To demonstrate the presence of TCTEX1D4 in normal and malignant prostate cells
- To identify TCTEX1D4/PPP1 complexes in normal and malignant prostate cells
- To perform subcellular and tissue localization studies of TCTEX1D4, PPP1 and other proteins associated with TGFβ signaling
- To identify variations in the expression levels of TCTEX1D4 and PPP1 between normal and malignant prostate cells

III. Materials and Methods

The composition of all solutions is presented in Appendix I.

III. 1. Cell culture

The following cell lines were used: RWPE-1, LNCaP, DU 145 and PC3. All cells were tested for the presence of mycoplasma and results were negative. Cells were split every week and medium renewal took place 2 to 3 times a week. Cell handling occurred according to the instructions presented by ATTC datasheets. Briefly, medium renewal occurred as follows: medium was removed by an aspiring pipette, cells were briefly washed with PBS and a suitable amount of medium was added (10 ml in 100 mm Petri dishes). To split the cells, medium as removed and cells washed with PBS, upon which a suitable amount of 0.25% trypsin-0.53mM EDTA was added (1-2 ml, in 100 mm Petri dishes). After cells have detached, a trypsin inhibitor solution (usually a 10% FBS-PBS solution) was added (8-9 ml, in 100 mm Petri dishes) and cells centrifuged for 3 minutes at circa 125 g. Then, supernatant was discarded and cells were re-suspended in 10 ml of fresh medium. Subcultivation ratio was usually between 1:10 and 4:10. Cells used in the experiments had between 10 and 30 passages. LNCaP and PC-3, and DU 145 cells were kind gifts from Dr. Rui Medeiros (IPO Porto) and Dr. Tobias Lunge (University Medical Center Hamburg-Eppendorf), respectively. RWPE-1 cells were acquired from ATCC. PBS, RPMI 1640, K-SFM and FBS were acquired from Invitrogen. More information about to the cell lines and cell culture conditions are in Tables 10 and 11.

Table 10 - Cell lines used and culture conditions. NOTE: Occasionally, 20% FBS was used in LNCaP cells to enhance adhesion. In the University Medical Center Hamburg-Eppendorf, cells were grown using FCS instead of FBS. K-SFM: Keratinocyte serum-free medium; EGF – Epidermal growth factor; BPE – Bovine Pituitary Extract; AA – penicillin/streptomycin.

Cell Line	Complete Medium	Adherent	Growth Conditions
RWPE-1	K-SFM with EGF (0.15 ng/ml), BPE (25 µg/ml) and 1% AA (v/v)	Yes	37º C / 5% CO_2
LNCaP **DU 145** **PC3**	RPMI 1640 with 10% (v/v) FBS and 1% AA (v/v)		

Table 11 - Cell lines characteristics regarding malignity, androgen responsiveness and site of metastasis upon extraction

Cell Line	Characteristics	Androgens	Tumorigenicity	Site of metastasis
RWPE-1	Normal Epithelial Cells	Sensitive	None	-
LNCaP	Prostate cancerous epithelial cells	Sensitive	Low	Lymph node
DU 145	Prostate cancerous epithelial cells	Insensitive	Moderate	Brain
PC3	Prostate cancerous epithelial cells	Insensitive	High	Bone

III. 2. Tissue Samples

Tissue samples used and information regarding their origin and preservation are found in Tables 12 and 13.

Table 12 - Tumor tissues and information regarding original cell line, type of cancer it represents and fixation method. PFA – paraformaldehyde solution (4%)

Originary cell line	Type of Cancer	Fixation
PC 3 **LNCaP** **DU 145** **LuCaP 23.1**	Prostate Cancer	PFA + Paraffin OR Flash Frozen
OH3 **H69**	Small Lung Cell Carcinoma	PFA+Paraffin

Table 13 - Normal tissues and information regarding origin of the samples and fixation method. PFA – Paraformaldehyde solution (4%); SG – Sucrose gradient; BPH – Benign prostatic hyperplasia

Tissue	Origin	Fixation
Prostate	Surgical procedure in patient with BPH	Flash Frozen OR PFA + SG + Cryopreservation
Lung	Biopsy	PFA + Paraffin
Placenta	Caesarian birth	Flash Frozen OR PFA + Paraffin OR PFA + SG + Cryopreservation

III. 3. Antibodies

For the present work the antibodies against the following proteins were used: TCTEX1D4 (C -terminal), TCTEX1D4 (N-terminal), PPP1CA, PPP1CC, Endoglin (CD 105), Smad 1, Smad 2 and Smad 3. Secondary antibodies against Mouse and Rabbit were also used. A detailed description is present in Tables 8 and 9.

Table 8 – List of primary antibodies used and information regarding origin, dilution range used and host. WB – Western Blot and IF - Immunfluorescence

Target	Company	Reference	Dilution Range (WB)	Dilution Range (IF)	Host
TCTEX1D4 (C-terminal)	Homemade	CBC8C	1/50-1/1000	1/150-1/500	Rabbit
TCTEX1D4 (N-Terminal)	Sigma-Aldrich	RP11269F19.9	1/50-1/1000	1/150-1/1000	Rabbit
PPP1CA	Homemade	CBC2C	1/2500	-	Rabbit
PPP1CC	Homemade	CBC3C	1/5000	1/500-1/1000	Rabbit
Endoglin	Abcam	ab114052	1/1000	1/300-1/500	Mouse
Smad 1	LifeSpan BioSciences	LS-C133298	1/300	1/50-1/300	Mouse
Smad 2	Novus Biologicals	H00004087-M05	1/500	1/50-1/300	Mouse
Smad 3	Sigma-Aldrich	SAB1404037	1/300	1/100-1/500	Mouse

Table 9 – List of secondary antibodies used and information dilution range used. WB – Western Blot and IF - Immunfluorescence

Target	Designation	Dilution Range (WB)	Dilution Range (IF)
Anti-Rabbit	Odissey 680 nm	1/5000	-
Anti-Mouse	Odissey 800 nm	1/5000	-
Anti-Rabbit	TrueBlot	1/1000	-
Anti-Rabbit	Cy3-labeled	-	1/500
Anti-Rabbit	Texas Red-labeled	-	1/300
Anti-Mouse	Cy3	-	1/500
Anti-Mouse	Alexa 488-labeled	-	1/300-1/500

III. 4. Primers

Primers for GAPDH, β-Actin, HPRT, PPP1CC and TCTEX1D4 were retrieved from the literature or designed using the Universal ProbeLibrary (Roche) or the Primer-Blast tool from NCBI, following the instructions required to design primers suitable for qPCR, including the fact that primers should span exon-exon junctions. Primers were synthesized at Eurofins MWG Operon. More information regarding is present in the Appendix II.

III. 5. Western Blotting

Sample preparation

The cell medium was removed; cells were washed with PBS and samples collected by adding boiling 1% SDS and gentle scrapping. The lysates were collected to microtubes and then boiled for 10 minutes and sonicated for 20 seconds.

BCA protein assay

The protein content of the samples was determined using BCA protein assay (Pierce). The standards for quantification were prepared as described in the table 14.

Table 14 - BCA Standards. BSA, bovine serum albumin; WR, Working reagent (to prepare WR 50 pars of BCA reagent A was mixed with 1 part of reagent B).

Standards	BSA (µl)	1%SDS (µl)	Protein mass (µg)	WR (ml)
P0	-	50	0	1
P1	1	49	2	1
P2	2	48	4	1
P3	5	45	10	1
P4	10	40	20	1
P5	20	30	40	1

Reactions were initiated by adding 1 ml of WR to 50 µL of each sample. The standards and samples were incubated at 37ºC for 30 minutes. The absorbance was then measured at 562 nm and a standard curve was prepared by plotting the value for each BSA standard against its concentration. Using this curve the protein concentration of each sample was determined.

SDS -PAGE

Samples (usually corresponding to 100 µg of protein) were subjected to SDS polyacrylamide gel electrophoresis (SDS-PAGE). Samples were prepared and 4x loading buffer added. Proteins were then resolved on a SDS-PAGE of variable percentage, most suitable to each case. Gels were run at 200V for approximately 1 hour.

Immunoblotting

After electrophoresis, proteins were transferred onto a solid support, while keeping their positions and then visualized with specific antibodies. Proteins were transferred to nitrocellulose membranes at 200 mA for circa 2 hours.

Immunoblotting of the transferred proteins was performed by initially blocking any possible non-specific binding of the primary antibody by immersing the membrane in 5% non-fat milk in TBS-T (blocking solution) for nearly 1 hour with shaking. Then, membranes were incubated with primary antibody (diluted in 3% non-fat milk, in TBS-T) and left for 1 hour to overnight depending on the antibody being used.

After removing the primary antibody, the membranes were washed three times, 10 minutes each, in TBS-T and the primary antibody was detected with a solution of the appropriate secondary antibody, diluted in 3% non-fat milk, in TBS-T for 1 hour with shaking. Membranes were again washed three times, for 10 minutes and the secondary antibody detected using either a chemiluminescence detection system (ECL; Amersham Pharmacia Biotech) or using the Odyssey detection System, accordingly to the secondary antibody used. In the first case, excess solution was drained by touching the edge of the membranes against tissue paper and the membrane was gently wrapped with cling-film, eliminating all air bubbles. In the dark room, the membrane was placed in a film cassette and an autoradiography film was placed on the top. The cassette was closed and the blot exposed over a certain period of time. The film was then removed and developed in a developing solution, washed in water and fixed in fixation solution. In the situations in which Odyssey detection system was used, membranes were scanned at 680 or 800 nm wavelength of excitation according to the secondary antibody used.

III. 6. Immunoprecipitation and Co-Immunoprecipitation

Sample Preparation

Usually two confluent 100 mm Petri dishes for each cell line were used. Cells were washed with PBS and samples lysed with RIPA lysis buffer (Millipore) containing protease inhibitors. The retrieving of the cells was done with a scrapper to a microtube, immediately put on ice. Samples were sonicated three times for 10 seconds and centrifuged at 16 000 g at 4ºC during 15 minutes. The pellet was re-suspended in SDS 1% becoming the Pellet Fraction. A small fraction of the supernatant was retrieved to a microtube originating the Soluble Fraction. The remaining supernatant was used in the following steps.

Pre-Clearence

The storage solution was removed from 20 µl of re-suspended dynabeads (Protein G Dynabeads, from Invitrogen) which were then washed 3 times with 500 µl of PBS. These were incubated 1 hour with rotation at 4ºC. After that, dynabeads were separated from the sample using a magnet (Dynal MPC). Dynabeads were re-suspended in SDS 1% thus forming the Dynabeads Bound Fraction. A small fraction of the supernatant was retrieved and labeled as Dynabeads Unbound Fraction. The remaining was used in the subsequent steps.

Precipitation

Samples from pre-clearance were incubated with primary antibody (3-5 µg) overnight with rotation at 4ºC. Re-suspended and washed dynabeads were used to incubate with the samples for 1h30 with rotation at 4ºC. An Unbound Immunoprecipitation Fraction was then retrieved upon dynabeads capture with the magnet. The remaining of the supernatant was discarded. Dynabeads were washed three times. Firstly, 100 µl of PBS was used and an aliquot was kept (Wash Fraction). The other 2 washing steps were carried out with 500 µl of PBS. The beads were then re-suspended in SDS 1% and boiled for 5 minutes, generating the Immunoprecipitation Fraction.

Fractions Preparation

Loading buffer 4x was added to all samples. Fractions were then boiled for 10 minutes.

SDS -PAGE

Samples were subjected to SDS polyacrylamide gel electrophoresis (SDS-PAGE) as previously described.

Immunoblotting

Samples were then immunoblotted as previously described. Notice that the difference between Immunoprecipitation and Co-Immunoprecipitation only occurs at this point. In the first, the primary antibody is the same (or against the same protein) which was used in the precipitation step. In the case of a Co-Immunoprecipitation the primary antibody used is against other protein, expected to interact and be bound to the one precipitated.

III. 7. Immunocytochemistry

Sample Preparation

A suitable number of cells were seeded into 6- or 12-well plates. Occasionally, a previous treatment of the coverslips was performed by briefly rinsing them with poly-L-ornithine (0.1 mg/ml, Invitrogen) in order to increase adherence. Cells are then left to rest until they are adherent to the coverslips, at least 24h.

Immunostaining

Afterwards, medium was removed and cells were gently washed with PBS. Cells were then fixed by adding paraformaldehyde 4% for 15 minutes. Cells were then treated with 0.2% Triton X-100 in PBS for 15 minutes. Cells were then blocked with a blocking solution, either a 3% solution of BSA in PBS or Immunoblock (Roth). Cells were then incubated with the primary antibody diluted in the blocking solution during 1 hour in a dark and wet chamber. Cells were then washed three times with PBS, followed by incubation with the secondary antibody, under the same conditions as the ones used for the primary antibody. DAPI is usually added to the secondary antibody dilution, however occasionally it was also used mounting medium with nuclear staining compound. After cells were washed three times with PBS the coverslips were mounted in the slides using a fluorescence mounting medium (either Mowiol, VectaShield or Dako were used). Slides were stored protected from the light at 4º C.

Image acquisition

Images were acquired using either fluorescence or confocal microscopes, with the assistance of Dr. Luís Korrodi, Dr. Georg Lüers and Dr. Ann-Kristin Brauns. The

devices used were a Olympus IX-81 inverted epifluorescence microscope and a Nikon A1 Confocal Laser Microscope.

III. 8. Immunohistochemistry

Sample Preparation

Three types of tissue samples were used: a) tumors generated in nude mice, via implantation of human cancer cell lines and further retrieval of the samples; b) normal placenta tissue previously prepared; c) fresh samples of normal tissues, namely from placenta and prostate. The first two types of samples were kindly provided by Dr. Tobias Lunge at the University Medical Center of Hamburg-Eppendorf. Fresh samples were treated by 3 different methods: a) flash frozen in liquid nitrogen; b) briefly treated with a 4% solution of paraformaldehyde in PBS, followed by paraffin embedding and c) treatment with a 4% solution of paraformaldehyde in PBS, followed by a prolonged sucrose gradient treatment. In this treatment, samples were submerged in a 10% sucrose solution for 1 hour, another hour in a 15% sucrose solution and then samples are left overnight at 4ºC in a 30% sucrose solution. Afterwards, samples were submersed in cold 2-methylbuthane and transferred into liquid nitrogen. Cryosections (5 µm) of the cryopreserved samples were prepared using Leica CM 3050 into Superfrost Plus (Hecht Assistant) slides using TissueTek (Sakura) as embedding material. Samples were stored at -20ºC or at -80ºC (long-term storage). The paraffin-embedded samples used were already sectioned.

Immunostaining

Firstly, in paraffin-embedded tissues paraffin was removed using the standard protocol of deparaffinization in a Varistain 24-4 (Thermo) device. On the other hand, cryopreserved samples were initially treated for 30 minutes with 0.2% TBS-TT. The next steps took place regardless of the type of sample and all incubation steps take place in a dark and wet-chamber, in order to avoid drying of the samples.

Tissues were blocked for 30 minutes with Immunoblock, after which were incubated with the primary antibody diluted in Immunoblock for 1 hour. Afterwards, samples were quickly washed twice with TBS-T and once with TBS, followed by 1 hour of incubation with the secondary antibody, diluted in the Immunoblock and with DAPI added. After another similar washing step, samples were mounted with Mowiol or Dako fluorescence mounting medium and stored in a cold and dark room.

Image acquisition

The devices used were an Olympus IX-81 inverted epifluorescence microscope and a Nikon A1 Confocal Laser Microscope with the assistance of Dr. Luís Korrodi, Dr. Georg Lüers and Dr. Ann-Kristin Brauns.

III. 9. RT-qPCR

Sample preparation

RNA samples from DU 145, LNCaP and PC3 cell lines were kindly provided by Dr. Tobias Lunge. Prostate and Placenta RNA samples were extracted from fresh flash-frozen samples.

RNA extraction

RNA was extracted from prostate and placenta tissues using the RNeasy Midi Kit (QIAGEN), following the instructions provided. Briefly, a small amount of the tissue (up to 250 mg) was kept frozen in liquid nitrogen and disrupted mechanically with a mortar and pestle followed by addition of a lysis buffer. After homogenization of the sample, a series of centrifugation steps through the RNeasy Midi Column using supplied buffers steps takes places and lastly the RNA is retrieved from the column centrifuging with RNase-free water. Long-term storage at-80 ºC.

Reverse transcription

The quality and concentration of the RNA samples from DU 145, LNCaP, PC3, Prostate and Placenta were assessed using the Nanodrop ND-1000 spectrophotomer device. Following that, reverse transcription was performed using RT2 First Strand Kit (QIAGEN) according to the instructions provided. Briefly, 1µg of RNA was used for the procedure and genomic DNA elimination buffer was used to increase the purity of the cDNA retrieved. Afterwards, a reverse transcription cocktail was prepared and incubated with the RNA during 15 minutes, at which point the reaction was terminated and nuclease-free water was added. Characterization of the cDNA in terms of concentration and purity was then performed using the Nanodrop ND-1000 device and samples were stored in -20ºC.

qPCR

A 96-well plate was used to sample the diverse components. A suitable amount of the forward and reverse primers (both between 0.2-0.6 µl of the 0.1 µmole solution) and cDNA (circa 150 ng) are mixed with 5µl of LCMM and qPCR-grade water is added to a final volume of 10 µl. Usually blanks for each primer pair (without the cDNA) and totally empty wells were included. The qPCR reaction and

quantification took place in the Roche LightCycler 480. The general protocol was as it follows: 1 – 1 cycle of 5 minutes of pre-incubation at 95º; 2 – 45 cycles of amplification (10 seconds at 95ºC), annealing (10 seconds at 60ºC) and extension (10 seconds at 72ºC); 3 – Melting curves.

Statistical analysis

Results were analyzed using the Relative Expression Software Tool – Multiple Condition Solver – version 2, which calculates the relative expression in qPCR using a pair-wise fixed reallocation randomization test.

III. 10. Mass Spectrometry

Immunoprecipitation was performed as previously described. IP fractions were then run through a 15% SDS-PAGE at 200V for circa 1 hour. Afterwards, the gel was fixed during 30 minutes using Fixing Solution and then washed with water. Then, the gel was stained for 30 minutes with Coomassie Blue Staining Solution upon which the gel was quickly washed with water and then submerged into Distaining Solution for 30 minutes. Distaining Solution is then renewed and gel is let to detain overnight, with renewal of the Distaining Solution being performed if necessary. All these steps take place with agitation.

Afterwards, the region of interest of the IP fraction was removed using a proper sterile cutting device and the sample retrieved was stored in -80ºC. Samples were then sent to Dr. Thorsten Muller at the Functional Proteomics Department (Ruhr-University Bochum) to be analyzed by liquid chromatography-electrospray tandem mass spectrometry.

IV. Results and Discussion

IV. 1. Confirm the existence of TCTEX1D4 in Prostate Cell Lines

IV. 1. 1. A Protein Level Approach

Preliminary results in our laboratory have suggested the presence of TCTEX1D4 in PCa cell lines, namely PC3 and LNCaP, using antibodies for TCTEX1D4 C- and N-terminus separately (Figure 8). In all cases, 100 µg of proteins from cell extracts were run in a 12% SDS-PAGE, immunoblotting with the respective primary antibodies was executed and detection was performed with Odissey 680 nm anti-rabbit antibodies. For a positive control, a bacterial extract of TCTEX1D4-expressing vector was used.

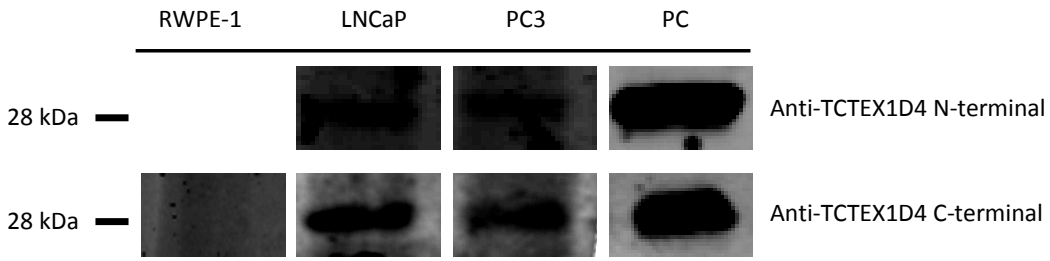

Figure 8 - TCTEX1D4 detection in prostate cancer cell lines LNCaP and PC3 and in immortalized normal prostate epithelial cells RWPE-1. PC - Positive Control

As depicted in Figure 8, a positive signal was detected in both cancer cell lines (PC3 and LNCaP) using both antibodies. Nevertheless, the signal is weak, particularly using the N-terminal TCTEX1D4 antibody which may indicate low expression. The bands are present in the expected area, since they appear slightly above the 25 kDa marker. Even though the predicted molecular weight for TCTEX1D4 is 23.4 kDa, it may appear between 24.9 and 28.4 kDa due to possible post-translational events such as phosphorylation and glycosylation, as previously reported by Korrodi-Gregório *et al [179]*.

Also depicted in Figure 8 is the result of TCTEX1D4 detection in RWPE-1 cells using the antibody against the C-terminal of TCTEX1D4. Unlike in the cases of the PCa cell lines, no signal was detected. This suggests that TCTEX1D4 protein levels in normal epithelial cells are even lower, leading to the speculation that this protein may be upregulated in PCa cells.

However, in order to confirm the presence of TCTEX1D4 in all cell lines, IP assays were performed to purify the sample and increase the concentration of the protein, thus expecting to provide a better signal.

Initially, IP with 5 µg of N-terminal TCTEX1D4 antibody was carried out. IP fractions were run through a 15% SDS-PAGE, immunoblotting was performed with anti-TCTEX1D4 (N-terminal) antibody (1:1000) and detection was achieved with Odissey 680 nm anti-rabbit antibody (1:1000). Results (Figure 9A) indicate the presence of TCTEX1D4 in both cell lines tested.

IP of TCTEX1D4 with 5µg of C-terminal TCTEX1D4 antibody also took place, followed by immunoblotting with the same antibody (1:1000). A TrueBlot secondary anti-rabbit antibody was used and detected using ECL. TrueBlot secondary antibodies mask the signal produced by immunoglobulins which are commonly detected in western blotting preceded by immunoprecipitation. Since TCTEX1D4 molecular weight is similar to the molecular weight of immunoglobulin light chains (25 kDa), it was important to use such strategy to assure that signal was obtained from TCTEX1D4. Results are presented in Figure 9B.

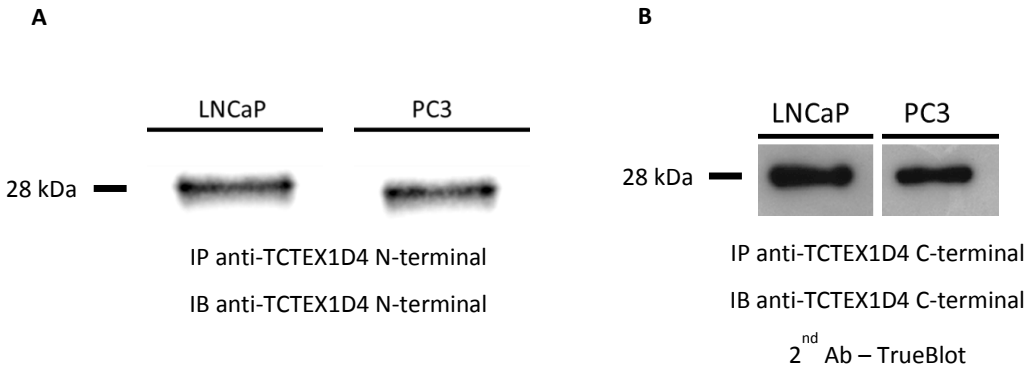

Figure 9 - Detection of TCTEX1D4 in LNCaP and PC3 cell lines upon immunoprecipitation using N-terminal and C-terminal antibodies in A and B, respectively.

Afterwards, a new detection of TCTEX1D4 protein was performed using IP with C-terminal antibody in order to evaluate the presence of TCTEX1D4 in RWPE-1 cells, in which signal was not detected in a standard western blotting procedure (Figure 8).

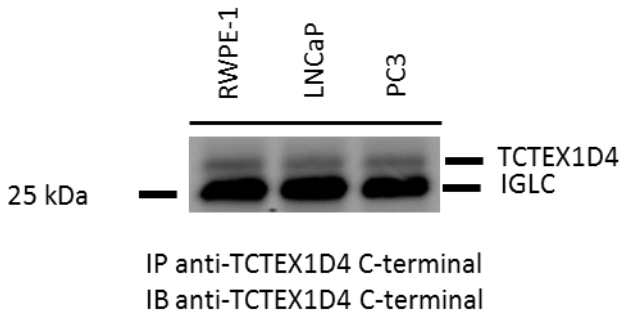

Figure 10 - Presence of TCTEX1D4 in RWPE-1, LNCaP and PC3 cell lines upon immunoprecipitation of TCTEX1D4 using C-terminal TCTEX1D4 antibody. IGLC – Immunoglobulin light chain

The results (Figure 10) after IP seem to indicate the existence of TCTEX1D4 in RWPE-1, LNCaP and PC3 cells. However, since no signal was detected in RWPE-1 cell line in a normal Western Blot (Figure 8), that may indicate that TCTEX1D4 protein levels in RWPE-1 cells are not sufficient for detection with that technique and only after IP it is detectable. Moreover, since it is readily detected in PCa cell lines prior to IP, this may indicate overexpression of TCTEX1D4 at the protein level in malignant cells.

The conjugation of the different results clearly demonstrates the existence of TCTEX1D4 at the protein level in all cell lines. Until now, TCTEX1D4 protein has never been described as present in prostate, either in normal or in pathological states. Nevertheless, TCTEX1D4 appears to be present at low levels and results seem to indicate that overexpression of TCTEX1D4 protein may occur in PCa cells.

IV. 1. 2. A mRNA level approach

Firstly, in order to optimize the relative quantification of TCTEX1D4 and PPP1CC mRNA using qPCR, a series of preliminary experiments took place with the following objectives:

a) Assess the quality of the available cDNA and decide the amount required to be used in the procedure, via the analysis of the results of the housekeeping genes;
b) Determine the possibility of formation of primer dimers;
c) Evaluate the quality of the primers, via the analysis of the obtained cp values and melting temperatures;
d) Optimize the qPCR conditions for each primer, performing qPCR experiments at different annealing temperatures, namely 58º, 60º and 64ºC
e) Evaluate the possibility of utilization of placenta cDNA as a positive control for TCTEX1D4.

After this optimization step was finished, some conclusions were drawn that helped design the subsequent experiments:

a) The quality of the cDNA was good and best results were achieved using 0.6 µl of cDNA (circa 150 ng);
b) Some primer pairs were excluded due to formation of primer dimers, low-performance or presence of multiple melting temperatures, namely GAPDH, GAPDH-001, b-actin_hs, PAPTEIE, PAPTT57, PAPTGCH and TCTEX1D4-201. Regarding the remaining, GAPDH_hs, PPP1CC96, TCTEX1D4-001 and TCTEX1D4_hs2 were considered to be the optimal primer pairs to use;
c) 45 annealing cycles at 60ºC conditions were chosen for qPCR;
d) Placenta cDNA was shown to be a suitable positive control.

TCTEX1D4 was detected in all situations and the ratio GAPDH/TCTEX1D4 seems to indicate a low expression of TCTEX1D4 even in the positive control (placenta), since the ratio GAPDH/TCTEX1D4 was 0.8. Since TCTEX1D4 expression is highly downregulated in the prostatic samples (vs. placenta), all data indicates a very low expression of TCTEX1D4 in the prostatic samples, coherent with the low expression detected at the protein level. Results are presented in Table 15 and Figures 11 and 12.

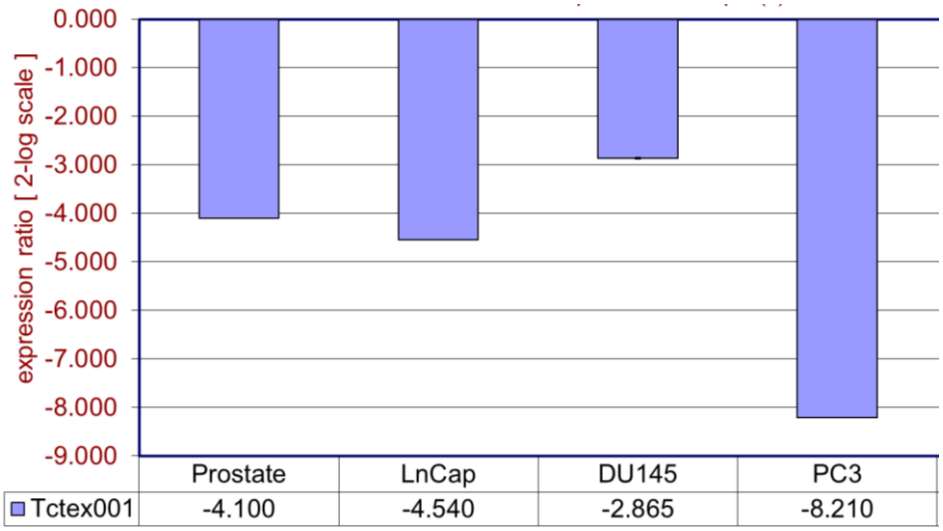

Figure 11 – Expression ratios for all the samples tested taking Placenta as positive control

The results indicate that TCTEX1D4 mRNA is present in all cell lines tested, as well as in the normal prostate tissue, although at very low levels. Such results are also a breakthrough since mammal EST analysis profiles for TCTEX1D4 available at UniGene do not contemplate TCTEX1D4 as being expressed at the mRNA level in prostate tissues.

IV. 1. 3. Assessing differences of TCTEX1D4 and PPP1CC expression

The REST tool was used to statistically analyze the results of qPCR. Figure 12 illustrates de expression ratios of TCTEX1D4 and PPP1CC between the cell samples analyzed vs. normal prostate sample.

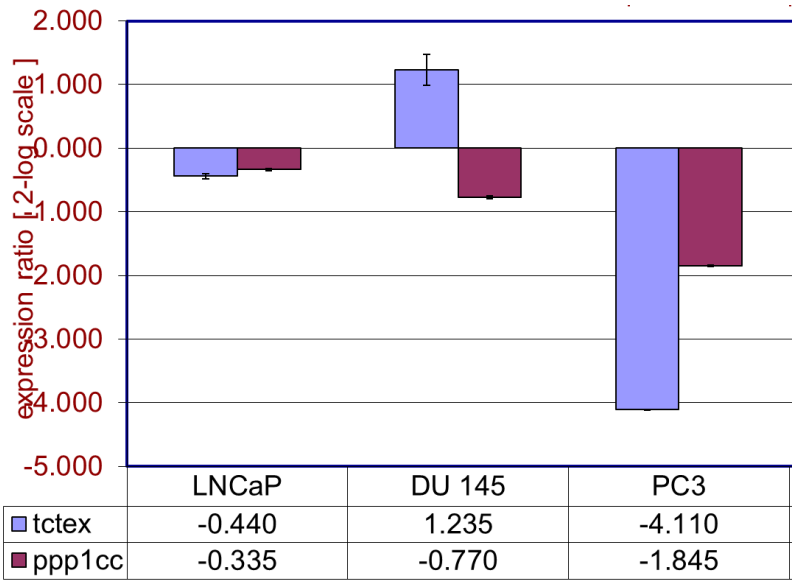

Figure 12 - Expression ratio of TCTEX1D4 and PPP1CC in LNCaP, DU 145 and PC3 cell, with prostate as control. Blue bar – TCTEX1D4 and Red bar – PPP1CC

In Table 15 are listed the expression ratios for each case. Results over 1.5-fold or below 0.75-fold are presented in bold and represent situations of intense upregulation or downregulation, respectively. The results seem to indicate a progressive downregulation of PPP1CC parallel to PCa progression. Concerning to TCTEX1D4 results, it appears that in both PC3 and LNCaP cell lines its expression is downregulated, whilst in DU 145 it is intensively upregulated. Since results at the protein level point towards overexpression in PC3 and LNCaP cell lines vs. RWPE-1, it may indicate a strong regulation of TCTEX1D4 levels in the post-translational level or in its catalytic rate. Proteins involved in signal transduction are often tightly regulated and its expression levels are commonly largely altered upon stimulation.

Table 15 – Expression ratios of TCTEX1D4 and PPP1CC between the tested samples and the normal prostate

Target	LNCaP	DU 145	PC3
TCTEX1D4	**0,737**	**2,354**	**0,058**
PPP1CC	0,793	**0,586**	**0,278**

Nevertheless, it is important to highlight two aspects which may introduce some difficulties in understanding the results: 1) the protein levels of both TCTEX1D4 and PPP1CC in DU 145 were never assessed during this work and, thereof, it is not possible to evaluate if the mRNA levels are coherent with the protein levels in this cell line; 2) Expression ratios were calculated vs. cDNA extracted from normal prostate tissue and not from isolated epithelial cells. Therefore, it may not be the most suitable control.

IV. 2. Confirm the existence of TCTEX1D4-PPP1 complex and evaluate the possible existence of other TCTEX1D4 complexes

The confirmation of existence of TCTEX1D4 in the cell lines created the need to evaluate TCTEX1D4 interactions in these cell lines. With only two articles published concerning this protein possible functions, the interactions already described are with PPP1 (in sperm) and with receptors of TGFβ signaling pathway, namely Endoglin (upon transfection in HEK293 cells). Thereof, it was of great interest to evaluate if such interactions are still present in prostate cell lines and, if possible, clarify such interactions and identify new ones. In order to do so, Co-IP and Mass Spectrometry techniques were applied.

IV. 2. 1. A Co-IP-based approach

TCTEX1D4-PPP1

TCTEX1D4 has been described as a PPP1 Interacting Protein (PIP). This interaction was firstly identified in a testis yeast two-hybrid and later confirmed by yeast co-transformation and co-immunoprecipitation in transfected cells. One of the hallmarks of interactions with PPP1 is the need for a physical binding between PPP1 and the PIP. Therefore, one of the strategies to detect PIPs is via Co-Immunoprecipitation assays. Henceforth, it was proposed to use co-immunoprecipitation in the available cell lines in order to confirm the referred interaction.

PPP1 is a ubiquitously expressed protein. Nevertheless, a confirmation of the existence of PPP1 in the samples has been carried out. Primarily, a Western Blot to detect both alpha and gamma isoforms of PPP1 was performed with the results being displayed in Figure 13. In this case, primary antibodies against PPP1CA and PPP1CC were used to detect their respective targets. 100 µg of protein sample of the cell extracts were run through a 12% SDS-PAGE.

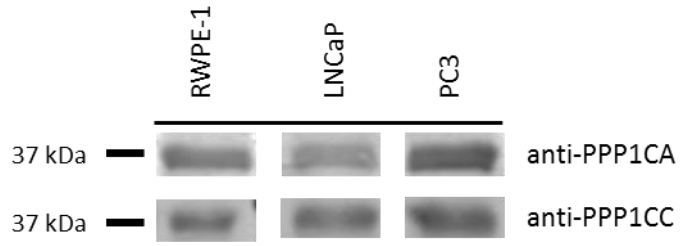

Figure 13 - PPP1CA and PPP1CC detection on RWPE-1, LNCaP and PC3 cell lines

As depicted in Figure 13, both isoforms were detected in all cell lines.

In order to confirm the possible interaction, TCTEX1D4 was precipitated using 5 µl of anti-C-terminal of TCTEX1D4 antibody, using as samples LNCaP and PC3 cells, in which TCTEX1D4 levels are proposed to be higher. Immunoblotting with the PPP1CA antibody was performed and detection took place using the Odissey 680 nm anti-rabbit antibody. Results are presented in the Figure 14A.

The absence of a band at 37 kDa (the molecular weight of PPP1) seems to indicate that no interaction was detected in PC3 cells. On the other hand, there appears to be a weak signal in the IP fraction of the LNCaP cells, which may indicate the presence of a complex between PPP1CA and TCTEX1D4 in that particular cell line.

Nonetheless, IP of PPP1CA using 3 µg of antibody anti-PPP1CA was performed followed by immunoblotting with antibody against C-terminal of TCTEX1D4 (Figure 14B). A double band at 25 kDa is visible, particularly perceptible in LNCaP cells, consistent with the idea of an interaction. Furthermore, the fact that this reversed approach to the complex granted much more clear results, may mean that the antibody for C-terminal of TCTEX1D4 is not particularly suitable for IP procedures.

Naturally, the next step was to verify the presence of a PPP1CC-TCTEX1D4 complex. In order to do so, IP of TCTEX1D4 was performed. Immunoblotting with anti-PPP1CC (1:5000) took then place and the results are presented in Figure 14C. The results may indicate the existence of PPP1CC in the IP fraction of LNCaP, despite the weakness of the signal obtained, which points towards the presence of interaction between PPP1CC and TCTEX1D4. Signal is absent in the IP fraction of PC3 cells. Once again, such results may be due to poor performance of the referred TCTEX1D4 C-terminal antibody in IP experiments.

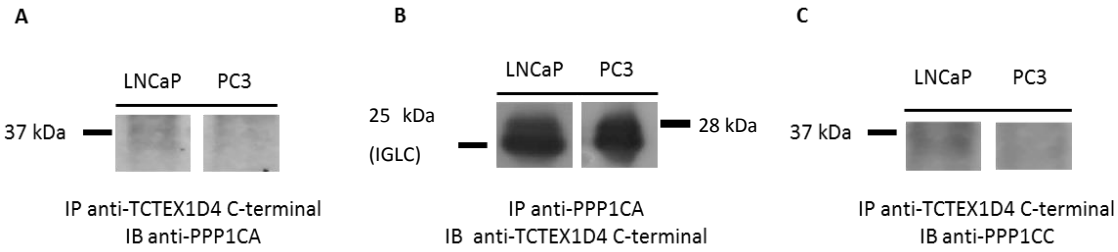

Figure 14 - Results of the Co-IPs performed to evaluate the possibility of existence of a TCTEX1D4-PPP1 complex.

TCTEX1D4-Endoglin

TCTEX1D4 has been found to interact with several members of the TGFβ-signaling pathway. Of particular relevance, it was found to associate with Endoglin, Betaglycan, ActRIIA and TβRII, all of which are commonly downregulated in cases of PCa. Therefore, since these cell lines represent prostate cancer cells, it was interesting to assess the possibility of an interaction between TCTEX1D4 and Endoglin. Although it is described as an endothelial co-receptor for TGFβ, intense alterations in Endoglin function have been described in PCa and therefore the possibility of existence of such interaction was evaluated. In order to do so, an IP of TCTEX1D4 using anti-TTCTEX1D4 C-terminal antibody was performed following the procedure already described. Afterwards, immunoblotting using antibody against Endoglin was performed. The results are depicted in Figure 15A.

Even though some signal appears in the 50 kDa area, which may correspond to the S-Endoglin form, it is way too weak. Therefore, the results do not clearly demonstrate the previously described interaction between Endoglin and TCTEX1D4. Nevertheless, poor performance of this antibody in IP may account for the absence of results. Moreover, the interaction may be indirect.

PPP1-Endoglin

Since Endoglin has been shown to bind to TCTEX1D4 and TCTEX1D4 is a PIP, it was proposed that it is the complex PPP1-TCTEX1D4 that binds to Endoglin. Therefore, it was decided to try to find evidences concerning the possibility of existence of a binding between PPP1 and Endoglin, thus forming a putative trimeric complex. In order to do so, IPs of PPP1CA and PPP1CC were prepared using 3 µg of the respective antibodies using as samples 2 Petri dishes of 100 mm of PC3 and LNCaP cells. Immunoblotting was then carried out using antibody against Endoglin which

was detected using 800 nm anti-mouse Odissey antibo dy. Results are presented in Figure 13B.

Once again, signal appears in the 55 kDa area, which may correspond to the S-Endoglin form. In this case, however, the signal is much stronger that in the previous one, thus pointing towards an interaction between both PPP1 isoforms and Endoglin in the cell lines tested.

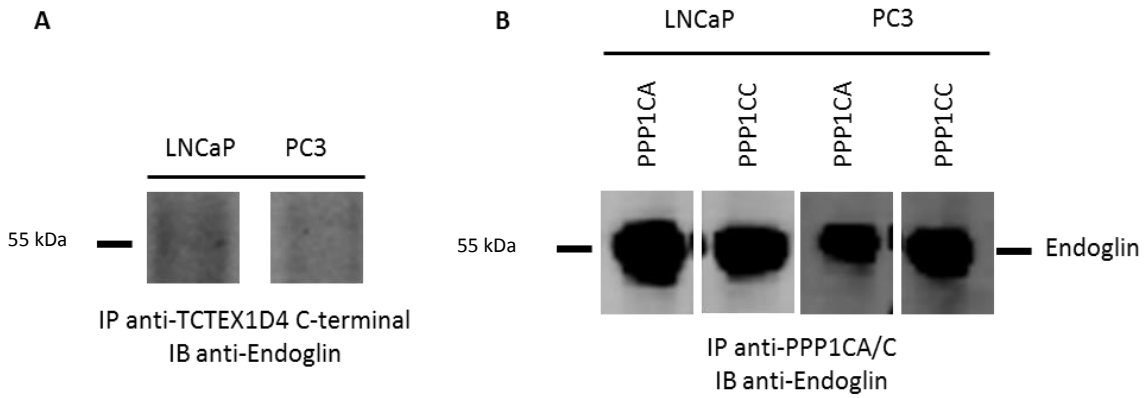

Figure 15 – Co-IP assays performed to evaluate the possibility of existence of Endoglin-TCTEX1D4 and Endoglin-PPP1CC complexes in LNCaP and PC3 cells

The results so far seem to strongly indicate an interaction between PPP1 and TCTEX1D4 and between PPP1 and Endoglin. The results do not clearly indicate a direct interaction between TCTEX1D4 and Endoglin, thus we raised the possibility of the existence of a trimeric complex of TCTEX1D4-PPP1-Endoglin, as depicted in Figure 16.

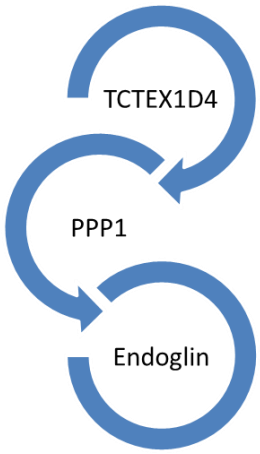

Figure 16 - Proposed trimeric complex of TCTEX1D4-PPP1-Endoglin

IV. 2. 2. A Mass Spectrometry-based approach

In order to identify the interaction between TCTEX1D4 and PPP1 a Mass Spectrometry analysis was performed. TCTEX1D4 IP using the C-terminal antibody was carried out. Extracts from PC3, LNCaP and RWPE-1 cells were used and the area of the IP fraction that was retrieved was between 25 and 40 kDa (Figure 17) to include not only the TCTEX1D4 but also the PPP1 region. Other possible interactors may also be identified using this technique

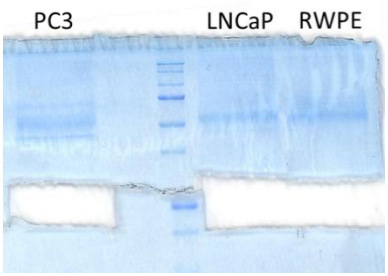

Figure 17 - Regions of the IP fractions of TCTEX1D4 sent to MS analysis

Regarding this subject, we are still currently awaiting the arrival of the results, which may not only confirm the proposed interaction between PPP1 and TCTEX1D4 but also identify other unpredicted complexes.

IV. 3. Localize TCTEX1D4 at the cellular and histological level

TCTEX1D4 localization in human sperm cells and upon transfection was previously described. Nonetheless, no studies regarding native TCTEX1D4 localization in human prostate cells were already published. Therefore it was of great interest to localize TCTEX1D4 in the normal and malignant prostate cells. In all cases, antibody against the C-terminal of TCTEX1D4 was used.

IV. 3. 1. TCTEX1D4 in normal and malignant prostate cells

Normal Cells - RWPE-1

RWPE-1 cells are commonly used to represent normal prostate epithelial cells, immortalized upon transfection of HPV-18. In these cells, TCTEX1D4 is present throughout the cytoplasm and nucleus, in a dispersed fine punctuate pattern (Figure 18).

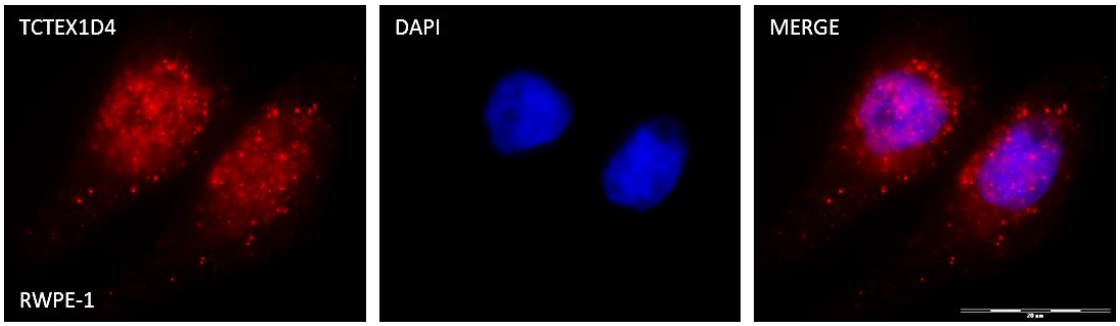

Figure 18 - Localization of TCTEX1D4 in RWPE-1 cells

Malignant cells – LNCaP and PC3

The androgen-dependent LNCaP cells represent moderately aggressive PCa cells. In this cell line, TCTEX1D4 localization (Figure 19) is delimited within the cytoplasm to a polarized and very restricted area in which large dots are visible producing a very intense signal. This may localize TCTEX1D4 to aggregates, vesicles or some organelle. The presence of signal in the nucleus is rather scarce (19B).

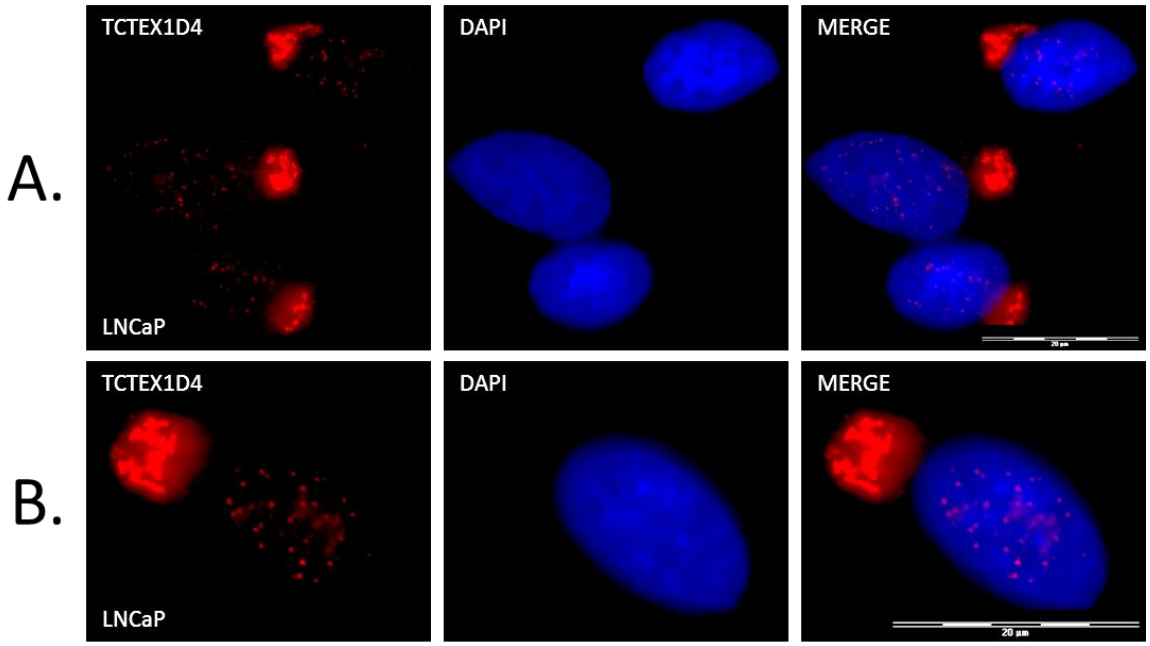

Figure 19 - Localization of TCTEX1D4 in LNCaP cells

PC3

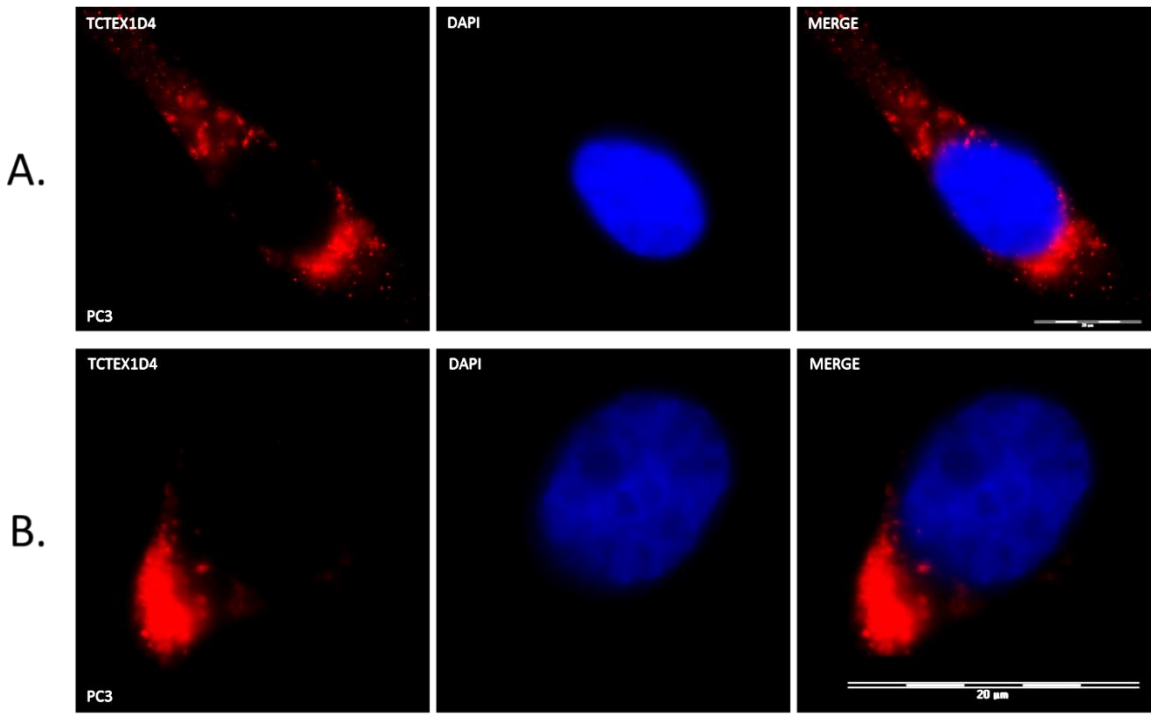

Figure 20 - Localization of TCTEX1D4 in PC3 cells

The androgen-independent PC3 cells represent highly aggressive PCa cells. In these cells, TCTEX1D4 was localized in the cytoplasm and almost completely absent from the nucleus. As depicted in Figure 20, the localization of TCTEX1D4 in this cell line appears to be less restricted than in LNCaP cells, but still much more localized if compared with normal cells. The pattern produced was of a number of medium-

sized dots which may also be restricted or correspond to some sort of vesicles, organelles or aggregates. In some cells in particular (20B), TCTEX1D4 was localized in a very restricted area in the vicinity of the nucleus, in a polarized manner, more similar to the pattern observed in LNCaP cells. As observed in LNCaP cells, the signal produced appeared to be more intense than the one present in RWPE-1 cells.

Korrodi-Gregório *et al. s*tudies [179] have localized TCTEX1D4 along the flagellum, in the region of the mitochondria and in the acrosome of sperm cells. Upon transfection, it was localized across the cytoplasm, in the microtubules and the MTOC of GC1-spg and COS-7 cells. Previously, Meng *et al.* [178] localized TCTEX1D4 in vesicles, microtubules and nucleus, upon transfection in HeLa cells.

Regarding TCTEX1D4 localization in RWPE-1, LNCaP and PC3 cells, the results seem to indicate a rather interesting difference in the pattern of its subcellular localization between normal and malignant cells (Table 16). Taking into account the limited published information regarding such matter, it may be suitable to speculate if in RWPE-1 cells the TCTEX1D4 observed throughout the cytoplasm is associated with microtubules and if the more restrictedly localized signals observed in PCa cells correspond to vesicles.

Other aspect that may be of interest is the fact that the TCTEX1D4 pattern in PC3 cells appears to be somewhat between the ones observed for LNCaP and RWPE-1 cells, despite the fact that PC3 represents a more aggressive PCa cell line than LNCaP. One factor that may contribute to elucidate this is that LNCaP cells, unlike PC3 cells, are often reported to be TGFβ-insensitive and absence of TGFβRs in LNCaP cells is commonly found. Therefore, since in LNCaP cells profound alterations of TGFβ signaling are present, and being TCTEX1D4 a proposed element of this signaling pathway, a more extended disruption of TCTEX1D4 localization or function in this cell line may be expected.

Table 16 - Differences in localization of TCTEX1D4 between the cell lines tested. "+" - abundant, "-" - scarce and "0" - absent

Signal of TCTEX1D4	RWPE-1	LNCaP	PC3
Cytoplasm	Throughout the cytoplasm	Very restricted and polarized	Restricted
Nucleus	+	-	0
Pattern	Fine punctuate	Large dots	Medium-sized dots
Intensity	Weaker	Strongest	Stronger

IV. 3. 2. TCTEX1D4 in normal prostate and in prostate tumors

In order to identify TCTEX1D4 in prostate tumor samples, a first round of immunohistochemistry was performed. As samples, tissue section from tumors generated in nude mice upon implantation of LNCaP, PC3, LuCaP 23.1 and DU 145 prostate tumor cells were used. These sections were paraformaldehyde-fixed and paraffin-embedded and were kindly provided by Dr. Tobias Lunge. Both antibodies against C- and N-Terminus of TCTEX1D4 were used and a range of dilutions (from 1:50 to 1:1000) was tested. Unfortunately, no positive results were found in any case (data not shown). In order to address this issue, optimization of the methodology was necessary and a key aspect noticed was the absence of a proper positive control. Since TCTEX1D4 localization in human tissues was never described before, a search for a positive control was undertaken in order to assess if the absence of results was due to lack of protein or due to some methodological problem.

TCTEX1D4 in Placenta – a positive control

A NCBI EST database analysis for TCTEX1D4 mRNA in mammals has found hits in female reproductive tract (ovary, oviduct, placenta, uterus and embryonic tissues), head-related tissues (such as brain and tongue) and lung. Thereof, the following tissues were initially tested as possible positive controls:

a) Normal lung
b) Tumors derived from the implantation of OH3 and H69 cancer cells in nude mice (small cell lung carcinoma cell lines)
c) Placenta

All the samples used so far had been fixed with paraformaldehyde and paraffin-embedded. Since once again the results were negative for all the tested samples, it was raised the possibility that was the treatment of the samples that constituted a problem that needed to be circumvented. It was also suggested that the use of cryopreserved samples may be more suitable, due to the fact that epitopes may be more accessible to the primary antibody.

Thusly, fresh placenta samples were collected and cryopreserved with either a previous brief fixation with paraformaldehyde followed by a sucrose gradient or flash frozen without any previous treatment. Immunhistochemistry experiments were performed, using antibody against the C-terminal of TCTEX1D4 as primary antibody (1:150 and 1:300 dilutions) and the secondary antibody used was anti-rabbit Cy3. Samples cryopreserved without fixation were defined as negative, but a

distinct positive signal was found in the fixed and then cryopreserved samples. A strong signal was found to be present in cells of the villi of the placenta (which may be compatible of DLC function of TCTEX1D4), particularly in the ones of central localization (Figure 21). The signal was of a non-nuclear fine punctuate pattern. From this point on, placenta has been regarded as a positive control for TCTEX1D4 at the tissue level.

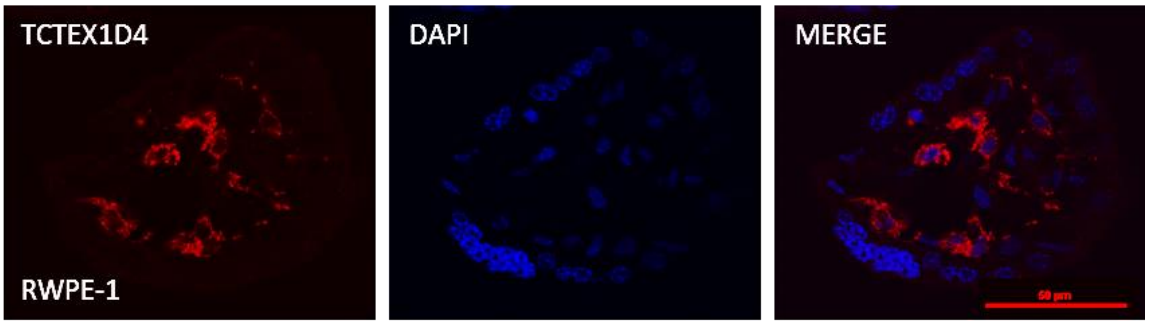

Figure 21 - TCTEX1D4 localization in the placenta tissue

TCTEX1D4 in Normal Prostate

Prostate samples were subjected to the same treatment that has revealed to be suitable for the placenta and the immunohistochemistry was performed under the same conditions. The results are presented in Figure 22. Positive signals were found and, like as observed in placenta, signals appeared as a non-nuclear fine punctuate pattern. However, it was not as intense or as common as observed in the placenta. Nevertheless, signal appears to be specific. Interestingly, only few cells per field demonstrate a positive signal. The low rate of positive cells stained may indicate that only a subset of prostate cells express TCTEX1D4. Stained cells did not appear to be glandular epithelial cells and the frequency of circa 5-10% positive cells may indicate that these cells correspond to a particular subset of cells, such as neuroendocrine or stem cells of the prostate.

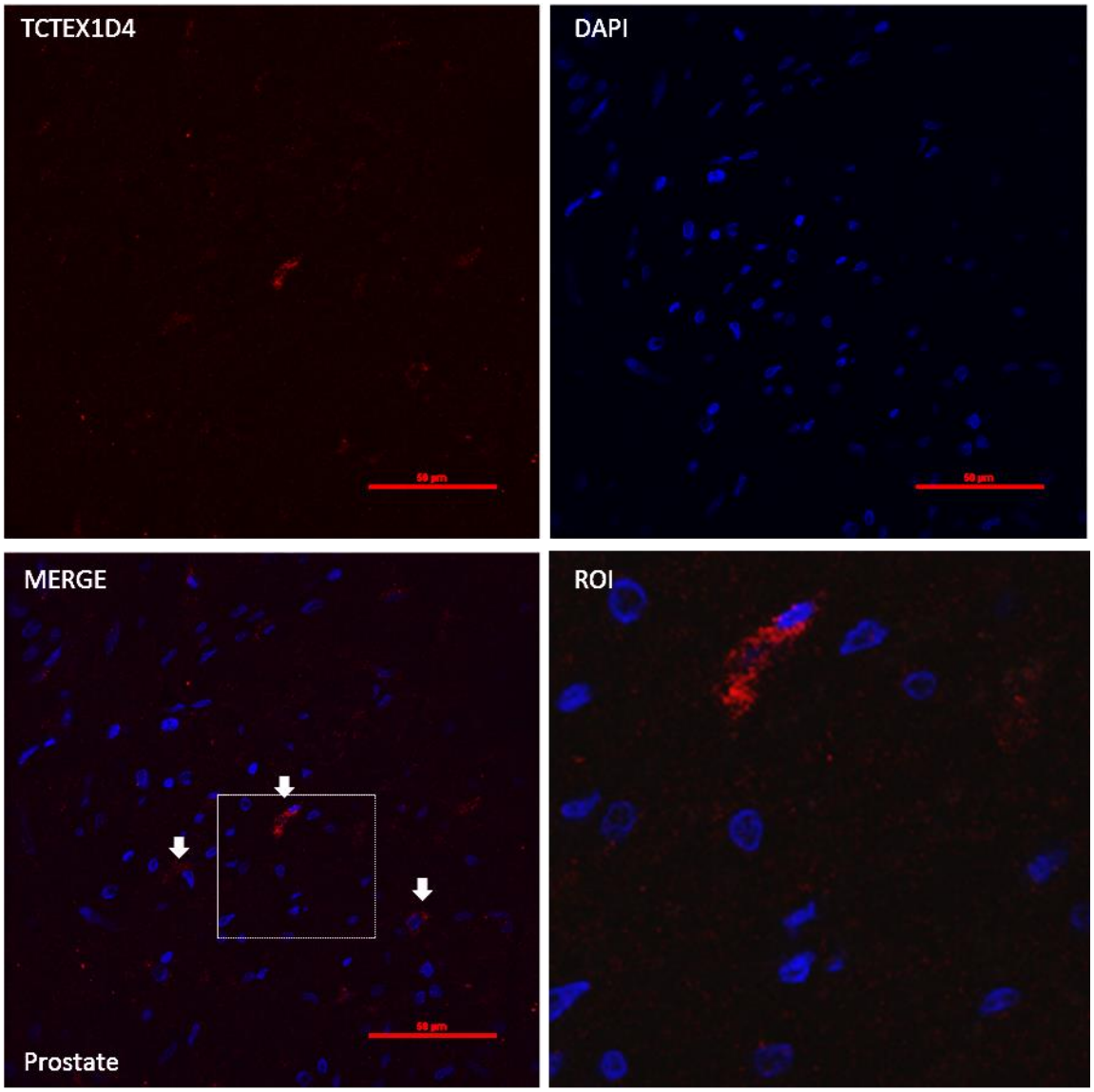

Figure 21 - TCTEX1D4 localization in the Prostate. In the merge image, arrows point out some cells stained positively. The box corresponds to the region of interest shown in the merge image.

TCTEX1D4 in Prostate Tumors

Cryopreserved tumor samples retrieved from xenografts generated upon implantation of LNCaP and PC3 cells in nude mice were available. IHC was performed in these samples under the established optimized conditions and the results are presented in Figure 22.

Unlike the observed in the normal prostate, in tumor samples TCTEX1D4 appears to be ubiquitous and distributed all throughout the tissue. Moreover, the signal produced is more intense and the pattern produced is also distinct: in these samples the signal appears as medium-sized dots which may correspond to vesicles

or aggregates, as previously proposed in the subcellular localization of TCTEX1D4 in PCa cell lines.

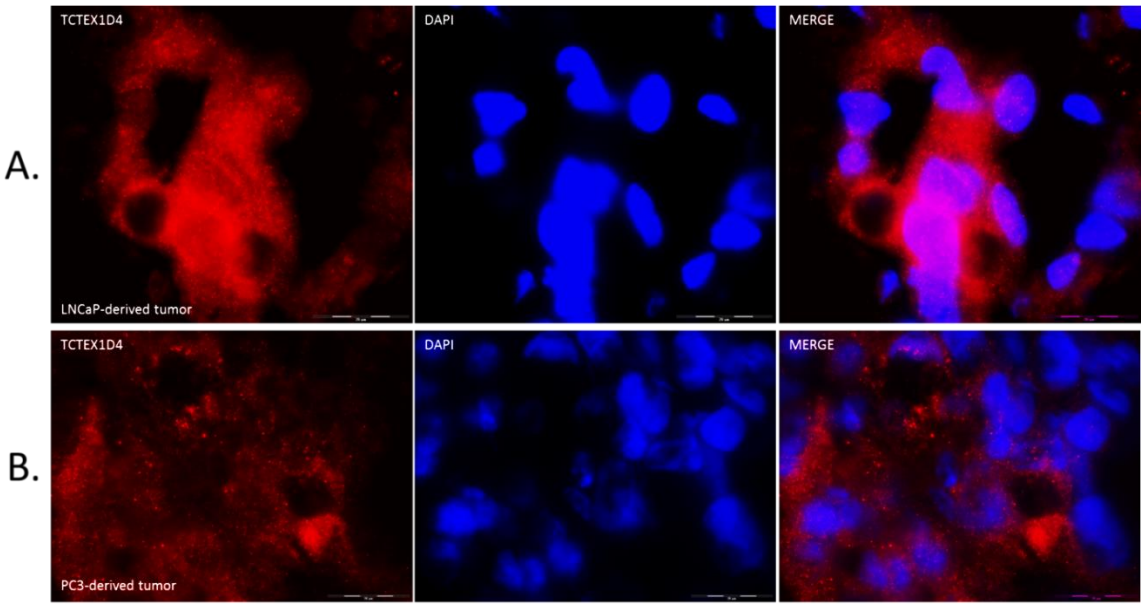

Figure 22 -TCTEX1D4 localization in LNCaP (A.) and PC3 (B.) derived tumors

Therefore, much like what it was observed for the prostate cells, encouraging differences in the tissue localization of TCTEX1D4 between the normal prostate and the tumor samples have been observed (Table 17). Furthermore, this work has established placenta as a TCTEX1D4 positive control for IHC.

Table 17 – Differences in the signal of TCTEX1D4 in the tissues analyzed

Signal of TCTEX1D4	Normal Placenta	Normal Prostate	Tumor samples
Distribution	Several cells of central localization	Restricted to a small subset of cells	Throughout all the sample
Pattern	Fine punctuate	Fine punctuate	Medium and large-sized dots
Intensity	Stronger	Weak	Stronges

IV. 4. Co-localize TCTEX1D4 and other proteins of interest

IV. 4. 1. Cellular level

In order to fulfill the objective of elucidating the role of TCTEX1D4 in the TGFβ signaling pathway in PCa, it is of crucial importance to figure out its place within the pathway. As DLC, it may play a role a role in the translocation of the signal towards the nucleus. Thus it may interact with receptors, co-receptors or with Smads. As its interaction and co-localization with Endoglin, TGFβRII and Betaglycan has already been studied, it was proposed to study its possible interaction with Smads. Due to the fact that it has been already demonstrated that it is possible that specific Smad pathways require specific DLCs, it was evaluated the co-localization of TCTEX1D4 with Smad 1, Smad 2 and Smad 3. Moreover, since the hypothesis of existence of a trimeric complex of TCTEX1D4-PPP1-Endoglin has been raised, co-localization studies with these proteins were also performed. All these studies were performed in RWPE-1, LNCaP and PC3 cell lines.

TCTEX1D4-ENDOGLIN

As predicted in the Co-IP procedures, the possible direct interaction between Tctex1d4 and Endoglin in PCa cells appears to be almost absent and very limited, since only a small number of co-localization spots were found in LNCaP and PC3 cells (Figures 24 and 25). On the other hand, in RWPE-1 cells such interaction may occur at an increased extent, since a much larger number of co-localization spots are observed, clearly depicted in Figure 23. This may indicate that there is a progressive decrease of TCTEX1D4-Endoglin interaction during malignant transformation. Notice that no Co-IP studies were performed in this cell line and, thereof, no other indication of such interaction is currently available.

Regarding the localization of that interaction, co-localization was found in cytoplasm. Notice that co-localization of Endoglin-TCTEX1D4 was previously reported by Meng *et al.* has been mostly localized in the cell membrane and cell protrusions and seldom in vesicles [178].

RWPE-1

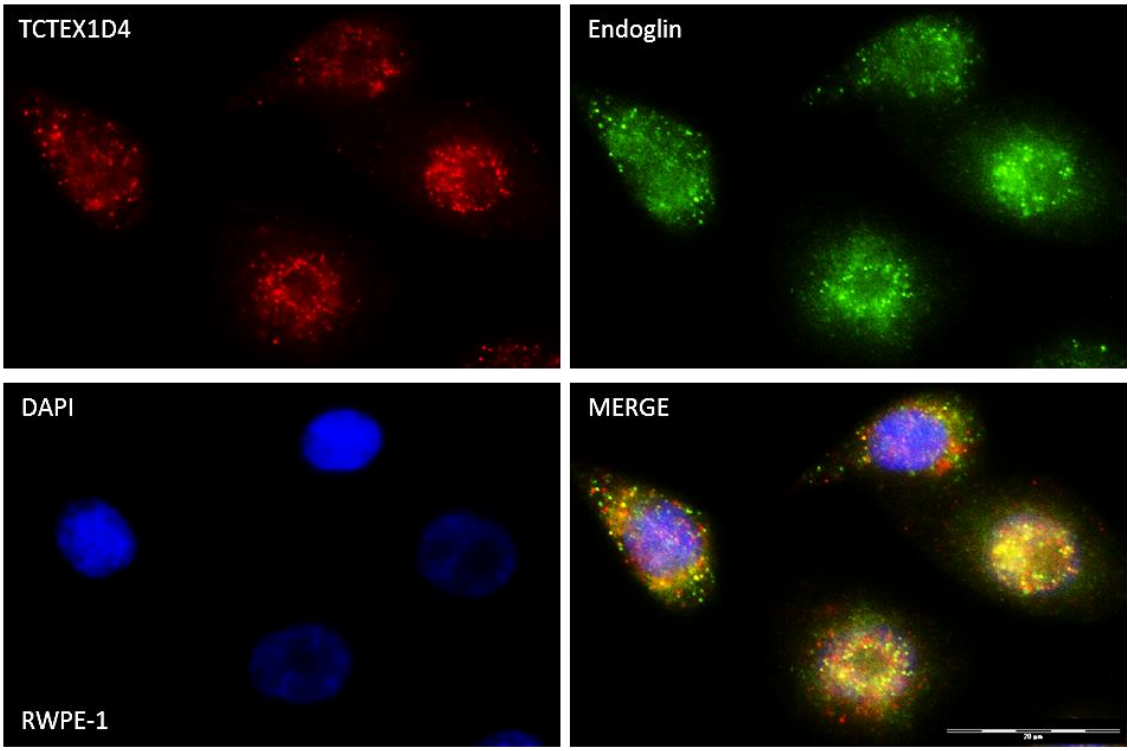

Figure 23 - Co-localization studies of TCTEX1D4 (red) and Endoglin (green) in RWPE-1 cells

LNCaP

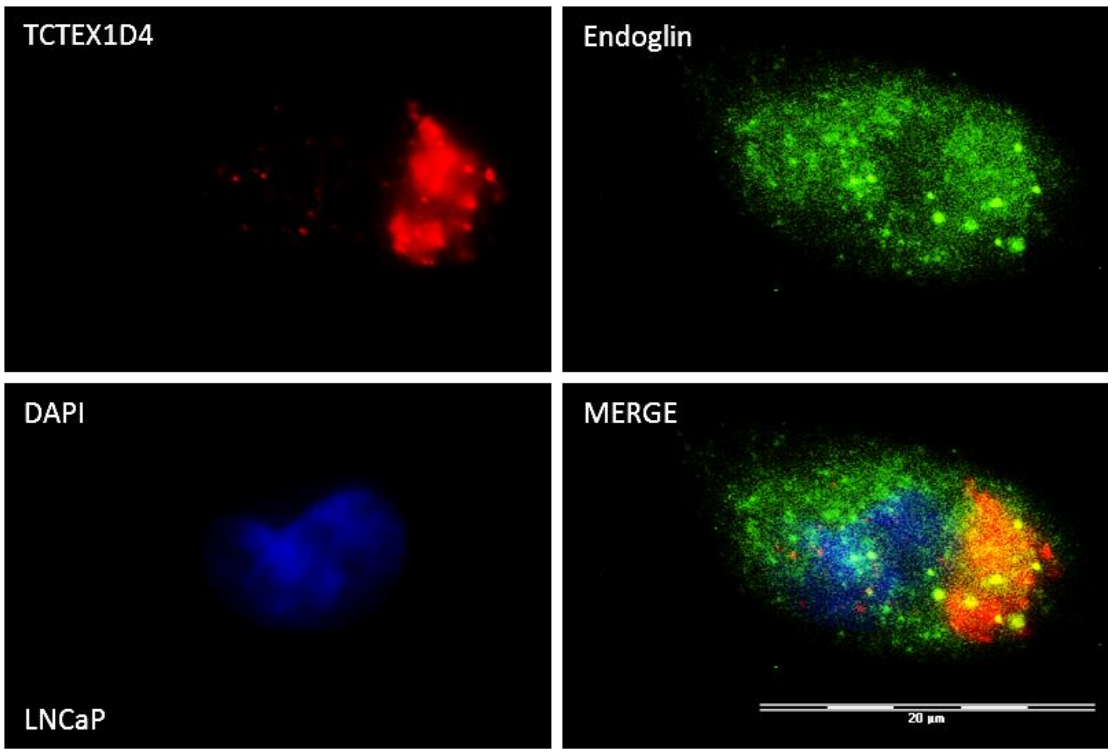

Figure 24 - Co-localization studies of TCTEX1D4 (red) and Endoglin (green) in LNCaP

PC3

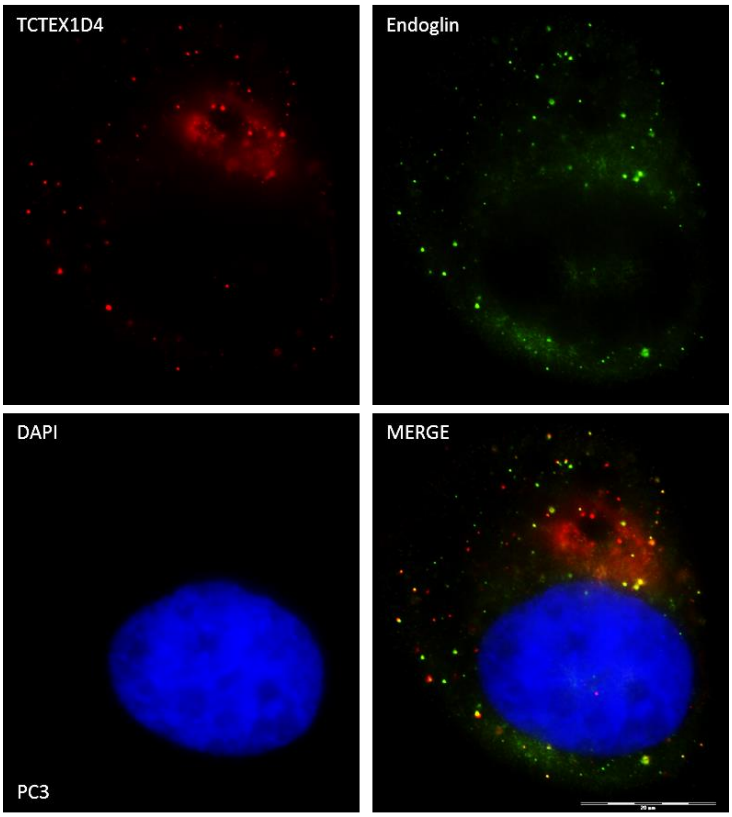

Figure 25 - Co-localization studies of TCTEX1D4 (red) and Endoglin (green) in PC3 cells

PPP1CC-ENDOGLIN

Co-localization between PPP1 and Endoglin has been observed in all cell lines, as predicted by the results of the Co-IP performed in PC3 and LNCaP cells lines which pointed towards an interaction between the two proteins (Figures 26 and 27). Regarding the localization of the co-localization it appears to be either in the cytoplasm (RWPE-1 and PC3, Figures 26 and 27 - right) or in the nucleus (LNCaP, Figure 27 - left). Nevertheless, it must be highlighted that the antibody against Endoglin performed poorly in all ICC experiments, producing weak signal and excessive background.

Once again, it appears to occur a decrease of the co-localization from normal to malignant cells. All the results so far seem to indicate a progressive disruption of the putative trimer TCTEX1D4-PPP1-Endoglin during acquisition of malignity, as co-localization is decreased in prostate cancer cells.

RWPE-1

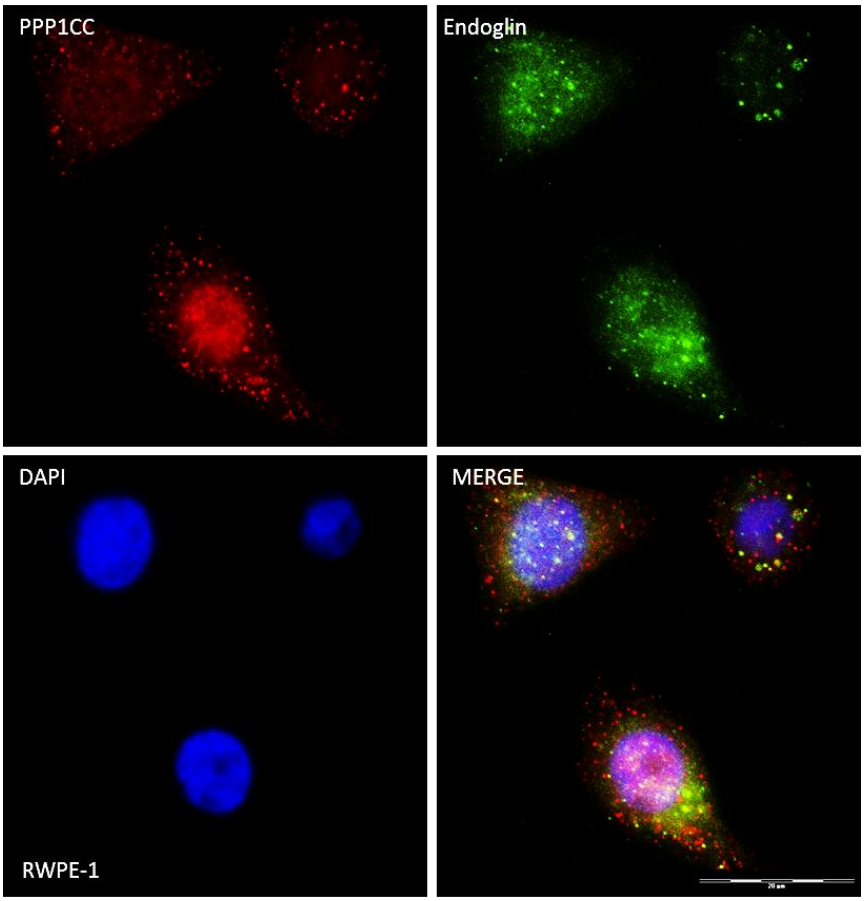

Figure 26 - Co-localization studies of PPP1CC (red) and Endoglin (green) in RWPE-1 cells

LNCaP and PC3

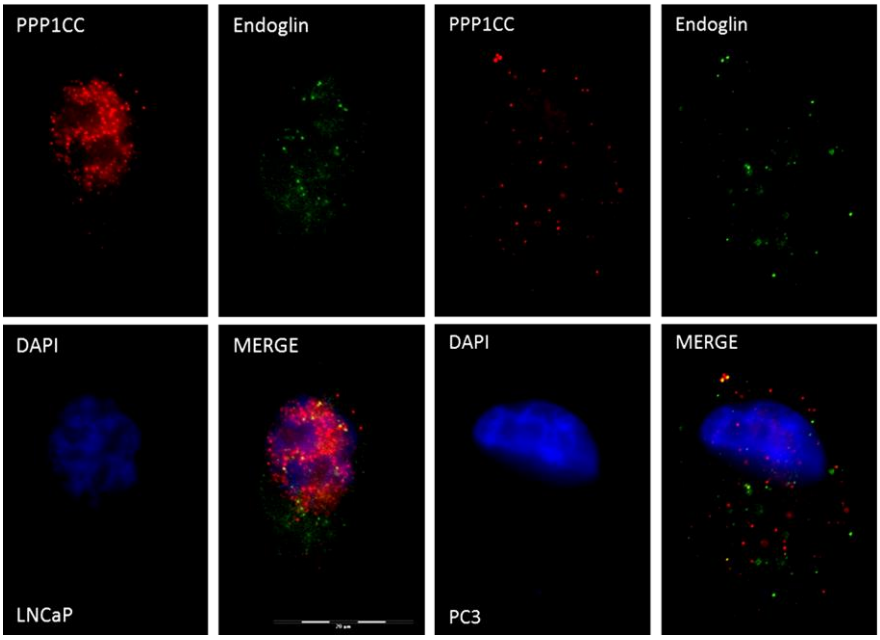

Figure 27 - Co-localization studies of PPP1CC (red) and Endoglin (green) in LNCaP (left) and PC3 (right) cells

Another interesting result is the fact that in LNCaP cells PPP1CC localization appears to be restricted to the nucleus, unlike in the other cell lines in which PPP1CC protein is also localized throughout the cytoplasm (Figure 28).

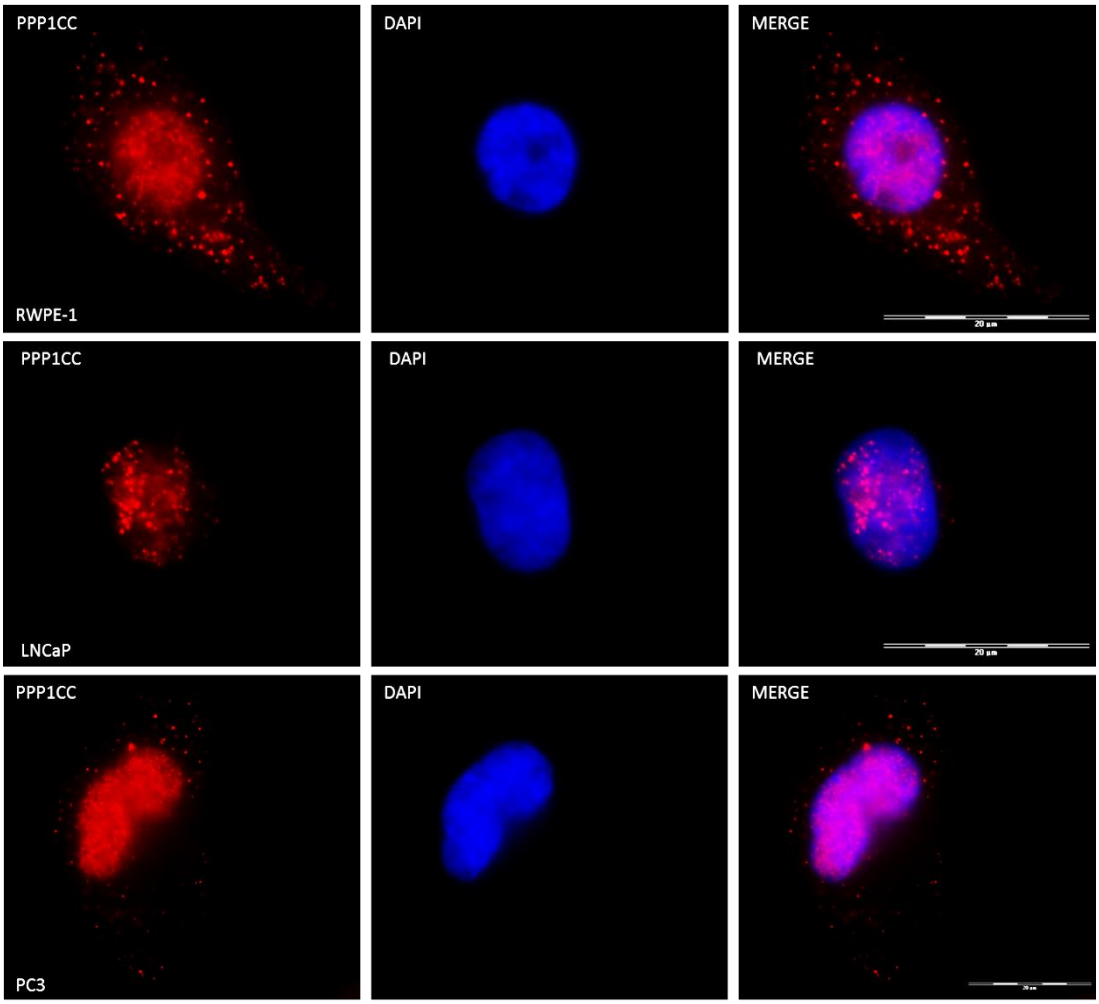

Figure 28 - Localization of PPP1CC in RWPE-1, LNCaP and PC3 cell lines

TCTEX1D4-SMADs

Co-localization studies of TCTEX1D4 with Smad 1, Smad 2 and Smad 3 were performed for RWPE-1, LNCaP and PC3. No signal for Smad 1 or Smad 2 was visible in any of the experiments (data not shown). Regarding co-localization of Smad 3 with TCTEX1D4 the results are presented in Figures 29, 30 and 31.

As depicted in Figure 29, co-localization of TCTEX1D4 and Smad 3 was observed in RWPE-1 cells but no co-localization was noticeable in both LNCaP and PC3 cells (Figures 30 and 31). This may indicate that TCTEX1D4 interacts with Smad 3 in normal cells and that this interaction is disrupted in malignant cells. In normal cells,

TCTEX1D4 may act as DLC in the Smad 3-mediated pathway intervening in the nuclear translocation of Smad 3, whilst in malignant cells TCTEX1D4 may be re-allocated to other non-canonical pathways. This is in accordance with the reported decrease in Smad 2/3 signaling observed in PCa.

RWPE-1

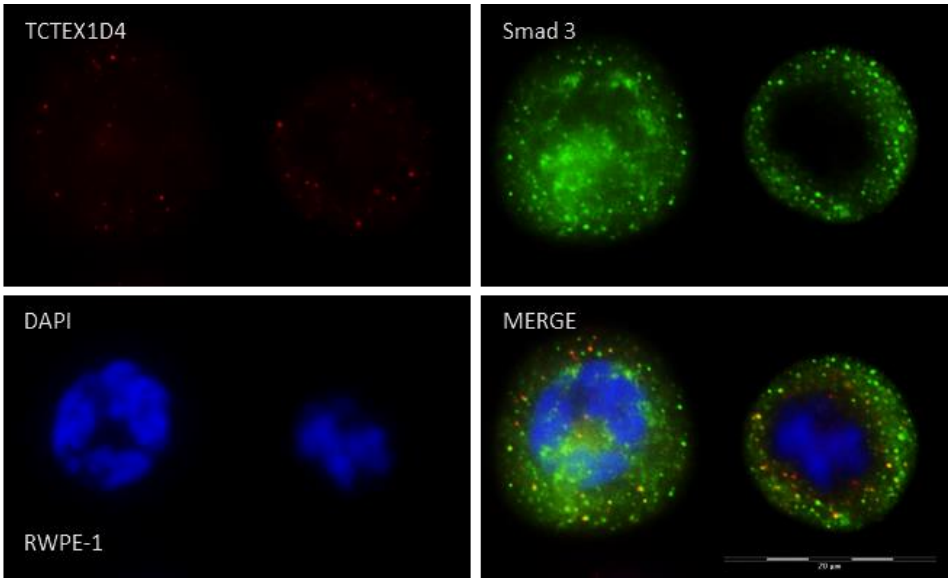

Figure 29 - Co-localization of TCTEX1D4 and Smad 3 in RWPE-1 cells

LNCaP

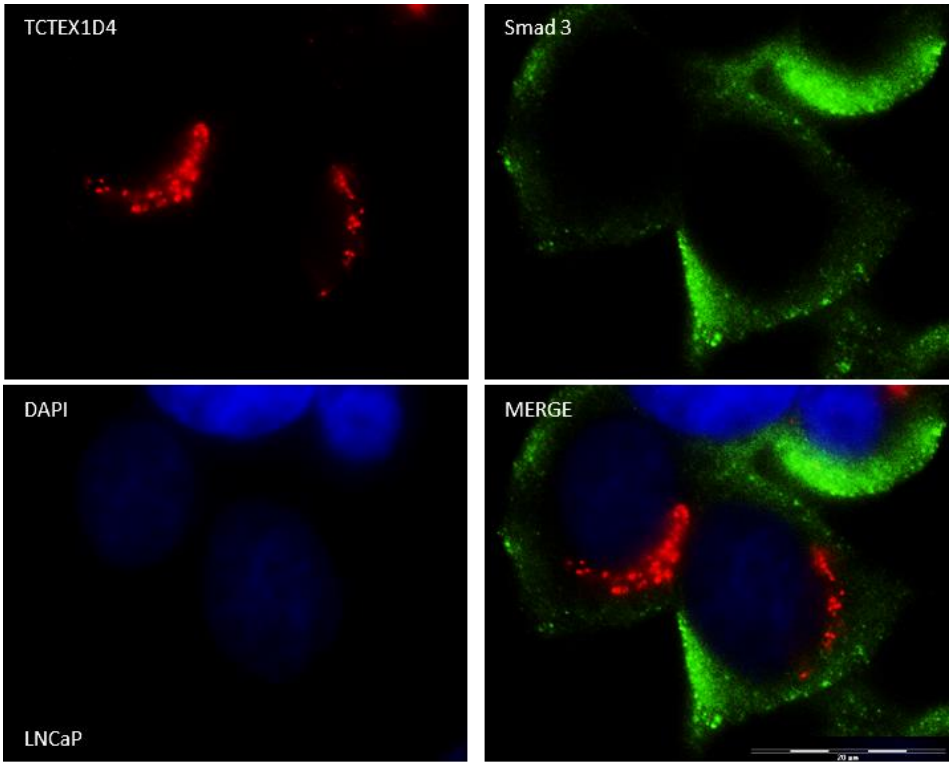

Figure 30 - Co-localization of TCTEX1D4 and Smad 3 in LNCaP cells

PC3

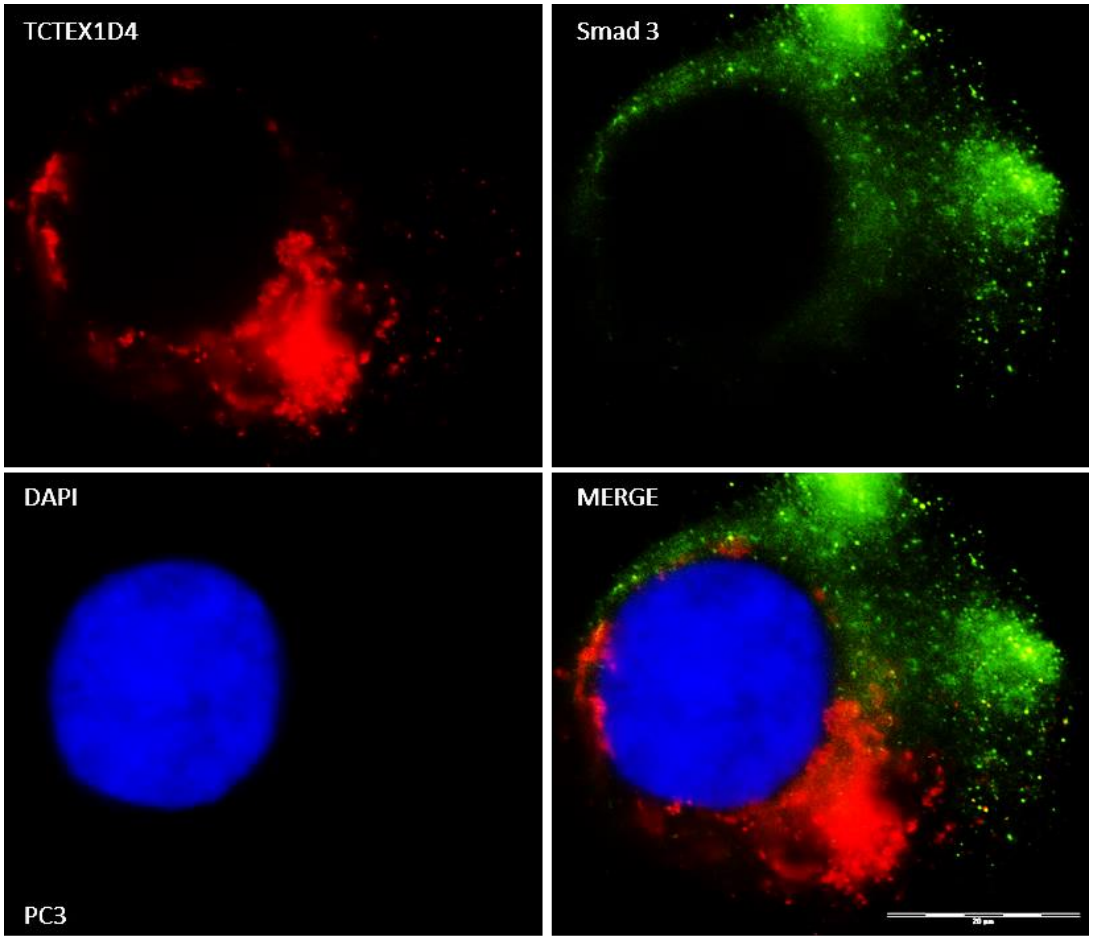

Figure 31 - Co-localization of TCTEX1D4 and Smad 3 in PC3 cells

IV. 4. 2. Prostate tissue

Co-localization studies of TCTEX1D4 and PPP1CC with Endoglin were performed. No signal for PPP1CC was observed and the signal of Endoglin in normal prostate and normal placenta tissue was restricted, as expected, to endothelial cells, producing a distinct signal in the apical membrane, depicted in Figures 32 and 33. Thus, no co-localization was observed in the normal prostate or in the normal placenta.

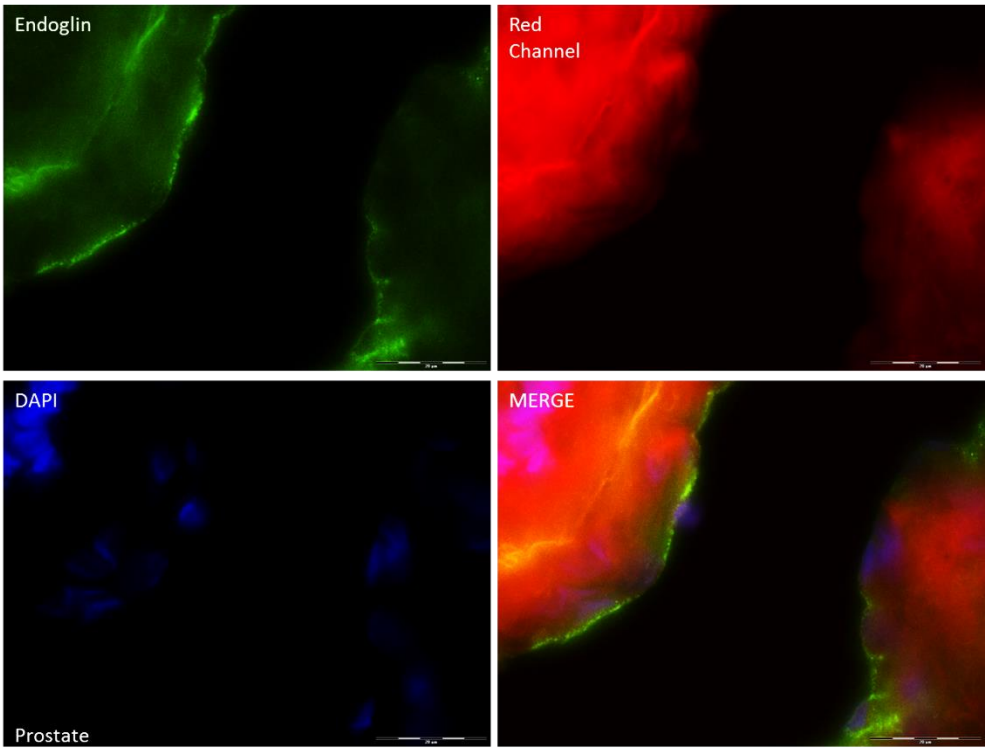

Figure 328 - Localization of TCTEX1D4 (green) in normal prostate

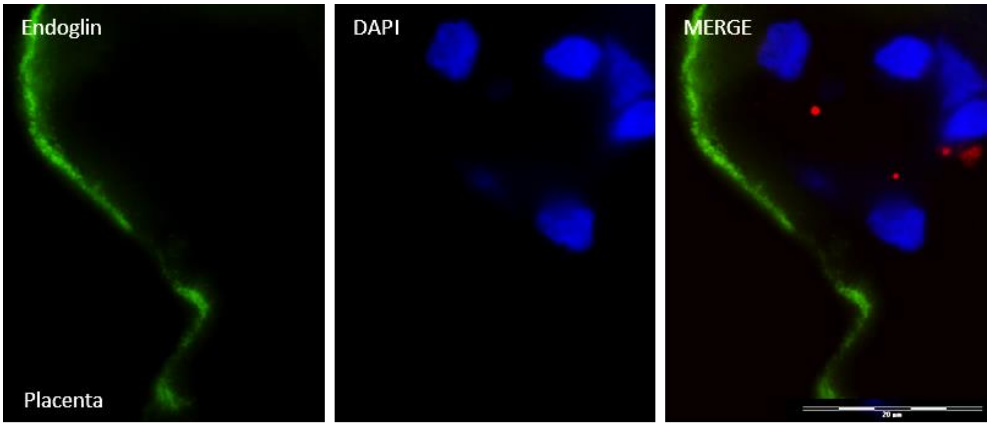

Figure 33 - Localization of TCTEX1D4 (green) in normal placenta

V. Conclusions and Future Work

V. 1. Conclusions

This work has contributed to increase our knowledge of TCTEX1D4 protein in human prostatic cells. Since this is a practically unknown protein, with only two published articles addressing its possible functions, this work faced many complications related to the fact that most techniques were not previously optimized for this protein. Moreover, the absence of information related to its functions, expression levels and localization made it more difficult to characterize it and give the results biological significance. Thereof, the results presented must be seen as preliminary and the proposed models, alterations and functions need to be further confirmed by proof-of-concept methodologies. Nevertheless, the main conclusions retrieved from this work are:

i. It was demonstrated, for the first time, the existence of TCTEX1D4 in normal prostate and in prostate cancer cells, both at the mRNA and at the protein levels
ii. TCTEX1D4 subcellular localization differs from normal prostate cells (throughout the cytoplasm and nucleus) to prostate malignant cells (polarized to a restricted region of the cytoplasm)
iii. TCTEX1D4 in normal prostate tissues was localized to a small subset of cell in which a fine punctuate weak signal is observed. On the contrary, in prostate tumors a strong TCTEX1D4 signal was detected in large dots all throughout the samples
iv. Placenta was defined as a suitable control for TCTEX1D4 at the mRNA level and for immunohistochemistry assays, in which a distinctive specific signal was for the first time described
v. Results from Co-Immunoprecipitation assays indicate the presence of a TCTEX1D4-PPP1 interaction.
vi. Results from Co-Immunoprecipitation assays and Immunocytochemistry co-localization studies point towards the presence of a PPP1-Endoglin interaction. Such interaction may be more common in normal cells
vii. Co-Immunoprecipitation assays did not indicate the presence of a TCTEX1D4-Endoglin interaction in prostate cancer cells. Furthermore, immunocytochemistry co-localization studies indicate that co-localization of Endoglin-TCTEX1D4 may be found in all cell lines but appears to occur at much higher extent in normal prostate cells (RWPE-1)
viii. TCTEX1D4-Smad 3 co-localization was found solely in normal cells (RWPE-1)

ix. At the tissue level, no co-localization was found between Endoglin and TCTEX1D4
x. A progressive decrease in mRNA levels of PPP1CC is found to occur parallel to progression of PCa cells malignity
xi. PPP1CC localization in LNCaP cells appears to be essentially nuclear, whilst PPP1CC is spread throughout the nucleus and cytoplasm in RWPE-1 and PC3 cells

In summary, this work raised several hypotheses:

- TCTEX1D4 subcellular localization is proposed to be altered in case of cancer. It is speculated if during the malignant transformation it is reallocated into a specific organelle, associated with microtubules or if it aggregates
- In normal prostate tissue TCTEX1D4 is restricted to a small number of cells, while in cancer it is detected throughout the sample. Therefore, it is speculated if there is a overexpression of TCTEX1D4 in prostate tumors, thus resulting in the presence of such differences in terms of staining patterns
- Results led to the proposed model of a trimeric complex of TCTEX1D4-PPP1-Endoglin. Furthermore, it is proposed that such complex may be disrupted during cancer progression, since co-localization of TCTEX1D4-Endoglin and PPP1-Endoglin are mostly observed in RWPE-1
- TCTEX1D4-Smad 3 complex may be disrupted during cancer progression. TCTEX1D4 may act in the nuclear translocation of Smad 3 in normal cells and the referred alteration in malignant cells may contribute to decrease Smad 3 signaling, a feature commonly observed in PCa

Other results from our laboratory indicate that TCTEX1D4 play a role in proliferation of prostate cells (both normal and malignant) and such effect may be modulated by phosphorylation events. Such results, in conjunction with the ones hereby presented, raise the possibility that TCTEX1D4 may play an active role during malignant transformation. Moreover, it appears that TCTEX1D4, via the interaction with Endoglin and Smad 3, may contribute to the disruption of TGFβ signaling, commonly observed in PCa.

V. 2. Future Work

The next steps necessary to test the hypotheses raised and further understand the role of TCTEX1D4 in TGFβ signaling and PCa are already defined.

Firstly, it is necessary to validate all the interactions proposed, using reversed Co-IP in the situations in which such was not yet performed.

Furthermore, TCTEX1D4 protein quantification is required, since results from qPCR were not easily understandable.

In order to describe precisely the subcellular localization of TCTEX1D4, the utilization of subcellular markers is necessary. Such strategy is also necessary to identify in which subset of cells TCTEX1D4 is detected in prostate tissue.

Moreover, to clarify the role of TCTEX1D4 in TGFβ pathway, luciferase assays will be performed to address by which Smad pathways the signal is translocated, upon transfection of TCTEX1D4 and stimulation with TGFβ, BMPs and Activins.

Regarding to its role in PCa, overexpression (by transfection) and knock-down (by shRNA) will be performed and the effects on cellular proliferation and migration will be evaluated.

Following these strategies, it is expected to achieve a better understanding of TCTEX1D4 role in TGFβ signaling pathway and in PCa development.

VI. References

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VII. Appendix

Appendix I - Solutions

Lower Gel Buffer

To 900 ml of deionized H2O add:

Tris 181.65 g

SDS 4 g

Mix until the solutes have dissolved. Adjust the pH to 8.9 and adjust the volume to 1L with deionized H2O.

Upper Gel Buffer

To 900 ml of deionized H2O add:

Tris 75.69 g

Mix until the solute has dissolved. Adjust the pH to 6.8 and adjust the volume to 1L with deionized H2O.

30% Acrylamide/0,8% Bisacrylamide

To 70 ml of deionized H2O add:

Acrylamide 29.2 g

Bisacrylamide 0.8 g

Mix until the solutes have dissolved. Adjust the volume to 100 mL with deionized water. Filter through a 0.2 µm filter and store at 4 °C.

10% Ammonium persulfate

In 10 ml of deionized H2O dissolve 1 g of APS.

10% SDS (Sodium dodecilsulfate)

In 10 ml of deionized H2O dissolve 1 g of SDS.

4x LB (Loading buffer)

1 M Tris solution (pH 6.8) 2.5 ml (250 mM)

SDS 0.8 g (0.8%)

Glycerol 4 ml (40%)

β-Mercaptoethanol 2 ml (2%)

Bromophenol blue 1 mg (0.01 %)

Adjust the volume to 10 ml with deionized H2O. Store in darkness at RT.

1 M Tris (pH 6.8) solution

To 150 ml of deionized H2O add:

Tris base 30.3 g

Adjust the pH to 6.8 and adjust the final volume to 250 ml.

10x Running Buffer

Tris 30.3 g (250 mM)

Glycine 144.2 g (2.5 M)

SDS 10 g (1%)

Dissolve in deionized H2O, adjust the pH to 8.3 and adjust the volume to 1 L.

10x Transfer Buffer

Tris 3.03 g (25 mM)

Glycine 14.41 g (192 mM)

Mix until solutes dissolution. Adjust the pH to 8.3 with HCl and adjust the volume to 800 ml with deionized H2O. Just prior to use add 200 ml of methanol (20%).

10x TBS

Tris 12.11 g (10 mM)

NaCl 87.66 g (150 mM)

Adjust the pH to 8.0 with HCl and adjust the volume to 1 L with deionized H2O.

10x TBS-T

Add 5 ml (0.05%) of Tween 20 to 1 L of 10x TBS

1x TBS-TT

Add 2 ml (0.2%) of Triton X-100 to 1 L of 1x TBS-T

1x PBS-Triton X-100

Add 2 ml (0.2%) of Triton X-100 to 1 L of 1x PBS

10% FBS-PBS

Add 50 ml of FBS to 450 ml of 1x PBS

4% paraformaldehyde-PBS

Add 4 grams of paraformaldehyde to 80 ml of 1x PBS

Adjust pH to 7.4 and the volume to 100 ml

3% milk-TBS-T solution

Add 30 g of powdered milk to 1 L of 1x TBS-T

5% milk-TBS-T solution

Add 50 g of non-fat powdered milk to 1 L of 1x TBS-T

3% Bovine Serum Albumin (BSA) solution

Add 30 g of BSA to 1 L of 1x TBST-T

1X RIPA lysis buffer with protease inhibitors

Mix the following (µl) in 8101 µl of deionized water:

10x RIPA lysis buffer	1000
PMSF (100X)	100,0
Benzamidine	500,0
Pepstatin A	100,0
Leupeptin	10,0
Aprotitin	88,8
EGTA	100,0

Protease inhibitiors preparation

Inhibitor	Weight (mg)	Volume of solvent (ml)	Solvent and storage
PMSF (100X)	17,42	1	Isopropanol, Ethanol or Methanol and store at -20°C
Benzamidine	24,03	5,13	H_20 and store at 4°C
Pepstatin A	0,34	5	Methanol or DMSO and store at 4°C
Leupeptin	0,93	1	H_20, ethanol or DMF and store at -20°C
Aprotitin	1,10	1	H_20 with 0,9% NaCl and 0,9% Benzyl-Alcohol and store at 4°C
EGTA	38,04	100	H_20, adjust the pH to 8.0 and store at RT

Fixing Solution

Add 500 ml of methanol and 100 ml of glacial acetic acid to 400 ml of deionized water

Staining solution

Add 1 ml of Coomassie Brilliant Blue R-250 to 1 L of Fixing Solution

Distaining solution

add 400 ml of methanol and 100 ml of glacial acetic acid to 500 ml of deionized water

Appendix II – Primers

Target Gene	Name	Sequences	Predicted Annealing (ºC)
GAPDH	GAPDH	f: 5'-aacgggaagcttgtcatcaatggaaa-3' r: 5'-aacgggaagcttgtcatcaatggaaa-3'	60
	GAPDH-001	f: 5'-ccccggtttctataaattgagc-3' r: 5'-caccttccccatggtgtct-3'	60
	GAPDH_hs	f: 5'-gacagtcagccgcatcttct-3' r: 5'-gcgcccaatacgaccaaatc-3'	61
beta-actin	b-actin_hs	f: 5'-ccacacaggggaggtgatag-3' r: 5'-agaccaaaagccttcatacatctca-3'	60
HPRT1	HPRT	f: 5'-gctataaattctttgctgacctgctg-3' r: 5'-aattacttttatgtcccctgttgactgg-3'	62
	HPRT-001	f: 5'-gaccagtcaacaggggacat-3' r: 5'-gtgtcaattatatcttccacaatcaag-3'	59
TCTEX1D4	TCTEX1D4-001	f: 5'-gtgagggggagtccaattct-3' r: 5'-cagacacttatttattgggatgtga-3'	59.5
	TCTEX1D4-201	f: 5'-gcctgggactatctgatcca-3' r: 5'-ccggagtctttggcattc-3'	59.5
	PAPTEIE	f: 5´- ccctgctcatgtgtctaacagcc-3' r: 5´- tttggcattctcctcctcctggc-3'	58
	PAPTT57	f: 5´- acagcctttaaccttctcagcacc-3' r: 5´- gcccggagtctttggcattctc-3'	57.5
	PAPTGCH	f 5´- aggagctcagtcggcaaggac-3' r: 5´- tggccatggacctgctgtgttttag-3'	59
	TCTEX1D4_hs	f: 5'-atcagaggccaaaggcagaag-3' r: 5'-ccttcaaccccacagatcct-3'	60
	TCTEX1D4_hs2	f: 5'-gtaggggagatctttgtgcca-3' r: 5'-cccgacttctctggaaggag-3'	60
PPP1CC	PPP1CC96	f: 5'- aacggctgctggaagtga – 3' r: 5'- agatttcacgagactttaagcaca- 3'	59.5
	PPP1CC64	f: 5' – caacatcgacagcattatcca – 3' r: 5' – gacattcttaccaggcttggac – 3'	59.5