Universidade de Aveiro
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Juliana Carina Cardoso Felgueiras Campos

Estudo do interatoma da proteína fosfatase 1: pesquisa de biomarcadores e potenciais alvos terapêuticos para o cancro da próstata

Unraveling the interactome of protein phosphatase 1: searching for biomarkers and therapeutic targets for human prostate cancer

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Bioquímica, realizada sob a orientação científica da Professora Doutora Margarida Fardilha, Professora Auxiliar com Agregação do Departamento de Ciências Médicas da Universidade de Aveiro, da Professora Doutora Carmen Jerónimo, Professora Catedrática Convidada do Departamento de Patologia e Imunologia Molecular Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto e do Professor Doutor José Fernando Mendes, Professor Catedrático do Departamento de Física da Universidade de Aveiro

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"Há gente que fica na história da história da gente"
("Chuva", autoria de Jorge Fernando, interpretado por Mariza)

Dedico este trabalho ao meu Avô Manuel

o júri

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palavras-chave

Proteína fosfatase 1, isoformas, cancro da próstata, sinalização celular, interação proteína-proteína, bioinformática

resumo

A próstata é uma importante glândula acessória do sistema reprodutor masculino. A sua génese, desenvolvimento e função são estritamente reguladas por vias de sinalização celular, cujo conhecimento permite uma melhor compreensão dos eventos moleculares e celulares subjacentes ao desenvolvimento de duas doenças frequentemente diagnosticadas em homens com mais de 50 anos: a hiperplasia benigna da próstata e o cancro da próstata (CaP). O CaP continua a ser uma das neoplasias malignas mais comuns em homens em todo o mundo e, apesar dos avanços recentes no seu diagnóstico e tratamento, a sua gestão clínica continua a ser desafiante.

A fosforilação reversível é um dos principais mecanismos de regulação do estado de ativação das proteínas e, consequentemente, das cascatas de sinalização celular por elas mediadas. A proteína fosfatase 1 (PP1)—um dos principais catalisadores celulares de reações de desfosforilação em resíduos de serina e treonina—participa em inúmeros processos celulares, desde o ciclo celular à dinâmica do citoesqueleto. A sua atividade, ou desregulação da mesma, tem sido também associada ao desenvolvimento e progressão de várias doenças, como o cancro. No CaP, sabe-se que a PP1 promove a atividade do recetor de androgénios (AR)—um fator de transcrição central na carcinogénese da próstata e o principal alvo das intervenções farmacológicas clinicamente aprovadas para o tratamento da doença em estadios iniciais. Contudo, são escassos os estudos dedicados à PP1 no CaP.

Assim, este estudo surge com o objetivo de caracterizar a expressão da PP1 no CaP e de identificar o seu interatoma para uma melhor compreensão da sua função e identificação de potenciais novos biomarcadores e/ou alvos terapêuticos para a doença. Neste sentido, analisamos a expressão das isoformas catalíticas PP1 em condições normais e tumorais da próstata e descobrimos que eram diferencialmente expressas. Além disso, os nossos resultados apontam para a existência de funções específicas de cada isoforma. Prosseguimos com a caracterização do interatoma da isoforma gama, PP-1G, no CaP, usando uma combinação de abordagens bioquímicas e bioinformáticas. Isto permitiu-nos identificar interatores particularmente relevantes para investigação detalhada em estudos futuros. Por último, desenhamos e sintetizamos péptidos derivados dos motivos de ligação à PP1 presentes na sequência de aminoácidos do AR, que mostramos serem internalizados por modelos celulares de CaP e diminuírem a viabilidade celular, mesmo em células resistentes à castração, quando usados em combinação.

keywords

Protein phosphatase 1, isoforms, prostate cancer, cell signaling, protein-protein interaction, bioinformatics

abstract

The prostate is an important accessory gland of the male reproductive system. Its genesis, development and function are strictly regulated by cell signaling pathways. Understanding such pathways is important to better understand the molecular and cellular events that underlie two frequently diagnosed diseases in men over 50—benign prostate hyperplasia and prostate cancer (PCa). PCa remains one of the most common malignancies in men worldwide and, despite recent advances in PCa diagnosis and treatment, clinical management is still challenging.

Reversible phosphorylation is one of the key cellular mechanisms for regulating the activation state of proteins and, consequently, their downstream signaling cascade(s). Protein phosphatase 1 (PP1)—one of the most relevant serine/threonine phosphatases—participates in several cellular processes, from cell cycle to cytoskeleton dynamics. Its activity, or deregulation, has also been associated with the development and progression of various diseases, including cancer. In PCa, PP1 is known to promote the androgen receptor (AR) activity—a central player in PCa development and progression and the main target of clinically-approved pharmacological interventions for the management of the disease at early castration-naïve stages. However, few studies have addressed PP1 in PCa.

Therefore, this study aimed to characterize the expression of PP1 in PCa and to identify its interactome for a better understanding of its function and identification of potential new biomarkers and/or therapeutic targets. To that end, we analyzed the expression of the PP1 catalytic isoforms in normal and tumoral conditions of the prostate and found them to be differentially expressed. In addition, our results provide evidence for potential isoform-specific roles, emphasizing the need for isoform-dedicated studies. We then proceed our study with the characterization of the PP-1G's (gamma isoform) interactome in PCa, using a combination of biochemical and bioinformatics approaches. This allowed us to highlight particularly relevant interactors that would be of interest to further address in future studies. Finally, we designed and synthesized cell-penetrating peptides based on the PP1-docking motifs present in the AR's amino acid sequence. The peptides were successfully internalized by PCa cells and decreased the viability of both androgen-dependent and castration-resistant PCa cells when used in combination.

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CHAPTER IV

ABBREVIATIONS AND ACRONYMS

4OHT	4-hydroxytamoxifen
ACK-1	Activated CDC42 kinase 1
ACYP2	Acylphosphatase-2
AdenoPCa	Prostate adenocarcinoma
ADRB2	Beta-2 adrenergic receptor
ADT	Androgen deprivation therapy
Akt	RAC-alpha serine/threonine-protein kinase
ALK	ALK tyrosine kinase receptor
AR	Androgen receptor
AR-V7	Androgen receptor variant 7
ARE	Androgen response elements
ARFGEF3	Brefeldin A-inhibited guanine nucleotide-exchange protein 3
Asp	Aspartic acid
ATR	Serine/threonine protein kinase ATR
BAD	Bcl-2-associated agonist of cell death
BAG-1	BAG family molecular chaperone regulator 1
Bcl2-L1	Bcl-2-like protein 1
BMP	Bone morphogenetic protein
BMPR	Bone morphogenetic protein receptor
BPH	Benign prostate hyperplasia
BRCA1	Breast cancer type 1 susceptibility protein
BSA	Bovine serum albumin
c-Fos	Proto-oncogene c-Fos
c-Jun	Transcription factor AP-1
C-terminal	Carboxy-terminal
Ca^{2+}	Calcium ion
CCND1	G1/S-specific cyclin-D1
CDK	Cyclin-dependent kinase
cDNA	Complementary deoxyribonucleic acid
CGRP	Calcitonin gene-related peptide
CHSY1	Chondroitin sulfate synthase 1
CLCF1	Cardiotrophin-like cytokine factor 1
CO_2	Carbon dioxide
COSMIC	Catalogue Of Somatic Mutations In Cancer
CRCP	Castration-resistant prostate cancer
CREB-1	Cyclic AMP-responsive element-binding protein 1
CTNNB1	Catenin beta-1
CUEDC2	CUE domain-containing protein 2
cycT1	Cyclin-T1
CYP17A1	Steroid 17 alpha hydroxylase/17,20 lyase
CYP19A1	Aromatase
DAB	3,3-diaminobenzidine
DGS	Directorate-General of Health [Direção-Geral da Saúde]

DHT	5α-dihydrotestosterone
DLL	Delta-like ligand
DNA	Deoxyribonucleic acid
E2	17β-estradiol
E2F1	Transcription factor E2F1
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EEF1A1	Elongation factor 1-alpha 1
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EIF2B4	Translation initiation factor eIF-2B subunit delta
EMT	epithelial-mesenchymal transition
ER	Estrogen receptor
ERK	Extracellular signal-regulated kinase
ERM	Ezrin-radixin-moesin complex
ERSPC	European Randomized Study of Screening for Prostate Cancer
ETS	Erythroblast transformation-specific
ETV	ETS translocation variant
F.E.	Fold enrichment
FGF	Fibroblast growth factor
FGFBP	Fibroblast growth factor-binding protein
FGFR	Fibroblast growth factor receptor
FOXO	Class O forkhead box transcription factor
FRP-1	Secreted frizzled-related protein 1
GADD34	Protein phosphatase 1 regulatory subunit 15A
GPCR	G-protein coupled receptor
GPER	G protein-coupled estrogen receptor
GRCh38	Genome Reference Consortium Human Build 38
HD6	Histone deacetylase 6
HIF-1-α	Hypoxia-inducible factor 1-alpha
His	Histidine
HPV	Human papillomavirus
HPV-16	Human papillomavirus type 16
HSP	Heat shock protein
IGF	Insulin-like growth factor
IGF1R	Insulin-like growth factor 1 receptor
IGF2R	Cation independent mannose-6-phosphate receptor
IGFBP	insulin-like growth factor-binding protein
IgG	Immunoglobulin G
IKK	Inhibitor of nuclear factor kappa-B kinase
IL	Interleukin
IRF	Interferon regulatory factor
IRS	Immunoreactive score
JAK	Tyrosine-protein kinase JAK
JNK	c-Jun N-terminal kinase
KCNC2	Potassium voltage-gated channel subfamily C member 2

LH/CG-R	Lutropin-choriogonadotropic hormone receptor
LIMS1	LIM and senescent cell antigen-like-containing domain protein 1
MAP3K7	Mitogen-activated protein kinase kinase kinase 7
MAPK	Mitogen-activated protein kinase
mCRPC	Metastatic castration-resistant prostate cancer
miRNA	Micro ribonucleic acid
mRNA	Messenger ribonucleic acid
MYPT1	Protein phosphatase 1 regulatory subunit 12A
N-terminal	Amino-terminal
NEPC	Prostate neuroendocrine carcinoma
NIPP-1	Nuclear inhibitor of protein phosphatase 1
NPM	Nucleophosmin
NPT	Normal prostate tissue
NSCLC	Non-small cell lung cancer
OT-R	Oxytocin receptor
p150	Tyrosine-protein kinase ABL1
p21	Cyclin-dependent kinase inhibitor 1
p53	Cellular tumor antigen p53
p65	Transcription factor p65
PAP	Prostatic acid phosphatase
PAR-3	Partitioning defective 3 homolog
PBS	Phosphate-buffered saline
PCa	Prostate cancer
PD-L1	Programmed cell death 1 ligand 1
PI3K	Phosphoinositide-3-kinase
PIN	Prostatic intraepithelial neoplasia
PKA C-alpha	cAMP-dependent protein kinase catalytic subunit alpha
PKC	Protein kinase C
PNUTS	Serine/threonine-protein phosphatase 1 regulatory subunit 10
PP-1A	Serine/threonine-protein phosphatase PP1-alpha catalytic subunit
PP-1B	Serine/threonine-protein phosphatase PP1-beta catalytic subunit
PP-1G	Serine/threonine-protein phosphatase PP1-gamma catalytic subunit
PP1	Protein phosphatase 1
PP1c	Protein phosphatase 1 catalytic subunit
PP2A	Protein phosphatase 2A
PPP	Phosphoprotein phosphatase
PPP1R11	E3 ubiquitin-protein ligase PPP1R11
PPP1R15B	Protein phosphatase 1 regulatory subunit 16B
PPP1R16B	Protein phosphatase 1 regulatory inhibitor subunit 16B
PR	Progesterone receptor
pRB	Retinoblastoma-associated protein
PRL-R	Prolactin receptor
PSA	Prostate-specific antigen
PTC	Protein patched homolog 1
PTK2	Focal adhesion kinase 1
PTM	Post-translational modification

RAD51	DNA repair protein RAD51 homolog 1
Raf-1	RAF proto-oncogene serine/threonine-protein kinase
RASSF5	Ras association domain-containing protein 5
RIPPO	Regulatory interactors of protein phosphatase 1
RNA	Ribonucleic acid
RNA-Seq	RNA-Sequencing
ROCK1	Rho-associated protein kinase 1
ROS	Reactive oxygen species
RPKM	Reads per kilobase per million reads
RT	Room temperature
S6K1	Ribosomal protein S6 kinase beta-1
SDS22	Protein phosphatase 1 regulatory subunit 7
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Ser	Serine
SHBG	Sex hormone-binding globulin
SHH	Sonic hedgehog protein
SHOC-2	Leucine-rich repeat protein SHOC-2
siRNA	Small interfering ribonucleic acid
SKP2	S-phase kinase-associated protein 2
SKR3	Serine/threonine-protein kinase receptor R3
Smad	Mothers against decapentaplegic homolog
SNP	Single nucleotide polymorphism
SRD5A1	3-oxo-5-alpha-steroid 4 dehydrogenase 1
SRD5A2	3-oxo-5-alpha-steroid 4 dehydrogenase 2
SSPN	Sarcospan
STAT	Signal transducer and activator of transcription
T3	Triiodothyronine
T4	Thyroxine
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline with Tween™ 20
TCGA	The Cancer Genome Atlas
tfm	Testicular feminization
TGF	Transforming growth factor
TGFR	Transforming growth factor receptor
Thr	Threonine
TLR	Toll-like receptor
TP53BP2	Apoptosis-stimulating of p53 protein 2
TRAF6	TNF receptor-associated factor 6
TRH	Thyrotropin-releasing hormone
Tyr	Tyrosine
UGE	Urogenital sinus epithelium
UGM	Urogenital sinus mesenchyme
UGS	Urogenital sinus
VDR	Vitamin D3 receptor
VEGF	Vascular endothelial growth factor
wt	Wild-type

WWTR1	WW domain-containing transcription regulator protein 1
ZEB1	Zinc finger E-box-binding homeobox 1
Zn^{2+}	Zinc ion

THESIS OVERVIEW

This thesis is composed by seven chapters, subdivided into Sections, when applicable: I – General introduction; II – Rationale, hypotheses and aims; III-V – Results chapters; VI – Concluding remarks and future perspectives; and, VII – References. The chapters dedicated to the general introduction and results of this thesis are written as publications (published/accepted for publication, submitted or soon to be submitted).

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Chapter Ib

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Chapter IVa

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Chapter III

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Disclosure: For the sake of thesis consistency, the versions presented here may not fully correspond to the published versions (i.e., efforts were made to uniformize abbreviations, acronyms and protein nomenclature; language (English USA/English UK); and tables and figures formatting throughout all chapters).

CHAPTER I

GENERAL INTRODUCTION

Section a

More than androgens: hormonal and paracrine signaling in prostate development and homeostasis

KEYWORDS

Steroid hormones
Androgens
Growth factors
Epithelial-stroma interactions

ABSTRACT

The prostate is the major exocrine gland of the male reproductive system. The prostatic epithelium secretes an alkaline fluid, the prostatic fluid, that constitutes about 20-30% volume of the seminal fluid. It provides proteins and ions essential to control the ejaculation process and to regulate proteins involved in sperm maturation (e.g., human kallikrein-related peptidases, phosphatases, polyamines, pepsinogen II, citrate, glucose, and Zn^{2+}, among others). The prostate exhibits some particularities when compared to other organs: it accumulates the highest levels of Zn^{2+} of any soft tissue; epithelial cells can produce energy by glycolysis (similarly to highly proliferative cells); and it is the only gland that tends to grow with aging, being associated with disorders of elderly, such as benign prostatic hyperplasia and carcinoma. Prostate development starts early in embryogenesis, but prostate maturation is only concluded in puberty. Specification of the prostate during human embryogenesis occurs before clear morphological evidence of a developing structure and involves the expression of signaling molecules that drive cells from the urogenital sinus to a prostatic cell fate. Prostate development and homeostasis are regulated by several hormones and growth factors and are highly dependent on autocrine and paracrine signaling. Efforts have been made to identify the mediators of prostate signaling as revised in this chapter, however this has been compromised by experimental constrains. Furthermore, most of the studies have been performed in rodent models, which makes extrapolations to other species difficult, given the interspecies variability on prostate anatomy and morphology.

Abbreviations: ACK-1, activated CDC42 kinase 1; ADT, androgen deprivation therapy; Akt, RAC-alpha serine/threonine-protein kinase; AR, androgen receptor; ARE, androgen response elements; BAD, Bcl-2-associated agonist of cell death; BAG-1, BAG family molecular chaperone regulator 1; BMP, bone morphogenetic proteins; BMPR, BMP receptor; BPH, benign prostate hyperplasia; CDK, cyclin-dependent kinase; c-Fos, proto-oncogene c-Fos; CGRP, calcitonin gene-related peptide; c-Jun, transcription factor AP-1; CRPC, castration-resistant prostate cancer; CTNNB1, catenin beta-1; CYP17A1, steroid 17 alpha hydroxylase/17,20 lyase; CYP19A1, aromatase; DHT, 5α-dihydrotestosterone; DLL, delta-like ligand; DNA, deoxyribonucleic acid; E2, 17β-estradiol; EGF, epidermal growth factor; EGFR, EGF receptor; ER, estrogen receptor; FGF, fibroblast growth factor; FGFBP, FGF-binding protein; FGFR, FGF receptor; FRP-1, secreted frizzled-related protein 1; GPCR, G protein-coupled receptor; GPER, G protein-coupled estrogen receptor; HIF-1-α, hypoxia-inducible factor 1-alpha; HSP, heat shock protein; IGF, insulin-like growth factor; IGF1R, IGF 1 receptor; IGF2R, cation-independent mannose-6-phosphate receptor (also known as IGF 2 receptor); IGFBP, IGF-binding proteins; IL, interleukin; JAK, tyrosine-protein kinase JAK; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; mRNA, messenger ribonucleic acid; OT-R, oxytocin receptor; PAP, prostatic acid phosphatase; PCa, prostate cancer; PI3K, phosphoinositide-3-kinase; PKC, protein kinase C; PR, progesterone receptor; PRL-R, prolactin receptor; PSA, prostate-specific antigen; PTC, protein patched homolog 1; PTK2, focal adhesion kinase 2; ROS, reactive oxygen species; Ser, serine; SHBG, sex hormone-binding globulin; SHH, sonic hedgehog protein; SRD5A1, 3-oxo-5-alpha-steroid 4 dehydrogenase 1; SRD5A2, 3-oxo-5-alpha-steroid 4 dehydrogenase 1; STAT, signal transducer and activator of transcription; T3, triiodothyronine; T4, thyroxine; tfm, testicular feminization; TGF, transforming growth factor; TGFR, TGF receptor; Thr, threonine; TRH, thyrotropin-releasing hormone; Tyr, tyrosine; UGE, urogenital sinus epithelium; UGM, urogenital sinus mesenchyme; UGS, urogenital sinus; wt, wild type; Zn2+, zinc ion.

1. Introduction

Most male internal genitalia derive from the embryogenic Wolffian duct (mesodermal origin), but not the prostate. The prostate gland has endodermal origin from a complex and heterogeneous part of the urogenital sinus (UGS) [1]. Prostate development follows gonads differentiation and involves several stages (Fig. Ia. 1) [2,3]. Gonadal-derived fetal androgens and interactions between UGS epithelium (UGE) and mesenchyme (UGM) are determinant throughout all prostate organogenesis. The underlying molecular mechanisms are far from being completely understood, but these requirements appear to be universal despite the anatomical dissimilarities observed in prostates from different species [4].

Several molecular pathways have been implicated in embryonic development of the prostate (Fig. Ia. 1) [5]. Most of the findings, nonetheless, resulted from studies using rodent knockout models and still lack confirmation in humans. The multiple reports on the restricted expression of the signaling mediators to one of the compartments (epithelial or mesenchymal) strengthened the hypothesis of vital paracrine signaling in the regulation of prostate growth and differentiation. Paracrine signaling occurs in both ways and secreted signaling molecules include members of diverse pathways: hedgehog, fibroblast growth factor (FGF), Wnt, Notch, among other pathways (Fig. Ia. 1) [6].

Despite the intensive prostate growth observed prenatally, the gland does not become static after birth. Prostate maturation is concluded during puberty, whereas androgens-mediated paracrine signaling between mesenchymal/stromal and epithelial compartments are maintained throughout life. This explains, at least in part, the tendency of prostate growth with ageing and its association with elderly disorders, such as benign prostate hyperplasia (BPH) and carcinoma [7,8]. Some of the developmental genes are also expressed in mature prostatic ducts. These include the homeobox protein Nkx-3.1 and the transcription factor SOX-9, which can be observed in luminal and basal cells, respectively, though their expression is lower when compared with phases of active growth [9].

The human adult prostate is a histologically heterogeneous gland. Several alternative models had emerged over decades to describe the adult prostate anatomy [10]. The currently accepted model considers three distinct glandular zones—peripheral, central, and transition—which are surrounded by a fibromuscular stroma [11]. The peripheral zone is the largest (~70% of the prostatic glandular tissue), constituting the site of origin of most prostate carcinomas (70–80%) and other prostatic disorders (e.g. chronic prostatitis and post-inflammatory atrophy) [8]. The central zone is the area that surrounds the ejaculatory ducts, whereas the transition zone surrounds the urethra and is the main responsible for prostate enlargement in BPH [8,11].

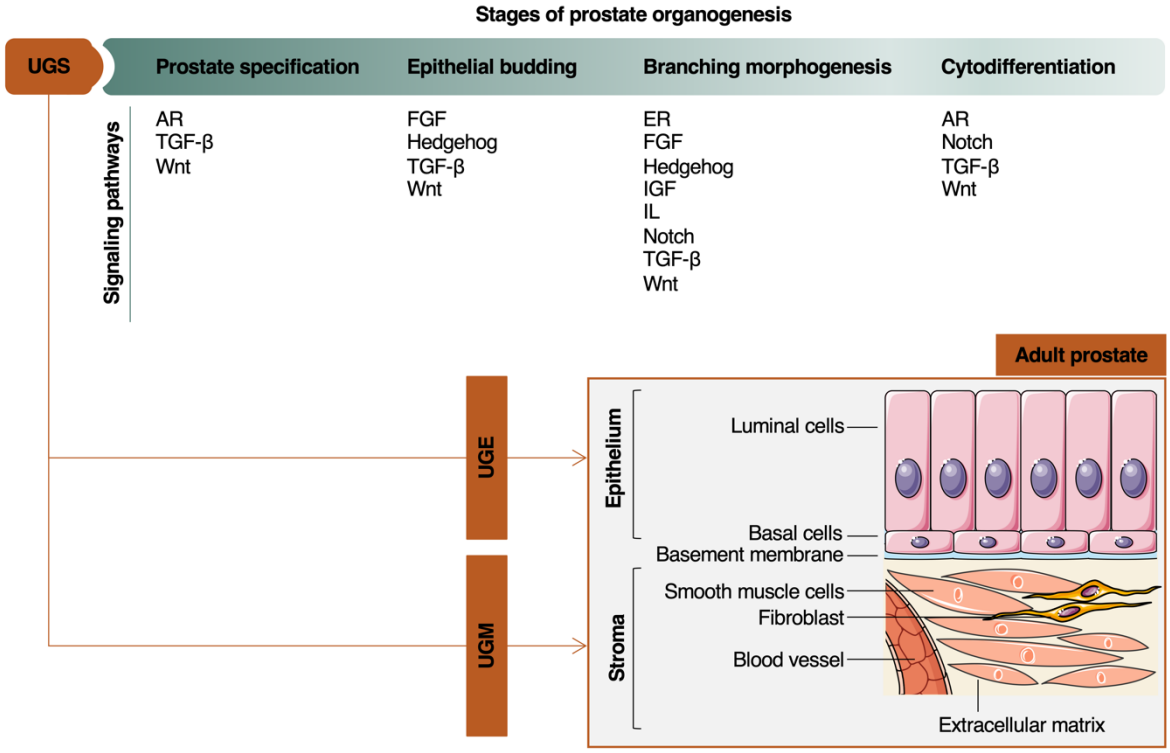

Fig. Ia. 1

Stages involved in prostate organogenesis, principal signaling pathways implicated in each stage and cellular composition of the adult gland. The mediators of the signaling pathways involved in each stage were mostly found in knockout studies using rodent models [2,3]. Figures were produced using Servier Medical Art (https://smart.servier.com). Abbreviations: AR, androgen receptor; ER, estrogen receptor; FGF, fibroblast growth factor; IGF, insulin-like growth factor; IL, interleukin; TGF-β, transforming growth factor beta; UGE, urogenital sinus epithelium; UGM, urogenital sinus mesenchyme; UGS, urogenital sinus.

The mature human prostate is composed by 30-50 tubuloalveolar glands specialized in the production and excretion of prostatic fluid. The central lumen is lined by a stratified epithelium of secretory tall columnar or luminal cells (major functional component of the prostate) and basal cells (Fig. Ia. 1). Other much less frequent cells include stem cells, which are believed to lay primarily in the basal cell population, and neuroendocrine cells, that can result from the differentiation of precursor stem cells. The luminal secretory cells are androgen-dependent and highly differentiated to produce prostatic fluid. On the other hand, basal cells are not entirely androgen-dependent (despite being androgen-sensitive) and are believed to sustain ductal integrity and survival of luminal cells [1,12]. The epithelial compartment is surrounded by a mesenchymal-derived stroma (Fig. Ia. 1), that differs between the different zones of the prostate and is affected by ageing. Although, the knowledge on stromal differentiation is still limited, smooth muscle cells and fibroblasts have been consistently referred as the main components. Immune cells, as well as vascular and neural components can also be found within the stroma [13].

2. Prostate dependence on androgens and mesenchymal/epithelial interactions: observations from tissue recombinant experiments

Androgens act by binding to the androgen receptor (AR), a ligand-dependent nuclear transcription factor that belongs to the nuclear steroid receptor superfamily. In the absence of functional AR or androgen deficiency, prostate development is largely impaired or nonexistent [2,4]. As shown by tissue recombinant experiments, the development of a fully functional gland occurs when wild-type (wt)-UGM and wt-UGE are combined (Table Ia. 1). In contrast, androgen-insensitive UGM from testicular feminization (tfm) mouse models[1] originates vagina-like structures when combined with UGE, and the prostate originated from tfm-UGE/wt-UGM combination lacks secretory function [2,4]. Hence, for prostate adequate growth, the mesenchyme must express AR and be androgen target organ (also corroborated by experiments that used mesenchyme from seminal vesicle and skin origin) (Table Ia. 1). Interestingly, wt-UGM was reported to induce the differentiation of bladder epithelium (a highly specialized non-glandular, AR-negative epithelium) into prostatic epithelium [4]. Altogether, these findings suggest the androgenic effects on prostatic epithelium morphogenesis are mediated through mesenchymal-epithelial interactions, rather than epithelial AR signaling itself.

Table Ia. 1
Experimental evidence of the requirement of androgens and mesenchymal-epithelial interactions for prostate development in male rodent models.

Tissue recombination			Epithelium				
			None	Seminal vesicle	Bladder	UGE	
						wt	tfm
Mesenchyme	None					Undifferentiated epithelium	
	Seminal vesicle					Prostate	
	Skin					Keratinized epithelium	
	UGM	wt	Fibromuscular tissue	Seminal vesicle	Prostate	Prostate	Prostate
		tfm				Vagina	Vagina

Abbreviations: tfm, testicular feminization; UGE, urogenital sinus epithelium; UGM, urogenital sinus mesenchyme; wt, wild-type.

In line with this thought, AR expression was shown to be restricted to the UGM prior to and during prostatic bud formation [4]. Recently, the ability of prostatic epithelium in inducing UGM differentiation into smooth muscle and to regulate smooth muscle architecture was also reported [4].

Two models have been proposed to explain how the epithelial budding into the surrounding UGM initiates: the andromedin model and the smooth muscle model [14]. According to the androme-

[1] *AR*-mutant mice, insensitive to androgens, that do not have prostate.

din hypothesis, AR-mediated signaling in the UGM leads to the production of one or more paracrine signaling factors, known as andromedins, that act on the UGE to promote growth and differentiation (Fig. Ia. 2A). Several molecules have been suggested as candidate andromedins, such as growth factors and members of the Wnt signaling pathway (see Section 5). The smooth muscle hypothesis defends the existence of localized and reciprocal mesenchymal-epithelial signaling and the presence of a smooth muscle layer that acts like a barrier between UGM and UGE to block excessive budding and outgrowth. In this model, androgens control epithelial budding by regulating the differentiation of smooth muscle [14]. The models are not mutually exclusive and might occur in simultaneous.

3. Androgens and androgen-mediated pathways in the prostate

Two natural androgens exist in mammals: testosterone, the major androgen secreted from testes, and 5α-dihydrotestosterone (DHT), the main androgen in prostate. Testosterone can be found in circulation bound to sex hormone-binding globulin (SHBG) (or other transporter proteins, such as albumin) or in its free and active form, which is able to translocate into prostatic cells (Fig. Ia. 2). Although both testosterone and DHT can bind to the AR, testosterone functions as a prohormone in prostate, where it is converted to DHT, a five-fold more potent androgen, by 5α-reductase enzymes (Fig. Ia. 2A) [15]. Two isoenzymes, 3-oxo-5-alpha-steroid 4-dehydrogenase 1 (SRD5A1) and 2 (SRD5A2), have been identified in both epithelial and stromal cells, but SRD5A2 has been indicated as the main isoenzyme expressed in stromal cells [16].

Androgens not only control fetal and neonatal prostate organogenesis, but they also participate in prostate growth during puberty and regulate the function and homeostasis of the mature adult gland [17,18]. In fact, the adult prostate remains exquisitely sensitive to withdrawal of circulating androgens and, in response, undergoes tissue atrophy, that can be reversed by their re-administration. To exert their functions, androgens can act directly on the prostatic epithelium via epithelial AR, which was shown to be expressed in the later phases of prostate development, or indirectly via stromal AR-induced secretion of paracrine mediators Fig. Ia. 2B). Unlike other organs, most of the mesenchymal/stromal-epithelial interactions in prostate are androgen-dependent, but the mechanisms responsible for tissue-specific AR signaling in physiological conditions remain largely unexplored. Nonetheless, the comparison between different androgen-responsive tissues from rodent models (including prostate, kidney, and epididymis) showed minimal overlap among AR-mediated transcription events [19]. Moreover, although several research teams have attempted to clarify which molecules are induced by androgen signaling in the prostate, the reduced similarities between data sets abrogated comparisons (most probably a reflection of the prostate tissue heterogeneity) [20–23]. Additionally, many studies that have examined androgen-mediated gene expression focused on epithelial cells and undervalued the mesenchymal/stromal compartment.

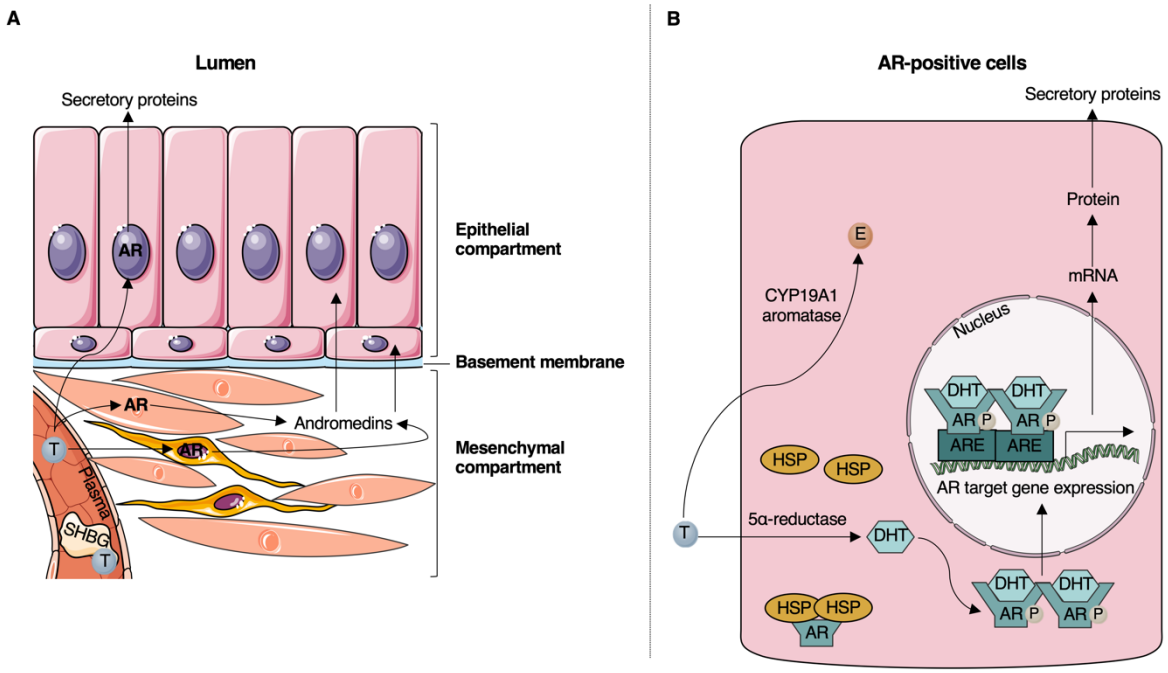

Fig. Ia. 2

Androgen-mediated genomic signaling in the prostate gland. **(A)** Circulating testosterone (T) can act directly in epithelial cells by binding to the androgen receptor (AR) or they can activate the AR signaling in stromal cells, resulting in the secretion of growth factors and survival molecules that act in luminal and basal cells from the epithelial compartment. **(B)** Androgen-mediated genomic pathway. Figures were produced using Servier Medical Art (https://smart.servier.com). Abbreviations: ARE, androgen response elements; DHT, 5α-dihydrotestosterone; E, estrogen; HSP, heat shock protein; P, phosphorylated; SHBG, sex hormone-binding globulin.

4. More for hormones in the prostate

Prostate morphology and physiology have long been thought to be almost exclusively dependent on hormonal control by androgens. Although androgen-mediated signaling is still considered the central player, it is currently known that other steroid and non-steroid hormones regulate complex gene networks involved in prostate organogenesis and homeostasis. To increase complexity, several studies reported the local production of hormones and hypothesize relevant autocrine and/or paracrine roles beside their conventional endocrine functions.

4.1. Sex steroid hormones

Estrogens and progesterone are mostly known for their actions on female reproduction, but they also exhibit important functions on males. The levels of 17β-estradiol (E2), the most potent estrogen in circulation, are maintained generally low, with peaks observed only during embryogenesis and aging [24]; while the circulatory levels of progesterone in males are quite similar to those observed in women (not considering the luteal phase) [25]. With aging, the levels of free circulating testosterone decrease with concomitant increase in the levels of free circulating E2, increasing the E2-to-testosterone ratio. This leads to the reactivation of prostate growth and is associated with malignant transformation [26].

The gonadal production of estrogens in males had long been thought to be restricted to Leydig cells. It was only in the 90s that other structures of the male reproductive tract became recognized as sources of estrogens (in fact, only about 20% are produced by testes) [26–28]. In the prostate, testosterone can be converted into estrogens in a reaction catalyzed by the aromatase enzyme CYP19A1, which is expressed by stromal cells (Fig. Ia. 2). Therefore, the estrogens function in the prostate is complex and diverse, involving both endocrine (via pituitary gland to decrease testosterone synthesis in testes) and prostate-specific actions, since estrogens can act independently of the circulatory levels via autocrine and/or paracrine signaling [24].

Like androgens, estrogens and progesterone can signal through genomic and non-genomic pathways. Classically, estrogens and progesterone bind to and activate estrogen receptors (ERs) and progesterone receptors (PRs), respectively, which act as transcription factors. The unbound receptors localize on the cytoplasm in complexes with heat shock proteins (HSPs). Upon ligand binding, the receptors are released from the complexes and undergo conformational changes that allow their translocation to the nucleus and binding to specific response elements in the DNA, thereby inducing the transcription of target-genes [26,29].

ERs have two isoforms, ER-α and ER-β, which are found in higher levels in the prostate than in neighboring structures, such as seminal vesicles and urethra [26]. Differential expression of the receptors is consistent with isoform-specific downstream signaling and distinct actions in the prostate (Table Ia. 2).

Table Ia. 2
Prostate-specific actions of estrogen receptors isoforms.

Receptor	Preferential localization	Effects on prostate	References
ER-α	Stroma	(+) Branching morphogenesis (fibroblast ER-α) (+) Stromal cell proliferation (smooth muscle ER-α) (+) Extracellular matrix deposition (smooth muscle ER-α) (+) Stem cell self-renewal (+) Progenitor cell proliferation (+) Prostate squamous metaplasia*	[26,30–33]
ER-β	Epithelium	(–) Growth (+) Epithelial cell differentiation (–) Epithelial-mesenchymal transition (+) Apoptosis (–) Inflammation (+) Progenitor cell differentiation (–) Stem cell self-renewal	[26,30,34,35]

* Condition characterized by the total replacement of the columnar secretory epithelium by layers of stratified squamous cells (reversible following removal of the estrogenic stimulus). It is a direct effect of ER-α in the prostate, in which ER-α-mediated paracrine signaling is required to elicit estrogen-induced prostatic squamous metaplasia: stromal ER-α stimulates epithelial proliferation, while epithelial ER-α mediates epithelial squamous differentiation.

Important findings concerning ER-β signaling have emerged mainly from studies on the prostate. ER-β is believed to oppose AR signaling on prostatic epithelium to restrain proliferation and inflam-

mation [30]. Prostatic epithelium also expresses ligand-independent ER-β isoform variants that can act as either constitutive activators, transcription enhancers, or dominant negative regulators of estrogen action. Additionally, estrogens can signal through G protein-coupled estrogen receptors (GPERs) and downstream mitogen-activated protein kinase (MAPK) signaling [26].

PRs also exist in two isoforms: PR-A and PR-B. Although the expression of PRs had been reported in stromal cells, particularly in a subset of fibroblasts and smooth muscle cells, their presence in the epithelial compartment is still controversial [36]. In humans, stromal PR suppresses prostate stromal cell proliferation by inhibiting cell cycle progression, despite isoform-specific regulation of gene transcription has been also described [37].

4.2. Non-steroid hormones

Several non-steroid hormones have also been implicated in prostate development and homeostasis. The prostate is responsive to prolactin and oxytocin, two polypeptide hormones secreted by the central nervous system that are mostly known for their roles during pregnancy and after childbirth [38,39]. Local production of both prolactin and oxytocin is observed in other tissues, including the prostate, which suggests potential autocrine and/or paracrine actions besides their classical endocrine routes [40].

Prolactin can be detected in males' circulation, though at lower levels than in females. It binds to the prolactin receptor (PRL-R), a member of the class I cytokine receptor superfamily, which is expressed by human prostate cells [40]. The intracellular signaling is then transduced by multiple non-receptor tyrosine (Tyr) kinases that interact with the PRL-R's intracellular domain, for the most common belonging to the Tyr-protein kinase JAK (JAK)/signal transducer and activator of transcription (STAT) pathway [41]. Similar to other tissues, prolactin signaling in the prostate seems to be primarily mediated through the long isoform of the PRL-R (despite the presence of a short PRL-R form is also reported [40]) and its downstream effectors from the canonical PRL-R/JAK2/STAT5 pathway. In addition, it may signal through non-canonical pathways involving the AR [38].

Several reports suggest that prolactin regulates prostate development, growth, and function [38]. Most studies, nonetheless, used rodent models and lack confirmation in humans. Prolactin induces growth and differentiation of the prostate epithelium [38,42]. These effects can be in part explained by a synergistic action with androgens, but also by androgen-independent mechanisms [38]. Prolactin activates two protein kinase C (PKC) isoforms, PKC-A and PKC-E, both identified in prostate epithelial cells. Prolactin-mediated activation of PKC-E is involved in the stimulation of the mitochondrial aspartate aminotransferase, a key citrate synthesis regulatory enzyme, and other metabolic entities [39]. Interestingly, these effects are likely to be cell-specific, since responsiveness to prolactin varied in rat cells derived from distinct prostate zones [39]. Even slight serum levels prolactin elevations (which tend to occur with aging [43]) were shown to produce significant changes in rats'

prostate epithelium, although no effect was observed regarding sexual behavior [44]. Moreover, it was shown that local overexpression of prolactin leads to the expansion of the stem cell subpopulation in rodents' prostate, which is likely to be involved in malignant transformation [45]. Prolactin was also shown to increase prostatic uptake and metabolism of testosterone in patients with prostate cancer (PCa) [42].

Oxytocin, oxytocin-associated neurophysin, and oxytocin receptor (OT-R) are present in both epithelial and stromal cells, despite the preferential localization in the epithelial compartment [46]. Oxytocin binds to the OT-R, which belongs to the G protein-coupled receptor (GPCR) superfamily and stimulates multiple signaling pathways [47].

Oxytocin has been suggested to promote prostate growth by stimulating mitosis and inhibiting apoptosis, but the mechanisms are not fully understood. Nonetheless, it has also been implicated in the regulation of prostate steroidogenesis and contractility, being a more effective constrictor than adrenergic agonists. Comprehensive reviews on the roles of oxytocin in prostate can be found elsewhere [48,49]. In humans, oxytocin expression, secretion, and mitogenic activities seem to be induced by complex interactions with androgens and estrogens [50,51]. These interactions are likely to be maintain in prostatic diseases [52,53].

The prostate is the major producer and secretor of the thyrotropin-releasing hormone (TRH) and TRH-like peptides among the organs from the male reproductive tract. According to ancient publications, TRH concentrations in rat ventral prostate could exceed those observed in the hypothalamus [54]. Its levels and biosynthesis are hormonally controlled by, for instance, thyroid hormones and androgens [54–56]—remarkably, this constituted the first evidence that a neuropeptide could be under hormonal control in an extra-hypothalamic site [54].

The existence of a prostate-thyroid axis has been demonstrated in rodent models. Prostatic TRH stimulates the secretion of thyroid hormones, both triiodothyronine (T3) and its prohormone thyroxine (T4), either directly or via the pituitary gland [57]. Thyroid hormones signaling in the prostate is far from being understood, but thyroid hormones receptors are expressed by human prostate cells [58]. Proper expression and activity of thyroid hormones during pre- and neonatal periods are determinant for AR status in adult prostate [59]. T4 acts directly on rat prostate gland to increase the release of calcitonin gene-related peptide (CGRP), which is produced by a subpopulation of neuroendocrine cells [60]. Calcitonin levels are also higher in the prostate than in other human organs (apart from those found in the thyroid gland, the main source of circulating calcitonin). These findings suggest that the prostate is the main source of the seminal calcitonin and that this peptide family may play important physiological roles [61]. Locally produced calcitonin may induce the release of the prostatic acid phosphatase (PAP) from rat prostate explants, possibly by interacting with the prostatic cholinergic system [62]. In contrast to the lack of information regarding physiological roles,

calcitonin involvement in prostate carcinogenesis has been widely reported [63]. Conversely, T3 stimulates the activity of prostatic glycosidases, which are important mediators of the glycoprotein metabolism [64]. Prostate hyperplastic conditions have been associated with high levels of T3, that induce cell proliferation and stimulate the expression of prostate-specific antigen (PSA) [65,66].

5. Prostate regulation by growth factor signaling

In addition to hormones, several growth factors are involved in the regulation of prostate growth, differentiation, and homeostasis. These regulators are mostly locally produced to act via autocrine and/or paracrine signaling (Fig. Ia. 3). As referred in Section 3, androgens stimulate stromal cells to synthesize growth and survival factors that are internalized by epithelial cells, where they activate signaling pathways that modulate cell proliferation (Fig. Ia. 2). The first evidence of such paracrine modulators belongs to the FGF family, although their assured relevance in prostate physiology, their classification as andromedins is still a focus of major discussion [67]. In fact, no growth factor expressed in the mesenchyme has been previously identified as being a direct target of androgenic regulation (*in vitro* and *in vivo* studies are not consistent).

In addition to FGFs, a whole range of growth factors has been reported in the prostate, including epidermal growth factors (EGFs), insulin-like growth factors (IGFs), and transforming growth factors (TGFs) [68,69]. It is worth mentioning that almost all these studies were performed in rodent models, whose prostates exhibit relevant anatomical and histological differences from humans' [70]. In fact, a study that analyzed human tissues, both fetal and adult (from BPH), reported differential expression of growth factors according to the developmental stage, with relevant differences to the findings in rodents: TGF-α, TGF-β1, TGF-β2, TGF-β3, and EGF were observed in human fetal prostate, but not FGF-7 or the receptors FGFR-2 and EGFR; on the other hand, all growth factors and corresponding receptors were found in human adult prostate, except for FGFR-2 [69]. Therefore, extrapolations should be done with care and efforts should be done to unveil human prostate organogenesis.

FGFs are key molecules for organ development in general and comprehensive reviews on their role in prostate development can be found elsewhere [67,71,72]. Among its members, FGF-7 and FGF-10 are particularly important for prostate development. Their highest concentrations are achieved during periods of active prostatic growth, and whilst some level of FGF-7 expression is maintained in the organ throughout life, FGF-10 expression in growth quiescent adult organ is residual. Both FGF-7 and FGF-10 act as mitogens for prostate epithelial cell via paracrine signaling (Fig. Ia. 3). FGFs bind to and activate transmembrane Tyr kinase FGFRs and the intracellular signaling is transduced mainly by MAPKs [73]. FGFR-2-mediated signaling is particularly important to maintain epithelial cell proliferation and branching of mice's developing prostate [71], as well as to prevent

stem cell differentiation (Fig. Ia. 3) [74]. Two isoforms of the FGFR-1 were identified in the prostate: FGFR-1A is primarily expressed by luminal epithelial cells, whereas FGFR-1B, which is believed to have greater affinity to member of the FGF family, is the main variant in smooth muscle cells and basal epithelial cells [75]. Additional members of the FGF signaling have been identified in the prostate, but their roles are not yet clarified (Fig. Ia. 3). As inferred from the ancient studies that suggested FGF-7 as an andromedin, FGF signaling is, at least in part, regulated by androgens [76], but FGF-7 was also shown to be capable of activating the androgen signaling pathway in epithelial cells [77].

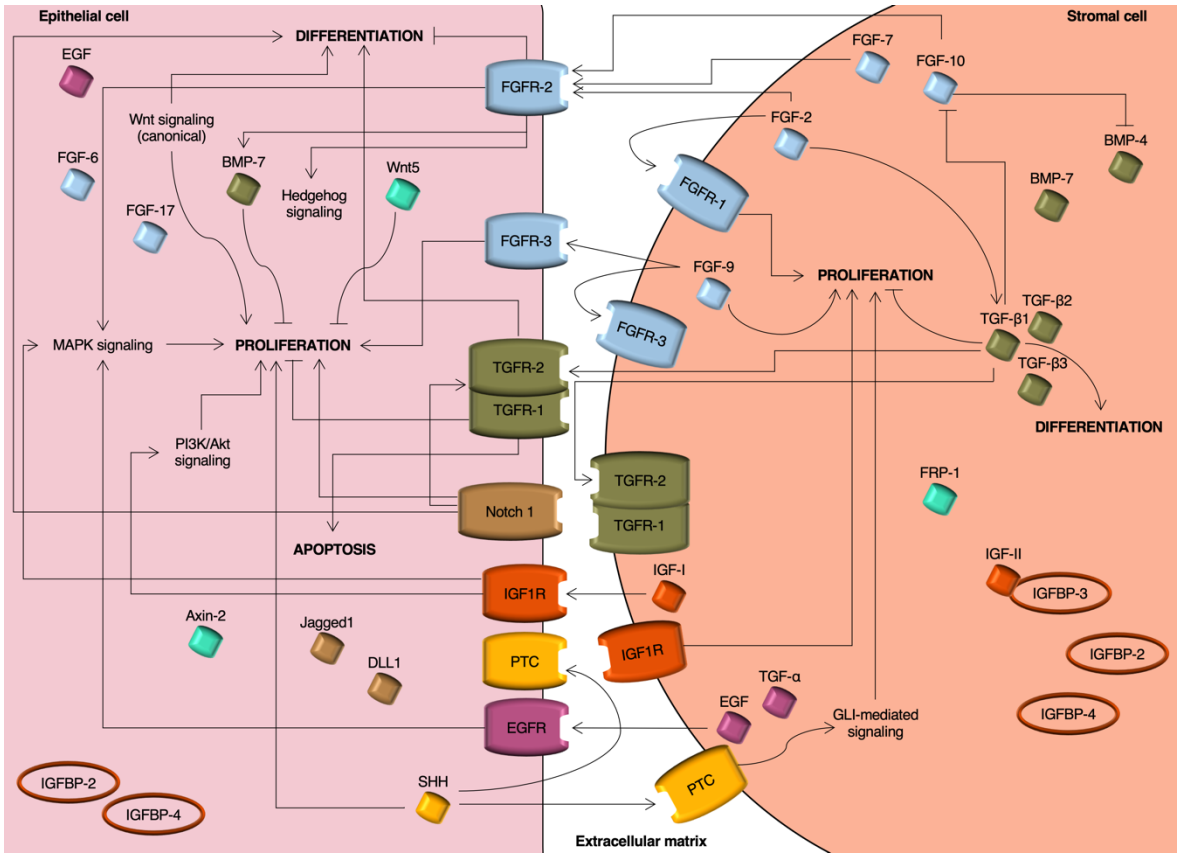

Fig. Ia. 3

Simplified schematic representation of the potential growth factor signaling network that regulates prostate homeostasis. Different colors correspond to different signaling pathways. Arrows indicate positive regulation and blind-ended arrow indicate negative regulation. Squares represent ligands, rectangles receptors and circles binding-proteins. Figures were produced using Servier Medical Art (https://smart.servier.com). Abbreviations: BMP, bone morphogenetic protein; DLL1, delta-like protein 1; EGF, epidermal growth factor; EGFR, EGF receptor; FGF, fibroblast growth factor; FGFR, FGF receptor; FRP-1, secreted frizzled-related protein 1; GLI, oncogene GLI; IGF, insulin-like growth factor; IGFBP, IGF-binding protein; IGF1R, IGF-I receptor; MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide-3-kinase; PTC, protein patched homolog 1; SHH, sonic hedgehog protein; TGF, transforming growth factor; TGFR, TGF receptor.

EGF and IGF signaling also have mitogenic effects on the prostate (Fig. Ia. 3). EGF and related polypeptides, such as TGF-α and amphiregulin, are expressed in normal prostate cells and are important regulators of prostate epithelium proliferation during early stages of development and of structural integrity in adult prostate. EGF and TGF-α bind to and activate the Tyr kinase receptor

EGFR, which is expressed on the basolateral surfaces of prostate epithelial cells [78]. While EGF is mainly produced by prostate epithelial cells and is secreted to the prostatic fluid at high concentration (the prostatic fluid contains the highest concentration of EGF in the human body), TGF-α is secreted in low amounts by prostate stromal cells (it is mainly secreted by tumor cells where it acts via autocrine signaling to promote growth) [78]. Therefore, EGF is believed to be the major EGF-related growth factor in the normal adult prostate [79]. When activated, EGFR signals through numerous intracellular pathways [78]. For instance, EGFR is required to interleukin-6 (IL-6)-mediated activation of MAPK signaling in prostate epithelial cells [80].

IGF signaling is complex; two highly homologous ligands—IGF-I and IGF-II—might signal through IGF receptors (IGF1R and IGF2R) and insulin receptor, and their effects are modulated by binding to IGF-binding proteins (IGFBPs) (in fact, most of the free IGF is bounded to IGFBPs). Several members of the signaling have been identified in prostate cells (Fig. Ia. 3). IGF ligands are produced by prostatic stromal cells (mainly smooth muscle cells) in response to androgen stimulation [81]. Since IGF1R is localized in epithelial cells, IGF signaling works in a paracrine manner to stimulate proliferation and induce basal to luminal differentiation of prostatic epithelial cells [82,83]. In terms of efficiency, IGF-I was shown to be a more potent mitogen than IGF-II or insulin [68]. The IGF-associate mitogenic effect is primarily mediated by the downstream activation of the phosphoinositide-3-kinase (PI3K)/RAC-alpha serine/threonine-protein kinase (Akt) and MAPK pathways [84].

In the opposing side of balanced growth regulation is the TGF-β signaling (Fig. Ia. 3). TGF-β is a large gene superfamily that encompass TGF-β, bone morphogenetic proteins (BMPs) and activins. Their effects in the prostate are complex since they can be either inhibitors or stimulators depending on the concentration of the mediators. For instance, TGF-β1 inhibits prostate growth, but was shown to promote branching morphogenesis in rat models, which support distinct roles according to prostate zones [85]. The function of the TGF-β signaling in the prostate, as well as crosstalk with other signaling pathways was reviewed in detail elsewhere [86–88]. TGF-β ligands bind to the TGF-β receptor type-2 (TGFR-2), which then form heterodimers with TGFR-1. The heterodimeric receptor has serine (Ser)/threonine (Thr) kinase activity and activates the downstream intracellular signaling through phosphorylation of protein Smads. Several studies support the major role of TGF-β1, BMP-4, and BMP-7 ligands in prostate development. BMP signaling through the receptor BMPR-1A regulates prostatic epithelial differentiation by controlling the *NKX-3.1* regulatory gene, one of the earliest markers of prostate development [89]. TGF-β signaling also cooperates with other signaling pathways to accomplish its functions. A positive feedback loop involving TGFR-1 and Notch signaling is linked to the homeostasis of prostate basal cells [90].

6. Additional pathways involved in prostate paracrine signaling

6.1. Hedgehog signaling pathway

Hedgehog signaling pathway regulates complex morphogenic processes during embryonic development of several tissues as well as adult organ homeostasis and regeneration [91,92]. Three ligands—sonic hedgehog (SHH), Indian hedgehog, and desert hedgehog—bind to the patched (PTC) transmembrane receptor and attenuate the smoothened receptor inhibitor. The intracellular signal transduction positively regulates members of the GLI-Kruppel family of transcription factors, resulting in proliferative stimulus [91,92].

Hedgehog signaling regulates prostate epithelial proliferation and ductal morphogenesis in a developmental stage- and hormonal environment-dependent way; paracrine signaling promotes epithelial proliferation and budding prenatally, while inhibits these processes postnatally [93]. SHH is the most abundantly expressed and is believed to be the master ligand in the developing prostate, despite functional redundancy among ligands is reported [94]. SHH expression level increases with the onset of ductal budding, peaks during active prostatic bud elongation (when it also re-localizes to sites of active growth of the UGE) and diminishes gradually until residual levels. Both SHH expression level and localization are dependent on the expression of testicular androgens [95]. SHH expression induces the expression of target genes in the adjacent UGM, including members of the Hedgehog signaling, thus establishing an autoregulatory feedback loop [93,95–97]. Notwithstanding the predominance of paracrine signaling, focal expression of PTC and GLI in the epithelium of growing prostatic duct tips also support the existence of autocrine signaling in prostate development [98], which is believed to promote the proliferation of progenitor cells at the bud tip [99]. The activity of the Hedgehog signaling pathway in the adult prostate is limited (and less understood), but at least the paracrine mode of action is thought to be preserved (Fig. Ia. 3). SHH is expressed from basal epithelial cells and binds to receptors in the surrounding stromal stem cells [100]. The association and function of Hedgehog signaling in prostate was recently reviewed by several authors [92,99,101].

6.2. Notch signaling pathway

The Notch signaling pathway mediates cell-cell interactions and is essential to maintain tissue integrity. The mammalian neurogenic locus notch homolog protein family consists of four highly conserved transmembrane receptors, Notch 1-4, which establish physical interactions with ligands that are expressed in the membrane of the neighboring cell. Five canonical ligands are identified in humans: two jagged ligands (Jagged1 and Jagged2), and three delta-like ligands (DLL1, DLL3 and DLL4). The ligand binding triggers a series of proteolytic cleavages on the receptor and the intracellular domain translocate to the nucleus where it takes part of a transcriptional activation complex that regulates several target genes' expression [102].

Differential expression of Notch signaling components has been shown in the human prostate epithelium, although with some discrepancies among studies. Most reports refer to Notch 1, whose expression was found in both cultured prostate cells and human prostate tissues, but Jagged1, Jagged2, DLL1, and Notch 2 expression was also found in cultured prostate cells [103].

Notch signaling is involved in prostate formation, development, and maintenance [104]. Functional studies in rodent models showed that it controls the growth of prostatic progenitor cells, promotes luminal cell differentiation, and downregulates AR activity [105,106]. Notch 1 is temporally and spatially regulated in rodent's prostates during normal development. It is essential for prostatic branching morphogenesis in the developing prostate and for prostatic re-growth in adults [107]. *Notch1*-inducible knockout mice displayed similar prostatic morphological alterations to those with *Nkx3-1* or the retinoblastoma-associated protein-coding gene *(Rb1*) deficiency, showing uncontrolled proliferation of prostatic epithelial cells and impaired differentiation [105]. *NOTCH1* knockdown was found to affect multiple signaling cascades, leading to significant mRNA levels increase of proto-oncogene c-Fos (c-Fos), transcription factor AP-1 (c-Jun), FGF-18, and prostate stem cell antigen [105].

6.3. Wnt signaling pathway

Wnt is a large family of secreted glycoproteins with multiple biological functions. Wnt canonical signaling is catenin beta-1 (CTNNB1)-dependent and is triggered by the binding of Wnt ligands to frizzled cell surface receptors [108].

Several studies have provided evidence for a central role of canonical Wnt signals in prostate formation [109–111]. A role for canonical Wnt signals in early prostate development has been suggested by the expression of numerous Wnt ligands in both UGM and UGE prior to and during prostate formation [109]. In fact, several Wnt ligands, as well as co-activators of the canonical Wnt pathway, display a sexually dimorphic expression pattern and are specifically detected in male UGS [111]. Furthermore, recent studies have shown that deletion of *CTNNB1* impairs prostate specification and bud formation, with only residual levels of the developmental genes *NKX3-1* and *HOXB13* being detected, suggesting an essential role for canonical Wnt signaling in prostate formation (Fig. Ia. 1) [110,112,113]. Importantly, Wnt signaling regulates many other processes in the developing prostate, including branching morphogenesis, proliferation of epithelial progenitor cells, and luminal cell differentiation [114].

Additionally, Wnt signal might be transduced through non-canonical, CTNNB1-independent pathways [115]. The non-canonical protein Wnt-5 was specifically associated with the regulation of prostate buds' position and size and it has a negative effect in epithelial proliferation and branching morphogenesis in rodent models [116]. The levels of secreted frizzled-related protein 1 (FRP-1), a Wnt antagonist, was also shown to be rather high in the developing mesenchyme and low in the adult

prostate of a mouse model [117]. Indeed, increased FRP-1 expression leads to enhanced epithelial proliferation and decreased expression of secretory proteins, indicating a signal is transduction through a non-canonical pathway [118].

7. Is it reasonable to talk about a prostate-specific proteome?

As reviewed in the prior sections, several signaling pathways were reported as essential for prostate-specific growth and differentiation, since the early steps that guide prostate specification from the UGS. Moreover, the prostate exhibits interesting features, such as its high capacity to store Zn^{2+} and its tendency to grow with aging. Given that proteins do most of the work in cells, regulating their morphology, function, and metabolism, it is reasonable to ask whether a prostate-specific proteome might exist.

According to the Human Protein Atlas database (date of access: 12/07/2018), 73% of all human proteins are expressed in the prostate, and despite the currently unknown existence of prostate exclusive proteins, the expression of 183 genes is higher in the prostate when compared with other tissues. From those, 20 are classified as prostate enriched genes (Table Ia. 3) and 51 are group enriched genes (most sharing expression with testis and cerebral cortex). The prostate enriched genes are expressed by the epithelial cells and mostly encode for secreted or membranous proteins [119], which is consistent with its dependence on autocrine and/or paracrine signaling.

Table Ia. 3
Prostate enriched genes.

Gene name	Protein name	Gene name	Protein name
ACPP	Prostatic acid phosphatase	*OR51E2*	Olfactory receptor 51E2
CHRNA2	Neuronal acetylcholine receptor subunit alpha-2	*RDH11*	Retinol dehydrogenase 11
		RFPL2	Ret finger protein-like 2
COL9A1	Collagen alpha-1(IX) chain	*RLN1*	Prorelaxin H1
KLK2	Kallikrein-2	*SLC30A4*	Zinc transporter 4
KLK3	Prostate-specific antigen	*SLC45A3*	Solute carrier family 45 member 3
KLK4	Kallikrein-4	*SP8*	Transcription factor Sp8
MSMB	Beta-microseminoprotein	*STEAP2*	Metalloreductase STEAP2
NCAPD3	Condensin-2 complex subunit D3	*TGM4*	Protein-glutamine gamma-glutamyltransferase 4
NEFH	Neurofilament heavy polypeptide		
NKX3-1	Homeobox protein Nkx-3.1	*TRPM8*	Transient receptor potential cation channel subfamily member 8

Data available from https://www.proteinatlas.org/humanproteome/prostate, v18 (date of access: 12/07/2018) [119].

8. So why does the 'prostate-specific signaling' matter?

The prostate gland is associated with major disorders of elderly, such as BPH and carcinoma. Over 80% of men aged more than 80 are likely to harbor BPH [120], whereas PCa is the most prevalent cancer in men worldwide [121]. Indeed, these facts, together with the increased population aging, constitute major health concerns.

BPH and PCa are characterized by abnormal growth of the gland. Hence, they might result, at least in part, from the unbalanced action of the signaling pathways involved in prostate development (giving emphasis to the need of a deeper understanding of prostate biology).

8.1. Androgen signaling as target for prostate cancer therapeutics

Similar to normal cells, AR signaling remains essential for growth and survival of PCa cells [122]. This dependency is exploited in androgen deprivation therapy (ADT), the mainstay therapeutic regimen, which encompasses surgical (orchidectomy) or chemical castration (by using luteinizing hormone-releasing hormone/gonadotropin-releasing hormone agonists or antagonists). This treatment aims to decrease circulating testicular androgens, depriving AR of its activating ligand and consequently hindering its pro-survival effects. Since adrenal glands also produce reduced amounts of androgens, the ADT is frequently complemented with the administration of antiandrogens [123]. These drugs act by competing with endogenous androgens for binding to the AR and often preclude AR access to the nucleus and to its transcriptional targets. Although, initially ADT is effective, about one third of the patients will relapse in 2-3 years with castration-resistant prostate cancer (CRCP), for which there is still no cure available. Even though CRPC becomes refractory to ADT, most CRPCs still express AR and its target PSA which implies that AR signaling is still active [124–126]. The reactivation of the AR signaling can be achieved through multiple mechanisms that may be broadly divided into ligand-dependent and ligand-independent. Ligand-dependent mechanisms include *AR* amplification and overexpression (leading to increased sensitivity to low levels of androgens), intra-tumoral steroidogenesis (*de novo* synthesis of androgens) and increased expression of AR co-factors (which facilitate AR's transcriptional function). Ligand-independent mechanisms include *AR* mutations (broaden AR's ligand-specificity) and constitutively active AR variants [121]. Hence, AR remains the main target in the context of CRPC.

Based on improved survival in Phase III clinical trials, two drugs have been approved for CRPC patients' treatment, abiraterone [127,128] and enzalutamide [129,130], which can be used in pre- or post-chemotherapy settings. Abiraterone is a selective and potent inhibitor of the steroid 17-alpha-hydroxylase/17,20 lyase (CYP17A1), an enzyme that is necessary for androgen biosynthesis and enzalutamide, a second-generation antiandrogen that competes with androgens for the binding to AR. Nevertheless, this therapy is not without caveats since not all CRPC patients respond to these drugs and resistance to treatment may develop with time [121]. Importantly, metastatic CRPCs frequently display AR expression heterogeneity, with cells exhibiting different AR expression levels or even AR loss. In fact, PCa with reduced, or even absent, expression of AR is increasingly more common, especially in patients previously treated with abiraterone and enzalutamide [131–133]. Thus, suggesting that at some point CRPCs evolve to become independent of the AR signaling, which poses serious concerns, due to the lack of an effective therapeutic option.

8.2. The complex crosstalk between signaling pathways in PCa

Non-androgenic pathways have also been shown to activate the AR. AR signaling may be activated by the crosstalk with signaling pathways (e.g., MAPK and PI3K/Akt) that are activated because of an extrinsic signal, namely growth factors (e.g., EGF and IGF-I) or cytokines (IL-6 and IL-8). Several peptide growth factors were reportedly increased during progression to CRPC and were suggested to regulate AR's transcriptional activity in androgen-depleted conditions [134]. They often do so by promoting the phosphorylation of AR itself or of its co-factors, increasing AR transcription [135].

On the other hand, AR signaling may also condition the expression of growth factors and a cooperative effect between growth factors and AR can be observed. This is the case of the androgenic induction of EGFR [136]. Cooperation between androgens and EGF promotes proliferation by stimulating the activity of cyclin-dependent kinase (CDK) 2, which facilitates G1-S transition [137]. Furthermore, IL-6 signals through EGFR modulate the expression of androgen-regulated *KLK3* [138]. The activation of AR by the EGFR-related receptor Tyr-protein kinase erbB-2 also seems to be required for optimal transcriptional activity of AR in PCa cells [139]. Indeed, heregulin-mediated erbB-2 activation induced activated CDC42 kinase 1 (ACK-1)-driven AR phosphorylation at Tyr-267/363, promoting AR's transcriptional activity independently of ligand stimulation [140].

IGF-I/IGF1R is an important growth promoting signaling pathway. In PCa, IGF-I signals via PI3K/Akt and MAPK/Ras GTPase signaling pathways to promote survival by phosphorylation (and consequent inactivation) of Bcl-2-associated agonist of cell death (BAD) [141]. Additionally, IGF-I or insulin promote PI3K/Akt-mediated phosphorylation of forkhead box protein O1, an AR co-repressor, rendering it inactive and consequently enhancing AR's transcriptional activity [141]. Conversely, upon ligand stimulation, AR can also regulate IGF-I/IGF1R signaling by modulating the expression of IGF-I [142], IGF1R [143] in PCa cells and IGFBPs [144] in normal fibroblasts.

In normal prostate, TGF-β released by stromal cells inhibits cell growth and promotes apoptosis, by inducing the expression of apoptosis regulator BAX, CDK inhibitor 1B and IGFBP3 and activating caspase-1. Conversely, TGFBR2 loss or mutation renders PCa cells refractory to the growth inhibitory effect of TGF-β signaling. Indeed, direct interactions between AR and TGF-β signaling mediators, Smad3 and Smad4, have been reported *in vivo* and *in vitro*. Nevertheless, there is still controversy on whether Smad3 has a negative or positive effect on the AR's transcriptional activity and on the dependency of a Smad3/Smad4 complex for a repressive effect [145,146]. The involvement of AR in the apoptotic effects mediated TGF-β signaling was demonstrated in a castration-resistant PCa cell line in which in the absence of DHT, AR expression reduced the TGF-β1/Smad transcriptional activity, thus preventing TGF-β1-induced apoptosis and growth inhibition [83]. In contrast, in an androgen-dependent PCa cell line, the treatment with DHT enhanced

TGF-β-induced apoptosis, via caspase-1 activation and targeting of the apoptosis regulator Bcl-2 [147]. Concurrently, bounded AR inhibits TGF-β signaling by preventing Smad3 binding to the Smad-binding elements [148].

In parallel, FGF/FGFR expression changes also seem to be associated with progression to CRPC. AR may alter the FGF-1, FGF2, FGF-8 and FGF-10 expression patterns' in both prostate tumor epithelial and stromal cells [83]. Remarkably, AR is up-regulated by paracrine FGF10 [149], which can induce the expression of FGF-2 and FGF-binding proteins (FGFBPs), hence facilitating the FGF-induced survival of PCa cells [150].

Moreover, vascular endothelial growth factor (VEGF), an angiogenic cytokine whose expression is induced by hypoxia-inducible factor 1-alpha (HIF-1-α) in response to low oxygen tension (hypoxia), might be activated by the AR. Specifically, in androgen sensitive tumors, AR promotes angiogenesis by inducing HIF-1-α which in turn activates VEGF [151]. Nonetheless, in androgen depleted conditions, intracellular reactive oxygen species (ROS) activate a small GTPase, Ras-related protein Ral-A, which, in turn, promotes vascular endothelial growth factor C upregulation. As a result, VEGFC increases the expression levels of BAG family molecular chaperone regulator 1 (BAG-1), an AR co-activator, thus contributing to increased AR transcriptional activity [152].

Interestingly, pro-inflammatory cytokines, IL-6 and IL-8, also seem to play a role in AR activation and were found overexpressed in PCa. IL-6 can act through the activation of different signaling pathways, namely c-Jun N-terminal kinase (JNK)/STAT3, MAPK and PI3K/Akt [153]. IL-6 treatment of androgen-dependent PCa cells led to AR transactivation through MAPK/STAT3 signal transduction pathways [138]. Additionally, IL-6 may also engage proto-oncogene Tyr-protein kinase Src-mediated direct phosphorylation of Tyr-534 of AR, which is a critical residue of AR's transcriptional activity in the presence of low doses of androgens [154]. Finally, IL-8 also activates AR and confers androgen-independent growth via Src and focal adhesion kinase 1 (PTK2) [155].

Altogether, these evidences suggest that despite being a central mediator in prostate biology, AR does not act alone; instead, it takes part of a complex interacting network capable of regulate the most diverse molecular events and biological processes that underlie cancer hallmarks. Therefore, the apparent re-awakening of key developmental signaling pathways and crosstalk during malignant transformation supports the relevance of characterizing, in-depth, such cascades in view of empowering translational medicine in the context of PCa.

Section b

Protein phosphatase 1 in tumorigenesis: is it worth a closer look?

KEYWORDS

Neoplasms
Cell signaling
Isoforms
Biomarkers
Therapeutics

ABSTRACT

Cancer cells take advantage of signaling cascades to meet their requirements for sustained growth and survival. Cell signaling is tightly controlled by reversible protein phosphorylation mechanisms, which require the counterbalanced action of protein kinases and protein phosphatases. Imbalances on this system are associated with cancer development and progression. Protein phosphatase 1 (PP1) is one of the most relevant protein phosphatases in eukaryotic cells. Despite the widely recognized involvement of PP1 in key biological processes, both in health and disease, its relevance in cancer has been largely neglected. Here, we provide compelling evidence that support major roles for PP1 in tumorigenesis.

Abbreviations*:* ADT, androgen deprivation therapy; AR-V7, androgen receptor variant 7; Asp, aspartic acid; Ca2+, calcium ion; CDK, cyclin-dependent kinase; CRPC, castration-resistant prostate cancer; C-terminal, carboxy-terminal; DNA, deoxyribonucleic acid; E2, 17β-estradiol; ECM, extracellular matrix; EMT, epithelial-mesenchymal transition; ERK, extracellular signal-regulated kinase; ERM, ezrin-radixin-moesin complex; FOXO, class O forkhead box transcription factor; His, histidine; HPV-16, Human papillomavirus type 16; HPV, Human papillomavirus; IRF, interferon regulatory factors; MAPK, mitogen-activated protein kinase; mCRPC, metastatic castration-resistant prostate cancer; miR, microRNA; mRNA, messenger RNA; N-terminal, amino-terminal; NSCLC, non-small cell lung cancer; PCa, prostate cancer; PKC, protein kinase C; PPP, phosphoprotein phosphatase; PP1, protein phosphatase 1; PP1c, PP1 catalytic subunit; PP2A, protein phosphatase 2A; PTM, posttranslational modification; RIPPO, regulatory interactors of PP1; RNA, ribonucleic acid; Ser, serine; siRNA, small interfering RNA; SNP, single nucleotide polymorphism; Thr, threonine; TLR, Toll-like receptor; Tyr, tyrosine; 4OHT, 4-hydroxytamoxifen.

1. Introduction

Protein phosphatases are a diverse group of proteins that catalyze the removal of phosphate groups from proteins through hydrolysis, thereby counterbalancing the action of protein kinases [156]. In eukaryotic cells, proteins are mainly phosphorylated on serine (Ser), threonine (Thr) and tyrosine (Tyr) residues; however, phosphorylation of histidine (His) and aspartic acid (Asp) residues, which was historically considered a prokaryotic style of protein regulation, has also been described [157,158]. Protein phosphatases are much more limited in number than protein kinases. According to a recent publication, the human genome includes about 189 genes that encode for protein phosphatases, and nearly 80 pseudogenes/retrogenes, some of which are predicted to have protein phosphatase domain and residual function. From the 189 protein phosphatases currently known, 40 have disease-associated variants and 12 are involved in cancer [159].

Protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) belong to the phosphoprotein phosphatase (PPP) superfamily of proteins and account for more than 90% of phosphatase reactions in eukaryotes. While the functions of PP2A in tumorigenesis have been extensively discussed [160-163], the roles of PP1 have mostly been overlooked. PP1 is a major Ser/Thr phosphatase, highly conserved in all eukaryotes and ubiquitously expressed. It regulates vital cellular processes, including cell cycle, meiosis, protein synthesis, apoptosis, cytoskeleton dynamics, glycogen metabolism, among many others [164]. PP1 has long been thought as a tumor suppressor due to early publications using phosphoprotein phosphatase inhibitors (e.g. okadaic acid, calyculin A, microcystin, among others) that were shown to promote tumorigenesis in several organs, including mouse skin, rat glandular stomach and rat liver [165]. Given the facts, the working hypothesis at the time seemed quite logical and straightforward: inhibition of PP1 and PP2A lead to sustained hyperphosphorylation and consequent loss of function of key tumor suppressor proteins [166]. Later, it was shown that tautomycin—a more potent inhibitor of PP1 than PP2A—did not exhibit tumor-promoting activities, motivating the idea that not all phosphoprotein phosphatase inhibitors would be tumor promoters [156]. Several studies followed, but the role of PP1 in tumorigenesis is still puzzling, with many contradictory findings—which may be one of the causes of PP1 neglect in cancer research.

The following sections provide a comprehensive review on the roles played by PP1 in tumors, aiming to clarify its relevance for cancer development and progression, as well as its potential as a biomarker and therapeutic target for cancer therapy. Since several proteins are referenced along the text, abbreviations, as well as the corresponding protein and gene names, are summarized in Suppl. Table Ib. 1.

2. PP1, a complex holoenzyme

PP1 is an oligomeric enzyme composed by a catalytic subunit (PP1c) and one or two regulatory subunits (known as regulatory interactors of PP1, RIPPOs). PP1c is encoded by three distinct genes, *PPP1CA* (11q13.2), *PPP1CB* (2p23.2) and *PPP1CC* (12q24.11), giving rise to three canonical isoforms—alpha (PP-1A or PP1α), beta (PP-1B or PP1β) and gamma (PP-1G or PP1γ), respectively [167]. Several transcripts are predicted to arise from each gene via splicing events. These include six protein-coding splice variants for *PPP1CA* and *PPP1CC* and seven for *PPP1CB* [168,169]. However, neither all transcripts have their full-length sequences uncovered, nor their existence is equally supported by the current knowledge. To date, only *PPP1CC* has two splice variants with ascertained biological relevance: gamma-1 (also known as PP1γ1), the canonical and ubiquitously expressed isoform; and gamma-2 (also known as PP1γ2), which seems to be restricted to testicular germ cells and spermatozoa [170]. The sequences of PP1c isoforms are highly similar, with minor differences observed only in the amino (N)- and carboxy (C)-terminal regions [171].

Back in the mid 90s, two groups resolved the crystal structure of two distinct PP1 complexes: PP-1A/microcystin and PP-1G/tungstate [172,173]. These studies provided the first clues about the structure of PP1's catalytic domain, but many followed to support and complement their findings by resolving additional complexes (a comprehensive review can be found elsewhere [174]). The catalytic domain has a central β-sandwich structure with a dimetal core in its active site. Each metal ion binds one of the oxygen atoms that surround the central phosphorus atom in the phosphate molecule of the substrate to mediate the dephosphorylation reaction [172,173]. The catalytic domain is highly conserved across PP1c isoforms and it is hardly changed even upon binding to other molecules [174].

The efficiency of PP1's catalytic machinery contrasts with its lack of substrate specificity. In fact, PP1c is not expected to exist freely within cells since this leads to uncontrolled and deleterious occurrence of dephosphorylation events [175]. It is currently known that PP1c has multiple grooves that function as anchoring sites for hundreds of structurally unrelated interacting proteins. Most of them have at least one RVxF-type[2] docking motif, which binds to a hydrophobic pocket away from the PP1's catalytic core, but a number of additional PP1-docking motifs has been described (reviewed in [174,176]). Distinct PP1-docking motifs can co-exist in the same RIPPO and each RIPPO can interact with one or many docking sites within the PP1c's surface, increasing the specificity of the interaction [174,176]. Hence, in 2012, Heroes E et al. came up with the interesting idea of a PP1 binding code (Fig. Ib. 1) [176].

Over 500 proteins are known to interact with PP1c (and many more may remain unknown) [177]. These proteins can be inhibitors, substrates, substrate specifiers and/or targeting subunits. Most bind

[2] R, arginine; V, valine; x, any amino acid (except proline); F, phenylalanine.

to all PP1 isoforms, but some show preference towards a specific isoform (as reviewed in [171]). This can be explained, at least in part, by the occurrence of interactions mediated by the C-terminal—the less conserved region among the isoforms [171,178,179]. The multiple possible combinations between catalytic and RIPPOs result in a wide variety of PP1 holoenzymes that can localize within distinct subcellular compartments and regulate virtually all cellular processes. In contrast to the catalytic subunits, the regulatory subunits are thought to exist as free entities and to have functions other than regulate PP1c. In fact, many of them have been widely explored in cancer research and addressed in other review papers [180,181]. Therefore, the following sections are particularly focused on PP1c—the active and often overlooked part of the enzyme.

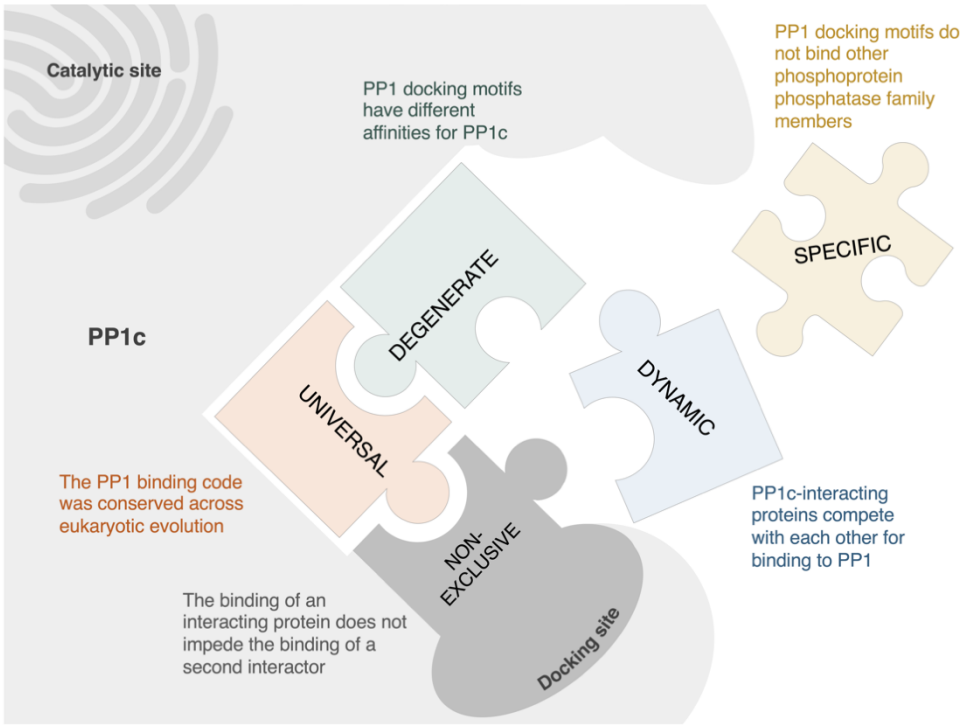

Fig. Ib. 1
Schematic representation of the PP1 binding code. These five principles (puzzle pieces) constitute the backbone for the binding of the catalytic domain (PP1c) to a myriad of distinct regulatory subunits, thereby explaining the diversity of PP1 holoenzymes.

3. Tumor-associated variations in PP1c-coding genes and transcripts

PP1c-coding genes are believed to be highly resistant to variation; however, a number of *de novo* variants has been described in patients with relevant clinical phenotypes, such as intellectual disability, neurodevelopmental delay, postnatal growth deficiency, macrocephaly, dysmorphic features, congenital heart disease, among others [182–184]. These observations raise the question of whether genetic variations on PP1c-coding genes may occur in cancer.

The analysis of 55 human cancer cell lines, including lung, colorectal, gastric and ovarian cancers, identified genetic variations in both PP1c- and RIPPOs-coding genes. Single nucleotide poly-

morphisms (SNPs) were observed in *PPP1CA* (C765T), *PPP1CB* (A201G) and *PPP1CC* (C819T)—all synonymous and predicted to be silent. Several variations, including non-synonymous nucleotide substitutions, were identified in RIPPOs-coding genes, which were corroborated by subsequent studies [185]. The study of genetic alterations in PP1c-coding genes is much more limited. According to data available at the cBioPortal for Cancer Genomics database, *PPP1CA* is the most frequently altered PP1c-coding gene in cancer (2.6%), followed by *PPP1CB* (1.2%) and *PPP1CC* (0.8%). Genetic alterations affect residues throughout all the nucleotide sequences, with exon 1 and exon 3 showing the lowest and highest numbers of residues affected, respectively (Fig. Ib. 2) [186,187].

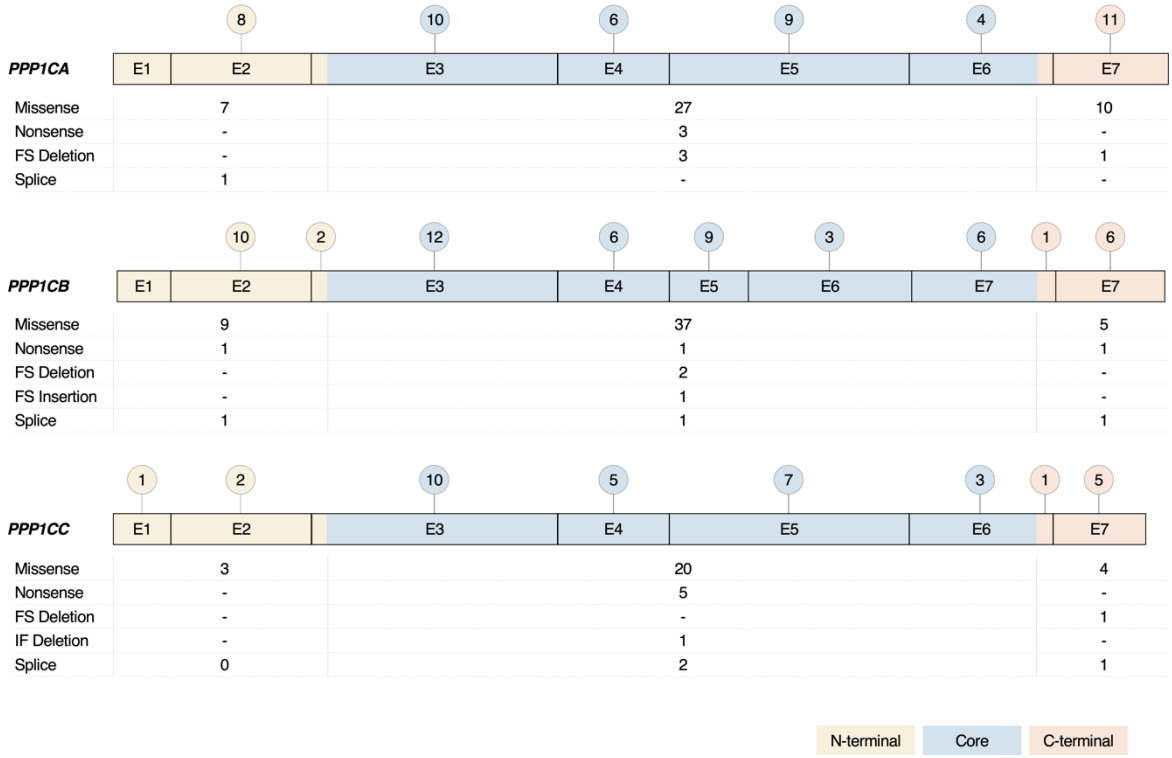

Fig. Ib. 2

Localization of genetic alterations within PP1c isoforms. The number of residues in each exon (E) affected by genetic alterations is shown inside circles. The impact of the alterations in the protein sequence is indicated below the schematic representation of each isoform. Data from TCGA PanCancer Atlas Studies, retrieved from the cBioPortal for Cancer Genomics database (https://www.cbioportal.org), v3.0.0 (date of access: June 2019) [186,187]. Schematic representations of PP1c isoforms were created according to data from Ensembl database (http://www.ensembl.org), release 96 (date of access: May 2019) [188]. Abbreviations: FS, frameshift; IF, in-frame.

Amplification is the most common alteration observed for the three PP1c-coding genes in cancer (Table Ib. 1) [186,187]. *PPP1CA* amplification was found in both localized and metastatic castration-resistant prostate cancers (mCRPC) (7% and 17% of the cases analyzed, respectively). The authors also reported a frequent co-occurrence with the amplification of the G1/S-specific cyclin-D1 gene (*CCND1*), suggesting that *PPP1CA* might function as a pro-metastatic proto-oncogene in prostate cancer (PCa) [189].

Table Ib. 1
Frequency of genomic alterations in the PP1c-coding genes.

		PPP1CA						***PPP1CB***						***PPP1CC***					
		Alteration Frequency (%)						Alteration Frequency (%)						Alteration Frequency (%)					
Cancer type	No. cases	Total	M	A	D	F	MA	Total	M	A	D	F	MA	Total	M	A	D	F	MA
Adrenocortical Carcinoma	92	4.35	2.17	2.17				2.17		2.17									
Bladder Urothelial Carcinoma	411	4.87	1.70	3.16				2.19	0.97	1.22				2.43	0.49	1.70	0.24		
Cervical Adenocarcinoma	46	6.52	2.17	4.35				2.17		2.17									
Cervical Squamous Cell Carcinoma	251	0.80		0.80				1.99	1.20	0.80				0.40	0.40				
Cholangiocarcinoma	36	5.56		5.56				2.78	2.78										
Colorectal Adenocarcinoma	594	0.67	0.34	0.17	0.17			1.18	1.18					0.84	0.84				
Diffuse Glioma	513	0.39		0.39				0.19	0.19					0.97		0.19	0.78		
Endometrial Carcinoma	586	4.78	1.88	2.90				5.46	3.41	2.05				1.71	1.54	0.17			
Esophageal Squamous Cell Carcinoma	95	7.37		6.32			1.05							1.05		1.05			
Esophagogastric Adenocarcinoma	514	3.89	0.58	3.11	0.19			0.97		0.78	0.19			1.17	0.78	0.39			
Glioblastoma	592	0.17		0.17				0.51	0.17	0.34				0.51	0.17	0.17	0.17		
Head and Neck Squamous Cell Carcinoma	523	6.31	0.38	5.74		0.19		0.19			0.19			0.38		0.38			
Hepatocellular Carcinoma	369	3.25	0.54	2.71				0.54		0.54				0.54	0.27	0.27			
Invasive Breast Carcinoma	1084	6.00		6.00				0.55	0.18	0.28	0.09			0.18	0.18				
Mature B-Cell Neoplasms	48	2.08	2.08																
Melanoma	448	4.24	2.01	2.01	0.22			3.13	2.46	0.45		0.22		2.01	1.56		0.45		
Non-Small Cell Lung Cancer	1053	1.71	0.57	1.04		0.09		1.33	0.57	0.57		0.09	0.09	0.76	0.28	0.19	0.28		
Ovarian Epithelial Tumor	585	3.08	0.17	2.91				3.59	0.17	3.25		0.17		0.85		0.85			
Pancreatic Adenocarcinoma	184	1.09	0.54	0.54															
Pheochromocytoma	147	1.36		1.36										0.68		0.68			
Prostate Adenocarcinoma	494	2.23	0.20	2.02				0.40		0.40				1.04		0.81	0.20		
Renal Non-Clear Cell Carcinoma	348	0.86	0.57			0.29		1.15	0.29	0.57	0.29			0.86	0.57	0.29			
Sarcoma	255	1.18		1.18				1.18	1.18					3.14		2.35	0.39		0.39
Undifferentiated Stomach Adenocarcinoma	13	7.69		7.69															

Data from TCGA PanCancer Atlas Studies, retrieved from the cBioPortal for Cancer Genomics database, https://www.cbioportal.org), v3.0.0 (date of access: June 2019) [186,187].
Abbreviations: A, amplification; D, deep deletion; F, fusion; M, mutation; MA, multiple alterations.

Furthermore, in oral squamous cell carcinoma cells, *PPP1CA* amplification was shown to be positively correlated with gene copy number and PP-1A expression [190]. In contrast, *PPP1CA* allelic loss was observed in thyroid, larynx, kidney and colorectal tumors [191]. Increased gene copy number and/or gene amplification of PP1c-coding genes would be a rational explanation for the PP1 upregulation observed in certain tumors. Chromosomal deletions and loss-of-function point mutations, on the other hand, would also be likely to occur in human cancers given the importance of PP1 in controlling cell growth and survival. In any case, these might determine the affinity of PP1c to bind RIPPOs, thus contributing to the rather diverse roles of PP1 in cancer.

Some fusion mutations have also been described. These include *CLCF1/PPP1CA* in both lung squamous cell carcinoma and papillary renal cell carcinoma; *CHSY1/PPP1CA* in head and neck squamous cell carcinoma; *ACYP2/PPP1CB* in lung squamous cell carcinoma; *PPP1CB/SSPN* in serous ovarian cancer; *PPP1CB/EIF2B4* in cutaneous melanoma; and *PPP1CC/KCNC2* in dedifferentiated liposarcoma (the proteins encoded by these genes can be consulted in Suppl. Table Ib. 1) [186,187]. In addition, the fusion protein PP-1B/ALK tyrosine kinase receptor was detected in a case of high grade glioma of infancy [192]. The clinical relevance of these fusions is still to be determined. Interestingly, RNA chimeras encompassing PP-1B and protein yippee-like 5 were observed in a series of 103 cases of chronic lymphocytic leukemia, without evidence of genomic fusion. These RNA chimeras were specifically observed in most of the cancer samples (95%) but not in paired normal samples, benign lymphocytes or even other types of cancer, such as prostatic carcinoma, gastric carcinoma and malignant melanoma. The resulting product is a truncated form of PP-1B with impaired phosphatase activity, which seems to be associated with increased proliferation and colony formation [193].

4. PP1c expression in tumors: a putative biomarker behind the spotlight

PP1c canonical isoforms are widely expressed in several types of tumors at both mRNA and protein levels. Their expression levels vary between tumoral and non-tumoral samples and also among tumors from different stages; however, the exact nature of the alterations is difficult to establish due to high variability in the results, in part explained by the intra- and inter-heterogeneity that characterize tumors.

Decreased PP-1A mRNA levels were observed in several types of tumors when compared with matched normal tissue. These include vulva, small intestine, kidney and prostate tumors and, in lower percentages, pancreas, lung, breast, stomach, small intestine and thyroid gland tumors [191]. Estrogen receptor (ER)-negative breast tumors were associated with low levels of PP-1A protein expression [194]. In contrast, PP-1A mRNA levels were found higher in maxillary sinus squamous cell carcinomas than in paired normal tissue [195]. Increased PP-1A protein expression was found in

glioblastomas and was associated with poor prognosis in tumors expressing the cellular tumor antigen p53 (p53) [196]. Enhanced PP-1A cytoplasmic staining was also correlated with high Gleason score in prostate tumors [197]. Interestingly, the urinary content of PP-1A mRNA was shown to have higher sensitivity than cytology to detect non-muscle invasive bladder cancer recurrence (both sensitivity and specificity were > 60%) [198].

Sporadic breast tumors showed decreased PP-1B and PP-1G mRNA expression when compared with normal breast tissue [194]. Nonetheless, upregulated levels were reported for other types of tumors. PP-1B was found to be overexpressed, at both mRNA and protein levels, in melanoma cell lines and tissues when compared with melanocytes and benign nevi. Herein, PP-1B was able to distinguish melanoma from nevi with 93% sensitivity and 65% specificity, thus suggesting its potential as a tumor marker [199]. PP-1G protein expression is increased in hepatocellular carcinomas compared with adjacent benign tissue and positively correlates with the expression of the proliferation marker protein Ki-67, as well as with histological grade and tumor size. Increased PP-1G protein expression levels were also observed in brain tumors. Likewise, PP-1G expression positively correlates with Ki-67 expression and hepatocellular carcinoma staging [200]. Thus, PP-1G has been proposed as a poor prognosis marker in hepatocellular cancer and glioma [200,201].

The predictive value of the three canonical isoforms was also assessed in non-small cell lung cancer (NSCLC) specimens. Squamous cell carcinomas with low PP1c mRNA levels, either considering the isoforms altogether or individually, were associated with a lower probability of survival. Conversely, in adenocarcinomas, mRNA expression of the three PP1c isoforms was not associated with patients' survival probability, whereas significant differences were apparent when considering each isoform individually. Whilst low levels of PP-1A or PP-1B were associated with higher risk of diminished survival, the inverse relationship was observed for PP-1G [202].

In addition to potential genetic variations that may underlie this differential expression as previously discussed (Section 3), it is also possible that PP1c-coding genes are targeted by epigenetic modifications. For instance, DNA methylation is usually associated with long-term repression of gene expression and PP1c's promoter methylation has been described in other pathophysiological contexts [203,204]. Although data on tumors is rather limited, decreased *PPP1CA* and *PPP1CB* methylation was reported in NSCLC specimens when compared to non-tumoral samples. In contrast, increased methylation was observed for *PPP1CC* [202]. This suggests that PP1c's promoters might be differentially regulated by methylation in NSCLC and possibly other cancers. Hence, it would be of interest to explore the possibility of methylation-mediated decrease in PP1c gene expression and PP1 activity when downregulation is observed.

5. Regulation of PP1c activity by post-translational modifications

As referred in Section 2, PP1c function is largely dependent on its interaction with RIPPOs [175]. Additionally, PP1c is long known to be directly regulated by phosphorylation. Back in the 80s, PP1c was shown to be phosphorylated on Tyr residues, *in vitro*, by the proto-oncogene tyrosine-protein kinase Src, with concomitant loss of phosphatase activity [205]. Subsequent studies provided further evidence for the regulation of PP1c by phosphorylation, not only in Tyr residues, but also in Ser and Thr residues [206–208]. All PP1c canonical isoforms were shown to be phosphorylated, and consequently inhibited, by cyclin-dependent kinases (CDKs) [206,208]. However, the study of PP1c regulation by phosphorylation is mostly limited to PP-1A$^{Thr\text{-}320}$ (though this residue is conserved among all isoforms (Fig. Ib. 3)), due to its role in determining PP-1A activation state during cell cycle [206,208,209]. However, additional residues are prone to regulation by post-translational modifications (PTMs), with some being targeted by more than one type of modification (Fig. Ib. 3).

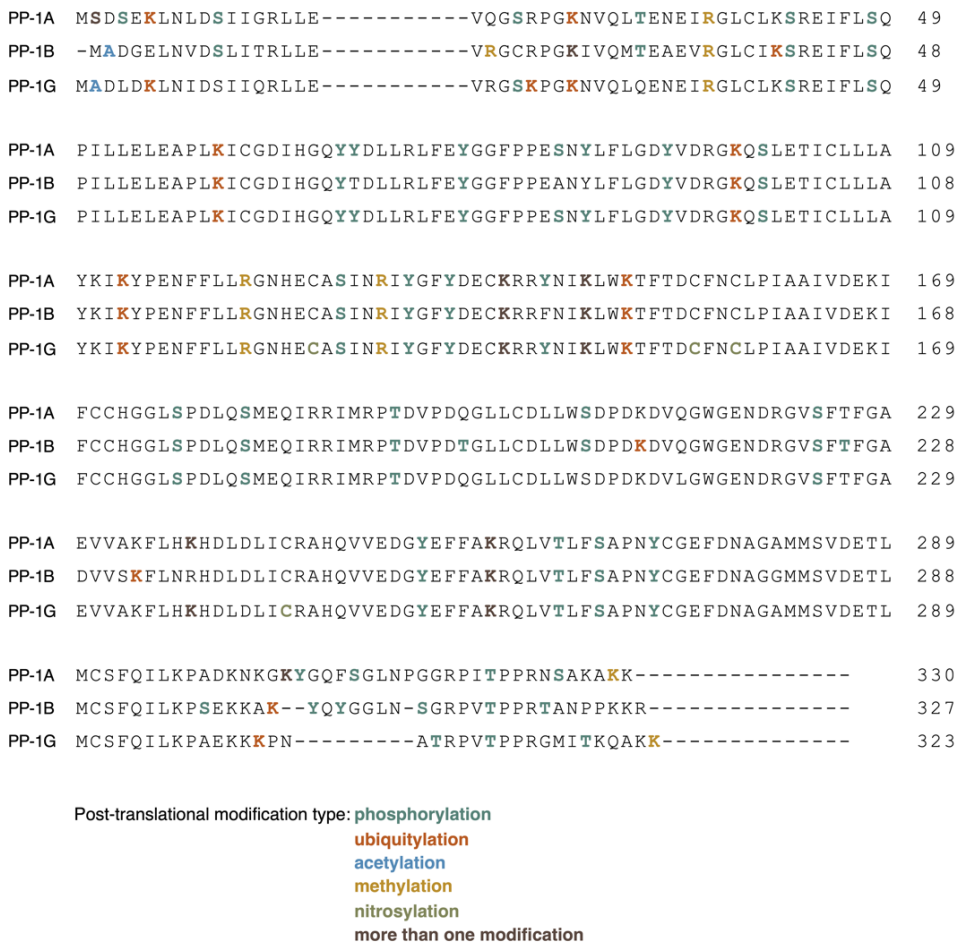

Fig. Ib. 3

PP1c's residues targeted by post-translational modifications (PTMs). Each color corresponds to a type of PTM. Data collected from PhosphoNET (http://www.phosphonet.ca), PhosphoSitePlus (https://www.phosphosite.org), dbPTM (http://dbptm.mbc.nctu.edu.tw) (date of access: May 2019).

Phosphorylation is the most common PTM in the regulation of PP1c, but the relevance of most phospho-residues for its function is still unknown. Some of them, nonetheless, have been identified in tumors (Table Ib. 2) and some correspond to sites affected by genetic alterations, mostly missense mutations [210,211]. This is interesting given the increasing number of reports showing that mutations affect phospho-events in human cancers by determining the gain and loss of phosphorylation sites [212,213].

Table Ib. 2
PP1c's phospho-residues identified in tumors and/or with known effect on the regulation of PP1c's activity.

Phospho-residue	Isoform	Tumor(s)	Regulatory protein(s)	Effect on protein
Non-conserved residues				
Ser-2	PP-1A	Breast, lung, ovarian	-	-
Ser-11	PP-1A	Breast, cervical	-	-
Ser-22	PP-1A	Breast, cervical	-	-
Tyr-306	PP-1A	Colorectal, gastric, leukemia, lung, melanoma	-	-
Ser-325	PP-1A	Breast, leukemia, lung	Nek2	(–) activity
Tyr-304	PP-1B	Breast, gastric, leukemia, lung, lymphoma	-	-
Tyr-306	PP-1B	Bile-duct, colorectal, esophageal, gastric, leukemia, liver, lung, lymphoma, neuroblastoma, ovarian, salivary gland, thymic	-	-
Ser-311	PP-1B	Cervical	-	-
Thr-307	PP-1G	-	Nek2	(–) activity
Thr-318	PP-1G	-	-	-
Conserved residues				
Ser-48	PP-1A	Breast	-	-
Ser-47	PP-1B	Breast	-	-
Ser-48	PP-1G	Breast	-	-
Tyr-93	PP-1A	Lung	-	-
Tyr-92	PP-1B	Lung	-	-
Tyr-93	PP-1G	Lung	-	-
Tyr-134	PP-1A	Leukemia	-	-
Tyr-133	PP-1B	Leukemia	-	-
Tyr-134	PP-1G	Leukemia	-	-
Tyr-137	PP-1A	Gastric, leukemia, lung	-	-
Tyr-136	PP-1B	Gastric, leukemia, lung	-	-
Tyr-137	PP-1G	Gastric, leukemia, lung	-	-
Tyr-255	PP-1A	Lung	-	-
Tyr-254	PP-1B	Lung	-	-
Tyr-255	PP-1G	Lung	-	-
Thr-320	PP-1A	Adrenal, pheochromocytoma, breast, cervical, gastric, leukemia, lung, lymphoma, melanoma, myeloma, ovarian	Akt, PP-1A	(–) activity
Thr-316	PP-1B	Breast, cervical, leukemia, hepatocellular, lung, lymphoma, ovarian, melanoma	-	-
Thr-311	PP-1G	Breast, colorectal, ovarian	ATR, CDK1, CDK2	(–) activity

(–) indicates decreased activity. Data collected from PhosphoNET (http://www.phosphonet.ca) and PhosphoSitePlus (https://www.phosphosite.org) (date of access: May 2019). Abbreviations: Akt, RAC-alpha serine/threonine-protein kinase; ATR, serine/threonine-protein kinase ATR; CDK, cyclin-dependent kinase; Nek2, serine/threonine-protein kinase Nek2; PP-1A, PP1-alpha catalytic subunit; PP-1B, PP1-beta catalytic subunit; PP-1G, PP1-gamma catalytic subunit; Ser, serine; Thr, threonine; Tyr, tyrosine.

In addition to the direct phosphorylation of PP1c there is also evidence of the phosphorylation of RVxF motifs in RIPPOs [214]. This mechanism was shown to be primarily regulated by aurora kinase B, which phosphorylates Ser and Thr residues within the RVxF of several RIPPOs during mitosis, although other protein kinases might also be involved. Interestingly, PP1c was shown to preferentially bind unphosphorylated RVxF motifs *in vitro* [214]. Therefore, RVxF phosphorylation could be an important mechanism to regulate the assembly of PP1 holoenzymes.

6. Cryptic roles for PP1 in tumorigenesis

The classification of PP1 as a tumor suppressor protein or an oncogene protein is still a subject of major debate. To this contributes the myriad of proteins with which PP1c is capable to interact with, as well as isoform-specific phenotypes. Complete PP1c loss-of-function is notably deleterious for mammalian cells and, not surprisingly, few phenotypes have been reported. The most eye-catching example is the impairment of spermiogenesis in *PPP1CC2*-knockout mice [215]. It is generally accepted that PP1c isoforms might have some overlapping role and can compensate for the lack of function from each other, thereby overcoming major negative effects; however, there is increasing evidence that they have non-redundant functions *in vivo*, stressing the need to consider isoform-specific phenotypes. For instance, PP-1B, but not the others, is important in the regulation of Ca^{2+} uptake by the sarcoplasmic reticulum of cardiomyocytes [216].

PP1c isoform-specific phenotypes in cancer are barely explored. This can be explained, in part, because a considerable number of studies that assess PP1c expression, regulation and roles in tumors does not discriminate between isoforms (e.g., use pan-specific antibodies and other molecular tools). However, a number of studies support non-redundant or even antagonizing functions for PP1c isoforms and, therefore, attention should be given to overgeneralization. Interesting phenotypic differences were observed in HeLa cervical carcinoma cells using PP1c isoform-specific small interfering RNAs (siRNAs): PP-1A-knockdown cells rounded up and showed defective cell proliferation and increased cell death; PP-1B-knockdown cells became flat, enlarged and rich in lamellipodia; and PP-1G-knockdown cells slightly rounded up, and showed increased population in S-phase and decreased population in G1 [217]. Evidence of antagonizing roles for PP-1A and PP-1G was seen in H1299 NSCLC cells. In contrast to HeLa cells, in which PP-1A is the most expressed isoform [217], PP-1G expression is about 4-fold higher than that of PP-1A in H1299 cells. PP-1G expression further increases upon PP-1A knockdown, while it decreases following PP-1A overexpression. PP-1G downregulation leads to decreased cell proliferation and compromises tumor formation in nude mice [218]. Nonetheless, the molecular mechanisms underlying these observations are far from being completely understood.

6.1. pRb and p53—not a tale of two substrates anymore

To date, the role played by PP1 in tumorigenesis has mostly been restricted to its recognition as regulator of two major tumor suppressors: the retinoblastoma-associated protein (pRb) and p53 [219].

pRb is known as the gatekeeper of the cell cycle as it prevents cells from premature entry in the S-phase. When phosphorylated, pRb becomes inactive and liberates E2F transcription factors to drive the expression of target genes involved in DNA replication and cell cycle progression [220]. Hyper-phosphorylation and constitutive inactivation of pRb are frequently observed in tumors, contributing to G1/S-phase transition and cell proliferation. In addition, pRb has been implicated in other molecular events, although some of its functions are still controversial (comprehensive reviews can be found elsewhere [221–223]). PP1c dephosphorylates pRb in several Ser and Thr residues (Table Ib. 3). All PP1c isoforms can bind to pRb, but they may exhibit preferences towards different phospho-residues [224]. At mitotic exit, PP1c-mediated dephosphorylation of pRb restores its growth-suppressive activity. Phosphorylation and consequent inactivation of PP1c by mitotic kinases in G1 renders pRb prone to hyperphosphorylation and determines cell cycle progression (detailed review of this interaction elsewhere [225]). Although PP1c/pRb interaction is central in cell cycle regulation, the role of PP1 in mitosis and mitotic exit is considerably deeper, with its activation/inactivation switch being essential for a precise cell cycle (as recently discussed in [226]). These observations strengthened the belief that PP1 could be an important tumor suppressor phosphatase since its activation on mitotic exit is determinant not only for maintaining pRb in its active state, but also for the overall temporal regulation of molecular events from anaphase to cytokinesis [226].

p53 acts as a lifeguard protein determining cell fate in response to multiple damaging stimulus. Depending on the extent of cellular stress, p53 can dictate DNA repair, cell cycle arrest, metabolic alterations or cell death [227]. p53-mediated signaling cascades have crucial inhibitory effects during cell malignant transformation and tumor progression to metastatic stages [228]. *TP53* mutations are observed in several cancers, which may result in loss-of-function or even confer it new oncogenic functions [229,230]. This translates into multiple and challenging p53-related scenarios in cancer. p53 activity is tightly controlled by PTMs, with emphasis on phosphorylation and acetylation. Phosphorylation is important not only in the regulation of wild-type p53, but also of its mutant forms, with phospho-p53 usually being the most stable and active version of the protein [231]. PP1 dephosphorylates two Ser residues in p53 that are involved in its activation (Table Ib. 3). Hence, in contrast to the pRb scenario, PP1 leads to p53 inactivation—which may seem paradoxical to a putative tumor suppressor phosphatase.

Table Ib. 3
Phospho-residues targeted by PP1c in pRb and p53.

Protein	Residue	Molecular effect(s) of phosphorylation / relevance in cancer	References
pRb	Ser-249	(–) p65 activity (–) PD-L1 expression (+) Cancer immunity (+) Tumor grade in lung cancer, squamous cell carcinoma subtype	[232,233]
	Thr-356	(–) pRb activity (–) pRb/E2Fs interaction (+) Cell cycle progression (+) Poor overall survival in HPV-negative squamous cell carcinoma of the head and neck	[234,235]
	Ser-608	(–) pRb activity (–) pRb/E2Fs interaction (+) Cell cycle progression	[234,236]
	Ser-788	(–) pRb/E2Fs/Dp-1 interaction (+) Cell cycle progression	[236]
	Ser-795	(–) pRb/E2Fs/DP-1 interaction (+) Cell cycle progression	[237]
	Ser-807	(–) pRb activity (+) pRb phosphorylation in other residues (–) pRb/p150 interaction (+) pRb/BAX interaction (–) Apoptosis	[238–241]
	Thr-821	(–) pRb/E2Fs/Dp-1 interaction (+) pRb/Sp1 interaction (–) pRb/LxCxE motif-containing proteins interaction (+) Elastogenesis (–) Apoptosis	[241–244]
	Thr-826	(–) pRb/E2Fs/Dp-1 interaction (–) pRb/LxCxE motif-containing proteins interaction	[241,244]
p53	Ser-15	(+) p53 activity (+) p53 transactivation (through interaction with co-activators) (+) p53 phosphorylation in other residues (–) p53/Mdm2 interaction (–) Aging-associated phenotypes (mouse models) (–) Malignancies development (mouse models) (+) Apoptosis	[245–249]
	Ser-37	(+) p53 activity (–) p53/Mdm2 interaction	[245–249]

(+) upregulation; (–) down-regulation. Abbreviations: BAX, apoptosis regulator BAX; E2Fs, E2F transcription factors; HPV, human papillomavirus; Mdm2, E3 ubiquitin-protein ligase Mdm2; p150, tyrosine-protein kinase ABL1; PD-L1, programmed cell death 1 ligand 1; p65, transcription factor p65; Sp1, transcription factor Sp1; Dp-1, transcription factor Dp-1.

6.1.1. PP1 in sustained proliferation and tumor growth

Malignant cells can revert the acquired differentiation pattern, returning to a stem-cell-like phenotype, which allows for sustained proliferation and other metabolic adaptations that support cell survival. They can overcome the tight control of the cell cycle in addition of being self-sufficient in terms of growth-promoting signals and impervious to growth-inhibitory signals [250]. A number of molecules and signaling pathways have been associated with the development of such hallmarks, with a great number of them resulting in alterations in pRb- and p53-mediated signaling.

Activation of mitogenic CDK/cyclin complexes and consequent inactivation of pRb by phosphorylation are commonly observed in human cancers to meet the requirements for uncontrolled cell

proliferation and tumor growth [250–252]. Since PP1c directly determines the activation state of pRb, it would be expected that PP1c inactivation and/or downregulation would occur during tumorigenesis to support cell proliferation. In fact, a number of oncogenic molecules, whose expression is upregulated in certain tumors, have been reported to decrease PP1c expression and/or activity towards pRb and other substrates. These include the microRNA (miR)-125b [253], the tyrosine-protein kinase Fer (Fer) [254] and caveolin-1 [255] (Fig. Ib. 4). In addition, tumor suppressor proteins known to enhance PP1c expression and/or activity are frequently downregulated in tumors, including neurabin-2 (Fig. Ib. 4) [256,257]. The expression levels of neurabin-2 and all PP1c canonical isoforms were shown to be coregulated in lung tumors and, interestingly, downregulation of PP-1A was shown to have the same effect of neurabin-2 downregulation in inducing cancer stem-like cell phenotype and increasing the proportion of cancer-initiating cells [202,258].

However, as indicated by expression studies (Section 4), PP1c downregulation is not always observed in tumors and its involvement in controlling the limitless replicative and proliferative potential of malignant cells is not that straightforward due to its high dynamism inside cells. In fact, the subcellular localization and activity of PP-1A and PP-1G was shown to vary in a cell cycle-dependent manner in human MG-63 osteosarcoma cells [259]. Also, PP1c association with different RIPPOs contributes to the multiple possible outcomes hitherto reported.

The Ser/Thr-protein phosphatase 1 regulatory subunits 10 (PNUTS) competes with pRb for binding to PP1c. Since the binding affinity of PNUTS to PP1c is much higher than that of pRb, the presence of PNUTS impairs PP1c-mediated dephosphorylation of pRb [260]. In addition, PNUTS may relocate PP1c towards other substrates involved in gene transcription. Studies in *Drosophila melanogaster* showed that PNUTS/PP1c holoenzyme associates with RNA polymerase II and supports normal cell proliferation and growth in developing tissues [261]. A recent study also found that the Myc proto-oncogene protein (Myc) is regulated by the PNUTS/PP1c holoenzyme (Fig. Ib. 4). Their co-amplification was observed in breast, lung and uterine carcinomas [262].

Several additional studies reported pro-proliferative and pro-growth functions for PP1c. All PP1c canonical isoforms were shown to dephosphorylate protein Mdm4 (Mdm4) at Ser-367, which increases its stability and promotes its inhibitory effect towards p53 (Fig. Ib. 4) [263]. In hepatocellular carcinoma cells, PP-1G was shown to upregulate Mdm4 and CCND1, while downregulated p53 and the CDK inhibitor 1 (p21). PP-1G downregulation led to decreased cell proliferation and colony formation, as well as cell cycle arrest at G1 [201]. PP-1G was also shown to promote cell proliferation via downregulation of NF-κB signaling in glioma cells (Fig. Ib. 4) Moreover, PP1c was shown to activate RAF proto-oncogene serine/threonine-protein kinase (Raf-1) and subsequent MAPK/extracellular signal-regulated kinase (ERK) signaling cascade (Fig. Ib. 4) [264]. As shown in myeloma cells, PP1c might also be involved in autocrine growth signaling mediated by IL-6 [265].

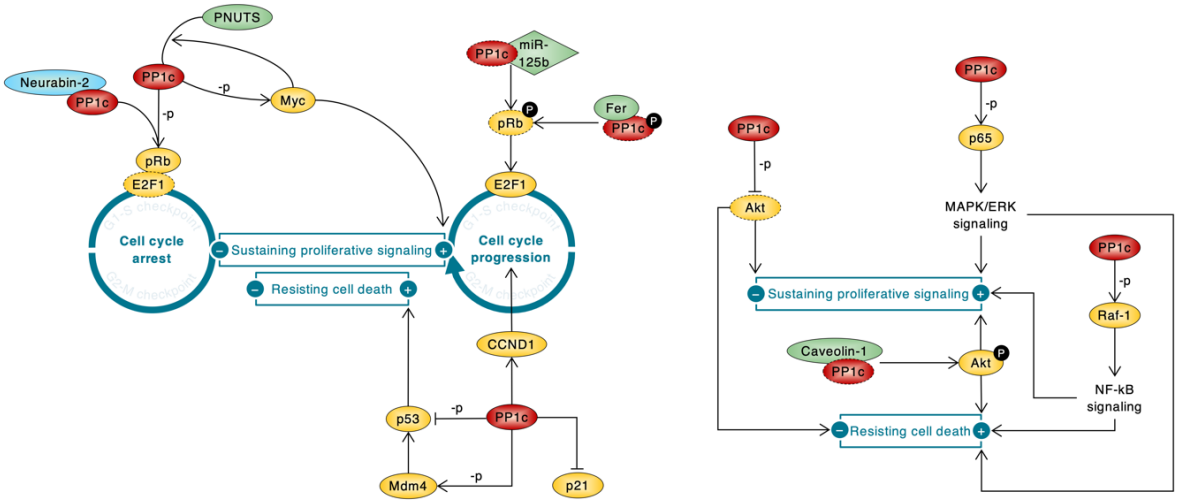

Fig. Ib. 4

PP1 in tumor growth. PP1 regulates cell cycle progression by maintaining pRb in its active state, which results in cell cycle arrest. Several oncogenic molecules (green), frequently upregulated in cancers, impair PP1c/pRb interaction. In opposition, tumor suppressor molecules (blue), usually downregulated in cancers, stabilize this interaction. PP1 also contributes to limit cell survival by inhibiting Akt-mediated signaling but upregulates MAPK/ERK and NF-κB signaling pathways, which contribute to tumor growth. PP1c is represented in red and other signaling mediators in yellow. p, phosphorylation; -p, dephosphorylation. Abbreviations: CCND1, G1/S-specific cyclin-D1; E2F1, transcription factor E2F1; Fer, tyrosine-protein kinase Fer; Mdm4, protein Mdm4; Myc, Myc proto-oncogene protein; p21, cyclin-dependent kinase inhibitor 1; p53, cellular tumor antigen p53; p65, transcription factor p65; PNUTS, PP1 regulatory subunit 10; Raf-1, RAF proto-oncogene serine/threonine-protein kinase.

6.1.2. PP1 in response to DNA damage and cellular stress

Genomic instability is a major trait of tumoral cells, which tend to accumulate DNA lesions. Depending on the type and extent of the damage, cells can either repair it and survive, enter a senescent state or activate death-related processes [266].

p53 is a central player in the regulation of DNA damage response and, as previously referred (Section 6.1.1), PP1c directly regulates its function. PP1c-mediated dephosphorylation of p53 down-regulates p53-mediated transcription and apoptosis, thus contributing to cell survival (Fig. Ib. 5) [201,267,268]. In addition, PP1c interacts with p53-binding proteins and other mediators of the apoptotic signaling (comprehensive reviews elsewhere [266,269]). Accordingly, PP-1A was shown to be a connecting molecule in the glioblastoma sub-network that contains p53 [270]. However, the apoptotic process in liver cancer was shown to be independent of PP1 [271].

PP1 has been gaining recognition as a controller of DNA repair-associated recombination and DNA damage checkpoint (recently reviewed in [266]). All PP1c isoforms were shown to interact with the breast cancer type 1 susceptibility protein (BRCA1)—a well-known tumor suppressor protein primarily implicated in breast and ovarian cancers [194]. The interaction is mediated by the RVxF motif ^{898}KVTF^{901} within BRCA1 fragment 4 [194,272,273]. BRCA1 is both a substrate and a regulator of PP-1A, which suggests a feedback mechanism for the regulation of BRCA1 phosphorylation state and/or an attempt to modulate PP-1A activity towards other substrates [272]. PP-1A

dephosphorylates BRCA1 at Ser-988, Ser-1423 and Ser-1524 [273] and enhances its E3 ubiquitin ligase activity (Fig. Ib. 5) [274].

PP-1A interaction with BRCA1 is required for cell survival after induced DNA damage (Fig. Ib. 5) [273,275]. Mutant BRCA1 lacking functional PP1-binding motif fails to relocalize efficiently within the nucleus and to recruit the DNA repair protein RAD51 homolog 1 (RAD51) to sites of DNA lesions [275]. Interestingly, a *BRCA1* K898E germline missense variant was found in two patients with breast and ovarian cancer. This variant abrogates the interaction between BRCA1 and PP1 and has a dramatic effect on BRCA1-mediated DNA repair, which raises the question of whether naturally-occurring mutations within PP1-binding motifs are potentially cancer-predisposing [276].

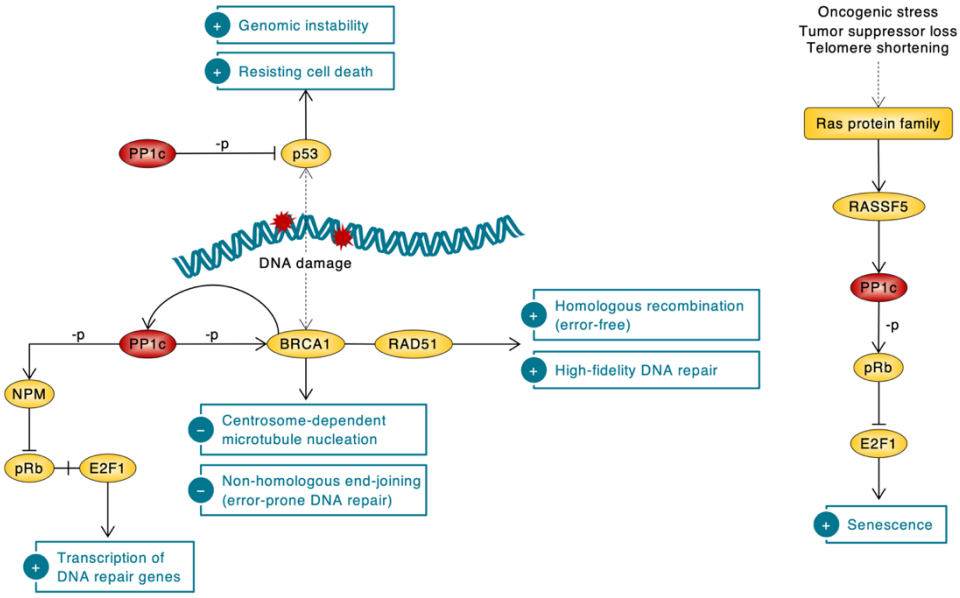

Fig. Ib. 5

PP1 in response to DNA damage and cellular stress. PP1c-mediated dephosphorylation and inactivation of the cellular tumor antigen p53 supports cell survival after DNA damage. PP1c-mediated dephosphorylation and activation of the breast cancer type 1 susceptibility protein (BRCA1) and nucleophosmin (NPM) promotes mechanisms of DNA repair after DNA damage. The axis PP1c/pRb induces cell senescence in response to cellular stress. PP1c is represented in red and other signaling mediators in yellow. -p, dephosphorylation. Figures were produced using Servier Medical Art (https://smart.servier.com). Abbreviations: E2F1, transcription factor E2F1; RAD51, DNA repair protein RAD51 homolog 1; RASSF5, Ras association domain-containing protein 5.

PP-1B was shown to dephosphorylate nucleophosmin (NPM) upon DNA damage in HeLa cervical carcinoma cells. PP-1B-mediated dephosphorylation of NPM, at Thr-199 and Thr-234/237 promotes its interaction with pRb and rescues the transcriptional activity of the transcription factor E2F1 to upregulate the expression of DNA repair genes (Fig. Ib. 5) [277].

In addition to DNA repair, PP1 has been implicated in tumor cell senescence (Fig. Ib. 5). PP-1A protein expression is upregulated by oncogenic Ras proteins [278]—a family of small GTPases that are key mediators of senescence in response to stimulus causing cellular stress [252]. Ras protein promotes the interaction between PP-1A and the Ras association domain-containing protein 5

(RASSF5), which belongs to a family of tumor suppressors and is a direct effector of the Ras family of proteins [279]. RASSF5 targets PP-1A to pRb, thereby promoting its dephosphorylation and cell cycle arrest. Constitutively active PP-1A, in turn, was shown to downregulate Ras oncogenic potential, as well as the cell cycle regulator CCND1 in mouse embryo fibroblasts. The same was not observed in cells overexpressing wild-type PP-1A probably due to the prompt phosphorylation and inactivation of the phosphatase during cell cycle progression [280].

6.2. PP1 in hormone-dependent signaling and endocrine resistance

Hormones are key regulators of cell division and control cell proliferation and metabolism. Accordingly, multiple cancers depend on hormones for successful establishment and growth, particularly at early stages [281].

PP1c was initially described as a positive regulator of the androgen receptor (AR) (Fig. Ib. 6A), a chief mediator of PCa development and progression [282]. PP-1A enhances AR nuclear localization and transcriptional activity through direct dephosphorylation of $AR^{Ser\text{-}650}$—a residue located at the hinge region whose phosphorylation is required for AR nuclear export [283]. The interaction between PP-1A and AR is supported by a positive feedback loop in which AR acts as a PP1c regulator besides being a substrate. AR targets PP-1A to chromatin, where it catalyzes the dephosphorylation of CDK9. As a result, positive transcriptional elongation factor b (composed by CDK9 and cyclin-T1 (CycT1)) is mobilized to induce AR transactivation (Fig. Ib. 6A) [284]. Furthermore, PP-1A restricts AR degradation by interacting with AR ligand-binding's domain and inhibiting its polyubiquitylation. This effect is upheld by PP-1A-mediated dephosphorylation and inactivation of Mdm2 and S-phase kinase-associated protein 2 (SKP2), which regulate AR ubiquitylation and degradation (Fig. Ib. 6A) [285]. PP-1B may also contribute through the interaction and downregulation of the serine/threonine-protein kinase PAK 6, a p21-activated kinase that mediates AR degradation through AR and Mdm2 phosphorylation [286,287]. This suggests putative cooperative roles for PP1c isoforms in regulating AR signaling in PCa.

Importantly, PP-1A-mediated mechanisms were shown to be independent of androgen stimulation and thus may sustain non-canonical AR signaling pathways in castration-resistant PCa (CRPC) [283,285,288]. Moreover, PP-1A-mediated inhibition of AR degradation was also found after enzalutamide and bicalutamide treatments—nonsteroidal antiandrogens used to treat mCRPC [285,288]. Altogether, this strongly suggests that PP-1A may contribute to therapeutic failure in PCa.

However, a recent phosphatase RNA interference screening uncovered distinct scenarios for PP1 holoenzymes in AR function. In this study, PP-1B was shown to have repressive roles towards AR function through myosin phosphatase—a tricomplex holoenzyme comprising PP-1B, the PP1 regulatory subunit 12A (MYPT1) and a smaller subunit with unknown function (Fig. Ib. 6). In contrast to the AR-promoting effects of PP-1A, myosin phosphatase reduces AR nuclear translocation, sta-

bility and transcriptional activity in both androgen-dependent and CRPC cells [289]. Therefore, given the prominent role of PP1c in regulating AR function, in spite of being as either positive or negative regulator, it would be of interest to better characterize the expression of PP1c isoforms in PCa, unravel their interactomes and better understand the role of PP1 holoenzymes in regulating AR signaling in both androgen-dependent and castration-resistant scenarios.

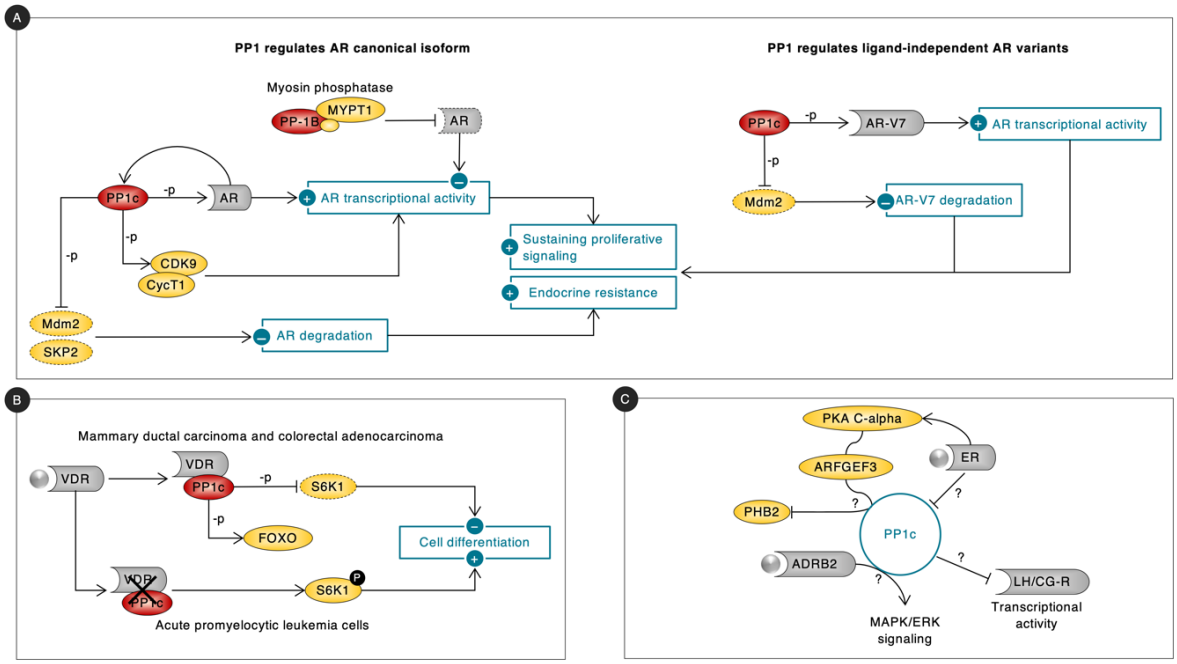

Fig. Ib. 6

PP1 regulates hormone-mediated signaling in cancer. (A) PP1c is a positive regulator of the androgen receptor (AR)-mediated signaling (both canonical and ligand-independent variants). However, recent evidence suggests that the holoenzyme myosin phosphatase inhibits the AR. **(B)** PP1c interacts with the vitamin D3 receptor (VDR) but the outcomes of the interaction are different on different cancers. **(C)** Few studies support the involvement of PP1c in other hormone-mediated signaling pathways, suggesting either direct or indirect association with the estrogen receptor (ER), the beta-2 adrenergic receptor (ADRB2) and the lutropin-choriogonadotropic hormone receptor (LH/CG-R). Receptors are represented in grey, PP1c is in red and other signaling mediators are in yellow. Circles represent receptor ligands. P, phosphorylated; -p, dephosphorylation. Abbreviations: AR-V7, AR variant 7; ARFGEF3, brefeldin A-inhibited guanine nucleotide-exchange protein 3; CDK9, cyclin-dependent kinase 9; CycT1, cyclin-T1; FOXO, class O of forkhead box; Mdm2, E3 ubiquitin-protein ligase Mdm2; PHB2, prohibitin-2; PKA C-alpha, cAMP-dependent protein kinase catalytic subunit alpha; PP-1B, PP1-beta catalytic subunit; S6K1, ribosomal protein S6 kinase beta-1; SKP2, S-phase kinase-associated protein 2.

The occurrence of AR splice variants has been proposed as a safeguarding system for cancer cells survival during androgen deprivation therapy (ADT), as well as a mechanism involved in the development of castration resistance [290]. AR variant 7 (AR-V7) was also shown to be upregulated by all PP1c isoforms [291]. PP1c dephosphorylates AR-V7$^{Ser\text{-}213}$ and decreases its Mdm2-mediated ubiquitylation and degradation (Fig. Ib. 6A) [291]. AR-V7 expression in circulating tumor cells is associated with resistance to therapy and poor prognosis in patients with PCa [292]. Hence, PP1 might be critical for PCa progression and metastization by supporting AR-mediated signaling and contributing to the stability of AR variants.

PP1 has been implicated in additional hormone-mediated events in cancer cells, but investigation is still scarce. Few studies suggest some kind of association with vitamin D3 receptor (VDR) [293-295], ER [194,272], beta-2 adrenergic receptor (ADRB2) [296] and lutropin-choriogonadotropic hormone receptor (LH/CG-R) [297] (Fig. Ib. 6B and 6C). VDR has been proposed as an anti-tumor factor, while ADRB2 has been associated with tumor progression and metastasis formation, though their roles are still in debate [298–300]. An inverse correlation was found between PP-1A expression and ER activation [194,272], but the role of PP1 in ER-mediated signaling is barely explored. A recent study reported the downregulation of PP-1A in response to ER stimulation by 17β-estradiol (E2) in rat myoblast cells [301]. On the other hand, the stimulation of ER-positive breast cancer cells with E2 indirectly promoted PP-1A-mediated dephosphorylation and inactivation of prohibitin-2, which has been proposed as a tumor promoter (Fig. Ib. 6C) [302,303]. PP-1A was also shown to be affected by the 4-hydroxytamoxifen (4OHT), the metabolite of tamoxifen, which is used to treat ER-positive breast cancer [304].

Hence, in addition of being an important regulator of AR-mediated signaling, increasing evidence sheds light on its involvement in other hormone-mediated events that might be particularly relevant for the development of hormone-sensitive cancers, as well as for progression to endocrine resistance.

6.3. PP1 in tumor microenvironment and metastatic cascade

Cancer progression ultimately leads to advanced metastatic stages—the main cause of cancer-related death. Despite recent advances in cancer therapeutics, the management of metastatic stages remains a major challenge in part because the underlying molecular mechanisms are not fully understood. Formation of metastasis involves an orchestrated sequence of events that enable tumor cells to detach from the primary tumor and invade neighboring tissues; enter the circulatory system and delude the immune system; and, eventually, cross the vessel's wall to seed in distant tissues [305]. Several studies implicate PP1 throughout the metastatic process by controlling molecular events in both malignant cells and cells from the microenvironment.

6.3.1. PP1 regulates epithelial integrity

Most cancers have epithelial origin (the so called carcinomas) [306]. In normal conditions, epithelial cells exhibit apical-basal polarity and are juxtaposed laterally by intercellular adhesion complexes [307]. As cancer progresses, loss of such contacts allows malignant cells to invade the neighboring stroma (Fig. Ib. 7) [308].

Cellular adhesions are composed by highly dynamic protein structures that are connected intracellularly to structures from the cytoskeleton (Fig. Ib. 7). By dephosphorylating key junctional and polarity-associated proteins, PP1 may be of fault for the loss of cell-to-cell contacts and apical-basal

polarity. PP-1A binds to the C-terminal region of occludin, one of the main integral membrane proteins in tight junctions. PP-1A-mediated dephosphorylation of occludin compromises its association with the tight junction protein ZO-1, which links the transmembrane complex to the actin cytoskeleton (Fig. Ib. 7A). This was shown to negatively regulate the assembly of tight junctions in human epithelial colorectal adenocarcinoma cells [309].

Interestingly, several studies support the existence of non-redundant functions for PP1c isoforms in regulating junctional and polarity-associated proteins. For instance, although all PP1c canonical isoforms can be found in close proximity to ZO-1 in tight junctions, PP-1A and PP-1G are mainly observed near distinct domains: while PP-1A is found near the N-terminus together with junctional proteins and polarity proteins from the PAR complex, PP-1G is found near the C-terminus together with cytoskeletal proteins and the polarity protein scribble homolog (scribble) [310]. The polarity-associated protein partitioning defective 3 homolog (PAR-3) was shown to interact with PP-1A and, to a lesser extent, PP-1G, but not with PP-1B. PP-1A dephosphorylates PAR-3 and mediates its binding to other regulatory proteins (Fig. Ib. 7A) [311]. Upregulation of PP1c and altered localization of PAR-3 are responsible for major alterations in the subcellular expression of ZO-1 and claudin family members, and were associated with disrupted integrity of the intestinal epithelial lining in coeliac disease [312].

Scribble binds to all PP1c canonical isoforms, though a preferential binding to PP-1A had been reported *in vitro* [313]. The interaction promotes scribble-mediated inhibition of MAPK/ERK signaling (Fig. Ib. 7A), but different mechanisms have been proposed. Scribble is a substrate and a regulator for PP-1G, with its loss leading to increased nuclear translocation of PP-1G and concomitant decrease in cytoplasmic and membrane pools [314]. PP-1A and PP-1B, on the other hand, take part in a macromolecular complex that encompasses scribble, the leucine-rich repeat protein SHOC-2 and the Ras-related protein M-Ras, both RIPPOs involved in the Ras signaling pathway. SHOC-2 and M-Ras promote PP1c-mediated dephosphorylation of Raf-1$^{Ser\text{-}259}$ (an inhibitory phospho-residue), leading to its activation and recruitment to the cell membrane to mediate the activation of the MAPK/ERK signaling pathway [313,315]. When scribble is present in the complex, it binds to SHOC-2 and PP1c, thereby preventing PP1c-mediated dephosphorylation of Raf-1 until M-Ras becomes active and recruits the complex to the cell membrane, where the competition for binding to PP1c takes place [313].

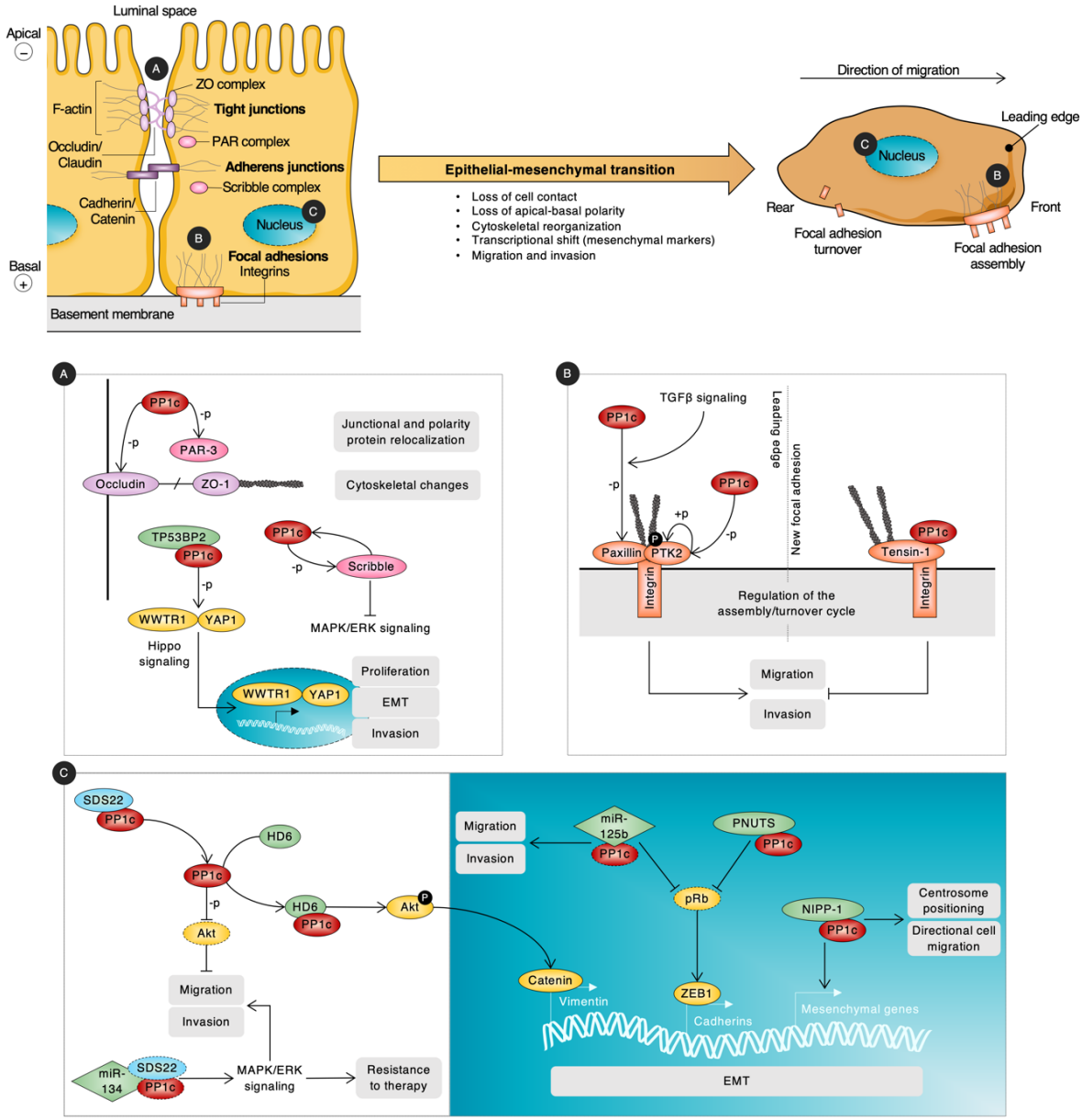

Fig. Ib. 7

PP1 regulates key molecular events during epithelial-mesenchymal transition (EMT). Epithelial cells exhibit cell-cell adhesion and apical-basal polarity. Disruption of epithelial integrity and transcriptional shift towards mesenchymal markers underlie EMT, which capacitates tumor cells with improved migration and invasion capabilities. **(A)** PP1 contributes to the loss of epithelial cell integrity by dephosphorylating key junctional and polarity-associated proteins, leading to major cytosketal changes. **(B)** PP1 regulates focal adhesion assembly/turnover cycle by regulating proteins co-localization with the actin cytoskeleton. **(C)** PP1 holoenzymes regulate the expression of mesenchymal genes and PP1 inhibition is likely to be necessary for the transcriptional shift during EMT. PP1 is in red (dash lining when inhibited); pink, purple and orange proteins are colored according to their localization (top scheme); blue, positive regulators of PP1c; green, negative regulators of PP1c; yellow, other signaling proteins. P, phosphorylated form; +p, phosphorylation; -p, dephosphorylation. Figures were produced using Servier Medical Art (https://smart.servier.com). Abbreviations: HD6, histone deacetylase 6; NIPP-1, nuclear inhibitor of PP1; PAR-3, partitioning defective 3 homolog; PNUTS, PP1 regulatory subunit 10; PP1c, PP1 catalytic subunit; PTK2, focal adhesion kinase 1; SDS22, protein phosphatase 1 regulatory subunit 7; TP53BP2, apoptosis-stimulating of p53 protein 2; WWTR1, WW domain-containing transcription regulator protein 1; YAP1, transcriptional coactivator YAP1; ZEB1, zinc finger E-box-binding homeobox 1; ZO-1, tight junction protein ZO-1.

PP1c is also a positive regulator of the Hippo signaling pathway at tight junctions. The apoptosis-stimulating of p53 protein 2 (TP53BP2) recruits PP1c to tight junctions to dephosphorylate the WW domain-containing transcription regulator protein 1 (WWTR1) and the transcriptional coactivator YAP1 (YAP1) (Fig. Ib. 7A) [316,317]. Like the above mentioned proteins, TP53BP2 preferentially binds to PP-1A [178]. PP1c-mediated dephosphorylation of WWTR1 and YAP1 enhances their nuclear accumulation and stimulates their transcriptional activity [316,317], which has been linked to increased cell proliferation, epithelial-mesenchymal transition (EMT) and cell invasion (Fig. Ib. 7A) [318,319]. Despite the pro-tumoral effects of this mechanisms, it has a negative impact in the survival of tumor cells, when occurring in other cells from tumor microenvironment. In differentiating osteoblasts, PP-1A is targeted to WWTR1 by the Na(+)/H(+) exchange regulatory cofactor NHE-RF1 and the consequent dephosphorylation and nuclear accumulation of WWTR1 affects the ability of the osteoblasts to support the survival of acute myeloid leukemia cells in bone marrow [320].

6.3.2. PP1 regulates focal adhesion dynamics

Paracrine signaling between components of the extracellular matrix (ECM) and tumor cells is critical for tumor progression and successful establishment of metastasis. All PP1c canonical isoforms are present at focal adhesions—major contact points between cells and the ECM—, but PP-1B, nonetheless, was shown to be particularly enriched at these sites. Members of the integrin receptor family and the focal adhesion kinase 1 (PTK2) are substrates for PP1c, albeit the downstream mechanisms of the interactions are not yet fully understood [321]. PP-1B dephosphorylates $PTK2^{Ser\text{-}722}$ in a cell cycle-dependent way, as their interaction is observed during G1, but not in mitotic cells [322]. PP-1B-mediated dephosphorylation of $PTK2^{Ser\text{-}722}$ triggers $PTK2^{Tyr\text{-}397}$ autophosphorylation, which was found upregulated in highly motile and invasive cells from multiple cancers (Fig. Ib. 7B) [323,324]. PTK2 activation by phosphorylation determines the assembly/turnover cycle of focal adhesions that allows cell directional movement and, therefore, PP1c/PTK2 interaction might contribute for cancer cell migration and invasion. PTK2 is recruited to the leading edge of migrating cells by the scaffold protein paxillin, which is itself a target for PTK2 kinase activity [325]. PP1c dephosphorylates paxillin and regulates its subcellular localization, being required for paxillin co-localization with actin (Fig. Ib. 7B). The interaction between PP1c and paxillin is upregulated by the transforming growth factor beta (TGF-β) signaling and was shown to mediate the TGF-β1-induced motility of epithelial cells from oral premalignant lesions (Fig. Ib. 7B) [326].

In contrast to the aforementioned pro-migratory roles of PP-1B through the PTK2-paxillin axis at the leading edge, PP-1A was found in newly formed focal adhesions of migrating cells, far from the leading edge, where it interacts with the scaffold protein tensin-1 (Fig. Ib. 7B) [327,328]. PP-1A binding to tensin-1 was shown to limit the migratory and invasive behavior of triple-negative breast

cancer cells (Fig. Ib. 7B) [328]. Henceforth, as discussed for tight junctions, PP1c isoforms may have distinct substrates and roles at focal adhesions. In fact, the N-terminal domain of tensin-1 binds to and targets PP-1A to focal adhesions, but not PP-1B; whereas PTK2 associates with PP-1B, but not with PP-1A [329]. This also suggests that PP1c isoforms may be differentially recruited to different types of focal adhesions to regulate specific stages of their assembly and turnover and, consequently, determine direction of migration [330].

6.3.3. PP1 in EMT, migration and invasion of cancer cells

EMT capacitates tumor cells with motile and invasive capabilities of mesenchymal cells [305,331]. Associations between PP1c and inhibitory RIPPOs have been demonstrated to enhance the expression of mesenchymal markers and to promote migration and invasion of cancer cells. These include PNUTS [332] and the nuclear inhibitor of protein phosphatase 1 (NIPP-1) [333,334], which are also PP1c substrate specifiers (Fig. Ib. 7C). Like PNUTS, which inhibits PP1c/pRb interaction, miR-125b targets this signaling axis and promotes migration and invasion of gastric cancer cells, being associated with metastases formation in lymph nodes and distant sites (Fig. Ib. 7C) [253].

Additional reports strengthened the hypothesis that PP1c inhibition may contribute to the activation of pro-metastatic pathways, such as Akt and MAPK/ERK signaling cascades. Histone deacetylase 6 (HD6) recruits PP1c from the PP1c/Akt complex and promotes EMT via Akt-mediated signaling (Fig. Ib. 7C) [335]. In contrast, the holoenzyme PP1c-protein phosphatase 1 regulatory subunit 7 (SDS22) impairs cell migration and invasion of breast cancer cells by inhibiting Akt-mediated signaling (Fig. Ib. 7C) [336]. Studies in *Drosophila melanogaster* showed that the PP1c/SDS22 holoenzyme downregulates the activity of myosin-2 and MAPK signaling, which help to maintain epithelial organization and downregulate invasiveness [337]. By targeting this holoenzyme, miR-134 upregulates MAPK/ERK signaling cascade and promotes migration, invasion and resistance to chemotherapy of ovarian cancer cells (Fig. Ib. 7C) [338].

6.3.4. Effects of tumor hypoxia in PP1

Tumor hypoxia has been implicated in long-term metabolic alterations that promote cancer hallmarks, as angiogenesis and metastasis [339]. It can induce temporary cell cycle arrest, which has been suggested to be a protective mechanism to allow cancer cells survival in hostile conditions, and has also been implicated in acquired resistance to therapy [340]. The effects on cell cycle are mediated via hypoxia-induced unbalance in pRb regulators, towards increased PP1c activity and maintenance of pRb in hypophosphorylated state (Fig. Ib. 8A) [341,342]. However, the influence that hypoxic environments exert on PP1c activity may be both isoform-specific and dependent on the degree of oxygen tension.

Pathological hypoxia was shown to upregulate PP-1B expression in pediatric cyanotic myocardium and in human umbilical vein endothelial cell cultures mimicking hypoxic environments [343]. Conversely, PP-1G was downregulated by hypoxia in colorectal carcinoma and cervical adenocarcinoma cell lines. The underlying molecular mechanism involves PP-1G/NIPP-1-mediated degradation of the cyclic AMP-responsive element-binding protein 1 (CREB-1) (Fig. Ib. 8A) [344,345]. PP1c/E3 ubiquitin-protein ligase PPP1R11 holoenzyme was also shown to induce EMT, migration and invasion of colorectal cancer cells under tumor hypoxia (Fig. Ib. 8A) [346].

6.3.5. PP1 in endothelial cell migration, angiogenesis and survival in circulation

Hypoxia eventually leads to the release of angiogenic signals by tumor cells that promote endothelial cell migration and angiogenesis [347]. Such signals downregulate PP1c activity in endothelial cells, as shown in human head and neck squamous cell carcinomas [348].

PP1 regulates the TGF-β signaling pathway cascade in endothelial cells, which is recognized by its pleiotropic outputs [349]. The mothers against decapentaplegic homolog 7 (Smad7) recruits PP-1A to the endothelial cell-restricted serine/threonine-protein kinase receptor R3 (SKR3) [350]. PP-1A dephosphorylates and inactivates SKR3, thereby limiting the downstream signaling via Smad1/5/9 (Fig. Ib. 8B). In turn, PP-1A transcription is induced upon SKR3 activation, which might indicate a negative feedback loop to regulate SKR3 activity (Fig. Ib. 8B).

As described for epithelial tumor cells (Section 6.3.2), PP-1B is an important regulator of focal adhesion dynamics and endothelial cell migration by controlling the activity of PTK2 (Fig. Ib. 8B). This regulatory mechanism is essential for tumor neovascularization [343]. Also, inhibition of PP1c in endothelial cells leads to delocalization of paxillin from focal adhesions and disrupts its association with the actin cytoskeleton, shaping major rearrangements and cellular rounding (Fig. Ib. 8B). However, this phenotypic alteration seems insufficient to induce endothelial cell migration, in part because the TGF-β signaling may somehow compensate for the lack of PP1 through mechanisms yet to be discovered [351].

In addition to its role in endothelial cell migration, PP1c might regulate other angiogenesis-related events, such as tubulogenesis and vascular permeability. PP-1A and PP-1G were identified in a screening as potential angiogenic targets for pharmaceutical intervention. Their knockdown in zebrafish embryos was followed by defects in vascular development, including in tubulogenesis [352]. In contrast, PP-1B downregulation in human umbilical vein endothelial cells did not affect endothelial tube formation [343].

Protein phosphatase 1 regulatory inhibitor subunit 16B (PPP1R16B), which belongs to the MYPT1 family, is highly expressed in endothelial cells where it assumes diverse functions (as reviewed in [353]). PPP1R16B/PP-1B holoenzyme regulates the phosphorylation state of several proteins that are critical to endothelial cell barrier, including the ezrin-radixin-moesin complex (ERM),

merlin, endothelin-1 and the elongation factor 1-alpha 1 (EEF1A1) (Fig. Ib. 8B) [353]. Using co-cultures of rat brain microvascular endothelial cells and rat glioma cells, Li Z et al. showed that PP1c is involved in a signaling cascade that promote blood-tumor barrier hyperpermeability (Fig. Ib. 8B) [354].

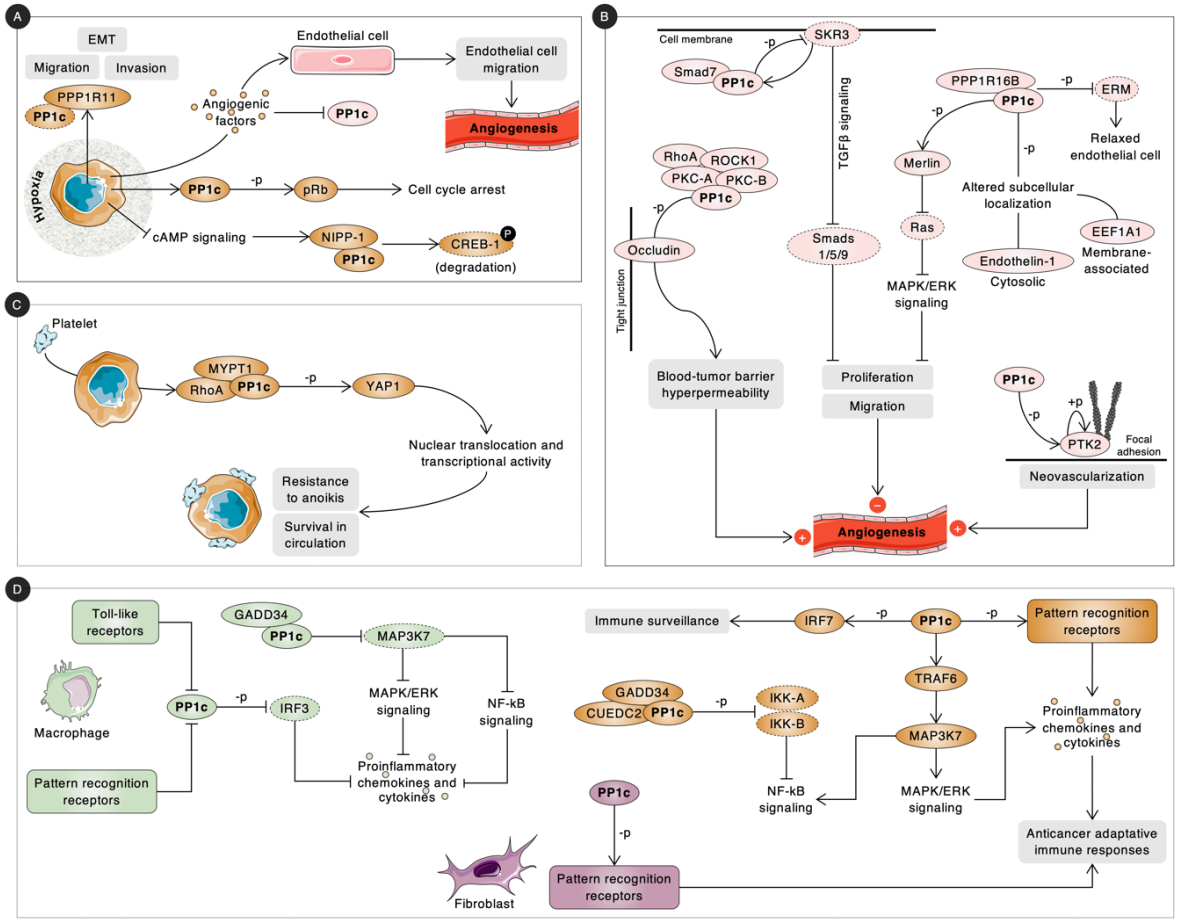

Fig. Ib. 8

PP1 in tumor microenvironment. (A) PP1 in response to hypoxia. **(B)** PP1 in endothelial cell dynamics and angiogenesis. **(C)** Paracrine signaling between platelets and tumor cells induce PP1-mediated activation of YAP1 transcriptional activity, contributing to tumor cell survival in circulation. **(D)** PP1 in inflammation and immune response. Different functions have been proposed in different cell types. Colors represent the cell type where the event takes place. P, phosphorylated form; +p, phosphorylation; -p, dephosphorylation. Figures were produced using Servier Medical Art (https://smart.servier.com). Abbreviations: CREB-1, cyclic AMP-responsive element-binding protein 1; CUEDC2, CUE domain-containing protein 2; EEF1A1, elongation factor 1-alpha 1; ERM, ezrin-radixin-moesin complex; GADD34, protein phosphatase 1 regulatory subunit 15A; IKK, inhibitor of nuclear factor kappa-B kinase; IRF, interferon regulatory factor; MAP3K7, mitogen-activated protein kinase kinase kinase 7; NIPP-1, nuclear inhibitor of protein phosphatase 1; PKC, protein kinase C; PP1c, protein phosphatase 1 catalytic subunit; PPP1R16B, protein phosphatase 1 regulatory inhibitor subunit 16B; pRb, retinoblastoma-associated protein; PTK2, focal adhesion kinase 1; RhoA, transforming protein RhoA; ROCK1, Rho-associated protein kinase 1; SKR3, serine/threonine-protein kinase receptor R3; Smad, mothers against decapentaplegic homolog; TRAF6, TNF receptor-associated factor 6.

Moreover, several studies have highlighted the relevance of PP1c in platelets, including its role in the cytoskeleton organization, Ca^{2+} influx and platelet aggregation [355–358]. Being present at the tumor microenvironment, platelets can induce the activation of the transforming protein

RhoA/MYPT1/PP1c axis in cancer cells to drive the transcription of genes involved in resistance to anoikis (i.e., apoptosis induced by cell detachment from the ECM) (Fig. Ib. 8C). This process was shown to contribute to thrombocytosis-induced increase in metastasis in ovarian and colorectal cancer models, which is linked to poorer prognosis [359].

6.3.6. PP1 in inflammation and immune response

The tumor microenvironment is characterized by sustained inflammation with several types of immune cells being found within the stroma. These are critical for cancer progression and also constitute the basis for cancer immunotherapy [360]. PP1c dephosphorylates several proteins engaged in innate immune response and inflammation, although these have been mostly studied during viral or bacterial infections, rather than in tumoral contexts [361–364]. It is worth to mention, nonetheless, that cancer cells can mimic viral infections to activate intracellular signaling pathways that produce interferon and proinflammatory cytokines [365]. Moreover, several viruses have been deeply implicated in the etiology of multiple cancers [366]. In this regard, PP-1G was shown to be a direct target of Human papillomavirus type 16 (HPV-16) oncoproteins in cervical cancers. In HPV-16-positive cervical tumors, PP-1G expression is reduced, with no nuclear expression and only residual cytoplasmic expression observed [367].

PP1 regulates two tightly related signaling pathways that connect inflammation and immunity to cancer development and progression—the Toll-like receptor (TLR) signaling and the NF-κB signaling. TLRs are key pattern recognition receptors involved in innate immunity that are expressed not only by immune cells, but also by other cell types, including tumor cells and cells from tumor microenvironment [368,369]. Conventionally, upon TLRs activation the signaling is transduced through downstream adapter molecules that mediate the activation of other signaling pathways (e.g. MAPK/ERK and NF-κB signaling) to upregulate the production of proinflammatory cytokines [370]. PP1c isoforms have both promoting and inhibitory effects on the regulation of these pathways, which might suggest isoform-specific roles in immunity and inflammation depending on the cell type and cellular context (Fig. Ib. 8D). GADD34 has been reported as a particularly relevant RIPPO in targeting PP1c to upstream regulators of MAPK/ERK and NF-κB signaling pathways in both macrophages and human epithelial cells (Fig. Ib. 8D) [371–373].

All PP1c canonical isoforms were shown to dephosphorylate several phospho-residues of the interferon regulatory factor 7 (IRF7) in Epstein-Barr virus-transformed lymphoblastic cells. PP1c-mediated dephosphorylation of IRF7 leads to its inactivation and impaired transcriptional activity during viral infection [374]. Interestingly, IRF7 downregulation in cancer cells seems to contribute to immune surveillance and occurrence of bone metastases in breast cancer and PCa (Fig. Ib. 8D) [375,376]. Furthermore, in response to RNA virus infection, PP-1A and PP-1G dephosphorylate the antiviral innate immune response receptor RIG-I at Ser-88 and the interferon-induced helicase C

domain-containing protein 1 at Ser-8/Thr-170 in human epithelial cells and fibroblasts, leading to their activation and production of interferon-β [363]. Activation of both sensors in cancer cells has been shown to trigger apoptosis, as well as to elicit the release of proinflammatory chemokines and cytokines that stimulate dendritic cells to initiate anticancer adaptative immune responses (Fig. Ib. 8D) [377,378]. Accordingly, treatment with PP1c inhibitors tautomycin and calyculin A induce calreticulin exposure in several tumor cell lines in a similar way to anthracyclins [379].

Hence, PP1c has several substrates engaged in inflammatory and immune responses and the few existing studies support both immune surveillance and antitumor immune responses as potential outcomes. In spite of the still limited knowledge, these studies stress the need for deeper investigation on the roles of PP1 in cancer immunity.

7. PP1 in the context of cancer therapy

7.1. PP1 contribution to chemosensitivity

PP1c activation has been observed in response to several treatments that promote unbalanced production of splice variants (Fig. Ib. 9). Ceramide is a potent tumor suppressor lipid that induces cell apoptosis in response to several chemotherapeutic agents (e.g. ectoposide, gemcitabine, cisplatin, daunorubicin, among others [380]). Both short and long chain ceramides were found to activate PP1c [381–383]. PP1c activity, in turn, mediates the phosphorylation state of splice variants that contain the evolutionarily conserved RVxF docking motif.

In lung adenocarcinoma cells, ceramide regulates the Bcl-2-like protein 1 (Bcl2-L-1) and caspase-9 alternative splicing, leading to the downregulation of the anti-apoptotic splice variants Bcl-x(L) and caspase-9b [384]. An improved liposome-based formulation of the short-chain cell permeable ceramide C6 was shown to inhibit cell survival and proliferation and to activate apoptosis in different melanoma cell lines via PP1c activation and consequent inactivation of Akt/mTOR signaling (Fig. Ib. 9) [385].

Like ceramide, emetine—a potent inhibitor of protein synthesis and effective chemotherapeutic agent [386]—also regulates Bcl2-L-1 alternative splicing towards an increase of pro-apoptotic variants in human cervical, breast and lung cancer cell lines; however, opposing effects have been reported for caspase-9 [387,388]. PP1c inhibition blocked emetine effects, which confirms the importance of PP1c in mediating emetine-induced cell death [387,388]. Tamoxifen is widely recognized as a chemopreventive and chemotherapeutic agent for ER-positive breast cancer [389]. Its metabolite, 4OHT, inhibits cancer cell proliferation and induces cell death by interfering with multiple signaling pathways and disturbing Ca^{2+} homeostasis. Although ER is the prime target for 4OHT, there is evidence that 4OHT upregulates PP-1A, while downregulates its phosphorylated and inactive form. This sensitizes breast cancer cells to tamoxifen cytotoxicity by increasing Ca^{2+} release from

the endoplasmic reticulum, an event mediated by the inositol 1,4,5-trisphosphate receptor type 1 (IP3R) (Fig. Ib. 9) [304].

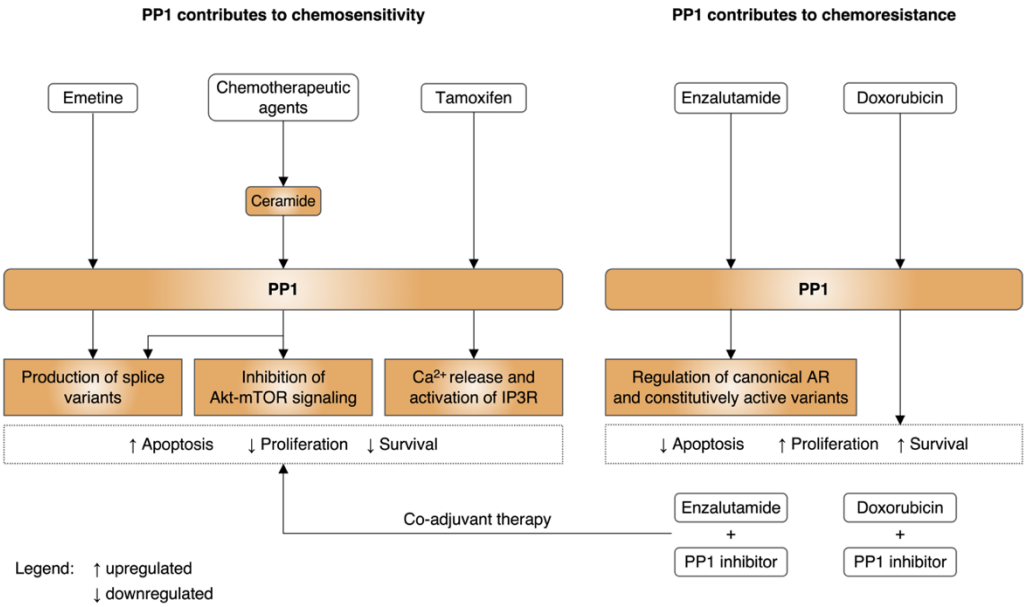

Fig. Ib. 9

PP1 in cancer therapy. PP1 contributes to chemosensitivity and chemoresistance through distinct mechanisms. The combination of PP1c inhibition with regularly used drugs, such as enzalutamide and doxorubicin, has been shown benefits as co-adjuvant therapy. Abbreviations: Akt, RAC-alpha serine/threonine-protein kinase; AR, androgen receptor; IP3R, inositol 1,4,5-triphosphate receptor type 1; mTOR, serine/threonine-protein kinase mTOR.

7.2. PP1 contribution to chemoresistance

The generation of AR variants, mainly AR-V7, has been implicated in castration resistance [390]. AR-V7 is constitutively active and may drive tumor progression even under enzalutamide and abiraterone antiandrogen treatments [391] and, as previously referred (Section 6.2), PP1c is a positive regulator of AR-V7 [291]. Enzalutamide was reported to induce cell type-dependent activation of PP1c and Akt in PCa cells to regulate AR phosphorylation status and Mdm2 activation. Collectively, these findings indicate that targeting PP1c may be useful during ADT and might be effective in the treatment of advanced CRPC. In fact, co-treatment of enzalutamide with a PP1 inhibitor was shown to reduce the expression levels of the prostate-specific antigen (PSA) and to impair the proliferation of PCa cells (Fig. Ib. 9) [285].

Recently, high expression of PP-1G was found to contribute to doxorubicin resistance (Fig. Ib. 9) [201]—one of the most potent antitumor agents [392]. Doxorubicin cytotoxic effects on hepatocellular carcinoma cells were enhanced when combined with PP-1G knockdown (Fig. Ib. 9) [201].

7.3. PP1 contribution to radiosensitivity

PP1 has also been associated with the response to radiotherapy. LIM and senescent cell antigen-like-containing domain protein 1 (LIMS1) was reported to be significantly upregulated in humor

tumors, being a critical regulator of cellular sensitivity to ionizing radiation and cytotoxic drugs. The interaction between LIMS1 and PP-1A, through the KFVEF binding motif, inhibits PP-1A activity and prevents Akt dephosphorylation at Ser-473 and Thr-308 residues. As a result, cellular resistance to ionizing radiation is enhanced [393]. Resistance to radiotherapy decreases after ceramide treatment and consequent PP1 activation [394]. Therefore, PP1 might be a promising therapeutic target to consider in future protocols of radiotherapy.

7.4. Is PP1 a potential target for the development of new cancer therapeutics?

As discussed in the previous sections, PP1c activity is influenced by several therapeutics and, in spite of not being recognized as the main therapeutic target, the modulation of its activity underlies many treatment responses (Fig. Ib. 9). So, the question is: is it feasible to use PP1 as target for the development of new cancer therapeutics?

Protein phosphatases had been considered ‘undruggable’ for many years due to their ubiquitous distribution and central role in controlling cell dynamics. However, recent findings brought back hope for phosphatase-directed therapeutics. A number of compounds targeting the tyrosine-protein phosphatase non-receptor type 11 and the tyrosine-protein phosphatase non-receptor type 1 have successfully made it into clinical trials [395]. Targeting PPP phosphatases, nevertheless, has been much more challenging mainly due to the high conservation of active sites within members of the family. This compromises the selectivity of allosteric inhibitors. For instance, LB-100, which was initially described as a PP2A inhibitor with potential chemo- and radiotherapy enhancing activity, was latter shown to also inhibit the Ser/Thr-protein phosphatase 5 [395].

PP1 presents an advantageous feature that might overcome the constrains of allosteric inhibition. As referred in Section 3, PP1 acts as holoenzymes and most RIPPOs have at least one RVxF-docking motif to mediate the binding to PP1c. This binding mechanism is not shared by the other members of the PPP family, which exhibit other types of short linear motifs for substrate recognition (hence the “specific” piece in the puzzle of the binding code (Fig. Ib. 1)). Therefore, targeting PP1 holoenzymes, instead of focusing on the PP1c's active site, is a promising solution to drug the ‘undruggable’. This was thought to be the case of Salubrinal, Guanabenz, Sephin1 and Raphin1, which interfere with protein synthesis by regulating the eukaryotic translation initiation factor 2A. Initial studies suggested that these molecules would directly inhibit PP1c/GADD34 and also PP1c/PPP1R15B in the case of Salubrinal and Raphin1. However, recent evidence indicates this may not be the case and current data does not support such straight interpretation [395].

A long journey is still ahead until the use of PP1 modulators in pharmacological protocols, but recent studies with small molecules that promote the assembly and stabilize PP2A holoenzymes shed light on the feasibility of the process [396,397]. Chatterjee J et al. developed the first cell-penetrating

peptide capable of selectively activate PP1 in living cells by competing with RVxF-containing RIPPOs [398]. The peptide was designed based on the NIPP-1 sequence and optimized versions have recently been developed, including a photoactivatable disrupting peptide [399,400]. In addition, a peptide was designed to target the interaction between PP1c and the leucine-rich repeat serine/threonine-protein kinase 2 [401]. Despite the progresses in the development of interfering peptides, functional studies are still lacking. However, given the huge number of PP1 holoenzymes involved in tumorigenesis, the characterization of their roles in specific cancer contexts is expected to uncover a myriad of opportunities for personalized cancer treatment.

8. Concluding remarks: is PP1 looking for a spotlight to stand in?

PP1 function has been neglected in cancer research despite all the interesting findings that have been reported. Its role in cancer biology is still unclear, with no consensus regarding the function of each PP1c isoform as either tumor-suppressor or tumor-promoter. Most probably, their expression and roles will be dependent on the type of cancer, the cancer staging and the RIPPOs they are interacting with. Dedicated analyses of PP1c isoforms expression and identification of their interactomes in tumors will certainly help to clarify these issues. Despite all the gaps in the current knowledge, it is undeniable that PP1 is involved in tumorigenesis, regulating several processes not only in tumor cells, but also in cells from the tumor microenvironment (Fig. Ib. 10).

Targeting PP1 holoenzymes is challenging but might add value to cancer therapeutics. The maintenance of PP1c/pRb interaction is likely to be essential for controlling unlimited replicative phenotype of cancer cells, as so is the inhibitory action of PP1c on Akt to control cell survival. Therefore, disruption of inhibitory interactions, such as caveolin-1/PP1c and Fer/PP1c, or stabilization pRb-activating holoenzymes, such as neurabin-2/PP1c, might provide some advantage in controlling tumor growth. The ability of PP1c to regulate AR-mediated signaling, as well as ligand-independent variants of the receptor makes these interactions convenient targets to explore new therapeutic approaches, particularly to mCRPC. Another interesting idea would be exploring the bittersweet contribution of PP1 for metastization. On the one hand, PP1c actions on cell-to-cell contacts seem to contribute to the disruption of epithelial integrity, which occurs during EMT; on the other hand, nuclear PP1c shows sign of helping to restring the transcription of mesenchymal markers, which is also essential for EMT.

A lot of questions remain to be answered, but in fact it is time for cancer research to put a spotlight on PP1.

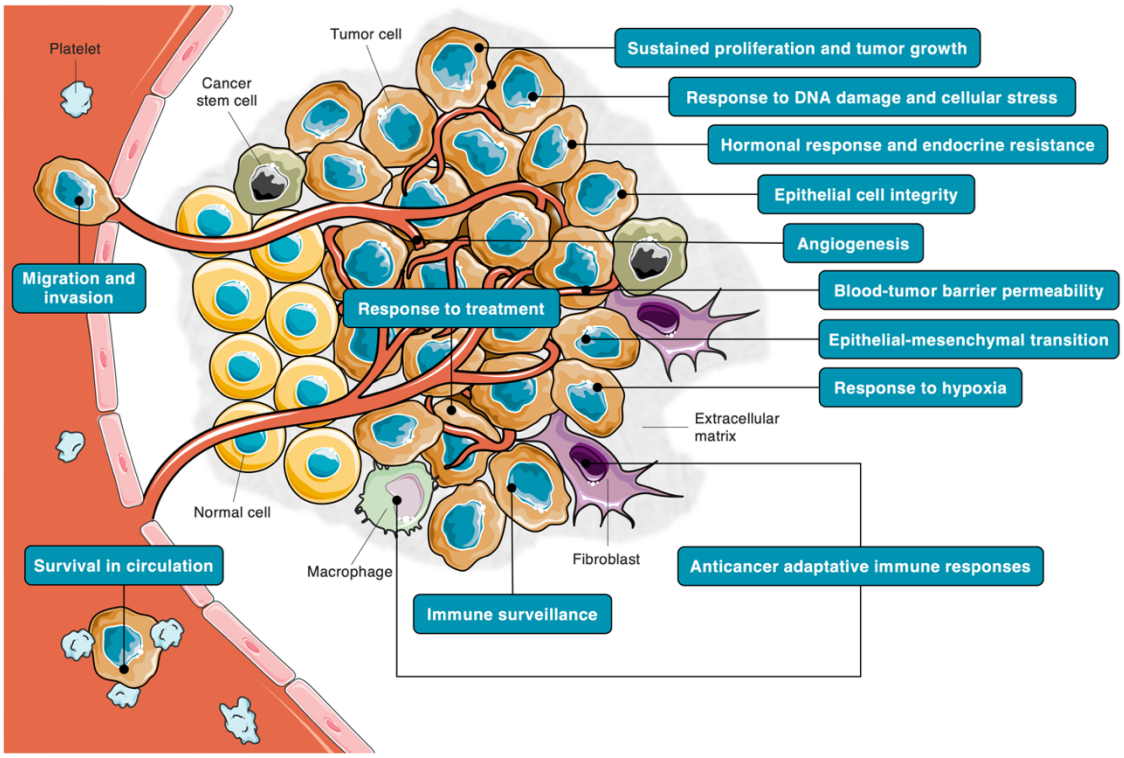

Fig. Ib. 10

Overview on the cancer-related processes in which PP1 has been implicated. Figures were produced using Servier Medical Art (https://smart.servier.com).

Suppl. Table Ib. 1
Nomenclature details of the proteins mentioned in the manuscript.

In the text	Recommended protein name	Alternative name(s)	Gene name	Synonyms	UniProtKB
ACYP2	Acylphosphatase-2	Acylphosphatase, muscle type isozyme; Acylphosphate phosphohydrolase 2	*ACYP2*	*ACYP*	P14621
ADRB2	Beta-2 adrenergic receptor	Beta-2 adrenoreceptor; beta-2 adrenoceptor	*ADRB2*	*ADRB2R B2AR*	P07550
Akt	RAC-alpha serine/threonine-protein kinase	Protein kinase B; PKB; Protein kinase B alpha; PKB alpha; Proto-oncogene c-Akt; RAC-PK-alpha	*AKT*	*PKB RAC*	P31749
ALK	ALK tyrosine kinase receptor	Anaplastic lymphoma kinase; CD_antigen: CD246	*ALK*		Q9UM73
Antiviral innate immune response receptor RIG-I	Antiviral innate immune response receptor RIG-I	DEAD box protein 58; Probable ATP-dependent RNA helicase DDX58; RIG-I-like receptor 1; RLR-1; Retinoic acid-inducible gene 1 protein; RIG-1; Retinoic acid-inducible gene I protein; RIG-I	*DDX58*		O95786
AR	Androgen receptor	Dihydrotestosterone receptor; Nuclear receptor subfamily 3 group C member 4	*AR*	*DHTR NR3C4*	P10275
ARFGEF3	Brefeldin A-inhibited guanine nucleotide-exchange protein 3	ARFGEF family member 3	*ARFGEF3*	*BIG3 C6orf92 KIAA1244*	Q5TH69
ATR	Serine/threonine-protein kinase ATR	Ataxia telangiectasia and Rad3-related protein; FRAP-related protein 1	*ATR*	*FRP1*	Q13535
Aurora kinase B	Aurora kinase B	Aurora 1; Aurora- and IPL1-like midbody-associated protein 1; AIM-1; Aurora/IPL1-related kinase 2; ARK-2; Aurora-related kinase 2; STK-1; Serine/threonine-protein kinase 12; Serine/threonine-protein kinase 5: Serine/threonine-protein kinase aurora-B	*AURKB*	*AIK2 AIM1 AIRK2 ARK2 STK1 STK12 STK5*	Q96GD4
BAX	Apoptosis regulator BAX	Bcl-2-like protein 4; Bcl2-L-4	*BAX*	*BCL2L4*	Q07812
Bcl2-L-1	Bcl-2-like protein 1 (Bcl2-L-1)		*BCL2L1*	*BCL2L BCLX*	Q07817
BRCA1	Breast cancer type 1 susceptibility protein	RING finger protein 53; RING-type E3 ubiquitin transferase BRCA1	*BRCA1*	*RNF53*	P38398
Calreticulin	Calreticulin	CRP55; Calregulin; Endoplasmic reticulum resident protein 60; ERp60; HACBP; grp60	*CALR*	*CRTC*	P27797
Caspase-9	Caspase-9 (CASP-9)	Apoptotic protease Mch-6; Apoptotic protease-activating factor 3; APAF-3; ICE-like apoptotic protease 6; ICE-LAP6	*CASP9*		P55211
Catenin	Catenin beta-1	Beta-catenin	*CTNNB1*	*CTNNB*	P35222

In the text	Recommended protein name	Alternative name(s)	Gene name	Synonyms	UniProtKB
Caveolin-1	Caveolin-1		*CAV1*	*CAV*	Q03135
CCND1	G1/S-specific cyclin-D1	B-cell lymphoma 1 protein; BCL-1; BCL-1 oncogene; PRAD1 oncogene	*CCND1*	*BCL1 PRAD1*	P24385
CDK1	Cyclin-dependent kinase 1 (CDK1)	Cell division control protein 2 homolog; Cell division protein kinase 1; p34 protein kinase	*CDK1*	*CDC2 CDC28A CDKN1 P34CDC2*	P06493
CDK2	Cyclin-dependent kinase 2	Cell division protein kinase 2; p33 protein kinase	*CDK2*	*CDKN2*	P24941
CDK9	Cyclin-dependent kinase 9	C-2K; Cell division cycle 2-like protein kinase 4; Cell division protein kinase 9; Serine/threonine-protein kinase PITALRE; Tat-associated kinase complex catalytic subunit	*CDK9*	*CDC2L4 TAK*	P50750
CHSY1	Chondroitin sulfate synthase 1	Chondroitin glucuronyltransferase 1; Chondroitin synthase 1; ChSy-1; Glucuronosyl-N-acetylgalactosaminyl-proteoglycan 4-beta-N-acetylgalactosaminyltransferase 1; N-acetylgalactosaminyl-proteoglycan 3-beta-glucuronosyltransferase 1; N-acetylgalactosaminyltransferase 1	*CHSY1*	*CHSY CSS1 KIAA0990*	Q86X52
CLCF1	Cardiotrophin-like cytokine factor 1	B-cell-stimulating factor 3; BSF-3; Novel neurotrophin-1; NNT-1	*CLCF1*	*BSF3 CLC NNT1*	Q9UBD9
CREB-1	Cyclic AMP-responsive element-binding protein 1 (CREB-1)		*CREB1*		P16220
CUEDC2	CUE domain-containing protein 2		*CUEDC2*	*C10orf66*	Q9H467
CycT1	Cyclin-T1 (CycT1, Cyclin-T)		*CCNT1*		O60563
Dp-1	Transcription factor Dp-1	DRTF1-polypeptide 1; DRTF1; E2F dimerization partner 1	*TFDP1*	*DP1*	Q14186
E2F1	Transcription factor E2F1 (E2F-1)	PBR3; Retinoblastoma-associated protein 1; RBAP-1; Retinoblastoma-binding protein 3; RBBP-3; pRB-binding protein E2F-1	*E2F1*	*RBBP3*	Q01094
EEF1A1	Elongation factor 1-alpha 1 (EF-1-alpha-1)	Elongation factor Tu; EF-Tu; Eukaryotic elongation factor 1 A-1; eEF1A-1; Leukocyte receptor cluster member 7	*EEF1A1*	*EEF1A EF1A LENG7*	P68104
EIF2B4	Translation initiation factor eIF-2B subunit delta	eIF-2B GDP-GTP exchange factor subunit delta	*EIF2B4*	*EIF2BD*	Q9UI10
Endothelin-1	Endothelin-1	Preproendothelin-1; PPET1	*EDN1*		P05305

In the text	Recommended protein name	Alternative name(s)	Gene name	Synonyms	UniProtKB
ER	Estrogen receptor (ER)	ER-alpha; Estradiol receptor; Nuclear receptor subfamily 3 group A member 1	*ESR1*	*ESR NR3A1*	P03372
Eukaryotic translation initiation factor 2A	Eukaryotic translation initiation factor 2A (eIF-2A)	65 kDa eukaryotic translation initiation factor 2A	*EIF2A*		Q9BY44
Ezrin	Ezrin	Cytovillin; Villin-2; p81	*EZR*	*VIL2*	P15311
Fer	Tyrosine-protein kinase Fer	Feline encephalitis virus-related kinase FER; Fujinami poultry sarcoma/Feline sarcoma-related protein Fer; Proto-oncogene c-Fer; Tyrosine kinase 3; p94-Fer	*FER*		P16591
GADD34	Protein phosphatase 1 regulatory subunit 15A	Growth arrest and DNA damage-inducible protein GADD34; Myeloid differentiation primary response protein MyD116 homolog	*PPP1R15A*	*GADD34*	O75807
HD6	Histone deacetylase 6 (HD6)	Tubulin-lysine deacetylase HDAC6	*HDAC6*	*KIAA0901*	Q9UBN7
Interferon-induced helicase C domain-containing protein 1	Interferon-induced helicase C domain-containing protein 1	Clinically amyopathic dermatomyositis autoantigen 140 kDa; CADM-140 autoantigen; Helicase with 2 CARD domains; Helicard; Interferon-induced with helicase C domain protein 1; Melanoma differentiation-associated protein 5; MDA-5; Murabutide down-regulated protein; RIG-I-like receptor 2; RLR-2; RNA helicase-DEAD box protein 116	*IFIH1*	*MDA5 RH116*	Q9BYX4
Interferon-β	Interferon beta (IFN-beta)	Fibroblast interferon	*IFNB1*	*IFB IFNB*	P01574
IKK-A	Inhibitor of nuclear factor kappa-B kinase subunit alpha (I-kappa-B kinase alpha, IKK-A, IKK-alpha, IkBKA, IkappaB kinase)	Conserved helix-loop-helix ubiquitous kinase; I-kappa-B kinase 1; IKK1; Nuclear factor NF-kappa-B inhibitor kinase alpha; NFKBIKA; Transcription factor 16; TCF-16	*CHUK*	*IKKA TCF16*	O15111
IKK-B	Inhibitor of nuclear factor kappa-B kinase subunit beta (I-kappa-B-kinase beta, IKK-B, IKK-beta; IkBKB)	I-kappa-B kinase 2; IKK2; Nuclear factor NF-kappa-B inhibitor kinase beta; NFKBIKB	*IKBKB*	*IKKB*	O14920
Interleukin-6	Interleukin-6 (IL-6)	B-cell stimulatory factor 2; BSF-2; CTL differentiation factor; CDF; Hybridoma growth factor; Interferon beta-2; IFN-beta-2	*IL6*	*IFNB2*	P05231
IP3R	Inositol 1,4,5-trisphosphate receptor type 1	IP3 receptor isoform 1; IP3R 1; InsP3R1; Type 1 inositol 1,4,5-trisphosphate receptor; Type 1 InsP3 receptor	*ITPR1*	*INSP3R1*	Q14643

In the text	Recommended protein name	Alternative name(s)	Gene name	Synonyms	UniProtKB
IRF3	Interferon regulatory factor 3		*IRF3*		Q14653
IRF7	Interferon regulatory factor 7		*IRF7*		Q92985
KCNC2	Potassium voltage-gated channel subfamily C member 2	Shaw-like potassium channel; Voltage-gated potassium channel Kv3.2	*KCNC2*		Q96PR1
Ki-67	Proliferation marker protein Ki-67	Antigen identified by monoclonal antibody Ki-67; Antigen KI-67; Antigen Ki67	*MKI67*		P46013
LH/CG-R	Lutropin-choriogonadotropic hormone receptor (LH/CG-R)	Luteinizing hormone receptor; LHR; LSH-R	*LHCGR*	*LCGR LGR2 LHRHR*	P22888
LIMS1	LIM and senescent cell antigen-like-containing domain protein 1	Particularly interesting new Cys-His protein 1; PINCH-1; Renal carcinoma antigen NY-REN-48	*LIMS1*	*PINCH PINCH1*	P48059
M-Ras	Ras-related protein M-Ras	Ras-related protein R-Ras3	*MRAS*	*RRAS3*	O14807
MAP3K7	Mitogen-activated protein kinase kinase kinase 7	Transforming growth factor-beta-activated kinase 1; TGF-beta-activated kinase 1	*MAP3K7*	*TAK1*	O43318
Mdm2	E3 ubiquitin-protein ligase Mdm2	Double minute 2 protein; Hdm2; Oncoprotein Mdm2; RING-type E3 ubiquitin transferase Mdm2; p53-binding protein Mdm2	*MDM2*		Q00987
Mdm4	Protein Mdm4	Double minute 4 protein; Mdm2-like p53-binding protein; Protein Mdmx; p53-binding protein Mdm4	*MDM4*	*MDMX*	O15151
Merlin	Merlin	Moesin-ezrin-radixin-like protein; Neurofibromin-2; Schwannomerlin; Schwannomin	*NF2*	*SCH*	P35240
Moesin	Moesin	Membrane-organizing extension spike protein	*MSN*		P26038
Myc	Myc proto-oncogene protein	Class E basic helix-loop-helix protein 39; bHLHe39; Proto-oncogene c-Myc; Transcription factor p64	*MYC*	*BHLHE39*	P01106
mTOR	Serine/threonine-protein kinase mTOR	FK506-binding protein 12-rapamycin complex-associated protein 1; FKBP12-rapamycin complex-associated protein; Mammalian target of rapamycin; mTOR; Mechanistic target of rapamycin; Rapamycin and FKBP12 target 1; Rapamycin target protein 1	*MTOR*	*FRAP FRAP1 FRAP2 RAFT1 RAPT1*	P42345
Myosin-2	Myosin-2	Myosin heavy chain 2; Myosin heavy chain 2a; MyHC-2a; Myosin heavy chain IIa; MyHC-IIa; Myosin heavy chain, skeletal muscle, adult 2	*MYH2*	*MYHSA2*	Q9UKX2

In the text	Recommended protein name	Alternative name(s)	Gene name	Synonyms	UniProtKB
MYPT1	Protein phosphatase 1 regulatory subunit 12A	Myosin phosphatase-targeting subunit 1; Myosin phosphatase target subunit 1; Protein phosphatase myosin-binding subunit	*PPP1R12A*	*MBS MYPT1*	O14974
Leucine-rich repeat serine/threonine-protein kinase 2	Leucine-rich repeat serine/threonine-protein kinase 2	Dardarin	*LRRK2*	*PARK8*	Q5S007
Na(+)/H(+) exchange regulatory cofactor NHE-RF1	Na(+)/H(+) exchange regulatory cofactor NHE-RF1	Ezrin-radixin-moesin-binding phosphoprotein 50; EBP50; Regulatory cofactor of Na(+)/H(+) exchanger; Sodium-hydrogen exchanger regulatory factor 1; Solute carrier family 9 isoform A3 regulatory factor 1	*SLC9A3R1*	*NHERF NHERF1*	O14745
Nek2	Serine/threonine-protein kinase Nek2	HSPK 21; Never in mitosis A-related kinase 2; NimA-related protein kinase 2; NimA-like protein kinase 1	*NEK2*	*NEK2A NLK1*	P51955
Neurabin-2	Neurabin-2	Neurabin-II; Protein phosphatase 1 regulatory subunit 9B; Spinophilin	*PPP1R9B*	*PPP1R6*	Q96SB3
NIPP-1	Nuclear inhibitor of protein phosphatase 1 (NIPP-1)	Protein phosphatase 1 regulatory inhibitor subunit 8	*PPP1R8*		Q12972
NPM	Nucleophosmin (NPM)	Nucleolar phosphoprotein B23; Nucleolar protein NO38; Nucleolar protein NO38	*NPM1*	*NPM*	P06748
Occludin	Occludin		*OCLN*		Q16625
p150	Tyrosine-protein kinase ABL1	Abelson murine leukemia viral oncogene homolog 1; Abelson tyrosine-protein kinase 1; Proto-oncogene c-Abl; p150	*ABL1*	*ABL JTK7*	P00519
p21	Cyclin-dependent kinase inhibitor 1	CDK-interacting protein 1; Melanoma differentiation-associated protein 6; MDA-6; p21	*CDKN1A*	*CAP20 CDKN1 CIP1 MDA6 PIC1 SDI1 WAF1*	P38936
p53	Cellular tumor antigen p53	Antigen NY-CO-13; Phosphoprotein p53; Tumor suppressor p53	*TP53*	*P53*	P04637
p65	Transcription factor p65	Nuclear factor NF-kappa-B p65 subunit; Nuclear factor of kappa light polypeptide gene enhancer in B-cells 3	*RELA*	*NFKB3*	Q04206
PAR-3	Partitioning defective 3 homolog (PAR-3, PARD-3)	Atypical PKC isotype-specific-interacting protein (ASIP), CTCL tumor antigen se2-5, PAR3-alpha	*PARD3*	*PAR3 PAR3A*	Q8TEW0
Paxillin	Paxillin		*PXN*		P49023

In the text	Recommended protein name	Alternative name(s)	Gene name	Synonyms	UniProtKB
PD-L1	Programmed cell death 1 ligand 1 (PD-L1, PDCD1 ligand 1)	B7 homolog 1; B7-H1; CD_antigen: CD274	*CD274*	*B7H1 PDCD1L1 PDCD1LG1 PDL1*	Q9NZQ7
Prohibitin-2	Prohibitin-2	B-cell receptor-associated protein BAP37; D-prohibitin; Repressor of estrogen receptor activity	*PHB2*	*BAP REA*	Q99623
PKA C-alpha	cAMP-dependent protein kinase catalytic subunit alpha (PKA C-alpha)		*PRKACA*	*PKACA*	P17612
PKC-A	Protein kinase C alpha type (PKC-A, PKC-alpha)		*PRKCA*	*PKCA PRKACA*	P17252
PKC-B	Protein kinase C beta type (PKC-B, PKC-beta)		*PRKCB*	*PKCB PRKCB1*	P05771
PNUTS	Serine/threonine-protein phosphatase 1 regulatory subunit 10	MHC class I region proline-rich protein CAT53; PP1-binding protein of 114 kDa; Phosphatase 1 nuclear targeting subunit; Protein FB19; p99	*PPP1R10*	*CAT53 FB19 PNUTS*	Q96QC0
PP-1A	Serine/threonine-protein phosphatase PP1-alpha catalytic subunit (PP-1A)	Protein phosphatase alpha; PP1α; PPP1CA	*PPP1CA*	*PPP1A*	P62136
PP-1B	Serine/threonine-protein phosphatase PP1-beta catalytic subunit (PP-1B)	Protein phosphatase beta; PP1β; PPP1CB; PPP1CD	*PPP1CB*		P62140
PP-1G	Serine/threonine-protein phosphatase PP1-gamma catalytic subunit (PP-1G)	Protein phosphatase gamma; PP1γ; PPP1CC; Protein phosphatase 1C catalytic subunit	*PPP1CC*		P36873
PPP1R11	E3 ubiquitin-protein ligase PPP1R11	Hemochromatosis candidate gene V protein; HCG V; Protein phosphatase 1 regulatory subunit 11; Protein phosphatase inhibitor 3	*PPP1R11*	*HCGV TCTE5*	O60927
PPP1R15B	Protein phosphatase 1 regulatory subunit 15B		*PPP1R15B*		Q5SWA1
PPP1R16B	Protein phosphatase 1 regulatory inhibitor subunit 16B	Ankyrin repeat domain-containing protein 4; CAAX box protein TIMAP; TGF-beta-inhibited membrane-associated protein; hTIMAP	*PPP1R16B*	*ANKRD4 KIAA0823*	Q96T49
pRb	Retinoblastoma-associated protein	p105-Rb; p110-RB1; pRb (Rb); pp110	*RB1*		P06400

In the text	Recommended protein name	Alternative name(s)	Gene name	Synonyms	UniProtKB
Proto-oncogene tyrosine-protein kinase Src	Proto-oncogene tyrosine-protein kinase Src	Proto-oncogene c-Src; pp60c-src; p60-Src	*SRC*	*SRC1*	P12931
PSA	Prostate-specific antigen (PSA)	Gamma-seminoprotein; seminin; Kallikrein-3; P-30 antigen; Semenogelase	*KLK3*	*APS*	P07288
PTK2	Focal adhesion kinase 1 (FADK 1)	Focal adhesion kinase-related nonkinase; FRNK; Protein phosphatase 1 regulatory subunit 71; PPP1R71; Protein-tyrosine kinase 2; p125FAK; pp125FAK	*PTK2*	*FAK FAK1*	Q05397
RAD51	DNA repair protein RAD51 homolog 1 (HsRAD51, hRAD51)	RAD51 homolog A	*RAD51*	*RAD51A RECA*	Q06609
Radixin	Radixin		*RDX*		P35241
Raf-1	RAF proto-oncogene serine/threonine-protein kinase	Proto-oncogene c-RAF; cRaf; Raf-1	*RAF1*	*RAF*	P04049
RASSF5	Ras association domain-containing protein 5	New ras effector 1; Regulator for cell adhesion and polarization enriched in lymphoid tissues; RAPL	*RASSF5*	*NORE1, RAPL*	Q8WWW0
RhoA	Transforming protein RhoA	Rho cDNA clone 12 (h12)	*RHOA*	*ARH12 ARHA RHO12*	P61586
ROCK1	Rho-associated protein kinase 1	Renal carcinoma antigen NY-REN-35; Rho-associated, coiled-coil-containing protein kinase 1; Rho-associated, coiled-coil-containing protein kinase I; ROCK-I; p160 ROCK-1; p160ROCK	*ROCK1*		Q13464
S6K1	Ribosomal protein S6 kinase beta-1 (S6K-beta-1, S6K1)	70 kDa ribosomal protein S6 kinase 1; P70S6K1; p70-S6K 1; Ribosomal protein S6 kinase I; Serine/threonine-protein kinase 14A; p70 ribosomal S6 kinase alpha; p70 S6 kinase alpha; p70 S6KA	*RPS6KB1*	*STK14A*	P23443
Scribble	Protein scribble homolog (scribble, hScrib)	Protein LAP4	*SCRIB*	*CRIB1 KIAA0147 LAP4 SCRB1 VARTUL*	Q14160
SDS22	Protein phosphatase 1 regulatory subunit 7	Protein phosphatase 1 regulatory subunit 22	*PPP1R7*	*SDS22*	Q15435
Serine/threonine-protein kinase PAK 6	Serine/threonine-protein kinase PAK 6	PAK-5; p21-activated kinase 6; PAK-6	*PAK6*		Q9NQU5

In the text	Recommended protein name	Alternative name(s)	Gene name	Synonyms	UniProtKB
Serine/threonine-protein phosphatase 5	Serine/threonine-protein phosphatase 5 (PP5)	Protein phosphatase T; PP-T; PPT	*PPP5C*	*PPP5*	P53041
SHOC-2	Leucine-rich repeat protein SHOC-2	Protein soc-2 homolog; Protein sur-8 homolog	*SHOC2*	*KIAA0862*	Q9UQ13
SKP2	S-phase kinase-associated protein 2	Cyclin-A/CDK2-associated protein p45; F-box protein Skp2; F-box/LRR-repeat protein 1; p45skp2	*SKP2*	*FBXL1*	Q13309
SKR3	Serine/threonine-protein kinase receptor R3 (SKR3)	Activin receptor-like kinase 1; ALK-1; TGF-B superfamily receptor type I; TSR-I	*ACVRL1*	*ACVRLK1 ALK1*	P37023
Smad1	Mothers against decapentaplegic homolog 1 (MAD homolog 1, Mothers against DPP homolog 1)	JV4-1; Mad-related protein 1; SMAD family member 1; SMAD 1; Smad1; hSMAD1; Transforming growth factor-beta-signaling protein 1; BSP-1	*SMAD1*	*BSP1 MADH1 MADR1*	Q15797
Smad5	Mothers against decapentaplegic homolog 5 (MAD homolog 5, Mothers against DPP homolog 5)	JV5-1; SMAD family member 5; SMAD 5; Smad5; hSmad5	*SMAD5*	*MADH5*	Q99717
Smad7	Mothers against decapentaplegic homolog 7 (MAD homolog 7, Mothers against DPP homolog 7)	Mothers against decapentaplegic homolog 8; MAD homolog 8; Mothers against DPP homolog 8; SMAD family member 7; SMAD 7; Smad7; hSMAD7	*SMAD7*	*MADH7 MADH8*	O15105
Smad9	Mothers against decapentaplegic homolog 9 (MAD homolog 9, Mothers against DPP homolog 9)	Madh6; SMAD family member 9; SMAD 9; Smad9	*SMAD9*	*MADH6 MADH9 SMAD8*	O15198
Sp1	Transcription factor Sp1		*SP1*	*TSFP1*	P08047
SSPN	Sarcospan	K-ras oncogene-associated protein; Kirsten-ras-associated protein	*SSPN*	*KRAG*	Q14714
Tensin-1	Tensin-1		*TNS1*	*TNS*	Q9HBL0
TGF-β1	Transforming growth factor beta-1 proprotein		*TGFB1*	*TGFB*	P01137
TP53BP2	Apoptosis-stimulating of p53 protein 2	Bcl2-binding protein; Bbp; Renal carcinoma antigen NY-REN-51; Tumor suppressor p53-binding protein 2; 53BP2; p53-binding protein 2; p53BP2	*TP53BP2*	*ASPP2, BBP*	Q13625

In the text	Recommended protein name	Alternative name(s)	Gene name	Synonyms	UniProtKB
TRAF6	TNF receptor-associated factor 6	E3 ubiquitin-protein ligase TRAF6; Interleukin-1 signal transducer; RING finger protein 85; RING-type E3 ubiquitin transferase TRAF6	*TRAF6*	*RNF85*	Q9Y4K3
Tyrosine-protein phosphatase non-receptor type 1	Tyrosine-protein phosphatase non-receptor type 1	Protein-tyrosine phosphatase 1B; PTP-1B	*PTPN1*	*PTP1B*	P18031
Tyrosine-protein phosphatase non-receptor type 11	Tyrosine-protein phosphatase non-receptor type 11	Protein-tyrosine phosphatase 1D; PTP-1D; Protein-tyrosine phosphatase 2C; PTP-2C; SH-PTP2; SHP-2; Shp2; SH-PTP3	*PTPN11*	*PTP2C SHPTP2*	Q06124
VDR	Vitamin D3 receptor (VDR)	1,25-dihydroxyvitamin D3 receptor; Nuclear receptor subfamily 1 group I member 1	*VDR*	*NR1I1*	P11473
WWTR1	WW domain-containing transcription regulator protein 1	Transcriptional coactivator with PDZ-binding motif	*WWTR1*	*TAZ*	Q9GZV5
YAP1	Transcriptional coactivator YAP1 (Yes-associated protein 1)	Protein yorkie homolog; Yes-associated protein YAP65 homolog	*YAP1*	*YAP65*	P46937
Protein yippee-like 5	Protein yippee-like 5		*YPEL5*		P62699
ZEB1	Zinc finger E-box-binding homeobox 1	NIL-2-A zinc finger protein; Negative regulator of IL2; Transcription factor 8; TCF-8	*ZEB1*	*AREB6 TCF8*	P37275
ZO-1	Tight junction protein ZO-1	Tight junction protein 1; Zona occludens protein 1; Zonula occludens protein 1	*TJP1*	*ZO1*	Q07157

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CHAPTER II

RATIONALE, HYPOTHESES AND AIMS

Cancer is a major societal burden with a considerable public health and economic impact globally. According to the Directorate-General of Health [*Direção-Geral da Saúde, DGS*], most premature deaths[3] in Portugal, in 2018, were attributed to neoplasms [402]. Prostate cancer (PCa) is one of the most common cancers worldwide. Age-adjusted incidence rates have dramatically increased over the last decades, with 1,276,106 new cases registered in 2018 [403]. In Europe, PCa accounted for 20% of the newly diagnosed cancer cases in men, ranking as the most incident male cancer (excluding non-melanoma skin cancer) [403]. According to the latest estimates from the Global Cancer Observatory[4], Portugal follows the European tendency, with 6,609 new diagnoses registered in 2018 (20.4% of the total number of cancers in men) [403].

The increase in incidence rates coincides with the widespread implementation of PCa screening programs, particularly based on the quantification of the plasma levels of the prostate-specific antigen (PSA) back in the 1980s. This has led to great debate within medical and scientific communities and it still is a controversial issue. There are studies reporting a positive impact of PSA-based screening in reducing PCa-associated mortality. For instance, the European Randomized Study of Screening for Prostate Cancer (ERSPC)—the largest study of its kind—reported a 21% reduction in the relative risk of PCa mortality at 13 years follow-up in men aged 55-69 years randomized to PSA testing [404]. Earlier detection and treatment of many asymptomatic PCa cases are on the basis of such observations—but they also became a challenge over the years. PSA is not a PCa-specific biomarker; so, overdiagnosis and overtreatment of potentially non-harmful PCa cases have great impact on patients' well-being following diagnosis and also imply considerable socioeconomic effects [405,406]. A comprehensive systematic review showed that nearly 20% of men received a false-positive test and 20-50% of the screen-detected cancers reflect overdiagnosis, which means that the disease would not have reached a clinical stage during these patients' lifetime and, thus, subsequent intervention could have been avoided [407]. Usually, a positive PSA test (i.e., PSA levels above the threshold) is followed by a biopsy—an invasive procedure with possible complications with an estimated hospitalization rate of 0.5-1.6% [407]. Risk stratification is then performed based on Gleason score (the main histopathological scoring system for PCa), clinical stage using the TNM system (which evaluates tumor (T), node (N) and metastasis (M) categories) and PSA levels [408].

To improve PCa diagnosis and staging, additional blood- and urine-based biomarkers have been proposed (as recently reviewed in [409]); however, there is still no agreement regarding their use in clinical practice. Some of these biomarkers might also be useful upon biopsy to aid the decision between treatment or active surveillance (e.g. Prolaris, Decipher and ProMark) [409]. This is of paramount importance to avoid overtreatment and associated risks. Localized PCa has been successfully

[3] Population aged ≥ 0 days and < 70 years old.
[4] A web-based platform that integrates global cancer statistics data from several projects of the International Agency for Research in Cancer's Section of Cancer Surveillance (http://gco.iarc.fr/).

treated with radical prostatectomy and/or radiotherapy [409]; however, both were associated with increased risk of erectile dysfunction and also bowel dysfunction, in the case of radical prostatectomy, which enhances psychological distress [407,410,411]. Hence, low- or intermediate-risk patients, particularly elderly men with a life-expectancy of < 5 years, would not benefit from intervention at all. A conservative management is recommended in these cases, including watchful waiting and/or active surveillance. In fact, neither radical prostatectomy nor radiotherapy were found to perform significantly better than active surveillance in reducing PCa mortality at a median of 10 years of follow-up [407]. The same randomized clinical trial, ProtecT (NCT02044172), reported, nonetheless, a lower risk of secondary outcomes such as disease progression and metastatic disease in men assigned to treatment groups (surgery and radiotherapy) [407].

Other treatment options are available, which might be indicated depending on cancer staging, and a few more are currently under clinical evaluation (comprehensive review in [412]). Androgen deprivation therapy (ADT) has become the gold-standard hormonal therapy for PCa. Like the normal prostate organogenesis needs androgens for the formation of a healthy gland, so does PCa to grow and survive (as discussed in Chapter Ia). Hence, ADT has been used either as a single treatment modality or in combination with other therapeutic approaches to palliate symptoms and improve overall survival of locally advanced or metastatic PCa patients [413]. However, as referred in Chapter Ia, in many patients disease progress to a castration-resistant cancer within a couple of years, even under effective ADT. Many molecular mechanisms have been proposed to underlie this refractory evolution, including androgen-based pathways. Accordingly, contemporary clinical research has been particularly focused on the development of new therapeutic options for the management of castration-resistant PCa (CRPC), as next generation endocrine agent and cytotoxic agents [414].

Interestingly, though, for such a common cancer, a lot of questions remain unanswered. This can be justified, at least in part, by the numerous knowledge gaps on PCa etiology and biology. Basic research in PCa has been challenging due to its multifactorial and heterogeneous nature. Age and race are the most well-characterized risk factors. Family history and genetics are also known to play an important role and several environmental and lifestyle-related behaviors have also been implied in PCa development [415]. Many developmental genes and signaling mediators involved in prostate organogenesis are also determinant for PCa onset and progression. And despite the central role of the androgen receptor (AR)-mediated signaling, there is a complex crosstalk between signaling pathways in PCa (as discussed in Chapter Ia). Interestingly, the activity of many of these signaling proteins, including the AR itself, and downstream signaling cascades are regulated by the protein phosphatase 1 (PP1). As discussed in Chapter Ib, the role of PP1 in cancer has unfairly been neglected; and this is particularly true in PCa. To date, only a few studies addressed PP1 expression, function and interaction with other proteins in PCa [416,417]. However, *PPP1CA* was found to be

amplified in both localized PCa and mCRPC, so it would be expected that PP-1A overexpression occurred during prostate carcinogenesis. However, PP-1A mRNA levels were found decreased in prostate tumors compared with paired normal tissue, suggesting that epigenetic mechanisms, such as promoter methylation, might be developed. There is also evidence for the amplification of *PPP1CB* and *PPP1CC* in prostate adenocarcinomas (albeit with a lower frequency than *PPP1CA*), as well as deep deletion of *PPP1CC* and *PPP1CA* mutation in a few samples of The Cancer Genome Atlas Prostate Adenocarcinoma (TCGA-PRAD) cohort. Therefore, we might hypothesize that PP1c isoforms are differentially expressed in PCa. In addition, PP-1A cytoplasmic staining was found to be correlated with high Gleason score. It is known that PP1 is ubiquitously expressed by cells and can be found at distinct cellular compartments where it plays a role in regulating signaling cascades and cellular processes. Hence, PP1c re-localization towards a specific cellular compartment might also occur during prostate carcinogenesis. Co-treatment of PCa cells with a PP1 inhibitor plus the nonsteroidal antiandrogen enzalutamide was shown to reduce the expression levels of PSA and to impair cell proliferation. This might be explained, at least in part, by PP1-mediated enhancement of the AR activity. In fact, the AR is one of the very few interactors of PP1 whose interaction was experimentally proved to exist and be relevant in PCa models [181]. Nonetheless, since PP1 overall inactivation might not be ideal for the reasons discussed in Chapter Ib, targeting PP1-mediated molecular events may present as an interesting alternative. To that end, unraveling the PP1 interactome in PCa is of great help to identify putative therapeutic targets. Also, these proteins, if enriched in prostate tissue or identified as cancer-testis antigens, might constitute potential biomarkers for the disease.

To improve the current knowledge on PP1 in human PCa, the following aims and tasks were proposed:

1) Evaluate the expression and potential relevance of PP1c isoforms in human PCa
   - Analyze the expression of PP-1A, PP-1B and PP-1G in human prostate specimens and prostate cell lines (normal and tumor)
   - Investigate the occurrence of genetic alterations and promoter methylation in PP1c-coding genes using publicly available PCa cohorts
   - Explore the association between the expression of PP1c isoforms and PCa-associated molecular events, as well as disease staging
2) Unravel the interactome of PP-1G in human PCa
   - Construct cDNA libraries from human prostate tissues (normal and tumor) and perform an Y2H screening using PP-1G as bait
   - Co-immunoprecipitate PP-1G and its interactors from human prostate tissues (normal and tumor) and identify the interactors by mass spectrometry

- Understand the currently available bioinformatics tools to collect, mine and interpret protein-protein interaction data
- Collect the already known PP-1G interactome from publicly available databases and identify the interactors known to be expressed in human prostate
- Construct a protein-protein interaction network for PP-1G in PCa and identify potential biomarkers and/or therapeutic targets

3) Assess the feasibility of using PP1-docking motif-mimetic cell-penetrating peptides to modulate prostate carcinogenesis
    - Design and synthesize potential cell-penetrating peptides based on the PP1-docking motifs in AR's primary sequence
    - Assess the internalization of the designed peptides in PCa cell lines
    - Assess the impact of the designed peptides in the viability of PCa cells and their effect in the expression of AR and its main target PSA
    - Test the effect of previously designed PP1-docking motif-mimetic cell-penetrating peptides on the viability of PCa cells.

CHAPTER III

PP1 CATALYTIC ISOFORMS ARE DIFFERENTIALLY EXPRESSED AND REGULATED IN HUMAN PROSTATE CANCER

PP1 catalytic isoforms are differentially expressed and regulated in human prostate cancer

KEYWORDS

Phosphatase
Isoforms
Phosphorylation
Mutations
Methylation

ABSTRACT

Protein phosphatase PP1 (PP1) has an effective catalytic machinery that is known to regulate the activity of multiple oncoproteins and tumor suppressive proteins, thereby controlling key signaling pathways for cancer development and progression. PP1 is a positive regulator of the androgen receptor (AR), which suggests major roles for PP1 in prostate carcinogenesis. However, studies dedicated to the characterization of PP1 in prostate cancer (PCa) are currently scarce. Here we analyzed the expression and localization of the PP1 catalytic isoforms (PP1c)—PP-1A, PP-1B and PP-1G—in formalin-fixed, paraffin-embedded tissue samples from twelve prostate tumor and four normal prostate tissue samples, as well as in androgen-dependent and castration-resistant PCa cell lines. We also performed a comprehensive data analysis from well-characterized PCa cohorts to assess transcript levels, genetic alterations and promoter methylation of PP1c-coding genes. We found that PP-1A is upregulated in PCa and that *PPP1CA* is frequently amplified, particularly in advanced stages, and identified its re-localization towards the nucleus in prostate tumors. In contrast, we found a downregulation of PP-1B transcripts in PCa and an association between PP-1G expression and Gleason score. PP-1B displayed the most distinctively distribution pattern in androgen-dependent cells and was found upregulated in a subset of tumors with *AR* amplification. We also found PP1c-coding genes to be rarely mutated in PCa and not prone to regulation by promoter methylation. On the other hand, protein phosphorylation might be an important mechanism for PP1cs' activity regulation in PCa cells. Taken together, our results suggest differential expression, localization and regulation of PP1c isoforms in PCa and support the need to investigate the roles of each isoform in prostate carcinogenesis in future studies.

Abbreviations*:* AdenoPCa, prostate adenocarcinoma; AR, androgen receptor; AR-V7, AR variant 7; BSA, bovine serum albumin; COSMIC, Catalogue Of Somatic Mutations In Cancer; CRPC, castration-resistant; PCa DAB, 3,3 diaminobenzidine; EDTA, ethylenediaminetetraacetic acid; ERG, transcriptional regulator ERG; ETS, erythroblast transformation-specific; ETV, ETS translocation variant; GRCh38, Genome Reference Consortium Human Build 38; IgG, immunoglobulin G; IRS, immunoreactive score; mCRPC, metastatic castration-resistant PCa; NEPC, prostate neuroendocrine carcinoma; NPT, normal prostate tissue; PAP, prostatic acid phosphatase; PBS, phosphate-buffered saline; PCa, prostate cancer; PIN, prostatic intraepithelial neoplasia; PP1, protein phosphatase 1; PP1c, protein phosphatase 1 catalytic subunit; PSA, prostate-specific antigen; RIPPO, regulatory interactor of PP1; RNA-Seq, RNA-Sequencing; RPKM, reads per kilobase per million reads; RT, room temperature; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; Ser, serine; SPOP, speckle-type POZ protein; TBS, tris-buffered saline; TBS-T, TBS with Tween™ 20; TCGA, The Cancer Genome Atlas; Thr, threonine; Tyr, tyrosine.

1. Introduction

Reversible protein phosphorylation is a primitive mechanism used by cells to promptly regulate protein activity and cell signaling [418]. Cancer cells master this system to meet their requirements for sustained cell growth and survival. Deregulated protein phosphorylation in cancer cells can result from several events, including alterations in its mediators—protein kinases and protein phosphatases—, either by altered gene expression or post-translational modifications, as well as occurrence of mutations that lead to gain or loss of phosphorylation sites in target proteins [419].

In the last decades, several studies have addressed the role of protein kinases in cancer development and progression, captivating the attention of the scientific community to explore their use as tumor markers and/or therapeutic targets [420]. The current knowledge is much more limited in what concerns protein phosphatases. Interesting, though, the foundations for the clinical use of tumor markers have laid on a protein phosphatase—the prostatic acid phosphatase (PAP). PAP had been widely used to aid in prostate cancer (PCa) diagnosis and staging, and to monitor treatment response, until the discovery of the prostate-specific antigen (PSA)—a turning point in PCa screening. Few additional phosphatases have been explored in the context of PCa, in particular members of the protein tyrosine (Tyr) phosphatases family (recently reviewed in [421]). However, proteins are mostly phosphorylated in serine (Ser) and threonine (Thr) residues (86.4% and 11.8% of the phosphorylation reactions, respectively) [422]. Therefore, most dephosphorylation events occurring within a cell in a given moment are expected to be catalyzed by Ser/Thr phosphatases.

Protein phosphatase 1 (PP1) was the first Ser/Thr phosphatase to be identified [423]. It regulates the activity of several tumor suppressor and oncogenic proteins, thus determining the flow of key oncogenic signaling cascades implied in the development of cancer hallmarks [180]. PP1 functions as an oligomer composed by a catalytically active and highly efficient subunit (PP1c) that is coupled to at least one regulatory subunit (RIPPO), which compensates for the lack of substrate specificity of the PP1c. In human cells, PP1c is encoded by three distinct genes—*PPP1CA* (11q13.2), *PPP1CB* (2p23.2) and *PPP1CC* (12q24.11)—giving rise to three highly homologous canonical isoforms: PP-1A, PP-1B and PP-1G, respectively. Their central cores are conserved and only minor differences are registered at terminal regions [424]. Canonical PP1c isoforms are believed to be ubiquitously expressed and their expression has been demonstrated in a variety of cancers at both transcript and protein levels. Several studies have reported differential PP1cs' expression between tumoral and non-tumoral samples, as well as among tumors from different stages [191,194,195,199-201].

In PCa, PP1 enhances the stability and transcriptional activity of the androgen receptor (AR), a central player in all stages of prostate carcinogenesis and progression to metastatic castration-resistant PCa (mCRPC)—the leading cause of mortality associated with the disease

[283-285,288,291]. Besides the canonical AR isoform, PP1 also upregulates the splicing variant AR-V7, which lacks ligand-binding domain and, therefore, has been implicated in cancer cell survival during androgen deprivation therapy [291]. Despite evidence suggesting major roles for PP1 in PCa, comprehensive studies devoted to the characterization of PP1c isoforms in PCa are still lacking.

In this study we aimed to investigate PP1c isoforms' expression in human PCa and to analyze their association with clinicopathological parameters using a combination of molecular biology studies with systematic data analysis from publicly available datasets. Our results show that PP1c isoforms are differentially regulated and expressed in normal prostate and PCa, also supporting the need for dedicated studies to decode their potentially non-redundant functions in prostate carcinogenesis. Moreover, we provide evidence for PP-1A and PP-1G worth as diagnostic and prognostic marker, respectively, which merit deeper investigation in future studies.

2. Materials and methods

2.1. Human samples

After approval by the institutional review board [Comissão de Ética para a Saúde do Instituto Português de Oncologia do Porto, CES-IPOFG_EPE 019/08], formalin-fixed, paraffin-embedded tissue samples were obtained from the archive of the Department of Pathology of the Portuguese Oncology Institute of Porto, IPO Porto, Portugal. Twelve cases of patients diagnosed with PCa and treated with radical prostatectomy at the Institution were randomly selected. Morphologically normal prostate tissue (NPT, n=4, confirmed histologically) were collected from the peripheral zone of prostates of patients submitted to cystoprostatectomy due to bladder cancer. Tissue collection and histopathological evaluation was performed according to institutional guidelines as previously described [425]. Prostate specimens were totally embedded and examined by a Uropathology-dedicated Pathologist for confirming the presence or absence of PCa in the selected samples. Relevant clinicopathological data for this study were collected retrospectively from medical records (Table III. 1).

2.2. Immunohistochemistry

Immunohistochemistry studies were performed using the Novolink™ Polymer Detection System (Leica Biosystems, Germany) as previously reported [426]. Briefly, paraffin-embedded tissues were first deparaffinized and rehydrated. Antigen retrieval was heat-induced in a microwave in the presence of 1× sodium citrate buffer solution, pH 6.0, for anti-PP-1G antibody, or 1× ethylenediaminetetraacetic acid (EDTA) buffer solution, pH 8.0, for anti-PP-1A and anti-PP-1B antibodies, as determined by initial optimization tests. Endogenous peroxidase activity was neutralized with

3% hydrogen peroxide for 10 min and non-specific binding was prevented by incubation with horse serum (1:50) for 20 min. Tissue sections were then incubated with the primary antibody (1:500) for 1 h at room temperature (RT), in a humidified chamber, following by incubation with Novocastra™ Post Primary (rabbit anti-mouse immunoglobulin G, IgG) and NovolinkTM Polymer, 30 min each. Slides were further incubated with 3,3-diaminobenzidine (DAB) in 1× phosphate-buffered saline (PBS)/0.05% hydrogen peroxide for 7 min, counterstained with hematoxylin solution and coverslipped using Entellan® (Sigma-Aldrich, USA).

Table III. 1
Clinicopathological data of the PCa patients.

Characteristics	Study population (N=12)
Age, years	64 (60–75)
Initial PSA, ng/mL	10.2 (4.8–17.5)
Pathological stage, n (%)	
pT2bN0M0	3 (25.0)
pT2cN0M0	2 (16.7)
pT3aN0M0	6 (50.0)
pT3bN0M0	1 (8.30)
Gleason score, n (%)	
6 (3+3)	5 (41.7)
7 (3+4)	5 (41.7)
8 (3+5)	2 (16.7)
Disease-free survival, months	114.2 (12.7-214)
Overall survival, months	150.3 (34.5-222.3)

Values are presented as median (range) unless otherwise indicated.

Three slides were used from each patient included in the study (one for each isoform) and the experiments were performed in parallel. Positive controls were selected from the analysis of the Tissue Atlas provided by The Human Protein Atlas database (https://www.proteinatlas.org, date of access: Feb, 2019) [427]. Slides were analyzed by an experienced Uropathologist regarding the percentage of positive cells, the staining intensity of immunoreactive cells, and the cellular localization of the signal. Immunostaining results were expressed as immunoreactive score (IRS) that reflected the positivity score (0: no positive cells; 1: 1 to 50% positive cells; 2: 51% to 90% positive cells; 3: more than 90% positive cells) plus the staining intensity score (0: no cell staining; 1: weak cell staining; 2: moderate cell staining; 3: strong cell staining). Hence, calculated IRS ranged from 0 to 6. Protein localization was classified as present in the cytoplasm, nucleus, or both.

2.3. Cell culture

Human prostate epithelial cells (PNT2 cell line) and PCa cells [LNCaP (androgen-dependent) and PC3 (androgen-independent) cell lines] were grown in RPMI 1640 Medium with L-glutamine, supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) penicillin-streptomycin solution (5,000 U/mL). All media and supplements were from Gibco™ (by Thermo Fisher Scientific, USA).

Cell cultures were maintained at 37°C in a humidified incubator with 5% CO_2 atmosphere. Cells were routinely checked for mycoplasma contamination and kept at low passage for the assays.

2.4. Antibodies

Rabbit polyclonal anti-PP-1A and anti-PP-1G antibodies were previously produced in-house [428]. Mouse monoclonal anti-PP-1B (sc-365678) and anti-tubulin beta chain (β-tubulin, sc-5274) antibodies were acquired from Santa Cruz Biotechnology, USA. Rabbit monoclonal anti-PP-1A phospho-Thr-320 antibody (ab62334) was obtained from Abcam (Cambridge, UK). Secondary antibodies IRDye® 800CW goat anti-mouse and IRDye 680RD® goat anti-rabbit were acquired from LI-COR® Biosciences, USA; goat anti-rabbit IgG (H+L) highly cross-adsorbed secondary antibody Alexa Fluor® 594 (A-11037) and goat anti-mouse IgG (H+L) highly cross-adsorbed secondary antibody Alexa Fluor® Plus 488 (A32723) were acquired from Invitrogen™ (by Thermo Fisher Scientific, USA).

2.5. Western blot

Cells were washed in 1× PBS, pH 7.4, and lysed in 1× RIPA lysis buffer [0.05 M Tris-HCl, pH 7.4, 0.15 M NaCl, 0.25% deoxycholic acid, 1% NP-40, 1mM EDTA (Merck, Germany) supplemented with 1× cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail (Roche, Switzerland) and 1% Phosphatase Inhibitor Cocktail II (Alfa Aesar by Thermo Fisher Scientific, USA). Whole-cell extracts were sonicated and centrifuged at 16,000 × g for 20 min at 4 ºC. Supernatants were collected and protein concentration was determined using the Pierce™ BCA Protein Assay (Thermo Scientific™ by Thermo Fisher Scientific, USA), according to the manufacturer's instructions. Equal amounts of protein (30 µg) were diluted in 4× Laemmli buffer, boiled for 5 min and separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

Gels were subsequently electrotransferred onto GE Healthcare Amersham™ Protran™ NC Nitrocellulose Membranes, 0.45 µm pore size (GE Healthcare, USA). Membranes were blocked in 5% Blotto non-fat dry milk (Santa Cruz Biotechnology, USA) or 5% bovine serum albumin (BSA), fraction V (for phospho-antibody), in tris-buffered saline (TBS), for 1 h at RT. Incubation with the primary antibodies occurred for 2 h at RT using the following dilutions: anti-PP-1A (1:5,000), anti-PP-1B (1:1,000), anti-PP-1G (1:5,000), anti-PP-1A^{Thr320} (1:1,000) and anti-β-tubulin (1:1,000). After being washed in TBS with 0.1% Tween™ 20 (TBS-T) (Fisher BioReagents™ by Thermo Fisher Scientific, USA), membranes were incubated with appropriate secondary antibodies, either IRDye® 800CW goat anti-mouse (1:10,000) or IRDye 680RD® goat anti-rabbit (1:20,000), for 1 h at RT. Membranes were scanned using the Odyssey Infrared Imaging System (LI-COR Biosciences, USA).

2.6. Immunofluorescence

Cells were washed in 1× PBS, pH 7.4, and fixed with 3.7% formaldehyde for 10 min. Following three washes, cells were permeabilized with 0.2% Triton™ X-100 (Fisher BioReagents™ by Thermo Fisher Scientific, USA) in 1× PBS for 15 min. Non-specific binding sites were blocked with 5% normal goat serum (Sigma-Aldrich, USA) and 1% BSA (NZYTech, Portugal) in 1× PBS/0.2% Triton™ X-100 for 1 h. Cells were then incubated for 1 h with the primary antibodies—anti-PP-1A (1:250), anti-PP-1B (1:100) or anti-PP-1G (1:500), all diluted in 1% BSA/1× PBS/0.2% Triton™ X-100. After three washing steps, cells were incubated with the appropriate secondary antibody—goat anti-Rabbit IgG, Alexa Fluor 594 (1:1,000) or goat anti-Mouse IgG, Alexa Fluor Plus 488 (1:1,000)—for 1 h, protected from light. Nucleus were stained with Hoechst 33258 (Abcam, UK) and coverslips were mounted onto microscope slides using Mowiol mounting medium. All steps were performed at RT. Images were acquired in an Olympus IX81 motorized inverted microscope (Olympus, Japan).

2.7. Databases and data mining

2.7.1. Catalogue Of Somatic Mutations In Cancer (COSMIC)

The occurrence of somatic point mutations in PP1c-coding genes in PCa was investigated through COSMIC database, v90, release date Sep 5, 2019 (https://cancer.sanger.ac.uk/cosmic) [429]. COSMIC is the largest repository of somatic mutations occurring human cancers and provides comprehensive data to explore their impact [429]. The search was performed using the Genome Reference Consortium Human Build 38 (GRCh38) genome version. *PPP1CA*, *PPP1CB* and *PPP1CC* were searched in all screen types, using the coordinate system 'Amino-acid', and the tissue filter specified to 'Prostate'.

2.7.2. cBioPortal for Cancer Genomics

Insights into PCa genomics were also obtained through comprehensive search in cBioPortal, v3.2.2, release date Feb 6, 2020 (https://www.cbioportal.org) [186,187,429]. This platform was developed at the Memorial Sloan Kettering Cancer Center to allow the integration and exploration of datasets from large-scale genomic studies. At the time of this study, cBioPortal integrated 21 PCa studies, accounting for a total of 6,836 samples from 6,550 patients. These included primary and metastatic samples from different cancer types, namely prostate adenocarcinoma (AdenoPCa), castration-resistant PCa (CRPC), and prostate neuroendocrine carcinoma (NEPC). We first queried cBioPortal for mutations and copy-number alterations in PP1c-coding genes in a global case set consisting of all samples analyzed in the 21 PCa studies. After determining the studies with relevant data for our study, we selected the most appropriate to use in each analysis considering the type of

genomic and clinical data provided by each dataset (Suppl. Table III. 1). Since some samples were common to more than one dataset, we also took this into account when choosing the datasets to avoid data duplication. Clinicopathological data of the combined study can be found in Suppl. Table III. 2.

2.7.3. UALCAN

Transcript levels of PP-1A, PP-1B and PP-1G were analyzed using The Cancer Genome Atlas (TCGA) datasets—the benchmark of cancer genomics [430]—through the web resource UALCAN (http://ualcan.path.uab.edu/index.html) [431]. UALCAN is a user-friendly tool that allows to perform differential analyses from RNA-Sequencing (RNA-Seq) Level-3 expression data. It includes two prostate datasets: AdenoPCa and metastatic PCa (MET500). By data mining these datasets we compared the expression of PP1cs between primary prostate tumors and normal prostate tissues, as well as across tumors with different Gleason scores and molecular signatures. We also analyzed promoter methylation levels, which were defined as beta value ranging from 0 (unmethylated) to 1 (fully methylated). Hypermethylation and hypomethylation were considered for beta values of 0.5-0.7 and 0.25-0.3, respectively.

2.8. Statistical analysis

Statistical significance of tissue- and cell-based assays was calculated using chi-square, Kruskal-Wallis, or Mann-Whitney tests, wherever appropriate. Statistical analysis was performed by using the IBM® SPSS® Statistics software, v25.0. Statistical analysis of genomic studies was performed via UALCAN database using the t-test to estimate the significance of difference in gene expression levels between groups [431]. The significance level was set to 0.05.

3. Results

3.1. Expression and localization of PP1c isoforms in NPT and PCa tissues

The expression of PP-1A, PP-1B and PP-1G was assessed in four NPT and twelve PCa cases by immunohistochemistry using isoform-specific antibodies (representative figures can be found in Fig. III. 1A). All samples were positively stained for the three isoforms, albeit with varying intensities (Suppl. Table III. 3). The IRS calculated for each isoform in each specimen was comparable in NPT—100% of the samples scored 4 for PP-1A and PP-1G, and 5 for PP-1B—, but variations were observed among PCa samples. Mean IRS comparison showed that PP-1A was overexpressed in PCa compared with NPT ($p=0.0039$; Fig. III. 1B). No significant alterations were observed for PP-1B and PP-1G (Fig. III. 1B).

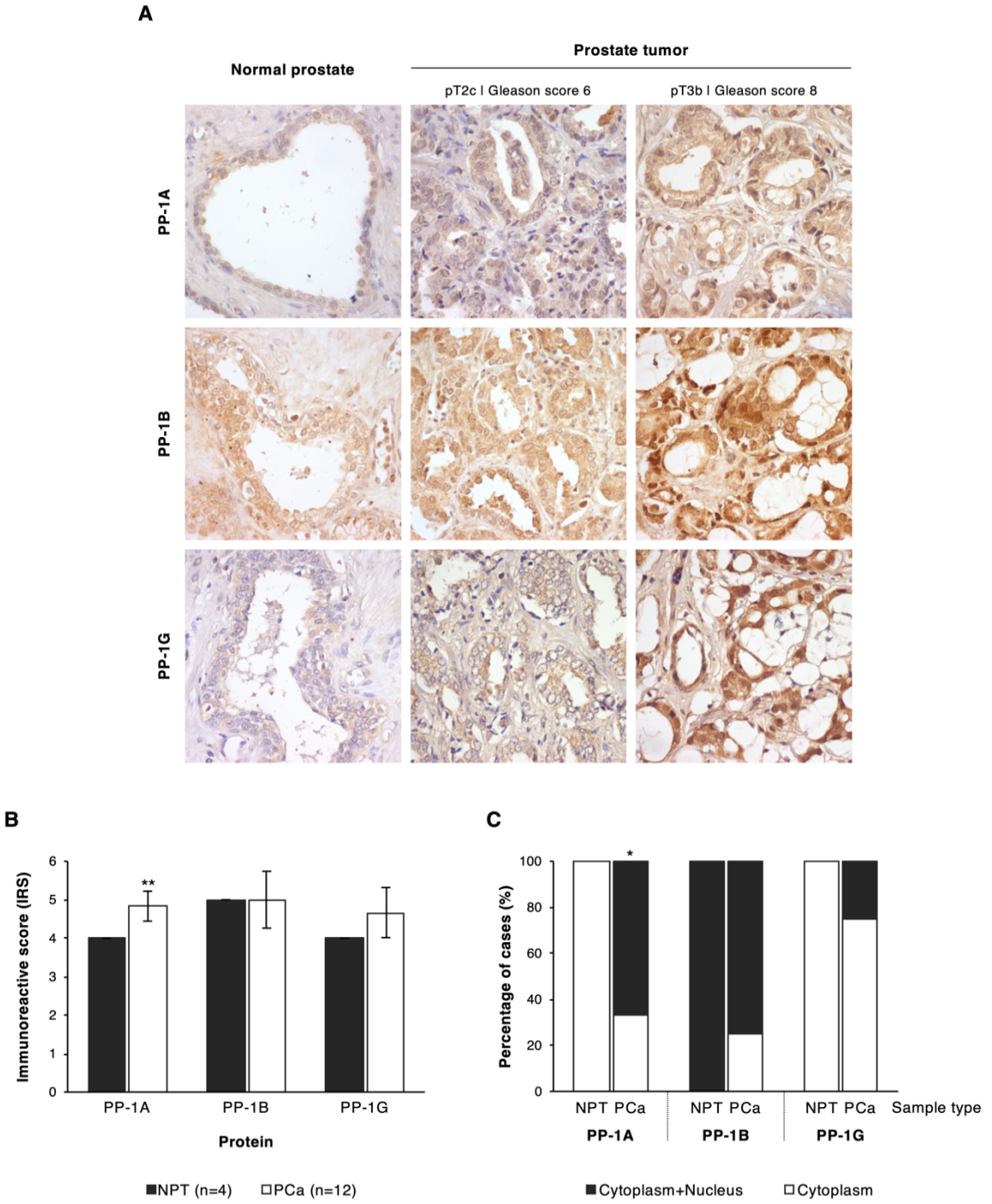


Fig. III. 1

Immunoexpression of PP1c isoforms in normal prostate (NPT) and prostate cancer (PCa) tissues. (A) Representative images of the immunostaining of PP-1A, PP-1B and PP-1G (top to bottom) in NPT and PCa with increasing aggressivity (left to right) (magnification 400×). Nuclei were counterstained with hematoxylin solution. Images in each column corresponds to the same patient. **(B)** Mean immunoreactive score of each isoform in NPT (n=4) and PCa (n=12). Results are expressed as mean ± SD. Statistical significance was calculated using the Mann-Whitney test. **(C)** Localization of the isoforms in the cases assessed. Results are expressed as percentage of cases observed in cytoplasm or in cytoplasm and nucleus. Statistical significance was calculated using the Chi-square test. * $p<0.05$, ** $p<0.01$.

NPT samples were also comparable in terms of protein localization: in 100% of the samples, PP-1A and PP-1G were detected in the cytoplasm, while PP-1B was detected in both cytoplasm and nucleus (Fig. III. 1C). In contrast, their localization varied among PCa samples. Cytoplasmic and nuclear localization was observed in 67%, 75% and 25% of the cases for PP-1A, PP-1B and PP-1G, respectively (Fig. III. 1C). Hence, PP-1A re-localization towards the nucleus was observed in PCa ($p=0.021$; Fig. III. 1C). In the remaining cases, positive staining was restricted to the cytoplasm (Fig. III. 1C). Of mention, in all studied cases at least one isoform was present in the nucleus (data not shown).

We then investigated potential associations between PP1c proteins' expression or localization and clinicopathological parameters, including pT stage and Gleason score. No significant associations were found for any isoform (Suppl. Fig. III. 1).

3.2. Expression and localization of PP1c isoforms in androgen-dependent and castration-resistant cell lines

PP1 had previously been identified as an AR positive regulator [283–285,288,291]. Hence, we analyzed the expression and localization of PP1c proteins in LNCaP (androgen-dependent cell line) and PC3 (castration-resistant cell line) using isoform-specific antibodies (Fig. III. 2). PNT2, an immortalized normal prostate epithelium cell line, was used as positive control for protein expression. Decreased PP-1A levels were observed in LNCaP cells when compared with PC3 cells (Fig. III. 2A). Similar expression levels were found for PP-1B and PP-1G (Fig. III. 2A).

Given the difference observed in the PP-1A expression, we went further to analyze its phosphorylation levels in the three cell lines, using a phospho-specific antibody for the most widely recognized phospho-residue, Thr-320. The results suggested that PP1c proteins might be differentially regulated between androgen-dependent and castration-resistant cells (Fig. III. 2B).

Differences were also identified in their cell distribution patterns (representative figures can be found in Fig. III. 2C). In LNCaP cells, PP-1A and PP-1G were found to be particularly localized in the nucleus, while PP-1B was minimally localized in the nucleus or totally absent (Fig. III. 2C). All isoforms were observed in the cytoplasm of both cell lines, albeit distinctive distribution patterns had been identified: PP-1A was more restricted to the perinuclear space than PP-1G, which was found widely dispersed within the cytoplasm; on the other hand, PP-1B showed cytoplasmic and membrane staining more consistent with filament network and cell-cell adhesion configurations (Fig. III. 2C).

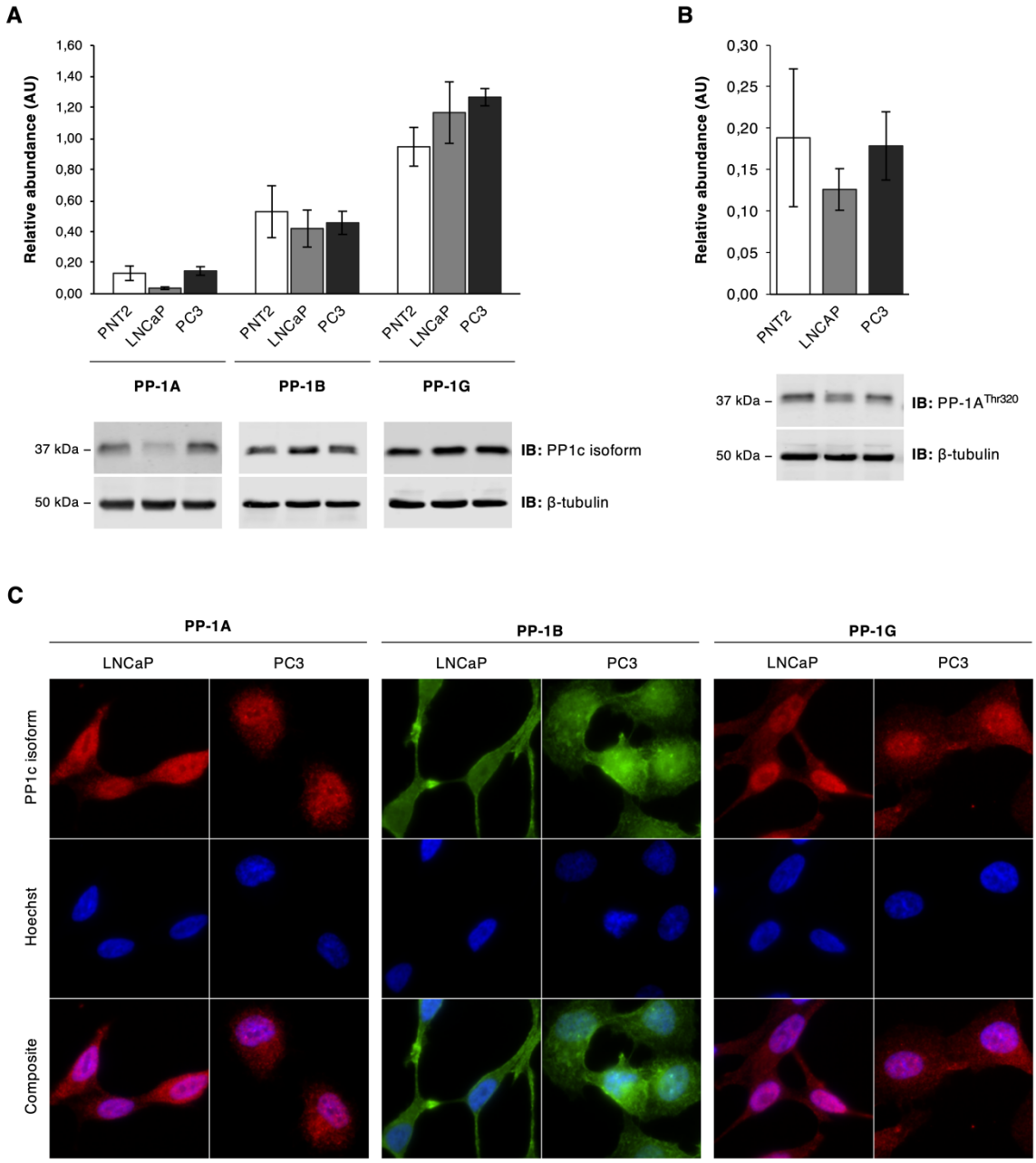


Fig. III. 2

Protein expression of PP1c isoforms in human PCa cell lines. Western blot analysis of PP-1A, PP-1B and PP-1G expression **(A)** and phosphorylation **(B)** in LNCaP (androgen-dependent) and PC3 (castration-resistant) cells using isoform-specific and phospho-specific antibodies, respectively. Immortalized normal prostate cells, PNT2, were used as positive control. Graphs depict the expression levels normalized to the loading control, β-tubulin, from triplicate experiments. Results are expressed as mean ± SD. **(C)** Representative images of PP-1A, PP-1B and PP-1G (top to bottom) in LNCaP and PC3 cells. Nucleus were stained with Hoechst. Images were acquired in an Olympus IX81 motorized inverted microscope (magnification 1000×).

3.3. Expression of PP1c transcripts in TCGA PCa cohorts

To investigate the expression of PP1c isoforms at transcript level we analyzed the TCGA AdenoPCa dataset through UALCAN portal. PP-1A was upregulated in prostate primary tumors compared with normal tissue (p=1.96E-05; Fig. III. 3A), whereas PP-1B was downregulated (p=0,009;

Fig. III. 3B). No significant alteration in PP-1G transcript levels were observed between the two conditions (Fig. III. 3C). However, an association between PP-1G expression and Gleason score was observed (Fig. III. 3D). Gleason score 6 tumors exhibited significantly lower PP-1G levels than Gleason score 7 (p=0.025), Gleason score 8 (p=0.009), Gleason score 9 (p=0.004) and Gleason score 10 tumors (p=8.19E-04) (Fig. III. 3D). PP-1G was also significantly overexpressed in Gleason score 10 tumors compared with Gleason score 7 tumors (p=0.017) (Fig. III. 3D). Conversely, no relevant associations with Gleason score were found for PP-1A and PP-1B (Suppl. Fig. III. 2A-B). Thus, despite the similar levels observed between normal prostate and primary prostate tumors, PP-1G was particularly overexpressed in PCa with higher Gleason score.

PCa heterogeneity is well-recognized and a major obstacle for the establishment of successful management strategies. In recent years, great effort has been put into defining molecular alterations that allow the identification of PCa subtypes. Hence, we analyzed the expression of PP-1A, PP-1B and PP-1G in association with known molecular signatures [432]. Given the relationship between PP1 and AR, we investigated PP1c transcripts levels in tumors with or without *AR* amplification in the metastatic PCa cohort (MET500 PRAD dataset) [433]. We found PP-1B overexpressed in tumors with *AR* amplification (p=0.042; Fig. III. 3E). Nonetheless, no significant differences were observed for PP-1A and PP-1G (Suppl. Fig. III. 2C-D). Additional associations were investigated in the TCGA AdenoPCa dataset. PP-1A mRNA expression levels were significantly higher in tumors with *SPOP* mutation (p=6.20E-04) and in tumors with *ETV1* fusion (p=0.017) compared to those with *ERG* fusion (Fig. III. 3F). In contrast, PP-1B mRNA expression levels were lower in tumors with *SPOP* mutation (p=0.007), as well as in tumors with *ETV4* fusion (p=0.046), than in those with *ERG* fusion (Fig. III. 3G). Similar expression levels were found for PP-1G in the different PCa molecular subtypes (Suppl. Fig. III. 2E).

3.4. Genetic alterations and promoter methylation of PP1c-coding genes in PCa

Seeking for understanding of the events underlying differential expression of PP1c isoforms, we investigated genetic alterations and promoter methylation of PP1c-coding genes. The occurrence of somatic mutations in *PPP1CA*, *PPP1CB* and *PPP1CC* was explored using COSMIC and cBioPortal databases. Point mutations were identified in prostate carcinomas, but not in hyperplasia, prostatic intraepithelial neoplasia (PIN) or adenomas (data not shown). The calculated mutation frequency was low for the three genes (Suppl. Table III. 4). With exception of one primary tumor sample that exhibited an intronic substitution in both *PPP1CB* and *PPP1CC* genes, no sample was mutated in more than one gene simultaneously (Suppl. Table III. 4). Also, only five samples displayed two distinct mutations in the same gene and only six mutations were observed in more than one sample (Suppl. Table III. 4).

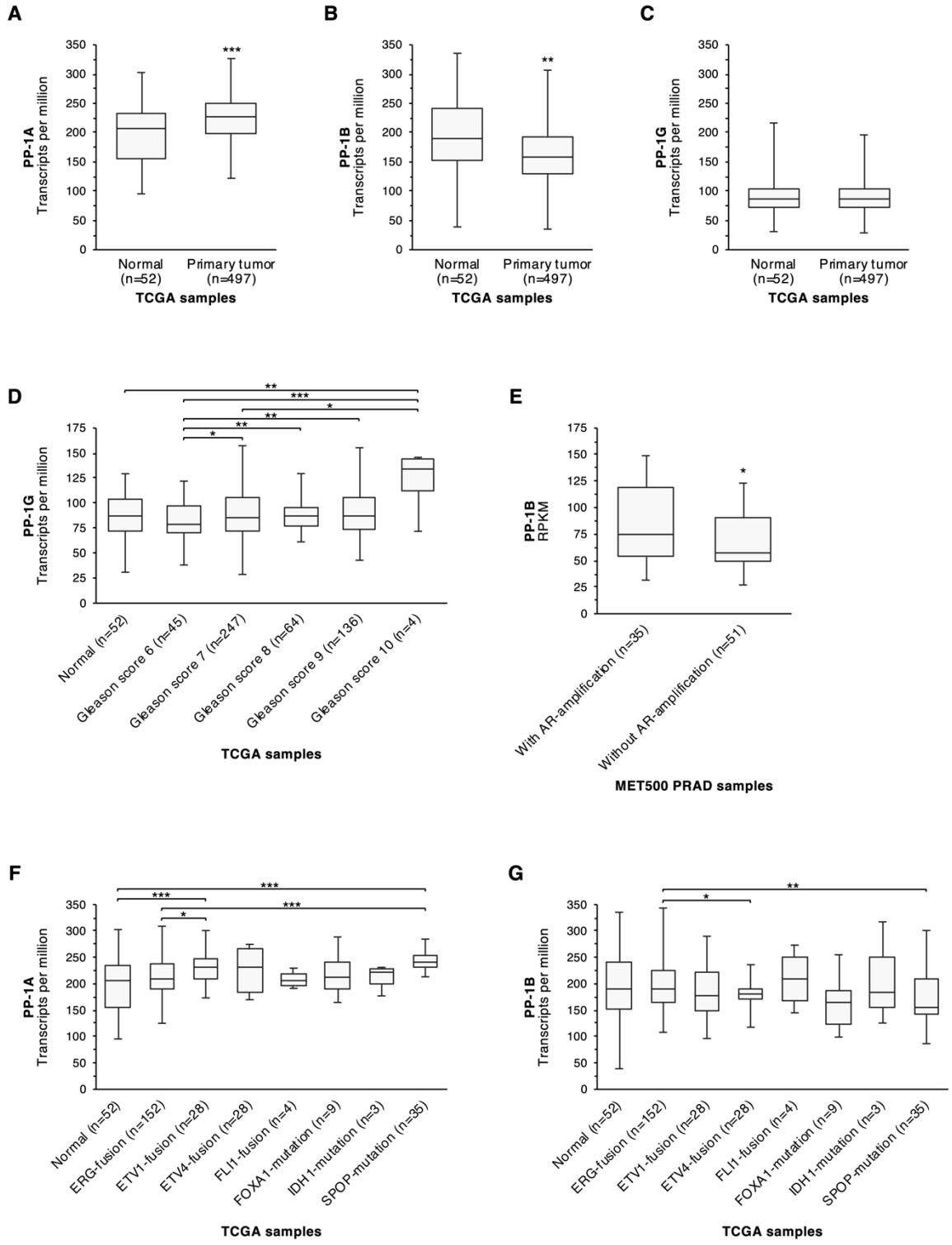


Fig. III. 3

Expression of PP1c transcripts in normal prostate and primary tumors from TCGA PCa cohorts. (A–C) mRNA expression levels of PP-1A **(A)**, PP-1B **(B)** and PP-1G **(C)** in normal prostate and primary tumors. **(D)** mRNA expression levels of PP-1G in tumors with different Gleason scores. **(E)** mRNA expression levels of PP-1B in tumors with or without androgen receptor (*AR*) amplification. Data is presented as reads per kilobase per million reads (RPKM). **(F–G)** mRNA expression levels of PP-1A **(F)** and PP-1B **(G)** in tumors with distinct molecular signatures. Data was reproduced and analyzed through UALCAN. ***p<0.001; **p<0.01; *p<0.05.

Few of the identified mutations had known impact on the amino acid sequence of the catalytic isoforms (Fig. III. 4A and Suppl. Table III. 4). Five missense mutations were identified in *PPP1CA*: three affecting the catalytic core sequence and two at the C-terminal (Fig. III. 4A and Suppl. Table III. 4). From those, two were found associated with copy gain—R221H and A299P (Fig. III. 4A and Suppl. Table III. 4). Two missense mutations were also identified in *PPP1CC*; one at the catalytic core sequence that was associated with copy gain (F227S) and one at the C-terminal (K319R) (Fig. III. 4A and Suppl. Table III. 4). In *PPP1CB*, a splicing site variant was detected in a metastasis sample (Fig. III. 4A and Suppl. Table III. 4).

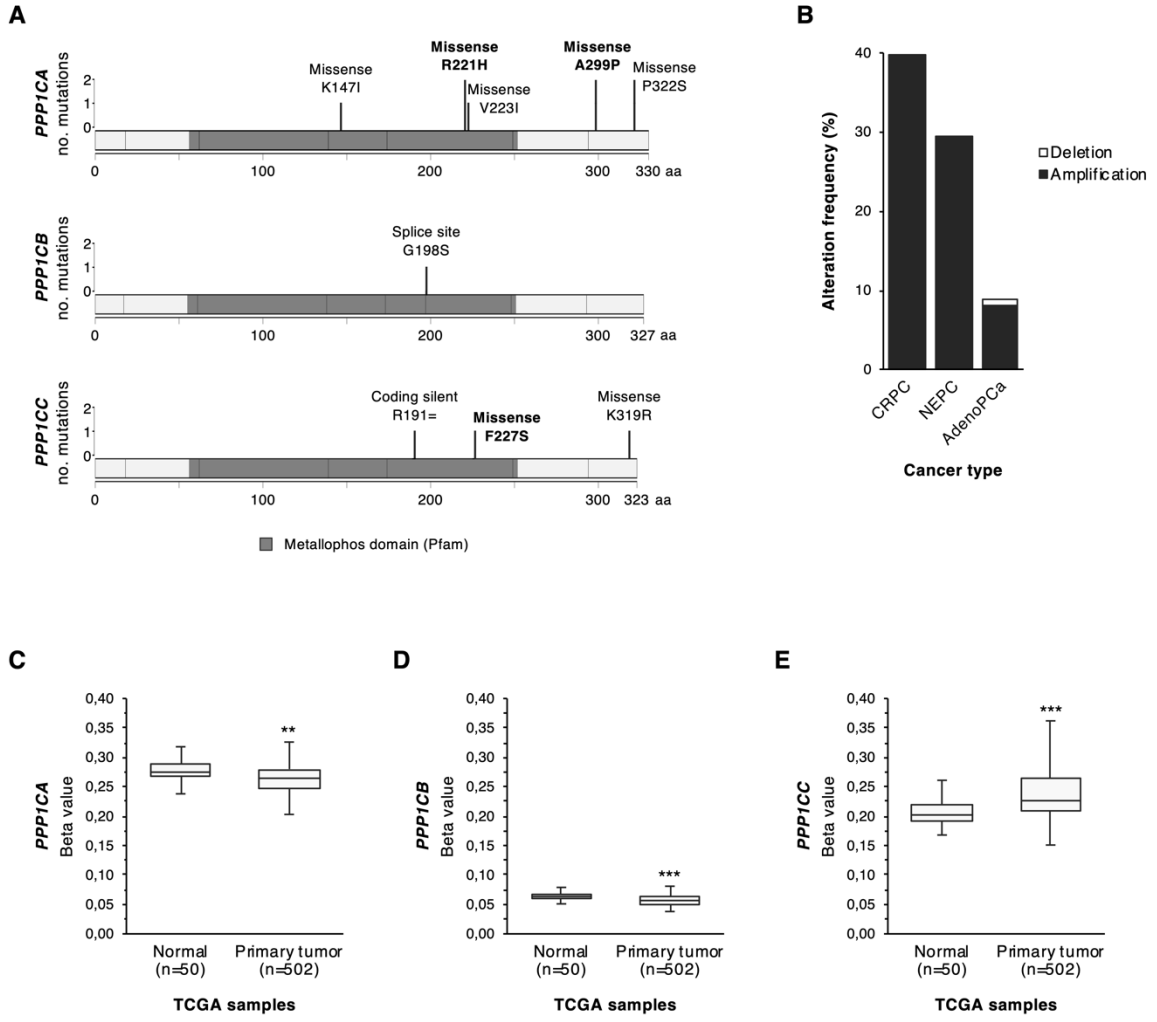


Fig. III. 4

Genetic alterations and promoter methylation of PP1c-coding genes in PCa. (A) Mutation diagram of *PPP1CA*, *PPP1CB* and *PPP1CC* genes in PCa. Mutations in bold are associated with copy gain. Data retrieved from COSMIC database, v90 (https://cancer.sanger.ac.uk/cosmic, accessed on Jan 9, 2020) and cBioPortal for Cancer Genomics, v3.2.2 (https://www.cbioportal.org, accessed on Jan 10, 2020). **(B)** Alteration frequency of PP1c-coding genes in castration-resistant prostate cancer (CRPC; N=63), prostate neuroendocrine carcinoma (NEPC; N=44), and prostate adenocarcinoma (AdenoPCa; N=1,443). Data retrieved from cBioPortal for Cancer Genomics, v3.2.2 (https://www.cbioportal.org, accessed on Jan 10, 2020). **(C–E)** Promoter methylation levels of *PPP1CA* **(C)**, *PPP1CB* **(D)** and *PPP1CC* **(E)** genes in normal prostate and primary tumors from TCGA PCa cohorts. Data was reproduced and analyzed through UALCAN. ***p<0.001; **p<0.01.

Copy number alterations in PP1c-coding genes were assessed through cBioPortal database. The analysis of eight non-redundant studies revealed that nearly 40% of CRPC samples and 30% of NEPC samples had amplified PP1c-coding genes (Fig. III. 4B). Both amplification and deletion were detected in AdenoPCa samples, though deletions occurred in a lower frequency (8,8% and 0,62%, respectively) (Fig. III. 4B).

Individual analysis of each gene showed that *PPP1CA* was the most frequently altered in all cancer subtypes, followed by *PPP1CC*, except for NEPC in which the amplification frequency of *PPP1CB* was slightly higher (Table III. 2). On the other hand, *PPP1CC* registered the highest number of deletions in AdenoPCa samples (Table III. 2).

Table III. 2
Copy number alterations in *PPP1CA*, *PPP1CB* and *PPP1CC* genes in PCa.

Cancer type	***PPP1CA***		***PPP1CB***		***PPP1CC***	
	Amp	Del	Amp	Del	Amp	Del
Castration-resistant prostate cancer (CRPC) (N=63)	19 (30.16)	0	8 (12.70)	0	13 (20.63)	0
Prostate neuroendocrine carcinoma (NEPC) (N=44)	9 (20.45)	0	8 (18.18)	0	7 (15.91)	0
Prostate Adenocarcinoma (AdenoPCa) (N=1,443)	75 (5.27)	1 (0.07)	29 (2.01)	2 (0.14)	36 (2.49)	7 (0.49)

Values are presented as number (%). Data retrieved from cBioPortal for Cancer Genomics, v3.2.2 (https://www.cbioportal.org, accessed on Jan 10, 2020). Datasets: NEPC (Multi-Institute 2016), Prostate (SU2C 2019), Prostate (MICH), Prostate (FHCRC, 2016), The MPC Project, Prostate (Eur Urol 2017), Prostate (TCGA PanCan 2018), Prostate (MSKCC 2010). Amp, amplification; Del, deletion.

We also investigated the promoter methylation levels of each PP1c-coding gene. In general, all genes exhibited residual or hypomethylated levels in both normal prostate and primary tumors (Fig. III. 4C-E). When comparing the two conditions, promoter methylation of *PPP1CA* (p=0.003) and *PPP1CB* (p=4.09E-06) was significantly lower in primary tumors than in normal prostate (Fig. III. 4C-D). On the other hand, primary tumors presented significantly higher *PPP1CC*'s promoter methylation than normal samples (p=1.63E-12; Fig. III. 4E).

4. Discussion

PP1 is involved in the regulation of virtually all biological processes by modulating the activity of a myriad of proteins [434]. Understanding its role in cancer has been challenging, with several findings supporting different functions and its classification as either tumor suppressor or oncogene protein still being debated. This can be easily explained by its structural complexity. PP1 functional multiplicity has been mostly attributed to PP1rs, which also exist as individual entities and have PP1-independent cellular functions [435]. However, increasing evidence suggests that, at the most

basic level, PP1 diversity is also determined by differential expression and localization of PP1c isoforms [428,436–438].

PP1c isoforms remain barely investigated in the context of cancer, in spite of increasing evidence showing their non-redundant and even antagonizing roles [217,218]. This can explain, at least in part, why different studies present contradictory findings. To the best of our knowledge, our study is the first providing a comprehensive characterization of the PP1c isoforms in PCa. By combining immunohistochemistry studies using isoform-specific antibodies with data mining of comprehensive TCGA AdenoPCa cohorts, we show that PP-1A is overexpressed in PCa at both transcript and protein levels (Fig. III. 1B and Fig. III. 3A). We also show a shift towards nuclear expression of PP-1A in PCa (Fig. III. 1C). Previous analyses using tissue microarrays had revealed higher expression of PP-1A in prostate tumors than in benign hyperplastic tissue [197]. The authors reported both cytoplasmic and nuclear localizations and identified a correlation between increased PP-1A cytoplasmic expression and higher Gleason score [197]. Nonetheless in our study, no significant associations were found between PP-1A expression and/or localization and tumor grading (Suppl. Fig. III. 1). This discrepancy might be explained in part by the small size of our cohort and the limited number of poorly differentiated tumors (Gleason score 8, n=2) compared with moderately differentiated tumors (Gleason score 6 and 7, n=10) (Suppl. Fig. III. 1). PP-1A overexpression has been reported in additional malignant tissues, including glioblastoma and bladder cancer tissues [196,270,439]. Consistent with our findings, these studies found PP-1A weakly expressed or absent from the nucleus of normal cells, but highly expressed in tumor cells [196,270,439]. Increased nuclear expression of PP-1A was particularly observed in mitotic cells and in more aggressive tumors [196,439].

In contrast to PP-1A, we found PP-1B mRNA expression to be lower in PCa (Fig. III. 3B). Though in our study we did not observe a corresponding reduction in PP-1B protein levels (Fig. III. 1B), previous studies reported significantly lower levels in PCa when compared with samples from benign prostatic hyperplasia [440]. In addition, PP-1B was the only isoform found in the nucleus of normal prostate cells, whereas 25% of the tumors showed nuclear exclusion of the protein (Fig. III. 1C). The redistribution of PP1c isoforms is not surprising as they are highly dynamic molecular entities and their presence have been described in various subcellular compartments [436,441]. Most likely, their localization depends on the cellular context (e.g., cell cycle stage) and their affinity for interacting proteins as suggested by several studies [442–444]. Depletion of PP-1B was shown to instigate massive nuclear abnormalities [445]. Nuclear lamina rupture occurred most probable as a consequence of the disruption of the interaction between PP-1B and the protein phosphatase 1 regulatory subunit 12A, a key targeting subunit to myosin light chain and other substrates that specifically interacts with PP-1B and PP-1G, but not with PP-1A [424,445]. This effect was shown

to be even more dramatic in cancer cell lines, which seem to be more sensitive to actomyosin-mediated nuclear dysmorphia [445].

No significant changes were identified for PP-1G both for mRNA and protein expression or in its distribution in NPT and PCa cells (Fig. III. 1 and Fig. III. 3C)—despite its nuclear translocation had been observed in 25% of the tumors (Fig. III. 1C). However, PP-1G was the only isoform whose transcript levels were found associated with tumor differentiation (Fig. III. 3D). The association between PP-1G expression and tumor grading has been reported for other malignancies, as hepatocellular carcinomas and brain tumors [200,201]. These reports provided evidence for the use of PP-1G as a marker of poor prognosis in hepatocellular cancer and glioma [200,201].

The ambiguity of the findings obtained when studying the role of PP1c isoforms in cancer somehow impairs the development of subsequent studies to address these divergences. However, increasing evidence suggests an association between the expression of PP1c isoforms and certain cancer molecular subtypes. For instance, low levels of PP-1A protein expression were associated with estrogen receptor-negative breast tumors [194], while its overexpression was associated with poor overall survival and progression free survival in *TP53*-expressing glioblastomas [196,439]. PCa is widely recognized as a disease with substantial inter- and intra-heterogeneity, and great effort has been putting into defining molecular features that aid tumor categorization. Henceforth, we hypothesized that the differences we observed in the expression of PP1cs could be related to distinct PCa molecular signatures. We found PP-1A and PP-1B to be differentially expressed in tumors with *SPOP* mutations, with PP-1A being increased and PP-1B being decreased in these tumors comparing with tumors with *ERG* fusion (Fig. III. 3F-G). *SPOP* mutations are the most commonly identified point mutations in primary PCa (up to 15% of the cases) and are considered driver lesions for the disease [446]. *SPOP* encodes for speckle-type POZ protein, a component of an E3 ubiquitin-protein ligase complex that regulates the ubiquitination and degradation of multiple proteins [446]. *SPOP* mutations have been recently shown to enhance cancer cell survival and resistance to docetaxel [447]. In PCa, *SPOP* mutations are mutually exclusive from rearrangements involving the erythroblast transformation-specific (ETS) family members, such as *ERG*, *ETV1* and *ETV4* [446]. These and other members of the ETS family of oncogenic transcription factors are frequently fused with androgen-regulated genes [446]. In our study, we also found PP-1A and PP-1B mRNA expression increased in tumors with *ETV1* and *ETV4* fusions, respectively, compared to tumors with *ERG* fusion (Fig. III. 3F-G). Whether ETS rearrangements are mutually exclusive among themselves is still dubious [448]; yet, they might support distinct oncogenic events in PCa. Interestingly, ETV1 but not ERG promotes AR's transcriptional activity and enhances autonomous testosterone production [449]. ETV1 and ETV4 seem to promote metastasis formation and have both specific and overlapping targets and functions [450]. In addition to the findings in primary tumors, we found the mRNA expression of

PP-1B higher in metastatic tumors with *AR* amplification than in tumors without *AR* amplification (Fig. III. 3E). *AR* amplifications are commonly observed in metastatic PCa, but not in most clinically localized PCas, and are believed to contribute to the resistance to AR-targeted therapies [451]. Altogether, these results are indicative of differential regulation of PP1c isoforms in PCa and disclose a potential association between their expression and specific PCa molecular subtypes.

These results prompted us to investigate putative differences in the expression and localization of PP1c isoforms in androgen-dependent and castration-resistant PCa cells. In our study, the most evident alteration was the reduced PP-1A levels in LNCaP cells comparing with those found for PC3 cells (Fig. III. 2A). The variation found in Thr-320 phospho-levels (Fig. III. 2B) also suggests that PP1cs are differentially regulated by phosphorylation between these two cell lines. Phosphorylation of Thr-320 inhibits PP1c activity and despite this phosphorylation event has been mostly explored as mechanism for PP-1A silencing during cell cycle progression, Thr-320 residue is conserved by all PP1c isoforms [206,208,209]. Hence, our results suggest that overall PP1c activity might be increased in androgen-dependent cells. These results would benefit from in-depth studies to identify and characterize PP1c phospho-forms in PCa. In addition, we found PP1c proteins to be widely distributed in LNCaP and PC3 cells (Fig. III. 2C). Similar staining patterns were identified for PP-1A and PP-1G, but not for PP-1B. Altogether, our data suggest that PP1c isoforms may play distinct roles in androgen-dependent and castration-resistant PCa cells. In fact, all PP1c isoforms were found to interact with AR, but only the interaction with PP-1A has been investigated in further detail [283,284]. PP-1A enhances AR nuclear localization and transcriptional activity through dephosphorylation of Ser-650 [283]. The interaction between PP-1A and AR is supported by a positive feedback loop in which AR acts as a PP1c regulator besides being a substrate. AR targets PP-1A to chromatin, where it catalyzes the dephosphorylation of cyclin-dependent kinase 9. As a result, positive transcriptional elongation factor b is mobilized to induce AR transactivation [284]. In addition, PP-1A inhibits AR polyubiquitylation by dephosphorylating and inactivating proteins involved in AR ubiquitylation and degradation [285]. PP-1B may also contribute to this effect through interaction and downregulation of the serine/threonine-protein kinase PAK 6, a cyclin-dependent kinase inhibitor 1-activated kinase that mediates AR degradation [286,287]. Henceforth, PP1c isoforms might play distinct roles, albeit cooperative at least in part, in regulating AR signaling in PCa.

Seeking for understanding the expression alterations we observed for PP1c isoforms, we went further to investigate genetic variations and changes in promoter methylation. We found that PP1c-coding genes are rarely mutated in PCa (Suppl. Table III. 4). This was not surprising since on the one hand the occurrence of point mutations in PCa is not frequent [446] and, on the other hand, PP1c-coding genes are believed to be highly resistant to mutation. However, de novo variants have

been described in patients with relevant clinical phenotypes [182–184]. The analysis of COSMIC and cBioPortal databases identified five missense mutation in *PPP1CA* and two in *PPP1CC*, distributed along the highly conserved catalytic core and the C-terminal (Fig. III. 4A), but the pathogenic relevance of these somatic mutations is still unknown. In contrast, PP1c-coding genes were found frequently amplified in PCa, specifically in advanced stages (Fig. III. 4B). Among the three genes, *PPP1CA* was the most frequently amplified (Table III. 2). *PPP1CA* is located on a frequently overrepresented chromosomal band [197]. Amplification of *PPP1CA* was recently described in both localized and metastatic castration-resistant prostate cancers (7% and 17% of the cases analyzed, respectively) [189]. The authors also reported a frequent co-occurrence with the amplification of G1/S-specific cyclin-D1 [189]. The amplification of *PPP1CA* might explain in part the overexpression of PP-1A in PCa, as previously suggested [197].

Gene's promoter region methylation is known to be part of a regulatory mechanism able to silence gene expression [452]. DNA methylation of PP1c genes' promoters have been described in other pathophysiological contexts [204,453]. Data on tumors is rather limited but decreased mean methylation was reported for both *PPP1CA* and *PPP1CB* in non-small cell lung cancer specimens when compared to non-tumoral samples. In contrast, increased mean methylation was observed for *PPP1CC* [202]. In our study, we found that PP1c-coding genes are nearly unmethylated or hypomethylated both in normal prostate and primary tumors (Fig. III. 4C-E). Even though, promoter methylation levels are significantly different between the two conditions, showing *PPP1CA* and *PPP1CB* decreased methylation levels (Fig. III. 4C-D), *PPP1CC* displayed increased methylation levels (Fig. III. 4E). Therefore, promoter methylation does not seem to be a crucial regulatory mechanism of PP1cs' expression in PCa.

5. Conclusion

Our study provides a first comprehensive characterization of PP1c isoforms in PCa and discloses the importance of investigating the roles of each isoform in prostate carcinogenesis. Our results, together with findings from previous studies, suggest that PP-1A might function as an oncoprotein in PCa, being involved in cell malignant transformation; PP-1B might have specific roles in the regulation of AR-mediated signaling; and PP-1G might be involved in PCa progression, given the association of its expression with Gleason score. These and other hypotheses would be of interest to address in future studies. Moreover, further studies in larger cohorts would be essential for determining the potential added-value of using PP-1A and PP-1G as diagnostic and prognostic markers for PCa, respectively, as well as to unravel potential associations of PP1c isoforms' expression with overall survival and disease-free survival.

Supplementary data

Suppl. Table III. 1

PCa datasets in cBioPortal with mutation and/or copy number alteration data for PP1c-coding genes.

Dataset	Samples	Alteration frequency (%)[c]	Sample details	Mutation data[d]	Copy number alteration data[d]	Study
The MPC Project	30	36.67	AdenoPCa	+	+	The Metastatic Prostate Cancer Project (Provisional, November 2019)[e]
NEPC (Multi-Institute 2016)	114	33.33	CRPC and NEPC		+	Neuroendocrine Prostate Cancer (Multi-Institute, Nat Med 2016) [454]
Prostate (SU2C 2019)	444	14.86	CRPC	+	+	Metastatic Prostate Adenocarcinoma (SU2C/PCF Dream Team, PNAS 2019) [455]
Prostate (FHCRC, 2016)	154	14.29	AdenoPCa (primary and metastasis)		+	Prostate Adenocarcinoma (Fred Hutchinson CRC, Nat Med 2016) [456]
Prostate (SU2C)	150	9.33	AdenoPCa (metastasis)	+	+	Metastatic Prostate Cancer (SU2C/PCF Dream Team, Cell 2015) [457]
PRAD (MSKCC/DFCI 2018)[a]	1013	9.28	AdenoPCa (primary and metastasis)	+	+	Prostate Adenocarcinoma (MSKCC/DFCI, Nature Genetics 2018) [458]
Prostate (Eur Urol 2017)	65	9.23	AdenoPCa		+	Prostate Adenocarcinoma (SMMU, Eur Urol 2017) [459]
Prostate (MICH)	61	8.20	AdenoPCa (primary and metastasis)		+	Metastatic Prostate Adenocarcinoma (MCTP, Nature 2012) [460]
Prostate (TCGA 2015)[b]	333	5.41	AdenoPCa (primary)	+	+	Prostate Adenocarcinoma (TCGA, Cell 2015) [432]
Prostate (TCGA)[b]	499	4.41	AdenoPCa (primary)	+	+	Prostate Adenocarcinoma (TCGA, Firehose Legacy) [461–467]
Prostate (TCGA PanCan 2018)[b]	494	3.44	AdenoPCa (primary)	+	+	Prostate Adenocarcinoma (TCGA, PanCancer Atlas) [461–467]
Prostate (MSKCC 2010)	213	2.82	AdenoPCa (primary and metastasis)		+	Prostate Adenocarcinoma (MSKCC, Cancer Cell 2010) [468]

Data accessed through cBioPortal for Cancer Genomics, v3.2.2 (https://www.cbioportal.org, accessed on Jan 10, 2020). [a] This study includes samples from other datasets: NEPC (Multi-Institute 2016), Prostate (FHCRC, 2016), Prostate (SU2C), and Prostate (TCGA 2015). [b] These studies have overlapping data. To avoid redundancy, only the most recent dataset (PanCan 2018) was used in our analyses. [c] Percentage of cases with genetic alterations in the queried genes (*PPP1CA*, *PPP1CB* and *PPP1CC*). [d] Sources of genetic data for the queried genes are indicated with +. [e] From the MPCproject (https://mpcproject.org/data-release). Abbreviations: AdenoPCa, prostate adenocarcinoma CRPC, castration-resistant PCa; NEPC, prostate neuroendocrine carcinoma.

Suppl. Table III. 2

Characterization of the study population from the combined study of PCa data cohorts.

Characteristics	Study population (N=1,442)
Age, years	64 (40–05)
NA (%)	38.3
PSA, ng/mL	197.6 (0–5691)
NA (%)	79.5
Pathological stage, %	
pT2cN0M0	1.3
pT3aN0M0	0.3
pT3bN0M0	0.3
pT4N0M0	0.2
pT2cN1M0	<0.1
pT3aN1M0	<0.1
pT3bN1M0	<0.1
NA	97.6
Gleason score, %	
6 (3+3)	3.6
7 (3+4)	7.2
7 (4+3)	4.9
8 (3+5)	0.2
8 (4+4)	2.7
8 (5+3)	0.1
9 (4+5)	2.0
10 (5+5)	0.4
NA	77.5
Disease-free survival, months	40.21 (0.10–165.2)
NA (%)	81.6
Overall survival, months	37.31 (0.8–218)
NA (%)	76.8

Data from the combined study resultant from the combination of the studies in Suppl. Table 1, collected from cBioPortal for Cancer Genomics, v3.2.2 (https://www.cbioportal.org, accessed on 10 Jan 2020). Values are presented as median (range) unless otherwise indicated. NA, not available.

Suppl. Table III. 3
PP1c isoforms' expression in NPT and PCa specimens.

	PP-1A		**PP-1B**		**PP-1G**	
	NPT	PCa	NPT	PCa	NPT	PCa
Positivity score						
0	0/4 (0)	0/12 (0)	0/4 (0)	0/12 (0)	0/4 (0)	0/12 (0)
1	0/4 (0)	0/12 (0)	0/4 (0)	0/12 (0)	0/4 (0)	0/12 (0)
2	0/4 (0)	0/12 (0)	0/4 (0)	0/12 (0)	0/4 (0)	0/12 (0)
3	4/4 (100)	12/12 (100)	4/4 (100)	12/12 (100)	4/4 (100)	12/12 (100)
Staining intensity score						
0	0/4 (0)	0/12 (0)	0/4 (0)	0/12 (0)	0/4 (0)	0/12 (0)
1	4/4 (100)	2/12 (17)	0/4 (0)	3/12 (25)	4/4 (100)	5/12 (42)
2	0/4 (0)	10/12 (83)	4/4 (100)	6/12 (50)	0/4 (0)	6/12 (50)
3	0/4 (0)	0/12 (0)	0/4 (0)	3/12 (25)	0/4 (0)	1/12 (8)
Immunoreactivity score (IRS)[1]						
4	4/4 (100)	2/12 (17)	0/4 (0)	3/12 (25)	4/4 (100)	5/12 (42)
5	0/4 (0)	10/12 (83)	4/4 (100)	6/12 (50)	0/4 (0)	6/12 (50)
6	0/4 (0)	0/12 (0)	0/4 (0)	3/12 (25)	0/4 (0)	1/12 (8)

Values are presented as number (%). [1]Calculated by adding the staining intensity score to the immunoreactivity percentage score.
Abbreviations: NPT, normal prostate tissue; PCa, prostate cancer.

Suppl. Table III. 4

Prostate carcinoma samples with profiled point mutations in *PPP1CA*, *PPP1CB* or *PPP1CC* genes.

Gene	COSMIC		cBioPortal[b]
	AdenoPCa[a] (N=1,462)	NS[a] (N=492)	AdenoPCa[a] (N=1,487)
PPP1CA	2 (0.14)	2 (0.41)	8 (0.54)
PPP1CB	28 (1.92)	6 (1.22)	1 (0.07)
PPP1CC	12 (0.82)	4 (0.81)	1 (0.07)

Mutations in detail:

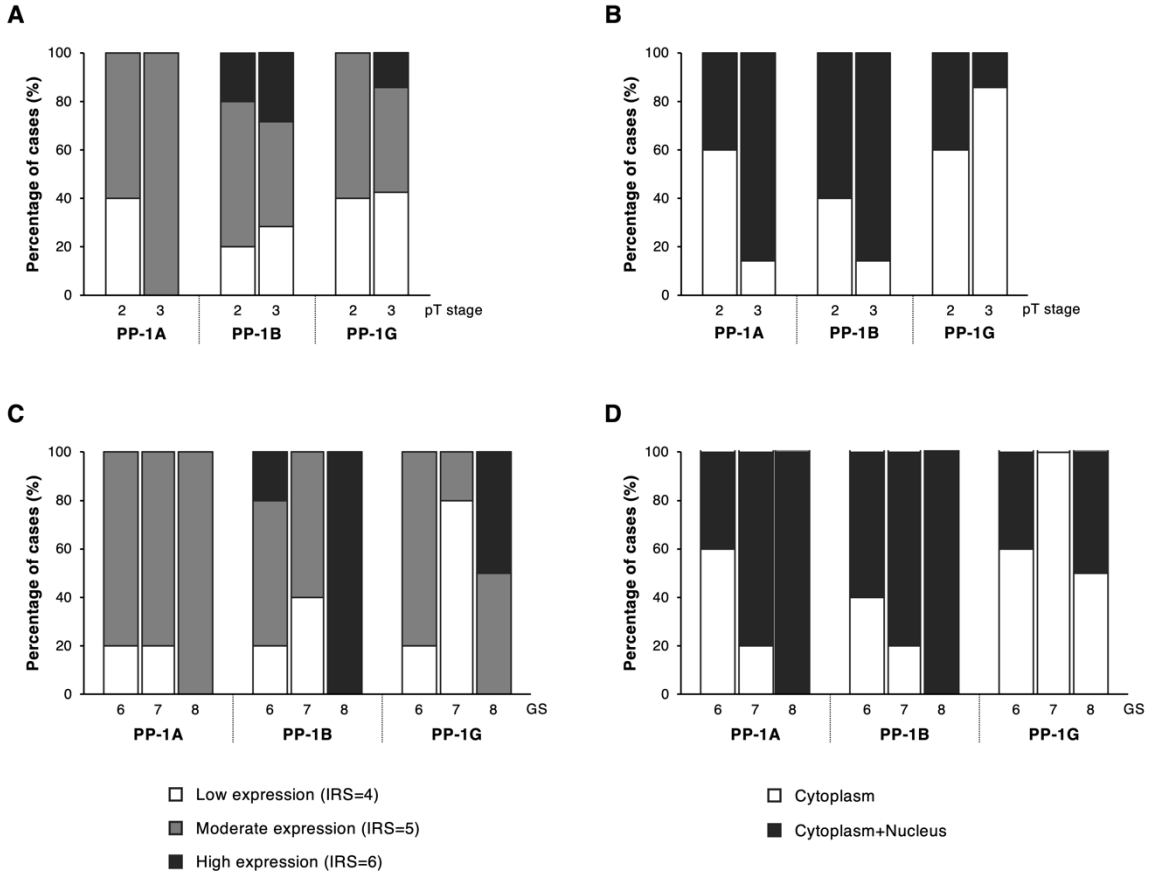
Gene	Mutation (CDS)	Mutation (AA)	Mutation type	Histological subtype	Sample source	Sample(s) ID	Database	Study
PPP1CA	*73del	Unknown	Deletion	Adenocarcinoma	Primary	CPCG0269-F1	COSMIC	ICGC(PRAD-CA): Prostate Adenocarcinoma - CA
	662G>A	R221H	Substitution - missense	Adenocarcinoma	Metastasis	PM189 PM189-TM	cBioPortal	Prostate Adenocarcinoma (MSKCC/DFCI, Nature Genetics 2018) Metastatic Prostate Adenocarcinoma (SU2C/PCF Dream Team, PNAS 2019)
	964C>T	P322S	Substitution - missense	Adenocarcinoma	Metastasis	SC_9047 SC_9047-Tumor	COSMIC cBioPortal	Metastatic Prostate Cancer (SU2C/PCF Dream Team, Cell 2015) Metastatic Prostate Adenocarcinoma (SU2C/PCF Dream Team, PNAS 2019)
	895G>C	A299P	Substitution - missense	Adenocarcinoma	Metastasis	SC_9068 SC_9068-Tumor	COSMIC cBioPortal	Metastatic Prostate Cancer (SU2C/PCF Dream Team, Cell 2015) Metastatic Prostate Adenocarcinoma (SU2C/PCF Dream Team, PNAS 2019)
	440A>T	K147I	Substitution - missense	Adenocarcinoma	Primary	TCGA-EJ-A65E-01	COSMIC cBioPortal	ICGC(PRAD-US): Prostate AdenoCarcinoma - TCGA, US Prostate Adenocarcinoma (TCGA, PanCancer Atlas)
	700G>A	V223I	Substitution - missense	Adenocarcinoma	Primary	AAPC-STID0000012568-Tumor-SM-2XU1M	cBioPortal	Prostate Adenocarcinoma (MSKCC/DFCI, Nature Genetics 2018)
PPP1CB	745-359_745-352del	Unknown	Deletion - intronic	Adenocarcinoma	Primary	0054_CRUK_PC_0054_T1_DNA	COSMIC	ICGC(PRAD-UK): Prostate Adenocarcinoma - UK
	879+1619_879+1623del	Unknown	Deletion - intronic	Adenocarcinoma	Primary	0059_CRUK_PC_0059_T1_DNA	COSMIC	ICGC(PRAD-UK): Prostate Adenocarcinoma - UK

Gene	Mutation (CDS)	Mutation (AA)	Mutation type	Histological subtype	Sample source	Sample(s) ID	Database	Study
PPP1CB	52+10562C>G	Unknown	Substitution - intronic	Adenocarcinoma	Primary	0114_CRUK_PC_0114_T1_DNA	COSMIC	ICGC(PRAD-UK): Prostate Adenocarcinoma - UK
	52+12132A>C	Unknown	Substitution - intronic	Adenocarcinoma	Primary	0164_CRUK_PC_0164_T1_DNA	COSMIC	ICGC(PRAD-UK): Prostate Adenocarcinoma - UK
	744+1011A>G	Unknown	Substitution - intronic	Adenocarcinoma	Primary	0168_CRUK_PC_0168_T1_DNA	COSMIC	ICGC(PRAD-UK): Prostate Adenocarcinoma - UK
	52+6569T>C	Unknown	Substitution - intronic	Adenocarcinoma	Primary	0175_CRUK_PC_0175_T1_DNA	COSMIC	ICGC(PRAD-UK): Prostate Adenocarcinoma - UK
	416-889A>G	Unknown	Substitution - intronic	Adenocarcinoma	Primary	0203_CRUK_PC_0203_T1_DNA	COSMIC	ICGC(PRAD-UK): Prostate Adenocarcinoma - UK
	520+590A>G	Unknown	Substitution - intronic	Adenocarcinoma	Primary	0206_CRUK_PC_0206_T1_DNA	COSMIC	ICGC(PRAD-UK): Prostate Adenocarcinoma - UK
	*2615A>G	Unknown	Substitution	Adenocarcinoma	Primary	0227_CRUK_PC_0227_T1_DNA	COSMIC	ICGC(PRAD-UK): Prostate Adenocarcinoma - UK
	52+9594G>A	Unknown	Substitution - intronic	Adenocarcinoma	Primary	0233_CRUK_PC_0233_T1_DNA	COSMIC	ICGC(PRAD-UK): Prostate Adenocarcinoma - UK
	52+5121T>G	Unknown	Substitution - intronic	Adenocarcinoma	Primary	CPCG0040-F1	COSMIC	ICGC(PRAD-CA): Prostate Adenocarcinoma - CA
	53-10821_53-10820del	Unknown	Deletion - intronic	Adenocarcinoma	Primary	CPCG0059-F1	COSMIC	ICGC(PRAD-CA): Prostate Adenocarcinoma - CA
	52+1284T>G	Unknown	Substitution - intronic	Adenocarcinoma	Primary	CPCG0090-F1	COSMIC	ICGC(PRAD-CA): Prostate Adenocarcinoma - CA
	593-628dup	Unknown	Insertion - intronic	Adenocarcinoma	Primary	CPCG0090-F1	COSMIC	ICGC(PRAD-CA): Prostate Adenocarcinoma - CA
	879+1764A>G	Unknown	Substitution - intronic	Adenocarcinoma	Not specified	CPCG0099-F1	COSMIC	ICGC(PRAD-CA): Prostate Adenocarcinoma - CA
	53-2934dup	Unknown	Insertion - intronic	Adenocarcinoma	Not specified	CPCG0103-F1	COSMIC	ICGC(PRAD-CA): Prostate Adenocarcinoma - CA
	52+7449dup	Unknown	Insertion - intronic	Adenocarcinoma	Primary	CPCG0127-F1 CPCG0243-F1 CPCG0324-F1 CPCG0336-F1 CPCG0339-F1 CPCG0361-F1	COSMIC	ICGC(PRAD-CA): Prostate Adenocarcinoma - CA

Gene	Mutation (CDS)	Mutation (AA)	Mutation type	Histological subtype	Sample source	Sample(s) ID	Database	Study
PPP1CB	521-568dup	Unknown	Insertion – intronic	Adenocarcinoma	Primary	CPCG0339-F1 CPCG0392-F1 CPCG0409-F1 CPCG0463-F1 CPCG0503-F1	COSMIC	ICGC(PRAD-CA): Prostate Adenocarcinoma - CA
	53-9679G>T	Unknown	Substitution - intronic	Adenocarcinoma	Primary	DA12003	COSMIC	ICGC(PRAD-FR): Prostate Cancer - Adenocarcinoma
	593-1958G>A	Unknown	Substitution - intronic	Adenocarcinoma	Primary	DA12007	COSMIC	ICGC(PRAD-FR): Prostate Cancer - Adenocarcinoma
	53-985G>C	Unknown	Substitution - intronic	Adenocarcinoma	Primary	DA1200E	COSMIC	ICGC(PRAD-FR): Prostate Cancer - Adenocarcinoma
	521-935T>C	Unknown	Substitution - intronic	Not specified	Primary	EOPC-043_tumor_01	COSMIC	ICGC(EOPC-DE): Early Onset Prostate Cancer - DE
	53-2363C>T	Unknown	Substitution - intronic	Not specified	Primary	EOPC-131_tumor_01	COSMIC	ICGC(EOPC-DE): Early Onset Prostate Cancer - DE
	53-10660T>A	Unknown	Substitution - intronic	Not specified	Primary	EOPC-149_tumor_01	COSMIC	ICGC(EOPC-DE): Early Onset Prostate Cancer - DE
	53-4285C>T	Unknown	Substitution - intronic	Not specified	Primary	EOPC-166_tumor_01	COSMIC	ICGC(EOPC-DE): Early Onset Prostate Cancer - DE
	52+604A>G	Unknown	Substitution - intronic	Not specified	Primary	CPCG_0183_Pr_P_P2	COSMIC	ICGC(EOPC-DE): Early Onset Prostate Cancer - DE
	53-12155C>G	Unknown	Substitution - intronic	Not specified	Primary	LOPC-022_tumor_01	COSMIC	ICGC(EOPC-DE): Early Onset Prostate Cancer - DE
	592G>A	G198S	Splice site	Adenocarcinoma	Metastasis	RP-1532_PCProject_0muduPhG_T1_v2_Exome_OnPrem	cBioPortal	The Metastatic Prostate Cancer Project (Provisional, November 2019)
PPP1CC	56-1203C>G	Unknown	Substitution - intronic	Adenocarcinoma	Primary	0004_CRUK_PC_0004_T1_DNA	COSMIC	ICGC(PRAD-UK): Prostate Adenocarcinoma - UK
	55+2654C>T	Unknown	Substitution - intronic	Adenocarcinoma	Primary	0014_CRUK_PC_0014_T1_DNA	COSMIC	ICGC(PRAD-UK): Prostate Adenocarcinoma - UK
	882+114del	Unknown	Deletion - intronic	Adenocarcinoma	Primary	0048_CRUK_PC_0048_T1_DNA	COSMIC	ICGC(PRAD-UK): Prostate Adenocarcinoma - UK
	55+2204A>T	Unknown	Substitution - intronic	Adenocarcinoma	Primary	0072_CRUK_PC_0072_T1_DNA	COSMIC	ICGC(PRAD-UK): Prostate Adenocarcinoma - UK

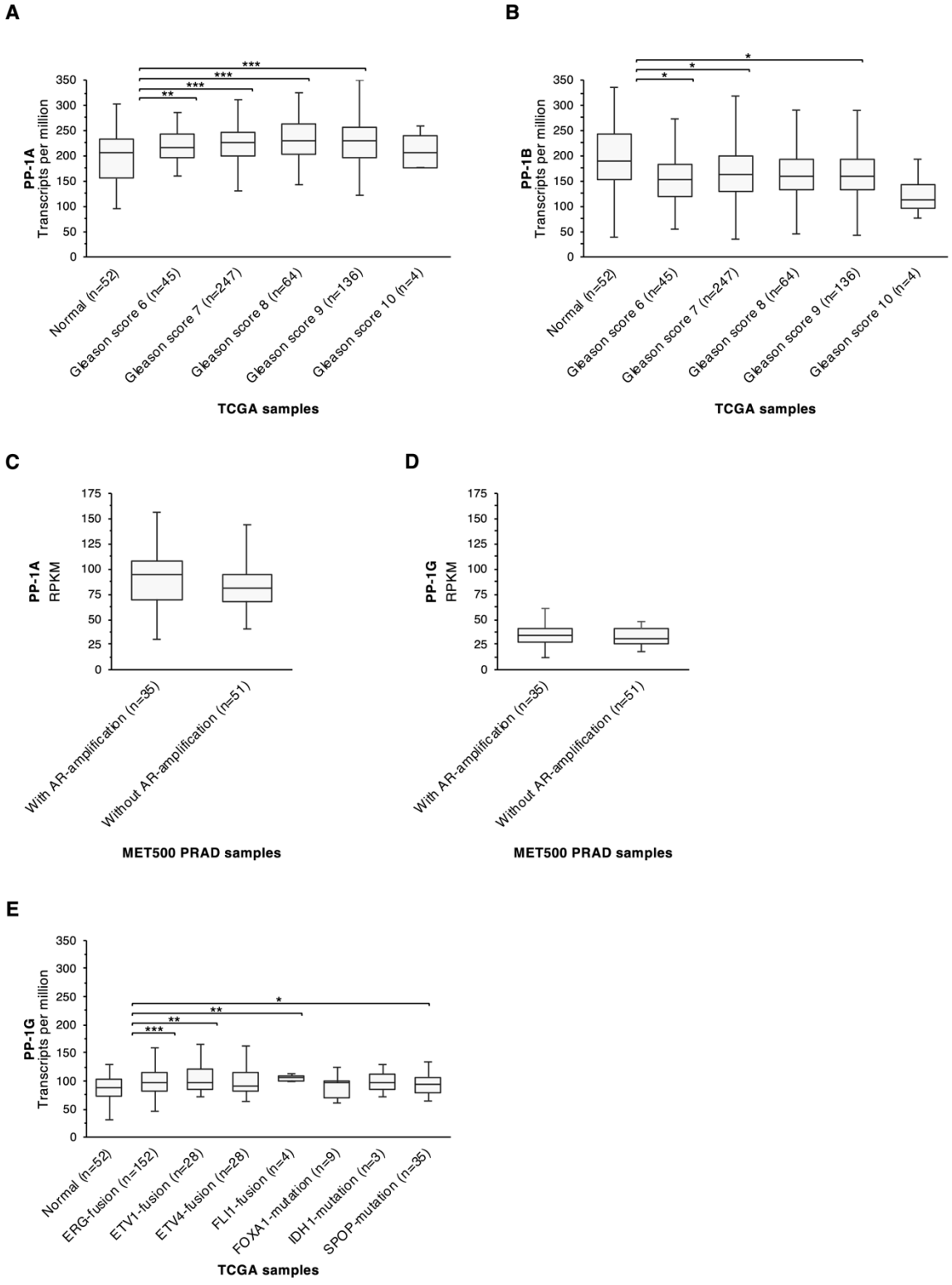
Gene	Mutation (CDS)	Mutation (AA)	Mutation type	Histological subtype	Sample source	Sample(s) ID	Database	Study
PPP1CC	956A>G	K319R	Substitution - missense	Adenocarcinoma	Primary	0149_CRUK_PC_0149_T1_DNA	COSMIC	ICGC(PRAD-UK): Prostate Adenocarcinoma - UK
	571C>A	R191=	Substitution - coding silent	Adenocarcinoma	Primary	06-081D3	COSMIC	Prostate Adenocarcinoma (Fred Hutchinson CRC, Nat Med 2016)
	419-1754T>G	Unknown	Substitution - intronic	Adenocarcinoma	Not specified	CPCG_0183_Pr_P_P2	COSMIC	ICGC(PRAD-CA): Prostate Adenocarcinoma - CA
	55+4940C>T	Unknown	Substitution - intronic	Adenocarcinoma	Primary	CPCG0117-F1	COSMIC	ICGC(PRAD-CA): Prostate Adenocarcinoma - CA
	56-3219G>T	Unknown	Substitution - intronic	Adenocarcinoma	Primary	CPCG0361-F2	COSMIC	ICGC(PRAD-CA): Prostate Adenocarcinoma - CA
	418+460dup	Unknown	Insertion - intronic	Adenocarcinoma	Primary	CPCG0396-F1	COSMIC	ICGC(PRAD-CA): Prostate Adenocarcinoma - CA
	55+5165_55+5175del	Unknown	Deletion - intronic	Adenocarcinoma	Primary	CPCG0397-F1	COSMIC	ICGC(PRAD-CA): Prostate Adenocarcinoma - CA
	55+1469dup	Unknown	Insertion - intronic	Adenocarcinoma	Primary	CPCG0407-F1	COSMIC	ICGC(PRAD-CA): Prostate Adenocarcinoma - CA
	56-3710T>C	Unknown	Substitution - intronic	Not specified	Primary	EOPC-046_tumor_01	COSMIC	ICGC(EOPC-DE): Early Onset Prostate Cancer - DE
	56-3632T>C	Unknown	Substitution - intronic	Not specified	Primary	EOPC-079_tumor_01	COSMIC	ICGC(EOPC-DE): Early Onset Prostate Cancer - DE
	56-3622A>T	Unknown	Substitution - intronic	Not specified	Primary	EOPC-079_tumor_01 EOPC-171_tumor_01	COSMIC	ICGC(EOPC-DE): Early Onset Prostate Cancer - DE
	56-3639C>T	Unknown	Substitution - intronic	Not specified	Primary	EOPC-171_tumor_01	COSMIC	ICGC(EOPC-DE): Early Onset Prostate Cancer - DE
	680T>C	F227S	Substitution - missense	Adenocarcinoma	Metastasis	PROS10448P01-SU2C.01115015-Tumor-SM-42W9C	cBioPortal	Metastatic Prostate Adenocarcinoma (SU2C/PCF Dream Team, PNAS 2019)
	-19G>T	Unknown	Substitution	Not specified	Metastasis	SC_9093	COSMIC	Metastatic Prostate Cancer (SU2C/PCF Dream Team, Cell 2015)
	187+201_187+202insT	Unknown	Insertion - intronic	Not specified	Metastasis	SC_9093	COSMIC	Metastatic Prostate Cancer (SU2C/PCF Dream Team, Cell 2015)

Values are presented as number (%). Data retrieved from COSMIC database, v90 (https://cancer.sanger.ac.uk/cosmic, accessed on 9 Jan 2020) and cBioPortal for Cancer Genomics, v3.2.2 (https://www.cbioportal.org, accessed on 10 Jan 2020). [a] Histological sub-classification of the prostate carcinoma. [b] Data from: The MCP Project, Prostate (SU2C 2019), and PRAD (MSKCC/DFCI 2018). Abbreviations: AdenoPCa, adenocarcinoma; NS, not specified.



Suppl. Fig. III. 1

Immunoexpression (A and C) and localization (B and D) of PP-1A, PP-1B and PP-1G in PCa tissues by pT stage or Gleason score (GS). Results are expressed as percentage of cases. Sample size: pT2 (n=5) and pT3 (n=7); GS 6 (n=5), 7 (n=5), and 8 (n=2). Statistical significance was calculated using the Chi-square test.



Suppl. Fig. III. 2

Expression of PP1c transcripts in normal prostate and primary tumors from TCGA PCa cohorts. (A–B) mRNA expression levels of PP-1A **(A)** and PP-1B **(B)** in tumors with different Gleason scores. **(C–D**) mRNA expression levels of PP-1A **(C)** and PP-1G (D) in tumors with or without androgen receptor (AR) amplification. Data is presented as reads per kilobase per million reads (RPKM). (E) mRNA expression levels of PP-1G in tumors with distinct molecular signatures. Data was reproduced and analyzed through UALCAN. ***p<0.001; **p<0.01; *p<0.05.

CHAPTER IV

UNRAVELLING THE INTERACTOME OF PP-1G IN HUMAN PROSTATE CANCER

Section a

Adding biological meaning to human protein-protein interactions identified by yeast two-hybrid screenings: a guide through bioinformatics tools

KEYWORDS

Protein-protein interaction networks
Databases
Pathways
Ontology
Tissue expression
Phenotypes

ABSTRACT

"*A man is known by the company he keeps*" is a popular expression that perfectly fits proteins. A common approach to characterize the function of a target protein is to identify its interacting partners and thus infer its roles based on the known functions of the interactors. Protein-protein interaction networks (PPINs) have been created for several organisms, including humans, primarily as results of high-throughput screenings, such as yeast two-hybrid (Y2H). Their unequivocal use to understand events underlying human pathophysiology is promising in identifying genes and proteins associated with diseases. Therefore, numerous opportunities have emerged for PPINs as tools for clinical management of diseases: network-based disease classification systems, discovery of biomarkers and identification of therapeutic targets. Despite the great advantages of PPINs, their use is still unrecognized by several researchers who generate high-throughput data to generally characterize interactions in a certain model or to select an interaction to study in detail. We strongly believe that both approaches are not exclusive and that we can use PPINs as a complementary methodology and rich-source of information to the initial study proposal. Here, we suggest a pipeline to deal with Y2H results using bioinformatics tools freely available for academics.

Abbreviations*:* ACSN, Atlas of Cancer Signaling Network; APID, Agile Protein Interactomes DataServer; ARN, Autophagy Regulatory Network; BioGRID, Biological General Repository for Interaction Datasets; BiNGO, Biological Networks Gene Ontology tool; BLAST, Basic Local Alignment Search Tool; CARFMAP, Cardiac Fibroblast Pathway Map; CCLE, Cancer Cell Line Encyclopedia; co-IP, co-immunoprecipitation; CPDB, ConsensusPathDB-human; CRM, cis-regulatory module; CTD, Comparative Toxicogenomics Database; DAVID, Database for Annotation, Visualization and Integrated Discovery; db2db, Database to Database Conversions; DDBJ, DNA Data Bank of Japan; DIP, Database of Interacting Proteins; EASE, Expression Analysis Systematic Explorer; EMBL, European Molecular Biology Laboratory; EMBOSS, European Molecular Biology Open Software Suite; ENCODE, Encyclopedia of DNA Elements; EST, expressed sequence tag; FANTOM5, Functional Annotation of Mammalian Genomes 5; GO, Gene Ontology; GTEx, Genotype-Tissue Expression; HAPPI, Human Annotated and Predicted Protein Interaction; HIPPIE, Human Integrated Protein-Protein Interaction rEference; HPA, Human Protein Atlas; HPRD, Human Protein Reference Database; HUPPO, Human Proteome Organization; IDD, Integrated Interactions Database; IMEx, International Molecular Exchange Consortium; IPI, International Protein Index; JEPETTO, Java Enrichment of Pathways Extended To TOpology; KEGG, Kyoto Encyclopedia of Genes and Genomes; LSID, Life Science Identifiers; MGI, Mouse Genome Informatics; MIM, Mendelian Inheritance in Man; MIMIx, Minimal Information About a Molecular Interaction; MINT, Molecular Interactions Database; miRNA, microRNA; MS, mass spectrometry; NAViGaTOR, Network Analysis, Visualization and Graphing Toronto; NCBI, National Center for Biotechnology Information; OMIM, Online Mendelian Inheritance in Man; PaGenBase, Pattern Gene Database; PANTHER, Protein ANalysis THrough Evolutionary Relationships (PANTHER); PDB, Protein Data Bank; PICR, Protein Identifier Cross-Reference Service; PPI, protein-protein interaction; PPIN, protein-protein interaction network; PCR, polymerase chain reaction; PSI, Proteomics Standard Initiative; PSICQUIC, Proteomics Standard Initiative Common QUery InterfaCe; PTM, post-translational modifications; RefSeq, Reference Sequence; RGD, Rat Genome Database; SAGE, serial analysis of gene expression; SEGUID, Sequence Globally Unique Identifiers; SIGNOR, SIGnaling Network Open Resource; SNP, single-nucleotide polymorphism; SPIKE, Signaling Pathway Integrated Knowledge Engine; STS, Sequence-Tagged Site; TiGER, Tissue-specific Gene Expression and Regulation; TSA, Transcriptome Shotgun Assembly; UniHI, Unified Human Interactome; WGS, Whole-Genome Sequencing; Y2H, yeast two-hybrid.

1. Introduction

Cell dynamics is determined by complex biological processes that involve the interaction of numerous molecular components. Such interactions can be represented in the form of dense biological networks, classified according to the molecules involved (e.g., protein-protein interaction networks (PPINs), metabolic networks and gene regulatory networks) [469].

Protein-protein interactions (PPIs) are crucial to virtually all biological processes, from cell division to cell metabolism. Protein interactomes depend on the cell type and are highly dynamic, adjusting in response to different stimuli and environmental changes [470]. PPIN are so strictly controlled that even a subtle deregulation in PPIs may lead to substantial perturbations in normal cell function, producing disease phenotypes [469,470]. Hence, identifying PPINs underlying different pathological conditions is of utmost importance to find potential targets for pharmaceutical intervention, for example. This notion has led to increasing use of high-throughput interaction mapping methodologies, such as yeast two-hybrid (Y2H) and co-immunoprecipitation (co-IP) coupled with mass spectrometry (MS).

The two-hybrid system allows to detect binary PPIs in an in vivo system (usually the yeast) by the activation of one or more reporter genes [471]. Since its development in 1989 by Fields and Song, the Y2H has contributed to the identification of thousands of PPIs and large interactomes due to its upgrade to an high-throughput version (according to the Proteomics Standard Initiative Common QUery InterfaCe (PSICQUIC) View (v1.4.6), around 17% of the PPIs described were detected by Y2H)[5] [471,472].

In an attempt to organize the massive interactome data that is being generated and make it easy and ready to use by researchers several databases have been developed. Here, we propose a general workflow to retrieve maximum information from Y2H results (Fig. IVa. 1), focusing on the study of human interactomes. We explain how to identify the positive clones obtained in an Y2H screening and explore multiple bioinformatics tools that enable the creation and visualization of PPINs and help adding biological meaning to the PPIs identified. The designation and website of all the resources referenced in the text can be consulted in Suppl. Table IVa. 1.

2. Identification of positive clones: obtaining a raw list of interactors from Y2H experiments

In a typical Y2H experiment, bait and prey proteins interact inside yeast cells so that reporter genes are expressed, and the interaction is detected in the appropriate growth media. Each positive clone is, therefore, a yeast colony that contains the Y2H plasmids encoding the interacting

[5] PSICQUIC View (v1.4.6) was queried in January 2016 using the following terms: “ptype:protein” (n=1,369,774) and “ptype:protein AND detmethod:yeast two hybrid” (n=241,156).

proteins: a known bait protein and an unknown prey protein (Fig. IVa. 2). The output of an Y2H screen can vary from few to hundreds of positive clones, which are organized in a master plate and given a code [473,474].

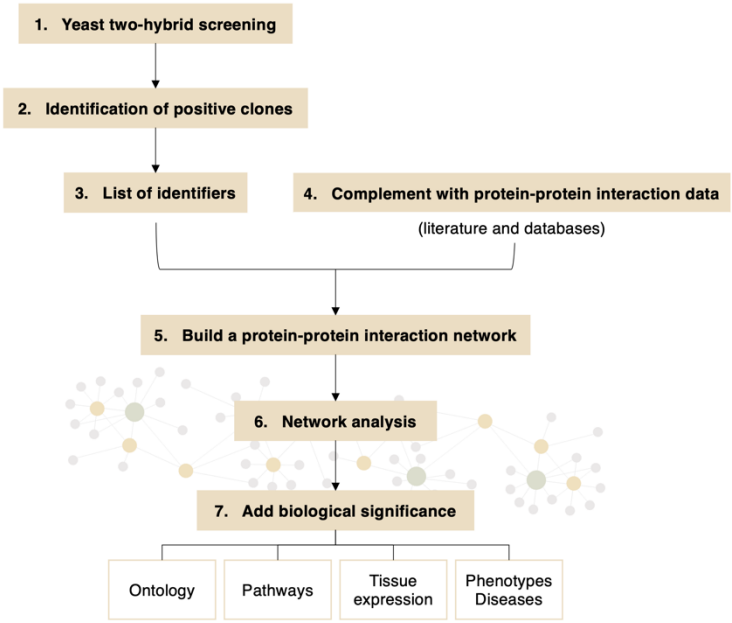


Fig. IVa. 1
General pipeline to add biological significance to yeast two-hybrid results *in silico*.

To eliminate duplicate clones, yeast colony polymerase chain reaction (PCR) analysis is then performed using a specific set of primers that hybridize to the multiple cloning site flanking sequences of the activation domain vector. The PCR products are analyzed by electrophoresis on agarose gels. To confirm that similar DNA bands contain the same insert, the PCR product is digested with frequent cutter restriction enzymes (such as AluI) and re-analyzed by electrophoresis on agarose gel. A representative clone of each different single band is used to identify the nucleotide sequence of the putative interacting protein. PCR products previously obtained—corresponding to single bands—are sequenced using an appropriate primer (e.g., T7 primer). The sequences of each putative positive clone are curated to remove the vector sequences and keep only the sequence that refers to the positive clone.

The sequences obtained for each clone are compared with a nucleotide database sequence using a local alignment sequence tool, such as Basic Local Alignment Search Tool (BLAST) [475] and European Molecular Biology Open Software Suite (EMBOSS) Water [476]. The different local alignments sequence tools share a common procedure: the query sequence is compared to a database sequence yielding a score that indicates the chance of homology. This comparison is repeated to all database sequences and the most reliable hits are reported. On the other hand, the tools differ in

speed, sensitivity and user interface. BLAST is the fastest and most popular, whereas EMBOSS Water (which uses the Smith-Waterman algorithm) is the most sensitive but takes longer to respond.

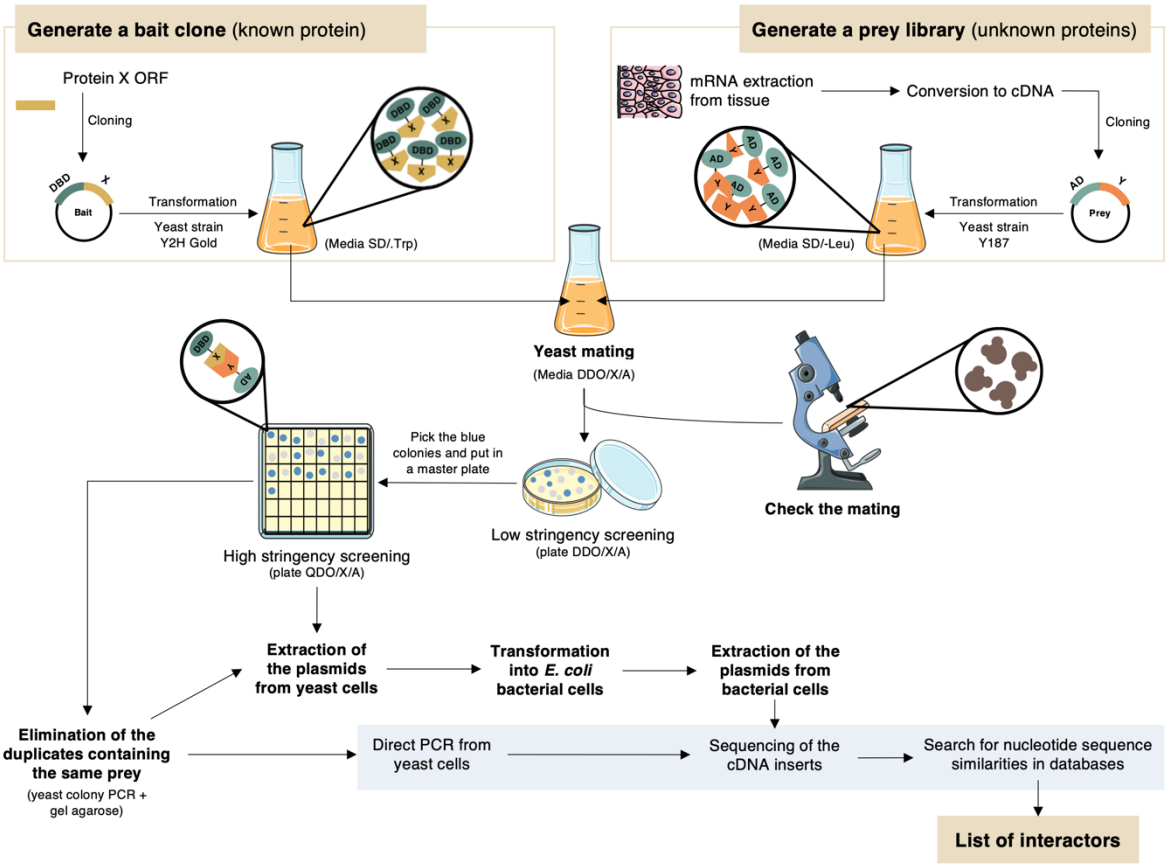


Fig. IVa. 2
Workflow of a yeast two-hybrid experiment (created according to the Matchmaker Gold Yeast Two-Hybrid System (Clontech Laboratories, Inc). Two alternative approaches are presented to recover plasmids from yeast cells for sequencing (highlighted in grey is the more recently developed method that involves direct PCR from yeast cells). Abbreviations: AD, activation domain; DBD, DNA-binding domain; Leu, leucine; Trp, tryptophan; SD, single dropout; DDO/X/A, double dropout medium: SD/–Leu/–Trp supplemented with X-a-Gal and Aureobasidin A; QDO/X/A, quadruple dropout medium: SD/–Ade/–His/–Leu/–Trp supplemented with X-a –Gal and Aureobasidin A. Figures were produced using Servier Medical Art from www.servier.com.

BLAST has been used extensively since its development in the early 90s to unravel what genes and proteins do. By simply submitting the query sequence, within seconds, a list of related sequences from many different organisms, as well as other helpful information, is displayed. BLAST finds regions of local similarity between sequences by comparing nucleotide (BLASTn) or protein (BLASTp) sequences to sequence databases and calculates the statistical significance of the matches. By default, BLASTn searches a nucleotide collection, which includes several sequence databases: GenBank, European Molecular Biology Laboratory (EMBL), DNA Data Bank of Japan (DDBJ), Protein Data Bank (PDB) and Reference Sequence (RefSeq) databases. The search excludes sequences from EST, Sequence-Tagged Site (STS), Genome Survey Sequence, Whole-Genome Sequencing (WGS), Transcriptome Shotgun Assembly (TSA), patent sequences and phase 0, 1 and 2

High-Throughput Genome Sequencing (for more information see [477]). To search BLAST, each nucleotide sequence is inserted (preferably in FASTA format) in the query box of the BLASTn panel, the appropriate organism is selected, and the default set up can be used. At this point the correct open reading frame is confirmed and the size of the insert, the existence of new splicing variants, among other sequence characteristics are analyzed. At the end of this task, a list of putative interactors is obtained, which corresponds to the interactome of the bait protein.

3. Researchers might ask: what's next for a protein interactors list?

Typically, the list of interactors is searched to select one or a few PPIs to be validated in human models (either cell lines or tissues), using common biochemical techniques (e.g., co-IP and co-localization). This selection is based on researchers' expertise or literature clues that might indicate that such PPIs could be relevant to the condition under study. Although this is the traditional approach that allowed the characterization of multiple key protein complexes for human health and disease, the amount of information that is 'lost' during this process can be huge. Efforts have been made to host interactomes in public databases so data can be easily accessed and readily used by researchers to improve the understanding of their own data.

3.1. Selecting the interactors' identifiers to be used in bioinformatics analysis

Biological databases present marked differences between each other, mainly because each of them focuses on a different subset of biological knowledge. Therefore, users are almost invited to query several databases to answer complex biological questions and later to integrate the information retrieved from the different sources. During the integration process, researchers face many challenges, starting with the absence of a global system of identifiers. This means that the same molecule may have multiple names, and the same name might identify multiple molecules. Efforts have been made in order to create a unified identifier system, such as the Life Science Identifiers (LSID) [478] and Sequence Globally Unique Identifiers (SEGUID) [479] initiatives, but none was particularly effective in solving the identifiers problem until now.

Each database includes a set of protein identifiers that users can use when querying (e.g., UniProt Accession, RefSeq and International Protein Index (IPI) identifiers) and, usually, they also accept gene names (which, nevertheless, can be ambiguous for the above-mentioned reasons). Thus, the list of interactors, usually retrieved as gene names from BLAST, should be first converted into protein identifiers. A number of web applications can help in the successful accomplishment of translating between different types of protein identifiers (whenever a one-to-one correspondence between identifiers is available), either by interactive or programmatic access (for an overview of biological database mapping tools see [480]).

When using an ID converter, users should define the query (input) and target (output) formats and select the species under consideration. The type of input/output allowed, as well as other features distinguish the tools available [480]. UniProt Retrieve/ID mapping tool is one of the most commonly used converters. In this case, one of the pairs (input or output) has to be the UniProt Accession (for instance, we can input the list of interactors' gene names and convert it into human UniProt by selecting *Homo sapiens* in the organism field) [481]. On the other hand, in the DAVID Gene ID Conversion Tool the input has to be a gene list and it can be converted to a number of identifiers, based on the DAVID gene concept [482]. Additional ID converter tools include the Database to Database Conversions (db2db) [483], the ID mapping from the iProClass database [484] and the Protein Identifier Cross-Reference Service (PICR) [485,486]. PICR is particularly useful since it allows to map gene or protein identifiers as well as protein sequences across over 100 different source databases in a single request. Moreover, the tool includes a PICR BLAST algorithm that enables a BLAST search to be done in parallel for each protein sequence queried and gives the user the opportunity to select the BLAST result for each protein sequence that can be used as entry point to the mapping-by-accession algorithm [485]. For details on the input/output formats available in each ID converter tool, see the online help at each web site (Suppl. Table IVa. 1).

3.2. Retrieving protein-protein interactions from public databases

As of December 2016, the Pathguide listed 257 PPI-related databases (and this number may be an underrepresentation as the database is not updated since 2013) [487]. A fundamental idea to keep in mind when searching PPI databases is that none of them provides full coverage of all known human PIPs. In fact, the overlap between PPI databases was shown to be relatively small and there is also little agreement in entry details [488–491].

PPI databases can be generally classified into three main types according to data collection: (1) primary databases, which collect experimental data from peer-reviewed scientific publications (these could be further subdivided according to organism-specificity and level of curation); (2) secondary databases, also known as meta-databases, which integrate data from several primary databases and could also include data from self-data mining; and (3) prediction databases, which include interactions predicted by computational methods [492].

When studying PPIs using bioinformatics methods it is important to have a clear distinction between experimentally detected and predicted interactions. While the first refers to an interaction detected using small-scale or large-scale molecular biology techniques, the second means the interaction was predicted to occur based on genomic context and structural information, network topology, text mining or machine learning algorithms using heterogeneous genomic/proteomic features [493,494]. For instance, when dealing with less-known proteins, the use of prediction data could be very useful to start unravelling the function of the protein; however, it could also introduce bias,

so the analysis should be done carefully. In our pipeline we will not include predictive tools, but deep knowledge on these can be acquired elsewhere [493,495,496].

Bioinformatics analysis of PPIs should be performed using high-confidence results, which implies, for example, filtering out potential false-positive interactions (all molecular biology techniques used to identify PPI generate false positive results). To this end, it is important to consider the PPI score and the database curation level. In each database, PPIs are classified using quantitative or qualitative scores. These scores are calculated by algorithms whose variables may include the method used to identify the interaction, the interaction type and the number of publications reporting that particular interaction. On the other hand, curation refers to the manual process of extracting information from scientific publications and converting it into a structured vocabulary that is made available. Different databases may use distinct levels of curation, varying from shallow curation (includes only key indications on the interactor identifier and the interaction detection method) to deep curation (includes a detailed description of both the interactor and the interaction itself), which follow different standards [492]. Therefore, if the aim is to use high-confidence data, it is advisable to search in deep curated databases and to use binary interactions experimentally detected with a high score.

The first databases developed had their own standards and data formats, making the task of combining datasets from different databases difficult and laborious. To solve this problem, several initiatives have emerged. In 2004, the Proteomics Standard Initiative group (PSI) of the Human Proteome Organization (HUPO) announced the development the PSI-MI XLM1.0 format for the exchange of protein interaction data [497] (v2.5 was later released to include interactions among other molecules and to provide a simpler format—the MITAB [498]). In what concerns data standards, a first model for the representation and exchange of PPIs data was also published in 2004—the Minimal Information About a Molecular Interaction (MIMIx) [499]. Years later, the International Molecular Exchange Consortium (IMEx) was created to implement a set of rules that must be followed by biocurators to deep curate scientific publications [500]. Due to the deep curation standards, the combination of IMEx partners' data is particularly useful when studying protein interactomes Table IVa. 1.

Secondary databases are also valuable tools to construct PPINs since they extract and integrate information from other databases and present them in a simple and unified language. Most of these databases are very user-friendly and allow the application of several filters, thereby conditioning the search according to user's requirements. However, secondary databases are not updated as frequently as primary databases and attention should be given to the set of primary databases where data is acquired from (e.g., level of curation, experimentally detected vs. predicted interactions).

Table IVa. 1
Databases that collect human protein-protein interactions.

Database	Interactions	Version	Last update	Features	Ref	Cytoscape[a]
HPRD	41,327	9.0	2010	Includes information on isoforms, post-translational modifications, subcellular localization, tissue expression, protein domains and association with human disease.	[501,502]	No
IntAct	658,369	4.2.6	2016	IMEx active member. In addition to its curation data, it includes data curated by other databases (e.g. MINT, UniProt, I2D, InnateDB, etc.) and acts as a common curation platform. Provides isoform information when data is available. Includes data for several organisms and other types of molecular interactions.	[503–505]	Yes
MINT	125,464	Beta	2013[b]	IMEx active member. It includes PPIs and other molecular interactions (e.g. promoter regions, mRNA) from 611 organisms. Associations to human diseases are highlighted.	[505–507]	Yes
DIP	81,731		2014	IMEx active member. It includes protein complexes retrieved from the Protein Data Bank and presents data for 10 organisms.	[508–510]	Yes
PDZBase	339		2004	Contains PDZ-domain-mediated PPIs found in human, rat, mouse, drosophila, *C. elegans*, xenopus and Synechocystis sp.	[511]	No
BioGrid	1,412,140	3.4.144	2017	Observer partner of IMEx. It includes protein and genetic interactions, PTMs and chemical associations from several species.	[512,513]	Yes
I2D	1,279,157	2.9	2015	IMEx active member. Includes experimental and predicted PPI data for *S. cerevisiae*, *C. elegans*, *D. melonogaster*, *R. norvegicus*, *M. musculus*, and *H. sapiens*.	[514,515]	Yes
InnateDB	27,039		2016	IMEx active member. Includes molecular innate immunity-related interactions from manual curation and other databases. Data are derived from *M. musculus*, *B. taurus* and *H. sapiens*.	[516,517]	Yes
VirHosNet	30,000	2.0	2016	Includes virus-virus, virus-host and host-host interactions. Manually curated data was complemented with data from high-confidence interaction databases and functional annotation.	[518,519]	Yes
UniProt		15.0	2016	IMEx active member. It is not a conventional PPI database; instead, data derives from IntAct and it is updated monthly. Present cross-references for multiple databases.	[520,521]	Yes
HPIDB	52,953	2.0	2016	IMEx active member Contains PPIs between 58 hosts (animal or plant) and 524 pathogens (virus, bacteria, fungi or other).	[522,523]	Yes
MatrixDB	15,018		2015	IMEx active member. Comprises interactions established by extracellular matrix proteins, matricryptins, glycosaminoglycans, lipids and cations.	[524,525]	Yes
STRING	932,553,897	10.0	2016	Includes known and predicted PPIs from more that 2,000 organisms.	[526,527]	Yes

[a] The column "Cytoscape" indicates the possibility of importing a network from the databases to the software.
[b] Since then it has been updated by the IntAct team.
Only freely accessible databases based on experimental evidence that remained active at the date of the publication were included.

For instance, Human Integrated Protein-Protein Interaction rEference (HIPPIE; v2.0, last update: 2016) [528,529], collects experimentally detected PPIs, while Unified Human Interactome (UniHI; v7.1, last update: 2014) [530,531] and Human Annotated and Predicted Protein Interaction (HAPPI) [532] also include predicted data. Agile Protein Interactomes DataServer (APID; last update: 2016) provides a comprehensive collection of experimentally validated PPIs for multiple organisms and allows the user to strict quality and coverage levels by including in the analysis all known interactions, only interactions proven by two or more experiments or only interactions proven by two or more publications [533,534]. HIPPIE and APID share major contributors, such as the Database of Interacting Proteins (DIP), the Human Protein Reference Database (HPRD), IntAct, the Molecular Interactions Database (MINT) and the Biological General Repository for Interaction Datasets (BioGRID), and both can be integrated with Cytoscape in order to create and visualize networks.

To provide an even more comprehensive and updated view of PPIs, PSICQUIC View was created [535,536]. This allows users to obtain all the molecular interactions to a given query that are hold in the 36 registered databases simultaneously (the PSICQUIC Registry summarizes the information about PSICQUIC View-registered databases) [536]. Currently, the PSICQUIC View web service states more than 150 millions of binary interactions, which include not only PIPs, but also protein-DNA, protein-chemical compounds and protein-RNA interactions found for numerous organisms [535,536]. The PSICQUIC View interface is based on a query system using simple codes or more complex languages that include multiple parameters, such as taxonomy identifiers and interaction detection method. Also interesting is that users can choose the databases they want to search from. This is particularly important if the user aims to restrict the PIPs to, for example, IMEx-curated databases. Upon retrieval of the binary PIPs, users can cluster the information into a single data file (a maximum of 5,000 binary interactions can be clustered at a time). When clustering, PSICQUIC View unifies the results obtained from each distinct database, thereby eliminating duplicates. Finally, users can download the binary interactions retrieved in a PSI-MITAB 2.5 format, which presents the information in a standard and controlled vocabulary, structured into 15 columns – each one providing relevant details on the PIP (e.g., ID of interactors, interaction detection method, publication identifier, taxon, etc.).

3.3. Creating a protein-protein interaction network

Network representation is based on the graph theory introduced by Euler in 1736 [537]. PPINs are typically undirected networks in which proteins are represented as nodes and interactions as edges [538].

Cytoscape is still one of the most widely used tools to create and analyze PPINs. It is an open-source software written in Java, which accepts several input formats and is connected to numerous biological databases, thereby enabling the integration of multiple data. Furthermore, it sup-

ports the installation of diverse plug-ins—that can be quickly and easily installed just by going to the "App manager" in the Apps menu of the software. The Cytoscape App Store, easily accessed through the Cytoscape website, contains all the plug-ins available.

When starting Cytoscape, users can import a previously saved network to explore topological properties or complement with other types of information or create a new network from a previously filled table or directly from public databases. In the latter case, users can query one or multiple identifiers simultaneously. The search reveals the active databases that have information on the interacting proteins of the query and users can select from which ones they want to import from (e.g., IMEx-complying datasets). If the option "Automatic Network Merge" is selected, a non-redundant network is created resulting from the combination of the various data sources selected (alternatively, the networks from each data source can be merged manually).

Cytoscape allows the creation of filters; these are very useful when producing human PPINs since users can create a filter to automatically exclude interactors detected in non-human organisms (just by selecting the nodes that respect the rule "taxonomy ID is 9606" and creating a new network from them) or to filter out molecules that are not proteins when the network was created using databases that include different types of molecular interactions (e.g., DNA-protein, drug-protein, etc.).

There are several other tools that can be used to create and visualize biological networks. OMICtools database lists more than 60 tools within the class "Network visualization", which greatly vary in the type of information they can display and present distinct strengths and limitations [539]. For instance, Pajek (v4.0, last update: 2016) is a popular software to handle huge networks (the number of nodes is virtually unlimited, unless by the memory size) [540]. Both Pajek and Network Analysis, Visualization and Graphing Toronto (NAViGaTOR) [541] support 2D and 3D layouts, can retrieve interactions from databases and allow the integration of plug-ins. Detailed information on these and other network visualization tools was reviewed elsewhere [542–544].

When working with highly complex PPINs, it could be useful to find clusters (i.e., groups of highly connected nodes), which are expected to reflect groups of proteins that share a similar function. Cluster analysis could be essential to highlight central proteins and interactions for the context under analysis [545]. Cytoscape App Store indicates 29 applications that can be used for clustering. Additional analysis of other networks' topological features (e.g., degree, shortest path length, betweenness, among others) reveal important biological information. Comprehensive reviews on networks' topological properties are available elsewhere [546,547].

4. Unravelling the biological significance of the protein-protein interaction network

Additional information could be added to PPINs to enhance the understanding of their biological role in the context under study. For this, it is important to address both the functions and the processes

in which the interactors are involved in, as well as their expression profiles and the phenotypes/diseases they are associated with. Conventionally, this task is performed by literature mining, with several general or biomedical-related web tools being used: PubMed, Google Scholar, ScienceDirect, Scopus, among many others that differ, for example, in content update and service provider profile. An extensive review of biomedical literature searching tools was published by Zhiyong Lu, in 2011 [548], whom data was complemented by members of the National Center for Biotechnology Information (NCBI) and integrated in a list that can be consulted in the NCBI website. Despite the undoubtable value of such information, this approach could be incredibly laborious and time-consuming, resulting in the retrieval of hundreds of entries that might be accurately assessed.

The task of unravelling the biological significance of PPINs can be facilitated by using the knowledge collected by several repositories in terms of functional annotation, tissue expression and association with phenotypes and diseases (which, nevertheless, do not overcome the usefulness of literature-mining; they are rather complementary methods).

4.1. Functional annotation

The most common approach to analyze a protein interactome is to check if it is enriched in a certain functional annotation. The most well-known functional database is the Gene Ontology (GO). The GO project emerged, in 1998, with three major goals: (1) to develop a group of vocabularies (known as ontologies) to describe key domains of molecular biology in a controlled and structured way; (2) to apply GO terms in the annotation of sequences, genes or gene products in biological databases; and, (3) to become a centralized public resource concerning ontologies, annotation data sets and software tools developed for use with GO data [549,550].

GO comprises three species-agnostic structured categories: (1) biological process, (2) molecular function and (3) cellular component. Each of these categories are organized as a tree structure, meaning that each term establishes relationships to one or multiple other terms [549,550]. The search on GO could be easily performed by pasting the list of UniProt Accessions and choosing the species and the ontology desired to be analyzed. This service connects to the analysis tool from the Protein ANalysis THrough Evolutionary Relationships (PANTHER) Classification System, which is maintained up to date with GO annotations [551].

4.2. Cell signaling pathways

As similar as PPI data, the developments in the comprehension of biochemical pathways have motivated several academic and commercial groups to create cell signaling maps, which were later incorporated in cell signaling pathway databases. The Pathguide meta-database lists 95 databases within the class “Signalling Pathways”, which greatly vary on data coverage, storage, visualization

and access [487]. It is important to highlight, nevertheless, that neither all of these databases remain active nor all include human-related data.

Signaling pathway databases can be generic, such as Kyoto Encyclopedia of Genes and Genomes (KEGG) PATHWAY [552,553], Reactome [554–556], WikiPathways [557,558], Pathway Commons [559], PANTHER PATHWAY [560], NetPath [561], Signaling Pathway Integrated Knowledge Engine (SPIKE) [562,563], ConsensusPathDB-human (CPDB) [564–566] and SIGnaling Network Open Resource (SIGNOR) [567], or related to a specific field, such as AlzPathway (for Alzheimer disease) [568,569], Atlas of Cancer Signaling Network (ACSN; for cancer) [570], Autophagy Regulatory Network (ARN; for autophagy) [571] or Cardiac Fibroblast Pathway Map (CARFMAP; for cardiac fibroblasts) [572]. Some databases also include data besides PPI, such as SignaLink, which incorporates resources to analyze signaling pathway cross-talks and contains information on pathway-specific transcription factors, miRNAs and regulatory enzymes [573,574].

An attention-grabbing review published in 2015 by Chowdhury S and Sarkar RR indicated 24 active (or at least in working state), open source databases that include human cell signaling-related data [575]. The authors compared the 24 databases regarding their pathway information and technical details. The list includes some of the most well-known, widely used and up-to-date cell signaling databases, including KEGG PATHWAY and Reactome databases (both literature-curated).

The KEGG PATHWAY database, launched in 1995 by Kanehisa M with the main objective of linking genes to functions, is thought as a pioneer amongst the human cell signaling pathway databases [552]. KEGG PATHWAY has a unique process of annotation that allows the generation of pathway maps that are manually drawn by inspiration in literature, textbooks and experts' knowledge. The database includes human diseases as one of the main categories, which in turn has several subcategories, such as cancer, immune, neurodegenerative and cardiovascular diseases, as well as drug resistance (for both antimicrobial and antineoplastic drugs). The recent update on KEGG architecture (October 2016) also introduced subject-oriented (e.g., KEGG Cancer) and organism-specific entry points in the homepage, making the database even more user-friendly for researchers that work in specific research areas [553]. The database can be searched using the KEGG Pathway ID, the pathway name or even the Gene ID. For a given queried pathway, the user can choose to see the reference pathway or an organism-specific version of the consulted pathway or, in the case of humans, the pathway with drug targets highlighted [553].

In Reactome, the “functional unit” is the reaction and multiple reactions are grouped into pathways which, in turn, are associated to biological processes [555]. Reactome classifies and catalogues molecular structures (it is not restricted to proteins and includes post-translational modified entities besides the original molecule), as well as presents information on their subcellular localizations and the transformations they can undergo (e.g. biochemical reaction, assembly to complexes, etc.)

[554-556]. One advantage when working with isoforms is that Reactome annotates specifically to protein isoforms when this information is available. The database uses the PSIQUIC View web service to overlay molecular interaction data from the Reactome Functional Interaction Network and external interaction databases (e.g. IntAct, ChEMBL, BioGRID and iRefIndex) [554–556]. All data from KEGG PATHWAY and Reactome are available for download.

The difficulty in choosing the signaling pathway resource(s) to be used, due to their different focus, specificity and level of detail, motivated Turei D and colleagues to develop OmniPath (released in late 2016) – an integrated resource of literature-curated human signaling pathways – and moving a step forward towards unification [490]. OmniPath integrates data from 27 public resources on signaling interactions that contain either enzyme-substrate, activity flow, undirected PPI or process description data. This way, OmniPath assures a four times higher coverage of interactions than the largest database it contains. Moreover, it contains information on the structure and mechanism of the interactions, drug targets, functional annotations and tissue-specific mutations [490]. The Pypath software (Python module) of OmniPath allows to assess large datasets, build custom signaling networks and combine them with other data. OmniPath also offers an ID converter tool (the Pypath's Mapper) [490].

4.3. Analysis of tissue expression

As previously stated, protein interactomes are highly dynamic and, accordingly, PPINs should be studied in conformity with biological context. This is particularly important when using Y2H screenings since the interactions are detected in an artificial environment. The addition of expression data to PPINs offers opportunity to better understand the network in tissue-specific context.

The most widely technologies used to map proteins present in a given tissue are based on measuring mRNA levels and include high-density oligonucleotide microarrays (DNA chips), RNA sequencing and ESTs. Fewer techniques exist for high-throughput measurement of protein levels (e.g., multiplexed immunohistochemical staining and MS) (for an overview of the technologies used for tissue expression mapping see [576]). However, oftentimes the data is not systematically compared and integrated.

When analyzing data from different tissue-expression databases it is important to take into consideration the varying names used for the same tissues (for tissue name standardization see the Brenda Tissue Ontology) [577].

The Human Protein Atlas (HPA) database includes millions of high-resolution images presenting the spatial distribution of proteins in 44 different normal human tissues, 20 different cancer types and 46 human cell lines. The HPA provides a global analysis of the secreted and membrane coding genes, complemented with analyses of various sub-proteomes, such as predicted secreted proteins (n=3171) and membrane-bound proteins (n=5570). Compiled lists of proteins expressed at elevated

levels in the different tissues are also available. HPA contains RNA data for 99.9% and protein data for 86% of the predictive human genes [578].

The Tissue-specific Gene Expression and Regulation (TiGER) provides data for tissue-specific gene expression and regulation in a variety of human tissues. The database contains tissue-specific gene expression profiles or EST data, cis-regulatory module (CRM) data and combinatorial gene regulation data. Presently, the database contains expression profiles for 19,526 UniGene genes, combinatorial regulations for 7,341 interacting transcription factor pairs and 6,232 putative CRMs for 2,130 RefSeq genes [579].

The Human Protein Reference Database (HPRD) contains information on the human proteome including PPIs, post-translational modifications (PTMs), domain architecture, disease association and tissue expression. The information is manually extracted from the literature by experts [502].

The Genotype-Tissue Expression (GTEx) portal includes data from 8555 postmortem samples covering 53 body sites from 544 individuals in order to examine the correlation between human genetic variation and tissue-specific gene expression in normal individuals [580–582].

The TISSUES web resource integrates data from many different technologies and sources, quantifies the reliability of each gene–tissue association, and thereby makes results from different sources comparable [576]. The web interface allows to search for a human gene and get a complete overview of where it may be expressed, the confidence that the gene of interest is expressed there and the evidence supporting the expression. TISSUES holds information for 21,294 genes and 5,305 different tissues and provides more than 2.2 million gene–tissue associations [576].

C-it is a knowledge database that focuses on uncharacterized, tissue-enriched genes in human and other species. C-It is based on EST expression profiles from the UniGene. PubMed articles and MeSH terms were used to classify uncharacterized genes. Additional expression studies from microarrays, serial analysis of gene expression (SAGE) and exon arrays were included to build a comprehensive source of expression analyses. It offers 3 different search methods: (1) by tissue, (2) by single gene and (3) by list of genes [583].

BioGPS is a centralized gene-annotation portal that enables researchers to access distributed gene annotation resources. One of the most popular plugins is the ‘Gene expression/activity chart’, which includes around 6000 datasets. BioGPS supports eight species and allows users contributions to the platform and great customizability [584,585].

Pattern Gene Database (PaGenBase) provides information for the collection of tissue- and time-specific pattern genes (specific genes, selective genes, housekeeping genes and repressed genes) of 11 model organisms identified from serial gene expression profiles of multiple physiological conditions. The database contains 906,599 pattern genes derived from the literature or from data mining of more than 1,145,277 gene expression profiles in 1,062 distinct samples [586].

The Expression Atlas includes highly-curated, quality-checked microarray and RNA sequencing data concerning gene and protein expression in animal and plant samples of different organism parts, developmental stages, cell types, diseases and other conditions such as a gene knock out and treatments with chemical compounds. The Baseline Atlas allows to recognize which genes are specifically expressed in a certain tissue and at what abundance. It incorporates baseline expression profiles of tissues from HPA, GTEx and Functional Annotation of Mammalian Genomes 5 (FANTOM5), and of cancer cell lines from the Encyclopedia of DNA Elements (ENCODE), Cancer Cell Line Encyclopedia (CCLE) and Genentech projects. The Differential Atlas allows to identify genes up- or downregulated in a certain condition conditions, such as a disease or treatment [587].

Integrated Interactions Database (IID) provides tissue-specific PPIs for human and model organisms. It covers six species and up to 30 tissues per species [588]. IID interaction data comprises three major sources: experimentally identified PPIs from major databases, orthologous PPIs and high-confidence computationally predicted PPIs. IID provides a total of 1,566,043 PPIs among 68,831 proteins, tissue annotations for interactions and several data visualization capabilities [588].

4.4. Phenotypes and disease association

The clear majority of diseases are now known to be caused by malfunction of multiple genes and consequent deregulation of molecular interactions. The first attempt to prioritize knowledge sharing on human gene-disease association was started by Dr Victor A. McKusick, who first published the book “Mendelian Inheritance in Man (MIM). A Catalog of Human Genes and Genetic Disorders” in 1966 [589]. The book gave birth to the Online Mendelian Inheritance in Man (OMIM), a genetic knowledgebase freely available and updated daily that is still the gold-standard source of human genotype-phenotype association information [590,591]. OMIM encompasses almost 6,000 phenotypes, which include single-gene mendelian disorders and traits, susceptibilities to cancer and complex diseases, variations that lead to abnormal but benign laboratory test values (referred as “nondiseases”), blood groups and somatic cell genetic diseases. When searching the database for a gene of interest, users obtain a table with gene-phenotype relationships that includes the phenotypes along with their inheritance and phenotype mapping key (that gives information on the evidence of the phenotype). Other types of information, as well as external links to a variety of databases can be found within the same page [592].

Many databases have emerged to cover maximum gene-disease associations. Some of them aim to be more comprehensive (e.g. DisGeNET aims to cover Mendelian, complex and environmental diseases [593], while others are restricted to a subject (e.g. the Psychiatric disorders Gene association NETwork – PsyGeNET [594]); some are based on text-mining approaches (e.g. Phenominer [595,596] and DISEASES [597]), whereas others hold manually curated data (e.g. DisGeNet [598] and Comparative Toxicogenomics Database (CTD) [599]).

DisGeNET (v4.0) contains 429,036 gene-disease associations resultant from the integration of information from various repositories (curated, predicted and variant databases) with data from literature (collected by different text-mining approaches) [593]. DisGeNET can be searched by disease, single-nucleotide polymorphism (SNP) or gene (the interface allows to search using single parameters or short lists). The database returns the Top 10 disease associations for the query (indicating the number of PubMed identifiers that support the association) and the Top 10 genes that share diseases with the query, specifying the number of diseases shared. By browsing details, users can check the complete lists and find details on the publications, diseases shared, among other information [593]. Its integration with Cytoscape is also possible through the DiGeNET Cytoscape plugin, which allows to represent and analyze human gene-disease association networks from an interactors list [600].

CTD overall goal is to understand the relationship between environmental exposures and human health. The database is composed by manually curated interactions (chemical-gene, chemical-disease and gene-disease), with the recent update (January 2017) guaranteeing the coverage of over 34,000 gene-disease associations (by both CTD and OMIM curation) [601]. This data is complemented by more than 20,000,000 inferred associations via curated chemical interaction. In addition, CTD includes information on chemical-gene-disease networks [599,601]. Gene-disease associations are presented in a tabular format, which further indicates the direct evidence (i.e. if the gene is a marker/mechanism and/or therapeutic) and can be filtered out to select by disease category and association type (curated versus inferred versus all) [599,601].

Genopedia is a gene-centered database that is regularly updated from PubMed to give information on human genetic associations [602]. Genopedia displays a table with the disease terms (MeSH terms) associated to the query along with the number of total publications and meta-analysis publications in which the gene-disease association is reported. By clicking the numbers, users can easily identify all the publications and the list can be further filtered by disease, gene, year, journal and country. Moreover, the database provides a list of connected genes (i.e., genes that are involved in the same disease) [602].

GeneCards (v3.0) functions like a ‘gene ID card’: it collects information from about 125 web services and integrates them to give the user a complete picture of the gene (e.g., in terms of function, expression, localization, etc.). Particularly interesting is the fact that GeneCards is linked to MalaCards to provide gene-disease associations. Therefore, when searching for a gene in GeneCards, one of the sections of the ‘card’ (named “disorders”) lists the disorders associated with the gene, the aliases of the disease and the information source [603]. MalaCards integrates human disorders and their annotations from 69 sources; these include major sources of genotype-phenotype associations, including OMIM, DISEASES, ClinVar (genomic variation-phenotype associations) [604] and Mouse Genome Informatics (MGI) [605]. Valuable data can be also obtained from knockout models,

with MGI and Rat Genome Database (RGD) [606] being two of the most used databases to study human phenotypes based on animal models.

4.5. Enrichment analysis tools

The necessity of attributing biological significance to large lists of molecules led to the development of several enrichment analysis tools. The degree of enrichment is calculated by comparing the interactors list against a certain background, which should be decided in accordance (i.e., typically, the enrichment analysis of a human PPIN is performed using the complete human genome as background; however, some analyses may benefit from using a tissue-specific background).

In 2009, a survey published by Huang DW and colleagues listed 68 enrichment tools [607]. The authors proposed a classification system to group the tools based on their algorithms: singular enrichment analysis (the most traditional and used strategy); gene set enrichment analysis (experimental groups are compared against each other (e.g. normal condition versus disease)); and modular enrichment analysis (similar to the first mentioned, but focusing on network relationships) [607].

Enrichment analysis tools may differ in the annotation sources (Table IVa. 2). Most of them are based on GO annotations; however, enrichment can also be defined based on the interactors' participation in signaling pathways, presence of protein features (e.g., domains), gene expression profiles and association to specific conditions.

When a broader enrichment analysis is desired, users should select a tool capable of collecting from multiple annotation sources, such as the Database for Annotation, Visualization and Integrated Discovery (DAVID) [607,608]. In addition to the web service, DAVID developers also produced a Windows desktop software application aiming the biological interpretation of gene lists derived from proteomic, microarray and SAGE experiments, called Expression Analysis Systematic Explorer (EASE). Functional analysis with EASE is very similar to that performed by DAVID, but it offers a few more statistical models to deal with multi-test comparison problems [609].

Another relevant difference between the enrichment analysis tools concerns the distinct statistical methods employed to quantitatively measure the enrichment, including Fisher's exact, hypergeometric, binomial and chi-square tests [610]. Moreover, the enrichment analysis tools may vary regarding the kind of input, the organisms supported, the requirements for local installation (e.g., GOSim is available as an R package [611]) and the presentation of the results. Table IVa. 2 summarizes currently active tools that can be used to identify enriched annotations within a list of interactors. Additional information on these tools can be consulted in [607,612].

Table IVa. 2
Enrichment tools to analyze the list of interactors resultant from the yeast-two hybrid screening.

Tool name	Annotation	Statistical methods	Multiple test correction	References
ConceptGen (v1.0)	Multiple sources	Modified Fisher's Exact	Benjamini-Hochberg	[613]
DAVID (v6.8)	Multiple sources	Modified Fisher's Exact	Bonferroni; Benjamini	[607,614]
Enrichr	Multiple sources	Fisher's Exact; Modified Fisher's Exact (z-score); Combined score (log(p)*z)		[615,616]
FatiGO/FatiGO+ (v5.0)	Multiple sources	Fisher's Exact	Westfall and Young; Benjamini-Yekutieli; Benjamini-Hochberg	[617]
FuncAssociate (v3.0)	GO	Fisher's Exact	Simulation-based correction	[618]
FunRich (v3.0)	Multiple sources	Hypergeometric	Bonferroni; Benjamini-Hochberg	[619]
g:Profiler	Multiple sources	Hypergeometric	g:SCS (Set Counts and Sizes)	[620,621]
GARNET	Multiple sources	Cohen's kappa	Benjamini-Hochberg	[622]
GeneCodis (v3.0)	Multiple sources	Hypergeometric; Chi-square	Simulation-based correction; Benjamini-Hochberg	[623]
GeneFuncster	GO and KEGG	Fisher's Exact		[624]
GeneTrail2 (v1.5)	Multiple sources	Hypergeometric; Fisher's Exact	Bonferroni; Benjamini-Hochberg	[625]
GO-2D	GO	Hypergeometric; Binomial	Bonferroni; FDR	[626]
GO-Elite (v1.2.5)	Multiple sources	Hypergeometric; Fisher's Exact	Benjamini-Hochberg	[627]
GO::TermFinder	GO	Hypergeometric	Bonferroni; Simulation-based correction	[628]
GOEAST	GO	Hypergeometric	Benjamini–Yekutieli	[629]
GoMiner	GO	Fisher's Exact		[630]
GOrilla	GO	Minimum hypergeometric; Hypergeometric		[631]
GOStat	GO	Fisher's Exact; Chi-square	Holm; Benjamini-Hochberg	[632]
KOBAS (v3.0)	Multiple sources	Binomial test; Chi-square; Fisher's Exact; Hypergeometric	FDR	[633,634]
MamPhEA	MGI	Fisher's Exact	Bonferroni	[635]
ProfCom	GO	Greedy heuristics	Monte–Carlo simulation	[636]
ToppFun	Multiple sources	Hypergeometric	Bonferroni	[532]

Only currently active, web-based tools that support singular enrichment analysis of *Homo sapiens* were considered.

Cytoscape also offers several applications to do enrichment analysis, such as Biological Networks Gene Ontology tool (BiNGO) [637]—one of the most downloaded Cytoscape plugin [638]—Java Enrichment of Pathways Extended To TOpology (JEPETTO) [639], Enrichment Map [640,641] and ClueGO [642]. Usually, these tools depict the overrepresented terms sorted in with p-values, as well as the number and identification of the interactors in the network that annotate to each term.

5. Practical examples that illustrate the potentials of combining Y2H with bioinformatics approach in the study of human interactomes

In previous works, we identified amyloid precursor protein (APP) and t-complex testis expressed protein 1 domain containing 4 (TCTEX1D4) interactomes using the Y2H system [643,644]. In both cases, a human testis cDNA library was used as prey and a bioinformatics approach was used to analyze the results.

The analysis of the positive clones in APP Y2H allowed us to identify 37 interacting proteins in human testis. Simultaneously, interaction databases (e.g., APID, BioGRID, DIP, HPRD, Intact) and published interactomes were queried for APP. The interactors were classified in terms of their expression in testis and spermatozoa according to information on tissue expression databases (e.g., C-It, TiGER, BioGPS, HPA, HPRD, Unigene) and published proteomes. Proteins associated with male infertility phenotypes were obtained from MGI, OMIM, Phenopedia and UniProt databases. The list of APP interactors was also explored by querying DAVID and KEGG for gene ontologies and pathways, respectively. Local and extended PPI networks were created by integrating all data and analyzed according to their topological properties (e.g., degree, betweenness centrality, clustering coefficient). The APP interaction network allowed the recognition of proteins complexes and modules crucial for several biological functions, such as cell adhesion [643].

The TCTEX1D4 Y2H screening allowed us to identify 40 new interactors. To complement the list of interactors, we used interactions retrieved using PSICQUIC View – a total of 44 interactors were then analyzed in more detail: tissue expression information was obtained from Unigene; male fertility-associated phenotypes from MGI and Phenopedia; ontologies from GO; and pathways from KEGG. DAVID was used to find enriched classes within our list [644].

Several studies from other authors have been published in the last few years that also successfully illustrate the potentials of analyzing Y2H results using bioinformatics tools. In a recent study, the interactome of the pro and active forms of caspase-6 (CASP6) was identified by matting bait strains with prey strains containing ~17,000 human sequence-validated non-redundant cDNAs from the human ORFeome collection. After excluding potential false-positive interactions, the authors obtained a list of 87 interactors that were either found to bind to all the CASP6 baits tested or only to the active ones. Besides CASP6 itself, only one of the interactors was previously identified as a

CASP6 substrate – huntingtin (HTT). PANTHER and GO databases were used to search the interactors list for enrichment in functions and pathways, while OMIM allowed to identify disease-related proteins. Since the authors aimed to further understand the potential roles of CASP6 interactors in the pathogenesis of Huntington disease (HD), they used previously published HD mRNA profiles to assess their expression levels in human HD postmortem brain tissue and perform an enrichment analysis using PANTHER. They identified a significant number of interactors altered in HD human brain tissue and an overrepresentation the insulin/IGF pathway-protein kinase B signaling cascade, p53 and PI3 kinase pathways. Using additional analyses, authors could identify caudate nucleus-specific HD network comprising six dysregulated CASP6 interactors. Bioinformatics approaches were used as a first step in order to prioritize the identification and characterization of possible CASP6 substrates involved in the pathogenesis of HD [645].

6. Conclusion

The study of human protein interactomes is laborious, but also incredibly useful to understand the role of PPIs in regulating biological processes and how their deregulation leads to pathological conditions. The process of characterizing an interactors list resultant from an Y2H screening involves not only the collection of all known interactions between them, but also to add biological significance to the network created using, for example, functional annotations, expression data and information on association to phenotypes/diseases. This kind of approach is also valuable for authors who use other methods to obtain their interactome data (for example, for mining lists of interactors obtained from Co-IP/MS or even lists the result from the combination of different biochemical methods). However, a major take-home message about the pipeline here proposed is that no database or tool is sufficiently broad to give all the information needed to perform this study. Therefore, researchers working in interactomics fields should have a good knowledge on the tools available and the potentials of their combination. Also, for the success of this kind of studies, it is of utmost importance that researchers take good care when publishing their interactomes, being detailed in the description of the methods used to identify PPIs and meticulous when identifying the interactors. These efforts combined with an increased proximity between researchers and the groups that maintain the databases are imperative to the evolution of the interactomics field.

7. Supplementary data

Suppl. Table IVa. 1
Links for the websites of the bioinformatics resources mentioned in the text.

Type	Resource	URL
General	OMICtools	https://omictools.com
	Pathguide	http://www.pathguide.org
Local alignment	BLAST: Basic Local Alignment Search Tool	https://blast.ncbi.nlm.nih.gov/Blast.cgi
	EMBOSS Water: European Molecular Biology Open Software Suite	http://www.ebi.ac.uk/Tools/psa/emboss_water/
ID Converter	DAVID Gene ID Conversion	https://david.ncifcrf.gov/conversion.jsp
	UniProt Retrieve/ID mapping	http://www.uniprot.org/uploadlists/
	db2db: Database to Database Conversions	https://biodbnet-abcc.ncifcrf.gov/db/db2db.php
	ID mapping from the iProClass database	http://pir.georgetown.edu/pirwww/search/idmapping.shtml
	PICR: Protein Identifier Cross-Reference Service	http://www.ebi.ac.uk/Tools/picr/
Protein-protein interactions	APID: Agile Protein Interactomes DataServer	http://cicblade.dep.usal.es:8080/APID/init.action
	BioGrid: Biological General Repository for Interaction Datasets	https://thebiogrid.org
	DIP: Database of Interacting Proteins	http://dip.doe-mbi.ucla.edu/dip/
	HAPPI: Human Annotated and Predicted Protein Interactions	http://discern.uits.iu.edu:8340/HAPPI/
	HIPPIE: Human Integrated Protein-Protein Interaction rEference	http://cbdm-01.zdv.uni-mainz.de/~mschaefer/hippie/
	HPIDB: Host Pathogen Interaction Database	http://www.agbase.msstate.edu/hpi/main.html
	HPRD: Human Protein Reference Database	http://www.hprd.org
	I2D: Interologous Interaction Database (I2D)	http://ophid.utoronto.ca/ophidv2.204/
	IMEx: The International Molecular Exchange Consortium	http://www.imexconsortium.org
	InnateDB	http://www.innatedb.com
	Intact	http://www.ebi.ac.uk/intact/
	MatrixDB	http://matrixdb.univ-lyon1.fr
	MINT: Molecular Interactions Database[1]	http://mint.bio.uniroma2.it
	PDZBase	http://abc.med.cornell.edu/pdzbase
	PSICQUIC Registry	http://www.ebi.ac.uk/Tools/webservices/psicquic/registry/registry?action=STATUS
	PSICQUIC View	http://www.ebi.ac.uk/Tools/webservices/psicquic/view/main.xhtml
	STRING	http://string-db.org
	UCL-BHF group	http://www.ucl.ac.uk/functional-gene-annotation/cardiovascular
	UniHI: Unified Human Interactome	http://www.unihi.org
	UniProt: Universal Protein Resource[1]	http://www.uniprot.org
	VirHosNet: Virus-Host Network	http://virhostnet.prabi.fr
	Cytoscape	http://www.cytoscape.org

Type	Resource	URL
Network visualization	NAVIGaTOR	http://ophid.utoronto.ca/navigator/index.html
	Pajek	http://mrvar.fdv.uni-lj.si/pajek/
Functional annotation	GO: Gene Ontology Consortium	http://www.geneontology.org
	PANTHER: Protein ANalysis THrough Evolutionary Relationships	http://www.pantherdb.org
Signaling pathways	ACSN: Atlas of Cancer Signaling Network	https://acsn.curie.fr
	AlzPathway	http://alzpathway.org/AlzPathway.html
	ARN: Autophagy Regulatory Network	http://autophagy-regulation.org
	CARFMAP: cardiac fibroblasts pathway map	http://visionet.erc.monash.edu.au/CARFMAP/
	CPDB: ConsensusPathDB-human	http://cpdb.molgen.mpg.de/CPDB
	KEGG PATHWAY: Kyoto Encyclopedia of Genes and Genomes	http://www.genome.jp/kegg/pathway.html
	NetPath	http://netpath.org
	OmniPath	http://omnipathdb.org
	PANTHER PATHWAY	http://www.pantherdb.org
	Pathway Commons	http://www.pathwaycommons.org/pc2/
	Reactome	http://reactome.org
	SignaLink	http://signalink.org
	SIGNOR: SIGnaling Network Open Resource	http://signor.uniroma2.it
	SPIKE: Signaling Pathway Integrated Knowledge Engine	http://www.cs.tau.ac.il/~spike/
	WikiPathways	http://www.wikipathways.org/index.php/WikiPathways
Tissue expression	HPA: Human Protein Atlas	http://www.proteinatlas.org
	TiGER: Tissue-specific Gene Expression and Regulation	http://bioinfo.wilmer.jhu.edu/tiger/
	TISSUES	http://tissues.jensenlab.org
	C-it	http://c-it.mpi-bn.mpg.de
	BioGPS	http://biogps.org/#goto=welcome
	Expression Atlas	https://www.ebi.ac.uk/gxa/home
	PaGenBase: Pattern Gene Database	http://bioinf.xmu.edu.cn/PaGenBase/
	IID: Integrated Interactions Database	http://iid.ophid.utoronto.ca/SearchPPIs/protein/#results
Phenotypes/ diseases association	ClinVar	https://www.ncbi.nlm.nih.gov/clinvar/
	CTD: Comparative Toxicogenomics Database	http://ctdbase.org
	DISEASES	http://diseases.jensenlab.org/Search
	DisGeNET	http://www.disgenet.org/web/DisGeNET/menu
	Genopedia	https://phgkb.cdc.gov/HuGENavigator/startPagePedia.do
	GeneCards	www.genecards.org
	MalaCards	http://www.malacards.org
	MGI: Mouse Genome Institute	http://www.informatics.jax.org
	OMIM: Online Mendelian Inheritance in Man	https://www.omim.org
	PhenoDigm	http://www.sanger.ac.uk/resources/databases/phenodigm/
	Phenominer	http://phenominer.mml.cam.ac.uk

Type	Resource	URL
	PsyGeNet: Psychiatric disorders Gene association NETwork	http://www.psygenet.org/web/PsyGeNET/menu;jsessionid=1aygrbmauhirevi0vngi2vx0r
	RGD: Rat Genome Database	http://rgd.mcw.edu/wg/home
Enrichment analysis	ConceptGen	http://conceptgen.ncibi.org
	DAVID: Database for Annotation, Visualization and Integrated Discovery	https://david.ncifcrf.gov
	Enrichr	http://amp.pharm.mssm.edu/Enrichr
	FatiGO/FatiGO+	http://www.babelomics.org
	FuncAssociate	http://llama.mshri.on.ca/funcassociate
	FunRich	http://www.funrich.org
	g:Profiler	http://biit.cs.ut.ee/gprofiler
	GARNET: Gene Annotation Relationship Network Tools	http://biome.ewha.ac.kr:8080/GSEAWebApp
	GeneCodis	http://genecodis.cnb.csic.es
	GeneFuncster	http://bioinfo.utu.fi/GeneFuncster
	GeneTrail	https://genetrail2.bioinf.uni-sb.de
	GO-2D	http://www.hrbmu.edu.cn/go-2d/index.htm
	GO-Elite	http://www.genmapp.org/go_elite/
	GO::TermFinder	http://search.cpan.org/dist/GO-TermFinder/
	GOEAST: Gene Ontology Enrichment Analysis Software Toolkit	http://omicslab.genetics.ac.cn/GOEAST/
	GoMiner	https://discover.nci.nih.gov/gominer/index.jsp
	Gorilla	http://cbl-gorilla.cs.technion.ac.il
	GOStat	http://gostat.wehi.edu.au
	KOBAS	http://kobas.cbi.pku.edu.cn
	MamPhEA: Mammalian Phenotype Enrichment Analysis	http://evol.nhri.org.tw/phenome/index.jsp?platform=mmus
	ProfCom: Profiling of Complex Functionality	http://webclu.bio.wzw.tum.de/profcom/
	ToppFun	https://toppgene.cchmc.org/enrichment.jsp

Section b

High-throughput analysis of the PP-1G interactome in human prostate cancer

KEYWORDS

Yeast two-hybrid
Co-immunoprecipitation
Mass spectrometry
Interactomics
Bioinformatics

ABSTRACT

Protein phosphatase 1 (PP1) is a complex holoenzyme that catalyzes dephosphorylation reactions in several proteins. Its efficiency depends on the association between the catalytic subunit and one or two regulatory subunits, which give specificity to the catalytic machinery. In contrast to the catalytic subunit, which is not believed to exist freely inside cells, regulatory subunits are proteins that usually have PP1-independent cellular roles. From regulatory subunits to substrates, hundreds of interactors have been identified throughout the years. With these findings, the hypothesis of highly dynamic PP1 interactomes, varying with cell types, cellular context and affinity to specific PP1 catalytic isoforms, has grown bigger. The study of PP1 interactomes has been covering several human tissues and pathological conditions, but not cancer. In this study, we identified and characterized the PP-1G interactome in human prostate cancer (PCa) using a combination of high-throughput techniques with comprehensive bioinformatics analysis. We used genomic and clinical data from large, publicly-available PCa cohorts to construct a differential protein-protein interaction network and identify interesting interactors with promising clinical application, as CCT2, YWHAG and CSRP1. Future studies to assess their role in prostate carcinogenesis would be of utmost importance.

Abbreviations*:* AP-MS, affinity-purification mass spectrometry; AR, androgen receptor; co-IP, co immunoprecipitation; DBD, DNA-binding domain; DDO, double dropout medium; FDR, false discovery rate; F.E., fold enrichment; GO, Gene Ontology; HIPPIE, Human Integrated Protein-Protein Interaction rEference; iMEX, The International Molecular Exchange Consortium; IP, immunoprecipitation; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LD-PCR, long distance PCR; Leu, leucine; PCa, prostate cancer; PFL, The Protein Frequency Library; PP1, protein phosphatase 1; QDO, quadruple dropout medium; RIPPO, regulatory interactors of PP1; SD, single dropout medium; TCGA, The Cancer Genome Atlas; TDO, triple dropout medium; Trp, tryptophan; Y2H, yeast two-hybrid.

1. Introduction

Protein phosphatase 1 (PP1) is a ubiquitous serine/threonine phosphatase involved in several cellular processes. Its multitude of roles is achieved through association with a broad spectrum of regulatory proteins, known as regulatory interactors of PP1 (RIPPOs), which bind to PP1 catalytic subunit (PP1c) with different affinities. These interactions create proficient holoenzymes in which PP1c is highly efficient in catalyzing dephosphorylation reactions and RIPPOs regulate PP1c activation state, provide specificity towards substrates and/or guidance throughout cellular compartments [175].

PP1 has been implicated in carcinogenesis, although conflicting results have been reported with studies showing both tumor-promoting and tumor-suppressing roles [416]. In fact, understanding PP1 function is challenging. PP1c is encoded by three distinct genes, giving rise to three canonical isoforms: PP-1A, PP-1B and PP-1G. Alternative splicing events originate additional isoforms, but with exception of gamma-2 (also known as PP1γ2), which is testis- and sperm-enriched, their biological relevance is unknown. Several RIPPOs are known to interact with all PP1c isoforms, whereas some isoform-specificity has also been suggested [171]. Also, between RIPPOs and substrates, PP1c is reported to interact with hundreds of proteins [175]. Therefore, the study of PP1 roles should focus not only on PP1c isoforms, but also their interactomes in specific cellular contexts.

Previous studies have uncovered PP1 interactomes in a variety of species and tissues, including *Saccharomyces cerevisiae* [646], *Drosophila melanogaster* [647], *Plasmodium falciparum* [648], mouse testis [649] and embryonic stem cells [650], rat brain [651], and human tissues using high-throughput techniques, such as yeast two-hybrid (Y2H) screenings and affinity-purification mass spectrometry (AP-MS). Y2H screenings were used to characterize the PP-1A interactome in human brain [473] and heart [652], and PP-1G (and its spliced isoform gamma-2) interactomes in human brain [653] and testis [474]. Additionally, PP1 gamma-2 was co-immunoprecipitated from human sperm samples using an isoform-specific antibody and its binding partners were identified by mass spectrometry (MS) [474]. A few studies have also unveiled the remodeling of PP1c interactome in pathological conditions, as paroxysmal atrial fibrillation [654] and heart failure [655]. In addition to improve the knowledge on the molecular events underlying pathophysiological conditions, the characterization of PP1c interactomes might highlight potential therapeutic targets.

PP1 is a positive regulator of the androgen receptor (AR) expression and transcriptional activity, which suggests it might play a central role in prostate cancer (PCa) development and progression. However, the function of PP1 in PCa has been poorly addressed. In addition to AR, few interactions have been characterized in PCa models [656]. Given that PP1c is known to interact with hundreds of proteins, including oncoproteins and tumor suppressors [180], it is most probable that PP1 interactome in PCa remains largely unknown.

Here, we identified and analyzed the PCa-associated PP-1G interactome by combining Y2H, MS and comprehensive bioinformatics analysis. As a result, we constructed a complex protein-protein interaction network, and we were able to identify a particularly relevant subnetwork for PCa. By integrating data from a large publicly available PCa cohort, we found that 60% of the PP-1G interactome was differentially expressed between primary prostate tumor and normal prostate tissue. In addition, we showed that the transcript levels of CCT2, YWHAG and CSRP1 were associated with poor prognosis. Altogether, these results contribute to improve the knowledge on the role of PP1 in PCa and pinpoints promising interacting partners to investigate in future studies.

2. Materials and methods

This study combined biochemical and bioinformatics approaches as represented in Fig. IVb. 1 (based on the approach discussed in Chapter IVa [657]).

2.1. Human samples

Prostate tissues were collected after PCa diagnosis and primary treatment with radical prostatectomy at the Portuguese Oncology Institute of Porto (IPO Porto, Portugal). Tissue collection and histopathological evaluation was performed according to institutional guidelines as previously described [425]. For construction of cDNA libraries, fresh-frozen tissue fragments from prostate tumor and adjacent benign areas from five patients were pooled per group (tumor vs. benign). For co-immunoprecipitation (co-IP) experiments, freshly-collected tissue specimens from tumoral and morphologically normal areas from three PCa patients were selected by an Uropathology-dedicated Pathologist and processed immediately upon surgery. Clinicopathological data of the PCa patients can be found in Table IVb. 1. The study received favorable opinion from the ethics committee (Comissão de Ética para a Saúde do Instituto Português de Oncologia do Porto, CES-IPOFG_EPE 019/08) and all participants provided their written informed consent.

Table IVb. 1
Clinicopathological data of the PCa patients.

	Age	Pathological stage	Gleason score	PSA (ng/mL)
cDNA library (n=5)				
1	69	pT3a	7	> 4
2	66	pT3a	7	14.14
3	52	pT3a	7	10
4	67	pT3a	7	4.7
5	61	pT3a	7	7.3
Co-immunoprecipitation (n=3)				
1	57	pT2	8	8.18
2	62	cT2	7	12.53
3	61	pT3a	7	6.94

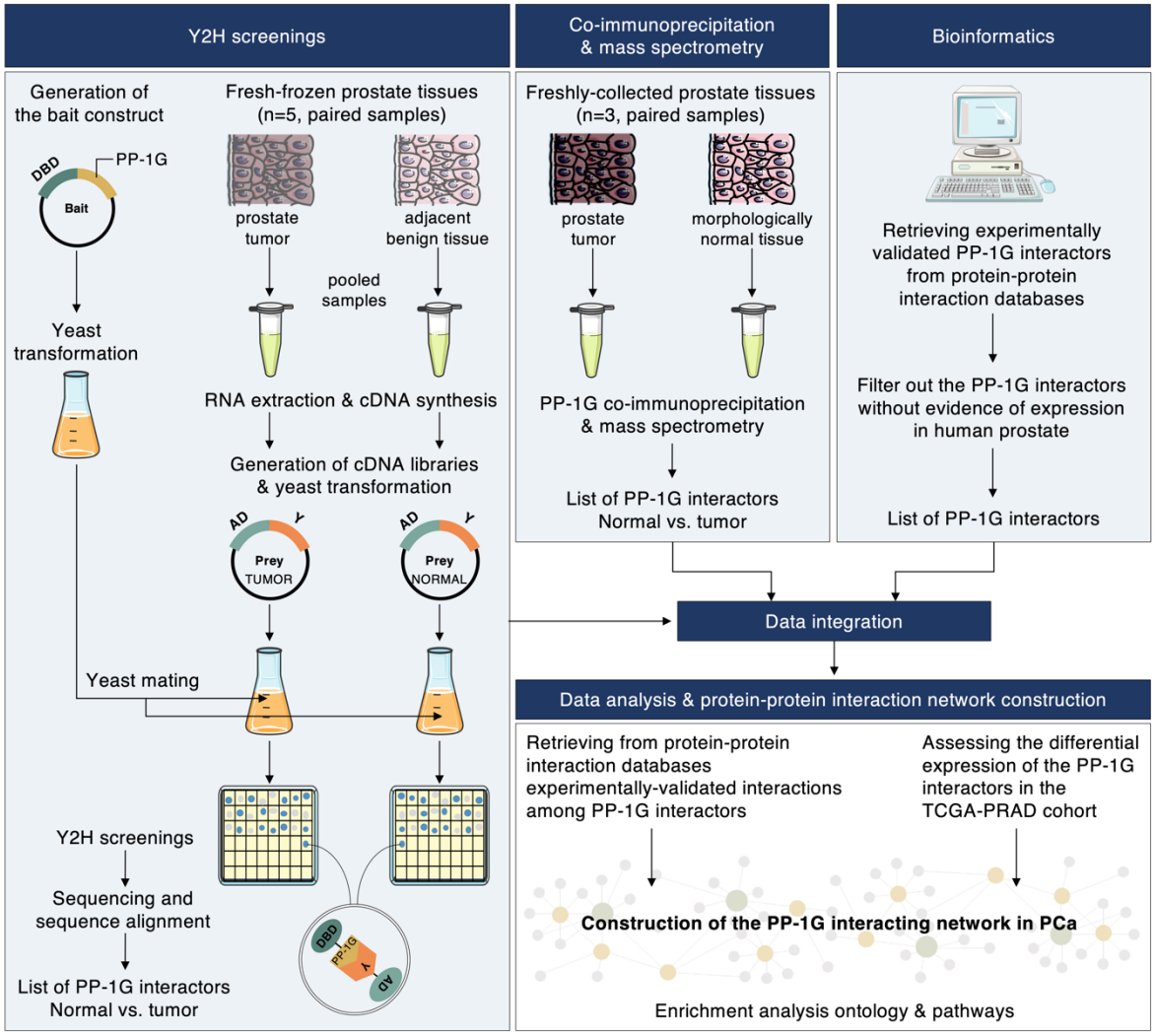


Fig. IVb. 1
Schematic representation of the methodology. Figures were produced using Servier Medical Art from www.servier.com and adapted from [657].

2.2. Yeast two-hybrid

2.2.1. Yeast strains and plasmids

pGBKT7 (containing the GAL4 DNA binding domain, DBD) and pGADT7 (containing the GAL4 activation domain, AD) were used as bait and prey vectors, respectively. pGBKT7-53 (which encodes the GAL4-DBD fused to the murine p53), pGBKT7-Lam (which encodes the GAL4-DBD fused to laminin C) and pGADT7-T (which encodes the GAL4-DBD fused to SV40 large T-antigen) were used in control conditions: the bait vector pGBKT7-53 and the prey vector pGADT7-T served as positive interaction control pair, while the bait vector pGBKT7-Lam and the prey vector pGADT7-T served as negative interaction control pair.

Bait vectors were transformed into Y2Hgold yeast cells [genotype: MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2 : : GAL1UAS–Gal1TATA–His3, GAL2UAS–Gal2TATA–

Ade2URA3 : : MEL1UAS–Mel1TATAAUR1-C MEL1], whereas prey vectors were transformed into Y187 yeast cells (genotype: MATα, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4Δ, gal80Δ, met–, URA3 : : GAL1UAS–Gal1TATA–LacZ MEL1). Yeast strains and media, Y2H vectors and all reagents necessary to the screenings were acquired from Clontech, USA, unless otherwise indicated.

2.2.2. Construction and characterization of PP-1G recombinant bait plasmid

pGBKT7 and pAS2-1/PP-1G were digested with SalI (New England BioLabs, USA) and XmaI (New England BioLabs, USA) restriction endonucleases. Linearized pGBKT7 and human PP-1G cDNA fragment were recovered from agarose gel using the NucleoSpin® Gel and PCR Clean-Up kit (Macherey-Nagel, Germany), according to the manufacturer's instructions, and used for ligation reaction. The recombinant plasmid and adequate controls were transformed into XL1 Supercompetent Cells (Stratagene, USA) and incubated overnight at 37 ºC. To check the cloning process, 10 colonies were randomly selected for purification, digested with XmaI endonuclease and analyzed in a 0.7% TAE agarose/GreenSafe gel alongside pGBKT7 vector and linearized pGBKT7 vector. Proper reading frame was validated by sequencing using the GAL4-DBD primer (5' TCATCGGAAGAGAGTAG 3') by the Eurofins Sequencing Services (France). pGBKT7/PP-1G bait plasmid was transformed into competent Y2Hgold yeast cells using the Yeastmaker™ Yeast Transformation System 2 (Clontech, USA), according to the manufacturer's instructions.

Bait was tested for auto-activation and toxicity in yeast cells. Also, bait protein expression was confirmed by preparing yeast cell extracts in cracking buffer following the urea/SDS method [658]. The expression of PP-1G bait protein was then assessed by western blot using an in-house produced anti-PP-1G-specific antibody [428].

2.2.3. Extraction of total RNA and cDNA synthesis

RNA extraction from pooled samples (~50 mg) was performed using the NucleoSpin® RNAII kit (Macherey-Nagel, Germany), following the manufacturer's instructions. Total RNA was eluted in RNase-free water and concentrated using the RNA Clean & Concentrator™ kit (Zymo Research, USA). RNA concentration and purity were determined with the DeNovix DS-11 Spectrophotometer (DeNovix, USA). First-strand cDNA was synthesized from 1.0-1.5 of µg total RNA with an oligo-dT primer by means of the SMART® cDNA synthesis technology (Clontech, USA), following the manufacturer's protocol. cDNA synthesis reactions carried out with or without 1 µg of mouse liver Poly A+ RNA was used as positive and negative controls, respectively. cDNA was then amplified by long distance PCR (LD-PCR) using the Advantage 2 Polymerase Mix (Clontech, USA) with the following thermal cycling parameters: (1) 95 ºC/30 s; (2) 95 ºC/10 s, 68 ºC/6 min with an increase of the extension time by 5 s with each successive cycle (26 cycles); (3) 68 ºC/5 min. Double-stranded

cDNA (7 µL) was analyzed on a 1.2% TAE agarose/GreenSafe gel alongside 0.25 µg of GeneRuler™ 1kb DNA Ladder (Fermentas Life Sciences, USA). The remaining double-stranded cDNA was purified with a CHROMA SPIN TE-400 column (Clontech, USA) and stored at -20 ºC until used.

2.2.4. Construction of cDNA libraries

Competent Y187 yeast cells were prepared using the Yeastmaker™ Yeast Transformation System 2 (Clontech, USA), according to the manufacturer's instructions. About 2 µg of double-stranded cDNA and 3 µg of SmaI-linearized pGADT7-Rec were co-transformed into competent Y187 yeast cells following the library-scale transformation protocol to construct the two libraries—prostate tumor (T) and adjacent benign tissue (N). cDNA libraries were created using the Make Your Own "Mate & Plate™" Library System (Clontech, USA), according to the manufacturer's instructions. To determine the number of independent clones in the libraries, 1/10 and 1/100 dilutions of transformed cells were spread onto single dropout medium lacking leucine (SD_{-Leu}) agar plates and the number of colonies was counted after incubation for 4 days at 30°C. Cell density was estimated using a hemocytometer and 1 mL aliquots of each library were stored at -80 ºC until used.

2.2.5. Yeast two-hybrid screenings

A concentrated Y2Hgold[pGBKT7/PP-1G] culture was combined with 1 mL aliquot of either Y187[pGADT7-Rec-T] or Y187[pGADT7-Rec-N] yeast two-hybrid libraries for mating according to the protocol described in the Matchmaker™ Gold Yeast Two-Hybrid User Manual (Clontech, USA). To calculate the titer of the libraries, 10 µL of each library was set aside and 1/100, 1/1,000 and 1/10,000 dilutions of each library were previously spread onto SD_{-Leu} agar plates to count the colonies after 5 days of incubation at 30 ºC.

Zygote formation after mating was monitored by phase contrast microscopy (40X). From the mated cultures, 1/10, 1/100, 1/1,000 and 1/10,000 dilutions were spread onto SD_{-Leu}, single dropout medium lacking tryptophan (SD_{-Trp}) and double dropout medium lacking both tryptophan and leucine (DDO) and incubated at 30 ºC for 5 days to measure viabilities, calculate the number of screened clones and determine mating efficiencies. The remaining culture solution was spread onto DDO/X/A (DDO supplemented with 40 µg/mL X-α-Gal (X) and 200 ng/mL Aureobasidin A (A)) and incubated at 30 ºC for 5 days. All blue colonies that appeared on DDO/X/A plates were then patched out and allowed to grow in higher stringency conditions: TDO/X/A (triple dropout medium lacking histidine, tryptophan and leucine and supplemented with 40 µg/mL X-α-Gal and 200 ng/mL Aureobasidin A) and QDO/X/A (quadruple dropout medium lacking adenine, histidine, tryptophan and leucine and supplemented with 40 µg/mL X-α-Gal and 200 ng/mL Aureobasidin A) plates.

2.2.6. Identification of positive clones

Prey library inserts from positive clones growing on QDO/X/A were amplified using the Matchmaker Insert Check PCR Mix 2 (Clontech, USA) with the following thermal cycling parameters: (1) 94 ºC 1 min; (2) 98 ºC 10 s (30 cycles); (3) 68 ºC 3 min. PCR products were analyzed on 1% TAE agarose/GreenSafe gel and then purified using the NucleoSpin® Gel and PCR Clean-Up kit (Macherey-Nagel, Germany) according to the manufacturer's instructions. Spin column-purified PCR products (20-80 ng/μL) were sequenced using the T7 promoter primer (5' TAATACGACTCACTATAGGG 3') (Thermo Fisher Scientific, USA) by the LightRun Sequencing Services (Germany). Individual sequences from good quality chromatograms were aligned using the Clustal Omega program for multiple sequence alignment to check for similarities (https://www.ebi.ac.uk/Tools/msa/clustalo/) [659]. Nonredundant clones' sequences were then searched for similarities against the human genome (Genome Reference Consortium Human Build 38 patch release 13, GRCh38.p13) through BLASTN or BLAT analyses in the Ensembl server (release 101, Aug 2020, http://www.ensembl.org/index.html) [660]. The search included known human genomic sequences, cDNAs (transcripts/splice variants) and non-coding RNA genes.

2.3. Co-immunoprecipitation and mass spectrometry

Dissected tissue specimens were incubated with a 1% collagenase type IV (Life Technologies, USA) solution for 1 h with rotation at 37 ºC and cross-linked with 1 mM dithiobis[succinimidylpropionate] (Thermo Scientific™ by Thermo Fisher Scientific, USA) for 30 min with rotation at room temperature according to the manufacturer's instructions. Protein extraction was performed by homogenizing thoroughly the samples in NP-40 lysis buffer with protease and phosphatase inhibitors using a Teflon pestle, followed by incubation overnight with constant agitation at 4 ºC. Samples were centrifuged at 4,500 ×g for 20 min at 4 ºC and total protein content was quantified in the supernatant by BCA assay (Thermo Scientific™ by Thermo Fisher Scientific, USA). A direct immunoprecipitation approach was followed using Dynabeads™ Protein G (Invitrogen™ by Thermo Fisher Scientific, USA) according to the manufacturer's instructions. Target antigen immunoprecipitation was performed by incubating the pre-cleared fraction (at least 3 mg of protein per assay) with 5 μg of either anti-PP-1G antibody (in-house production [428]) or rabbit IgG for control. After gentle washing steps, the immunoprecipitated complexes were resuspended in trypsin digestion buffer and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) at the VIB Proteomics Core (VIB Center for Medical Biotechnology, Ghent University, Belgium). To confirm the immunoprecipitation procedure, 10% of the final volume of the eluates was analyzed by western blot using the anti-PP-1G antibody.

2.3.1. Analysis of the mass spectrometry results

LC-MS/MS runs of all 6 samples were searched together using the MaxQuant algorithm (v1.6.3.4.) with default search settings, including a false discovery rate (FDR) set at 1% at both peptide and protein levels. Spectra were searched against human proteins in the UniProt database (UniProtKB/Swiss-Prot UniProt release v2019_01, https://www.uniprot.org) [168].

Principal component analysis of the expression matrix was applied to analyze sample clustering. The raw list of proteins was filtered to keep the proteins consistently quantified in at least one condition for further analyses (i.e., proteins detected in at least two samples from a given condition). Also, proteins that appeared only in IgG controls were filtered out. The resultant list was subjected to two statistical approaches: (1) pairwise comparisons between IP and IgG control conditions using the limma statistical package in paired mode; (2) two-way ANOVA. Statistical significance was set at 0.05 (i.e., p-value < 0.05 or -log10 p-value > 1.301).

2.4. Bioinformatics analysis

2.4.1. Protein-protein interaction network

Gene and protein nomenclatures were retrieved from the UniProt Knowledgebase (UniProtKB), UniProt, release 2020_04 (https://www.uniprot.org) [168]. Experimentally validated PP-1G interactors were retrieved from publicly available protein-protein interaction databases, including the Human Integrated Protein-Protein Interaction rEference (HIPPIE) database [661], v2.2 (release Feb 14, 2019), and The International Molecular Exchange Consortium (iMEX) [662]. To retrieve the network data from HIPPIE, interaction's confidence score was set to 0.63. The network from iMEX consortium was directly imported to the Cytoscape software, v3.8.1 (release Sep 24, 2020) [663], through PSICQUIC Services (as indicated in the tutorial in Chapter IVa [657]). Networks merging and analysis of characteristic properties were performed using Cytoscape. MCODE plugin of Cytoscape, v1.6.1, was used to identify functionally-related modules [664].

2.4.2. Enrichment analysis

Enrichment analysis was performed through the Gene Ontology (GO) knowledgebase (release Sep 10, 2020), powered by PANTHER Classification System, v15.0., to search for biological processes, molecular functions and cellular components. The analysis used the PANTHER Overrepresentation Test (release 20200728) with the annotation version DOI:10.5281/zenodo.3980761 or the Reactome version 65, released 2020-11-17, for Reactome Pathways. The queried list was searched against the *Homo sapiens* reference list (all genes in database) to determine the fold enrichment (F.E.) and results' significance was assessed using the Fisher's Exact test with Bonferroni correction for multiple testing. Significance level was set to 0.05.

2.4.3. Expression data

Human prostate proteome was retrieved from The Human Protein Atlas, v19.3 (release Mar 6, 2020, freely available at https://www.proteinatlas.org/humanproteome/tissue/prostate). Transcript levels in normal prostate tissue (n=52) and primary tumor (n=497) were analyzed in The Cancer Genome Atlas Prostate Adenocarcinoma (TCGA-PRAD) cohort, via UALCAN web resource (http://ualcan.path.uab.edu/index.html) [431]. Statistical significance was assumed when $p<0.01$ to identify differentially expressed genes between the two condition. Transcripts with extremely low values of transcripts per million (TPM) were classified as 'Not Applicable' (NA).

3. Results

3.1. Bait sub-cloning and testing

PP-1G cDNA was removed from the pAS2-1/PP-1G construct by restriction digestion with XmaI/SalI and directionally cloned into XmaI/SalI-digested pGBKT7 vector. A unique fragment of approximately 8,400 bp was observed on the 0.7% TAE agarose/GreenSafe gel as expected, due to the shift of the incorporation of the PP-1G fragment (~1,120 bp) in the pGBKT7 vector (~7,300 bp) (Fig. IVb. 2A). Two colonies (numbers 2 and 3 in Fig. IVb. 2A) were subsequently processed for sequencing, which confirmed that the PP-1G cDNA was cloned in-frame with the GAL4-DBD of the pGBKT7 bait plasmid (Fig. IVb. 2B).

Prior to the Y2H screenings, the bait was tested for autoactivation and toxicity. The bait was found as not toxic, with the Y2HGold[pGBKT7/PP-1G] colonies growing in SD_{-Trp} agar plates being similar, in both number and size, to the Y2HGold[pGBKT7] control colonies (Fig. IVb. 2C). Also, the bait was not capable of autoactivation, since distinct pale pink colonies appeared in SD_{-Trp} and $SD_{-Trp/X}$ agar plates and no colonies were observed in $SD_{-Trp/X/A}$ agar plates (in none of the dilutions tested), whereas diploid positive controls showed brilliant blue colonies (Fig. IVb. 2C). Bait protein expression in pGBKT7/PP-1G-transformed Y2HGold yeast cells was analyzed by western blot using a anti-PP-1G antibody. A band of the expected molecular weight confirmed the expression of the PP-1G/GAL4-DBD fusion protein in Y2HGold[pGBKT7/PP-1G]-transformed cells (Fig. IVb. 2D).

3.2. Construction and assessment of the cDNA libraries

Total RNA was obtained from human prostate tumor biopsies and adjacent benign tissue. An average amount of 730 ng and 530 ng of total RNA was used to generate the prostate normal (N) and tumoral (T) cDNA libraries, respectively. After LD-PCR amplification, a continuous smear ranging from 200 to 1,500 bp was observed on agarose gel for both conditions (Fig. IVb. 3A). The amount of double-stranded cDNA obtained for each condition after column purification with CHROMA SPIN TE-400 columns was 116 ng/μL (T) and 122 ng/μL (N). cDNA libraries were produced directly

in yeast, via *in vivo* recombination between the purified double-stranded cDNA (~2 µg) and the prey vector pGADT7-Rec (SmaI-linearized). After 5 days of incubation at 30 ºC, the number of independent clones assessed on SD_{-Leu} agar plates was $2.4x10^6$ and $1.5x10^5$ for T and N cDNA libraries, respectively. Final cell density was $2.28x10^8$ cells/mL and $2.45x10^8$ cells/mL for T and N, respectively.

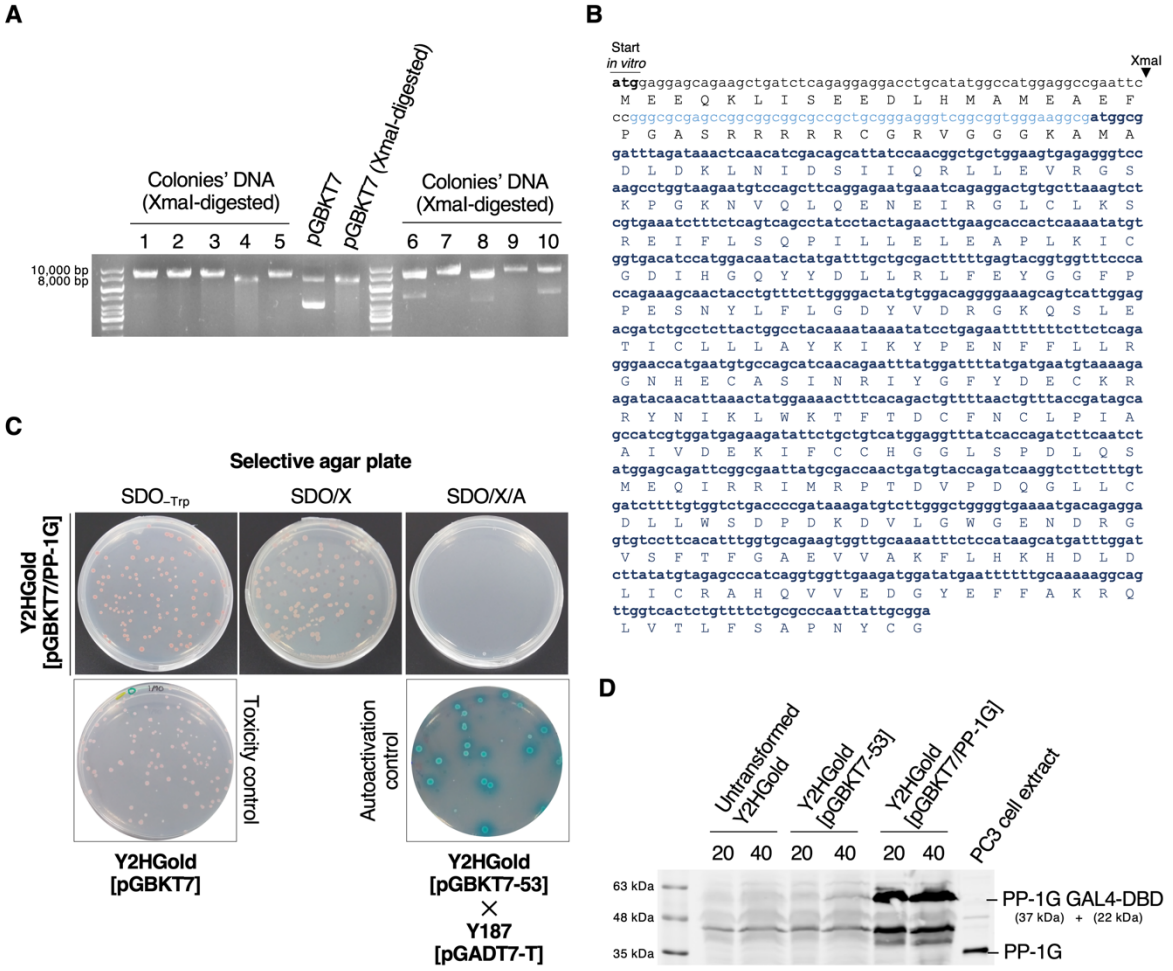


Fig. IVb. 2

Bait construction and testing. PP-1G cDNA was removed from the pAS2-1/PP-1G construct by restriction digestion with XmaI/SalI enzymes and directionally cloned into the XmaI/SalI-digested pGBKT7 vector. The recombinant plasmid was transformed into XL1 Supercompetent Cells overnight at 37 ºC. **(A)** Randomly selected colonies (1-10) were purified, digested with XmaI endonuclease and analyzed in a 0.7% TAE agarose/GreenSafe gel alongside pGBKT7 vector and linearized pGBKT7 vector to check for the incorporation of the PP-1G fragment in the bait vector. **(B)** Partial nucleotide and amino acid sequences of the pGBKT7/PP-1G recombinant plasmid (corresponding to colony 2 in A). Letters in black are part of the vector sequence; light blue corresponds to a non-coding sequence in the human PP-1G; and darker blue represents the partial PP-1G sequence collected from the chromatogram. **(C)** pGBKT7/PP-1G bait construct was assessed for autoactivation and toxicity in Y2HGold yeast strain. Y2HGold[pGBKT7] was used as control for toxicity and the mating between Y2HGold[pGBKT7 53] and Y187[pGADT7-T], which produces positive clones, was used as control for autoactivation. **(D)** Bait protein expression in yeast cells was analyzed by western blot using an in-house produced anti-PP-1G antibody.

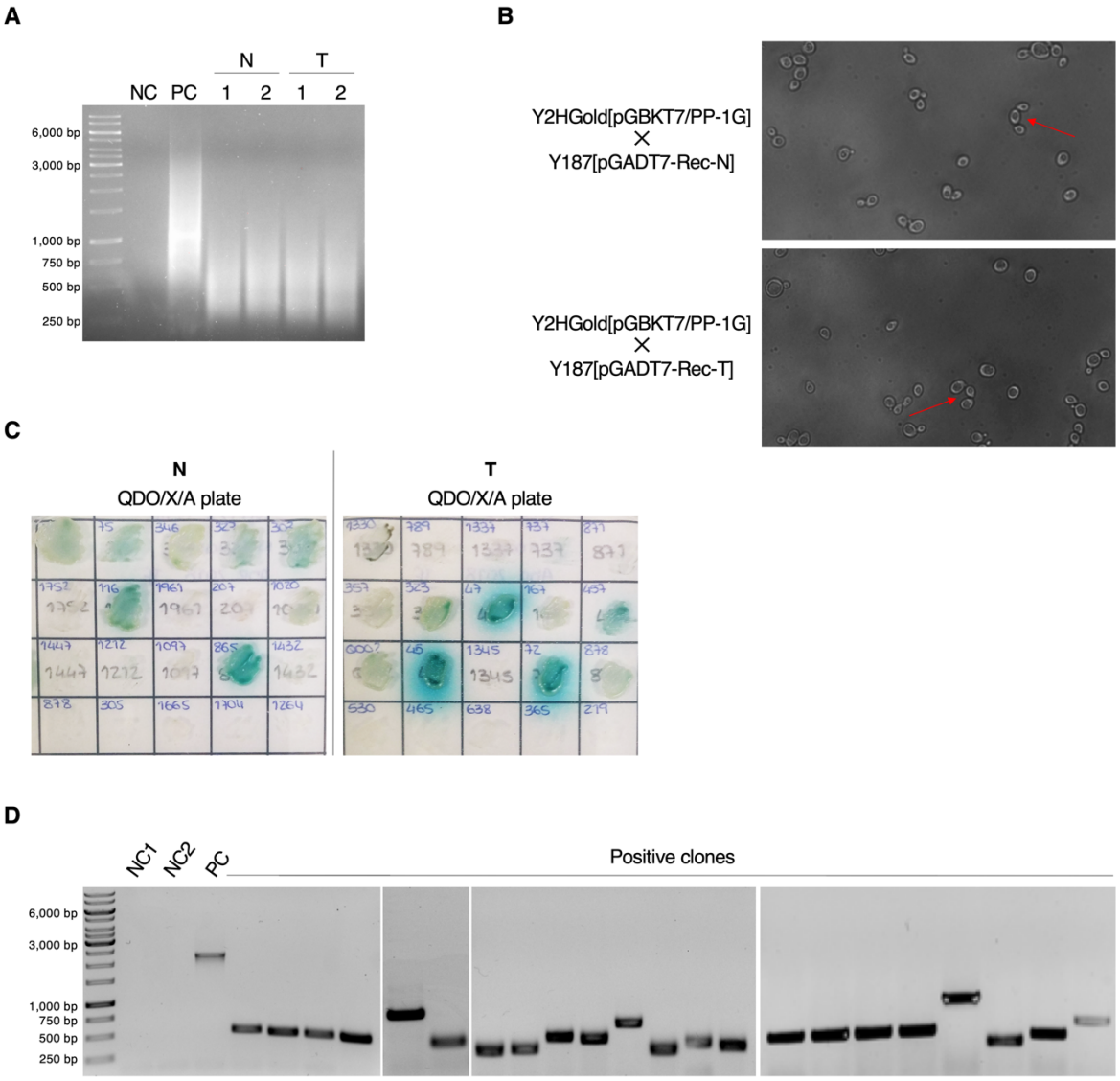


Fig. IVb. 3

cDNA libraries construction and Y2H screenings. (A) cDNA libraries were prepared from paired normal (N) and tumoral (T) pooled tissues. After long distance PCR amplification, a continuous smear ranging from 200 to 1,500 bp was observed on agarose gel for both conditions. Mouse liver Poly A+ RNA was used as positive control. **(B)** Bait and prey yeast strains were allowed to mate, and zygote formation (red arrows) was confirmed by microscopy (representative images; magnification 400×). **(C)** The mating products were spread onto dropout medium plates with increasing stringency conditions, with the highest being QDO/X/A, to sort out the positive (blue) clones (representative images). **(D)** Prey inserts from positive clones were amplified and analyzed on agarose gel to check for integrity and sent for sequencing (representative images). Abbreviations: NC, negative control; PC, positive control.

3.3. Analysis of the PP-1G interactome by Y2H screenings

After confirming zygotes formation (Fig. IVb. 3B), the mating products were spread on DDO/X/A agar plates for incubation at 30 °C. Blue yeast colonies were patched out onto higher stringency conditions (TDO/X/A and QDO/X/A). After restreaking each potentially positive clone to enhance the segregation of positive interactors, 21 and 30 clones were recovered from T and N screenings, respectively (Fig. IVb. 3C). Prey inserts were amplified and assessed in an agarose gel

before being sent for sequencing (Fig. IVb. 3D). Sequences from good quality chromatograms with more than 100 bp of insert length (upon vector trimming) were aligned to assess for redundancy. Only one of the clones was amplified from more than one colony (Table IVb. 2). Also, except for one of the sequences, all encompassed a polyadenylate tail (data not shown).

Nonredundant sequences were then searched against the human genome to identify the potential interactors. Each sequence produced several results, some with very similar scores, which hindered the task of attributing an interactor to each sequence. Also, most of the highest-scoring alignments corresponded to non-coding regions or clones (Table IVb. 2). Hence, we considered NOP10, NLRX1, NUP188, PRKN, ZNF793 and MYH9 for the bioinformatics analysis (section 3.6). From the highlighted sequences, only MYH9 is a known PP-1G interactor and PRKN has an RVxF-type PP1-docking motif.

Table IVb. 2
Highest-scoring alignments for positive clones obtained in the Y2H screenings.

ID	N[a]	Match	E-value	Coverage (%)	Identity (%)	Accession
Normal prostate cDNA library						
C1N	1	Homo sapiens chromosome 17 clone VMRC59-312C04, complete sequence	3e-160	100	89.67	AC279067.1
C2N	5	Homo sapiens NOP10 ribonucleoprotein (NOP10), RefSeqGene (LRG_345) on chromosome 15[*]	3e-91	94	100	NG_011562.1
C3N	1	Homo sapiens 3 BAC RP11-572M11 (Roswell Park Cancer Institute Human BAC Library) complete sequence	3e-144	83	97.99	AC078785.22
C4N	1	Human DNA sequence from clone RP11-22P2 on chromosome Xq23-24, complete sequence	0.0	98	99.68	AL590376.7
C5N	1	JP 2013138686-A/8: Genetic polymorphism associated with coronary event and drug response, method of detection and use thereof	0.0	95	100	HW593821.1
C6N	1	Homo sapiens NLR family member X1 (NLRX1), RefSeqGene on chromosome 11[*]	6e-70	58	100	NG_047185.1
C7N	1	Homo sapiens chromosome 7 clone RP11-344L16, complete sequence	5e-141	100	97.96	AC018646.3
		Homo sapiens clone RPC11-98D12 from 7q31, complete sequence	5e-141	100	97.96	AC008154.6
Prostate tumor cDNA library						
C1T	1	Homo sapiens nucleoporin 188 (NUP188), RefSeqGene on chromosome 9[*]	3e-97	100	94.47	NG_033111.1
C2T	1	Homo sapiens parkin RBR E3 ubiquitin protein ligase (PRKN), RefSeqGene on chromosome 6[*,b]	1e-81	100	93.69	NG_008289.2
C3T	1	Homo sapiens chromosome 10 clone RP11-45D20, complete sequence	2e-99	100	98.56	AC026395.11
		JP 2009519002-A/334 “Novel therapeutic targets in cancer” (patent sequence)	1e-99	100	98.56	DM254082.1
C4T	1	Homo sapiens zinc finger protein 793 (ZNF793), mRNA	2e-59	97	92.12	NM_001013659.3
C5T	1	Homo sapiens myosin heavy chain 9 (MYH9), RefSeqGene (LRG_567) on chromosome 22[*,b]	1e-102	100	90.53	NG_011884.2

ID	N[a]	Match	E-value	Coverage (%)	Identity (%)	Accession
C6T	1	Homo sapiens X BAC RP11-86A5 (Roswell Park Cancer Institute Human BAC Library) complete sequence	1-54	98	96.27	AC121342.6

Alignments were performed by BLAST analysis through the National Library of Medicine website (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Nucleotide collection (nr/nt), expressed sequence tags (est) and patent sequences (pat) databases were searched using the algorithm optimized for highly similar sequences (megablast). * Non-coding region. [a] Number of colonies. [b] Has PP1-docking motif(s).

3.4. Analysis of the PP-1G interactome by co-IP/MS

To enhance the coverage of the PP-1G interactome, co-IP/MS was selected as a complementary approach. After radical prostatectomy, the prostate glands from three patients were carefully evaluated by an Uropathology-dedicated Pathologist to select representative samples from tumoral and non-tumoral regions. Samples were immediately processed and cross-linked to avoid degradation and loss of protein-protein interactions. After pre-clearance, each sample was split into two: PP-1G was immunoprecipitated in half of the sample, while the remaining half was incubated with rabbit IgG to be used as control. PP-1G was successfully immunoprecipitated from prostate tissue samples as assessed by western blot (Fig. IVb. 4A).

The twelve resultant eluates were analyzed by LC-MS/MS (Suppl. Fig. IVb. 1). A total of 5,137 peptides and 1,430 proteins were identified in at least one of the twelve samples (Suppl. Table IVb. 1). Corroborating the western blot results, PP-1G peptides were detected in all IP fractions (Suppl. Table IVb. 1). Limited overlap of proteins was observed between samples from different patients; out of the 1,430 proteins, 1,021 proteins were detected in only one sample and 1,136 in either one or two samples (Suppl. Table IVb. 1). Principal component analysis applied to the protein expression matrix showed a clear clustering of samples per patient along the first principal component (Fig. IVb. 4B).

By filtering out the proteins that only appeared in IgG controls, we obtained 1,287 potential interactors, 271 of which only detected in co-IP outputs—from those, 200 were only detected in T-IP eluates and 27 in N-IP eluates (Suppl. Table IVb. 1). To proceed with the analysis, we selected the proteins consistently quantified in at least one condition (i.e., all proteins detected in at least two samples from a given condition). This selection process resulted in a list of 201 proteins (excluding PP-1G itself); 7% were already known PP-1G interactors and 10% were described as either PP-1A or PP-1B interactor (Suppl. Table IVb. 2). Two of the proteins were exclusively detected in N-IP, five in T-IP and three in both IP conditions (Fig. IVb. 4C and Table IVb. 3). PPP1R7 and CCT2 were already known PP-1G interactors and have at least one PP1-docking motifs, likewise ACO3, RACK1, KCTD3, YWHAG and PPP1R7 (Table IVb. 3) [528]. In addition, eight proteins showed a significant difference in their quantification when comparing co-IP eluates with IgG controls. None

of them have previously been identified as a PP-1G interactor but, except for XCCR5, all present one to four PP1-docking motifs (Table IVb.4).

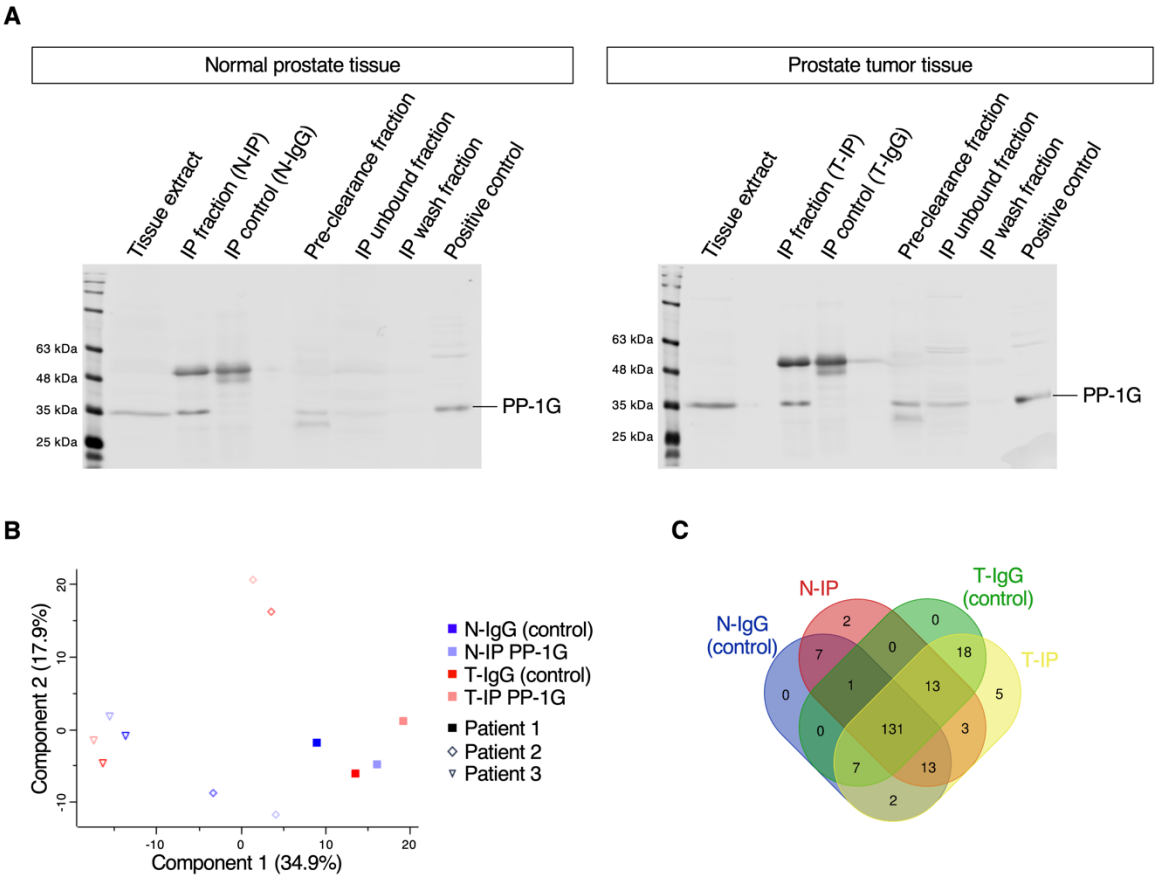


Fig. IVb. 4

PP-1G co-immunoprecipitation and mass spectrometry analysis. PP-1G was immunoprecipitated from freshly-collected human prostate tissues (paired normal and tumor tissue from three patients) using a non-denaturant protocol to preserve protein-protein interactions. The resultant eluates were analyzed by mass spectrometry. **(A)** To check for the efficiency of PP-1G immunoprecipitation, 10% of the final eluate volume was analyzed by western blot using an anti-PP-1G antibody. Adequate immunoprecipitation controls were included and total protein extract from LNCaP prostate cancer cell line was used as positive control in the immunoblot. **(B)** The protein expression matrix was analyzed using principal component analysis. **(C)** Venn diagram of the proteins consistently quantified in at least one condition. Abbreviations: IgG, immunoglobulin G; IP, immunoprecipitation; N, normal; T, tumor.

Additionally, to better understand the influence of each variable in the results, we used a 2-way ANOVA test, taking into consideration the co-IP effect (i.e., PP-1G vs IgG control), the patient effect (i.e., patient 1 vs. patient 2 vs. patient 3) and the interaction between these two variables. No significant result was obtained considering the p-value correction after multiple hypothesis testing, but some returned p-value<0.05 or -log10(p-value)>1.301 (Suppl. Table IVb. 3). Together with the above-mentioned proteins, the interaction between PP-1G and these proteins might be of particular interest to validate in human prostate tissue samples (normal and tumor).

Table IVb. 3
Proteins that were exclusively found in co-IP conditions.

Gene name	UniProt	Protein name	Known PP-1G interactor?	PP1-docking motifs	Function
Normal tissue					
ANXA4	P09525	Annexin A4	No	No	Calcium/phospholipid-binding protein
AOC3	Q16853	Membrane primary amine oxidase	No	2 RVxF motifs	Cell adhesion
Tumor tissue					
CCT2	P78371	T-complex protein 1 subunit beta	Yes	1 RVxF motif and 1 apoptotic signature motif	Chaperone
DDAH2	O95865	N(G),N(G)-dimethylarginine dimethylamino-hydrolase 2	No	No	Regulates the generation of nitric oxide
KCTD3	Q9Y597	BTB/POZ domain-containing protein KCTD3	No	2 RVxF motifs and 1 apoptotic signature motif	Accessory subunit of the hyperpolarization-activated potassium channel
RACK1	P63244	Receptor of activated protein C kinase 1	No	1 RVxF motif	Scaffold protein
RPL11	P62913	60S ribosomal protein L11	No	No	RNA- and rRNA-binding in ribosome
Normal and tumor tissues					
ATP5F1C	P36542	ATP synthase subunit gamma, mitochondrial	No	No	ATP synthesis and ion transport
PPP1R7	Q15435	Protein phosphatase 1 regulatory subunit 7	Yes	1 apoptotic signature motif	Regulatory subunit of PP1
YWHAG	P61981	14-3-3 protein gamma	No	1 apoptotic signature motif	Adapter protein

3.5. Expression and prognostic significance of the proteins uniquely found or enriched in co-IP conditions

To assess the relevance of the proteins exclusively found or enriched in co-IP eluates (Table IVb. 3 and Table IVb. 4), we analyzed TCGA-PRAD transcriptome sequencing data. ANXA4 and AOC3 (exclusively detected in co-IPs from morphologically normal tissue) were found significantly downregulated in primary prostate tumors (Fig. IVb. 5A-B). On the other hand, CCT2, RACK1 and RPL11 (exclusively detected in co-IPs from tumor tissue) were found significantly upregulated in primary prostate tumors (Fig. IVb. 5C-E). A slight downregulation was observed for DDAH2 (Fig. IVb. 5F) and no alteration was found for KCTD3 (data not shown).

YWHAG, CSRP1, PGK1 and FASN were also found to be differentially regulated in PCa: primary tumors showed higher expression of YWHAG and FASN, as well as reduced expression of PGK1 and CSRP1 when compared with normal tissue (Fig. IVb. 5G-J). Moreover, FASN and CSRP1 were among the top-250 transcripts up- and downregulated in primary prostate tumors, respectively. No significant alterations were observed for DBT, C1QB, HIBADH, XRCC5 and XRCC6 (data not shown).

Table IVb. 4
Proteins differentially quantified between co-IP conditions and IgG controls.

Gene name	UniProt	Protein name	p-value	-log (p-value)	Known PP1 interactor?	PP1-docking motifs	Function
Normal tissue							
C1QB	P02746	Complement C1q subcomponent subunit B	3,19E-02	1,50	No	2 RVxF motifs	Complement pathway and immunity
CSRP1	P21291	Cysteine and glycine-rich protein 1	2,26E-02	1,65	No	1 RVxF motif	Zinc ion-binding and actin cytoskeleton organization
DBT	P11182	Lipoamide acyltransferase component of branched-chain alpha-keto acid dehydrogenase complex, mitochondrial	3,15E-02	1,50	No	1 RVxF motif and 1 apoptotic signature motif	Branched-chain amino acid catabolic process
HIBADH	P31937	3-hydroxyisobutyrate dehydrogenase, mitochondrial	4,30E-02	1,37	No	1 SILK motif	Branched-chain amino acid catabolism
PGK1	P00558	Phosphoglycerate kinase 1	4,69E-02	1,33	No	3 RVxF motifs	Glycolysis
Tumor tissue							
XRCC5	P13010	X-ray repair cross-complementing protein 5	2,92E-02	1,53	No	No	
Normal and tumor tissues							
FASN	P49327	Fatty acid synthase	2,22E-03	2,65	No	4 RVxF motifs and 1 apoptotic signature motif	Lipid biosynthesis and metabolism
XRCC6	P12956	X-ray repair cross-complementing protein 6	1,40E-02	1,85	No	2 RVxF motifs	Single-stranded DNA-dependent ATP-dependent helicase

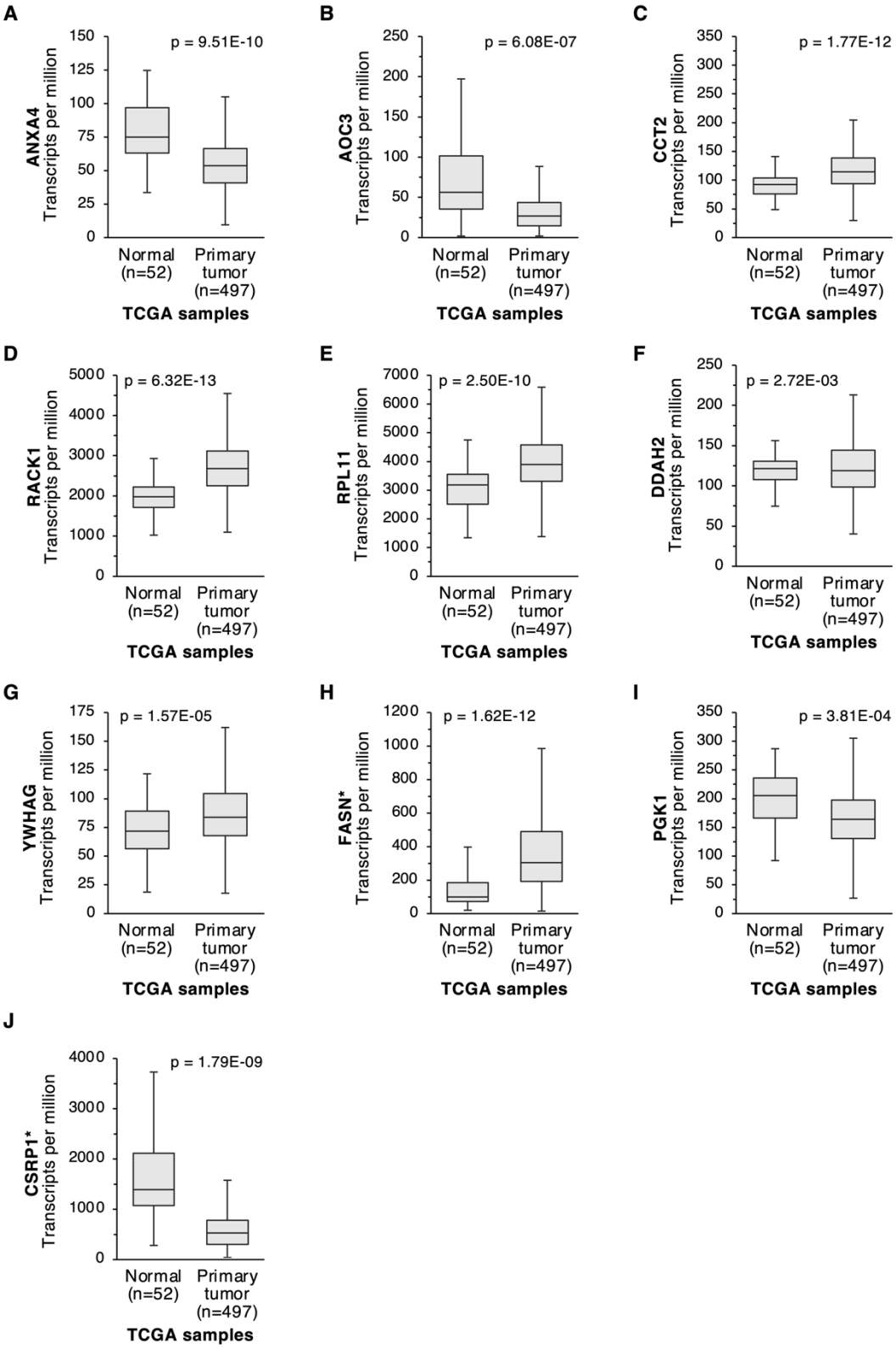


Fig. IVb. 5

Expression of ANXA4 (A), AOC3 (B), CCT2 (C), RACK1 (D), RPL11 (E), DDAH2 (F), YWHAG (G), FASN (H), PGK1 (I) and CSRP1 (J) in the TCGA-PRAD cohort. Data was reproduced and analyzed through UALCAN (http://ualcan.path.uab.edu/index.html) and is presented as transcripts per million. * Ranked in the top-250 up- or down-regulated transcripts in TCGA-PRAD cohort.

In addition, we analyzed the prognostic significance of the differentially regulated transcripts. Associations were observed between Gleason score and the transcript levels of CCT2, YWHAG and CSRP1 (Fig. IVb. 6A-C). High levels of CCT2 were also found associated with decreased survival probability (Fig. IVb. 6D).

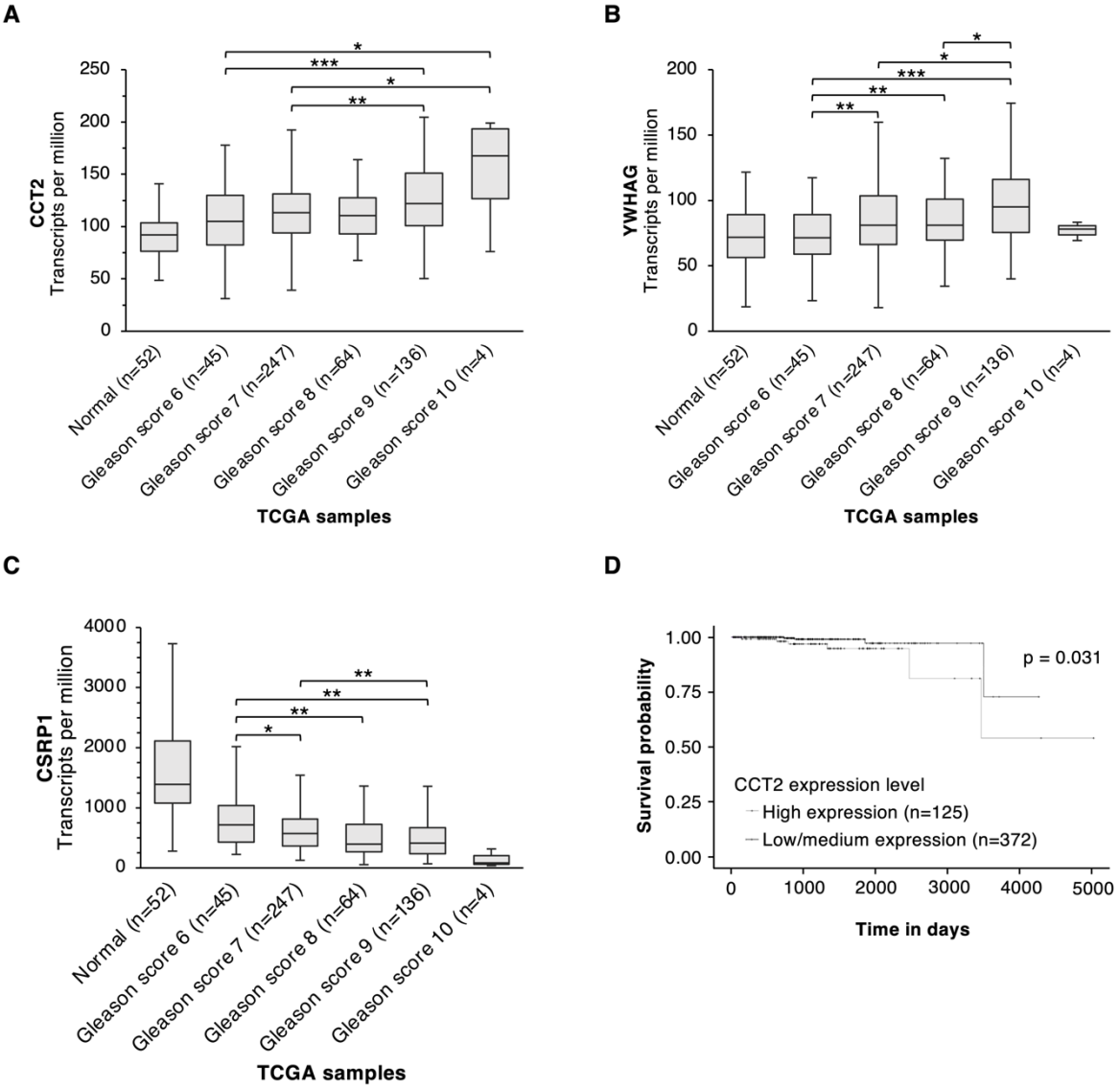


Fig. IVb. 6

Prognostic analysis of CCT2, YWHAG and CSRP1 in PCa. Transcriptome sequencing data from the TCGA-PRAD cohort showing the association between Gleason score and CCT2 **(A)**, YWHAG **(B)** and CSRP1 **(C)** expression. **(D)** Survival analysis of CCT2 in the TCGA-PRAD cohort. Data was reproduced and analyzed through UALCAN (http://ualcan.path.uab.edu/index.html) and is presented as transcripts per million.

3.6. PP-1G interaction network in human PCa

To improve the coverage of the PP-1G interactome in human PCa and better understand the relationship between interactors, we performed a comprehensive bioinformatics analysis to construct a PP-1G protein-protein interaction network in PCa. We retrieved all the experimentally validated

PP-1G interactors from publicly accessible databases and searched for their expression in human prostate proteome using The Human Protein Atlas. Only PP-1G interactors expressed in human prostate were analyzed with the 202-protein list obtained from the co-IP/MS experiments (for this purpose we considered the majority protein IDs indicated in Suppl. Table IVb. 2, column B) and the six proteins from the Y2H screenings. The protein-protein interaction network resultant from this data integration was composed of 560 nodes and 9,075 edges, revealing a high inter-connectivity (Suppl. Fig. IVb. 2). We then added information regarding nodes' expression in normal prostate and primary prostate tumor (data from the TCGA-PRAD cohort) and found that 60% of PP-1G's interactome was differentially expressed (Fig. IVb. 7A). Furthermore, 2% and 4% were ranked in the top-250 up- and downregulated in primary prostate tumors, respectively (Fig. IVb. 7A).

Given the network complexity, we searched for functionally related modules using the MCODE plug-in of Cytoscape. The most significant module includes 44 nodes, including PP-1G itself, and 258 edges (Fig. IVb. 7B). In addition to PP-1G, PCLAF and TP53 are the main hubs of the subnetwork, showing a degree of 30, followed by BRCA1 and PP-1A (PPP1CA in the network, the official gene name) (Fig. IVb. 7C). CCT2, FLNA, HSPA8 and ACTB—already known PP-1G interactors that we also detected in the co-IP experiments—are among the mediators of this subnetwork (Fig. IVb. 7B), as so are FASN, HSPA9, CAPZA2, EEF1A1, FLOT2, LGALS3BP, VIM, C1QBP, TUBA1A, HSPD1 and YWHAG (Fig. IVb. 7B), which have not previously been reported as PP-1G interactors but were co-immunoprecipitated with PP-1G in our experiments. From these, most (8/11) have at least one PP1-docking motif (Fig. IVb. 7B).

3.7. Enrichment analysis of the PCa-associated PP-1G interactome

Enrichment analysis was performed to determine the most representative ontologies and pathways in the PP-1G interactome. “Phosphatase modulator” (F.E. 12.81, p=1.60E-10) was the most enriched protein class, followed by significance by “ribosomal protein” (F.E. 4.98, p=1.53E-07) and “transmembrane signal receptor” (F.E. 0.27, p=8.08E-04) protein classes (Fig. IVb. 8). Key terms related to phosphatase activity regulation were among the most enriched biological processes and molecular functions, including “Positive regulation of phosphoprotein phosphatase activity” (F.E. 14.06, p=5.35E-03), “Protein phosphatase inhibitor activity” (F.E. 12.30, p=1.94E-08), “Protein phosphatase activator activity” (F.E. 17.03, p=1.91E-02) and “Protein phosphatase 1 binding” (F.E. 22.15, p=2.94E-13) (Fig. IVb. 8). Accordingly, “PTW/PP1 phosphatase complex” (F.E. 31.64, p=7.09E-04) and “Protein phosphatase type 1 complex” (F.E. 27.07, p=3.11E-08) were enriched cellular compartments (Fig. IVb. 8). “HSF1 activation” (F.E. 27.68, p=2.66E-06) and “MAP3K8 (TPL2)-dependent MAPK1/3 activation” (F.E. 20.76, p=1.68E-05) were the two most enriched pathways.

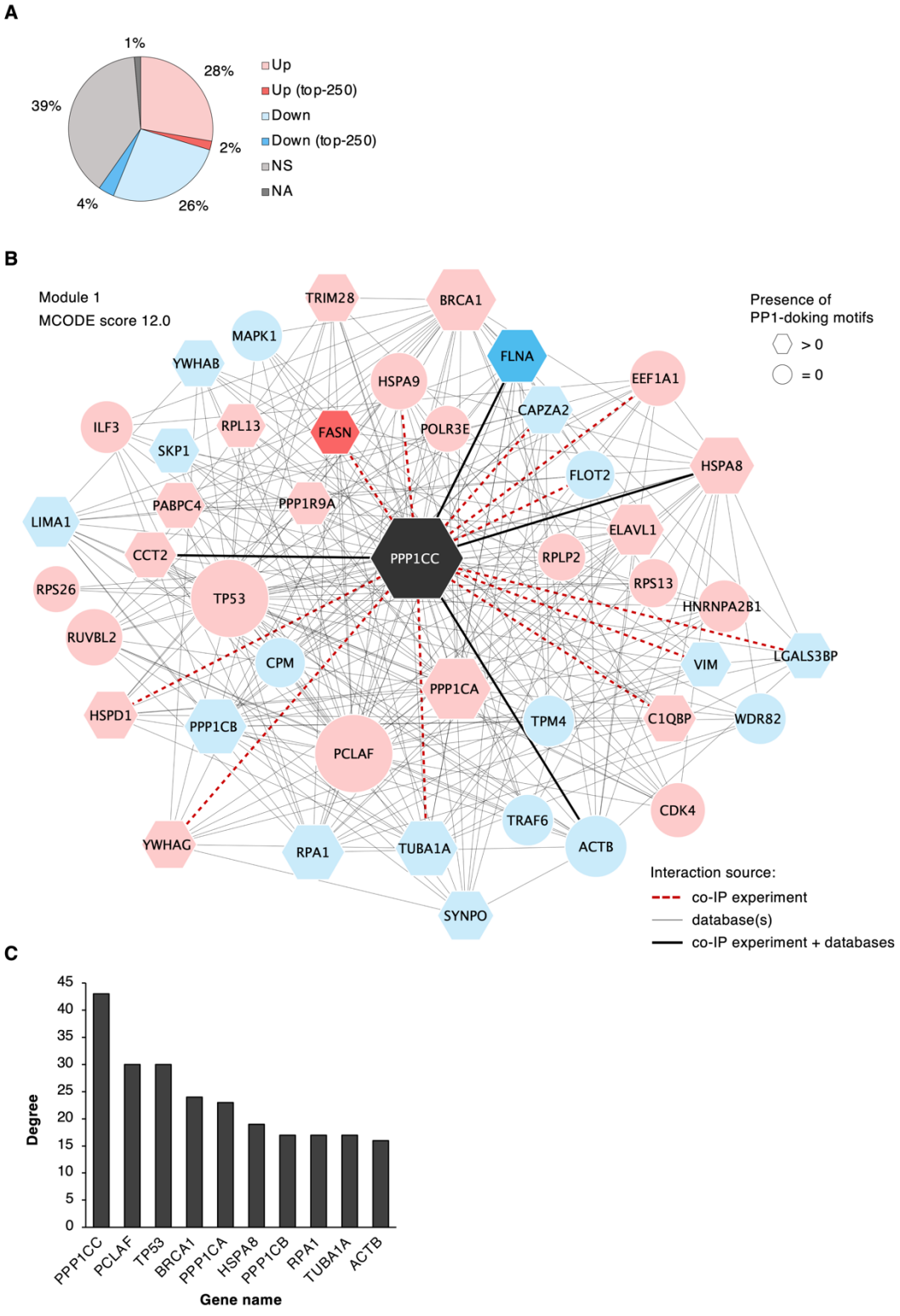


Fig. IVb. 7

Analysis of the PP-1G interactome in human PCa. (A) Percentage of the PP-1G interactome upregulated, downregulated and not statistically different (NS) between primary prostate tumors and normal prostate tissues (expression data from the TCGA-PRAD cohort). NA, not available. **(B)** Functionally related subnetwork with the highest MCODE score. PP-1G is highlighted in a black node (PPP1CC, the official gene name). Nodes' size reflects nodes' degree and colors indicate upregulation (rose; darker when in top-250) and downregulation (blue; darker when in top-250). **(C)** Top-10 hub nodes from the functionally related subnetwork.

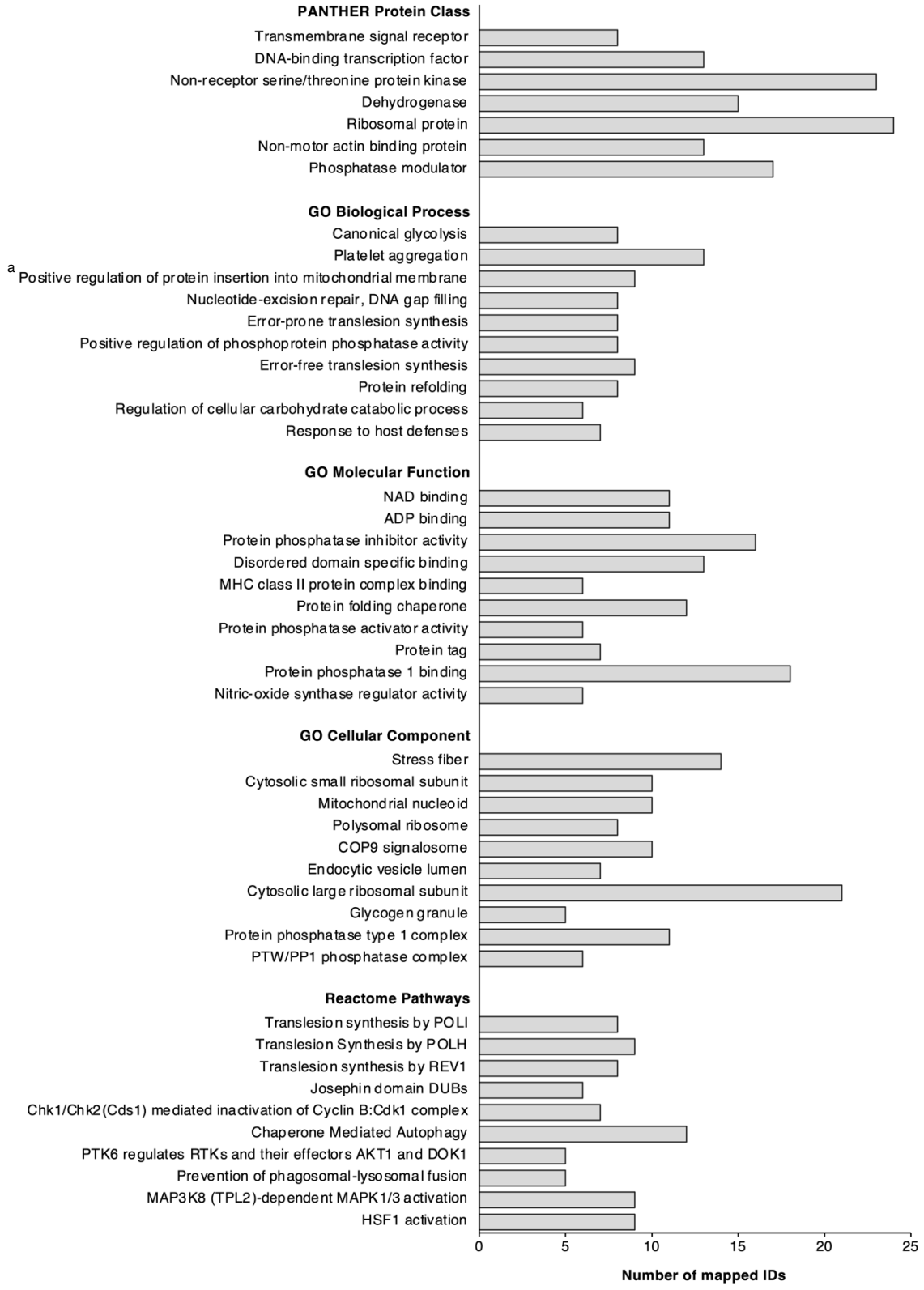


Fig. IVb. 8

Enrichment analysis of the PP-1G interaction network. Except for PANTHER Protein Class, all results correspond to the top-10 child terms in the analysis. Data was retrieved from the Gene Ontology (GO) knowledgebase (release Sep 10, 2020) and powered by PANTHER Classification System. [a] Involved in apoptotic signaling pathway

4. Discussion

In the present study, we used three complementary approaches to characterize the PP-1G interactome in human prostate cancer. Two experimental methods were elected for identifying differentially regulated interactions between prostate tumor tissue and paired normal tissue: YH2 and co-IP/MS. The combination of these two high-throughput approaches were already reported to improve the coverage of protein interactome analysis [665].

For the Y2H screenings, we started by constructing cDNA libraries from fresh-frozen prostate tumor and morphologically normal tissues from 5 radical prostatectomy specimens. Tissue fragments were pooled per group—tumor and normal—for maximization of the starting material. Despite the relatively limited size range of both libraries (200-1,500 bp, Fig. IVb. 3C), our findings are in agreement with previous studies that constructed cDNA libraries from prostatic intraepithelial neoplasia specimens [666], as well as from other organs (e.g. benign and malignant thyroid tissues [667]). Then, a PP-1G bait construct (which was first proved to be non-toxic and unable to auto-activate the screening system in yeast cells, Fig. IVb. 2C) was used to fish for interacting partners within each cDNA library. Despite the high initial number of colonies growing in DDO/X/A, these were severely reduced upon striking to high stringent environments as expected. The genetic material from positive clones was amplified and sequenced to identify the potential interactor; however, the reduced number of base pairs recovered from chromatograms upon vector trimming compromised the accurate alignment with the human genome, since several results with similar scores were obtained for each sequence. Also, most of the matches corresponded to non-coding regions and, thus, their biological relevance needs to be further investigated. Increasing evidence suggests that alterations in non-coding sequences of several genes might be relevant for carcinogenesis. Indeed, *FOXA1* non-coding regions were recently suggested as promising therapeutic targets for PCa management [668]. In fact, PCa is a pioneer in the field of research of non-coding RNAs [669]. Non-coding RNAs can be similar to mRNAs in that they can be transcribed by polymerase II, have a 5'-cap and a 3'-polyadenylated tail (the latter observed in most of our sequences) and accumulate in the cytoplasm. When associated with ribosomes, their open-reading frames can be translated into small peptides that show low expression levels and are weakly conserved across different species [670]. Whether PP1 may play a role in the regulation of such structures is still to be determine, but phosphatases and non-coding RNAs were previously shown to interplay [671].

For the co-IP/MS experiment, PP-1G and its interacting partners were co-immunoprecipitated from freshly collected prostate tumor and paired morphological normal prostate tissues from three patients undergoing radical prostatectomy. The developed protocol allowed for PP-1G isolation and identification in its native state and its interactome from the tissue lysates under physiological

conditions. The identification of PP-1G and already reported interactors support the efficiency of the method; however, the high heterogeneity found among the protein matrix obtained from different patients impacted in the statistical analysis, precluding significant results. It is widely recognized that prostate tumors exhibit high intra- and inter-tumoral heterogeneity, with tumors staging Gleason score 7 (as two of our patients) being particularly challenging [672]. This might explain the limited protein overlap observed for different patients and indicates the need of increasing the number of samples to be used in future experiments. Nonetheless the above-mentioned limitations, we were able to identify 10 proteins exclusively in co-IP eluates: two in N-IP (ANXA4 and AOC3), five in T-IP (GNB2L1, CCT2, KCTD3, DDAH2 and RPL11) and three in both (YWHAG, ATP5C1 and PPP1R7).

PPP1R7, also known as SDS22, is one of the most conserved RIPPOs, although with mostly unknown function. It has been proposed as a targeting subunit and/or substrate specifier but also as an activator or inhibitor [673]. PP1/PPP1R7 holoenzyme have been implicated in carcinogenesis, particularly in the regulation of epithelial integrity [416]. Our results suggest that PPP1R7 might be an important regulator of PP1 activity in PCa.

CCT2 was also previously identified as a PP-1G interactor, but the biological relevance of their association is still to be determined. CCT2 is a chaperone involved in the correct folding of several proteins (e.g., actin and tubulin) and its overexpression has been implicated in the development and progression of various cancers, including PCa [674]. Although its role in prostate carcinogenesis is not fully understood, its expression seems to be associated with poor prognosis (Fig. IVb. 6). Also, previous studies suggest it might be a good therapeutic target [675,676].

In addition to CCT2, the transcript levels of YWHAG were also found to be associated with Gleason score (Fig. IVb. 6). YWHAG belongs to the 14-3-3 family of proteins, which are signaling mediators and bind to several proteins. PP1 regulates YWHAG interaction with other proteins [679]. Hence, the modulation of PP1/YWHAG interaction might be a promising target to affect the dynamics of additional proteins and signaling pathways. Though its role in PCa is still far from being completely understood, YWHAG was reported to promote breast cancer cell motility [677], as well as epithelial-mesenchymal transition and metastatic potential of non-small cell lung cancer cells [678].

In contrast to YWHAG and CCT2, CSRP1 was not previously reported as a PP-1G interactor; however, it does contain a PP1-docking motif (Table IVb. 4). Investigations on CSRP1 in cancer are rather scarce and somewhat controversial. CSRP1 was suggested as a tumor suppressor gene in colorectal cancer and its inactivation by aberrant methylation was proposed as a candidate diagnostic marker for liver cancer. Our findings are also supportive of a tumor suppressor role for CSRP1 in PCa (Fig. IVb. 6). In fact, it is one of the top-250 downregulated transcripts in primary prostate

tumors and lower levels are associated with higher Gleason score. Also, we were only able to identify CSRP1 in co-immunoprecipitates from normal prostate tissue. Whether a direct interaction between PP-1G and CSRP1 exists and it is lost during prostate carcinogenesis, or it is a consequence of the reduced expression of CSRP1 in tumor tissues to undetectable levels, is still to be determined.

To enhance our characterization of the PP-1G interactome in PCa, we complemented the data obtained in the biochemical studies with experimentally validated interactions with proteins expressed in the prostate proteome. We then integrated expression data from the TCGA-PRAD cohort to construct a differentially expressed protein-protein interaction network (Suppl. Fig. IVb. 2). The identification of a functional module within this network sustains their partnered involvement in the regulation of biological processes and allowed us to stress a PP-1G-mediated subnetwork with particular relevance in PCa. Attention should be given, nonetheless, to the presence of 11 putative new interactors that need to be further validated using additional methods (Fig. IVb. 7B).

Regarding the biological relevance of the PCa-associated PP-1G interactome, various proteins were found to regulate the same biological process and/or molecular function and/or to be involved in the same signaling pathways (Fig. IVb. 8). Not surprisingly, events related to phosphatase regulation and localization within PP1 complexes were found enriched in our network. The existence of several cytoskeletal and actin-binding motor proteins in the network also supports the relevance of PP-1G in modulating cell polarity and epithelial integrity (as revised in [416]).

Altogether, we unveiled and characterized the interactome of PP-1G in human PCa and identified new potential interactions that, upon validation by additional methods, might be of great interest to explore as therapeutic targets. We also showed that some interactors would be of interest to further explore as candidate diagnostic and/or prognostic marker.

5. Supplementary data

Suppl. Table IVb. 1

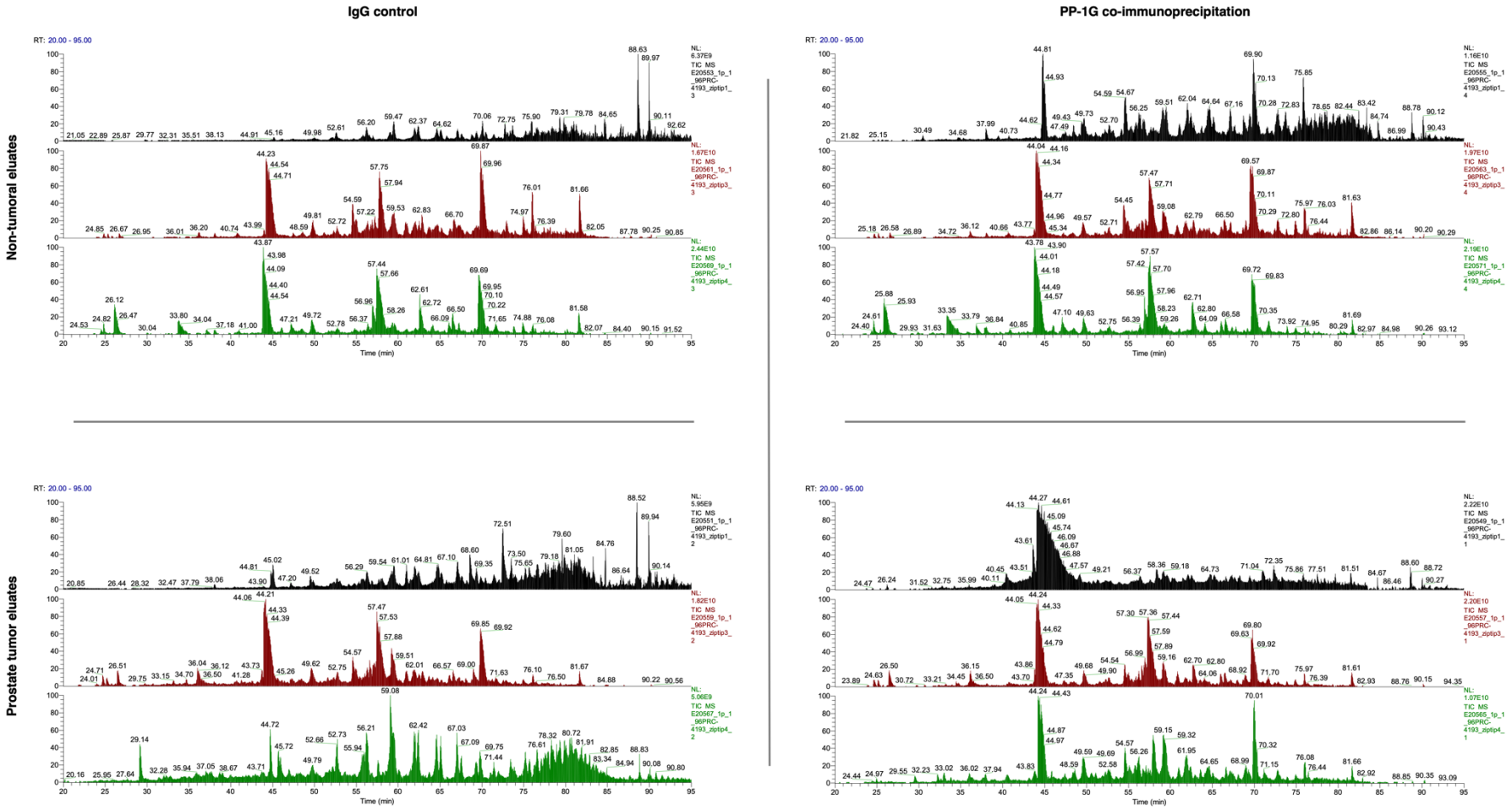
Raw list of peptides and proteins identified by co-IP/MS. To access full data, please consult the file in https://uapt33090-my.sharepoint.com/:x:/g/personal/julianacfelgueiras_ua_pt/Ea69tpegliNKunOdtgJpneABizfaj4ue07Vm3CzypY-s_A?e=u5KhXd. Data will be submitted the PRoteomics IDEntifications (PRIDE) database (http://www.ebi.ac.uk/pride) [680] upon article acceptance.

Suppl. Table IVb. 2

Proteins consistently quantified in at least one condition. All proteins detected in at least two samples from a given condition). To access full data, please consult the file in https://uapt33090-my.sharepoint.com/:x:/g/personal/julianacfelgueiras_ua_pt/EfZDEYm_-BZOr4_0FwWgGNQBU6US5EME1MeQRUzBC24KXQ?e=9gZgqg.

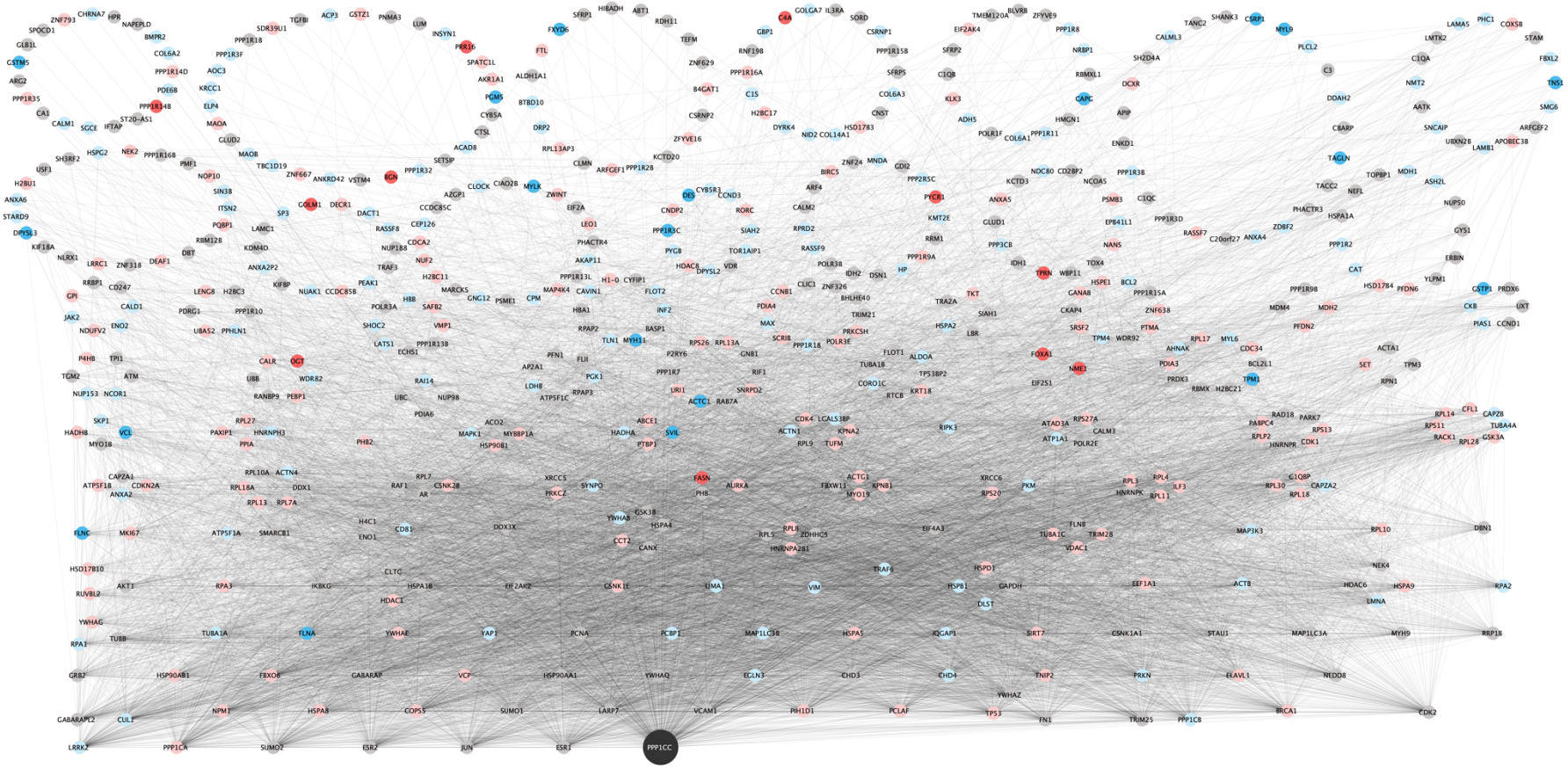
Suppl. Table IVb. 3

Proteins with 2-way ANOVA significant results. The interaction of PP-1G with these proteins might be of particular relevance to validate in additional studies using human prostate models (normal and tumoral). To access full data, please consult the file https://uapt33090-my.sharepoint.com/:x:/g/personal/julianacfelgueiras_ua_pt/EQsvbCgYPE9Fi9B6r1c1yOsBaRrlixuoEsIMEusALzktSw?e=aNZgdZ.



Suppl. Fig. IVb. 1

Total ion chromatograms of the PP-1G co-immunoprecipitation eluates from paired tumor and morphologically normal prostate tissues from PCa patients (n=3), and respective technical controls, analyzed by LC-MS/MS.



Suppl. Fig. IVb. 2

PP-1G interaction network in human PCa. PP-1G is highlighted in a black node (PPP1CC, the official gene name). Nodes are distributed according to their degree (descendent degree from the bottom to the top of the network) and, except for PPP1CC, are colored according to the differential expression observed in normal (n=52) and primary tumor tissues (n=497) from the The Cancer Genome Atlas Prostate Adenocarcinoma cohort (TCGA-PRAD): blue, downregulated in PCa; red, upregulated in PCa; grey, not significantly altered. The nomenclature of the nodes corresponds to the official gene name annotated in UniprotKB, release 05_2020.

CHAPTER V

FEASIBILITY OF USING PP1-DOCKING MOTIF-MIMETIC CELL-PENETRATING PEPTIDES TO MODULATE PROSTATE CARCINOGENESIS

Feasibility of using PP1-docking motif-mimetic cell-penetrating peptides to modulate prostate carcinogenesis

KEYWORDS

Prostate cancer
Protein phosphatase 1
Androgen receptor
Protein-protein interaction
Bioportide

ABSTRACT

In contrast to protein kinases, their counterpart protein phosphatases have long been considered ‘undrugabble’. Recent data have been changing this paradigm, showing that protein phosphatases and the interaction interfaces they establish with substrates and/or regulatory proteins are interesting therapeutic targets, albeit challenging. The serine/threonine-protein phosphatase 1 (PP1) is a major cellular catalyzer of dephosphorylation reactions that regulates hundreds of proteins and signaling cascades. Its interaction with the androgen receptor (AR) in prostate cancer (PCa) cells increases AR expression and transcriptional activity even in castration-resistant contexts. Therefore, PP1/AR interaction is likely to be determinant for PCa development and progression. Here, we designed and synthesized candidate bioportides that mimic the PP1-docking motifs in the AR's primary sequence. We show that these peptides, particularly when used synergistically, can reduce the viability of both androgen-dependent and castration-resistant PCa cells. Additionally, we show that MSS1 and mitoparan bioportides also have a negative influence upon the viability of PCa cells.

Abbreviations: ADT, androgen deprivation therapy; AKAP4, A-kinase anchor protein 4; AR, androgen receptor; AR-V7, AR variant 7; BS, binding site; CPP, cell-penetrating peptide; DBD, DNA-binding domain; DIC, differential interference contrast; DMF, N,N-dimethylformamide; Fmoc, 9-fluorenylmethoxycarbonyl; GUSB, beta-glucuronidase; HPLC, high-performance liquid chromatography; LBD, ligand-binding domain; mitP, mitoparan; PCa, prostate cancer; PP1, serine/threonine-protein phosphatase 1; PSA, prostate-specific antigen; TAMRA, 5(6)-carboxytetramethylrhodamine.

1. Introduction

Cancer therapeutics is a rapidly evolving field of research. The introduction of the targeted therapy concept, back in the 80s, was a major turning point in the effectiveness of cancer treatment, contributing to a better management of the disease and increased survival rates [681]. In recent years, protein-protein interactions have emerged as interesting therapeutic targets since they constitute the backbone of dense and complex signaling networks that determine cancer cells' behavior [682]. However, targeting protein-protein interactions is challenging due to intrinsic features of their interface (e.g., large size and minimal or inexistent structural pockets to dock small molecules). No natural ligands exist for protein-protein interactions' interface and many therapeutic strategies using small-molecule modulators or protein-based approaches are, therefore, difficult to apply. Moreover, targeting intracellular protein-protein interactions adds another layer of complexity to the subject since it requires modulators to cross the cell membrane, which is beyond the abilities of many [683].

Cell-penetrating peptides (CPPs) are short amino acid sequences (typically <30 amino acids, polycationic) developed to efficiently deliver bioactive cargoes intracellularly. Initially, they were designed to be relatively inert with the sole purpose of acting as carriers or pharmacokinetic modifiers. Hence, most studies employ sychnologically-organized tandem constructs, with an active cargo (message) combined with an inert CPP (functionally discrete and continuous address). However, some CPPs have their own intrinsic bioactivity—the so called bioportides—and, therefore, can directly regulate cellular processes [684]. These bioportides usually derive from native protein sequences and are rhegnylogically-organized (i.e., cellular penetration and bioactivity are discontinuously distributed within the primary sequence of the peptide). For instance, Cyt $c^{77–101}$, derived from cytochrome c, promotes caspase-3-mediated cell apoptosis; camptide, derived from calcitonin receptor, modulates insulin secretion and infectivity of hepatitis C virus; and, nosangiotide, derived from endothelial nitric oxide synthase, has antiangiogenic properties [685,686]. Henceforth, the use of linear CPPs to target cancer-related intracellular signaling mediators and transcription factors has been given increasing attention (as recently revised in [687]).

The androgen receptor (AR) is a ligand-activated transcription factor and a major player in prostate cancer (PCa) development and progression. It is also the therapeutic target *per excellence* in PCa, with androgen deprivation therapy (ADT) being the gold-standard treatment for patients with locally advanced disease [688]. However, the vast majority of PCa cases progress to a castration-resistant state that fail to respond to therapy. Compelling evidence suggests that AR splicing variants could have important mechanistic roles in this process [689]. The serine/threonine-protein phosphatase 1 (PP1) is a positive regulator of AR expression and transcriptional activity, acting by direct dephosphorylation and enhancement of AR nuclear localization and/or decreasing AR degradation [690,691]. Besides regulating AR canonical isoform, PP1 was shown to promote the activity of

AR variant 7 (AR-V7) [291], which lacks the ligand-binding domain (LBD), being constitutively active [692]. These findings suggest that the interaction between PP1 and AR might be a promising target for pharmacological interventions in both locally advanced and metastatic-castration resistant PCa.

Recently, we have successfully designed an optimized bioportide, MSS1, that mimics the PP1-docking motif in A-kinase anchor protein 4 (AKAP4) [693]. This peptide was shown to disrupt the interaction between AKAP4 and PP1γ2, a testis- and sperm-specific PP1 isoform, with a significant impact in sperm cell motility [693]. Here we designed and synthesized bioportides that mimic the PP1-docking motifs in AR and assessed their ability to translocate into PCa cells, modulate their viability and affect the expression of AR and its pivotal downstream target gene, the prostate-specific antigen (PSA). We also evaluated the effect of MSS1 and mitoparan (mitP), a mitochondriotoxic and apoptogenic bioportide, in PCa cells viability. This study emphasizes the potentials of using bioportides to modulate prostate carcinogenesis.

2. Materials and methods

2.1. Cell culture

LNCaP (androgen-dependent) and PC3 (castration-resistant) PCa cells were continuously grown in RPMI 1640 medium with L-glutamine, supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) penicillin-streptomycin solution (5,000 U/mL), all acquired from Gibco™ (Thermo Fisher Scientific, USA). RPMI 1640 medium without phenol red and with no supplementation was used during the assays as further detailed in the next sections. Cell cultures were checked for mycoplasma contamination and routinely maintained at 37 °C in a humidified incubator with 5% CO_2 atmosphere. Cells were kept at low passage for the assays and cell viability was frequently monitored by Trypan Blue staining (Thermo Fisher Scientific, USA) to ensure a pre-treatment viability superior to 90%.

2.2. Design, synthesis and purification of the bioportides

AR's amino acid sequence was searched for PP1-docking motifs using the ScanProsite tool (freely available at https://prosite.expasy.org/scanprosite/) [694]. Potential CPP sequences were predicted *in silico* using CellPPD (freely available at http://crdd.osdd.net/raghava/cellppd/) [695,696] and CPPpred (freely available at http://bioware.ucd.ie/cpppred) [697]. The water solubility of peptides was estimated using Peptide Property Calculator provided by Innovagen AB (freely available at https://pepcalc.com).

Peptides were synthesized at a 0.1 mmol scale using a 9-fluorenylmethoxycarbonyl (Fmoc)-based solid-phase peptide synthesis strategy on a Liberty Blue™ Automated Microwave Peptide Synthesizer (CEM Microwave Technology Ltd, UK) as previously described [698,699].

Briefly, rink-amide methylbenzylhydrylamine resin (0.59 mmol/g) was used as solid support to obtain C-terminally amidated peptides. Couplings of N-α-Fmoc-protected amino acids (0.2 M) were carried out in N,N-dimethylformamide (DMF) using N,N-diisopropylcarbodiimide as activator and 1 M OxymaPure® plus 0.1 M N,N-diisopropylethylamine as additives to provide higher yields with less racemization. Special coupling cycles were used for arginine (double coupling, 30 W/75 °C/300 s) and cysteine/histidine (single coupling, 30 W/50 °C/600 s) to reduce δ-lactam formation of arginine and racemization of cysteine/histidine, respectively [699]. Fmoc removal was done with 20% (v/v) piperidine in DMF. Peptides were cleaved from the resin using a standard cleavage solution consisting of trifluoroacetic acid/triisopropylsilane/water (95:2.5:2.5) under gentle agitation for 3 h at room temperature. Fluorescent versions of the peptides were obtained by treating the resin-bound peptides with 0.1 M 5(6)-carboxytetramethylrhodamine (TAMRA) under gentle agitation for 3 h at room temperature.

Peptides were purified by semi-preparative reverse-phase high-performance liquid chromatography (HPLC) on a PerkinElmer® FlexarTM with UV/Vis Detector system fitted with a C18 column (Zorbax 300SB-C18). A gradient of 5−100% acetonitrile/water containing 0.1% TFA was applied as previously [698–700]. Pure peptides were freeze dried and stored with desiccant at -20 ºC until used. The predicted masses of all peptides were confirmed by mass spectrometry.

In addition to the peptides specifically designed in this study, we also used the newly designed sychnologic peptide, MSS1—a molecular modelling-guided improvement of a peptide derived from the primary sequence of the PP1 interactor AKAP4 (YRSVITFVAV sequence covalently coupled to penetratin [693]); AKAP4-BM M, an Ala-substituted homologue of the AKAP4 sequence lacking the PP1-docking motif (GQQDQDRAAAAAVAVSTLNV sequence covalently coupled to penetratin [693]); mitP [701]; and the widely-recognized CPP Tat.

2.3. Live-cell confocal microscopy

TAMRA-conjugated peptides were reconstituted in ultrapure water to obtain 1 mM stock solutions, which were filter-sterilized (0.2 μm) and stored into aliquots at -20 ºC until used. Peptides translocation into cells was assessed by live-cell confocal microscopy as previously described [699,700]. Briefly, cells were grown in standard growth conditions to 60-70% confluence in 35-mm sterile glass base dishes. Peptides were diluted in RPMI-1640 medium without phenol red and added to cells, previously washed in RPMI-1640 medium without phenol red, to a final concentration of 5 μM. Cells were incubated for 1 h at 37 °C, protected from light, in a humidified incubator with 5% CO_2 atmosphere. Following the incubation time, cells are gently washed in RPMI-1640 medium without phenol red and left in 2 mL medium for microscopic examination. Untreated cells and cells incubated with TAMRA-conjugated Tat were included as background reading and positive control,

respectively. Images were acquired in a Zeiss LSM 510M confocal microscope (CarlZeiss Microimaging GmbH, Germany) equipped with an environmental chamber for adequate temperature and CO_2 control of living cells.

2.4. Quantitative uptake of fluorescent bioportides

Quantitative analysis of peptides translocation was performed using TAMRA-conjugated peptides. Cells were grown in standard growth conditions to 80% confluence in 6-well plates. Cells were then washed in RPMI-1640 medium without phenol red and incubated with 5 μM TAMRA-labelled peptides for 1 h, as indicated for live-cell confocal microscopy. After, cells were collected as previously described with few modifications [699,700]. Briefly, cells were washed four times with Hanks' Balanced Salt Solution without phenol red (Sigma-Aldrich, USA), detached with 10% (w/v) trypsin without phenol red (Sigma-Aldrich, USA), at 37 °C, and collected by centrifugation at 6,000 rpm for 5 min at 4 °C. Pelleted cells were resuspended in 300 μL of 0.1 M NaOH and lysed overnight at -20 °C. To calculate fluorescence intensity, 250 μL of each lysate were transferred to a 96-well black plate and analyzed in an Infinite® 200 PRO microplate reader (Tecan, Switzerland) using the settings λAbs 544 nm/λEm 590 nm. Three replicates were prepared per condition in three independent experiments.

2.5. Cell viability

Peptide stock solutions of 1 mM were prepared as described for fluorescent versions. Cells were grown in standard growth conditions to 60-70% confluence in 96-well plates. Sequential dilutions of the peptides were prepared in RPMI-1640 medium without phenol red and added to cells at 1–20 μM. Cells were then incubated for 24 or 48 h at 37 °C in a humidified incubator with 5% CO_2 atmosphere. PrestoBlue™ Cell Viability Reagent (Thermo Fisher Scientific, USA) was diluted in RPMI-1640 medium without phenol red (1/10) and added to cells for 30 min according to the manufacturer's instructions. Fluorescence intensity (λAbs 560 nm/λEm 600 nm) was measured in an Infinite® 200 PRO microplate reader (Tecan, Switzerland). Three to five replicates of each condition were performed in three independent experiment.

2.6. Real-time PCR

Cells were incubated with 10 μM of AR-BS1, AR-BS2, AR-BS3 or a combination of the three peptides in RPMI-1640 medium without phenol red for 24 h at 37 °C in a humidified incubator with 5% CO_2 atmosphere. RNA was isolated from cells using TRIzol® Reagent (Invitrogen, USA) and first strand cDNA synthesis was carried out using the RevertAid RT Reverse Transcription Kit (Thermo Fisher Scientific, USA), according to manufacturer's instructions. Each sample was analyzed in triplicate for the expression of AR and PSA using the NZYSpeedy qPCR Green Master

Mix (NZYTech, Portugal) and normalized to the expression of the beta-glucuronidase (GUSB) housekeeping gene. RNA levels were quantified in a 7500 Real-Time PCR system (Applied Biosystems, USA) and quantitative data was analyzed using the relative standard curve method. The experiment was performed in duplicate.

2.7. Statistical analysis

Statistical analysis was performed using IBM® SPSS® Statistics software, v25.0 (New York, USA), by applying the Kruskall-Wallis test or the Mann-Whitney U test for pairwise comparisons. The significance level was set at 0.05.

3. Results

3.1. Synthesis of linear peptides derived from the PP1-docking motifs in AR's primary sequence

AR's amino acid sequence contains three PP1-docking motifs, which are all RVxF sequences: ^{581}KVFF^{584}, ^{715}HVVKW^{719} and ^{913}KPIYF^{917} (Fig. V. 1A). While the binding site (BS) 1 is localized at the DNA-binding domain (DBD) and is conserved among all AR isoforms, BS2 and BS3 are localized at the LBD and are only shared by AR full length (the canonical isoform) and AR variant 45 (Fig. V. 1B). These sequences and additional flanking residues were analyzed using CPP prediction databases and peptides' solubility was calculated as a first approach to their design. The first two sequences were identified as candidate rhegnylogic bioportides and only a small amino acid change was applied in each of them to improve solubility (Table V. 1 and Suppl. Table V. 1). Due to its predicted poor performance as a CPP (Suppl. Table V. 1), the third sequence was coupled to Tat sequence (Table V. 1 and Suppl. Table V. 1), a well-known and efficient CPP [702].

Table V. 1
AR-BS peptides' sequences.

Peptide	Sequence	Length (AA)	Mass (g/mol)
AR-BS1[a]	GSC**KVFF**KRAAKGKQK-NH_2	16	1782.17
AR-BS2[b]	RQLV**HVVKW**AKKL-NH_2	13	1605.01
AR-BS3	KV**KPIYF**HTGRKKRRQRRRPPQ-NH_2	22	2832.40

[a] Amino acid change: E to K. [b] Amino acid change: A to K. PP1-docking motifs are in bold. Amino acid changes from the primary amino acid sequence are underlined. Tat sequence is within the rectangle.
AA, amino acid.

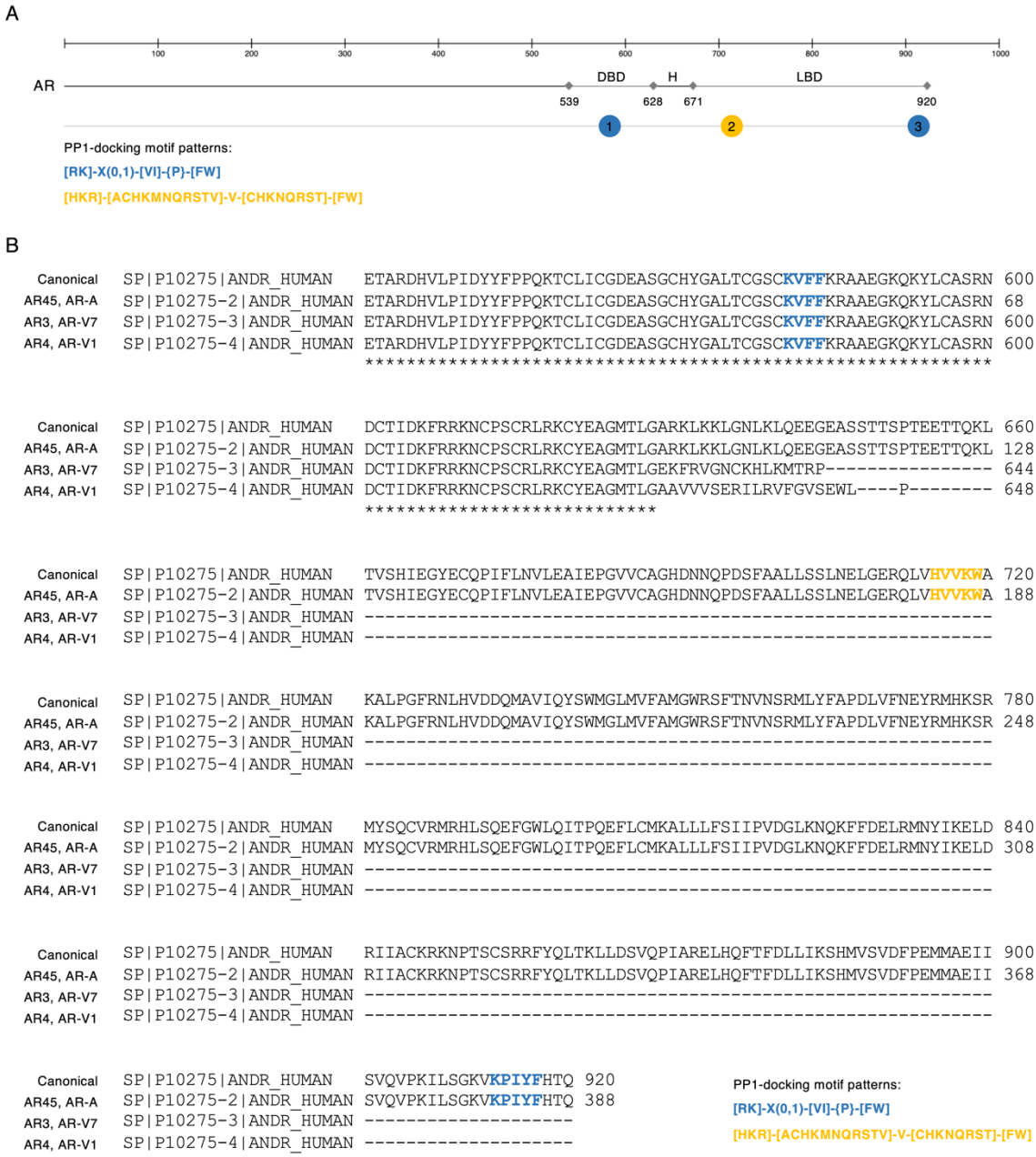


Fig. V. 1

Partial AR's primary sequences and PP1-docking motifs. (A) AR's primary sequence contains three PP1-docking motifs (numbered circles): one localized at the DNA-binding domain (DBD) and two at the ligand-binding domain (LBD). These two regions are separated by a small hinge region (H). **(B)** Alignment of partial AR isoforms' primary sequences depicting the conservation of the PP1-docking motifs among isoforms. PP1-docking motifs are colored according to their pattern, as indicated in the figure.

3.2. Translocation of the AR-BS peptides into PCa cells

Fluorophore-conjugated versions of the AR-BS peptides were employed to visualize their intracellular distribution by live-cell confocal microscopy and to assess their internalization quantitatively. The three peptides demonstrated cellular penetration after 1 h of incubation, mostly showing a vesicular distribution throughout the cytoplasm (Fig. V. 2A).

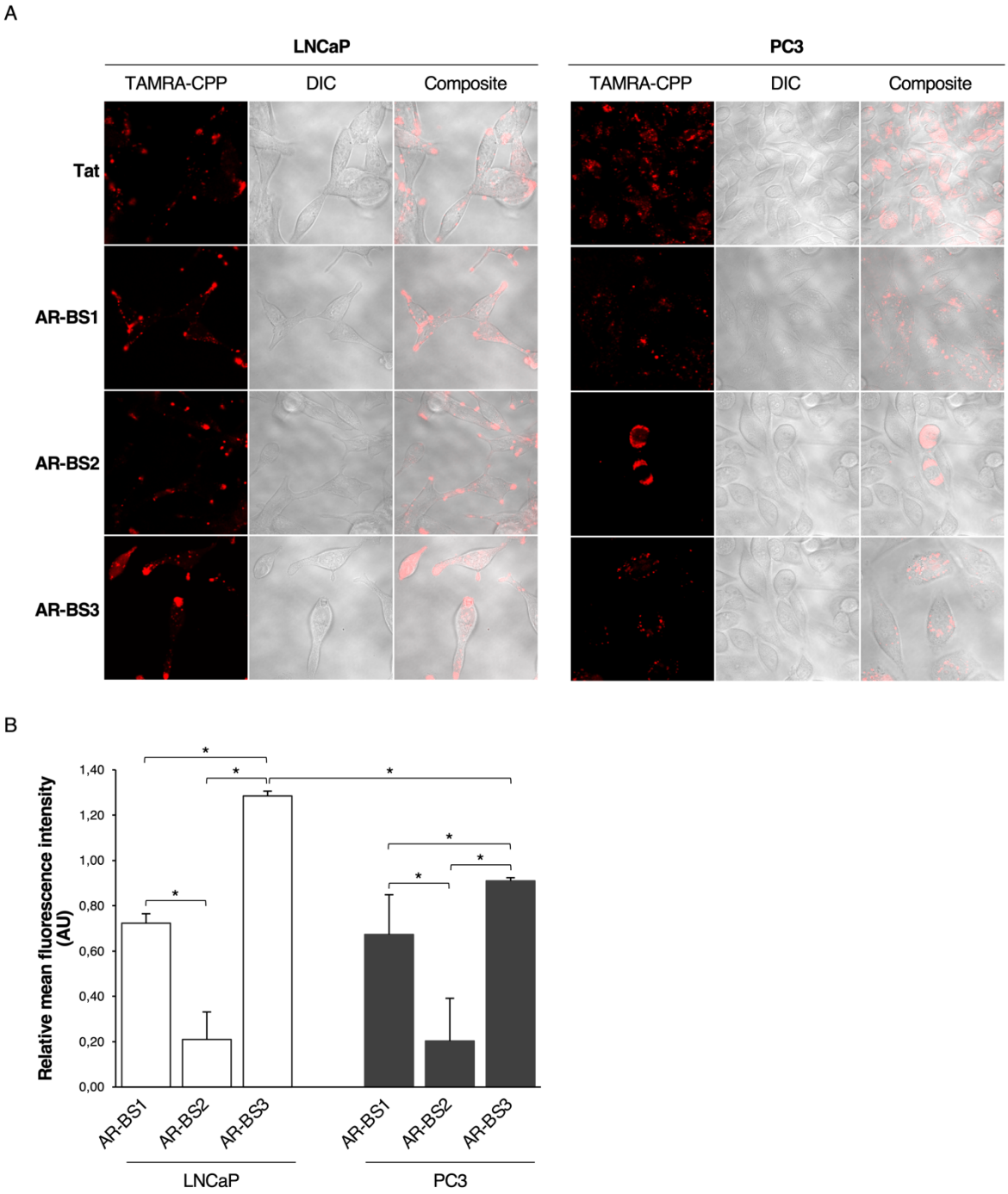


Fig. V. 2

Translocation of AR-BS peptides into PCa cells. LNCaP and PC3 cells were treated with TAMRA-labelled AR-BS peptides (5 µM) and incubated for 1 h at 37 °C in a humidified incubator with 5% CO_2 atmosphere. Cells incubated with TAMRA-Tat CPP at the same conditions were used as positive control for CPP internalization. Cells were then washed and visualized by live-cell confocal microscopy (magnification 1000×)**(A)** or lysed for internal fluorescence intensity measurements **(B)**. Normalized data is expressed as the ratio of mean fluorescence (minus background) to Tat fluorescence (minus background) ± SD from three independent experiments performed in triplicates. Comparisons between peptides internalization were assessed using the Mann-Whitney U statistical test. *p<0.05. AU, arbitrary units; CPP, cell-penetrating peptide; DIC, differential interference contrast.

Punctual localization within the nucleus and nucleolus was found particularly for AR-BS3 (Fig. V. 2A). Quantitative uptake experiments showed the peptides were differentially internalized by both

LNCaP (p=0.0273) and PC3 cells (p=0.0273). AR-BS3 was the most efficiently internalized among the three, with a translocation efficacy of 0.91 in PC3 cells and 1.29 in LNCaP cells (p=0.0495), performing better than Tat CPP in LNCaP cells (Fig. V. 2B). AR-BS1 was moderately cell--penetrating, with an efficacy index of 0.72 and 0.67 in LNCaP and PC3 cells, respectively (Fig. V. 2B). AR-BS2 showed less efficient cell-penetrating properties, with a translocation efficacy of about 0.20 in both cell lines (Fig. V. 2B).

3.3. Effect of AR-BS peptides, MSS1 and mitP on PCa cells' viability

An exploratory study was performed by incubating cells with different concentrations of AR-BS peptides (1, 3, 5, 10 and 20 μM) for different incubation periods (24 or 48 h). At the concentrations studied, we found no significant difference between the two time points (data not shown). Also, no effect was observed in cell viability when using the lowest concentrations of 1 and 3 μM (data not shown). The analysis of cell viability after 24 h treatment with 5, 10 or 20 μM of each AR-BS peptide showed no dose-dependent effect (Suppl. Table V. 2). Hence, for subsequent analyses, we treated LNCaP and PC3 cells with 10 μM of AR-BS1, AR-BS2, AR-BS3, AKAP4 BM M, MSS1 or mitP for 24 h. Differences in cell viability were observed for both LNCaP (p=0.0352) and PC3 (p=0.0298) cells. LNCaP cells showed decreased cell viability in response to treatment with AR-BS3 (mean 96%, p=0.0369), MSS1 (mean 66%, p=0.0369) and mitP (mean 32%, p=0.0369) when compared to cells with no treatment (Fig. V. 3A). Decreased viability of PC3 cells was observed in response to the three AR-BS peptides in similar proportion (AR-BS1 mean 89%, AR-BS2 mean 87% and AR-BS3 mean 88%; p=0.0369) when compared to cells with no treatment, as well as after treatment with MSS1 (mean 57%, p=0.0339) and mitP (mean 26%, p=0.0369) (Fig. V. 3A). The mutant AKAP4 peptide lacking the PP1-docking motif (AKAP4 BM M) revealed no effect on cell viability in all cell lines (Fig. V. 3A). These results indicate that treatments with mitP and MSS1 have a pronounced impact in PCa cells' viability, while treatments with AR-BS peptides have a minor effect.

3.4. Synergetic effect of AR-BS peptides on PCa cells' viability

To assess the potential synergetic effect of AR-BS peptides we treated cells with combinations of the peptides (5 or 10 μM of each peptide) for 24 h. All combinations, regardless of the concentration, significantly decreased the viability of both LNCaP and PC3 cells when compared to controls (cells with no treatment) (Fig. V. 3B). Only the combinations AR-BS2+AR-BS3 in PC3 cells (p=0.0463) and AR-BS1+AR-BS2+AR-BS3 in both LNCaP (p=0.0495) and PC3 cells (p=0.0463) showed a statistically significant dose-dependent decrease in cell viability (Fig. V. 3B). The synergetic effects of the peptides upon cell viability were more pronounced in LNCaP cells than in PC3 cells (Fig. V. 3B). The combination of the three peptides, at 10 μM each, was the most

effective in reducing the viability of LNCaP (mean 68%, p=0.0369) and PC3 cells (mean 80%, p=0.0369) (Fig. V. 3B). Altogether, the results support the existence of a synergetic effect of the AR-BS peptides in modulating PCa cells' viability, mainly when the three peptides are combined.

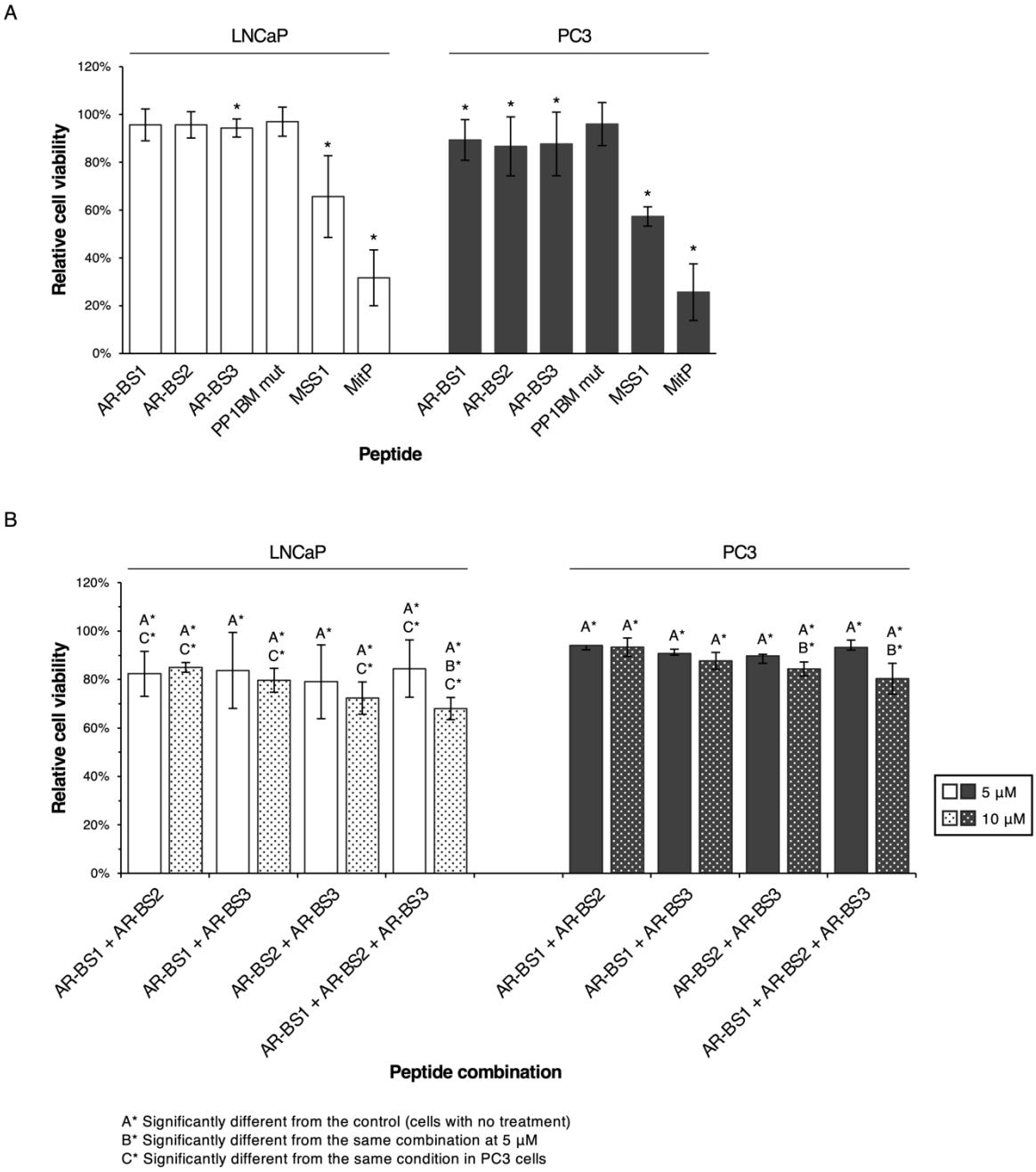


Fig. V. 3

Viability of PCa cells in response to peptides treatment. LNCaP and PC3 cells were treated with AR-BS1, AR-BS2, AR-BS3, AKAP4 BM M, MSS1 or mitP (10 µM) for 24 h **(A)** or treated with combinations of AR-BS peptides (5 or 10 µM of each peptide) for 24 h to assess their synergetic effect **(B)**. Cell viability was evaluated using the PrestoBlue Cell Viability Reagent according to the manufacturer's instructions. Percentage cell viability was calculated from the ratio between treatment condition and control condition (cells with no treatment). Results are expressed as mean percentage ± SD from three independent experiments with five replicates per condition. Comparisons between peptides internalization were assessed using the Mann-Whitney U statistical test. *p<0.05.

3.5. Effect of AR-BS peptides on AR and PSA transcriptional levels

Since PP1 was reported to increase AR expression and transcriptional activity, we went further to assess the transcriptional levels of AR and PSA in LNCaP cells in response to treatment with each AR-BS peptide (10 μM) or the combination of the three (10 μM each). No significant differences were observed in the levels of neither AR nor PSA after each treatment (Fig. V. 4).

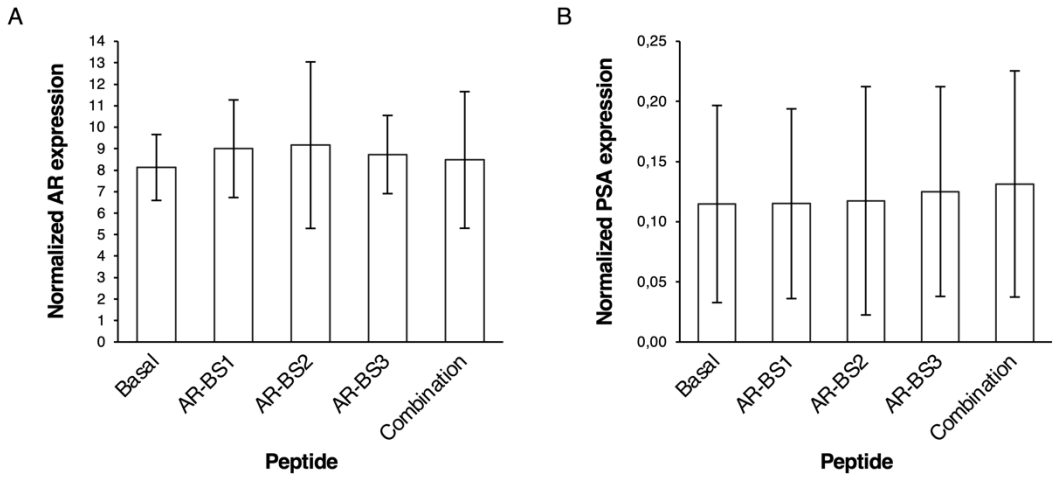


Fig. V. 4

AR and PSA transcriptional levels in response to treatment with AR-BS peptides. LNCaP cells were treated with 10 μM of AR-BS1, AR-BS2 or AR-BS3, or a combination of the three (10 μM each) for 24 h. Total RNA was extracted, and real-time PCR analysis was performed to quantify the levels of AR **(A)** and PSA **(B)**. Expression values were normalized to the levels of the BGUS housekeeping gene. Each condition was performed in triplicates and the experiment was repeated twice.

4. Discussion

In recent years, CPPs have been proven to be useful tools to target intracellular protein-protein interactions in cancers [687]. The relevance of AR-mediated signaling for PCa development and progression to life-threaten metastatic castration-resistant stages entitles it as the most enthused and challenging target for therapeutic purposes [688]. PP1 is a positive regulator of AR expression and activity [690,691]. PP1 and AR can interact directly (though the occurrence of indirect interactions cannot be excluded), but the nature of the binding is not yet fully understood.

Bioinformatics analysis of AR's primary sequence revealed the existence of three PP1-docking motifs (Fig. V. 1). In this study, we predicted and synthesized three potential bioportides to mimic the three PP1-docking motifs—AR-BS1, AR-BS2 and AR-BS3, numbered according to the correspondent binding site. All peptides were shown to be internalized by both androgen-dependent and castration-resistant PCa cells, albeit with different efficacies (Fig. V. 2). Several studies showed that CPPs are internalized by direct membrane translocation and/or energy-dependent endocytosis. Once inside cells, additional events might determine the achievable intracellular concentration, including premature proteolysis [703]. Also, intracellular accretion of CPPs and bioportides in

organelles, macromolecular entities and specific proteins has been discussed [704] as shown, for instance, for Tat, which accumulates within acidic secretory granules [705]. AR-BS peptides also displayed a typical vesicular distribution within the cells (Fig. V. 2A). However, quantitative uptake analysis indicates a clearly lesser intracellular concentration of AR-BS2 (Fig. V. 2B). From the sequence point of view, this is surprising since CPP prediction algorithm scored AR-BS2 as the most likely CPP (Suppl. Table V. 1). It is possible then that intracellular events determine its behavior. Although all binding sites are RVxF motifs—the most frequently observed motif in PP1-interacting proteins—BS2 has a distinct pattern from the other two: BS1 and BS3 share the pattern [RK]-X(0,1)-[VI]-{P}-[FW], which has high sensitivity but low specificity for PP1; whereas BS2 presents the pattern [HKR]-[ACHKMNQRSTV]-V-[CHKNQRST]-[FW], which has lower sensitivity but higher specificity (Suppl. Table V. 1) [706]. In spite of the diminished intracellular accumulation of AR-BS2 when compared to AR-BS1 and AR-BS3, the resultant decrease in cell viability was identical (Fig. V. 3A). Therefore, an improved characterization of AR-BS2 uptake and follow-up inside cells might help to determine the optimal conditions to maximize its intracellular concentration and cellular effect.

The relevance played by each motif in regulating AR/PP1 interaction might also influence the intracellular concentration and distribution of AR-BS peptides within cells. Previous reports showed that PP1 preferably associates with AR's LBD and decreases its polyubiquitylation and subsequent degradation [707]. Also, PP1 was shown to dephosphorylate AR^{Ser650} in the LBD, thereby contributing to the maintenance of AR in the nucleus and promoting its transcriptional activity [690]. Due to these observations, we also analyzed the transcriptional levels of AR and its downstream target, PSA, in response to treatment with the AR-BS peptides but no alterations were observed (Fig. V. 4). It cannot be excluded, though, possible alterations in AR expression at the protein levels, since PP1 was shown to impair AR degradation [707]. On the other hand, PP1 was also reported as a positive regulator of AR-V7, which only shares the KVFF motif with the canonical AR isoform and lacks the LBD (Fig. V. 1B). Despite the fact that a direct association between PP1 and AR-V7 is yet to be confirmed, PP1 was shown to dephosphorylate AR^{Ser213} and prevent its proteasome-mediated degradation [291]. Given what is already known for other PP1-interacting proteins, the binding to PP1 can primarily occur via a specific groove but further enhanced by additional interaction sites [708]. Therefore, the three RVxF motifs in AR might contribute to a precise binding code that increases the specificity of the binding in cellular context where PP1-interacting proteins compete with each other for binding to PP1. In fact, we observed a higher reduction in cell viability when using AR-BS peptides synergistically (Fig. V. 3B), which might indicate a better performance in interfere with substrate recruitment. Future *in vitro* studies to assess the ability of AR-BS peptides to disrupt AR/PP1 interaction will help to highlight these issues.

MSS1 was developed as an optimized AKAP4-mimetic sequence peptide to modulate sperm motility [693]. *In vitro* studies confirmed its ability to disrupt the interaction between PP1γ2, a testis- and sperm-specific PP1 isoform, and AKAP4 [693]. In this study, we also show that MSS1 decreases the viability of PCa cells (Fig. V. 3A). Interestingly, AKAP4 is a cancer/testis antigen and was previously observed in the cytoplasm and cell membrane of LNCaP cells, as well as in human PCa biopsies [709]. We have also detected the presence of AKAP4 in PC3 cells by immunoblotting (data not shown). On the other hand, we could not detect PP1γ2 neither in LNCaP nor in PC3 cells. Hence, it is possible that MSS1 disrupts the interaction between other PP1 isoforms and AKAP4, if existent, or even other substrates. MitP is a mitochondriotoxic bioportide with demonstrated pro-apoptotic properties in glioblastoma astrocytoma and urinary bladder cancer cells [710]. Here, we also report mitP as a cytotoxic agent for both androgen-dependent and castration-resistant PCa cells (Fig. V. 3A). Given these results, the effects of both MSS1 and mitP in prostate carcinogenesis is worth further investigation.

5. Conclusion

We strongly believe that AR/PP1 interaction is an enthusiastic therapeutic target in PCa and that future optimization in the design of our peptides would be of utmost importance to achieve higher and broader efficacy and improve the hitherto described results. Several strategies could be employed to improve the delivery efficiency and the intracellular stability of our peptides as already applied in previous studies that aimed to optimize existing CPPs (as recently revised in [711]). For instance, CPPs can be modified to prevent either their recognition or access to cleavable sites by hydrolytic enzymes. Different approaches have successfully been applied to overcome these issues, including amino acid substitutions (e.g., substitute protease cleavage sites by protease-resistant residues), stereochemical modifications (i.e., replace L-amino acids by D-amino acids) and shielding strategies (e.g., polymer conjugation with polyethylene glycol), among others. Additionally, conformational stabilization (e.g., by cyclization, disulfide-induced dimerization and dendrimer formation) could enhance cellular uptake and endosomal escape. Another promising idea is to conjugate the bioportides with tumor-homing sequences to accomplish selectivity towards tumor cells [711]. Overall, the results are promising in showing the potential of using PP1-docking motif-mimetic bioportides to modulate prostate carcinogenesis.

6. Supplementary data

Suppl. Table V. 1
Cell-penetrating peptides prediction based on the PP1-docking motifs in AR's primary sequence.

Peptide sequence	Score	Charge	Molecular weight
Binding site 1			
GSC**KVFF**KRAA	0.290	3.00	1213.60
GSC**KVFF**KRAAEGKQK	0.421	4.00	1784.33
GSC**KVFF**KRAAKGKQK	0.570	6.00	1783.39
Binding site 2			
RQLV**HVVKW**AKAL	0.750	3.50	1548.11
RQLV**HVVKW**AKKL	0.793	4.50	1605.21
Binding site 3			
SGKV**KPIYF**HTQ	0.106	2.50	1404.81
SGKV**KPIYF**HTGRKKRRQRRRPPQ	0.736	10.50	2977.87

The potential peptides were selected from the analysis of AR's primary sequence. PP1-docking motifs (bold) and flaking residues were analyzed through CPP prediction databases and changes were introduced to improve their scores. Scores were obtained from CPPpred (http://bioware.ucd.ie/cpppred [697]) and are interpreted as follows: 0-0.5, the peptide is very unlikely to be cell-penetrating; 0.5-1.0, the peptide is predicted to be cell-penetrating (the closer to 1.0 the more confident that the peptide will be cell-penetrating). Charge and molecular weight were calculated using CellPPD (http://crdd.osdd.net/raghava/cellppd/ [695,696]). AR, androgen receptor; PP1, serine/threonine-protein phosphatase PP1.

Suppl. Table V. 2
Cell viability in response to treatment with different concentrations of AR-BS peptides for 24 h.

		LNCaP			PC3		
		5 µM	10 µM	20 µM	5 µM	10 µM	20 µM
AR-BS1	Mean	97%	96%	94%	89%	89%	81%
	SD	0,08	0,07	0,05	0,09	0,09	0,12
AR-BS2	Mean	95%	96%	83%	93%	87%	81%
	SD	0,09	0,06	0,18	0,08	0,12	0,17
AR-BS3	Mean	91%	94%	99%	79%	88%	84%
	SD	0,11	0,04	0,07	0,16	0,13	0,10

Percentage cell viability was calculated from the ratio between treatment condition and control condition (cells with no treatment). Results are expressed as mean ± SD from three independent experiments with five replicates per condition.

CHAPTER VI

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

This study was motivated by robust supporting evidence for the involvement of PP1 in prostate carcinogenesis yet addressed in a very limited number of studies. Our main goal was to unravel the PP1 interactome to identify potential biomarkers and therapeutic targets for PCa. To fulfill the main goal, we established a set of secondary goals and tasks to guide our investigation and allow us to better characterize the three PP1c canonical isoforms in human PCa models, identify the PP-1G interactome in human PCa and address the feasibility of using PP1-docking motif-mimetic cell-penetrating peptides to modulate prostate carcinogenesis. The hypotheses, aims, findings and final considerations of our work are summarized in Fig. VI. 1.

We started by analyzing the expression and localization of PP1c isoforms in human prostate tissues (normal and tumoral) and human prostate cell lines (preneoplastic, androgen-dependent and castration-resistant) in Chapter III. To improve our characterization, we complement the biochemical approach with comprehensive data mining of large PCa cohorts from The Cancer Genome Atlas (TCGA) program. Altogether, the results presented in Chapter II show that PP-1A, PP-1B and PP-1G are differentially expressed in PCa, providing the first comprehensive characterization of PP1c isoforms in PCa. As observed in other cancer types (Chapter Ib), the results also suggest that PP1c isoforms may have different roles in PCa-associated molecular events. To better clarify each PP1c-specific relevance for prostate carcinogenesis, phenotypic studies are a must-include part in future investigation.

In Chapter IV, we unraveled the PP-1G interactome in PCa using a combination of high-throughput techniques—Y2H and co-IP/MS—with bioinformatics analysis. To assist in our work, we started by compiling information regarding the tools available to analyze an interactome data set in the form of a tutorial (Chapter IVa). This allowed us to improve the workflow in order to retrieve relevant information from the data obtained by the experimental approaches, which is presented in Chapter IVb. The experimental search for PP-1G interactors was performed using paired normal and tumoral tissues to better mimic *in vivo* conditions; however, the construction of the respective cDNA libraries to produce easily identifiable positive clones in the Y2H screenings was proven to not be an easy task. This compromised the obtention of complete sequences for a reliable identification of the expressed proteins. Also, the inter-individual and inter-/intra-tumor heterogeneity was an issue for the quantification of the expression of PP-1G interactors by MS. Therefore, the increase of the sample size in future studies will be essential to unveil differentially regulated interactions in normal and tumoral conditions of the prostate. In spite of the experimental constrains, we were still able to identify at least part of the PP-1G interactome and to highlight interesting interactions that should be further validated by other methods and analyzed in dedicated studies.

In Chapter V, we designed and synthesized three cell-penetrating peptides derived from the three PP1-docking motifs in the AR amino acid sequence. The three peptides are internalized by human PCa cells, albeit with different efficacies. This was expected since two of them were rhegnylogically-designed, while one was coupled to the Tat sequence, which has widely been used with successful internalization. Though the peptides showed minor or no effect in reducing PCa cells' viability when assessed individually, their combination improved their performance. Additionally, we tested the recently optimized MSS1 bioportide, which significantly decreased the cell viability of both androgen-dependent and castration-resistant PCa cells. This result further strengthens our belief that PP1 interactions might be valuable therapeutic targets. Therefore, in future studies, we aim to assess the ability of the AR-BS peptides to disrupt AR/PP1 interaction and optimize their structure in view of significant impact in PCa cells dynamics.

In conclusion, the work described in this thesis is a further step into the characterization of PP1 expression, function and interactome in PCa—an ongoing investigation with several research opportunities and more questions than answers.

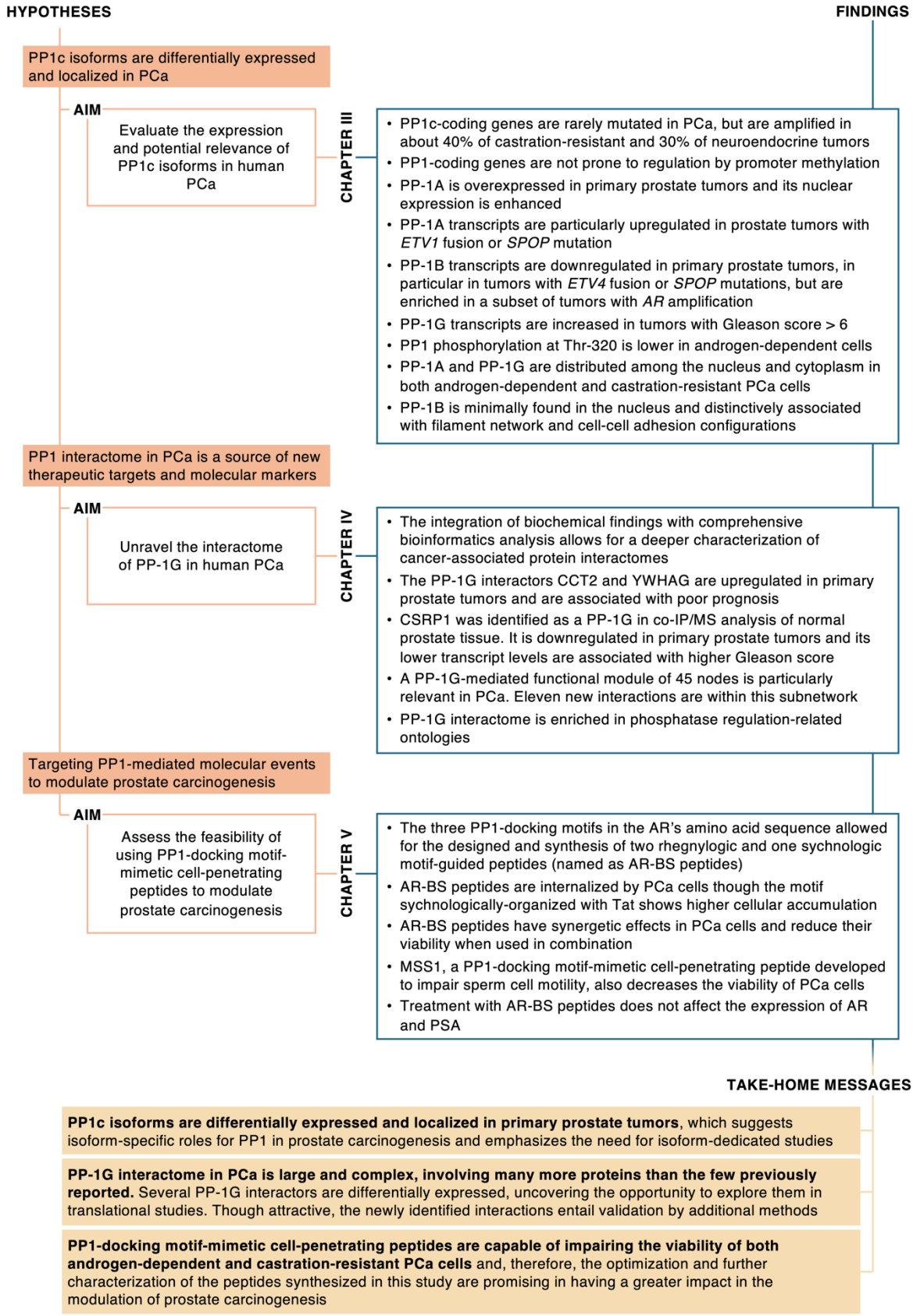


Fig. VI. 1
Hypotheses, aims, findings and final considerations of the present work.

CHAPTER VII

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