Androgen actions regulating TRIB1/3 in prostate cancer: effects on cell proliferation, survival, and metabolism

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"Science knows no country, because knowledge belongs to humanity, and is the torch which illuminates the world. Science is the highest personification of the nation because that nation will remain the first which carries the furthest the works of thought and intelligence."

— Louis Pasteur

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Resumo

O cancro da próstata (PCa) é uma neoplasia sensível aos andrógenos, sendo a sobrevivência e proliferação celular dependentes da regulação androgénica. Nesta fase, os casos de PCa podem ser tratados com terapias de privação de androgénios (ADT), as quais visam diminuir os níveis circulantes de androgénios ou bloquear as suas ações. No entanto, alterações genéticas incluindo a fusão de genes, amplificação de oncogenes, mutações e supressões de genes concorrem para promover o avanço da doença para formas mais agressivas. A seguir, ocorrem alterações fenotípicas/de comportamento, tais como a perda de sensibilidade aos androgénios, o que permite que as células cancerosas cresçam mesmo que os níveis de androgénios circulantes se mantenham baixos. É o chamado cancro da próstata resistente à castração (CRPC). Está bem estabelecido em vários tipos de células cancerosas a existência do chamado "efeito de Warburg", que consiste na reprogramação metabólica que permite às células cancerosas sobreviver, proliferar e formar metástases, utilizando elevadas quantidades de glicose como substrato energético, tanto em condições aeróbias como anaeróbias. As células tumorais utilizando glicólise para manter as necessidades energéticas, produzem subsequentemente grandes quantidades de lactato, o qual é exportado para o meio extracelular. No caso do PCa, a reprogramação metabólica para o fenótipo mais glicolítico acompanha a progressão da doença para o CRPC. Foi igualmente demonstrado que os androgénios são importantes reguladores do metabolismo das células de PCa, estimulando a glicólise, bem como o metabolismo lípidico.

A família de pseudocinases Tribbles (TRIBs), que inclui a TRIB1, TRIB2 e TRIB3, foi descrita como tendo um papel no controlo da diferenciação, transcrição, proliferação e metabolismo. Estas proteínas caracterizam-se por funcionar como moléculas de tipo andaime ou adaptadoras em muitas redes de vias de sinalização, incluindo módulos de proteína cinase activada por mitógeno (MAPK) e fosfinoinositede 3-cinase (PI3K-Akt). Consistentes com as ações descritas e o controlo da sinalização intracelular, as TRIBs têm estado envolvidas no desenvolvimento de patologias diversas, bem como estão implicadas em vários cancros. As evidências indicam o papel oncogénico da TRIB1 e TRIB3 e o seu estado de elevada expressão na iniciação e progressão de tumores, especialmente no melanoma, pulmão, fígado, e leucemias agudas, mas também no PCa. No entanto, os mecanismos que controlam a expressão

das TRIBs no PCa ainda são mal compreendidos. Além disso, foi demonstrado o envolvimento da TRIB1 e TRIB3 na homeostase dos lípidos.

O principal objetivo desta tese é investigar o papel dos androgénios na regulação da expressão da TRIB1 e TRIB3 no PCa e lançar luz sobre a influência das TRIBs no destino das células da próstata. A modulação androgénica das TRIBs em células PCa foi determinada usando 10 nM 5α-dihidrotestosterona (DHT), na presença ou ausência do anti-androgénio bicalutamida (10 μM). O tratamento com DHT diminuiu a expressão da proteína e mRNA das TRIB1 e TRIB3 nas células de PCa sensíveis aos androgénios, LNCaP e 22Rv1, sem alterações observadas nas células não neoplásicas PNT1A. A bicalutamida (10 μM) bloqueou o efeito da DHT na supressão da expressão da TRIB1 em células LNCaP e 22Rv1, o que sugere o envolvimento do recetor de androgénios (AR). Estes resultados foram validados pela análise da expressão do gene padrão alvo dos androgénios, o antigénio específico da próstata (PSA), confirmando-se o aumento da sua expressão nas células tratadas com DHT e o bloqueio dos efeitos pela bicalutamida.

Para aprofundar o estudo do impacto do AR na regulação da expressão da TRIB1, realizámos o knockdown (KD) do AR em células LNCaP e 22Rv1 e avaliámos a expressão da TRIB1. O KD do AR resultou num aumento significativo da expressão do mRNA da TRIB1 em células 22Rv1. Estes resultados estão em linha com os obtidos *in vivo*, uma vez que ratos castrados exibiram níveis de expressão do mRNA de TRIB1 aumentados em comparação com o controlo, o que realça os efeitos reguladores dos androgénios na expressão das TRIBs no PCa. Além disso, utilizando a análise ChiP-seq em tumores primários humanos *versus* tecidos sólidos normais, foi demonstrado que o AR se liga directamente ao *locus* dos genes *TRIB1* e *TRIB3*. As células LNCaP tratadas com o androgénio sintético r1881 também apresentaram um padrão semelhante de regulação das TRIBs. Em conjunto, estas descobertas proporcionam uma visão útil das ações do AR na regulação da expressão da TRIB1 no PCa. Quanto aos efeitos fisiológicos sobre o destino das células de PCa, observou-se uma maior viabilidade celular, proliferação e migração nas células tratadas com androgénio, enquanto que não se observaram alterações na atividade da caspase-3.

As TRIBs estão associadas a vias de sinalização e alvos moleculares como o pERK e o pAkt, conhecidos por terem papéis proeminentes na sobrevivência, apoptose, e diferenciação em resposta a uma gama de estímulos, incluindo hormonas esteróides.

Foi assim também medida a sua expressão após tratamento com 10 nM DHT, sendo observada uma desregulação da sua expressão.

A TRIB1 demonstrou ser sobre-expressa no PCa e associada à progressão da doença. Contudo, não é claro se os níveis de expressão aumentados da TRIB1 promovem a transformação maligna das células da próstata. Portanto, foi investigada a influência dos níveis de expressão da TRIB1 nas células da próstata não-neoplásicas (PNT1A) e neoplásicas (PC3). A sobreexpressão da TRIB1 (OE) em células PNT1As aumentou a proliferação e migração celular, sem alterações na atividade da caspase-3 e na viabilidade celular. O efeito da TRIB1 OE no metabolismo lipídico também foi medido tendo-se observado um aumento significativo de gotículas lipídicas nas célula PNT1A com OE TRIB1. Isto foi acompanhado pelo aumento da expressão da sintetase de ácidos gordos (FASN), uma proteína alvo chave no metabolismo lipídico em condições neoplásicas. Globalmente, a OE de TRIB1 mudou as características das células não neoplásicas de PNT1A para um fenótipo semelhante ao cancro. Curiosamente, não foram observadas alterações sobre a viabilidade, proliferação e metabolismo das células PC3 com silenciamento da TRIB1. Estes resultados implicam a TRIB1 na regulação do metabolismo, bem como sugerem o seu papel como "força motriz" para a iniciação de tumores e progressão da doença nas fases iniciais do desenvolvimento da PCa. Em resumo, esta dissertação demonstrou as ações androgénicas na regulação da expressão das TRIBs em células de PCa. Revelando a ligação entre as TRIBs, androgénios e AR no desenvolvimento e progressão do PCa. Coletivamente, mais investigação sobre estas interações permitirá justificar as TRIBs como base para o desenvolvimento de novas abordagens terapêuticas para a PCa.

Palavras-chave

Androgénios, Tribbles, TRIB1, TRIB3, cancro da próstata.

Resumo alargado

O cancro da próstata (PCa) é um cancro comum entre os homens, representando o segundo cancro mais diagnosticado e a quinta causa de morte por cancro em todo o mundo. Inicialmente, o PCa é sensível aos androgénios com a sobrevivência celular e a proliferação das células tumorais dependente da ação desta classe de hormonas esteroides. Nesta fase, o PCa pode ser controlado com o recurso às terapias de privação de androgénios (ADT), as quais funcionam diminuindo ou bloqueando os níveis de androgénios circulantes. Os pacientes geralmente respondem bem à ADT, no entanto, a resistência à terapia ocorre após administração continuada do tratamento por períodos de tempo entre 2 a 3 anos. A progressão do PCa com a perda de sensibilidade androgénica representa a evolução para uma forma mais agressiva da doença, a qual é acompanhada por alterações genéticas incluindo a fusão de genes, amplificação de oncogenes, mutações e supressões de genes. , Estas alterações sustentam o crescimento e progressão do PCa independentemente dos níveis de androgénios circulantes, levando ao desenvolvimento do cancro da próstata resistente à castração (CRPC), em que as células tumorais são capazes de sobreviver e metastatizar mesmo que na ausência de androgénios. O CRPC está geralmente relacionado com elevadas taxas de mortalidade e limitadas opções terapêuticas. Além disso, várias alterações genéticas e metabólicas têm sido identificadas como necessárias para a progressão para a fase agressiva de CRPC. A reprogramação metabólica é uma alteração observada em células cancerosas permitindo-lhes sobreviver, proliferar e metastizar, ainda que em ambientes desfavoráveis. Estas alterações foram primeiramente descritas naquilo que é conhecido como o efeito de Warburg, Os seus estudos pioneiros em 1927 mostraram que as células cancerosas usam altas quantidades de glicose como substrato energético produzindo grandes quantidades de lactato, o que acontece tanto em condições aeróbicas como anaeróbias. O lactato produzido e exportado para o espaço extracelular leva a uma acidificação do ambiente tumoral que tem ações importantes na supressão imune e favorecimento da progressão tumoral. No caso do PCa, este apresenta um metabolismo muito particular em que a reprogramação metabólica para o fenótipo mais glicolítico acompanha a progressão da doença para o CRPC. Na verdade, apenas os estádios mais avançados da doença se caracterizam por apresentar o efeito de Warburg. Estudos anteriores do grupo de investigação e outros, demonstraram que os androgénios são importantes reguladores do metabolismo das células de PCa,

estimulando a glicólise, assim como o processamento de lípidos. No caso do PCa o metabolismo lipídico apresenta alterações importantes e é uma via associada à carcinogénese.

As Tribbles (TRIBs) são pseudoenzimas, ou seja, são estruturalmente relacionadas com enzimas ativas, no entanto, não têm atividade catalítica. Esta família de pseudoquinases, que inclui TRIB1, TRIB2, e TRIB3, tem sido descrita em vários estudos como tendo um papel no controlo da diferenciação, transcrição, proliferação, e metabolismo, além de estar envolvida no desenvolvimento de diversas doenças. As TRIBs têm vindo a ser implicadas em vários tipos de cancros devido, por exemplo, à sua elevada sobreexpressão na iniciação e progressão do tumor. As suas ações oncogénicas têm vindo a ser reportadas no melanoma, cancro do pulmão, cancro do fígado, e leucemias agudas, mas também no PCa. Contudo, vários aspetos da atividade dos TRIBs parecem ser específicos de alguns tipos de células e organismos. Os três membros da família TRIBs funcionam como moléculas andaimes ou adaptadores em muitas vias de sinalização, incluindo os módulos de quinase proteica ativada por mitogénios (MAPK) e 3-quinase (PI3K-Akt). Ao longo dos anos, têm vindo a ser exploradas os mecanismos de atuação e condições que alteram a atividade das TRIBs, nomeadamente, o seu papel na ativação das vias de sinalização. No entanto, os mecanismos que controlam a expressão das TRIBs, particularmente no PCa, continuam mal compreendidos. Por outro lado, foi demonstrado o envolvimento da TRIB1 e TRIB3 na homeostase dos lípidos, o que levanta a questão sobre a sua relação com o metabolismo do PCa e a regulação androgénica.

O objetivo desta tese é investigar o papel dos androgénios na regulação da expressão da TRIB1 e TRIB3 no PCa e clarificar a influência das TRIBs no destino das células prostáticas. A modulação androgénica das TRIBs em células PCa foi determinada usando 10 nM 5α-dihidrotestosterona (DHT), um androgénio clássico não aromatizável e que tem uma ação crucial na fisiologia prostática. As experiências de estimulação com DHT foram realizadas na presença ou ausência do anti-androgénio bicalutamida numa concentração de 10 μM. Esta concentração tem sido testada em diferentes cenários, tendo demonstrado ser efetiva na inibição dos efeitos da DHT.

O tratamento com DHT diminuiu a expressão da proteína e mRNA das TRIB1 e TRIB3 nas células de PCa sensíveis aos androgénios, LNCaP e 22Rv1, sem alterações observadas nas células não neoplásicas PNT1A. Estes resultados demonstraram ainda

num painel de células da próstata que, i) tanto as células não neoplásicas como as neoplásicas têm uma expressão basal de TRIB1 e TRIB3; e que ii) a expressão mais elevada das TRIBs foi observada em células de PCa mais agressivas. Confirmou-se também que a bicalutamida (10 μM) bloqueou o efeito da DHT na supressão da expressão da TRIB1 em células LNCaP e 22Rv1, o que sugere o envolvimento do recetor de androgénios (AR) nesta regulação. Estes resultados foram validados pela análise da expressão do gene padrão alvo dos androgénios, o antigénio específico da próstata (PSA), confirmando-se o aumento da sua expressão nas células tratadas com DHT e o bloqueio dos efeitos pela bicalutamida.

Para aprofundar o estudo do impacto do AR na regulação da expressão da TRIB1, realizámos o knockdown (KD) do AR em células LNCaP e 22Rv1 e avaliámos a expressão da TRIB1. O KD do AR, efetuado com o uso de siRNAs, resultou num aumento significativo da expressão do mRNA da TRIB1 em células 22Rv1, o que suporta a regulação pelo AR. Estes resultados estão em linha com os obtidos *in vivo*, uma vez que ratos castrados e, portanto, com níveis de androgénios circulantes diminuídos, apresentaram expressão aumentada do mRNA de TRIB1 em comparação com o controlo, o que igualmente realça os efeitos reguladores dos androgénios na expressão das TRIBs no PCa. A proteína Homeobox Nkx3.1, um gene regulado pelos androgénios e que é expresso na maioria dos adenocarcinomas primários da próstata, apresentou níveis de expressão do mRNA diminuídos em animais castrados, o que confirmou o sucesso da castração.

Além disso, utilizando a análise ChiP-seq em tumores primários humanos *versus* tecidos sólidos normais, foi demonstrado que o AR se liga directamente ao *locus* dos genes *TRIB1* e *TRIB3*. As células LNCaP tratadas com o androgénio sintético r1881 também apresentaram um padrão semelhante de regulação das TRIBs. Em conjunto, estas descobertas proporcionam uma visão útil das ações do AR na regulação da expressão da TRIB1 no PCa. Quanto aos efeitos fisiológicos sobre o destino das células de PCa, observou-se um aumento da viabilidade celular, proliferação e migração nas células tratadas com androgénios, enquanto que não se observaram alterações na atividade da caspase-3.

As TRIBs estão associadas a vias de sinalização e alvos moleculares como o pERK e o pAkt, conhecidos por terem papéis proeminentes na sobrevivência, apoptose, e diferenciação em resposta a uma gama de estímulos, incluindo hormonas esteróides.

Foi assim também medida a sua expressão após tratamento com 10 nM DHT, sendo observada uma desregulação da sua expressão. Estes resultados sugerem que a alteração da expressão das TRIBs em resposta à DHT tem consequências no cenário de comunicação intracelular e vias de sinalização ativas.

A TRIB1 demonstrou ser sobre-expressa no PCa e associada à progressão da doença. Contudo, não é claro se os níveis de expressão aumentados da TRIB1 promovem a transformação maligna das células da próstata. Assim, foi investigada a influência da TRIB1 nas células da próstata não-neoplásicas (PNT1A) e neoplásicas PC3. Como mencionado anteriormente, as células PNT1A apresentam baixa expressão de TRIB. Portanto, a expressão de TRIB1 foi manipulada sobreexpressando-a em células PNT1A e silenciando-a nas células PC3. A sobreexpressão da TRIB1 em células PNT1 aumentou a proliferação e migração celular, sem alterações na atividade da caspase-3 e na viabilidade celular. O efeito da sobre-expressão da TRIB1 no metabolismo lipídico também foi medido tendo-se observado um aumento significativo de gotículas lipídicas nas células PNT1A com níveis aumentados de TRIB1. A acumulação de lípidos intracelulares foi acompanhada pelo aumento da expressão da sintetase de ácidos gordos (FASN). A FASN é uma proteína chave no metabolismo lipídico, cuja expressão está bastante aumentada em condições neoplásicas, sendo determinante para suportar a proliferação celular e geração de membrana através do aumento da síntese se ácidos gordos. Globalmente, a sobre-expressão da TRIB1 mudou as características das células não neoplásicas PNT1A para um fenótipo semelhante ao cancro. Curiosamente, não foram observadas alterações na viabilidade, proliferação e metabolismo das células PC3 com silenciamento da TRIB1. Estes resultados implicam a TRIB1 na regulação do metabolismo, bem como sugerem o seu papel como "força motriz" para a iniciação de tumores e progressão da doença nas fases iniciais do desenvolvimento da PCa. As evidências aqui reportadas suportam o papel oncogénico da TRIB1 e investigação adicional para clarificar as suas ações.

Em resumo, esta dissertação demonstrou que os androgénios regulam a expressão das TRIBs em células de PCa, e que a ligação TRIBs, androgénios e AR pode contribuir para o desenvolvimento e progressão do PCa. Coletivamente, mais

investigação sobre estas interações permitirá justificar as TRIBs como base para o desenvolvimento de novas abordagens terapêuticas para a PCa.

Palavras-chave

Androgénios, Tribbles, TRIB1, TRIB3, cancro da próstata.

Abstract

Prostate cancer (PCa) is an androgen-sensitive neoplasia with cell survival and proliferation being dependent on androgenic regulation. At this stage, PCa cases can be treated with androgen deprivation therapy (ADT), which aims to decrease the circulating levels of androgens or block their actions. However, genetic alterations including the fusion of genes, amplification of oncogenes, gene mutations and deletions concur promoting the advance of the disease to more aggressive forms. Following this, phenotypic/behaviour changes such as loss of androgen sensitivity occur, allowing the cancer cells grow even if circulating androgens are low. It is the so-called castrate resistant prostate cancer (CRPC). A well-established observation 'in several cancer cell types is the Warburg effect', the metabolic reprogramming that enables cancer cells to survive, proliferate and metastasize, using high amounts of glucose as energy substrate, both in aerobic and anaerobic conditions. Tumour cells using glycolysis to maintain energy needs, subsequently produce high amounts of lactate that is exported to the extracellular medium. In the case of PCa, the metabolic reprogramming towards the more glycolytic phenotype accompanies the progression of disease to the CRPC. It was demonstrated that androgens are important regulators of PCa cells metabolism, enhancing glycolysis as well as lipid handling.

The pseudokinase family Tribbles (TRIBs), which includes TRIB1, TRIB2 and TRIB3, has been described to have a role in controlling differentiation, transcription, proliferation, and metabolism. These pseudokinase proteins are characterized by functioning as scaffold-like or adaptor molecules in many signalling pathway networks including mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K-AKTf) modules. Consistent with the described actions and the control of intracellular signalling, TRIBs have being involved in disease development, as well as are implicated in several cancers. Evidence indicates the oncogenic role of TRIB1 and TRIB3 and their highly expressed state in tumour initiation and progression, especially in melanoma, lung, liver, and acute leukemia, but also in PCa. Yet, the mechanisms that control TRIBs expression in PCa are still poorly understood. Moreover, the involvement of TRIB1 and TRIB3 in lipids homeostasis has been demonstrated.

The main goal of this thesis was to investigate the role of androgens in regulating TRIB1 and TRIB3 expression in PCa and to shed light on the influence of TRIBs on

prostate cell fate. The androgenic modulation of TRIBs in PCa cells was determined using 10 nM 5α-dihydrotestosterone (DHT), in the presence or absence of the anti-androgen bicalutamide (10 μM). DHT treatment decreased TRIB1 and TRIB3 expression at a protein and mRNA level in the androgen sensitive LNCaP and 22Rv1 cells with no changes observed in the non-neoplastic PNT1A cells. Bicalutamide (10 μM) blocked the effect of DHT in down-regulating TRIB1 expression in LNCaP and 22Rv1 cells, which suggests the involvement of the androgen receptor (AR). These findings were validated by the expression analysis of the standard androgen-responsive gene prostate-specific antigen (PSA), confirming its increased expression in DHT treated cells and blocking of effects by bicalutamide. To further study the impact of the AR in TRIB1 regulation, we performed a knockdown (KD) of the AR in LNCaP and 22Rv1 cells and measured TRIB1 expression. AR KD resulted in a significant TRIB1 increase in 22Rv1 cells at an mRNA level. These outcomes are in line with those obtained *in-vivo*, as castrated mice displayed increased TRIB1 mRNA expression levels compared to control, which highlights the androgens' regulatory effects of TRIBs in PCa. Moreover, using ChiP-seq analysis in human primary tumours vs normal solid tissues it was shown that AR directly binds to the TRIB1 and TRIB3 gene locus. LNCaP cells treated with the synthetic androgen r1881 also showed a similar pattern of TRIBs downregulation. Together these findings provide useful insight into AR actions in regulating TRIB1 expression in PCa. Concerning the physiological effects on PCa cell fate, enhanced cell viability, proliferation and migration were observed in androgen treated cells, whereas no alterations were seen on caspase-3 activity.

TRIBs associated signalling pathways targets such as pERK and pAKT, prominently known to have roles in survival, apoptosis, and differentiation in response to a range of stimuli including steroid hormones were also measured after 10 nM DHT treatment and downregulation of their expression was observed.

TRIB1 has been shown to be overexpressed in PCa and associated with the progression of disease. However, it is not clear if is the TRIB1 increased expression levels that promote the malignant transformation of prostate cells. Therefore, the influence of TRIB1 expression levels in non-neoplastic (PNT1A) and neoplastic (PC3) prostate cells fate was investigated. TRIB1 overexpression (OE) on PNT1As increased cell proliferation and migration, with no changes on caspase-3 activity and cell

viability. The effect of TRIB1 OE on lipid metabolism was also measured and a significant increase of lipid droplets was observed in PNT1A cell OE TRIB1. This was accompanied by the increased expression of fatty acid synthase (FASN), a key target protein in lipid metabolism in neoplastic conditions. Overall, TRIB1 OE changed the features of non-neoplastic PNT1A cells to a cancer-like phenotype. Interestingly, no changes were observed on the viability, proliferation and metabolism of TRÎB1 knockdown PC3 cells. These results implicate TRIB1 in the regulation of metabolism, as well as suggest its role as a 'driving force' for tumour initiation and progression of disease in the early stages of PCa development. In summary, this dissertation demonstrated the androgen actions in the regulation of TRIBs in PCa cells. Revealing the link between TRIBs, androgens and AR in PCa development and progression. Collectively, further research on these interaction warrants TRIBs as a basis for the development of new therapeutic approaches for PCa.

Keywords

Androgens, Tribbles, TRIB1, TRIB3, prostate cancer.

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List of Abbreviations

ACC	Acetyl coenzyme A carboxylase
ADT	Androgen Deprivation Therapy
AFS	Anterior fibromuscular stroma
AKT	Protein kinase B
AML	Acute myeloid leukaemia
AP-1	Activator protein 1
AP-4	Activator protein 4
APL	Acute promyelocytic
APPL1	Adaptor Protein, Phosphotyrosine Interacting with PH Domain & Leucine Zip
AR	Androgen Receptor
ARE	Androgen Response Elements
ATF4	Activating transcription factor 4
ATF6	Transcription factor 6
BMP	bone morphogenetic proteins
BPH	Benign prostatic hyperplasia
C/EBP	CCAAT/enhancer binding protein
CHOP	CCAAT/enhancer-binding protein Homologous Protein
c-MYC	Multifunctional transcription factor NKX3.1
COP1	E3 Ubiquitin Ligase
CRC	Colorectal cancer
CRPC	Castration Resistant Prostate Cancer
CT	C-terminal
CZ	Central Zone
DBD	DNA Binding Domain
DHT	5α-dihydrotestosterone
E2F	E2 factor
EGFR	Epidermal Growth Factor Receptors
EMT	Epithelial Mesenchymal Transition
ER	Estrogen Receptor
ERK	Extracellular Signal-Regulated Kinases
EVI1	Ecotropic viral integration site-1
FASN	Fatty acid synthase
FOG	Folded gastrulation

FOXO	Forkhead box O
GATA2	GATA-binding factor 2
GPCR	G-protein coupled receptors
GSK3β	Glycogen synthase kinase 3 beta
GSTP1	Glutathione S-transferase pi
HCC	Hepatocellular carcinoma
HDAC1	Histone deacetylase-1
HDL	High-density lipoprotein
HIF-1α	Hypoxia-inducible factor-1α
HNF4A	Hepatocyte nuclear factor 4 Alpha
HOXA9	Homeobox A9
IGF-1	Insulin-like Growth Factor Receptor
IL-3	Interleukin-3
JNK	c-Jun N-terminal kinases
KD	Knockdown
KGF	Keratinocyte Growth Factor Receptor
KO	Knockout
LBD	Ligand Binding Domain
MAPK	Mitogen-activated Protein Kinase
MEIS1	MEIS Homeobox 1
MSCR1	Macrophage Scavenger Receptor 1
mTOR	mammalian target of rapamycin
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NKX3.1	NK3 Homeobox 1
NOTCH1	Notch homolog 1
NT	N-terminal
NTD	N-Terminal Domain
OE	Overexpression
pAKT	Phosphorylated Protein kinase B
PAP	Prostatic Acid Phosphatase
PCa	Prostate Cancer
pERK	Phosphorylated Extracellular Signal-Regulated Kinases
PERK	Phosphorylation of protein kinase-like ER kinase
PI3K	Phosphoinositide 3-kinases

PIA	Proliferative Inflammatory Atrophy
PIN	Prostatic intraepithelial neoplasia
PIP3	PI (phosphoinositide) 3-phosphatase
PITX1	Paired-like homeodomain 1
PML-RARα	Promyelocytic leukemia-retinoic acid receptor α
PPARγ	Peroxisome proliferator-activated receptor gamma
PSA	Prostate Specific Antigen
PTEN	Phosphatase and Tensin Homolog
PZ	Peripheral Zone
RCC	Renal cell carcinoma
RNASEL	Ribonuclease L
SNIP1	Smad Nuclear Interacting Protein 1
SREBP-1	Sterol regulatory element binding proteins 1
TCF4	Transcription factor 4
TF	Transcription Factor
THC	Delta-9-tetrahydrocannabinol
TLR	Toll-like receptors
TRIB	Tribbles
TZ	Transition Zone
vLDL	Very-low-density lipoprotein
XBP1	X-box binding protein 1
ZEB1-AS1	Zinc finger E-box-binding homeobox 1-antisense 1

Chapter I:

General Introduction

1. The Prostate: an overview of the gland and cancer aetiology

1.1. Anatomy and physiology of the prostate gland

The prostate is an accessory gland of the male reproductive system, situated at the base of the bladder and including the proximal portion of the urethra [1]. The prostate comprises of mainly three regions including; central, peripheral, and transition zone [2] (Fig. I.1). Approximately 70% of the prostate tissue is made up of the peripheral zone [3]. The remaining is shared between the central zone (25%), which begins at the seminal vesicles and run down to the prostatic urethra [2], and the transition zone (5%) that surrounds the prostatic urethra, and with age it increases in its size [4]. The lateral and posterior areas of the prostate are surrounded by a fibrous connective tissue [2]. The anterior fibromuscular stroma is a non-glandular section, which forms a thick layer that binds and shields the three glandular zones [5]. The prostate gland zones can also be divided under common anatomical orientation in anterior, dorsal, lateral and ventral lobes [3].

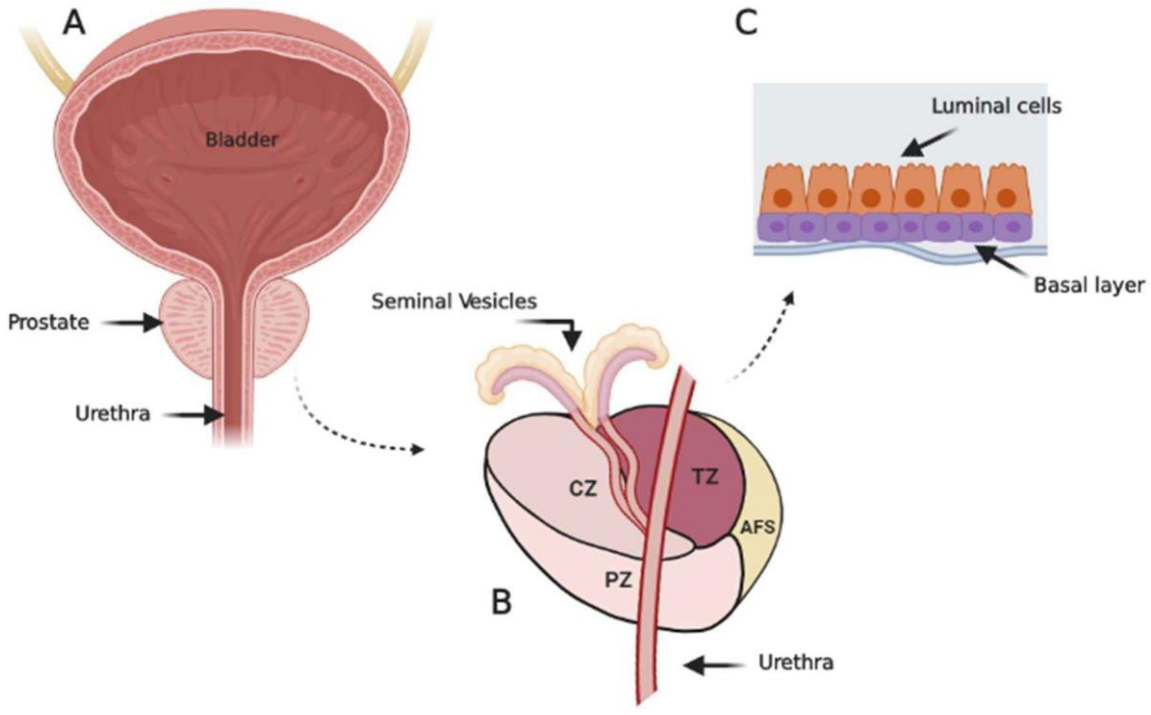

Figure I.1. Schematic representation of prostate anatomy and glandular epithelium.

(A) Diagram showing the location of the prostate and its relationship with the bladder and urethra. (B) Anatomical divisions of the prostate gland. The anterior and apical compartment is bounded by anterior fibromuscular stroma (AFS). Furthermore, the prostate gland is divided into three different zones; the largest section (70 %) which is the peripheral zone (PZ). 25 % belongs to the central zone (CZ), also having ejaculatory ducts and the urethra proximally run through it and lastly the transition zone (TZ),

which contains 5 % of the prostate gland. (C) Organization of prostate epithelium. The luminal layer comprises of columnar cells which produce prostatic secretion, prostate specific antigen and keratinocyte growth factor receptor. Directly below lies the basal layer that consists of a stem cell-like cells, which contain numerous growth factors responsible for normal cellular growth. The basal cells can also differentiate into the columnar secretory cells. Several other cell-types are present within the epithelial layer, namely, neuroendocrine cells, and intermediate cells (not shown).

Two epithelial layers form the unique sporadically shaped gland regions (Fig, I.1). The luminal epithelial internal layer comprises of lengthy columnar cells vital for producing prostatic secretion, namely the prostate specific antigen (PSA), and characterized by expressing the androgen receptor (AR), cytokeratin 8 and 18,, and keratinocyte growth factor receptor (KGF) [6]. The external layer of cuboidal epithelial cells divides the basal cells and stroma. These cells produce and express TP63, cytokeratin 5 and 14, epidermal growth factor receptors (EGFR) insulin-like growth factor receptor (IGF-1), estrogen receptor (ER), and keratin permitting healthy cellular growth [7, 8]. The stroma is made up of a number of cells namely; nerves, fibroblasts, lymphatics and smooth muscle, involved in producing growth factors that develop prostate cells and regulating cell growth and secretion [9]. The basal layer (Fig. I.1) comprises of a pool of cells comparably stem cell-like, having the ability to differentiate into the columnar cells and regulate the flow of luminal cells to and from the extracellular region.

The prostate gland secretes numerous factors namely PSA, prostaglandins, prostatic acid phosphatase (PAP), and citric acid [10], that are key components of the seminal fluid [1]. These factors partake in processes like sperm motility, survival and delivery, highly contributing to a successful fertilization.

In male adults, the prostate gland size is maintained by balancing cellular regeneration and death through hormonal homeostasis ensured by the endocrine glands [11]. The main group of circulating hormones involved in regulating prostate physiology are the androgens; testosterone, and 5α-dihydrotestosterone (DHT), which is a metabolite of testosterone by the activity of 5α-reductase isoenzyme [12]. Another steroid hormone not only present but preserves homeostatic control is 17β-estradiol (E_2), belonging to the family of estrogens [13]. Testosterone and E_2 levels change as aging occur, yet such changes are not age-related. However, the alteration in the levels of DHT are constantly observed. Such hormonal shifts are believed to cause abnormality within the prostate such as benign prostatic hyperplasia (BPH), being also implicated in the malignant transformation of prostate and emergence of prostate cancer (PCa) [12, 14].

Sex steroid hormones can also be synthesized intraprostatically and the enhanced expression and activity of steroidogenic enzymes has been describe in PCa [12].

1.2. Prostate carcinogenesis: molecular and cellular events

Various lines of evidence have shown that continuous abnormal development of the prostate gland can be as a result of chronic inflammation, which is one of the drivers commonly observed from pre-neoplastic to malignant prostate progression [15]. Prostatic inflammatory response is composed of recruiting and expanding leukocytes to the prostate, namely; lymphocytes, myeloid cells and macrophages [16]. Irrespective of prostate etiology, inflammation is understood to incite carcinogenesis via triggering cell and genomic damage, and generating a microenvironment containing a vast number of cytokines and growth factors that can increase cellular replication, angiogenesis and tissue repair [15]. Interestingly, areas with prostatic atrophy can frequently be identified in aging men, which is often associated with an inflammatory response [17]. The areas typically show increased epithelial proliferation and are termed "proliferative inflammatory atrophy" (PIA) [18]. Areas of PIA are also usually situated in proximity with prostatic intraepithelial neoplasia (PIN) and adenocarcinoma, and therefore PIA is also presented as a precursor lesion for PCa [19]. PIN development is characterized by interactions of the prostate cells with inflammatory cells, and is considered the first histological change seen in PCa [20]. Generally, PIN lesions form in the epithelial cells located in the PZ. Whereas, BPH which is non-malignant overgrowth of stroma and epithelial cells usually occur in the TZ [21]. The most frequently found lesions in patients are PIN and high-grade PIN which can be considered precursors of PCa [22].

Initially, PCa is confined in the prostate zones, the primary PCa. However, as the disease progresses multiple factors tend to shift causing an independence and overdrive of cancer cell proliferation, thereby increasing chances of cancer cell infiltration into surrounding tissues [23]. A critical step in the malignant progression of PCa occurs when epithelial cells undergo epithelial mesenchymal transition (EMT), giving the cells the ability to reach other tissues [24]. In fact, the spread is commonly detected in nearby lymph nodes and also distant organs such as the liver, bone marrow, lungs and brain [25].

Mutations or deletions in key targets genes are common events in PCa, which result in the alteration of protein expression pattern underlying the progression of disease. The molecular aetiology of prostate carcinogenesis is not entirely understood. However,

there are multiple factors that are differentially expressed in the various stages of PCa progression contributing to tumour growth, cellular survival, migration and invasion. The most well-known and frequently detected genes identified in having roles in the initiation of PCa include GSTP1, P53 and PTEN. GSTP1 encodes the pi-class glutathione S-transferase that belongs to the class of detoxifying enzymes and play an important role in the detoxification of a number of exogenous and endogenous carcinogens by conjugation with glutathione [26]. It was shown to be epigenetically inactivated by CpG island promoter hypermethylation in PCa [18, 27]. PTEN is a PI (phosphoinositide) 3-phosphatase tumour suppressor, which functions as an inhibitor and suppressor of cell proliferation, growth, survival and motility by inhibiting the PI 3-kinase (PI3K) [28]. Generally, PTEN is inactivated by gene deletion or mutation in multiple cancers including PCa [28, 29]. P53 is a well-established tumour suppressor gene also found to be mutated in PCa. It has a fundamental antiproliferative action by activating or repressing vital effector genes in cell cycle regulation and apoptosis [30].

Other somatic molecular modifications that arise prior to or at the onset of PIN include the epigenetic silencing of other genes, such the NKX3.1 [31], and the activation of proto-oncogene MYC [32]. In a subset of patient cases, oncogenic ETS family transcription factors are triggered by gene fusions, which are frequently resulting in the ERG gene and TMPRSS2 gene fusion on chromosome 21 and/or at the onset of invasive adenocarcinoma [32].

At cellular level, classically, the epithelial luminal cells are considered the primary cells in the origin of PCa, while basal cells are regarded as a protective barrier of luminal cells against any oncogenic drive [33]. Nonetheless, basal cells are continuously in contact with the stroma that interacts with cytokines, which can steer cancer signalling to the epithelial cells [34]. During the development of metastatic PCa, basal cells are understood to contribute to the process as they present high levels of genes involved in the EMT, thus are immensely responsive to alterations leading to oncogenic signalling [33, 35]. Another major cell type implicated in the malignant transformation of prostatic tissues are stem cells. Prostate stem cells are the target cells of oncogenic transformation, suggesting a role of PCa initiation as they are present in both basal and luminal layers [36]. Overall, PCa is a multifaceted pathology involving numerous possible processes that control the initiation and progression of the disease, which are yet to be clarified.

1.3. The role of androgens in prostate cancer

Androgens and AR signalling have a vital role in PCa development and progression [37]. For androgens to function they are required to bind the AR [38], a transcription factor, controlling the expression of target genes that regulate cellular process like proliferation, growth and differentiation [36]. The AR is a member of the nuclear receptor superfamily of transcription factors and is the key player in androgen signalling [39]. The AR protein is structurally organized into functional domains: i) the N-terminal domain (NTD) with transcriptional regulatory functions; ii) the DNA-binding domain (DBD) responsible for interaction with the DNA; and iii) the ligand-binding domain (LBD), that coordinates interaction with androgens [40]. Testosterone and its metabolite DHT obtained by the activity of 5α-reductase are the main AR ligands. However, DHT has higher binding affinity to the AR than testosterone.

Generally, AR is located in the cytoplasm forming a complex with chaperone family members, the heat shock proteins [37]. In the presence of androgens, several events occur (Fig. I.2) namely binding of the androgens to the AR and the complex undergoes a conformational change. Heat-shock proteins are dissociated, and dimerization of the receptor occurs. AR dimers bind to the androgen-response elements (AREs) in the promoter and enhancers regions of target genes (Fig. I.2) [41]. Co-regulatory proteins such as co-activators or co-repressors form the AR-transcriptional machinery, which regulates transcription. Genomic alterations such as gain of function of AR coactivators, and loss of AR corepressors are in the AR pathway are frequent seen in PCa development [42]. It is also described that the AR can regulate transcription by interacting with other transcription factors, not binding DNA directly [43].

Non-genomic AR signalling is an alternative pathway which does not rely on AR nuclear translocation or AR-DNA binding. It can be mediated by a membrane-anchored or -recruited AR that interacts, for example, with the tyrosine-protein kinase Src thereby activating the kinase domain. The activated Src then induces Ras-mediated MAPK/ERK cascade and rapid non-genomic effects. However, the activated ERK may phosphorylate AR and its coactivators, therefore enhancing AR genomic activity [44]. In general, non-genomic signalling induction is dependent on low to physiological androgen concentrations [45].

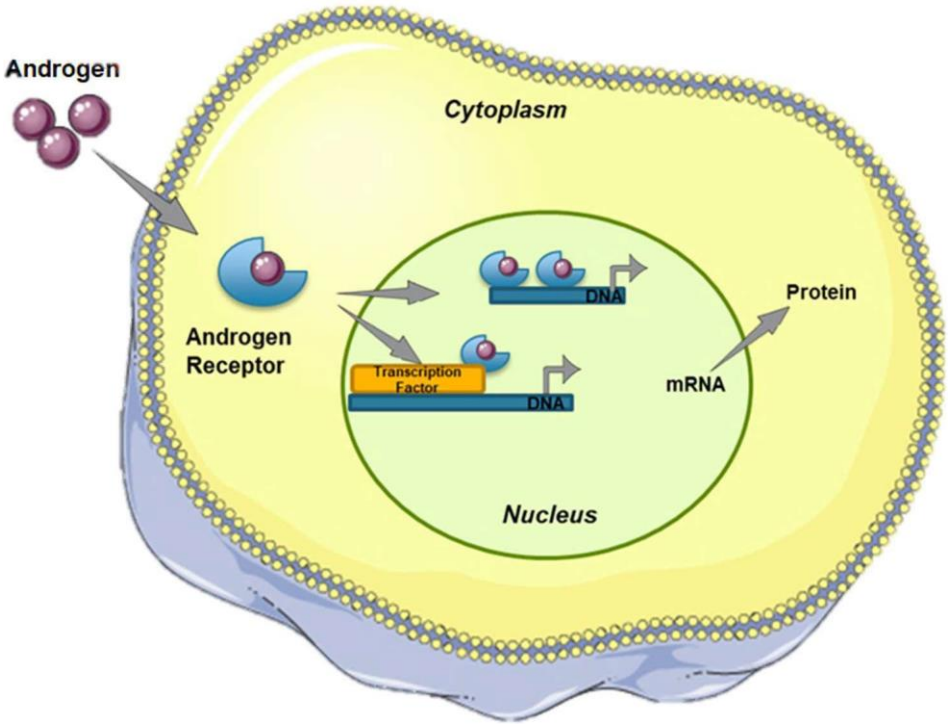

Figure I.2. Schematic illustration of the classical (genomic) AR signalling mechanisms.

Androgens bind to the AR that undergoes a conformational change and translocates to the nucleus. Within the nucleus ligand-AR complexes bind to the androgen response elements (AREs) in the promoter regions of target genes. Upon recruitment of coregulators (not shown), the transcriptional machinery is assembled, and AR-regulation of gene expression occurs. Alternatively, the AR can modulate transcription by interacting with other transcription factor already bind to DNA.

Several genes within the AR transcriptional network encode proteins involved in PCa growth [37]. In the initial phase of PCa, the cancer cells are highly dependent on androgens activity for proliferation and survival; this cancer stage is known as androgen sensitive. For this reason, androgen deprivation therapy (ADT) by reducing levels or blocking circulating androgens can be used to control androgen-sensitive PCa [46]. Anti-androgen drugs (synthetic steroid or non-steroidal pure antiandrogens) and orchiectomy (surgical castration) are the types of ADT often used for PCa treatment [41]. Although ADT is generally the first line of treatment because of its effective androgen suppression, the treatment comes with multiple adverse effects including metabolic changes, increased body fat, reduced bone density, loss of muscle mass, erectile dysfunction, and vasomotor symptoms. For several years bicalutamide (BIC), nilutamide and flutamide were the main anti-androgens used. The most common amongst them is the nonsteroidal antiandrogen BIC. BIC acts by inhibiting the AR,

thereby blocking androgen effects and flare [47]. For patients with clinically localized, advanced, recurrent, lymph node metastases, or asymptomatic metastatic PCa, an antiandrogen in combination with surgical or chemical castration is the general treatment [48]. Due to the adverse effects which BIC has, another common anti-androgen was developed to improve the survival rate and side effects of the treatment. Enzalutamide, a second-generation orally administered AR antagonist binds to the AR ligand-binding domain, blocking androgens binding and further action by preventing AR nuclear translocation, impairing DNA binding, and affecting coactivator recruitment [49].

Surprisingly, in locally recurrent PCa tissue the androgen levels remain high, which is enough to activate the AR, even after castration. Therefore, some PCa cells may become resistant to ADT [50]. Despite the castrate levels of androgens in serum, the AR target gene PSA also remains expressed. Due to prolonged administration of ADT the cells survive, proliferate and metastasize in the presence or absence or androgens [51]. The term used to describe this cancer stage is castration-resistant prostate cancer (CRPC). The molecular mechanisms underlying CRPC development are not fully understood, therefore clarifying the intracellular pathways that are involved in the acquisition of the androgen-resistant phenotype is crucial for significant advances in PCa research. Nevertheless, it is known that AR gene amplification is one of the most common events in CRPC development [52]. The AR overexpression allows activation of the AR pathway even in the absence or low levels of androgens. In many patient samples, post-translational modifications such as phosphorylation, acetylation, ubiquitination, methylation, and sumoylation of the AR protein have been detected in CRPC tissues. Though, their effect on AR activity and CRPC remains to be elucidated [53]. Additionally, several other mechanisms have been linked with the initiation and progression of CRPC; point mutation of AR gene, alterations in androgen biosynthesis in consequence increased 5α-reductase, for example, AR variants and co-factor change [54].

Among the other alterations, the progression to CRPC is characterized by a metabolic reprogramming with the establishment of the so-called Warburg effect, i.e. intensive use of glucose with the production of lactate even in the presence of oxygen [55]. Our research group demonstrated that CRPC has a more glycolytic phenotype than the androgen-responsive stage of disease, linking the metabolic alteration with the aggressiveness of PCa [56, 57]. Moreover, studies demonstrating the androgenic regulation of lipid metabolism, glycolysis and glutaminolysis in PCa have been

published over the years [58-60]. Androgens have been reported to contribute to the metabolic adaptation of PCa cells stimulating lipid *de novo* synthesis, uptake and oxidation [61]. In glucose metabolism androgens are known to stimulate glucose uptake and lactate production through the alteration of multiple targets in the glycolytic flux such as glucose transporters (GLUT1 and GLUT3), hexokinase, phosphofrutokinase 1 and monocarboxylate transporter 4 [59]. Also, AR signalling has been shown to play a role in glutamine metabolism enhancing the glutamine transporter ASCT2 expression, thereby controlling the growth and survival of androgen sensitive PCa [58]. Overall, available knowledge supports androgens as metabolic regulators in PCa.

Concerning the treatment of CRPC, in most cases its development is highly correlated with the metastatic PCa failing to respond to ADT treatment [62]. Generally, after several months of ADT administration (e.g., BIC or enzalutamide), treatment options are modified either in concentration, addition of combination treatment or completely changed to other therapies such as chemotherapy [63]. Docetaxel is a chemotherapeutic agent commonly used for CRPC. Over the years, it has shown a positive increase in survival rates of patients. Furthermore, a few chemotherapy agents have been developed which are based on docetaxel. These agents are used in rare cases of docetaxel resistant PCa [64]. Thus, the importance of the AR role in the development of metastatic CRPC is fundamental in understanding the mechanisms of disease and enabling novel therapies that could improve treatment effectiveness.

1.4. Epidemiology and risk factors of prostate cancer

PCa is a highly heterogeneous and multifactorial disease. Being the second most diagnosed cancer in men. It accounts for 7.3% (1,414,259) of new cases and 3.8% (375,304) cancer deaths worldwide in 2020 [65]. In Portugal, 20.4% of male cancers cases are PCa, with 6609 new cases in 2018 [66]. Numerous endogenous and exogenous factors such as genetic and epigenetic changes, race, aging, hormones, growth factors, obesity, diet and the environment, have been shown to contribute and indicated as risk factors for the development of PCa [64, 67].

The early stage of PCa may be asymptomatic and it is often having indolent progression, but generally, incidence is increased with age and in specific populations. African descents are the major race affected by PCa. Rates of cases and mortality are lower in other races such as Asians, Hispanics and Caucasians. Hormones, lifestyle,

lack of access to health institutions and genetic variation may underlie and explain the incidence and mortality rates of African descent population [68].
Also, familial aggregation is considered a strong risk factor in PCa. The inheritable disease gives rise to genetic susceptibility of developing it. Additionally, modified inheritable genes such as, macrophage scavenger receptor 1 (MSR1), Ribonuclease L (RNASEL), X chromosome mutation encoding for AR, can also give rise to poor prognosis, which is why many are used as markers of predisposition diseases [69].
The prevalence of PCa cases is commonly seen in men over 40 years of age, and it greatly increases in ages above [70]. Several changes such as the development of metabolic disorders, including diabetes, central obesity and hypertension occur with aging which are associated with prostate inflammation and changes in sex steroid molecular pathways [71]. In spite of that, it is well established that the male hormone and its receptor, androgens and AR, are strongly linked to the development of PCa. The growth of cancer cells becomes independent of hormone levels. Intraprostatic and serum concentrations of androgens are often found high, which is a positive association with the risk of PCa [72].
Many more suggested risk factors that affect the aetiology and pathogenesis of PCa are environmental factors. Lifestyle and obesity are a growing societal issue [73]. More than ever, obesity is the world's leading metabolic disorder, hyperglycaemia and insulin deregulation follow. The adipose tissue produces molecules, which are continually secreted and deregulate the homeostasis of prostate tissue, for example, adipokines and cytokines [74]. Many of these secreted molecules drive the imbalance of hormone levels, cell proliferation, angiogenesis, migration, invasion and metastasis. Other controversial factors include smoking, excessive alcohol consumption and exposure to toxic chemicals, which also may contribute to PCa.

Finally, persistent chronic inflammation and urinary infectious or sexual obtained agents have also been suggested to increase the incidence and pathogenesis of prostatic inflammation and PCa. Lack of tissue repair and inflammatory response can increase delivery of cytokines and growth factors and increase the production of reactive species, thereby aiding the PCa development [75].

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2. The Tribbles pseudokinases family: from structure-expression to the regulation of molecular mechanisms and disease

2.1. Introduction

Tribbles (TRIBs) are the most well-studied human pseudoenzymes which belong to the pseudokinase family, having a distinct characteristic of an inactive catalytic kinase domain [1]. Several studies have shown TRIBs to be critical controllers of many physiological and pathological processes such cancer, metabolism, energy homeostasis, cell cycle control, development, immunity [2, 3], functioning as modulators and mediators of signalling cascades and scaffold adaptor proteins. Over the recent years, TRIBs research has advanced and revealed many unidentified biological functions of the three TRIB isoforms. From their discovery in having a role in coordinating morphogenesis in fruit flies, to the identification of typical mammalian tissue expression profiles in distinct physiological conditions, and the emergence of datasets and clinical studies of altered expression, TRIBs proteins are now indicated as potential biomarkers for prediction, diagnosis, and prognosis of many disease types.

Interestingly, TRIBs' role in the biology of malignancy has been a major research focus on the last years, with studies addressing the involvement of various TRIB isoforms in pathology and their associated target genes and molecular cascades. In general, TRIBs upregulation is associated with unfavourable tumour stage, metastasis, bad prognosis, and recurrence of disease. In fact, TRIB1, TRIB2 and TRIB3 have been described as master oncogenic factors, having increased protein and mRNA expression in cancers such as, breast [4], leukaemia [5, 6], melanoma [7], prostate [8, 9], oral tongue squamous cell [10], lung [11], colorectal [12], gastric [13], thyroid [14], liver [15], renal cell [16]. Nevertheless, TRIBs function is not limited to oncogenic actions, in some cases they can act as a tumour-suppressors [17]. TRIBs have also been described to have a role in non-neoplastic disorders and in regulating multiple biological processes, such as hypoxia, cell fate, stress response, development, and embryogenesis [18-22]. Other suggested roles for TRIBs protein are related with the inflammatory response and regulation of immune system. TRIBs have been implicated in the regulation of inflammation by modulating the activity of important signalling network targets that trigger innate immune responses [23-25]. However, there is still unresolved mechanisms of TRIBs action on inflammation and how TRIBs contribute to immune disease development.

Also, a major function for TRIBs pseudokinases family, namely TRIB1 and TRIB3 has been related with the regulation of liver and adipose tissue function, with implications in the control of cardiometabolic parameters such as triglycerides, low and high-density lipoprotein cholesterol *in vivo* [26]. Moreover, TRIB3 has been associated with insulin resistance and is a functional regulator of insulin sensitivity and mitochondrial glucose oxidation [27], being suggested as a potential target for type 2 diabetes treatment. Indeed, the TRIBs' functional impact on many biological systems and their function as scaffold adaptor proteins and modulators of signalling cascades renders them interesting pharmacological targets. However, it is still apparent that extensive research is required to unlock more in-depth cellular processes which TRIBs are associated with.

The present review highlights the TRIBs expression pattern and its deregulation in various disease backgrounds, detailing the signalling networks and molecular mechanisms associated with TRIBs actions. The usefulness of TRIBs as disease biomarkers and the possibility of manipulating their expression levels for therapeutic intervention also is emphasized.

2.2. Tribbles pseudokinase family

Tribbles (TRIBs) pseudokinases were first described in a *Drosophila melanogaster* mutant screening study, which identified regulatory genes involved in cell proliferation and division [17]. Thereafter, three mammalian homologues were identified, TRIB1, TRIB2 and TRIB3 and their structure and biological activity have been characterized. TRIBs are part of the human protein kinase family that have a conserved kinase-like domain, which is a unique feature across species [28]. For kinases to function, three highly conserved motifs should be present: the i) N-terminal VAIK-motif, which has a role of interacting with α and β phosphates during ATP-binding, the ii) HRD centre-domain, which contains the catalytic aspartic acid residue that plays a proton acceptor role resulting in protein transfer, and the iii) DFG-domain located in the C-terminal in which the binding of aspartic acid to Mg^{2+} occurs, thereby interacting with the β and γ phosphate of ATP in the binding loop [21]. On the contrary, TRIBs contain a degenerate HRD motif in the catalytic site and totally lack both the VAIK and DFG domains, which makes them pseudokinases [20]. All TRIBs

proteins typically have three-domains (Fig. II.1) that consist of a variable N-terminal (NT) region, a pseudokinase domain, and the adjacent C-terminal (CT) region [17]. The NT domain is characterized by the presence of a putative PEST sequence, and the CT domain contains MEK1- and COP1-binding motifs, respectively.

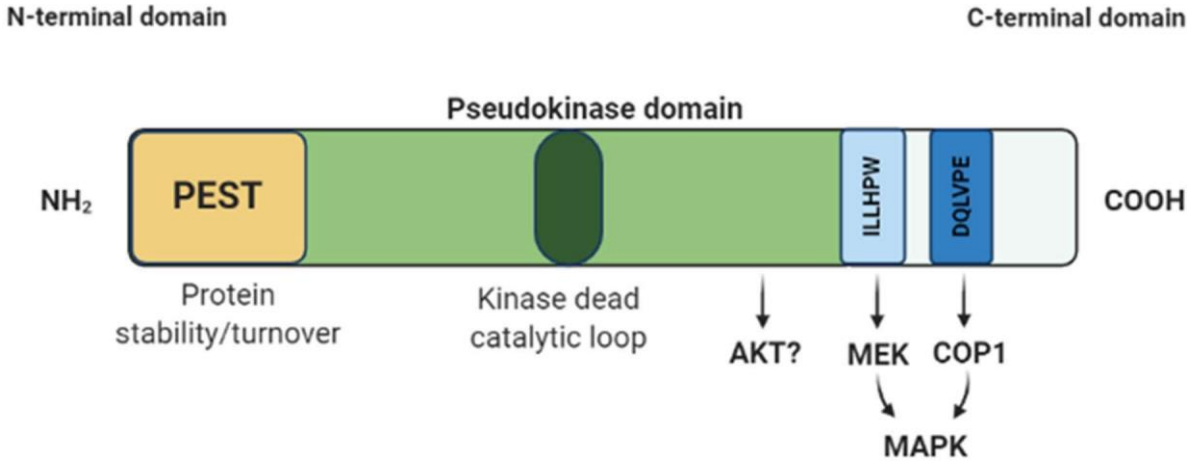

Figure II.1. General protein structure of tribbles proteins with the N-terminal, pseudokinase and C-terminal domains.

The pseudokinase domain has the so-called kinase dead catalytic loop ([29]). The N-terminal and C-terminal domains contain a putative PEST sequence, and MEK1- and COP1-binding motifs, respectively. Furthermore, C-terminal domain comprises of two regulatory regions; ILLHPW motif, which mediates MEK signalling and DQLVPE motif that directly binds to the E3 ubiquitin ligases. Pseudokinase domain/AKT modules are thought to interact, however its regulatory action is not fully known.

2.2.1. N-terminal region

In most organisms, the TRIBs proteins' NT domain contains approximately 60–80 residues [30]. However, organisms, such as *D. melanogaster, Drosophila pseudoobscura, Gallus gallus* and *Pan troglodytes* can have longer NT domains [31]. The conservation of the NT domain in TRIB1 and TRIB3 subgroups is limited to a few protein motifs, which contrasts with TRIB2 that has remarkable conservation among different species.

Within 43 TRIB proteins amino acid sequences analysed; two evolutionary conserved peptide motifs present in the NT domain of TRIB subfamilies were identified [32]. The first peptide motif is located at the end of the NT segment and is comprised of two sections, which are divided by one or more amino acids. The first section includes <4 positively charged amino acids, and the second section is defined by the consensus

[K/R]2×2[D/E]X[D/E] nuclear localization sequence [31]. This sequence pattern is found in all three TRIB subfamilies [20]. A unique feature that only appears in TRIB1 and TRIB2 proteins is a second consensus motif, the G-S-P, which is situated near the kinase-like domain [32]. Work by Kadam *et al* (2000) has previously shown that the human SMAD nuclear interacting protein (SNIP1) contains a highly conserved G-S-P motif in a similar position within its NT segment [33]. SNIP1 is a nuclear protein linked with several targets namely; Slbo [34] and C/EBP Homologous Protein (CHOP) [19] from the CCAAT/enhancer binding protein (C/EBP) transcription factor protein family, which have been reported to interact with the TRIB subfamily. Thus far, no other role for the G-S-P motif has been described.

Nevertheless, the NT region of most of the TRIB proteins investigated is mainly characterized by the abundance of proline (6-23%) and serine (7-24%) amino acids, which increases the number of potential phosphorylation sites for proline-dependent kinases. TRIB1 has been shown to contain several possible phosphorylation sites for proline-dependent kinases [31], but contrastingly, the NT region of TRIB2 subfamily proteins has a limitation to potential AKT phosphorylation sites. These proline-rich regions may also serve as anchorage sites for proteins harbouring SH3 or WW domains [35].

The abundant proline and serine amino acids of the NT domains of TRIBs are highly featured in the so-called PEST regions (Fig. II.1). A prediction study by *Hegedus et al.* revealed that 34 TRIBs proteins sequences (out of a total of 50 analysed) contained PEST motifs [31]. Moreover, the ratio of TRIBs proteins containing PEST sequences was higher amongst vertebrate species. The presence of PEST motifs has been implicated in the control of proteins half-life time through affecting their susceptibility for degradation and rapid turnover [36-38]. Accordingly, several reports have been showing the instability of TRIB proteins [17, 37, 39, 40]. Such findings have been related to the TRIBs role in regulating intracellular signalling and cell division, biological processes that strictly depend on the rapid protein turnover switching on and off specific molecular targets.

2.2.2. Kinase-like domain

The TRIB's pseudokinase domain (Fig, II.1) is a serine/threonine rich site with the absence of a canonical kinase activity, which serves as a scaffold for binding of substrate proteins [41]. *Hegedus et al.* investigated TRIB kinase domain structures

from a variety of species using bioinformatic tools, which revealed that the kinase-like domain is present in all TRIB proteins [31], though missing some of the motifs present in catalytically active kinases.

The pseudokinase domain folds into a two-lobed 3-dimensional structure and comprises of 12 subdomains (10–30 amino acids) several of which consist of characteristic, evolutionary conserved residues of functional importance [42, 43]. The amino-terminal lobe contains I–IV subdomains, which mostly antiparallel β-sheets and primarily act as the ATP binding site (phosphate donor nucleotide). All TRIBs' subdomain II has the lysine crucial for ATP binding. The VIA–XI subdomains are mainly located in the α-helical carboxy-terminal lobe, functioning as docking sites of the phosphate acceptor peptide substrate [31]. The connecting 'hinge' region situated between the two lobes contains the V subdomain, which provides a suitable environment for the catalytic reaction. A significant difference between the typical kinases and the kinase-like domain of TRIB proteins is the lack of DFG metal-binding motif (E[S/N]LED) from subdomain VII [31]. Amongst the several kinases identified thus far, the most have the full set of kinase signature motifs [44]. This evidence supports that TRIB proteins do not have kinase activity. However, the high conservation of TRIBs' kinase-like domain during evolution indicates their functional relevance.

The involvement of TRIBs pseudokinase domain in ATP binding seems to differ among the family. In TRIB2, ATP interacts with the pseudokinase domain without the presence of metal ions and undergoes weak hydrolysis [43, 45]. It has been suggested that TRIB2 contains numerous conserved hydrophobic residues, which readily attach a trapped complex formed from an active ATP conformation stabilizing the domain structure suitable for catalytic processes. This occurrence is seen in TRIB2 and TRIB3 but not in TRIB1, which lacks affinity for ATP [46, 47]. The implication of TRIB2 and TRIB3 ATP-binding, though vestigial, is yet to be elucidated and is a matter of controversy. Binding assays, co-precipitation and mutagenesis studies demonstrated that AKT signalling modules also have a binding site within the TRIBs pseudokinase domain (Fig. II.1) [17, 46]. Moreover, it has been demonstrated that TRIBs control the activity of AKT-dependent signalling pathways. The reduction of TRIB1 levels regulate activity of the PI3K-AKT pathway [48]. High expression levels of TRIB2 increase AKT phosphorylation, activating its effects [49]. On the contrary, TRIB3 negatively regulates AKT modules [50]. Its inhibition occurs by TRIB3 binding AKT, thereby

preventing its activation via upstream kinases [51]. Numerous molecular interactions of AKT/TRIB1-3 are mentioned, however, its mechanism of action is yet to be known. Nevertheless, the functional relevance of TRIBs-AKT interaction has been highlighted in multiple cell types (to be discussed below).

TRIB pseudokinases were suggested to have an unusual substrate specificity, and their interacting partners still are being disclosed. A number of substrates use the TRIBs central kinase-like domain as a binding platform [17, 36]. A well-studied example is the anchorage and positioning of substrates for degradation by the E3 ubiquitin ligase. The TRIB pseudokinase domain is adjacent to a CT ubiquitin E3 ligase- targeting motif (Fig. II.1), and these regions were proposed to interact [52].

2.2.3. C-tail region

In most cases, the CT fragments of TRIB proteins are approximately 35-45 residues long and contain abundant charged amino acids. This region is highly conserved among TRIBs but very distinctive from other kinases [17]. Present in all TRIB protein flanking regions is the [DE]-Q-x-V-P-[DE] motif (Fig. II.1), a highly evident characteristic, which is followed by many negatively charged amino acids [31]. The CT region is thought to be important for protein-protein interactions [53] and it has been implicated in the ubiquitin ligases binding (Fig. II.1). The conserved CT region supports the coupling of E3 ubiquitin ligases such as COP1 binding peptide motif that identifies specific ubiquitin chains (i.e., K48-linked) in proteins like C/EBPα, thus controlling protein stability through the ubiquitin proteasome pathway [17]. COP1's autoinhibitory properties that target the C/EBPα allow TRIB proteins to govern the level of C/EBPα by modifying COP1 substrate specificity to engage C/EBPα for

Ubiquitination [54]. This is through COP1 selectively binding C/EBPα within a recognition sequence [47]. Another conserved motif (ILLHPWF) in the CT region is a binding site for MAPK/MEK family members (Fig. II.1), which allows TRIBs modulation of these signal transduction pathways.

2.3. Signalling networks associated with tribbles actions

The protein structure and the interacting molecular partners determine the signalling networks that have been related to TRIBs' role in signal transduction. Overall, besides

binding E3 ubiquitin ligases, TRIBs are known to act as adaptors proteins modulating signalling pathways linked with several biological processes, which mainly includes the MAPK and AKT pathways (Fig. II.2).

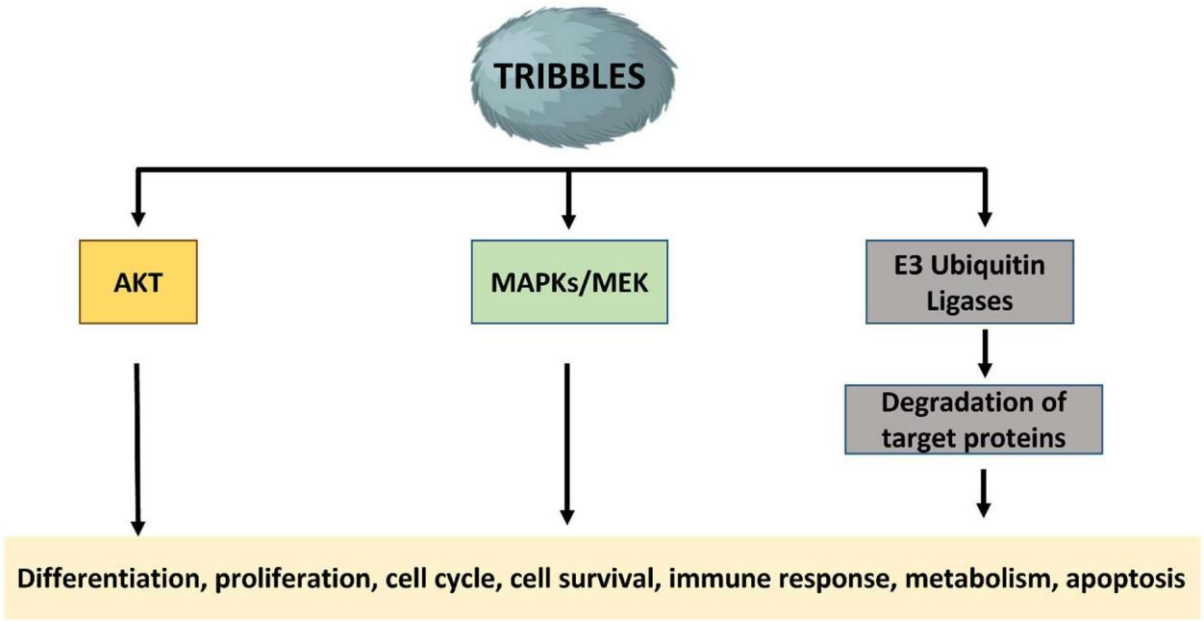

Fig. II.2. Overview of molecular pathways associated with Tribbles and major effects.
TRIBs interact with MAPKs family and E3-ubiquitin ligases affecting biological processes such as differentiation, proliferation, cell cycle, cell survival, stress and immune responses, and metabolism.

2.3.1. MAPK pathway

While not having kinase activity, TRIB1, 2 and 3 have been shown to interact with MAPKs cascade, controlling its activation [24, 33]. TRIBs/MAPK interaction has been identified in many cell types and tissues namely, monocytes, lymphocytes, macrophage-like, smooth muscle cells, hepatocytes, epithelial cells, fibroblasts, embryonic kidney, embryonic stem cells, and mouse embryo [2, 55-58]. As adaptor proteins, TRIBs have been implicated in the MAPKs prominent role controlling physiological and pathophysiological cellular responses namely; cell differentiation, survival, and proliferation, development, inflammation/stress and apoptosis [59]. Currently, there are three main groups of MAPKs that have been characterized in mammalians, which include; extracellular signal-regulated kinase (ERKs), Jun kinase (JNKs), and p38 MAPK [63]. Within the MAPK cascade, TRIBs regulate both the phosphorylation of MEK/ERK and the degradation of C/EBP transcription factor [57].

In mammalian cells, the C/EBP family of transcription factors (TFs) are among the gene expression regulators negatively regulated by TRIBs.

In renal cell carcinoma cells, modulation of TRIB3 by hypoxia-inducible factor-1α (HIF-1α) increased cell viability, proliferation, and invasiveness through activating the MAPK network [16]. It appears that TRIB1 and TRIB3 are linked to MEK/ERK pathway. TRIB1 was not only related with the ERK signalling but also augmented its phosphorylation, which consequently depleted interleukin-3 (IL-3) levels thereby decreasing apoptosis in acute myeloid leukaemia (AML) cells [57]. Furthermore, in hepatocytes TRIB1 upregulation is mediated by ERK1/2 at a transcriptional level [58]. Also, it has been demonstrated that TRIB3 positively regulates ERK signalling during proliferation and osteogenic differentiation [16]. Both TRIB1 and TRIB3 positively regulate ERK, while TRIB2 has a negative regulatory role in ERK signalling [55, 60]. All three TRIBs interact with MEK1 via its C-terminal ILLHPWF motif (Fig. 1), consequentially enhancing ERK phosphorylation [57].
The relationship of TRIBs with the degradation of the transcription factor C/EBPα also seems to be MEK-dependent. C/EBPα degradation occurs through the C-terminal DQXVP[D/E] peptide motif, which is directly associated with E3 ligase COP1-binding resulting in stabilization of TFs and proteasomal degradation Furthermore, TRIB1 induction of C/EBPα degradation mediated by MEK was reported in AML cells [57]. Also, TRIB2 overexpression was shown to decrease C/EBPα protein levels, which resulted in myeloid differentiation [61]. Moreover, TRIB2 increased degradation of C/EBPβ in 3T3-L1 preadipocytes, suppressing adipocytic differentiation. A similarly pattern was seen in lung and liver cancer cells, where elevated expression of TRIB2 correlate with decreased levels of C/EBPα [4, 62]. Despite the fact that all TRIBs contain the DQXVP[D/E] motif, TRIB3 seems to lack in functionality and ability to facilitate C/EBP degradation [17]. In myeloid cells, TRIB1 and TRIB2, can bind and degrade C/EBPα to block differentiation, and, on the contrary, TRIB3 cannot [15]. Nevertheless, existent evidence demonstrates the robust correlation of TRIB-induced C/EBPα degradation [15, 20, 63]. Also, it has been suggested that TRIB1 physiological functions, as for example, the modulation of innate immunity can be achieved by the control of C/EBPβ expression levels, which is a significant transcription factor for metabolic alterations such as inflammation and endoplasmic reticulum (ER) stress [41].

2.3.2. PI3K/AKT pathway

PI3/AKT is the other major regulatory pathway which TRIBs have been shown to interact with [64]. In response to extracellular signals, PI3/AKT activation mainly promotes proliferation, cell survival, growth, angiogenesis, and metabolic actions [50]. Among many other genes, TRIB1 has been indicated as a downstream effector of PI3K signalling cascade [65]. It was observed that TRIB1 knockdown inhibits the activity of numerous targets of the PI3K pathway, within and out of the nucleus, which demonstrates the relevance of TRIB1 in signal transduction in this pathway [48]. Also for TRIB2, it was shown that it directly activates the PI3K/AKT signalling by interacting with AKT and subsequently increasing enzymatic activity. This occurs by TRIB2 repressing forkhead box O (FOXO) activation, thereby disrupting the p53/MDM2 axis . FOXOs are downstream targets of the PI3K/AKT pathway, and another family of TFs regulated by TRIBs [7, 66-68]. A wide range of FOXOs are known to mediate the response to extracellular signals, as a result several changes occur, namely; regulation of cell-cycle progression, cell survival, and cell type-specific responses [69]. In consequence of PI3K/AKT axis activation, FOXOs undergo AKT-mediated phosphorylation, which in turn promotes downstream binding of 14-3-3 protein, CRM1-mediated nuclear export and cytoplasmic sequestration. Conditions such as stress and lack of growth factors inhibit the PI3K/AKT pathway, resulting in the translocation of FOXO proteins to the cell nucleus, and, consequently, in the implementation of their transcriptional activity [70]. In melanoma cells, TRIB2 was shown to stimulate growth and survival via the repealing of FOXO3a function [71]. AKT activation by TRIB2 can also arise even in the presence of a PI3K inhibitor, which was proposed as causing resistance to anticancer drugs [49, 72].

In the liver, a report demonstrated that TRIB3 interacts with AKT blocking its activation. It has been suggested that such interaction suppress, for example, insulin signalling [50]. In neuron cells, *Saleem et al* [66]. observed that augmented levels of TRIB3 interact with AKT thereby inhibiting its activity and consequentially activating FOXO1 [66, 73]. In addition, TRIB3 upregulation and the subsequent AKT inhibition, triggered by administration of various antitumour agents, was shown to promote apoptosis of pancreatic and tongue squamous cell carcinoma [74, 75]. However, the exact molecular action of TRIB3-AKT regulation and whether loss of TRIB3

contributes to the initiation and progression of cancer remains to be elucidated. While TRIBs are linked with the PI3K/AKT signalling network, a cell-specific activity seems to occur, with distinct cross-talks existing depending on the cellular context.

2.4. Tribble's regulation of transcription factors activity

The landscape of signalling pathways in the interplay of TRIBs actions could be increasingly diverse, as other interacting proteins have been identified (Table 1). TFs like the peroxisome proliferator-activated receptors have been linked with TRIBs isoforms [10]. For example, TRIB3 was shown to interact with the nuclear receptor peroxisome proliferator-activated receptor gamma (PPARγ) [76], an important regulator of adipogenesis, insulin signalling and lipid metabolism [76, 77]. PPARγ promotes the expression of adipocyte related genes inducing intracellular storage of fat [78], and the involvement of TRIB3 in adipocyte differentiation is thought to be mainly governed by PPARγ. TRIB3 has been shown to suppress PPARγ signalling through protein-protein interaction. Conversely, TRIB3 knockdown induces adipocyte differentiation [10, 76]. Interestingly, in 3T3-L1 pre-adipocytes Takahashi *et al* [77] reported a direct binding to the full-length PPAR-γ by TRIB3, prompting a decline in PPAR-γ and CCAAT enhancer binding protein α (CEBPα) production, eventually impeding adipocyte differentiation [76]. Additionally, TRIB3 has been shown to act as negative regulator of activating transcription factor 4 (ATF4) via inhibiting ATF4 transcriptional activity in stress response setting and type 2 diabetes [79, 80].

Immune homeostasis is a physiological condition, which TRIBs are associated with, and that includes the relationship with inflammatory signalling networks such as the Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB). NF-κB are a family TFs that regulate cellular responses such as inflammation and proliferation, cell survival and innate/adaptive immune developments [81]. NF-κB are activated by Toll-like receptors (TLRs) [82] and TRIBs have been shown to play a role at this level. TRIB1 has been shown to negatively regulate the transcription of the nuclear factor for IL-6 expression (NF-IL6), and consequently NF-IL6–dependent gene expression, in TLR-mediated signalling [83]. TRIB1 overexpression in macrophages prevented NF-IL6-dependent gene expression, thereby reducing its protein levels. Inversely, TRIB1-deficient cells displayed increased NF-IL6 DNA-binding activity with augmented levels of NF-IL6 proteins. A study by Wei *et al* [83] revealed that TRIB2 binds and

regulates TLR5 signalling pathway, leading to inhibition of NF-κB activity in inflammatory bowel disease [82]. In *Porphyromonas endodontalis* lipopolysaccharide-treated mouse osteoblasts, TRIB3 overexpression enhanced NF-κB phosphorylation, while TRIB3 knockdown inhibited NF-κB phosphorylation and Wnt5a expression [84]. Furthermore, a similar pattern showing TRIB3 induction during ER stress in INS-1 cells and primary rodent pancreatic islets cells resulted in the activation of NF-κB pathway, thereby enhancing pancreatic β cell apoptosis. Whereas, NF-κB pathway inhibition significantly diminished responses for pancreatic β cell apoptosis [85]. This indicates a complex inverse correlation between TRIBs and NF-κB signalling *in vitro* and *in vivo*.

Both TRIB1 and TRIB3 have been shown to play a regulatory role in the AP-1 pathway via binding to MAPKs. In HeLa cells, upregulation of endogenous TRIB1 and TRIB3, as well as their overexpression was shown to inhibit AP-1 activity [86]. In colorectal cancer, TRIB2 has been shown to interact with AP-4, enhancing AP-4-mediated transcriptional activity. The physical interaction of TRIB2-AP-4 increased binding of AP-4 on p21 promoter thereby, negatively regulating p21 expression [87], which was proposed to have a role in colon tumorigenesis.

Evidence of the regulation of NFκB by TRIB1 was demonstrated by Gendelman *et al* [48] where they showed that knockdown of TRIB1 inhibits NFκB-responsive promoter activity, whereas TRIB1 overexpression increases promoter activity. Certainly, the relationship between TRIBs and all the described signalling pathways and transcription factors present a dynamic and valuable olecular prospect for development of new drugs targeting several human diseases.

Table II.1. Transcription factors whose activity is regulated by the different TRIBs isoforms.

Tribbles	Transcription factor	Disease	Reference
TRIB1	AP-1	Inflammatory bowel diseases	[82, 86]
	NF-κB	Type 2 diabetes, pancreatic cancer	[24, 48, 88]
TRIB2	AP-4	Colorectal cancer	[87]
TRIB3	AP-1	Inflammatory bowel diseases	[82, 86]
	NF-κB	Type 2 diabetes, pancreatic cancer	[24, 48]
	PPARγ	Insulin resistance and obesity	[10, 89]

2.5. Transcription factors regulating tribbles expression

A fundamental process in eukaryotic organisms is the transcriptional regulation of gene expression, as it ultimately enables cells to carry out their specialized functions [77]. The transcription of the three TRIBs isoforms has been shown to be controlled by a combinational regulation of several TFs [2]. Several investigations have disclosed the TFs that regulate TRIBs expression in distinct physiological conditions, though a substantial amount of information came from AML (Table 2). Identified TFs are, for example, ATF4, C/EBPα, C/EBPβ, CHOP, E2 factor (E2F), FOXO1, FOXO3a, FOXP3, paired-like homeodomain 1 (PITX1), folded gastrulation (FOG), GATA-binding factor 2 (GATA2), Notch homolog 1 (NOTCH1), MEIS Homeobox 1 (MEIS1), Homeobox A9 (Hoxa9), and Ecotropic viral integration site-1 (Evi1).

Table II.2. Transcription factors that bind to the TRIBs promoter region regulating TRIB expression.

Tribbles	Transcription factors	Disease	Reference
TRIB3	ATF4	Type 2 diabetes, insulin resistance	[19, 79, 80]
TRIB1, TRIB2, TRIB3	C/EBPα, C/EBPβ, CHOP	AML, Stress related & neurodegenerative diseases, type I diabetes	[6, 19, 76] [90]
TRIB2	E2F	AML	[91]
TRIB1, TRIB3	FOXO1, FOXO3a, FOXP3	Melanoma cells, metabolic syndromes	[71, 73] [92]
TRIB2	PITX1	Leukaemia	[93, 94]
TRIB1, TRIB2	MEIS1, Hoxa9, Evi1	AML	[95-97]
TRIB2	NOTCH1	AML	[98]
TRIB2	FOG and GATA2	Acute leukaemia	[99]

In the context of leukaemia, when C/EBP homologs such as CHOP, C/EBPα and C/EBPβ are bound to the TRIB3 promoter region, upregulation of its transcription occurs [57]. In turn, TRIB3 exerts a negative feedback mechanism inhibiting the expression of CHOP [99]. Meaning there could be a regulatory loop between the C/EBP family of TFs and the TRIBs proteins. Conversely, TRIB3 is induced via AFT4/CHOP pathway during unfolded protein response [100]. Present in the TRIB3 promoter (specifically in tunicamycin response region) are amino-acid response elements which overlap the binding site of CHOP. Activation of this promoter activity is via Tunicamycin treatment and interaction with CHOP and ATF4. Meaning, CHOP or ATF4 knockdown supresses tunicamycin-initiated TRIB3 induction [19]. In AML, the E2F family members also have the ability to bind and induce TRIB2 promoter

activity, demonstrating the direct regulation of TRIB2 expression by E2F proteins [91]. PITX1, FOG, GATA2 and NOTCH1 have also been mentioned to regulate TRIB2 in leukaemia through directly binding to the TRIB2 promoter region [97, 98, 101]. Other TFs that have shown activity in the TRIB isoforms promoters belong to the FOXO family. The presence of putative FOXO-binding sites has been shown in the TRIB3 promoter region, having the ability to enhance its expression [66]. Furthermore, in melanoma, TRIB2 has been demonstrated as a repressor of FOXOs. Thus, providing an advantageous setting for cellular growth and survival [71]. In some circumstances, a combination of TFs may work together in modulating the transcription of various TRIBs isoforms. For instance, CHOP and ATF4 amalgamate to activate TRIB3 promoter activity in kidney cells during ER stress. Nevertheless, ATF4 overexpression alone also causes TRB3 promoter transcriptional activation[19]. Other evidence of co-operative TFs regulating TRIB1 and TRIB2 to accelerate AML are Evi1, MEIS1 and Hoxa9. These genes have been identified to have similar integration sites thereby coactivate with one another [95]. *In-vivo*, Meis1 was shown to contain an in occupancy on TRIB2s regulatory sequence [94].

2.6. Tribble's expression and function in distinct physiological conditions

TRIB proteins have been described as important regulators of cell cycle, cell proliferation and differentiation, inflammation, metabolism, and stress cell responses. The following sections address the TRIBs' tissue expression and function in distinct biological processes and physiological conditions as reflected in these specific areas.

2.6.1.Development

The TRIBs' role in embryogenesis and cell differentiation has been studied in multiple vertebrate and invertebrate models, including; rodents, *Xenopus, Drosophila spp*. and *Caenorhabditis elegans* [21]. However, the most consistent development research has focused on *Drosophila*. Nevertheless, the first identification of TRIBs was during gene screening, in which the mutations controlling cell division and migration in the development of embryonic *Drosophila* were investigated [94, 102]. Such research has revealed that TRIBs are critical regulators of *Drosophila* development in oogenesis. The number of germ cell division and oocyte determination is affected by TRIB levels. TRIB acts through proteasomal degradation of String and Twine which are CDC25 mitotic activators. TRIBs controlling CDC25 initiates mitosis and cell fate

determination [30]. During the development of *C. elegans* TRIBs ortholog NIPI-3 acts as a negative regulator of the CEBP-1 transcriptional expression in numerous tissues [103]. NIPI-3 inhibits CEBP-1, thereby enabling its development and maintaining proliferation of larval. Moreover, NIPI-3 is an important defence factor against translational inhibitors such as ToxA in *C. elegans* [103]. Also, NIPI-3 has a significant role in immunity, it acts by enabling survival of host tissue from pathogen-mediated damage. Within intestinal innate immune signalling, it has been shown that NIPI-3 negatively regulates C/EBP-1, sequentially negatively regulating downstream protective immune responses. In genetic mutation studies of NIPI-3 mutant, NIPI-3 negatively regulates PMK-1/p38 signalling by transcriptional suppression of CEBP-1 [104]. In murine models, TRIB3 has been linked to embryonic brain development via increasing its expression in early prenatal state. Neuronal characterization also revealed enlarged lateral ventricles in the absence of TRIB3 [104]. During an *in-vivo* study in zebrafish embryos, increased ectopic TRIB2 expression promoted primary erythrocyte differentiation by increasing erythropoiesis-related genes. TRIB2 is also crucial for haemolytic tolerance during development [105]. Finally, a study in xenopus embryos of TRIB2 homolog Xtrb2 suggested a role in cell movements. Xtrb2 expression has been observed in multiple development stages, namely, maternally, blastula and gastrula, dorsal neural tube, eyes, and cephalic neural crest [106]. A morpholino injection (prevents the mid-blastula transition delay) was administered into the embryos which resulted in involution defects within in the dorsal mesoderm[107]. This is consistent with the previous findings in gastrulation defects in *Drosophila* trbl mutant. Knockdown of Xtrb2 delays mitosis progression in the embryo thereby disrupting G1/S associated delamination and migration, closure of the neural tube, development of eyes and segmentation of somites [106].

Although TRIBs function is not fully understood, the family members have an important role in the differentiation of macrophages, adipocytes, and bone and muscular tissue. TRIB1 is vital for controlling M2-like macrophage differentiation and its deficiency causes irregularity in macrophage differentiation [108] TRIB3 is important in the regulation of bone morphogenetic proteins (BMPs) signalling in embryonic cells [16]. Also, downregulation of TRIB3 reduces BMP-mediated osteoblast differentiation of mouse myoblast cells and other cellular responses like smooth muscle cell phenotype maintenance [109]. In adipocyte production, prior to the growth arrest period mitotic clone expansion, TRIB2 and TRIB3 expression are

briefly suppressed leading to a simultaneous return into the cell cycle, which is accompanied by the alteration of gene expression pattern to produce the typical phenotype of mature adipocytes [110]. This decreased expression of TRIBs is vital for fat cell formation. Continuous TRIB2 and TRIB3 expression has been found to block cell adipogenesis, and contrastingly these TRIBs' knockdown causes premature differentiation and mitotic clone expansion [77, 111]. Regulation of cell cycle in preadipocytes by TRIBs is unclear. Nevertheless, TRIBs inhibitory effects on differentiation can occur through numerous actions such as Suppression of C/EBPβ, C/EBPα activity [104]. Additionally, TRIB1 contributes to adipogenesis regulation via binding to liver-enriched transcriptional activator protein (LAP), thereby activating isoform of C/EBPβ. Degradation of an enzyme like Acetyl coenzyme A carboxylase (ACC), as it regulates fatty acid synthesis [111].

Overall, many contradictions and knowledge gaps still are found in the development stage studies using various animal models such as the role of TRIB mutant phenotypes and tissue specificity concerning TRIBs action.

2.6.2. Inflammatory stimulation/immune system

TRIBs have a direct and indirect involvement in the regulatory signal mechanisms of immune cells that further develop into chronic inflammatory diseases. Early studies show TRIB isoforms regulate inflammatory signalling pathway, through binding to c-Jun N-terminal kinases (JNKs), and p38 thus regulating IL-8 production [112]. Additionally, reports have also shown TRIBs to regulate the MAPK pathway activation pathways in response to a range of inflammatory signals [86, 113]. Within various cell types and tissues TRIBs may act as either pro or anti-inflammatory [23]. TRIB1 has been mainly observed to play an anti-inflammatory role in many biological processes. In macrophages, TRIB1 protein was associated with innate inflammation signalling through its interaction with multiple key proteins such as COP1 and C/EBPα, whereas M2-macrophage polarisation is seen in a TRIB1 deficient microenvironment [23, 114]. Furthermore, loss of macrophage-Trib1 expression in the arterial wall is linked with the promotion of a combination of inflammation in the atherosclerotic plaque, atheroma formation and/or impaired resolution of inflammation [23]. In chronic inflammation of human atherosclerotic arteries, TRIB1 expression is increased, thereby, lowering cell proliferation and chemotaxis of vascular smooth

muscle [8, 55].

TRIB2 has been shown as a novel regulator of inflammatory activation of monocyte [55]. Decreased TRIB2 levels enhanced LPS-induced IL-8 production through increased activation of the MAPK pathway. Furthermore, modified LDL extremely down-regulated TRIB2 expression, consequently, contributing to the inflammatory processes in vascular disease development [55]. Upregulation of TRIB2 in human atherosclerotic plaques is also evident. TRIB2 decreases IL-10 mRNA expression in primary human monocyte-derived macrophages, which suggests that TRIB2 may be important for plaque instability and inflammation [87]. Also, numerous inflammatory signalling targets and cascades which TRIBs regulate are triggered by toll/interleukin 1 receptors (TLR/IL-1R) ligands [115]. Particularly, there is a feedback regulation loop of TRIB2 inhibiting TLR-5 signalling during flagellin (an TLR-5 ligand) cell stimulation, causing TRIB2 to be upregulated. Relatively, TRIB2 is substantially diminished in inflammatory bowel disease compared with the inactive disease [115].

Considering TRIB3, a study in lymphocytes suggested that it inhibits inflammation, improving acute and chronic kidney disease outcomes [25, 116]. TRIB3 complex tissue specific signalling inflammation has been reported. In oesophageal cancer cells, TRIB3 interacts with RelA, an NF-kB family member, inhibiting NF-kB signalling [83]. Furthermore, indirect inhibit of NF-kB can occur through TRIB3 downstream effector actions in the ATF4-CHOP signalling cascade [117, 118]. In contrast, TRIB3 enhances NF-kB activity during apoptotic induction in pancreatic β cells [119]. Further identification of the key drivers in the regulatory pathways associated with TRIBs' actions would enable a better understanding of the protein's role in inflammation.

2.6.3. Insulin resistance

Insulin signalling is among the highlighted molecular mechanisms in which TRIB family members are involved in, particularly TRIB3 isoform [120]. It has been reported TRIB3 impairs the actions of insulin being associated with type 2 diabetes and insulin resistance [86, 121, 122]. In skeletal muscle and liver, TRIB3 expression is induced by conditions like metabolic stress, and also plays a vital role in regulating AKT protein homeostasis [123]. This occurs by TRIB3 directly interacting with AKT, a key modulator of insulin action in target cells [124]. TRIB3 acts by blocking the

phosphorylation of AKT, thereby disrupting insulin signalling and inhibiting the suppression process of hepatic glucose homeostasis [125].The overexpression of TRIB3 in skeletal muscle directly inhibits insulin signalling, therefore contributing to insulin resistance [123] and suggesting TRIB3 as a potential insulin sensitivity target in patients with type 2 diabetes. Furthermore, in nutrient excess conditions insulin resistance is linked with TRIB3 increase in skeletal muscles which inhibits glucose uptake and enhances mitochondrial glucose oxidation subsequently decreasing TRIB3 expression in the adipose tissue that would increase glucose transport required for triglyceride storage. Whereas nutrient deprivation reduces TRIB3 levels in the skeletal muscle, enhancing glucose transport and reduces glucose oxidation [27].

In pancreatic β cells and peripheral tissues, insulin signalling is inhibited by TRIB3 action binding to AKT/PKB, and reduction of AKT2 activity [120]. Not only is this effect seen in human cells but also in mice hepatocytes where TRIB3 overexpression triggered insulin resistance, whereas TRIB3 knockdown increased sensitivity to insulin signalling [123, 125]. Interestingly, there are converse effects of TRIB3 which are yet to be understood; for example, in adipocytes TRIB3 has a positive effect of increasing fatty acid oxidation [126]; on the other hand, ER stress induced TRIB3 expression has a negative effect in the liver through decreasing insulin signalling via AKT binding, in turn inhibiting AKT actions [127]. Also, bear in mind that the inhibitory effect of TRIB3 on AKT activation is regulated by an adaptor protein, phosphotyrosine interacting with PH domain and leucine zipper 1 (APPL1), which is an adaptor molecule that contains a NH2-terminal Bin/Amphiphysin/Rvs domain [128]. In primary rat hepatocytes, AKT competitively interacts with both TRIB3 and APPL1. APPL1 overexpression eradicates the impaired effects of insulin caused by TRIB3 overexpression, indicating that APPL1 can directly antagonize the inhibitory actions of TRIB3 on insulin and AKT signalling [128]. Apart from AKT, MAPK is another signalling pathway important in selective insulin resistance [19]. Since TRIB3 functions as a molecular switch regulating three classes of MAPK activation [86], the TRIB3/MAPK signalling axis also was implicated in insulin responsiveness. TRIB3 upregulation contributes to insulin resistance binding to and regulating MAPK kinases activity [55, 129]. The correlation described between TRIB3 and insulin resistance also was demonstrated in obese patients[130]. Transcript levels of *TRIB3* were measured in hepatic, subcutaneous and visceral tissue. An increased abundance

of *TRIB3* transcript was observed in all three tissue types, including associated markers of insulin resistance such as *SREBP-1c,* which functions as a gene regulator required for glucose metabolism [130]. Altogether, the existent studies in varying tissues strongly confirm the role of TRIB3 in insulin resistance.

2.6.4. Obesity

Obesity is a major risk factor for multiple diseases including insulin resistance and type-2 diabetes, hypertension, cardiovascular diseases and aggressive cancers [131]. The adipose tissue in obesity has been shown to promote cancer cells' growth and aggressiveness by providing a dysregulated microenvironment with increased growth factor activation and secretory proteins (e.g. pro-inflammatory) that enable invasion and migration of cancer cells [132]. Cancer cells are also characterized by undergoing a set of metabolic dysfunctions and an adjustment of energy metabolism that enhance cell proliferation, invasion, and survival, limiting or avoiding apoptosis [133]. This metabolic reprogramming is one of the established hallmarks of cancer, and relies mainly on the upregulation of aerobic glycolysis, glutaminolysis, mitochondrial biogenesis, and lipid metabolism [134]. Therefore, high-lipid availability in obesity, hypercholesterolemia and elevated serum triglycerides have been established as risk factors for several types of cancer [135]. Interestingly, multiple metabolic malfunctions in mammalian cells have been associated with changes in TRIB3 expression levels. TRIB3 was implicated to play an important part in obesity, particularly in signalling that regulate hepatic lipid homeostasis and glucose metabolism [136, 137]. A study clarified the mechanisms by which TRIB3 affects lipid metabolism leading to obesity by using a TRIB3 knockout mice model, in which common targets of TRIB3 action, namely CEBPα, PPARα, AKT and triglyceride content, were measured. Compared to WT mice, altered expression of these targets was found in theTRIB3 knockout mice liver [131]. The TRIB3 deficient mice exhibited obesity with phenotypically obese livers, displaying also increased GLUT2 levels and triglyceride storage, and elevated plasma levels of very-low-density lipoprotein (vLDL) and cholesterol [131]. In a different context, TRIB3 has been demonstrated to mediate obesity-induced interference in brown adipocyte function and differentiation, as TRIB3 inhibition resulted in improved function of brown adipocytes. Transgenic mice with an obese phenotype and high-fat diet fed animals showed increased TRIB3 in brown adipocytes which consequentially impaired tissue function [138].

Furthermore, it has also been shown that obesity through high-fat feeding in mice and type 2 diabetes in humans significantly increases TRIB3 expression in skeletal muscle, which suggests that glucose metabolism and lipid hepatocytes are via TRIB3 regulation [123, 139]. Moreover, increased levels of TRIB3 during fasting have been observed in transgenic mice expressing TRIB3 in the adipose tissue, which promotes lipolysis by initiating the ACC degradation. Also, TRIB3 triggers ubiquitination of ACC via association with the E3 ligase COP1 [137]. Suggesting that TRIB3 contributes to controlling numerous molecular pathways associated with adipose tissue differentiation and lipid storage by acting as a negative regulator of both processes in mature adipocytes [137].

TRIB1 is another family member reported to be involved in processes such as lipogenesis and glycogenesis which may lead to obesity [140]. In hepatic cells, TRIB1 actions have been demonstrated to indirectly regulate lipid metabolism via associated genes namely fatty acid synthase (FASN), ACC1 and stearoyl-coenzyme A desaturase1 (SCD) [141]. In addition, TRIB1 has been shown to regulate triglycerides and vLDL levels in mice [142]. Overexpression of TRIB1 in hepatic tissue significantly decreased plasma triglycerides and cholesterol levels by reducing vLDL production. Inversely, TRIB1 knockout mice showed increased levels of plasma triglycerides and cholesterol due to augmented vLDL production [142].
Also, *TRIB1* polymorphisms have been linked to dysregulated serum lipids levels [143, 144]. A study in a Chinese Han cohort showed that *TRIB1* polymorphism affected plasma lipid metabolism. A comparison of TRIB1 rs17321515 genotype carriers and non-carriers with or without non-alcoholic fatty liver disease and/or coronary heart disease demonstrated that patients with the rs17321515 allele had higher serum triglycerides and total cholesterol levels, as well as high fasting plasma glucose and high-density lipoprotein (HDL) levels [144]. The same TRIB1 rs17321515 SNP in a Spanish familial hypercholesterolemia (FH) cohort revealed an association with dyslipidemia (i.e., HDL, vLDL-cholesterol imbalance, and high total cholesterol concentrations). Anthropometric measures of patients with genetic diagnosis of FH showed considerably higher waist circumference and dyslipidemia compared to minor allele carriers of FH [143]. Altered serum lipid levels is typical of obesity phenotypes and known to increase the risk of non-alcoholic fatty liver disease and coronary heart disease. However, further studies are required to fully understand how TRIB1

influences lipid metabolism and its contribution to obesity.

2.6.5. Stress response

The ER is a large eukaryotic organelle essential for calcium storage, synthesis, transport and folding of proteins [145]. Disruption of its function can lead to ER stress, thereby impairing protein folding and many other functions [146]. Characterization of the ER stress may be through increased expression of multiple associated TFs such as phosphorylation of protein kinase-like ER kinase (PERK), activating X-box binding protein 1 (XBP1, transcription factor 6 (ATF6), and CCAAT/enhancer-binding protein homologous protein (CHOP) [147]. Several intracellular and extracellular modifications can cause ER stress triggers. TRIB3 was indicated as a novel ER-related protein found to be upregulated during the ER stress response. For example, high doses of ethanol, which suppress insulin signalling, showed to elevate TRIB3 levels [148]. The mechanism by which TRIB3 expression is induced is not entirely known. However, TRIB3 mediated ER stress by preventing the activation of pathways associated with effectors such as Glycogen synthase kinase 3 beta (GSK3β), and Sterol regulatory element binding proteins 1 (SREBP-1) [36, 146]. Consistent with He *et al.* [146], Ohoka *et al.* [127] suggested that TRIB3 is induced via ATF4-CHOP pathway. Previous reviews have shown that TRIB3 interaction with ER stress and the mTORC pathway [141]. It is known that TRIB3 blocks the phosphorylation of AKT at Ser473, which indicates that TRIB3 may inhibit the function of mTORC2. This may be through binding to mTOR and the rapamycin-insensitive companion of mTOR (Rictor), thereby modulating its activity [149]. Within the multifaceted signalling networks, TRIB3 acts as a juncture of ER stress and mTORC function.

TRIB1 has also been linked to ER signalling in prostate cancer and was described to control ER chaperone Glucose regulatory protein 78 (GRP78) expression, a protein essential for prostate tumorigenesis. Additionally, knockdown of TRIB1 significantly decreased the mRNA expression levels of numerous ER chaperones and associated genes in prostate cancer cells [88]. ER stress can also trigger autophagy, which is a regulated degradation process whereby dysfunctional cellular components are removed. Reports of reciprocal inhibition between autophagic and proteasomal degradation have been shown in cancer and diabetes via TRIB3 mediation and insulin-IGF1, thus promoting a malignant disease. Insulin-IGF1-suppressed

autophagic flux is restored when TRIB3 is silenced, therefore reducing tumour growth and metastasis [117]. In glioma cells, TRIB3 is induced by ER stress, which in turn modifies autophagic induction. Effects of TRIB3 on AKT can modulate mTORC, subsequently activating autophagy [25]. Autophagy may facilitate with the ER-associated degradation systems to diminish misfolded proteins, thus improving viability of healthy cells. Such findings provide a better insight on TRIB–ER axis and its involvement in multiple signalling networks and disease progression.

2.6.6. Hypoxia

Hypoxia, a well-studied hallmark of solid tumours associated with resistance mechanisms and poor prognosis after treatment [150], has been related with TRIBs' actions. TRIB3 has been reported to be overexpressed in multiple human tumours which was linked to hypoxic environments [16, 18, 36]. A study demonstrated that hypoxic growth condition (48 h and 72 h) produced an accumulation of endogenous TRIB3 protein and also upregulated its mRNA transcript levels in colorectal and prostate cancer cells [36]. Furthermore, a co-localization study of TRIB3 with a hypoxia marker pimonidazole revealed an upregulation of TRIB3 after hypoxia in breast cancer cells, tissue and xenografts [18]. Also, in pulmonary arterial endothelial cells in acute hypoxia (6 or 8 h) TRIB3 protein levels significantly increased compared with normal conditions, which was shown to be a time-dependent effect [151]. The mechanisms that lead to TRIB3 overexpression in hypoxia started to be clarified and implicate changes in the microenvironment with the activation of cellular stress response pathways for instance, ER stress and unfolded protein response [16]. Moreover, the HIF-1α was shown to upregulate TRIB3 expression levels in renal cell carcinoma (RCC) cells, which enhanced cell proliferation, migration, and invasion via MAPK pathway [16].

Considering TRIB1, analysis in a transgenic mice model has demonstrated that TRIB1 overexpression decreases the infiltration of hypoxic tumour-associated macrophages in the tumour microenvironment [152]. Consequently, accelerated breast tumour growth was only seen at a later stage compared to the TRIB1 knockout mice [152]. Increased presence of TRIB1, TRIB3 and their associated genes in hypoxic conditions indicate their importance in modulating tumour cell growth. TRIBs expression and actions over the tumorigenesis process are detailed in the following section.

2.7. Tribbles' actions in cancer

In humans, recent research has demonstrated TRIBs key role in tumorigenesis and disease progression in numerous cancer types namely, acute and chronic leukaemia [5, 153], renal cell [16], colorectal [154], liver [155], glioma [156], lung [4, 157], pancreatic [158], thyroid [14], ovarian [11], breast [152] and prostate [88, 159]. The exact relationship between tribbles and these cancers is yet to be understood. Nevertheless, TRIBs are found to influence various transcriptional networks, which control critical signalling pathways that regulate substrate degradation, cell proliferation, and differentiation. TRIBs are generally associated with imbalanced cellular systems and have been identified as irregular drivers of transcription, translation, or protein turnover in multiple tissues. Interestingly, one of the highest TRIB1 expression is found in the thyroid gland, and this TRIB isoform was suggested to be involved in the aetiology of thyroid cancer [14]. However, published data has not shown yet the mechanisms underlying its action.

In colorectal cancer (CRC), TRIB1 was correlated with the metastization process, as it was shown to promote cell motility and invasion by activating MMP-2 via FAK/Src and ERK pathways [154]. Considering TRIB2, a report demonstrated its action blocking cellular senescence through AP4/p21 signalling and activating AKT, which promoted resistance of CRC to therapy, being associated with poor prognosis [160]. The involvement of TRIB2 in promoting drug resistance seems not to be exclusive of CRC, as similar outcomes were found in other cancer cell types namely, melanoma, colon, pancreatic, breast, renal and osteosarcoma [49]. Hill *et al.* showed that TRIB2 is a key regulatory target of the PI3K signalling complex exerting a direct interaction with AKT, which ablates forkhead box O activation disrupting the p53/MDM2 regulatory axis and conferring resistance to chemotherapy. Moreover, the significantly increased TRIB2 expression in patient tumour tissues was associated with an enhanced phosphorylation of AKT, MDM2, FOXO3a and diminished response to therapeutics [49]. Also, TRIB3 was shown to increase stem cell-features and CRC tumorigenesis through its interaction with β-catenin, a central component of the cadherin protein complex, and transcription factor 4 (TCF4) [9].

In RCC, no reports exist of TRIB1 and TRIB2 actions, but the oncogenic role of TRIB3 has been proposed. Elevated TRIB3 expression levels were seen in RCC and associated with advanced tumour stage and negative prognosis [16]. Moreover, TRIB3

overexpression significantly enhanced RCC cell proliferation, invasion and migration, while knocking down its expression inhibited these biological processes [16]. Overexpression of TRIB2 and TRIB3 are also detected and frequently amplified in lung cancer [4, 157]. *In vitro* studies knocking down TRIB2 demonstrated the suppression of cell proliferation and tumour growth, which implicates TRIB2 in lung tumorigenesis [4]. Similarly, TRIB3 knockdown significantly inhibited malignancy, cell proliferation, invasion and tumour growth whereas TRIB3 upregulation in lung cancer cases correlates with metastasis, recurrence, and poor patient survival [88, 157]. TRIBs pseudokinases also show significant regulation and partake in mechanisms that occur during the development and progression of numerous subtypes of leukaemia's, such as myeloid, chronic lymphocytic and acute promyelocytic (APL). TRIB-mediated degradation of TFs like C/EBP is one of the most understood pathways linking TRIBs with the promotion of oncogenesis, which has been detailed in the context of leukaemia. Many AML patients with C/EBPα mutations display an interesting mechanism where C/EBPα p42 isoforms are degraded by both TRIB1 and TRIB2, which results in an overabundance of p30 [153, 161]. C/EBPα degradation by TRIBs influences AML pathogenesis and patient's response to therapeutics, as TRIB1s repression of C/EBPα generally maintains myeloid and stem cell homeostasis [188]. Also, elevation of TRIB3 promotes APL, which occurs via its interaction with promyelocytic leukemia-retinoic acid receptor α (PML-RARα), preventing modifications such as sumoylation, ubiquitylation, and degradation [162].

Studies indicating TRIB1, TRIB2, and TRIB3s association with pancreatic cancer have been recently published. The previously mentioned Chinese Han population case study showed that TRIB1 single nucleotide polymorphisms contributed to the development of pancreatic cancer, however its prevalence remains unknown [158]. TRIB2 protein by its interaction with AKT, inhibition of FOXO activation, and disruption of the p53/MDM2 axis promotes resistance to anti-cancer therapies [49, 61]. Also, it is suggested that pancreatic cancer cell growth, viability, and invasion is enhanced through a long non-coding RNA, zinc finger E-box-binding homeobox 1-antisense 1 (ZEB1-AS1) by regulating miR-505-3p/TRIB2 axis [163]. In terms of TRIB3, its overexpression significantly increased NF-kB activity which lead to the induction of apoptosis in pancreatic β cells [119]. Indeed, the TRIB3 and NF-kB complex relationship has been previously reported as detailed before in this review.

TRIBs dysregulation is also seen in the development of cancer of male and female reproductive systems. Although the area lacks extensive research, the relationship between TRIBs and breast, ovarian, and prostate cancer has been investigated. It is important to note that TRIBs are frequently overexpressed in reproductive cancers, which gives rise to notion that TRIBs are relevant for tumorigenesis. TRIB1 was shown to be overexpressed in triple-negative breast cancer, regulating NF-κB/AP1-dependent transactivation of cyclin D1. This reveals that TRIB1 has an important role in regulating cell cycle and cell survival, which is NFkB-mediated [48]. Augmented levels of TRIB3 have also been described in human breast cancer patients [100, 101, 164, 165]. Wennemers *et al* [101] showed a relation with TRIB3 mRNA levels and poor prognosis of breast cancer patients, which may be influenced by AKT inhibition. High TRIB1 expression is also associated with a poorer survival rate in ovarian cancer patients [11]. TRIB1 overexpression activates ERKs which in turn promotes ascites to regulate OV-90 cell invasion, suggesting that TRIBs participate in the control of tumour cell behaviour [11], likely being involved in metastization. Noteworthy, a report in glioma cells suggested that TRIB1 also seems to play an important role in the development of radio-resistance. Human glioma cells display upregulated TRIB1 upon irradiation, which resulted in a significantly higher radioresistance [156]. A formed complex of TRIB1 with histone deacetylase-1 (HDAC1) binds to the p53 promoter which interferes with the p53 expression in glioma cells. Thus, TRIB1 overexpression promotes radioresistance by the repression of TP53 expression [156]. In the case of hepatocellular carcinoma (HCC), all three TRIB subtypes are associated with it. TRIB1 has been described to play key role in liver function through CEBPα degradation due its ubiquitin ligase activity [166]. Also, TRIB1 interaction with hepatic regulatory networks targets such as hepatocyte nuclear factor 4 Alpha (HNF4A) and p53 promotes tumorigenesis and invasiveness of HCC [155, 167]. In what concerns TRIB2, its elevated expression levels were shown to be essential for liver cancer cell growth, survival and malignant transformation via Wnt/β-catenin, a signalling network that regulates crucial cellular functions like genetic stability, proliferation, differentiation, stem cell renewal, migration, and apoptosis [168-170]. Similar to TRIB2, upregulation of TRIB3 promotes cell growth in both *in vitro* and *in vivo* models of HCC. Furthermore, clinical observations show high TRIB3 levels correlate with large tumour size and poor prognosis [171].

Lastly, studies of the role of TRIBs in prostate cancer progression identified

upregulation in both TRIB1 and TRIB3 mRNA and proteins levels [88, 114, 172, 173]. TRIB1 overexpression in prostate cancer cells has been shown to promote cell survival and tumorigenesis through the TRIB1-ER chaperone axis [88]. TRIB1 was shown to regulate GRP78, an important ER chaperone with a crucial role in the unfolded protein response, which is involved in maintaining the prostate tumour-propagating cells and associated with tumour recurrence [88]. In line with this, a previously published report demonstrated TRIB1 contribution to prostate cancer via analysing the effect of its overexpression in murine models [159]. *Pten* $^{pc-/-}$ mice overexpressing TRIB1 displayed increased incidence of prostate adenocarcinoma and high-grade intraprostatic epithelial lesions. Moreover, TRIB1 was shown to be overexpressed in human prostate cancer cases and linked to the 8q24 amplicon that is involved in the oncogenesis of prostate cancer. The transcription factor and oncogene *cMYC*, which is a marker of tumour aggressiveness and poor outcome in a wide range of cancers [174], is amplified in chromosome 8q24. An interesting fact reported in this work was not only was TRIB1 co-amplified with the *cMYC* amplicon, but it is also transcriptionally regulated by *cMYC*, suggesting a dependency between these genes [159]. There is a lack of information on the role of TRIB3 in PCa, therefore more studies are required to define the regulatory actions of TRIB3 in this urological cancer.Investigations on miRNAs modulating TRIB1 levels and altering gene expression profile of a number of inflammatory targets in human macrophages and prostate cancer cells have been previously seen [8, 88, 114, 172]. It has been shown that silencing or downregulating miRNAs such as miR-101-3p and miR-132-3p increase TRIB1 expression levels in prostate cancer cells. Furthermore, overexpression of TRIB1 in human macrophages significantly enhanced genes linked with activated macrophages [114], which can be a crucial response in the case of tumour's microenvironment. Indeed, a significant increase in tumour infiltrating macrophages was observed in the prostate epithelium of TRIB1 overexpressing transgenic mice model, which suggests that the tumour-promoting actions of TRIB1 are possibly correlated to non-cell autonomous function [159]. Advances in studies of the mechanistic function of abnormally expressed TRIBs mRNA and proteins, and clarifying their relationship with other oncogenes, such as the *cMYC*, could be potentially useful for diagnostic and prognostic processes of cancer.

2.8. Final remarks

TRIB research has come a long way beginning from *Drosophila* to advanced mammalian studies. Over the years, demonstrations of TRIBs involvement in several cellular systems and signalling pathways have been published. Many reports have highlighted TRIBs as relevant prospects for their benefit as therapeutic targets and biomarkers, due to their involvement in a variety of cellular processes in supporting the degradation of target proteins and regulating several signalling networks. Herein, we have focused on the complex role of TRIB proteins in regulatory mechanisms leading to disease development and progression. The information assembled in this review strengthens the theory that TRIB proteins have a highly unique characteristic of expression that is multifaceted and ambiguous, having different isoforms oncogenic and/or tumour suppressive functions which are tissue specific. Moreover, elevated expression levels of all three TRIBs have been identified as contributors of radio or chemotherapeutic resistance through pro-survival and anti-apoptotic actions. Nevertheless, studies in several cell types and animal models have shown TRIB proteins to be the driving force of many deregulated physiological conditions and pathologies such as cardiovascular diseases, neurological and metabolic disorders [2, 26]. Therefore, it has been envisaged that manipulating TRIBs (possibly in the initial stages of neoplastic and non-neoplastic diseases) could be used as a treatment approach.

Research efforts on the characterization of TRIBs structure via crystallographic analysis has revealed a potential to design a promising and valuable small-molecules inhibitors capable of modulating its activity and function [175, 176].

Also, external stimuli can regulate TRIBs activity. It is for example the case of cannabinoids, used for their anti-tumour actions in various cancer models, and capsaicin, a main chemical compound in chili peppers that has various physiological effects on processes such as inhibition of proliferating cells [46, 47]. Based on their anti-proliferative and pro-apoptotic properties, the potential of these compounds as chemotherapeutic agents in several human cancers has been explored. *In vivo* studies using cannabinoids have revealed TRIB3 as a key regulator of the delta-9-tetrahydrocannabinol (THC) anti-cancer activity [46]. Genetic inhibition of TRIB3 was shown to prevent both cannabinoid-induced autophagy and apoptosis *in vivo*, which strongly implicates TRIB3 in the anti-cancer action of cannabinoids.

Also, capsaicin has been shown to target TRIB3 by upregulating its expression, resulting in the promotion of apoptotic cell death in various gastric cancer cells [47]. TRIB3 overexpression increased capsaicin-induced apoptosis, while knockdown revealed the correlation of capsaicin effects in apoptotic cell death with the induction of TRIB3 in gastric cancer cells. Suggesting that the combination of capsaicin and chemotherapeutic agents may provide an effective approach for the treatment of human gastric cancer. Even so, further research is necessary to understand their mechanism of action prior to developing them as therapeutics for cancer treatment.

Undeniably, the knowledge of TRIB isoforms has improved greatly in the past two decades, which is currently the building blocks for better understanding of the unidentified signalling modules and networks that regulate diverse cellular processes. Nonetheless, attaining more in-depth insight of the regulatory roles of TRIB isoforms in several biological processes will enable the development of novel disease biomarkers and targeting specific molecular players for therapeutic purposes.

2.9. References

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Chapter II:

2. Hypothesis and aims of the thesis

PCa is a hormone-dependent cancer, highly dependent on the actions of sex steroid hormones, namely, androgens. In the last years, it has been shown that, besides controlling PCa cell proliferation and survival, androgens also act as important metabolic regulators. Androgens play a relevant role in reprogramming the metabolism of PCa, modulating glycolysis, glutaminolysis and lipid metabolism. Moreover, this regulation accompanies the progression of disease and the establishment of CRPC. TRIBs proteins are a family of pseudokinases that play a key role in controlling immunity and lipid metabolism. Also, TRIB1 and TRIB3 actions have been implicated in several types of cancer, but their role in PCa is poorly understood. This project aims to clarify the relationship between TRIB1/3 expression and its androgenic modulation in PCa. Cell lines and *in vivo* models of disease were used to obtain valuable data, which may provide information supporting TRIB1/3 as novel therapeutic targets in PCa.

This thesis aims to elucidate the TRIBs association with PCa by undertaking the following:

1. Determine how androgens/anti-androgens affect TRIB1 (and TRIB3) expression levels and activity in neoplastic and non-neoplastic prostate cell line models;
2. Determine the effect of the androgenic regulation of TRIB1/3 expression in prostate cell intracellular signalling activity, cell proliferation, apoptosis, migration and epithelial mesenchymal transition.
3. Characterize the effect of silencing or overexpressing TRIB1/3 in cell intracellular signalling activity, prostate cell proliferation, apoptosis, migration, epithelial mesenchymal transition, and lipid metabolism.
4. Validate informative findings in PCa mouse models with or without altered TRIB1 or TRIB3 expression.

In **Chapter I**, it is described the general overview of the prostate gland. Additionally, the stages of PCa from initial to metastatic and how androgens affect its progression are presented. Furthermore, the epidemiology of PCa and risk factors are summarized. Additionally, a review of TRIBs pseudokinases structure and function, and expression pattern in distinct physiological conditions and diseases. **Chapter II**

outlines the hypothesis and aims of the thesis. The four Chapters thereafter are devoted to the aims and objectives of the thesis:

Chapter III characterizes TRIBs expression in PCa cell lines models.

Chapter IV investigates androgen actions in regulating TRIB1 and TRIB3 in LNCaP and 22RV1 PCa cells. The effects of androgens and anti-androgens on TRIB1 and TRIB3 expression, as well as the impact on signalling pathways activation, cell proliferation and migration. Additionally, the expression of key genes regulating lipid metabolism are analysed

Chapter V compares the effects of TRIB1 overexpression and TRIB1 KD in normal prostate epithelial cells and PCa cells.

Chapter VI entails a final summary and future perspectives of the project.

Chapter III:

3. Characterization of Tribbles expression in prostate cancer cell lines models

Abstract

Tribbles (TRIBs) are a family of pseudokinases that have been indicated as regulators of multiple biological processes such as transcription, cell survival, differentiation, and proliferation, inflammation, and metabolism. Also, TRIBs actions have been associated with processes contributing to the progression of several cancer types. However, the function and mechanism of action of TRIBs in prostate cancer (PCa) is poorly understood. This chapter aimed to investigate the basal endogenous expression of TRIB1/3 and to confirm validity of TRIB antibodies. To tease it apart a range of cells were used, including human non-neoplastic epithelial prostate cells PNT1A, neoplastic androgen-sensitive LNCaP and 22RV1, and androgen-insensitive PC3 and DU145 cells. Cells were seeded at 500,000 cells/well and starved for 24 h. siRNA TRIB1 knockdown and plasmid overexpression were also performed for 24 h before protein extraction for Western blot analysis. Observation of the results showed that TRIB1 and TRIB3 are expressed at basal level in both non-neoplastic and neoplastic cells. Knockdown of TRIB1 in PC3 and overexpression of TRIB1 in PNT1A also confirmed antibody specificity. Based on this analysis further work on androgenic regulation of TRIBs in PCa is more likely to provide more information which continues in chapter V.

Keywords: Tribbles; TRIB1; TRIB3; prostate cancer; androgen-sensitive prostate cancer cells; androgen-insensitive prostate cancer cells.

3.1. Introduction

Tribbles (TRIBs) are a serine/threonine pseudokinase family, which uniquely contain a catalytically inactive kinase domain [1]. TRIBs proteins have been described as important regulators of processes such as cell proliferation, differentiation, and survival, as well as metabolism, inflammation, and cancer development, among others [2]. Within the TRIBs pseudokinase family three TRIB proteins are known, TRIB1, TRIB2, and TRIB3. Their main mechanistic function is to act as adaptor molecules for signalling networks such as MAPK and PI3K/AKT modules [3].

TRIBs are found to be overexpressed in several cancers, specifically in acute myeloid leukaemia, melanoma, and liver and lung cancer [3, 4]. However, their role in cancer progression, and particularly in prostate cancer (PCa), is yet to be clarified. This chapter aims to characterize the expression of TRIB1 and TRIB3 in the PCa cell line models to be used in the present thesis. A panel of prostate cells (Table III.1) was selected to mimic the various stages of PCa from androgen-sensitive to -insensitive metastatic disease. Prostate cell lines under study were as follows; non-neoplastic epithelial cell line PNT1A, and neoplastic cell lines LNCaP, 22Rv1, PC3 and DU145. LNCaP and 22Rv1 express the AR and are both androgen-sensitive cell lines. In contrast, the more aggressive PCa cell lines PC3 and DU145 lack androgen-responsiveness (Table III.1) and have been widely used as CRPC models [5, 6].

PTEN is a well-known tumour suppressor gene, which negatively regulates the PI3K/AKT signalling network, thereby controlling cell growth and survival [7]. In PCa, loss of PTEN occurs causing continuous activation of its downstream target AKT, and consequently has been linked with enhanced cell proliferation and inhibition of apoptosis [7, 8] In this thesis, cell lines with the presence (22Rv1 and DU145) and absence of PTEN (LNCaP and PC3) were used (Table III.1).

Table III.1. Immortalized human prostate cell lines under study.

Cell line	Morphology	AR	PTEN	Origin
PNT1A	Epithelial	+	+/+	Non-neoplastic
LNCaP	Epithelial	+	-/-	Lymph node metastasis
22Rv1	Epithelial	+	+/+	Primary tumour xenograft
PC3	Epithelial	-	-/-	Bone metastasis
DU145	Epithelial	-	+/-	Central nervous system metastasis

3.2.Materials and Methods

3.2.1. Cell culture

Immortalized non neoplastic prostate epithelial cell line PNT1A was purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK). Prostate cancer cell lines LNCaP, 22Rv1, PC3, DU145 were purchased from the ATCC and maintained in Roswell Park Memorial Institute medium 1640 medium (RPMI) (Gibco, Life Technologies, Paisley, UK) supplemented with 10 % fetal bovine serum (FBS, Biochrom, Berlin, Germany) and 1 % penicillin (100U/ml)/streptomycin (Sant a Cruz Biotechnology, Heidelberg, Germany), and incubated with 5 % CO_2 at 37 °C. For experimental use, the cells were harvested with a trypsin solution buffered with EDTA (0.025 %).

3.2.2. Protein extraction

Total protein was extracted from PNT1A, LNCaP, 22Rv1, PC3 and DU145 cells using RIPA buffer (150 mM NaCl, 1 % Nonidet-P40 substitute, 0.5 % Na-deoxycholate, 0.1 % SDS, 50 mM Tris pH 8 and 1 mM EDTA) supplemented with 1 % protease inhibitors cocktail (Sigma-Aldrich, St Louis, MO, USA) and 10 % PMSF (Sigma-Aldrich). Protein concentration was determined by the bicinchoninic acid assay (Bio-Rad, Rockford, IL, USA) Protein extraction followed as per protocol in Vaz *et al*, 2012 [6].

3.2.3.Western blot analysis

40 μg of human prostate cell line protein extracts were resolved by SDS-PAGE on 12 % gels. The samples were then electro-transferred to a PVDF membrane (GE Healthcare, Buckinghamshire, UK). Membranes were incubated overnight at 4 °C with rabbit anti-TRIB 1 (1:1000, 09-126, Millipore, Temecula, CA, USA), rabbit anti-TRIB 3 (1:1000, ab75846, abcam, Cambridge, UK) and mouse anti-β-actin (1:40,000, A5441, Sigma-Aldrich) primary antibodies. Goat anti-rabbit IgG-HRP (1:40,000, sc-2004, Santa Cruz Biotechnology, CA, USA) or goat anti-mouse IgG-HRP (1:40,000, sc-516102, Santa Cruz Biotechnology, CA, USA) were used as secondary antibodies. Membranes were incubated with Clarity™ Western ECL substrate (Bio-Rad) for 5 min and immunoreactive bands visualized using the ChemiDoc™ MP Imaging System (Bio-Rad). Band densities were obtained according to standard methods using the Image Lab 5.1 Software (Bio-Rad) and normalized by division with the respective β-actin band density.

3.2.4. TRIB1 knockdown and overexpression: transient transfection

Transfection was carried out using Lipofectamine3000 (Invitrogen, Carlsbad, CA, USA). In 80% confluent cells, TRIB1-knockdown in PC3 and LNCaP cells by TRIB1 specific siRNA (2500 ng) transfection (SMARTpool: ON-TARGETplus TRIB1 siRNA, L-003633-00-0010, Dharmacon, Cambridge, UK) and TRIB1-overexpression in PNT1A was performed using 2 μg of the hTRIB1 4/TO/myc-his recombinant pcDNA (Invitrogen, V1030-20) plasmid expression vector (gifted by Endre Kiss-Toth laboratory). PC3 and LNCaP cells were also transfected with siRNA nonspecific to TRIB1 (ON-TARGET plus non-targeting siRNA, D-001810-01-05, (Dharmacon) as controls. All cells were maintained in a humidified incubator (5% CO2, 37 □C) for 24 h. Protein expression analysis was performed using Western blot

3.3. Results

3.3.1.TRIB1 and TRIB3 are expressed both in neoplastic and non-neoplastic human prostate cells

To analyse the endogenous protein levels of TRIBs in the prostate cell lines under study (Table III.1), a Western blot (WB) analysis was performed. Fig. III.1 shows the representative immunoblots illustrating the basal expression of TRIB1 and TRIB3 in non-neoplastic (PNT1A) and neoplastic (LNCaP, 22Rv1, PC3, DU145) human prostate cells. Both TRIB1 and TRIB3 proteins were detected in neoplastic and non-neoplastic cells lines. Higher expression levels of TRIB1 seem to exist in the androgen-sensitive LNCaP cells and -insensitive PC3 cells compared to 22Rv1 and DU145 (Fig. III.1). TRIB3 expression generally showed to be less variable among cells lines. Non-neoplastic prostate cells PNT1A also seem to exhibit low levels of TRIBs expression (Fig. III.1).

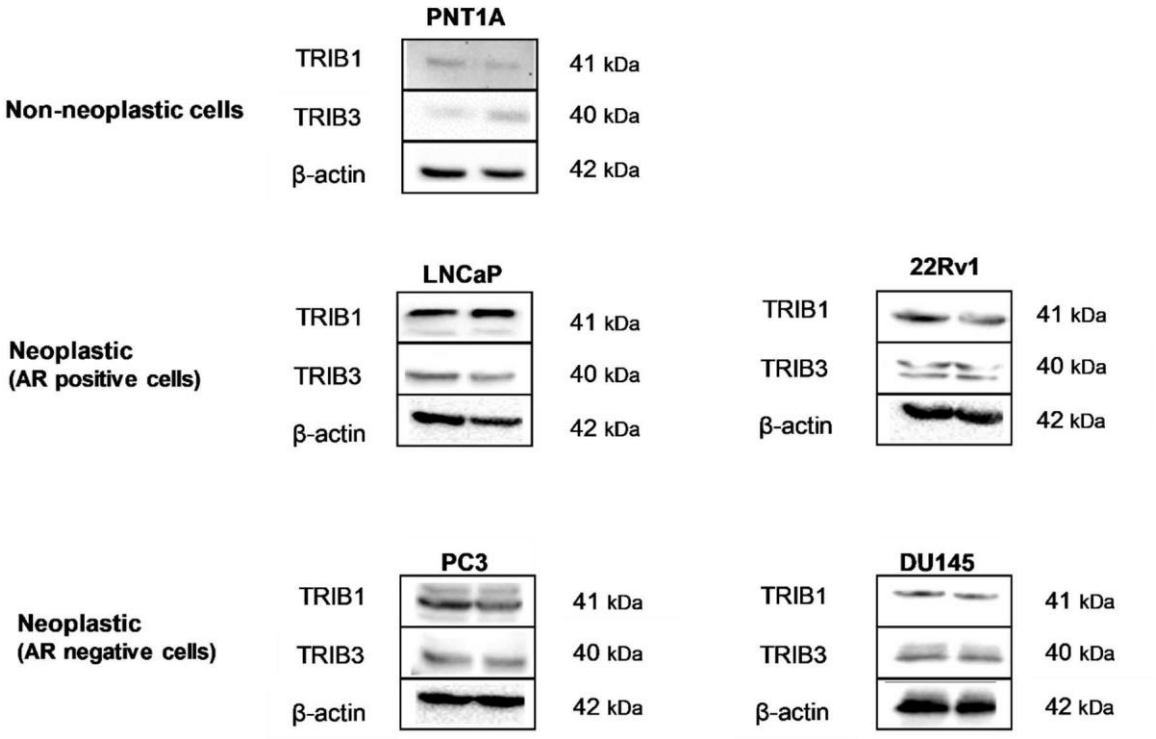

Figure III.1. TRIB1 and TRIB3 expression in non-neoplastic PNT1A, and neoplastic LNCaP, 22Rv1, DU145 and PC3 human prostate cells.

80 % confluent cells (passage number (5-90) were maintained in culture for 24 h. Protein was extracted, and TRIBs expression was determined by WB analysis. β-actin was included as a protein loading control. Representative immunoblots of duplicates of three independent experiments are shown.

3.3.2.TRIB1 knockdown and overexpression both in human prostate cells and macrophages confirm antibody specificity.

Specificity of antibodies is an important factor during antibody selection. Within the Tribbles Research and Innovation Network (TRAIN) consortium, a number of commercially available antibodies specific to TRIBs were under scrutiny as they are relatively new in the market. Further analysis was required to establish quality and specificity of the antibodies. Numerous TRIB antibodies were tested from various companies in different institutes among the TRAIN consortium, under varying conditions and the best antibodies with clear specific signalling were chosen. This included the TRIB1 Millipore (09-126) and TRIB3 abcam (ab75846) antibodies. Herein, we used a standard WB analysis in PCa cells knockdown (KD) or overexpressing TRIBs to test and validate the TRIB1 antibody (Fig. III.2). TRIB1 was overexpressed in non-neoplastic PNT1A cells, and neoplastic AR positive LNCaP cells. Also, TRIB1 was KD in the metastatic AR negative PC3 cells. WB analysis in prostate cells was carried out comparatively with macrophages, a cell line known to highly express TRIB1 [9].

TRIB1-siRNA, KD the TRIB1 protein expression in PC3 cells at the precise molecular weight of 41 kDa (Fig. III.2). TRIB1 overexpression was successful in PNT1A cells being detected a band of approximately 55 kDa, likely by the addition of a c-Myc-His tag in the expressed recombinant protein. Additionally, the macrophages showed an immune-reactive band at the expected molecular weight (41 kDa), in line with that detected in PCa cells. Moreover, WB data analysis showed no background or other non-specific binding (Fig. III.2), indicating that the anti-TRIB1 antibody is specifically binding.

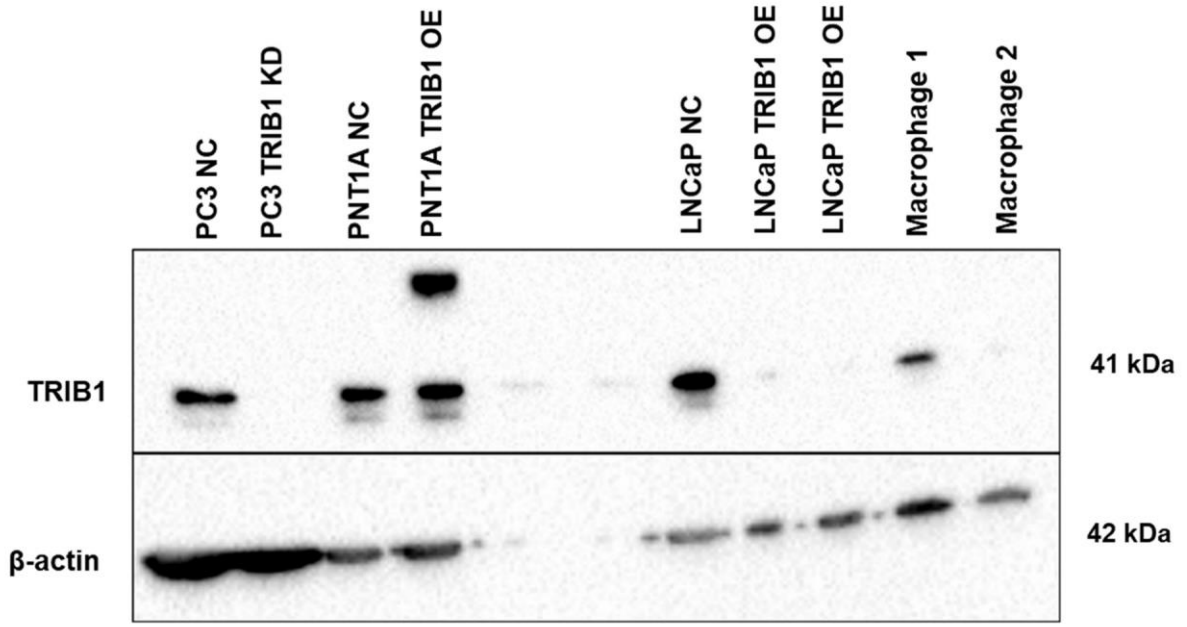

Figure III.2. Immunodetection of TRIB1.
TRIB1 protein expression in non-neoplastic (PNT1A) and neoplastic (LNCaP and PC3) human prostate cells (negative controls, NC) and TRIB1-knockdown (KD) or -overexpression (OE) cells compared with macrophages using WB analysis. Cells were treated with TRIB1-siRNA (50 nM) for KD and TRIB1 expression plasmid (2 μg) for overexpression for 24 h. Total protein was extracted, and WB using the Millipore anti-TRIB1 (09-126) antibody was performed. Representative immunoblots of three independent experiments are shown.

3.4. Discussion

To date, many studies have shown TRIB1 and TRIB3 as mediators of important signalling systems in several diseases and playing vital roles such increasing survival, proliferation, migration, metabolism and altering immune response [10-19]. As a starting point to study the role of TRIB1 and TRIB3 in prostate cells, the basal expression levels of these proteins in a panel of PCa cells mimicking different stages of disease (Table III.1) was determined (Fig. III.1). Also, a WB analyses was performed to test the specificity of TRIB1 antibody (Fig. III.2).

Within the panel of prostate cells used, generally PCa cells seem to display high TRIB1, as well as TRIB3 expression levels, though both proteins were also detected in PNT1A cells (Fig. III.1). These findings clear evidence that TRIBs are endogenously present in non-neoplastic prostate cells (Fig. III.1). At least for our knowledge, this is the first study analysing TRIBs expression in non-cancer cell line models. Other previous

studies have shown the upregulation of TRIB1 expression in PCa cases [20, 21]

However, currently it is unknown why such changes in TRIBs expression levels are seen in neoplastic microenvironments. Nevertheless, altered TRIBs expression are thought to support cell proliferation and survival and these changes are seen in numerous cancers, namely, colorectal, lung, melanoma, breast, pancreatic, leukaemia [22-24]. However, TRIBs functionality in cancer in general, and particularly in PCa, are yet to be clarified.

Analysing TRIBs basal proteins levels established the presence of TRIBs in the prostate cell line models under study. Within the TRAIN consortium strategy, our next aim was to confirm the specificity of the selected anti-TRIB antibody. As expected, a specific TRIB1-immunoreactive protein was detected in PNT1A, LNCaP and PC3 cells, as it disappears with TRIB1 siRNA KD and was detected in the positive control macrophages cells (Fig. III.2). Macrophages have been shown to express TRIB1, which has been linked to their biology and function [9]. Therefore, macrophages protein samples are suitable as positive controls.

Overall, this study confirmed TRIB1 and TRIB3 proteins expression in non-neoplastic and neoplastic human prostate cells, and the suitability of anti-TRIB1 antibody for WB applications.

3.5. Acknowledgements

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Chapter IV:

4. Androgen actions in regulating TRIB1 and TRIB3 in LNCaP and 22RV1 prostate cancer cells

Abstract

Prostate cancer (PCa) is one of the leading causes of death in men worldwide. Androgens, the male sex steroid hormones, and androgen receptor (AR) play an important role in supporting PCa cell proliferation and tumour growth. Therefore, androgen deprivation therapy is a gold standard in PCa treatment. However, as PCa advances tumours lose its responsiveness to androgens, and continue growing independently of androgen ablation. The transition from androgen-sensitive to –insensitive stage of disease is underpinned by multiple molecular alterations in AR and other associated genes. Also, a metabolic reprogramming with the enhancement of aerobic glycolysis characterizes the advance of PCa to androgen-insensitive stage and drives the aggressiveness of disease. Androgens have been shown to stimulate the glycolytic metabolism of PCa cells, being also linked with the regulation of lipid metabolism, i.e., uptake, fatty acid *de novo* synthesis, and β-oxidation. Tribbles (TRIBs) are a family of pseudokinases (TRIB1, TRIB2 and TRIB3) that have been described to have a major role in controlling cell fate and their oncogenic or tumour suppressor actions in multiple cancers including PCa have been suggested. Furthermore, TRIB1 and TRIB3 have been identified as crucial molecular targets in the regulation of lipid metabolism, which raises the curiosity about their relationship with androgens actions. This chapter aimed to investigate the androgenic modulation of TRIB1 and TRIB3 levels in human prostate cells and analyse cell fate upon changes to TRIBs expression induced by androgens. The non-aromatizable androgen 5α-dihydrotestosterone (DHT) downregulated TRIB1 and TRIB3 expression, which underlined the increased viability and proliferation of the PCa cells. No effects were seen concerning apoptotic cell death. The anti-androgen bicalutamide (BIC) blocked DHT effects in controlling TRIB1 expression, suggesting the involvement of AR. Moreover, *in-silico* time series experiment predicted TRIB1 and TRIB3 as androgen targets. Also, ChiP-seq results demonstrated the direct interaction between AR and TRIB1/3 gene locus and the existence of AR binding sites at the TRIB1 locus in human PCa samples. Additionally, AR silencing augmented TRIB1 expression in 22Rv1 cells, further confirming the AR involvement. These results demonstrate that androgens and the AR have a direct role in regulating TRIBs expression in PCa cells and implicate this molecular interaction in PCa development.

Keywords: Androgens; 5α-dihydrotestosterone; TRIB1; TRIB3; prostate cancer;

PNT1A cells.

4.1. Introduction

Despite recent advances in knowledge and progresses in therapy, prostate cancer (PCa) remains a major health problem in males, accounting for a significant number of deaths every year [1]. PCa growth strongly depends on the circulating levels of sex steroid hormones androgens and androgen receptor (AR) signalling. Subsequently, androgen deprivation therapy (ADT) is the main form of systemic therapy for the management of PCa [2]. The aim of ADT is to target all the transcription and translation AR-associated activities, suppressing PCa cell proliferation and tumour growth [3]. However, androgen-unresponsive tumours arise after a mean time of 2-3 years of anti-androgen therapy [4]. The transition from androgen-sensitive to -insensitive, invasive, and metastatic PCa is an occurrence that is yet to be fully understood [5].

Nevertheless, PCa development and growth can be defined as a gradual process requiring a series of genetic and phenotypic modifications, consequence of loss or gain of function in target cell regulators that are then maintained by the interaction with the tumour microenvironment [6]. These molecular alterations are related with structural rearrangements, point mutations, allelic loss, somatic copy number alterations, abnormal miRNAs regulation and the existence of single nucleotide polymorphisms in AR and other target genes, which altogether determine enhanced proliferation, avoidance of apoptosis and the migration and invasion properties of PCa cells [6].

Another feature associated with the progression of PCa is the metabolic reprogramming, with the dysregulated metabolism being a vital driver of tumour aggressiveness [7]. Contrary to initial stages of disease, advanced PCa display a more glycolytic phenotype relying on aerobic glycolysis, the 'Warburg effect' [8-10]. As disease progresses, PCa cells display high rates of glucose consumption along with increased lactate production [5, 11], a feature that was shown to be stimulated by androgens [12, 13]. Moreover, the role of androgens as metabolic drivers also encompasses the regulation of glutaminolysis and lipid metabolism [12, 14]. Androgens actions have been shown to enhance lipid uptake and fatty acid *de novo* synthesis, as well as favouring its oxidation [15].

The Tribbles (TRIBs) family are a pseudokinase protein group, composed of TRIB1, TRIB2, TRIB3 isoforms [16], which are characterized by the lack of catalytic activity,

though playing relevant roles as scaffold or adaptors molecules [17]. TRIBs act as modulators of intracellular signalling, namely the PI3K/AKT and MAPK pathways being involved in the control of several biological processes, namely gene transcription, cell differentiation, and proliferation and cell death [16]. These actions controlling cell fate, not surprisingly, sustain the identification of TRIBs family members as tumour suppressors proteins or oncogenes [18]. Available studies have also highlighted the importance of TRIBs, mainly TRIB1 and TRIB3, in the regulation of lipid metabolism [19-21], which altogether raises the curiosity about their actions in PCa and the putative relationship and dependency on androgen regulation. Indeed, only few studies have linked TRIBs to PCa, where they show the overexpression of specifically TRIB1 isoform driving tumorigenesis via promoting cell survival and proliferation of PCa cells [22-24]. However, no published data has investigated the regulatory mechanisms that maintain TRIBs expression levels in PCa. This study aims to investigate the androgenic regulation of TRIBs in non-neoplastic PNT1A and androgen sensitive LNCaP and 22Rv1 PCa cells.

4.2. Materials and Methods

4.2.1. In-silico analysis

Prediction data of the androgenic regulation of TRIB1 and TRIB3 in PCa cells was obtained through *in-silico* analysis on published datasets, namely using the transcriptome tool analysis of the Nuclear Receptor Signalling Atlas (NURSA) [25]. Additionally, Betastasis, which is an independent genomics analysis and visualization tool (betastasis.com/prostate_cancer/massie_et_al_2011/) was also used to explore *in silico* the regulation of TRIBs by androgens.

4.2.2. Cell culture and treatments

Immortalized non-neoplastic prostate epithelial cell line PNT1A was purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK. PCa cell lines LNCaP and 22Rv1 were purchased from the ATCC and maintained in Roswell Park Memorial Institute medium 1640 medium (RPMI 1640) (Gibco, Life Technologies, Paisley, UK) supplemented with 10 % fetal bovine serum (FBS, Biochrom, Berlin, Germany) and 1 % penicillin (100U/ml)/streptomycin (Santa Cruz Biotechnology, Heidelberg, Germany), in an atmosphere with 5 % CO_2 at 37 °C. For experimental use the cells were trypsinized with a trypsin solution buffered with EDTA (0.025 %).

For hormonal stimulation experiments PNT1A, LNCaP and 22Rv1 cells were grown up to 60 % confluency and maintained for an additional 24 h in phenol red-free RPMI (Gibco) supplemented with 5 % charcoal-stripped FBS (Gibco). Cells were exposed to 1, 10 or 100 nM of DHT (Sigma-Aldrich, St Louis, USA), a non-aromatizable androgen, for 24 h. Stimulation with 10 nM DHT was repeated in the presence of 3, 5 and 10 μM of AR antagonist Bicalutamide (BIC, Sigma-Aldrich) for the same time period. The inhibitor was added to cells 30 min before hormonal stimulation. All assays were carried out in 6 biological replicates. After treatment, cells were scraped and harvested for total RNA and protein extraction.

4.2.3.Animals

Mice prostates were collected from adult PTEN knockout (KO) and PTEN wild-type (WT) 6 days after orchiectomy and TRIB1 expression analysed in castrated animals compared with non-castrate [26]. Sample collection and qPCR data analysis were acquired through close collaboration with Dr Parastoo Shahrouzi under Dr Arkaitz Carracedo group at CIC bioGUNE, Spain. All mouse experiments, including the procedures of castration, were carried out following the ethical guidelines established by the Biosafety and Welfare Committee at CIC bioGUNE (Details are available from the authors, [26]).

4.2.4.ChiP-sequencing analysis

ChIP-seq data was obtained through collaborative means within the TRAIN research team and the Prof Wilbert Zwart of the Netherlands Cancer Institute. ChIP-seq was performed as described by the authors using LNCaP cells treated with R1881 and prostate tumour tissue [27, 28].

4.2.5. Protein extraction and Western blot analysis

Total protein was extracted from PNT1A, LNCaP and 22Rv1 cells using RIPA buffer (150 mM NaCl, 1 % Nonidet-P40 substitute, 0.5 % Na-deoxycholate, 0.1 % SDS, 50 mM Tris pH 8 and 1 mM EDTA) supplemented with 1 % protease inhibitors cocktail (Sigma-Aldrich) and 10 % PMSF (Sigma-Aldrich). Protein concentration was determined by the bicinchoninic acid assay (Bio-Rad), and 40 μg of protein extracts were resolved by SDS-PAGE on 10 and 12 % gels. The samples were then electro-transferred to a PVDF membrane (GE Healthcare). Membranes were incubated

overnight at 4 °C with rabbit anti-TRIB 1 (1:1000, 09-126, Millipore, CA, USA), rabbit anti-TRIB 3 (1:1000, ab75846, Abcam, Cambridge, United Kingdom), rabbit anti-AKT (1:1000, no.9272; Cell Signalling Technology, Danvers, MA, USA), rabbit anti-phospho-AKT (1:1000, no.9271; Cell Signalling Technology), rabbit anti-phospho-p44/42 MAPK (Erk1/2) (1:1000, no.9101; Cell Signalling Technology), and mouse anti-β-actin (1:1,000, A5441, Sigma-Aldrich) antibodies. Goat anti-rabbit IgG-HRP (1:40,000, sc-2004, Santa Cruz Biotechnology, CA, USA) or anti-mouse-IgGκ HRP-linked (1:20000, sc-516102, Santa Cruz) were used as secondary antibodies. Membranes were incubated with Clarity™ Western ECL substrate (Bio-Rad) for 5 min and immunoreactive bands visualized using the ChemiDoc™ MP Imaging System (Bio-Rad, Hercules, CA, USA). Band densities were obtained according to standard methods using the Image Lab 5.1 Software (Bio-Rad) and normalized by division with the respective β-actin band density. Protein extraction followed as per protocol in Vaz *et al*, 2016 [12].

4.2.6.RNA extraction and qPCR

Total RNA was extracted from LNCaP and 22Rv1 cells using tripleXtractor reagent (Grisp, Portugal) following manufacturer's instructions. The quantity and quality of total RNA were assessed by spectrophotometry at 260 and 280 nm (Pharmacia Biotech, Ultrospec 3000, Demark). One microgram of total RNA was reverse transcribed using the First-Strand cDNA synthesis kit (NZYTech, Lisboa, Portugal) in a final volume of 20 µl. cDNA synthesis reaction was initiated by a 10-min incubation at 25 °C, followed by 30 min at 50 °C and an inactivation step at 85 °C for 5 min. l µl of RNase H was added, and incubation proceeded at 37 °C for 20 min. The reaction was stopped by cooling to 4 °C, and the synthesized cDNA was stored at −20 °C until further use. The quality and integrity of the cDNA samples were assessed by amplification of the 18S housekeeping gene. The mRNA expression of TRIB1/3 in DHT treated LNCaP and 22Rv1 cells was determined by RT-qPCR using specific primers (Table IV.1). β-actin and B2M were used as internal controls to normalize gene expression levels (Table IV.1). RT-qPCR was carried out in an iQ5 system (Bio-Rad), and efficiency of the amplifications was determined for all primer sets using serial dilutions (1, 1:3, 1:6 and 1:9) of LNCaP and 22Rv1 cDNA. Primer concentration and annealing temperature for each primer set were optimized, and the specificity of amplicons was determined by melting curve analysis. Amplification reactions were

carried out using 1 µL of synthesized cDNA in a final volume of 20 µL containing 10 µL SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad) and 500 nM of sense and antisense primers for each gene. Cycling conditions comprised 5-min denaturation at 95 °C, followed by 40 cycles at 95 °C for 10 s, a specific annealing temperature for each gene for 30 s and 72 °C for 10 s. Samples were run in triplicate in each qPCR assay.

Table IV.1. PCR primer sequences, amplicon size base pairs (bp), and annealing temperature (AT).

Gene	Primer sequence	Amplicon size (bp)	AT
AR	Fwd: AGGCCTTCCCTGTACACCAA	132	64
	Rev: GTCTTGGCCTGGTCATTTCC		
PSA	Fwd: CCAGGGACCATGTTTTGCC	126	60
	Rev: CGAAGACGACAAGATGGACAA		
TRIB1	Fwd: CTTCTGGTTGGACGATACCC	101	55
	Rev: TTCCAAGACGGACTCAAACC		
TRIB3	Fwd: TTTGTCTTCGCTGACCGTGA	98	55
	Rev: TGCTTGTCCCACAGGGAATC		

4.2.7. siRNA transient transfection

Cell seeding (80% confluency) was performed and the culture was incubated in a humidified incubator (5% CO2, 37°C) for 24 h. The next day, the TRIB1 and AR were knocked down in LNCaP and 22Rv1 cells by siRNA transfection. TRIB1 (SMARTpool: ON-TARGETplus TRIB1 siRNA, L-003633-00-0010, Dharmacon) and AR (SMARTpool: ON-TARGETplus AR siRNA, L-003400-00-0005, Dharmacon) specific siRNAs (50 nM) were used to transfect cells by using either Viromer blue (lipocalyx,

Cambridge Bioscience, Cambridge, UK) or lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) as a transfection reactive as per manufacturer's instructions. Control cells were transfected with siRNA nonspecific to TRIB1 and AR (ON-TARGET plus non-targeting siRNA, D-001810-01-05, Dharmacon) and maintained in culture in a humidified incubator (5% CO2, 37°C) for 24 h. TRIB1 and AR gene expression analysis were performed using qPCR and Western blot.

4.2.8. MTT assay

Cell viability of DHT-treated cells and controls was determined by the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The conversion of MTT compound to the coloured formazan product was detected at 490 nm in a xMark™ Microplate Absorbance Spectrophotometer (Bio-Rad). The relative number of viable cells in each experimental condition was calculated by normalizing the absorbance to that of the control without DHT. All experiments were repeated at least six times, and each experiment was carried out in triplicates.

4.2.9. Ki67 immunofluorescence

LNCaP and 22RV1 cells were seeded on 8 chamber glass slides and treated as previously described. Cells were fixed for 15 min in 4% formaldehyde in Phosphate Buffered Saline (PBS). Cell permeabilization was achieved with PBS containing 0.1% TRITON X-100 for 15 min. Blocking was performed by incubating with PBS containing 0.1% (w/v) Tween-20 (PBS-T) and 20% FBS for 1 h. After blocking, cells were incubated with the rabbit monoclonal anti-Ki67 antibody (1:250, ab16667, Abcam, Cambridge, United Kingdom) for 1-24 h at room temperature. Alexa 594 goat anti-rabbit IgG (1:500, A-11012, Invitrogen) was used as a secondary antibody for 1 h at room temperature. Four washes of 5 minutes in PBS were performed after each incubation. Cells were mounted in DakoCytomation Fluorescent Mounting Medium supplemented with DAPI. The slides were examined by using a Widefield inverted microscope (Leica AF6000LX).

4.2.10. Caspase-3-like activity assay

Caspase-3-like enzymatic activity was determined spectrophotometrically at 405 nm by detecting the presence of p-nitro-aniline (pNA), upon cleavage of caspase-3

substrate (Ac-DEVD-pNA). Briefly, 50 µg of total protein extract was incubated overnight at 37 °C in reaction buffer (25 mM HEPES, 0.1% 3CHAPS, 10% sucrose, and 10 mM DTT, pH 7.5) and 200 µM of Ac-DEVD-pNA. The amount of generated pNA was calculated by extrapolation with a standard curve.

4.2.11. Migration assays

Migration assay was performed using 8 µM pore size inserts (35224, SPL, Life Sciences, Naechon-Myeon Pocheon, South Korea). Briefly, PNT1A, LNCaP and 22Rv1 cells (3.0 x105 cells/well) treated with DHT (10 nM), were placed into the upper chambers in serum-free media. The lower chambers contained 20% FBS. After 24 h, cells on the lower surface of membrane were fixed with PFA 4% and stained blue with Hoechst 33342. Cells were then counted in 10, randomly selected, 20x magnification fields per transwell.

4.2.12. Statistical analysis

The statistical significance of differences between experimental groups was evaluated by unpaired t-test and one-way ANOVA with Dunnett's test, using GraphPad Prism v7.00 (GraphPad Software, Inc., La Jolla, CA, USA). $P < 0.05$ was considered statistically significant. All experimental data are shown as mean ± standard error of the mean (S.E.M).

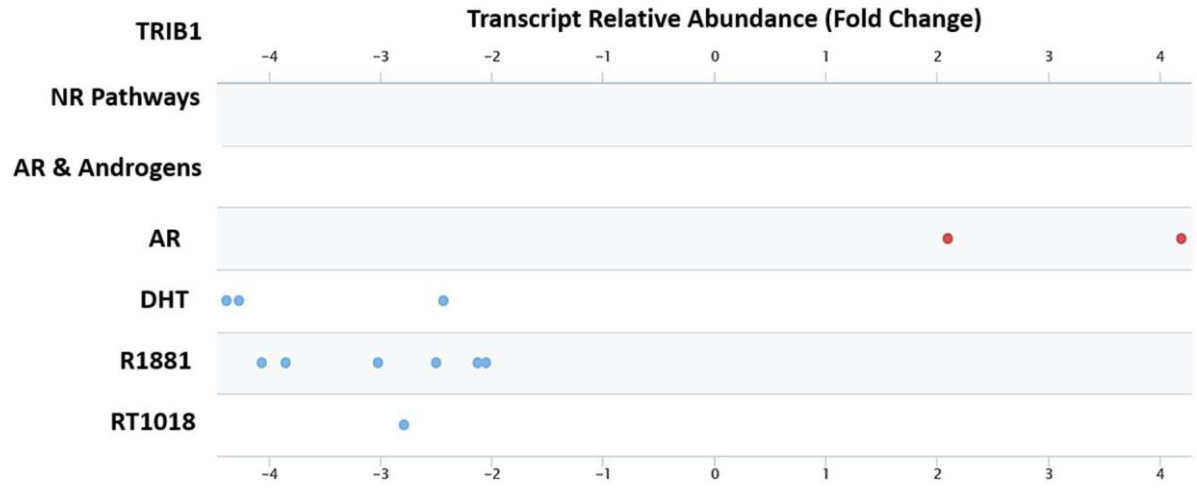
4.3. Results

4.3.1. Androgens downregulate TRIB1 and TRIB3 expression levels in human prostate cancer cells

To determine whether androgens regulateTRIB1 and TRIB3 expression in prostate cells, we started with an *in-silico* approach. Using the NURSA transcriptome analysis web tool, TRIB1 and TRIB3 genes were predicted as targets of androgens and AR signalling pathway. Fig. IV.1. shows data points from human cell line model experiments, and from clinical data sets for TRIB1 and TRIB3 expression. The blue dots indicate the downregulation of TRIB1 after treatment of LNCaP cells with various concentrations of DHT, or synthetic androgens R1881 or RT1018 (Fig. IV.1), and red data dots mean an upregulation of TRIB1 after AR knockdown in LNCaP cells. Contrary to TRIB1, the transcriptome analysis showed the opposite feature for TRIB3 gene expression regulation by androgens/AR. High concentrations of DHT or R1881

upregulated TRIB3 expression, while a downregulation occurred when the AR was silenced (Fig. IV.1). Tables IV.2 and IV.3 summarise the different experiments generating the scatter plots of Fig. IV.1 for TRIB1 and TRIB3.

A



B

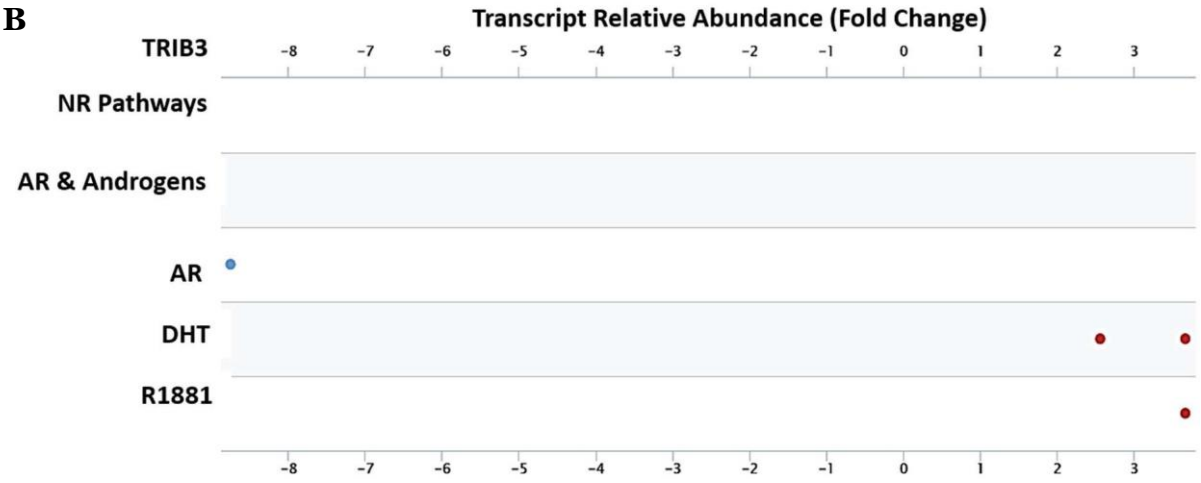


Figure IV.1. TRIB1 and TRIB3 are predicted targets of androgens and AR in human tissues and cells.

Scatter plots obtained from NURSA Transcriptomine analysis tool (https://nursa.org/nursa/index.jsf [25]) identifying TRIB1 (A) and TRIB3 (B) as targets of androgens and AR in human tissues and cells. Separate dots are data points from individual human cell line models experiments or clinical data sets collected from various articles published. Red dots mean an upregulation of TRIB1 (A) or TRIB3 (B) after AR knockdown in LNCaP cells, and blue dots indicate the downregulation of TRIB1 after treatment of LNCaP cells with various concentrations of DHT, or synthetic androgens R1881 or RT1018 [25].

Table IV.2. Data on the androgen regulation of TRIB1 expression in human cell lines and tissues obtained from NURSA Transcriptomine analysis tool.

Experiment	Regulatory Molecule	Concentration	Time of Treatment	Fold Change	P-value	PMID*
R1881 vs Veh (UXT KD)	R1881	10 nM	18 h	-2.051804942	2.394E-07	19318562
AR KD vs NC	AR	-	-	2.096244619	0.000786986	21330406
R1881 vs Veh	R1881	1 nM	12 h	-2.128740365	0.000336619	21602788
DHT vs Veh	DHT	1 nM	16 h	-2.437180124	0.00857938	17566103
R1881 vs Veh	R1881	1 nM	24 h	-2.496248383	4.528E-07	17010675
RTI018 vs Veh	RTI018	10 nM	24 h	-2.787532727	0.000024853	16574741
R1881 vs Veh – 24 h	R1881	1 nM	24 h	-3.020945171	1.3097E-06	21602788
ELK1 KD + R1881 vs Veh	R1881	1 nM	48 h	-3.853310811	2.97133E-05	23426362
R1881 vs Veh	R1881	1 nM	48 h	-4.070611381	2.43393E-05	23426362
AR KD vs NC – LNCaP	AR	-	-	4.194956694	0.020796512	19632176
DHT vs Veh	DHT	30 nM	24 h	-4.272261631	0.0001	-
DHT vs Veh	DHT	1 nM	24 h	-4.386286354	0.00009	-

* PMID PubMed Unique Identifier, https://nursa.org/nursa/index.jsf [25].

Table IV.3. Data on the androgen regulation of TRIB3 expression in human cell lines and tissues obtained from NURSA Transcriptome analysis tool.

Experiment	Regulatory Molecule	Concentration	Time of Treatment	Fold Change	P-value	PMID*
DHT vs Veh	DHT	100 nM	16 h	2.557625134	0.007870993	19632176
DHT vs Veh	DHT	100 nM	16 h	3.66860089	0.028024911	19632176
Celastrol vsR1881 vs Veh	R1881	1 nM	24 h	3.673194505	9.1552E-06	17010675
AR KD vs NC	AR	-	-	-8.756734908	0.000876448	19632176

*PMID PubMed Unique Identifier, https://nursa.org/nursa/index.jsf [25].

From the NURSA web tool analysis, our hypothesis that androgens regulate TRIB1/3 expression levels in human PCa cells was developed. Thus, human PCa cells LNCaP

and 22Rv1 and prostate epithelial cells PTN1A were treated with various concentrations of DHT (1, 10, 100 nM) for 24 h. The TRIB1 and TRIB3 protein (Fig, IV.2) and mRNA (Fig, IV.3) expression were analysed using the Western blot (WB) and qPCR methods, respectively. The specificity of the TRIB1 antibody was tested by WB analysis (Fig. III.2) in a panel of prostate cells OE or KD TRIB1 and macrophages. TRIB1-immunoreactive protein was seen in PNT1A, LNCaP and 22Rv1 prostate cell lines, as well as in macrophages, with observable differences upon OE or KD. No presence of non-specific binding or background was observed, confirming that the anti-TRIB1 antibody is suitable for WB analysis.

At a protein level, LNCaP and 22Rv1 cells treated with DHT (10 nM) for 24 h displayed a significantly diminished expression of TRIB1 (Fig. IV.2A and 2B, respectively). Also, TRIB3 protein levels were decreased in LNCaP (Fig. IV.2D) and 22RV1 (Fig. 2E) DHT- treated cells compared to control. DHT-treatment had no significant changes in the expression of TRIB1 and TRIB3 in PNT1A cells compared to control (Fig. IV.2C, F). Additionally, increased expression of the androgen-target gene PSA was seen in DHT- treated LNCaP cells compared to control (Fig. IV.2G).

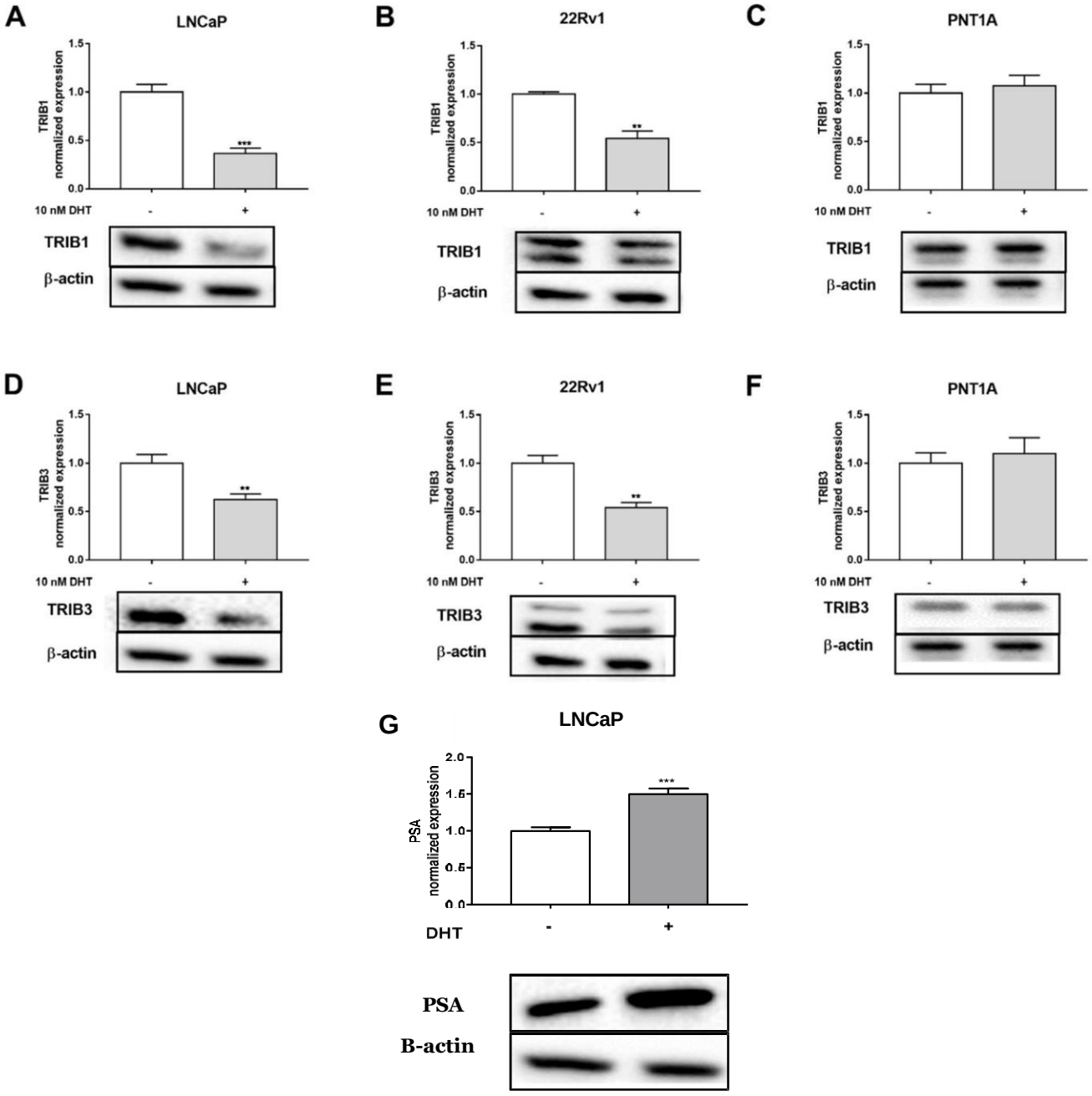


Figure IV.2. Protein expression of TRIB1 and TRIB3 in neoplastic and non- plastic cells

TRIB1, TRIB3 and PSA protein expression in neoplastic LNCaP (A, D) and 22Rv1 (B, E), and non-neoplastic PNT1A (C, F, G) human prostate cells treated with DHT (10 nM) for 24 h. Data are represented as mean ± S.E.M after normalization of WB results with β-actin (n=6). All results are expressed as fold variation relative to the control. $^{**}p < 0.01$ and $^{***}p < 0.001$. Representative immunoblots are shown as bottom panels.

The modulation of TRIB1 and TRIB3 mRNA levels by androgens in LNCaP and 22Rv1 cells was determined, after treating cells with increasing concentrations of DHT (0, 1, 10 and 100 nM) for 24 h. A significant downregulation of TRIB1 and TRIB3 mRNA expression was seen in both LNCaP and 22Rv1 cell lines (Fig. IV.3). 10 and 100 nM DHT downregulated TRIB1 and TRIB3 mRNA expression in LNCaP cells (Fig. IV.3A and C, respectively). Moreover, the results also showed that DHT specifically on TRIB1 in LNCaP cells had a concentration dependent effect (10 nM vs 100 nM DHT, p <0.05). Similarly, a downregulation of TRIB1 and TRIB3 mRNA levels was observed in 22Rv1 cells (Fig. IV.3B and D, respectively) with a significant reduction in the 10 and 100 nM DHT. No effects were perceived with 1 nM DHT (Fig. IV.3).

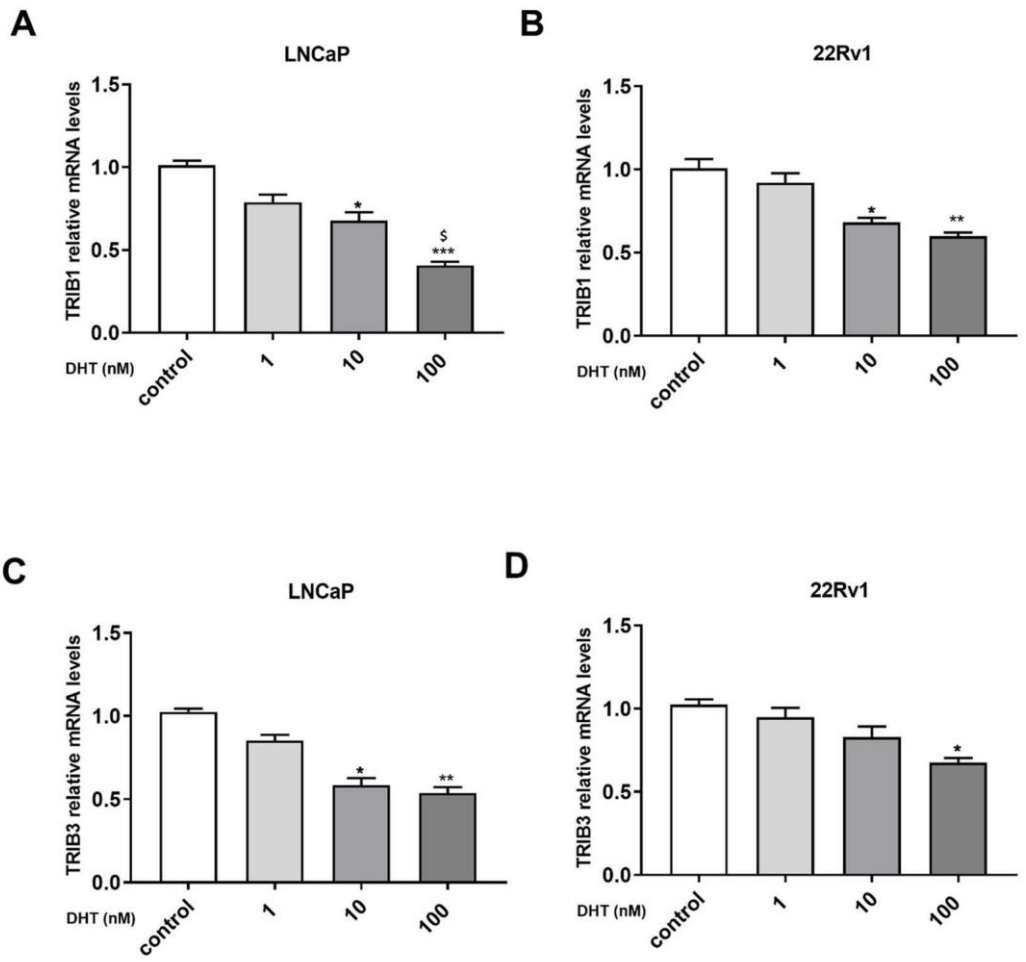


Figure IV.3. mRNA expression of TRIB1 and TRIB3 in neoplastic and non- plastic cells

TRIB1 and TRIB3 mRNA expression in LNCaP **(A, C)** and 22Rv1 **(B, D)** human PCa cells treated with DHT (1, 10 and 100 nM) for 24 h determined by qPCR. Data are

represented as mean ± S.E.M after normalization of results with that of the housekeeping gene β-actin (n=6). All results are expressed as fold variation relative to the control. *p <0.05, **p <0.01 and ***p <0.001 and $^{\$}p$ <0.05 when compared to 10 nM DHT.

4.3.2. Androgen receptor is involved in the regulation of TRIB1 expression in prostate cancer cells

Using a number of *in-silico* analyses and published data, additional information on whether androgens are involved in regulating TRIB1 and TRIB3 expression levels in PCa cells was obtained. Fig. IV.4 is an *in-silico* analysis from Betastasis, an online genomics analysis and visualization tool [13] that allowed obtaining predictive information of TRIBs expression using a time series qPCR experiment in which LNCaP cells were treated with the synthetic androgen R1881 (1 nM) or 0.1% ethanol as a control. Orange lines (1 nM R1881) indicated average expression of TRIB1 (Fig. IV.4A) and TRIB3 (Fig. IV.4B) relative to untreated (blue, 0.1 % ethanol) LNCaP cells. From approximately 3 hours onwards, R1881-treated LNCaP cells displayed reduced TRIB1 expression, including the 24 h experimental timeframe (Fig. IV.4A). Concerning TRIB3 expression, though presenting a general trend to decrease in response to R1881, the effect was less consistent than that observed for TRIB1 (Fig.IV.4B).

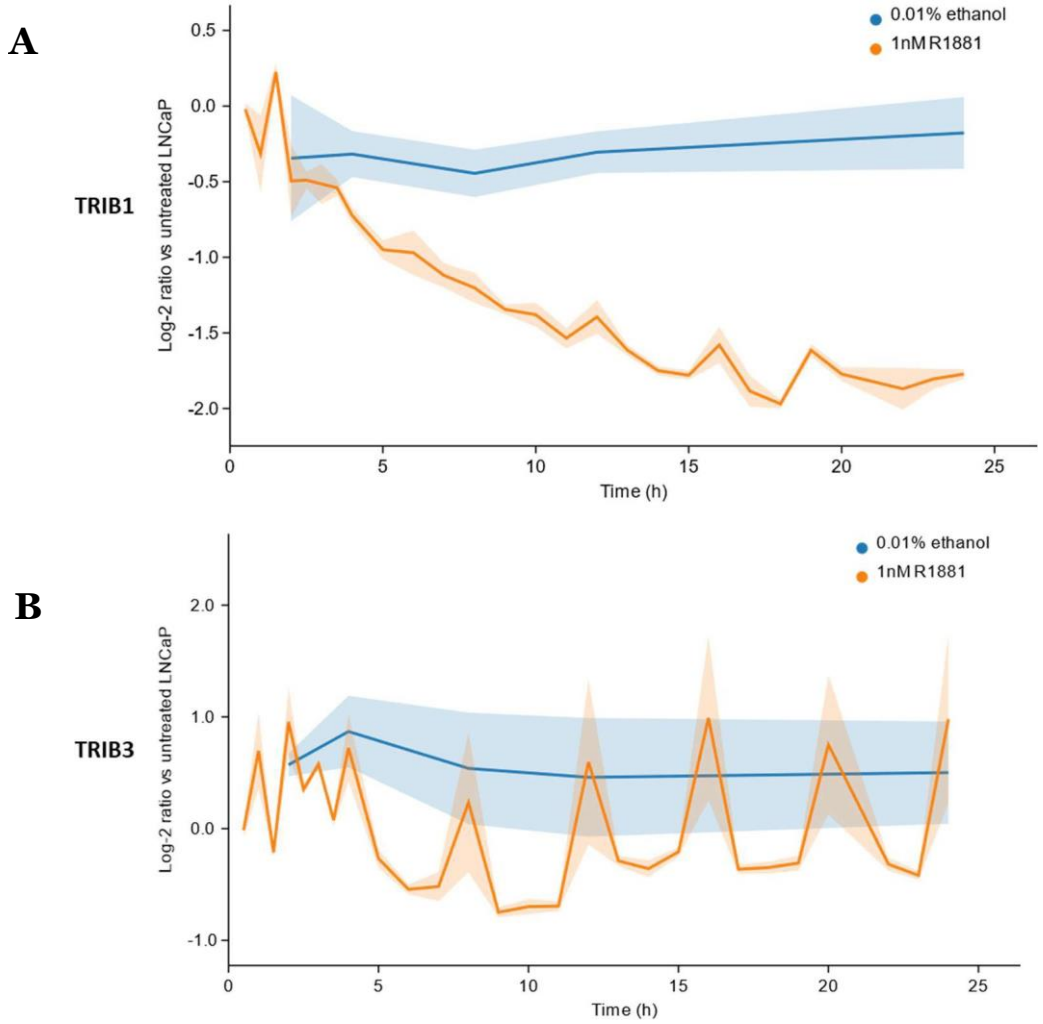


Figure IV.4. TRIB1 and TRIB2 mRNA expression in LNCaP cells in-silico.

In-silico analysis of TRIB1 **(A)** and TRIB3 **(B)** relative expression in LNCaP cells after exposure to 0.1% ethanol or 1 nM R1881 [13]. A total of 48 RNA samples were harvested from LNCaP cells grown for 72 h in steroid depleted medium (RPMI supplemented with 10% charcoal dextran stripped FBS). Samples were taken at 2, 4, 8, 12 and 24 h; and the (R1881)-treated samples were taken every 30 min for 4 h then every hour until 24 h following treatment. Blue (0.1 % ethanol) and orange (1 nM R1881) lines indicate average expression of TRIB1 and TRIB3 relative to untreated LNCaP cells. Corresponding shaded areas indicate the S.E.M.

To infer about the involvement of the AR in regulating TRIB1 expression in PCa cells, DHT treatment (10 nM) was repeated in the presence of the androgen receptor inhibitor BIC (10 μM). Results obtained in both LNCaP and 22Rv1 cells, showed that DHT treatment significantly decreased TRIB1 expression at 24 h (Fig. IV.5A and B, respectively), which confirms the previous results (Fig. IV.3A, B). Moreover, BIC blocked the effect of DHT in downregulating TRIB1 expression both in LNCaP

(Fig.IV.5A) and 22RV1 (Fig. IV.5B) cells. The experimental approach and AR responsiveness was validated by analysing the expression of the standard androgen target gene PSA. Increased PSA expression was observed in DHT-treated LNCaP (Fig. IV.5C) and 22RV1 (Fig. IV.5D) cells; effects that were blocked by BIC (Fig. IV.5C, D).

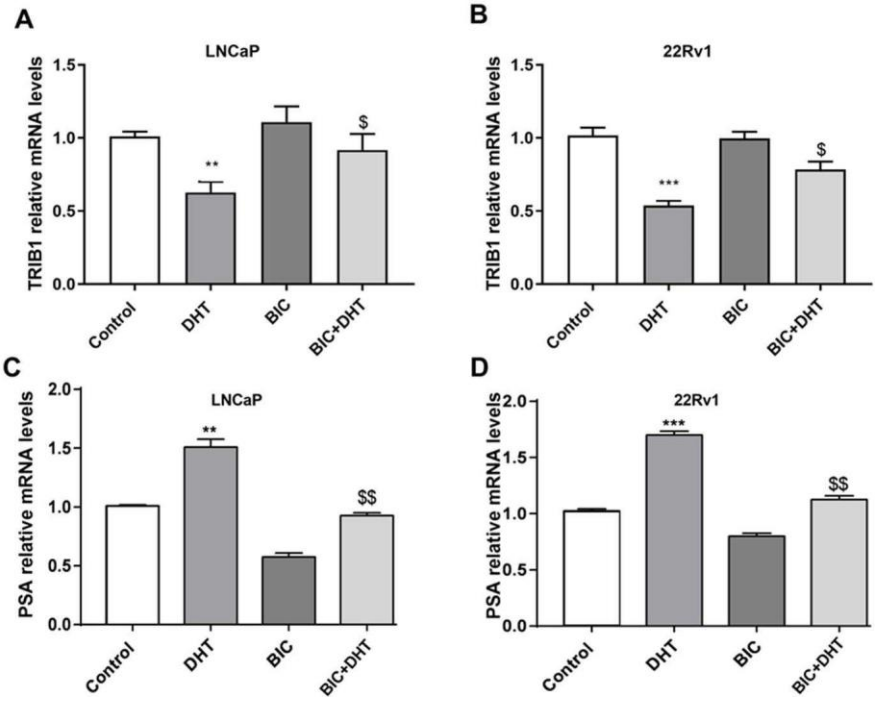


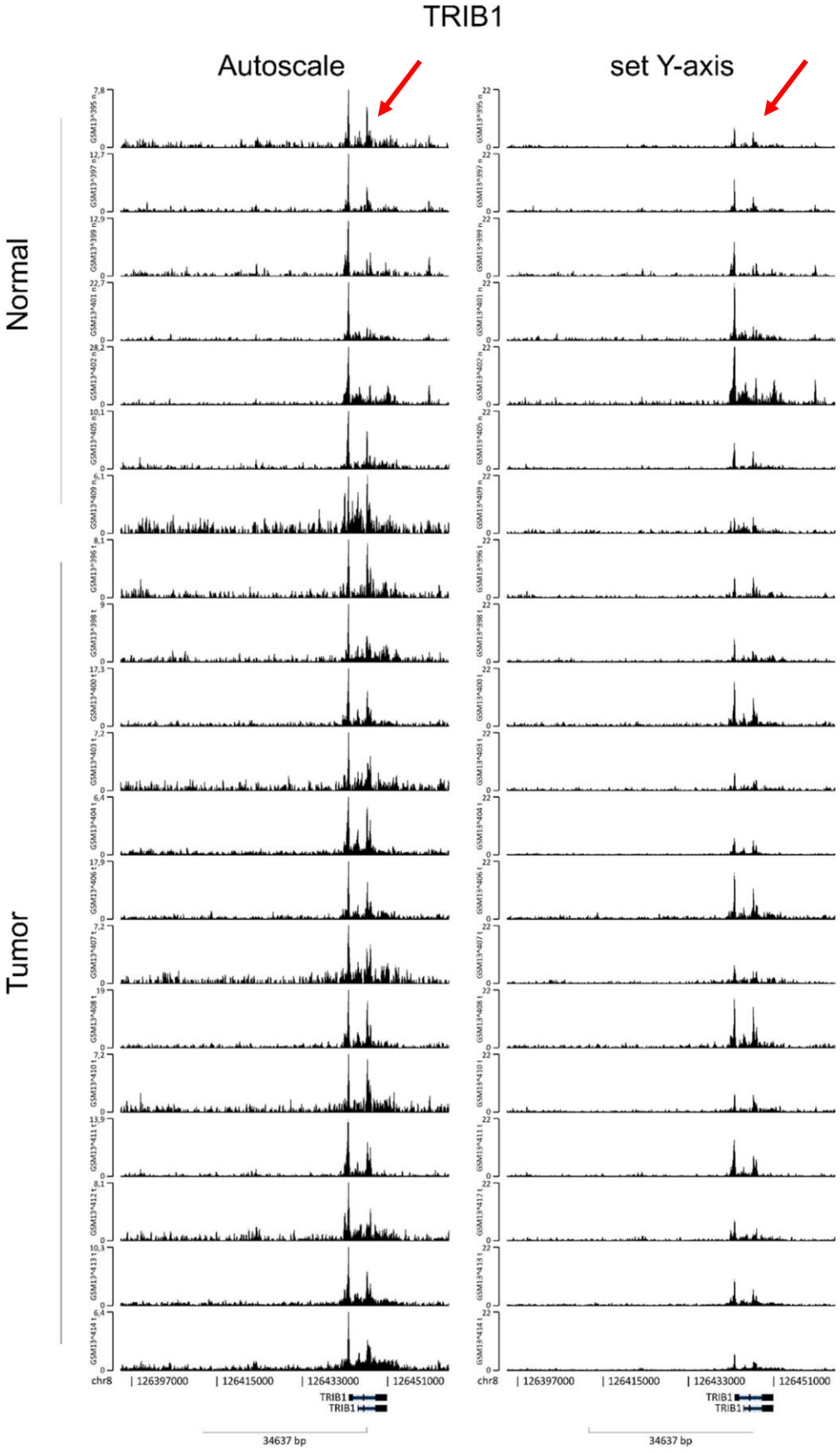
Figure IV.5. Assessment of TRIB1 after treatment with Bicalutamide (+/- DHT).

TRIB1 mRNA expression in LNCaP **(A)** and 22Rv1 **(B)** human PCa cells treated with DHT (10 nM) in the presence or absence of BIC (10 μM) for 24 h determined by qPCR. Expression analysis of the androgen target gene PSA in LNCaP **(C)** and 22RV1 **(D)** treated cells was included as a control of experimental procedures. Data are represented as mean ± S.E.M after normalization of results with that of the housekeeping gene β-actin (n=6). Results are expressed as fold-variation compared to control. $^{**}p$ <0.01 and $^{***}p$ <0.001 when compared with untreated control. $^{\$}p$ <0.05 and $^{\$\$}p$ <0.01 when compared to DHT (10 nM).

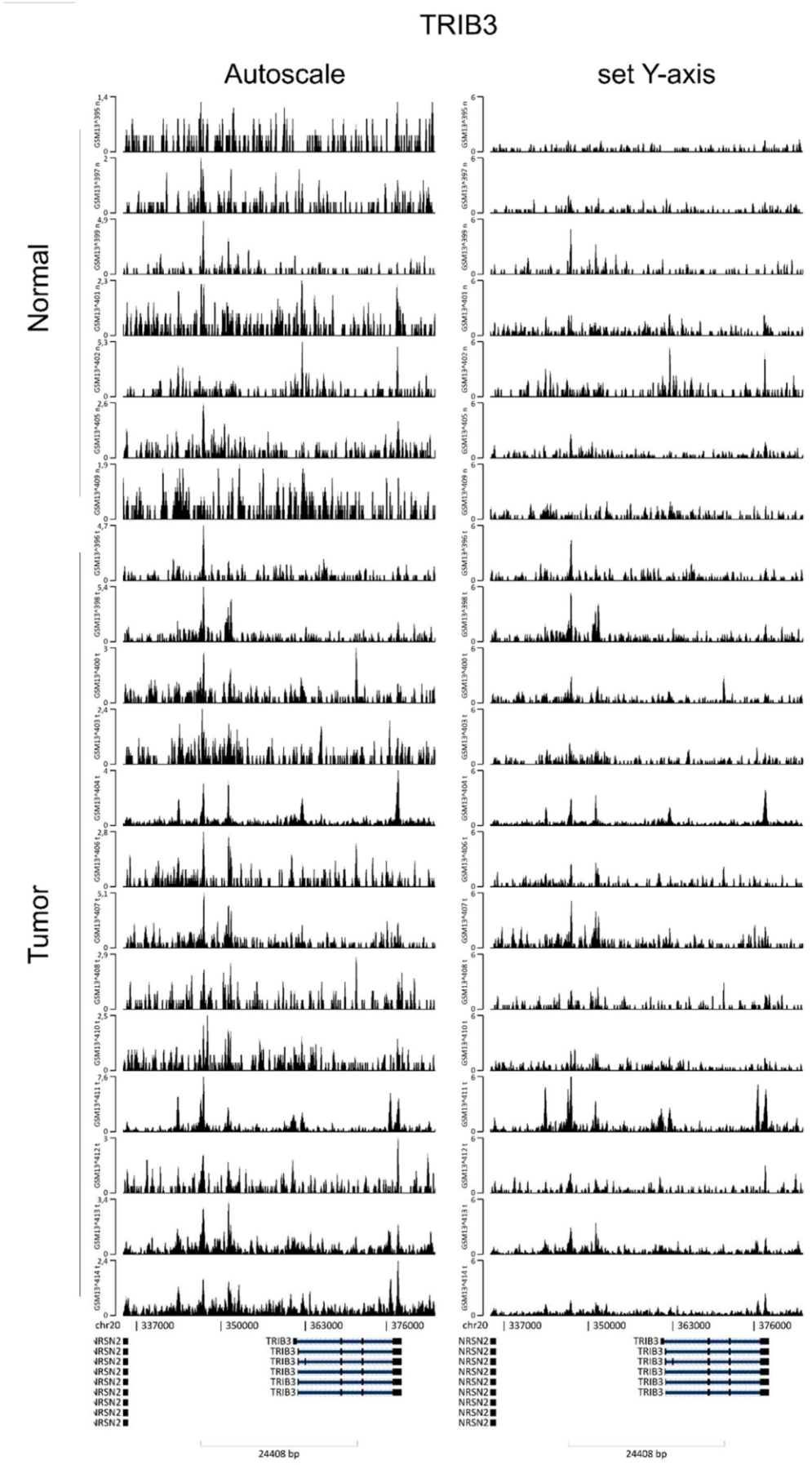
To confirm whether AR interacts with TRIBs genes regulatory regions, a ChiP-seq analysis was performed in collaboration with Prof Wilbert Zwart research group at the The Netherlands Cancer Institute [27, 28]. Fig. IV.6A and B is a visual inspection of the ChiP-seq profile in primary prostate tumours *vs* normal solid tissues based on Pomerantz et al data [29]. The snapshot demonstrates the existence of AR binding regions within the TRIB1 and TRIB3 genes loci. Moreover, a stronger increased peak

intensity was seen for TRIB1 compared to the weak signal of TRIB3 (Fig. IV.6A, B).
A genome browser snapshot in LNCaP cells treated with the synthetic androgen R1881 [27] also revealed the binding of the AR to TRIBs regulatory regions, as indicated by the comparison with the vehicle-treated cells (Fig. IV.6C). Furthermore, other interesting transcription factors have been shown to interact with TRIBs gene locus. It is the case of the Forkhead box protein A1 (FOXA1), a transcription factor frequently mutated in PCa [30]. It is also required to facilitate chromatin accessibility at specified binding sites for AR. Here we saw highly shared peaks of AR with FOXA1 (+/- R1881) (Fig.IV.6C).

A


TRIB1
Autoscale
set Y-axis
Normal
Tumor
chr8
126397000
126415000
126433000
126451000
TRIB1
TRIB1
34637 bp

B


TRIB3
Autoscale
set Y-axis
Normal
Tumor
chr20
337000
350000
363000
376000
NRSN2
TRIB3
24408 bp

C

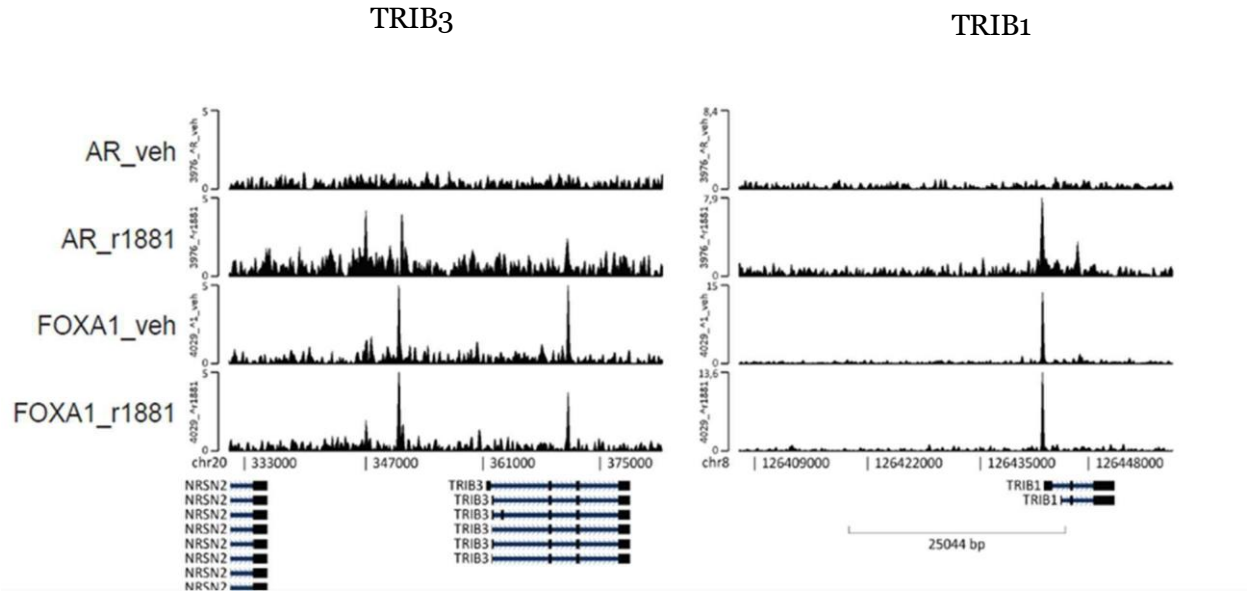


Figure IV.6. ChiP-seq analysis

Snapshots of AR and FOXA1 ChiP-seq analysis across TRIB1 and TRIB3 genes loci in human primary prostate tumours *vs*. normal solid tissues **(A, B)** and LNCaP **(C)** cells treated with the synthetic androgen R1881or vehicle (veh). Genomic coordinates are indicated.

4.3.4. Androgen receptor knockdown increased TRIB1 expression levels in human prostate cancer cells

To further confirm the involvement of the AR in regulating TRIB1 expression in PCa cells, silencing of the AR was performed using siRNA (transient transfection) for 24 h. The mRNA and protein expression levels of TRIB1 in AR-knockdown LNCaP and 22Rv1 cells were determined by qPCR and WB analysis, respectively (Fig. IV.7). In LNCaP cells no significant difference was observed in TRIB1 expression after silencing the AR gene (Fig. IV.7A). Whereas, in the *PTEN*$^{+/+}$ 22Rv1 cells silencing the AR gene significantly increased TRIB1 mRNA expression (Fig. IV.7B). Besides AR, TRIB1 knockdown was included as a control to confirm its expression in these cell lines and the efficiency of the knockdown approach. Reduction of TRIB1 (Fig. IV.7A, B) and AR (Fig, IV.7C) mRNA expression was seen after siRNA transfection confirming the successful knockdown. However, no apparent changes were seen at a protein level in both LNCaP and 22Rv1 cells (Fig. IV.7D, E).

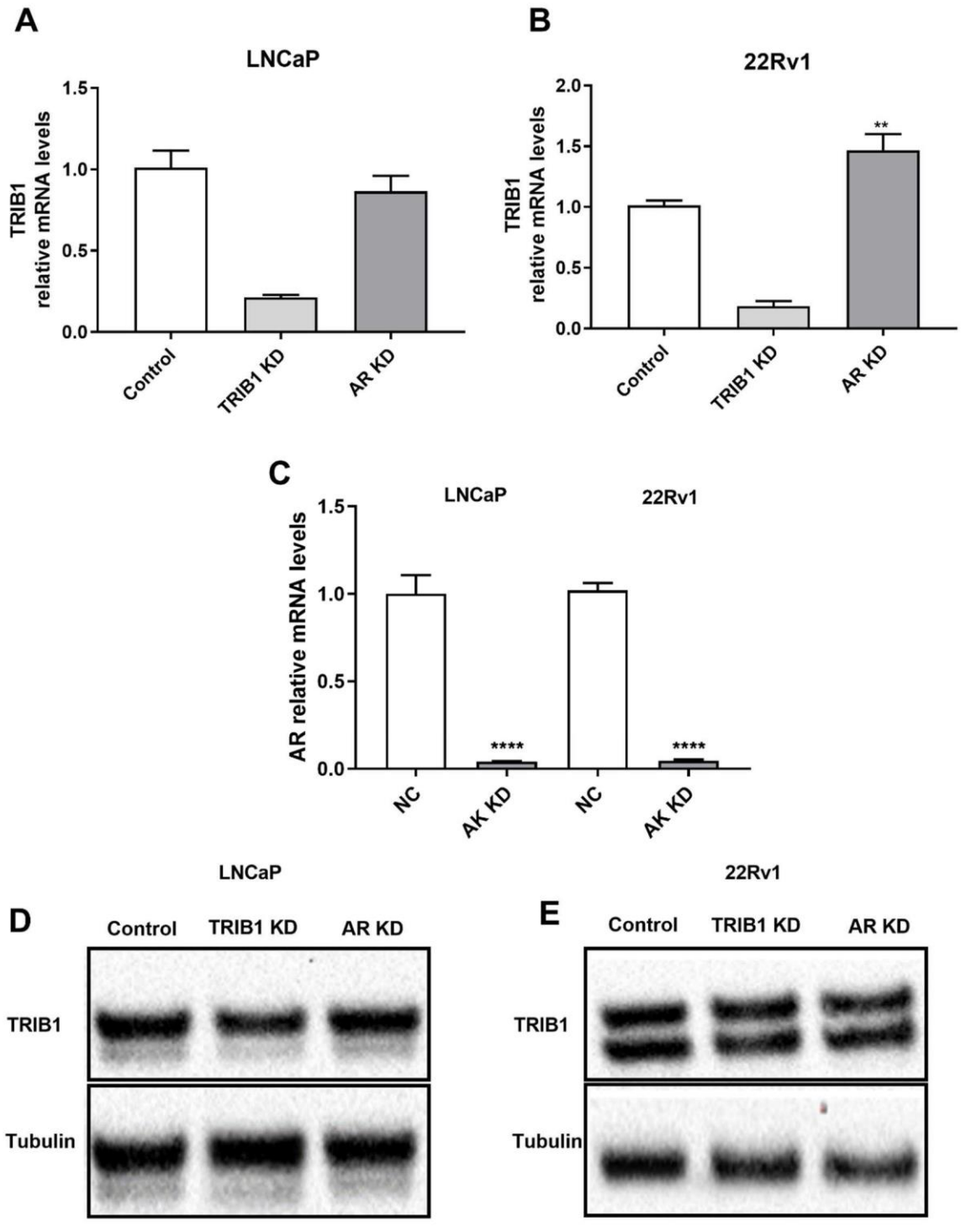


Figure IV.7. Protein and gene expression of TRIB1 and AR after siRNA knockdown.

TRIB1 **(A, B)** and AR **(C)** mRNA expression, and TRIB1 protein levels **(D, E)** after siRNA knockdown of AR in LNCaP and 22Rv1 human prostate cancer cells for 24 h. qPCR data are represented as mean ± S.E.M after normalization with β-actin. (n=3). TRIB1 expression after AR KD was determined by WB analysis. Tubulin was included as a protein loading control. All results are expressed as fold variation relative to the non-targeting control $^{**}p < 0.01$ and $^{****}p < 0.0001$.

4.3.5. TRIB regulation by androgens occurs in vivo

The expression levels of *TRIB1* in the prostate of castrated and precastrated PTEN KO and WT mice was investigated. Castration had no statistically significant effect on *Trib1* expression in the prostate of PTEN WT mice (Fig. IV.8A), but interestingly, PTEN KO castrated mice exhibited significantly increased *Trib1* levels (Fig. IV.8C). NK3 Homeobox *1* (Nkx3.1), a prostatic tumour suppressor gene, which is androgen regulated was used as a control to confirm successful castration. Significantly diminished levels of *Nkx3.1* were seen in castrated mice compared to non-castrated, both in PTEN KO and WT animals (Fig. 8B and Fig. 8D, respectively).

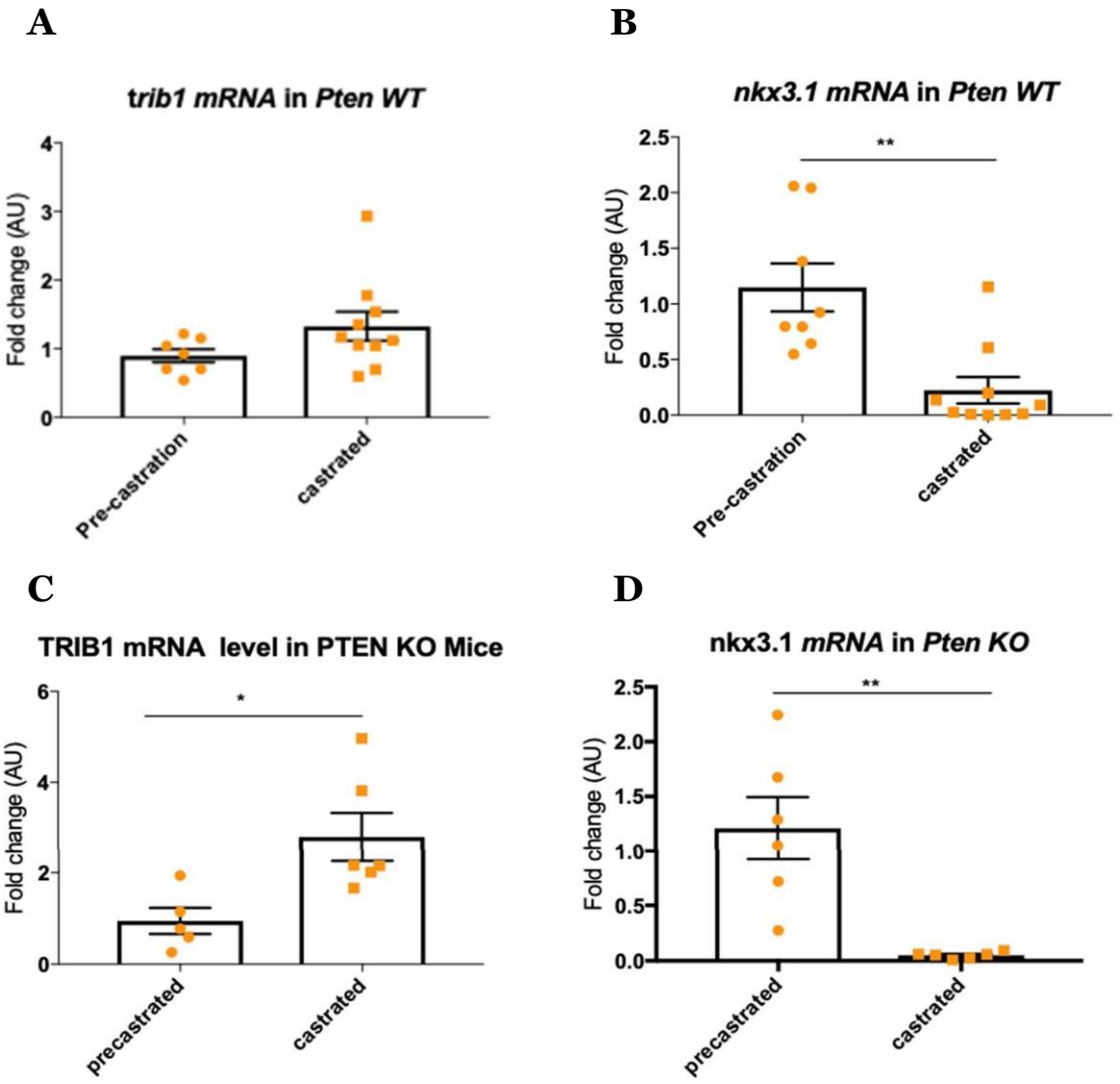


Figure IV.8. Gene expression of in-vivo studies

TRIB1 **(A, C)** and *Nkx3.1* **(B, D)** relative gene expression levels in castrated and precastrated PTEN WT and PTEN KO mice (n=5-8) determined by qPCR 6 days after orchiectomy. Expression Values were normalized to *Gapdh*; a.u. = arbitrary unit. * p <0.05, ** p <0.01.

4.3.6. Androgens effects modulating TRIBs expression are associated with enhanced cell proliferation without affecting apoptosis in prostate cancer cells

The MTT assay was used to evaluate the prostate cell proliferation upon androgens actions diminishing TRIB1 expression. A significant increase in cell proliferation was observed in PNT1A, LNCaP and 22RV1 cells treated with DHT (10 nM) for 24 h compared to control (Fig. IV.9A). Ki67 fluorescent immunocytochemistry was used to assess proliferation of LNCaP (Fig. IV.9B) and 22Rv1 PCa cells (Fig. IV.9C) in response to DHT treatment, which was shown to diminish TRIB1 expression (Fig. IV.3). A significant increase in the number of Ki67 positive cells was observed in LNCaP cells treated with DHT (10 nM) compared to control (Fig. IV.9B). Similar to LNCaP, 22Rv1 DHT-treated cells had significantly increased Ki67 labelling compared to control (Fig. IV.9C). Representative images of the Ki67 staining, DAPI and the merged images in DHT-treated and control cells are presented in Fig. IV.9D.

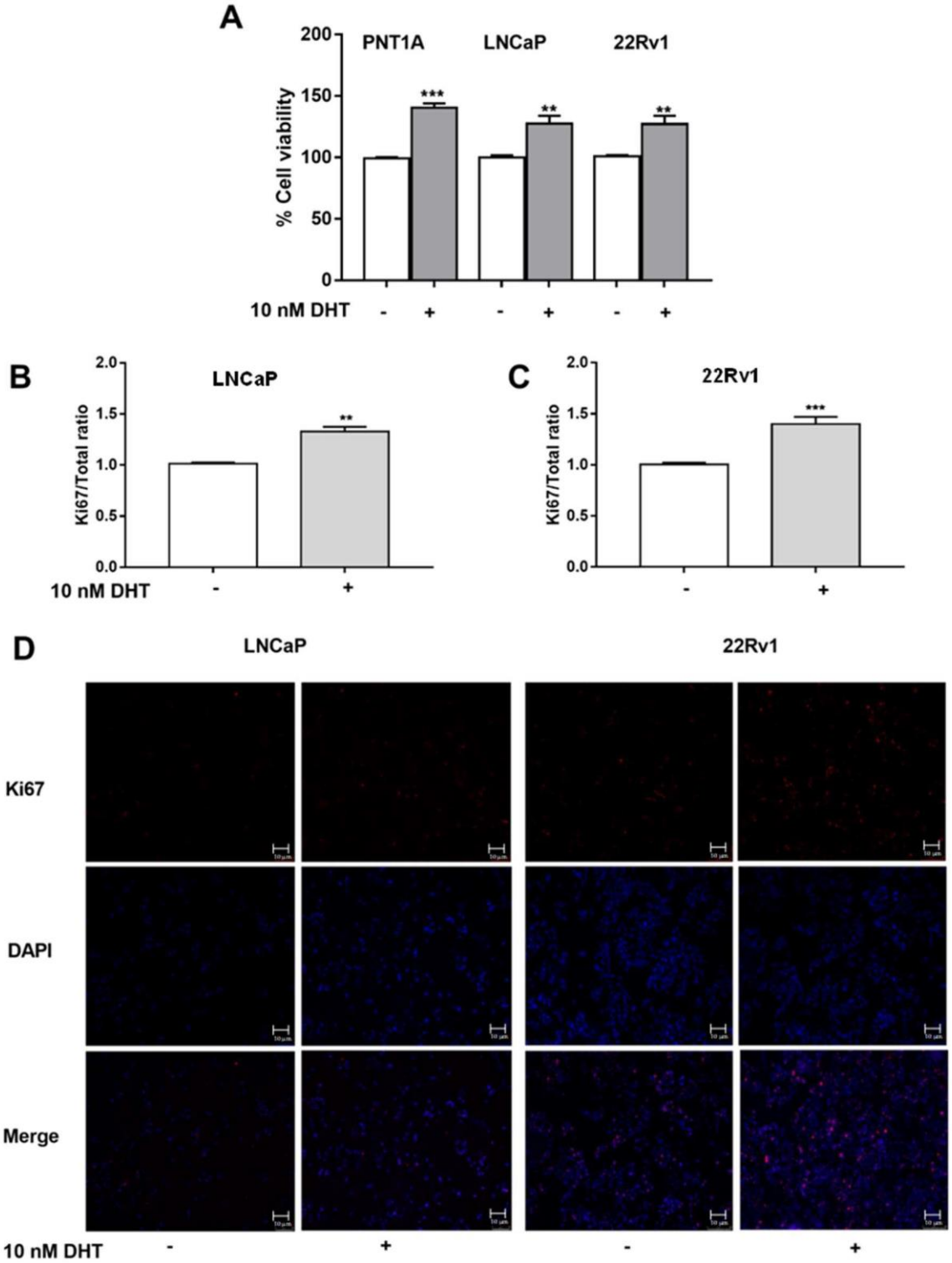


Figure IV.9. Cell proliferation in DHT treatment cells.

PNT1A, LNCaP and 22Rv1 human prostate cells proliferation after treatment with DHT (10 nM) for 24 h. **(A)** Cell proliferation determined via MTT assay **(B, C)** Cell proliferation evaluated by the Ki67 fluorescent immunocytochemistry. The Ki67/Total ratio corresponds to the percentage of Ki67 positive cells relative to total of LNCaP **(B)** and 22Rv1-treated cells **(C)** (10 random fields analysed in each condition). Results are expressed as fold-variation relative to each control. Error

bars indicate mean ± SEM, $^{**}p<0.01$, $^{***}p<0.001$ relative to control. **(D)** Representative images of Ki67 positive proliferative cells (red), nucleus stained blue with DAPI and merged labelling. Scale: 10 μM.

To determine whether androgens and reduced TRIB1 expression affect prostate cells survival, caspase-3-like activity was measured. Caspase-3 is an executioner of apoptosis activated by both the intrinsic and extrinsic pathways [31], and thus a good indicator of cell death. Concerning the measure of caspase-3-like activity (Fig. IV.10) in PNT1A, LNCaP and 22Rv1 human prostate cells, no changes were observed, which indicated that DHT had no apoptotic effects.

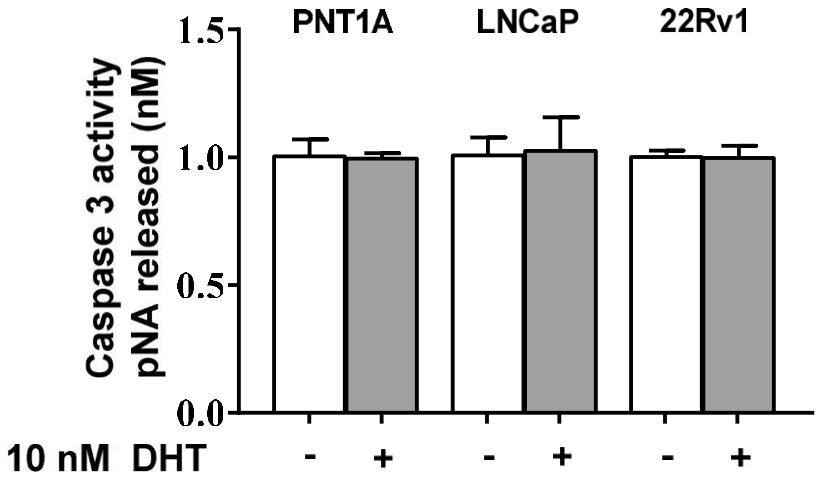


Figure IV.10. Caspase-3 activity in prostate cells.

Caspase-3 activity in prostate cells PNT1A, LNCaP and 22Rv1 treated with DHT (10 nM) for 24 h. Enzyme activity was determined spectrophotometrically (405 nM) based on the release of the product pNA chromophore, (n = 6). Error bars indicate mean ± S.E.M. Results are expressed as fold-variation relative to control.

4.3.7. Androgens increased migration of human prostate cells

Several studies have demonstrated that DHT can promote PCa cell migration [32]. Figure IV.11 supports the findings of increased migration in the PNT1A, LNCaP and 22Rv1 prostate cells in response to androgen treatment. DHT treatment increased PNT1A, LNCaP and 22Rv1 migration by 27, 20, and 40%, respectivelly (Fig. IV.11A). Representative images showing the increased number of migrated cells after DHT (10

nM) treatment are shown (Fig. IV.11B).

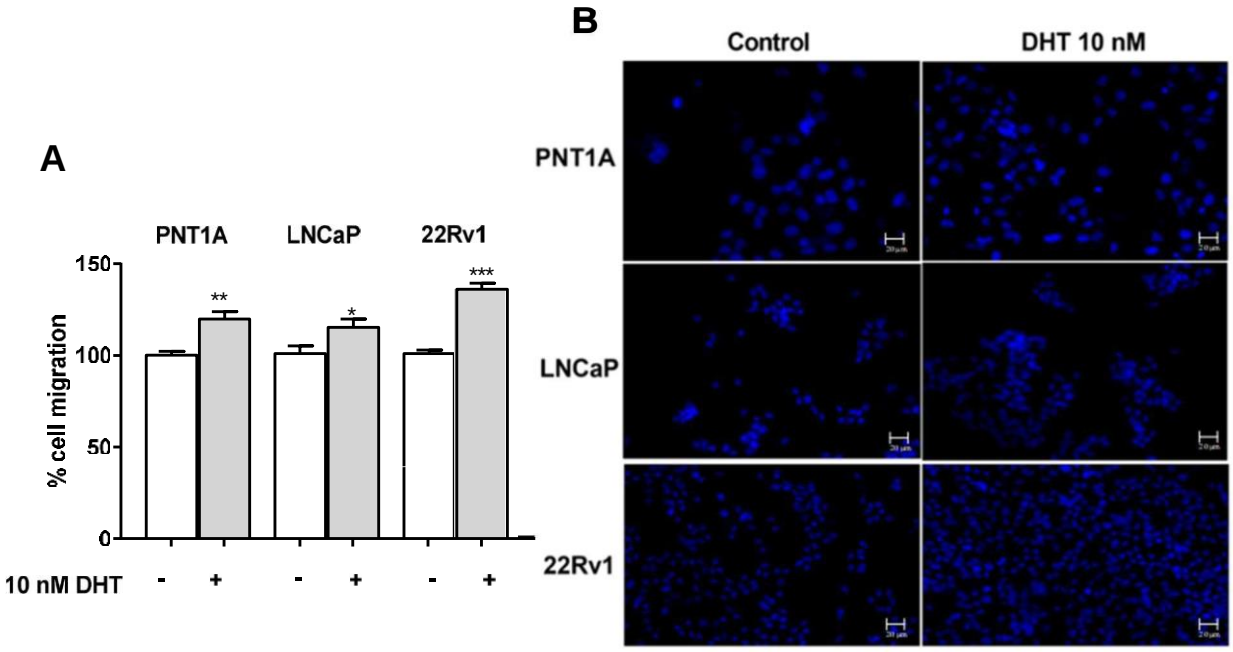
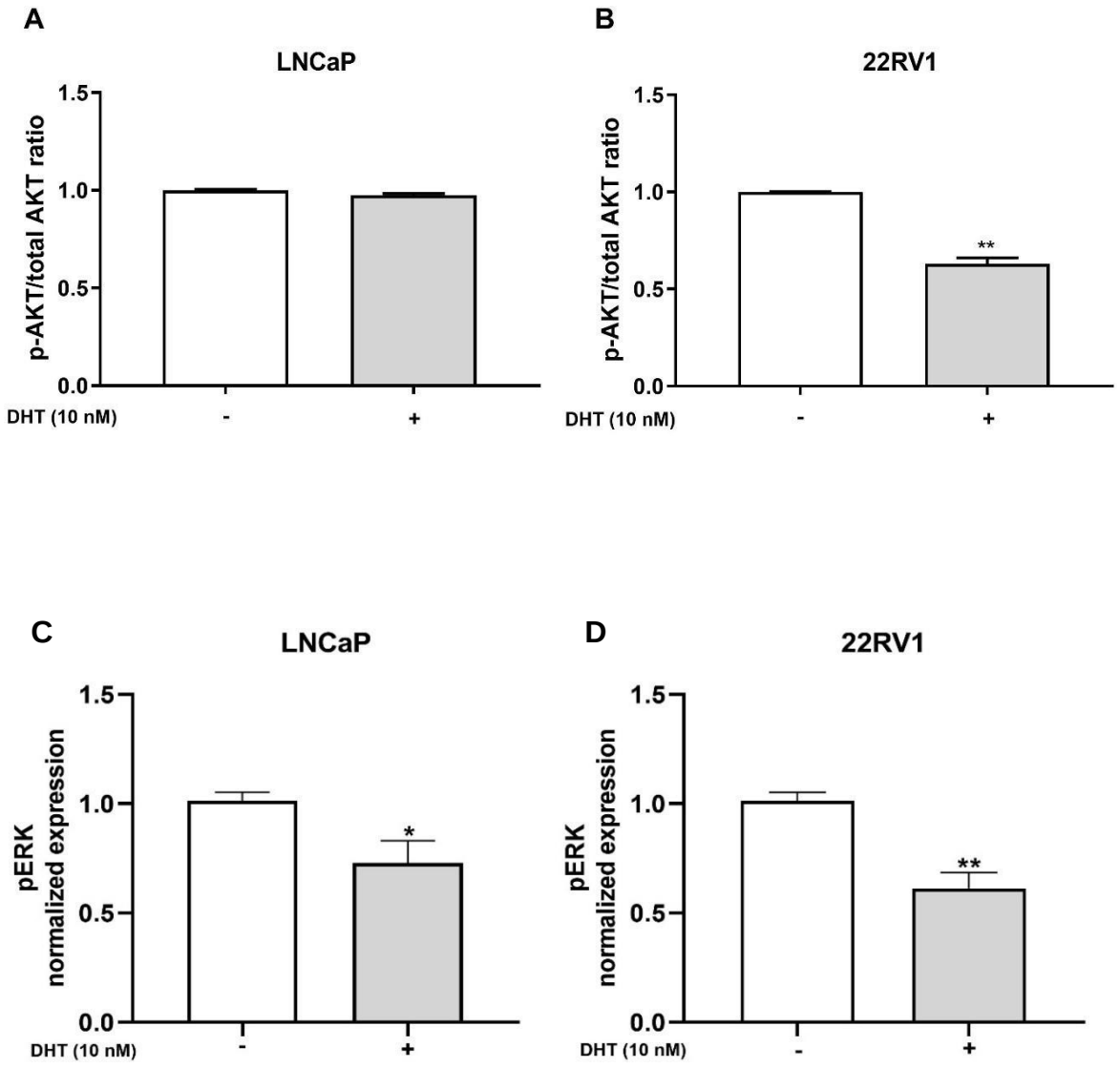


Figure IV.11. Migration assay of prostate cells treated with DHT.

Migration of PNT1A, LNCaP and 22Rv1 cells treated with DHT (10 nM) for 24 h. (**A**) Cell migration determined using the trans-well assay. The upper chamber contained cells with serum-free medium and +/- DHT. The lower chamber contained FBS complete medium, which acted as a chemoattractant. Data are expressed as the average number of migrating cells per field (10 fields per condition, 20x magnification). Cells were fixed with PFA 4% and stained with Hoechst. Error bars indicate mean ± S.E.M (n = 6), $*p$ <0.05, $**p$ <0.01 and $***p$ <0.001 relative to control. (**B**) Representative images of stained cells. Scale: 20 μM.

TRIB1 and TRIB3 are associated with several signalling pathways. However, the most essential signalling transducers are the PI3K/AKT and MAPK (ERK) [16]. These signalling pathways are known to play a prominent role in survival, apoptosis, and cell differentiation in response to a range of stimuli's including steroid hormones. [33]. As shown in the previous results, DHT downregulated TRIB1 and TRIB3 expression in PCa cells (Fig. IV.3), which raised the curiosity about the expression of associated signalling targets. Therefore, the expression of AKT and MAPK (ERKs) in LNCaP and 22Rv1 cells treated with DHT (10 nM) was measured using WB analysis.

It showed no changes in pAKT/AKT ratio in LNCaP cells cells (Fig. IV.12 A, C). However, a significant reduction of pAKT/AKT ratio in 22Rv1 cells (Fig. IV.12 B, D) was observed. In addition, a decrease in pERK1/2 was also seen both in LNCaP and 22Rv1 (Fig. IV.12).



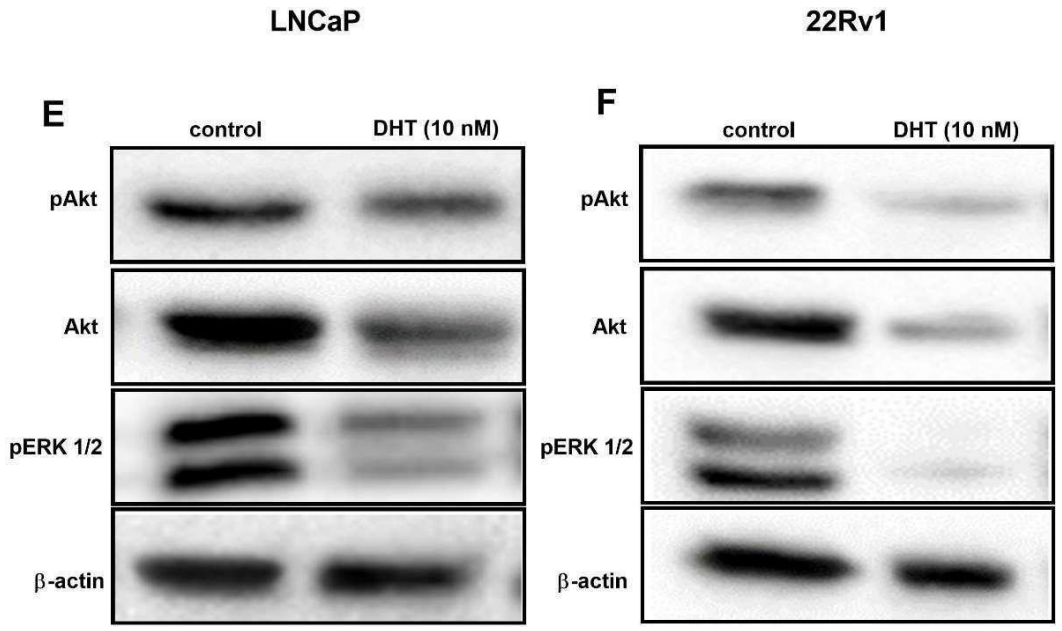


Figure IV.12. Protein expression of target genes associated with TRIBs.

pAKT, AKT and pERK1/2 expression in LNCaP **(A, C)** and 22RV1 **(B, D)** human PCa cells treated with DHT (10 nM) for 24 h. Data are represented as mean ± S.E.M after normalization of WB results with β-actin (n=6). All results are expressed as fold variation relative to the control, $^{**}p < 0.01$. Representative immunoblots of pAKT, AKT and pERK1/2 staining in control and DHT-treated cells are shown in panels E and F.

4.4. Discussion

In recent years, TRIBs proteins have gained popularity due to their unique and unclear involvement in multiple regulatory pathways and cancer. In the present study, we explored the androgen regulation of TRIB1 and TRIB3 expression in androgen responsive human PCa cells, focusing on the treatment with DHT, a potent metabolite of testosterone, as well as on AR inhibition. Hence, we looked at the impact of the various DHT concentrations on TRIB1 and TRIB3 expression at both mRNA and protein level. *In-silico* analyses (Fig. IV.1, Fig. IV.4) indicated the downregulation of TRIB1 after androgen treatment at various experimental concentrations and timepoints, which supported further investigating the androgen regulation of TRIBs expression levels in human prostate cells. We demonstrated that DHT decreased TRIB1 expression at both protein and mRNA levels in the androgen sensitive cell line models used LNCaP and 22RV1 (Fig. IV.2 and IV.3). To the best of our knowledge, no other studies were specifically designed to investigate the role of androgens in regulating the expression of TRIBs. However, other two independent studies have

also shown that DHT or R1881 treatment repressed TRIB1 expression levels in LNCaP PCa cells [29, 30]. Moreover, TRIB1 expression regulation by androgens has been previously reported in a microarray assay study for characterizing the AR response in hormonal therapy resistance in PCa cells [31]. Our results also showed that TRIB3 expression levels are downregulated by DHT, at least for 24 h treatment (Fig. IV.2, 3). Thus far, and in contrast with TRIB1 that showed consistent decreased expression in PCa cells after androgen treatment, TRIB3 expression in response to androgens seems to be somewhat instable as could be seen in the *in-silico* analysis (Fig. V.4). This was why TRIB3 analysis was not considered in further experiments.

After confirming the inverse correlation between TRIB1 expression and DHT treatment in PCa cells *in vitro* (Fig. IV.2, Fig. IV.3) we sought to explore if these effects could occur *in vivo*. Indeed, castration increased TRIB1 expression in PTEN KO mice (Fig. IV.8). Interestingly, no significant alteration was found on TRIB1 expression after castration in the WT mice. It is possible that this observation is influenced by the PTEN-AR relationship as a previous study has demonstrated PTEN to suppress AR activity via an PI3K/AKT-independent pathway in early passage LNCaP cells, having direct links by AR directly interacting with PTEN [32]. This interaction inhibited AR nuclear translocation and promoted the AR protein degradation, which sustained reduced AR transactivation [32]. Moreover, this study also showed that in higher passage number LNCaP cells, PTEN suppresses AR activity via the PI3K/Akt- dependent pathway. In depth studies will be needed to clarify if a cross-talk exists between PTEN (and the PI3K/AKT) and the AR in the regulation of TRIB1 expression.

Our next goal was to investigate whether the AR is involved in mediating the DHT effects regulating TRIB1 expression in prostate cells. The use of the antagonist bicalutamide, employed to inhibit the AR actions, resulted in blocking DHT effects, which supports the involvement of AR in regulating TRIB1 expression at the transcriptional level (Fig. IV.5).

Following the findings gained from AR inhibition by the antagonist bicalutamide, KD experiments performed also implicated AR in the regulation of TRIB1 expression. Silencing AR significantly increased TRIB1 levels in 22Rv1 cells (Fig.IV.7), also supporting the direct regulation of *TRIB1* gene. Interestingly, AR KD do not affect TRIB1 expression in the PTEN -/- LNCaP cells.

The putative interaction of AR with TRIBs genes was further understood by utilising a ChiP-seq analysis. Both in primary prostate tumours (Fig. IV.6A) and LNCaP cells treated with the synthetic androgen R1881 (Fig. IV.6C), a strong increased peak intensity indicated the AR binding within the TRIB1 locus, which supports that the AR directly interacts with *TRIB1* gene. Less peak intensity seen in TRIB3 indicates a weaker interaction of the AR with the *TRIB3* gene and supports the inconsistency of the regulation found in the *in-silico* analysis.

Androgens are well-known agents increasing viability, and survival of PCa cells, and acting as a driving force in tumour development and growth [33]. It is also known that TRIBs have the ability to regulate cell growth and proliferation through their association with cellular pathways like MAPK and PI3k/AKT which control these mechanisms [34]. Thus, we analysed PCa cells proliferation and migration in response to androgens in the context of TRIB1 downregulation (10 nM DHT for 24 h). In these conditions, DHT treatment enhanced the proliferation and migration of LNCaP and 22Rv1 PCa cells (Fig. IV.9 and IV.11), though not altering the apoptotic activity, as indicated by the unaltered caspase-3-like activity (Fig. IV.10).Indeed, this followed the published studies reporting the androgens actions inhibiting cell death via acting as a survival factor for prostate cells [35]. However, androgens also significantly enhanced the proliferation and migration features of non- neoplastic PNT1A cells (Fig. IV.9 and IV.11), in experimental conditions without altered TRIB1 levels (Fig. IV.3C). Nevertheless, altogether the obtained findings strongly suggest that androgens actions regulating PCa cell fate may depend on the downregulation of TRIB1. However, reports link TRIBs overexpression with the promotion of prostate tumorigenesis and TRIB1 has been described as overexpressed in PCa cases [36]. Different perspectives could contribute to this, namely the fact that androgens are not required in the later stages of PCa progression thereby this switch may induce TRIB1' abundant expression. The majority of PCa cases are also characterized by PTEN loss [37], and similar to the previously mentioned in LNCaP cells, the up- and down-regulation of TRIB1 may depend on the PCa stage and the differential PTEN-AR relationship.

Importantly, PI3K/AKT and MAPK signalling pathways are highly essential for normal prostate growth and differentiation, and their oncogenic activation is implicated in PCa [38]. Since they are also widely known as the main signalling

cascades downstream TRIB actions [39], the effect of DHT on TRIB-related targets such as ERK and AKT was measured. DHT reduced pERK, pAKT and AKT expression levels in PCa cells (Fig. IV.12). Similarly, a previously published study has shown a repressive role of androgens in the activation of AKT, a trend of the downregulation of pAKT expression in DHT (10 nM) stimulated LNCaP cells at an earlier timepoint of 8 h [40] suggesting a differential expression of these genes in PCa. Despite the suppression of the PI3K/AKT activation, as indicated by the reduced levels of active phosphorylated AKT, PCa cells proliferation was increased indicating that other survival signalling pathways are active.

In summary, this study has demonstrated the androgenic regulation of TRIB1 and TRIB3 in androgen responsive LNCaP and 22Rv1 cells, with the direct involvement of the AR. Moreover, present findings suggest a cross-talk of AR with the PTEN and transcription factors in this regulation. Further research efforts will clarify the complex signalling relationship of androgens/AR in regulating TRIBs expression, and how altered TRIBs levels affects PCa cell fate. Understanding these molecular interactions will help defining the role and potential of TRIBs as prognosis biomarkers for PCa.

4.5. Acknowledgements

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Chapter V:

5. The effects of manipulating TRIB1 expression in non- neoplastic prostate epithelial cells and prostate cancer cells

Abstract

Tribbles-1 (TRIB1) is a pseudokinase protein that has been suggested to be implicated in the initiation, development, and progression prostate cancer (PCa). However, there is a lack of insight into the role and contribution of TRIB1 in PCa, namely if are its increased expression levels that promote the malignant transformation of prostate cells, or if they are a consequence of the carcinogenic process itself. This study is aimed to explore whether TRIB1 overexpression (OE) has any physiological effects altering the behaviour of non-neoplastic (PNT1A) prostate cells. The effect of TRIB1 knockdown (KD) in the human PCa PC3 cell line was also analysed.. Cell viability, proliferation, migration, and glucose and lipid metabolism were assessed after OE or KD of TRIB1. TRIB1 OE significantly increased the proliferative activity and migration of PNT1A cells, with no apoptotic effects. A disruption of lipid metabolism, with increased lipid droplets accumulation was also seen in PNT1A cells OE TRIB1, along with an upregulated FASN protein expression. No significant changes in proliferation, viability, migration, and apoptosis were seen upon TRIB1 KD in PC3 cells, suggesting that increased expression of TRIB1 in non-neoplastic prostate cells has more impact than the KD in neoplastic ones. Moreover, changes in the physiological characteristics of non-neoplastic cells into a cancer-like phenotype after altering TRIB1 expression suggests that TRIB1 may play an important part in PCa initiation. Further investigations are required to disclose the role of TRIB1 in prostate tumorigenesis and its usefulness as a biomarker or therapeutic target.

Keywords: TRIB1; prostate cancer; PNT1A cells, lipid metabolism.

5.1. Introduction

Tribbles-1 (TRIB1) is a protein kinase belonging to the three-membered pseudokinase family which are catalytically inactive [1]. The evolutionary conserved pseudoenzymes are known to control diverse signalling networks such as MAPK, PI3K/AKT [1, 2], with TRIBs functioning as scaffold or adapter molecules enabling binding or interaction with a broad range of targets [2]. These pseudokinases possess a COPI-binding domain at the C-terminal that regulates protein stability via ubiquitination and proteasomal degradation [1, 3-5]. As a consequence of being regulators of basic cellular and molecular process, TRIBs are strongly implicated in differentiation, inflammation, cellular stress, glycolytic metabolism, lipogenesis, cell survival, and apoptosis [6]. For this reason, TRIBs have been pointed out as targets in several pathologies. In particular, TRIB1 has been linked with metabolic traits, namely, lipid metabolism control. It was associated with plasma lipoproteins, adiponectin triglyceride and cholesterol levels [7, 8]. Moreover, TRIB1 is one of the pseudokinases that has been pointed out as an oncogene. The high expression of TRIB1 in prostate cancer (PCa,) cases was reported and there is emerging evidence suggesting its involvement in the initiation, development, and progression of disease [9]. Recent studies have described TRIB1 as a modulator and target of numerous miRNAs thereby altering the inflammatory profile of human PCa [10]. Previous studies also have demonstrated TRIB1 to be a critical factor for PCa cell survival and to regulate target genes involved in prostate tumour-propagation [9]. Moreover, TRIB1 was described to be associated with the regulation of the oncogene cMYC and the tumour suppressor p53 protein [11], which are strongly associated with aggressive tumorigenesis and poor clinical outcomes [11, 12]. However, it is still unclear if are the TRIB1 increased expression levels that promote the malignant transformation of prostate cells, or if they are a consequence of the carcinogenic process itself.

This study investigated the effect of TRIB1 overexpression (OE) in non-neoplastic PNT1A human prostate cell fate, by analysing cell viability, proliferation, and migration. Also, the effect on lipid and glycolytic metabolism was investigated. The effect of TRIB1 knockdown (KD) in the human PCa PC3 cell line was also analysed. Exploring of the effects of manipulating (silencing or upregulating) TRIB1 expression levels in prostate cells may help in understanding the role of this pseudokinase in PCa development and progression.

5.2. Methods and Materials

5.2.1. Cell culture

Immortalized non-neoplastic prostate epithelium cell line PNT1A was purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK). PCa cell lines LNCaP and PC3 were purchased from the ATCC and cultured in Roswell Park Memorial Institute medium 1640 medium (RPMI 1640, Gibco, Life Technologies, Paisley, UK) supplemented with 10 % fetal bovine serum (FBS, Biochrom, Berlin, Germany) and 1 % penicillin (100U/ml)/streptomycin (Santa Cruz Biotechnology, Heidelberg, Germany), in an atmosphere with 5 % CO2 at 37 °C. For experimental use the cells were trypsinized with a trypsin solution buffered with EDTA (0.025 %).

5.2.2. Cell Transfection

Transient transfection was carried out using Lipofectamine3000 (Invitrogen, Carlsbad, CA, USA). In 80% confluent cells, TRIB1 OE in PNT1A cells was performed using 2 μg of the hTRIB1 4/TO/myc-his recombinant pcDNA (Invitrogen, Carlsbad, CA, V1030-20) expression vector coupled with GFP (gifted by Endre Kiss-Toth laboratory). TRIB1 KD in PC3 cells was performed by TRIB1 specific siRNA (2500 ng) transfection (SMARTpool: ON-TARGETplus TRIB1 siRNA, L-003633-00-0010, Dharmacon, Cambridge, UK). PC3 cells were also transfected with siRNA nonspecific to TRIB1 (ON-TARGET plus non-targeting siRNA, D-001810-01-05, (Dharmacon) as mock-transfected control. All cells were maintained in a humidified incubator (5% CO2, 37 ºC) for 24 h.

5.2.3. Protein extraction and Western blot analysis

Total protein was extracted from PNT1A, and PC3 cells using RIPA buffer (150 mM NaCl, 1 % Nonidet-P40 substitute, 0.5 % Na-deoxycholate, 0.1 % SDS, 50 mM Tris pH 8 and 1 mM EDTA) supplemented with 1 % protease inhibitors cocktail (Sigma-Aldrich) and 10 % PMSF (Sigma-Aldrich, St Louis, MO, USA). Protein concentration was determined by the bicinchoninic acid assay (Bio-Rad, Rockford, IL, USA), and 40 μg of protein extracts were resolved by SDS-PAGE on 10 and 12 % gels. The samples were then electro-transferred to a PVDF membrane (GE Healthcare). Membranes were incubated overnight at 4 °C with rabbit anti-TRIB1 (1:1000, 09-126, Millipore, CA, USA), rabbit anti-Fatty Acid Synthase (FASN) (1:1000, no.3180; Cell Signaling Technology, Danvers, MA, USA) and mouse anti-β-actin (1:1,000, A5441, Sigma-

Aldrich) antibodies. Goat anti-rabbit IgG-HRP (1:40,000, sc-2004, Santa Cruz Biotechnology, CA, USA) or anti-mouse-IgGκ HRP-linked (1:20000, sc-516102, Santa Cruz) were used as secondary antibodies. Membranes were incubated with Clarity™ Western ECL substrate (Bio-Rad) for 5 min and immunoreactive bands visualized using the ChemiDoc™ MP Imaging System (Bio-Rad). Band densities were obtained according to standard methods using the Image Lab 5.1 Software (Bio-Rad) and normalized by division with the respective β-actin band density. Protein extraction followed as per protocol in Vaz *et al*, 2016 [13].

5.2.4. MTT assay

Cell viability was determined by the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The conversion of MTT compound to the coloured formazan product was detected at 490 nm in a xMark™ Microplate Absorbance Spectrophotometer (Bio-Rad). The relative number of viable cells in each experimental condition was calculated by normalizing the absorbance to that of the mock control. All experiments were repeated at least six times, and each experiment was carried out in triplicates.

5.2.5. Caspase-3-like activity assay

Caspase-3-like enzymatic activity was determined spectrophotometrically at 405 nm by detecting the presence of p-nitro-aniline (pNA), upon cleavage of caspase-3 substrate (Ac-DEVD-pNA). Briefly, 50 μg of total protein extract was incubated overnight at 37 °C in reaction buffer (25 mM HEPES, 0.1% 3CHAPS, 10% sucrose, and 10 mM DTT, pH 7.5) and 200 μM of Ac-DEVD-pNA. The amount of generated pNA was calculated by extrapolation with a standard curve.

5.2.6.Ki67 immunofluorescence

After 24 h transfection, PNT1A cells OE TRIB1, TRIB1 KD PC3 cells and mock-transfectants were fixed for 15 min in 4% formaldehyde in Phosphate Buffered Saline (PBS). Cell permeabilization was achieved with PBS containing 0.1% TRITON X-100 for 15 min. Blocking was performed by incubating with PBS containing 0.1% (w/v) Tween-20 (PBS-T) and 20% FBS for 1 h. After blocking, cells were incubated with the rabbit monoclonal anti-Ki67 antibody (1:250, ab16667, Abcam, Cambridge, United

Kingdom) for 1-24 h at room temperature. Alexa 594 goat anti-rabbit IgG (1:500, A-11012, Invitrogen) was used as a secondary antibody for 1 h at room temperature. Four washes of 5 minutes in PBS were performed after each incubation. Cells were mounted in DakoCytomation Fluorescent Mounting Medium supplemented with DAPI. The slides were examined by using a Widefield inverted microscope (Leica AF6000LX).

5.2.7. Migration assays

Migration assay was performed using 8 μM pore size inserts (35224, SPL, Life Sciences, Naechon-Myeon Pocheon, South Korea). Briefly, PNT1A cells (3.0 x105 cells/well) OE TRIB1 were placed into the upper chambers in serum-free media. The lower chambers contained 20% FBS. After 24 h, cells on the lower surface of membrane were fixed with PFA 4% and stained blue with Hoechst 33342. Cells were then counted in 10, randomly selected, 40x magnification fields per transwell.

5.2.8. Oil-Red-O Assay

PNT1A cells OE TRIB1, TRIB1 KD PC3 cells and mock-transfectants (5.0 x105 cells/well) seeded in 12-well plates were fixed with 4% PFA for 30 min. Cells were washed twice with distilled water and rinsed with 60% isopropanol for 5 min. Cells were then stained with Oil Red O for 20 min. Microscope images were obtained. The lipid content (O-red-o stained) quantification was carried out by diluting dye with 100 % isopropanol for 5 min with gentle agitation. Absorbance was measured using xMark™ Microplate Absorbance Spectrophotometer (Bio-Rad) at 492 nm.

5.2.9. Quantification of glucose and lactate

Glucose and lactate concentration in the cell culture medium of PNT1A OE TRIB, TRIB1 KD PC3 and mock-transfectant cells for 24h and 48 h of culture were determined by spectrophotometric analysis using commercial kits (Spinreact, Girona, Spain) as described previously [13]. The glucose consumption and lactate production were calculated relatively to initial glucose and lactate concentrations at 0 h of treatment. Measure acquired were then normalized to the total number of cell in each experimental condition.

5.2.10. Statistical analysis

The statistical significance of differences between experimental groups was evaluated by unpaired t-test and one-way ANOVA with Dunnett's test, using GraphPad Prism

v7.00 (GraphPad Software, Inc., La Jolla, CA, USA). $P < 0.05$ was considered statistically significant. All experimental data are shown as mean ± standard error of the mean (S.E.M).

5.3. Results

5.3.1. TRIB1 overexpression in PNT1A cells confirmed by Western blot

To investigate the behavior of non-neoplastic prostate cells OE TRIB1, PNT1A cells were transfected with the hTRIB1 4/TO/myc-his recombinant pcDNA plasmid or a mock plasmid for 24 h. Western blot analysis with the control LNCaP cells confirmed TRIB1 overexpression in PNT1A cells, and the results obtained adhere to Chapter III findings which show native TRIB1 of approximately 41 kB, and an additional extra band of higher size (Fig. V.1). GFP fluorescence image (Fig. V.1B) allowed the detection of GFP tagged plasmid confirming TRIB1 OE. Since PC3 cells highly express TRIB1, KD PC3 cells was included in the following analysis to compare differences in non-neoplastic vs neoplastic cell line, respectively OE or with TRIB1 KD. Details and confirmation of TRIB1 KD in PC3 cells can be seen in Chapter III (Fig. III.2.).

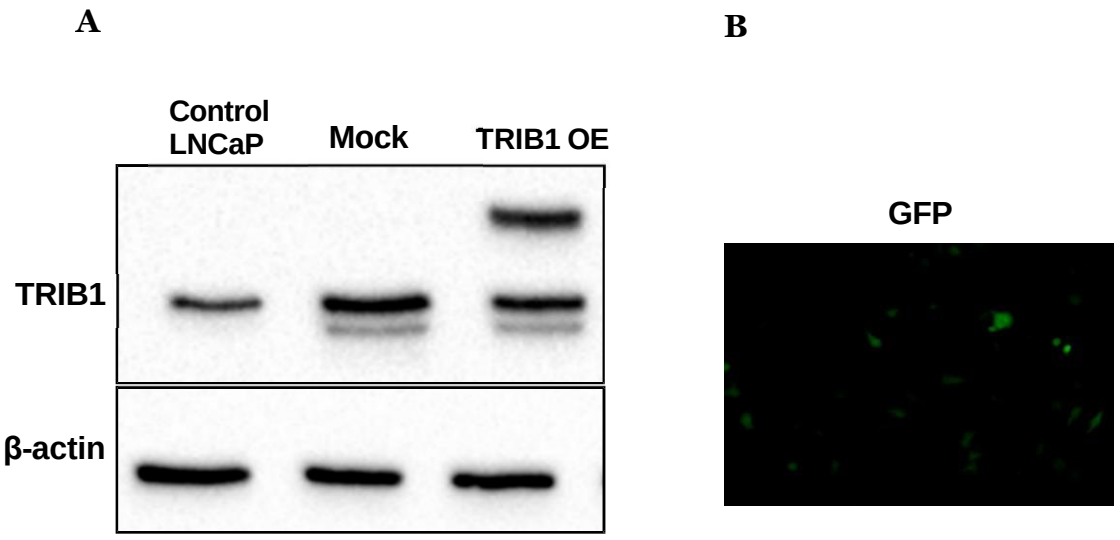

Figure V.1 Overexpression of TRIB1 in non-neoplastic cells.

TRIB1 OE in non-neoplastic PNT1A cells. (A) WB analysis of TRIB1 expression in mock-transfected and PNT1A OE cells after 24 h transfection. Neoplastic LNCaP prostate cells were used as a control. (B) GFP fluorescence was used as a control to

confirm successful OE. Representative image obtained in the Zeiss LSM 710 laser scanning confocal microscope (Carl Zeiss, Gottingen, Germany) 40 x magnification is shown.

5.3.2. TRIB1 overexpression did not alter PNT1A cell viability and survival

Cell viability and cell death in PNT1A cells OE TRIB1, were measured using the MTT (Fig. V.2) and caspase-3 (Fig. V.3) activity assays, respectively. PC3 cells with TRIB1 KD were included to compare caspase-3 activity in non-neoplastic and neoplastic cells lines which, respectively, are KD or overexpress TRIB1. No changes were seen in both cell viability (Fig. V.2) and caspase-3 (Fig. V.3) activity in PNT1A and PC3 cells, regardless of transfection time for 24 h or 48 h.

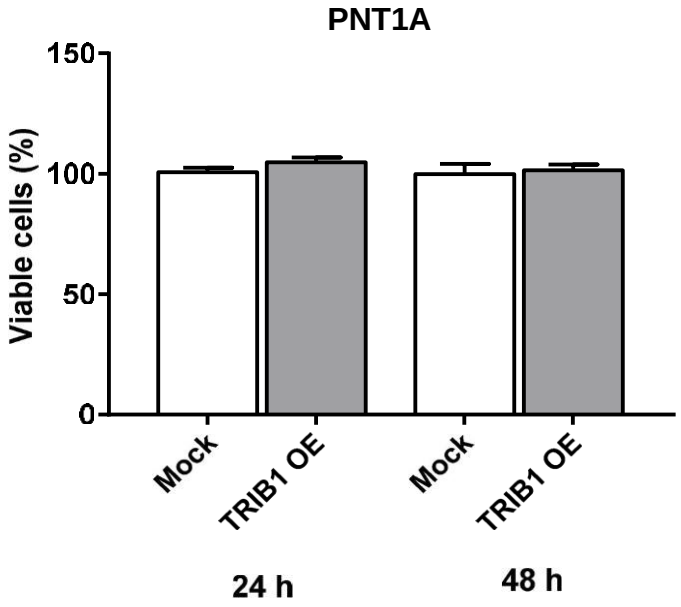

Figure V.2. Cell viability of PNT1A cells overexpressed with TRIB1.

Viability of mock transfected and PNT1A cells OE TRIB1 after 24 h and 48 h of transfection determined via MTT assay. Error bars indicate mean ± S.E.M. (n = 6). Results are expressed as fold-variation relative to control.

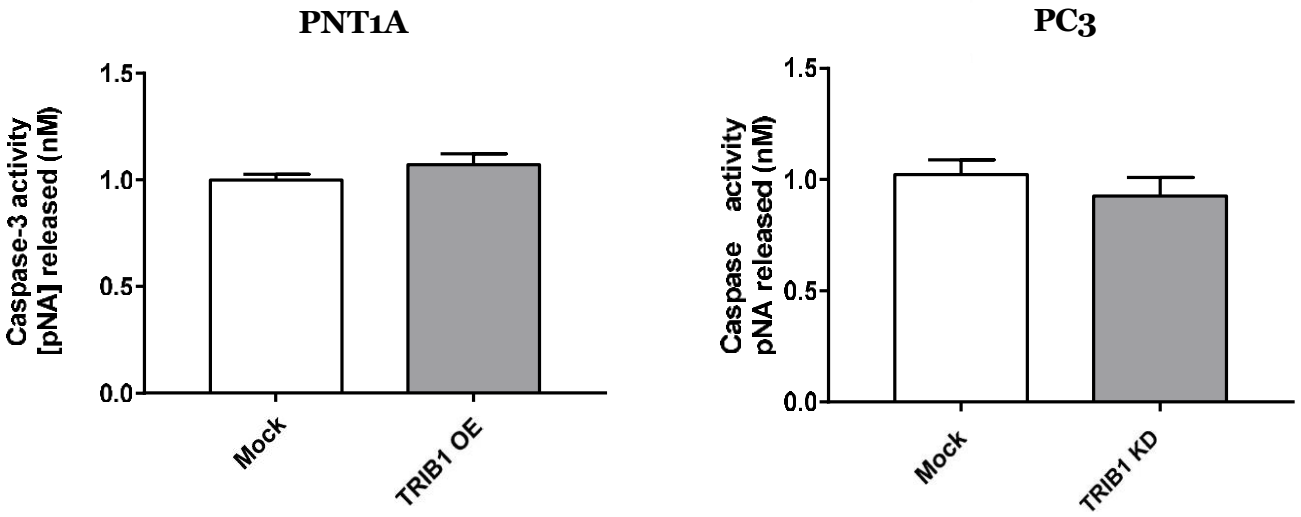

Figure V.3. Caspase-3 activity in PNT1A cells OE TRIB1 and -KD PC3 cells.

Caspase-3 activity in PNT1A cells OE TRIB1, TRIB1 KD PC3 cells, and mock-transfected cells after 24 h. Enzyme activity was determined spectrophotometrically (405 nM) based on the release of the reaction product pNA chromophore (n = 6). Error bars indicate mean ± S.E.M. Results are expressed as fold-variation relative to control.

5.3.3. TRIB1 overexpression increased PNT1A cell proliferation

To explore the proliferative capacity of PNT1A cells OE TRIB1 and TRIB1 KD PC3 cells, the Ki67 nuclear proliferation marker fluorescent immunocytochemistry analysis was used (Fig. V.4). The results obtained showed that the number of Ki67 positive cells relative to total cell count is increased in PNT1A cells OE TRIB1 (Fig. V.4A), which is visible in the fluorescent immunocytochemistry images (V.4B). No changes on the Ki67 proliferative index were seen in TRIB1 KD PC3 cells (Fig. V.4A and V.4B).

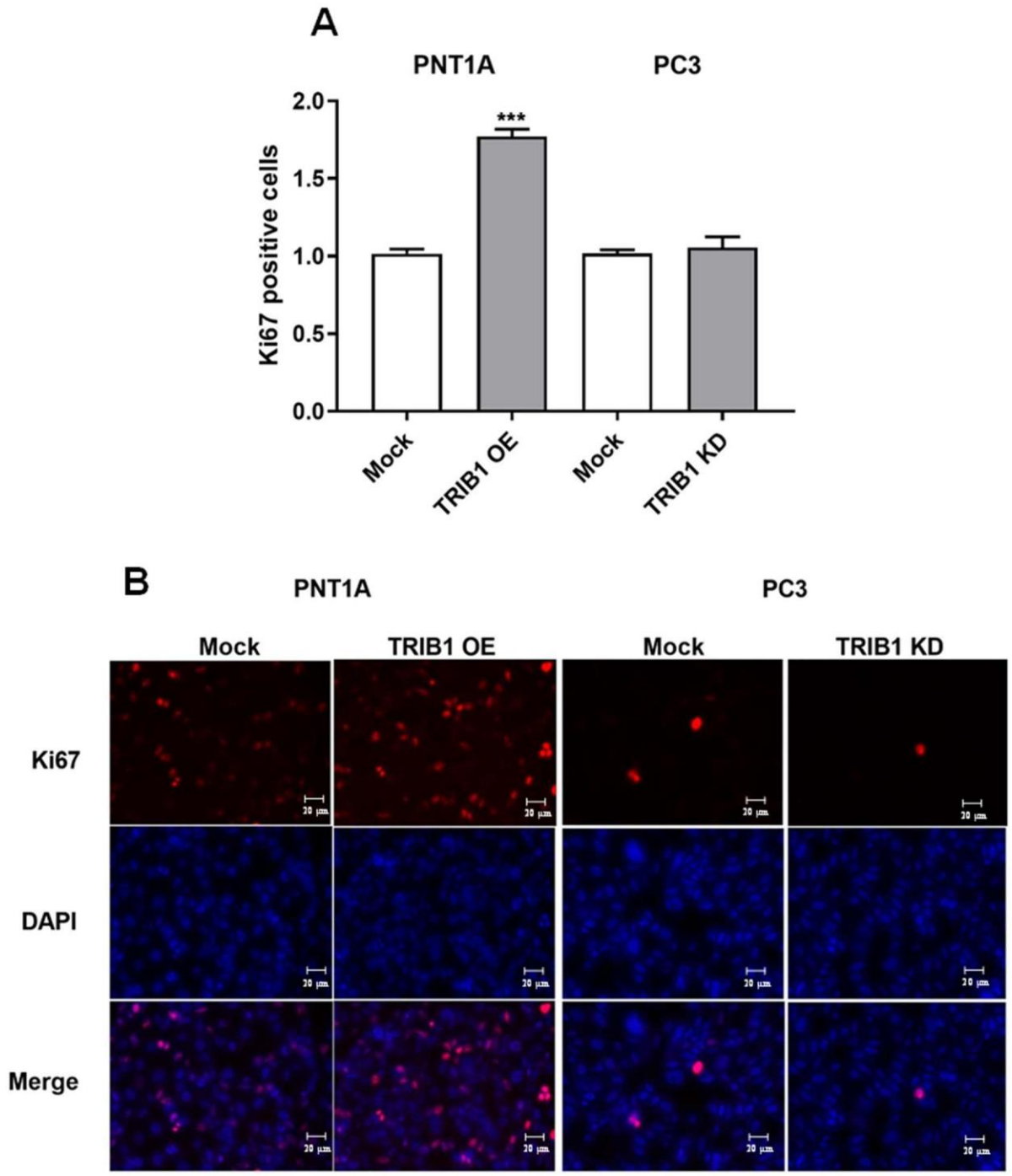

Figure V.4. Ki67 proliferation in PNT1A cells OE TRIB1 and -KD PC3 cells.

Proliferation of PNT1A OE TRIB1 and PC3 TRIB1 KD cells determined by Ki67 fluorescent immunocytochemistry after 24 h transfection. **(A)** Percentage of Ki67 positive cells relative to the total cell number of each corresponding cell line. Results are expressed as fold-variation relative to mock-transfected cells. Error bars indicate mean ± SEM, ***p <0.001 relative to control. **(B)** Representative images of Ki67 positive proliferative cells (red), nucleus stained blue with DAPI, and merged labelling. Scale: 20 µM.

5.3.4. TRIB1 overexpression increased migration of PNT1A cells.

To better understand the effects of TRIB1 OE on PNT1A cell migration, Transwell assays were performed. The results obtained showed a significant increase of migration (> 50% variation) in PNT1A cells OE TRIB1 compared to mock control (Fig. V.5).

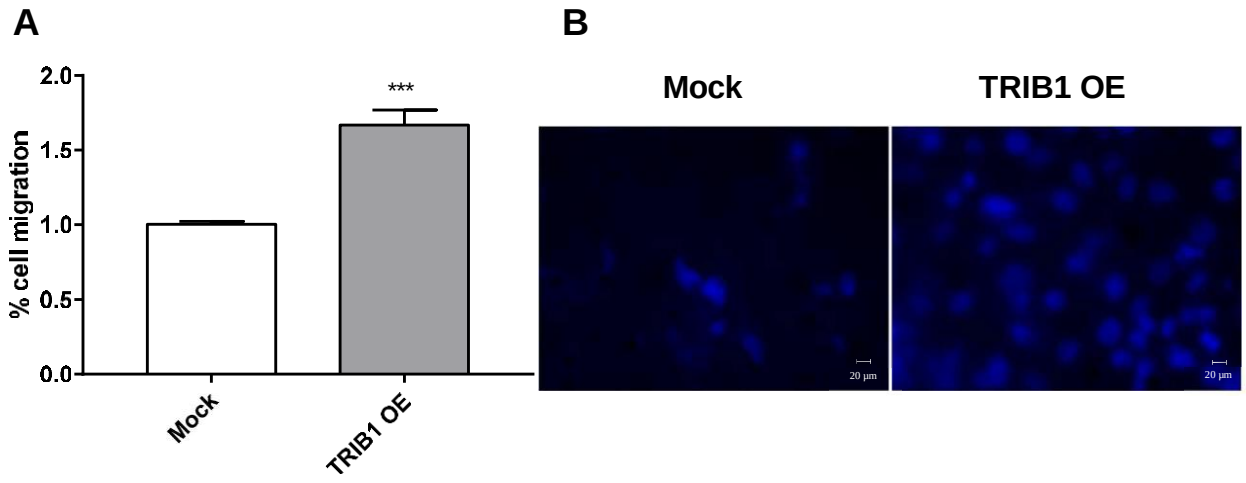

Figure V.5. Migration assay in PNT1A cells OE TRIB1 cells.

Migration of mock transfected and PNT1A cells OE TRIB1 OE ls after 24 h transfection. **(A)** Cell migration determined by the trans-well migration assay. The upper chamber contained cells with serum-free. The lower chamber contained FBS complete medium, which acted as a chemoattractant. Data express the average number of migrating cells per field (10 fields per condition, 40x magnification). Cells were fixed with PFA 4% and stained with Hoechst. Results are expressed as fold-variation relative to mock-transfected cells. Error bars indicate mean ± S.E.M (n = 6), ***p <0.001 relative to control. **(B)** Representative images of cells with Hoechst-stained nuclei. Scale: 20 µM.

5.3.5. TRIB1 overexpression altered PNT1A lipid metabolism into PCa-like characteristics

In vitro and *in vivo* studies have been associating TRIB1 with the regulation of lipid metabolism, namely influencing plasma lipid levels and triglycerides [13]. As enhanced lipid metabolism is a feature of PCa that is strictly dependent on androgens regulation [14] we sought to investigated lipid metabolism upon manipulating TRIB1 levels. Oil-red-o staining indicated that TRIB1 OE significantly increased the lipid droplets content in PNT1A cells (Fig V.6A, B). No changes were observed in oil-red

staining (Fig. V.6B) and estimated lipid content (Fig. V.6A) in TRIB1 KD PC3 cells. Furthermore, protein levels of fatty-acid synthase (FASN), a key target associated with the increased lipid metabolism in PCa were also determined. WB results demonstrated that TRIB1 OE significantly increased FASN expression in PNT1A cells (Fig. V.7).

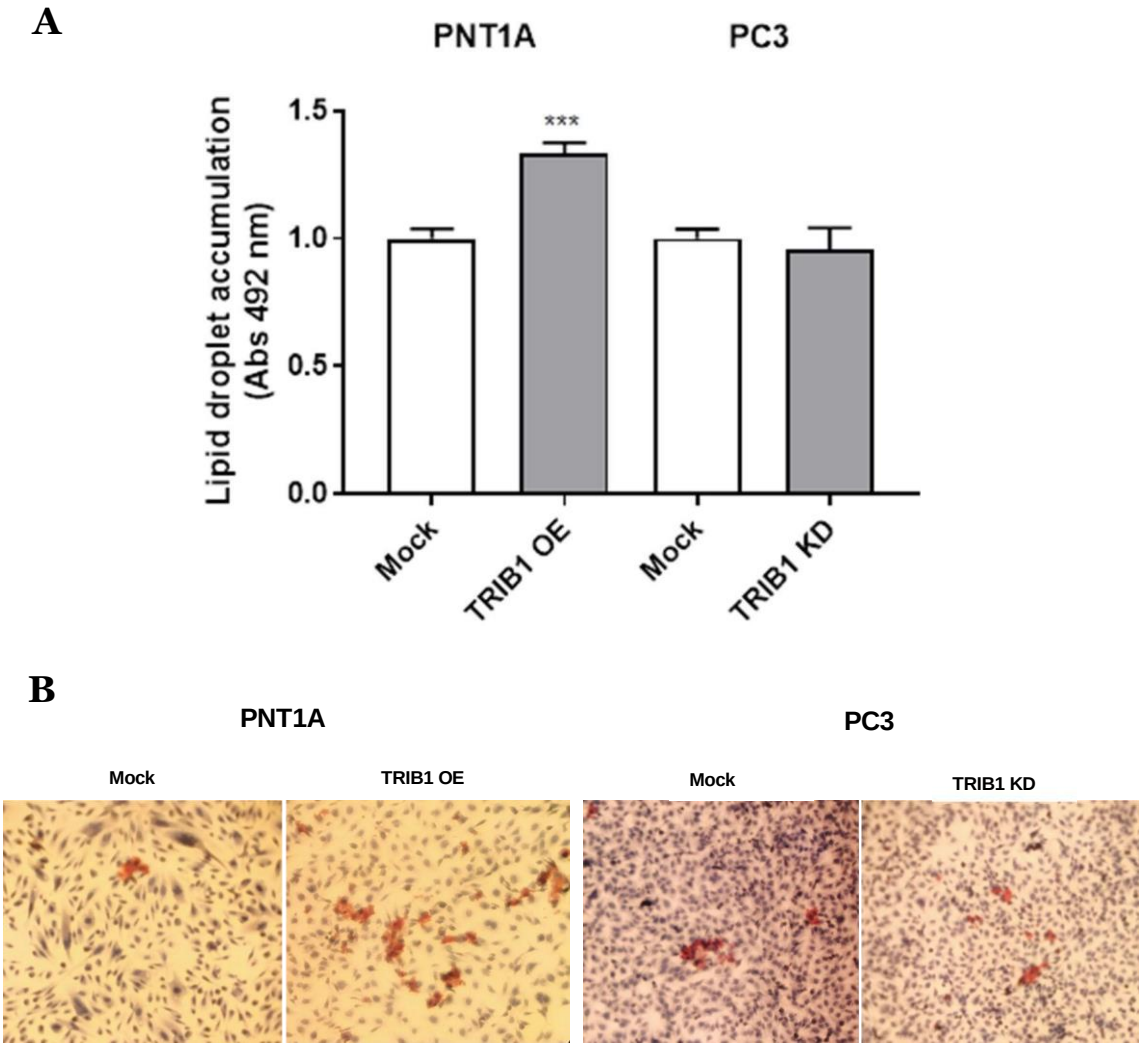

Figure V.6. Lipid droplet accumulation in PNT1A cells OE TRIB1 and -KD PC3 cells.

Lipid droplet content in PNT1A cells OE TRIB1 and PC3 KD TRIB1 cells after 24 h transfection. (A) Lipid droplet quantification determined using the Oil Red-O assay. Results are expressed as fold-variation relative to mock-transfected cells. Error bars indicate mean ± S.E.M (n = 6), ***p <0.001 relative to mock. (B) Representative images of Oil Red staining.

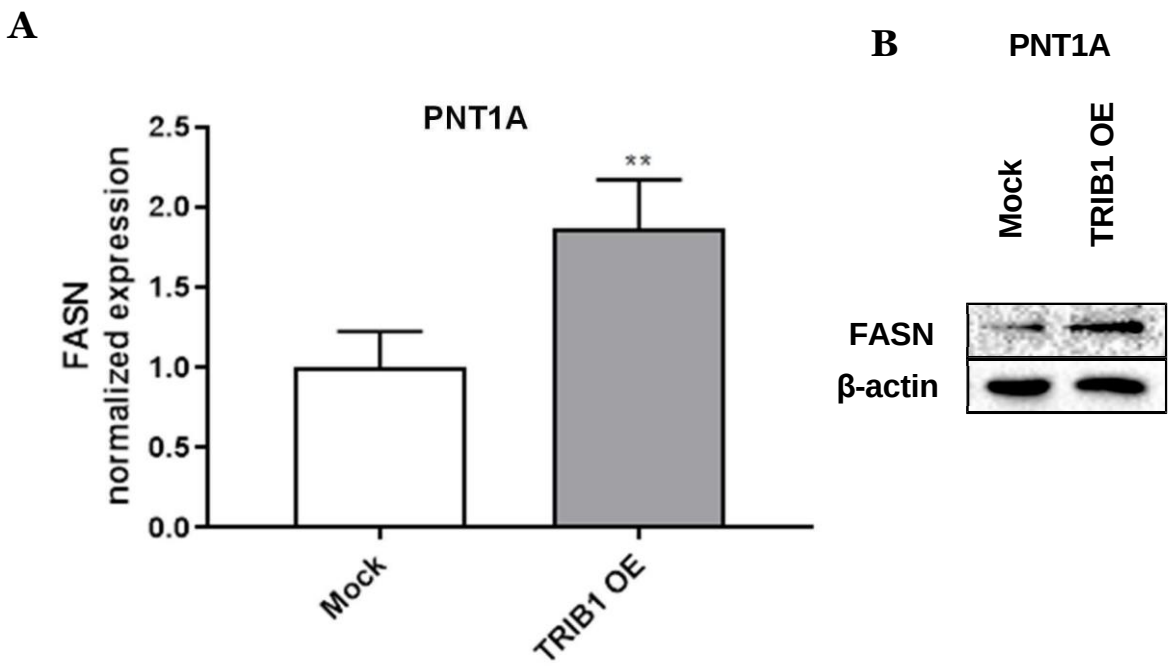

Figure V.7 FASN expression in PNT1A cells OE TRIB1 cells.

FASN protein expression levels in mock-transfected and PNT1A cells OE TRIB 1 after 24h h transfection. **(A)** Results of WB analysis after normalization expression with β-actin. Results are expressed as fold-variation relative to mock-transfected cells. Error bars indicate mean ± S.E.M (n = 6), $^{**}p$ <0.01. **(B)** Representative images of WB.

5.3.6. Glycolytic metabolism is not affected by manipulating TRIB1 levels in prostate cells.

Metabolic shifts like increased rate of glycolysis occur during PCa progression, enabling sufficient energy for the cancer cells to grow [15]. To investigate the glycolytic profile of PNT1A and PC3 cells after increasing or suppressing TRIB1 expression, glucose and lactate content in cell culture medium was determined. Glucose consumption and lactate production were estimated for 24 h and 48 h transfection compared with mock-transfectants. No effect was seen in both glucose consumption (Fig. V.8A) and lactate production (Fig. V.8B) in either PNT1A cells OE TRIB1 or TRIB1 KD PC3 cells.

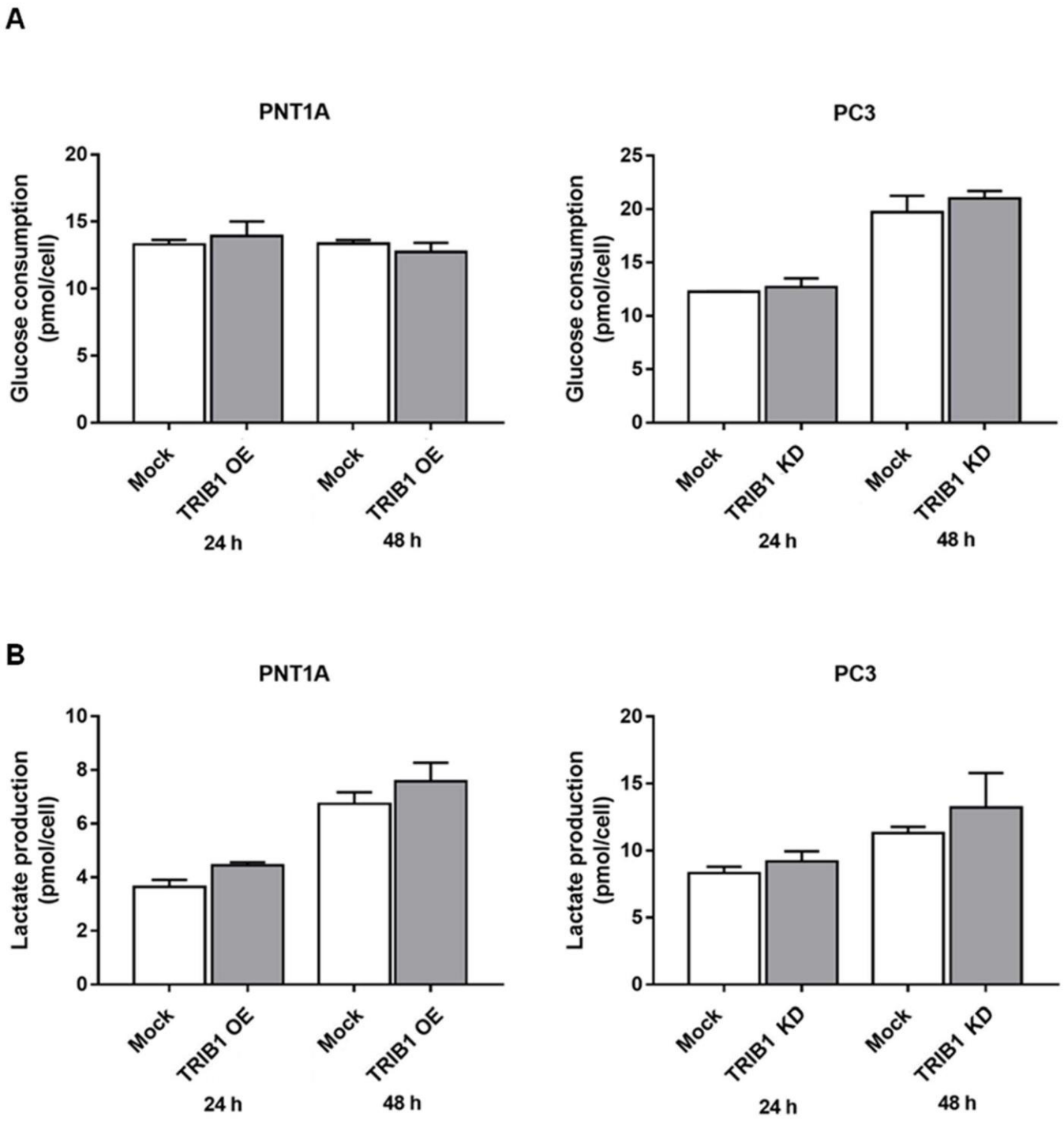

Figure V.8 Metabolic activity in PNT1A cells OE TRIB1 and -KD PC3 cells.

Glucose consumption **(A)** and lactate production **(B)** in PNT1A cells OE TRIB1 and PC3 TRIB1 KD cells for 24 h and 48 h transfection. Results are expressed as foldariation relative to mock-transfected cells. Error bars indicate mean ± S.E.M (n= 6).

5.4. Discussion

TRIBs' roles in controlling cell growth, proliferation, and differentiation make them interesting targets in the initial development stages of the carcinogenic process. Indeed, the TRIB1 isoform was shown to be overexpressed in PCa and associated with the progression of disease [9, 15]. Such studies propelled the interest to investigate the oncogenic effects and prostate cell fate upon altering TRIB1 expression levels in non-neoplastic and neoplastic cells via OE or KD. TRIB1 OE in non-neoplastic PNT1A prostate epithelial cells was confirmed by detecting the expected band of 41 kB and deemed efficient in producing the OE required (Fig. V.1). At least to our knowledge, this is the first study overexpressing TRIB1 in a non-neoplastic cell line model. It will be crucial to understand the role of this TRIBs' isoform potentially leading to PCa, namely, whether there are the TRIB1s increased expression levels that promote the malignant transformation of prostate cells, or if they are a consequence of the carcinogenic process itself.

Gene alterations are expected to result in physiological changes that could affect cell behaviour, namely, their proliferative and migration capabilities, as well as the metabolic status. Herein, we investigated the effect of TRIB1 OE in disrupting the viability, proliferation, and migration properties of PNT1A cells.

Modifying TRIB1 expression did not cause alterations in PNT1A viability (Fig. V.2). Also, TRIB1 KD in the PC3 cell line which endogenously overexpresses TRIB1 did not change cell viability. Accordingly with the results of cell viability, no changes were seen in caspase-3-like activity in both PNT1A cells OE TRIB1 and PC3 TRIB1 KD (Fig. V.2 and V.3).

Concerning proliferation, and contrary to cell viability results, TRIB1 OE altered the proliferative behavior of PNT1A cells increasing the number of Ki67positive cells (Fig. V.4). Similar to our findings, TRIB1 OE significantly increased HepG2 and Huh7 hepatocellular carcinoma cell proliferation after 24 hours [12]. Furthermore, published work has shown that: i) TRIB1 transgenic OE significantly increased prostate epithelial cell proliferation *in vivo* and ii) injection of PC3 cells OE TRIB1 significantly increased tumors growth [11, 16] However, TRIB1 KD in PC3 cells showed no impact on cell proliferation. Interestingly, similar findings were previously described either by knocking down or knocking in TRIB expression in PCa cell, PC3 or DU145, respectively [11], which suggests that TRIB1 OE is more likely to alter the fate

of non-neoplastic prostate cells. Moreover, the present results also suggest that TRIB1 promotes the proliferative characteristics of PCa cells, possibly being implicated in the initiation of the development of PCa.

TRIB1 actions have also been linked with promoting cell migration in colorectal cancer and hepatocellular carcinoma, while silencing TRIB1 reduced cell migration [12, 17]. Information on TRIB1 influencing migration and invasion of PCa did not alter anchorage-independent growth or invasive growth in three-dimensional systems [11]. Our findings are very interesting, as they have shown that TRIB1 OE induced a significant increase in the migration rate of non-neoplastic PNT1A cells (Fig. V.5). Taken together, the obtained findings support the hypothesis that the alteration of TRIB1 expression pattern, and its augmented levels, that shift prostate cell fate towards the tumour-like features of increased proliferation and migration capacity, rather than altering the behaviour of already transformed PCa cells. They link TRIB1 with prostate tumorigenesis and collectively demonstrate the possible role of TRIB1 in tumour initiation and development of PCa.

Metabolic adaptation is one of many outlined hallmarks of cancer that enables cancer development by maintaining continuous sources of energy and building blocks, ensuring the survival of cancer cells [18-20]. Among the diverse peculiarities of cancer cells metabolism, a rewiring in the use of lipids has been described. For example, accumulation of lipid droplets can be identified during initiation, promotion, and progression processes of cancer development [21]. In PCa cells, the enhanced uptake and storage of lipids, which provide the building blocks for membrane synthesis, facilitating proliferation and growth, has been described [22]. Previous reports revealed the association of TRIB1 with lipogenesis and triglyceride metabolism in animal models and human samples [23, 24], but the relationship of TRIB1 with lipid metabolism in the prostate in unknown. Herein, we demonstrated that TRIB1 OE alters the phenotype of non-neoplastic prostate cells to the neoplastic-like metabolic characteristics. Interestingly, TRIB1 OE significantly increased lipid droplet accumulation in PNT1A cells (Fig. V.6), whereas manipulating TRIB levels in the neoplastic PC3 cells had no effect concerning the amount of lipids stored. Moreover, increased expression of FASN was seen in non-neoplastic PNT1A TRIB1 OE cells compared to control (Fig. V.7). In general, FASN expression is elevated in PCa cells and associated with poor prognosis and cancer development [25, 26]. Studies in mice

have demonstrated a significant downregulation of FASN in TRIB1 KO animals and an increase of FASN in TRIB1 OE [27], which indicates that TRIB1 can be a target regulator of FASN. It also suggests that TRIB1 OE followed by the increased levels of FASN support *de novo* synthesis of fatty acids and lipid accumulation, which is required to sustain cancer cells growth.

Glycolysis plays a pivotal role in cancer progression. PCa cells in advanced stages of disease exploit the altered energy metabolism where there is increased conversion of glucose into pyruvate followed by increased lactate production [13]. Here, our results showed that TRIB1 OE did not alter the glycolytic profile of PNT1A cells, as no significant changes in glucose consumption and lactate production (Fig. V.8) were perceived. Also, TRIB1 KD did not affect glucose and lactate handling by PC3 cells (Fig. V.8). Noteworthy, PC3 cells, regardless of TRIB1 expression, are much more glycolytic than PNT1A cells, displaying higher glucose consumption and lactate production. This observation follows and reiterates the previous findings of our research group demonstrating that castration-resistant PCa cells have higher rates of glycolysis than androgen-sensitive and non-neoplastic cells [14]. In light of present results, TRIB1 OE does not necessarily drive the glycolytic flux of non-neoplastic prostate cells, which is in line with its putative role as an early-stage tumorigenic factor, as the metabolic shift of PCa towards a more glycolytic phenotype occurs in advanced stages of disease.

In sum, this study first demonstrated that TRIB1 OE altered the phenotype of non-neoplastic PNT1A cells, enhancing proliferation and migration, and stimulating PCa-like lipid metabolism. Moreover, the absence of significant changes in the survival, and proliferative and migration behaviour of TRIB1 KD PC3 cells supports the possible role of TRIB1 in the onset and early development of PCa. The obtained results stimulate further research to disclose TRIB1's involvement in prostate tumorigenesis and its role as a biomarker or therapeutic target.

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Chapter VI:

6. Final summary and future perspectives

Worldwide, the morbidity and mortality rates associated with PCa remain prevalent despite many advances made in diagnosis and therapy. Continual improvement has been seen over the years due to several research contributions, however greater understanding of the unresolved molecular basis of PCa is required in the clinical detection, diagnostic approaches, and treatment options. Initially, androgens are the primary regulators of PCa cells, as it progresses a switch in characteristics occurs to an androgen-insensitive phenotype which is related to the lethality of the disease.

Studies presented in this thesis contributed to obtaining insight on the androgenic regulation of TRIB1 and TRIB3 in PCa cells, and how sex steroid hormones and the androgen receptor are involved. Also, TRIB1 influence phenotypic changes of normal prostate cells. Chapter III of the thesis focused on the characterization of TRIB1 and TRIB3 in normal prostate (PNT1A) and androgen sensitive (LNCaP, 22Rv1) and CPRC (PC3, DU145) cell line models. We established the varying basal protein expression levels which displayed a dependency on androgen sensitivity. LNCaP and 22Rv1 cells showed less TRIB1 and TRIB3 protein expression compared to the more aggressive CRPC DU145 and PC3 cells that exhibited high protein TRIB1 and TRIB3 expression levels. We also confirmed the suitability of the anti-TRIB1 antibody for protein analysis. These findings demonstrated that normal prostate cells and PCa cells have endogenous TRIB1 and TRIB3 expression, with levels changing due to loss of androgenic sensitivity. In Chapter V, we investigated androgen actions in controlling TRIB1 and TRIB3 expression in LNCaP and 22RV1 PCa cells. The effects of androgens (DHT) and anti-androgens (Bicalutamide) on TRIB1 and TRIB3 expression, as well as the impact on signalling pathways activation, cell proliferation, and migration were investigated. Neoplastic LNCaP and 22RV1 cells displayed a diminished TRIB1/TRIB3 protein expression after DHT treatment, which was underpinned by the downregulation of TRIB-associated targets such as ERK1/2 and pAKT, AKT. All three cell lines (PNT1A LNCaP, 22RV1) showed increased cell viability upon DHT stimulation. Bicalutamide blocked androgen effects in regulating TRIB1 expression. Moreover, its effectiveness was validated with a standard biomarker PSA. In addition, AR silencing enhanced TRIB1 expression in 22Rv1 cells, confirming the AR involvement. TRIB1 alteration demonstrated increased activity in the proliferation and migration of PNT1A cells. Future efforts focussing more on *in vivo* models could potentially reveal whether the presence or absence of TRIBs affects tumour growth in PCa progression. With this rationale we aimed to investigate tumour development in

transgenic mice with TRIB1 overexpression (Appendix I). For this purpose, as part of secondment studies at the University of Sheffield, mice were subjected to an intraprostatic injection of luciferase-tagged TRAMP-C1 cells (Fig. AI.1), and tumour left to grow for a period of 7-21 days. After that, bioluminescence imaging was used to measure tumour growth (Fig. AI.2). Tumour cells were visualised in the first few days via bioluminescence imaging (Fig. AI.2), however after 1 week tumour cells could no longer be detected and H&E staining (Fig. AI.3) did not show any cancer cells present. qPCR analysis of TRIB1 and TRIB3 mRNA expression in wild type and transgenic mice prostate tissue was also measured which showed increased expression of both TRIB1 and TRIB3 in Transgenic model (Fig. AI.4).

The complex nature of PCa involves other modifications such as genetic and metabolic alterations that trigger the neoplastic transformation and progression of disease to aggressive forms. Several studies showed that PCa cells have distinctive metabolic features compared with normal cells. The Warburg effect is a well-established and published feature of cancer cells which is the rewiring of metabolism that promotes cell proliferation, growth, and survival. Enhanced glucose uptake and fermentation of glucose to produce lactate even while oxygen is present have been continually observed in cancer cells. This phenomenon of increased glycolytic activity is known as one of the hallmarks that shapes the progression of cancer. The reprogramming of energy metabolism and androgen deprivation therapy resistance in PCa cells is generally related to more aggressive forms of PCa such as CPRC development. Moreover, it is related with increased rates of glycolytic metabolism. Thus, elucidating the metabolic alterations that underly early PCa development and the progression to CPRC would provide information of utmost relevance for the detection, treatment and disease prognosis.

In the context of metabolic regulation, our experiments showed lipid droplets accumulation in PNT1A cells overexpressing (OE) TRIB1, which indicates its involvement in lipid metabolism. These results reveal the AR, androgens and TRIBs unique regulatory role in PCa cells and an interesting perspective for the exploitation of targeting TRBs and the AR in future therapeutic studies. Chapter VI evaluated the effect of TRIB1 OE and TRIB1 KD in normal prostate epithelial cells and PCa cells. First, characterisation of TRIB1 OE and KD were performed in non-neoplastic PNT1A and neoplastic PC3 cell lines. Following that, the physiological cell fate was assessed

by measuring the cell viability, proliferation, migration, and metabolism. Results obtained in TRIB1 OE PNT1A cells showed a significant increase in proliferative activity and migration, with no apoptotic effects.

Interestingly, the disruption of lipid metabolism upon TRIB1 OE was paralleled by the upregulated FASN protein expression. Along with an upregulated FASN protein expression. Differences in physiological characteristics of non-neoplastic cells into a cancer-like phenotype after manipulating TRIB1 was observed, suggesting that TRIB1 plays an important part in PCa initiation, therefore further investigations are necessary to utilize TRIB1 as a therapeutic target of PCa.

The work presented in this thesis identified and inveterate the vital androgen actions in the regulation of TRIB1 and TRIB3 in PCa, highlighting the sex steroid hormone influence on targets genes and tumour microenvironment of PCa. Regarding the relationship between TRIBs and PCa, the findings of this thesis demonstrated that TRIBs could be involved in the initiation stages of PCa development. Meaning, TRIBs could be potentially utilized as inhibitors or biomarkers in future clinical treatment of early-stage carcinogenesis. Considering the outcome of increased protein expression of FASN in PNT1A after TRIB1 OE, TRIBs interaction with cellular metabolism would be an interesting future study. An exciting aspect to explore would be understanding the trio relationship of androgens, TRIBs and the metabolism as future treatment approaches. In conclusion, the study highlighted the significance of androgenic modulation of mainly TRIB1 and TRIB3 in PCa. The data presented also identified TRIB1 as a possible driver of physiological alterations of non-neoplastic cells to a more neoplastic phenotype. Altogether, these findings have built on the understanding and emphasized on the TRIBs oncogenic roles in PCa. Thus, more work is required to determine the molecular role and mechanism of action of TRIBs, to utilise them as therapeutic biomarker targets improving PCa outcome.

Appendix I

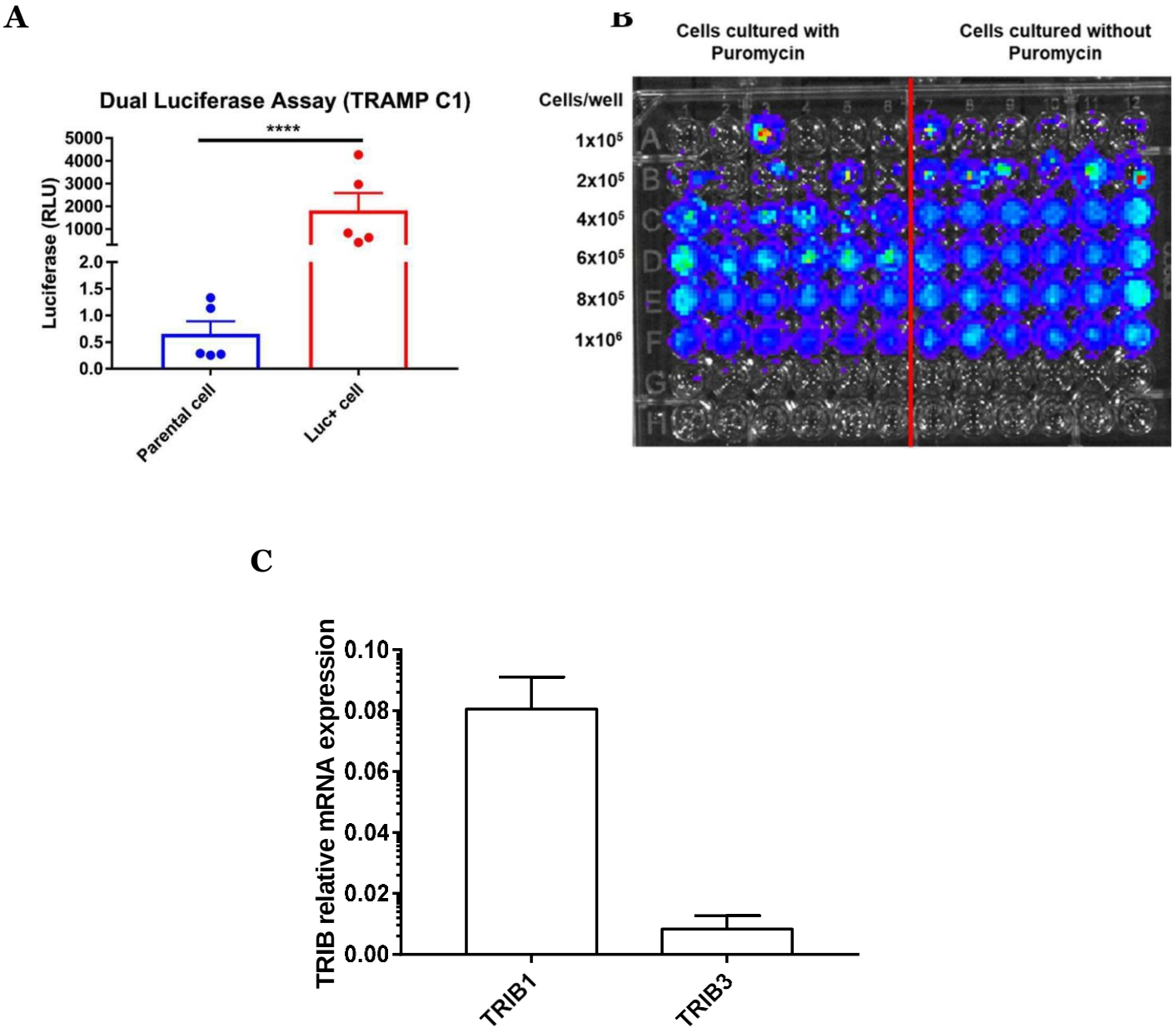

Figure AI.1. Luciferase assay, puromycin selection and TRIBs mRNA expression in TRAMP C1 cells. (A) Measure of luciferase expression in TRAMP C1 cells with luciferase reporter gene using the Dual luciferase assay. TRAMP-C1 cells were transfected with a luciferase reporter gene for 24 h, then cells were washed with PBS 1X twice and lysed using 35 ml of 1X Passive Lysis Buffer (Promega); 5 ml of lysate was transferred onto a Nunc 384-well white polystyrene microplate. The substrates of Firefly luciferase (LAR II, Promega) and Renilla luciferase (Stop & Glo, Promega) were added to the cell lysates in a ratio of 1:1. Luminescence was measured first at 560 nm for firefly luciferase and at 480 nm for Renilla luciferase using a microplate reader (Thermo Fisher Scientific). All the readings were normalized to the readings generated by parent cells to subtract the luminescence background and then Renilla/Firefly ratio was calculated. (B) TRAMP C1 cells cultured with or without puromycin to measure selection of cells expressing luciferase reporter gene. (C) TRIB1 and TRIB3 mRNA expression levels in TRAMP C1 cells, n=3.

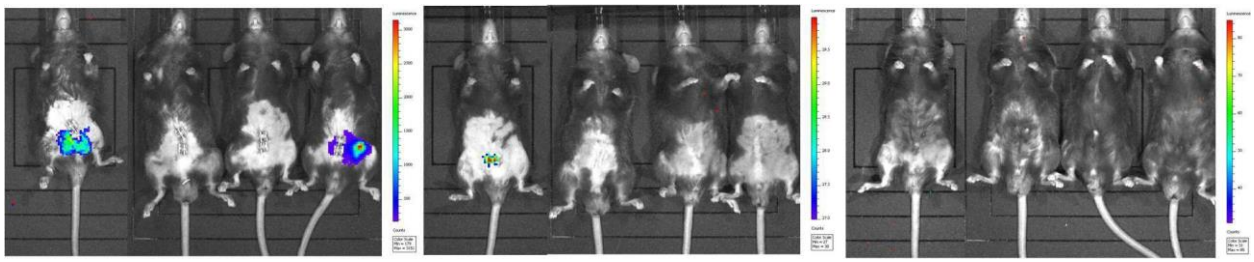

Figure AI.2. Bioluminescence imaging of transgenic mice after intraprostatic injection of TRAMP-C1 luciferase transfected cells. IVIS bioluminescence imaging of TRIB1 wild type (WT) and TRIB1 transgenic (Tg) mice after intraprostatic injection of the TRAMP-C1 cells into the myeloid-specific *Trib1* transgenic mice. Automated exposure was used to measure luminescence.

In vivo imaging was performed in TRIB1 Tg mice, which were subjected to an intraprostatic injection of luciferase tagged TRAMP-C1. Mice were anesthetized using ketamine and placed inside the camera box of the IVIS Spectrum imager. D-Luciferin was injected intra-peritoneally 5 min before imaging. Luminescence of the luciferin was obtained by automatic exposure. Representative images were taken over a period of 7-21 days.

Generation of myeloid specific Trib1 over-expressing transgenic mice was made by crossing Rosa26.Trib1 mice with Lyz2Cre recombinase transgenic mice (www.jax.org/strain/004781). Result; Rosa26.Trib1 x Lyz2Cre (Trib1mTg) over-expressing Trib1 transgene by ~2.5 fold [1]. Wild- type litter mates (Trib1mWT) were used as controls. Mice utilised were congenic on a C57BL/6J background. Housing was in a controlled environment with a 12-h light/dark cycle, at 22°C in OptiMICE rack individually ventilated cage system and provided with free access to H_2O and a standard chow diet (#2918; Harlan Teklad). Mice were sacrificed humanely by Schedule 1 method at 10–13 weeks of age. Mice handling was in accordance with UK legislation (1986) Animals (Scientific Procedures) Act. All mouse experiments were approved by the University of Sheffield Project Review Committee and carried out under a UK Home Office Project License (70/8670).

Prior to the day of implantation, TRAMP C1-Luciferase cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). On the day of implantation, cells were collected by 0.25% trypsin-EDTA, centrifuged down the cell pellet and washed with DMEM without FBS or P/S.Cells were counted and resuspend in PBS at a concentration of $2x10^7$ cells/mL (4×10^5 cells/20 µL). Equal volume of matrigel was added to create a 1:1 PBS/matrigel cell suspension at a final concentration of 4×10^5 cells/40 µL per mouse. The cell mix was kept on ice until injection to prevent solidification. Mice were anesthetized with isoflurane (5% for induction via chamber, 1-3% for maintenance via nose cone). Full induction was verified by the loss of toe pinch reflex. The abdomen was shaved and sterilized by three rounds of circular application of surgical scrub using sterile pads followed by sterile alcohol wipes and dried. A lower midline incision was made using a 29G gauge needle. Sutures were performed to close the inner abdominal musculature with absorbable monofilament violet 5-0 17mm 3/8 circle taper point needle PDS II. The outer abdominal skin was closed with sterile wound clippers. 0.1 mg/kg of the analgesic buprenorphine s.c. was administered immediately after surgery. The photon flux of the tumour was monitored by IVIS imaging system on ventral side, and then IVIS imaging is performed once a week until experimental endpoint. Imaging was

performed by intra-peritoneally injecting mice with D-luciferin (150mg luciferin/kg body weight).

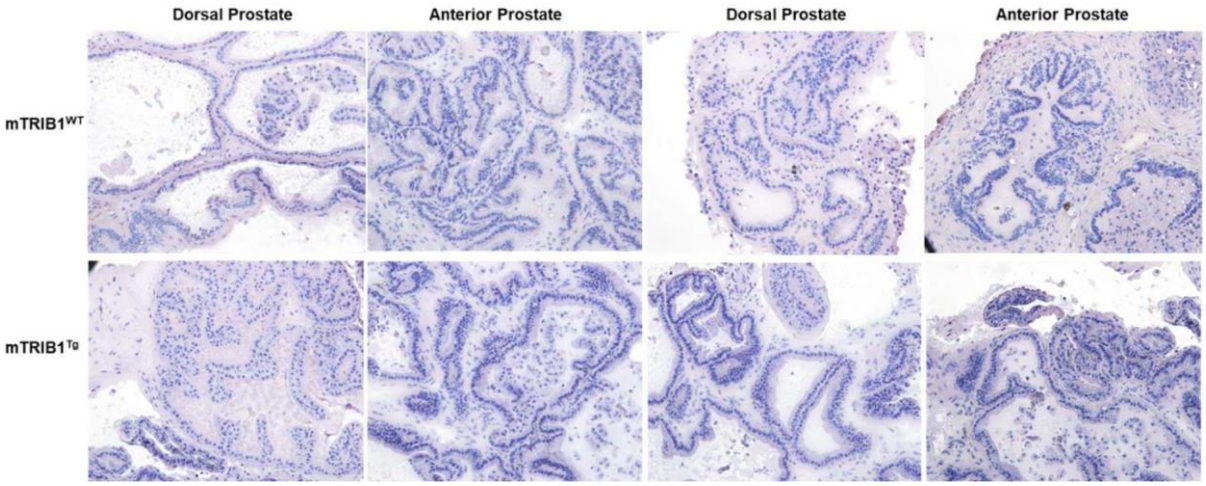

Figure AI.3. Haematoxylin and eosin (H&E) staining of wild type (WT) and transgenic (Tg) mice prostates.
Representative images of mice prostate sections with magnification x20 (WT, n=5 and Tg, n= 6). H&E staining was performed on TRIB1 WT and TRIB1 Tg mice. Frozen sections were cut to the thickness of 16 µm. Briefly, thawed frozen sections glass slides were fixed with 70% ethanol for 10 second, washed in deionized water, submerged in fresh MAYERS hematoxylin for 30 second, washed in water, immersed in Hematoxylin for 30 seconds, wash in 70% ethanol, submerged in eosin for 90 seconds, dehydrated sections were then washed twice 10 seconds in 95% ethanol and washed twice 10 seconds with 100% ethanol, and finally placed in xylene for 30 seconds.

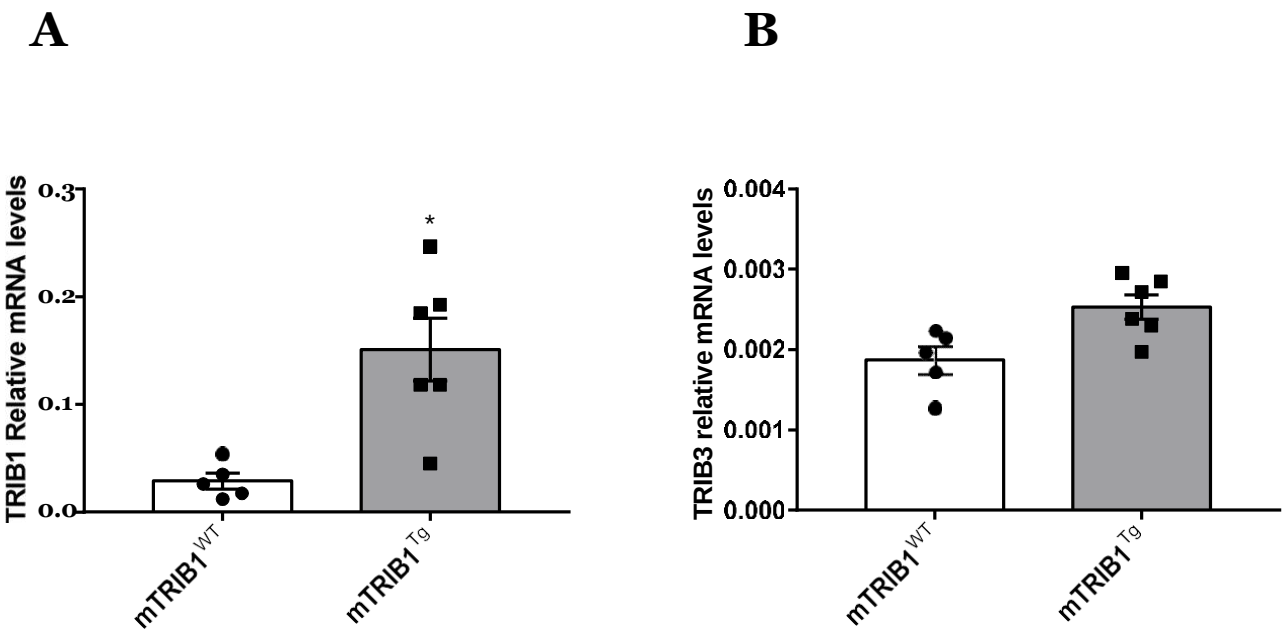

Figure AI.4. TRIB1 (A) and TRIB3 (B) mRNA expression in wild type (WT) and transgenic (Tg) mice prostate tissue.
Results were determined by qPCR analysis. Data are represented as mean ± S.E.M after normalization with β-actin housekeeping genes, *p <0.05 relative to WT control. (WT, n=5 and Tg, n= 6).

References

1. Johnston, J.M., et al., *Myeloid Tribbles 1 induces early atherosclerosis via enhanced foam cell expansion*. Science Advances, 2019. **5**(10): p. eaax9183.