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GERMLINE VARIANTS IN GENES INVOLVED IN THE CHROMOSOME SEGREGATION MACHINERY ASSOCIATED WITH INHERITED PROSTATE CANCER PREDISPOSITION

Maria Pedro Silva

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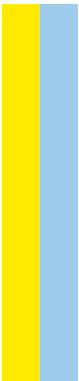


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D.ICBAS 2024

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Maria Pedro Silva



MARIA PEDRO PESSOA DE BARROS PEREIRA DA SILVA

**GERMLINE VARIANTS IN GENES INVOLVED IN THE CHROMOSOME
SEGREGATION MACHINERY ASSOCIATED WITH INHERITED PROSTATE CANCER
PREDISPOSITION**

Dissertation for applying to the degree of
Doctor of Philosophy in Pathology and
Molecular Genetics

Supervisor –Manuel R. Teixeira, MD, PhD

Category – Guest Full Professor

Affiliation – School of Medicine and Biomedical
Sciences (ICBAS), University of Porto, Portugal

Co-supervisor – Paula Cristina Martins dos
Santos Paulo, PhD

Category – Assistant Investigator

Affiliation – Portuguese Oncology Institute of
Porto (IPO Porto)

Co-supervisor – Helder Maiato, PhD

Category – Principal Investigator

Affiliation – Institute for Research and Innovation
in Health (i3S), University of Porto, Portugal



*"Work hard and never admire your own
science.
It can always be better".*

Jacob Hanna

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DECLARAÇÃO DE HONRA

Declaro que a presente tese é de minha autoria e não foi utilizada previamente noutro curso ou unidade curricular, desta ou de outra instituição. As referências a outros autores (afirmações, ideias, pensamentos) respeitam escrupulosamente as regras da atribuição, e encontram-se devidamente indicadas no texto e nas referências bibliográficas, de acordo com as normas de referência. Tenho consciência de que a prática de plágio e auto-plágio constitui um ilícito académico.

Maria Pedro Pessoa de Barros Pereira da Silva

TABLE OF CONTENTS

SUMMARY	13
RESUMO	15
PUBLICATIONS.....	17
LIST OF ABBREVIATIONS	19
INTRODUCTION	23
1. CANCER: A DOUBTLESS MAJOR HEALTH PROBLEM	23
1.1 THE EPIDEMIOLOGY OF PROSTATE CANCER (PRCA)	24
1.2 PRCA RISK FACTORS.....	26
2. PROSTATE CANCER SCREENING, DIAGNOSIS, STAGING AND TREATMENT	27
2.1 PROSTATE CANCER SCREENING	27
2.2 PROSTATE CANCER DIAGNOSIS AND GRADING	28
2.3 PROSTATE CANCER STAGING	29
2.4 PROSTATE CANCER TREATMENT	31
3. HEREDITARY PROSTATE CANCER (HPC)	32
3.1 THE <i>HOXB13</i> GENE	33
3.2 THE DDR GENES <i>BRCA1</i> AND <i>BRCA2</i>	34
3.3 THE MMR GENES	34
3.4 OTHER WELL-ESTABLISHED DDR GENES	35
3.5 <i>BRIP1</i> , <i>NBS1</i> , <i>RAD51C</i> AND <i>RAD51D</i>	35
4. CRITERIA FOR GENETIC COUNSELLING, GUIDELINES FOR GENETIC TESTING AND SURVEILLANCE.....	36
5. LANDSCAPE OF SOMATIC ALTERATIONS IN PROSTATE CANCER.....	37
6. CHROMOSOMAL INSTABILITY AND CANCER	37
7. MITOSIS AND FAITHFUL CHROMOSOME SEGREGATION	39
7.1 MITOSIS – OVERVIEW	39
7.2 MITOTIC SPINDLE ASSEMBLY	40
7.3 KINETOCHORE-MICROTUBULE ATTACHMENTS AND DYNAMICS	41
7.4 SPINDLE ASSEMBLY CHECKPOINT	42
7.5 <i>BUB1B</i>	44
7.6 <i>CEP57</i>	47
7.7 MOSAIC VARIEGATED ANEUPLOIDY 1 AND 2	48
AIMS.....	51
METHODOLOGY	53

CHAPTER I	55
CHAPTER II	101
GENERAL DISCUSSION	128
CONCLUSIONS	132
FUTURE PERSPECTIVES	134
REFERENCES	136

SUMMARY

BACKGROUND: Prostate cancer (PrCa) is the second most frequent cancer in men and the fifth leading cause of cancer-related deaths globally. Despite its high prevalence, the genetic factors underlying ~15% of PrCa cases linked to familial aggregation or early-onset disease remain largely unexplored. Current research accounts for only ~5% of these cases, leaving the majority without a definitive molecular diagnosis. Understanding the genetic basis of hereditary PrCa is crucial for improving prevention, prognosis and treatment, as it facilitates predictive testing and targeted screening for individuals with predisposing germline mutations. Additionally, it provides reassurance to non-carriers, confirming their population-level risk despite a family history of the disease.

AIMS: This PhD thesis sought to clarify the potential of heterozygous germline variants in genes involved in chromosome segregation, microtubule-kinetochore attachment and spindle dynamics to explain some of the missing heritability in hereditary/familial PrCa. We aimed to assess the prevalence of deleterious variants in the most promising candidate genes, evaluating potential founder effects, and clarify their biological role in triggering prostate carcinogenesis.

METHODOLOGY: To identify the most promising candidate genes, we integrated data from two next-generation sequencing (NGS) approaches: whole-exome sequencing (WES) and targeted panel sequencing. Our analysis focused on mutated genes that segregated with the disease in affected families and were involved in critical processes such as chromosome segregation, spindle dynamics, and microtubule-kinetochore (MT-KT) attachment. Initially, we prioritized genes with heterozygous germline variants and previously associated with autosomal recessive syndromes that are characterized by aneuploidy, evaluating their contribution to inherited PrCa predisposition within a cohort of 462 early-onset and/or familial PrCa patients. Subsequently, we focused on genes carrying deleterious variants segregating with PrCa in a WES discovery dataset of duos/trios from 45 families, and then validated the findings in the entire series of patients. To further investigate the functional role of one top candidate in PrCa carcinogenesis, we employed a CRISPR/Cas9-engineered cell model and conducted in vitro functional assays.

RESULTS AND DISCUSSION: We identified five novel PrCa predisposition genes, namely *BUB1B*, *CEP57*, *CEP290*, *EML4* and *TUBGCP5*, with deleterious variants observed in 5.1% of families presenting early-onset and/or familial disease. This represents the first report of pathogenic or likely pathogenic germline variants in these genes among PrCa patients, specifically in those with early-onset/familial disease. Our findings contribute significantly to the yet scarce understanding of the genetic basis underlying PrCa susceptibility. Validation in larger cohorts is necessary, but these results have the potential to inform genetic screening strategies and influence personalized treatment approaches.

RESUMO

INTRODUÇÃO: O cancro da próstata (CaP) é o segundo cancro mais frequente em homens e a quinta principal causa de morte por cancro em todo o mundo. Apesar da sua alta prevalência, os fatores genéticos subjacentes a aproximadamente 15% dos casos de CaP relacionados com agregação familiar ou doenças de início precoce permanecem largamente inexplorados. A investigação atual explica apenas cerca de 5% desses casos, deixando a maioria dos doentes sem um diagnóstico molecular definitivo. Compreender a base genética do CaP hereditário é crucial para melhorar a prevenção, melhorar o prognóstico e o tratamento, pois facilita o teste preditivo e o rastreio direcionado para indivíduos com variantes germinativas predisponentes. Além disso, oferece segurança para os não portadores, confirmando o risco populacional, apesar da sua história familiar da doença.

OBJETIVOS: Esta tese de doutoramento teve como objetivo esclarecer o potencial das variantes germinativas heterozigóticas em genes envolvidos na segregação cromossômica, ligação microtúbulo-cinetocoro e dinâmica do fuso mitótico para explicar parte da hereditariedade não resolvida no CaP hereditário/familiar. Pretendemos avaliar a prevalência de variantes deletérias nos genes candidatos mais promissores, avaliando os potenciais efeitos fundadores, e clarificar o seu papel biológico no desencadeamento da carcinogénese prostática.

METODOLOGIA: Para identificar os genes candidatos mais promissores, integrámos dados de duas abordagens de sequenciação de nova geração (NGS): sequenciação do exoma completo (WES) e sequenciação de painel direcionado de genes. A nossa análise focou-se em genes mutados que segregavam com a doença nas famílias afetadas e que estavam envolvidos em processos críticos, como a segregação cromossômica, a dinâmica do fuso mitótico e a ligação microtúbulo-cinetocoro (MT-KT). Inicialmente, priorizámos genes, com variantes germinativas heterozigóticas e previamente associados a síndromes autossômicas recessivas, que são caracterizadas por aneuploidias, avaliando a sua contribuição para a predisposição hereditária para CaP numa série de 462 doentes com PrCa de início precoce e/ou familiar. Em seguida, focámo-nos em genes com variantes deletérias segregando com a doença num conjunto de dados de exomas de duos/trios de 45 famílias (série de descoberta), validando posteriormente os resultados em toda a série de doentes. Para investigar mais a fundo o papel funcional de um dos principais candidatos

na carcinogénese do CaP, utilizámos um modelo celular através da engenharia de edição do genoma *CRISPR/Cas9* e realizámos ensaios funcionais *in vitro*.

RESULTADOS E DISCUSSÃO: Identificámos cinco novos genes predisponentes para CaP, nomeadamente: o *BUB1B*, a *CEP57*, a *CEP290*, o *EML4*, e o *TUBGCP5*, com variantes deletérias observadas em 5.1% das famílias com doença de início precoce e/ou familiar. Este é o primeiro relato de variantes germinativas patogénicas ou provavelmente patogénicas nestes genes em doentes com CaP, especificamente nos casos de início precoce/familiar. Os nossos resultados contribuem significativamente para o entendimento, ainda limitado, da base genética da suscetibilidade para CaP. A validação em coortes maiores é necessária, mas estes resultados têm o potencial de orientar estratégias de rastreio genético e influenciar abordagens de tratamento personalizado.

PUBLICATIONS

According to “Artigo nº 34, do Decreto-Lei nº 115/2013”, results contained in the following published works were used for this thesis:

PAPER I

***BUB1B* monoallelic germline variants contribute to prostate cancer predisposition by triggering chromosomal instability**

Maria P. Silva[†], Luísa T. Ferreira[†], Natércia F. Brás, Lurdes Torres, Andreia Brandão, Manuela Pinheiro, Marta Cardoso, Adriana Resende, Joana Vieira, Carlos Palmeira, Gabriela Martins, Miguel Silva, Carla Pinto, Ana Peixoto, João Silva, Rui Henrique, Sofia Maia, Helder Maiato, Manuel R. Teixeira[†] & Paula Paulo[†]. *Journal of Biomedical Science* (2024) 31:74

[†] Contributed equally/Joint-first authors.

PAPER II

Deleterious germline variants in mitotic spindle formation genes in early-onset/familial prostate cancer patients

Maria P. Silva, Andreia Brandão, Marta Cardoso, Sofia Maia, Manuel R. Teixeira, Paula Paulo. *In preparation*

LIST OF ABBREVIATIONS

ACMG	American College of Medical Genetics and Genomics
ACS	American Cancer Society
ADT	Androgen-deprivation therapy
AJCC	American Joint Committee on Cancer
APC/C	E3 ubiquitin ligase anaphase-promoting complex/cyclosome
AR	Androgen receptor gene
AS	Active surveillance
ASR	Age-standardized rate
ATM	Ataxia Telangiectasia Mutated gene
B3BD	Bub3-binding domain
BER	Base excision repair
<i>BRCA1</i>	Breast cancer gene 1
<i>BRCA2</i>	Breast cancer gene 2
<i>BRIP1</i>	<i>BRCA1</i> - interacting Protein C-terminal helicase 1 gene
<i>Bub1</i>	Budding uninhibited by benzimidazoles 1 Homolog gene
<i>BUB1B</i>	Budding uninhibited by benzimidazoles 1 Homolog Beta gene
<i>Bub3</i>	Budding uninhibited by benzimidazoles 3 Homolog gene
BubR1	Budding uninhibited by benzimidazoles 1 Related-1
Cas9	CRISPR associated protein 9
CRC	Colorectal cancer
Cdc20	Cell division cycle 20 gene
cdk1	cyclin dependent kinase 1 gene
<i>CEP</i>	Centrosomal protein
<i>CEP57</i>	Centrosomal protein, 57 KDa gene
<i>CEP290</i>	Centrosomal protein, 290 KDa gene
CIN	Chromosomal instability
<i>CHEK2</i>	Checkpoint Kinase 2 gene
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DDR	DNA damage repair
DRE	Digital rectal examination
DSB	Double-strand break
EAU	European Association of Urology
EMA	European Medicines Agency
<i>EML4</i>	EMAP Like 4 gene
EPCAM	Epithelial cell adhesion molecule gene

LIST OF ABBREVIATIONS

<i>ERG</i>	v-ets erythroblastosis virus E26 oncogene homolog gene
ETS	Erythroblastosis virus E26 transformation-specific
FDA	Food and Drug Administration
FH	Family history
FRR	Familial relative risk
GGG	Gleason grade group
GS	Gleason score
GWAS	Genome-wide association studies
HE	Hematoxinilin and eosin
<i>HOXB13</i>	Homeobox B13 gene
HPC	Hereditary Prostate Cancer
HR	Hazard ratio/ Homologous recombination
Kn11	kinetochore scaffold 1
KT	Kinetochore
LS	Lynch syndrome
Mad1	Mitotic arrest-deficient 1like 1
Mad2	Mitotic arrest-deficient 2 like 1
MAP	Microtubule-associated protein
MCC	Mitotic checkpoint complex
<i>MLH1</i>	mutL homologue 1 gene
mCRPC	Metastatic castration-resistant PrCa
MIS12	MIS12 Kinetochore Complex Component
MMR	Mismatch repair
Mps1	Monopolar spindle 1
<i>MSH2</i>	mutS homologue 2 gene
<i>MSH6</i>	mutS homologue 6 gene
MSI-H	Microsatellite instability-high
MT	Microtubule
MVA	Mosaic variegated aneuploidy syndrome
<i>MYC</i>	Myc myelocytomatosis viral oncogene homolog gene
<i>NBS1</i>	Nijmegen Breakage Syndrome 1 gene
<i>NBN</i>	Nijmegen breakage syndrome 1 (nibrin) gene
NCCN	National Comprehensive Cancer Network Guidelines
Ndc80	NDC80 Kinetochore Complex Component
NHEJ	Non-homologous end joining
Nuf2	NUF2 Component Of NDC80 Kinetochore Complex
OR	Odds ratio

<i>PALB2</i>	The Partner and Localizer of BRCA2 gene
PARP	Poly adenosine diphosphate-(ADP) ribose polymerase
PARPi	Poly adenosine diphosphate-(ADP) ribose polymerase inhibitor
<i>PCA3</i>	Prostate Cancer Associated 3 gene
PCS	Premature chromatide separation
PD-1	Programmed cell death protein-1
PD-L1	Ligand programmed death ligand-1
PMS2	Postmeiotic segregation increased 2
PrCa	Prostate Cancer
PSA	Serum Prostate Antigen
<i>PTEN</i>	Phosphatase and tensin homolog gene
<i>RAD51C</i>	RAD51 Paralog C gene
<i>RAD51D</i>	RAD51 Paralog D gene
RR	Relative risk
RT	Radiation therapy
SAC	Spindle assembly checkpoint
SNP	Single-nucleotide polymorphisms
Spc24	SPC24 Component Of NDC80 Kinetochore Complex
Spc25	SPC25 Component Of NDC80 Kinetochore Complex
<i>SPDEF</i>	SAM Pointed Domain Containing ETS Transcription Factor gene
<i>SPOP</i>	Speckle type BTB/POZ protein gene
<i>TMPRSS2</i>	Transmembrane protease serine II gene
TNM	Tumor-Node-Metastasis
TPR	Tetratricopeptide domain
<i>TP53</i>	Tumor protein p53 gene
TSG	Tumor supressor gene
<i>TUBGCP5</i>	Tubulin Gamma Complex Component 5 gene
UICC	International Union for Cancer Control
USPSTF	US Preventive Task Force
VUS	Variants of unknown significance
WHO	World of Health Organization

INTRODUCTION

1. Cancer: A Doubtless Major Health Problem

Cancer is an alarming public health and economic issue as it is one of the main leading causes of death worldwide. According to the World of Health Organization (WHO) for the year 2019, cancer is the most prevalent leading cause of death, even when compared with the high mortality rates of stroke and ischemic heart diseases [1] [2]. An estimated 20.0 million new cancer cases and 9.7 million deaths were related with cancer in 2022 [3] and, due to the increase in the population life expectancy, 30.2 million cases are estimated by 2040 [4].

Regardless of the diverse risk factors associated with cancer [5], [6], [7], [8], it is well established that it arises from molecular alterations in genes involved in cell survival, growth, proliferation, and differentiation. These genetic alterations may lead to changes in the cell division process, in which cells are able to escape from the mechanisms that regulate cell cycle, forming their own ecosystem (tumor) that can interfere with organ physiology, ultimately resulting in organ failure and, if not treated, death [9]. Cancer can be treated by general cell death-inducing agents, which are commonly accompanied with severe side effects, reducing the patients' quality of life [10]. Since cancer is an heterogenous genetic disease, even in patients that share the same type of cancer [11], targeted or personalized therapies have emerged in the last years as an alternative cancer treatment, which interfere with the dysregulated molecular pathways caused by the genetic alterations [12]. Currently, the genetic characterization of tumors necessary to obtain information for a proper therapy selection and prognosis prediction is based on tissue biopsies of the primary tumor or metastases. However, tumors are a heterogeneous agglomeration of cell subpopulations, each carrying a distinct set of molecular alterations. Indeed, different metastatic lesions at different locations in the body can have a different genetic profile [13]. This may result in therapy resistance as only a fraction of the tumor or metastatic sites will be targeted, while other subpopulations that harbour other mutations can thrive and promote cancer progression [14].

To cope with this intratumor and intertumor heterogeneity, the scientific and medical communities are constantly debating the power of surrogate biomarkers, which potentially have a broader impact in reducing the cancer burden, since they can improve cancer detection at early stages and identify individuals at higher risk to develop the disease, thereby improving patient's survival.

1.1 The epidemiology of Prostate Cancer (PrCa)

PrCa represents a significant challenge to the clinical community worldwide, as it ranks as the second most common cancer (14.2%; after lung cancer) and the fifth deadliest cancer (7.3%) in men worldwide [3][15].

PrCa incident rates are three-fold higher in developed countries compared with developing countries (35.5 and 12.6 per 100,000 individuals, respectively). The age-standardized rate (ASR) is highest in Northern and Western Europe, the Caribbean, Australia/New Zealand, Northern America, and Southern Africa. Moreover, the cases are increasing in Japan and Singapore where, historically, this cancer had a low incidence rate [16]. PrCa remains relatively rare in Asia and Northern Africa. The mortality rates are less variable though (7.3 and 6.6 per 100,000 individuals, developed *versus* developing countries, respectively), with higher rates in the Caribbean, southern, middle and western Africa (Figure 1).

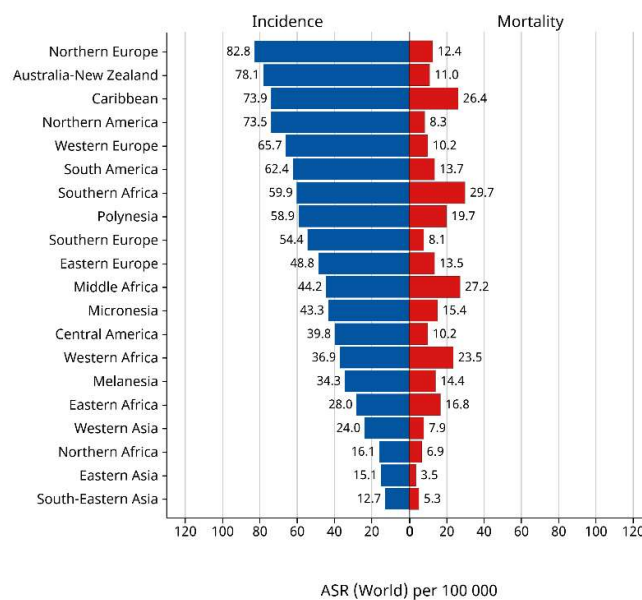
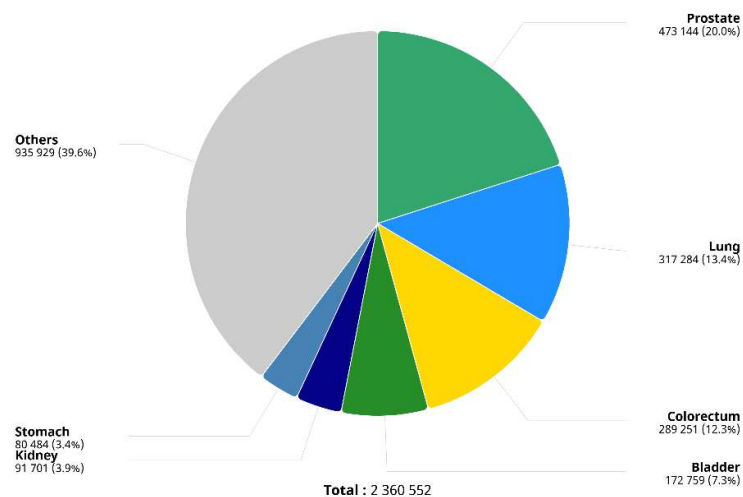


Figure 1: Region-specific incidence and mortality. Age-standardized PrCa incidence and mortality rates by world area (W). Rates are shown in descending order of the world. Source: GLOBOCAN 2022 <https://gco.iarc.fr> [Accessed on 11 July 2025].

In Europe, PrCa is the most frequently diagnosed cancer in men, accounting for 20.0% of all cancers in 2022 (Figure 2A), and the fourth leading cause of cancer-related mortality by cancer site (10.5%), after lung (23.0%), colorectal (12.2%) cancers [16]. In Portugal, PrCa accounts for 20% of all cancers among men (Figure 2B) and is the fourth leading cause of cancer-related deaths (10.7%) [3] [16].

A)



B)

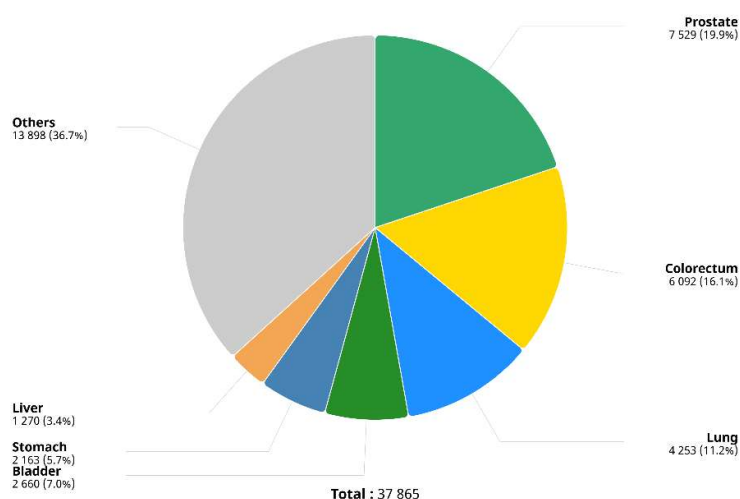


Figure 2: Estimated cancer incident in Europe (A) and Portugal (B) (number of newly diagnosed cases and proportion of each cancer comparing with all types of cancer). Prostate cancer is the first most common cancer in both Europe and Portugal. SOURCE GLOBOCAN, 2022. [Accessed on 11 July 2025].

The high PrCa incidence rates can be partially attributed to the use of Serum Prostate Antigen (PSA) testing for PrCa screening. PSA has a propensity to detect a large amount of pre-clinical cancers, many of which may be clinically insignificant, and is poor at discriminating between such men and those harbouring lethal disease who would certainly benefit from a more precise screening and treatment [17]. Indeed, recent

data estimates that around 20-40% of the PrCa cases suffer from overdiagnosis through extensive PSA testing [18], [19].

1.2 PrCa risk factors

Despite the substantial health burden due to PrCa, its etiology remains quite baffling as consistent evidence is available for only a few risk factors such as, aging, African ancestry, and family history (FH) of the disease [15], [20], [21].

Age is a key risk factor for PrCa as incidence and mortality rates are strongly associated with aging, with an average age at diagnosis of 66 years [22]. These rates vary widely across regions and populations. Epidemiological data have shown that African-Americans, Caribbeans, and black men living in Europe have the highest incidence of PrCa worldwide and are more prone to develop PrCa earlier in life when compared to other racial and ethnic groups, such as those of Asian ancestry [23], [24], [25]. Although PrCa incidence rates in Africa are low, the individuals of African ancestry might share a common genetic background more prone to the development of high-risk disease [26]. In contrast to the overdiagnosis through massive PSA testing in developed countries, the low incidence rates in Africa may be attributed to underdiagnosis, differences in screening protocols [27] as well as disparities in healthcare access [28].

Heritability, as in most cancers, is another important risk factor for PrCa. PrCa is a polygenic disease with a large amount of heritability whereby men have a different risk of developing the malignancy [29]. Men with a brother or father with PrCa have at least a two-fold increased risk of developing the disease compared to men without family history of the disease. The risk further increases three- to five-fold if the affected first-degree relative had early-onset PrCa (≤ 55 years) [30]. Similarly, first-degree relatives of a patient who died from PrCa have a two-fold increased risk of death from the disease compared with men diagnosed with PrCa without a PrCa family history [22]. Although to a lesser extent and, yet, not consistently shown in the literature, the diet (specially meat consumption) and other lifestyle factors, including sedentarism and smoking, have also been proposed to contribute to the makeup of this disease [5], [6], [7], [8].

Among all the risk factors that are associated with increased risk of developing PrCa, the most important are genetic factors. In fact, about 5-15% of the PrCa cases have been related with a hereditary component due to familial aggregation of the disease. However, the underlying genetic causes are still largely unknown. Pathogenic germline variants in genes that are consistently associated to PrCa susceptibility include the Lynch syndrome (LS) genes *MLH1*, *MSH2*, *MSH6*, and *PMS2*, involved in mismatch repair, and the Hereditary Breast and Ovarian Cancer (HBOC) genes *BRCA1*, *BRCA2*,

ATM, *PALB2*, and *CHEK2*, involved in homologous recombination [31]. Hereditary PrCa (HPC) will be discussed in more detail in Chapter 3.

2. Prostate cancer screening, diagnosis, staging and treatment

2.1 Prostate cancer screening

Localized prostate cancer is asymptomatic and when symptoms arise the disease is commonly in an advanced stage. Therefore, screening has particular importance, as it provides an opportunity to diagnose the disease at a curable stage. Screening through digital rectal examination (DRE) and prostate-specific antigen (PSA) testing are the most common strategies for PrCa screening.

PSA remains the most widely used biomarker to detect PrCa early, despite not being a cancer-specific marker. As most individuals with elevated PSA levels do not present PrCa, PSA has turned out to be an organ-specific biomarker [32]. PSA is a serine protease produced by epithelial cells lining the prostatic ducts and is secreted directly into the prostatic ductal system in concentrations million times higher than in plasma. In PrCa patients, damage occurring in the secretory pathways leads to PSA leaking into the extracellular space [32]. Consequently, increased PSA level in serum can be observed as a sign of either inflammatory or neoplastic processes in the prostate [32]. The risk of PrCa is higher with an increased PSA level, but there is no level of PSA below which the risk of PrCa can be discarded. Despite the cut-off value of PSA levels not being uniform across ethnicities, in western countries the widely accepted threshold value of PSA is 4.0ng/mL. PSA levels above this cut-off value should indicate that additional studies are needed, including prostatic biopsy, to confirm or discard the presence of cancer [32]. The US Preventive Task Force (USPSTF) released updated recommendations in 2018 that suggest no PSA-based PrCa screening in individuals aged >70 years, and informed decision-making regarding PrCa screening in individuals aged 55 to 69 years. Specifically, for men in this younger age group, clinicians should inform about the potential harms and the benefits of the PSA screening. The USPSTF does not provide guidance for individuals younger than 55 years [33]. Instead, the National Comprehensive Cancer Network (NCCN) guidelines recommend two-to-four-year intervals for men aged 45-74 years with PSA levels <1ng/mL, and one-to-two-year intervals for those with PSA of 1-3ng/mL [17]. Notwithstanding the widely use of the PSA test for PrCa screening, it can provide false positive data leading to unnecessary painful prostate biopsies and diagnosis of low-risk PrCa. This can ultimately lead to overtreatment of early-detected PrCa that otherwise would not threaten life expectancy,

resulting in unnecessary side-effects that impair patients' quality of life and increase health care expenditures.

Prostate digital palpation through the rectum has long been an important clinical tool for the detection of PrCa. However, DRE suspicious for malignancy is not particularly sensitive, nor specific, for a diagnosis of carcinoma. DRE has a lower positive predictive value for detection of PrCa when compared to PSA test, and misses one-quarter to one-half of the PrCa cases diagnosed in the prostate biopsy as a consequence of serum PSA elevation [34]. These observations suggest that DRE may not contribute to significantly reduce mortality, but instead may result in a high number of false positives leading to unnecessary invasive diagnostic tests.

2.2 Prostate cancer diagnosis and grading

When PrCa is suspected based on the results of PSA screening test or symptoms, the definitive diagnosis requires a tumor biopsy for histopathologic evaluation. Transrectal ultrasound-guided biopsy has been the standard diagnostic test for PCa, to obtain 10 to 12 tissue samples in a grid-like pattern [35], [36]. These samples are then analyzed by a pathologist for the establishment of a histopathological diagnosis through light microscopic examination of hematoxylin and eosin (HE) stained tissue samples. Additionally, the pathologist will assign one Gleason score to both the most predominant and the second most predominant dedifferentiation cells' pattern in a tumor biopsy. The final Gleason Score (GS) is determined based on the sum of the two Gleason score patterns. These patterns are then assigned into a Gleason Grade from 1 to 5 (Table 1). The Gleason scoring system is the most frequently used grading system and is one of the strongest predictor systems for determining PrCa prognosis and for clinical decision-making [37]. GS ≤ 6 represents the best overall prognosis (grade group 1). A GS of 7 includes both a Gleason 3+4 (grade group 2) and a Gleason 4+3 (grade group 3), with the latter being associated with a significantly worse prognosis. GS 8 tumors comprise grade group 4, and GS 9 or 10 correspond to the worst prognostic group (grade group 5) [38].

Table 1: Grade group by corresponding Gleason score.

Grade group	Gleason score
1	≤ 6 (3+3)
2	7 (3+4)
3	7 (4+3)

4	8 (4+4; 3+5; 5+3)
5	9, 10 (4+5, 5+4, 5+5)

As PrCa is a heterogeneous disease, ranging from small, indolent, low-grade tumors to large and aggressive tumors, the primary goal after establishing the diagnosis of PrCa is to evaluate local and distant disease extension by staging the disease, to assess its aggressiveness [35].

2.3 Prostate cancer staging

Staging is the grouping of diseases into broad categories based on the extent of the disease, being helpful for the prediction of prognosis, as well as to delineate the more appropriate therapeutic strategy. Therefore, the most widely used staging system for PrCa is the Tumor-Node-Metastasis (TNM) proposed by the American Joint Committee on Cancer (AJCC) and the International Union for Cancer Control (UICC) (Table 2). This system considers the extent of the primary tumor (T), the involvement of regional lymph node (N) and the presence or absence of metastasis at distant sites of the body (M). Stage could also be reported based on the clinical evidence obtained before the treatment, by using minimally invasive methodologies (clinical stage), or through pathological findings after surgical removal of the prostate gland (pathological stage). The latter could estimate the prognosis [39]. It is relevant to distinguish intracapsular tumors (stages T1, T2) from extracapsular tumors (T3a, T3b), because it has a strong impact in the therapeutic decision, since only organ confined tumors have a potentially curative treatment [40].

Table 2: Overview of the TNM / pTNM staging system for PCa (adapted from [39]).

Primary Tumor (T)	
Tx	Primary tumor cannot be assessed
T0	No evidence of primary tumor
T1	Clinically inapparent tumor not palpable or visible by imaging
T1a	Tumor incidental histologic finding in 5% or less of tissue resected
T1b	Tumor incidental histologic finding in more than 5% of tissue resected
T1c	Tumor identified by needle biopsy (e.g. because of elevated PSA)
T2	Tumor confined within prostate
T2a	Tumor involves one half of one lobe or less
T2b	Tumor involves more than half of one lobe, but not both lobes

T2c	Tumor involves both lobes
T3	Tumor extends beyond the prostate
T3a	Extracapsular extension (unilateral or bilateral)
T3b	Tumor invades seminal vesicle(s)
T4	Tumor is fixed or invades adjacent structures other than seminal vesicles such as external sphincter, rectum bladder, levator muscles, and/or pelvic wall

Pathological (pT)*

pT2	Organ confined
pT2a	Unilateral, involving one-half of one lobe or less
pT2b	Unilateral, involving more than one-half of one lobe but not both lobes
pT2c	Bilateral disease
pT3	Extraprostatic extension
pT3a	Extraprostatic extension
pT3b	Seminal vesicle invasion
pT4	Invasion of bladder, rectum

Regional Lymph nodes (N)

Nx	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Regional lymph node metastasis

Pathologic (pN)

pNx	Regional nodes not sampled
pN0	No positive regional nodes
pN1	Metastases in regional node(s)

Distant metastasis (M)

M0	No distant metastasis
M1	Distant metastasis
M1a	Non-regional lymph node(s)
M1b	Bone(s)
M1c	Other site(s) with or without bone disease

**There is no pathologic T1 classification*

2.4 Prostate cancer treatment

The treatment options for PrCa depend on the risk category into which disease was classified. For a patient with clinically low-risk PrCa, and for some patients with intermediate-risk disease, clinicians frequently decide for active surveillance (AS) [41]. Low-intermediate-risk patients are treated with minimally invasive ablative therapies, such as radiation therapy (RT) or radical prostatectomy, with curative intent [42]. Patients with localized or metastatic high-risk disease are typically treated with RT combined or not with hormonal therapy, while patients with recurrent or metastatic, castrate-resistant disease can be treated with chemotherapy [43], [44], [45], [46].

The potential of targeted therapies based on leveraging the patient's immune system to treat PrCa is currently being actively investigated. FDA has recently approved the PD-1 inhibitor pembrolizumab for the treatment of unresectable or metastatic microsatellite instability-high (MSI-H)/MMR-deficient solid tumors, including PrCa [47]. Likewise, current NCCN guidelines support the use of pembrolizumab in patients with MSI-H or MMR-deficient metastatic castration resistant PrCa (mCRPC), whose disease has progressed through at least one line of systemic therapy [48]. Indeed, it would be of a great value the development of a biomarker panel that predicts the benefit and response of checkpoint inhibitors [47], particularly for metastatic PrCa patients that are no longer responding to classic therapies.

Defects in DNA damage repair (DDR) genes are common in PrCa and these alterations provided an opportunity for precision oncology approaches considering the approval of Poly (ADP-ribose) polymerase inhibitor (PARPi). PARP is an enzyme that plays a crucial role in the repair of DNA single-strand breaks. The inhibition of this enzyme leads to the accumulation of single-strand breaks, as well as double-strand breaks (DSBs) which are normally repaired by homologous recombination (HR). When there is a deficiency in the HR repair system, the accumulation of unrepaired DSB leads to cell death [49]. This phenomenon is called “synthetic lethality”, being the mechanism underlying the efficacy of PARPi occurring in cells with deficiency in HR repair [50].

In May 2020, the Food and Drug Administration (FDA) approved the PARPi Olaparib for patients with mCRPC carrying pathogenic germline or somatic variants in HR genes, namely *BRCA1*, *BRCA2*, *ATM*, *BARD1*, *BRIP1*, *CDK12*, *CHEK1*, *CHEK2*, *FANCL*, *PALB2*, *RAD51B*, *RAD51C*, *RAD51D*, and *RAD54L*. In the same year, the European Medicines Agency (EMA) approved Olaparib for mCRPC patients carrying somatic or germline variants in *BRCA1* or *BRCA2* only [51].

3. Hereditary prostate cancer (HPC)

As previously mentioned, the proportion of PrCa expected to occur in a hereditary/familial context has been estimated to be of 5–15% [52]. HPC follows a Mendelian pattern of inheritance (typically autosomal dominant), thought to result from the inheritance of a single highly penetrant gene variant in each family [53] [54]. HPC, predicted to account for 3-5% of all PrCa cases, is formally used to describe families with a strong history of PrCa, specifically, families fulfilling the Johns Hopkins criteria, which include: 1) three or more first-degree relatives diagnosed with PrCa, 2) three successive generations with the disease, or 3) at least two relatives diagnosed with early-onset PrCa (<55 years) [55]. On the other hand, the term familial PrCa is a more inclusive concept, which is used to define familial cluster of the disease that does not entirely fulfil the Johns Hopkins HPC criteria [56].

Albeit 43% of the patients diagnosed before the age of 56 may carry a high-risk allele for HPC [55], the identification of the genes involved in inherited predisposition has been a challenge when compared with other hereditary cancers.

The simultaneous analysis of multiple susceptibility genes has led to the identification of rare variants in several genes of moderate- to high-penetrance in hereditary cancer syndromes [57], [58]. In fact, most of the genes contributing to an inherited predisposition to PrCa were identified by the observed occurrence of PrCa cases in families with known hereditary cancer syndromes, namely hereditary breast and ovarian cancer (HBOC) and Lynch syndrome (LS) [57], [58], [59], [60].

To date, the genes more consistently associated with HPC susceptibility and reported by the NCCN Guidelines for genetic testing include HBOC-associated genes (*BRCA1* and *BRCA2*, *CHEK2*, *ATM*, and *PALB2*) and LS-associated genes (*MLH1*, *MSH2*, *MSH6*, and *PMS2*). Additionally, *HOXB13*, *BRIP1*, and *NBS1* are also recommended in specific populations [17]. Overall, in 0.6 to 6.25% of cases, hereditary predisposition to PrCa is due to *HOXB13* variants [60], in 1.2 to 5.3% to *BRCA2* variants, in 1.8 to 2.8% to *CHEK2* variants, in 1.6 to 2.7% to *ATM* variants, in 0.7 to 1.74% to MMR variants, in 0.9 to 1.25% to *BRCA1* variants, in 0.4 to 0.5% to *PALB2* variants, in 0.1 to 0.2% to *BRIP1* and *NBS1* variants [27], [61], [62], [63], [64]. The remaining cases are attributed to other, yet unidentified, genetic factors (Figure 3).

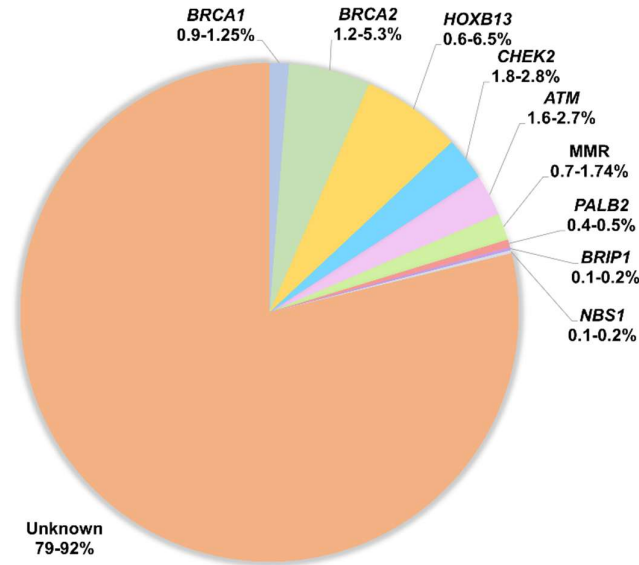


Figure 3: Frequency of reported deleterious variants in the established and potentially actionable genes predisposing to HPC. About 7-21% represent variant frequencies known so far, while 79-92% encompass the uncharted genes. Adapted from [31].

3.1 The *HOXB13* gene

HOXB13 encodes a homeobox transcription factor that plays a critical role in the regulation of cellular growth and differentiation during normal development of the prostate gland [65]. Variants in this gene are responsible for early-onset PrCa cases [66]. By studying a sample of 5,083 unrelated European subjects who were diagnosed with PrCa, Ewing et al. found that the carrier rate of the *HOXB13* G84E variant was increased by ~20-fold compared with that of 1,401 control subjects. This variant was significantly more common in men diagnosed at younger age (<55 years) and with a positive FH, than those without a FH and diagnosed after 55 years of age [67]. Additionally, a study conducted by our group, which focused on 462 early-onset/familial PrCa cases, showed that the *HOXB13* G84E variant is rare (if present) in the Portuguese population, but other *HOXB13* variants, such as A128D, F240L, and G135E (the latter present in the Chinese population [68]) may play a role in PrCa predisposition [69], [70]. Altogether, these reports show that *HOXB13* is the most replicated and the single PrCa-specific susceptibility gene [71]. Interestingly, the *HOXB13* G84E variant has been observed almost exclusively in men of European ancestry, suggesting a possible founder effect [72].

3.2 The DDR genes *BRCA1* and *BRCA2*

It is well known that *BRCA* genes are considered the major biomarkers for the genetic study of HBOC syndrome, an inherited disorder that predisposes to a substantial lifetime risk of developing breast and ovarian cancers [73]. Among the germline variants, *BRCA1* and *BRCA2* tumor suppressor genes (TSGs) are inherited in an autosomal dominant manner with incomplete penetrance [74], [75]. The encoded proteins of *BRCA1* and *BRCA2* genes hold a crucial role in DNA damage repair (DDR) of DSBs through the HR repair pathway, cell cycle checkpoint control and maintenance of genomic stability [76]. Germline alterations in these genes have been associated with an increased risk of other malignancies, including PrCa, pancreatic cancer, and melanoma [27], [62], [77], [78]. Specifically, in PrCa, the germline *BRCA1* variants increase the RR to develop PrCa by 1.8- to 3.8-fold in men aged <55 years [79], [80]. Although variants in both genes have been associated with a more aggressive phenotype linked with a poor disease prognosis, alterations in *BRCA2* are more well-established in PrCa predisposition. Pathogenic variants in *BRCA2* may represent about 5% of the familial clustering of PrCa and confer an increased RR of 2.5- to 8.6-fold by the age of 65 years old [81], [82], [83], [84]. Men ≤55 years carrying a *BRCA2* variant seem to be considerably more susceptible to develop PrCa with a RR ranging from 7.8 to 23 [81], [83]. Therefore, germline alterations in the *BRCA2* gene act as independent predictors of younger age of diagnosis, more aggressive phenotype, and higher mortality rate compared with non-carriers [83], [85], [86], [87], [88].

3.3 The MMR genes

Lynch Syndrome is one of the major causes of inherited predisposition to a large spectrum of cancers, mainly colorectal cancer [89], [90]. This autosomal dominant cancer-susceptibility syndrome is characterized by inheritance of inactivating variants in MMR genes, such as *MLH1*, *MSH2*, *MSH6* and *PMS2*, or large deletions of the *EPCAM* gene that is located upstream of the *MSH2* gene [91], [92], [93], [94], [95]. These genetic alterations impair the ability of the MMR system to recognise and repair DNA mismatches, thereby leading to microsatellite instability (MSI) and an increased risk of developing cancer [96]. LS primarily predisposes to colorectal and endometrial cancers, although several other extracolonic cancers have been reported, including gastric, small bowel, pancreatic, brain, prostate and urothelial [97], [98]. Interestingly, patients carrying variants in the MMR genes have a 2.0- to 3.7-fold increased RR of HPC [99]. Haraldsdottir *et al.* have reinforced PrCa as a possible product of LS by reporting a nearly five-fold increased risk of developing the disease in men with LS, but without earlier onset

or a more aggressive disease [100]. Moreover, a considerably higher PrCa risk for *MSH2* variant carriers has been reported compared to *MLH1* and *MSH6* [20].

3.4 Other well-established DDR genes

ATM and *CHEK2* encode TSGs involved in the ATM–Chk2–p53 DNA DSBs repair signalling pathway. The “sensor” of DNA damage is the *ATM* serine threonine kinase which activates a DNA response by phosphorylating downstream proteins involved in DNA repair and/or cell-cycle control [101]. *CHEK2* is a cell-cycle checkpoint protein kinase that, upon ATM-mediated activation, triggers DNA repair or cell cycle arrest/apoptosis through TP53 activation [102]. Homozygous germline alterations in *ATM* cause ataxia telangiectasia syndrome, which is characterized by an increased predisposition for several types of cancer, namely breast, colorectal, gastric, and pancreatic cancers [103]. *CHEK2* genetic variants have been consistently associated with increased cancer risk, including breast, colon, thyroid, and kidney cancer [27]. *ATM* and *CHEK2* are among the first DNA-repair genes for which germline loss-of-function variants were found in families with an aggregation of PrCa [104], [105], [106], [107], [108]. Pathogenic germline *CHEK2* variants are associated with an increased risk to develop HPC, with an overall risk of 1.9-3.3 [52], while the estimated RR of deleterious *ATM* germline variants is ~1.29 [109].

PALB2 acts as a bridge between *BRCA1* and *BRCA2* to form a “*BRCA* complex” essential for the HR repair mechanism [110]. While biallelic loss-of function variants in this gene predispose to Fanconi anemia [111], heterozygous germline alterations have been associated with an increased risk of breast and pancreatic cancers [111], [112], [113]. Pathogenic germline variants in *PALB2* are present in about 0.56% HPC [114]. A recent large study of PrCa families, all with a *PALB2* pathogenic germline variant, reported an estimated risk for developing PrCa to be 0.42 [115].

3.5 *BRIP1*, *NBS1*, *RAD51C* and *RAD51D*

The *BRIP1* gene is involved in DSB repair as it encodes a DNA helicase that binds directly to the BRCT repeats of *BRCA1* [116]. To date, few studies have reported potentially pathogenetic *BRIP1* variants in PrCa patients. Specifically, Kote-Jarai et al. found a moderate risk of PrCa (OR 2.4) in a set of familial and early-onset PrCa patients carrying a recurrent *BRIP1* truncating (c.2392C>T) variant [117]. This variant was subsequently reported to occur in other two PrCa families, further highlighting the involvement of the *BRIP1* gene in familial PrCa predisposition [106].

Another gene of interest is *NBS1* or *NBN*, which encodes a protein involved in the DSB repair complex [118]. *NBS1* has been proposed as a candidate PrCa susceptibility gene, particularly for the familial/hereditary subset. A specific founder variant (c.657del5), estimated to account for 1.4% of all PrCas in Poland, has been associated with a three-fold increased risk of PrCa in Polish patients under 60 years, a four-fold increased risk for male carriers with a positive FH and an apparent significant effect on overall survival after adjusting for age, stage, and tumor grade [104]. Similarly, in a recent analysis for the contribution of *NBS1* founder alleles to PrCa specific survival and risk, the 657del5 variant was associated with significantly worse survival (hazard ratio 1.6) [119].

RAD51C and *RAD51D* repair genes have been reported as having inherited alterations in several HPC studies [60], [62], [106], [114]. Unfortunately, data supporting the presence of such associations is still scarce. As such, new strategies are further required to unveil the heterogeneous hereditary component behind the early-onset/familial PrCa to validate these and other proposed candidates as HPC susceptibility genes. Also, the strategy of multigene panel testing reinforces the possible pan-cancer implication of key signaling pathways in hereditary predisposition, instead of cancer type-specific drivers. The hypothesis of identifying novel targetable pathways or genes, with clinically actionable germline variants and transversal to hereditary syndromes, might decrease inter-patient heterogeneity and provide a significant value information in patients with advanced disease that run out of approved therapeutic options.

4. Criteria for genetic counselling, guidelines for genetic testing and surveillance

Early-onset of aggressive PrCa, combined with FH of the disease or other heritable cancers, are strong predictors for a hereditary component arguing that those patients are candidates to undergo genetic testing. In fact, genetic testing in PrCa patients is important to identify relatives carrying pathogenic germline variants in genes associated with increased risk of developing cancer. Aligned with emerging evidence sustaining the importance of the genetic landscape in the clinical management of the disease, there has been an effort to establish guidelines for the genetic risk assessment of patients with PrCa.

According to the updated version of the NCCN guidelines for PrCa early detection (version 1.2022) [17], referral for genetic counseling and genetic testing should be considered for all PrCa patients with a FH of cancer, or with an Ashkenazy Jewish

heritage, due to high prevalence of *BRCA1* and *BRCA2* germline variants. In addition, patients with a suspicious FH, defined as having a heavy FH of PrCa (with direct relative, diagnosed before the age of 60 years, or multiple family members diagnosed with breast cancer and PrCa at any age), or with three or more cancer cases (breast, colorectal, endometrial, gastric, ovarian, pancreatic, prostate, melanoma, kidney, bile duct, small bowel, or urothelial cancer) on the same side of the family before the age of 50, also meet the criteria. With the approval of PARPi, all mCRPC patients have been referred for genetic testing (germline and somatic) to guide treatment decisions in addition to familial risk assessment regardless of FH of cancer [17].

Upon the identification of a pathogenic germline alteration in a moderate-to high-risk gene, referral for genetic counseling is recommended to evaluate the risk of second primary tumors, as well as possible therapeutic options [120]. Furthermore, the confirmation of hereditary cancer predisposition can prompt cascade counseling and the genetic testing of additional at-risk family members, which can guide implementation cancer screening and/or prophylaxis [17], [120].

5. Landscape of somatic alterations in prostate cancer

While this thesis does not primarily address this topic, it is important to highlight that the range of somatic genetic alterations in PrCa tumors plays a critical role in clinical decision-making. Current NCCN guidelines advocate for multigene panel testing to identify somatic mutations in homologous recombination (HR) and mismatch repair (MMR) genes, especially for patients with regional or metastatic PrCa. Additionally, evidence suggests that somatic tumor sequencing can uncover underlying germline variants. For instance, it was demonstrated that approximately 8% of alterations identified in mCRPC tumors occurred in the germline [121]. As a result, when somatic variants in HR or MMR genes are detected, genetic counseling is strongly advised to explore potential associations with HBOC or LS [48]. More studies are needed to understand the molecular mechanisms through which germline and somatic variants interact and cooperate to drive PrCa tumorigenesis.

6. Chromosomal instability and cancer

Chromosomal instability (CIN) is a hallmark of human cancer and results from errors in chromosome segregation during mitosis, leading to structural and numerical chromosomal abnormalities. An important distinction should be drawn between aneuploidy and CIN. The first means a state of abnormal chromosome number whereas the latter refers to ongoing chromosome segregation errors throughout consecutive cell

divisions. The two entities often co-occur in human cancers. Tumors presenting high levels of chromosome copy number alterations also exhibit chromosome missegregation [122]. Yet, stable aneuploidies can exist in the absence of CIN as seen in some hematologic malignancies [123]. A significant proportion of the defects underlying CIN in cancer is a mitotic phenotype whereby cells undergoing anaphase exhibit chromosomes that lag in the spindle midzone, referred as lagging chromosomes, resulting from erroneous attachments of microtubules (MTs) at the kinetochores (KTs) [124].

The cell cycle is tightly regulated and its checkpoints control transitions between phases, by detecting external or internal cellular issues. Therefore, the failure of these safe mechanisms may underly CIN, including defects in the mitotic spindle assembly checkpoint (SAC), sister chromatid cohesion, KT-MT attachment, or in centrosome replication [122], [125], [126]. Under normal circumstances, the proper functioning of these mechanisms governs the fidelity of chromosome segregation during cell division, whereby defects lead to chromosome lagging and premature initiation of anaphase before all the chromosomes are aligned and attached to the spindle MTs, ultimately leading to an increased risk of chromosome missegregation (Figure 4). Compounding this, defects in the DDR pathway impair the activation of checkpoints such as G1/S or G2/M, allowing damaged cells to bypass repair processes. This enhances CIN, promotes tumorigenesis, and prevents apoptosis, further destabilizing genome integrity and cell cycle regulation. Together, these interlinked failures underscore the interplay between DDR, SAC, and centrosomal integrity in regulating the cell cycle and preventing CIN, which is central to maintaining genomic stability [127], [128], [129].

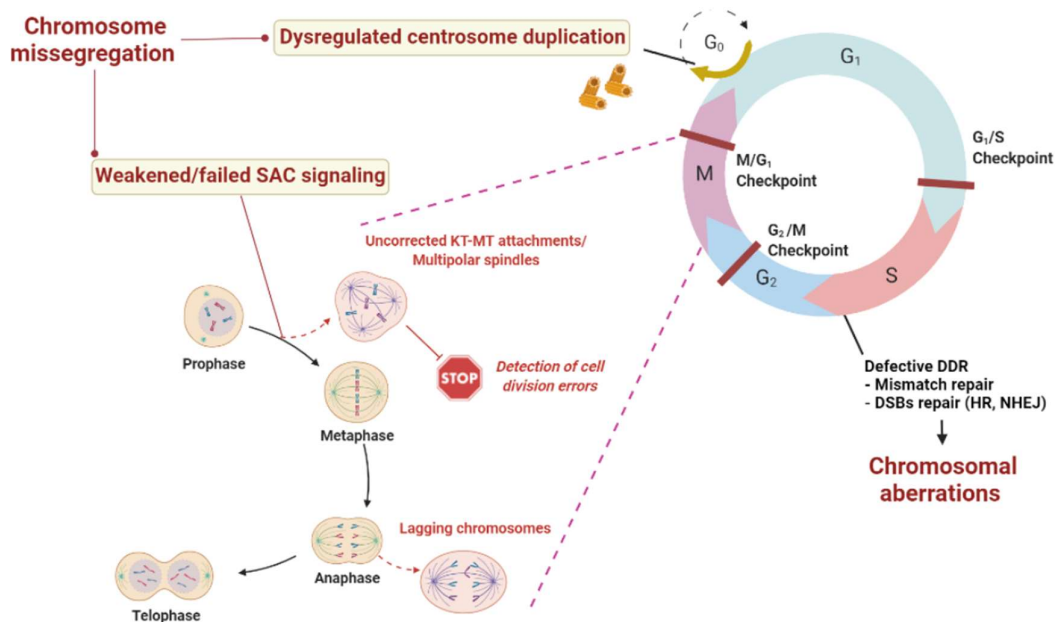


Figure 4: Defects of cell cycle regulation and CIN. Chromosomal aberrations: defective DNA damage response and repair during DNA replication phase, particularly in the mismatch and DSBs repair, results in chromosomal aberrations such as deletions, amplifications, inversions and translocations. Chromosome missegregation: impaired SAC signalling, uncorrected KT - MT attachments and multipolar spindles due to dysregulated centrosome duplication contribute to lagging chromosomes in anaphase, which leads to chromosomal missegregation during cell division. These abnormalities predispose the cell to CIN. DDR: DNA damage response; HR: homologous recombination; NHE: non-homologous end joining.

7. Mitosis and faithful chromosome segregation

7.1 Mitosis – Overview

All living organisms rely on cell reproduction to grow and survive. Thus, the main goal of a cell is to divide. Briefly, the cell is either growing and preparing for division (interphase) or dividing (M phase) (Figure 4). During interphase, the cell synthesizes proteins and RNA. The chromosomal DNA, instead, is replicated during S phase (synthetic phase) and is equally partitioned to the daughter cells during mitosis. The remaining two phases are gaps between mitosis and the S phase (G₁) or between the S phase and the following M phase (G₂) (Figure 4). These GAP phases are important to give the cell extra time to grow and are also important regulatory transitions when specific biochemical switches occur to control cell cycle progression. Importantly, G₁ is the

moment when the cell either commits to divide or, in the presence of inhibitory growth conditions, exits from the cell cycle. When the latter occurs, the cell stops in a non-dividing state, also known as G0 [130].

Mitosis consists in a physical reorganization of the cellular contents to form two new cells that are faithful replicas of their mother. It is characterized by specific states of chromosome condensation and the formation of the mitotic spindle, a highly complex machinery responsible for chromosome movement, separation of the chromatids, their translocation to the spindle poles, and distribution to the dividing daughter cells. Mitosis is conventionally divided into five stages known as prophase, prometaphase, metaphase, anaphase, and telophase. The first visible mitotic event occurs in the nucleus, with the onset of chromosome condensation which defines the beginning of prophase. In this phase DNA condenses into densely packed structures called chromosomes and centrosomes separate and start to build up the mitotic spindle. The breakdown of the nuclear envelope, the MTs of the mitotic spindle invasion of nuclear space and interaction with chromosomes, which are translocated to the cell equator (chromosome congression), marks the beginning of prometaphase. Once all chromosomes are aligned at the equator, which characterizes metaphase, the cell is allowed to exit mitosis, and the sister chromatids migrate to opposite poles (chromosome segregation) during anaphase. At each pole, the nuclear envelope reforms around the decondensing sister chromatids resulting in two daughter nuclei, being the last stage of mitosis, the telophase [131]. Cytokinesis proceeds mitosis, in which cytoskeleton is divided, and two individual daughter cells are originated from a single mother.

Mitosis has been studied since the early 1880s, and we have a detailed, yet incomplete, description of spindle dynamics and mechanics, a sense of mechanisms at a molecular level, and a long list of proteins [132].

7.2 Mitotic spindle assembly

Once cells enter mitosis, all interphase MTs disassemble and re-organize into a complex structure composed of a bipolar antiparallel array of MTs, known as the mitotic spindle. The migration of chromosomes during mitosis is entirely dependent on this structure, which makes it the engine of mitosis. However, the assembly of the MTs into a bipolar spindle depends on other vital components, namely the centrosomes, the KTs and the MT-associated motor and non-motor proteins (MAPs). A family of centrosomal (CEP) proteins was found to play a vital role for the formation of a bipolar spindle [133]. Among these proteins, CEP57 is associated with MTs and is essential for the maintenance of spindle MTs anchorage at KTs, and its accumulation at the centrosome is thought to be involved in MT nucleation [134], together with CEP290, and TUBGCP5,

a gamma-tubulin complex-associated protein [135]. Also, a myriad of microtubule-associated proteins (MAPs) is required to orchestrate the spindle architecture and dynamics [136]. EML4 is required for spindle organization and for the proper attachment of KTs to spindle MTs [137].

Missegregation of a single chromosome, during mitosis, would generate two aneuploid cells, which can have dire consequences for the cells and for the individual [138]. Therefore, defects in spindle motor-associated proteins disrupt the balance of spindle MT-mediated forces, which affect the overall spindle assembly. Notwithstanding, the composition and function of the mitotic spindle are not fully understood, and a more complete inventory of its components would be an important advance.

7.3 Kinetochore-microtubule attachments and dynamics

Because the encounter between the KTs and the MTs has a stochastic component, a cell cannot “tell” precisely how long it will take for all KTs to properly attach to the spindle. This explains why a quality control mechanism is required to assure accurate chromosome segregation. Not only are KTs the sites of attachment of spindle MTs, they also are the platform on which the SAC apparatus (the quality control) is assembled. KTs thus play a central role in assuring faithful chromosome segregation. As incorrect attachments lead to mitotic errors that lead to genetic instabilities, correct KT-MT attachments should remain stable while incorrect attachments should be destabilized and corrected [139].

The foremost question regarding KT-MT attachment is how can MTs remain firmly attached to the KTs and simultaneously grow and shrink, allowing chromosomes to move back and forth during mitosis. We will focus only on the outer KT components believed to interact with SAC components, namely BubR1. When a cell enters mitosis, the components of KTs are assembled at the centromeres of each chromatid. A set of proteins called the KMN network (KNL1, Mis12, and Ndc80 protein subcomplexes) forms the evolutionarily conserved core of KT structure involved in MT binding and SAC function [140] (Figure 5). Mis12 and Ndc80 are themselves assemblies of several proteins. The Ndc80 complex, for instance, is comprised by Ndc80, Nuf2, Spc24, and Spc25 proteins, which together form the principal anchor for mature K-MT attachments, while Knl1 also has a microtubule binding activity [141], [142], [143], [144], [145]. The KMN network in turn recruits SAC components (Bub1, BubR1, Mps1, Mad1, Mad2) and associated proteins, which play important roles in establishing the proper attachment and biorientation of chromosomes on the mitotic spindle. Once assembled during prophase, the KMN network is present throughout mitosis, whereas many of the SAC

and motor components are only abundant on unattached KTs [146], [147], [148], [149]. As the KMN network is the platform for both MT-binding and the SAC components, it has a dual role in coordinating K–MT attachment and the spindle checkpoint response. Knl1 subunit of the KMN network appears to be the major binding protein recruiting both Bub1 and BubR1 to KTs (Figure 5).

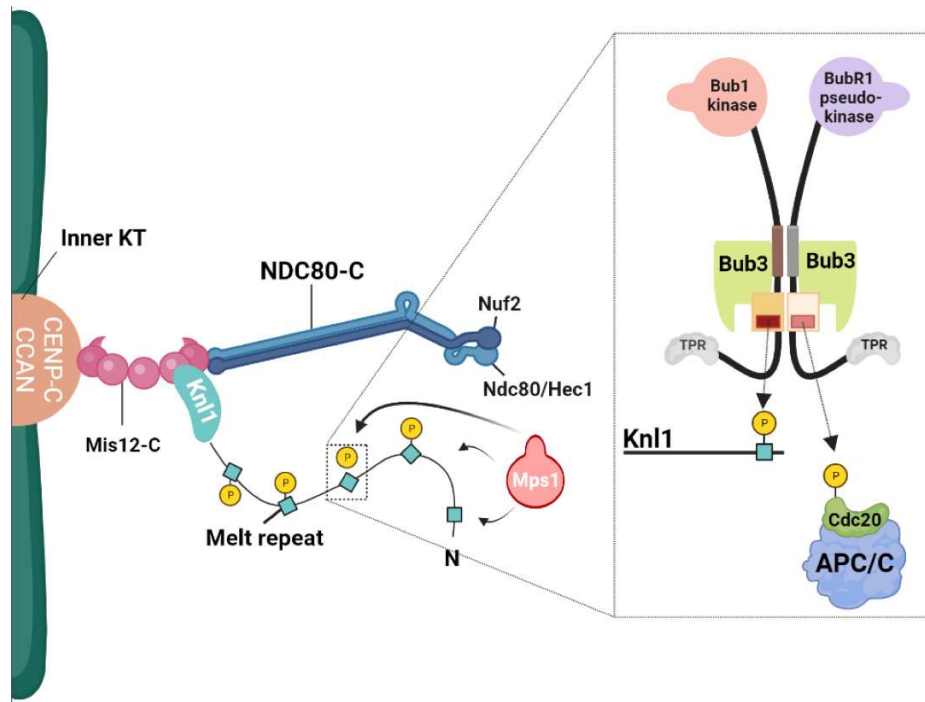


Figure 5: Schematic depiction of the outer kinetochore. Knl1, a component of the outer KT KMN network (Knl1, Mis12, and Ndc80 complexes), acts as a platform for Bub1 and Bub3 localization. MELT repeats in Knl1 are phosphorylated by the checkpoint kinase Mps1 and recruit Bub1:Bub3, which, in turn, recruits BubR1:Bub3 involving equivalent regions of Bub1 and BubR1 comprising the Bub3-binding domain (B3BD). The TPR regions of human Bub1 and BubR1 bind to non-conserved short motifs of Knl1. Knl1: Kinetochore scaffold 1; Bub1: Budding uninhibited by benzimidazoles 1 Homolog; Bub3: Budding uninhibited by benzimidazoles 3 Homolog; MELT: Met-Glu-Leu-Thr; BubR1: Budding uninhibited by benzimidazoles 1 - related 1. Adapted from (Overlack et al, 2017).

7.4 Spindle assembly checkpoint

The main purpose of mitosis is to equally segregate the genetic material to the daughter cells. This implies that each KT must be correctly attached to MTs emanating from opposite poles during anaphase onset. To ensure an accurate chromosome segregation, the cell is equipped with a surveillance system named SAC, a quality control mechanism that prevents anaphase until all chromosomes are stably attached to the spindle MTs. The SAC does not function by monitoring the spindle assembly *per se* but

rather the status of each KT - MT attachment. Unattached KTs generate SAC signaling and anaphase is inhibited. On the other hand, SAC is switched-off when all KTs are stably bound to MTs, and the inhibition of anaphase is then relieved, and the cell cycle can continue [150] (Figure 6).

The unattached KTs recruit SAC proteins resulting in the generation of the mitotic checkpoint complex (MCC). This mitotic effector is a diffusible tetrameric protein complex composed by Mad2, BUBR1/Mad3 and Bub3, at the unattached KT, where they sequester Cdc20 [151], [152], [153], [154]. Cdc20 is a co-factor of the ubiquitin ligase anaphase-promoting complex/cyclosome (APC/C) that targets cyclin B and securin for proteasomal degradation. Securin inhibits separase, a protease required for the cleavage of the cohesin complex that holds sister chromatids together [155]. When all KTs are correctly attached to MTs the “wait anaphase signal” is shut-down, the MCC complex is striped from the KTs and releases Cdc20. Once released, Cdc20 is free to activate the APC/C, which will target securin and cyclin B for destruction, which inactivates Cdk1 and promotes mitotic exit (Peters 2006, Musacchio and Salmon 2007).

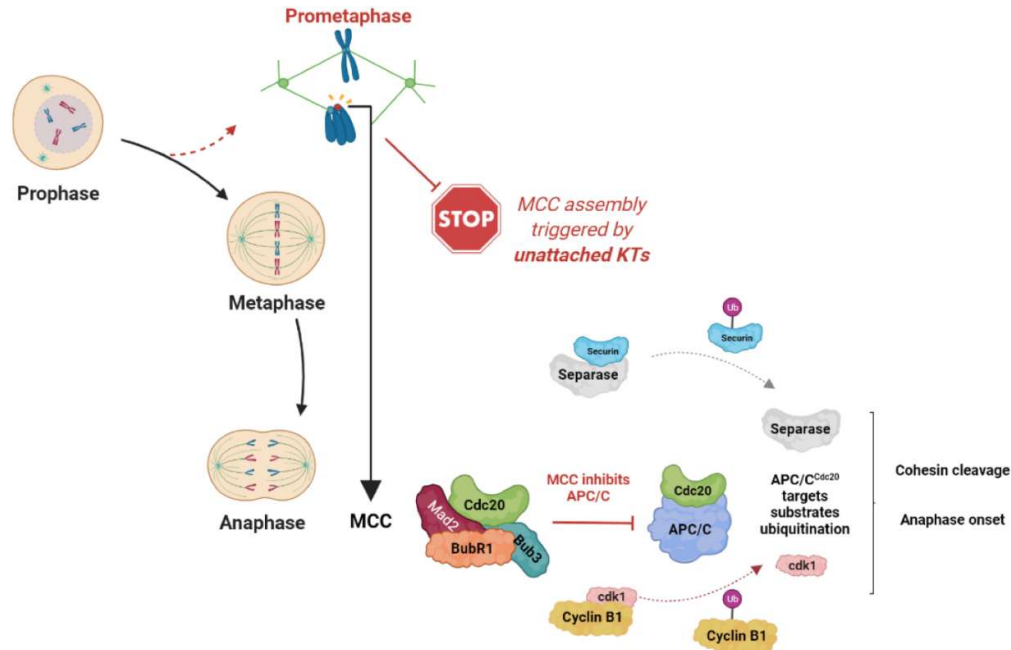


Figure 6: The mitotic checkpoint prevents premature chromosome segregation by inhibiting APC/C activity. In prometaphase, mitotic checkpoint signaling is triggered by unattached KT, leading to the assembly of MCC, the effector of the checkpoint, composed by Cdc20, BubR1, Mad2, and Bub3. APC/C ubiquitination activity is blocked by the MCC. After all chromosomes are correctly attached to MTs, APC/C is relieved and catalyzes anaphase onset. As an E3 ubiquitin ligase, APC/C is responsible for targeting securin and cyclin B1 for degradation. Degradation of securin releases separase, whereas cyclin B1 degradation leads to Cdk1 inactivation. The cascade of degradation events initiates chromosome segregation and mitotic exit. APC/C: E3 ubiquitin ligase anaphase-promoting complex/cyclosome; KT: Kinetochore; MT: microtubule; MCC: Mitotic checkpoint complex; Cdc20: Cell division cycle 20; BubR1: Budding uninhibited by benzimidazoles 1 Related-1; Mad2: Mitotic arrest-deficient 2; Bub3: Budding uninhibited by benzimidazoles 3; cdk1: cyclin dependent kinase 1.

7.5 BUB1B

BubR1, encoded by gene *BUB1B*, is the mammalian homologue of Mad3, one of the six original SAC proteins identified in genetic screens in yeast 20 years ago. It was the first SAC protein shown to have functions beyond SAC, namely in other cellular processes both in and out of mitosis. Besides mitosis, BubR1 plays important roles in ciliogenesis, neural differentiation, and DNA repair [156], [157].

The intense scrutiny of BubR1 is based on its key role not only in the spindle checkpoint but also because it is required for the efficient attachment of KT to spindle MTs. BubR1 is an inactive pseudokinase that contributes directly to the SAC and to APC/C inhibition as a subunit of the MCC, together with Bub3, Cdc20, and Mad2 [158]. The organization of the functional domains of BubR1 supports its dichotomized biological

functions during mitosis. The N-terminal region of BubR1 (≈ 420 amino acids) is the most highly conserved across eukaryotes and is involved in KT-binding and checkpoint signaling [159], while the middle and C-terminal regions are involved in promoting stable KT - MT attachments, during chromosome segregation [160] (Figure 7).

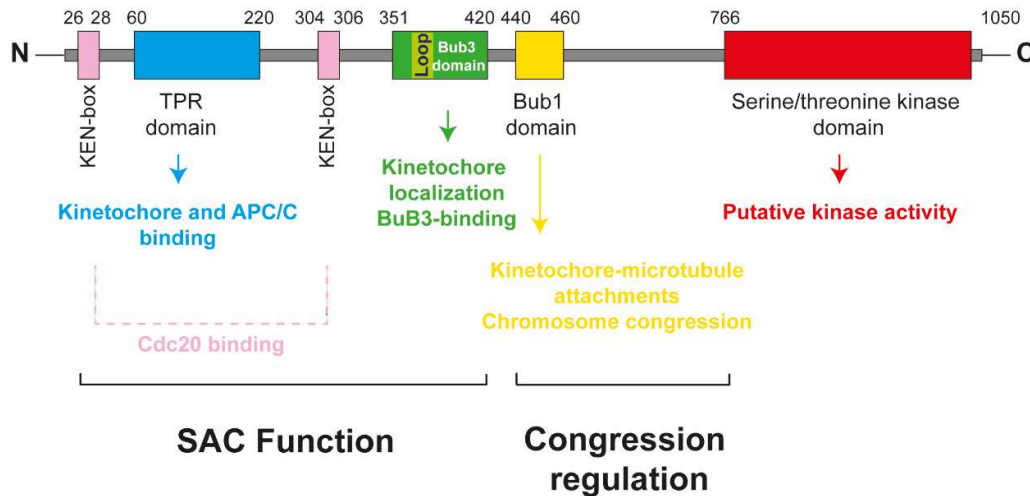


Figure 7: Schematic representation of BubR1 domain organization. The main function of each domain is highlighted. The N-terminus half of BubR1 (~ 420 residues) include domains that regulate SAC signaling. Domains responsible for protein-protein interactions include tetratricopeptide repeat motif (TPR), and Bub3-binding domain (B3BD). The Bub1-interacting domain, whose kinase activity may be strictly required for chromosome alignment, but not for SAC signaling is found within the C-terminal half of BubR1. This region is responsible for regulate chromosome congression. The remaining length of the C-terminus is a putative kinase domain, whose activity is debated in the literature.

The N-terminal region of BubR1 contains two Lys-Glu-Asn motifs (KEN) box motifs (26-28, 304-306 residues, respectively) required for effective SAC function, allowing BubR1 to interact with the APC/C through its Cdc20 recognition subunit and physically block APC/C activation [31, 32]. The BubR1 tetratricopeptide repeat (TPR) motif (≈ 60 -220 residues) has been shown to bind to the outer KT protein Knl1 [161], [162] and may contribute to the recruitment of BubR1 to KTs (Figure 5). TPR domain also contributes to the binding of BubR1 to Cdc20 [163]. Residues 351-420 encode the Bub3-binding domain (also called GLEBS), important for BubR1 recruitment to the KT [159], [164], [165]. Within Bub3 domain, there is a Loop region (368-379 residues), a direct APC/C binder that promotes stable association of the BubR1:Bub3 MCC proteins with the APC/C [166]. Spanning the C-terminus region of BubR1 is the kinase domain, which is not essential for SAC control [167], but its function in mitosis is one of the most controversial topics. The question on whether this domain is indeed catalytic, with several evidence suggesting that BubR1 is an inactive pseudokinase [168], while other

studies support a role for regulating BubR1 stability and abundance as well as catalytically acting upon multiple targets during mitosis [168], is yet to be clarified.

BubR1 is unique amongst mitotic regulators as both under- and overexpression result in drastically different phenotypes [169], [170]. Complete loss of BubR1 is lethal, since it causes early embryonic death [171], while variants that reduce BubR1 protein levels result in aneuploidy [170] and increased predisposition to cancer in mice [172]. Conversely, high levels of BubR1 protect against age-related phenotypes in tissues that are prone to increased aneuploidy rates with age and extends life- and health span in mice [169].

In humans, biallelic germline variants in *BUB1B* have been causally implicated in mosaic variegated aneuploidy (MVA1, OMIM: #257300), an autosomal recessive disorder defined by constitutional CIN in the form of mosaic aneuploidies, mostly trisomies and monosomies in >5% of cells in different tissues [173], [174], [175]. Clinically, individuals with MVA1 display microcephaly, growth- and mental retardation. In addition, MVA patients are more prone to develop childhood cancers including rhabdomyosarcoma, Wilms tumor and leukemia [173], [176]. As of now, it is still poorly understood how these MVA-related variants shape BubR1 function to promote aneuploidy and cancer predisposition [177]. The pattern of BubR1 biallelic variants found in MVA families was of one truncated BubR1 allele paired with a missense variant in the other allele [173]. Also, missense variants mapping in or near the kinase domain were shown to reduce overall BubR1 levels by destabilizing the protein [177], suggesting that compromised expression of BubR1 may predispose to constitutional CIN and increased risk of tumor development [178].

Additionally, monoallelic *BUB1B* germline variants were initially reported in seven Japanese families with premature chromatid separation syndrome (PCS, OMIM #176430), a rare autosomal disorder characterized by premature separation of all sister-chromatids in more than 50% of mitotic lymphocytes [179], [180] and phenotypically associated with MVA syndrome [174]. Subsequent follow-up analyses showed that these families carried another *BUB1B* missense variant that segregated with the MVA disorder [181]. Germline variants in *BUB1B* are also implicated in elevated risk for adult cancers. A germline homozygous intronic variant in *BUB1B* was identified in a patient with history of multiple primary gastrointestinal cancers, without any variants detected for the known gastrointestinal-associated cancer genes [182]. More recently, a higher frequency of deleterious germline *BUB1B* variants in patients with familial pancreatic cancer provides support for *BUB1B* as a candidate for pancreatic cancer predisposition gene [183]. Another *BUB1B* deleterious variant was recently found in an individual with three independent primary tumors (melanoma, breast cancer and mesothelioma) [184].

Although *BUB1B* germline variants have been previously identified in two of those tumors [185], [186], there are still no reports of *BUB1B* germline variants associated with these cancer types. *In vivo* mice studies have shown that SAC function relies on the tightly orchestrated expression of its different components, in which an imbalance of one of them is sufficient to promote chromosome segregation defects, senescence, and, therefore, tumor initiation [187], [188], [189]. Interestingly, monoallelic germline variants in *Bub1* and *Bub3* kinases were shown to confer an increased risk to develop early-onset colorectal cancer (CRC) in which *BUB1B* variants were rare [190], [191]. This further suggests that genetic variants in different SAC genes promote cancer development, and that different tissue features may rule the predisposition for oncogenic transformation driven by deleterious germline variants in SAC genes.

7.6 CEP57

The assembly of a bipolar mitotic spindle is a crucial requirement for faithful chromosome segregation and cell division, and, in animal cells, bipolarity of the spindle emerges through MT nucleation from two centrosomes. The faithful segregation of chromosomes during anaphase is also dependent on the proper attachment of chromosome KTs to MTs.

One of the proteins suggested to affect chromosome segregation by interfering with KT - MT dynamics is centrosomal protein, 57KDa (CEP57). CEP57 encodes a cytoplasmic protein called translokin, which localizes to the centrosome and has a function in MT stabilization [192]. CEP57 also plays a role in SAC signalling [193]. The N-terminus (59-239 residues) coiled-coil domain is within a region required for localization of CEP57 to the centrosome and for multimerization of the protein. The C-terminal half of CEP57 (~278-491 residues), including the second coiled-coil domain, is required for nucleating, bundling and anchoring microtubules to the centrosomes [194] (Figure 8).

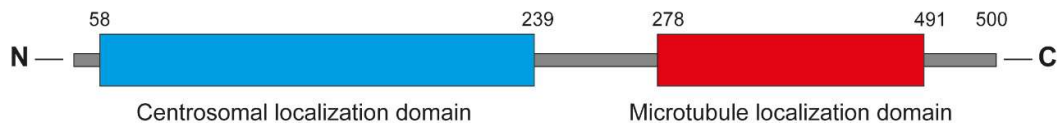


Figure 8: Schematic representation of key domains within CEP57. The dual role of CEP57 is explained by the organization of the domains through the protein. The N-terminus is responsible for centrosomal localization of CEP57, while the C-terminus is implicated in the localization of this protein in the MTs.

CEP57 localizes also to KTs through its interaction with the KT - MT protein Mis12 (Figure 5) and activates SAC signaling by recruiting the MCC heterodimer Mad1:Mad2

to the KT, thus ensuring correct chromosome segregation [193]. This CEP57 role in mitosis explain the aneuploidies found in MVA syndrome, in which absence of a functional CEP57 protein result in a weakened SAC signalling, premature metaphase-anaphase transition and an increased rate of lagging chromosomes [193]. Considering that CEP57 is localized to spindle poles, its depletion results in an increased percentage of cells with multipolar spindles [133], which may also induce chromosome lags in the spindle, the major mitotic phenotype underlying CIN. Biallelic loss-of-function variants in *CEP57* have only be described in twelve patients [192] and predispose to MVA2 syndrome (OMIM # 614114). The set of clinical features of MVA2 include growth retardation, short limb ends, congenital hypothyroidism and congenital heart disease [175]. Even though MVA1 patients are known to have an increased risk of childhood malignancies such as Wilms tumor, rhabdomyosarcoma and leukemia [173], [176], no such predisposition has been observed for the 12 patients with MVA2 (reviewed in [192]). However, *CEP57* was proposed to be a haplo-insufficient tumor suppressor (TSG) by observing spontaneous and carcinogen-induced cancer phenotypes in the heterozygous *CEP57^{+/-}* and *CEP57^{+/-}* mice. In fact, variants or mis-regulation of a variety of TSGs or oncogenes are correlated with centrosome amplification [195], further promoting aneuploidies and malignant behaviour in tumor cell lines, mouse tumor models, human tumors and hematological cancers (Levine et al. 1991; Lingle et al. 1998; D'Assoro et al. 2002; Pihan et al. 2003; Giehl et al. 2005).

To the best of our knowledge, there is only one report of a heterozygous carrier of a deleterious variant in *CEP57*, who developed early-onset PrCa [108]. This variant was further identified in one patient with metastatic PrCa at diagnosis for our group in a parallel study (data not published). These data suggest that further studies are needed to address whether homozygous or heterozygous carriers with truncating variants in *CEP57* have cancer predisposition.

7.7 Mosaic variegated aneuploidy 1 and 2

Mosaic aneuploidies on chromosome metaphases are a hallmark of MVA syndrome. While phenotypes associated with *BUB1B* and *CEP57* variants are broadly similar, with growth retardation, facial dysmorphism, normal or only mildly delayed development, absence of cancer predisposition in *CEP57* variant carriers and lack of PCS might be distinguishing features, helpful in the differential diagnosis of these two disorders (Figure 8).

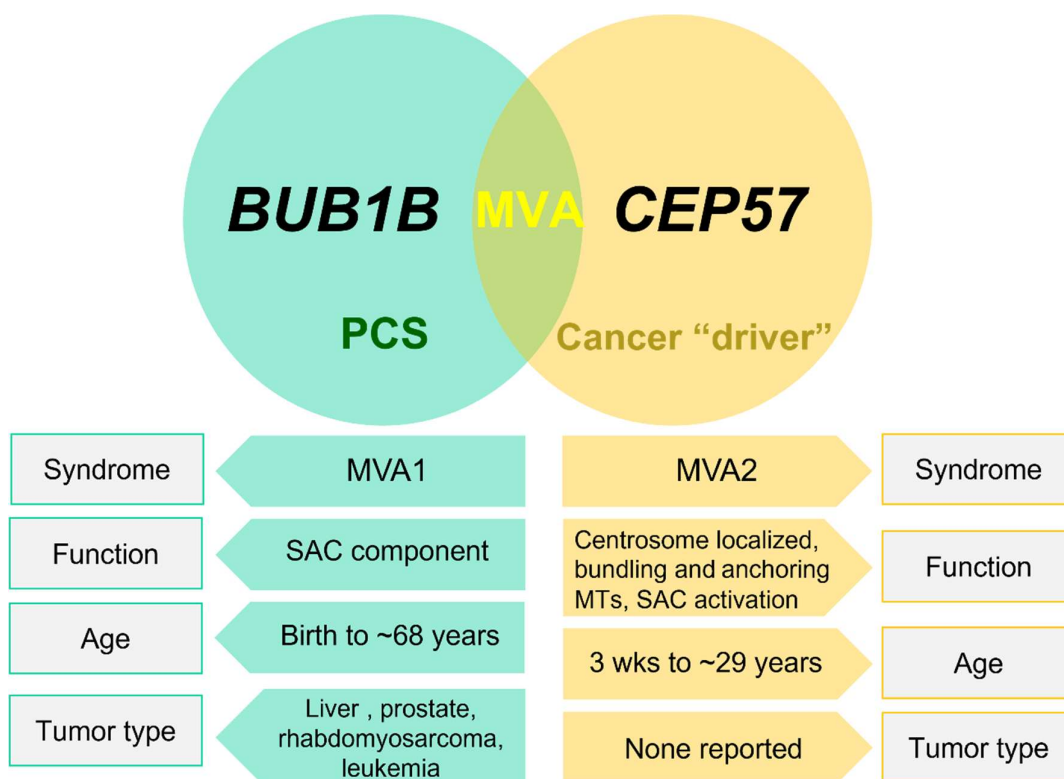


Figure 8: Schematic representation of the characteristics of MVA caused by biallelic variants in *BUB1B* and *CEP57* genes.

AIMS

The main goal of this thesis is to clarify the potential of heterozygous germline variants in genes involved in chromosome segregation, microtubule-kinetochore attachment and spindle dynamics to explain some of the missing heritability in hereditary/familial PrCa. To accomplish this goal, we will combine full-sequencing strategies, targeted genome-editing, molecular and cellular biology, and computational approaches to address the following specific aims:

1. To evaluate the role of heterozygous germline variants in the *BUB1B* and *CEP57* genes, both involved in the autosomal recessive mosaic variegated aneuploidy syndromes, for PrCa inherited predisposition in a series of 462 patients with early-onset and/or familial disease.
2. To identify other genes involved in *BUB1B*- and/or *CEP57*-related pathways that show deleterious variants segregating with PrCa in a whole-exome sequencing discovery dataset of duos/trios from 45 families, and then validate the findings in the entire series of patients.
3. To look for founder effects for the most frequent variants by using haplotype and identical-by-descent analyses with high-density genome-wide SNPs.
4. To clarify the biological mechanisms by which variants affecting the chromosome segregation machinery might trigger carcinogenesis in human cells, as well as their possible therapeutic implications, by combining in vitro, ex vivo and in silico approaches.

METHODOLOGY

To identify the most promising candidate genes, we integrated data from two next-generation sequencing (NGS) approaches: whole-exome sequencing (WES) performed in duos and trios from 45 families, and targeted sequencing using a custom gene panel in our entire cohort of 462 probands with suspected hereditary prostate cancer (HPC).

Our analysis focused on mutated genes that segregated with the disease in affected families and were involved in critical biological processes such as chromosome segregation.

Initially, we prioritized classical chromosome segregation genes associated with cancer predisposition and assessed how heterozygous variants could contribute to hereditary predisposition to PrCa in a cohort of 462 patients with early-onset and/or familial PrCa.

We then focused on other chromosome segregation genes carrying deleterious variants segregating with the disease in exome data from duos/trios of 45 families (discovery cohort), subsequently validating the findings in the full patient cohort.

CHAPTER I

***BUB1B* monoallelic germline variants contribute to prostate cancer predisposition by triggering chromosomal instability**

Maria P. Silva, Luisa T. Ferreira, Natercia F. Bras, Lurdes Torres, Andreia Brandao, Manuela Pinheiro, Marta Cardoso, Adriana Resende, Joana Vieira, Carlos Palmeira, Gabriela Martins, Miguel Silva, Carla Pinto, Ana Peixoto, Joao Silva, Rui Henrique, Sofia Maia, Helder Maiato, Manuel R. Teixeira, and Paula Paulo

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RESEARCH

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BUB1B monoallelic germline variants contribute to prostate cancer predisposition by triggering chromosomal instability

Maria P. Silva^{1†}, Luísa T. Ferreira^{1†}, Natércia F. Brás², Lurdes Torres^{1,3}, Andreia Brandão¹, Manuela Pinheiro¹, Marta Cardoso¹, Adriana Resende^{1,3}, Joana Vieira^{1,3}, Carlos Palmeira⁴, Gabriela Martins⁴, Miguel Silva^{1,3}, Carla Pinto^{1,3}, Ana Peixoto^{1,3}, João Silva^{1,3}, Rui Henrique⁵, Sofia Maia¹, Helder Maiato^{6,7,8}, Manuel R. Teixeira^{1,3,9†} and Paula Paulo^{1*†} 

Abstract

Background Prostate cancer (PrCa) is the most frequently diagnosed cancer in men. Variants in known moderate- to high-penetrance genes explain less than 5% of the cases arising at early-onset (< 56 years) and/or with familial aggregation of the disease. Considering that BubR1 is an essential component of the mitotic spindle assembly checkpoint, we hypothesized that monoallelic *BUB1B* variants could be sufficient to fuel chromosomal instability (CIN), potentially triggering (prostate) carcinogenesis.

Methods To unveil *BUB1B* as a new PrCa predisposing gene, we performed targeted next-generation sequencing in germline DNA from 462 early-onset/familial PrCa patients and 1,416 cancer patients fulfilling criteria for genetic testing for other hereditary cancer syndromes. To explore the *pan*-cancer role of *BUB1B*, we used in silico BubR1 molecular modeling, in vitro gene-editing, and ex vivo patients' tumors and peripheral blood lymphocytes.

Results Rare *BUB1B* variants were found in ~ 1.9% of the early-onset/familial PrCa cases and in ~ 0.6% of other cancer patients fulfilling criteria for hereditary disease. We further show that *BUB1B* variants lead to decreased BubR1 expression and/or stability, which promotes increased premature chromatid separation and, consequently, triggers CIN, driving resistance to Taxol-based therapies.

Conclusions Our study shows that different *BUB1B* variants may uncover a trigger for CIN-driven carcinogenesis, supporting the role of *BUB1B* as a (*pan*)-cancer predisposing gene with potential impact on genetic counseling and treatment decision-making.

Keywords *BUB1B*, BubR1, Cancer predisposition, Premature chromatid separation, Chromosomal instability, Taxol response

[†]Maria P. Silva, Luísa T. Ferreira, Manuel R. Teixeira and Paula Paulo contributed equally to this work.

*Correspondence:

Paula Paulo

paula.paulo@ipoporto.min-saude.pt

Full list of author information is available at the end of the article



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Introduction

Prostate cancer (PrCa) is the second most frequently diagnosed cancer and the fifth leading cause of male cancer deaths worldwide [1]. Familial aggregation, Hereditary Breast and Ovarian Cancer Syndrome, and Lynch Syndrome are among the most important risk factors for PrCa development, in addition to age, race, ethnicity, and environmental factors. Nevertheless, little is known about the genetic determinants that underlie PrCa development in patients with early-onset and/or familial aggregation of the disease [hereafter referred to as HPC (hereditary prostate cancer) cases] [2]. In fact, previous screenings in our series of 462 HPC cases revealed that <5% of the patients are carriers of variants in the DNA damage response or *HOXB13* genes known to be associated with PrCa predisposition [3–5]. As such, new strategies are required to unveil the heterogeneous genetic components underlying hereditary PrCa development.

Aiming to provide new insights into the missing heritability of PrCa, we previously unveiled *CEP57* as a potential PrCa predisposing gene [5]. Biallelic deleterious variants in *CEP57* were described in 2011 as a new cause of Mosaic Variegated Aneuploidy (MVA), leading to the classification of the MVA2 syndrome, which contrasts with the MVA1 syndrome, caused by biallelic variants in the *BUB1B* gene. Although aneuploidy is a known cancer hallmark, there are no reports of cancer development in MVA2 patients, with only thirteen cases reported to date [6]. For MVA1 syndrome, the frequency of cancers (mostly in childhood) is not higher than 40% [7], eventually biased by the high rates of early mortality. Although scarce, carriers of *BUB1B* heterozygous deleterious variants have been reported in some cancer patient cohorts, namely, early-onset colorectal cancer [8], familial pancreatic cancer [9], and cutaneous melanoma with multiple primary carcinomas [10]. Nevertheless, the role of *BUB1B* as a cancer-predisposing gene is not yet clarified.

BubR1 (Budding uninhibited by benzimidazole-related 1), encoded by the *BUB1B* gene, is an essential component of the Spindle Assembly Checkpoint (SAC), a surveillance mechanism that prevents persistent chromosome missegregation (defined as Chromosomal Instability or CIN) and aneuploidy [11, 12]. SAC function relies on the proper assembly of a mitotic checkpoint complex (MCC) catalyzed at unattached kinetochores (KTs). The MCC is composed of Mad2, Bub3, BubR1, and Cdc20, and acts by inhibiting APC/C (anaphase promoting complex/cyclosome) activation, which is essential for mitotic exit [13]. Several studies have shown that partial reduction of the MCC is sufficient to severely impair SAC signaling leading to chromosome segregation fidelity defects, aneuploidy and CIN, hallmarks of cancer [14,

15]. MVA1 syndrome and the Premature Chromatid Separation (PCS) trait, caused by biallelic and monoallelic *BUB1B* variants, respectively, are the strongest evidence for a causal effect of CIN in cancer development arising from SAC defects [16–19]. However, solid and mechanistic evidence establishing a link between specific *BUB1B* monoallelic variants and cancer predisposition are lacking. Additionally, considering the association between CIN levels and the response to taxane-based chemotherapeutic drugs [20, 21], the identification of carriers of deleterious *BUB1B* variants may also have significant implications for treatment decision-making.

In this work, we aimed to assess the frequency of carriers of germline variants in *BUB1B* among patients with criteria for hereditary PrCa and to clarify the biological mechanisms by which *BUB1B* variants might trigger carcinogenesis in human cells, as well as to evaluate their possible therapeutic implications. By combining *in vitro*, *ex vivo* and *in silico* approaches, we show that rare *BUB1B* variants are prevalent in early-onset/familial PrCa and in other cancer syndromes, and trigger oncogenic transformation by destabilizing BubR1, leading to PCS and CIN, and, consequently, taxane-resistant proliferation. Altogether, our results provide novel insights into the underlying mechanisms of (prostate) carcinogenesis and highlight *BUB1B* gene in the roadmap of genetic counselling and precision medicine.

Materials and methods

HPC samples

This study used germline DNA from 462 index cases of early-onset/familial PrCa (HPC) cases, previously recruited [4]. The patients were recruited based on two criteria: early-onset disease, with PrCa diagnosis before the age of 56, and/or familial/hereditary PrCa, with more than one case with PrCa and at least one of them diagnosed before the age of 66. Of the 462 HPC cases, 240 (51.9%) fulfilled the early-onset disease criterion, and 311 (67.3%) fulfilled the family history criterion, with 89 (19.3%) fulfilling both criteria. Demographic and clinicopathological characteristics of all the carriers are listed in Table S1.

Targeted next-generation sequencing (T-NGS)

We used a customized gene panel designed with Agilent SureDesign (Agilent Technologies, Santa Clara, CA, USA) to sequence germline DNA from the index patients of all 462 PrCa cases. The panel covered, among other PrCa genes under investigation, the coding and splicing regions of *BUB1B* (NM_001211.5). Capture and sequencing, data processing, variant annotation, and prioritization were performed as previously described

[22]. Detailed information is described in Supplementary Methods.

Dataset of patients tested for other hereditary cancer syndromes

To gain insights into the possible *pan*-cancer role of pathogenic/likely pathogenic variants in *BUB1B*, we searched for *BUB1B* carriers among 1,416 cancer patients referred for molecular diagnosis of multiple inherited cancer syndromes at the Department of Genetics of IPO Porto, already screened with the TruSight Cancer Panel v.1 (Illumina). Variant annotation and filtering were performed as described for the custom T-NGS panel mentioned above and detailed in Supplementary Methods.

Control samples

To estimate the risk between the carrier status for the c.1171_1173del variant and PrCa development, we used germline DNA from 459 healthy male individuals (mean age 48.3 years; SD $\pm$ 10.2 years) as control samples, including 288 blood donors from the Portuguese Oncology Institute of Porto with no personal history of cancer at the time of blood collection and 171 healthy relatives with negative predictive genetic testing (each from independent families). To estimate the global risk for cancer development, germline DNA from 416 healthy females (mean age 48.6 years; SD $\pm$ 9.7 years) was also used, which included 243 blood donors and 173 healthy relatives with negative predictive genetic testing.

To assess the basal levels of the PCS trait in our population, we performed PCS on normal lymphocyte metaphase spreads of ten age-matched healthy males aged between 42 and 78 years (mean age 52.6 years; SD $\pm$ 10.7 years), specifically recruited for this purpose, under signed informed consent.

KASP genotyping

Kompetitive Allele Specific PCR (KASP) genotyping, with variant-specific KASP probes, was performed according to the manufacturer's instructions. Assay primers (Metabion, Köln, Germany) summarized in Table S2 were designed using the Primer-BLAST design tool from the National Center for Biotechnology Information (NCBI) [23], and the PCRs were run on a LightCycler 480 Real-Time instrument (Roche Life Sciences, Basel, Switzerland). For data analysis, LightCycler 480 Software 1.5.0 was used.

Haplotype analyses

The T-NGS data were phased using BEAGLE 4.1 [24], and IBD haplotypes were determined using the BEAGLE Refined IBD algorithm [25]. The lengths of the shared haplotype segments were estimated by the distance

between the last two shared markers flanking the variants. A similar IBD and haplotype approach was applied to the high-density SNP genotype data from the Portuguese early-onset/familial PrCa sample collection (374 PrCa cases and 180 controls) obtained with the Infinium OncoArray-500 K BeadChip (Illumina) as part of the PRACTICAL consortium, as previously described [26].

Microsatellite haplotype analysis was performed using nine polymorphic microsatellite markers flanking the gene, namely, D15S118, D15S1012, D15S1044, D15S146, D15S214, TR20GT, D15S968, AFM196XB8, and D15S781. A total of seven probands carrying the *BUB1B* c.1171_1173del variant were genotyped, including all five HPC patients, the two additional carrier patients found among the 1,416 screened for multiple hereditary cancer syndromes (Table 2), and seven unaffected family members. Primers were designed using the Primer-BLAST tool [23] (Table S2) and acquired from Metabion. All markers were assayed by PCR using fluorescently end-labeled primers, and PCR products were run on a 3500 Genetic Analyzer together with the fluorescence-labeled DNA fragment size standard 600-LIZ (Thermo Fisher Scientific, Waltham, MA, USA). Haplotype construction was performed manually based on the genotypes obtained from probands and family members.

Next-generation DNA and RNA sequencing of FFPE tumor samples

To assess the impact of *BUB1B* germline variants on *BUB1B* mRNA expression and transcriptomic profiles in the corresponding prostate carcinomas, available prostate tissue samples from carriers were submitted to RNA sequencing. For this purpose, matched tumor and normal RNA were extracted from $\sim$ 5 μ m sections of formalin-fixed paraffin-embedded (FFPE) prostate tissues after deparaffinization using xylene and ethanol, according to the recommendations of the High Pure FFPE RNA Isolation Kit (Roche Life Sciences). For each library preparation, 100 ng of total RNA was used as input for the TruSeqTM RNA Exome (Illumina) protocol, according to the manufacturer's instructions. Libraries were sequenced on an Illumina NextSeq 500 using the NextSeq High Output v2.5 kit using the pair-end run mode with a run setup of 2 $\times$ 76 bps. Data was processed for both transcript expression and variant calling, as described in Supplementary Methods.

To look for a somatic "second hit" in *BUB1B* in the tumors of HPC patients carrying germline variants, DNA was extracted from $\sim$ 5 μ m sections of FFPE tumor tissues using the Cobas[®] DNA Sample Preparation Kit (Roche Life Sciences) after deparaffinization with xylene and ethanol, according to the manufacturer's recommendations. A T-NGS custom panel covering, among other genes, the

coding and splicing regions of *BUB1B* (NM_001211.5), designed with Agilent SureDesign, was used. Capture, sequencing, data processing, and variant annotation were performed as described for germline DNA.

Generation of prostate in vitro cell models carrying the *BUB1B* variant c.1171_1173del

The human non-tumorigenic prostate-derived cells RWPE-1 were kindly provided by Prof. Margarida Fardilha (University of Aveiro, Portugal). Wild-type RWPE-1 and derived monoclonal cell populations were cultured in sterile conditions, at 37°C, 5% CO₂, and a humidified atmosphere, in Keratinocyte Serum-Free (KSF) Medium (Gibco) supplemented with L-glutamine, 0.005 ng/μL Human Recombinant Epidermal Growth Factor (EGF), and 50 ng/μL of Bovine Pituitary Extract (BPE), and 1% penicillin-streptomycin (Gibco).

To induce specific editing of the *BUB1B* gene in RWPE-1 cells, we used the Alt-R CRISPR/Cas9 System from Integrated DNA Technologies (IDT, Coralville, IA, USA) following the manufacturer's instructions. Among 153 isolated clones, gene-editing leading to the presence of the recurrent *BUB1B* variant c.1171_1173del was observed in four independent clones, in which gene-editing also led to the occurrence of a second in-frame variant (c.1133_1156del) in the other allele, predicted to lead to loss of amino acids from Proline 378 to Histidine 385 [p.(Pro378_His385del)]. No heterozygous clones with monoallelic gene-editing for the c.1171_1173del variant were found. Thus, we randomly selected two of the four clonal populations with the same genotype (*BubR1*^{Δ391/Δ378-385}) – named C1 and C2 – for further phenotypic evaluation. Detailed information is described in Supplementary Methods.

Sanger sequencing

For Sanger sequencing, monoclonal cell populations were lysed directly in the well of a 96-well plate with 10 μL of reaction buffer mixture of the Xpert directXtract Lysis Buffer (GRiSP, Porto, Portugal) after a D-PBS wash (Gibco, Thermo Fisher Scientific). Cell lysates were incubated at 75°C for 5 min, followed by heat-inactivation at 95°C for 10 min. The cell lysate was diluted 5× for PCR amplification of the *BUB1B* in-frame deletion, as previously described [5]. Specific primers were designed using Primer-BLAST [23] (Table S2) and acquired from Metabion. The PCR products were purified with Exo/SAP Go (GRiSP) and forwarded for sequencing PCR using the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific), according to the manufacturers' instructions. Samples were run in a 3500 Genetic Analyzer (Thermo Fisher Scientific).

Western blot

To assess steady-state BubR1 expression levels, total protein extracts were obtained from 90% confluent cells. Briefly, adherent cells were washed twice with ice-cold D-PBS and scrapped with ice-cold lysis buffer (50 mM Tris HCl, pH 7.5, 150 mM NaCl, and 0.1% NP-40). After incubation on ice for 15 min, soluble fractions were collected by centrifugation at 13,000 rcf for 30 min.

To obtain mitotic extracts, 90% confluent cells in T75 flasks were synchronized with 0.5 μM nocodazole. Mitotic cells were harvested by shake-off and centrifugation at 1200 rpm for 5 min. Cell pellets were washed once with D-PBS and resuspended in ice-cold lysis buffer (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% NP-40, and 0.5% Triton X-100) supplemented with a cocktail of protease inhibitors (Roche Life Sciences). Proteins (50 μg) were separated in a 10% SDS-PAGE system and transferred to a nitrocellulose membrane using the Trans-Blot Turbo Transfer System (Bio-Rad Laboratories, Hercules, CA, USA). BubR1, Bub3, Cdc20 and β-actin were probed using the following antibodies: rabbit anti-BubR1 (1:1,000; ab70544, Abcam, Cambridge, UK), anti-Bub3 (1:2,000; 27,073-1-AP, Proteintech, Rosemont, IL, USA), mouse anti-cdc20 (1:250; sc-5296, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-β-actin antibody (1:10,000; A1978, Sigma-Aldrich). Anti-rabbit (1:10,000) and anti-mouse (1:2,500) HRP-conjugated secondary antibodies (Sigma-Aldrich and Bio-Rad Laboratories, respectively) were visualized using Clarity Western ECL Substrate (Bio-Rad Laboratories).

Cycloheximide (CHX) chase assay

Cells at 80–90% confluence in T25 flasks were treated with 20 μg/mL of cycloheximide (Sigma-Aldrich; C4859) without medium change. At different time points (0 h, 4 h, 8 h and 16 h), total protein extracts were obtained and analyzed by western blot, as described above. Two independent experiments were performed for each clonal cell line paired with WT cells. Images were acquired in the ChemiDoc™ XRS System and data quantified in ImageJ.

Quantitative PCR (qPCR)

To evaluate the expression levels of *BUB1B* in the gene-edited cell line models and WT cells, total RNA was extracted from each cell population using the RNeasy Mini Kit (QIAGEN, Hilden, Germany), and cDNA was synthesized using the H-minus RevertAid cDNA synthesis kit (Fermentas, part of Thermo Fisher Scientific) with oligo-dT primers, according to the manufacturers' protocols. Primers and probes (TaqMan) for *BUB1B* were

designed using Primer3 software (v4.1, <https://primer3.ut.ee>) and acquired from Metabion (Martinsried, Germany) (Table S2). The beta-glucuronidase (*GUSB*) housekeeping gene was used as an endogenous control for normalization of the expression levels, and the primers/probe mix was acquired as a pre-developed TaqMan Gene Expression Assay from Applied Biosystems (part of Thermo Fisher Scientific). Relative expression levels were obtained by calibrating *GUSB* normalized *BUB1B* expression values from each population for the expression levels of the WT control population.

Proliferation and apoptosis assays

Cellular proliferation/viability and apoptosis levels were evaluated with MTT (Sigma-Aldrich) and APOPercent-age (Biocolor, Carrickfergus, UK) colorimetric assays, respectively, as previously described [27, 28]. Growth rate (GR) was calculated from the absorbance values obtained with the MTT assay using the formula $GR = (T96 - T0) / T0$. To evaluate Taxol growth inhibition, complete growth medium containing 10 nM Taxol or an equivalent volume of the drug vehicle (DMSO) was added to cells at T0 (h), and the MTT assay was evaluated at T96 (h). For each cell population, the percentage of growth inhibition was calculated using the formula $\% \text{ Inhibition} = 100 \times [1 - (T96 - T0)_{\text{Taxol}} / (T96 - T0)_{\text{Vehicle}}]$, and the percentage of apoptosis was obtained by correcting apoptotic cells to the “total cells” (sum of viable and apoptotic cells) at T96 (h). Statistical data was obtained from four independent experiments.

Time-lapse microscopy

Live cell analysis was performed under conditions of mitotic arrest generated by persistent unattached KTs using 0.5 μM nocodazole (Sigma-Aldrich) for 16 h. Mitotic controls and *BUB1B*-edited cell clones were imaged by phase-contrast microscopy every 20 min for 36 h using an IN Cell Analyzer 2000 microscope (GE Healthcare, CH, IL, USA) at 37 °C in complete KSF medium. Image processing and quantification were performed in ImageJ.

Premature Chromatid Separation (PCS) analysis

Peripheral blood samples were cultured for 72 h in RPMI 1640 medium with GlutaMAX-I (Gibco) supplemented with 20% fetal bovine serum (Gibco) and stimulated with Phytohaemagglutinin-M (Biological Industries, Israel). Colcemid™ (100 ng/mL, Gibco) was added 90 min before cell harvesting by trypsinization and cells were processed for G-banding with Leishman staining, according to standard protocols. The analysis of PCS in patients

HPC343 and HPC369 carrying missense variants was not performed due to the absence of biological material.

For analysis of PCS levels in cell lines, cells at 90% confluence in T75 flasks were Colcemid™-synchronized for 16 h before harvesting and G-banding processing.

Automatic capture of metaphases from each case was performed using the microscope slide scanning system GSL-120 (CytoVision version 7.4; Leica Biosystems, Baden-Württemberg, Germany). The percentage of metaphases with PCS, corresponding to cells with separate and splayed chromatids with discernible centromeres, involving all or most chromosomes of a metaphase cell, was quantified manually by a certified cytogeneticist.

High-throughput screening of lagging chromosomes

Quantification of lagging chromosomes was performed using 50–500 images of contiguous fields acquired in an IN Cell Analyzer 2000 microscope (GE Healthcare, CH, IL, USA) with a Nikon 40 $\times$ /0.95 NA Plan Fluor objective (binning 2 $\times$ 2) using a large chip CCD Camera (CoolSNAP K4) with a pixel array of 1,024 $\times$ 1,024 (2.7027 pixel/ μm resolution), as previously described [29]. All early to late anaphase figures were classified regarding the presence or absence of lagging chromosomes. Any DAPI-positive material between the two chromosome masses, but distinguishably separated from them, was counted as lagging chromosomes. DNA bridges were excluded from this analysis.

Immunofluorescence

RWPE-1 cells were fixed with 4% paraformaldehyde in PBS (Electron Microscopy Sciences) for 10 min and permeabilized with 0.5% Triton X-100 (Sigma-Aldrich) for another 5 min. BubR1, Cdc20 and ACA were immunostained using the following antibodies: rabbit anti-BubR1 1:1,000; ab70544, Abcam, Cambridge, UK, mouse anti-cdc20 (1:100; sc-5296, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-centromere antibodies (ACA, 1:5,000; kind gift from B. Earnshaw, Wellcome Trust Centre for Cell Biology, University of Edinburgh, Edinburgh, UK). Alexa Fluor 488, 568 and 647 (1:1000, ThermoFisher) were used as secondary antibodies, and DNA was counterstained with 1 $\mu\text{g}/\text{ml}$ DAPI (Sigma-Aldrich).

Quantification of fluorescence intensity at the kinetochores

The fluorescence intensity signal of BubR1 and Cdc20 was measured directly at KTs in a circular ROI (region of interest) using ImageJ and normalized to the intensity signal of ACA in the same ROI. Background fluorescence was measured outside the ROI and subtracted from each

KT. The mean values of all quantified KTs quantified were plotted in a scattered dot plot.

Computational analysis of BubR1 variants

Because there is no experimental structure of the human mitotic checkpoint serine/threonine-protein kinase BubR1 (EC:2.7.11.1) in the Protein Data Bank (<http://www.rcsb.org>), the molecular structure of the wild-type protein was first constructed by homology modeling using MODELLER software [30] (detailed in Supplementary Methods).

The molecular dynamics simulations were performed to assess the molecular dynamics of the BubR1 proteins in explicit solvent at atomistic resolution. The Amber 18.0 simulation package (parm14SB force field) was used to carry out the optimizations and MD simulations (detailed in Supplementary Methods).

The bioinformatic tools MUpro [31] and I-Mutant 2.0 [32] were used to predict the effect of the currently studied variants (BubR1^{R120Q}, BubR1^{I147T}, BubR1^{R244C}, BubR1^{Δ391} and BubR1^{R416Q}) and the control variants (BubR1^{F175G}, BubR1^{F175L}, BubR1^{E413K}) on the structural stability of the protein. Starting from the amino acid sequence data, both web servers use a set of machine learning methods to automatically predict protein stability changes upon single-site mutations. These indicators provide fast, quite accurate (77–80% correct previsions) and quantitative previsions of the effects of the variants.

Results

Rare *BUB1B* variants are recurrently found in early-onset/familial PrCa and other cancers and carriers

of the c.1171_1173del variant share a common ancestor

The frequency of *BUB1B* variants in our 462 HPC cases was assessed by NGS using a customized targeted gene panel (T-NGS). Overall, six patients were carriers of truncating variants (Table 1, Fig. 1), representing 1.3% of the full HPC series and 1.7% of the patients with early-onset disease (<56 years at diagnosis). The variant c.1171_1173del, p.(Glu391del), was identified in five patients (Fig. 1A) and the frameshift variant c.2481del, p.(Gln827HisfsTer13), was found in one patient (Fig. 1B). Three of the five families with the recurrent in-frame variant fulfilled both the early-onset and family history criteria (HPC119, HPC154 and HPC262), representing 3.4% of 89 such families. Segregation with the disease was performed in family HPC262, whose index patient had metastatic disease at diagnosis and the carrier father had two additional primary carcinomas (skin and stomach) eight years after the PrCa diagnosis.

Of the 875 non-cancer controls genotyped using KASP (Kompetitive Allele-Specific PCR) technology, the variant c.1171_1173del was absent in the 459 male controls, suggesting an increased risk for PrCa development for carriers (OR=10.98, CI: 0.61–200.39; *p*=0.074). This ~ten-fold increased risk for PrCa development is strengthened when compared with the 41 carriers among 36,058 male controls from the Non-Finish European

Table 1 List of *BUB1B* germline variants identified in HPC patients

Variant position (GRCh37)	SNP ID	cDNA change	Protein change	MAF ^a	Patient ID	HPC criteria	Other cancers in family ^c (gender, age ^b)
15:40,462,857	rs1349349252	c.359G>A	p.(Arg120Gln)	0.0016%	HPC450	FH	PrCa (83y)
15:40,468,733	rs763623522	c.440T>C	p.(Ile147Thr)	0.0083%	HPC369	Age	None
15:40,488,854	rs778590557	c.1171_1173del	p.(Glu391del)	0.0569%	HPC63	Age	GaCa (M, 75y), LuCa (M, 57y)
					HPC119	Age + FH	PrCa (52y), PrCa (60y), PrCa (74y), PrCa (?)
					HPC154	Age + FH	BrCa (F, 68y), BrCa (F, 63y), CoCa (M, 70y), GaCa (M, 86y) LuCa (F, 21y), LuCa (M, 80y), PrCa (78y), SkCa (F, 58y)
					HPC227	FH	GaCa (M, 55y), PrCa (58y)
					HPC262	Age + FH	PrCa (70y), PrCa (73y) + GaCa (81y) + SkCa (81y)
15:40,488,934	rs763272400	c.1247G>A	p.(Arg416Gln)	0.0031%	HPC343	Age	PaCa (M, 62y)
15:40,504,793	rs865779865	c.2481del	p.(Gln827HisfsTer13)	NR	HPC278	FH	CoCa (M, 77y), CoCa (M, 85y), ReCa (M, 58y)

^aAge stands for early-onset diagnosis of PrCa (<56 years) and "FH" stands for positive family history of PrCa BrCa- Breast cancer, CoCa Colon cancer, GaCa Gastric cancer, LuCa Lung cancer, PaCa Pancreatic cancer, ReCa Rectal cancer, SkCa Skin cancer

^b Minor Allele Frequency (MAF) in male NFE (Non-finish European) obtained from gnomAD; NR—Not reported

^c at diagnosis

^d 1st and 2nd degree relatives

^e Signet ring cell carcinoma

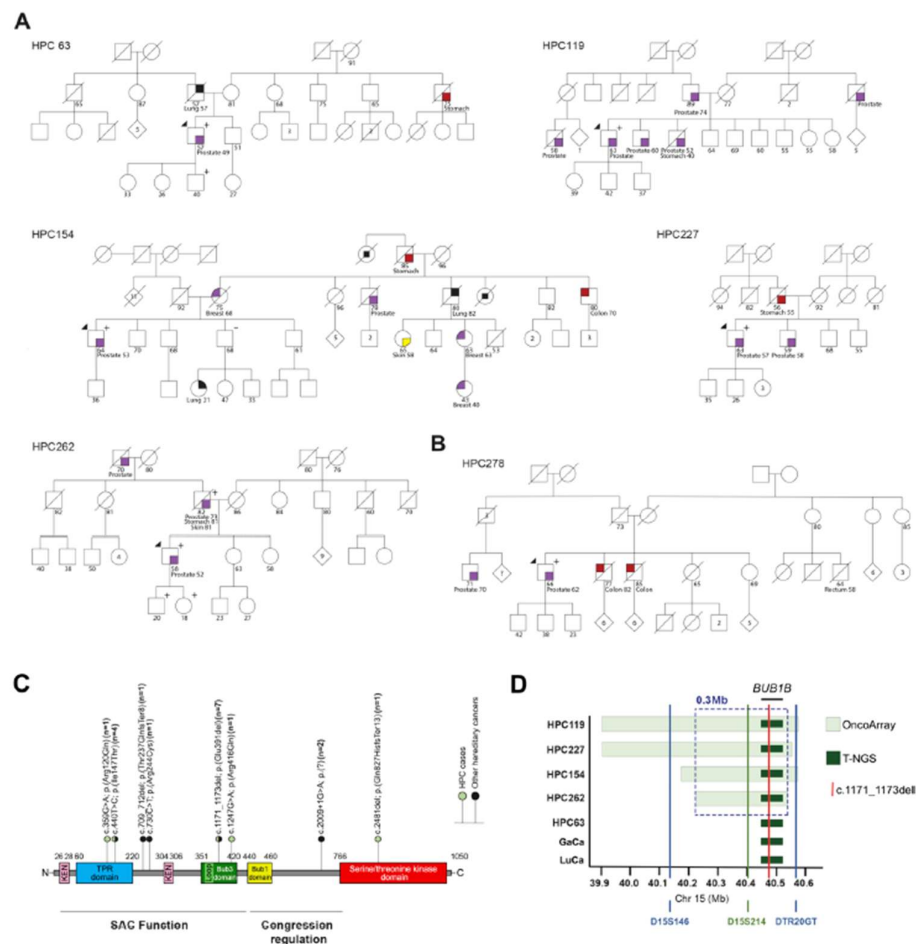


Fig. 1 Profile of the genetic variation found in the *BUB1B* gene and flanking genomic region. **A** Pedigree of the patients carrying the *BUB1B* in-frame variant c.1171_1173del; p.(Glu391del). **B** Pedigree of patient HPC278 carrying the *BUB1B* frameshift variant c.2481del, p.(Gln827HisfsTer13). Carrier patients are labeled with a "plus" mark. Squares represent the males, circles the females and diamonds unknown gender. Deceased individuals are represented by a diagonal line through a symbol, and the affected individuals are highlighted by colored symbols. The index case is indicated by an upper left arrow, and the cancer type and age at diagnosis are indicated whenever known. **C** Spectrum and predicted protein consequences of germline *BUB1B* variants identified in HPC patients and patients fulfilling criteria for other hereditary cancers. For each variant, the full number of carrier patients is shown. The main BubR1 functional domains and known functions are highlighted. The lollipop color code indicates the patient group in which variants were identified. **D** Graphical representation of the 0.3 Mb core haplotype shared by the seven carriers of the *BUB1B* c.1171_1173del variant. The dark-green region represents the shared haplotype identified by analysis of T-NGS data, whereas the light-green region represents the haplotypes identified using the high-density SNP Oncoarray genotype data. Microsatellite analysis corroborated the IBD analysis, showing conservation of the microsatellite marker D15S214, and microsatellite variability up- and downstream of the core haplotype boundaries (markers D15S146 and DTR20GT)

(NFE) population (OR=9.61, CI: 3.78–24.43; $p < 0.0001$; gnomAD Database – <https://gnomad.broadinstitute.org>). The frameshift variant c.2481del is not reported in the gnomAD database.

Additionally, three patients were carriers of missense variants (Table 1 and Fig. S1), increasing the frequency

of *BUB1B* variants to 1.9% in the whole HPC series and to 2.5% among patients with early-onset disease. Two of these variants [c.359G>A, p. (Arg120Gln); and c.440 T>C, p.(Ile147Thr)] are located in the tetratricopeptide (TPR) domain, while the third variant [c.1247G>A, p.(Arg416Gln)], as well as the recurrent

in-frame variant, is located in the Bub3 binding domain (Fig. 1C).

Available data from 1,416 patients screened with the TruSight Cancer Panel at the Department of Laboratory Genetics of IPO Porto for different hereditary cancer syndromes showed nine carriers of *BUB1B* variants (Table 2 and Fig. 1C). The in-frame variant c.1171_1173del, p.(Glu391del), and the missense variant c.440 T>C, p.(Ile147Thr), also identified in HPC patients, were present in two and three patients, respectively, thus being the most frequent *BUB1B* variants identified in this study.

Among the 875 non-cancer controls genotyped using KASP, the variant c.1171_1173del was found in two females, resulting in an ~4.8-fold increased risk for cancer development for carriers (CI: 0.92–24.71; $p=0.052$).

Considering the high frequency of the c.1171_1173del variant in our cohorts, we investigated a potential founder effect by Identical-by-descent (IBD) analysis. Using data from the T-NGS – custom panel for HPC samples and TruSight Cancer panel for samples from patients with other cancers – a shared haplotype harboring the c.1171_1173del variant was identified among all the seven carriers/families, strongly suggesting a common founder origin (Fig. 1D). To verify whether the shared haplotype extended beyond the *BUB1B* gene, we performed IBD analysis using high-density genome-wide small nucleotide polymorphism (SNP) data available for 374 of the 462 HPC cases as part of the PRACTICAL consortium [26]. IBD analysis identified a shared core haplotype of ~0.3 Mb flanking the variant (chr15:40,225,465–40,528,155) in the four carriers profiled, which was not conserved in the 370 non-carriers. Additionally, microsatellite analysis in all carriers and

available healthy relatives (Table S3) showed phased haplotypes in two informative families [family HPC262 (Fig. S1) and the GaCa patient family (Fig. S2)], which revealed a conserved region of ~113 Kb, comprising the region from the marker D15S214 up to the full *BUB1B* gene (Table S3). This region was also conserved in patients HPC63 and HPC119, and the four remaining carriers (HPC227, HPC154, the LuCa patient, and the healthy son of patient HPC63), despite being heterozygous for the marker D15S2014, harbored the allele consistent with the conserved region. These results corroborate the IBD analysis, showing a conserved microsatellite (D15S2014) within the shared haplotype and microsatellite variability up and downstream of the core haplotype (~0.3 Mb) boundaries (D15S146 and DTR20GT) (Fig. 1D), further supporting the existence of a common ancestor.

Both the recurrent in-frame and missense *BUB1B* variants are predicted to lead to protein instability and impair proper MCC assembly

To determine whether the recurrent in-frame variant and the four missense variants, predicted to result in the proteins p.(Glu391del) (BubR1^{Δ391}), p.(Arg120Gln) (BubR1^{R120Q}), p.(Ile147Thr) (BubR1^{I147T}), p.(Arg244Cys) (BubR1^{R244C}), and p.(Arg416Gln) (BubR1^{R416Q}), respectively, may impact the structural stability of the protein, we used the bioinformatic tools Mupro [31] and I-Mutant 2.0 [32]. The predicted free energy changes of two known unstable missense variants, BubR1^{E413K} and BubR1^{F175G} [33–35], and the well-known stable variant BubR1^{F175L} [33, 34], were used as positive and negative cut-off values for interpretation of instability effects. Both bioinformatic tools predict that BubR1^{I147T} greatly destabilizes

Table 2 List of *BUB1B* germline variants identified in non-prostate cancer patients fulfilling criteria for genetic testing

Variant position (GRCh37)	SNP ID	cDNA change	Protein change	MAF ^a	Index cancer history (gender, age ^b)	Other cancers in family ^c (gender, age ^b)
15:40,468,733	rs763623522	c.440 T>C	p.(Ile147Thr)	0.0083%	BrCa (F, 35y) BrCa (F, 58y) PCC (F, 47y)	PrCa (70y) BrCa (F, 42y) None
15:40,476,040	rs992789522	c.709_712del	p.(Thr237GlnfsTer8)	NR	BrCa (F, 32y)	PrCa (55y), OvCa (35y), GaCa (M, 50y)
15:40,476,063	rs867444045	c.730C>T	p.(Arg244Cys)	0.0014%	BrCa (F, 45y)	LuCa (F, 65y)
15:40,488,854	rs778590557	c.1171_1173del	p.(Glu391del)	0.0569%	GaCa* (F, 38y) LuCa (M, 68y)	CoCa (M, 57y), LuCa (M, 75y) LuCa (M, 46y), LuCa (M, 20y)
15:40,498,660	rs1028243319	c.2009+1G>A	p.(?)	NR	BrCa (F, 40y, 44y) GaCa* (F, 34y)	PrCa (70y), PrCa (79y), GaCa (M, 47y) None

BrCa Breast cancer, CoCa Colon cancer, GaCa Gastric cancer, LuCa Lung cancer, OvCa Ovarian cancer, PCC Pheochromocytoma

^a Minor Allele Frequency (MAF) in male NFE (Non-finish European) obtained from gnomAD; NR—Not reported

^b at diagnosis

^c 1st and 2nd degree relatives

* Signet ring cell carcinoma

the protein conformation, while the other missense variants have a rather small effect, if any (Table 3). Mupro also envisages that the in-frame variant BubR1^{Δ391} reduces protein stability, which could then cause protein misfolding, aggregation and degradation.

To provide structural details on the impact of the same variants in the BubR1 protein, we performed Molecular Dynamics (MD) simulations on explicit solvated models of BubR1. The structural analysis was focused on specific regions of the highly conserved N-terminus of BubR1, specifically, in two Lys-Glu-Asn motifs (called KEN-1 and KEN-2 boxes), which have been implicated in Cdc20 binding and are required for effective APC/C inhibition and SAC function [36]; in the tetratricopeptide (TPR) domain, which is essential for SAC function via interaction with Knl-1 [34]; and in the Bub3 binding domain (B3BD) [37]. Figure 2A illustrates the molecular model of BubR1^{WT}, highlighting the TPR and B3BD regions of the four variants under study by superimposition of the various protein structures. The mutants BubR1^{F175G}, BubR1^{F175L} and BubR1^{E413K} were also studied both as structural controls and to validate the computational molecular model. The main structural differences observed in all mutants relative to the BubR1^{WT} are shown in Table 3 (detailed in Tables S4-S7 and Fig.

S3). BubR1^{Δ391} and BubR1^{R416Q} show high fluctuation in the atomic position of the TPR residues, while the B3BD region of BubR1^{I147T} has greater mobility than that of BubR1^{WT} ($\Delta\text{RMSF} > 3 \text{ \AA}$). Regarding the TPR region, the BubR1^{F175G} is known to disrupt the TPR structure and abolish the binding to Knl-1 (herein used as a positive control) [33, 34]. As the substitution of the same amino acid by a leucine (BubR1^{F175L}) maintains the interaction with Knl-1 [33, 34], we used this mutant as a negative control. Our data indicate that the variants BubR1^{Δ391}, BubR1^{I147T}, BubR1^{R244C} and BubR1^{R416Q} have at least two similar structural changes to the BubR1^{F175G}, suggestive of TPR disruption and loss of Knl-1 binding. The BubR1^{Δ391}, BubR1^{I147T}, and BubR1^{R244C} also show variations in the secondary structure, indicating intrinsic disorder of this region in solution. Additionally, the BubR1^{I147T} loses intramolecular hydrogen bonds between the α -helices of TPR, modifying the overall conformation of TPR, which, subsequently, may also interfere with the interaction with Knl-1 (Fig. S3, detailed in Table S7).

Concerning the B3BD, the structural analysis was focused on the *Loop* (368–379 residues) because this region is a direct APC/C binder and promotes stable association of the BubR1:Bub3 proteins with the APC/C. Indeed, in vitro studies have shown that BubR1^{Loop}

Table 3 Predicted effect of the different amino acid substitutions on BubR1 stability and structural protein properties

BubR1 Mutant	MUpuro		I-mutant 2.0		TPR domain				Loop region			Full protein	
	$\Delta\Delta G$ (kcal/mol)	Protein structure stability	$\Delta\Delta G$ (kcal/mol)	Protein structure stability	SASA ($\text{\AA}^2$)	Interhelical distance ($\text{\AA}$)	Δ RMSF ($\text{\AA}$)	Δ secondary structure (%)	SASA ($\text{\AA}^2$)	Δ RMSF ($\text{\AA}$)	Δ secondary structure (%)	SASA ($\text{\AA}^2$)	Δ secondary structure (%)
BubR1 ^{WT}	-	-	-	-	0 ± 103	0.0 ± 0.9	-	-	0 ± 64	-	-	0 ± 1016	-
BubR1 ^{R129Q}	-0.81	decrease	-0.58	decrease	-79 ± 100	1.5 ± 0.8	< 3	-2.1	50 ± 82	< 3	-15.7	607 ± 928	-1.7
BubR1 ^{I147T}	-2.16	decrease	-4.36	decrease	-173 ± 112	1.2 ± 0.8	< 3	22.4	285 ± 76	> 3	-6.2	-52 ± 1017	-0.8
BubR1 ^{R244C}	-0.81	decrease	-0.05	no effect	-451 ± 114	-0.4 ± 0.8	< 3	12.8	-31 ± 76	< 3	20.3	-3201 ± 2523	1.4
BubR1 ^{Δ391}	-1.14	decrease	NM	NM	-270 ± 104	-0.2 ± 0.8	> 3	11.5	94 ± 73	< 3	9.4	-2935 ± 1084	1.2
BubR1 ^{R416Q}	-0.59	decrease	-0.89	decrease	-37 ± 112	-4.9 ± 0.9	> 3	4.9	328 ± 90	< 3	9.9	238 ± 1196	0.9
BubR1 ^{F175G}	-1.93	decrease	-2.26	decrease	-238 ± 103	-4.0 ± 0.8	> 3	-18.1	142 ± 62	< 3	22.4	-1978 ± 1033	1.3
BubR1 ^{F175L}	-0.49	decrease	0.00	no effect	-351 ± 113	-0.2 ± 0.8	< 3	9.4	93 ± 80	< 3	6.8	-754 ± 1373	0.9
BubR1 ^{E413K}	-0.99	decrease	-1.48	decrease	-168 ± 105	-5.6 ± 1.1	> 3	19.3	183 ± 89	> 3	25.3	-3099 ± 1358	1.2

Note: Negative/stable and positive/unstable mutant controls are colored at green and red, respectively, while differences higher than the negative control or statistically valid are highlighted in bold

$\Delta\Delta G$ change in free energy, TPR tetratricopeptide, SASA solvent accessible surface area, RMSF root-mean-square fluctuation, NM not measured

mutants have a reduced ability to inhibit the APC/C [35]. Herein, the BubR1^{E413K} located in the B3BD, was used as a positive control due to its known capability to prevent the Bub3 binding and disrupt SAC signaling [35]. Our results denote some similar structural changes in BubR1^{I147T} and BubR1^{R416Q} variants as the BubR1^{E413K}, suggesting that these mutants may have comparable effects by interfering with the BubR1:Bub3 association. Despite the proximity of the residue deleted by the in-frame variant (E391) to the Loop, BubR1^{Δ391} does not significantly affect the structure of the Loop region. However, BubR1^{Δ391} shows variations in the solvent exposure area and intramolecular bonds of both KEN-1 and KEN-2 residues (Fig. 2B, Tables S4 and S7). Since KEN-1 is essential for the binding with Cdc20 and Mad2 and the KEN-2 promotes the binding of another Cdc20 [35], these modifications suggest that BubR1^{Δ391} could interfere with the recognition or binding of Cdc20 and Mad2 proteins. All mutants show slight changes in the protein secondary structure, with BubR1^{Δ391}, BubR1^{R120Q}, and BubR1^{R244C} being the most similar to the positive controls; however, the overall structure is still comparable to the structure of BubR1^{WT}.

In-frame deletions affecting the BubR1 B3BD reduce overall BubR1 abundance and promote proliferation without compromising its recruitment to the kinetochore
Single nucleotide substitutions have been linked to SAC defects caused by severe or partial loss of BubR1 expression in families carrying *BUB1B* biallelic (MVA patients) or monoallelic variants (parents of MVA patients), respectively [19]. This observation led us to hypothesize that the monoallelic *BUB1B* variants found in our study could contribute to (prostate) cancer predisposition through a similar mechanism. To test this hypothesis, and considering their predicted impact on BubR1 structure in silico, we focused on the most frequent variant identified in our study – the in-frame variant c.1171_1173del, p.(Glu391del). For that purpose, we generated prostate cell models with genome editing for the c.1171_1173del variant derived from non-tumorigenic RWPE-1 cells using homology-directed repair (HDR)-mediated CRISPR/Cas9. Since we found no viable cell clones homozygous for the c.1171_1173del variant, we selected monoclonal populations exhibiting a compound heterozygous genotype containing this in-frame *BUB1B* variant (BubR1^{Δ391/Δ378–385}) to assess variant's pathogenicity. Thus, in addition to c.1171_1173del, all the four clones exhibit a second in-frame variant (c.1133_1156del) with a predicted similar impact (located in BubR1 B3BD but outside the *Loop* region; Fig. S4), from which we selected two (named C1 and C2) to use in the following experiments.

Quantitative analysis of BubR1 expression both at protein and mRNA levels showed a slight decrease (20–30%) in BubR1 expression in all gene-edited cell clones compared to WT cells (Fig. 3A, Fig. S4). To evaluate whether the established cell models could represent the biology of tumors from carriers of the *BUB1B* in-frame variant c.1171_1173del, we performed RNA sequencing in tumor/normal matched prostate tissues from patient HPC63, carrying the germline variant c.1171_1173del, as well as in prostate tissues from patient HPC278, carrying the frameshift variant c.2481del, and from three additional HPC patients negative for known *BUB1B* germline variants. As observed in our in vitro models, RNA sequencing analysis showed decreased *BUB1B* expression in the prostate cancer tissue relative to the normal counterpart in both *BUB1B* carriers, while tumor/normal matched prostate tissues from non-carriers showed an inverse pattern (Fig. 3B). In vitro, decreased *BUB1B* in C1 and C2 clones led to significantly increased proliferation rates compared with WT cells (Fig. 3C), supporting the oncogenic cancer-predisposing role of this variant.

To gain further insights into the biological mechanism underlying the oncogenic behaviour of *BUB1B*-edited cell clones, we performed western-blot analysis of mitotic protein extracts collected upon cell-cycle arrest with nocodazole for 16 h. In line with decreased mRNA levels, C1 and C2 clones showed a very significant decrease in BubR1 protein expression (Fig. 3D) and a moderate and a slight decrease in Cdc20 (two isoforms) and Bub3 expression levels, respectively, suggestive of premature disassembly and degradation of the MCC components. As free MCC levels (not KT-bound) are critical for SAC signaling [13], we investigated whether the “instability” of the MCC was due to improper recruitment of BubR1 and Cdc20 to the KTs. For this purpose, we quantified the expression levels of BubR1 and Cdc20 at KTs in the absence of microtubules (unattached KTs) in randomly selected mitotic cells treated with nocodazole for 4 h. Similar expression levels were observed in edited and control cells (Figs. 3E–F), although edited cells showed a tendency for higher expression of both BubR1 and Cdc20 at KTs, reaching statistical significance in the C2 population.

To assess whether reduced BubR1 protein levels in *BUB1B* gene-edited cell populations may be caused by increased protein degradation, as predicted in silico, we measured the half-life of BubR1 by cycloheximide (CHX) chase in both WT and gene-edited cell clones. We consistently observed lower BubR1 levels in both C1 and C2 cell populations in every time point comparing with WT cells (Fig. 3G and Fig. S5), which are more pronounced after 16 h of CHX treatment (Fig. 3H). This tendency is observed within each assay, despite the variability observed when combining the two experiments. The

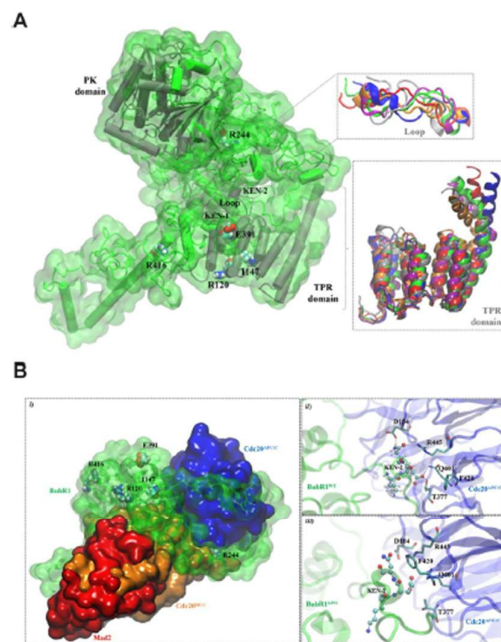


Fig. 2 Conformational BubR1 changes and MCC interactions predicted to result from non-truncating *BUB1B* variants. **A** 3D conformational structure of the BubR1^{WT} model showing the localization of the TPR, Loop, KEN boxes, and PK (pseudokinase) domains, as well as the residues predicted to be affected by the missense and in-frame *BUB1B* variants identified in this study. Changes in the conformation of the Loop and TPR domains are highlighted after the superimposition of the BubR1^{WT} (green) model with the BubR1^{R120Q} (red), BubR1^{I147T} (blue), BubR1^{R244C} (silver), BubR1^{Δ391} (purple) and BubR1^{R416Q} (orange) models. **B** 3D structure of the MCC showing the interaction between BubR1^{WT}, Mad2, and two Cdc20 molecules, as well as the structural position of the residues predicted to be affected by the missense and in-frame *BUB1B* variants identified in this study (i). Close-up of the secondary structure of the KEN-1 motif and surrounding residues showing conformational changes leading to different residue interactions when comparing BubR1^{WT} (ii) and BubR1^{Δ391} (iii) models

same tendency was observed in a 3rd independent clone with the same genotype (C3; Fig. S5).

These results suggest that, as predicted in silico, the recurrent *BUB1B* in-frame deletion c.1171_1173del (BubR1^{Δ391}) compromises the stability of the MCC but not the ability to recruit BubR1-Cdc20 to unattached KTs.

In-frame deletions affecting the BubR1 B3BD impair SAC signaling and trigger chromosomal instability, driving resistance to Taxol treatment

To test SAC function in our cell models, we performed a well-established assay based on the measurement of

mitotic duration in the presence of persistently unattached KTs upon treatment with nocodazole. Our live-cell analysis revealed that C1 and C2 cells exit mitosis significantly earlier (11.6 ± 1.4 h and 10.8 ± 1.1 h, respectively) than WT cells (15.4 ± 1.1 h and 14.9 ± 0.7 h, respectively) (Fig. 4A–B). Moreover, the impact of the *BUB1B* variants on SAC function was as severe as the acute SAC disruption obtained upon Mps1 inhibition (Fig. 4B). Cytogenetic examination and quantification of premature chromatid separation (PCS) showed a significant increase in PCS in both *BUB1B*-edited clones (38–56%) compared to WT cells (32%) (Fig. 4C–D). Similarly, HPC patients carrying the monoallelic *BUB1B* c.1171_1173del variant exhibited 34–54% PCS metaphases compared to 0.75–8.74% found in age-matched male controls (Table S8, Fig. 4C). High percentages of PCS were also observed in patient HPC278, carrying the frameshift variant c.2481del, in patient HPC450, carrying the missense variant c.359G > A, and in the healthy sons of patients HPC63 and HPC262, both carrying the c.1171_1173del variant (Table S8).

The efficacy of taxane-based chemotherapies, currently approved by the United States Food and Drug Administration (FDA) for the treatment of several advanced cancers, including metastatic castration-resistant prostate cancer [38], is limited by intrinsic and acquired drug resistance associated with higher CIN [20, 21]. Therefore, we questioned whether increased PCS in *BUB1B* carriers would be associated with increased CIN and whether CIN levels would correlate with response to Taxol treatment. We observed a dramatic resistance to Taxol-induced growth inhibition and apoptosis in *BUB1B*-edited cells compared with WT cells (Fig. 4E). Complementarily, immunofluorescence analysis revealed a significantly higher percentage of lagging chromosomes in anaphase in *BUB1B*-edited cells (Fig. 4F–G). Altogether, these observations establish a link between BubR1 in-frame deletions affecting B3BD, SAC defects, CIN and Taxol resistance, sustaining the pathogenicity of the recurrent variant c.1171_1173del (BubR1^{Δ391}).

Discussion

In this work, we identified nine carriers of rare germline variants in the *BUB1B* gene among 462 PrCa patients fulfilling the criteria for hereditary prostate cancer. This makes *BUB1B* one of the most frequently mutated genes described in our HPC patients, accounting for 1.9% of the early-onset/familial PrCa patients and 2.5% of the patients with early-onset disease (diagnosis < 56 years). Additionally, 0.6% of the cancer patients fulfilling the criteria for genetic testing for other hereditary cancer syndromes were also carriers of rare *BUB1B* germline

variants, following the *pan*-cancer profile observed for known moderate- to high-penetrance risk genes.

Considering the broad dissemination of NGS technology throughout genetic laboratories worldwide, the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG/AMP) have published guidelines for variant interpretation in genes associated with Mendelian disorders, narrowing the translatability of genetic findings [39]. However, in genes associated with recessive syndromes (caused by two deleterious variants), as is the case of *BUB1B* in MVA, the pathogenicity of monoallelic variants may be harder to clarify, since the effect of isolated variants may not reflect that of variant combination. *BUB1B*, however, has an advantage over other genes in this category since heterozygous *BUB1B* variants are associated with an autosomal dominant trait – PCS (OMIM # 176,430). In fact, fundamental studies have defined a link between loss of BubR1 expression, mitotic checkpoint defects, PCS and CIN [17, 18]. Additionally, both truncating and non-truncating variants in *BUB1B* have been associated with loss of protein expression and mitotic defects, determinants of the MVA phenotype [19, 40, 41]. Nevertheless, despite the potential of spindle checkpoint genes as tumor suppressors and the first validation of a causal link between *BUB1B* variants and lung and colorectal cancer development in BubR1[±] mouse models in 2004 [41], the cancer-predisposing role of *BUB1B* remains largely elusive.

Several studies have explored the functional relevance of BubR1 variants identified in MVA patients, which mainly cluster in the kinase domain (C-terminal) [19, 42]. In our study, aside from the frameshift variant c.2481del and the splicing variant c.2009 + 1G > A, the *BUB1B* variants identified, both in HPC patients and in patients with other cancers, cluster at or near the TPR and B3BD domains (N-terminal). Thus, we have started by investigating the potential consequences of

the identified *BUB1B* variants at the protein level using in silico molecular modeling and molecular dynamics simulations of all non-truncating *BUB1B* variants, using known BubR1 mutants as controls. Our analysis shows that all the identified variants are predicted to lead to mild to severe loss of BubR1 stability and to TPR disruption, probably impairing Knl-1 binding and leading to SAC dysfunction. To validate this assumption, we focused on the most frequent variant identified in our study, and one of the two variants here described, and ever reported, to be recurrently found in cancer patients – c.1171_1173del (BubR1^{Δ391}) – representing 38.9% of all *BUB1B* variant carriers and 55.5% of HPC index patients who carry a *BUB1B* variant. Hahn et al. (2016), in an attempt to clarify the phenotypic implications of two *BUB1B* variants (c.1171_1173del and c.2834G > A) identified in the germline of early-onset colorectal cancer cases, have induced overexpression of both mutants and WT *BUB1B* in HEK293 and HeLa cells and found no differences in protein expression, localization (at KTs), or Bub3 binding between the three cell models [8]. Aiming to look beyond Hahn et al. observations, we used CRISPR/Cas9 to generate clonal cell populations derived from the non-tumorigenic RWPE-1 prostate cells carrying the variant c.1171_1173del (BubR1^{Δ391}). Despite the high success rate of genomic editing by homology-directed repair (~26.8%; detailed in Supplementary Methods), heterozygous (BubR1^{WT/Δ391}) or homozygous (BubR1^{Δ391/Δ391}) clonal populations were not found. Instead, the four clonal populations that persisted to grow harbored a compound heterozygous genotype (BubR1^{Δ391/Δ378–385}) in which the second variant was also in-frame and localized in B3BD (c.1133_1156del). Although speculative, it is possible that, as described for other *BUB1B* truncating variants, the homozygosity for the c.1171_1173del, or its co-occurrence with a truncating variant, are not compatible with life, in

(See figure on next page.)

Fig. 3 Impact of in-frame deletions in BubR1 B3BD on proliferation and mitosis. **A** Quantification of BubR1 expression at both transcript and protein level in BubR1^{WT/WT} (WT) and BubR1^{Δ391/Δ378–385} (C1 and C2) RWPE-1 cells by western-blot and qRT-PCR, respectively. **B** Normalized *BUB1B* expression levels in matched tumor/normal prostate tissues from HPC patients obtained by RNA sequencing. HPC63 and HPC278 are carriers of germline *BUB1B* variants [p.(Glu391del) and p.(Gln827HisfsTer13), respectively] and HPC64, HPC235 and HPC387 are non-carriers of known *BUB1B* variants. **C** Growth rate assessed by the MTT assay at 96 h in culture (**p* < 0.05; two tailed, paired *t* test). **D** Protein levels from mitotic cells collected by shake-off upon 16 h of nocodazole treatment. Cell lysates from WT and C1/C2 cell populations were immunoblotted for BubR1 (targeting B3BD), Cdc20 and Bub3. β-actin was used as a loading control. **E–F** Subcellular localization (**E**) and quantification (**F**) of BubR1 and Cdc20 at KT, normalized to ACA levels, upon 4 h of nocodazole treatment. Scale bars, 5 μm. The pool of three independent experiments (45–47 cells) from each cell population is shown (ns- no statistical significance (*p* > 0.05), **p* < 0.05; two tailed, unpaired *t* test). **G–H** BubR1 expression levels were assessed by western blot before (0 h) and after blocking de novo protein synthesis with cycloheximide (CHX) for 4 h, 8 h and 16 h (**G**), and quantified by densitometry analysis (**H**). Data were obtained from two independent experiments. A representative blot is shown (**G**), and differences between WT and clonal cell populations are depicted for each WT-paired clonal population after 16h of CHX treatment (**H**). The % of BubR1 expression was obtained by normalizing BubR1 levels to those of the corresponding β-actin and adjusting to the expression levels obtained before CHX treatment (at 0h). For comparison, different colored circles highlight matched clone/WT pairs from the same assay

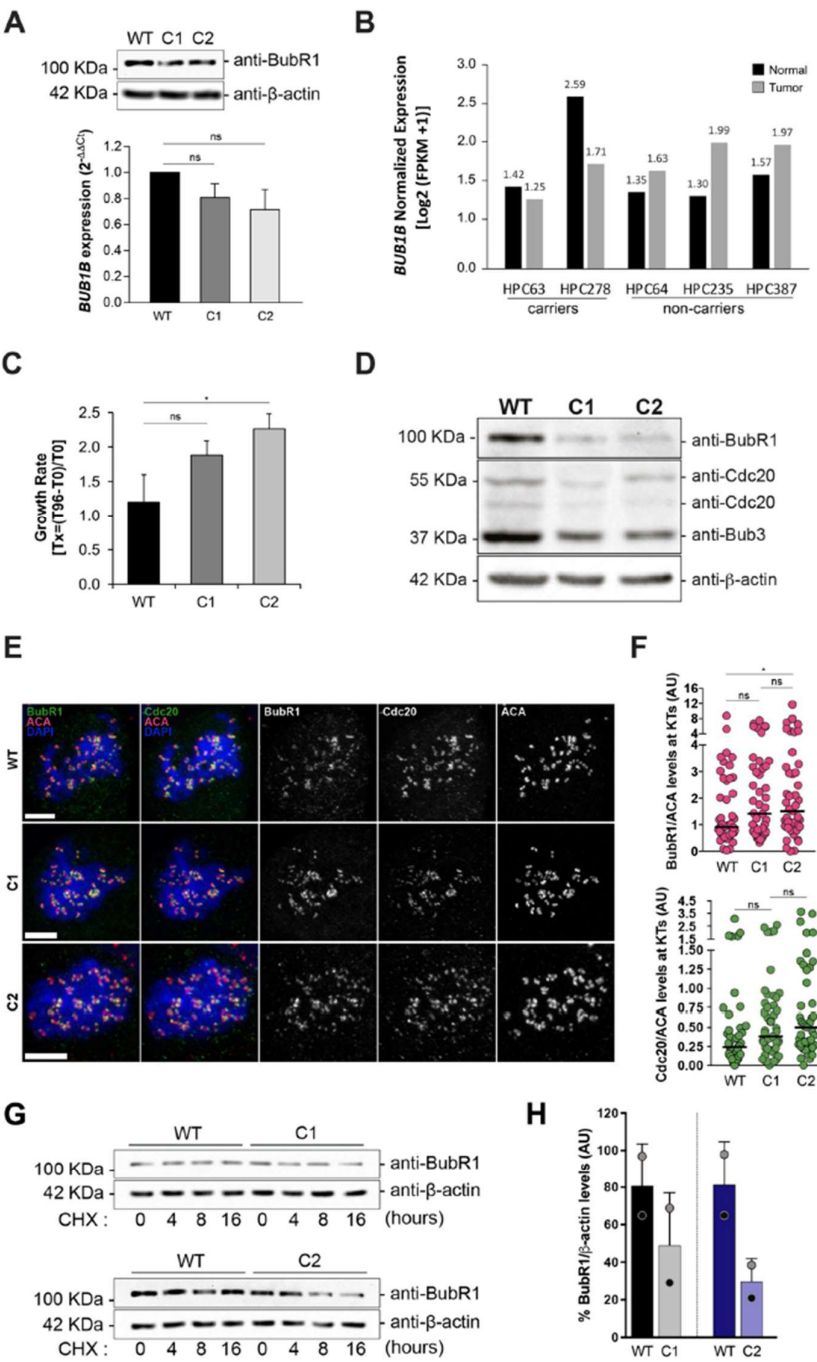


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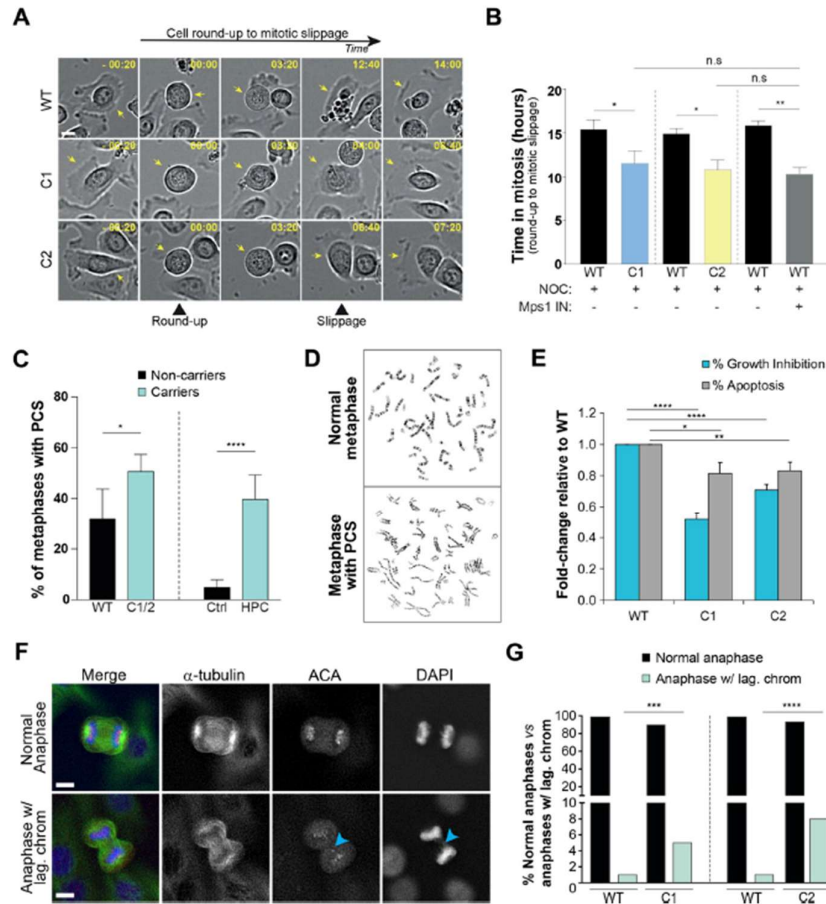


Fig. 4 *BUB1B* in-frame variants in B3BD impair SAC robustness leading to PCS and Taxol-resistant chromosomal instability. **A** Representative images of phase contrast microscopy time-series illustrating mitotic entry (cell round-up), mitotic arrest by nocodazole treatment and mitotic exit in WT and *BUB1B*-edited cells (C1 and C2). Images were acquired every 20 min. Scale bar, 10 μm. Time, hr:min. **B** Quantification of mitotic duration of the cells represented in A. Bars indicate mean values and the error bars represent the SD from three independent experiments (* $p < 0.05$, ** $p < 0.01$, two tailed, unpaired t test). **C** Bar plot of the frequency of metaphase cells with PCS comparing *BUB1B*-edited and WT cells (left) and human blood metaphase cells in age-matched male controls compared to HPC patients carrying the *BUB1B* c.1171_1173del variant (right). Error bars represent the SD of the mean of three independent experiments (* $p < 0.05$, **** $p < 0.0001$, two tailed, unpaired t test). At least 100 metaphase cells were analyzed per condition. **D** Representative images of normal metaphase and PCS events in carriers of the *BUB1B* c.1171_1173del variant. **E** Impact on growth inhibition and apoptosis after 96 h of treatment with Taxol (10 nM; * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0005$, two tailed, paired t -test). **F** Representative images of anaphase cells immunostained with antibodies against α-tubulin and centromere proteins (ACA), depicting normal anaphase and chromosome missegregation in anaphase (lagging chromosome) highlighted by a blue arrow. DNA was counterstained with DAPI. Scale bars, 10 μm. **G** Quantification of the percentage of normal anaphases vs anaphases with lagging chromosomes from a pool of six independent experiments (*** $p < 0.001$, **** $p < 0.0001$, Chi-squared test)

which case a pathogenic role would be attributed to the c.1171_1173del. On the other hand, the absence of heterozygous cell populations ($BubR1^{WT/\Delta391}$) may indicate a technical issue related with the CRISPR/Cas9 approach used. It is possible that finetuning the

Cas9 activity or, alternatively, using a high fidelity Cas9 would improve monoallelic gene-editing [43, 44]. However, considering the recessive nature of *BUB1B* variants and the fine regulation of *BUBR1* activity, with a barely measurable phenotypic impact observed in

the studied compound heterozygous cell populations (BubR1^{Δ391/Δ378–385}), it is also possible that monoallelic gene-editing would result in unperceived phenotypic changes in the timeframe of these assays.

The established cell models validate the observations from Hahn et al. [8] by showing that in cells carrying the variant c.1171_1173del, both BubR1 and Cdc20 are efficiently recruited to unattached KTs, yet we further show that this is not sufficient to ensure proper SAC function and chromosome segregation fidelity, probably as a result of a decrease in both protein expression and stability of KT-independent MCC. As a consequence, *BUB1B*-edited cells showed an increase in both PCS and lagging chromosomes, markers of CIN [12, 17], which correlated with decreased sensitivity to Taxol-induced growth inhibition, as observed in other cancer models [20, 45, 46]. Although we cannot exclude possible cumulative effects of the two in-frame variants present in our in vitro cell models, in vitro PCS levels are consistent with increased PCS levels observed in lymphocytes derived from *BUB1B* carriers and with the autosomal dominant effect of heterozygous *BUB1B* variants described for the PCS trait, strongly suggesting that the variant c.1171_1173del is sufficient to trigger oncogenic transformation. Additionally, *BUB1B*-edited clonal cell populations showed a partial to severe loss of *BUB1B* transcripts, recapitulating the observations from RNAseq analysis of tumor samples from patients carrying *BUB1B* variants, and also a severe loss of protein abundance, respectively, leading to proliferation-prone premature mitotic exit in a checkpoint-dependent manner, similar to what has been described for MVA variants [19] and supporting the hypomorphic nature of the variant c.1171_1173del. Although increased *BUB1B* expression has been described as a common mechanism underlying carcinogenesis in different sporadic cancer types [47–49], our study shows that a different oncogenic-driver underlying mechanism operates in cells with decreased *BUB1B* expression triggered by a rare monoallelic variant. Moreover, our observation of decreased Taxol sensitivity in *BUB1B*-edited cells is in line with the finetuned balance between CIN levels and cell fate, where high CIN levels compromise response to Taxol-based therapies [45, 46], potentially highlighting the ineffectiveness of Taxol-based therapies for the treatment of carcinomas arising in carriers of “deleterious” *BUB1B* variants.

Notwithstanding the identification of a core haplotype of 0.3 Mb among carriers of the variant c.1171_1173del, sustaining the hypothesis of a founder effect in northern Portugal, it is possible that the high fluctuation in the percentage of PCS among carriers (19–70%) is related to genetic variation outside the boundaries of the shared haplotype, as reported for different haplotypes found in

Japanese families with PCS syndrome [18]. Further studies assessing the prevalence of carriers and the corresponding haplotypes across diverse genetic backgrounds holds immense promise in elucidating the origins of this variant and expanding our comprehension of its pivotal role in driving cancer development.

Conclusions

Our data provide solid evidence that monoallelic *BUB1B* variants are sufficient to cause a large spectrum of sub-clinical progressive chromosomal instability that predisposes carriers to (prostate) cancer development, supporting the classification of the variants identified in this study as “likely pathogenic” and pinpointing *BUB1B* as a (*pan*)cancer predisposing gene. Altogether, our studies suggest that patients carrying the variant c.1171_1173del, or potentially other “deleterious” variants in the *BUB1B* gene, may benefit from pretherapeutic assessment of CIN/PCS to optimize treatment stratification and clinical trial design.

Abbreviations

ACMG/AMP	American College of Medical Genetics and Genomics and the Association for Molecular Pathology
B3BD	Bub3 binding domain
BubR1	Budding uninhibited by benzimidazole-related 1
CIN	Chromosomal instability
HPC	Early-onset/familial (hereditary) PrCa
KT	Kinetochore
MVA	Mosaic Variegated Aneuploidy
MCC	Mitotic checkpoint complex
PCS	Premature Chromatid Separation
SAC	Spindle Assembly Checkpoint
TPR	Tetratricopeptide (domain)

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12929-024-01056-z>.

Additional file 1. Supplementary Methods.

Additional file 2. Supplementary Tables (Tables S1–S8).

Additional file 3. Supplementary Figures (Figures S1–S5).

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Not applicable.

Authors' contributions

M.P.S. and L.T.F. established the in vitro cell models with *BUB1B* gene editing, performed in vitro experiments and KASP genotyping, and wrote the manuscript. N.F.B. performed computational analysis of BubR1 variants. L.T. performed Premature Chromatid Separation (PCS) analysis in both patients and in vitro cell populations. A.B. performed haplotype analyses using T-NGS and SNP OncoArray genotype data, and analysed RNA sequencing data. M.P. performed microsatellite haplotype analysis. M.C. participated in T-NGS and gene-editing optimization. A.R. and M.S. participated in RNA sequencing. J.V. participated in the validation of *BUB1B* gene editing. C.Pa. and G.M. participated in the establishment of the in vitro cell models with *BUB1B* gene editing. C.Pl. and A.P. performed NGS analysis with the TruSight Cancer Panel in non-prostate cancer patients fulfilling criteria for genetic testing. J.S. was involved in the recruitment of control samples for PCS analysis. R.H. performed histopathological analysis of FFPE tissues. S.M. and M.R.T. were involved in

patient recruitment and genetic counselling for both patients and all interested relatives. H.M. supervised time-lapse microscopy and high-throughput screening of lagging chromosomes. P.P. designed and supervised the study, participated in T-NGS of both germline and FFPE DNA, and in RNA sequencing, and revised the manuscript.

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Availability of data and materials

All data and associated protocols are included in the Manuscript and Supplementary Information and available to the readers. Cell lines generated in this study are available upon request.

Declarations

Ethics approval and consent to participate

Patient samples were collected under ethics approval with reference CES 38-010 revised by the IPO Porto Ethics Committee. Informed consent was obtained from all subjects involved in the study.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

¹Cancer Genetics Group, IPO Porto Research Center (CI-IPOP) / RISE@CI-IPOP (Health Research Network), Portuguese Oncology Institute of Porto (IPO Porto) / Porto Comprehensive Cancer Center, Porto, Portugal. ²LAQV, REQUIMTE, Department of Chemistry and Biochemistry, Faculty of Sciences, University of Porto, Porto, Portugal. ³Department of Laboratory Genetics, Portuguese Oncology Institute of Porto (IPO Porto) / Porto Comprehensive Cancer Center, Porto, Portugal. ⁴Department of Immunology, Portuguese Oncology Institute of Porto (IPO Porto) / Porto Comprehensive Cancer Center, Porto, Portugal. ⁵Department of Pathology, Portuguese Oncology Institute of Porto (IPO Porto) / Porto Comprehensive Cancer Center, Porto, Portugal. ⁶Chromosome Instability & Dynamics Group, Instituto de Investigação e Inovação em Saúde, University of Porto / Porto Comprehensive Cancer Center, Porto, Portugal. ⁷Cell Division Group, Experimental Biology Unit, Department of Biomedicine, Faculty of Medicine, University of Porto, Porto, Portugal. ⁸Instituto de Biologia Molecular e Celular, University of Porto, Porto, Portugal. ⁹School of Medicine and Biomedical Sciences (ICBAS), University of Porto, Porto, Portugal.

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References

- Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA Cancer J Clin*. 2021;71:209–49. <https://doi.org/10.3322/caac.21660>.
- Brandão A, Paulo P, Teixeira MR. Hereditary predisposition to prostate cancer: From genetics to clinical implications. *Int J Mol Sci*. 2020;21:5036. <https://doi.org/10.3390/ijms21145036>.
- Maia S, Cardoso M, Paulo P, Pinheiro M, Pinto P, Santos C, et al. The role of germline mutations in the BRCA1/2 and mismatch repair genes in men ascertained for early-onset and/or familial prostate cancer. *Fam Cancer*. 2016;15:111–21. <https://doi.org/10.1007/s10689-015-9832-x>.
- Maia S, Cardoso M, Pinto P, Pinheiro M, Santos C, Peixoto A, et al. Identification of two novel HOXB13 germline mutations in Portuguese prostate cancer patients. *PLoS ONE*. 2015;10: e0132728. <https://doi.org/10.1371/journal.pone.0132728>.
- Paulo P, Maia S, Pinto C, Pinto P, Monteiro A, Peixoto A, et al. Targeted next generation sequencing identifies functionally deleterious germline mutations in novel genes in early-onset/familial prostate cancer. *PLoS Genet*. 2018;14: e1007355. <https://doi.org/10.1371/journal.pgen.1007355>.
- Feng B, Chang G, Zhang Q, Li X, Tang Y, Gu S, et al. A novel CEP57 variant associated with mosaic variegated aneuploidy syndrome in a Chinese female presenting with short stature, microcephaly, brachydactyly, and small teeth. *Mol Genet Genomic Med*. 2022;10:1951. <https://doi.org/10.1002/mgg3.1951>.
- García-Castillo H, Vázquez-Velázquez AI, Rivera H, Barros-Núñez P. Clinical and genetic heterogeneity in patients with mosaic variegated aneuploidy: Delineation of clinical subtypes. *Am J Med Genet A*. 2008;146A:1687–95. <https://doi.org/10.1002/ajmg.a.32315>.
- Hahn MM, Vreede L, Bemelmans SASA, van der Looij E, van Kessel AG, Schackert HK, et al. Prevalence of germline mutations in the spindle assembly checkpoint gene BUB1B in individuals with early-onset colorectal cancer. *Genes Chromosomes Cancer*. 2016;55:855–63. <https://doi.org/10.1002/gcc.22385>.
- Roberts NJ, Norris AL, Petersen GM, Bondy ML, Brand R, Gallinger S, et al. Whole genome sequencing defines the genetic heterogeneity of familial pancreatic cancer. *Cancer Discov*. 2016;6:166–75. <https://doi.org/10.1158/2159-8290.CD-15-0402>.
- Pritchard AL, Johansson PA, Nathan V, Howie M, Symmons J, Palmer JM, et al. Germline mutations in candidate predisposition genes in individuals with cutaneous melanoma and at least two independent additional primary cancers. *PLoS ONE*. 2018;13: e0194098. <https://doi.org/10.1371/journal.pone.0194098>.
- Orr B, Godek KM, Compton D. Aneuploidy. *Curr Biol*. 2015;25:R538–42. <https://doi.org/10.1016/j.cub.2015.05.010>.
- Bakhoum SF, Swanton C. Chromosomal instability, aneuploidy, and cancer. *Front Oncol*. 2014;4:161. <https://doi.org/10.3389/fonc.2014.00161>.
- Sudakin V, Chan GKT, Yen TJ. Checkpoint inhibition of the APC/C in HeLa cells is mediated by a complex of BUBR1, BUB3, CDC20, and MAD2. *J Cell Biol*. 2001;154:925–36. <https://doi.org/10.1083/jcb.200102093>.
- Heinrich S, Geissen EM, Kamenz J, Trautmann S, Widmer C, Drewe P, et al. Determinants of robustness in spindle assembly checkpoint signalling. *Nat Cell Biol*. 2013;15:1328–39. <https://doi.org/10.1038/ncb2864>.
- Giam M, Rancati G. Aneuploidy and chromosomal instability in cancer: A jackpot to chaos. *Cell Div*. 2015;10:3. <https://doi.org/10.1186/s13008-015-0009-7>.
- Ochiai H, Miyamoto T, Kanai A, Hosoba K, Sakuma T, Kudo Y, et al. TALEN-mediated single-base-pair editing identification of an intergenic mutation upstream of BUB1B as causative of PCS (MVA) syndrome. *Proc Natl Acad Sci*. 2014;111:1461–6. <https://doi.org/10.1073/pnas.1317008111>.
- Matsuura S, Ito E, Tauchi H, Komatsu K, Ikeuchi T, Kajii T. Chromosomal instability syndrome of total premature chromatid separation with mosaic variegated aneuploidy is defective in mitotic-spindle checkpoint. *Am J Hum Genet*. 2000;67:483–6. <https://doi.org/10.1086/303022>.
- Matsuura S, Matsumoto Y, Morishima K, Izumi H, Matsumoto H, Ito E, et al. Monoallelic BUB1B mutations and defective mitotic-spindle checkpoint in seven families with premature chromatid separation (PCS) syndrome. *Am J Med Genet A*. 2006;140:358–67. <https://doi.org/10.1002/ajmg.a.31069>.
- Suijkerbuijk SJE, Van Osch MHJ, Bos FL, Hanks S, Rahman N, Kops GJPL. Molecular causes for BUBR1 dysfunction in the human cancer predisposition syndrome mosaic variegated aneuploidy. *Cancer Res*. 2010;70:4891–900. <https://doi.org/10.1158/0008-5472.CAN-09-4319>.
- Swanton C, Nicke B, Schuett M, Eklund AC, Ng C, Li Q, et al. Chromosomal instability determines taxane response. *Proc Natl Acad Sci*. 2009;106:8671–6. <https://doi.org/10.1073/pnas.0811835106>.
- Scribano CM, Wan J, Esbona K, Tucker JB, Lasek A, Zhou AS, et al. Chromosomal instability sensitizes patient breast tumors to multipolar divisions

- induced by paclitaxel. *Sci Transl Med*. 2021;13:eabd4811. <https://doi.org/10.1126/scitranslmed.abd4811>.
22. Paulo P, Cardoso M, Brandão A, Pinto P, Falconi A, Pinheiro M, et al. Genetic landscape of homologous recombination repair genes in early-onset/familial prostate cancer patients. *Genes Chromosomes Cancer*. 2023. <https://doi.org/10.1002/gcc.23190>.
 23. Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden TL. Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics*. 2012;18:134. <https://doi.org/10.1186/1471-2105-13-134>.
 24. Browning SR, Browning BL. Rapid and accurate haplotype phasing and missing-data inference for whole-genome association studies by use of localized haplotype clustering. *Am J Hum Genet*. 2007;81:1084–97. <https://doi.org/10.1086/521987>.
 25. Browning BL, Browning SR. Improving the accuracy and efficiency of identity-by-descent detection in population data. *Genetics*. 2013;194:459–71. <https://doi.org/10.1534/genetics.113.150029>.
 26. Brandão A, Paulo P, Maia S, Pinheiro M, Peixoto A, Cardoso M, et al. The CHEK2 variant c.349A>G is associated with prostate cancer risk and carriers share a common ancestor. *Cancers*. 2020;12:3254. <https://doi.org/10.3390/cancers12113254>.
 27. Cardoso M, Maia S, Paulo P, Teixeira MR. Oncogenic mechanisms of HOXB13 missense mutations in prostate carcinogenesis. *Oncoscience*. 2016;3:288–96. <https://doi.org/10.18632/oncoscience.322>.
 28. Mesquita D, Barros-Silva JD, Santos J, Skotheim RL, Lothe RA, Paulo P, et al. Specific and redundant activities of ETV1 and ETV4 in prostate cancer aggressiveness revealed by co-overexpression cellular contexts. *Oncotarget*. 2015;6:5217–36. <https://doi.org/10.18632/oncotarget.2847>.
 29. Ferreira LT, Orr B, Rajendraprasad G, Pereira AJ, Lemos C, Lima JT, et al. α -Tubulin dephosphorylation impairs mitotic error correction by suppressing MCAK centromeric activity. *J Cell Biol*. 2020;219: e201910064. <https://doi.org/10.1083/jcb.201910064>.
 30. Martí-Renom MA, Stuart AC, Fiser A, Sánchez R, Melo F, Sali A. Comparative protein structure modeling of genes and genomes. *Annu Rev Biophys Biomol Struct*. 2000;29:291–325. <https://doi.org/10.1146/annurev.biophys.29.1.291>.
 31. Cheng J, Randall A, Baldi P. Prediction of protein stability changes for single-site mutations using support vector machines. *Proteins*. 2006;62:1125–32. <https://doi.org/10.1002/prot.20810>.
 32. Capriotti E, Fariselli P, Casadio R. I-Mutant2.0: predicting stability changes upon mutation from the protein sequence or structure. *Nucleic Acids Res*. 2005;33:W306–310. <https://doi.org/10.1093/nar/gki375>.
 33. Lara-Gonzalez P, Scott MIF, Diez M, Sen O, Taylor SS. BubR1 blocks substrate recruitment to the APC/C in a KEN-box-dependent manner. *J Cell Sci*. 2011;124(Pt 24):4332–45. <https://doi.org/10.1242/jcs.094763>.
 34. Kiyomitsu T, Obuse C, Yanagida M. Human Blinkin/AF15q14 is required for chromosome alignment and the mitotic checkpoint through direct interaction with Bub1 and BubR1. *Dev Cell*. 2007;13:663–76. <https://doi.org/10.1016/j.devcel.2007.09.005>.
 35. Overlack K, Bange T, Weissmann F, Faesen AC, Maffini S, Primorac I, et al. BubR1 Promotes Bub3-Dependent APC/C Inhibition during Spindle Assembly Checkpoint Signaling. *Curr Biol*. 2017;27:2915–2927.e7. <https://doi.org/10.1016/j.cub.2017.08.033>.
 36. Elowe S, Dulla K, Uldschmid A, Li X, Dou Z, Nigg EA. Uncoupling of the spindle-checkpoint and chromosome-congression functions of BubR1. *J Cell Sci*. 2010;123(Pt 1):84–94. <https://doi.org/10.1242/jcs.056507>.
 37. Taylor SS, Ha E, McKeon F. The human homologue of Bub3 is required for kinetochore localization of Bub1 and a Mad3/Bub1-related protein kinase. *J Cell Biol*. 1998;142:1–11. <https://doi.org/10.1083/jcb.142.1.1>.
 38. Ojima I, Lichtenthal B, Lee S, Wang C, Wang X. Taxane anticancer agents: a patent perspective. *Expert Opin Ther Pat*. 2016;26:1–20. <https://doi.org/10.1517/13543776.2016.1111872>.
 39. Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet Med*. 2015;15:405–24. <https://doi.org/10.1038/gim.2015.30>.
 40. Dai W, Wang Q, Liu T, Swamy M, Fang Y, Xie S, et al. Slippage of mitotic arrest and enhanced tumor development in mice with BubR1 haploinsufficiency. *Cancer Res*. 2004;64:440–5. <https://doi.org/10.1158/0008-5472.can-03-3119>.
 41. Hanks S, Coleman K, Reid S, Plaja A, Firth H, Fitzpatrick D, et al. Constitutional aneuploidy and cancer predisposition caused by biallelic mutations in BUB1B. *Nat Genet*. 2004;36:1159–61. <https://doi.org/10.1038/ng1449>.
 42. Sieben CJ, Jegannathan KB, Nelson GG, Sturmlechner I, Zhang C, Van Deursen WH, et al. BubR1 allelic effects drive phenotypic heterogeneity in mosaic-variegated aneuploidy progeria syndrome. *J Clin Invest*. 2020;130:171–88. <https://doi.org/10.1172/JCI126863>.
 43. Kawamata M, Suzuki HI, Kimura R, Suzuki A. Optimization of Cas9 activity through the addition of cytosine extensions to single-guide RNAs. *Nat Biomed Eng*. 2023;7:672–91. <https://doi.org/10.1038/s41551-023-01011-7>.
 44. Idoko-Akoh A, Taylor L, Sang HM, McGrew MJ. High fidelity CRISPR/Cas9 increases precise monoallelic and biallelic editing events in primordial germ cells. *Sci Rep*. 2018;8:15126. <https://doi.org/10.1038/s41598-018-33244-x>.
 45. Janssen A, Kops GJPL, Medema RH. Elevating the frequency of chromosome mis-segregation as a strategy to kill tumor cells. *Proc Natl Acad Sci U S A*. 2009;106:19108–13. <https://doi.org/10.1073/pnas.0904343106>.
 46. Replogle JM, Zhou W, Amaro AE, McFarland JM, Villalobos-Ortiz M, Ryan J, et al. Aneuploidy increases resistance to chemotherapeutics by antagonizing cell division. *Proc Natl Acad Sci U S A*. 2020;117:30566–76. <https://doi.org/10.1073/pnas.2009506117>.
 47. Ando K, Kakeji Y, Kitao H, Iimori M, Zhao Y, Yoshida R, et al. High expression of BUBR1 is one of the factors for inducing DNA aneuploidy and progression in gastric cancer. *Cancer Sci*. 2010;101:639–45. <https://doi.org/10.1111/j.1349-7006.2009.01457.x>.
 48. Maciejczyk A, Szelachowska J, Czapiga B, Matkowski R, Halon A, Györfy B, et al. Elevated BUBR1 expression is associated with poor survival in early breast cancer patients: 15-year follow-up analysis. *J Histochem Cytochem*. 2013;61:330–9. <https://doi.org/10.1369/0022155413480148>.
 49. Qiu J, Zhang S, Wang P, Wang H, Sha B, Peng H, et al. BUB1B promotes hepatocellular carcinoma progression via activation of the mTORC1 signaling pathway. *Cancer Med*. 2020;9:8159–72. <https://doi.org/10.1002/cam4.3411>.

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Supplementary Information

Supplementary Methods

BUB1B monoallelic germline variants contribute to prostate cancer predisposition
by triggering chromosomal instability

Maria P. Silva^{1,#}, Luísa T. Ferreira^{1,#}, Natércia F. Brás², Lurdes Torres^{1,3}, Andreia Brandão¹, Manuela Pinheiro¹, Marta Cardoso¹, Adriana Resende^{1,3}, Joana Vieira^{1,3}, Carlos Palmeira⁴, Gabriela Martins⁴, Miguel Silva^{1,3}, Carla Pinto^{1,3}, Ana Peixoto^{1,3}, João Silva^{1,3}, Rui Henrique⁵, Sofia Maia¹, Helder Maiato^{6,7,8}, Manuel R. Teixeira^{1,3,9,&}, Paula Paulo^{1,&*}

¹Cancer Genetics Group, IPO Porto Research Center (CI-IPOP) / RISE@CI-IPOP (Health Research Network), Portuguese Oncology Institute of Porto (IPO Porto) / Porto Comprehensive Cancer Center, Porto, Portugal;

²LAQV, REQUIMTE, Department of Chemistry and Biochemistry, Faculty of Sciences, University of Porto, Porto, Portugal;

³Department of Laboratory Genetics, Portuguese Oncology Institute of Porto (IPO Porto) / Porto Comprehensive Cancer Center, Porto, Portugal;

⁴Department of Immunology, Portuguese Oncology Institute of Porto (IPO Porto) / Porto Comprehensive Cancer Center, Porto, Portugal;

⁵Department of Pathology, Portuguese Oncology Institute of Porto (IPO Porto) / Porto Comprehensive Cancer Center, Porto, Portugal;

⁶Chromosome Instability & Dynamics Group, Instituto de Investigação e Inovação em Saúde, University of Porto / Porto Comprehensive Cancer Center, i3S, Porto, Portugal;

⁷Cell Division Group, Experimental Biology Unit, Department of Biomedicine, Faculty of Medicine, University of Porto, Porto, Portugal;

⁸Instituto de Biologia Molecular e Celular, University of Porto, Porto, Portugal;

⁹School of Medicine and Biomedical Sciences (ICBAS), University of Porto, Porto, Portugal.

#,& Equal contributions

*Corresponding author, Paula Paulo

Email: paula.paulo@ipopoporto.min-saude.pt

Targeted next-generation sequencing (T-NGS)

Variants with a frequency above 10% in the whole dataset, a minor allele frequency (MAF) >0.1% in the non-Finnish European population (NFE; gnomAD database [1]; <https://gnomad.broadinstitute.org>) or in the Iberian population (IBS, 1000G phase III project database [2]) or predicted to be synonymous were excluded. Additionally, non-synonymous variants predicted to be deleterious by less than 9 of the 11 pathogenicity predictors (SIFT, Polyphen2-HDIV, LRT, MutationTaster2, MutationAssessor, FATHMM, MetaSVM, MetaLR, CADD, VEST4, and MutPred) or a conservation score less than 0.5 in two of the four conservation tools (PhyloP, phastCons, GERP++, and SiPhy), all available at dbNSFP (<http://database.liulab.science/dbNSFP#database> [3]), were excluded. All the described *BUB1B* variants were validated by Sanger sequencing.

Analysis of RNA sequencing data from FFPE tumor samples

The analysis of *BUB1B* expression was performed according to the protocol described by Pertea et al. (2016) [4]. Briefly, the raw sequence reads were aligned to the human reference genome (GRCh38) using HISAT2 v2.2.1 [5]. The HISAT2 index for the reference genome was built using the same reference genome. The resulting SAM files were converted to sorted and indexed BAM files using Samtools v1.11 [6]. The alignments were assembled into full and partial transcripts and quantified using StringTie v2.1.4 [4]. The reference annotation file (Homo_sapiens.GRCh38.108.gtf) obtained from Ensembl was used as a guide for transcript assembly. The expression levels of each gene and transcript were quantified as FPKM (fragments per kilobase of transcript per million mapped reads).

Generation of prostate *in vitro* cell models carrying the BubR1^{A391} variant

The design of gRNAs targeting *BUB1B* exon nine, and the HDR donor template was performed using the Alt-R HDR Design Tool from IDT (<https://www.idtdna.com/pages/tools/alt-r-crispr-hdr-design-tool>). The HDR donor included the *BUB1B* in-frame deletion and homologous arms with 40 bp in each side of the variant. A synonymous variant (G>A) was also introduced to modify the PAM and prevent Cas9 recutting of the target after gene editing. The sequences of the gRNAs and HDR donor template used are listed in **Table S8**.

For paired nicking experiments, each RNP-bound gRNA was formed separately, and two RNPs were mixed at an equal molar ratio prior to adding to the cells at the time of transfection. For cationic lipid delivery of RNP complexes, we used the CRISPRMAX reagent (Thermo Fisher Scientific), and the reverse transfection method was used for delivery to RWPE-1 cells in a 6-well plate. To increase the activity of the homology-directed repair (HDR) system, after 48 h, cells were collected, and 5×10^5 cells were seeded in a 6-well plate for cell cycle synchronization for 16 h with nocodazole ($0.033 \mu\text{M}$, Sigma-Aldrich, Burlington, MA, USA), followed by 3 h with aphidicolin ($2 \mu\text{g/mL}$, Sigma-Aldrich) [7,8]. The efficiency of enrichment for cells in S+G2/M was evaluated by flow cytometry with propidium iodide staining, as described by others [9]. Synchronized cells were transfected with $7.5 \mu\text{g}$ (180 pmol) of Alt-R HDR template donor (from IDT) containing the *BUB1B* c.1171_1173del variant with Xfect transfection reagent (Clontech, Mountain View, CA, USA) according to the manufacturer's instructions. An Alt-R HDR enhancer (3 mM , from IDT) was co-delivered with the Alt-R HDR template to the cells to a final concentration of $30 \mu\text{M}$ [10]. The 6-well plate was transferred to a 32°C , 5% CO_2 incubator (cold-shock) for 4 h [11], after which both nanoparticle complexes and HDR enhancer were removed from cells and replaced by KSF complete medium containing $30 \mu\text{M}$ HDR enhancer only. The plates were returned to the 32°C , 5% CO_2 incubator for another 16 h, after which the medium was replaced by complete KSF medium for optimal cell response. The cells were kept at 32°C for another 24 h and then maintained at 37°C . Monoclonal populations were isolated by the serial dilution technique in 384-well plates. After expansion in 96-well plates, replicates from clonal populations were genotyped using Sanger sequencing and screened for HDR-mediated gene-specific editing using Synthego's ICE (Inference of CRISPR Edits) tool. Of the 153 isolated clones, ~26.8% showed genomic integration of the synonymous variant included in the HDR donor, and approximately half of those (19/41) had 30-50% of the sequencing reads with insertion of the c.1171_1173del (predicted by ICE). Among those, the large majority (13/19) entered a senescence/apoptotic state and were discarded. Of the six remaining clones that were viable and expanded, two were discarded for having a complex genotype, eventually as a result of a polyclonal population, and all four remaining clones harbored a second in-frame variant (c.1133_1156del) predicted to also affect the B3BD [p.(Pro378_His385del)] (**Figure S4**). Two of these four clones were selected for further studies, namely, clone #47 (referred to as C1) and clone #131 (referred to as C2). Quantitative analysis of the *BUB1B* transcriptomic profile by droplet digital PCR showed that wild-type (WT) *BUB1B* was not expressed, 54-58% of the *BUB1B* transcripts harbored the c.1171_1173del variant and 42-46% harbored the c.1133_1156del variant.

Droplet digital PCR (ddPCR)

Cells at ~90% confluence in T25 flasks were nocodazole-arrested (0.5 μ M, Sigma-Aldrich) for 16 h, washed using D-PBS (GRiSP, Porto, Portugal) and trypsinized with TrypLE™ Express Enzyme (Gibco, Thermo Fisher Scientific). Cells were harvested and centrifuged at 300×g for 5 min and the pellet was washed twice using D-PBS before RNA extraction using the RNeasy® Mini Kit (Qiagen, Frederick, MD, USA) following the manufacturer's instructions. RNA was quantified using a Qubit RNA Assay (Thermo Fisher Scientific, Carlsbad, CA, USA). For cDNA synthesis, 1 μ g of RNA was subjected to reverse transcription with random hexamers using the H-minus RevertAid cDNA synthesis kit (Thermo Fisher Scientific) according to the manufacturer's instructions. ddPCR was performed with the QuantStudio 3D Digital PCR System (Applied Biosystems, Waltham, MA, USA), and assays were run in duplicate for each sample. The reaction mix for two chips was composed of 17.4 μ l of QuantStudio 3D Digital PCR Master Mix v2, 1.74 μ l of the respective primer/probe assay, 3.66 μ l of DNase/RNase-free water and 12.0 μ l of cDNA (~1 ng/ μ l). Then, 14.5 μ l of the reaction mixture was loaded on a QuantStudio 3D Digital PCR Chip v2 using QuantStudio 3D Digital PCR Chip Loader. The chips were placed in a GeneAmp PCR System 9700 for thermal cycling under the following conditions: 96°C for 10 min, followed by 39 cycles of a two-step thermal profile at 60°C for 2 min, and 98°C for 30 s and a final extension step at 60°C for 2 min. Thereafter, the chips were read with the QuantStudio 3D Digital PCR Instrument and analyzed using the QuantStudio 3D AnalysisSuite Cloud Software (Thermo Fisher Scientific; <https://apps.thermofisher.com/quantstudio3d/>).

Computational analysis of BubR1 variants

Molecular modeling. The BubR1 (chain S) present in the APC/C-MCC complex (PDBID 5LCW), the BubR1 (PDBID 2 WVI) and the HMP-1 M domain (PDBID 5H5M), the Karyopherin nuclear state - RNA binding protein (PDBID 3ICQ) and the Bub1 kinase domain (PDBID 4QPM) were used as templates to generate the model (with discrete optimized protein energy (DOPE) varying between -0.59 to 0.80). On the other hand, the 3D structure of BubR1 predicted by the AlphaFold v2.0 (AF-O60566-model), an artificial intelligence approach used across diverse applications equally well compared to experimentally determined structures [12], was also used in further studies. Both models have high yield quality factors of ERRAT analysis (<http://servicesn.mbi.ucla.edu/ERRAT/>) (88.4 and 94.3, respectively), and their reliability has also been evaluated by their superimposition with the N-terminus of BubR1 present in the APC/C-MCC complex (PDBID 6TLJ). Although the two

fit rather well in the crystallographic complex, the latter maximizes the interactions with the Cdc20^{MCC}, Cdc20^{APC/C} and Mad2 proteins and represents an average of protein conformational substates, so it was used in further simulations. Starting from the wild-type (BubR1^{WT}) geometry, various BubR1 mutants (R120Q, F175G, F175L, I147T, R244C, Δ 391, E413K and R416Q) were modelled using the X-leap tool of the Amber 18.0 simulation package [13].

The interaction ability between the SAC components and the APC/C co-activator Cdc20 was also assessed by modeling a tetrameric complex composed of BubR1¹⁻⁴²⁰, Cdc20^{MCC}, Cdc20^{APC/C} and Mad2. The X-ray APC/C-MCC complex (PDBID 6TLJ) was used as the starting geometry because it has the two well-defined Cdc20 subunits, the Mad2 and the N-terminus of BubR1 [14]. The latter was modeled until the amino acid 420 due to its reported essential role in SAC function [15].

Molecular Dynamics (MD) simulations. Several MD simulations at 310 K were performed to assess the molecular dynamics of the BubR1 proteins in explicit solvent at atomistic resolution. The Amber 18.0 simulation package (parm14SB force field) was used to carry out the optimizations and MD simulations. The force field parameters developed by Sticht et al. (2006) [16] and Kaxiras et al. (2012) [17] were assigned for the modified phosphorylated amino acids (phosphoserine and phosphothreonine) and acetylated lysine, respectively. The protonation states of the ionizable residues were determined by the PropKa tool [18]. Re-equilibrated TIP3P water molecules were used, filling a rectangular box with a minimum distance of 15 Å between the box faces and any atom of each system. First, each system was minimized in two steps: a harmonic restraint of 20 kcal mol⁻¹ Å⁻² was kept on protein for 10000 steps, and then, an unrestrained minimization for 30000 steps was performed with the steepest descent and conjugate gradient methods. Then, they were equilibrated for 100 ps, followed by 100 ns of production MD simulation. To increase the sampling, 3 replicas, starting from different initial velocities, were simulated for each system. The pressure and the temperature were controlled by using the Berendsen barostat and the Langevin thermostat [19]. The SHAKE algorithm [20] was employed to constrain the bond lengths involving hydrogen atoms. Periodic boundary conditions were considered. Non-bonded interaction pairs were calculated within 10 Å. Beyond that, Coulomb interactions were analyzed by the Particle-Mesh Ewald (PME) algorithm [21], and vdW interactions were truncated. The MD trajectories were analyzed with the CPPTRAJ module [22] of the Amber 18.0 simulations package, combined with the visual molecular dynamics (VMD 1.9.2) program for visualization, analysis and image rendering [23].

References

1. Karczewski KJ, Francioli LC, Tiao G, Cummings BB, Alföldi J, Wang Q, et al. The mutational constraint spectrum quantified from variation in 141,456 humans. *Nature*. 2020;**581**:434–443. doi: 10.1038/s41586-020-2308-7.
2. Fairley S, Lowy-Gallego E, Perry E, Flicek P. The International Genome Sample Resource (IGSR) collection of open human genomic variation resources. *Nucleic Acids Res*. 2020;**48**(D1):D941–D947. doi: 10.1093/nar/gkz836.
3. Liu X, Li C, Mou C, Dong Y, Tu Y. dbNSFP v4: a comprehensive database of transcript-specific functional predictions and annotations for human nonsynonymous and splice-site SNVs. *Genome Med*. 2020;**12**:103. doi: 10.1186/s13073-020-00803-9.
4. Pertea M, Kim D, Pertea GM, Leek JT, Salzberg SL. Transcript-level expression analysis of RNA-seq experiments with HISAT, StringTie and Ballgown. *Nat Protoc*. 2016;**11**:1650–1667. doi: 10.1038/nprot.2016.095.
5. Kim D, Paggi JM, Park C, Bennett C, Salzberg SL. Graph-based genome alignment and genotyping with HISAT2 and HISAT-genotype. *Nat Biotechnol*. 2019;**37**:907–915. doi: 10.1038/s41587-019-0201-4.
6. Danecek P, Bonfield JK, Liddle J, Marshall J, Ohan V, Pollard MO, et al. Twelve years of SAMtools and BCFtools. *Gigascience*. 2021;**10**:giab008. doi: 10.1093/gigascience/giab008.
7. Heyer WD, Ehmsen KT, Liu J. Regulation of homologous recombination in eukaryotes. *Annu Rev Genet*. 2010;**44**:113–139. doi: 10.1146/annurev-genet-051710-150955.
8. Lin S, Staahl BT, Alla RK, Doudna JA. Enhanced homology-directed human genome engineering by controlled timing of CRISPR/Cas9 delivery. *Elife*. 2014;**3**:e04766. doi: 10.7554/eLife.04766.
9. Vasconcelos-Nóbrega C, Pinto-Leite R, Arantes-Rodrigues R, Ferreira R, Brochado P, Cardoso ML, et al. In vivo and in vitro effects of RAD001 on bladder cancer. *Urol Oncol*. 2013;**31**:1212–21. doi: 10.1016/j.urolonc.2011.11.002.
10. Skarnes WC, Pellegrino E, McDonough JA. Improving homology-directed repair efficiency in human stem cells. *Methods*. 2019;**164–165**:18–28. doi: 10.1016/j.ymeth.2019.06.016.
11. Guo Q, Mintier G, Ma-Edmonds M, Storton D, Wang X, Xiao X, et al. “Cold shock” increases the frequency of homology directed repair gene editing in induced pluripotent stem cells. *Sci Rep*. 2018;**8**:2080. doi: 10.1038/s41598-018-20358-5.

12. Jumper J, Evans R, Pritzel A, Green T, Figurnov M, Ronneberger O, Tunyasuvunakool K, Bates R, Židek A, Potapenko A, et al. 2021. Highly accurate protein structure prediction with AlphaFold. *Nature* **596**: 583–589.
13. Case DA, Ben-Shalom IY, Brozell SR, Cerutti DS, Cheatham III TE, Cruzeiro VWD, et al. Amber 2018. San Francisco: University of California; 2018.
14. Alfieri C, Tischer T, Barford D. A unique binding mode of Nek2A to the APC/C allows its ubiquitination during prometaphase. *EMBO Rep.* 2020;**21**:e49831. doi: 10.15252/embr.201949831.
15. Suijkerbuijk SJE, Van Osch MHJ, Bos FL, Hanks S, Rahman N, Kops GJPL. Molecular causes for BUBR1 dysfunction in the human cancer predisposition syndrome mosaic variegated aneuploidy. *Cancer Res.* 2010;**70**:4891–4900. doi: 10.1158/0008-5472.CAN-09-4319.
16. Homeyer N, Horn AH, Lanig H, Sticht H. AMBER force-field parameters for phosphorylated amino acids in different protonation states: phosphoserine, phosphothreonine, phosphotyrosine, and phosphohistidine. *J Mol Model.* 2006;**12**:281–289. doi: 10.1007/s00894-005-0028-4.
17. Papamokos G V, Tziatzos G, Papageorgiou DG, Georgatos SD, Politou AS, Kaxiras E. Structural role of RKS motifs in chromatin interactions: a molecular dynamics study of HP1 bound to a variably modified histone tail. *Biophys J.* 2012;**102**:1926–33. doi: 10.1016/j.bpj.2012.03.030.
18. Dolinsky TJ, Nielsen JE, McCammon JA, Baker NA. PDB2PQR: an automated pipeline for the setup of Poisson-Boltzmann electrostatics calculations. *Nucleic Acids Res.* 2004;**32**:W665–W667. doi: 10.1093/nar/gkh381.
19. Larini L, Mannella R, Leporini D. Langevin stabilization of molecular-dynamics simulations of polymers by means of quasisymplectic algorithms. *J Chem Phys.* 2007;**126**:104101. doi: 10.1063/1.2464095.
20. Ryckaert JP, Ciccotti G, Berendsen HJC. Numerical integration of the cartesian equations of motion of a system with constraints: molecular dynamics of n-alkanes. *J Comput Phys.* 1977;**23**:327–341.
21. Essmann U, Perera L, Berkowitz ML, Darden T, Lee H, Pedersen LG. A smooth particle mesh Ewald method. *J Chem Phys.* 1998;**103**:8577.

22. Roe D, Cheatham 3rd T. PTRAJ and CPPTRAJ: Software for Processing and Analysis of Molecular Dynamics Trajectory Data. *J Chem Theory Comput.* 2013;**9**:3084–3095. doi: 10.1021/ct400341p.
23. Humphrey W, Dalke A, Schulten K. VMD: visual molecular dynamics. *J Mol Graph.* 1996;**14**:27–28, 33–8. doi: 10.1016/0263-7855(96)00018-5.

Supplementary Tables

BUB1B monoallelic germline variants contribute to prostate cancer predisposition by triggering chromosomal instability

Maria P. Silva^{1,#}, Luísa T. Ferreira^{1,#}, Natércia F. Brás², Lurdes Torres^{1,3}, Andreia Brandão¹, Manuela Pinheiro¹, Marta Cardoso¹, Adriana Resende^{1,3}, Joana Vieira^{1,3}, Carlos Palmeira⁴, Gabriela Martins⁴, Miguel Silva^{1,3}, Carla Pinto^{1,3}, Ana Peixoto^{1,3}, João Silva^{1,3}, Rui Henrique⁵, Sofia Maia¹, Helder Maiato^{6,7,8}, Manuel R. Teixeira^{1,3,9,&}, Paula Paulo^{1,&*}

¹Cancer Genetics Group, IPO Porto Research Center (CI-IPOP) / RISE@CI-IPOP (Health Research Network), Portuguese Oncology Institute of Porto (IPO Porto) / Porto Comprehensive Cancer Center, Porto, Portugal;

²LAQV, REQUIMTE, Department of Chemistry and Biochemistry, Faculty of Sciences, University of Porto, Porto, Portugal;

³Department of Laboratory Genetics, Portuguese Oncology Institute of Porto (IPO Porto) / Porto Comprehensive Cancer Center, Porto, Portugal;

⁴Department of Immunology, Portuguese Oncology Institute of Porto (IPO Porto) / Porto Comprehensive Cancer Center, Porto, Portugal;

⁵Department of Pathology, Portuguese Oncology Institute of Porto (IPO Porto) / Porto Comprehensive Cancer Center, Porto, Portugal;

⁶Chromosome Instability & Dynamics Group, i3S - Instituto de Investigação e Inovação em Saúde, Universidade do Porto / Porto Comprehensive Cancer Center, Porto, Portugal;

⁷Cell Division Group, Experimental Biology Unit, Department of Biomedicine, Faculty of Medicine, University of Porto, Porto, Portugal;

⁸Instituto de Biologia Molecular e Celular, Universidade do Porto, Porto, Portugal;

⁹School of Medicine and Biomedical Sciences (ICBAS), University of Porto, Porto, Portugal.

^{#,&} Equal contributions

*Corresponding author, Paula Paulo

Email: paula.paulo@ipoporto.min-saude.pt

Table S1. Demographic and clinicopathological characteristics of the HPC patients carrying *BUB1B* germline variants.

Patient ID	Age ^a	PSA ^a (ng/ml)	Gleason score	TNM stage	Treatment scheme ^a	Nr. PrCa in the family ^b	Nr. Cancers in the family ^b
HPC63	49	16.03	6(3+3)	pT2cNxMx	RP+RT	1	3
HPC119	55	10.51	6(3+3) ^c	cT3aN0M0	RT+ADT	6	6
HPC154	53	4.0	7(3+4)	pT2aNxM0	RP+RT	2	6
HPC227	57	10.64	8(4+4)	pT2cNxM0	RP+RT+ADT	2	3
HPC262	52	331	7(3+4) ^c	cT4N0M1	ADT+CT	3	4
HPC278	62	17.37	8(4+4)	pT3aNxM0	RP+RT	2	4
HPC343	47	17.7	7(4+3)	pT3bN1M0	RP+RT	1	2
HPC369	54	7.22	4(2+2)	pT2NxMx	RP	1	1
HPC450	61	10.0	7(3+4) ^c	cT2cN0M0	RT+ADT	2	3

^a at/upon diagnosis; ^b Family history considering both maternal and paternal lineages; ^c Biopsy is the source of the Gleason score. RP- Radical prostatectomy; ADT- Androgen deprivation therapy; RT- Radiotherapy; CT- Chemotherapy.

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Table S2. Sequences of the primers/oligos used in the different methods.

Primer name/application	5'→3' Sequence
<i>KASP Genotyping</i>	
c.1171_1173 - Forward	AAGAAGGAGATCCTCTACAAAGGG
c.1171_1173 - Reverse Wt specific	<u>GAAGGTGACCAAGTTCATGCTA</u> AATACATCAT CTTCTCTTTCTTCTCCTC
c.1171_1173 - Reverse Mut specific	<u>GAAGGTCGGAGTCAACGGATTA</u> AATACATCAT CTTCTCTTTCTTCTCA
<i>Conventional PCR and Sanger sequencing</i>	
Exon 9 - Forward	GAACATAATTATTGATGGCCCTTGT
Exon 9 - Reverse	AGGTCAGCTCAACCTTCACA
<i>qRT-PCR</i>	
Exon 2 - Forward	GGGAGATGAATGGGAACTGA
Exon 3 - Reverse	CCAAACATCCAGAGGGTCAT
Exon 2/3 TaqMan Probe (5'FAM, 3'TAMRA)	ATTCAAATGCCCGTTTCTGCTGCTG
<i>Droplet digital PCR (ddPCR)</i>	
Exon 9 - Forward	AGCACCAGAAAGCCTGGAAA
Exon 10 - Reverse	CTCTGCACTGGTCAATAGCT
c.1171_1173del TaqMan Probe (5'FAM, 3'VIC)	TCTTTCTTCTCAGACGCTTGCTGATGG
c.1133_1156del TaqMan Probe (5'FAM, 3'VIC)	ATTCAAATGCCCGTTTCTGCTGCTG
<i>CRISPR/Cas9</i>	
sgRNA1	TGGCTCTGAACCCTTTGTAG
sgRNA2	AGAGAAGATGATGTATTGTA
CRISPR/Cas9 HDR donor (c.1171_1173del)	

AAGGAGAATTCCCCTACTCCTGCATAAATCTTCTCTTTACAATACATCATCTTCTCTTTCTTCTCAGACGC
TTGCTGATGGCTCTGAACCCTTTGTAGAGGATCTCCTTCTTCCTTTCCAGGCTTTCTGGTGCTT

Note: Tails in KASP primers are underlined (not gene specific).

Table S3. Microsatellite markers of the seven patients, and available relatives, carrying the *BUB1B* variant c.1171_1173del

Index patient/relative		Microsatellite Markers									
		D15S118	D15S1012	D15S1044	D15S146	D15S214	<i>BUB1B</i> [#]	DTR20GT	D15S968	AFM196XB8	D15S781
HPC63	Index*	217/ 221	153 /153	193 /205	206 /210	267 /267	+/-	157 /159	142 /144	223/ 233	182/ 184
	son*	221 /221	153 /159	193 /193	206 /210	261/ 267	+/-	157 /159	142 /144	223 /227	184 /184
HPC119	Index*	221/221	153/159	193/193	206/210	267 /267	+/-	137/161	134/142	221/225	182/184
HPC154	Index*	213/225	157/163	199/203	208/208	261/ 267	+/-	137/159	140/146	211/225	182/184
HPC227	Index	213/221	159/159	199/201	208/210	263/ 267	+/-	163/163	144/146	221/225	184/186
HPC262	Index*	215 /221	163 /163	195/ 203	206 /206	267 /267	+/-	137 /163	138 /142	221 /225	184 /184
	Daughter	215 /217	163 /169	193/ 203	206 /208	265/ 267	+/-	137 /139	138 /142	219/ 221	182/ 184
	Son*	215 /221	163 /163	193/ 203	206 /208	267 /267	+/-	137 /163	134/ 138	221 /227	184 /184
GaCa	Index	215/ 221	157 /163	201 /201	208 /210	267/ 267	+/-	159 /159	140 /140	225 /225	182/ 184

	Father*	213/ 221	157 /159	193/ 201	204/ 208	261/ 267	+/-	137/ 159	130/ 140	225 /225	182/ 184
	Mother*	215/225	153/163	201/203	206/210	261/267	-/-	159/161	140/142	225/225	182/182
	Brother*	213/215	159/163	193/201	204/210	261/267	-/-	137/159	130/140	225/225	182/182
	Sister	213/225	153/159	193/203	204/206	261/261	-/-	137/161	130/142	225/225	182/182
LuCa	Index	221/225	153/157	201/201	204/208	261/ 267	+/-	161/161	140/144	211/223	184/186

* PCS analysis performed (Table S6); #Genotype for the *BUB1B* variant c.1171_1173del; Conserved region is marked in shadowed grey. Possible shared haplotype in each family is marked in bold letters. GaCa- Gastric Cancer; LuCa- Lung Cancer.

Table S4. Variation of the SASA values of key BubR1 regions in mutant proteins relatively to WT.

Variant	KEN-1	KEN-2	TPR3 (150-185)	Loop (368-379)	Bub1 (440-460)	PK (766-1050)	Full protein
BubR1 ^{WT}	0 ± 46	0 ± 59	0 ± 103	0 ± 64	0 ± 145	0 ± 492	0 ± 1016
BubR1 ^{R120Q}	-62 ± 47	-116 ± 58	-79 ± 100	50 ± 82	-159 ± 139	-499 ± 436	607 ± 928
BubR1 ^{I147T}	-28 ± 38	-98 ± 53	-173 ± 112	285 ± 76	-80 ± 156	316 ± 440	-52 ± 1017
BubR1 ^{R244C}	15 ± 38	34 ± 55	-451 ± 114	-31 ± 76	-140 ± 137	-584 ± 433	-3201 ± 2523
BubR1 ^{Δ391}	-166 ± 37	51 ± 56	-270 ± 104	94 ± 73	-182 ± 128	-519 ± 414	-2935 ± 1084
BubR1 ^{R416Q}	-146 ± 39	1 ± 57	-37 ± 112	328 ± 90	-58 ± 136	613 ± 450	238 ± 1196
BubR1 ^{F175G}	20 ± 40	-147 ± 45	-238 ± 103	142 ± 62	-210 ± 125	339 ± 518	-1978 ± 1033
BubR1 ^{F175L}	-166 ± 48	-8 ± 53	-351 ± 113	93 ± 80	-459 ± 143	265 ± 475	-754 ± 1373
BubR1 ^{E413K}	-104 ± 40	-96 ± 50	-168 ± 105	183 ± 89	311 ± 206	-534 ± 462	-3099 ± 1358

SASA: solvent accessible surface area;

Notes: SASA values are in Å³. Statistically different values to BubR1^{WT} are highlighted in bold.

Table S5. Distance variation between the main helical structures of the TPR in mutant proteins relatively to WT.

Variant	TPR1-A to TPR1-B	TPR2-A to TPR2-B	TPR3-A to TPR3-B	C-term- α H to TPR3-A	C-term- α H to TPR3-B	TPR1 to TPR2	TPR2 to TPR3
BubR1 ^{WT}	0.0 $\pm$ 0.2	0.0 $\pm$ 0.3	0.0 $\pm$ 0.2	0.0 $\pm$ 0.7	0.0 $\pm$ 0.9	0.0 $\pm$ 0.3	0.0 $\pm$ 0.3
BubR1 ^{R120Q}	0.0 $\pm$ 0.3	-0.2 $\pm$ 0.3	0.0 $\pm$ 0.2	2.0 $\pm$ 0.8	1.5 $\pm$ 0.8	0.2 $\pm$ 0.4	-0.1 $\pm$ 0.3
BubR1 ^{I147T}	-0.1 $\pm$ 0.2	0.3 $\pm$ 0.3	0.1 $\pm$ 0.2	3.0 $\pm$ 0.7	1.2 $\pm$ 0.8	-0.7 $\pm$ 0.4	-0.1 $\pm$ 0.3
BubR1 ^{R244C}	0.0 $\pm$ 0.3	-0.2 $\pm$ 0.3	0.1 $\pm$ 0.2	-0.3 $\pm$ 1.0	-0.4 $\pm$ 0.8	-0.4 $\pm$ 0.3	-0.2 $\pm$ 0.3
BubR1 ^{A391}	0.1 $\pm$ 0.2	0.2 $\pm$ 0.3	0.1 $\pm$ 0.2	-0.2 $\pm$ 0.7	-0.2 $\pm$ 0.8	0.0 $\pm$ 0.4	-0.2 $\pm$ 0.3
BubR1 ^{R416Q}	0.0 $\pm$ 0.2	0.2 $\pm$ 0.3	0.1 $\pm$ 0.2	-1.3 $\pm$ 0.7	-4.9 $\pm$ 0.9	-0.8 $\pm$ 0.3	-0.4 $\pm$ 0.3
BubR1 ^{F175G}	0.0 $\pm$ 0.2	-0.1 $\pm$ 0.3	0.0 $\pm$ 0.2	-0.2 $\pm$ 0.6	-4.0 $\pm$ 0.8	-0.2 $\pm$ 0.3	-0.1 $\pm$ 0.3
BubR1 ^{F175L}	0.2 $\pm$ 0.3	0.3 $\pm$ 0.3	0.0 $\pm$ 0.2	-2.1 $\pm$ 0.7	-0.2 $\pm$ 0.8	-0.3 $\pm$ 0.4	-0.7 $\pm$ 0.3
BubR1 ^{E413K}	-0.2 $\pm$ 0.2	0.0 $\pm$ 0.3	0.0 $\pm$ 0.2	-0.9 $\pm$ 0.7	-5.6 $\pm$ 1.1	-0.5 $\pm$ 0.3	-0.5 $\pm$ 0.3

Notes: Distance values are in Å. Statistically valid differences are highlighted in bold.

Table S6. Variation (%) of the secondary structural elements of all BubR1 mutants relatively to the WT.

Region	Variant	Secondary structural elements				
		β -sheet	3-10 α -helix	α -helix	Turn	Bend
TPR domain	R120Q	0.0	0.0	1.6	-2.1	0.6
	I147T	0.0	-14.7	22.4	-9.9	2.2
	R244C	0.3	-8.2	12.8	-2.7	-2.1
	Δ 391	0.0	-6.5	11.5	-2.3	-2.6
	R416Q	0.0	-4.6	4.9	1.4	-1.7
	F175G	0.0	-18.1	13.2	-1.5	6.4
	F175L	0.0	-7.4	9.4	-2.8	0.9
	E413K	0.0	-4.6	19.3	-12.4	-2.3
KEN-1	R120Q	0.0	-2.2	-22.0	22.0	2.3
	I147T	0.0	-7.1	9.9	-3.7	0.9
	R244C	0.0	13.3	-2.7	-9.3	-1.3
	Δ 391	0.0	38.0	-44.7	8.8	-2.1
	R416Q	0.0	18.1	-45.9	22.2	5.6
	F175G	0.0	-2.6	-7.2	9.5	0.4
	F175L	0.0	18.5	-22.9	-12.8	17.2
	E413K	0.0	-7.5	-7.4	13.9	1.1
KEN-2	R120Q	0.0	-8.8	-17.6	8.5	17.9
	I147T	0.0	4.8	-33.1	25.9	2.4
	R244C	0.0	-38.4	6.1	3.7	28.6
	Δ 391	0.0	-31.5	17.8	12.1	1.5
	R416Q	0.0	-24.4	-33.1	37.8	19.7
	F175G	0.2	-46.6	-15.1	62.0	-0.5
	F175L	0.0	-4.8	-23.4	24.0	4.2
	E413K	0.0	-38.4	6.1	3.7	28.6
Loop	R120Q	1.4	-6.1	13.0	-15.7	7.3
	I147T	-6.2	-2.0	-1.7	5.4	4.4
	R244C	-6.3	-6.1	2.0	-9.9	20.3
	Δ 391	-4.0	-4.8	7.9	-8.5	9.4
	R416Q	-2.4	-1.7	-4.4	-1.4	9.9
	F175G	-6.3	-5.9	4.6	-14.8	22.4
	F175L	-6.3	6.1	-2.7	-3.8	6.8
	E413K	-6.1	-4.7	-5.7	-8.8	25.3

Full protein	R120Q	-0.3	1.4	-1.7	0.9	-0.2
	I147T	-0.3	0.5	-0.8	0.6	0.1
	R244C	-0.9	-0.6	0.2	-0.1	1.4
	Δ391	-0.9	-0.2	0.1	-0.2	1.2
	R416Q	-0.7	0.1	-0.8	0.9	0.5
	F175G	-1.0	-0.7	0.0	1.3	0.1
	F175L	-0.8	-0.3	0.8	-0.6	0.9
	E413	-0.8	0.0	-0.2	-0.3	1.2

Note: Higher differences are highlighted in bold numbers and shown in the main manuscript. The negative and positive mutant controls are colored in green and red, respectively.

Table S7. Intramolecular contacts made by key residues of the different BubR1 mutants.

Residue 1	Residue 2	Frequency of interaction (%)								
		WT	R120Q	I147T	R244C	Δ 391	R416Q	F175G	F175L	E413K
K26	W22	50	37	52	30	-	45	40	37	38
N28	S25	-	-	-	-	-	41	30	-	-
R120	D73	47	-	74	51	62	65	53	65	56
	D118	63	-	85	88	93	49	83	63	48
I147	L142	40	39	-	46	37	37	39	35	-
	Q145	-	-	35	-	-	-	-	-	-
E161	R33	52	-	61	34	33	39	31	62	31
N306	A303	33	-	34	36	-	37	34	-	-
Q386	V382	40	32	-	39	39	40	-	33	-
	E391	30	30	-	33	-	-	-	31	34
R416	F412	49	58	-	53	37	33	39	34	57
	E413	30	36	30	-	62	-	-	30	-
	F420	-	35	-	-	38	-	-	-	-
D610	A614	52	-	-		43	-	55	-	45
F611	A615	55	43	41		52	-	-	49	45
S670	K668	32	35	-	73	-	61	32	-	45
R727	S915	74	-	43	-	-	-	30	-	35
	Q921	52	-	-	-	-	-	-	-	-
	L731	49	-	45	43	35	50	46	33	44
	P721	45	37	43	58	-	-	38	-	37
	C723	43	47	57	52	50	53	58	55	50
	S913	-	53	40	52	64	82	43	53	46
D777	S798	34	-	-	-	30	34	-	-	-
	S797	-	-	-	46	35	-	-	58	38
Y778	D911	59	43	94	-	59	67	42	32	30
	C775	57	45	33	34	49	48	-	48	50
K795	V834	66	74	89	62	69	75	81	77	69
D882	S884	68	50	49	-	44	49	39	36	50

R931	58	38	31	49	-	34	-	-	-
R886	50	-	31	-	31	-	46	58	33

Note: Only the H-bonds with frequency higher than 30% along the simulations were considered.

Table S8. Premature chromatid separation (PCS) frequencies of healthy controls and HPC carriers of *BUB1B* variants.

Sample	Age^a	cDNA change	Protein change	PCS (%)
Control 1	50	-/-	-/-	0.75%
Control 2	51	-/-	-/-	1.36%
Control 3	51	-/-	-/-	2.41%
Control 4	52	-/-	-/-	2.57%
Control 5	45	-/-	-/-	4.29%
Control 6	67	-/-	-/-	4.41%
Control 7	42	-/-	-/-	5.67%
Control 8	78	-/-	-/-	8.64%
Control 9	45	-/-	-/-	8.72%
Control 10	45	-/-	-/-	8.74%
HPC63 son (healthy)	40	c.1171_1173del	p.(Glu391del)	19%
HPC262 son (healthy)	25	c.1171_1173del	p.(Glu391del)	70%
HPC63	67	c.1171_1173del	p.(Glu391del)	34%
HPC119	72	c.1171_1173del	p.(Glu391del)	37%
HPC154	72	c.1171_1173del	p.(Glu391del)	34%
HPC262	67	c.1171_1173del	p.(Glu391del)	54%
HPC278	72	c.2481del	p.(Gln827HisfsTer13)	28%
HPC450	72	c.359G>A	p.(Arg120Gln)	61%

^a at the time of blood collection. -/-, negative genotype for the variant c.1171_1173del; p.(Glu391del).

Supplementary Figures

BUB1B monoallelic germline variants contribute to prostate cancer predisposition
by triggering chromosomal instability

Maria P. Silva^{1,#}, Luísa T. Ferreira^{1,#}, Natércia F. Brás², Lurdes Torres^{1,3}, Andreia Brandão¹, Manuela Pinheiro¹, Marta Cardoso¹, Adriana Resende^{1,3}, Joana Vieira^{1,3}, Carlos Palmeira⁴, Gabriela Martins⁴, Miguel Silva^{1,3}, Carla Pinto^{1,3}, Ana Peixoto^{1,3}, João Silva^{1,3}, Rui Henrique⁵, Sofia Maia¹, Helder Maiato^{6,7,8}, Manuel R. Teixeira^{1,3,9,&}, Paula Paulo^{1,&*}

¹Cancer Genetics Group, IPO Porto Research Center (CI-IPOP) / RISE@CI-IPOP (Health Research Network), Portuguese Oncology Institute of Porto (IPO Porto) / Porto Comprehensive Cancer Center, Porto, Portugal;

²LAQV, REQUIMTE, Department of Chemistry and Biochemistry, Faculty of Sciences, University of Porto, Porto, Portugal;

³Department of Laboratory Genetics, Portuguese Oncology Institute of Porto (IPO Porto) / Porto Comprehensive Cancer Center, Porto, Portugal;

⁴Department of Immunology, Portuguese Oncology Institute of Porto (IPO Porto) / Porto Comprehensive Cancer Center, Porto, Portugal;

⁵Department of Pathology, Portuguese Oncology Institute of Porto (IPO Porto) / Porto Comprehensive Cancer Center, Porto, Portugal;

⁶Chromosome Instability & Dynamics Group, i3S - Instituto de Investigação e Inovação em Saúde, Universidade do Porto / Porto Comprehensive Cancer Center, Porto, Portugal;

⁷Cell Division Group, Experimental Biology Unit, Department of Biomedicine, Faculty of Medicine, University of Porto, Porto, Portugal;

⁸Instituto de Biologia Molecular e Celular, Universidade do Porto, Porto, Portugal;

⁹School of Medicine and Biomedical Sciences (ICBAS), University of Porto, Porto, Portugal.

#,& Equal contributions

*Corresponding author, Paula Paulo

Email: paula.paulo@ipoporto.min-saude.pt

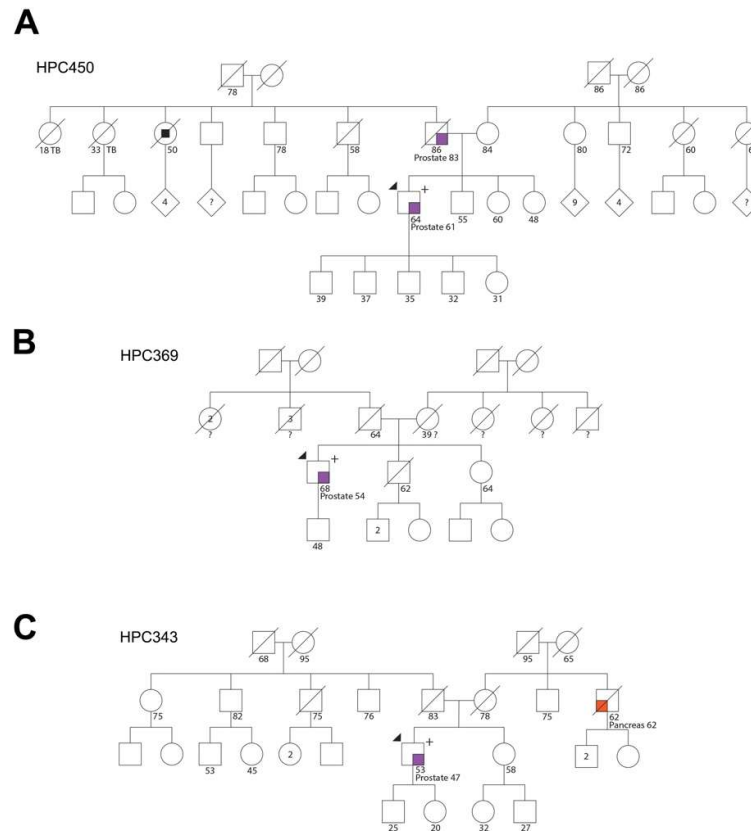


Figure S1. Pedigrees of the HPC cases carrying missense *BUB1B* variants. A) Carrier of the *BUB1B* missense variant c.359G>A; p.(Arg120Gln). **B)** Carrier of the *BUB1B* missense variant c.440T>C; p.(Ile147Thr). **C)** Carrier of the *BUB1B* missense variant c.1247G>A; p.(Arg416Gln). Carrier patients are labeled with a “plus” mark. Squares represent the males, circles the females and diamonds unknown gender. Deceased individuals are represented by a diagonal line through a symbol and the affected ones are highlighted by colored symbols. The index case is indicated by an upper left arrow and the cancer type and age at diagnosis are indicated whenever known.

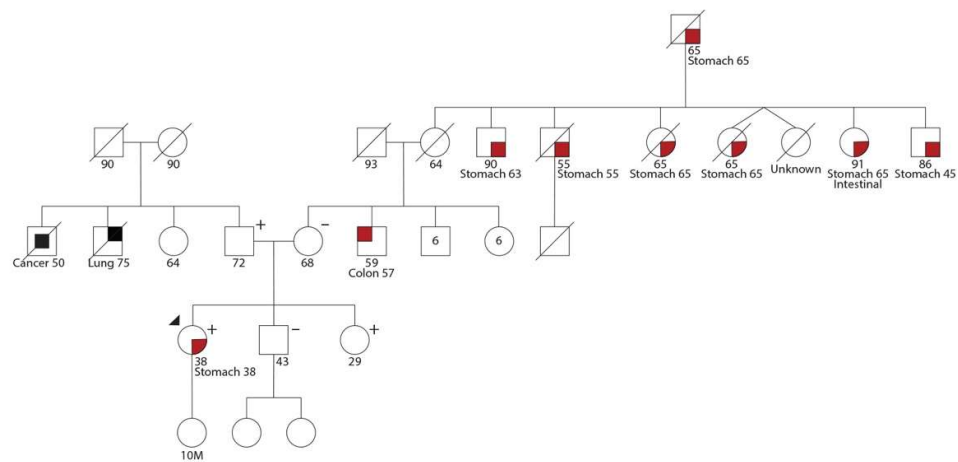


Figure S2. Pedigree of the gastric cancer patient with phased haplotype carrying the in-frame variant c.1171_1173del.

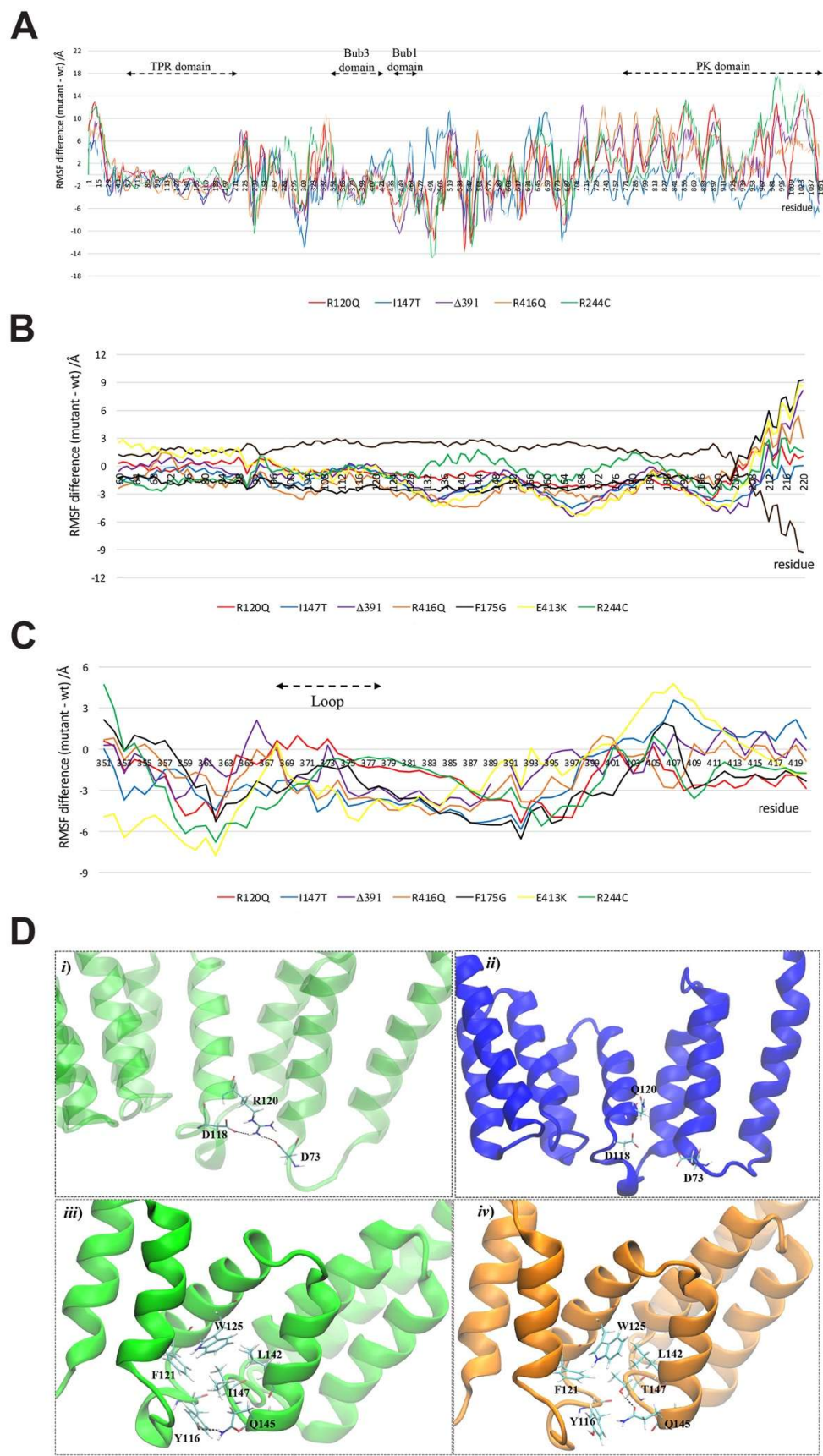


Figure S3. MD simulations highlight structural differences between BubR1 mutants and WT proteins. MD simulations for **A)** complete BubR1 protein, **B)** BubR1^{TPR} (60 – 220 residues) and **C)** BubR1^{Bub3} (351 – 420 residues) showing variations in RMSF values. **D)** representative MD structures of the TPR region for the BubR1^{WT} (green, *i* and *iii*), BubR1^{R120Q} (blue, *ii*) and BubR1^{I147T} (orange, *iv*). Proteins are depicted as cartoon, while the residues are represented in sticks and colored by atom type. Some key distances are also indicated.

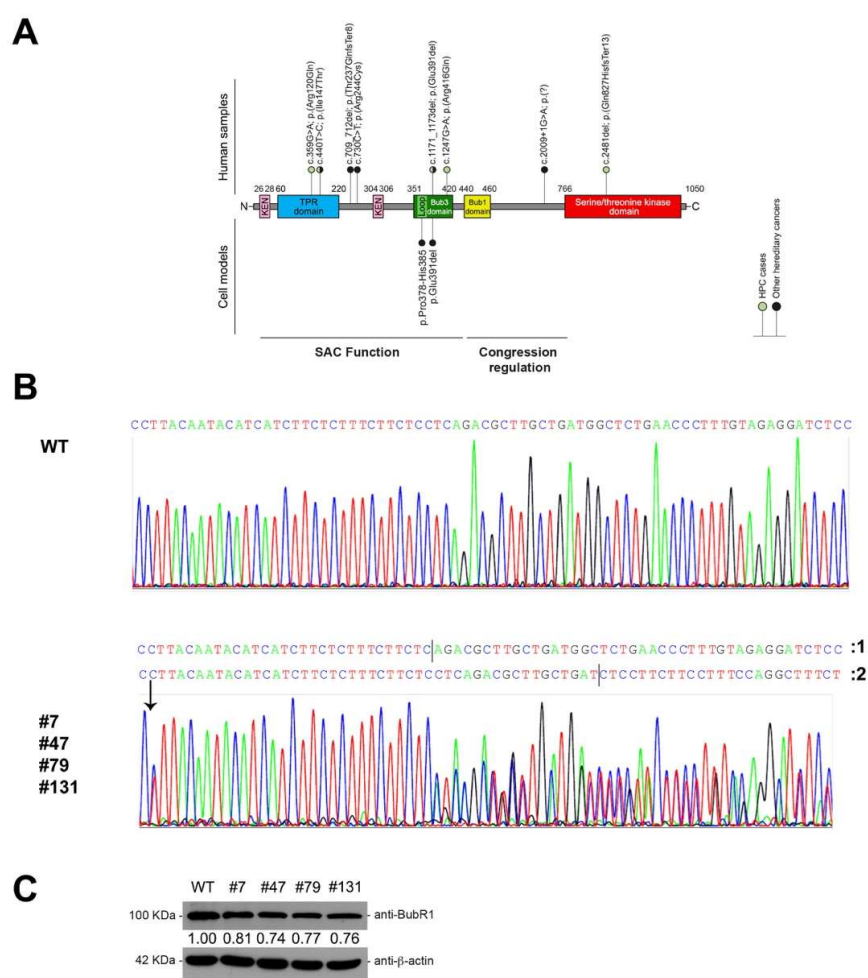


Figure S4. *BUB1B* gene-edited cell clones used in phenotypic assays. A) Schematic representation of the BubR1 protein showing the location of the variants identified in patients and the *in vitro* cell models used in this study. **B)** Representative Sanger sequencing electropherograms for the WT population and the *BUB1B*-edited clones containing the c.1171_1173del variant (read :1). The four clones (#7, #47, #79 and #131) revealed to, additionally, harbour the variant c.1133_1156del (read :2). An arrow highlights the synonymous variant included in the PAM sequence of the HDR template to avoid cleavage by Cas9. Deletion

sites are shown by a vertical line in each sequence. **C)** BubR1 expression levels in all clonal populations comparing with WT cells, assessed by western-blot. Relative BubR1 expression in each cell population were obtained by correcting to its respective β -actin levels and normalized to WT. Clones #47 and #131 were used in all experiments and named C1 and C2, respectively, for simplification. In the chase experiments, clone #79 (named C3) was also used.

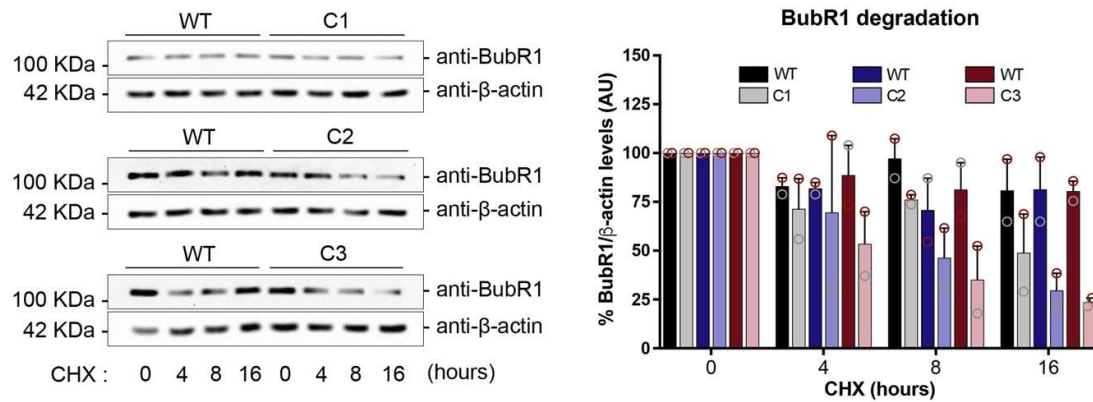


Figure S5. Analysis of BubR1 expression upon cycloheximide (CHX) treatment in WT and *BUB1B* gene-edited cells. Representative images from western blot analysis of BubR1 expression before (0h) and after 4h, 8h and 16h of treatment with CHX are shown at *left*. Blots for C1 and C2 clones are the same shown in the main manuscript, here used for comparison with data obtained for a 3rd independent clone (named C3) harboring the same genotype. Densitometry analysis of western blot images is shown at *right*. For every time point, BubR1 expression levels are shown for each WT-paired clonal cell population (C1, C2 and C3). The % of BubR1 expression was obtained by normalizing BubR1 levels to those of the corresponding β -actin and adjusting to the expression levels obtained before CHX treatment (at 0h). For comparison, different colored circles highlight matched clone/WT pairs from the same assay.

CHAPTER II

Deleterious germline variants in mitotic spindle formation genes in early-onset/familial prostate cancer patients

Maria P. Silva¹, Andreia Brandão¹, Marta Cardoso¹, Sofia Maia¹, Manuel R. Teixeira^{1,2,3,&}, Paula Paulo^{1,*}

¹ Cancer Genetics Group, IPO Porto Research Center (CI-IPOP) / RISE@CI-IPOP (Health Research Network), Portuguese Oncology Institute of Porto (IPO Porto) / Porto Comprehensive Cancer Center, Porto, Portugal;

² Department of Laboratory Genetics, Portuguese Oncology Institute of Porto (IPO Porto) / Porto Comprehensive Cancer Center, Porto, Portugal;

³ School of Medicine and Biomedical Sciences (ICBAS), University of Porto, Porto, Portugal.

*Corresponding author, Paula Paulo

Email: paula.paulo@ipoporto.min-saude.pt

Keywords: prostate cancer predisposition, *CEP57*, *CEP290*, *EML4*, *TUCGCP5*, mitotic spindle formation

Abstract

Background: Prostate cancer (PrCa) is among the most heritable human cancers, yet only a small proportion of patients have been found to harbor deleterious variants in established cancer predisposition genes.

Methods: A pathway-based approach of candidate genes obtained by Whole-exome sequencing (WES), previously conducted and analyzed on multiple affected members from 45 PrCa families, was used to search for shared pathways between *BUB1B* and *CEP57* genes. Genes common to the pathways were prioritized and subsequently analyzed using targeted next-generation sequencing (T-NGS) across the entire cohort of 462 early-onset and/or familial PrCa patients.

Results: Fifteen index PrCa patients were shown to carry germline pathogenic/likely pathogenic variants in the *CEP57*, *CEP290*, *EML4* and *TUBGCP5* genes. Cross-referencing these findings with TCGA data identified somatic mutations in the same genes among PrCa patients, reinforcing their relevance in cancer biology. The most frequently mutated gene in our study was *CEP290*, exhibiting truncating variants in three families and missense variants in another two.

Discussion: This is the first report of pathogenic/likely pathogenic germline variants in *CEP290*, *EML4* and *TUBGCP5* in PrCa patients, namely in those with early-onset/familial disease. Together with *CEP57*, germline variants in mitotic machinery genes were found in 3.2% of the PrCa families. Our findings reinforce the hypothesis that defects in mitotic pathways, particularly those regulating mitotic spindle formation, may significantly contribute to early-onset/familial PrCa development, and underscore the importance of pathway-based approaches to unveil the heterogeneity of PrCa predisposition.

Introduction

Hereditary prostate cancer (HPC) comprises 5-15% of all prostate cancer (PrCa) cases [52], which overall ranks second among the most common non-skin cancers worldwide [15]. While PrCa has a significant heritability component, only age, race, and family history (FH) are well established risk factors [28]. Advances in Next Generation Sequencing (NGS) have identified several PrCa susceptibility genes, including DNA repair genes associated with moderate- to high-penetrance for hereditary breast and ovarian cancer (*BRCA1*, *BRCA2*, *ATM*, *CHEK2*, or *PALB2*) or Lynch syndrome (*MLH1*, *MSH2*, *MSH6*, or *PMS2*). Targeted NGS (T-NGS) screening in our series of 462 patients fulfilling criteria for HPC showed that less than 5% of the patients are carriers of deleterious/truncating variants in known DNA repair genes [108], [196], [197], leaving much of HPC's molecular basis unexplained.

Previous studies from our group, aiming to uncover new PrCa predisposing genes, have highlighted the putative involvement of genes linked to microtubule (MT) dynamics and chromosome segregation in PrCa predisposition [108], [198]. Using a panel of 94 genes involved in cancer-predisposing syndromes to screen 121 suspected HPC patients with a heavy personal or familial history of cancer, we have previously reported one patient carrying a stop variant in *CEP57* [108]. The *CEP57* gene encodes a 57 kDa member of the CEP family of centrosomal proteins and plays a key role in MT stabilization at the centrosome, crucial for faithful chromosomal segregation during cell division [194]. More recently, we have shown that monoallelic variants in *BUB1B* occur in 1.9% of suspected HPC families [198]. The *BUB1B* gene encodes BubR1, an essential component of the mitotic checkpoint complex, along with Mad2, Bub3 and Cdc20, and which activity is essential for proper chromosome segregation during mitosis [126], [199].

To gain further insights into the general contribution of *BUB1B*- and/or *CEP57*-related pathways in PrCa predisposition, we used a pathway-based approach to query our list of candidate PrCa predisposing genes obtained upon whole-exome sequencing (WES) of duos/trios from 45 HPC families [197]. The *CEP290*, *EML4*, and *TUBGCP5* genes were identified as players in *BUB1B*- and/or *CEP57*-related pathways, specifically involved in coordinating spindle formation during mitosis. We then used a custom targeted NGS (T-NGS) approach to screen the entire series of 462 early-onset and/or familial PrCa patients for variants in *CEP57*, *CEP290*, *EML4*, and *TUBGCP5* genes and have identified deleterious/potentially pathogenic variants in ~3.2% of the cases.

Materials and Methods

Early-onset/familial PrCa cohort

This study involved 462 index cases from a previously described series of early-onset/familial PrCa cases. Patients were recruited based on two criteria: early-onset disease, with PrCa diagnosis before the age of 56, and/or family history (FH) of PrCa, with more than one case having a PrCa diagnosis and at least one of them having a diagnosis before the age of 66. Of the 462 index patients, 240 (51.9%) fulfilled the early-onset disease criterion, 311 (67.3%) fulfilled the FH criterion, and 89 (19.3%) fulfilled both criteria. Demographic and clinicopathological characteristics are shown in Supplementary Table 1.

Pathway-based approach of candidate genes obtained by WES

Data from whole-exome sequencing (WES) of 96 PrCa patients from 45 families (39 duos and 6 trios) was previously obtained [200]. For the purpose of this study, we retained only truncating variants (frameshift, stop or splicing) segregating with the disease in the affected duos/trios (179 variants in 173 genes).

The list of 173 mutated genes found by WES was used as an input to search for *BUB1B*- and/or *CEP57*-associated genes in PathCards (<https://pathcards.genecards.org>). Briefly, we searched for shared pathways between *BUB1B* and *CEP57* genes and identified two main pathways: the “Cell Cycle, Mitotic Superpath” and the “Mitotic Prometaphase” pathway (part of the “EML4 and NUDC in mitotic spindle formation Superpath”). Then, we crossed our list of mutated genes with both the list of genes associated with mitotic cell cycle (n=693) and the list of genes involved in mitotic prometaphase (n=205). The genes *CEP290*, *EML4* and *TUBGCP5* were shared by the three gene lists (Supplementary Figure 1), being selected together with *CEP57* for T-NGS. The entire series has previously been analyzed for *BUB1B* [198].

T-NGS

To access the prevalence of germline variants in the three prioritized candidate genes from the WES discovery series, as well as that in *CEP57*, we performed T-NGS in the whole series of 462 early-onset/familial PrCa patients using a customized gene panel designed with Agilent SureDesign, which covered the coding and splicing regions of *CEP57* (NM_014679), *CEP290* (NM_025114), *EML4* (NM_001145076) and *TUBGCP5* (NM_001102610).

Capture, sequencing, data processing, and variant annotation and prioritization were performed as previously described [201]. Additionally, an *in-house perl*-based script adapted from the ANNOVAR software was created to gather information for the minor allele frequency (MAF) in non-Finnish European (NFE), from the Genome Aggregation Database [gnomAD v2.1.1, June 2021], and for the pathogenicity predictors at dbNSFP (v3.5). To determine the pathogenicity of the splicing variants, the *in-silico* predictors scSNV, including dbscSNV_RF_SCORE and dbscSNV_ADA_SCORE [202], [203], SPIDEX [204] and SpliceAI [205], were employed.

All the variants were validated by Sanger sequencing.

KASP genotyping

To evaluate the frequency in the general Northern Portuguese population of the missense variants recurrently identified in our series of HPC patients, we used KASP technology genotyping (KBioscience, Herts, UK) in a series of 875 healthy individuals, following manufacturer's recommendations. KASP assay primers (Supplementary Table 2) were designed using the Primer-BLAST [206] design tool from NCBI, and data were analyzed in the LightCycler 480 Software 1.5.0.

Results

Of the 462 early-onset and/or familial PrCa patients analyzed, 15 presented germline variants in *CEP57*, *CEP290*, *EML4* or *TUBGCP5*, representing 3.2% of the patients (Table 1 and Supplementary Table 3). Of those, 8 patients showed truncating variants (frameshift, stop, in-frame deletion, or splicing), and the remaining 7 patients harbored missense variants.

CHAPTER II

Table 1 - List of germline truncating/potentially pathogenic variants in *CEP57*, *CEP290*, *EML4* and *TUBGCP5* genes found in early-onset/familial PrCa patients.

Gene (RefSeq)	Variant position (GRCh37)	SNP ID	cDNA change; protein change	Variant type	Number of HPC carriers (%)	MAF (NFE) ^a	Patient ID	Family relationship of WES carriers	Predicted as Pathogenic ^b
<i>CEP57</i> (NM_014679)	11:95546149	rs1164730107	c.256G>C; p.(Glu86Gln)	Missense	2 (0,433%)	0,0000%	HPC146 HPC437	N/A	12/15
<i>CEP57</i> (NM_014679)	11:95555126	rs368470481	c.791C>G; p.(Ser264Ter)*	Stop-gain	1 (0,216%)	NR	HPC421	N/A	N/A
<i>CEP57</i> (NM_014679)	11:95564300	NR	c.1386_1388del; p.(Lys463del)	In-frame	1 (0,216%)	NR	HPC508	N/A	N/A
<i>CEP290</i> (NM_025114)	12:88465563	rs386834159	c.5850del; p.(Phe1950fs)	Frameshift	1 (0,216%)	NR	HPC32	son + father	N/A
<i>CEP290</i> (NM_025114)	12:88476852	rs756302731	c.4966_4967del; p.(Glu1656AsnfsTer3)	Frameshift	1 (0,216%)	0,0032%	HPC470	2 first cousins	N/A
<i>CEP290</i> (NM_025114)	12:88477682	N/A	c.4754A>G; p.(His1585Arg)	Missense	1 (0,216%)	0,0211%	HPC423	N/A	13/15
<i>CEP290</i> (NM_025114)	12:88487688	rs760503950	c.3162_3167del; p.(Ile1055_Ser1056del)	In-frame	1 (0,216%)	0,0028%	HPC14	N/A	N/A
<i>CEP290</i> (NM_025114)	12:88530499	N/A	c.362A>G; p.(Glu121Gly)	Missense	1 (0,216%)	NR	HPC375	N/A	12/15

<i>EML4</i> (NM_001145076)	2:42510055	NR	c.887_890del; p.(Asn296MetfsTer16)	Frameshift	2 (0,433%)	NR	HPC382 HPC502	2 first cousins (HPC502)	N/A
<i>EML4</i> (NM_001145076)	2:42528414	rs143911025	c.1523A>G; p.(His508Arg)	Missense	1 (0,216%)	0,0612%	HPC493	N/A	12/15
<i>TUBGCP5</i> (NM_001102610)	15:22836062	rs748417578	c.201-1G>T; p.(?)	Splice-site	1 (0,216%)	0%	HPC62	2 brothers	N/A
<i>TUBGCP5</i> (NM_001102610)	15:22846945	rs199607815	c.820T>A; p.(Thr274Ser)	Missense	1 (0,216%)	0,0256%	HPC103	N/A	6/6
<i>TUBGCP5</i> (NM_001102610)	15:22866795	NR	c.2407A>T; p.(Tyr803His)	Missense	1 (0,216%)	NR	HPC131	N/A	6/6

Index patients from WES families are highlighted in bold.

^a Minor Allele Frequency (MAF) in male NFE (Non-finish European) obtained from gnomAD; NR – Not Reported; N/A – Not applicable; NP – Not Performed

^c Fraction of functional predictors where the variant is expected to be pathogenic among the total number of tools considered for analysis (depending on data availability)

* Variant previously described by our group [108] .

Germline variants in the centrosomal protein genes *CEP57* and *CEP290*

The stop-gain variant c.791C>G found in the *CEP57* gene, as well as the family's pedigree, was previously described (Table 1, Figure 1A) [108]. Additionally, an in-frame deletion in *CEP57* (c.1386_1388del) was found in patient HPC508 (Table 1, Figure 1B), diagnosed at the early age of 55. The patient has also FH of the disease, with a maternal uncle diagnosed with PrCa at the age of 78. Moreover, a missense variant in *CEP57* (c.256G>C) was found in two unrelated patients, HPC146 and HPC437 (Supplementary Figure 2A and 2B), both fulfilling the FH criterion (Table 1). This variant occurs at a highly conserved nucleotide (as predicted by the four conservation predictors) and is considered potentially pathogenic by 8 out of the 11 pathogenicity predictors (Supplementary Table 3). Patient HPC146 was diagnosed at the age of 59 and has three relatives affected with PrCa, a brother and both paternal and maternal uncles, diagnosed at the age of 53, 58 and 65, respectively. All affected relatives are deceased, except his 90-year-old maternal uncle, rendering segregation analysis difficult to perform due to his advanced age. The patient HPC437 was diagnosed at the age of 60, thus fulfilling the FH criterion only, with both his father and a paternal uncle (deceased) having been diagnosed with PrCa at the ages of 60 and 75, respectively. This recurrent variant was the only one screened in healthy subjects to further clarify a potential disease association, being found in one male. Nevertheless, this variant was not present among the 4,310 male controls from the Non-Finish European (NFE) population in the gnomAD Database (<https://gnomad.broadinstitute.org>).

Three index patients were identified as carriers of truncating/likely pathogenic variants in *CEP290* (Table 1). The *CEP290* variant c.4966_4967del was carried by a patient with FH of PrCa (HPC470), segregating with the disease in his paternal cousin (HPC512), diagnosed at the age of 73 (Table 1, Figure 1C). The other frameshift variant in *CEP290*, (c.5850del) was shared by HPC32, a patient that fulfills both the early-onset and the FH criteria, and his affected father, who was diagnosed at the age of 70 (Table 1, Figure 1D). An in-frame deletion variant in *CEP290* (c.3162_3167del) was additionally found (Table 1) in an early-onset patient (HPC14), who was diagnosed at the age of 52, without FH of PrCa (Figure 2E).

Regarding the missense variants found in *CEP290*, both the variant c.4754A>G and the variant c.362A>G are considered to be potential pathogenic by 9 of the 11 pathogenicity predictors. The first, was detected in a patient (HPC423) with FH of PrCa from the maternal side, with an affected first-degree cousin deceased at the age of 64 (Table 1, Supplementary Table 3, Supplementary Figure 2C). The second, was found in patient HPC375, who was diagnosed at the age of 59, but fulfills the FH criterion with two affected

relatives, his father (diagnosed at the age of 76) and a paternal uncle (unknown age of diagnosis) (Table 1, Supplementary Table 3, Supplementary Figure 2D).

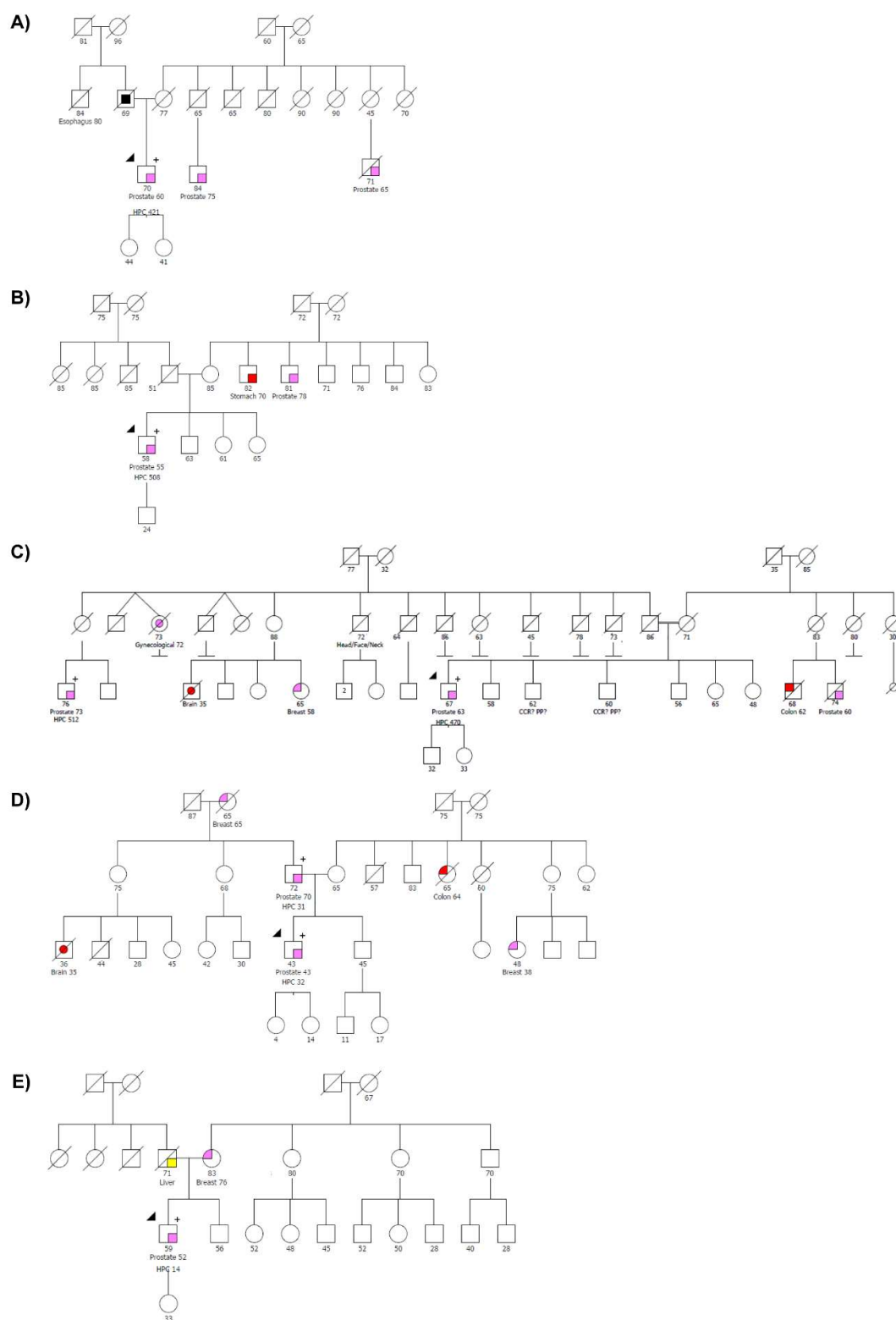


Figure 1 - Family pedigrees of patients carrying unique truncating variants in *CEP57* and *CEP290* genes. **A)** Patient HPC421, harboring the *CEP57* stop-gain variant c.791C>G. **B)** Patient HPC508, harboring the *CEP57* in-frame variant c.1386_1388del. **C)** Patient HPC470, harboring the *CEP290* frameshift variant c.4966_4967del, which co-segregates with the disease in the paternal cousin HPC512. **D)** Patient HPC32, harboring the *CEP290* frameshift variant c.5850del, segregating with the disease in his father (HPC31). **E)** Patient HPC14, harboring the *CEP290* in-frame variant c.3162_3167del. Males are represented by a square, females by a circle and unspecified gender by a diamond. A diagonal line through a symbol indicates that the individual is deceased, and colored symbols represent affected individuals. The index case is indicated by an arrow, and the cancer type and age at diagnosis are indicated when known.

Germline variants in *EML4* and *TUBGCP5*

The frameshift variant c.887_890del in *EML4* was initially detected when screening the WES series and is carried by the index patient (HPC502) and his affected paternal cousin (HPC136), diagnosed at the ages of 61 and 65, respectively (Table 1, Figure 2A). This variant was latter found in patient HPC382 by T-NGS, who was diagnosed at the early-age of 46 and has FH of PrCa (Figure 2B). The *EML4* c.1523A>G missense variant, considered potentially pathogenic by 7 of the 11 pathogenicity predictors (Supplementary Table 3), was identified in a patient (HPC493) fulfilling both the early-onset and FH criteria, being diagnosed at the age of 55 (Table 1). This patient had an affected paternal uncle, but segregation analysis was not possible to be performed, as the patient is deceased (Supplementary Figure 3A).

Lastly, our WES results revealed the splice variant c.201-1G>T in *TUBGCP5*, carried by an early-onset patient with FH of the disease (HPC62, Table 1). His affected brother (HPC21), diagnosed at the age 55, also carries this *TUBGCP5* variant (Figure 2C). To further address whether this variant, which occurs in a highly conserved splicing region [207] upstream of exon 3, interferes with normal splicing, we queried various *in silico* software tools. This variant was predicted to be disease-causing by MutationTaster [208] and to impact splicing through the loss of an acceptor splice site by all four *in silico* tools queried (Supplementary Table 4).

Additionally, three missense variants were found in *TUBGCP5*, however, information from the *in silico* pathogenicity predictors is scarce, with only six or seven of the eleven pathogenic predictors with available information (Table 1, Supplementary Table 3). The *TUBGCP5* variants c.820T>A; p.(Thr274Ser) and c.2407A>T; p.(Tyr803His), were found to occur at highly conserved nucleotides and were considered potentially pathogenic by 6 out of 6 *in silico* predictors. The first was found in patient HPC103, diagnosed at the age of 61 and has two relatives affected with PrCa, his father, diagnosed at the age of 74, and his paternal uncle (unknown diagnosed age), both deceased (Supplementary Figure 3B). The latter was found in patient HPC131, who fulfills the early-onset criteria, diagnosed

at the age of 54 and with an affected brother (unknown age of diagnosis) (Supplementary Figure 3C) The remaining missense variant, found in patient HPC165, was not further explored due to the unconserved nature of the involved nucleotides (Supplementary Table 3).

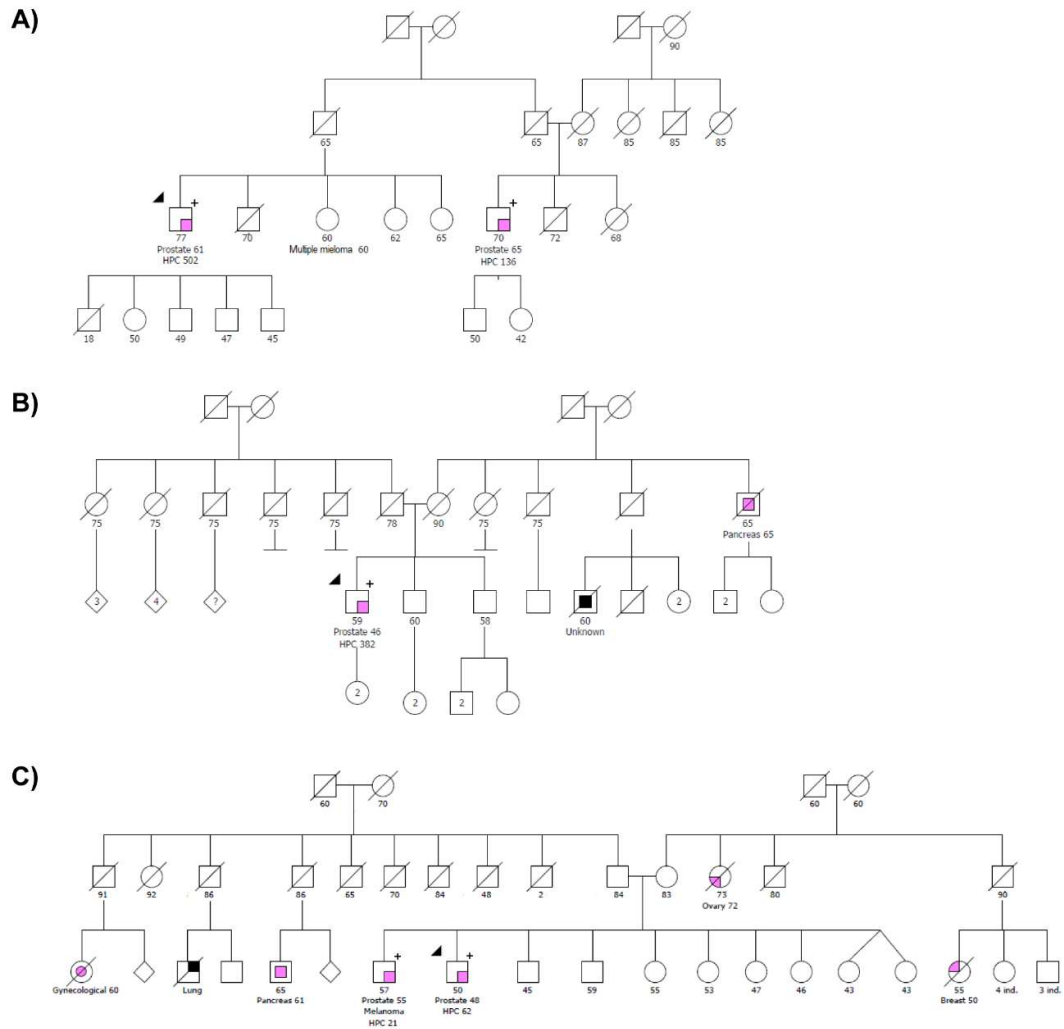


Figure 2 - Family pedigrees of patients carrying unique truncating variants in *EML4* and *TUBGCP5* genes. A) Patient HPC502, harboring the *EML4* frameshift variant c.887_890del, co-segregating with the disease in his cousin (HPC136). **B)** Patient HPC382 harboring the *EML4* frameshift variant c.887_890del. **C)** Patient HPC62, harboring the *TUBGCP5* splice variant c.201-1G>T, segregating with the disease in his brother (HPC21). Males are represented by a square, females by a circle, and unspecified gender by a diamond. A diagonal line through a symbol indicates that the individual is deceased, and colored symbols represent affected individuals. The index case is indicated by an arrow, and the cancer type and age at diagnosis are indicated when known.

Somatic variants and differential gene expression of mitotic pathway genes in Prostate Cancer

When exploring available data from the TCGA-PRAD dataset for the four genes under study, twelve carriers of somatic variants were found, overall representing 2.4% of the 501 PrCa patients. These include two missense variants in *CEP57* (Figure 3A), one truncating and three missense variants in *CEP290* (Figure 3B), four missense variants in *EML4* (Figure 3C), and one truncating and one missense variants in *TUBGCP5* (Figure 3D).

Expression patterns reported by TCGA reveal that *CEP57* exhibits an inverse expression pattern compared to other three genes, with higher expression in normal prostate tissue than in PrCa samples (Supplementary Figure 2A), suggesting a potential tumor-suppressor role. Interestingly, this differential expression is also observed in other cancers, including breast, renal, thyroid, and uterine cancers, where *CEP57* expression is reduced in tumor samples. Contrarily, the expression levels of *CEP290* and *EML4* are significantly higher in PrCa relative to the normal prostate tissue (Supplementary Figures 1B and 1C), suggesting oncogenic activity of overexpressed proteins. In the case of *TUBGCP5*, although its expression levels are slightly increased in PrCa, the increase is not statistically significant from the expression of the normal tissue (Supplementary Figure 2).

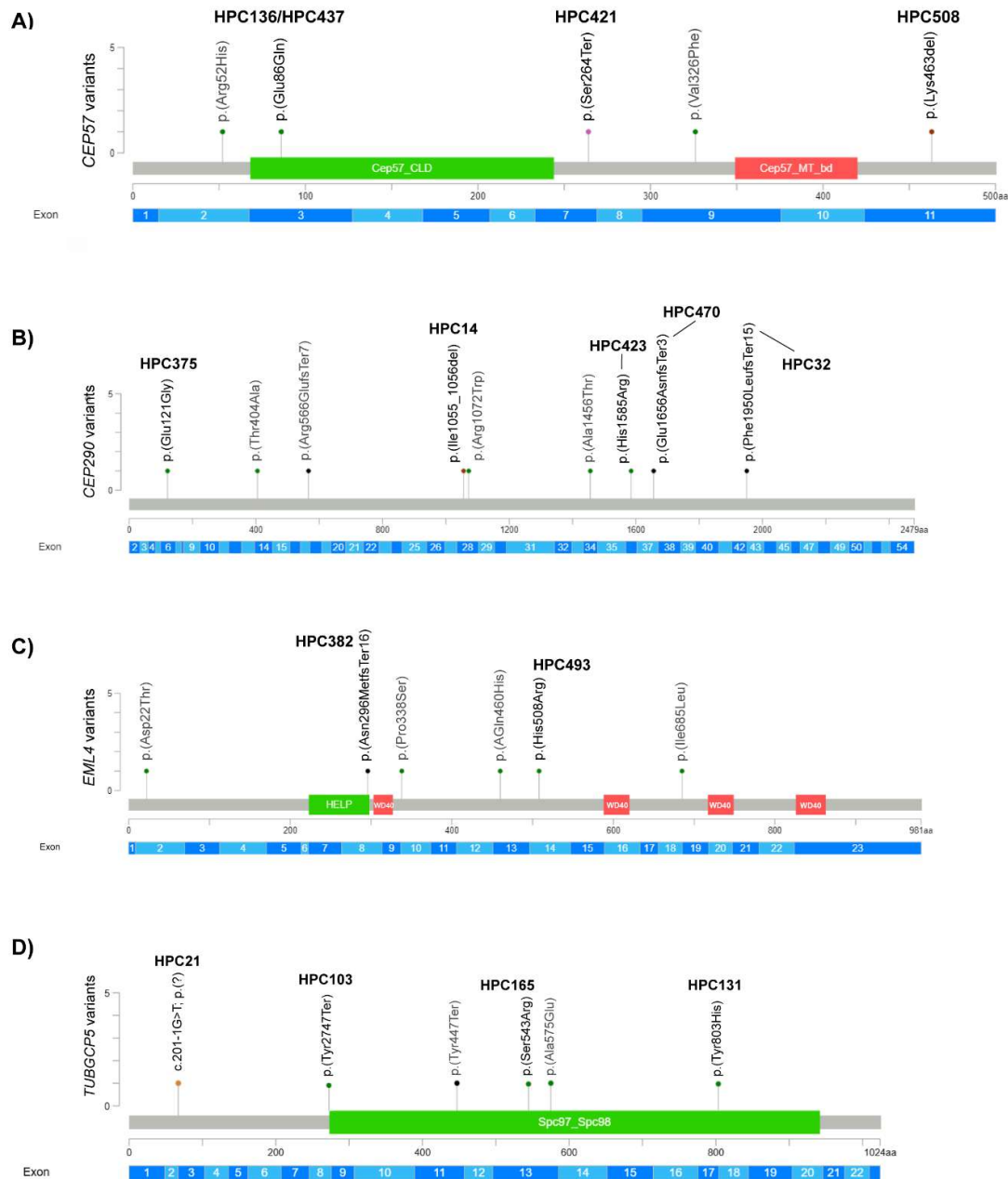


Figure 3 - Mutation map showing the predicted protein localization of the TCGA-PRAD somatic variants and the germline variants identified in this study. A) CEP57, B) CEP290, C) EML4 and D) TUBGCP5. Variants found in TCGA-PRAD (Firehose Legacy study, n=501 samples) [209], [210] are shown in grey, and variants found in this study are shown in black, with the corresponding patient ID. Variant circle color code stands for deleteriousness: black, truncating; red, yellow, potentially truncating (splicing); and green, missense. The main functional protein domains are shown.

Discussion

In this study, we aimed to identify novel genes contributing to inherited PrCa predisposition. In an earlier study from our group in a cohort of 121 index patients from the 462 PrCa families, T-NGS for known PrCa predisposing genes allowed us to identify a deleterious variant in *CEP57* [108], which encodes a protein that localizes to the centrosome and has a function in microtubular stabilization [175]. Furthermore, we have recently shown that monoallelic variants in *BUB1B* are recurrently found in early-onset/familial PrCa [11], a gene that encodes an essential component of the mitotic checkpoint complex, BubR1. The fact that biallelic variants in *BUB1B* and *CEP57* are linked to recessive syndromes characterized by mosaic trisomies and monosomies (MVA1, OMIM #257300, and MVA2, OMIM #614114, respectively) led us to explore our WES data from 96 PrCa patients across 45 families with early-onset or familial disease, prioritizing genes harboring truncating variants (frameshift, stop or splicing) that co-segregated with the disease within affected duos/trios and that are involved in pathways shared between *BUB1B* and *CEP57*, namely the “Cell Cycle, Mitotic Superpath” and “Mitotic Prometaphase”. This analysis revealed three overlapping genes, *CEP290*, *EML4*, and *TUBGCP5*, all of them involved in MT-KT attachment and identified as key players of the mitotic cell machinery, coordinating spindle formation during mitosis [135], [211], [212], [213].

CEP290 (The Centrosomal Protein 290, encoded by the *CEP290* gene, co-localizes on the centrosome (main MT-organizing center) and cilia, playing a critical role in cilia biogenesis [214]. The common loss of primary cilia in preinvasive and invasive PrCa [215], as well as the tight association between cancer and ciliopathies, suggests that defects in cilia biogenesis might be a transversal defect leading to PrCa development and progression [216]. In fact, variants in *CEP290* have shown to impair centrosome cohesion and ciliogenesis [217], leading to multipolar spindles, chromosome missegregation, and chromosomal instability (CIN) [218], which drive aneuploidy and tumor progression. Additionally, biallelic germline variants in *CEP290* predispose to the autosomal recessive disorder Joubert Syndrome (OMIM #610188).

The *EML4* gene, which encodes a member of the echinoderm MT-associated protein-like (EMAP) family, is essential for the formation and stability of MTs [219], [220], being also required for the organization of the mitotic spindle and for the proper attachment of KTs to MTs [211]. In addition to its involvement in the *EML4-ALK* fusion in lung adenocarcinomas, resulting in aberrant activation of signaling pathways such as PI3K/AKT and RAS/ERK promoting cell survival, proliferation, and metastasis [221], *EML4* variants can disrupt MT stability, enhancing chromosomal missegregation [221].

The *TUBGCP5* gene encodes the Tubulin Gamma Complex Component 5, a gamma-tubulin protein essential for MT nucleation at the centrosome, enabling MT-binding activity [135]. Variants in this gene can disrupt centrosome function leading to CIN through impaired spindle formation and abnormal chromosome segregation [222].

In the TCGA-PRAD dataset, a limited number of somatic variants have been documented in PrCa patients. Germline and somatic variants in these genes contribute to carcinogenesis by disrupting critical processes in centrosome regulation, spindle assembly, and chromosome segregation. Collectively, these genetic alterations foster genomic instability and tumor evolution, hallmark processes in cancer. Studies on centrosomal proteins have demonstrated their importance in cell cycle regulation and response to genomic stress, highlighting how defects in components like *TUBGCP5* contribute to tumorigenesis [129], [217].

To the best of our knowledge, this is the first report of germline variants in *CEP290*, *EML4* and *TUBGCP5* genes as possible PrCa predisposing genes. In fact, when querying VarElect from GeneCards with the list of genes included in “Cell Cycle, Mitotic Superpath” and “Mitotic Prometaphase” for “hereditary cancer”, none of these genes (*CEP290*, *EML4* and *TUBGCP5*) was listed as a gene reported to be associated with hereditary cancer predisposition. In fact, apart from the known cancer-predisposing DNA-repair (DDR) genes, which have previously been related to known hereditary cancer syndromes and with early-onset/familial PrCa, namely *BRCA2*, *ATM*, *BRCA1* and *CHEK2* [20], the *BUB1B* gene was listed to be directly associated with this phenotype [198]. Browsing the list of genes associated with “hereditary cancer” we found *MAD1L1*, which encodes a component of the spindle-assembly checkpoint that prevents the onset of anaphase until all chromosomes are properly aligned at the metaphase plate [223], [224], [225]. In fact, germline biallelic mutations in *MAD1L1* were described as a cause of aneuploidy with high tumor susceptibility [226]. This observation reinforces a potential link between mitotic pathway dysregulation and hereditary cancer risk, emphasizing the importance of these pathways in furthering our understanding of tumorigenesis.

In this study, we report germline deleterious/potentially pathogenic monoallelic variants in *CEP57*, *CEP290*, *EML4* and *TUBGCP5* in 3.2% of the early-onset and/or familial PrCa cases, extending to 5.1% (with the previously described 1.9% of *BUB1B* carriers) the fraction of early-onset/familial PrCa patients carrying rare germline variants in mitotic spindle genes.

Altogether, our findings reinforce the hypothesis that defects in mitotic pathways, particularly those regulating mitotic spindle formation, may significantly contribute to early-onset/familial PrCa development, and underscore the importance of pathway-based approaches to unveil the heterogeneity of PrCa predisposition. Upon validation, these

genes could enable predictive testing and targeted PrCa screening for carriers, while avoiding overdiagnosis in non-carriers.

Additional Information

Ethics approval and consent to participate

Patient samples were collected under ethics approval with reference CES 38–010 revised by the IPO Porto Ethics Committee. Informed consent was obtained from all subjects involved in the study.

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References

- [1] I. Heidegger *et al.*, “Hereditary prostate cancer – Primetime for genetic testing?,” Dec. 01, 2019, *W.B. Saunders Ltd.* doi: 10.1016/j.ctrv.2019.101927.
- [2] H. Sung *et al.*, “Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries,” *CA Cancer J Clin*, vol. 71, no. 3, pp. 209–249, May 2021, doi: 10.3322/caac.21660.
- [3] P. Rawla, “Epidemiology of Prostate Cancer,” *World J Oncol*, vol. 10, no. 2, pp. 63–89, 2019, doi: 10.14740/wjon1191.
- [4] S. Maia *et al.*, “The role of germline mutations in the BRCA1/2 and mismatch repair genes in men ascertained for early-onset and/or familial prostate cancer,” *Fam Cancer*, vol. 15, no. 1, pp. 111–121, Jan. 2016, doi: 10.1007/s10689-015-9832-x.
- [5] S. Maia *et al.*, “Identification of two novel HOXB13 germline mutations in Portuguese prostate cancer patients,” *PLoS One*, vol. 10, no. 7, Jul. 2015, doi: 10.1371/journal.pone.0132728.
- [6] P. Paulo *et al.*, “Targeted next generation sequencing identifies functionally deleterious germline mutations in novel genes in early-onset/familial prostate cancer,” *PLoS Genet*, vol. 14, no. 4, Apr. 2018, doi: 10.1371/journal.pgen.1007355.
- [7] M. P. Silva *et al.*, “BUB1B monoallelic germline variants contribute to prostate cancer predisposition by triggering chromosomal instability,” *J Biomed Sci*, vol. 31, no. 1, Dec. 2024, doi: 10.1186/s12929-024-01056-z.
- [8] K. Momotani, A. S. Khromov, T. Miyake, P. T. Stukenberg, and A. V. Somlyo, “Cep57, a multidomain protein with unique microtubule and centrosomal localization domains,” *Biochemical Journal*, vol. 412, no. 2, pp. 265–273, Jun. 2008, doi: 10.1042/BJ20071501.
- [9] S. Heinrich *et al.*, “Determinants of robustness in spindle assembly checkpoint signalling,” *Nat Cell Biol*, vol. 15, no. 11, pp. 1328–1339, Nov. 2013, doi: 10.1038/ncb2864.
- [10] M. Giam and G. Rancati, “Aneuploidy and chromosomal instability in cancer: A jackpot to chaos,” May 20, 2015, *BioMed Central Ltd.* doi: 10.1186/s13008-015-0009-7.
- [11] M. Cardoso *et al.*, “Exome sequencing of affected duos and trios uncovers PRUNE2 as a novel prostate cancer predisposition gene,” *Br J Cancer*, vol. 128, no. 6, pp. 1077–1085, Apr. 2023, doi: 10.1038/s41416-022-02125-6.
- [12] P. Paulo *et al.*, “Genetic landscape of homologous recombination repair genes in early-onset/familial prostate cancer patients,” *Genes Chromosomes Cancer*, Jul. 2023, doi: 10.1002/GCC.23190.
- [13] Q. Li and K. Wang, “InterVar: Clinical Interpretation of Genetic Variants by the 2015 ACMG-AMP Guidelines,” *Am J Hum Genet*, vol. 100, no. 2, p. 267, Feb. 2017, doi: 10.1016/J.AJHG.2017.01.004.
- [14] X. Jian, E. Boerwinkle, and X. Liu, “In silico prediction of splice-altering single nucleotide variants in the human genome,” *Nucleic Acids Res*, vol. 42, no. 22, p. 13534, Dec. 2014, doi: 10.1093/NAR/GKU1206.

- [15] H. Y. Xiong *et al.*, "The human splicing code reveals new insights into the genetic determinants of disease," *Science*, vol. 347, no. 6218, p. 1254806, Jan. 2015, doi: 10.1126/SCIENCE.1254806.
- [16] K. Jaganathan *et al.*, "Predicting Splicing from Primary Sequence with Deep Learning," *Cell*, vol. 176, no. 3, pp. 535-548.e24, Jan. 2019, doi: 10.1016/j.cell.2018.12.015.
- [17] J. Ye, G. Coulouris, I. Zaretskaya, I. Cutcutache, S. Rozen, and T. L. Madden, "Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction," 2012. [Online]. Available: <http://www.biomedcentral.com/1471-2105/13/134>
- [18] A. Anna and G. Monika, "Splicing mutations in human genetic disorders: examples, detection, and confirmation," Aug. 01, 2018, *Springer Verlag*. doi: 10.1007/s13353-018-0444-7.
- [19] J. M. Schwarz, D. N. Cooper, M. Schuelke, and D. Seelow, "Mutationtaster2: Mutation prediction for the deep-sequencing age," 2014, *Nature Publishing Group*. doi: 10.1038/nmeth.2890.
- [20] J. Gao *et al.*, "Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal," *Sci Signal*, vol. 6, no. 269, Apr. 2013, doi: 10.1126/scisignal.2004088.
- [21] E. Cerami *et al.*, "The cBio Cancer Genomics Portal: An open platform for exploring multidimensional cancer genomics data," *Cancer Discov*, vol. 2, no. 5, pp. 401–404, May 2012, doi: 10.1158/2159-8290.CD-12-0095.
- [22] K. Snape *et al.*, "Mutations in CEP57 cause mosaic variegated aneuploidy syndrome.," *Nat Genet*, vol. 43, no. 6, pp. 527–9, Jun. 2011, doi: 10.1038/ng.822.
- [23] D. Chen *et al.*, "EML4 promotes the loading of NUDC to the spindle for mitotic progression," *Cell Cycle*, vol. 14, no. 10, pp. 1529–1539, Jan. 2015, doi: 10.1080/15384101.2015.1026514.
- [24] J. CY. Song H, Park JE, "DDA3 targets Cep290 into the centrosome to regulate spindle positioning," *Biochem Biophys Res Commun*, vol. 463, no. (1-2), pp. 88-94., 2015.
- [25] C. Zhu and W. Jiang, "Cell cycle-dependent translocation of PRC1 on the spindle by Kif4 is essential for midzone formation and cytokinesis.," *Proc Natl Acad Sci U S A*, vol. 102, no. 2, pp. 343–8, Jan. 2005, doi: 10.1073/pnas.0408438102.
- [26] N. Izumi, K. Fumoto, S. Izumi, and A. Kikuchi, "GSK-3beta regulates proper mitotic spindle formation in cooperation with a component of the gamma-tubulin ring complex, GCP5.," *J Biol Chem*, vol. 283, no. 19, pp. 12981–91, May 2008, doi: 10.1074/jbc.M710282200.
- [27] A. Brandão, P. Paulo, and M. R. Teixeira, "Hereditary predisposition to prostate cancer: From genetics to clinical implications," Jul. 02, 2020, *MDPI AG*. doi: 10.3390/ijms21145036.
- [28] C. Villarroya-Beltri *et al.*, "Biallelic germline mutations in MAD1L1 induce a syndrome of aneuploidy with high tumor susceptibility," 2022.
- [29] C. Alfieri *et al.*, "Molecular basis of APC/C regulation by the spindle assembly checkpoint," *Nature*, vol. 536, no. 7617, pp. 431–436, Aug. 2016, doi: 10.1038/nature19083.

- [30] E. S. Fischer *et al.*, “Molecular mechanism of Mad1 kinetochore targeting by phosphorylated Bub1,” *EMBO Rep*, vol. 22, no. 7, Jul. 2021, doi: 10.15252/embr.202052242.
- [31] A. C. Faesen *et al.*, “Basis of catalytic assembly of the mitotic checkpoint complex,” *Nature*, vol. 542, no. 7642, pp. 498–502, Feb. 2017, doi: 10.1038/nature21384.
- [32] Adib R *et al.*, “Mitotic phosphorylation by NEK6 and NEK7 reduces the microtubule affinity of EML4 to promote chromosome congression.,” *Sci Signal*, vol. 12, no. (594):eaaw2939, Aug. 2019.
- [33] E. N. Firat-Karalar, “The ciliopathy gene product Cep290 is required for primary cilium formation and microtubule network organization,” *Turkish Journal of Biology*, vol. 42, no. 5, pp. 371–381, 2018, doi: 10.3906/biy-1805-25.
- [34] N. B. Hassounah, R. Nagle, K. Saboda, D. J. Roe, B. L. Dalkin, and K. M. McDermott, “Primary Cilia Are Lost in Preinvasive and Invasive Prostate Cancer,” *PLoS One*, vol. 8, no. 7, Jul. 2013, doi: 10.1371/journal.pone.0068521.
- [35] L. Fabbri, F. Bost, and N. M. Mazure, “Primary cilium in cancer hallmarks,” Mar. 02, 2019, *MDPI AG*. doi: 10.3390/ijms20061336.
- [36] M. Pollmann, R. Parwaresch, S. Adam-Klages, M. L. Kruse, F. Buck, and H. J. Heidebrecht, “Human EML4, a novel member of the EMAP family, is essential for microtubule formation,” *Exp Cell Res*, vol. 312, no. 17, pp. 3241–3251, Oct. 2006, doi: 10.1016/j.yexcr.2006.06.035.
- [37] Z. Wu *et al.*, “Cep131-Cep162 and Cby-Fam92 complexes cooperatively maintain Cep290 at the basal body and contribute to ciliogenesis initiation,” *PLoS Biol*, vol. 22, no. 3, Mar. 2024, doi: 10.1371/journal.pbio.3002330.
- [38] G. G. Slaats *et al.*, “DNA replication stress underlies renal phenotypes in CEP290-Associated Joubert syndrome,” *Journal of Clinical Investigation*, vol. 125, no. 9, pp. 3657–3666, Sep. 2015, doi: 10.1172/JCI80657.
- [39] S. R. Sabir, S. Yeoh, G. Jackson, and R. Bayliss, “EML4-ALK variants: Biological and molecular properties, and the implications for patients,” Sep. 05, 2017, *MDPI AG*. doi: 10.3390/cancers9090118.
- [40] N. Teixidó-Travesa, J. Roig, and J. Lüders, “The where, when and how of microtubule nucleation - one ring to rule them all,” *J Cell Sci*, vol. 125, no. 19, pp. 4445–4456, 2012, doi: 10.1242/jcs.106971.
- [41] M. Lin, S. S. Xie, and K. Y. Chan, “An updated view on the centrosome as a cell cycle regulator,” Dec. 01, 2022, *BioMed Central Ltd*. doi: 10.1186/s13008-022-00077-0.
- [42] T. Li *et al.*, “TIMER2.0 for analysis of tumor-infiltrating immune cells,” *Nucleic Acids Res*, vol. 48, no. W1, pp. W509–W514, Jul. 2020, doi: 10.1093/NAR/GKAA407.

Supplementary Information

Supplementary Table 1 - Demographic and clinicopathological characteristics of the HPC patients carrying truncating/potentially pathogenic variants in *CEP57*, *CEP290*, *EML4* and *TUBGCP5*.

Patient ID	Age ^a	HPC criteria	PSA ^a (ng/ml)	Gleason score	TNM stage	Treatment ^a	Total PrCa in the family ^b	Total Cancers in the family ^b
HPC14	52	Age	35.5	9 (4+5)	pT3aNxMx	Surg+RT+ADT	1	3
HPC32	43	Age+FH	5.69	7 (3+4) ^b	cT1cN0M0	RT	2	6
HPC62	48	Age+FH	5.05	6 (3+3)	pT2cNxMx	Surg	2	9
HPC103	61	FH	9.0	7 (4+3)	pT2NxM0	Surg	3	8
HPC131	54	Age+FH	9.5	6 (3+3) ^b	cT1cN0M0	RT	2	4
HPC146	59	FH	4.45	6 (3+3) ^b	cT2bNxM0	BT	4	4
HPC375	59	FH	3.7	7 (4+3) ^b	cT3NxM0	RT+ADT	3	4
HPC382	46	Age	9.2	6 (3+3)	pT2NxM0	Surg	1	3
HPC421	60	FH	11.4	6 (3+3)	pT3aNxMx	Surg+RT	3	5
HPC423	57	FH	5.92	7 (4+3)	pT3aN0M0	Surg	2	3
HPC437	60	FH	14.86	5 (2+3) ^b	cT2aN0M0	RT+ADT	3	7
HPC470	63	FH	3.92	7 (4+3)	pT3aNxMx	Surg	3	8
HPC493	55	Age+FH	49.17	7 (4+3) ^b	cT2cN0M0	RT+ADT	2	3
HPC502	61	FH	7.65	6 (3+3)	pT3aNxMx	Surg+RT	2	2
HPC508	55	Age+FH	9.68	7 (3+4) ^b	cT2cNxM0	BT	2	3

^a at/upon diagnosis; ^b FH considering both maternal and paternal lineages; ^c Biopsy is the source of the Gleason score. RP- Radical prostatectomy; ADT- Androgen deprivation therapy; RT- Radiotherapy; CT- Chemotherapy.

Supplementary Table 2 - Sequences of the primers used for KASPar SNP genotyping.

Primer name/application	5'→3' Sequence
<i>CEP57</i> c.256G>C - Reverse	TCCCTTTCTGTATCTGTTTCATCC
<i>CEP57</i> c.256G>C - Forward Wt specific	GAAGGTGACCAAGTTCATGCTGATTCGACGCTTGGAACCTTG
<i>CEP57</i> c.256G>C - Forward Mut specific	GAAGGTCGGAGTCAACGGATTGATTCGACGCTTGGAACCTTC

Supplementary Table 3 - *In silico* pathogenicity and conservation predictions for missense variants found in *CEP57*, *CEP290*, *EML4* and *TUBGCP5*.

Gene (RefSeq)	Variant	SIFT	Polyphen2_HDIV	LRT	Mutation Taster	Mutation Assessor	FATHMM	PROVEAN	VEST3 ^b	Meta SVM	Meta LR	CADD ^{a, b}	Total (pathogenicity)	GERP ++ ^b	phyloP100way ^b	phastCons 100way ^b	SiPhy_29way ^b	Total (conservation)	TOTAL ^c
<i>CEP290</i> (NM_025114)	c.362A>G; p.(Glu121Gly)	D	D	D	D	M	T	D	0.467	D	D	24.5	9/11	0.485	0.615	0.715	0.680	3/4	12/15
<i>CEP290</i> (NM_025114)	c.4754A>G; p.(His1585Arg)	T	D	D	D	M	D	N	0.771	D	D	24.2	9/11	0.789	0.764	0.715	0.778	4/4	13/15
<i>CEP57</i> (NM_014679)	c.256G>C; p.(Glu86Gln)	D	D	D	D	M	T	D	0.863	T	T	26.8	8/11	0.971	0.763	0.715	0.991	4/4	12/15
<i>EML4</i> (NM_001145076)	c.1523A>G; p.(His508Arg)	D	D	D	D	H	T	D	0.745	T	T	25.4	8/11	0.524	0.927	0.715	0.562	4/4	12/15
<i>TUBGCP5</i> (NM_001102610)	c.820T>A; p.(Thr274Ser)	-	D	-	-	-	-	-	0.834	-	-	22.9	3/3	0.643	0.757	0.715	0.690	4/4	7/7
<i>TUBGCP5</i> (NM_001102610)	c.1629G>C; p.(Ser543Arg)	-	-	-	-	-	-	-	0.328	-	-	20.4	1/2	0.332	0.513	0.715	0.395	2/4	3/6
<i>TUBGCP5</i> (NM_001102610)	c.2407A>T; p.(Tyr803His)	-	-	-	-	-	-	-	0.994	-	-	28.8	2/2	0.603	0.897	0.715	0.580	4/4	6/6

D- probably damaging/deleterious; T- tolerated; M/H- Medium/High Probability of being pathogenic; N/A- not available.

^a Variants with a CADD score above 20 were considered as possibly damaging (P) [108].

^b Rankscore values.

^c Fraction of functional predictors where the variant is expected to be pathogenic, which includes the classifications D, M and P, and the conservation predictors with score >0.5, among the 15 tools tested.

Supplementary Table 4 - *In silico* pathogenicity prediction scores of the splicing variant.

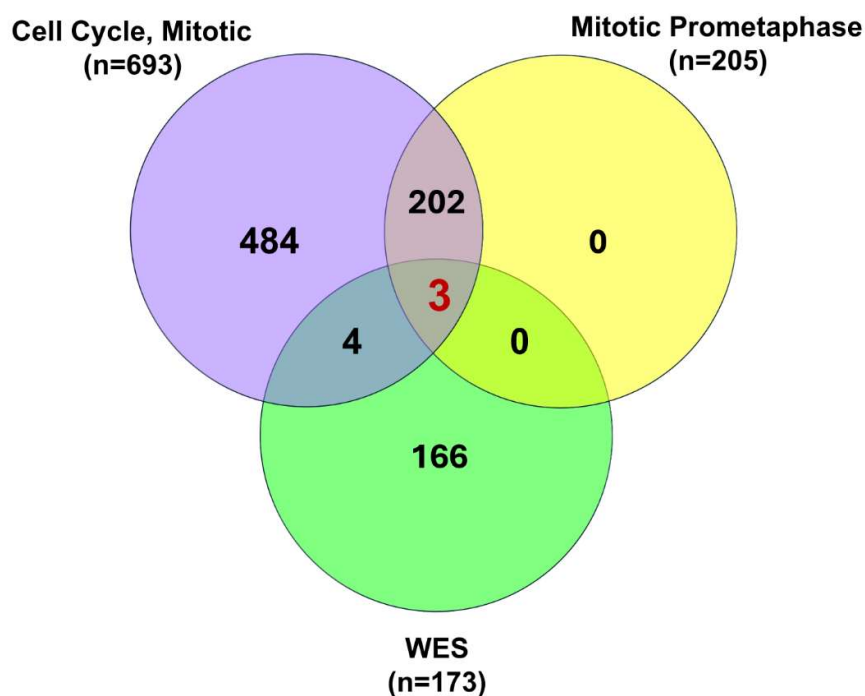
Variant	<i>TUBGCP5</i> c.201-1G>T
Prediction	scSNV (dbscSNV_ADA_SCORE) ^a : 1.000 scSNV (dbscSNV_RF_SCORE) ^a : 0.948 SPIDEX (dpsi_zscore) ^b : - 3.379 SpliceAI (Δ score) ^c : 0.980

^a SCORE > 0.6, the variant is predicted to impact splicing [202], [203];

^b absolute values of the score >2 are considered to be deleterious [204];

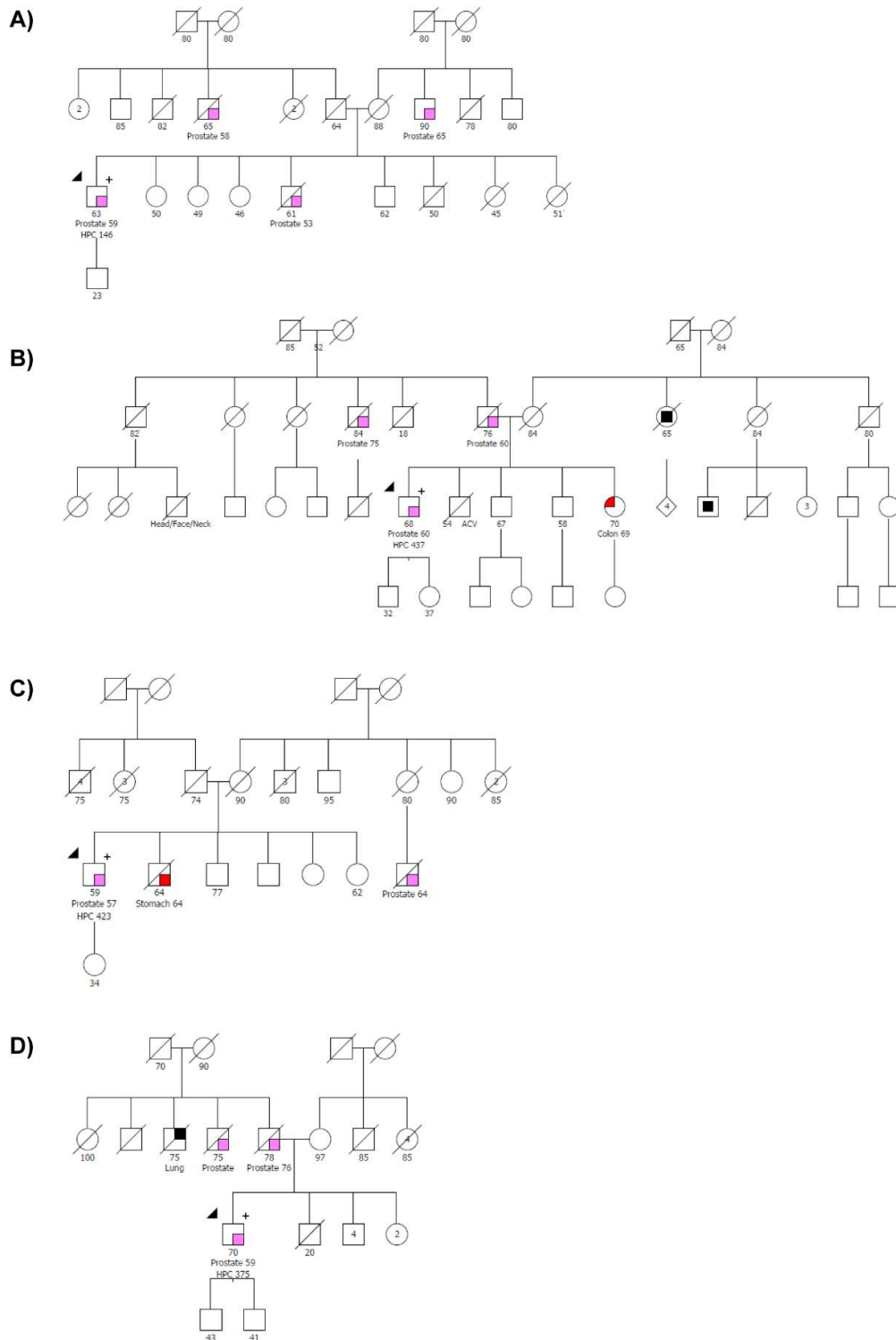
^c Δ score ≥ 0.8 , the variant is predicted to impact splicing [205].

Supplementary Figures

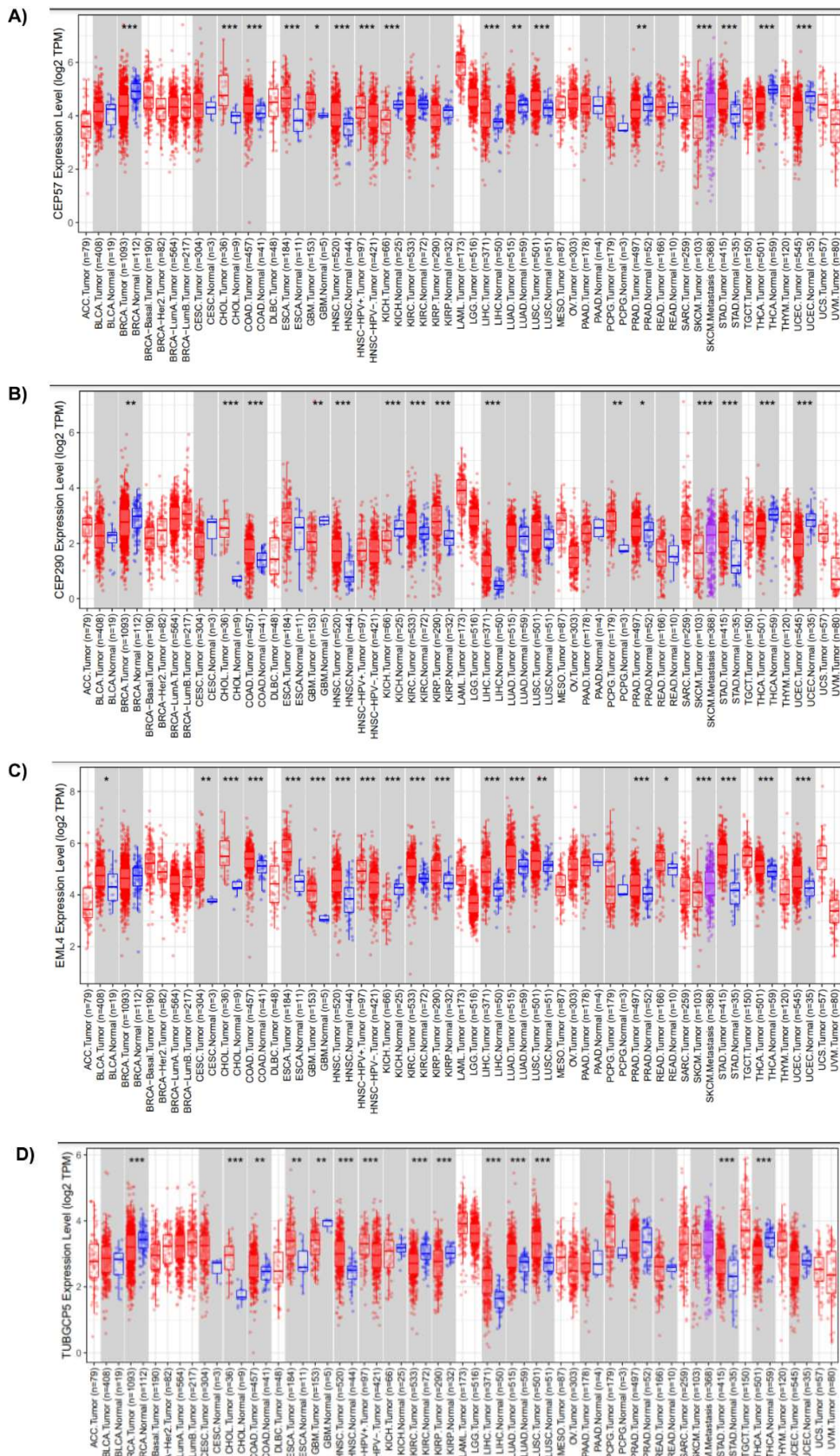


Supplementary Figure 1 - Venn diagram illustrating the strategy used for gene prioritization.

The 173 genes found by WES (green circle) were crossed with the two gene lists from PathCards (<https://pathcards.genecards.org>) clustered in “Cell Cycle, mitotic” (n=693), and “Mitotic Prometaphase” (n=205) pathways, shared by *BUB1B* and *CEP57*. Three genes were identified to be involved in both mitotic spindle formation and mitosis, namely, *CEP290*, *EML4* and *TUBGCP5*.



Supplementary Figure 2 - Family pedigrees of patients carrying unique missense variants in *CEP57* and *CEP290* genes. A) Patient HPC146, and B) HPC437 harboring the *CEP57* recurrent missense variant c.256G>C. C) Patient HPC423, harboring the *CEP290* missense variant c.4754A>G. D) Patient HPC375, harboring the *CEP290* missense variant c.362A>G. Males are represented by a square, females by a circle, and unspecified gender by a diamond. A diagonal line



Supplementary Figure 4 - Differential gene expression between tumor and adjacent normal tissues for across all TCGA tumors. Distributions of **A) *CEP57*, B) *CEP290*, C) *EML4*, and D) *TUBGCP5*** expression levels are displayed by box plots, build using TIMER 2.0 [227]. The statistical significance computed by the Wilcoxon test is annotated by the number of asterisks (*: p-value < 0.05; **: p-value <0.01; ***: p-value <0.001). ACC: Adrenocortical carcinoma; BLCA: Bladder Urothelial Carcinoma; BRCA: Breast invasive carcinoma; CESC: Cervical squamous cell carcinoma and endocervical adenocarcinoma; CHOL: Cholangiocarcinoma; COAD: Colon adenocarcinoma; DLBC: Lymphoid Neoplasm Diffuse Large B-cell Lymphoma; ESCA: Esophageal carcinoma; GBM: Glioblastoma multiforme; HNSC: Head and Neck squamous cell carcinoma; KICH: Kidney Chromophobe; KIRC: Kidney renal clear cell carcinoma; KIRP: Kidney renal papillary cell carcinoma; LAML: Acute Myeloid Leukemia; LGG: Brain Lower Grade Glioma; LIHC: Liver hepatocellular carcinoma; LUAD: Lung adenocarcinoma; LUSC: Lung squamous cell carcinoma; MESO: Mesothelioma; OV: Ovarian serous cystadenocarcinoma; PAAD: Pancreatic adenocarcinoma; PCPG: Pheochromocytoma and Paraganglioma; PRAD: Prostate adenocarcinoma; READ: Rectum adenocarcinoma; SARC: Sarcoma; SKCM: Skin Cutaneous Melanoma; STAD: Stomach adenocarcinoma; TGCT: Testicular Germ Cell Tumors; THCA: Thyroid carcinoma; THYM: Thymoma; UCEC: Uterine Corpus Endometrial Carcinoma; UCS: Uterine Carcinosarcoma; UVM: Uveal Melanoma.

GENERAL DISCUSSION

PrCa represents a significant challenge to the clinical community worldwide, with a subset of men presenting an inherited predisposition to develop this disease. Up to 15% of the newly diagnosed PrCa cases are expected to arise from germline variants in genes that predispose individuals for this malignancy. The identification of deleterious/pathogenic variants is mandatory to a better clinical management of PrCa. Knowledge of a man's germline status provides valuable information regarding prognosis [29], [228], has treatments implications because deleterious variants can guide the selection of targeted treatments [229], [230], [231], and enables cascade testing for family members.

Contrarily to other hereditary cancer syndromes, little is known about the genetic determinants that underlie PrCa development in patients with early-onset and/or familial aggregation of the disease. In fact, not all men are at equal risk for developing PrCa, which may be due to the polygenic nature of the disease, its high heterogeneity, incomplete penetrance, the presence of phenocopies, and pleiotropy [232], [233], [234], [235]. Additionally, it is often difficult to obtain samples from multiple affected patients from the same family for genetic testing, especially for more than two generations [236], which does not allow to establish robust genotype-phenotype correlations.

Aiming to identify part of the genes masked within inherited PrCa predisposition, our group has recently gathered a series of 462 PrCa probands and 55 affected relatives with early-onset and/or familial/hereditary disease [70]. Previous studies by our group, employing genetic testing of specific genes [70], [196] and T-NGS for a multigene panel [108], have enabled the identification of multiple families carrying germline variants in established cancer predisposition genes. However, when we started this PhD thesis, most of the families remained without an identified causative variant, after screening for known predisposing genes [198], [223], highlighting the need for new strategies. Whole-exome sequencing (WES), in particular, has been instrumental in identifying the causes of various genetic diseases, including rare Mendelian disorders and inherited cancer predisposition syndromes [237], [238], [239], [240]. In Portugal, particularly in the north, the population has a relatively homogenous genetic background. Our research, along with contributions from others, has led to the identification of several Portuguese founder mutations linked to different cancer predisposition syndromes [241], [242], [243], [244], [245] [246], [247] highlighting how our population's genetic structure can aid in prioritizing candidate genes through a genome-wide approach. Consequently, we initiated a pilot study using WES on duos and trios of PrCa patients from 45 of the 462 previously recruited families. Targeted NGS panels function as "customized" exon sequencing tools, enabling the screening of

specific phenotype-related genes at an affordable cost, while reducing both the volume and complexity of output data [248].

In this project, we aimed to identify new genes with deleterious germline variants that might account for a significant portion of inherited PrCa predisposition. We combined information from two NGS approaches (exome and targeted panel) to select the most promising candidates and we aimed to identify genes involved in chromosome segregation, spindle dynamics and MT-KT attachment. To validate the best candidate genes, we extended our analysis to the whole series of 462 PrCa patients using T-NGS.

The first paper was focused on *BUB1B* gene as it was the best candidate among those involved in the chromosome segregation machinery. In fact, it is most frequently mutated gene in the series, with nine rare variants (9/462) identified representing 1.9% of early-onset/familial PrCa cases (and ~0.6% of other cancer types). Most clinical/translational research linking *BUB1B* variants to cancer has been conducted within the framework of MVA syndrome, a condition with a high penetrance caused by homozygous or compound heterozygous variants in *BUB1B*. Individuals with MVA syndrome often exhibit various abnormalities, including susceptibility to several types of cancer, particularly during childhood. In contrast, our study focuses on a cohort of individuals meeting testing criteria for hereditary PrCa and no previous history of cancer-associated syndromes, where we identified germline *BUB1B* variants in heterozygosity. Here, we propose that the presence of a recurrent variant, and potentially other less frequent *BUB1B* variants, in one single allele is sufficient to induce subclinical chromosomal instability, thus increasing susceptibility to PrCa. The identification of heterozygous *BUB1B* variants consistently associated with hereditary PrCa may contribute to explain the missing heritability of PrCa beyond the well-established predisposition genes. Moreover, our study extends beyond a comprehensive analysis of *BUB1B* variants in PrCa to encompass also potential therapeutic implications.

The second paper results from querying our WES data for deleterious variants in genes involved in shared pathways between the *BUB1B* and *CEP57* genes. We have identified three genes, namely *CEP290*, *EML4*, and *TUBGCP5*, with variants segregating with the disease in four (8.9%) of the 45 families. Screening the entire series revealed additional rare truncating and potential pathogenic missense variants in these genes, as well as validated the previously identified truncating variant in *CEP57* [108] and further revealed a recurrent and potential pathogenic missense variant in this gene. Altogether, 15 (3.2%) of early-onset/familial PrCa cases were shown to carry potentially deleterious variants in the *CEP57*, *CEP290*, *EML4*, and *TUBGCP5* genes. To the best of our knowledge, this work describes the highest frequency of variants in genes involved in spindle formation and MT-KT attachment in early-onset/familial PrCa reported so far.

Apart from *BUB1B*, for which heterozygous variants predispose to the PCS trait (OMIM #176430), there are no reports on the biological impact of truncating/deleterious heterozygous variants in the remaining four genes (*CEP57*, *CEP290*, *EML4* and *TUBGCP5*). Moreover, all these proteins, together with *BUB1B*, participate in the “Cell Cycle, Mitotic” and “Mitotic Prometaphase” pathways and errors in chromosome segregation during anaphase may lead to aneuploidy, a hallmark of cancer. Considering that most genetic causes underlying early-onset/familial PrCa development are yet unknown, the finding of different variants in different genes encoding members of the same cellular pathway deserves further investigation. In fact, 5.1% of the index patients with early-onset and/or familial PrCa were shown to present a potential deleterious variant in one of the five genes here reported, a proportion that is similar to the mutation rate that has been reported in the literature for genes involved in homologous recombination repair.

The success of this research is attributed to two main factors: First, careful sample selection and size played a crucial role. Patients involved in this study were from a genetically homogenous population in northern Portugal and recruitment was independent of clinicopathologic features (besides the defined age of diagnosis and the family history criteria), ensuring an unbiased and representative sample (at least of those that were able to respond to the study invitation). Concerning sample size, this series is one of the largest early-onset and familial PrCa reported so far, enhancing statistical power, strengthening the reliability and generalizability of findings on genetic factors linked to PrCa predisposition. Second, having multiple affected family members in each family within the WES series was essential for narrowing down potential new genes contributing to PrCa predisposition.

CONCLUSIONS

In this PhD thesis, we have made a significant contribution to unraveling the molecular basis of PrCa predisposition by identifying germline variants in *BUB1B*, *CEP57*, *CEP290*, *EML4*, and *TUBGCP5*, collectively present in 5.1% of the families in our cohort. To the best of our knowledge, this is the first report of these genes as candidate PrCa susceptibility genes. If validated, these discoveries could pave the way for predictive genetic testing and pre-symptomatic cancer screening strategies for healthy carriers of predisposing variants, while also providing reassurance to non-carriers with a FH of the disease, thereby reducing the risk of overdiagnosis and overtreatment. Additionally, this work emphasizes the significance of expanding genetic screening to include mitotic pathway genes for better understanding and managing PrCa predisposition, paving the way for predictive testing and personalized interventions.

FUTURE PERSPECTIVES

This study has opened new avenues for future research. First, we aim to replicate these findings in a larger cohort by leveraging our involvement in the global PRACTICAL consortium to analyze WES data from a diverse set of PrCa patients and controls across multiple populations. Additionally, we plan to expand the segregation analysis of the *CEP57*, *CEP290*, *EML4* and *TUBGCP5* to other family members affected by PrCa or other cancers. We also seek to characterize the mutational signature and tumor mutational burden in carriers of these variants.

The interplay between CIN and response to taxane-based therapies is critical in metastatic settings, where high CIN levels compromise response to these therapies. Given our observation of decreased taxol sensitivity in *BUB1B*-edited cells, we already extended the screening for "deleterious" variants in *BUB1B* in metastatic PrCa samples. *CEP57* was also included in the customized panel, as the involvement of *CEP57* in spindle MT dynamics may modulate the effectiveness of taxane-based therapies in metastatic PrCa patients as well. This emphasizes the importance of integrating genomic profiling into the management of metastatic PrCa to optimize therapeutic outcomes.

For *CEP57*, as already performed for *BUB1B*, we aim to identify mechanisms underlying potential inactivation of the second allele in tumors of germline variant carriers and to conduct functional analyses to understand the impact of *CEP57* deleterious variants on PrCa development and progression.

Lastly, the other spindle-associated genes with deleterious germline variants identified in this study require further investigation to elucidate their potential roles in inherited PrCa predisposition

REFERENCES

- [1] World Health Organization (WHO), "Global Health Estimates 2020: Deaths by Cause, Age, Sex, by Country and by Region, 2000-2019. ."
- [2] F. Bray, M. Laversanne, E. Weiderpass, and I. Soerjomataram, "The ever-increasing importance of cancer as a leading cause of premature death worldwide," *Cancer*, vol. 127, no. 16, pp. 3029–3030, Aug. 2021, doi: 10.1002/cnrc.33587.
- [3] F. Bray *et al.*, "Global cancer statistics 2022: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries," *CA Cancer J Clin*, vol. 74, no. 3, pp. 229–263, May 2024, doi: 10.3322/caac.21834.
- [4] H. Sung *et al.*, "Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries," *CA Cancer J Clin*, vol. 71, no. 3, pp. 209–249, May 2021, doi: 10.3322/caac.21660.
- [5] Jemal A and et al, "The Cancer Atlas," *American Cancer Society*, 2019.
- [6] L. Q. Rogers, K. S. Courneya, R. Paragi-Gururaja, S. J. Markwell, and R. Imeokparia, "Lifestyle behaviors, obesity, and perceived health among men with and without a diagnosis of prostate cancer: A population-based, cross-sectional study," *BMC Public Health*, vol. 8, 2008, doi: 10.1186/1471-2458-8-23.
- [7] E. M. John, M. C. Stern, R. Sinha, and J. Koo, "Meat consumption, cooking practices, meat mutagens, and risk of prostate cancer," *Nutr Cancer*, vol. 63, no. 4, pp. 525–537, May 2011, doi: 10.1080/01635581.2011.539311.
- [8] I. N. Benke, M. F. Leitzmann, G. Behrens, D. Schmid, and D. Schmid, "Physical activity in relation to risk of prostate cancer: a systematic review and meta-analysis Running head: Physical activity and prostate cancer risk," 2018, doi: 10.1093/annonc/mdy073/4904101.
- [9] D. Mandair, R. E. Rossi, M. Pericleous, T. Whyand, and M. E. Caplin, "Prostate cancer and the influence of dietary factors and supplements: a systematic review," 2014. [Online]. Available: <http://www.nutritionandmetabolism.com/content/11/1/30>
- [10] D. Hanahan and R. A. Weinberg, "Hallmarks of cancer: The next generation," Mar. 04, 2011. doi: 10.1016/j.cell.2011.02.013.
- [11] A. S. Choudhari, P. C. Mandave, M. Deshpande, P. Ranjekar, and O. Prakash, "Phytochemicals in cancer treatment: From preclinical studies to clinical practice," 2020, *Frontiers Media S.A.* doi: 10.3389/fphar.2019.01614.
- [12] R. Fisher, L. Pusztai, and C. Swanton, "Cancer heterogeneity: Implications for targeted therapeutics," Feb. 19, 2013. doi: 10.1038/bjc.2012.581.
- [13] V. Schirrmacher, "From chemotherapy to biological therapy: A review of novel concepts to reduce the side effects of systemic cancer treatment (Review)," *Int J Oncol*, vol. 54, no. 2, pp. 407–419, Feb. 2019, doi: 10.3892/ijo.2018.4661.
- [14] J. Fares, M. Y. Fares, H. H. Khachfe, H. A. Salhab, and Y. Fares, "Molecular principles of metastasis: a hallmark of cancer revisited," Dec. 01, 2020, *Springer Nature*. doi: 10.1038/s41392-020-0134-x.

- [15] I. Dagogo-Jack and A. T. Shaw, "Tumour heterogeneity and resistance to cancer therapies," Feb. 01, 2018, *Nature Publishing Group*. doi: 10.1038/nrclinonc.2017.166.
- [16] Ferlay J *et al.*, "Global Cancer Observatory: Cancer Today. Lyon, France: International Agency for Research on Cancer. Available from: <https://gco.iarc.fr/today>, accessed [22-Nov-2021]," Prostate Cancer Fact Sheets.
- [17] "National Comprehensive Cancer Network. NCCN Clinical Practice Guidelines in Oncology—Prostate Cancer Early Detection, Version 1.2022 [/www.nccn.org/professionals/physician_gls/pdf/prostate_detection.pdf](http://www.nccn.org/professionals/physician_gls/pdf/prostate_detection.pdf) (Accessed on 1 March 2022)".
- [18] G. Draisma *et al.*, "Lead time and overdiagnosis in prostate-specific antigen screening: Importance of methods and context," *J Natl Cancer Inst*, vol. 101, no. 6, pp. 374–383, Mar. 2009, doi: 10.1093/jnci/djp001.
- [19] M. Quinn and P. Babb, "Patterns and trends in prostate cancer incidence, survival, prevalence and mortality. Part I: international comparisons."
- [20] A. Brandão, P. Paulo, and M. R. Teixeira, "Hereditary predisposition to prostate cancer: From genetics to clinical implications," Jul. 02, 2020, *MDPI AG*. doi: 10.3390/ijms21145036.
- [21] C. H. Pernar, E. M. Ebot, K. M. Wilson, and L. A. Mucci, "The epidemiology of prostate cancer," *Cold Spring Harb Perspect Med*, vol. 8, no. 12, 2018, doi: 10.1101/CSHPERSPECT.A030361.
- [22] A. Brandt, J. L. Bermejo, J. Sundquist, and K. Hemminki, "Age at Diagnosis and Age at Death in Familial Prostate Cancer," *Oncologist*, vol. 14, no. 12, pp. 1209–1217, Dec. 2009, doi: 10.1634/theoncologist.2009-0132.
- [23] P. Kheirandish and F. Chinegwundoh, "Ethnic differences in prostate cancer," *Br J Cancer*, vol. 105, no. 4, pp. 481–485, Aug. 2011, doi: 10.1038/bjc.2011.273.
- [24] H. E. Taitt, "Global Trends and Prostate Cancer: A Review of Incidence, Detection, and Mortality as Influenced by Race, Ethnicity, and Geographic Location," Nov. 01, 2018, *SAGE Publications Inc*. doi: 10.1177/1557988318798279.
- [25] T. R. Rebbeck *et al.*, "Global Patterns of Prostate Cancer Incidence, Aggressiveness, and Mortality in Men of African Descent," *Prostate Cancer*, vol. 2013, pp. 1–12, 2013, doi: 10.1155/2013/560857.
- [26] D. G. Bostwick *et al.*, "Human prostate cancer risk factors," Nov. 15, 2004. doi: 10.1002/cncr.20408.
- [27] J. T. Zhen *et al.*, "Genetic testing for hereditary prostate cancer: Current status and limitations," Aug. 01, 2018, *John Wiley and Sons Inc*. doi: 10.1002/cncr.31316.
- [28] P. Rawla, "Epidemiology of Prostate Cancer," *World J Oncol*, vol. 10, no. 2, pp. 63–89, 2019, doi: 10.14740/wjon1191.
- [29] H. Ni Raghallaigh and R. Eeles, "Genetic predisposition to prostate cancer: an update," Jan. 01, 2022, *Springer Science and Business Media B.V.* doi: 10.1007/s10689-021-00227-3.
- [30] O. Bratt, "Hereditary prostate cancer Historical background and genetic principles."

REFERENCES

- [31] M. T. Vietri *et al.*, "Hereditary prostate cancer: Genes related, target therapy and prevention," Apr. 01, 2021, *MDPI AG*. doi: 10.3390/ijms22073753.
- [32] M. Akizhanova *et al.*, "PSA and Prostate Health Index based prostate cancer screening in a hereditary migration complicated population: Implications in precision diagnosis," *J Cancer*, vol. 8, no. 7, pp. 1223–1228, Apr. 2017, doi: 10.7150/jca.18012.
- [33] "Screening for Prostate Cancer: U.S. Preventive Services Task Force Recommendation Statement." [Online]. Available: www.annals.org
- [34] R. L. Grubb *et al.*, "Prostate cancer screening in the Prostate, Lung, Colorectal and Ovarian cancer screening trial: Update on findings from the initial four rounds of screening in a randomized trial," *BJU Int*, vol. 102, no. 11, pp. 1524–1530, Dec. 2008, doi: 10.1111/j.1464-410X.2008.08214.x.
- [35] J. L. Descotes, "Diagnosis of prostate cancer," Apr. 01, 2019, *Editorial Office of Asian Journal of Urology*. doi: 10.1016/j.ajur.2018.11.007.
- [36] M. S. Litwin and H. J. Tan, "The diagnosis and treatment of prostate cancer: A review," Jun. 27, 2017, *American Medical Association*. doi: 10.1001/jama.2017.7248.
- [37] L. Brunese, F. Mercaldo, A. Reginelli, and A. Santone, "Formal methods for prostate cancer Gleason score and treatment prediction using radiomic biomarkers," *Magn Reson Imaging*, vol. 66, pp. 165–175, Feb. 2020, doi: 10.1016/j.mri.2019.08.030.
- [38] J. K. Sehn, "Prostate Cancer Pathology: Recent Updates and Controversies."
- [39] S. B. Edge and C. C. Compton, "The american joint committee on cancer: The 7th edition of the AJCC cancer staging manual and the future of TNM," Jun. 2010. doi: 10.1245/s10434-010-0985-4.
- [40] "Prostate Cancer Eur Urol 2000;38:74-78," 2000. [Online]. Available: www.karger.com
- [41] R. C. Chen *et al.*, "Active surveillance for the management of localized prostate cancer (Cancer Care Ontario guideline): American society of clinical oncology clinical practice guideline endorsement," *Journal of Clinical Oncology*, vol. 34, no. 18, pp. 2182–2190, Jun. 2016, doi: 10.1200/JCO.2015.65.7759.
- [42] N. Mottet *et al.*, "EAU-ESTRO-SIOG Guidelines on Prostate Cancer. Part 1: Screening, Diagnosis, and Local Treatment with Curative Intent," *Eur Urol*, vol. 71, no. 4, pp. 618–629, Apr. 2017, doi: 10.1016/j.eururo.2016.08.003.
- [43] P. Cornford *et al.*, "EAU-ESTRO-SIOG Guidelines on Prostate Cancer. Part II: Treatment of Relapsing, Metastatic, and Castration-Resistant Prostate Cancer," *Eur Urol*, vol. 71, no. 4, pp. 630–642, Apr. 2017, doi: 10.1016/j.eururo.2016.08.002.
- [44] T. Golabek *et al.*, "Evidence-based recommendations on androgen deprivation therapy for localized and advanced prostate cancer," 2016, *Polish Urological Association*. doi: 10.5173/ceju.2016.812.
- [45] Lorente D, Fizazi K, Sweeney C, and de Bono JS, "Optimal Treatment Sequence for Metastatic Castration-resistant Prostate Cancer," *Eur Urol Focus*, vol. 2, no. 5, pp. 488–498, 2016.

- [46] Gravis G *et al.*, “Chemotherapy in hormone-sensitive metastatic prostate cancer: Evidences and uncertainties from the literature,” *Cancer Treat Rev.*, vol. 55, pp. 211–217, 2017.
- [47] P. Isaacsson Velho and E. S. Antonarakis, “PD-1/PD-L1 pathway inhibitors in advanced prostate cancer,” May 04, 2018, *Taylor and Francis Ltd.* doi: 10.1080/17512433.2018.1464388.
- [48] J. L. Mohler *et al.*, “Prostate cancer, version 2.2019,” *JNCCN Journal of the National Comprehensive Cancer Network*, vol. 17, no. 5, pp. 479–505, 2019, doi: 10.6004/jnccn.2019.0023.
- [49] D. Sigorski, E. Iżycka-Świeszewska, and L. Bodnar, “Poly(ADP-Ribose) Polymerase Inhibitors in Prostate Cancer: Molecular Mechanisms, and Preclinical and Clinical Data,” Dec. 01, 2020, *Adis*. doi: 10.1007/s11523-020-00756-4.
- [50] A. Ashworth, “A synthetic lethal therapeutic approach: Poly(ADP) ribose polymerase inhibitors for the treatment of cancers deficient in DNA double-strand break repair,” 2008. doi: 10.1200/JCO.2008.16.0812.
- [51] J. de Bono *et al.*, “Olaparib for Metastatic Castration-Resistant Prostate Cancer,” *New England Journal of Medicine*, vol. 382, no. 22, pp. 2091–2102, May 2020, doi: 10.1056/nejmoa1911440.
- [52] I. Heidegger *et al.*, “Hereditary prostate cancer – Primetime for genetic testing?,” Dec. 01, 2019, *W.B. Saunders Ltd.* doi: 10.1016/j.ctrv.2019.101927.
- [53] Yasin Bhanji, William B Isaacs, Jianfeng Xu, and Kathleen A Cooney, “Prostate Cancer Predisposition,” *Urol Clin North Am.*, vol. 3, no. 48, pp. 283–296, Aug. 2021.
- [54] T. R. Rebbeck, “Prostate Cancer Genetics: Variation by Race, Ethnicity, and Geography,” Jan. 01, 2017, *W.B. Saunders*. doi: 10.1016/j.semradonc.2016.08.002.
- [55] B. S. Carter *et al.*, “Hereditary prostate cancer: Epidemiologic and clinical features,” *Journal of Urology*, vol. 150, no. 3, pp. 797–802, 1993, doi: 10.1016/S0022-5347(17)35617-3.
- [56] J. L. Stanford and E. A. Ostrander, “Familial Prostate Cancer,” 2001. [Online]. Available: <http://epirev.oxfordjournals.org/>
- [57] H. L. Rehm, “Disease-targeted sequencing: A cornerstone in the clinic,” Apr. 2013. doi: 10.1038/nrg3463.
- [58] S. M. Domchek, A. Bradbury, J. E. Garber, K. Offit, and M. E. Robson, “Multiplex Genetic Testing for Cancer Susceptibility: Out on the High Wire Without a Net?,” 2013. [Online]. Available: www.jco.org
- [59] E. K. Bancroft *et al.*, “A prospective prostate cancer screening programme for men with pathogenic variants in mismatch repair genes (IMPACT): initial results from an international prospective study,” *Lancet Oncol*, vol. 22, no. 11, pp. 1618–1631, Nov. 2021, doi: 10.1016/S1470-2045(21)00522-2.
- [60] P. Paulo *et al.*, “Validation of a Next-Generation Sequencing Pipeline for the Molecular Diagnosis of Multiple Inherited Cancer Predisposing Syndromes,”

REFERENCES

- Journal of Molecular Diagnostics*, vol. 19, no. 4, pp. 502–513, Jul. 2017, doi: 10.1016/j.jmoldx.2017.05.001.
- [61] A. O. Sokolova and H. H. Cheng, “Genetic Testing in Prostate Cancer,” Jan. 01, 2020, *Springer*. doi: 10.1007/s11912-020-0863-6.
 - [62] C. C. Pritchard *et al.*, “Inherited DNA-Repair Gene Mutations in Men with Metastatic Prostate Cancer,” *New England Journal of Medicine*, vol. 375, no. 5, pp. 443–453, Aug. 2016, doi: 10.1056/nejmoa1603144.
 - [63] S. H. Tan, G. Petrovics, and S. Srivastava, “Prostate cancer genomics: Recent advances and the prevailing underrepresentation from racial and ethnic minorities,” Apr. 22, 2018, *MDPI AG*. doi: 10.3390/ijms19041255.
 - [64] M. Pritzlaff *et al.*, “Diagnosing hereditary cancer predisposition in men with prostate cancer,” doi: 10.1038/s41436.
 - [65] J. D. Norris *et al.*, “The Homeodomain Protein HOXB13 Regulates the Cellular Response to Androgens,” *Mol Cell*, vol. 36, no. 3, pp. 405–416, Nov. 2009, doi: 10.1016/j.molcel.2009.10.020.
 - [66] C. Prevention, G. Hereditary, and A. Epidemiology Accepted On, “The Role of BRCA Testing in Hereditary Pancreatic and Prostate Cancer Families,” 2019, doi: 10.1200/EDBK_.
 - [67] C. M. Ewing *et al.*, “Germline Mutations in HOXB13 and Prostate-Cancer Risk,” *New England Journal of Medicine*, vol. 366, no. 2, pp. 141–149, Jan. 2012, doi: 10.1056/nejmoa1110000.
 - [68] C. Stanislaw, Y. Xue, and W. R. Wilcox, “Genetic evaluation and testing for hereditary forms of cancer in the era of next-generation sequencing,” Mar. 01, 2016, *Cancer Biology and Medicine*. doi: 10.28092/j.issn.2095-3941.2016.0002.
 - [69] M. Cardoso, S. Maia, P. Paulo, and M. R. Teixeira, “Oncoscience 288 www.impactjournals.com/oncoscience Oncogenic mechanisms of HOXB13 missense mutations in prostate carcinogenesis,” 2016. [Online]. Available: www.impactjournals.com/oncoscience
 - [70] S. Maia *et al.*, “Identification of Two Novel HOXB13 Germline Mutations in Portuguese Prostate Cancer Patients.,” *PLoS One*, vol. 10, no. 7, p. e0132728, 2015, doi: 10.1371/journal.pone.0132728.
 - [71] J. Wei *et al.*, “Germline HOXB13 G84E mutation carriers and risk to twenty common types of cancer: results from the UK Biobank,” *Br J Cancer*, vol. 123, no. 9, pp. 1356–1359, Oct. 2020, doi: 10.1038/s41416-020-01036-8.
 - [72] Z. Chen *et al.*, “The G84E mutation of HOXB13 is associated with increased risk for prostate cancer: Results from the REDUCE trial,” *Carcinogenesis*, vol. 34, no. 6, pp. 1260–1264, Jun. 2013, doi: 10.1093/carcin/bgt055.
 - [73] M. T. Vietri *et al.*, “Five italian families with two mutations in brca genes,” *Genes (Basel)*, vol. 11, no. 12, pp. 1–15, Dec. 2020, doi: 10.3390/genes11121451.
 - [74] K. Gudmundsdottir and A. Ashworth, “The roles of BRCA1 and BRCA2 and associated proteins in the maintenance of genomic stability,” Sep. 25, 2006. doi: 10.1038/sj.onc.1209874.

- [75] H. Tulinius, V. Egilsson, G. H. Olafsdottir, and H. Sigvaldason, "PAPERS Risk of prostate, ovarian, and endometrial cancer among relatives of women with breast cancer."
- [76] R. Roy, J. Chun, and S. N. Powell, "BRCA1 and BRCA2: Different roles in a common pathway of genome protection," Jan. 2012. doi: 10.1038/nrc3181.
- [77] Z. J. Ren *et al.*, "First-degree family history of breast cancer is associated with prostate cancer risk: A systematic review and meta-analysis," *BMC Cancer*, vol. 19, no. 1, Sep. 2019, doi: 10.1186/s12885-019-6055-9.
- [78] F. R. Schumacher *et al.*, "Association analyses of more than 140,000 men identify 63 new prostate cancer susceptibility loci," *Nat Genet*, vol. 50, no. 7, pp. 928–936, Jul. 2018, doi: 10.1038/s41588-018-0142-8.
- [79] D. Leongamornlert *et al.*, "Germline BRCA1 mutations increase prostate cancer risk," *Br J Cancer*, vol. 106, no. 10, pp. 1697–1701, May 2012, doi: 10.1038/bjc.2012.146.
- [80] D. Thompson and D. F. Easton, "Cancer Incidence in BRCA1 Mutation Carriers." [Online]. Available: <http://www.insightful.com>
- [81] S. M. Edwards *et al.*, "Two Percent of Men with Early-Onset Prostate Cancer Harbor Germline Mutations in the BRCA2 Gene," 2003.
- [82] I. Agalliu *et al.*, "Rare germline mutations in the BRCA2 gene are associated with early-onset prostate cancer," *Br J Cancer*, vol. 97, no. 6, pp. 826–831, Sep. 2007, doi: 10.1038/sj.bjc.6603929.
- [83] Z. Kote-Jarai *et al.*, "BRCA2 is a moderate penetrance gene contributing to young-onset prostate cancer: Implications for genetic testing in prostate cancer patients," *Br J Cancer*, vol. 105, no. 8, pp. 1230–1234, Oct. 2011, doi: 10.1038/bjc.2011.383.
- [84] "Cancer Risks in BRCA2 Mutation Carriers." [Online]. Available: <http://jnci.oxfordjournals.org/>
- [85] E. C. Page *et al.*, "Interim Results from the IMPACT Study: Evidence for Prostate-specific Antigen Screening in BRCA2 Mutation Carriers," *Eur Urol*, vol. 76, no. 6, pp. 831–842, Dec. 2019, doi: 10.1016/j.eururo.2019.08.019.
- [86] E. Castro *et al.*, "Effect of BRCA Mutations on Metastatic Relapse and Cause-specific Survival after Radical Treatment for Localised Prostate Cancer," *Eur Urol*, vol. 68, no. 2, pp. 186–193, Aug. 2015, doi: 10.1016/j.eururo.2014.10.022.
- [87] H. Thorne *et al.*, "Decreased prostate cancer-specific survival of men with BRCA2 mutations from multiple breast cancer families," *Cancer Prevention Research*, vol. 4, no. 7, pp. 1002–1010, Jul. 2011, doi: 10.1158/1940-6207.CAPR-10-0397.
- [88] D. J. Gallagher *et al.*, "Germline BRCA mutations denote a clinicopathologic subset of prostate cancer," *Clinical Cancer Research*, vol. 16, no. 7, pp. 2115–2121, Apr. 2010, doi: 10.1158/1078-0432.CCR-09-2871.
- [89] H. T. Lynch, C. L. Snyder, T. G. Shaw, C. D. Heinen, and M. P. Hitchens, "Milestones of Lynch syndrome: 1895-2015," *Nat Rev Cancer*, vol. 15, no. 3, pp. 181–194, Feb. 2015, doi: 10.1038/nrc3878.

REFERENCES

- [90] S. Haraldsdottir *et al.*, "Comprehensive population-wide analysis of Lynch syndrome in Iceland reveals founder mutations in MSH6 and PMS2," *Nat Commun*, vol. 8, 2017, doi: 10.1038/ncomms14755.
- [91] S. W. ten Broeke *et al.*, "JOURNAL OF CLINICAL ONCOLOGY Cancer Risks for PMS2-Associated Lynch Syndrome," *J Clin Oncol*, vol. 36, pp. 2961–2968, 2018, doi: 10.1200/JCO.
- [92] V. Bonadona *et al.*, "Cancer Risks Associated With Germline Mutations in MLH1, MSH2, and MSH6 Genes in Lynch Syndrome." [Online]. Available: <http://www.jama>
- [93] J. Balmañ *et al.*, "Prediction of MLH1 and MSH2 Mutations in Lynch Syndrome." [Online]. Available: <https://jamanetwork.com/>
- [94] K. Tuttlewska, J. Lubinski, and G. Kurzawski, "Germline deletions in the EPCAM gene as a cause of Lynch syndrome - literature review," Aug. 12, 2013. doi: 10.1186/1897-4287-11-9.
- [95] M. J. L. Ligtenberg, R. P. Kuiper, A. Geurts Van Kessel, and N. Hoogerbrugge, "EPCAM deletion carriers constitute a unique subgroup of Lynch syndrome patients," Jun. 2013. doi: 10.1007/s10689-012-9591-x.
- [96] K. Li, H. Luo, L. Huang, H. Luo, and X. Zhu, "Microsatellite instability: A review of what the oncologist should know," Jan. 13, 2020, *BioMed Central Ltd*. doi: 10.1186/s12935-019-1091-8.
- [97] S. A. Cohen and A. Leininger, "The genetic basis of lynch syndrome and its implications for clinical practice and risk management," Jul. 22, 2014, *Dove Medical Press Ltd*. doi: 10.2147/TACG.S51483.
- [98] M. T. Vietri *et al.*, "Prevalence of mutations in BRCA and MMR genes in patients affected with hereditary endometrial cancer," *Medical Oncology*, vol. 38, no. 2, Feb. 2021, doi: 10.1007/s12032-021-01454-5.
- [99] C. Prevention, G. Hereditary, and A. Epidemiology Accepted On, "The Role of BRCA Testing in Hereditary Pancreatic and Prostate Cancer Families," 2019, doi: 10.1200/EDBK_.
- [100] S. Haraldsdottir *et al.*, "Prostate cancer incidence in males with Lynch syndrome," *Genetics in Medicine*, vol. 16, no. 7, pp. 553–557, 2014, doi: 10.1038/gim.2013.193.
- [101] J. H. Lee and T. T. Paull, "Activation and regulation of ATM kinase activity in response to DNA double-strand breaks," Dec. 10, 2007. doi: 10.1038/sj.onc.1210872.
- [102] "bartek2003".
- [103] M. Choi, T. Kipps, and R. Kurzrock, "ATM mutations in cancer: Therapeutic implications," Aug. 01, 2016, *American Association for Cancer Research Inc*. doi: 10.1158/1535-7163.MCT-15-0945.
- [104] C. Cybulski *et al.*, "An inherited NBN mutation is associated with poor prognosis prostate cancer," *Br J Cancer*, vol. 108, no. 2, pp. 461–468, Feb. 2013, doi: 10.1038/bjc.2012.486.

- [105] E. H. Seppälä *et al.*, "CHEK2 variants associate with hereditary prostate cancer," *Br J Cancer*, vol. 89, no. 10, pp. 1966–1970, Nov. 2003, doi: 10.1038/sj.bjc.6601425.
- [106] D. Leongamornlert *et al.*, "Frequent germline deleterious mutations in DNA repair genes in familial prostate cancer cases are associated with advanced disease," *Br J Cancer*, vol. 110, no. 6, pp. 1663–1672, Mar. 2014, doi: 10.1038/bjc.2014.30.
- [107] X. Dong *et al.*, "Mutations in CHEK2 Associated with Prostate Cancer Risk," 2003.
- [108] P. Paulo *et al.*, "Targeted next generation sequencing identifies functionally deleterious germline mutations in novel genes in early-onset/familial prostate cancer," *PLoS Genet*, vol. 14, no. 4, Apr. 2018, doi: 10.1371/journal.pgen.1007355.
- [109] D. Thompson *et al.*, "Cancer risks and mortality in heterozygous ATM mutation carriers," *J Natl Cancer Inst*, vol. 97, no. 11, pp. 813–822, Jun. 2005, doi: 10.1093/jnci/dji141.
- [110] M. T. Vietri *et al.*, "Analysis of PALB2 in a cohort of Italian breast cancer patients: identification of a novel PALB2 truncating mutation," Sep. 08, 2015, *Kluwer Academic Publishers*. doi: 10.1007/s10689-015-9786-z.
- [111] S. Reid *et al.*, "Biallelic mutations in PALB2 cause Fanconi anemia subtype FA-N and predispose to childhood cancer," *Nat Genet*, vol. 39, no. 2, pp. 162–164, Feb. 2007, doi: 10.1038/ng1947.
- [112] M. C. Southey *et al.*, "PALB2, CHEK2 and ATM rare variants and cancer risk: Data from COGS," *J Med Genet*, vol. 53, no. 12, pp. 800–811, Dec. 2016, doi: 10.1136/jmedgenet-2016-103839.
- [113] N. Rahman *et al.*, "PALB2, which encodes a BRCA2-interacting protein, is a breast cancer susceptibility gene," *Nat Genet*, vol. 39, no. 2, pp. 165–167, Feb. 2007, doi: 10.1038/ng1959.
- [114] P. Nicolosi *et al.*, "Prevalence of Germline Variants in Prostate Cancer and Implications for Current Genetic Testing Guidelines," in *JAMA Oncology*, American Medical Association, Apr. 2019, pp. 523–528. doi: 10.1001/jamaoncol.2018.6760.
- [115] D. Wokołarczyk *et al.*, "PALB2 mutations and prostate cancer risk and survival," *Br J Cancer*, vol. 125, no. 4, pp. 569–575, Aug. 2021, doi: 10.1038/s41416-021-01410-0.
- [116] S. Seal *et al.*, "Truncating mutations in the Fanconi anemia J gene BRIP1 are low-penetrance breast cancer susceptibility alleles," *Nat Genet*, vol. 38, no. 11, pp. 1239–1241, Nov. 2006, doi: 10.1038/ng1902.
- [117] Z. Kote-Jarai *et al.*, "A recurrent truncating germline mutation in the BRIP1/FANCDJ gene and susceptibility to prostate cancer," *Br J Cancer*, vol. 100, no. 2, pp. 426–430, Jan. 2009, doi: 10.1038/sj.bjc.6604847.
- [118] S. Qiu and J. Huang, "MRN complex is an essential effector of DNA damage repair," Jan. 01, 2021, *Zhejiang University Press*. doi: 10.1631/jzus.B2000289.
- [119] B. Rusak *et al.*, "Inherited NBN mutations and prostate cancer risk and survival," *Cancer Res Treat*, vol. 51, no. 3, pp. 1180–1187, Jul. 2019, doi: 10.4143/crt.2018.532.

REFERENCES

- [120] V. N. Giri *et al.*, "Germline genetic testing for inherited prostate cancer in practice: Implications for genetic testing, precision therapy, and cascade testing," *Prostate*, vol. 79, no. 4, pp. 333–339, Mar. 2019, doi: 10.1002/pros.23739.
- [121] D. Robinson *et al.*, "Integrative clinical genomics of advanced prostate cancer," *Cell*, vol. 161, no. 5, pp. 1215–1228, May 2015, doi: 10.1016/j.cell.2015.05.001.
- [122] S. F. Bakhoum and L. C. Cantley, "The Multifaceted Role of Chromosomal Instability in Cancer and Its Microenvironment," Sep. 06, 2018, *Cell Press*. doi: 10.1016/j.cell.2018.08.027.
- [123] K. Paulsson and B. Johansson, "Trisomy 8 as the sole chromosomal aberration in acute myeloid leukemia and myelodysplastic syndromes," *Pathologie Biologie*, vol. 55, no. 1, pp. 37–48, Feb. 2007, doi: 10.1016/j.patbio.2006.04.007.
- [124] D. Cimini, B. Howell, P. Maddox, A. Khodjakov, F. Degrossi, and E. D. Salmon, "Merotelic Kinetochore Orientation Is a Major Mechanism of Aneuploidy in Mitotic Mammalian Tissue Cells 7," 2001. [Online]. Available: <http://www.jcb.org/cgi/content/full/153/3/517>
- [125] S. L. Thompson, S. F. Bakhoum, and D. A. Compton, "Mechanisms of Chromosomal Instability," Mar. 23, 2010. doi: 10.1016/j.cub.2010.01.034.
- [126] M. Giam and G. Rancati, "Aneuploidy and chromosomal instability in cancer: A jackpot to chaos," May 20, 2015, *BioMed Central Ltd*. doi: 10.1186/s13008-015-0009-7.
- [127] K. S. Lawrence, T. Chau, and J. A. Engebrecht, "DNA Damage Response and Spindle Assembly Checkpoint Function throughout the Cell Cycle to Ensure Genomic Integrity," *PLoS Genet*, vol. 11, no. 4, Apr. 2015, doi: 10.1371/journal.pgen.1005150.
- [128] F. Luna-Maldonado, M. A. Andonegui-Elguera, J. Díaz-Chávez, and L. A. Herrera, "Mitotic and DNA Damage Response Proteins: Maintaining the Genome Stability and Working for the Common Good," Dec. 13, 2021, *Frontiers Media S.A.* doi: 10.3389/fcell.2021.700162.
- [129] M. Lin, S. S. Xie, and K. Y. Chan, "An updated view on the centrosome as a cell cycle regulator," Dec. 01, 2022, *BioMed Central Ltd*. doi: 10.1186/s13008-022-00077-0.
- [130] D. O. Morgan, *The cell Cycle: Principles of control*. London, UK: New Science Press., 2007.
- [131] J. Y. Ong and J. Z. Torres, "Phase Separation in Cell Division," Oct. 01, 2020, *Cell Press*. doi: 10.1016/j.molcel.2020.08.007.
- [132] T. J. Mitchison and E. D. Salmon, "Mitosis: a history of division," 2001. [Online]. Available: <http://cellbio.nature.com>
- [133] Q. Wu *et al.*, "Cep57, a NEDD1-binding pericentriolar material component, is essential for spindle pole integrity," *Cell Res*, vol. 22, no. 9, pp. 1390–1401, Sep. 2012, doi: 10.1038/cr.2012.61.
- [134] A. Kumar, V. Rajendran, R. Sethumadhavan, and R. Purohit, "CEP proteins: The knights of centrosome dynasty," *Protoplasma*, vol. 250, no. 5, pp. 965–983, 2013, doi: 10.1007/s00709-013-0488-9.

- [135] N. Izumi, K. Fumoto, S. Izumi, and A. Kikuchi, "GSK-3 β regulates proper mitotic spindle formation in cooperation with a component of the gamma-tubulin ring complex, GCP5.," *J Biol Chem*, vol. 283, no. 19, pp. 12981–91, May 2008, doi: 10.1074/jbc.M710282200.
- [136] S. Gadde and R. Heald, "Mechanisms and molecules of the mitotic spindle," Sep. 21, 2004. doi: 10.1016/j.cub.2004.09.021.
- [137] D. Chen *et al.*, "EML4 promotes the loading of NUDC to the spindle for mitotic progression," *Cell Cycle*, vol. 14, no. 10, pp. 1529–1539, Jan. 2015, doi: 10.1080/15384101.2015.1026514.
- [138] S. L. Prosser and L. Pelletier, "Mitotic spindle assembly in animal cells: A fine balancing act," Mar. 01, 2017, *Nature Publishing Group*. doi: 10.1038/nrm.2016.162.
- [139] I. M. Cheeseman and A. Desai, "Molecular architecture of the kinetochore-microtubule interface," Jan. 2008. doi: 10.1038/nrm2310.
- [140] D. Varma and E. D. Salmon, "The KMN protein network - Chief conductors of the kinetochore orchestra," Dec. 15, 2012. doi: 10.1242/jcs.093724.
- [141] J. G. DeLuca, W. E. Gall, C. Ciferri, D. Cimini, A. Musacchio, and E. D. Salmon, "Kinetochore Microtubule Dynamics and Attachment Stability Are Regulated by Hec1," *Cell*, vol. 127, no. 5, pp. 969–982, Dec. 2006, doi: 10.1016/j.cell.2006.09.047.
- [142] J. P. I. Welburn *et al.*, "Aurora B Phosphorylates Spatially Distinct Targets to Differentially Regulate the Kinetochore-Microtubule Interface," *Mol Cell*, vol. 38, no. 3, pp. 383–392, May 2010, doi: 10.1016/j.molcel.2010.02.034.
- [143] R. R. Wei, J. Al-Bassam, and S. C. Harrison, "The Ndc80/HEC1 complex is a contact point for kinetochore-microtubule attachment," *Nat Struct Mol Biol*, vol. 14, no. 1, pp. 54–59, Jan. 2007, doi: 10.1038/nsmb1186.
- [144] J. Espeut, D. K. Cheerambathur, L. Krenning, K. Oegema, and A. Desai, "Microtubule binding by KNL-1 contributes to spindle checkpoint silencing at the kinetochore," *Journal of Cell Biology*, vol. 196, no. 4, pp. 469–482, 2012, doi: 10.1083/jcb.201111107.
- [145] I. M. Cheeseman, J. S. Chappie, E. M. Wilson-Kubalek, and A. Desai, "The Conserved KMN Network Constitutes the Core Microtubule-Binding Site of the Kinetochore," *Cell*, vol. 127, no. 5, pp. 983–997, Dec. 2006, doi: 10.1016/j.cell.2006.09.039.
- [146] B. J. Howell *et al.*, "Cytoplasmic dynein/dynactin drives kinetochore protein transport to the spindle poles and has a role in mitotic spindle checkpoint inactivation," *Journal of Cell Biology*, vol. 155, no. 7, pp. 1159–1172, Dec. 2001, doi: 10.1083/jcb.200105093.
- [147] D. B. Hoffman, C. G. Pearson, T. J. Yen, B. J. Howell, and E. D. Salmon, "Microtubule-dependent Changes in Assembly of Microtubule Motor Proteins and Mitotic Spindle Checkpoint Proteins at PtK1 Kinetochores," 1995.
- [148] "Spindle Checkpoint Protein Dynamics at Kinetochores in Living Cells", doi: 10.1016/j.

REFERENCES

- [149] J. F. Dorn and P. S. Maddox, "Kinetochore dynamics: How protein dynamics affect chromosome segregation," Feb. 2012. doi: 10.1016/j.ceb.2011.12.003.
- [150] P. Lara-Gonzalez, F. G. Westhorpe, and S. S. Taylor, "The spindle assembly checkpoint," Nov. 20, 2012. doi: 10.1016/j.cub.2012.10.006.
- [151] S. H. Kim, D. P. Lin, S. Matsumoto, and A. Kitazono, "Fission Yeast Slpl: An Effector of the Mad2-Dependent Spindle Checkpoint results in a delay of chromosome decondensation. Therefore, Mad2 was expected to abolish the metaphase arrest seen in the Tomohiro Matsumoto*." [Online]. Available: www.sciencemag.org/SCIENCEVOL.27913FEBRUARY1998
- [152] M. A. Hoyt, L. Totis, and B. T. Roberts, "S. cerevisiae Genes Required for Cell Cycle Arrest in Response to Loss of Microtubule Function," 1991.
- [153] R. Li't and A. W. Murray', "Feedback Control of Mitosis in Budding Yeast," 1991.
- [154] L. H. Hwang *et al.*, "Budding Yeast Cdc20: A Target of the Spindle Checkpoint Author," 1998.
- [155] J. M. Peters, "The anaphase promoting complex/cyclosome: A machine designed to destroy," Sep. 19, 2006. doi: 10.1038/nrm1988.
- [156] R. E. Karess, K. Wassmann, and Z. Rahmani, "New insights into the role of BubR1 in mitosis and beyond," in *International Review of Cell and Molecular Biology*, vol. 306, Elsevier Inc., 2013, pp. 223–273. doi: 10.1016/B978-0-12-407694-5.00006-7.
- [157] V. M. Bolanos-Garcia and T. L. Blundell, "BUB1 and BUBR1: Multifaceted kinases of the cell cycle," Mar. 2011. doi: 10.1016/j.tibs.2010.08.004.
- [158] S. J. E. Suijkerbuijk *et al.*, "The Vertebrate Mitotic Checkpoint Protein BUBR1 Is an Unusual Pseudokinase," *Dev Cell*, vol. 22, no. 6, pp. 1321–1329, Jun. 2012, doi: 10.1016/j.devcel.2012.03.009.
- [159] P. Lara-Gonzalez, M. I. F. Scott, M. Diez, O. Sen, and S. S. Taylor, "BubR1 blocks substrate recruitment to the APC/C in a KEN-box-dependent manner," *J Cell Sci*, vol. 124, no. 24, pp. 4332–4345, Dec. 2011, doi: 10.1242/jcs.094763.
- [160] C. R. Bloom and B. J. North, "Physiological relevance of post-translational regulation of the spindle assembly checkpoint protein BubR1," Dec. 01, 2021, *BioMed Central Ltd*. doi: 10.1186/s13578-021-00589-2.
- [161] T. Kiyomitsu, C. Obuse, and M. Yanagida, "Human Blinkin/AF15q14 Is Required for Chromosome Alignment and the Mitotic Checkpoint through Direct Interaction with Bub1 and BubR1," *Dev Cell*, vol. 13, no. 5, pp. 663–676, Nov. 2007, doi: 10.1016/j.devcel.2007.09.005.
- [162] V. Krenn, A. Wehenkel, X. Li, S. Santaguida, and A. Musacchio, "Structural analysis reveals features of the spindle checkpoint kinase Bub1-kinetochore subunit Knl1 interaction," *Journal of Cell Biology*, vol. 196, no. 4, pp. 451–467, 2012, doi: 10.1083/jcb.201110013.
- [163] W. C. H. Chao, K. Kulkarni, Z. Zhang, E. H. Kong, and D. Barford, "Structure of the mitotic checkpoint complex," *Nature*, vol. 484, no. 7393, pp. 208–213, Apr. 2012, doi: 10.1038/nature10896.

- [164] K. Overlack *et al.*, "A molecular basis for the differential roles of Bub1 and BubR1 in the spindle assembly checkpoint", doi: 10.7554/eLife.05269.001.
- [165] S. S. Taylor, E. Ha, and F. Mckee, "The Human Homologue of Bub3 Is Required for Kinetochore Localization of Bub1 and a Mad3/Bub1-related Protein Kinase," 1998. [Online]. Available: <http://www.jcb.org>
- [166] K. Overlack *et al.*, "BubR1 Promotes Bub3-Dependent APC/C Inhibition during Spindle Assembly Checkpoint Signaling," *Current Biology*, vol. 27, no. 19, pp. 2915-2927.e7, Oct. 2017, doi: 10.1016/j.cub.2017.08.033.
- [167] S. J. E. Suijkerbuijk, M. H. J. van Osch, F. L. Bos, S. Hanks, N. Rahman, and G. J. P. L. Kops, "Molecular causes for BUBR1 dysfunction in the human cancer predisposition syndrome mosaic variegated aneuploidy," *Cancer Res*, vol. 70, no. 12, pp. 4891–4900, Jun. 2010, doi: 10.1158/0008-5472.CAN-09-4319.
- [168] S. J. E. Suijkerbuijk, M. Vleugel, A. Teixeira, and G. J. P. L. Kops, "Integration of Kinase and Phosphatase Activities by BUBR1 Ensures Formation of Stable Kinetochore-Microtubule Attachments," *Dev Cell*, vol. 23, no. 4, pp. 745–755, Oct. 2012, doi: 10.1016/j.devcel.2012.09.005.
- [169] D. J. Baker *et al.*, "Increased expression of BubR1 protects against aneuploidy and cancer and extends healthy lifespan," *Nat Cell Biol*, vol. 15, no. 1, pp. 96–102, Jan. 2013, doi: 10.1038/ncb2643.
- [170] D. J. Baker *et al.*, "BubR1 insufficiency causes early onset of aging-associated phenotypes and infertility in mice," *Nat Genet*, vol. 36, no. 7, pp. 744–749, Jul. 2004, doi: 10.1038/ng1382.
- [171] Q. Wang *et al.*, "BUBR1 deficiency results in abnormal megakaryopoiesis," *Blood*, vol. 103, no. 4, pp. 1278–1285, Feb. 2004, doi: 10.1182/blood-2003-06-2158.
- [172] D. J. Baker, K. B. Jegannathan, L. Malureanu, C. Perez-Terzic, A. Terzic, and J. M. A. van Deursen, "Early aging-associated phenotypes in Bub3/Rae1 haploinsufficient mice," *Journal of Cell Biology*, vol. 172, no. 4, pp. 529–540, Feb. 2006, doi: 10.1083/jcb.200507081.
- [173] S. Hanks *et al.*, "Constitutional aneuploidy and cancer predisposition caused by biallelic mutations in BUB1B," vol. 36, no. 11, pp. 1159–1161, 2004, doi: 10.1038/ng1449.
- [174] S. Matsuura *et al.*, "Monoallelic BUB1B mutations and defective mitotic-spindle checkpoint in seven families with premature chromatid separation (PCS) syndrome," *Am J Med Genet*, vol. 140 A, no. 4, pp. 358–367, Feb. 2006, doi: 10.1002/ajmg.a.31069.
- [175] K. Snape *et al.*, "Mutations in CEP57 cause mosaic variegated aneuploidy syndrome," *Nat Genet*, vol. 43, no. 6, pp. 527–9, Jun. 2011, doi: 10.1038/ng.822.
- [176] S. Jacquemont, M. Bocéno, J. M. Rival, F. Méchinaud, and A. David, "High risk of malignancy in mosaic variegated aneuploidy syndrome," *Am J Med Genet*, vol. 109, no. 1, pp. 17–21; discussion 16, Apr. 2002, Accessed: Jun. 09, 2016. [Online]. Available: <http://www.ncbi.nlm.nih.gov/pubmed/11932988>
- [177] S. J. E. Suijkerbuijk, M. H. J. van Osch, F. L. Bos, S. Hanks, N. Rahman, and G. J. P. L. Kops, "Molecular causes for BUBR1 dysfunction in the human cancer

REFERENCES

- predisposition syndrome mosaic variegated aneuploidy," *Cancer Res*, vol. 70, no. 12, pp. 4891–4900, Jun. 2010, doi: 10.1158/0008-5472.CAN-09-4319.
- [178] G. J. P. L. Kops, B. A. A. Weaver, and D. W. Cleveland, "On the road to cancer: Aneuploidy and the mitotic checkpoint," Oct. 2005. doi: 10.1038/nrc1714.
- [179] T. Kajii *et al.*, "Mosaic Variegated Aneuploidy With Multiple Congenital Abnormalities: Homozygosity for Total Premature Chromatid Separation Trait," 1998.
- [180] S. Matsuura, E. Ito, H. Tauchi, K. Komatsu, T. Ikeuchi, and T. Kajii, "Chromosomal Instability Syndrome of Total Premature Chromatid Separation with Mosaic Variegated Aneuploidy Is Defective in Mitotic-Spindle Checkpoint," *Am. J. Hum. Genet.*, vol. 67, pp. 483–486, 2000.
- [181] H. Ochiai *et al.*, "TALEN-mediated single-base-pair editing identification of an intergenic mutation upstream of BUB1B as causative of PCS (MVA) syndrome," *Proc Natl Acad Sci U S A*, vol. 111, no. 4, pp. 1461–1466, Jan. 2014, doi: 10.1073/pnas.1317008111.
- [182] T. Rio Frio *et al.*, "Homozygous BUB1B Mutation and Susceptibility to Gastrointestinal Neoplasia Sum m a r," 2010.
- [183] N. J. Roberts *et al.*, "Whole genome sequencing defines the genetic heterogeneity of familial pancreatic cancer," *Cancer Discov*, vol. 6, no. 2, pp. 166–175, Feb. 2016, doi: 10.1158/2159-8290.CD-15-0402.
- [184] A. L. Pritchard *et al.*, "Germline mutations in candidate predisposition genes in individuals with cutaneous melanoma and at least two independent additional primary cancers," *PLoS One*, vol. 13, no. 4, Apr. 2018, doi: 10.1371/journal.pone.0194098.
- [185] O. D. Røe *et al.*, "Genome-wide profile of pleural mesothelioma versus parietal and visceral pleura: The emerging gene portrait of the mesothelioma phenotype," *PLoS One*, vol. 4, no. 8, Aug. 2009, doi: 10.1371/journal.pone.0006554.
- [186] K. A. Myrie, M. J. Percy, J. N. Azim, C. K. Neeley, and E. M. Petty, "Mutation and expression analysis of human BUB1 and BUB1B in aneuploid breast cancer cell lines." [Online]. Available: www.atcc.org
- [187] D. J. Baker, F. Jin, K. B. Jeganathan, and J. M. van Deursen, "Whole Chromosome Instability Caused by Bub1 Insufficiency Drives Tumorigenesis through Tumor Suppressor Gene Loss of Heterozygosity," *Cancer Cell*, vol. 16, no. 6, pp. 475–486, Dec. 2009, doi: 10.1016/j.ccr.2009.10.023.
- [188] R. Sotillo *et al.*, "Mad2 overexpression promotes aneuploidy and tumorigenesis in mice."
- [189] R. M. Ricke, K. B. Jeganathan, L. Malureanu, A. M. Harrison, and J. M. van Deursen, "Bub1 kinase activity drives error correction and mitotic checkpoint control but not tumor suppression," *Journal of Cell Biology*, vol. 199, no. 6, pp. 931–949, Dec. 2012, doi: 10.1083/jcb.201205115.
- [190] M. M. Hahn *et al.*, "Prevalence of germline mutations in the spindle assembly checkpoint gene BUB1B in individuals with early-onset colorectal cancer," *Genes*

- Chromosomes Cancer*, vol. 55, no. 11, pp. 855–863, Nov. 2016, doi: 10.1002/gcc.22385.
- [191] R. M. de Voer *et al.*, “Germline mutations in the spindle assembly checkpoint genes BUB1 and BUB3 are risk factors for colorectal cancer,” *Gastroenterology*, vol. 145, no. 3, pp. 544–7, Sep. 2013, doi: 10.1053/j.gastro.2013.06.001.
 - [192] B. Feng *et al.*, “A novel *CEP57* variant associated with mosaic variegated aneuploidy syndrome in a Chinese female presenting with short stature, microcephaly, brachydactyly, and small teeth,” *Mol Genet Genomic Med*, Apr. 2022, doi: 10.1002/mgg3.1951.
 - [193] H. Zhou, T. Wang, T. Zheng, J. Teng, and J. Chen, “Cep57 is a Mis12-interacting kinetochore protein involved in kinetochore targeting of Mad1-Mad2,” *Nat Commun*, vol. 7, 2016, doi: 10.1038/ncomms10151.
 - [194] K. Momotani, A. S. Khromov, T. Miyake, P. T. Stukenberg, and A. V. Somlyo, “Cep57, a multidomain protein with unique microtubule and centrosomal localization domains,” *Biochemical Journal*, vol. 412, no. 2, pp. 265–273, Jun. 2008, doi: 10.1042/BJ20071501.
 - [195] K. Fukasawa, “p53, cyclin-dependent kinase and abnormal amplification of centrosomes,” Sep. 2008. doi: 10.1016/j.bbcan.2008.04.002.
 - [196] S. Maia *et al.*, “The role of germline mutations in the BRCA1/2 and mismatch repair genes in men ascertained for early-onset and/or familial prostate cancer,” *Fam Cancer*, vol. 15, no. 1, pp. 111–121, Jan. 2016, doi: 10.1007/s10689-015-9832-x.
 - [197] S. Maia *et al.*, “Identification of two novel HOXB13 germline mutations in Portuguese prostate cancer patients,” *PLoS One*, vol. 10, no. 7, Jul. 2015, doi: 10.1371/journal.pone.0132728.
 - [198] M. P. Silva *et al.*, “BUB1B monoallelic germline variants contribute to prostate cancer predisposition by triggering chromosomal instability,” *J Biomed Sci*, vol. 31, no. 1, Dec. 2024, doi: 10.1186/s12929-024-01056-z.
 - [199] S. Heinrich *et al.*, “Determinants of robustness in spindle assembly checkpoint signalling,” *Nat Cell Biol*, vol. 15, no. 11, pp. 1328–1339, Nov. 2013, doi: 10.1038/ncb2864.
 - [200] M. Cardoso *et al.*, “Exome sequencing of affected duos and trios uncovers PRUNE2 as a novel prostate cancer predisposition gene,” *Br J Cancer*, vol. 128, no. 6, pp. 1077–1085, Apr. 2023, doi: 10.1038/s41416-022-02125-6.
 - [201] P. Paulo *et al.*, “Genetic landscape of homologous recombination repair genes in early-onset/familial prostate cancer patients,” *Genes Chromosomes Cancer*, Jul. 2023, doi: 10.1002/GCC.23190.
 - [202] Q. Li and K. Wang, “InterVar: Clinical Interpretation of Genetic Variants by the 2015 ACMG-AMP Guidelines,” *Am J Hum Genet*, vol. 100, no. 2, p. 267, Feb. 2017, doi: 10.1016/J.AJHG.2017.01.004.
 - [203] X. Jian, E. Boerwinkle, and X. Liu, “In silico prediction of splice-altering single nucleotide variants in the human genome,” *Nucleic Acids Res*, vol. 42, no. 22, p. 13534, Dec. 2014, doi: 10.1093/NAR/GKU1206.

REFERENCES

- [204] H. Y. Xiong *et al.*, "The human splicing code reveals new insights into the genetic determinants of disease," *Science*, vol. 347, no. 6218, p. 1254806, Jan. 2015, doi: 10.1126/SCIENCE.1254806.
- [205] K. Jaganathan *et al.*, "Predicting Splicing from Primary Sequence with Deep Learning," *Cell*, vol. 176, no. 3, pp. 535-548.e24, Jan. 2019, doi: 10.1016/j.cell.2018.12.015.
- [206] J. Ye, G. Coulouris, I. Zaretskaya, I. Cutcutache, S. Rozen, and T. L. Madden, "Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction," 2012. [Online]. Available: <http://www.biomedcentral.com/1471-2105/13/134>
- [207] A. Anna and G. Monika, "Splicing mutations in human genetic disorders: examples, detection, and confirmation," Aug. 01, 2018, *Springer Verlag*. doi: 10.1007/s13353-018-0444-7.
- [208] J. M. Schwarz, D. N. Cooper, M. Schuelke, and D. Seelow, "Mutationtaster2: Mutation prediction for the deep-sequencing age," 2014, *Nature Publishing Group*. doi: 10.1038/nmeth.2890.
- [209] J. Gao *et al.*, "Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal," *Sci Signal*, vol. 6, no. 269, Apr. 2013, doi: 10.1126/scisignal.2004088.
- [210] E. Cerami *et al.*, "The cBio Cancer Genomics Portal: An open platform for exploring multidimensional cancer genomics data," *Cancer Discov*, vol. 2, no. 5, pp. 401–404, May 2012, doi: 10.1158/2159-8290.CD-12-0095.
- [211] D. Chen *et al.*, "EML4 promotes the loading of NUDC to the spindle for mitotic progression," *Cell Cycle*, vol. 14, no. 10, pp. 1529–1539, Jan. 2015, doi: 10.1080/15384101.2015.1026514.
- [212] J. CY. Song H, Park JE, "DDA3 targets Cep290 into the centrosome to regulate spindle positioning," *Biochem Biophys Res Commun*, vol. 463, no. (1-2), pp. 88-94., 2015.
- [213] C. Zhu and W. Jiang, "Cell cycle-dependent translocation of PRC1 on the spindle by Kif4 is essential for midzone formation and cytokinesis," *Proc Natl Acad Sci U S A*, vol. 102, no. 2, pp. 343–8, Jan. 2005, doi: 10.1073/pnas.0408438102.
- [214] E. N. Firat-Karalar, "The ciliopathy gene product Cep290 is required for primary cilium formation and microtubule network organization," *Turkish Journal of Biology*, vol. 42, no. 5, pp. 371–381, 2018, doi: 10.3906/biy-1805-25.
- [215] N. B. Hassounah, R. Nagle, K. Saboda, D. J. Roe, B. L. Dalkin, and K. M. McDermott, "Primary Cilia Are Lost in Preinvasive and Invasive Prostate Cancer," *PLoS One*, vol. 8, no. 7, Jul. 2013, doi: 10.1371/journal.pone.0068521.
- [216] L. Fabbri, F. Bost, and N. M. Mazure, "Primary cilium in cancer hallmarks," Mar. 02, 2019, *MDPI AG*. doi: 10.3390/ijms20061336.
- [217] Z. Wu *et al.*, "Cep131-Cep162 and Cby-Fam92 complexes cooperatively maintain Cep290 at the basal body and contribute to ciliogenesis initiation," *PLoS Biol*, vol. 22, no. 3, Mar. 2024, doi: 10.1371/journal.pbio.3002330.

- [218] G. G. Slaats *et al.*, “DNA replication stress underlies renal phenotypes in CEP290-Associated Joubert syndrome,” *Journal of Clinical Investigation*, vol. 125, no. 9, pp. 3657–3666, Sep. 2015, doi: 10.1172/JCI80657.
- [219] M. Pollmann, R. Parwaresch, S. Adam-Klages, M. L. Kruse, F. Buck, and H. J. Heidebrecht, “Human EML4, a novel member of the EMAP family, is essential for microtubule formation,” *Exp Cell Res*, vol. 312, no. 17, pp. 3241–3251, Oct. 2006, doi: 10.1016/j.yexcr.2006.06.035.
- [220] Adib R *et al.*, “Mitotic phosphorylation by NEK6 and NEK7 reduces the microtubule affinity of EML4 to promote chromosome congression. ,” *Sci Signal*, vol. 12, no. (594):eaaw2939, Aug. 2019.
- [221] S. R. Sabir, S. Yeoh, G. Jackson, and R. Bayliss, “EML4-ALK variants: Biological and molecular properties, and the implications for patients,” Sep. 05, 2017, *MDPI AG*. doi: 10.3390/cancers9090118.
- [222] N. Teixidó-Travesa, J. Roig, and J. Lüders, “The where, when and how of microtubule nucleation - one ring to rule them all,” *J Cell Sci*, vol. 125, no. 19, pp. 4445–4456, 2012, doi: 10.1242/jcs.106971.
- [223] C. Alfieri *et al.*, “Molecular basis of APC/C regulation by the spindle assembly checkpoint,” *Nature*, vol. 536, no. 7617, pp. 431–436, Aug. 2016, doi: 10.1038/nature19083.
- [224] E. S. Fischer *et al.*, “Molecular mechanism of Mad1 kinetochore targeting by phosphorylated Bub1,” *EMBO Rep*, vol. 22, no. 7, Jul. 2021, doi: 10.15252/embr.202052242.
- [225] A. C. Faesen *et al.*, “Basis of catalytic assembly of the mitotic checkpoint complex,” *Nature*, vol. 542, no. 7642, pp. 498–502, Feb. 2017, doi: 10.1038/nature21384.
- [226] C. Villarroja-Beltri *et al.*, “Biallelic germline mutations in MAD1L1 induce a syndrome of aneuploidy with high tumor susceptibility,” 2022.
- [227] T. Li *et al.*, “TIMER2.0 for analysis of tumor-infiltrating immune cells,” *Nucleic Acids Res*, vol. 48, no. W1, pp. W509–W514, Jul. 2020, doi: 10.1093/NAR/GKAA407.
- [228] E. Castro *et al.*, “Germline BRCA mutations are associated with higher risk of nodal involvement, distant metastasis, and poor survival outcomes in prostate cancer,” *Journal of Clinical Oncology*, vol. 31, no. 14, pp. 1748–1757, May 2013, doi: 10.1200/JCO.2012.43.1882.
- [229] J. Russo and V. N. Giri, “Germline testing and genetic counselling in prostate cancer,” Jun. 01, 2022, *Nature Research*. doi: 10.1038/s41585-022-00580-7.
- [230] S. Das, S. S. Salami, D. E. Spratt, S. D. Kaffenberger, M. F. Jacobs, and T. M. Morgan, “Bringing Prostate Cancer Germline Genetics into Clinical Practice,” Aug. 01, 2019, *Lippincott Williams and Wilkins*. doi: 10.1097/JU.0000000000000137.
- [231] J. Mateo *et al.*, “Accelerating precision medicine in metastatic prostate cancer,” *Nat Cancer*, vol. 1, no. 11, pp. 1041–1053, Nov. 2020, doi: 10.1038/s43018-020-00141-0.
- [232] A. Dias, Z. Kote-Jarai, C. Mikropoulos, and R. Eeles, “Prostate Cancer Germline Variations and Implications for Screening and Treatment,” *Cold Spring Harb Perspect Med*, vol. 8, no. 9, Sep. 2018, doi: 10.1101/CSHPERSPECT.A030379.

REFERENCES

- [233] S. Kommu, S. Edwards, and R. Eeles, "The Clinical Genetics of Prostate Cancer," *Hered Cancer Clin Pract*, vol. 2, no. 3, p. 111, 2004, doi: 10.1186/1897-4287-2-3-111.
- [234] D. J. Schaid, "The complex genetic epidemiology of prostate cancer," *Hum Mol Genet*, vol. 13, no. suppl_1, pp. R103–R121, Apr. 2004, doi: 10.1093/HMG/DDH072.
- [235] J. Simard, M. Dumont, P. Soucy, and F. Labrie, "Perspective: Prostate Cancer Susceptibility Genes," *Endocrinology*, vol. 143, no. 6, pp. 2029–2040, Jun. 2002, doi: 10.1210/ENDO.143.6.8890.
- [236] "Perspective: Prostate Cancer Susceptibility Genes," 2002. [Online]. Available: <https://academic.oup.com/endo/article/143/6/2029/2989354>
- [237] P. N. Robinson, "Whole-exome sequencing for finding de novo mutations in sporadic mental retardation," *Genome Biol*, vol. 11, no. 12, Dec. 2010, doi: 10.1186/gb-2010-11-12-144.
- [238] S. B. Ng *et al.*, "Exome sequencing identifies the cause of a mendelian disorder," *Nat Genet*, vol. 42, no. 1, pp. 30–35, Jan. 2010, doi: 10.1038/ng.499.
- [239] I. Donner, T. Kiviluoto, A. Ristimäki, and et al., "Exome sequencing reveals three novel candidate predisposition genes for diffuse gastric cancer.," *Fam Cancer*, vol. 14, pp. 241–246, 2015.
- [240] C. Esteban-Jurado *et al.*, "Whole-exome sequencing identifies rare pathogenic variants in new predisposition genes for familial colorectal cancer," *Genetics in Medicine*, vol. 17, no. 2, pp. 131–142, Feb. 2015, doi: 10.1038/gim.2014.89.
- [241] Pinto P *et al.*, "Implementation of next-generation sequencing for molecular diagnosis of hereditary breast and ovarian cancer highlights its genetic heterogeneity.," *Breast Cancer Res Treat.*, vol. 2, no. 159, pp. 245–256, 2016.
- [242] A. Peixoto *et al.*, "The role of targeted BRCA1/BRCA2 mutation analysis in hereditary breast/ovarian cancer families of Portuguese ancestry," *Clin Genet*, vol. 88, no. 1, pp. 41–48, Jul. 2015, doi: 10.1111/cge.12441.
- [243] M. Pinheiro *et al.*, "The MSH2 c.388_389del mutation shows a founder effect in Portuguese Lynch syndrome families.," *Clin Genet*, vol. 84, no. 3, pp. 244–50, Sep. 2013, doi: 10.1111/cge.12062.
- [244] A. Peixoto *et al.*, "International distribution and age estimation of the Portuguese BRCA2 c.156_157insAlu founder mutation.," *Breast Cancer Res Treat*, vol. 127, no. 3, pp. 671–9, Jun. 2011, doi: 10.1007/s10549-010-1036-3.
- [245] R. G. Martins *et al.*, "A founder SDHB mutation in Portuguese paraganglioma patients," Dec. 2013. doi: 10.1530/ERC-12-0399.
- [246] M. Pinheiro *et al.*, "A novel exonic rearrangement affecting MLH1 and the contiguous LRRFIP2 is a founder mutation in Portuguese Lynch syndrome families," *Genetics in Medicine*, vol. 13, no. 10, pp. 895–902, Oct. 2011, doi: 10.1097/GIM.0b013e31821dd525.
- [247] Manuela Pinheiro *et al.*, "The nonsense mutation MSH2 c.2152C>T shows a founder effect in Portuguese Lynch syndrome families," *Genes Chromosomes Cancer*, vol. 58, no. 9, pp. 657–664, Sep. 2019.

- [248] C. S. Ku *et al.*, “Technological advances in DNA sequence enrichment and sequencing for germline genetic diagnosis,” *xpert Review of Molecular Diagnostics*, vol. 12, no. 2, pp. 159–173, 2012.