Universidade do Minho
Escola de Medicina

Céline Saraiva Gonçalves

Deciphering key roles of WNT6 in glioblastoma: mechanistic, functional, and clinical insights

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Tese de Doutoramento em Ciências da Saúde

Trabalho efetuado sob a orientação do
Doutor Bruno Marques Costa

julho de 2018

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University of Minho, July 31, 2018

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"Always aim high, work hard, and care deeply about what you believe in. And, when you stumble, keep faith. And, when you're knocked down, get right back up and never listen to anyone who says you can't or shouldn't go on." – Hillary Clinton

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Summary/Resumo

Summary

Malignant gliomas are one of the deadliest cancers worldwide, for which no curative treatment is yet available. Patients with glioblastoma (GBM), the most frequent and malignant form of glioma, present a median overall survival of approximately 15 months after diagnosis. This is mainly due to the poor and unpredictable response to the standard-of-care therapy that includes radio- and chemotherapy, after surgical resection. To improve the clinical management of these patients, several efforts are being made to molecularly characterize gliomas, which would allow to improve patients' stratification, develop targeted therapies and thus improve patients' clinical outcome. However, the mechanisms underlying glioma pathophysiology are still underexplored, hampering the identification of new therapeutic targets.

Alterations in signaling pathways required to preserve the embryonic determination may play an important role in the tumorigenic process. The WNT pathway has been described to influence nearly all aspects of embryonic development, from a single cell – establishing its polarity – to an organism – specifying the entire body axis. At the cellular level, the WNT pathway regulates cell motility, apoptosis and their capacity to self-renew. In adults, the WNT pathway is responsible for tissue homeostasis, which might explain why its aberrant activation has been reported in several tumor types, including GBM. In tumor cells, the WNT pathway has been implicated in tumor initiation, growth, invasion, metastasis, immune evasion, stem cell capacity, and therapy resistance. In humans, there are 19 WNT ligands capable of activating the WNT pathway and with nearly unique roles in development. Among them, WNT6 was recently associated with chemoresistance in gastric and bladder cancers, poor prognosis of esophageal squamous cell carcinoma and osteosarcoma patients, and increased risk to develop colorectal adenoma. However, nothing was known about the relevance of WNT6 in gliomas.

This thesis aimed to unveil the relevance of WNT6 in GBM, particularly focusing on the mechanisms regulating its expression, its downstream effector signaling pathways, and its clinical significance in GBM patients. We demonstrated that WNT6 is significantly overexpressed in GBM when compared to lower-grade gliomas, independently of IDH mutations and 1p/19q co-deletion status, and in a gene-dosage independent manner. Moreover, WNT6 expression was associated with increased GBM cell viability, proliferation, invasion, migration, glioma stem cell capacity, and resistance to temozolomide-based chemotherapy, implicating WNT6 as an important oncogenic factor in glioma. Using *in vivo* intracranial GBM mice models with both WNT6 overexpressing and silencing GBM cell models, we demonstrated that WNT6-high tumors presented increased features of tumor aggressiveness, which was ultimately associated with mice shorter overall survival. Moreover, we identified the WNT/β-catenin, SFK

and STAT3 pathways as WNT6-mediated signaling mechanisms in GBM. The association between WNT6 and stem cell markers or key cancer related pathways was also confirmed in GBM patients. Importantly, in GBM patients from several independent datasets, WNT6 was a strong prognostic biomarker of shorter overall survival, independently of other well-known prognostic factors.

Since the first step for the design of new targeted therapies requires not only the identification of new molecular targets but also the understanding of their activation mechanisms, we further investigated the potential upstream regulators underlying WNT6 overexpression in glioma. We showed that DNA methylation of specific regions in the *WNT6* locus regulates its expression in glioma. Additionally, we observed that *HOXA9*, an oncogenic transcription factor in GBM, is co-expressed with *WNT6* in gliomas. Interestingly, we found that HOXA9 directly binds to the *WNT6* promoter region, transcriptionally activating its expression. Thus, *WNT6* DNA methylation and HOXA9 are upstream regulators of WNT6 expression in gliomas, creating new hints for innovative therapeutic approaches.

In conclusion, the work summarized in this thesis describes WNT6 as a novel critical oncogene in human GBM and identifies its upstream regulators (DNA methylation and HOXA9) and downstream molecular mechanisms (namely WNT, SFK and STAT3 pathways), which may have an impact on prognostic stratification and in the design of novel attractive therapeutic options for this deadly disease.

Resumo

Os gliomas malignos são um dos cancros mais mortíferos a nível mundial, para os quais não existe ainda um tratamento curativo. Pacientes com glioblastoma (GBM), o tipo de glioma mais maligno e frequente, apresentam uma sobrevida de aproximadamente 15 meses após o diagnóstico. Este facto deve-se maioritariamente à resposta fraca e imprevisível que estes pacientes apresentam quando tratados com a terapia convencional, que inclui radio- e quimioterapia após ressecção cirúrgica do tumor. Ao longo dos últimos anos, têm-se efetuado esforços no sentido de caracterizar molecularmente os gliomas de forma a melhorar a estratificação dos pacientes, desenvolver terapias direcionadas e, deste modo, melhorar o seu tratamento. Contudo, os mecanismos subjacentes à sua fisiopatologia estão ainda pouco explorados, o que impede a identificação de novos alvos terapêuticos.

Alterações em vias de sinalização celulares necessárias para a embriogénese poderão ter um papel fundamental no processo tumorigénico. Em específico, a via WNT controla diversos aspetos do desenvolvimento embrionário, desde a definição da polaridade de uma única célula num tecido até à especificação do eixo corporal de um organismo. Ao nível celular, a via WNT regula a motilidade, a apoptose e a capacidade de autorrenovação. Em adultos, a via WNT é responsável pela homeostasia dos tecidos, o que pode explicar o facto da sua ativação aberrante ter sido observada em diversos tipos tumorais, incluindo GBM. Em células tumorais, a via WNT tem vindo a ser associada com a iniciação, o crescimento, a invasão, a metastização, a evasão imune, a capacidade de células estaminais e a resistência à terapia do tumor. Em humanos, existem 19 ligandos da via WNT capazes de a ativar, os quais têm um papel singular no desenvolvimento. Entre estes, o WNT6 foi recentemente associado com a resistência à quimioterapia em cancro do intestino e da bexiga, com o pior prognóstico de pacientes com carcinoma de células escamosas do esôfago e osteossarcoma, e com o risco aumentado para desenvolver adenomas colorretais. Contudo, nada se sabe acerca da relevância do WNT6 em gliomas.

Esta tese tem por objetivo desvendar a relevância do WNT6 em GBM, focando particularmente nos mecanismos que regulam a sua expressão, nas vias de sinalização efetoras da sua ação, e na sua significância clínica em pacientes com GBM. Para tal, demonstrou-se que o WNT6 está significativamente sobre-expresso em GBM quando comparado a gliomas de menor grau, independentemente da existência de mutações nos genes IDH ou da co-delecção 1p/19q, e independentemente do número de cópias de ADN. Além disso, a expressão de WNT6 foi associada com o aumento da viabilidade celular, proliferação, invasão, migração, capacidade de células estaminais de glioma e resistência à quimioterapia baseada em temozolomida, estabelecendo o WNT6 como um importante fator oncogénico em glioma. Com

recurso a modelos GBM intracraniais em ratinho e usando modelos de células de GBM com expressão de WNT6 diferencial, demonstrou-se que tumores com maior expressão de WNT6 apresentam um aumento de características associadas à agressividade tumoral, sendo observado uma menor sobrevida destes ratinhos. Além disso, identificamos as vias de sinalização WNT/β-catenina, SFK e STAT3 como potenciais mecanismos de sinalização mediados pelo WNT6 em GBM. A associação entre WNT6 e marcadores de células estaminais ou vias relacionadas com cancro foi ainda corroborada em pacientes com GBM. Com recurso a várias bases de dados independentes de pacientes com GBM, mostrou-se que o WNT6 é um forte biomarcador de prognóstico, associado com a menor sobrevida dos pacientes, e independente de outros fatores de prognóstico bem conhecidos.

Uma vez que o primeiro passo para o desenho de novas terapias direcionadas requer não só a identificação de novos alvos moleculares, mas também a compreensão dos seus mecanismos de ativação, foram ainda investigados os potenciais mecanismos reguladores responsáveis pelo aumento da expressão do WNT6 em glioma. Por um lado, mostrou-se que a metilação do ADN em regiões específicas do *locus* do *WNT6* regula a sua expressão em glioma. Por outro lado, observou-se que o *HOXA9*, um fator de transcrição oncogénico em GBM, está co-expresso com o *WNT6* em gliomas. De facto, demonstrou-se que o HOXA9 se liga diretamente à região promotora do *WNT6*, ativando transcripcionalmente a sua expressão. Deste modo, tanto a metilação do ADN ao nível do *WNT6* como o HOXA9 são potenciais reguladores da expressão do WNT6 em glioma, e podem assim ser utilizados para o desenvolvimento de abordagens terapêuticas inovadoras.

Em suma, este trabalho descreve o WNT6 como um novo oncogene em GBM humano, identificando alguns dos mecanismos que regulam a sua expressão (a metilação do ADN e o HOXA9), bem como os mecanismos moleculares que são responsáveis pela sua ação (nomeadamente, as vias de sinalização WNT, SFK e STAT3). Deste modo, os resultados obtidos poderão ter um impacto crucial na estratificação prognóstica dos pacientes com GBM, e na conceção de novas opções terapêuticas mais atrativas para esta doença altamente mortífera.

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Abbreviations List

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#	5-Aza	5–Aza-2′-deoxycytidine
	β-TrCP	F-box-containing protein E3 ubiquitin ligase
A	ACC	adrenocortical carcinoma
	ADP	adenosine diphosphate
	AKT	AKT serine/threonine kinase
	ALT	alternative lengthening of telomeres
	AMP	adenosine monophosphate
	ANOVA	analysis of variance
	APC	adenomatosis polyposis coli
	ARF	ADP ribosylation factors
	ATM	ATM serine/threonine kinase; formerly ataxia telangiectasia mutated
	ATR	ATR serine/threonine kinase; formerly ataxia telangiectasia and Rad3 related
	ATRX	α-thalassemia/mental retardation syndrome X-linked
B	BBB	blood brain barrier
	BCA	bicinchoninic acid
	BCL2/9	B-cell CLL/lymphoma 2/9
	BCR	Biospeciment Core Resources
	b-FGF	basic FGF
	BLCA	bladder urothelial carcinoma
	bp	base pairs
	BRAF	B-Raf proto-oncogene, serine/threonine kinase; formerly v-raf murine sarcoma viral oncogene homolog B
	BRCA	breast invasive carcinoma
	BrdU	bromodeoxyuridine; 5-bromo-2'-deoxyuridine
	BSA	bovine serum albumin
C	CAMKII	calmodulin-dependent protein kinase 2
	cAMP	cyclic adenosine monophosphate
	Cas9	CRISPR associated protein 9
	CAV1	caveolin 1
	CBP	CREB binding protein; CREBBP
	CBTRUS	Central Brain Tumor Registry of the United States
	CCND1/2	cyclin D1/2
	CD90	THY1 cell surface antigen
	CDK4/6	cyclin dependent kinase 4/6
	CDKN2A/B/C	cyclin dependent kinase inhibitor 2A/B/C
	cDNA	complementary DNA
	CESC	cervical squamous cell carcinoma and endocervical adenocarcinoma
	CFSE	carboxyfluorescein diacetate succinimidyl ester
	ChIP	chromatin immunoprecipitation
	CHOL	cholangiocarcinoma
	CI	confidence interval
	CIC	capicua transcriptional repressor

	CK1	casein kinase 1
	CMA	Comprehensive Meta Analysis
	CNS	central nervous system
	CO_2	carbon dioxide
	COAD	colon adenocarcinoma
	CRD	cysteine-rich domain
	CREB	cAMP response element-binding protein
	CRISPR	clustered regularly interspaced short palindromic repeats
	CSC	cancer stem cells
	CT	computed tomography
	CTLA4	cytotoxic T-lymphocyte associated protein 4
	CX3CL1	C-X3-C motif chemokine ligand 1
D	DAB	3,3'-diaminobenzidine
	DAPI	4′,6-diamidino-2-phenylindole
	DCT	dopachrome tautomerase
	DGAV	*Direcção Geral de Alimentação e Veterinária*
	DKK1/3	dickkopf WNT signaling pathway inhibitor 1/3
	DLBC	lymphoid neoplasm diffuse large B-cell lymphoma
	DMEM	Dulbecco modified eagle medium
	DMSO	dimethyl sulfoxide
	DNA	deoxyribonucleic acid
	DNMT	DNA methyltransferase
	DVL	dishevelled segment polarity proteins
E	ECL	enhanced chemiluminescence
	EDTA	ethylenediamine tetraacetic acid
	EGF	epidermal growth factor
	EGFR	EGF receptor
	EGFRvIII	EGFR variant III
	ELDA	extreme limiting dilution assay
	ELF	extremely low frequency
	ELISA	enzyme-linked immunosorbent assay
	eNOS	endothelial nitric oxide synthase
	ES	enrichment score
	ESCA	esophageal carcinoma
F	FBS	fetal bovine serum
	FDR	false discovery rate
	FELASA	Federation for Laboratory Animal Science Associations
	FGF	fibroblast growth factor
	FGFR	FGF receptor
	FISH	fluorescent *in situ* hybridization
	FITC	fluorescein isothiocyanate
	FPKM-UQ	fragments per kilobase of transcript per million mapped reads–upper quartile
	FUBP1	far upstream element binding protein 1
	FZD	G protein-coupled receptors, class F frizzled

G	GBM	glioblastoma
	GBM-O	glioblastoma with oligodendroglial component
	g-CIMP	glioma CpG island methylator phenotype
	GFAP	glial fibrillary acidic protein
	GFP	green fluorescent protein
	GJA1	gap junction protein alpha 1
	GLUT1	solute carrier family 2 member 1
	GO	gene ontology
	gRNA	guide RNA
	GSC	GBM stem cells
	GSEA	Gene Set Enrichment Analysis
	GSK3	glycogen synthase kinase 3
	GTP	guanosine triphosphate
H	H&E	hematoxylin & eosin
	H3 K27	histone H3, lysine 27
	HB	Hospital Braga
	HIF1α	hypoxia inducible factor 1 subunit alpha
	HNSC	head and neck squamous cell carcinoma
	HOXA9	homeobox A9
	HR	hazard ratio
	HRP	horseradish peroxidase
	HSA	Hospital Santo António
	HSM	Hospital Santa Maria
	HSP	heat shock protein
I	ICI	immune checkpoint inhibitor
	IDH	isocitrate dehydrogenase
	IF	immunofluorescence
	IFNγ	interferon gamma
	IgE/G	immunoglobulin E/G
	IHC	immunohistochemistry
	IL2	interleukin 2
	$Ins(1,4,5)P_3$	inositol-1,4,5-trisphosphate
	Int-1	integration site 1
J	JNK	jun N-terminal kinase
	JUN	jun proto-oncogene, AP-1 transcription factor subunit
K	KICH	kidney chromophobe
	KIRC	kidney renal clear cell carcinoma
	KIRP	kidney renal papillary cell carcinoma
	KLRG1	killer cell lectin like receptor G1
	KPS	Karnofsky performance status
L	L1CAM	L1 cell adhesion molecule
	LAG3	lymphocyte activating 3
	LAML	acute myeloid leukemia
	LEF	lymphoid enhancer factor

	LGG	lower grade glioma
	LIHC	liver hepatocellular carcinoma
	LOH	loss of heterozygosity
	LRP	low density lipoprotein receptors
	LUAD	lung adenocarcinoma
	LUSC	lung squamous cell carcinoma
M	MAPK	mitogen-activated protein kinase
	MDM2	MDM2 proto-oncogene; formerly human homolog of mouse double minute 2
	MDM4	MDM4, p53 regulator; formerly human homolog of mouse double minute 4
	MESO	mesothelioma
	MGMT	O-6-methylguanine-DNA methyltransferase
	MMR	mismatch repair
	MMTV	mouse mammary tumor virus
	MRI	magnetic resonance imaging
	mRNA	messenger RNA
	MSCV	murine stem cell virus
	MSigDb	Molecular Signature database
	MSP	methylation-specific PCR
	mTOR	mechanistic target of rapamycin kinase
	MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
	MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
	MVP	microvascular proliferation
	MYC	MYC proto-oncogene, bHLH transcription factor
N	NADP+	nicotinamide adenine dinucleotide phosphate
	NADPH	nicotinamide adenine dinucleotide phosphate reduced form
	NESTIN	neuroectodermal stem cell marker
	NFAT	nuclear factor associated with T cells
	NF-kB	nuclear factor kappa B
	NK	natural killer cell
	NKD1/2	naked cuticle homolog 1/2
	NOS	not otherwise specified
	NP-40	nonyl phenoxypolyethoxylethanol
	ns	non-statistically significant
	NSG	NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ
	NTRK2	neurotrophic receptor tyrosine kinase 2
O	O/N	overnight
	Oct4	POU class 5 homeobox 1; POU5F1
	OS	overall survival
P	p14	ribonuclease P/MRP subunit p14; RPP14
	PAAD	pancreatic adenocarcinoma
	PBS	phosphate-buffered saline
	PCPG	pheochromocytoma and paraganglioma
	PCR	polymerase chain reaction

	PCV	procarbazine, lomustine, and vincristine
	PD1	programmed cell death 1; PDCD1
	PDGFR	platelet-derived growth factor receptors
	PE	plating efficiency
	PEG3/Pw1	paternally expressed 3
	PI	propidium iodide
	PI3K	phosphoinositide 3-kinase; phosphatidylinositol-4,5-bisphosphate 3-kinase
	PKC	protein kinase C
	PLC	phospholipase C
	PRAD	prostate adenocarcinoma
	PTEN	phosphatase and tensin homolog
Q	qPCR	quantitative PCR
	qRT-PCR	quantitative reverse transcription-PCR
R	RAF	rapidly accelerated fibrosarcoma kinase family
	RAS	rat sarcoma kinase family
	RB	retinoblastoma
	READ	rectum adenocarcinoma
	RGD	Arg-Gly-Asp tripeptide
	RHO	rho family GTPases
	RNA	ribonucleic acid
	RNAseq	RNA sequencing
	ROR	RTK-like orphan receptor
	RTK	receptor tyrosine kinase
	RT	room temperature
	RYK	receptor-like tyrosine kinase
S	SARC	sarcoma
	SD	standard deviation
	SDS	sodium dodecyl sulfate
	SEER	Surveillance, Epidemiology and End Results program
	SFK	SRC family kinases
	SFRP	secreted frizzled-related proteins
	SHH	sonic hedgehog
	shRNA	short-hairpin RNA
	siRNA	small interfering RNA
	SIRT6	sirtuin 6
	SKCM	skin cutaneous melanoma
	SNP	single nucleotide polymorphism
	SOX2/10	SRY-box 2/10
	SRC	SRC proto-oncogene, non-receptor tyrosine kinase
	SRY	sex-determining region Y
	STAD	stomach adenocarcinoma
	STAT	signal transducer and activator of transcription
T	TBP	TATA-box binding protein
	TBS	tris-buffered saline

	TBXT	T-box transcription factor T
	TCF	T-cell factor
	TCGA	The Cancer Genome Atlas
	TERT	telomerase reverse transcriptase
	TET	ten-eleven translocation
	TGCT	testicular germ cell tumors
	THCA	thyroid carcinoma
	THYM	thymoma
	TIM3	hepatitis A virus cellular receptor 2; HAVCR2
	TMA	tissue microarray
	TMZ	temozolomide
	TNF	tumor necrosis factor
	TP53	tumor protein p53
	TRIS	tris(hidroximetil)aminometano
	TSC1/2	tuberous sclerosis complex subunit 1/2
	TSS	transcription start site
U	UCA1	urothelial cancer associated 1
	UCS	uterine carcinosarcoma
	UVM	uveal melanoma
V	VEGF	vascular endothelial growth factor
	VEGFR	VEGF receptor
W	WB	western blot
	WHO	World Health Organization
	WNT	wingless-related integration site

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Aims and thesis outline

Aims and thesis outline

Gliomas are particularly devastating primary brain tumors owing to their localization and remarkable invasive growth. Glioblastoma (GBM) is the most common and lethal glioma in adults. Despite being only palliative, surgery, radiotherapy and temozolomide-based chemotherapy are still the gold standard treatments. Significant advances have been made to understand the molecular pathogenesis of gliomas. Indeed, the recent identification of new diagnostic markers significantly improved the classification of glioma tumors, from a merely histology-based classification to one incorporating both histologic and molecular information. However, the identification of new prognostic and therapeutic molecular targets, in the context of personalized strategies based on tumor intrinsic signaling pathways, are still of paramount importance to ultimately improve the outcome of glioma patients. The aberrant activation of the WNT signaling pathway has been reported in several tumors, including GBM, and has been shown to present an important role for tumor aggressiveness.

In this context, the **aim** of this thesis was to understand the relevance of WNT6, an activator of the WNT pathway for which little is known in cancer, in the pathophysiology of GBM, by characterizing its functional, mechanistic and clinical role. To this end, this thesis is divided into four interconnected chapters, each one with a specific goal as described below:

Chapter 1 introduces the general context of this thesis, aiming to prepare the reader for the following chapters. In this sense, a brief overview is given on the epidemiology of primary brain and central nervous system tumors. An extended description on the histological and molecular classification of gliomas follows. Moreover, a description of the clinically-used and some new promising molecular prognostic factors in glioma, as well as some of the most characterized oncogenic pathways in GBM, is then summarily presented. The chapter concludes by presenting an overview on the WNT pathway, with specific emphasis on its role in gliomas.

Chapter 2 and **3** are composed by two distinct research works focusing on WNT6, a ligand and activator of the WNT pathway. The WNT6 ligand was recently associated with chemoresistance in gastric and bladder cancer, poor prognosis of esophageal squamous cell carcinoma patients, and increased risk to develop colorectal adenoma, being also reported to present oncogenic functions in colon cancer. However, the relevance of WNT6 in human gliomas was still unknown. In this context, in **Chapter 2**, we aimed to explore the functional (*in vitro* and *in vivo*) and clinical relevance of WNT6 in GBM, as well as to

identify its downstream effector pathways. The manuscript presented in this chapter was accepted for publication in Theranostics (doi:10.7150/thno.25025). Although Chapter 2 demonstrates that WNT6 is overexpressed in a subset of GBM patients, leading to their poor prognosis, the mechanisms underlying WNT6 aberrant expression in gliomas were still unexplored. In **Chapter 3**, we aimed to study some potential upstream regulators of WNT6 expression in gliomas, analyzing *WNT6* copy number alterations, DNA methylation and its link with potential direct transcriptional regulators. Additionally, the independent prognostic value of *WNT6* in GBM was also evaluated. In Chapter 3 we identified *WNT6* DNA methylation and HOXA9, which we previously described to be an oncogenic transcription factor in GBM, as upstream regulators of WNT6 expression. The work presented in this chapter is under preparation for submission to an international peer-reviewed journal.

Chapter 4 covers a general discussion of the major results of the presented work (Chapter 2 and 3), which are here debated in an integrated manner, on the light of other relevant published work in the field. Moreover, suggestions for future perspectives are also discussed. Finally, a brief conclusion of this thesis is presented.

Chapter 1

General Introduction

This chapter introduces the general context of this thesis, including a brief overview on the epidemiology of primary brain and central nervous system tumors, and an extended description on the histological and molecular classification of gliomas. Next, molecular prognostic factors and oncogenic pathways are presented, concluding with an overview on the WNT pathway in gliomas.

Contents

1.1. Primary brain and central nervous system tumors: epidemiology

Malignant primary brain tumors represent approximately 32% of all brain tumors and are diagnosed in more than 250 000 individuals each year worldwide[1], being the 17th most common cancer type [1]. In Portugal, 932 new cases were reported in 2012 [1]. Despite their relatively low incidence when compared to other primary cancers (e.g., lung, breast, prostate and colorectal cancers) or to metastatic brain tumors, they constitute a major source of morbidity and mortality, being the 12th most deadly cancer worldwide with almost 190 000 estimated death per year [1] and ranking first in average of years of life lost among all tumor types [2]. In Portugal, 718 estimated deaths were reported in 2012 [1], being in the top-10 of most fatal cancers.

As reported for other cancers, the overall incidence of brain tumors, in adults, increases with aging [1, 3], which might be a result of the time required for the occurrence of genetic alterations that will lead to the malignant transformation and/or to the diminished immune surveillance that also occurs with aging. Over the past three decades, the incidence of brain tumors has increased, which might be due to the increased life-expectancy or a reflect of improved: i) classification and reporting; ii) access to diagnostic imaging; and, iii) medical practices, among others [4]. Attempts to interpret geographic and ethnic variations regarding the incidence and death rates of primary brain tumors are difficult due to the worldwide differences in the availability and access to medical care. Indeed, the regions with the highest rates of reported cases and death due to primary malignant brain tumors have better access to medical imaging (e.g., Northern Europe, white population in North America, and Israel) than the areas with the lower rates (e.g., India and Philippines), suggesting that the incidence is probably underestimated in developing countries [5, 6]. One exception to this are the rate of malignant brain tumors observed in Japan, considered an economically prosperous country, which are less than half the rate observed in Northern Europe, suggesting differences in risk factors based on ethnic variations in inherited susceptibility and/or cultural or geographic differences [5].

Although unexplained, the most consistent brain tumor epidemiology result is the 1.3-fold increased risk in males vs. females for developing or dying from a brain tumor [1]. In fact, the worldwide estimated age-standardized[2] incidence rate is 3.9 in males vs. 3.0 in females, while the estimated age-standardized mortality rate is 3.0 in males vs. 2.1 in females. Similar values are observed when considering Portugal only [1].

[1]The estimated number of incident cases was 256 213, for both sexes, worldwide in 2012.

[2]Age-standardized rates are necessary when comparing populations with different age-distributions, as age has a powerful effect on cancer risk and it is expressed per 100 000 individuals.

Although the potential contribution of several environmental epidemiologic risk factors has been analyzed, only ionizing radiation (resulting from therapeutic X-irradiation or found in Hiroshima survivors) was associated with increased risk for brain tumor [7-11], more specifically, glioma, and history of allergies/atopic disease with a decreased risk [6, 12-22]. In its turn, the impact of the socioeconomic status and education level, as well as certain occupations (e.g., physicians, firefighters, farmers, rubber manufacturers, etc.) [23-32], diet [6], smoking [33, 34], electromagnetic fields [35-41] and cell phones exposure [42-47], among others [48, 49], remains inconclusive. A summary of these studies can be found in Figure 1.1.

1.2. Gliomas: histological and molecular WHO classification

For many decades, gliomas, which represent a large majority of malignant brain tumors (81%), have been traditionally classified based on light microscopic features as astrocytic, oligodendroglial, oligoastrocytic (mixed), or ependymal tumors, as described in the 2007 central nervous system (CNS) World Health Organization (WHO) classification guidelines [50]. This classification evolved from the work by Baily and Cushing (1926), where brain tumors were classified based on extensive histological examination combined with the clinical course of the patient [51]. In this classification, tumors were named after the normal cells from which they resembled and that, at the time, was presumed to be their cell of origin. In addition to the lineage name, the term anaplastic was added if histological atypia was observed, which reflected the tumor progression status. This classification was widely accepted as it proved to be clinically relevant. Baily/Cushing classification was improved afterwards but always retained the main principle: presumed origin and progression status. Two decades later, Kernohan *et al.* proposed what latter became the WHO grading system (grade I – lower malignancy – to IV – higher malignancy), which significantly improved the progression status description, and was based on the histological presence/absence of cytological atypia, anaplasia, mitotic activity, microvascular proliferation (MVP) and necrosis [52, 53].

However, this traditional histologically-based classification [54] was subject to considerable interobserver variability, particularly in the context of diffusely infiltrating gliomas (e.g., differences in the classification of astrocytoma vs. oligodendroglioma vs. oligoastrocytoma) [50, 55, 56]. Moreover, advances in (epi)genetics and transcriptomic analyses have shed light on the biological and clinical variability observed within each histologically defined glioma entity [56, 57]. These observations suggested that some of the molecular alterations underlying this variability might in fact be used as biomarkers for more accurate glioma classification [57]. Indeed, recent studies demonstrated that this

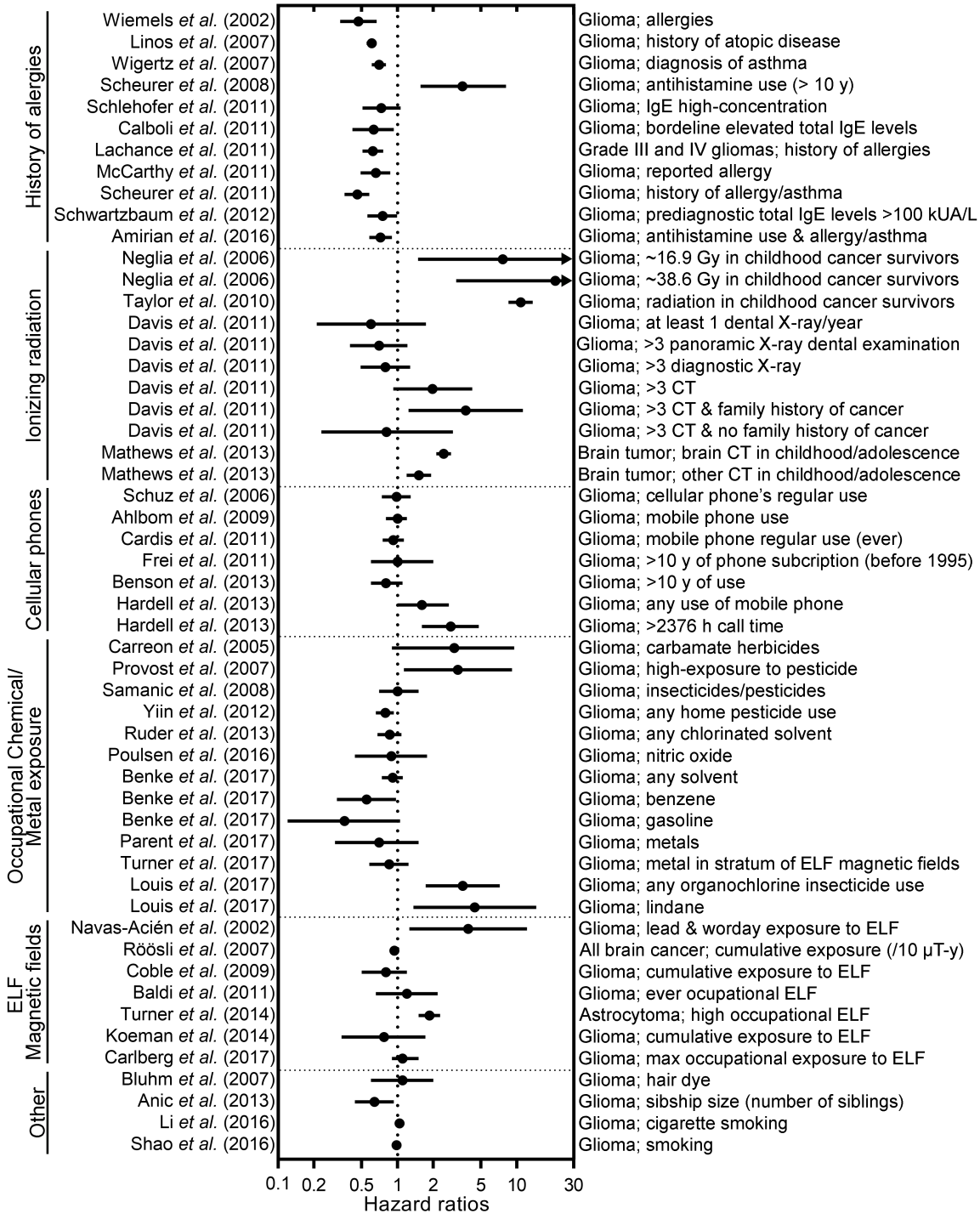

Figure 1.1. Forest plot of selected studies on potential risk factors for glioma.
CT = Computed Tomography; ELF = Extreme Low Frequency magnetic fields; IgE = Immunoglobulin E.

molecular-based classification is more diagnostically relevant than the traditional system, which contributed to the 2016 revision of the guidelines [50].

The resulting 2016 CNS WHO classification introduced the concept of "integrated diagnosis" (Figure 1.2) by incorporating both microscopic and molecular parameters in the glioma classification [56]. This resulted in more homogeneous glioma entities and increased diagnostic objectivity/accuracy,

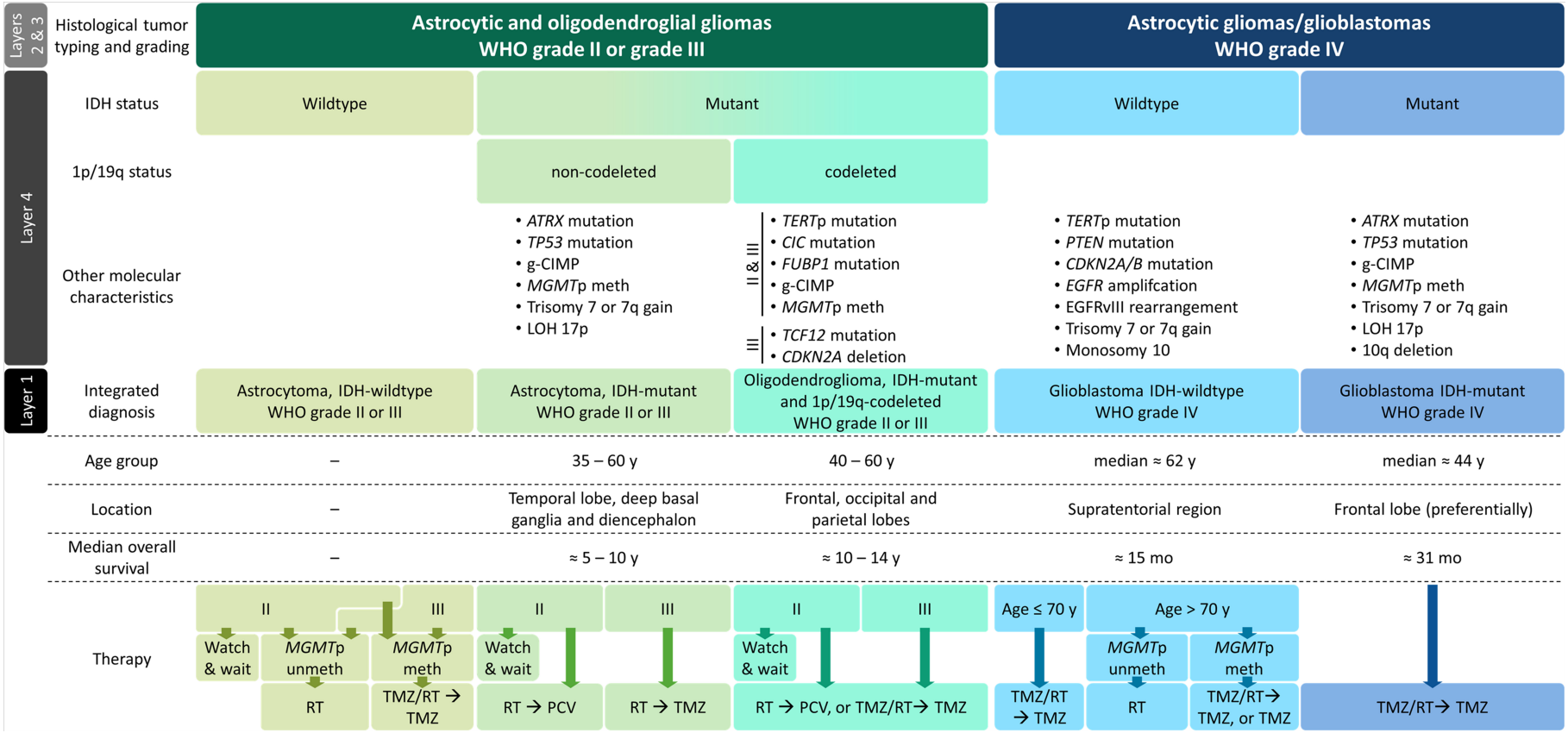

Figure 1.2. Diagnostic approach, molecular features and current post-surgery treatment strategies for adult diffuse gliomas according to the 2016 WHO classification of tumors of the CNS.

Besides the histological characterization (typing and grading; layer 2 and 3), diffuse gliomas are molecularly characterized according to IDH mutations and 1p/19q co-deletion status (layer 4), culminating in the integrated diagnosis (layer 1). IDH-wildtype WHO grade II or III astrocytoma are considered an uncommon diagnosis in the 2016 WHO classification of CNS tumors and should be avoided. LOH: Loss of Heterozygosity; meth: methylated; *MGMT*p: *MGMT* promoter; PCV: Procarbazine, Lomustine, and Vincristine; RT: Radiotherapy; *TERT*p: *TERT* promoter; TMZ: temozolomide; unmeth: unmethylated.

which may ultimately lead to better patient management. Nowadays, the diagnosis of glioma is "layered", with the histologic classification as layer 2, WHO grading as layer 3, and molecular characterization as layer 4, culminating in an "integrated diagnosis" at layer 1 [56]. One caveat to this "workflow" is that the diagnosis will not necessarily proceed from layer 2 (histologic) to layer 4 (molecular), as the molecular characterization might superimpose the histological classification in the final "integrated diagnosis" [56]. This new classification led to a profound restructuration of many brain tumor categories (Figure 1.3), creating new entities/subgroups and eliminating others [50, 56]. Whenever molecular testing is unfeasible or inconclusive, either due to tissue availability limitation, low tumor content, or other circumstances impeding reliable molecular testing, the diagnosis should be performed using the histologic classification followed by the term "not otherwise specified (NOS)" [56].

The following paragraphs summarize the most important features of circumscribed and diffuse gliomas considering the 2016 CNS WHO classification, with a special emphasis on diffuse gliomas – the focus of this dissertation.

1.2.1. Circumscribed gliomas

Circumscribed gliomas, also known as non-diffuse gliomas, are slow-growing tumors with little infiltration of the adjacent brain parenchyma and may be found particularly in younger ages [50]. This category includes three main entities: pilocytic astrocytoma, subependymal giant cell astrocytoma and pleomorphic xanthoastrocytoma.

The improvement in the molecular characterization of these tumors allowed the implementation of effective molecularly targeted therapies [58, 59]. For example, pilocytic astrocytoma (WHO grade I), the most common non-diffuse glioma, particularly incident in children and adolescent, lacks mutations in the *IDH1/2* (isocitrate dehydrogenase 1 and 2; IDH refers to both isoforms) genes, but present mutations in the fibroblast growth factor receptor 1 (*FGFR1*) or in the neurotrophic receptor tyrosine kinase 2 (*NTRK2*) genes, or tandem duplications in a v-Raf murine sarcoma viral oncogene homolog B (*BRAF*) fusion gene [58]. Considering the impact of these genetic alterations in the mitogen-activated protein kinase (MAPK) pathway, pilocytic astrocytoma is considered a single pathway disease and the MAPK inhibitor selumetinib has been pointed as a potential targeted therapy [60-62].

Similarly, mutations in the tumor suppressor genes *TSC1* and *TSC2*, which affect the mTOR signaling, were described to be driven mutations of subependymal giant cell astrocytomas (WHO grade I; mostly diagnosed in the first two decades of life), against which everolimus, a mTOR inhibitor, has shown to be effective [63-68].

Finally, pleomorphic xanthoastrocytomas (WHO grade II) are the rarest and consequently less

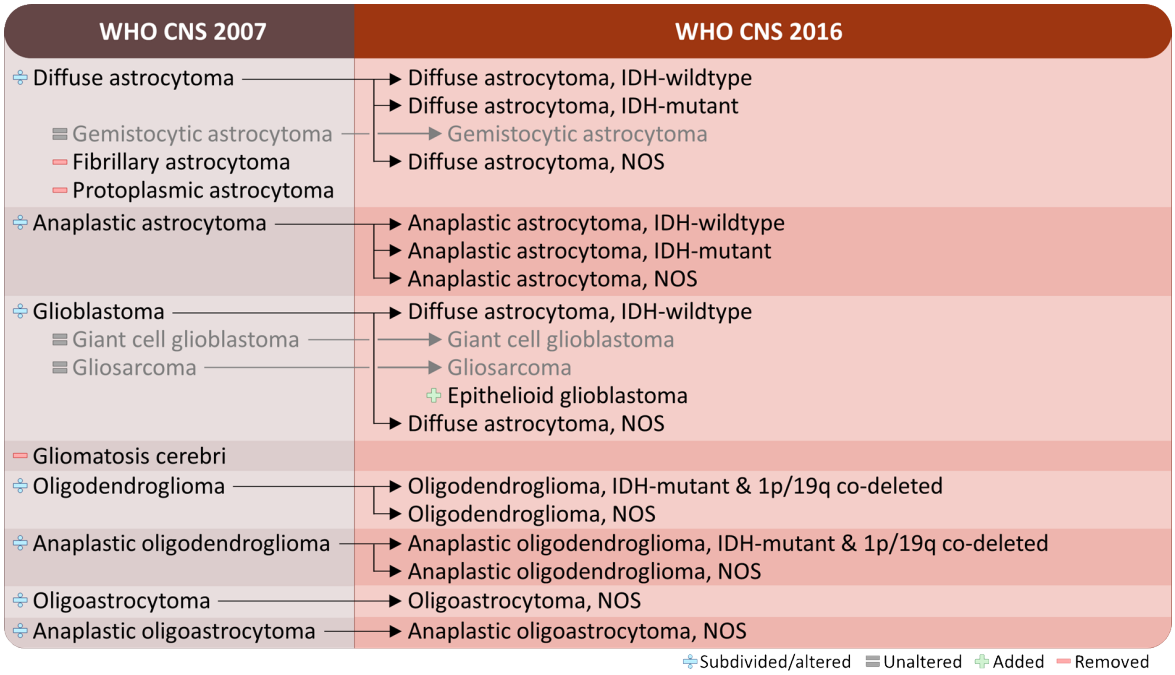

Figure 1.3. Alterations observed between the WHO classification of CNS tumors 2007 and 2016. Entities were subdivided/altered, others were unaltered or removed, and new entities were added. Oligoastrocytoma and anaplastic oligoastrocytoma are provisional entities in the 2016 classification. NOS: not otherwise specified.

characterized glioma entity, most often diagnosed in children and young adults. However, more than half of these tumors present activating $BRAF^{V600E}$ mutations [58, 59, 69], which respond well to vemurafenib, a BRAF inhibitor [70, 71].

1.2.2. Diffuse gliomas

Diffuse gliomas are by far the most frequent primary brain tumors, especially in adults [3]. The nosological shift to the integrated diagnosis paradigm (combining molecular and histological characteristics; Figure 1.2) affected in many ways the classification of these tumors (Figure 1.3) [56]. While until then astrocytomas had been considered a group of tumors (encompassing both circumscribed and diffuse astrocytomas), ignoring the molecular and prognostic divergences between them, now, all diffusely infiltrating gliomas (independently of their phenotype, i.e. astrocytic or oligodendroglial) are grouped together [56]. This gathered gliomas with similar growth patterns, behaviors and genetic drivers (such as IDH mutations), which are genetically and prognostically similar, and consequently opened the possibility for the development of guided therapies and thus better patient management. This broad category includes therefore the diffuse astrocytoma (WHO grade II), anaplastic astrocytoma (WHO grade III), diffuse oligodendrogliomas (WHO grade II), anaplastic oligodendrogliomas (WHO grade III) and glioblastoma (GBM; WHO grade IV)[3] [56].

[3]Note that, the diffuse midline glioma, H3 K27M-mutant, predominantly found in children, is also included in this category, but is beyond the scope of this dissertation.

In opposition to circumscribed gliomas, diffuse gliomas are histologically characterized by diffusely infiltrative growth along existing brain structures, including the brain parenchyma, with tumor cells invading individually or as cell aggregates forming a network throughout the neuropil [50]. This characteristic invasion is often accompanied by the so-called "Scherer's secondary structures" that are pathognomonic of diffuse gliomas and comprise the aggregation of tumor cells around neurons (perineuronal satellitosis), blood vessels, white matter tracts and under the pial membrane [50]. Furthermore, diffuse gliomas might invade along myelinated fiber tracts, commonly crossing the corpus callosum in direction to the contralateral hemisphere ("butterfly glioma" pattern) [50]. In addition, WHO grade II and III diffuse gliomas carry an inherent tendency for recurrence and malignant progression, and present recurrent point mutations in *IDH1/2* [57]. These mutations are also present in GBMs originating from lower-grade tumors (secondary GBMs), which may constitute a potential initiating somatic aberration [57]. However, studies demonstrated that IDH mutation *per se* is not sufficient for the induction of tumor growth, suggesting that additional genetic hits are required for glioma development [72]. In the case of diffuse and anaplastic astrocytomas, mutations in *TP53* and *ATRX* genes are required, while 1p/19q co-deletion[4] and *TERT* promoter mutation occur in oligodendroglial tumors [56, 73, 74]. Indeed, these genetic profiles are mutually exclusive and considered pathognomonic of astrocytic vs. oligodendroglial lineage tumors (Figure 1.2).

IDH-wildtype tumors histologically graded as WHO grade II and III are considered an uncommon diagnosis, and must only be performed if i) the immunohistochemistry (IHC) for the mutant R132H IDH1 protein, and ii) the sequencing of *IDH1* codon 132 and *IDH2* codon 172 gene mutations are both negative or if the latter alone is negative [56]. Care must be taken to avoid misdiagnosis. Since IDH-wildtype grade II and especially grade III diffuse gliomas show molecular characteristics of GBM and behave like them [73, 75], these cases might reflect underestimation of the histologically-based malignancy grade. In opposition, they might also be a result of malignancy overestimation, as a subset of these IDH-wildtype diffuse astrocytomas have been reported to be associated with better survival outcomes, and show molecular characteristics of grade I pilocytic astrocytoma or other low-grade gliomas [76]. In this context, additional molecular characterization of genetic aberrations associated with both IDH-wildtype GBM (e.g., *EGFR* amplification, loss of chromosome 10, etc.) or pilocytic astrocytoma (i.e. *BRAF* fusion gene) should be performed in initially diagnosed IDH-wildtype diffuse grade II or III, to discard the possibility of misdiagnosis [56, 57]. Because of this and the fact that it is believed that molecular analyses in the future

[4]Note that, 1p/19q co-deletion refers to whole-arm deletion of both chromosomes, typically caused by an unbalanced translocation [*t*(1;9)(q10;p10)]. Partial deletions on either chromosome are not sufficient to perform a diagnosis [145].

will lead to the reclassification of IDH-wildtype grades II/III diffuse gliomas in other entities, they are considered provisional entities in the new 2016 WHO CNS classification [56].

For all diffuse gliomas, whenever IDH testing is not available or incomplete (no sequencing data), the term NOS should be added to the histologic classification [56].

More details about each category of diffuse glioma can be found in the sections below.

1.2.2.1. Diffuse astrocytic and anaplastic astrocytic gliomas

Diffuse astrocytic (grade II) and anaplastic astrocytic (grade III) gliomas are divided in three categories: the IDH mutant (the great majority), the IDH-wildtype (very rare) and NOS [56]. These tumors occur predominantly in adults (peak incidence: 35 to 60 years) and are characterized by a rapid clinical progression with focal deficits, with a median overall survival of 5 to 10 years (for IDH-mutant tumors) [77, 78]. Regarding their localization in the brain, they are preferentially found on the temporal lobe, deep basal ganglia and diencephalon [78].

Histologically, astrocytic gliomas present nuclear irregularities and hyperchromasia, while, molecularly, IDH-mutant astrocytomas are defined by two additional mutations in *TP53* and *ATRX* (mutually exclusive to 1p19q co-deletion[5]) [50, 57, 78]. Both TP53 and ATRX mutations might be easily identified by IHC standard techniques. The mutation of ATRX, which presents an important role in chromatin remodeling and regulation of telomere length (more details in section 1.3.4), leads to the loss of its nuclear expression [57, 78].

Histological examination remains the gold-standard technique for glioma grading (Figure 1.4) and includes the assessment of the mitotic activity, MVP, and necrosis [50, 79]. WHO grade II astrocytomas present none of these features. However, grade III are the ones presenting increased mitotic activity (without a specific cut-off, ≥ 3 mitoses per 10-high-power-fields is sufficient). The additional presence of MVP and/or necrosis automatically confers the diagnosis of glioblastoma [50, 79]. However, some genetic alterations were already associated with the progression from grade II to grade III/IV astrocytoma. These genetic alterations include *CDKN2A* and *ARF* deletion (due to 9p21 deletion), *CDKN2B* deletion (due to 19q deletion), and MYC and RTK/RAS/PI3K pathways activation, among others [57, 63]. Although recent studies suggested that the prognostic differences between astrocytomas grade II and III are not as significant as historically reported [80-83], for now, the WHO grading system was retained in the new 2016 classification.

The fibrillary astrocytoma and protoplasmic astrocytoma entities were removed from the 2016

[5]Note that, in some cases, ATRX mutation assessment (through IHC) is being used rather than 1p/19q co-deletion assessment (that needs more sophisticated techniques) to distinguish astrocytic vs. oligodendroglial lesions.

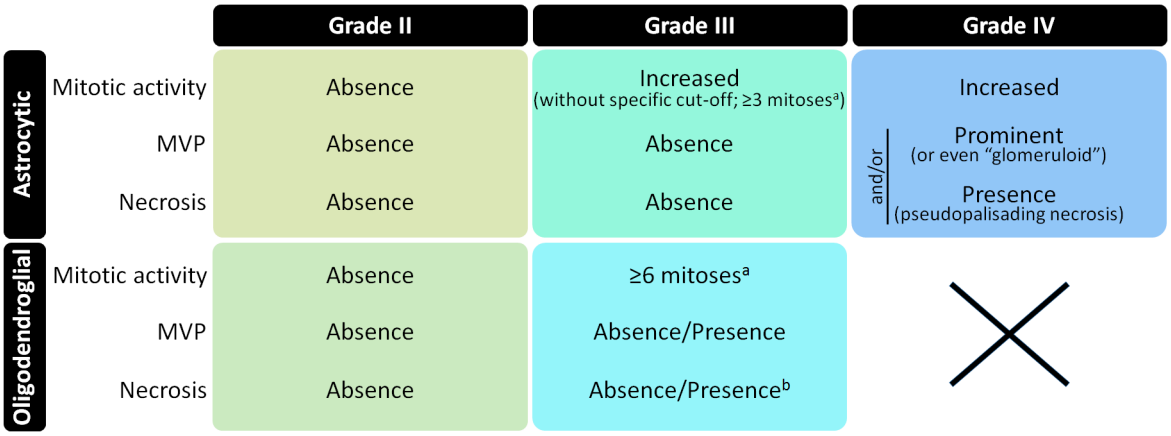

Figure 1.4. WHO grading system for diffuse gliomas.
Histological examination remains the gold-standard technique for glioma grading and includes the assessment of the mitotic activity, microvascular proliferation (MVP) and necrosis. [a] in 10-high-power-field; [b] in the 2007 classification, oligodendroglial tumors with necrosis were classified as GBM with oligodendroglial component (GBM-O). Now GBM-O cases would be converted in GBM, IDH-wildtype; GBM, IDH-mutant; or anaplastic oligodendroglioma, IDH-mutant, 1p/19q co-deleted.

classification for their lack of clinical relevance (Figure 1.3). Gliomatosis cerebri, until now defined by its extreme infiltrative growth pattern (involving at least three cerebral lobes), is also no longer a distinct entity in the new classification, rather being considered a growth pattern found in all diffuse gliomas subtypes (Figure 1.3) [56].

The single variant of diffuse astrocytic tumors maintained in the 2016 WHO CNS tumor classification is the gemistocytic astrocytoma (Figure 1.3). As the name implies, these tumors are characterized by significant gemistocytic astrocytic cells (> 20% of cells with abundant eccentrically placed cytoplasm). These tumors are also characterized by a rapid malignant progression and present perivascular lymphocytic infiltrates [50, 56].

1.2.2.2. Oligodendroglial and anaplastic oligodendroglial gliomas

As for astrocytic tumors, based on the 2016 WHO classification, oligodendroglioma (grade II) and anaplastic oligodendroglioma (grade III) are divided in three categories: the IDH-mutant (the great majority), the IDH-wildtype (very rare) and NOS [56]. Tumors from the oligodendroglial lineage occur predominantly in adults (peak incidence: 40 to 60 years) and are characterized by an indolent growth pattern, seizures, an improved prognosis (compared to astrocytic tumors of the same grade) and sensitivity to both radio- and chemotherapy [84-87]. Grade II oligodendrogliomas are more common in younger patients, as the case of all IDH-mutant grade II and III diffuse gliomas (independently of the 1p/19q co-deletion) [77]. While the median overall survival of oligodendrogliomas may be higher than 10 years, they are almost invariably fatal and should not be considered benign [77].

Although they may arise throughout the CNS, oligodendrogliomas appear mostly in the white matter of cerebral hemispheres with predominance in the frontal, occipital and parietal lobes. At the

recurrence, leptomeningeal spread may occur, but extra-CNS metastases are very rare. However, in contrast to astrocytoma, areas of remarkably sharp borders with surrounding brain tissue can often be found [77].

Histologically, grade II oligodendrogliomas present uniformly round to oval cells, with round nuclei, which are uniform and present a crisp nuclear membrane, delicate chromatin, and small to inconspicuous nucleoli. In more anaplastic (grade III) tumors, they also present an increased cell size and pleomorphism with a more i) prominent nucleoli, ii) vesicular chromatin pattern, and iii) epitheloid features [77].

Molecularly, and as aforementioned, oligodendrogliomas are defined by IDH mutation (present in 90% of the cases) in combination to 1p/19q co-deletion (considered pathognomonic of the oligodendroglial lineage in the new classification). However, *TERT* promoter activating mutations (in hot spot regions: C228T and C250T) are present in more than 95% of these tumors. Other common but not defining genetic alterations are the mutation in *CIC* (on 19q13.2; leading to the inactivation of a transcriptional repressor; in 50–70% of the cases) and *FUBP1* (on 1p31.1; up-regulating *MYC* expression; in 30% of the cases; Figure 1.2) [77].

In the past, diffuse gliomas that histologically appeared as both astrocytic and oligodendroglial were classified as mixed gliomas or oligoastrocytomas [54]. However, with the use of molecular analyses, most of the mixed gliomas have shown to present pathognomonic features of either astrocytoma or oligodendroglioma [56]. Therefore, in the 2016 version of the WHO CNS classification, diagnoses of oligoastrocytoma and anaplastic oligoastrocytoma are discouraged, with the molecular information superimposing the histological classification (i.e. tumors histologically resembling oligodendroglial but with no 1p/19q co-deletion, are classified as an astrocytic glioma) [56]. Whenever molecular analyses are not possible or are inconclusive, histologically "mixed" tumors might be diagnosed as oligoastrocytoma, NOS [56].

The WHO grading of oligodendroglial and mixed tumors is assessed using the same histologic features of astrocytic tumors but with some discrepancies (Figure 1.4). As for astrocytic tumors, grade II oligodendroglial and mixed tumors are the ones without any of these histologic features. However, grade III is only classified when mitotic activity is present in ≥ 6 cells per 10 high-power fields (rather than the ≥ 3 mitoses for anaplastic astrocytoma) [56, 77]. Moreover, contrarily to diffuse astrocytic tumors, in the new classification, pure oligodendroglial tumors remain grade III lesions even if they present MVP and/or necrosis [56]. In the past, oligodendroglial tumors presenting necrosis were preferentially diagnosed as glioblastoma with oligodendroglial component (GBM-O) [54]. However, following the WHO 2016

guidelines, and upon molecular studies, such tumors would be rather classified as GBM, IDH-wildtype or IDH-mutant, or anaplastic oligodendroglioma, IDH-mutant, 1p19q co-deleted [56]. Note that, following this new classification, grade II oligodendrogliomas may progress to grade III oligodendrogliomas, but never to grade IV glioblastoma, which seems to always derive from an astrocytic precursor (Figure 1.4) [56].

1.2.2.3. Glioblastoma

GBMs correspond to more than half of all malignant brain tumors and to 58% of all gliomas, being the most common and malignant form of glioma [5]. Although the annual incidence rate is relatively low when compared to cancers from other primary sites, they are deadly tumors with low percentages of 1 to 10-years survival rates (Figure 1.5 and Figure 1.6) [3]. As for brain tumors in general, males are more affected by GBM than females (1.6:1), and whites more than blacks (2:1) [5]. Moreover, hereditary cancer syndromes are most likely not associated with GBM, as only a small subset (1%) may be explained by heredity, including neurofibromatosis types 1 and 2, Turcot and Li-Fraumeni syndromes [5].

As previously mentioned, GBM may be classified in IDH-mutant (in this case, the rarest type), IDH-wildtype (the most common; 90% of all GBM), and NOS [56]. However, the classification of these tumors remains histologically based rather than molecular. Indeed, GBM are the tumors with astrocytic phenotype, featuring nuclear atypia, MVP and necrosis, consequently designated as grade IV gliomas by histologic analyses (Figure 1.4) [56].

IDH-wildtype GBMs preferentially occur in elderly patients (median age at the diagnosis: 62 years), are localized in the supratentorial region[6], and correspond to primary GBM that develop *de novo* (i.e. with a short clinical history – less than 3 months before diagnosis – and without a pre-existing lower-grade precursor lesion) [56, 57]. They are associated with copy number gains on chromosome 7, monosomy of the chromosome 10, *EGFR* amplification, and mutations in *PTEN*, *TERT*, *CDKN2A* and *CDKN2B* genes. As in grade II and III diffuse gliomas, *TERT* promoter mutation is mutually exclusive to *ATRX* mutation, which are common in IDH-mutant GBM (Figure 1.2) [56].

IDH-mutant GBMs typically occur in younger adults (median age at the diagnosis: 44 years), are preferentially localized in the frontal lobe, and include the majority of secondary GBM (the ones that develop from a pre-existing diffuse or anaplastic astrocytoma) [56, 57]. Consequently, these tumors present a molecular profile similar to IDH-mutant astrocytomas, including copy number gains on chromosome 7, loss of heterozygosity of the 17p arm, *TP53* and *ATRX* mutations, and g-CIMP phenotype

[6]The supratentorial region of the brain is the area located above the tentorium cerebelli, i.e. the cerebrum. The cerebrum is a large part of the brain containing the cerebral cortex (frontal, parietal, temporal, and occipital lobes), as well as several subcortical structures, including the hippocampus, basal ganglia, and olfactory bulb.

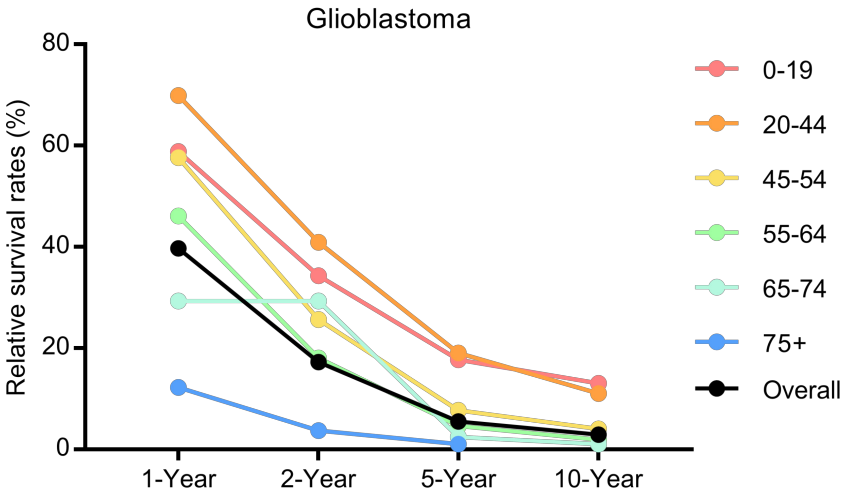

Figure 1.5. Glioblastoma one to ten years relative survival rate per age ranges.
Source: Central Brain Tumor Registry of the United States (CBTRUS; *www.cbtrus.org/*)

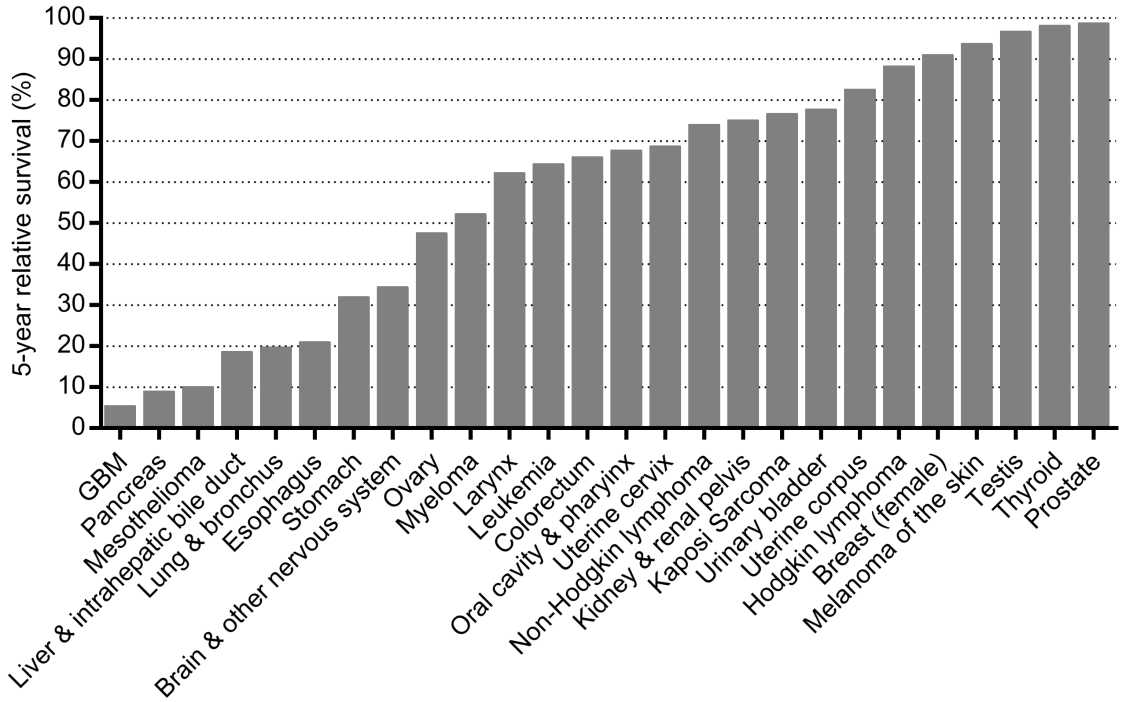

Figure 1.6. Five-years relative survival rates for several cancers.
Source: SEER Cancer Statistics Review 1975-2015, through the Surveillance, Epidemiology and End Results program by National Cancer Institute (*https://seer.cancer.gov/csr/1975_2015/*); and CBTRUS (*www.cbtrus.org/*)

[56, 57]. Note that, not all IDH-mutant GBM present a history of a pre-existing lower-grade lesion, while progression from IDH-wildtype gliomas (e.g., IDH-wildtype anaplastic astrocytoma or pleomorphic xanthoastrocytoma) to secondary GBM may also occur (Figure 1.2) [56].

The division of IDH-wildtype and IDH-mutant GBM was one of the most important improvements of the new classification. Although they cannot be discriminated under the microscope, they are not only biologically distinct but also associated with completely different clinical features, including prognosis, age at onset, among others. Indeed, the survival of patients with IDH-mutant GBM may be equal to or

even better than the survival of IDH-wildtype anaplastic astrocytoma (grade III) [88].

In GBM patients older than 55 years, IDH1 R132H IHC is sufficient to discriminate between IDH-wildtype and IDH-mutant, while in younger patients and in patients with clinical evidence of a pre-existing less malignant lesion, IDH sequencing should be performed when the IHC result is negative [56].

GBM tumors are highly infiltrative, with a great percentage of them in adults (approximately 50%) infiltrating more than one lobe and a low percentage (5%) growing multifocally [89]. Although rare, in the late stages of the disease, leptomeningeal dissemination may occur [90]. Many years ago, when GBM IDH-wildtype and IDH-mutant were considered as a single GBM entity, a study demonstrated that patients with frontal lobe GBM survived longer (11.4 months) when compared to patients with temporal or parietal lobe GBM (9.1 and 9.6 months, respectively) [91]. However, this might be related to the fact that IDH-mutant GBM, which are associated with a more favorable prognosis (IDH mutation, higher rates of gross total resection and younger ages), preferentially occur in the frontal lobe [92-94].

Personality changes and mood disorders are frequent in patients with frontal-lobe tumors, but these symptoms are frequently mistakenly associated with psychogenic disorders or as part of the normal aging process, delaying the diagnosis. In brain tumors, their location is undoubtedly critical for the type of first presenting symptoms [95]. Although headache is the first symptom associated with brain tumor (present in 64% of the patients), a low percentage of patients (8%) present headache as the only symptom [95]. Epilepsy is one of the main presenting symptoms in GBM patients occurring in 24 to 68% of the patients at the time of diagnosis and developing later in 19 to 38% of the others [95]. However, epilepsy is mainly characteristic of slow growing tumors [96], as lower-grade gliomas. Sensorimotor deficits are the next most frequently presenting symptom (occur in 20% of the cases), and a minority (5%) present aphasia derived from tumors affecting the speech-dominant hemisphere (left side) [95].

Giant-cell glioblastoma and gliosarcoma are histologic variants of IDH-wildtype GBM (Figure 1.3) [56]. The first one represents only 1% of all GBM, occurs in younger adults (median age at diagnosis: 51 years) and is characterized by large cells (> 500 μm of diameter) with multiple nucleus and presenting lymphocytic infiltration [97]. In its turn, gliosarcoma represents only 2% of all GBM, and is characterized by both malignant glial and sarcomatous elements (i.e. shows signs of fibroblastic, cartilaginous, osseous, smooth and striated muscle, or adipose cell lineage). These tumors occur preferentially in the temporal lobe, with patients having an age at diagnosis and a clinical outcome similar to other GBM, IDH-wildtype tumors [98]. However, contrarily to other GBM, gliosarcoma tumors may spread into the body (e.g., to lungs, liver and lymph nodes), although the phenomenon is still rare [99].

In the context of GBM, the variant epithelioid GBM was provisionally introduced in the new

classification under the GBM, IDH-wildtype arm (Figure 1.3) [56]. This variant is characterized by large epithelioid cells with abundant eosinophilic cytoplasm, vesicular chromatin, and prominent nucleoli. Moreover, they present the $BRAF^{V600E}$ mutation (in 50% of the cases) and occur frequently in children and young adults [56, 97].

Notwithstanding being invariably fatal, the standard treatment (described below) is able to temporarily keep stable or even improve the quality of life and the cognitive function of GBM patients, even in most impaired patients (i.e. the elderly) [100-103]. However, as soon as the first-line treatment fails, patients experience a severe impact on their quality of life [100-103].

1.2.2.4. Current Therapy

Despite the recent advances in the characterization of the genetic and epigenetic profile of diffuse gliomas, which culminated in the molecularly based 2016 WHO CNS tumor classification, molecularly targeted therapies have, to date, failed in phase III clinical trials in patients with gliomas [63]. Throughout decades, several studies focused on comparing treatment options for diffuse glioma. However, in the case of grade II and III diffuse glioma, which may present survivals above 10 years, the results obtained now are from trials started decades ago. Therefore, the gold-standard therapy for the majority of diffuse glioma remains the classic treatment regimen of surgery, radiotherapy and/or chemotherapy (with "old" drugs), which is still inadequate [63].

When applied, gross total resection in all diffuse gliomas is associated with improved survival of patients, independently of grade or patient age [104, 105]. However, due to their invasive phenotype, diffuse gliomas almost invariably recur after resection, requiring additional treatment to prolong the patient's survival [63]. The treatment and timing of treatment applied to each patient depends on patient age, clinical performance status, tumor entity, and molecular biomarkers [63].

Similar to the surgery, postsurgical radiotherapy showed to be beneficial in diffuse gliomas of any grade, when compared to surgery alone [106]. However, some studies clearly showed that radiotherapy leads to patients' progressive cognitive decline [107]. In this sense, in the setting of WHO grade II diffuse gliomas and for patients aged under 40 with favorable prognostic features, delayed radiotherapy until radiologic progression is used – the "watch and wait" procedure – as it was reported that this does not compromise the overall survival of the patients (Figure 1.2) [108-110].

Regarding chemotherapy, 1p/19q co-deletion (i.e. in oligodendroglial tumors) was predictive of long-term benefit from PCV polychemotherapy (procarbazine, lomustine, and vincristine) [63]. However, some tumors lacking 1p/19q co-deletion also responded well to PCV treatment, being unknown if this benefit is linked to IDH mutation or *MGMT* promoter methylation [63]. Based on three large trials showing

improved outcome if PCV is added to radiotherapy, the standard of care of grade III 1p/19q co-deleted patients and grade II IDH-mutant requiring post-operative treatment is now radiotherapy with adjuvant PCV (Figure 1.2) [63]. Due to the high toxicity observed in patients treated with PCV, some trials tested the substitution of PCV by temozolomide (TMZ), a better tolerated and easier to administrate agent. Preliminary data showed good results in patients with grade III gliomas 1p/19q non-co-deleted [63].

From all diffuse gliomas, GBMs present the best-defined standard of care (Figure 1.2), consisting of postoperative radiotherapy (60 Gy in 30 fractions) with concomitant daily TMZ, followed by 6 cycles of adjuvant TMZ – "the Stupp protocol" [111]. However, concomitant and adjuvant TMZ only improves the survival of GBM patients from ≈12 months (10% of 2-year survival) to ≈15 months (26% of 2-year survival), as assessed in patients aged under 70 years [111]. For patients over 70 years, characterized by a poorer prognosis, shorter radiotherapy regimens, as well as TMZ administration based on the *MGMT* promoter methylation, showed increased efficacy [63]. Thus, patients with *MGMT* promoter unmethylated with an IDH-wildtype GBM (if aged over 70 years) or an IDH-wildtype grade II/III tumor might be dispensed from TMZ treatment (Figure 1.2).

1.3. Molecular prognostic factors of glioma

Despite the advances in the understanding of these extremely heterogeneous tumors, a question of paramount importance remains: how can the GBM treatment be optimized for the benefit of individual patients?

Since most of GBM patients are unresponsive to the current standard of care, the identification of these patients is of paramount importance in order to keep them away from deleterious, expensive and innefective therapies, and which will eventually allow to redirect them to alternative therapies for which they might present a better response.

As discussed before, in 2016, new diagnostic biomarkers were included in the WHO classification of gliomas; the next sections will briefly describe their role in glioma, as well as the roles of one class of promissing putative molecular markers (HOX genes).

1.3.1. *IDH1* and *IDH2* mutations

The identification of IDH mutations' role in glioma significantly impacted the new WHO CNS tumors classification, as discussed above. These mutations were identified 10 years ago by Parsons *et al.* in a small cohort of GBM and were associated with patients' survival differences [112]. These observations were further validated in a large cohort [113, 114] and in subsequent retrospective studies [115]. Approximately 30% of all gliomas present IDH mutations in the enzymatic active site, which are

more common in WHO grade II and III gliomas (60 – 90%) than in GBM (5 – 10%) [114, 116]. Most of them concern the *IDH1* gene (its protein is found at the cytoplasm and peroxisomes) or, less commonly (4 – 5% of the cases), the *IDH2* gene (codify to a mitochondrial enzyme), which are mutually exclusive [57, 117]. Virtually all *IDH1* mutations (>90%) occur in the codon 132 (c.395G>A; p.R132H)[7], while *IDH2* mutations occur in its analogue residue in the codon 172 (almost 60% of the times, this mutation corresponds to a c.515G>A; p.R172K[8]) [118]. These mutations occur early in the tumorigenic process, and are somatic and hemizygous preserving one copy of wildtype [119, 120]. In daily practice, IDH1 R132H mutation can be reliably detected by IHC. However, 10% of IDH mutations are missed [121, 122]. Indeed, sequencing to detect other *IDH1* and *IDH2* mutations is recommended for WHO grade II and III, and for young adult GBM patients with negative IDH1 R132H IHC [56].

In a normal physiologic condition, IDH enzymes catalyze the oxidative decarboxylation of isocitrate, producing α-ketoglutarate and CO_2, and resulting in the reduction of $NADP^+$ to NADPH (Figure 1.7) [123]. However, the mutant IDH enzyme presents an altered product affinity catalyzing the reduction of α-ketoglutarate to R(-)-2-hydroxyglutarate and using NADPH as cofactor. In this context, IDH-mutant gliomas present increased levels of R(-)-2-hydroxyglutarate and decreased levels of NADPH (Figure 1.7). While the latter predisposes cells to oxidative stress [123], R(-)-2-hydroxyglutarate accumulation contributes to tumorigenesis [124]. This potential role in the tumorigenesis might be due to its competitive inhibition of α-ketoglutarate-dependent dioxygenases activity, including histone demethylases and the ten-eleven translocation (TET) family of 5-methylcytosine hydroxylases [125]. Consequently, increased

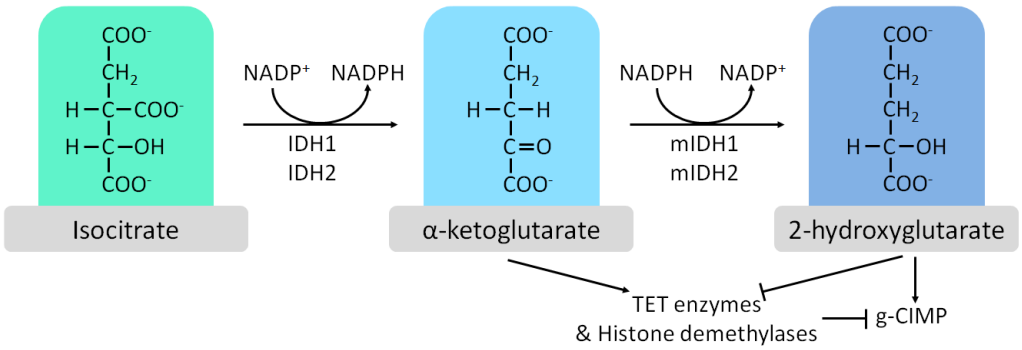

Figure 1.7. Biochemical transformation of isocitrate to 2-hydroxyglutarate and its biological impact. IDH enzymes (IDH1/2) catalyze the oxidative decarboxylation of isocitrate to α-ketoglutarate (reducing $NADP^+$ to NADPH). In contrast, IDH mutant enzymes (mIDH1/2) produce 2-hydroxyglutarate from α-ketoglutarate (oxidizing NADPH to $NADP^+$). This may lead to the inhibition of TET enzymes and histone demethylases, which in turn promote the glioma CpG island methylator phenotype (g-CIMP). Note that, α-ketoglutarate is a co-factor of TET enzymes and 2-hydroxyglutarate competes with its binding site.

[7]The substitution in *IDH1* gene of a G to a A nucleotide at position 395 is responsible for the missense R132H mutation, which corresponds to the amino acid substitution at position 132, from an arginine (R) to a histidine (H) [118].

[8]Other possible mutations are p.R132C, p.R132G, p.R132S and p.R132L [118].

histone methylation and hypermethylation of multiple CpG islands in the DNA (referred as glioma CpG island methylator phenotype – g-CIMP), which includes the *MGMT* gene promoter, is an epigenetic characteristic of IDH-mutant gliomas. Another described hypothesis is that the reduction of α-ketoglutarate in the cells may increase HIF-1α levels resulting in the activation of hypoxia-response element genes, which are described to have a role in angiogenesis, proliferation and motility [126, 127].

Although i) IDH-mutant gliomas present an improved outcome when compared to IDH-wildtype gliomas of the same histological grade; ii) no major prognostic differences were observed between IDH-mutant grade II and III gliomas [82]; and iii) IDH-wildtype grade II and III gliomas can be as aggressive as IDH-wildtype GBM and have a prognosis similar to these grade IV gliomas [128], the WHO histological grading scheme was maintained in the revision of the WHO CNS tumors classification. In addition to the prognostic significance of IDH mutations, they presented a predictive role for therapy. Indeed, IDH-mutant grade II and III gliomas have a better response to chemotherapy or the combination of chemo- and radiotherapy when compared to radiotherapy alone, than IDH-wildtype ones [86, 129]. Moreover, IDH mutant proteins might be used as therapeutic targets. Indeed, several molecules targeting them have been developed and showed potential therapeutic effects in glioma [124, 130-138].

1.3.2. 1p/19q co-deletion

1p/19q co-deletion in glioma presents not only a diagnostic value, but also a prognostic and predictive one[9] [139].

This loss of heterozygosity (LOH) on the short arm of chromosome 1 (1p) and the long arm of chromosome 19 (19q) was described, for the first time, in 1998, to be present in 90% of oligodendrogliomas and 50–70% of anaplastic oligodendrogliomas [140-142]. Now, in the new classification, 1p/19q co-deletion was considered pathognomonic of the oligodendroglial lineage, and this diagnosis can only be performed in the presence of this LOH. Tumors presenting 1p/19q co-deletion commonly harbor additional activating mutations in the promoter region of *TERT*. In contrast, 1p/19q co-deletion and mutations in *ATRX* and *TP53* genes are mutually exclusive [56].

Mechanistically, the combined loss of the 1p and 19q arm (Figure 1.8) is the result of a still poorly understood balanced translocation [*t*(1;19)(q10;p10)] with the breakpoint around the centromere [143-145]. Briefly, this results in a chromosome derivative by the fusion of the 1p and 19q arms [*t*(1;19)] at the centromere (q10;p10). Usually, translocations in cancer occur in a gene-specific breakpoint

[9]Note that only whole-arm co-deletion presents predictive value. Partial deletions on either 1p or 19q chromosome arm are associated with poor patient outcome [139].

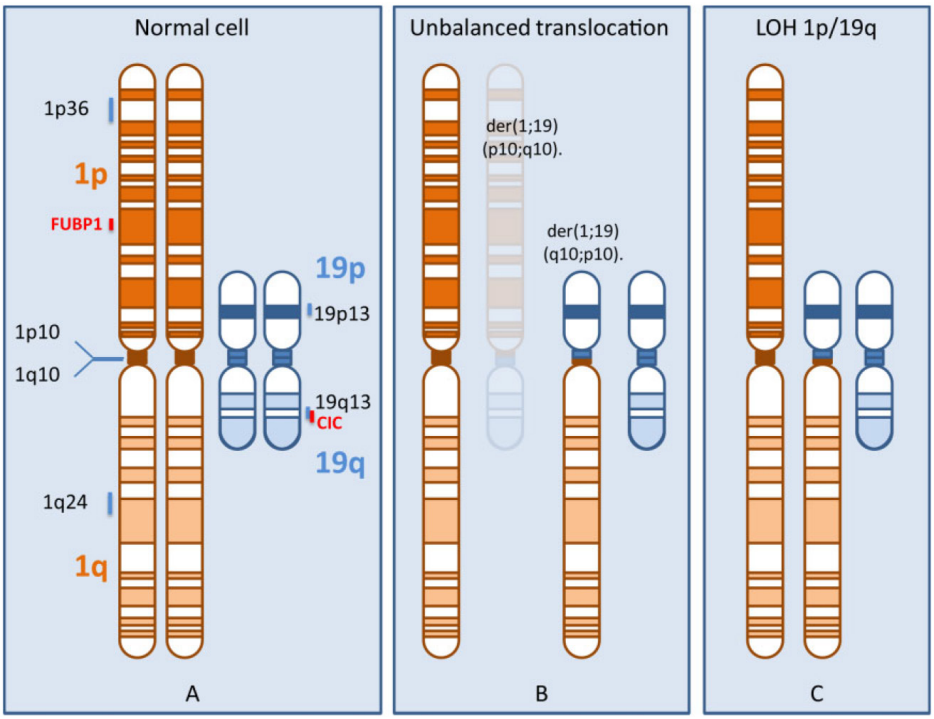

Figure 1.8. Chromosomal translocations leading to 1p/19q co-deletion.
(A) Normal cells contain 2 copies of chromosomes 1 and 19. **(B)** Unbalanced translocation leads to a transposition at the centromere level of 19q to 1p (shaded) and its subsequent loss **(C)**, and of the formation of a derivative chromosome consisting of 1q and 19p. Reproduced with permission from Brandner and Deimling (2015) [145].

producing fusion gene products. However, this breakpoint occurs in a gene-poor centromeric region and is constantly associated with co-deletion [143, 144]. A recent study performed whole exome sequencing of oligodendrogliomas and identified inactivating mutations of the *CIC* gene (homologue of the Drosophila gene capicua) present at the 19q arm and of the *FUBP1* gene (far-upstream element binding protein 1) present at the 1p arm, in 60 and 30% of the cases, respectively [146, 147]. However, these frequencies are lower than expected to justify the complete 1p/19q co-deletion and are unlikely to be the primary target of this LOH.

The prognosis of 1p/19q co-deleted glioma patients (oligodendroglial lineage) is more favorable than the ones from the astrocytic lineage. Moreover, the progression from an oligodendroglioma to GBM is unlikely to occur (Figure 1.4) [63].

The predictive value of 1p/19q co-deletion (already discussed in section 1.2.2.4) is associated with the observed benefit of patients with anaplastic oligodendroglioma to the addition of PCV chemotherapy to radiotherapy alone in two phase III trials [142, 148]. These results were further validated in multiple studies [149-151]. Although at the time of these trials less toxic regimens were preferred, these results re-defined PCV as the standard of care for tumors from the oligodendroglial lineage (Figure 1.2). However, the mechanisms underlying this favorable outcome are still poorly understood [63].

In clinical routine, 1p/19q co-deletion is most commonly assessed by fluorescent *in situ*

hybridization (FISH) and microsatellite analysis for LOH. As FISH allows to preserve the tissue architecture, it is the preferred technique by neuropathologists [57].

1.3.3. *MGMT* promoter methylation

MGMT (O-6-methylguanine-DNA methyltransferase) promoter methylation is one of the glioma biomarkers with prognostic and predictive application, but with no diagnostic value. A significant number of GBM (40–70%) display *MGMT* promoter methylation [152-155], being it also frequent in anaplastic gliomas (50–80%) [156, 157].

MGMT is a DNA repair protein, which counteracts the DNA damage induced by alkylating agents, such as TMZ[10] [158, 159]. In this sense, *MGMT* transcriptional silencing, due to the methylation of the *MGMT*-associated 5'-CpG island (at the promoter), results in the sensitivity to alkylating chemotherapeutic agents [159].

Briefly, the MGMT protein acts by removing the alkyl group from the guanine O^6 position, a target site of DNA alkylation by alkylating chemotherapies. In this process, the MGMT protein is consumed by the transference of the alkyl group to itself, which leads to its proteasomal degradation, with cells needing to be continuously restoring it. Therefore, MGMT expression levels directly correlate with the repair capacity of the cells [159].

When unrepaired, chemotherapy-induced lesions, like O^6-methylguanine, sensitize the cells to radiation and may alone trigger cell apoptosis [160]. Therefore, from its first description, *MGMT* promoter methylation rapidly became one of the most interesting and studied biomarker in neuro-oncology [159]. The interest in this biomarker considerably increased after the phase III clinical trial publication from Hegi *et al.* associating *MGMT* promoter methylation to longer overall survival in GBM patients, independently of the treatment [161]. Moreover, among patients with *MGMT* promoter methylated tumors, a survival benefit was observed for those treated with TMZ and radiotherapy when compared to those that received radiotherapy alone. These results were further validated in elderly GBM patients (> 65 years), which led to the inclusion of *MGMT* promoter methylation status as a decision-guide for therapy selection in these patients (Figure 1.2) [116, 159, 162]. However, this observed differential benefit might be restricted to IDH-wildtype gliomas, as in IDH-mutant and g-CIMP gliomas, which already present a better response to therapy, *MGMT* promoter methylation is not able to predict treatment response [163]. Since alterations

[10]TMZ mechanism of action consists in DNA alkylation/methylation, most often at the O^6 and N^7 sites on guanine and N^3 site on adenine. O^6-Methylguanine production leads to the insertion of a thymine base instead of cytosine during the cellular replication, which is recognized by mismatch repair (MMR) enzymes. The action of the MMR cause DNA single-strand breaks, which are converted to double-strand breaks and ultimately lead to the failure of DNA replication and cell death [158, 159].

in *MGMT* promoter methylation status at recurrence have not been observed and its status maintains the predictive role for TMZ response, patients with recurrent GBM and prolonged progression-free survival, whose first-line treatment consisted in radiochemotherapy, might be re-challenged with TMZ [164, 165].

MGMT methylation status might be evaluated through i) methylation-specific PCR (MSP); ii) pyrosequencing; or iii) array-based DNA methylation approaches. The first two techniques need sodium bisulfite-modified DNA but are the most commonly used ones given their simplicity and cost-efficiency [159]. Despite its clinical significance, *MGMT* promoter methylation status definition is still challenging. In fact, the thresholds for selecting *MGMT* promoter methylated tumors are difficult to define and the absence of a standardized testing method render its assessment prone to variability [159].

1.3.4. *ATRX* mutation

ATRX (α-thalassemia/mental retardation syndrome X-linked) codify to a central component of chromatin remodeling complex, important in DNA replication, telomere stability and gene transcription. This enzyme is involved with the telomerase-independent alternative lengthening of telomeres (ALT) mechanism, by which most gliomas undergo telomere repair [166, 167]. Since it is present on the X chromosome, a single inactivating mutation (at the active allele in the case of females) is sufficient for the loss of ATRX function [168, 169]. *ATRX* mutations were only recently described in pediatric and adult GBMs [170]. These mutations occur in 70–90% of IDH-mutant astrocytic gliomas (without 1p/19q co-deletion) and result in telomere lengthening and probably genetic instability. In opposition to IDH mutations, *ATRX* mutations do not have hot spot regions, may be either truncating or missense, may be subclonal, may present different type of mutations within the tumor, and may be different between the original tumor and the recurrence [168]. In most cases, *ATRX*, IDH and *TP53* mutations occur simultaneously in the tumor. In contrast, they are mutually exclusive with *TERT* promoter mutations and 1p/19q co-deletion. In IDH-mutant astrocytic gliomas, *ATRX* mutation is associated with improved progression-free and overall survival [171, 172].

Since *ATRX* mutations can be easily assessed by IHC (loss of ATRX staining – normally present in all cells) or by sequencing, its assessment might be used instead of 1p/19q co-deletion to help in the diagnosis of astrocytic vs. oligodendroglial lesions [173]. However, further studies are required to make sure that this substitution is valid. For now, the WHO 2016 CNS tumor classification explicitly does not accept ATRX IHC as an alternative to diagnose 1p/19q co-deleted oligodendrogliomas [56].

1.3.5. HOX genes

HOX genes are part of the homeobox family, which encodes transcription factors crucial in both

embryo development and post-development regulation [174-179]. In humans, there are 39 HOX genes, which are divided into four clusters (HOXA to HOXD) localized in chromosome 7, 17, 12 and 2, respectively [174]. HOX genes, which may present tumor suppressive or oncogenic functions, are frequently altered in cancer, including breast [180-182], brain [183-188], lung [189, 190], colon [191], cervix [192], bladder [193, 194], kidney [195], and leukemia [196-200]. Abdel-Fattah *et al.* [186] were the first reporting the aberrant expression of HOX genes in gliomas. In 2008, Murat *et al.* [185] suggested a HOX-dominated gene cluster as an independent predictive factor of therapy resistance in GBM. In 2010, our group described that a subset of GBM patients present an aberrant expression of HOXA genes when compared to less malignant gliomas or normal brain tissues [183]. Moreover, the PI3K pathway was identified as the HOXA genes upstream regulatory mechanism [183]. Among them, *HOXA9* emerged as the only one whose expression was associated with GBM patients' poor prognosis, independently of other well-known prognostic factors, including *MGMT* promoter methylation [183]. Indeed, in patients with *MGMT* promoter methylation (associated with better prognosis, as detailed acima), *HOXA9* was still able to identify a subset of patients with poor prognosis [183]. More recently, we validated the prognostic value of *HOXA9* in GBM and showed that functionally it leads to increased GBM cell viability, invasion, stemness and resistance to TMZ, and decreased cell death [201]. *In vivo*, we demonstrated that HOXA9 leads to mice shorter survival [201]. Interestingly, when overexpressed in non-tumorigenic human immortalized astrocytes, we showed that HOXA9 contributes to tumor initiation and causes mice death [201].

Taking this into account, HOXA9 is a promising putative molecular marker in glioma, for which further studies are warranted.

1.4. Oncogenic pathways in glioblastoma

As explained in section 1.2, advances in the (epi)genetics characterization of diffuse gliomas resulted in the 2016 WHO CNS tumor classification, an improvement over the previous one (2007 WHO classification). Notwithstanding, current therapies for diffuse gliomas are still palliative, and the patients sooner or later unfortunately succumb to the disease [111]. In this context, the better understanding of the molecular oncogenic events underlying the disease is still crucial, as it may lead to the identification of new diagnostic and/or prognostic biomarkers, or even to the identification of new targeted therapies.

In gliomas, there are three major signaling pathways frequently altered: the RB, p53 and RTK/RAS/PI3K pathways [202], which will be explored below.

1.4.1. RB pathway

The RB (retinoblastoma) pathway, a negative regulator of the cell cycle, is commonly aberrantly

inactivated in gliomas [203], specifically in 78% of GBM tumors. Indeed, alterations in several components of this pathway were detected. While *RB* mutation or deletion were observed in a small percentage of GBM, *RB* promoter methylation was observed in a larger extent and was more common in IDH-mutant GBM when compared to IDH-wildtype ones. Moreover, inactivating mutations of its upstream negative regulator (*CDKN2A* in 52%, *CDKN2B* in 47%, or *CDKN2C* in only 2% of the cases), or activating mutations in the downstream positive regulators (*CDK4* in 18%, *CDK6* in 1% and *CCND2* in 2% of the cases) are also commonly found [204, 205]. These alterations have been associated with decreased survival of patients with anaplastic astrocytoma [206]. On the other hand, p16 (codified by *CDKN2A* gene) was associated with increased survival of patients treated with chemoradiotherapy [207].

Nonetheless, the prognostic value of RB pathway alterations is considered to be borderline [208].

1.4.2. p53 pathway

The p53 pathway presents a major role in several cellular processes, such as cell cycle regulation, apoptosis, differentiation, and DNA damage response [209]. In this sense, it is not surprising that p53 pathway alterations are implicated in almost every cancer, including glioma. Indeed, p53, the "guardian of the genome", is one of the most well-established tumor suppressor protein [209]. Similar to the RB pathway, the observed alterations might be found directly in p53 (deletions), in upstream negative regulators (*MDM2* in 14%, *MDM4* in 7% and *p14* in 49% of the cases) or in downstream regulators (*ATM* and *ATR*) [205, 210-212]. Alterations in the p53 pathway directly due to p53 mutation are present in 27% of IDH-wildtype and 81% of IDH-mutant GBM tumors [56]. Moreover, they are also found in lower-grade gliomas (1p/19q non-co-deleted) and are associated with progression to higher grades [213-215]. Consequently, besides the absence of a predictive value for treatment, and despite being associated mostly with astrocytic tumors (1p/19q non-co-deleted), p53 lacks diagnostic specificity. However, due to its tumor suppressive role, p53 is marginally associated with increased patient survival [216].

1.4.3. RTK/RAS/PI3K pathway

Between the RB, p53 and RTK/RAS/PI3K pathways, the latter is considered one of the most suitable pathways for pharmacological intervention, because it is easier to inhibit activation events than to replace lost tumor-suppressive functions (as in the RB and p53 pathways) [217]. The RTK/RAS/PI3K pathway is altered in 88% of malignant gliomas [218]. RTKs are a broad family of receptor tyrosine kinases, which includes the EGFR, PDGFR, VEGFR, FGFR, among other subfamilies [219]. The RAS and PI3K pathways are downstream effectors of RTKs. PI3K alone has over 40 downstream targets [219]. Thus, there is a vast number of components of the RTK/RAS/PI3K pathway and, consequently, an

increased opportunity of alterations. In this sense, alterations in several components are typically observed, being the most frequent: *EGFR* amplifications (in 45% of GBMs), PI3K gain of function (in 15%), and *PTEN* loss (in 36%; most common in IDH-wildtype GBM) [218, 220]. Beside its prognostic value, PTEN expression may sensitize the tumors to chemoradiotherapy [221], and is predictive of the response to EGFR inhibitors [222]. Indeed, a huge number of EGFR inhibitors were created and already tested in clinical trials [220]. However, despite the promising results *in vitro,* none of them showed satisfactory results for the treatment of GBM patients, contrarily to the observed in other cancers [223-230]. Monoclonal antibodies against EGFR constitutively active mutant variant (EGFRvIII; present in 50% of *EGFR* amplified GBM) are also being explored for GBM treatment and have led to an improved survival of some patients [231-235]. Moreover, inhibitors of the PI3K pathway started to be created and are being tested in preclinical and phase II studies [236, 237]. Several inhibitors of PDGFR, an activator of the RAS/RAF/MAPK pathway, which is also altered in gliomas [238, 239], have also been introduced in clinical trials, but none of them demonstrated significant effect in the context of glioma treatment [240-247].

1.5. The WNT pathway: overview

Recent breakthroughs in the cancer stem cell field redirected our attention from the RB, p53 and RTK/RAS/PI3K pathways to the WNT, Hedgehog, and Notch pathways [248, 249]. These pathways present an important role in development and stem cell control, and, unsurprisingly, their deregulation has been widely described in cancer, including gliomas [248]. Given its relevance for this thesis, in this section, and subsections therein, a brief overview of the WNT pathway will be presented, together with a description of its role in cancer, and more specifically in glioma. An emphasis on the WNT6 ligand, the focus of this thesis, and its role in cancer will also be given.

In 1982, Nusse and Varmus described the first mammalian member of the WNT family [250], the *WNT1* gene (then named *Int-1*, to denote the first common integration site [251]), as a gene activated by integration of mouse mammary tumor virus (MMTV) proviral DNA in virally induced breast tumors [252-254]. These authors also described that Int-1 might be a secreted protein and, thus, possibly an extracellular growth factor. However, until 2003, no one was able to produce or isolate significant amounts of Int-1 protein. Still in the ‘80s, it was found that the human Int-1 protein sequence is 99% similar to the mouse homologue [255] and an Int-1 related sequence was also found in *Drosophila melanogaster* [252]. In 1988, Tsukamoto and Varmus described that *Int-1* is a *bona fide* proto-oncogene independently of the virus infection, as mice expressing *Int-1* developed mammary gland cancer at about 6 months of age

[256]. Back to the '80s, studies in *Drosophila* unveiled genes essential for embryo development [257]. One of them were the *wingless* genes, that were identified before, in 1976, as a weak mutant allele that leads to loss of wing tissue [258]. At that time, several studies were performed demonstrating that the sequence of *Int-1* gene and *Wingless* were identical [259, 260]. In this context, one of the first gene described to be involved in embryo development was also found to be activated in cancer, which raised for the first time the hypothesis that cancer may arise from cells that escaped from the normal developmental constraints on proliferation and differentiation [261].

In the '90s, the int and wingless nomenclature became confuse and inadequate, because: i) other MMTV activated genes were identified and named as *Int-2*, *Int-3*, and *Int-4*, but they were not necessarily linked to *Int-1* (e.g., *Int-2* was a member of the FGF family and *Int-3* was shown to be related to Notch signaling) [262-266]; and ii) PCR-based homology screens identified a large family of genes related to *Int-1* independently of being activated or not by the MMTV [267]. Thus, to avoid further confusion, a portmanteau of Int and Wingless was proposed: WNT (from Wingless-related integration site) [268]. In this sense, *Int-1* is now *WNT1*, *Int-2* is *FGF3*, *Int-3* is *Notch4* and *Int-4* is *WNT3a*.

At the end of the 20th century, after some of the major players from the WNT pathway were recognized (i.e. WNT ligands, APC, β-catenin, GSK3), it became clear that this signaling pathway was unusual compared to the other known signaling pathways at that time, which consisted mostly of successive protein phosphorylations [251]. Indeed, the regulation of the WNT signaling pathway (as for the Hedgehog and NF-kB signaling pathways) depends on the degradation of a key pathway component (in this case β-catenin) through ubiquitination/proteasomal degradation after its phosphorylation [251]. Later, β-catenin independent pathways were also described, dividing the WNT signaling pathway in two major groups: the β-catenin dependent pathway (also known as canonical pathway; Figure 1.9) and the β-catenin independent pathways (also known as non-canonical pathways) [251]. This introduction will focus mainly in the canonical pathway (as it is the most characterized and described in cancer), but a brief section describing the non-canonical pathways may also be found below[11].

1.5.1. Canonical pathway

In unstimulated cells, β-catenin levels are kept low by a "destruction complex" that includes two constitutively active serine-threonine kinases, GSK3α/β (Glycogen synthase kinase 3, α/β) and CK1α/δ (casein kinase 1, α/δ), and two tumor suppressors, APC (adenomatous polyposis coli) and AXIN, the last

[11]Throughout this chapter, whenever the canonical or non-canonical terms are not specified, the term "WNT pathway" refers to the canonical WNT pathway (β-catenin dependent).

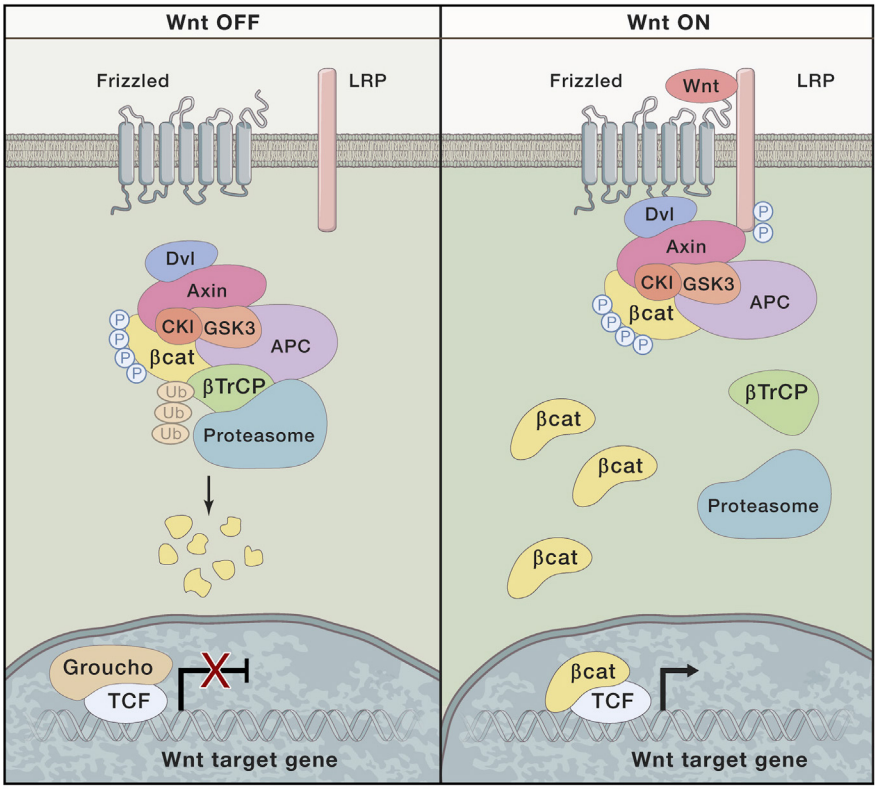

Figure 1.9. WNT signaling pathway.
WNT OFF (left): In unstimulated cells, β-catenin is degraded by the "destruction complex" that includes two constitutively active serine-threonine kinases, GSK3α/β and CK1α/δ, and two tumor suppressors, APC and AXIN. The complex creates a β-TrCP recognition site on β-catenin by phosphorylation, inducing its ubiquitination and thus its proteasomal degradation. In the nucleus, Groucho and TCF are repressing WNT target genes. **WNT ON** (right): WNT ligands bind to FZD and its co-receptor LRP5/6; the latter is phosphorylated by GSK3 and CK1γ promoting the binding of AXIN to LRP. This results in β-catenin release and accumulation in the cells, which will enter the nucleus and bind to TCF, displacing Groucho, and transcriptionally activating the WNT target genes. Adapted with permission from Nusse and Clevers (2017) [269].

acting as a scaffold (Figure 1.9) [269]. CK1 and GSK3 sequentially phosphorylates β-catenin at a series of N-terminal Ser/Thr moieties, tagging it for proteasomal degradation. CK1 phosphorylates at Ser45, while GSK3 phosphorylates at Thr41, Ser37, and Ser33 residues [270]. This phosphorylation motif is recognized by the F-box-containing protein E3 ubiquitin ligase β-TrCP inducing β-catenin ubiquitination and subsequent proteasomal degradation [271, 272]. Besides that, β-catenin plays a second major role in cells, contributing to cell-to-cell adhesion complexes containing cadherins, such as E-cadherin and α-catenin, being another way of controlling β-catenin levels [273]. However, it is thought that these distinct functions of β-catenin are most likely independent, as in some organisms, for example, *C. elegans*, they are performed by distinct homologs [274].

The canonical pathway is initiated through the interaction of a WNT ligand to the heterodimeric complex formed by one FZD receptor and its LRP5/6 co-receptor at the cell surface (Figure 1.9). FZD proteins (a family of 10 mammalian FZDs) are seven-transmembrane receptors with a large extracellular N-terminal cysteine-rich domain (CRD) where WNT ligands bind [275]. This CRD contains a hydrophobic

groove that interacts with a lipid on the WNT molecule [276], with affinities to WNT proteins in the nM range [277]. The simplicity of this interaction may explain why WNT-FZD interactions are promiscuous (i.e. one WNT ligand may bind to several FZD and vice versa) [275, 276]. Only recently the importance of the LRP5/6 co-receptor was understood. Indeed, Gong *et al.* described that LRP6 may present separate binding sites for different classes of WNT proteins [278], introducing some specificity in the WNT-FZD-LRP interaction. Upon WNT binding and FZD/LRP dimerization, GSK3 and CK1γ phosphorylate the cytoplasmic tail of LRP, promoting the binding of AXIN to LRP [279-282]. In its turn, DVL (Dishevelled) interacts with the cytoplasmic part of FZD, which facilitates the interaction between LRP and AXIN [283]. This new complex formed (including DVL, AXIN and GSK3) hampers β-catenin phosphorylation by GSK3[12], stabilizing β-catenin, which is released and accumulates in the cell [284].

Looking to the nucleus, when the WNT pathway is OFF (Figure 1.9), TCF/LEF (T cell factor/lymphoid enhancer-binding factor) is bound to Groucho and acts as a repressor of the WNT target genes [285-287]. However, when the WNT pathway turns ON, β-catenin, which is accumulated in the cytoplasm, enters the nucleus and binds to TCF/LEF transcription factors, displacing Groucho, and transcriptionally activating the same target genes that were being repressed before [288, 289]. Other key players of this transcriptional activation are BCL9, Pygopos and CBP. Several genes, such as *MYC* [290], *CTLA4* [291], *CCND1* [292, 293], *JUN* [294], *TERT* [295], *GJA1* [296], and *TBXT* [297, 298], were found to have TCF/LEF binding sites in their promoters, likely being WNT target genes at least in some particular cell types. Some of these target genes have been shown to play an important role in cancer [299-307].

1.5.2. Non-canonical pathways

Non-canonical WNT signaling pathways (also known as β-catenin-independent pathways) are continuously being re-described as new receptors and functions are identified. From these, the best characterized are the planar cell polarity and WNT-Ca^{2+} pathways [308-310].

The activation of the planar cell polarity non-canonical pathway through the binding of WNT ligands to FZD receptors leads to the activation of RHO and RAC (small GTPases), and JUN-N-terminal kinase (JNK) inducing asymmetrical cytoskeletal rearrangements and polarized cell migration. The planar cell polarity and β-catenin dependent pathway are antagonists (i.e., the inhibition of one of them will lead to the overactivation of the other) [311, 312]. However, similar to the canonical pathway (described acima), the planar cell polarity pathway also presents a role in cancer; in this case, by mediating cell

[12]Note that GSK3 also participate in other pathways, including the mTOR/AKT pathway, which may explain the high interaction between the WNT pathway and these other pathways.

motility events [313, 314].

The second best characterized non-canonical pathway is the WNT-Ca^{2+} pathway [315]. This pathway is activated by the binding of WNT ligands to FZD receptors and results in G-protein-dependent calcium release from intracellular stores. Briefly, heterotrimeric G proteins activate PLC (phospholipase C), which in turns activates the production of diacylglycerol and Ins(1,4,5)P_3 (inositol-1,4,5-trisphosphate). Ins(1,4,5)P_3 leads to the intracellular calcium release, which activates both protein kinase C (PKC) and calmodulin-dependent protein kinase 2 (CAMKII). At the end, this leads to the activation of the transcriptional regulator NFAT (nuclear factor associated with T cells). This pathway was described to have a role in inflammation, neurodegeneration and cancer [315].

A less well understood mechanism involves the binding of WNT ligands to ROR and RYK (members of the tyrosine kinase family), which activates the JNK and SRC kinases, respectively. WNT ligands interactions with these receptors often lead to β-catenin independent actions (non-canonical WNT pathway) [316].

1.5.3. WNT proteins and post-translational modifications

In mammals, like humans and mice, there are at least 19 WNT genes divided in 12 subfamilies. Most of these subfamilies are conserved throughout the animal kingdom; for example, 11 of them occur in Cnidaria (a sea anemone) and even sponges present a few WNT genes in their genome [317]. Interestingly, WNT genes are not present in the genome of single-cell organisms, suggesting the WNT pathway may present an important role in the evolutionary origin of multicellular animals [318].

WNT proteins are approximately 40 kDa in size and their sequence consist of 350 to 400 amino acids, with a signal sequence for secretion at the N-terminal and 22 conserved cysteine residues [319]. The latter is characteristic of all WNT ligands and may lead to the globular secondary structure of these proteins. Despite the crucial importance of the WNT proteins in some normal and disease contexts, their biochemical properties, and thus their mode of action, have been poorly characterized and our knowlegde about these proteins is still undeveloped. For several years, researchers tried to purify WNT ligands but without success. At that time, two main questions emerged: “why WNT proteins were so difficult to purify?” and “where they insoluble or highly adherent?”. WNT3a was the first successfully purified WNT ligand and revealed that they are lipid modified [320], explaining their insolubility. However, more studies are needed to better understand how, where and when the post-translational modifications found in WNT proteins occur. Nowadays, there are two recognized modifications common to all WNTs: glycosylation and acylation. However, they may vary between WNT ligands; for example, WNT1 presents four glycosylations, while WNT3a carries two N-linked glycosylations [321]. Glycosylations are tought to be

important for WNT ligand protein folding and secretion. In its turn, acylation was described to be critical for WNT activity and secretion [322]. At least one acylation may occur and consists in a mono-unsaturated fatty acid (palmitoleic acid) attached to the conserved serine [320, 323-325]. Porcupine, a palmitoyl transferase, was described to be the potential critical effector of this transfer of a lipid to the WNT conserved serine [326]. The lipid is primarily used by the WNT protein as a binding motif to the FZD receptors, but it is also crucial for WNT secretion as it confers hydrophobicity to this protein and may tether it to the cell membrane. Considering their hydrophobicity, how WNT proteins travel from the secretory cell to the target cell remains uncertain. Some authors observed that WNT proteins may be incorporated into secretory vesicles or exosomes [327-330] or may travel through direct contact between the cells [331, 332]. These findings have been reviewed in Takada *et al.* [333].

For convenience, we are constantly trying to associate each WNT ligand to the canonical or non-canonical pathway, and, indeed, some of them will preferentially activate one of these pathways; for example, it has been described that WNT1, WNT3a and WNT8 are commonly associated with the canonical pathway, while WNT5a and WNT11 are generally linked with the non-canonical pathway [334]. However, WNT ligands cannot be rigorously subdivided according to the pathway that they activate, since it depends on the cellular and molecular context [334]. Indeed, despite the constant association of WNT5a and WNT11 with the non-canonical pathway, they have also been described to activate the β-catenin-dependent pathway in certain contexts [335-337]. For the receptors, some of them are well established; for example, FZD can activate both WNT pathways, while LRP5/6 are found in the canonical one and ROR1/2 in the non-canonical one. Indeed, more than the sequence and structural differences in the WNT proteins, the canonical or non-canonical role of these ligands is more likely to be conferred by the repertoire of expressed receptors and signal transducers [322].

1.5.4. WNT6 ligand

The mouse *WNT6* (located at chromosome 1) was identified for the first time by Gavin *et al.* in 1990 [267]. Although previously identified and partially cloned by Rankin *et al.* in 1999 [338], only in 2001, Kirikoshi *et al.* completely cloned the human *WNT6* genomic sequence (located at chromosome 2) [339]. Throughout the animal kingdom, *WNT6* has been frequently clustered together with *WNT10a*. In human, specifically, the *WNT6* gene may be found in the 2q35 chromosomal region, only 7 kb apart from *WNT10a* [339].

Similar to other WNT ligands, the WNT6 protein is 39.7 kDa in weight and its sequence consists of 365 amino acids, with a signal sequence for secretion at the N-terminal and an RGD motif [339]. Regarding post-translational modifications, the WNT6 sequence presents two N-linked asparagine

glycosylations (at positions 86 and 311) and one predicted serine acylation (at the position 228) [339]. Similar to other WNT proteins, the WNT6 protein sequence is conserved between species; for example, the human protein is 98% identical to the mouse ant rat homologue [340]. From all WNT proteins, in humans, WNT1 (47.4% amino-acid identity) and WNT4 (43% amino-acid identity) are the most homologous to WNT6 [339].

1.5.5. Emerging roles of the WNT pathway in cancer

Although the first mammalian member of the WNT pathway (*Int-1*, now *WNT1*) was described in the context of cancer [250], only around 1990, with the identification of *APC* mutations as a cause for hereditary forms of intestinal cancer, in both mouse and human, the WNT pathway was recognized to have an important role in cancer [341-343]. However, given the importance of the WNT pathway in embryogenesis and in adult stem cells [344], it is not so surprising that this pathway has been implicated in tumor initiation, growth, invasion, metastasis, dormancy, immunity, response to therapy and cancer stem cell maintenance [345]. Aberrant activation of the WNT pathway, induced by mutations, overexpression of either ligands, receptors or β-catenin, or repression of natural inhibitory molecules, have been described in several cancers, including glioma, melanoma, colon, liver, skin, breast, and bone marrow [346, 347]. APC and β-catenin mutations are the most frequently observed in cancer. For example, APC somatic mutations were found in 85% of all sporadic colorectal cancers. On the other hand, 5 to 10% of these cancer present mutations in β-catenin that removed or changed the phosphorylation sites that target it for degradation [348-350]. Note that, WNT aberrant activation in cancer is not only restricted to cancer cells, but also implicate cells from the microenvironment and immune system [346].

Although the role of the WNT canonical pathway in cancer is well recognized, whether the non-canonical pathways present a role or not is still under discussion. However, as explained acima, some reports described that WNT non-canonical pathway might be activated in cancer cells to help in their migration and metastization, through PKC and other effectors of the WNT/Ca^{2+} pathway [351, 352].

The following subsection focus on the molecular alterations of the WNT signaling players found in glioma, and their functional and clinical implications.

1.5.5.1. WNT pathway in glioma

Although β-catenin is frequently described to be accumulated in the nucleus of glioma cells, these tumors rarely present mutations in APC or β-catenin itself, suggesting that other mechanisms are more likely to be responsible for WNT aberrant activation in gliomas [347]. Nonetheless, relatively few alterations in other components, such as ligands, receptors, and natural inhibitors of the pathway, have been reported [347]. These alterations are detailed below.

Out of the 19 WNT ligands, five (WNT1, WNT2, WNT3a, WNT5a and WNT11) have been reported to present an important role in gliomas [353-364]. WNT1, WNT2, WNT3a and WNT5a were shown to be expressed in a graded manner in gliomas, and, as WNT11, they were associated with increased proliferation, invasion/migration and stemness capacity of GBM cells [353-359, 361, 363, 364]. *In vivo*, WNT2, WNT3a and WNT5a were associated with higher tumor aggressiveness [353, 356, 359, 362, 363]. Interestingly, WNT5a, which seems to act in a non-canonical manner, was also associated with increased resistance to TMZ-based chemotherapies, with the presence of microglial cells in the tumor and with a proinflammatory signature [357, 359]. Mechanistically, it was shown that WNT5a depends of RYK for the induction of its invasive function [355]. Mir-30a and mir-129-5p were further described as upstream direct-regulators of WNT5a in glioma [359, 362]. WNT11 in its turn was reported to be induced by hypoxia and directly regulated by HIF1α [364]. However, only WNT5a showed to be prognostically valuable, correlating with the poor prognosis of GBM patients [359].

Although in glioma no mutations were found in β-catenin, its expression was described to increase in a graded manner [365-368]. Moreover, β-catenin silencing in glioblastoma was associated with decreased proliferation, invasion, and increased apoptosis [365, 368, 369]. *In vivo*, β-catenin silencing delayed the tumor growth in a subcutaneous model and prolonged the mice' survival in an orthotopic model [369]. As expected, β-catenin silencing resulted in the decreased expression of WNT target genes [370]. Clinically, β-catenin expression was associated with the poor prognosis of GBM patients [368, 371, 372].

Naturally occurring WNT inhibitors, such as SFRP1, SFRP2, SFRP4, SFRP5, DKK1, DKK3, NKD1, NKD2 and PEG3/Pw1, are frequently epigenetically silenced in glioma through DNA hypermethylation of their promoters [373-386]. Curiously, promoter hypermethylation of SFRP and PEG3/Pw1 are more frequently found in primary GBMs, while promoter hypermethylation of DKKs is most frequent in secondary GBMs.

However, as nothing is known about WNT6 in glioma, the focus of this thesis, in the next section a summary of the relevance of WNT6 in cancer can be found.

1.5.6. WNT6 in cancer

The importance of WNT6 in tissue development and cellular differentiation has been well recognized [340, 387-409]. Notwithstanding, little is known regarding WNT6 roles in cancer. In 2011, the first report relating WNT6 and cancer described that *WNT6* polymorphism was associated with increased risk of colorectal adenoma [410]. However, only in 2013, Yuan *et al.* described for the first

time the functional impact of WNT6 expression in cancer, specifically, in gastric cancer [411]. They described that, in gastric cancer cells, WNT6 was regulated by CAV1 and associated with increased resistance to anthracycline chemotherapeutics. In patients, WNT6 expression was inversely correlated with the response to Epirubicin, Cisplatin and 5-Fluorouracil, and positively associated with tumor stage and nodal status [411]. One year later, WNT6 was also associated with cisplatin resistance in bladder cancer cell lines, upon *UCA1* regulation [412]. Later, WNT6 expression was shown to be predictive of poor prognosis and recurrence in esophageal cancer [413]. Moreover, *WNT6* DNA methylation was associated with better prognosis of children with osteosarcoma and was inversely correlated with *WNT6* expression [414]. In 2018, WNT6 and WNT10a were associated with post-transplant smooth muscle tumor growth through the activation of the non-canonical WNT pathway [415]. Finally, WNT6 expression in colon cancer was functionally associated with increased proliferation, cell cycle, and migration, and with decreased cell apoptosis [416].

Regarding its canonical or non-canonical functions, little is known in cancer; however, WNT6 regulation of the epithelium formation, adhesion, cell-cell communication and development of several tissues like heart muscle has been associated with the canonical pathway [340, 391-393, 396, 402, 406-409, 417], while WNT6 signals through the non-canonical pathway in the neural crest induction, in the skeletal muscle, dental pulp and macrophage differentiation, and in post-transplant smooth muscle tumor growth [390, 403-405, 415].

1.5.7. The WNT pathway as a therapeutic target

Given the important role of the WNT pathway in cancer, there has been a growing interest in identifying and creating compounds for its inhibition for cancer treatment [418]. The recently described role of this pathway in the regulation of immune cell infiltration into the tumor reinforced its interest, as its modulation might be useful in the context of immunotherapies [419]. Several companies (e.g., Novartis, Bayer, OncoMed, Prism Biolab, Enzo Life Sciences, Tocris Bioscience, EMD Millipore, Abmole Bioscience) have been investing in the creation of distinct compounds (e.g., small inhibitory molecules, monoclonal antibodies, peptides, fusion proteins) with the aim of specifically targeting some of the major players of the WNT pathway (e.g., WNT ligands, FZD receptors, porcupine, β-catenin, TCF/CBP) [418]. Some of these compounds are being tested in preclinical studies, and a great number are already in clinical trials (Phase I/II) in the context of several solid tumors (e.g., melanoma, colorectal, breast, pancreatic, ovarian, and hepatocellular cancers), lymphoid malignancies or diseases unrelated with cancer, where aberrant WNT pathway is well-established [418]. Figure 1.10 illustrates some of these compounds, and their targets and mechanism of action within the WNT pathway [345]. Although none of

them have yet been approved for clinical use, the data obtained from phase I/II clinical trials is promising and these drugs may soon become an option for cancer treatment [418]. Since the WNT pathway has been associated with chemoresistance and cancer stem cells (associated with therapy resistance and tumor recurrence), most of the trials are combining WNT inhibition with conventional therapies, and synergistic effects are being observed [418]. Moreover, given the recent association of the WNT pathway with immune evasion, there may be a rationale to include WNT inhibitors with immunotherapies to improve the clinical benefit of these therapies, which has been limited to a small subset of patients [420].

In the era of personalized medicine, biomarkers for predicting the subset of patients which will most probably benefit form a given therapy are being investigated. However, there is still no clinically relevant molecule or signature that may predict for WNT pathway aberrant activation.

For further details about WNT inhibitory compounds, the reader is kindly referred to the reviews in Zhan *et al.* [345], and Krishnamurthy and Kurzrock [418], or directly at *www.clinicaltrials.gov*.

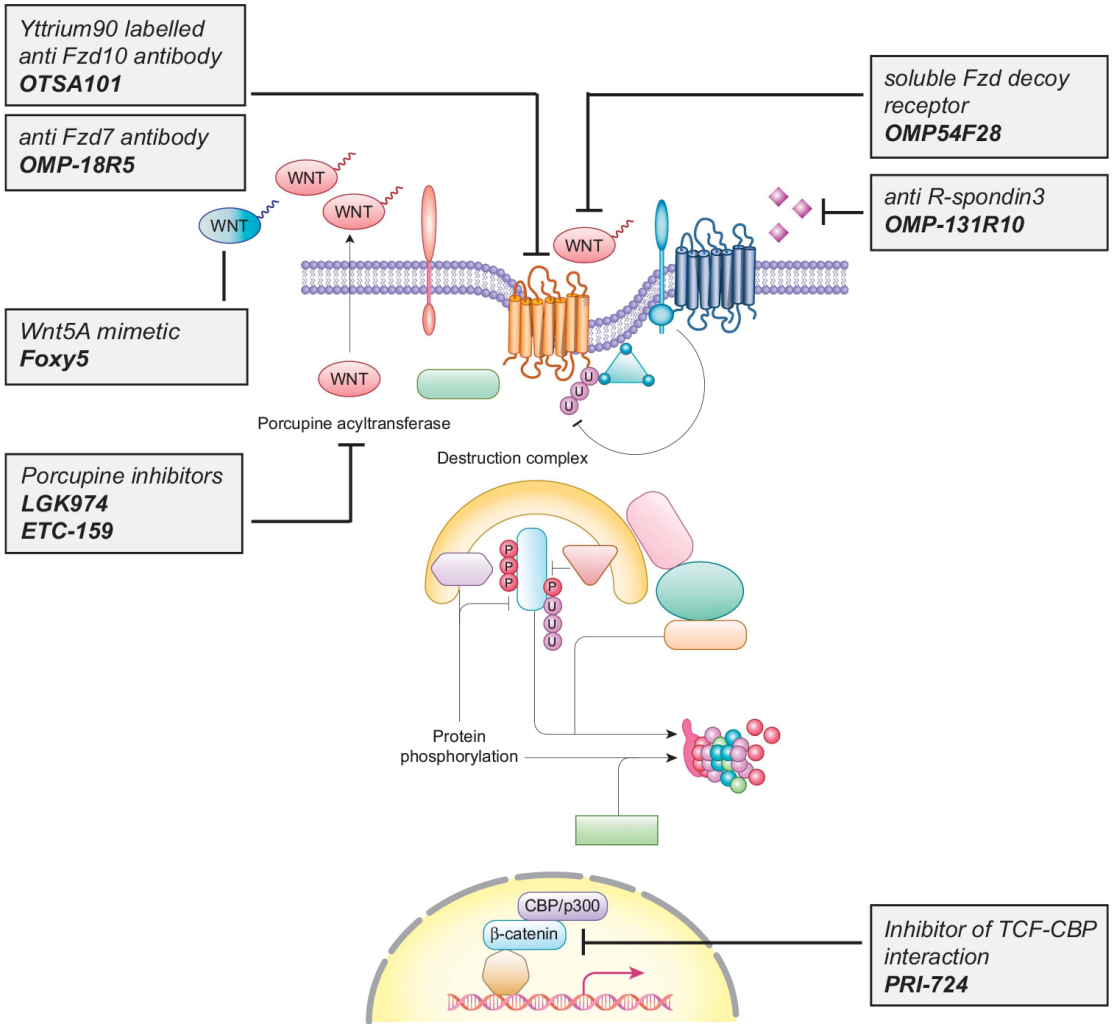

Figure 1.10. Compound under investigation for the inhibition of the WNT pathway.
Schematic representation of the WNT (canonical) pathway and some of the WNT inhibitors in phase I/II clinical trials for the treatment of several types of cancer. Reproduced with permission from Zhan *et al.* (2017) [345].

Overall, data implicating the WNT pathway in cancer aggressiveness, combined with the sparse investigation on the relevance of WNT6 in cancer, which just started to be uncovered, are compelling arguments to further investigate its role in the pathophysiology of glioma, where nothing is known. This may stimulate the identification of new targets and the creation of new directed therapies for this deadly disease, which may also be applicable to other cancer types where WNT6 oncogenic role was already defined (section 1.5.6 for more details).

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Chapter 2

WNT6 is a novel oncogenic prognostic biomarker in human glioblastoma

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Contents

WNT6 is a novel oncogenic prognostic biomarker in human glioblastoma

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Abstract

Glioblastoma (GBM) is a universally fatal brain cancer, for which novel therapies targeting specific underlying oncogenic events are urgently needed. While the WNT pathway has been shown to be frequently activated in GBM, constituting a potential therapeutic target, the relevance of WNT6, an activator of this pathway, remains unknown.

Methods: WNT6 protein and mRNA levels were evaluated in GBM. WNT6 levels were silenced or overexpressed in GBM cells to assess functional effects *in vitro* and *in vivo*. Phospho-kinase arrays and TCF/LEF reporter assays were used to identify WNT6-signaling pathways, and significant associations with stem cell features and cancer-related pathways were validated in patients. Survival analyses were performed with Cox regression and Log-rank tests. Meta-analyses were used to calculate the estimated pooled effect.

Results: We show that WNT6 is significantly overexpressed in GBMs, as compared to lower-grade gliomas and normal brain, at mRNA and protein levels. Functionally, WNT6 increases typical oncogenic activities in GBM cells, including viability, proliferation, glioma stem cell capacity, invasion, migration, and resistance to temozolomide chemotherapy. Concordantly, in *in vivo* orthotopic GBM mice models, using both overexpressing and silencing models, WNT6 expression was associated with shorter overall survival, and increased features of tumor aggressiveness. Mechanistically, WNT6 contributes to activate typical oncogenic pathways, including Src and STAT, which intertwined with the WNT pathway may be critical effectors of WNT6-associated aggressiveness in GBM. Clinically, we establish WNT6 as an independent prognostic biomarker of shorter survival in GBM patients from several independent cohorts.

Conclusion: Our findings establish WNT6 as a novel oncogene in GBM, opening opportunities to develop more rational therapies to treat this highly aggressive tumor.

2.1. Introduction

Glioblastoma (GBM) is the most lethal tumor of the central nervous system in adults. Despite recent advances in the understanding of the oncogenic molecular mechanisms, and improvements in neuroimaging technologies, surgery and adjuvant treatments, patients with GBM still exhibit a rapid unfavorable clinical evolution. The median survival of patients is 15 months after diagnosis, a scenario that has not changed significantly in the last decades [1-4]. In combination with their resistance to most conventional therapies, including radiotherapy and temozolomide (TMZ), GBMs are highly infiltrative and diffuse, impeding complete surgical resections. In this context, understanding the key underlying molecular mechanisms contributing to the aggressiveness of this tumor may be a critical step in the design of new and more effective precision therapies.

The WNT signaling pathway displays pleiotropic physiological functions, ranging from neurogenesis to stem cell proliferation, and has been implicated in various human cancers, including glioma [5-8]. This pathway is particularly relevant in the context of cancer stem cells (CSC), which have been pointed as critical mediators of cancer recurrence and resistance to radio- and chemotherapy [7, 9-11]. Therefore, novel therapeutic strategies targeting particular components of the WNT pathway have been widely explored [9, 10, 12]. The WNT6 ligand is an activator of the WNT pathway, which only recently was associated with chemoresistance in gastric and bladder cancers [13, 14], poor prognosis of esophageal squamous cell carcinoma patients [15], and increased risk to develop colorectal adenoma [16]. Importantly, no studies to date have explored the relevance of WNT6 in human glioma.

In this work, we show WNT6 is overexpressed in GBM, the most common and malignant glioma, and displays typical hallmark oncogenic functions in both *in vitro* and *in vivo* GBM models by affecting the activity of classic oncogenic signaling pathways, including WNT, SFK and STAT pathways. Critically, we provide data from several independent GBM patient cohorts establishing WNT6 as a prognostic biomarker associated with shorter overall survival.

2.2. Results

2.2.1. WNT6 is overexpressed in primary GBM tissues

While high WNT6 expression levels were previously observed in different human cancer cell lines [13, 17, 18], little is known about its specific roles in tumors, particularly in GBM. In order to address this, we first analyzed gene expression array data from normal brains, lower-grade gliomas (LGG, WHO grades II and III) and GBM (WHO grade IV) patients deposited in TCGA [19]. When compared to normal samples, *WNT6* was not overexpressed in any of the LGG patients (0/27), while 15.6% of GBM patients

(89/572) presented high *WNT6* levels (Figure 2.1A; $p = 0.026$). Concordantly, testing the protein levels of WNT6 by immunohistochemistry (IHC) in another dataset of glioma tissues (from Hospital Santo António; HSA) showed that only GBMs present high expression of WNT6 protein (16.3%; Figure 2.1B bottom; $p = 0.037$). WNT6 immunoreactivity expression in glioma showed to be mainly cytoplasmic

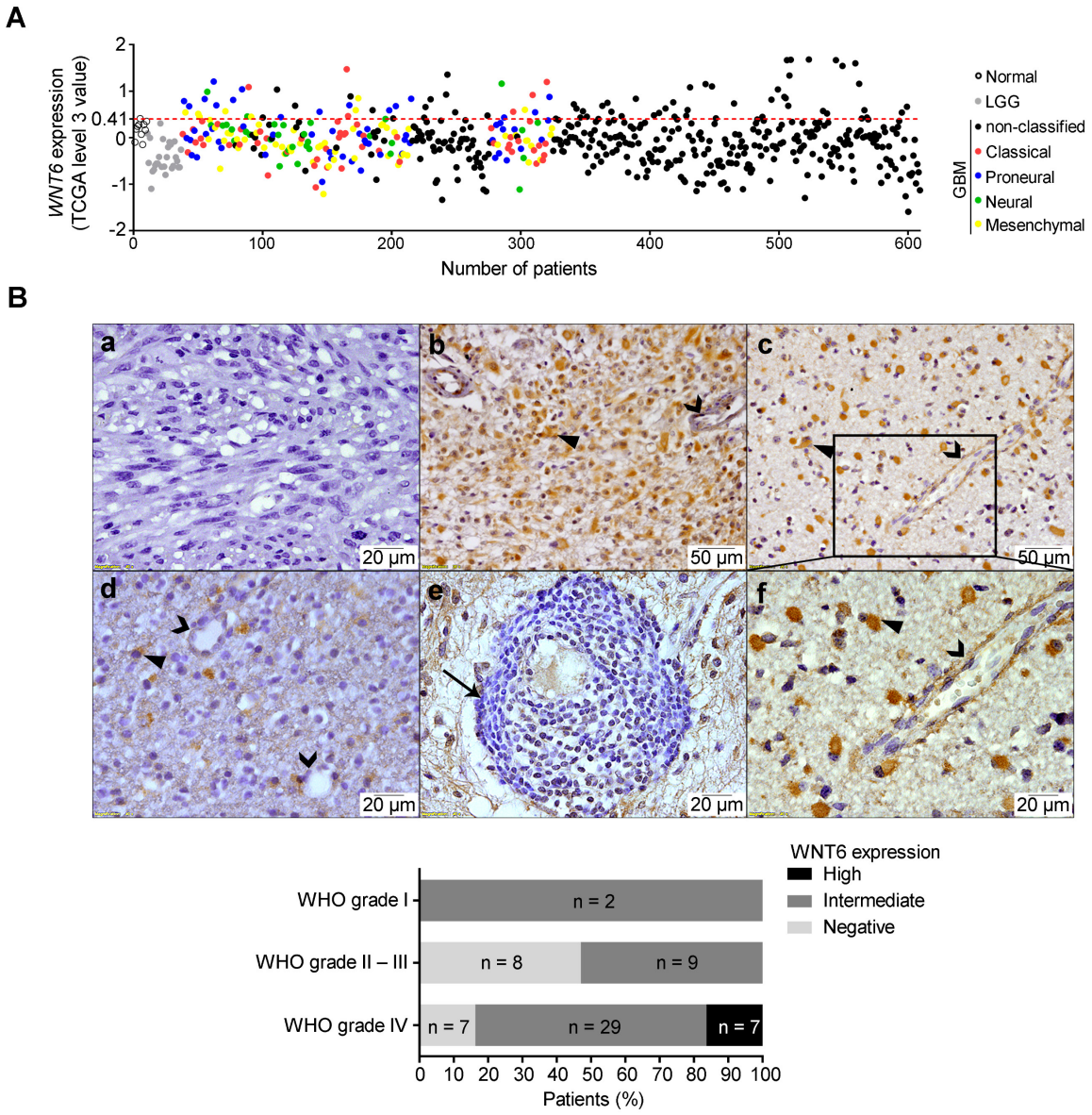

Figure 2.1. WNT6 is overexpressed at the mRNA and protein levels in GBM.
(A) Expression levels of *WNT6* in 27 lower-grade gliomas (LGG; grey dots), 572 glioblastomas (GBM, WHO grade IV; black and colored dots) and 10 unmatched normal brains (black unfilled dots) from TCGA. GBM molecular subtypes are represented as colors (red = classical; blue = proneural; green = neural; yellow = mesenchymal). *WNT6* is overexpressed (TCGA data level 3 values ≥ 0.41; above red dashed line) in 16% (n = 89) of GBM samples. **(B)** WNT6 protein expression in WHO grades I-IV glioma samples from Hospital Santo António cohort assessed by IHC (n = 63; $p = 0.037$; chi-squared test). Representative images are shown for a WNT6-negative GBM (a), high WNT6 expression in GBM (b) and WHO grade III anaplastic oligoastrocytoma (c), and a WHO grade II diffuse oligodendroglioma with intermediate levels of WNT6 expression (d). WNT6 staining was mostly cytoplasmic in glioma cells (closed arrowheads) and not present in lymphocytes (e; arrow), being almost exclusively negative for endothelial cells (b-d and f; open arrowheads). Bottom graph summarizes IHC data for the whole dataset. LGG: lower-grade glioma; TCGA: The Cancer Genome Atlas; WHO: World Health Organization.

(closed arrowheads), with a diffuse pattern where almost all tumor cells are positive (Figure 2.1B, b and c), or a more scattered pattern (Figure 2.1B, d). Tumor-infiltrating lymphocytes were negative for WNT6 expression (arrow; Figure 2.1B, e), with endothelial cells being negative or showing some faint immunoreactivity (open arrowheads; Figure 2.1B, b, c, d, and f). Representative images of positive and negative controls, and hematoxylin and eosin stainings are displayed in Figure 2.S1. Together, these results show WNT6 mRNA and protein levels associate with high glioma grade, suggesting it may be important in the pathophysiology of glioma.

2.2.2. High *WNT6* expression is indiscriminately present in all molecular subtypes of GBM

Several efforts have been made to stratify GBM into molecular subgroups [20-27]. We evaluated the levels of *WNT6* expression among the GBM subtypes described by Verhaak *et al* (classical, mesenchymal, neural and proneural) [28] in a total of 4 independent cohorts, totaling 201 patients from TCGA, 59 from Freije, 159 from Gravendeel and 26 from Vital datasets. High levels of WNT6 were detected with no significant differences in subsets of patients of each GBM molecular subtype in all datasets (Figure 2.1A and Figure 2.S2), suggesting WNT6 activation in GBM is independent of these molecular signatures.

2.2.3. WNT6 has oncogenic functions and promotes GBM aggressiveness *in vitro*

To address whether WNT6 expression influences typical cancer hallmark features of GBM cells, WNT6 expression was silenced in U373MG and SNB19 cell lines by shRNA, resulting in significantly decreased mRNA and protein levels (Figure 2.2A-B), as well as overexpressed in U87MG cells, which do not express *WNT6* endogenously (Figure 2.S3A). WNT6-low cells presented a significantly lower viability when compared with the corresponding WNT6-high counterparts (Figure 2.2C-D and Figure 2.S3B-C). Concomitantly, WNT6 silencing significantly decreased proliferation of GBM cells (Figure 2.2E). Considering GBM cells display remarkable migration and invasion potentials, we tested how WNT6 affects these hallmark features of GBM. Wound healing migration assays and matrigel invasion assays showed that WNT6 significantly increases the capacity of GBM cells to migrate and invade (Figure 2.2F-K and Figure 2.S3D-E). Together, these data suggest WNT6 is an important factor regulating several cancer hallmarks, which might influence GBM aggressiveness.

2.2.4. WNT6 increases resistance to temozolomide chemotherapy in GBM cells

Previous reports have described the relevance of the WNT pathway in increased resistance of many cancers to radio- and chemotherapy [7-9, 15]. In this context, we tested whether WNT6 expression

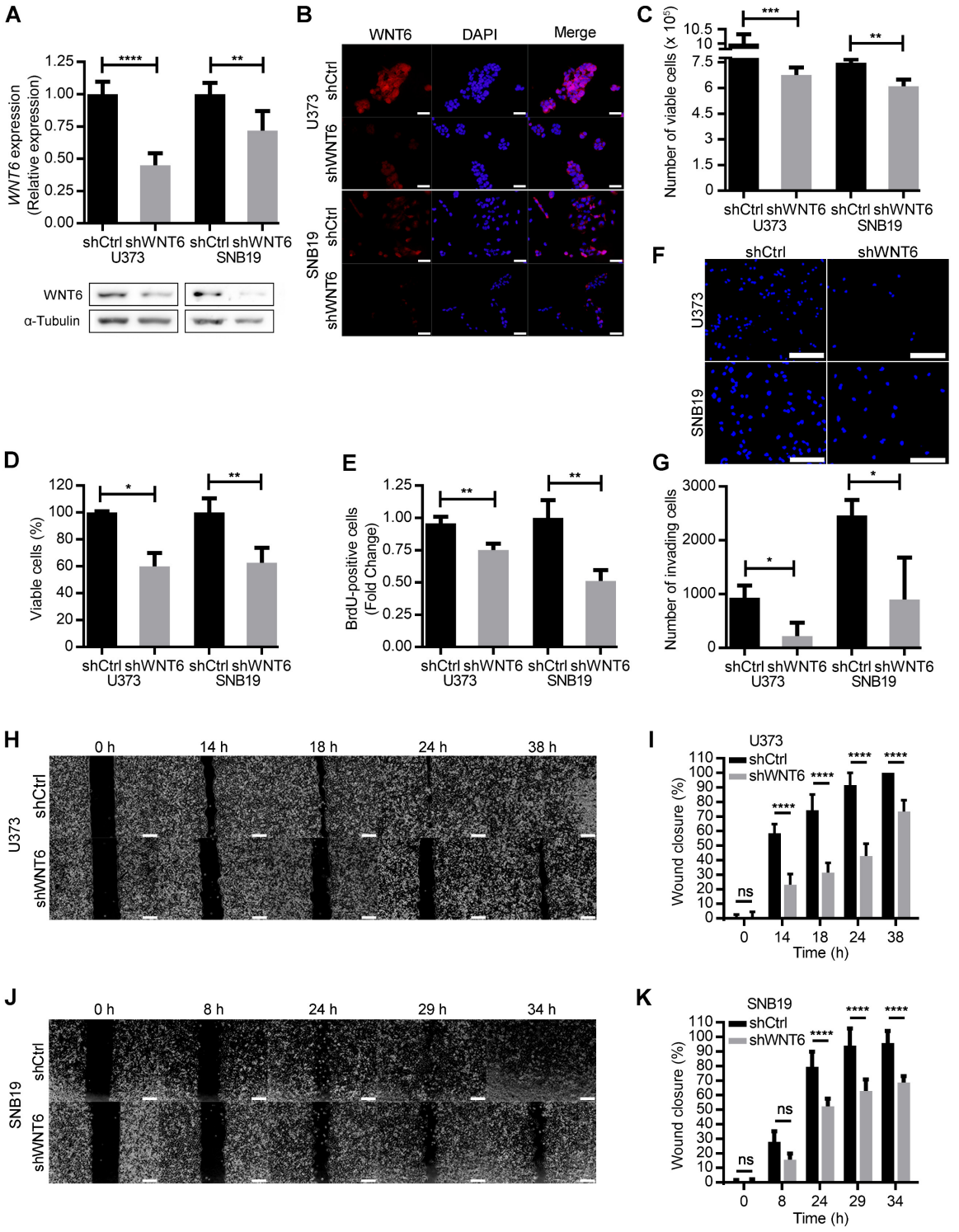

Figure 2.2. WNT6 promotes GBM aggressiveness *In vitro*.

(A-B) The efficiency of WNT6 silencing in U373 and SNB19 glioblastoma cells was analyzed by qRT-PCR and WB (A), and immunofluorescence (B). (A) Top: *WNT6* expression levels were normalized to *TBP*. Bottom: WB images are representative of 3 independent assays; α-tubulin was used as reference protein. (B) DAPI was used to stain the nucleus (40x magnification; scale bar = 50 μm). **(C-D)** Cell viability was measured by trypan blue (C) and MTT (D) assays in shCtrl and shWNT6 cells. **(E)** Cell proliferation was evaluated by BrdU incorporation. **(F-G)** Matrigel invasion assays were used to assess the cells' invasion capacity. Representative images (F) with cell nuclei stained with DAPI (scale bar = 250 μm) and quantification (G) of invasive U373 and SNB19 shCtrl and shWNT6 cells. **(H-K)** Migration (wound healing) assays were

(continued on next page)

(cont.) performed in U373 (H-I) and SNB19 (J-K) shCtrl and shWNT6 cells to assess the cells' migration capacity. Representative micrographs throughout time for U373 (H) and SNB19 (J) cells (40x magnification; scale bar = 250 µm). The wound gap was measured and expressed as a percentage of wound closure for U373 (I) and SNB19 (K) cells (**** p <0.001; two-way ANOVA post-hoc Sidak's test). Results represent data from at least 3 independent experiments (mean ± SD). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$ and ****, $p < 0.001$ (unless otherwise stated, a two-sided unpaired *t*-test with Welch's correction was applied when homoscedasticity was not verified).

may have an impact in the sensitivity of GBM cells to clinically relevant therapies, including TMZ chemotherapy and radiation. Interestingly, WNT6-silenced GBM cells were significantly more sensitive to TMZ-mediated cell death than their respective control cells (Figure 2.3A-B). In contrast, WNT6 did not affect sensitivity of GBM cells to radiation treatments (Figure 2.S4). Overall, these results suggest WNT6 promotes resistance of GBM cells to TMZ, with potential clinical implications as TMZ is still the standard-of-care for these patients.

2.2.5. WNT6 promotes glioblastoma stem cell features

Due to the critical roles of GBM stem cells (GSC) in the pathophysiology and clinical outcome of

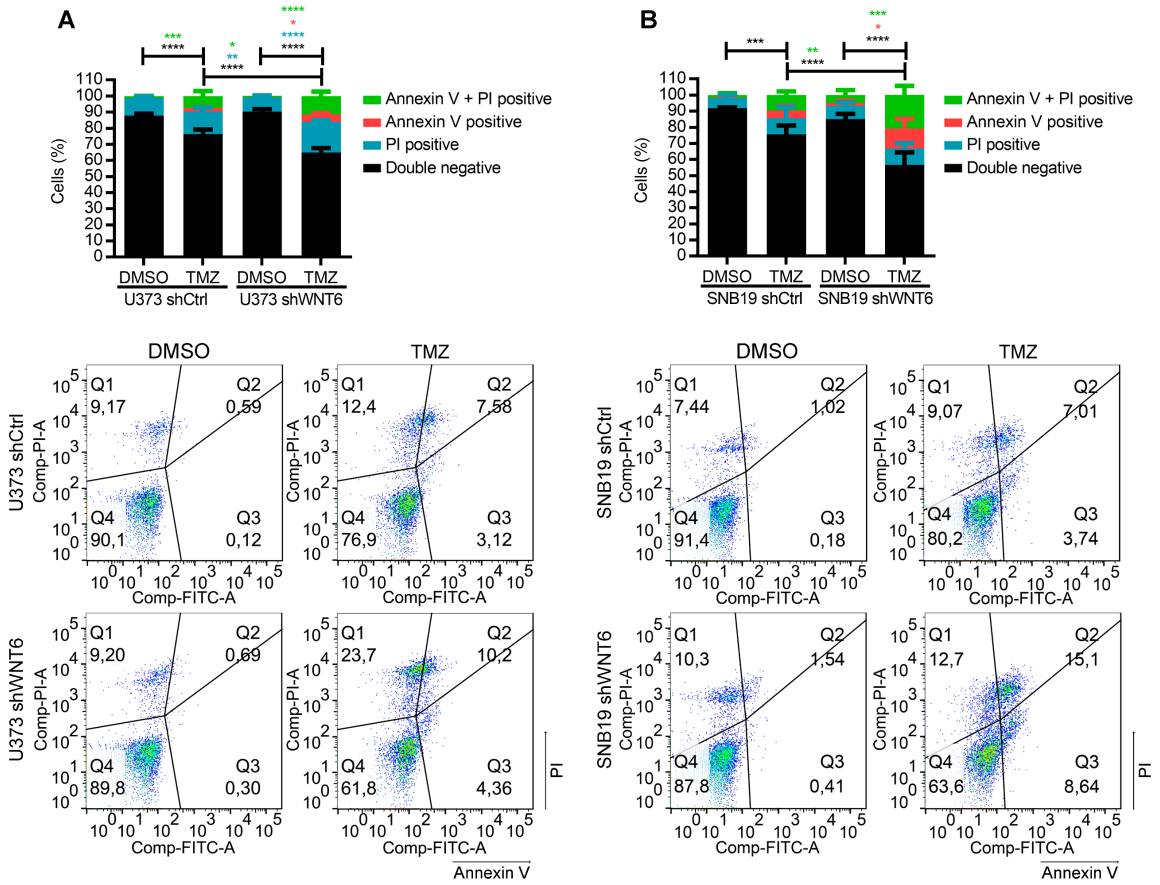

Figure 2.3. WNT6 promotes resistance to temozolomide chemotherapy in GBM cells.
(A-B) U373 (A) and SNB19 (B) shCtrl- and shWNT6-transfected cells were treated with vehicle or TMZ for 6 days, and cell death was measured by Annexin-V and PI. The graphs present data of 3 independent assays (mean ± SD). Representative dot plots are displayed below. *, p <0.05; **, p <0.01; ***, $p < 0.001$ and ****, $p < 0.0001$ (two-way ANOVA post-hoc Tukey's test). DMSO: dimethyl sulfoxide; PI: propidium iodide; TMZ: temozolomide.

GBM patients, we investigated how WNT6 may affect GBM cells' stemness. Interestingly, WNT6-silenced U373 and SNB19 cells showed prominent decreased levels of NESTIN and SOX2 proteins, two common markers of GSCs (Figure 2.4A). In addition, WNT6 expression resulted in significantly higher capacities of U373, SNB19 and U87 cells to form neurospheres when cultured under GSC conditions (Figure 2.4B-C and Figure 2.S3F-G), functionally suggesting WNT6 contributes to the stem cell phenotype of GBM cells. Concordantly, WNT6 expression resulted in significantly increased neurosphere-forming frequency in a limiting dilution sphere-forming assay (1/3.81 for shCtrl vs. 1/16.11 for shWNT6; $p = 0.01$, likelihood ratio test, Figure 2.4D for U373 cells; SNB19 cells with WNT6-silencing did not form spheres in the tested conditions – data not shown; 1/62.3 for U87-Ctrl vs. 1/24.6 for U87-WNT6; $p = 0.004$, likelihood ratio test, Figure 2.S3H), an assay that allows the quantification of cells with stem-like properties that are capable of forming a new neurosphere derived from a single cell.

In addition to sphere-based stem cell assays, we then tested U373 and SNB19 cells cultured under specific adherent conditions that allow GSC expansion (neurosphere medium supplemented with B27, EGF and FGF in laminin-coated wells) [29]. GBM cells acquired a more elongated phenotype in these adherent stem cell conditions (Figure 2.4E), as previously described [29, 30]. Concordantly with our findings in "non-stem cell" conditions (Figure 2.2C-D), WNT6 silencing significantly impaired cell viability of U373 and SNB19 GBM cells grown in adherent stem cell-like conditions (Figure 2.4F). Globally, these data suggest WNT6 has an important functional role in the formation and maintenance of GSCs.

2.2.6. *WNT6* is associated with stem cell-related genes in GBM clinical samples

To further translate our results to the clinical setting, we evaluated correlations between the expression levels of *WNT6* and typical stem cell-related genes in GBM patients from TCGA. Interestingly, heatmap analyses revealed common patterns of gene expression between various stem cell-related genes and *WNT6* (Figure 2.4G), and individual correlation graphs showed statistically significant positive correlations between the expression levels of *WNT6* and these genes ($p < 0.0001$; Figure 2.4H and Figure 2.S5). These data from GBM patients fit well with our *in vitro* findings, further suggesting WNT6 contributes to the stem cell phenotype of GBM cells.

2.2.7. High WNT6 levels accelerate GBM-associated death in intracranial mice models

To expand our findings to *in vivo* models, U373-shCtrl and U373-shWNT6 (Figure 2.5), or U87-Ctrl and U87-WNT6 cells (Figure 2.S3I-L) were orthotopically injected in the brain of NSG mice. Importantly, all mice bearing WNT6-low tumors presented significantly delayed evidences of GBM-related neurological symptoms and slower body weight loss than mice bearing WNT6-high tumors (Figure 2.5A

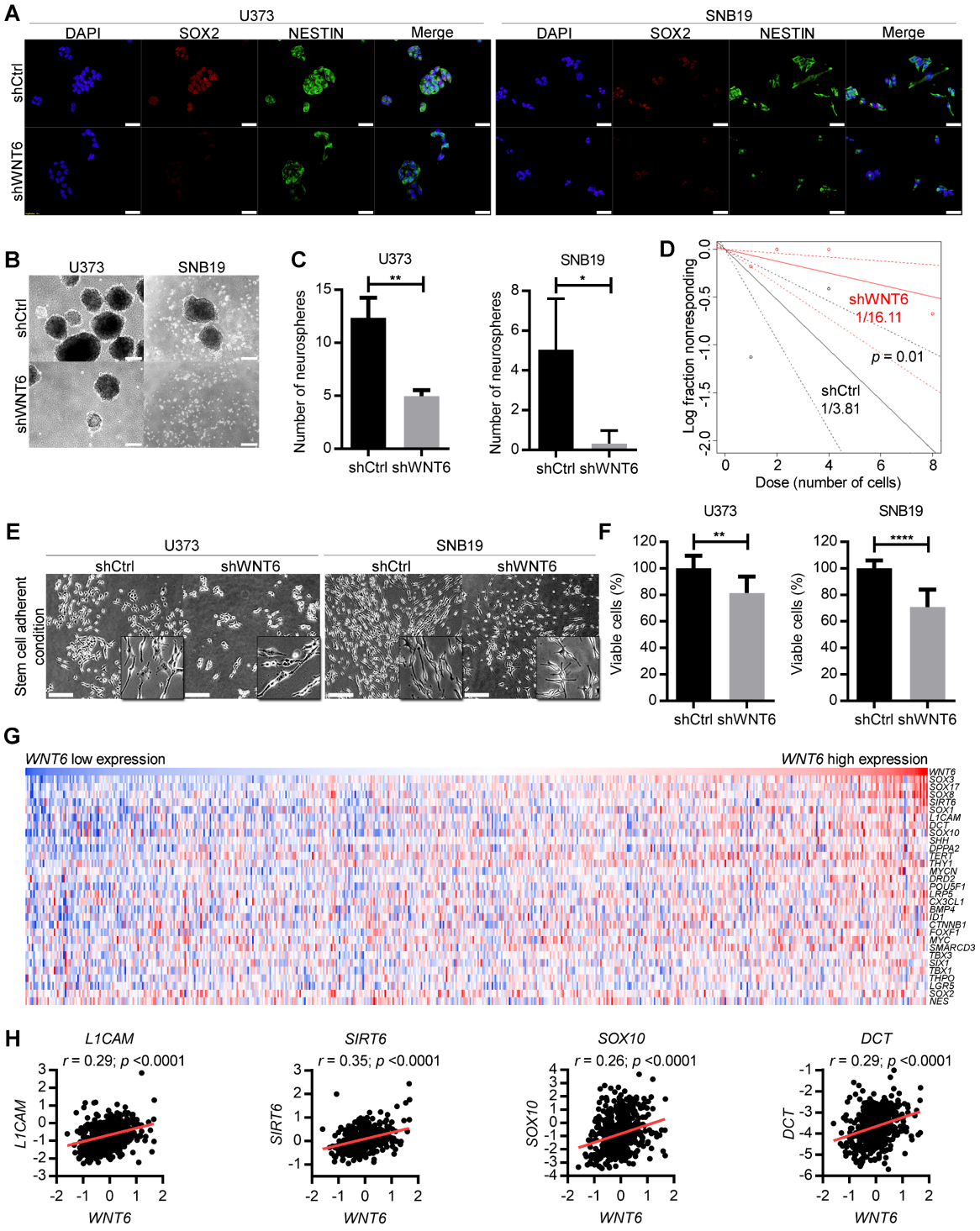

Figure 2.4. WNT6 silencing decreases GBM stem cell features.

(A) SOX2 and NESTIN double-immunostaining in U373 (left) and SNB19 (right) shCtrl/shWNT6 cells. DAPI was used to stain cell nuclei (40x magnification; scale bar = 50 µm). **(B)** Representative images of U373 (left) and SNB19 (right) shCtrl/shWNT6 neurospheres at days 10 and 15 after plating, respectively (40x magnification; scale bar = 250 µm). **(C)** Quantification of neurospheres formation (n = 3 independent assays; mean ± SD; *, $p < 0.05$; **, $p < 0.01$, two-sided unpaired *t*test with Welch's correction being applied when homoscedasticity was not verified). **(D)** Limiting dilution assays in U373-shCtrl (black) and -shWNT6 (red) cells to assess their sphere-forming capacity. The graph is representative of 3 independent assays with similar results. The trend lines represent the estimated active cell frequency ($p = 0.01$, likelihood ratio test). **(E)** Representative images of U373 (left) and SNB19 (right) shCtrl/shWNT6 cells cultured under stem cell-adherent conditions (scale bar = 250 µm). **(F)** MTT assay evaluating U373 (left) and SNB19 (right) shCtrl/shWNT6 cell

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(cont.) viability when cultured under stem cell-adherent conditions (n = 3 independent assays; mean ± SD; **, $p < 0.01$; **** $p < 0.0001$; two-sided unpaired *t*-test with Welch's correction applied when homoscedasticity was not verified). **(G)** Heatmap representation of expression levels of *WNT6* and 30 stem cell-related genes in 573 GBM patients' samples from TCGA. Each column corresponds to a patient and each line to a gene. Expression values increase from darker blue (low levels) to darker red (high levels). **(H)** Correlation graphs between *WNT6* expression (x-axis) and the expression of stem cell genes selected based on the heatmap (y-axis; Pearson's correlation test; *r* and *p* values are indicated).

and Figure 2.S3I). This was accompanied by a significantly longer overall survival of U373-shWNT6 and U87-Ctrl mice as compared to mice injected with U373-shCtrl and U87-WNT6 cells, respectively (Figure 2.5B and Figure 2.S3J), implicating high levels of WNT6 as a negative prognostic biomarker in orthotopic GBM *in vivo* models. Post-mortem hematoxylin & eosin (H&E) analyses confirmed GBM formation in all animals (Figure 2.5C and Figure 2.S3K), with a very invasive phenotype typical of GBM. IHC confirmed long-term WNT6 silencing and overexpression in U373-shWNT6 and U87-WNT6 tumors, respectively (Figure 2.5D and Figure 2.S3L). Histologically, shWNT6 GBM tumors also presented: i) lower proliferative activity, as observed by decreased Ki-67 and Cyclin D1 immuno-stainings (Figure 2.5E); ii) diminished expression of the stem cell marker SOX2, despite no alteration in the expression of NESTIN (Figure 2.5F); and iii) decreased expression of BCL2 protein, a critical anti-apoptotic molecule (Figure 2.5G). Together, these data support WNT6 as a critical mediator of GBM aggressiveness *in vivo*, identifying some molecular mediators that might sustain this aggressive phenotype, in line with what was observed in the *in vitro* assays.

2.2.8. WNT6 acts through β-catenin-dependent WNT signaling and downstream targets of SFKs/STAT pathways

To explore the underlying molecular mechanisms by which WNT6 influences GBM aggressiveness, the activation status of a large variety of cancer-related kinases was evaluated in U373 and SNB19 cell lines using a human phospho-kinase antibody array (Figure 2.6A-B). The phosphorylation levels of 4 proteins belonging to the non-receptor tyrosine Src family kinases [SFK; namely p-Fgr (Y412), p-Yes (Y426), p-Fyn (Y420) and p-Src (Y419)], and p-HSP27 (S78/S82), a member of the heat shock protein (HSP) family, were significantly decreased upon WNT6 silencing in U373 and SNB19 GBM cells (Figure 2.6A-B). In addition, the total levels of β-catenin were also decreased upon WNT6 silencing in both cell lines (Figure 2.6A-B).

Of note, other signaling pathways were concordantly affected upon WNT6 silencing in both cell lines, but at the level of different proteins. For example, the PI3K/AKT/mTOR pathway activation was decreased in U373 cells by directly affecting the phosphorylation of activating residues of AKT and mTOR, two of the major mediators of this pathway. While these specific proteins were not significantly altered in

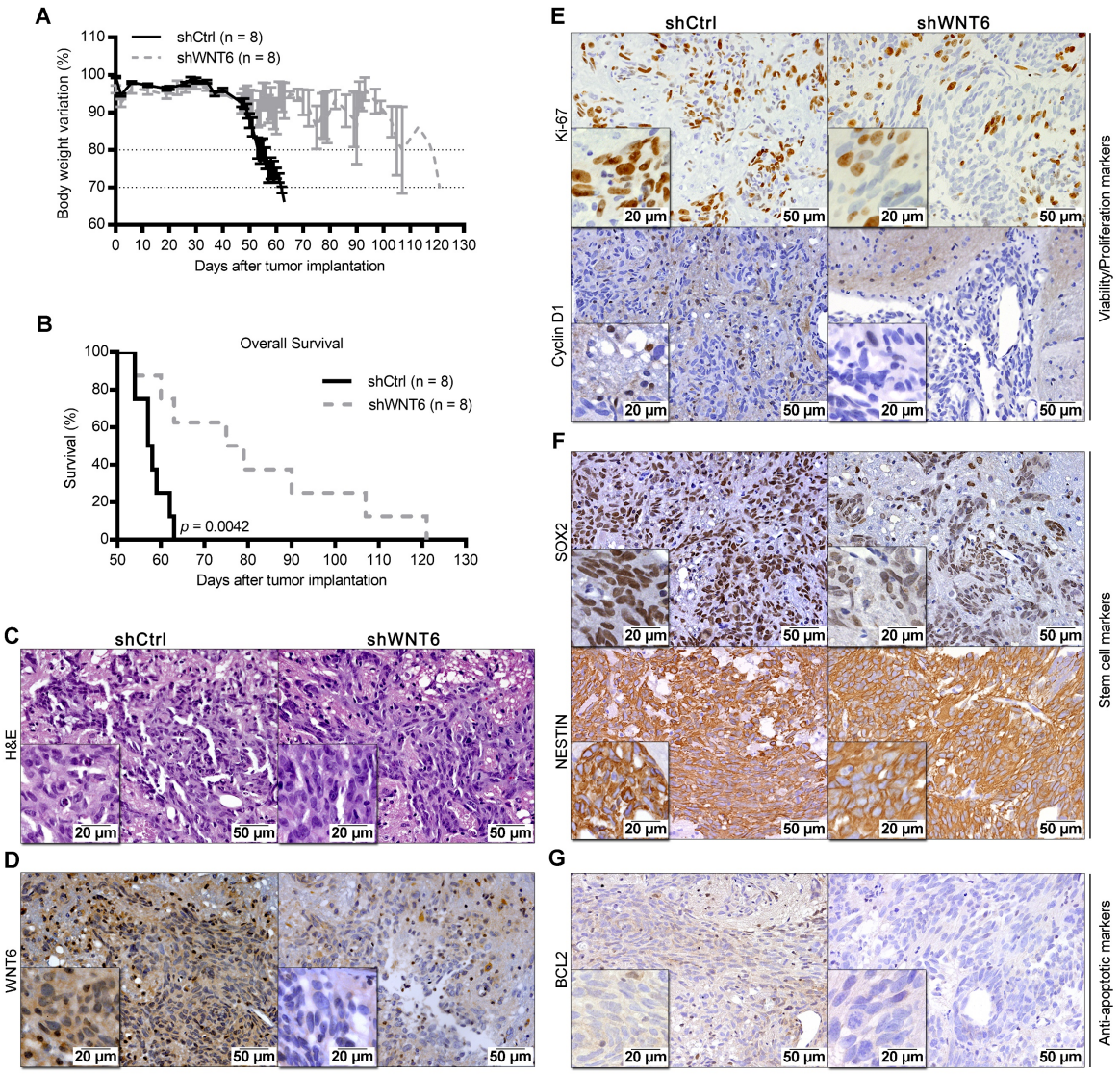

Figure 2.5. WNT6 accelerates GBM-associated death in an intracranial mouse model.
U373 transfected cells were orthotopically injected in the brain of NSG mice (n = 8 per group). **(A)** Weight curves after tumor implantation. **(B)** Kaplan-Meier overall survival curves of mice ($p = 0.0042$; Log-rank test). **(C-G)** Post-mortem brain histological and molecular analyses. (C-D) H&E and anti-WNT6 IHC staining were used as controls for GBM formation and successful long-term WNT6 silencing, respectively. (E-G) IHC for cell proliferation/viability markers Ki-67 and Cyclin D1 (E), stem cell markers SOX2 and NESTIN (F), and anti-apoptotic marker BCL2 (G). Scale bars' values are shown in each microphotograph (20 µm or 50 µm). H&E: hematoxylin and eosin.

SNB19 cells, other downstream targets of this pathway, such as p70 S6K and eNOS, showed decreased phosphorylation levels, globally resulting in similar decreased signaling of the PI3K pathway. It is also interesting to note that STAT3 phosphorylation levels were altered in WNT6-silenced U373 and SNB19 cells, but at different residues: serine 727 (S727) phosphorylation was increased in U373 cells, while tyrosine 705 (Y705) phosphorylation was decreased in SNB19 cells (Figure 6A and B). Despite the contrasting phosphorylation levels of these STAT3 residues, the biological meaning is the same, as S727 phosphorylation inactivates, while Y705 phosphorylation activates STAT3 activity [31].

In addition to these signaling pathways concordantly affected in both cell lines, cell-line specific

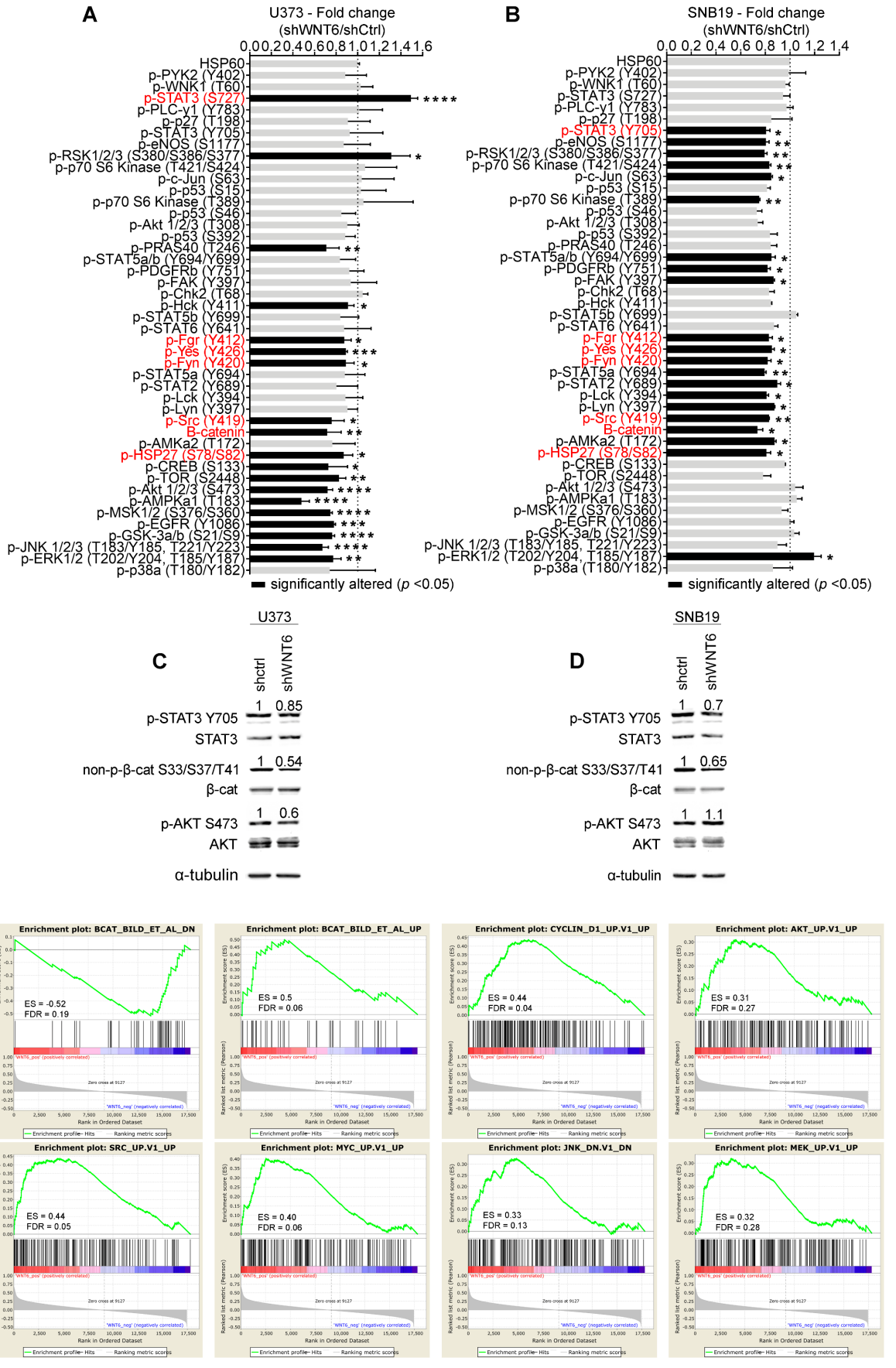

Figure 2.6. WNT6 influences the activation status of WNT, SFK, and STAT pathways in GBM.

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(cont.) Phospho-kinase arrays were performed to evaluate the phosphorylation status of cancer-related kinases in U373 **(A)** and SNB19 **(B).** Each array includes 2 technical replicates; 2 and 1 biological replicates were used for U373 and SNB19, respectively. Fold changes between phosphorylation levels of proteins in shWNT6 vs. shCtrl cells are shown (mean ± SD). HSP60 was used as reference protein. Similar alterations in the phosphorylation of particular proteins for U373 and SNB19 cells are highlighted in red. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$; ****, $p < 0.0001$; two-sided unpaired *t*-test. **(C-D)** The phosphorylation status of STAT3, β-catenin, and AKT, and their respective total forms were assessed in U373 (C) and SNB19 (D) shCtrl and shWNT6 cells by WB (images are representative of 3 independent assays; α-tubulin was used as reference protein for normalization; relative expression levels are shown above each band). **(E)** GSEA analyses identifying signatures of genes significantly associated (ES > 0.3, and FDR < 0.3) with *WNT6* in GBM patients from TCGA. ES: enrichment score; FDR: false discovery rate.

effects were also observed, such as alterations in the phosphorylation of JNK, GSK3a/b, and EGFR in U373 cells, and of STAT2, STAT5a, STAT5a/b, PDGFRb, and c-Jun in SNB19 cells. Again, despite these examples of cell-specific effects, the silencing of WNT6 consistently resulted in a decreased activation status of these cancer-related pathways in GBM.

To further validate the results obtained in the phospho-kinase arrays, the phosphorylation status of 3 proteins was assessed by western blot (WB) and normalized to α-tubulin (reference protein; Figure 2.6C-D), as performed in the arrays. Interestingly, WBs show a slight decrease of p-STAT3 Y705 in U373 cells, and a more prominent decrease in SNB19 cells, as evidenced in the phospho-arrays. Similarly, a significant decrease of non-p-β-cat S33/S37/T41 levels upon WNT6 silencing was observed in both cell lines, while only U373 presented decreased p-AKT S473 phosphorylation levels, further validating the phospho-arrays data.

To confirm that WNT6 activates the canonical WNT/β-catenin signaling pathway, TCF/LEF reporter assays were performed in both U373 shCtrl/shWNT6 cells and in U87 Ctrl/WNT6 cells. Concordantly to the aforementioned results, GBM cells with high levels of WNT6 presented a significantly increased activation of the WNT canonical pathway as compared to their negative counterparts (Figure 2.S6A-D).

Together, these results suggest that WNT6 acts not only through β-catenin-dependent WNT signaling, but also through the activation of downstream targets of SFKs/STAT pathways, highlighting WNT6 as a critical molecule involved in a variety of crucial signaling pathways in GBM, which associate with the observed oncogenic functional effects *in vitro* and *in vivo*, and reveal putative novel targets for therapeutic interventions in WNT6-high GBMs.

2.2.9. *WNT6*-correlated genes are enriched for WNT, AKT, SRC, MYC, JNK and MAPK signaling pathways in GBM patients

To expand the WNT6-associated molecular mechanisms detected *in vitro* (Figure 2.6A-D) to clinical samples of GBM, gene set enrichment analysis (GSEA) were performed to identify transcriptomic

signatures reminiscent of *WNT6*-associated genes in patients (Figure 2.6E). The gene expression profiles of GBM patients from TCGA (n = 573) were gathered, and *WNT6* expression used as a continuous label (*i.e.*, all genes were ranked based on their correlation with *WNT6*). *WNT6*-positively correlated genes revealed to be significantly enriched for genes up-regulated upon activation of WNT, AKT, SRC, MYC, JNK and MAPK signaling (all enrichment scores, ES > 0.3, and false discovery rates, FDR < 0.3; Figure 2.6E). Concordantly, *WNT6*-negatively correlated genes were enriched for genes down-regulated upon WNT signaling activation (ES > 0.3 and FDR < 0.3; Figure 2.6E top left). Overall, these transcriptomic results derived from GBM samples fit well with our *in vitro* phosphorylation data, where the same pathways were shown to be altered upon WNT6 silencing, further supporting the critical links between WNT6 and key oncogenic signaling pathways associated with glioma aggressiveness.

2.2.10. High levels of *WNT6* expression associate with shorter overall survival in GBM patients

Since our data demonstrated critical roles of WNT6 in GBM aggressiveness *in vitro* and *in vivo*, and its association with GBM's key signaling pathways, we investigated the clinical significance of *WNT6* expression in a variety of GBM patient datasets. In our Portuguese dataset (Hospital Braga and Hospital Santa Maria, n = 51), high levels of *WNT6* mRNA were significantly associated with shorter overall survival (OS) of GBM patients ($p = 0.032$; log-rank test; Figure 2.7A). Importantly, this association between high *WNT6* mRNA levels and poor prognosis in GBM patients was further validated in 3 additional independent datasets: Freije (n = 59) [24], Gravendeel (n = 159) [32] and Vital (n = 26) [33] datasets ($p = 0.006$, $p = 0.029$, $p = 0.02$, respectively; Figure 2.7B-D). These findings were then validated in 407 GBM patients with available survival data from TCGA (Table 2.1), in which a multivariable Cox model showed a statistically significant association between higher values of *WNT6* expression and shorter OS of GBM patients ($p = 0.030$), independently of other prognostic variables, including patient age ($p < 0.0001$), Karnofsky performance status (KPS; $p = 0.001$), gender ($p = 0.037$) and treatment with chemo- or radiotherapy ($p < 0.0001$; Table 2.1). Moreover, high levels of WNT6 protein expression, evaluated by IHC in a GBM Brazilian cohort (n = 87), also showed an evident trend for shorter OS ($p = 0.069$; Figure 2.S7), highlighting that both mRNA and protein levels of WNT6 may be used as a clinically valuable biomarker. To reinforce this association, a meta-analysis including all independent datasets showed that WNT6-high expression is globally associated with shorter survival of GBM patients (Hazard Ratio = 1.637; 95% confidence interval: 1.285 – 2.086; $p < 0.0001$; Figure 2.7E). Together, these results suggest that *WNT6* expression may be a new prognostic factor useful for the stratification of GBM patients.

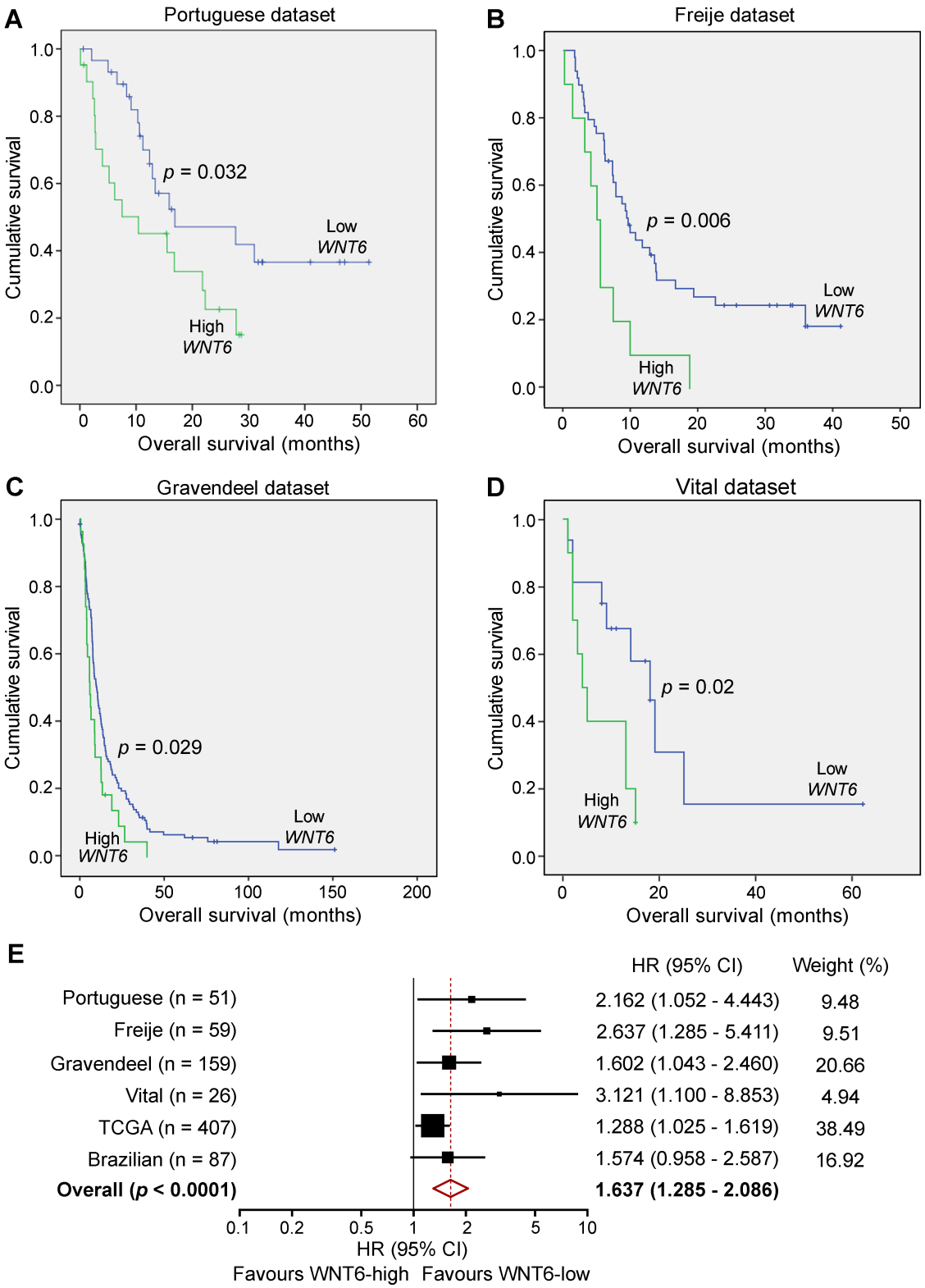

Figure 2.7. *WNT6* expression is prognostically valuable in GBM patients.
Kaplan-Meier survival curves of WNT6-low and WNT6-high GBM patients derived from qRT-PCR data **(A)** or microarray **(B-E)** data. (A) Portuguese dataset, n = 51, median OS 16.9 vs. 10.4 months (low vs. high *WNT6* expression, respectively; $p = 0.032$, Log-rank test). (B) Freije dataset, n = 59, median OS 9.6 vs. 5 months ($p = 0.006$, Log-rank test). (C) Gravendeel dataset, n = 159, median OS 10.1 vs. 6 months ($p = 0.029$, Log-rank test). (D) Vital dataset, n = 26, median OS 18 vs. 4 months ($p = 0.02$, Log-rank test). (E) Forest plot of hazard ratios (HR), illustrating the associations between WNT6 expression and overall survival in GBM patients. Each cohort is accompanied by its HR (black square; the size of the

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(cont.) square represents the weight of the study in the meta-analysis) and 95% confidence intervals (extending lines). The diamond represents the estimated pooled effect (HR = 1.637; 95% confidence interval: 1.285 – 2.086; $p < 0.0001$). CI: confidence interval; HR: hazard ratio.

Table 2.1. High levels of *WNT6* expression are significantly associated with shorter survival of GBM patients (n = 407; microarray data)

	Overall survival	
	***p*-value**	**Exp(B)**
***WNT6* expression**	0.03	1.288
Age	<0.0001	1.029
KPS	0.001	0.984
Gender[a,b]	0.037	0.771
Chemo- or Radiotherapy[a,c]	<0.001	0.192

[a]Gender and Chemo- or Radiotherapy was used as categorical variables;
[b]Female (n=154) vs. Male (n=253);
[c]Treatment (n=383) vs. No treatment (n=24).

2.3. Discussion

Deregulated signaling of the WNT pathway has been reported in several human cancers, including GBM, and has been associated with treatment resistance [13, 34-36]. This is partly due to stem cell features sustained by WNT signaling, such as increased proliferation and migration [34]. The present study is the first dissecting the functional roles and underlying molecular mechanisms mediated by WNT6 activation in human GBM, establishing it as a clinically relevant prognostic biomarker (Figure 2.7, Figure 2.8, Table 2.1, and Figure 2.S7). Due to its critical functions during embryogenesis [37-42], deregulated expression of WNT6 in adults may favor tumorigenesis [13-16]. Our data show that the expression of WNT6 in gliomas is grade-dependent, at the gene and protein levels (Figure 2.1), similarly to what was previously described for other WNT ligands [5, 6, 43-46], thus associating WNT6 with increased malignancy of gliomas. In addition, our findings demonstrate prominent effects of WNT6 on GBM cell viability, proliferation, glioma stem cell capacity, invasion, migration and sensitivity to TMZ-based chemotherapy, comprehensively implicating WNT6 as an important oncogenic factor in glioma (Figure 2.2, Figure 2.3, Figure 2.4, and Figure 2.S3). This is in agreement with the known effects of other WNT ligands in GBM [5, 6, 44, 46, 47]. According to the definition of CSC [48], we showed that WNT6 expression not only correlates with the expression of cancer stem cell-associated genes, but also functionally impacts the self-renewal capacity of GSC (Figure 2.4 and Figure 2.S3F-H). Concordant with the functional effects, molecular data also showed WNT6 associates with typical GSC markers, both in GBM cells and patient samples.

Considering the global effects of WNT6 in GSC phenotypes (Figure 2.4 and Figure 2.S3F-H), cell

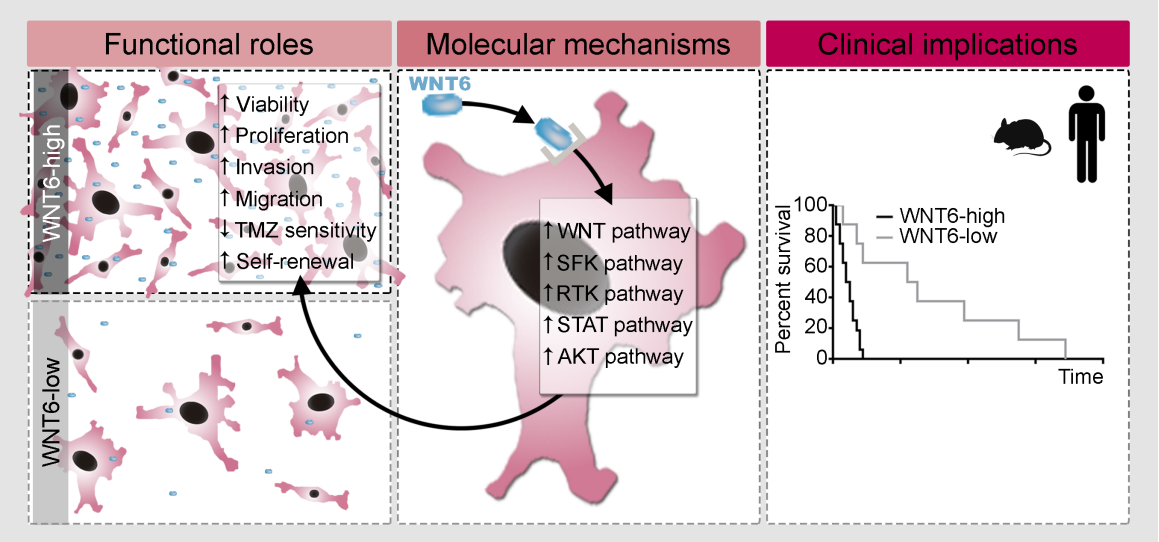

Figure 2.8. Proposed model for the functional, molecular and clinical impact of WNT6 in GBM. WNT6-high GBM cells present increased proliferation, invasion, migration, TMZ resistance and stem cell self-renewal capacities, which are accompanied by activation of the WNT, SFK, RTK, STAT and AKT pathways. Clinically, WNT6-high tumors cause shorter overall survival (both in mouse GBM models and human patients).

motility capacity (Figure 2.2 and Figure 2.S3D-E), and resistance to chemotherapy (Figure 2.3), it will be interesting to evaluate its potential value as a biomarker for tumor recurrence, which is of paramount importance to the clinical management of GBM as these patients recur almost universally after treatment. Importantly, we also demonstrated that WNT6 is a novel biomarker of GBM prognosis, as patients with WNT6-high tumors from a variety of independent datasets presented a significantly shorter OS than WNT6-low tumors (Figure 2.7, Table 2.1, and Figure 2.S7), a clinical finding that is in agreement with our *in vivo* data (Figure 2.5 and Figure 2.S3I-J). This might be linked to the WNT6 oncogenic activities, such as increased invasion/migration, hampering complete tumor resection, increased tumor relapse probability due to its roles in GSC maintenance (Figure 2.4 and Figure 2.S3F-H), and the response to TMZ treatment (Figure 2.3), which is still the gold-standard chemotherapeutic drug used for GBM patients. These findings fit well with previous reports describing that WNT6 increases the chemoresistance of gastric [13] and bladder [14] cancer, and associates with the overall and disease-free survivals of esophageal squamous cell carcinoma patients [15]. Together, these data implicate WNT6 as a novel prognostic biomarker and putative therapeutic target for GBM patients. The fact that WNT6 levels can be easily assessed at the mRNA or protein levels with routine molecular biology techniques may also facilitate the widespread implementation of this biomarker in the clinical settings.

Taking into consideration that (i) the first step for designing novel therapeutic strategies requires not only the identification of new molecular targets, but also the understanding of their functional mechanisms, and (ii) nothing was known of WNT6-mediated signaling mechanisms in GBM or any other cancer type, our phospho-kinase arrays provide the first data on the activation status of several cancer-

related pathways that may be regulated by WNT6 (Figure 2.6). The effects observed in β-catenin in U373 and SNB19 cells clearly suggest that WNT6 might act through the canonical WNT pathway in GBM, which was further validated with the TCF/LEF reporter assays in U373 and U87 cells (Figure 2.S6). In addition, WNT6 silencing led to reduced phosphorylation/activation states of 4 non-receptor tyrosine Src kinases (Fgr, Yes, Fyn and Src), which have been described to contribute to the activation of downstream targets of RTKs, such as EGFR, influencing glioma aggressiveness [49-51]. WNT6 silencing also resulted in decreased phosphorylation of HSP27, an anti-apoptotic protein [52] associated with increased cell proliferation and decreased Fas-induced apoptosis in prostate cancer [53], and was shown to mediate the activation of p38/ERK (MAPK) pro-survival signaling in cervical cancer cells [54]. Nevertheless, other cell line-specific alterations in WNT6-silenced cells, namely in p-AKT, p-TOR and p-PRAS40 in U373 cells, clearly demonstrate the association between WNT6 and the activation of the PI3K/AKT/mTOR pathway, which is known to have a major role in the oncogenic process of GBM cells [55]. Regarding p-STAT3 alterations upon WNT6 silencing, it was reported that reduced p-STAT3 S727 or increased p-STAT3 Y705 lead to more aggressive glioma tumors *in vivo*, and enhanced glioma proliferation and invasion *in vitro* [31], which also fits well with a WNT6-mediated oncogenic role in GBM. Importantly, GSEA data derived from clinical GBM samples further supported the molecular links between WNT6 and these cancer-related pathways. In this context, our results, together with the reports associating the WNT pathway with the MAPK, PI3K and STAT3 pathways [56-63], strongly support a role of WNT6 in the regulation of these cancer-related pathways and suggest that WNT6 acts, at least partly, through the canonical WNT signaling, independently of the expression of other well-known canonical WNT ligands (WNT1 and WNT3a; Figure 2.S8). Future work should focus on the potential predictive value of WNT6 for targeted therapies with specific inhibitors of WNT-related pathways (e.g., LGK974, currently tested in clinical trials for several solid tumors; NCT01351103).

In summary, this work provides significant contributions to the neuro-oncology field, profiting from the understanding of WNT6-mediated signaling pathways and from the portrayal of the functional roles and clinical value of WNT6 in GBM, potentially impacting prognostic stratification and aiding in rationale treatment decisions for GBM patients.

2.4. Materials and methods

2.4.1. TCGA data analysis in glioma patients

All gene expression data from samples hybridized by the University of North Carolina, Lineberger Comprehensive Cancer Center, using Agilent G4502A 244K, were downloaded from TCGA

(*https://portal.gdc.cancer.gov/*) [19], including 572 GBMs, 27 LGGs, and 10 unmatched non-tumoral patient samples. To prevent duplicated entries from the same patient—when more than one portion per patient was available—the median expression value was used. Three probe sets hit *WNT6* gene (A_23_P119916, A_32_P159877 and A_24_P208513). The provided value was pre-processed and normalized according to "level 3" specifications of TCGA. *WNT6* overexpression in glioma samples was considered when higher than that in non-tumoral samples ("level 3" value ≥ 0.41). Clinical data of each patient was provided by the biospecimen core resources and includes information about patients' age at diagnosis, gender, KPS and days to death and to last follow-up.

2.4.2. Freije, Gravendeel and Vital datasets

WNT6 expression microarray data from Freije (n = 59) [24], Gravendeel n = 159 [32], and Vital (n = 26) [33] datasets of GBM patients were obtained in the GlioVis data portal [64]. The top 17% of tumors expressing highest levels of *WNT6* were classified as WNT6-high in Freije (n = 10/59) and Gravendeel (n = 27/159) datasets, similar to the cutoff used for TCGA, and the top 39% for the Vital (n = 10/26) dataset due to the small number of patients. Clinical data included GBM subtype classification, OS and vital status of the patients.

2.4.3. Glioma primary tissues

Glioma tumor specimens were obtained from patients who underwent a craniotomy for tumor removal or stereotaxic biopsy at 4 different hospitals: Hospital Braga (HB), Hospital Santa Maria (HSM, Centro Hospitalar Lisboa Norte; samples requested from Biobanco-iMM, Lisbon Academic Medical Center, Lisbon, Portugal) and Hospital Santo António (HSA, Centro Hospitalar Porto), Portugal, and Hospital de Câncer de Barretos, Brazil. Samples from HB and HSM were reserved for RNA-based studies, transported in dry ice to the lab and stored at -80 °C. Samples from HSA and Brazil were formalin-fixed and paraffin-embedded and used for IHC analysis. Samples from Brazil were organized in triplicates into tissue microarrays (TMAs) [65]. Tumors were classified according to WHO 2007 [66] and only patients with glial tumor histological diagnosis were included in the study.

2.4.4. Immunohistochemistry

Tissues sections were deparaffinized and rehydrated by xylene and ethanol series. Sodium citrate buffer (10 mM, 0.05% Tween 20, pH 6) was used for antigen retrieval. Endogenous peroxidase activity was blocked with 3% H_2O_2 in TBS 1x for 10 min. Immunohistochemical staining was performed using the LabVision kit (UltraVision Large Volume Detection System Anti-polyvalent, HRP) according to the manufacturer's instructions. WNT6, Ki-67, Cyclin D1, SOX2, NESTIN, GFAP and BCL2 antibodies were

used (see Table 2.S1 for details). Concerning Ki-67 staining, before antigen retrieval, tissues were permeabilized using TBS-Tween 0.5%, for 10 min. DAB substrate (DAKO) was used as chromogen, followed by counterstaining with hematoxylin. The TMA from the Brazilian cohort was blind-scored by a pathologist based on WNT6 staining intensity: 1 = weak, 2 = moderate and 3 = strong. The average of the triplicate spots was calculated and used for the survival analysis. An average > 2 was considered as WNT6 high expression.

2.4.5. Cell lines and culture conditions

Human GBM cell lines U373MG and U87MG (kindly provided by Dr. Joseph Costello, University of California, San Francisco), and SNB19 (obtained from German Collection of Microorganisms and Cell Cultures) were cultured in DMEM (Biochrom) supplemented with 10% Fetal Bovine Serum (FBS; Biochrom). Cells were maintained in a humidified atmosphere at 37 °C and 5% (v/v) CO_2. These conditions were maintained throughout the studies, unless otherwise stated. Testing for mycoplasma contamination was performed every month.

2.4.6. WNT6-silencing by shRNA in GBM cells

U373MG and SNB19 cell lines were plated at 70 000 cell/well (24-well plate) and 100 000 cells/well (12-well plate), respectively. Cells were transfected with *WNT6* gene-specific shRNA expression vector inserted in a pRS plasmid (TR308360, clones TI333434 and TI333435 from Origene; named U373-shWNT6 or SNB19-shWNT6, respectively) or with a scrambled negative control non-effective shRNA in a pRS plasmid (TR30012, Origene; named U373-shCtrl or SNB19-shCtrl). Lipofectamine 3000 (Invitrogen) transfection reagent was used according to the manufacturer's recommendations (ratio plasmid/lipofectamine, 1:2.5). Stable transfection was achieved by selecting transfected clones with puromycin (1 μg/mL).

2.4.7. WNT6 overexpression in GBM cells

U87MG cells were plated at 200 000 cell/well (6-well plate). Cells were transfected with the pcDNA-Wnt6 vector (gift from Dr. Marian Waterman; Addgene plasmid #35913) [67] or with the respective empty vector (pcDNA3.2 GW delCMV; gift from Dr. Edward Hsiao; Addgene plasmid #29496) [68]. Lipofectamine 3000 (Invitrogen) transfection reagent was used according to the manufacturer's recommendations (4 μL of Lipofectamine 3000 per 1 μg of DNA). Stable transfection was achieved by selecting transfected clones with geneticin (800 μg/mL).

2.4.8. qRT-PCR

Total RNA was extracted from GBM patients' tissues and cell lines using the TRIzol method (Invitrogen) and cDNA from 1 µg of the total RNA was then synthesized (RT-Phusion Kit, Thermo Scientific). The levels of *WNT6* and *TBP* (reference gene) were assessed by quantitative reverse transcription-PCR (qRT-PCR; KAPA SYBR® FAST qPCR Kit, KAPABIOSYSTEMS or PowerUp™ SYBR™ Green Master Mix, ThermoFisher Scientific, for cell lines and GBM patient's samples, respectively) with the following sets of primers: *WNT6* Fwd 5'–GACGAGAAGTCGAGGCTCTTT–3' and Rev 5'–CGAAATGGAGGCAGCTTCT–3'; *TBP* Fwd 5'–GAGCTGTGATGTGAAGTTTCC–3' and Rev 5'–TCTGGGTTTGATCATTCTGTAG–3'. The annealing temperature was 60 °C for both. The levels of *WNT1* and *WNT3a* were assessed using Taqman gene expression assays (Hs00180529_m1 and Hs00263977_m1, respectively) and normalized against *TBP* (Hs00427620_m1), following manufacturer's instructions. Levels were determined based on the $2^{-\Delta\Delta Ct}$ method, as described in [69].

2.4.9. Immunofluorescence (IF)

U373 (shCtrl/shWNT6) and SNB19 (shCtrl/shWNT6) cells were plated on coverslips in DMEM supplemented with 10% FBS. Cells were fixed with paraformaldehyde 4% and then incubated in 1% BSA in PBS/0.1% Tween for 1 h. Afterwards, cells were incubated overnight at 4 °C with the primary antibody against WNT6 or SOX2. Alexa fluor® 594 goat anti-rabbit IgG (H+L; A-11012, ThermoFisher Scientific) secondary antibody was used. Additionally, SOX2-coverslips were incubated with anti-NESTIN primary antibody. Next, Alexa Fluor® 488 goat anti-mouse IgG (H+L; A-11001, ThermoFisher Scientific) secondary antibody was used. DAPI (VECTASHIELD® Mounting Medium with DAPI, Vector Laboratories) was used to stain the cell nucleus (blue) at a concentration of 1.5 µg/mL. Further details about the antibodies used are provided in Table 2.S1.

2.4.10. Cell viability assays

Cell viability was determined 6 days after plating by both trypan blue and MTT (U373 and SNB19 cells) or MTS (U87 cells) assays. For trypan blue assay, cells were plated, in triplicate, at an initial density of 20 000 cells/well in 6-well plates. For MTT/MTS assay, transfected cells were plated in 12-well plates, in triplicate, at an initial density of 6 000 (for U373 and U87 cells) or 10 000 cells/well (SNB19).

For the MTT assay, cells were incubated with 0.5 mg of MTT (ThermoFisher Scientific) per mL of PBS 1x, for up to 2 h in a humidified atmosphere at 37 °C and 5% CO_2. Subsequently, the formed crystals were dissolved using an acidified isopropanol solution (0.04 M HCl in isopropanol), as described in [70], and the absorbance was read at 570 nm. For the MTS assay, cells were incubated with 10% of the MTS

solution (CellTiter 96® AQueous One Solution Cell Proliferation Assay; Promega) in DMEM supplemented with 10% FBS, for up to 1 h in a humidified atmosphere at 37 °C and 5% CO_2. Absorbance was recorded at 490 nm.

2.4.11. Cell proliferation

Cell proliferation was evaluated based on the measurement of BrdU incorporation during DNA synthesis (Cell Proliferation ELISA, BrdU colorimetric assay, Roche), as recommended by the manufacturer. Briefly, cells were cultured in DMEM (Gibco®) supplemented with 10% FBS (Biochrom) in 96-well plates (cell density: 2 000 cells/well) for 4 days. After that, BrdU was added to the cells and they were re-incubated for 4 h.

2.4.12. Cell migration

Ibidi 2-well inserts (Ibidi) with a cell-free gap were used in 12-well plates to evaluate cell migration by wound healing assay. U373 and SNB19 transfected cells were plated in triplicate, at an initial density of 70 000 and 60 000 cells in each side of the insert, respectively, and left to adhere overnight. After that, inserts were removed (0 h time-point) and wells were washed with PBS to remove dead cells and debris. The migration of the cells into the gap was imaged (2 images/well) over hours until wound closure was achieved (images were captured at the same locations over time). The gap size (pixels) was measured using an automated software (beWound – Cell Migration Tool, v1.7, BESURG, Portugal; *www.besurg.com*) and manually verified and corrected when deemed necessary. At least 15 lines equally spaced across the image and perpendicular to the gap main axis were used for increased measurement representativeness.

2.4.13. Cell invasion

Cell invasion was assessed using the BD BioCoat™ Matrigel™ Invasion Chambers (Corning®) as recommended by the manufacturer. Briefly, cells were seeded into the top (insert) at the initial density of 50 000 cells/well (U373 and SNB19) or 25 000 cells/well (U87; 24-well chambers) in DMEM (Gibco®) supplemented with 1% FBS. EGF was added to the medium of the lower chamber (DMEM supplemented with 10% FBS) at a concentration of 20 ng/mL, as chemoattractant. Cells were incubated for 22 h. Next, cells that invaded through the membrane were stained using VECTASHIELD Mounting Medium with DAPI (Vector Laboratories) and a picture of the entire membrane was obtained using an Olympus Upright BX61 microscope. The total cell number was counted with the help of ImageJ software (version 1.49).

2.4.14. Cell death

Cell death was evaluated after 6 days of treatment with TMZ (400 μM and 800 μM, for U373

and SNB19 transfected cells, respectively) or DMSO (Dimethyl sulfoxide; Vehicle). TMZ treatment was applied 24 h after plating and was renewed 2 days after. Cells were stained with Annexin V-FITC (BD Bioscience) and propidium iodide (PI; TermoFisher Scientific), according to the manufacturer's protocol, followed by flow cytometry analysis. At least 10 000 events were acquired. Results were analyzed using FlowJo software (version 10).

2.4.15. Colony formation assay

For clonogenic cell survival assay, cells were seeded at a density of 1 000 to 2 000 per 25-cm^2 flask, in triplicates. Twenty-four hours after seeding, irradiation was performed at RT in single exposure doses, delivered by a Novalis TX® linear accelerator, with a nominal energy of 6 MV at a dose rate of about 400 cGy/min. Doses of 2 and 4 Gy were calculated using a dedicated treatment planning system to a depth of water of 1.5 cm. The plating efficiency (PE) represents the percentage of seeded cells that grew into colonies. Colonies with more than 50 cells were counted by microscopic inspection and PE, as well as the radiation-surviving fraction (PE of experimental group/PE of control group), were determined. Survival data were obtained with data from 3 independent experiments.

2.4.16. Cell viability assay (MTT) under stem cell conditions

Culture vessels were coated with 10 µg/mL natural mouse laminin (Invitrogen) for 3 h at 37 °C or overnight at 4 °C, as previously described [29]. U373 and SNB19 transfected cells were plated in 12-well laminin-coated plates, in triplicate, at an initial density of 6 000 and 10 000 cells per well, respectively. Cells were cultured in serum-free stem cell medium composed by DMEM-F12 (Gibco) supplemented with 1x B27 (Invitrogen), 20 ng/mL of EGF (Invitrogen), and 20 ng/mL of basic fibroblast growth factor (b-FGF, Invitrogen). After 4 days in culture, cells were incubated with MTT as detailed above (section "Cell viability assays").

2.4.17. Neurosphere formation assay

U373, SNB19 and U87 transfected cells were plated at the maximum density of 4 000 cells/mL in low-attachment 24-well plates (Corning). Cells were cultured in serum-free stem cell medium, as detailed above. Neurospheres were supplemented with fresh media every 4–5 days (250 µL/well). The number of neurospheres was counted after 10 days for U373 and U87 cells and 15 days for SNB19.

2.4.18. Limiting dilution assay

Cell numbers of U373 and U87 transfected cells were adjusted to a starting concentration of 40 000 cells/mL and 160 000 cells/mL, respectively, from which multiple serial dilutions were

performed and plated in low-attachment 96-well plates (Corning). At the end, cell densities ranged from 1 000 to 1 cells per well in a final volume of 100 µL for U373 cells and from 4 000 to 8 cells per well for U87 cells. Cells were cultured in serum-free stem cell medium, as detailed above. Cultures were disturbed only for media supplementation every 5 days. After 15 days, the fraction of wells not containing neurospheres was determined for each condition and plotted against the initial plated cell density. Stem cell frequencies and statistical significance were calculated using the Extreme Limiting Dilution Assay (ELDA) software available at *http://bioinf.wehi.edu.au/software/elda/* [71].

2.4.19. Stereotactic orthotopic xenografts

NSG (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ) mice aged 4 months were anesthetized with a mixture of ketamine (75 mg/kg) and medetomidine (1 mg/kg) supplemented with butorphanol (5 mg/kg) for its analgesic properties and placed into the digital 3-axis stereotaxic apparatus (Stoelting). A total of 5×10^5 U373 or 2×10^5 U87 transfected cells (resuspended in 5 µL PBS 1x) were injected into the brain striatum using a 10 µL Hamilton syringe with a point style 4 beveled 26s-gauge needle, as previously described [72]. A total of 8 male and 6 female mice per group were used for U373 and U87 transfected cells, respectively. U373 and U87 cells were used as no reports were found for SNB19 tumorigenic capacity when orthotopically injected in immunocompromised mice. Mice were maintained under standard laboratory conditions, which included an artificial 12 h light/dark cycle, controlled ambient temperature (21 ± 1 °C) and a relative humidity of 50–60%. The confirmation of specified pathogen-free health status of sentinel mice maintained within the same animal room was performed according to FELASA guidelines. Experimental mice were always manipulated in a flow hood chamber, except during the surgery. Mice's body weight was evaluated 3 times per week, while general behavior and symptomatology were registered daily. Sacrifice at the pre-established humane endpoint was performed when mice presented severe weight loss (> 30% from maximum weight). Death was used for Kaplan-Meyer representation.

2.4.20. Human phospho-kinase antibody array

Human phospho-kinase antibody arrays (ARY003B; R&D systems) were used to simultaneously detect the relative levels of phosphorylation of 43 kinase phosphorylation sites and 2 related total proteins. Capture and control antibodies were spotted in duplicate on nitrocellulose membranes. The protocol was done following the manufacturer's recommendations. In this case, 400 µg of total protein from all cell lines (U373 shCtrl/shWNT6 and SNB19 shCtrl/shWNT6) was used. The obtained chemiluminescence was measured using the ChemiDoc imaging system (Biorad). Adjusted volume per spot (*i.e.*, average pixel intensity of the spot times its area, corrected for the background) was measured using the Quantity

One image analysis software. Background was eliminated using the local option. The average of each duplicated spot was calculated, normalized to HSP60 (reference protein) and used to determine the relative change in phosphorylated kinase proteins between shCtrl and shWNT6 conditions. Two sets of arrays were performed for U373 shCtrl/shWNT6, while only 1 was used for SNB19 shCtrl/shWNT6.

2.4.21. Western blot analysis

Cells were washed with PBS 1x and removed by scratch in a lysis buffer containing 50 mM Tris pH 8, 2 mM EDTA pH 7.4, 0.1% SDS, 1% NP-40 and 150 mM NaCl, inhibitors of proteases 1x (B14001, Biotool) and phosphatases 1x (B15001, Biotool). After a 15-min incubation on ice, whole cell lysate was centrifuged at 13 000 × *g* for 15 min at 4 °C. The total protein concentration was determined from the obtained supernatant using the Pierce™ BCA protein assay kit (ThermoFisher Scientific), according to the manufacturer's protocol. Protein extracts (denatured and reduced; 20 µg/lane) were separated in a 10% SDS-polyacrylamide gel by electrophoresis and transferred to nitrocellulose membranes (Hybond-C Extra, GE Healthcare Life Sciences), using the Trans-blot turbo transferring system (Bio-rad), following the manufacturer's instructions. A protein ladder (Page ruler prestained protein ladder; Fermentas) was used to determine the approximate protein size and to monitor the progress of the electrophoretic run and the success of the transfer. Before starting the immunodetection, membranes were blocked with BSA (5% in TBS 1x), for 1 h at RT. The immunodetection was achieved using antibodies against p-STAT3 Y705, STAT3, non-p-β-catenin S33/S37/T41, β-catenin, p-AKT S473, AKT, and α-tubulin (see Table 2.S1 for details). Blots were revealed with peroxidase-conjugated secondary anti-mouse and anti-rabbit IgG antibodies (see Table 2.S1 for details), followed by enhanced chemiluminescence (ECL) solution (SuperSignal West Femto Chemiluminescent Substrate; Thermo Scientific). The obtained chemiluminescence was measured using the ChemiDoc imaging system (Biorad). If necessary, membranes were stripped using the Restore™ PLUS Western Blot Stripping Buffer (Thermo Fisher Scientific), following the manufacturer's recommendations. As for the phospho-kinase arrays, adjusted volume per band was measured using the Quantity One image analysis software. Phosphorylated protein values were normalized to α-tubulin (reference protein).

2.4.22. TCF/LEF reporter assay

The Cignal TCF/LEF Reporter Assay Kit (GFP; Qiagen) was used to quantify the specific activation of β-catenin-dependent WNT signaling (canonical pathway). The protocol was done following the manufacturer's recommendations. Briefly, U373-shCtrl, U373-shWNT6, U87-Ctrl and U87-WNT6 cells were reverse transfected with i) a TCF/LEF reporter vector, which encodes a monster GFP under the

control of a TATA box (basal promoter) joined to TCF/LEF responsive elements; ii) a negative control vector, which encodes GFP under the control of a TATA box; or iii) a positive control vector, which constitutively expresses the monster GFP for visual confirmation of the transfection efficiency. For this, cells were plated in triplicate at an initial density of 20 000 cells/well in 96-well plates with black walls and clear bottoms (Glass SPL Black plate). Lipofectamine 3000 (Invitrogen) transfection reagent was used according to the manufacturer's recommendations (0.25 µL of Lipofectamine 3000 per 100 ng of DNA). After 16 h of transfection, the medium was renewed and 24 h post-transfection, cells were treated with 50 mM of LiCl. GFP expression was verified 24 h after treatment under the microscope and quantified using a Varioskan Flash (Thermo Fisher Scientific) plate reader (excitation at 488 nm and emission at 515 nm). GFP expression in the reporter assay condition was normalized against the negative controls (as blank) and the respective positive controls (to control for transfection efficiency).

2.4.23. Gene Set Enrichment Analysis (GSEA)

The raw expression data profile (Agilent G4502A 244K) of all GBM patients from TCGA (n = 573) was extracted as detailed above. GSEA (*www.broad.mit.edu/gsea/*) software was then used [73]. In this work, a continuous phenotype profile was employed to find gene sets that correlate with *WNT6* (gene neighbors). Accordingly, Pearson's correlation was used to score and rank the genes. Gene sets from the Molecular Signature Database (MSigDb) C6 collection were used. Default options were used, unless otherwise stated. Significant enrichments were considered when false discovery rate (FDR) < 0.30.

2.4.24. Statistical analyses

Homoscedasticity was verified with Levene's test and differences between groups were assessed by a two-sided unpaired *t*-test, with Welch's correction applied accordingly. For the wound healing assay and radiation treatment sensitivity assay, a two-way ANOVA followed by the post-hoc Sidak's test for multiple comparison testing was used. For the TMZ treatment cell death assay, a two-way ANOVA followed by the post-hoc Tukey's test for multiple comparison testing was used. The correlation between the expressions of *WNT6* and stem-related genes (from the gene ontology gene-set GO_Positive_regulation_of_stem_cell_proliferation) was calculated using Pearson's correlation coefficient (r). The effects of WNT6 on mouse OS was assessed by log-rank test. These analyses were performed with GraphPad Prism 6.01 software (GraphPad software, Inc.).

The Chi-square test was used to assess the difference between the distributions of tumors with high and low *WNT6* expression stratified for LGGs and GBMs. To evaluate the prognostic value of *WNT6* expression, univariate or multivariate analyses of survival were performed using, respectively, the log-rank

test or the Cox proportional hazard model, where the potential confounding effect of some variables is considered. These analyses were made with SPSS 22.0 software (SPSS, Inc.). A meta-analysis including all datasets was performed with the Comprehensive Meta Analysis (CMA) v3 software (Biostat, Inc.). Hazard ratios and 95% confidence intervals from the Cox univariate or, when possible, multivariate analyses were used. A random effects statistical model was applied.

For all statistical tests, significance was considered when $p < 0.05$.

2.4.25. Study approval

Written informed consent for investigation purposes was obtained from all patients. All procedures were in accordance with institutional ethics standards. Clinical samples in which patient information was collected were obtained from the Hospital Braga and Hospital Santa Maria, Portugal, and Hospital de Barretos, Brazil, with their respective ethical approvals (SECVS 150/2014 and CNPD 7435/2011). All animal procedures were conducted in accordance with the guidelines for the care and use of laboratory animals (European Directive 2010/63/EU) and approved by the Direcção Geral de Alimentação e Veterinária (DGAV, reference 017761), the competent national authority for animal protection.

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Author contributions

CSG, NS and BMC designed research studies. CSG, JVC, MP, EPM, SQ, and EC conducted experiments. CSG, JVC, MP, EPM, SQ, EC, RT, CC (C. Clara), NS, and BMC analyzed data. RT, MMP,

AAP, FP, CC (C. Custódia), CCF, and RMR provided patient samples. CSG and BMC wrote the manuscript. All authors reviewed and approved the manuscript.

Competing interests

The authors have declared that no competing interest exists.

Appendixes

Appendixes including:

- Supplementary Figures and Legends;
- Supplementary Tables.

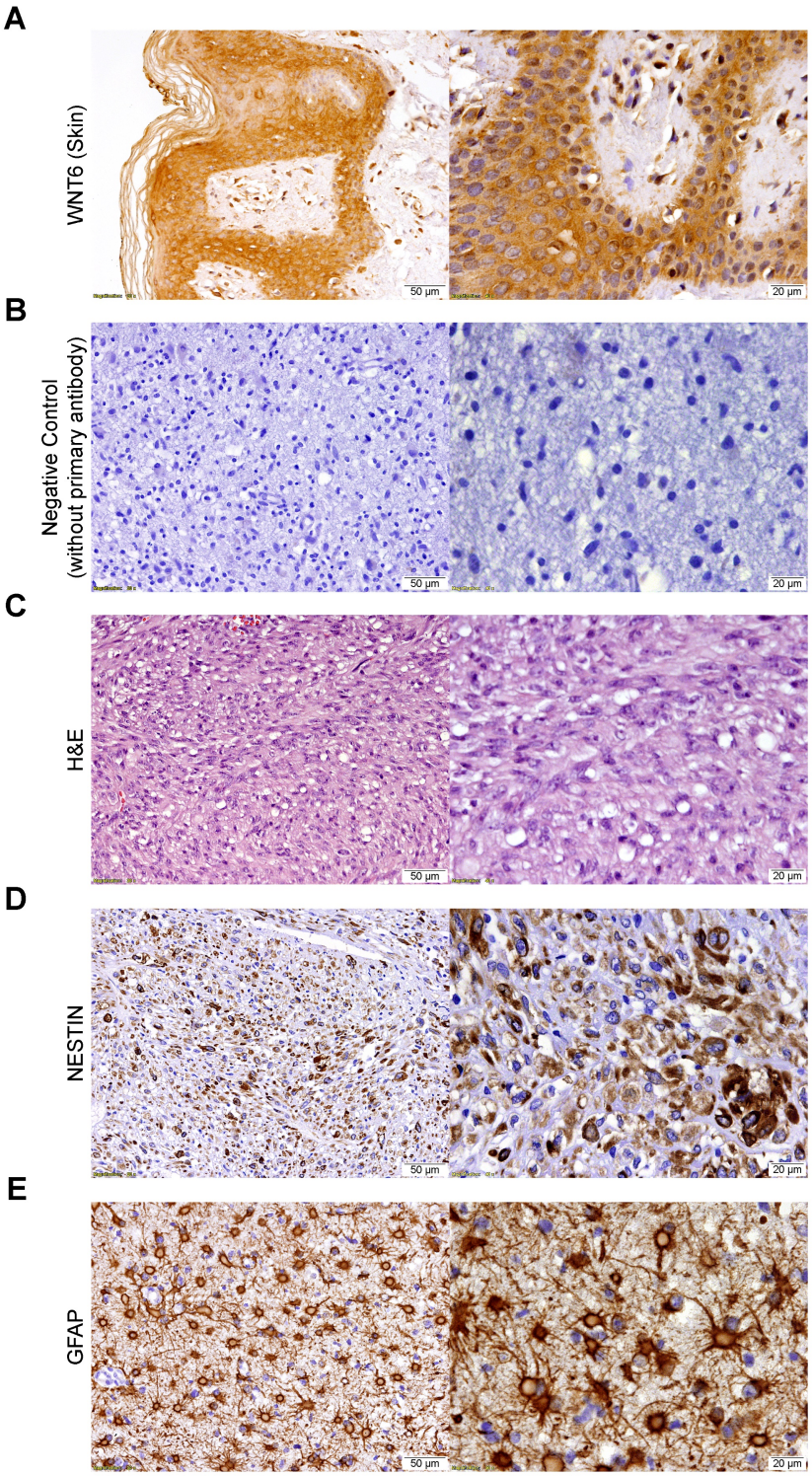

Figure 2.S1. Representative images of immunohistochemistry controls for HSA tissues.
Representative images are shown for: **(A)** WNT6 stainings in skin tissues, used as both negative and positive control, for its specific WNT6 expression patterns, where WNT6 expression might be found in the epidermis and its derivative appendages (i.e. hair follicle) and not in the other subsequent layers, to ensure for the specificity of the primary antibody; **(B)** Negative controls without primary antibody incubation; **(C)** Hematoxylin and Eosin stainings to confirm the presence of tumor; **(D)** NESTIN and **(E)** GFAP stainings were used as typical GBM markers.

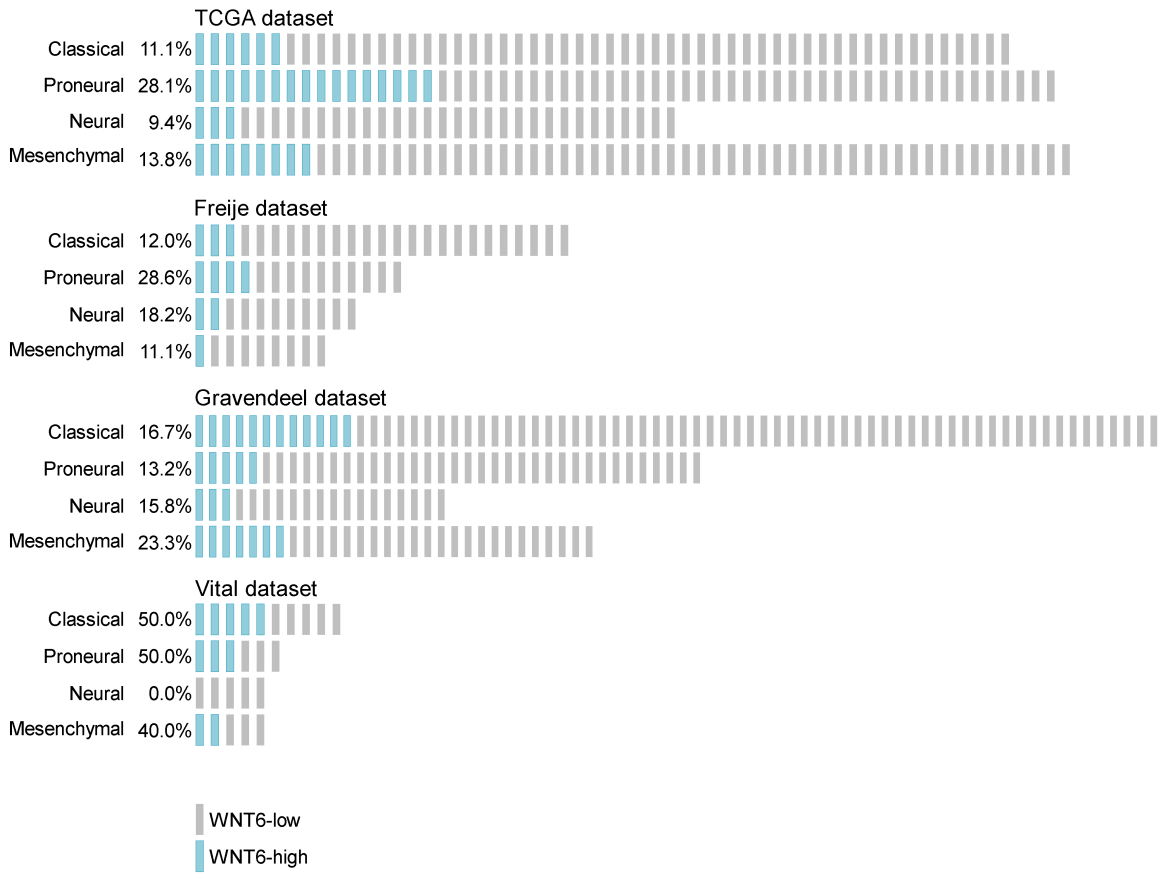

Figure 2.S2. *WNT6* expression is present in all 4 molecular subtypes of GBM.
Distribution of WNT6-high patients among GBM molecular subtypes from the TCGA (n = 201), Freije (n = 59), Gravendeel (n = 159) and Vital (n = 26) datasets.

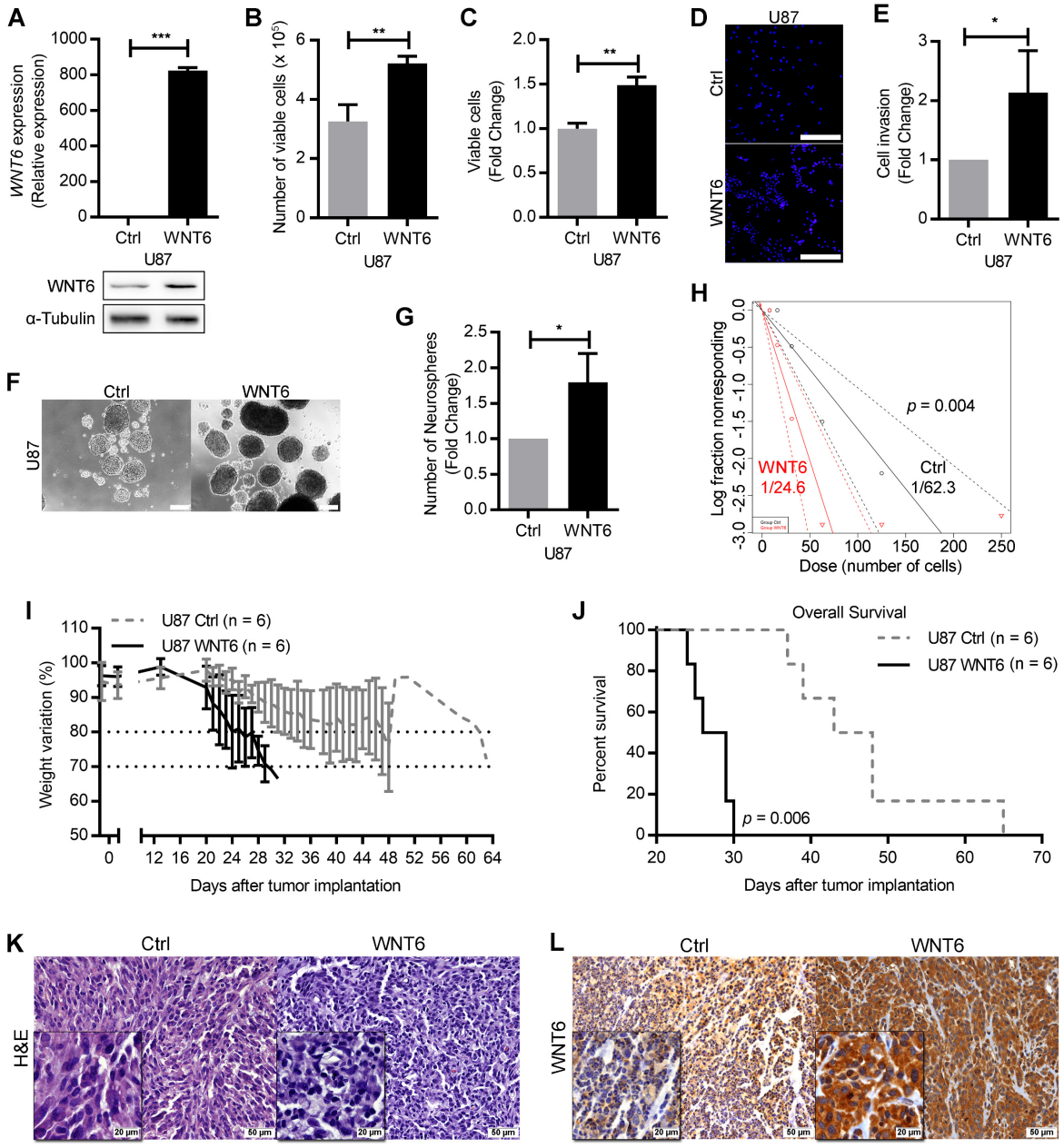

Figure 2.S3. WNT6 overexpression promotes GBM aggressiveness *In vitro* and *In vivo*.
(A) The efficiency of WNT6 overexpression in U87 glioblastoma cells was analyzed by qRT-PCR (top) and WB (bottom). (Top) *WNT6* expression levels were normalized to *TBP*. (Bottom); α-tubulin was used as reference protein. **(B and C)** Cell viability was measured by trypan blue **(B)** and MTS **(C)** assays in Ctrl and WNT6 cells. **(D and E)** Matrigel invasion assays were used to assess the cells' invasion capacity. Representative images (**D**; cell nuclei stained with DAPI; scale bar = 300 µm) and quantification **(E)** of invasive U87 Ctrl and WNT6 cells. **(F)** Representative images of U87 Ctrl/WNT6 neurospheres at day 10 after plating (40x magnification; scale bar = 200 µm). **(G)** Quantification of neurospheres formation. **(H)** Limiting dilution assays in U87-Ctrl (black) and U87-WNT6 (red) cells to assess their sphere-forming capacity. The trend lines represent the estimated active cell frequency (n = 3 independent assays; $p = 0.004$, likelihood ratio test). **(I-L)** U87 transfected cells were orthotopically injected in the brain of NSG mice (n = 6 per group). **(I)** Mice weight curves after tumor implantation. **(J)** Kaplan-Meier overall survival curves of mice ($p = 0.006$; Log-rank test). **(K and L)** Post-mortem brain histological and molecular analyses. H&E **(K)** and anti-WNT6 IHC staining **(L)** were used as controls for GBM formation and successful long-term WNT6 overexpression, respectively. Results represent data from at least 3 independent experiments (mean ± SD). *, $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.005$ (otherwise stated, a two-sided unpaired *t*test with Welch's correction being applied when homoscedasticity was not verified).

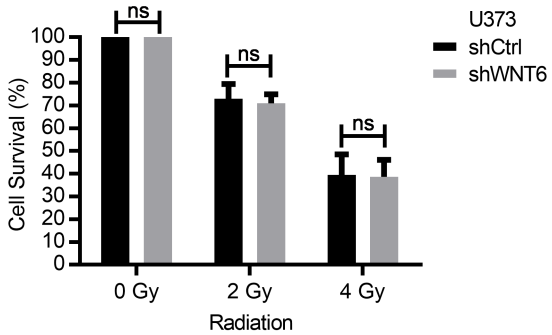

Figure 2.S4. WNT6 does not affect sensitivity of GBM cells to radiotherapy.
U373 shCtrl and shWNT6 cells were treated with increasing doses of radiation (0, 2, and 4 Gy) and cell survival was analyzed by colony formation assays. ns = non-statistically significant (two-way ANOVA post-hoc Sidak's test).

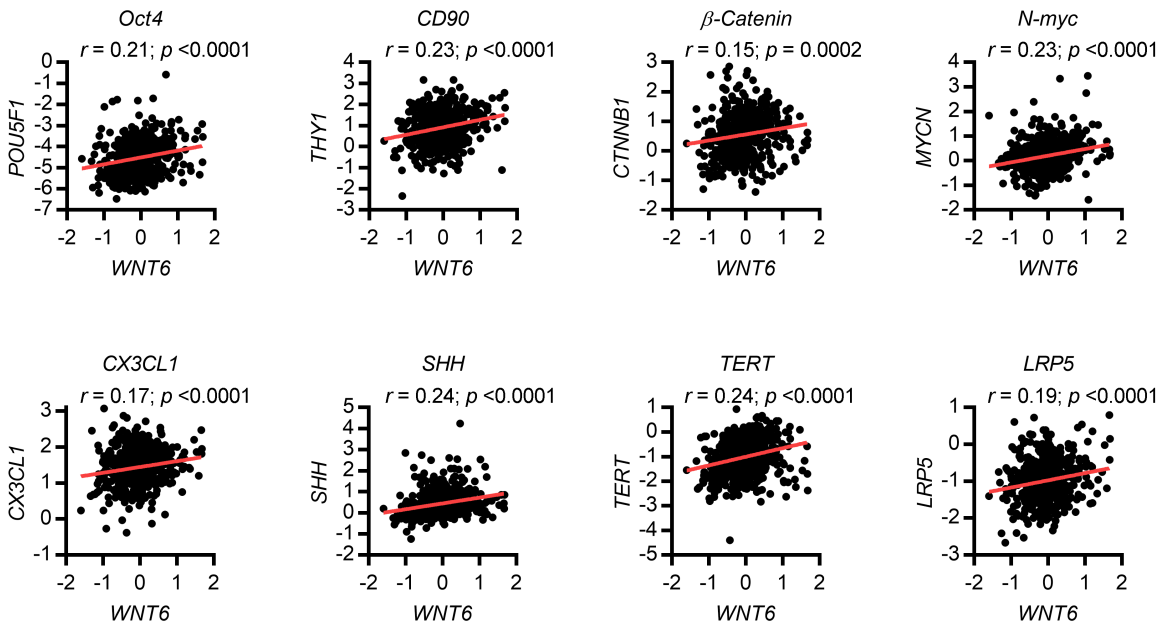

Figure 2.S5. *WNT6* correlates with stem cell genes.
Correlation graphs between *WNT6* expression (x-axis) and the expression of stem cell genes selected based on the heatmap in Figure 4 (y-axis; Pearson's correlation test *r* and *p* values are indicated).

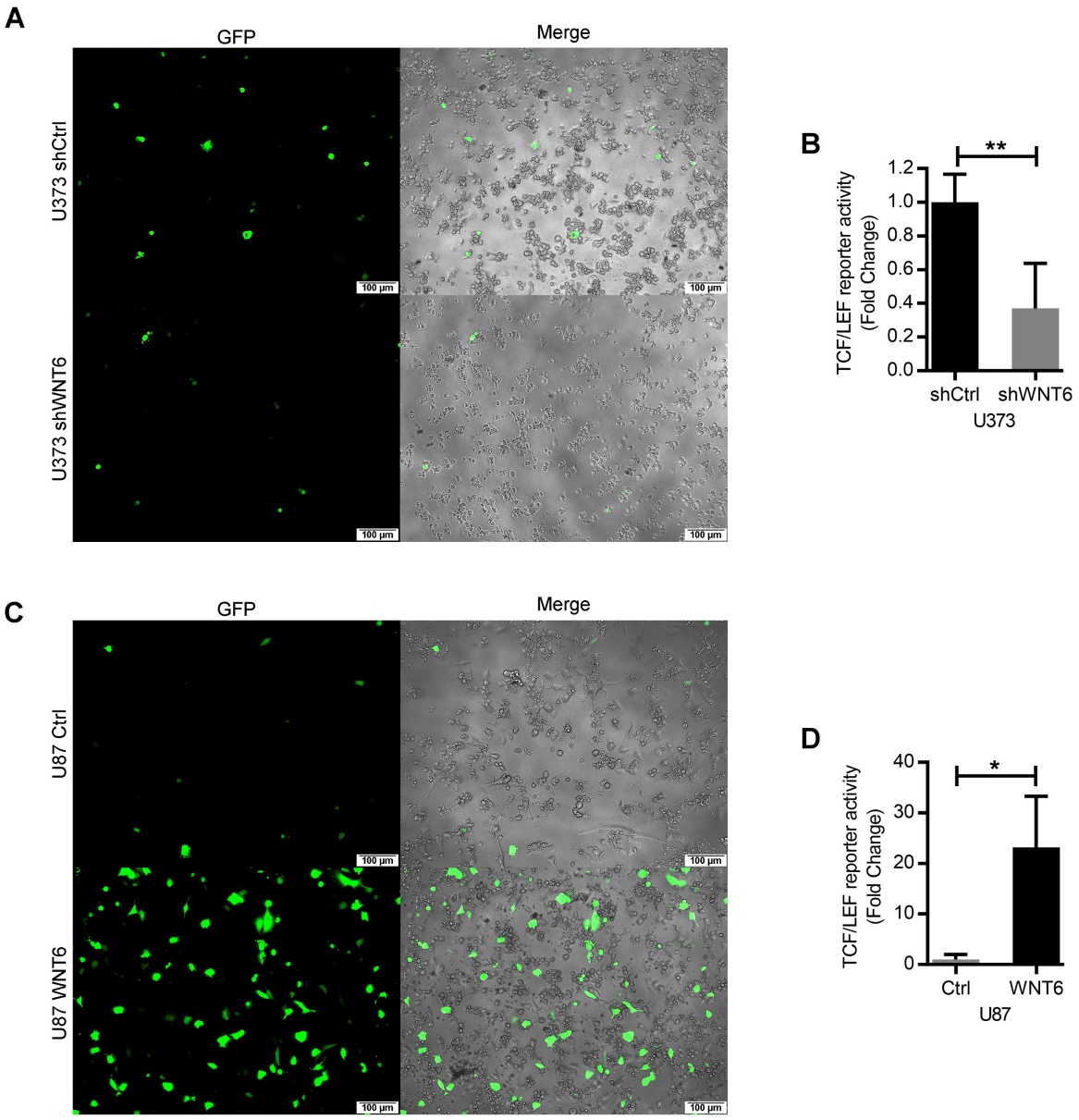

Figure 2.S6. WNT6 activates the WNT canonical signaling pathway.
TCF/LEF reporter assays in U373 shCtrl/shWNT6 **(A and B)** and U87 Ctrl/WNT6 cells **(C and D)**. **(A and C)** Representative images are displayed (100x magnification; scale bar = 100 µm). **(B and D)** GFP expression was used as a measure of TCF/LEF promoter activity and was normalized against negative and positive controls. (n = 3 independent assays; mean ± SD; *, $p < 0.05$ and **, $p < 0.01$, two-sided unpaired *t*-test).

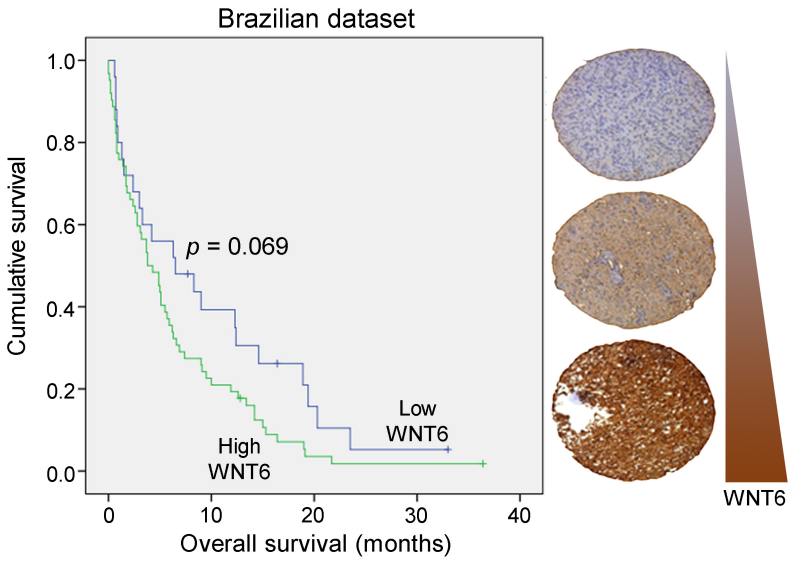

Figure 2.S7. Expression levels of WNT6 protein are prognostically valuable in GBM patients.
Kaplan-Meier survival curves of WNT6-low and WNT6-high GBM patients derived from IHC (protein) data from a Brazilian dataset (n = 87; median OS 6.5 vs. 3.8 months, low vs. high WNT6 expression, respectively; $p = 0.069$, Log-rank test). Representative IHC microphotographs showing different levels of WNT6 protein expression in particular tumors are shown.

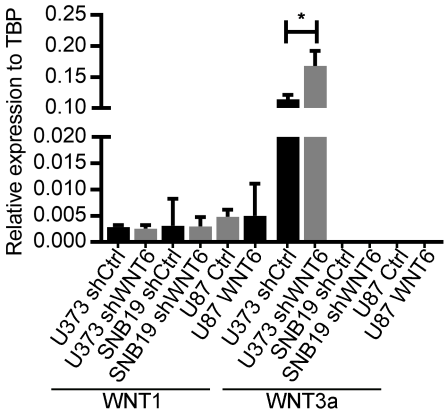

Figure 2.S8. Manipulation of WNT6 expression levels does not concomitantly affect levels of *WNT1* or *WNT3a*.

WNT1 and *WNT3a* expression was evaluated by qRT-PCR in U373 shCtrl/shWNT6, SNB19 shCtrl/shWNT6 and U87 shCtrl/shWNT6 cells (n = 3 independent assays; mean ± SD; *, $p < 0.05$, two-sided unpaired *t*-test).

Table 2.S1. List of antibodies used for immunohistochemistry, immunofluorescence, and Western blot

Protein	Reference	Source	Technique	Dilution	Incubation
WNT6	ab50030	Abcam	IHC	1:450	O/N at 4˚C
			IF	1:1 000	O/N at 4˚C
	ab154144		WB	1:500	O/N at 4˚C
Ki-67	550609	BD Biosciences	IHC	1:200	O/N at 4˚C
Cyclin D1	2978	Cell Signaling	IHC	1:100	O/N at 4˚C
SOX2	AB5603	EMD Millipore	IHC	1:500	O/N at 4˚C
			IF	1:300	O/N at 4˚C
BCL2	2870	Cell Signaling	IHC	1:200	O/N at 4˚C
NESTIN	MAB5326	EMD Millipore	IF	1:100	1h at RT
rabbit IgG (H+L) – Alexa fluor® 594	A-11012	ThermoFisher Scientific	IF	1:1 000	1h at RT in the dark
mouse IgG (H+L) – Alexa fluor® 488	A-11001	ThermoFisher Scientific	IF	1:1 000	1h at RT in the dark
p-STAT3 Y705	9145	Cell Signaling	WB	1:2 000	O/N at 4˚C
STAT3	9132	Cell Signaling	WB	1:1 000	O/N at 4˚C
non-p-β-catenin S33/S37/T41	4270	Cell Signaling	WB	1:5 000	O/N at 4˚C
β-catenin	610153	BD Bioscience	WB	1:2 000	O/N at 4˚C
p-AKT S473	4051	Cell Signaling	WB	1:1 000	O/N at 4˚C
AKT	2920	Cell Signaling	WB	1:2 000	O/N at 4˚C
α-tubulin	sc-23948	Santa Cruz Biotechnology	WB	1:1 000	1h at RT
mouse IgG – HRP conjugated	sc-2031	Santa Cruz Biotechnology	WB	1:1 000	1h at RT
rabbit IgG – HRP conjugated	sc-2004	Santa Cruz Biotechnology	WB	1:1 000	1h at RT

IHC: Immunohistochemistry; IF: Immunofluorescence; WB: Western Blot; O/N: Overnight; RT: Room Temperature

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Chapter 3

Molecular mechanisms regulating WNT6 expression in glioma

The results presented in this chapter are under preparation to be submitted for publication as an original article in an international peer reviewed journal:

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Contents

Molecular mechanisms regulating WNT6 expression in glioma

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Abstract

Gliomas represent the majority of malignant brain tumors, being glioblastoma (GBM) the most frequent and malignant form. Despite all efforts to improve treatment strategies, from surgery to radio- and chemotherapy, including immunotherapies, patients with GBM still present a very poor prognosis. In this context, it is crucial to better understand glioma pathogenesis to identify new potential molecular targets. WNT6 has been recently identified as a new oncogenic molecule in GBM, with prognostic value in patients; however, the mechanisms underlying WNT6 aberrant expression in gliomas are still unknown.

Methods: *WNT6* mRNA levels were evaluated in glioma and stratified according to the new WHO glioma classification. *WNT6* copy number aberrations were assessed in glioma patients using an SNP array. DNA methylation was assessed in glioma patients using a DNA methylation array and in glioma cells by MSP after sodium bisulfite treatment. Glioma cell lines were treated with 5-Aza-2'-deoxycytidine to assess the impact of global DNA methylation in *WNT6* expression. *In silico* analyses were performed to identify potential *WNT6* transcriptional activators. ChIP analyses were used to test the direct transcriptional regulation of *WNT6* by HOXA9. Immuno-stainings and TCF/LEF reporter assays were used to assess the impact of HOXA9 expression in the activation of the WNT/β-catenin signaling pathway. Co-expression analyses were performed in patients. Patient survival analyses were performed with the Cox regression model adjusted for patient's age, Karnofsky Performance Status, gender, therapy, and *HOXA9* expression.

Results: *WNT6* is overexpressed in a subset of gliomas, independently of IDH mutation, 1p/19q co-deletion status, and *WNT6* gene copy number. Interestingly, *WNT6* expression associates with the DNA methylation levels of particular CpG regions at both the promoter and the gene body in glioma patients. Concordantly, 5-Aza global demethylation increased *WNT6* expression in glioma cells. The homeoprotein transcription factor HOXA9 was identified as a direct transcriptional regulator of *WNT6* in GBM, binding to its promoter region, and activated the WNT/β-catenin signaling pathway in *in vitro* and *in vivo* models of GBM. In various cohorts of glioma patients, *WNT6* and *HOXA9* mRNA levels were significantly correlated, extending our *in vitro* findings into the clinical setting. Interestingly, *WNT6*/*HOXA9* co-expression was not limited to glioma, having been observed also in patients with leukemia and pancreatic cancer, where HOXA9 has been shown to have oncogenic functions. Clinically, we validated *WNT6* as a prognostic factor of poor prognosis in GBM, independently of *HOXA9* expression, which has been previously associated with poorer clinical outcome.

Conclusion: Our findings describe for the first time *WNT6* regulatory mechanisms in GBM, establishing particular DNA methylation patterns and HOXA9 as novel regulators of WNT6 expression in

glioma. This knowledge, at the light of the clinical relevance of the WNT6/HOXA9 axis in GBM patients, may contribute to develop new targeted therapies for this deadly disease.

3.1. Introduction

Gliomas represent 81% of all malignant brain tumors, and have been traditionally classified by the World Health Organization (WHO) according to histological features into four malignancy grades (I to IV) [1, 2]. Glioblastoma (GBM, grade IV) is the most common and lethal glioma in adults, with a median survival of approximately 15 months after diagnosis [3, 4]. Although their clinical response is poor and unpredictable, patients with GBM are still equally treated with a standardized approach that includes surgery, radiotherapy and chemotherapy, mostly with the alkylating agent temozolomide (TMZ) [5, 6]. In the last years, substantial progress in the understanding of the molecular pathogenesis of gliomas has been achieved [6]. Although these advances resulted in improvements in the diagnosis and classification of glioma tumors, improvements in therapies that truly impact on patient outcomes are still needed [4]. In this context, there has been an increasing interest in the development of targeted therapies for the treatment of this deadly disease. This is because targeted therapies are potentially less toxic due to their increased tumor specificity, and the direct blockade of altered oncogenic pathways may have a direct impact on tumor cell proliferation. Unfortunately, the success of these new therapies has been mostly disappointing [7-18].

WNT6, a ligand and activator of the WNT pathway, was recently described to be overexpressed in GBM, having been associated with patients' poor prognosis (Chapter 2). Functionally, WNT6 expression was associated with increased GBM cell viability, proliferation, invasion, migration, resistance to TMZ, and stemness capacity (Chapter 2). *In vivo*, WNT6 accelerated mice GBM-associated death. Moreover, WNT6 was shown to contribute to the activation of the WNT, SFK and STAT pathways, which might be critical effectors of WNT6-associated aggressiveness in GBM (Chapter 2). Although the impact of WNT6 overexpression in GBM, as well as its downstream mechanisms, are now better understood, the molecular mechanisms underlying WNT6 overexpression in GBM remain unknown. In this context, this study aims to investigate the impact of *WNT6* gene copy number alterations and DNA methylation on its expression, as well as to identify novel potential direct regulators of WNT6 in GBM. Combining this information will further allow the development of more rational therapeutic approaches that may revert WNT6 activation in highly aggressive WNT6-positive GBMs.

3.2. Results

3.2.1. *WNT6* expression in glioma is independent of IDH mutation and 1p/19q co-deletion status

While we previously described that WNT6 expression increases with glioma grade (Chapter 2),

whether this is associated or not with IDH mutation and 1p/19q co-deletion status – the diagnostic molecular factors used in the new 2016 CNS tumor classification [4] – was still unknown. Here, we confirmed that *WNT6*-high expression increases with grading in patients from TCGA (RNAseq data; 226 grade II, 240 grade III and 161 grade IV gliomas; Figure 3.1A), and that this is independent of IDH

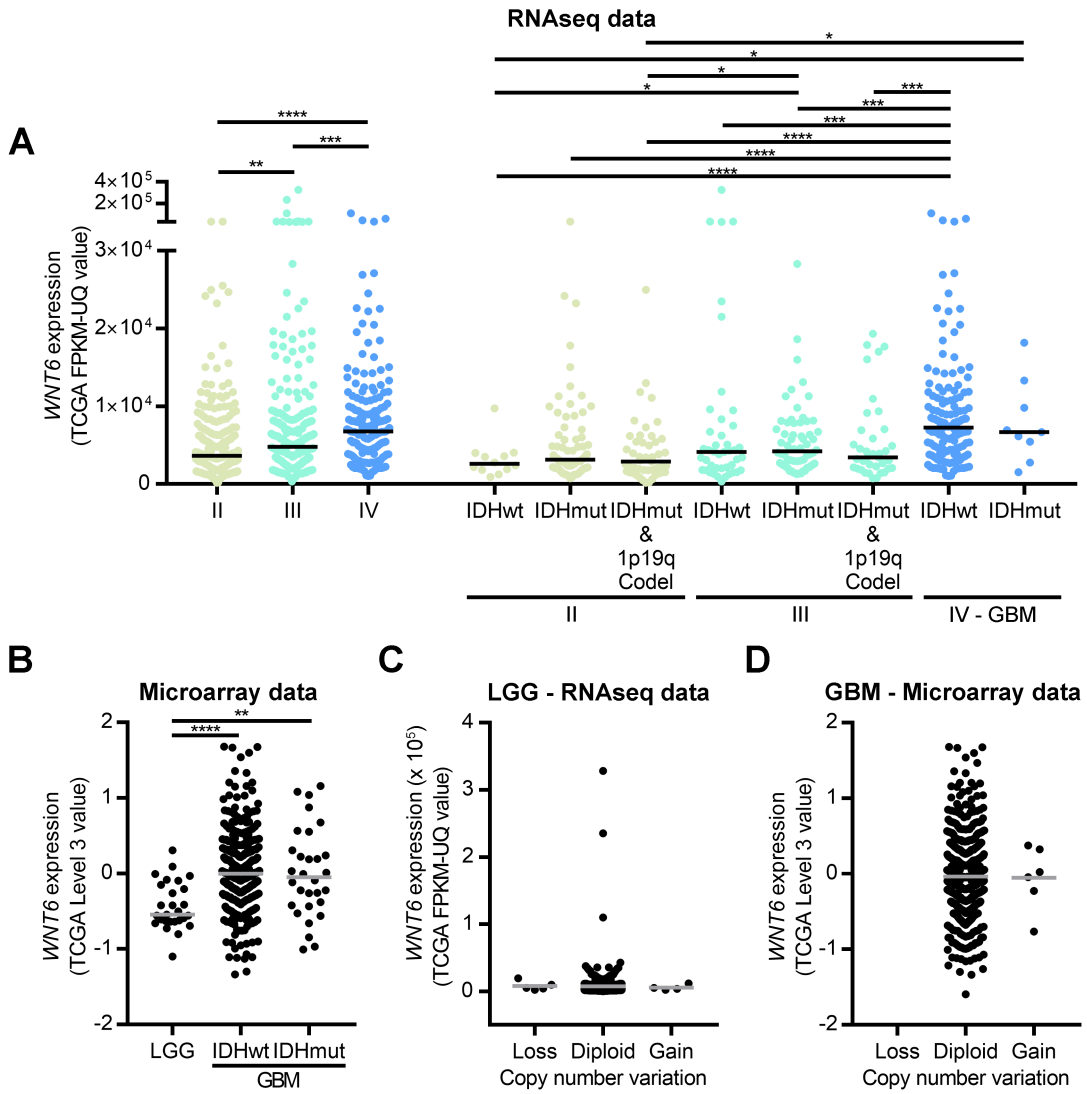

Figure 3.1. *WNT6* is overexpressed in a subset of gliomas, independently of IDH mutation, 1p/19q co-deletion status, and *WNT6* copy number.
(A) RNA sequencing expression levels of *WNT6* in grade II gliomas (12 IDH-wildtype, 75 IDH-mutant non-co-deleted, and 47 IDH-mutant co-deleted), in grade III gliomas (43 IDH-wildtype, 62 IDH-mutant non-co-deleted, and 37 IDH-mutant co-deleted), and in GBM (143 IDH-wildtype, 9 IDH-mutant) patients from TCGA. *WNT6* is highly expressed in 23.5%, 31.7% and 50% of grade II, III and IV gliomas, respectively; and in 8.3%, 22.7% and 15% of IDH-wildtype, IDH-mutant non-co-deleted and IDH-mutant co-deleted grade II, in 25.6%, 27.4% and 27% of IDH-wildtype, IDH-mutant non-co-deleted and IDH-mutant co-deleted grade III, and in 52.4% and 44% of IDH-wildtype and IDH-mutant grade IV (GBM). **(B)** Microarray expression levels of *WNT6* in 27 lower-grade gliomas (LGG; grades II and III) and in GBM (368 IDH-wildtype and 30 IDH-mutant) patients from TCGA. *WNT6* is highly expressed in 7.4% of LGG, in 50% of IDH-wildtype GBM and in 47% of IDH-mutant GBM. **(C)** *WNT6* RNAseq expression levels stratified by copy number aberration in LGG patients (5 with gene deletion, 500 with no alterations and 4 with gene amplification). **(D)** *WNT6* microarray expression levels stratified by copy number aberration in GBM patients (563 with no alterations and 2 with gene amplification; no deletions were found). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$; and ****, $p < 0.0001$ (Mann-Whitney test). IDHwt: IDH-wildtype; IDHmut: IDH-mutant; 1p19q Codel: 1p/19q co-deletion.

mutation and 1p/19q co-deletion status (12 IDH-wildtype, 75 IDH-mutant non-co-deleted, and 47 IDH-mutant co-deleted grade II; 43 IDH-wildtype, 62 IDH-mutant non-co-deleted, and 37 IDH-mutant co-deleted grade III; and 143 IDH-wildtype and 9 IDH-mutant GBM for TCGA RNAseq data, Figure 3.1A; and 27 lower-grade II and III glioma – LGG; and 368 IDH-wildtype and 30 IDH-mutant GBM for TCGA microarray data, Figure 3.1B). Due to the limited number of LGG patients with available microarray data (n = 27), these cases were not divided according to the 2016 CNS classification (Figure 3.1B); instead, RNAseq data was used to verify the relationship between *WNT6* expression and the new diagnostic molecular factors in LGG (Figure 3.1A), including a total of 466 LGG patients, and 161 GBM cases. Together, these results show that *WNT6* expression associates with higher glioma grade and is independent of IDH mutation and 1p/19q co-deletion status.

3.2.2. *WNT6* expression is regulated by DNA methylation in gliomas

Gene and chromosomal copy number alterations are a major mechanism of gene expression deregulation in cancer [19-21]. Oncogenic molecules, as *EGFR*, are commonly amplified in GBM, while tumor suppressor genes, like *PTEN*, are frequently deleted [22]. To understand the mechanisms responsible for *WNT6* overexpression in glioma, we started by investigating copy number alterations of *WNT6* locus in LGG (n = 509) and GBM (n = 565) patients from TCGA (Figure 3.1C-D). In LGG, only 4 patients (0.79%) presented *WNT6* gene amplification, while the region encompassing *WNT6* was deleted in 5 cases (0.98%; Figure 3.1C). In GBM, only 2 patients (0.35%) presented *WNT6* gene amplification (Figure 3.1D), and no deletions were observed. Both in LGG and GBM, *WNT6*-amplified cases did not present high expression levels of *WNT6*. Thus, considering the infrequency of *WNT6* amplification and the absence of an association with *WNT6* expression levels, copy number aberrations are not likely a major mechanism mediating *WNT6* overexpression in GBM.

DNA methylation is an epigenetic regulatory mechanism that also plays important roles in cancer [23, 24]. For example, *MGMT* promoter methylation is a well-established mechanism of *MGMT* silencing in glioma, associating with patients' better response to alkylating chemotherapeutic drugs, particularly TMZ (as discussed in section 1.3.3, Chapter 1). In this context, we also evaluated *WNT6* DNA methylation in glioma patients, and investigated whether it might be associated with *WNT6* expression levels (Figure 3.2). By evaluating a total of 28 methylation sites within the *WNT6* locus, in 516 LGG and 141 GBM patients, we identified regions that are consistently hypomethylated (e.g., from the 4th probe [cg16256504] to the 8th probe [cg02175741]) or hypermethylated (e.g., 16th probe [cg05618201]) both in LGG and GBM (Figure 3.2A and Figure 3.S1).

To further clarify whether there is a direct association between *WNT6* DNA methylation and

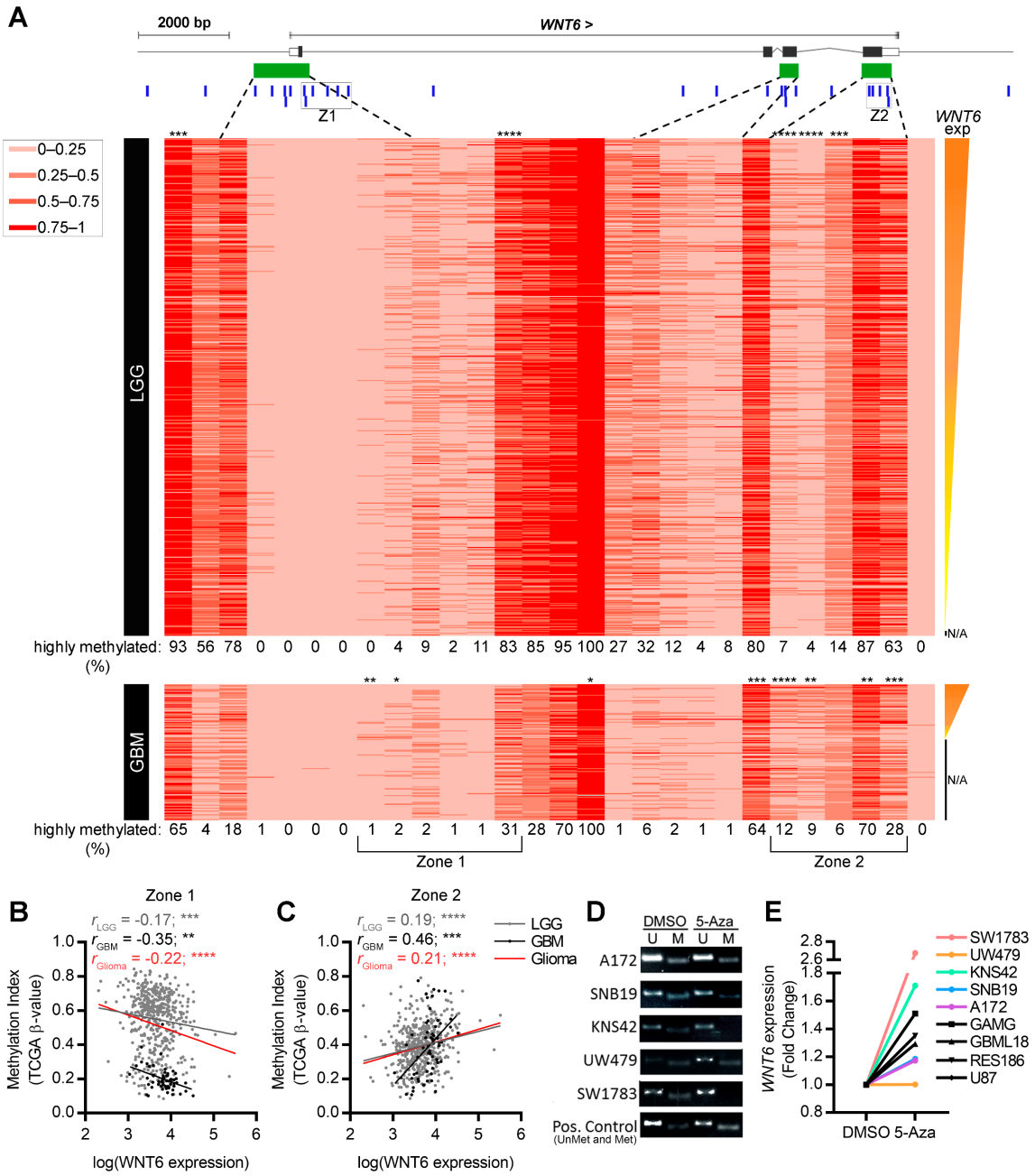

Figure 3.2. *WNT6* is transcriptionally regulated by DNA methylation in gliomas.

(A) Heatmap representation of DNA methylation levels (TCGA β-values) corresponding to *WNT6* locus in 516 LGG (top) and 141 GBM (bottom) patients from TCGA. Each column corresponds to a probe and each line to a patient. A total of 28 methylation probes (vertical blue bars) were assessed. CpG islands >300 bp are represented in green. Coding exons are represented by blocks connected by lines representing introns. The white rectangles at the ends (left and right) represents the 5' and 3' UTR, respectively. The methylation color code with TCGA β-values is shown on the top left. Patients are ranked based on *WNT6* expression, as shown at the right. The percentage of highly methylated cases (TCGA β-values ≥ 0.5) are depicted for LGG and GBM below their respective heatmap. Probes whose methylation levels were statistically correlated with *WNT6* expression levels ($|r| > 0.15$) are marked with * above the respective column (Pearson's or Spearman's correlation, according to the normality of the samples, as tested by D'Agostino and Pearson normality test). **(B-C)** Correlation graph between *WNT6* expression (TCGA FPKM-UQ value) of 511 LGG (grey dots and linear regression line), 58 GBM (black dots and linear regression line) or 569 gliomas (red linear regression line) and DNA methylation index (TCGA β-values) of the probes from both selected regions (Zone 1 and 2). **(D)** MSP functional assessment of 5-Aza treated cells. **(E)** *WNT6* expression levels in glioma cell lines treated with DMSO or 5-Aza (the impact on DNA methylation was not assessed in cell lines represented in black). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$; and ****, $p < 0.0001$; N/A = Not available; Z1 = Zone 1; Z2 = Zone 2.

expression, statistical correlation analyses were performed for each probe in 511 LGG and 58 GBM for which both expression (TCGA RNAseq data) and DNA methylation data were available (Figure 3.2A). Interestingly, the DNA methylation of 5 probes (representing 1 CpG dinucleotide) were significantly correlated with *WNT6* expression in LGG patients (2 inversely correlated and 3 positively correlated; Figure 3.S1A-E). Similarly, in GBM, significant correlations were observed in 8 CpG sites (2 inversely correlated and 6 positively correlated; Figure 3.S1F-M). When we performed integrated analyses gathering information of DNA methylation of probes in the vicinity of those that presented significant correlations, we found that the DNA methylation of the first selected region (Zone 1) located closely downstream of the promoter (Figure 3.2B) was inversely correlated with *WNT6* expression in GBM (r = -0.35; p < 0.01) and glioma (r = -0.22; p < 0.0001) patients. On the other hand, the DNA methylation of the second selected region (Zone 2; encompassing the last CpG island in *WNT6* gene body) is positively correlated with *WNT6* expression in GBM (r = 0.46; p < 0.001) and glioma (r = 0.21; p < 0.0001) patients (Figure 3.2C). Similar results were obtained using TCGA GBM microarray data (including a higher number of GBM patients; n = 117; r = -0.25, p < 0.01; r = 0.32, p < 0.001, for the first and second region, respectively; data not shown).

Having characterized *WNT6* DNA methylation levels in the large cohort from TCGA based in microarray data, we further characterized *WNT6* DNA methylation levels in an independent Portuguese cohort (Hospital Santo António, n = 18) using methylation-specific PCR (MSP; Figure 3.2A). Primers for MSP were designed to detect the CpG's recognized by the first probes of selected zone 1 (Figure 3.2B; cg11175192 and cg06157334). As observed in the methylation array from TCGA, a great percentage of GBM patients (55.6%) presented *WNT6* hypomethylation in this region as assessed by MSP (Figure 3.S2).

To elucidate the role of global *WNT6* DNA methylation in its expression, a panel of glioma cell lines were treated with 5-Aza-2'-deoxycytidine (5-Aza), a global DNA demethylating agent (Figure 3.2D-E). 5-Aza mediated demethylation was validated through MSP in 5 treated cell lines (Figure 3.2D). Interestingly, 5-Aza treatment successfully increased *WNT6* expression in most, but not all, cell lines (7 out of 9; Figure 3.2E). Together, these results suggest that *WNT6* DNA methylation levels may at least partially contribute to regulate *WNT6* expression in glioma. Notwithstanding, other potential mechanisms are likely to play critical regulatory roles, namely some *WNT6* promoter binding transcription factors.

3.2.3. *WNT6* is transcriptionally activated by HOXA9 in GBM

It was previously shown that *WNT6* is regulated by CAV1 (a scaffolding protein) and *UCA1* (a long non-coding RNA) in gastric and bladder cancer, respectively [25, 26]. However, we found that, in the case of GBM, *CAV1* and *WNT6* are not correlated (r = -0.03, p = 0.66), and *UCA1* and *WNT6* are inversely

correlated (r = -0.29, p < 0.001). Thus, these molecules are unlikely responsible for promoting *WNT6* expression in GBM.

Searching for potential transcription factors, *in silico* analyses using the MatInspector tool (Genomatix) were performed, which revealed a significant number of potential transcription factors for *WNT6* (Figure 3.3A). Among them, we found potential binding sites for HOXA9 (Figure 3.3B), which, interestingly, our group has previously showed to present oncogenic roles and to be a prognostic biomarker in GBM [27, 28].

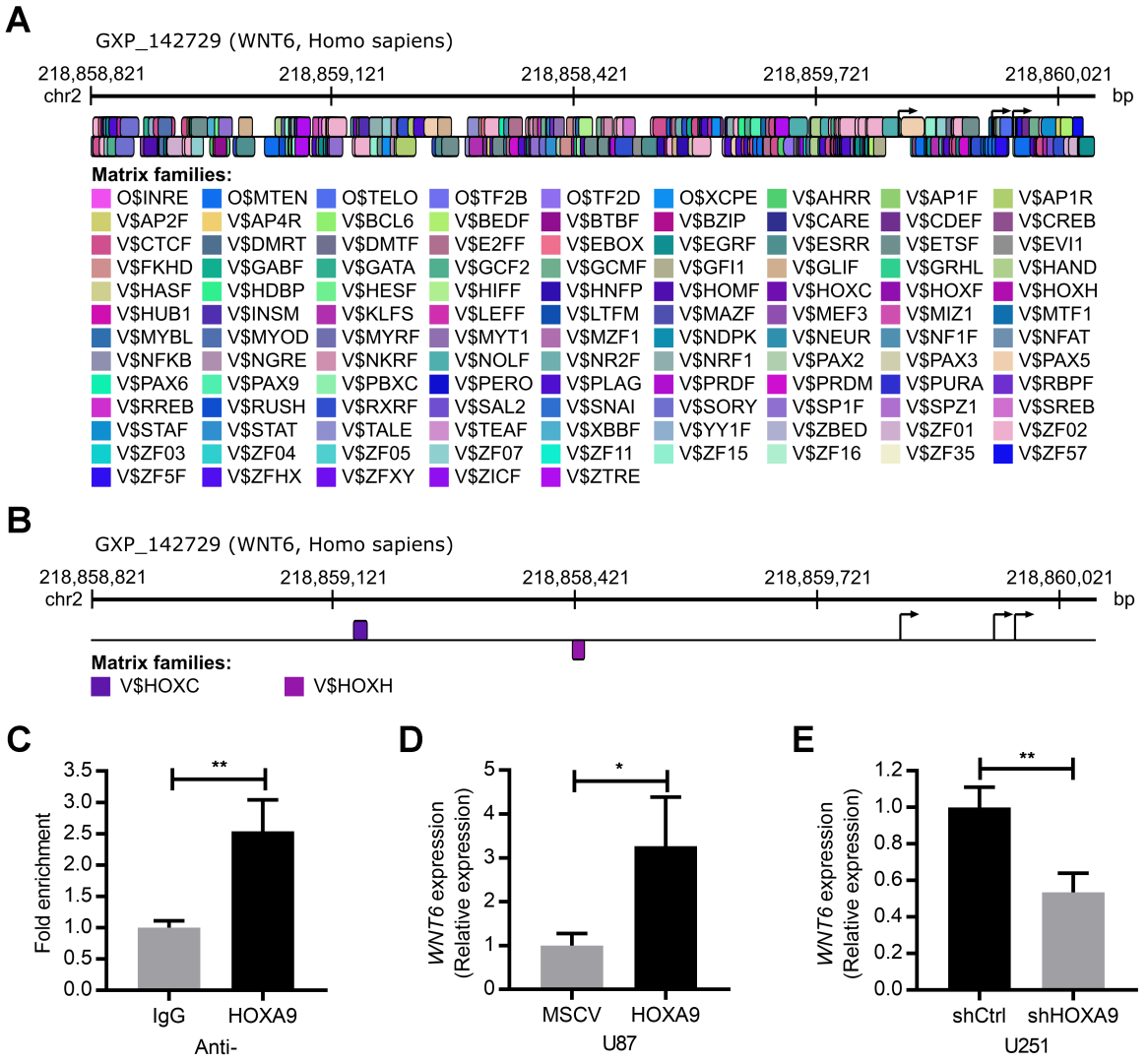

Figure 3.3. *WNT6* is a direct target of HOXA9 in GBM.
(A) MatInspector (Genomatix) representation of potential matrix families of transcription factors in *WNT6* promoter region. Each matrix match is represented by a colored rectangle and each color represents a matrix family. **(B)** MatInspector representation of specific potential HOXA9 binding sites within *WNT6* promoter region. Violet and pink represent the matrices HOX_PBX.01 (matrix sim = 0.83, sequence: ggtgG*GATggctg*gggg and family HOXC) and MEIS1A_HOXA9.01 (matrix sim = 0.86, sequence: *TGACaggttt*gttg*a* and family HOXH), respectively. Base pairs in italic appear in a position with a high conservation profile in the matrix (ci-value > 60). Base pairs in capital letters represent the core sequence used by the program. Matches represented on top of the sequence line were found on the positive strand, while matches found on the negative strand reside below the sequence line. (A-B) Putative transcription start sites (TSS) are marked by an

(continued on next page)

(cont.) arrow. Color codes for the matrix families are depicted below. **(C)** Chromatin Immunoprecipitation (ChIP) was performed to assess the putative binding of HOXA9 to the promoter region of *WNT6*, followed by qPCR. The fold enrichment presented is normalized to the input (DNA not exposed to immunoprecipitation – PCR positive control) and to the IgG background signal (ChIP negative control). Three independent experiments (mean and standard deviation) are represented. **(D-E)** *WNT6* qRT-PCR was performed in U87 cells transfected to overexpress HOXA9 (U87-HOXA9) and its negative counterparts (U87-MSCV; D), and in U251-shCtrl (HOXA9-high) and U251-shHOXA9 (HOXA9-low) cells (E). Three independent experiments (mean and standard deviation) are represented.

To test whether HOXA9 may effectively bind to the promoter region of *WNT6*, anti-HOXA9 chromatin immunoprecipitation (ChIP) on U251 GBM cells (endogenously expressing *HOXA9*) was performed (Figure 3.3C). We found that HOXA9 binds to *WNT6* promoter region in GBM cells ($p < 0.01$; Figure 3.3C), therefore demonstrating that WNT6 is a direct target of HOXA9 in GBM.

In this context, to validate that HOXA9 binding to the promoter region of *WNT6* activates its expression, *WNT6* qRT-PCR was performed in the previously described HOXA9 GBM cell models [27, 28]. Interestingly, *WNT6* expression increased upon *HOXA9* overexpression in U87 cells (Figure 3.3D), and decreased upon *HOXA9*-silencing in U251 cells (Figure 3.3E).

3.2.4. HOXA9 activates the WNT/β-catenin pathway

As previously seen in Chapter 2, WNT6 is an activator of the WNT/β-catenin pathway in GBM. Thus, we aimed to understand if HOXA9 may also increase the activity of this pathway. For this, we started by performing β-catenin immunofluorescences in U87-HOXA9 cells and their negative counterparts (MSCV; Figure 3.4A). Interestingly, β-catenin protein expression increased in U87-HOXA9 cells and showed to be mainly perinuclear. This association was not only observed *in vitro*, as U87+/-HOXA9 *in vivo* tumors grown subcutaneously in mice also showed significantly higher expression of WNT6 in HOXA9-positive tumors when compared to negative counterparts (Figure 3.4B), and higher expression of β-catenin, mainly in the nucleus. In addition, Cyclin D1, a transcriptional target of the WNT pathway, was also found upregulated in HOXA9-positive tumors when compared to negative tumors (Figure 3.4B).

To confirm that HOXA9 is able to activate the canonical WNT pathway, TCF/LEF reporter assays were performed in U87-MSCV and U87-HOXA9 cells. Concordantly to the aforementioned results, HOXA9-high GBM cells presented a significantly increased activation of the WNT canonical pathway as compared to their negative counterparts (U87-MSCV; Figure 3.4C-D).

Together, these results point out HOXA9 as a direct transcriptional activator of *WNT6*, consequently activating the WNT/β-catenin pathway in GBM.

3.2.5. *WNT6* and *HOXA9* are co-expressed in glioma patients

To validate the association of *WNT6* and *HOXA9* in the clinical context, the expression of these

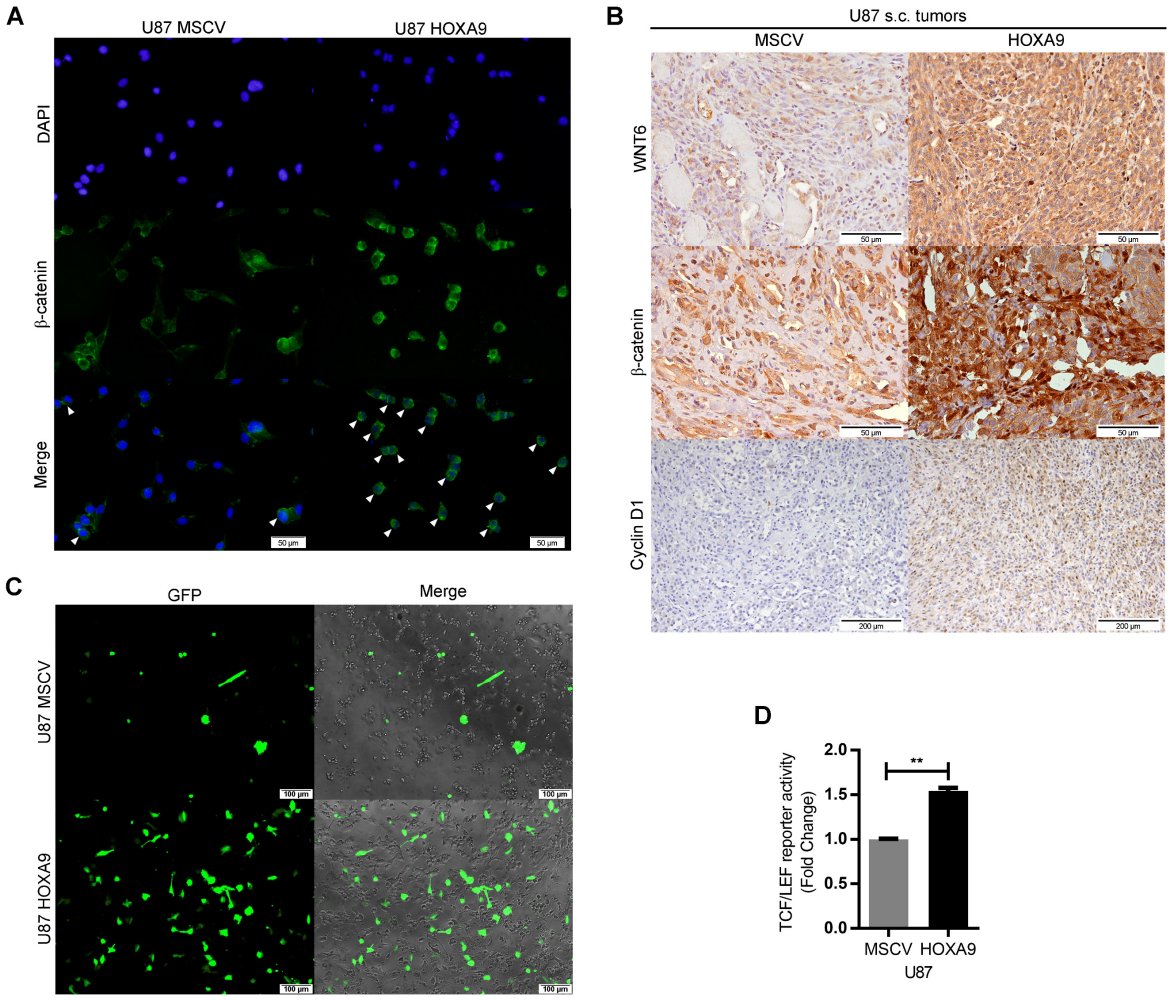

Figure 3.4. HOXA9 activates the WNT/β-catenin pathway in *In vitro* and *In vivo* models of GBM.
(A) β-catenin expression and sub-cellular localization in HOXA9-negative (U87-MSCV) and HOXA9-positive (U87-HOXA9) GBM cells was observed by immunofluorescence. White arrows indicate peri-nuclear stained cells. Representative images are displayed (200x magnification; scale bar = 50 μm). **(B)** WNT6, β-catenin and Cyclin D1 expression was analyzed by immunohistochemistry in subcutaneous tumors formed upon injection of HOXA9-negative and HOXA9-positive U87 cells in immunocompromised mice. Representative images are displayed (scale bars are specified in each image). **(C-D)** TCF/LEF reporter assay in U87-MSCV (HOXA9-negative) and U87-HOXA9. (C) Representative images are displayed (100x magnification; scale bar = 100 μm). (D) GFP expression was used as a measure of TCF/LEF promoter activity and was normalized against negative and positive controls. (n = 3 independent assays; mean ± SD; **, $p < 0.01$, two-sided unpaired *t*test).

two genes was analyzed in our glioma patients' cohort from Hospital Braga, Portugal (n = 31; Figure 3.5A), and in 3 additional larger independent cohorts (TCGA, n = 666; Bao, n = 274 and Gill, n = 75; Figure 3.5B-D). In all cohorts, *WNT6* and *HOXA9* levels were highly co-expressed ($r = 0.77$, $p < 0.0001$; $r = 0.22$, $p < 0.0001$; $r = 0.55$, $p < 0.0001$; $r = 0.25$, $p < 0.05$, for the Portuguese, TCGA, Bao and Gill datasets, respectively).

To understand if this association is exclusive of glioma tumors, the same analyses were performed in all cancer types from TCGA with available RNAseq expression data (Table 3.1 and Figure 3.6). Interestingly, *WNT6* and *HOXA9* were found to be co-expressed in other cancer types, including

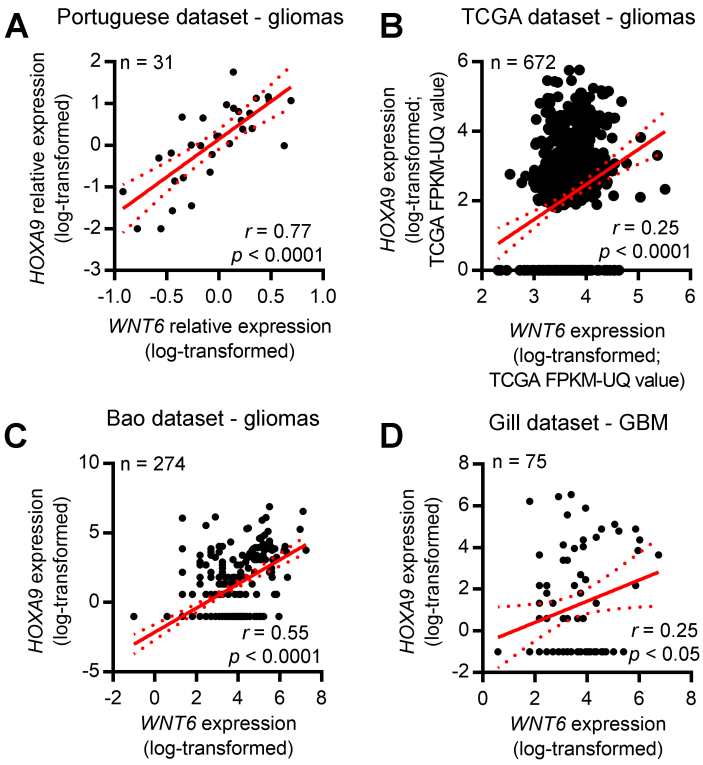

Figure 3.5. *WNT6* and *HOXA9* are co-expressed in glioma patients.
Correlation graphs between *WNT6* and *HOXA9* expression in glioma patients from **(A)** our Portuguese cohort (qRT-PCR data; n = 31; $r = 0.77$, $p < 0.0001$), **(B)** the TCGA dataset (RNAseq data; n = 672; $r = 0.25$, $p < 0.0001$), **(C)** Bao dataset (Microarray data; n = 274; $r = 0.55$, $p < 0.0001$), and **(D)** Gill dataset (Microarray data; n = 75; $r = 0.25$, $p < 0.05$). The red line represents the linear regression line, while dashed curved lines represent the 95% confidence interval.

leukemia, testicular germ cell tumor, melanoma and cholangiocarcinoma (Table 3.1 and Figure 3.6).

3.2.6. *WNT6* is prognostically valuable independently of *HOXA9* expression in LGG and GBM patients

We previously described that WNT6 (Chapter 2) and HOXA9 [27, 28] are each associated with decreased overall survival of GBM patients. In this work, we also investigated whether they would maintain their clinical significance independently of each other. Thus, the clinical impact of *WNT6* in GBM was evaluated using a multivariable Cox model to adjust to the potential confounding effect of other putative prognostic factors, namely patient age, KPS, gender and therapy, as well as *HOXA9* expression (Table 3.2). Interestingly, *WNT6* expression associated with shorter survival of GBM patients independently of *HOXA9* expression in GBM patients from the TCGA (n = 405; $p = 0.04$), with *HOXA9* also maintaining its clinical impact ($p = 0.02$; Table 3.2). This result suggests that both *WNT6* and *HOXA9* are critical and informative prognostic factors in GBM patients.

3.3. Discussion

WNT ligands are morphogen molecules important during embryogenesis [29, 30], whose

Table 3.1. *WNT6* and *HOXA9* co-expression in all cancer types with available RNAseq data in TCGA

Cancer code	Cancer designation	#	Correlation value	*p*-value
ACC	Adrenocortical carcinoma	79	0.20	0.077
BLCA	Bladder urothelial carcinoma	408	-0.11	**0.029**
BRCA	Breast invasive carcinoma	1091	0.21	**<0.0001**
CESC	Cervical squamous cell carcinoma and endocervical adenocarcinoma	304	0.00	0.989
CHOL	Cholangiocarcinoma	36	**0.38**	**0.021**
COAD	Colon adenocarcinoma	456	0.05	0.313
DLBC	Lymphoid neoplasm diffuse large B-cell lymphoma	48	0.02	0.892
ESCA	Esophageal carcinoma	161	-0.07	0.367
HNSC	Head and neck squamous cell carcinoma	500	0.07	0.108
KICH	Kidney chromophobe	65	0.07	0.596
KIRC	Kidney renal clear cell carcinoma	530	-0.01	0.845
KIRP	Kidney renal papillary cell carcinoma	288	-0.10	0.088
LAML	Acute myeloid leukemia	151	**0.53**	**<0.0001**
LIHC	Liver hepatocellular carcinoma	371	0.09	0.061
LUAD	Lung adenocarcinoma	513	0.23	**<0.0001**
LUSC	Lung squamous cell carcinoma	501	0.17	**0.000**
MESO	Mesothelioma	86	0.20	0.058
PAAD	Pancreatic adenocarcinoma	177	0.15	**0.044**
PCPG	Pheochromocytoma and paraganglioma	179	0.04	0.579
PRAD	Prostate adenocarcinoma	495	-0.07	0.104
READ	Rectum adenocarcinoma	166	0.07	0.359
SARC	Sarcoma	259	-0.05	0.416
SKCM	Skin cutaneous melanoma	466	**0.26**	**<0.0001**
STAD	Stomach adenocarcinoma	375	-0.07	0.172
TGCT	Testicular germ cell tumors	150	**0.45**	**<0.0001**
THCA	Thyroid carcinoma	502	0.17	**0.0001**
THYM	Thymoma	119	-0.22	**0.018**
UCS	Uterine carcinosarcoma	56	-0.22	0.108
UVM	Uveal melanoma	80	0.04	0.718

deregulated expression has been described in cancer, including GBM [31-37]. We recently showed that the WNT6 ligand is overexpressed in GBM and is associated with tumor aggressiveness *in vitro* and *in vivo* (Chapter 2). However, the mechanisms underlying WNT6 overexpression in GBM were still unknown. In this study, we investigated the upstream mechanisms regulating WNT6 in GBM, establishing *WNT6* DNA methylation and HOXA9 as potential regulators of WNT6 expression. Additionally, as we previously described that HOXA9 has a prognostic value in GBM [27, 28], we further investigated whether the prognostic value of *WNT6* expression in GBM patients was dependent on the status of *HOXA9*.

Our data show that *WNT6*-high expression in glioma increases with grade independently of IDH

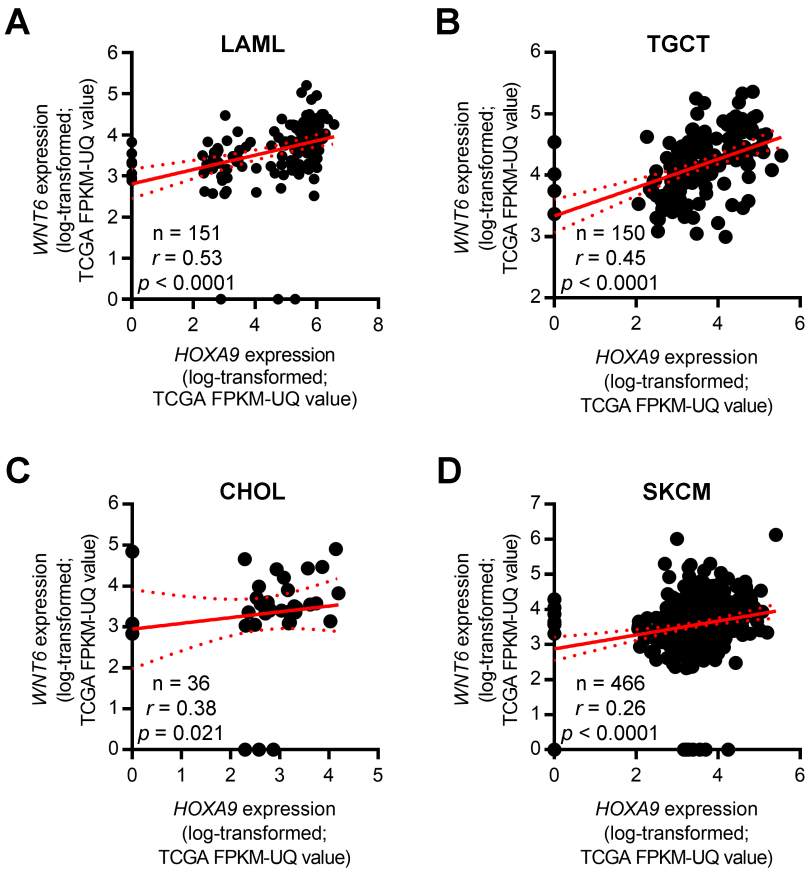

Figure 3.6. *WNT6* and *HOXA9* co-expression is not exclusive of glioma tumors.
Correlation graphs between *WNT6* and *HOXA9* expression in **(A)** acute myeloid leukemia (LAML; n = 151; $r = 0.53$, $p < 0.0001$); **(B)** testicular germ cell tumor (TGCT; n = 150; $r = 0.45$, $p < 0.0001$); **(C)** cholangiocarcinoma (CHOL; n = 36; $r = 0.38$, $p = 0.021$); and **(D)** skin cutaneous melanoma (SKCM; n = 466; $r = 0.26$, $p < 0.0001$). The red line represents the linear regression line, while dashed curved lines represent the 95% confidence interval.

Table 3.2. Cox multivariate analysis in GBM patients from TCGA

	Overall Survival		
	***p*-value**	**HR**	**95% CI**
***WNT6* expression**[a]	**0.041**	1.27	1.01-1.60
Age at diagnosis[a]	**<0.0001**	1.03	1.02-1.04
KPS[a]	**0.001**	0.98	0.97-0.99
Gender[b]	**0.03**	0.76	0.60-0.97
Treatment[c]	**<0.0001**	0.19	0.12-0.30
***HOXA9* expression**[d]	**0.022**	1.82	1.09-3.04

[a] *WNT6* expression, age and KPS were used as continuous variables; [b]Female vs. Male; [c]non-treated vs. Treated; [d] *HOXA9*-low vs. *HOXA9*-high expression.

mutation and 1p/19q co-deletion status (Figure 3.1). This result suggests that *WNT6* expression associates with glioma increased malignancy independently of the potential differences observed between glioma molecular subtypes (astrocytoma IDH-wildtype, astrocytoma IDH-mutant and oligodendroglioma). Considering this result and the previous description of WNT6 association with resistance to chemotherapy

(Chapter 2), it would be interesting to address the potential value of patient stratification based on WNT6 expression not only for prognosis, but also for therapy decision, similarly to what has been done for *MGMT* promoter methylation status within IDH-wildtype gliomas, in which patients whose tumors lack *MGMT* promoter methylation are treated with radiotherapy only [4].

To reach our goal of understanding the mechanisms underlying *WNT6* activation in glioma, we integrated data from (epi)genetic and *in silico* analyses from patients and cell lines. First, we observed that *WNT6* was expressed in a gene dosage independent manner in glioma (Figure 3.1). Contrarily, our findings demonstrate that DNA methylation, a critical epigenetic mechanism, may be tightly regulating *WNT6* expression in glioma (Figure 3.2 and Figure 3.S1), similarly to what was observed for other WNT ligands in other cancer types [38-42]. In particular, we observed that higher levels of promoter DNA methylation are associated with *WNT6* silencing, while gene body methylation is positively associated with its expression (Figure 3.2 and Figure 3.S1). This is in agreement to the known effect of promoter and gene body DNA methylation in gene expression regulation in normal and cancer cells [43-46]. Interestingly, most of the CpG sites are more frequently methylated in LGG than GBM patients (19 out of 28), suggesting that *WNT6* locus may be globally hypomethylated during tumor progression (Figure 3.2).

Although DNA methylation was clearly associated with the regulation of *WNT6* expression in glioma, this association was not universal. Thus, after ruling out the potential regulation of *WNT6* by CAV1 and *UCA1* in GBM, contrasting to what was described in gastric and bladder cancer [25, 26], we showed that HOXA9 is one of its direct transcriptional regulators in GBM (Figure 3.3), as it binds to *WNT6* promoter region, and that its expression correlates with and increases the levels of *WNT6* expression in GBM patients and cell models. HOXA9 is an oncogenic transcription factor whose expression has been associated with increased GBM aggressiveness and patients' poor survival [27, 28]. Interestingly, when performing gene set enrichment analysis (GSEA; Chapter 2, section 2.2.9) to identify transcriptomic signatures reminiscent of *WNT6*-associated genes in GBM patients, we observed that *WNT6*-negatively correlated genes were enriched for genes up-regulated in acute myeloid leukemia cells upon HOXA9 knockdown (ES = -0.26 and FDR = 0.18; data not shown), further supporting the link between WNT6 and HOXA9. Indeed, *WNT6* and *HOXA9* were not only co-expressed in glioma (Figure 3.5), but also in leukemia, testicular germ cell tumors, cholangiocarcinoma and melanoma (Figure 3.6 and Table 3.1). However, the roles of either WNT6 or HOXA9 in these tumors are largely unknown, except in leukemia where the oncogenic role of HOXA9 is well established [47-50]. Indeed, it would be interesting to evaluate if, in acute myeloid leukemia, HOXA9 also activates *WNT6* expression, and whether WNT6 displays oncogenic roles, as we observed in GBM. Since the role of WNT6 is not known in leukemia, our results

suggest that, similarly to what is observed in glioma (Chapter 2), WNT6 might be regulated by HOXA9 and may thus potentially have an important role in this tumor. Further studies are warranted to study the role of both WNT6 and HOXA9 in the remaining tumors.

Moreover, we demonstrated that WNT6 and HOXA9 cooperate in activating the WNT/β-catenin signaling pathway in GBM, suggesting WNT6 and the WNT/β-catenin signaling pathway as effectors of HOXA9-mediated aggressiveness in GBM [27, 28]. Nonetheless, we showed that *WNT6* is prognostically valuable independently of *HOXA9* in GBM patients (Table 3.2), suggesting that it may have an important oncogenic role and mechanisms of transcriptional regulation apart from those dependent on HOXA9 function. For example, WNT6 may influence patient outcome by modulating the tumor microenvironment/immunologic landscape [51], or even by regulating the glioma stem cells population [52], thus influencing tumor pathophysiology/recurrence and ultimately patient prognosis. Future works should explore the potential therapeutic value of targeting the HOXA9/WNT6/WNT pathway axis in GBM, for which novel treatments are urgently needed.

In summary, this work provides significant insights on the mechanisms underlying WNT6 and HOXA9 aggressiveness in GBM, which may potentially influence GBM patients' management by aiding treatment decisions and prognostic stratifications, while also opening new opportunities to identify or develop potentially more effective therapies for these highly resistant tumors.

3.4. Materials and methods

3.4.1. TCGA data analysis in glioma patients

The Cancer Genome Atlas (TCGA; *https://portal.gdc.cancer.gov/*) was used to obtain information about gene expression from LGG (n = 27), GBM (n = 572), adrenocortical carcinoma (ACC; n = 79), bladder urothelial carcinoma (BLCA; n = 408), breast invasive carcinoma (BRCA; n = 1091), cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC; n = 304), cholangiocarcinoma (CHOL; n = 36), colon adenocarcinoma (COAD; n = 456), lymphoid neoplasm diffuse large B-cell lymphoma (DLBC; n = 48), esophageal carcinoma (ESCA; n = 161), head and neck squamous cell carcinoma (HNSC; n = 500), kidney chromophobe (KICH; n = 65), kidney renal clear cell carcinoma (KIRC; n = 530), kidney renal papillary cell carcinoma (KIRP; n = 288), acute myeloid leukemia (LAML; n = 151), liver hepatocellular carcinoma (LIHC; n = 371), lung adenocarcinoma (LUAD; n = 513), lung squamous cell carcinoma (LUSC; n = 501), mesothelioma (MESO; n = 86), pancreatic adenocarcinoma (PAAD; n = 177), pheochromocytoma and paraganglioma (PCPG; n = 179), prostate adenocarcinoma (PRAD; n = 495), rectum adenocarcinoma (READ; n = 166), sarcoma (SARC; n = 259), skin cutaneous

melanoma (SKCM; n = 466), stomach adenocarcinoma (STAD; n = 375), testicular germ cell tumors (TGCT; n = 150), thyroid carcinoma (THCA; n = 502), thymoma (THYM; n = 119), uterine carcinosarcoma (UCS; n = 56), and uveal melanoma (UVM; n = 80). Agilent G4502A 244K data was used for LGG and GBM (WNT6-high expression was considered when TCGA level 3 value ≥ 0 [GBM median value]), while RNAseq data (Illumina HiSeq 2000 sequencing system) was download for all cancers (WNT6-high expression was considered when TCGA FPKUM-UQ value ≥ 6800 [GBM median value]) [53]. In the Agilent microarray, three probe sets hit *WNT6* gene (A_23_P119916, A_32_P159877 and A_24_P208513) and one hits *HOXA9* (probe A_23_P500998). To prevent duplicated entries from the same patient – when more than one portion per patient was available – the median expression value was used. The provided value was pre-processed and normalized according to "level 3" specifications of TCGA.

Gene copy number alterations, and DNA methylation status, as well as clinical information, when available, were also obtained from LGG and GBM patients [53].

Gene copy number data from 372 GBM and 514 LGG samples was assessed using Affymetrix Genome-Wide Human SNP Array 6.0. Gene amplifications or deletions were considered for Log_2 copy number tumor/normal ≥ 0.32 (gene copy number ≥ 2.5) or ≤ -0.42 (gene copy number ≤ 1.5), respectively.

DNA methylation data was evaluated using Illumina Infinium Human DNA Methylation 450 array and includes the methylation status of 141 GBM and 516 LGG samples. For this study, 28 probes that span from 5 000 bp upstream to 5 000 bp downstream of the *WNT6* gene were selected. This region of approximately 24 000 bp includes 3 CpG islands.

Clinical data of each patient was provided by the Biospecimen Core Resources (BCRs) and includes information about patients' age at diagnosis, gender, Karnofsky performance status (KPS) and days to death and to last follow-up.

3.4.2. Glioma primary samples

Glioma tumor specimens were obtained from patients who performed a craniotomy for tumor removal or stereotaxic biopsy at 2 different hospitals: Hospital Santo António (HSA, Centro Hospital Porto) and Hospital Braga (HB), Portugal, in a total of 18 GBM and 31 glioma patients, respectively. HSA samples were reserved for DNA-based studies, while HB samples were used for RNA-based studies. All samples were transported in dry ice to the lab and stored at -80 °C. Only patients with glial tumor histological diagnosis were included in the study.

Written informed consent for investigation purposes was obtained from all patients. All procedures were in accordance with institutional ethics standards. No patient information was collected.

WNT6 and *HOXA9* expression microarray data from Bao (n = 274) [54] and Gill (n = 75) [55] datasets of glioma patients were obtained from the GlioVis data portal [56].

3.4.3. GBM cell lines

The commercially-available pediatric glioma cell lines (KNS42, Res186, and UW479) were cultured in DMEM/F12 (Gibco) supplemented with 10% Fetal bovine serum (FBS; Biochrom). Commercially available adult cell lines (A172, GAMG, SNB19, SW1783, and U87MG) and the primary GBM-derived culture (GBML18; established in our lab) were cultured in DMEM (Biochrom) supplemented with 10% FBS. The U87MG cell line was previously [27] genetically retrovirally infected with murine stem cell virus (MSCV) containing the *HOXA9* coding region to overexpress this gene (U87-HOXA9) or with an empty vector (U87-MSCV, control). While U251 cells, which presents endogenous levels of *HOXA9*, were previously [28] transfected with a shRNA against *HOXA9* to silence its expression (U251 shHOXA9) or with a non-effective shRNA vector (U251 shCtrl). All cells were maintained in a humidified atmosphere at 37 °C and 5% (v/v) CO_2.

3.4.4. 5–Aza-2′-deoxycytidine (5-Aza) treatment

Glioma cell lines (SW1783, UW479, KNS42, SNB19, A172, GAMG, RES186, and U87MG) and a primary cell line (GBML18; established in the lab) were plated in T25-flasks at an initial concentration of 75 000 cells per T25. Treatment with 5 µM 5-Aza (Sigma-Aldrich®) or Dimethyl sulfoxide (DMSO; Sigma-Aldrich®) was performed for 72 hours with daily renewal. Next, cells were collected by trypsinization, and DNA and RNA were extracted by the TRIzol method (Invitrogen).

3.4.5. Sodium bisulfite treatment

DNA was extracted from 18 GBM primary tumor and glioma cell lines using the TRIzol method (Invitrogen). After quantification, it was subjected to sodium bisulfite treatment – conversion of unmethylated cytosines to uracil residues –, according to manufacturer's instructions (EZ DNA Methylation-Gold™ Kit; Zymo Research).

3.4.6. Methylation-Specific PCR (MSP)

WNT6 DNA methylation was evaluated by MSP on bisulfite converted DNAs, using the following sets of primers: unmethylated set, Fwd 5'-TTTTGTGTTCGGCGTACGT-3' and Rev 5'-AATCTATCCTAAATCCCGAA-3'; methylated set, Fwd 5'-TGTTGTTGTTTTTGTGTTTGGTGTAT-3' and Rev 5'-CCCCAATCTATCCTAAATCCCA-3'. Touchdown MSP was performed (AmpliTaq Gold 360; annealing temperature for unmethylated or methylated primers at 62 °C or 60 °C, respectively – decrement of 1 °C

per cycle for 10 cycles – and 52 °C or 50 °C, respectively, for additional 28 cycles). A bisulfite-treated blood DNA of a control subject (NB569) was used as an unmethylated control for MSP reactions. The same DNA was *in vitro* methylated (CpG Methyltransferase M.SssI; New England Biolabs Inc.) according to manufacturer's protocol, followed by sodium bisulfite treatment, and used as a methylated control. All MSP products were loaded onto a 3.5% agarose gel.

3.4.7. qRT-PCR

Total RNA was extracted from cell lines using the TRIzol method (Invitrogen), and cDNA from 1 µg of the total RNA was synthesized (RT-Phusion Kit, Thermo Scientific) [57]. *WNT6* and *TBP* (reference gene) levels were assessed by quantitative reverse transcription-PCR (qRT-PCR; KAPA SYBR® FAST qPCR Kit, KAPA BIOSYSTEMS) with the following sets of primers: *WNT6* Fwd 5'– GACGAGAAGTCGAGGCTCTTT–3' and Rev 5'–CGAAATGGAGGCAGCTTCT–3'; *TBP* Fwd 5'– GAGCTGTGATGTGAAGTTTCC–3' and Rev 5'–TCTGGGTTTGATCATTCTGTAG–3'. For both sets, the annealing temperature was 60 °C. Levels were determined based on the $2^{-\Delta\Delta Ct}$ method, as previously described [58]. The expression data from the Portuguese glioma dataset was log-transformed (log[relative expression + 1]).

3.4.8. Genomatix analysis

MatInspector from Genomatix software [59] (*www.genomatix.de*) was used to investigate putative binding sites for transcription factors in the *WNT6* gene. This *in silico* tool identifies transcription factor binding sites in nucleotide sequences based on a large library of weight matrices [59, 60]. A perfect match gets a matrix similarity of 1 when the tested sequence corresponds to the most conserved nucleotide at each position of the matrix. A good match to the matrix was considered when matrix similarity > 0.80. The Ci-value (consensus index) for the matrix represents the degree of conservation of each position within the matrix. A Ci-value of 100 is reached by a position with total conservation of one nucleotide.

3.4.9. Chromatin Immunoprecipitation (ChIP)

ChIP experiments were done as previously described [61]. In brief, cells were cross-linked with 1.42% formaldehyde for 15 minutes, followed by quenching with 125 mM glycine for 5 minutes. Cells were lysed with immunoprecipitation buffer (150 mM NaCl, 50 mM Tris-HCl, 5 mM EDTA, 0.5% NP-40, 1% Triton X-100) and chromatin was sheared by sonication (Sonics Vibra Cell VC70T, 21 cycles for 15 seconds) to obtain DNA fragments of approximately 0.5-1 kb. The volume of sheared chromatin equivalent to 2 million cells was incubated with the required antibody in an ultrasonic bath for 15 minutes, followed by incubation with protein A-agarose beads (Amersham) and Chelex 100 (Bio-Rad). The following

antibodies were used to immunoprecipitate chromatin: 4 μg anti-HOXA9 (Santa Cruz), 2 μg anti-Histone H3 (H3; Abcam), 3 μg anti-Immunoglobulin G (IgG; Sigma). DNA amplification was done by qPCR (Maxima SYBR Green, Fermentas) with the following set of primers for *WNT6*: Fwd 5'-CAGGGGCATCAAAGACATTT-3' and Rev 5'-TCAAGAGATCGAGGGGTCAG-3', designed to amplify a portion of the promoter region 1000bp upstream the transcription start region. The annealing temperature was 60°C. Anti-histone H3 and anti-IgG were used as ChIP positive and negative controls, respectively. The input represents a control for the amount of DNA used in precipitations. The level of *WNT6* was calculated for each experiment using the $2^{-\Delta\Delta Ct}$ method as previously described [58]. For each cell line, three biological replicates were tested, and each qPCR experiment was done in triplicate.

3.4.10. Immunofluorescence (IF)

For β-Catenin IF (610153, BD Transduction Laboratories, 1:200), U87-MSCV and U87-HOXA9 cells were fixed in 95% ethanol and 5% acetic acid (v/v). Alexa Fluor® 488 Goat Anti-mouse IgG (H+L; Alfagene) secondary antibody (green) was used. DAPI (VECTASHIELD® Mounting Medium with DAPI, Vector Laboratories) was used to stain the cell nucleus (blue) at a concentration of 1.5 μg/ml.

3.4.11. Immunohistochemistry (IHC)

Tissues sections were deparaffinized and rehydrated by xylene and ethanol series, as described in [52]. Immunohistochemical staining was performed using the LabVision kit (UltraVision Large Volume Detection System Anti-polyvalent, HRP) according to the manufacturer's instructions. WNT6 antibody from abcam (ab50030; 1:450), β-Catenin antibody from BD Transduction Laboratories (610153; 1:150) and Cyclin D1 antibody from Cell Signaling (2978S; 1:100) were used. DAB substrate (DAKO) was used as chromogen, followed by counterstaining with hematoxylin.

3.4.12. Mice GBM subcutaneous injection

The procedures for the establishment of the mice GBM subcutaneous models were previously described by Pojo *et al.* [28].

3.4.13. Statistical analysis

Correlation values were calculated using the Pearson's or Spearman's correlation coefficient, according to the normality of the samples (tested by the D'Agostino and Pearson normality test). Homoscedasticity was verified with the Levene's test, and differences between groups were assessed by a two-sided unpaired *t*-test, with Welch's correction being applied accordingly. GraphPad Prism 6.01 software was used (GraphPad software, Inc.).

Survival analyses were performed by multivariate analysis using the Cox proportional hazard model, where the potential confounding effect of some variables is considered. These analyses were made with SPSS 22.0 software (SPSS, Inc.)

For all statistical tests, significance was considered when $p < 0.05$.

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Author contributions

CSG, NS and BMC designed research studies. CSG, AXM, MP, and EPM conducted experiments. CSG, AXM, MP, EPM, CP, NS, and BMC analyzed data. AAP, MMP, RR provided patient samples. CSG and BMC wrote the manuscript.

Competing interests

The authors have declared that no competing interest exists.

Appendixes

Appendixes including:

- Supplementary Figures and Legends.

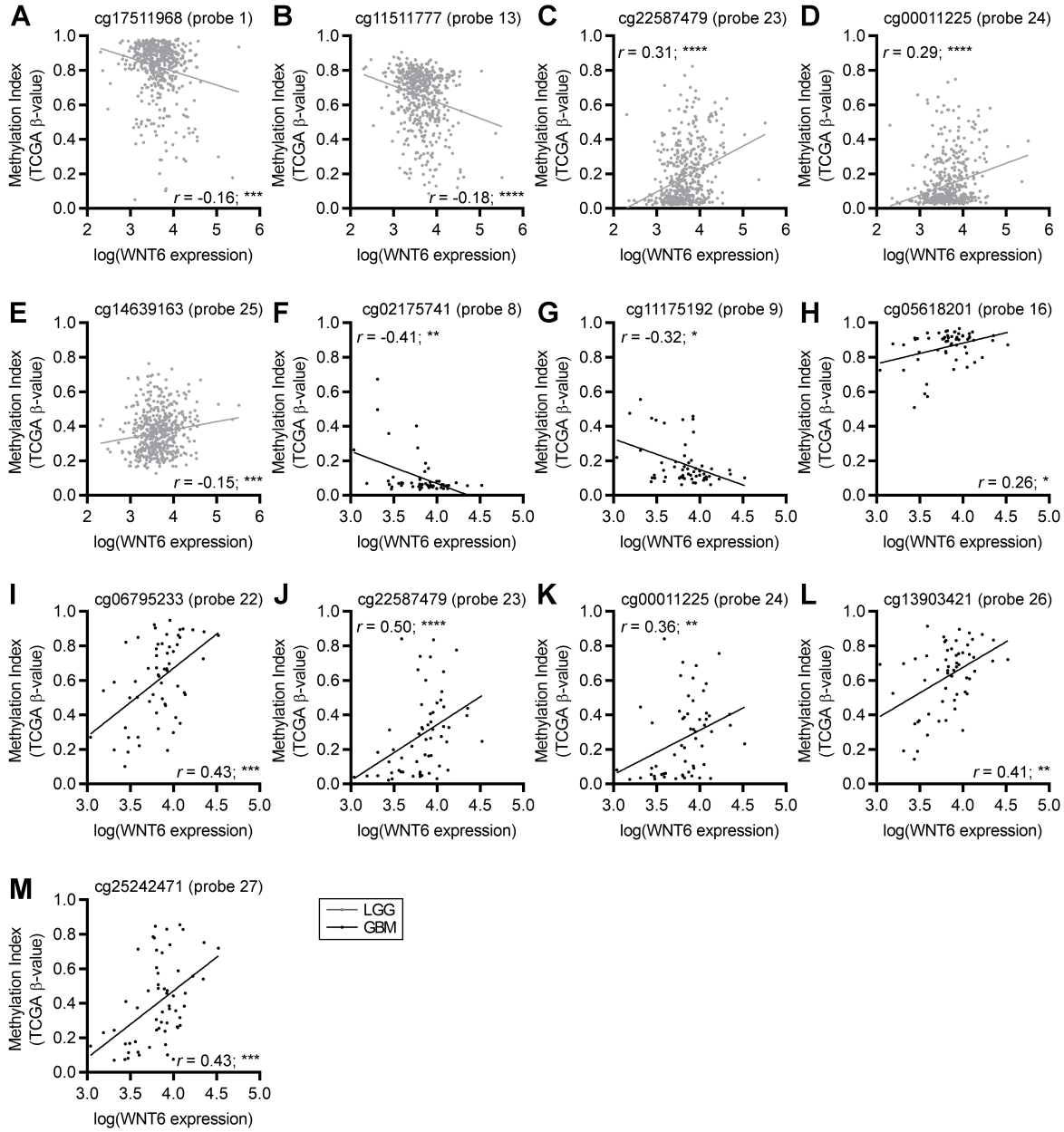
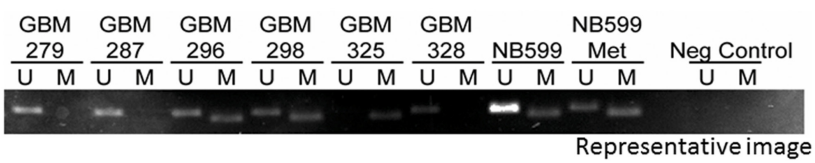


Figure 3.S1. *WNT6* DNA methylation correlates with *WNT6* expression in gliomas.
Correlation graphs between *WNT6* expression (x-axis) and its DNA methylation from different CpG sites (y-axis) in LGG **(A-E)** and GBM **(F-M)**. The indicated probe number is based on Figure 3.2. Spearman's correlation test *r* are indicated. *, $p < 0.05$, **, $p < 0.01$; ***, $p < 0.005$; and ****, $p < 0.001$.



WNT6 methylation status	Unmethylated	Methylated
Number of patients	10/18	8/18
Percentage	55.6%	44.4%

Figure 3.S2. *WNT6* DNA methylation in a GBM Portuguese cohort.
MSP analyses of 18 GBM patients from Hospital Santo António. Hypermethylation was considered whenever a methylated band was observed. Top: representative image. Bottom: summary of the results.

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Chapter 4

General discussion & future perspectives

This chapter presents an integrated general discussion of the major results of this thesis, contextualizing them within the recent literature, and discussing potential future questions to address. At the end, a brief conclusion of the thesis is given.

Contents

4.1. General discussion

Gliomas are the most frequent type of primary malignant brain tumors and constitute a major source of morbidity and mortality, ranking first in average of years of life lost among all tumor types [1-3]. Despite the advances in the understanding of their pathophysiology, there are still no curative therapies available [4]. Therefore, the identification of new molecular markers with prognostic and potential therapeutic value is urgent. As mentioned before, in physiological conditions, the WNT pathway controls cell fate, proliferation, migration, polarity, and death [5]. Deregulated WNT pathway has been reported in several cancers, including GBM, the most malignant type of glioma, and has been implicated in promoting tumor initiation, growth, invasion/metastasis, immune evasion, and resistance to current therapies [6-8]. This increased resistance has been attributed to stem cell-like features sustained by WNT signaling, such as increased self-renewal capacity, and tumorigenic potential [9]. While the importance of the WNT pathway has been largely explored in several tumor types, little is known about the roles of WNT6, a ligand of this pathway, in cancer [10-13]. Indeed, this thesis compiles the first studies focusing on WNT6 functional role, and WNT6 upstream regulatory and downstream effector mechanisms in human gliomas (Chapter 2 and Chapter 3).

In our first study (Chapter 2), we found that WNT6 is overexpressed in GBM, when compared to less malignant gliomas, associating it with increased glioma malignancy. By genetically manipulating WNT6 expression in several GBM cell models, we studied the functional impact of WNT6 expression in this deadly disease. We observed that WNT6 influences several cancer-associated aggressiveness features, such as viability, proliferation, invasion, migration, sensitivity to TMZ-based chemotherapy, and cancer stem cell features, independently of other well-known oncogenic WNT ligands (WNT1 and WNT3a; Chapter 2). These results implicate *WNT6* as a novel oncogene in human gliomas, similarly to what was previously described for other WNT ligands [14-18]. Indeed, we are aware of the potential redundancy of WNT ligands in cancer. Notwithstanding, although *WNT6* is co-expressed with some WNT ligands in GBM (Table 4.1), it was still significantly associated with GBM patients' shorter overall survival independently of all other WNT ligands, except for *WNT8b* (only marginally above the significance level: $p = 0.052$; n = 403; TCGA dataset) and *WNT10a*, which were not *per se* prognostic biomarkers for GBM patients (Table 4.2). Moreover, besides *WNT6*, only *WNT5b* was significantly associated with poor prognosis of these patients ($p = 0.017$; n = 403; TCGA dataset). Importantly, the prognostic value of WNT6 was validated in 5 additional independent cohorts, in which WNT6 expression was assessed using different techniques (Agilent and Affymetrix microarrays, qRT-PCR, and IHC). The consistency of these findings across multiple

Table 4.1. *WNT6* co-expression with other WNT ligands in glioma

	LGG		GBM	
WNT ligand	***r***	***p*-value**	***r***	***p*-value**
WNT1	0.05	ns	0.07	ns
WNT2	0.02	ns	-0.10	*
WNT2b	0.08	ns	-0.01	ns
WNT3	-0.07	ns	-0.09	*
WNT3a	0.10	*	**0.38**	****
WNT4	0.19	****	0.19	****
WNT5a	-0.11	*	0.00	ns
WNT5b	0.12	**	0.04	ns
WNT7a	-0.04	ns	-0.04	ns
WNT7b	-0.05	ns	0.17	****
WNT8a	0.02	ns	0.04	ns
WNT8b	0.02	ns	0.11	**
WNT9a	0.05	ns	0.19	****
WNT9b	-0.07	ns	0.09	*
WNT10a	0.17	****	**0.48**	****
WNT10b	0.05	ns	0.15	***
WNT11	**0.31**	****	**0.33**	****
WNT16	-0.02	ns	-0.05	ns

RNAseq data with 511 samples were used for LGG, while Agilent data with 570 samples were used for GBM. Spearman correlation test was used. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$; and ****, $p < 0.001$.

GBM datasets of various world regions/institutions is crucial to establish WNT6 as a clinically-useful biomarker. Concordantly, this clinical finding is in agreement with our *in vivo* data (Chapter 2), in which both WNT6-silencing and WNT6-overexpression models showed a marked influence of WNT6 in the aggressiveness and prognosis of GBM xenografts. In this context, these data strengthen our hypothesis that WNT6 presents an important role in GBM pathophysiology. Given the interesting association obtained between *WNT5b*, which was previously reported to be amplified in pediatric grade III and IV astrocytomas [19], and the prognosis of GBM patients, further studies are important to understand the functional role of WNT5b in glioma, as performed for WNT6.

WNT6 co-expression with *WNT10a* in GBM fits well with previous reports in colorectal cancer [20], where these two genes were found to be co-expressed. Indeed, in humans, *WNT6* and *WNT10a* are clustered in the 2q35 chromosomal region, less than 7 kb apart. With respect to *WNT3a* or *WNT11*, there are no reports describing a potential association between *WNT6* and these genes in development or cancer, being thus required further studies to clarify this link. In Chapter 2, we already evaluated the expression of *WNT3a* in our WNT6-manipulated *in vitro* models, including both WNT6-silencing and WNT6-

Table 4.2. Cox multivariate analysis in GBM patients from TCGA

	Overall Survival (GBM patients)			
	WNT6 [a]		tested WNT ligand[b]	
WNT ligand	***p*-value**	**HR**	***p*-value**	**HR**
WNT1	**0.031**	1.286	0.461	0.791
WNT2	**0.034**	1.280	0.537	1.037
WNT2b	**0.045**	1.266	0.341	1.069
WNT3	**0.035**	1.279	0.415	0.921
WNT3a	**0.041**	1.288	0.888	0.968
WNT4	**0.030**	1.300	0.616	0.955
WNT5a	**0.030**	1.286	0.074	0.872
WNT5b	**0.031**	1.287	**0.017**	1.234
WNT6	-	-	**0.030**	1.288
WNT7a	**0.042**	1.270	0.542	1.040
WNT7b	**0.022**	1.310	0.207	0.861
WNT8a	**0.042**	1.269	0.310	0.855
WNT8b	0.052	1.257	0.225	1.193
WNT9a	**0.020**	1.317	0.111	0.742
WNT9b	**0.033**	1.284	0.780	0.908
WNT10a	0.157	1.214	0.453	1.075
WNT10b	**0.049**	1.263	0.328	1.071
WNT11	**0.036**	1.284	0.893	0.984
WNT16	**0.027**	1.292	0.085	1.068

[a]Confounding factors used in the multivariate analysis: *WNT6* expression, age, KPS, gender, treatment, and the expression of each one of the other ligands. [b]Confounding factors used in the multivariate analysis: age, KPS, gender, treatment, and the expression of each one of the other ligands. Significant *p*-values are in bold. HR = Hazard Ratio

overexpressing models. *WNT3a* levels neither decreased upon WNT6 silencing, nor increased upon WNT6 overexpression (Figure 2.S8). In fact, *WNT3a* expression was only detected in U373 cells, and its levels increased upon WNT6 silencing. These data suggest that, despite the observed correlation in GBM patients, there is no association between *WNT3a* levels and the WNT6 functional effects that we reported in this thesis.

Considering the need for new precision therapies, it is crucial not only to identify new prognostic factors, but also to understand the molecular mechanisms underlying its associated aggressiveness. In this context, in Chapter 2, we focused on identifying WNT6-mediated signaling mechanisms in GBM; importantly, this has not been described for any cancer type, giving potentially critical insights into the mechanisms of action of WNT6 in cancers where it is overexpressed [11-13, 20-22]. Complementary, in

Chapter 3, we focused on the identification of WNT6 upstream regulators in gliomas, as only *UCA1* and CAV1 molecules have previously been pointed as potential regulators of WNT6 in bladder and gastric cancer, respectively [11, 12].

There are at least three intracellular signaling pathways activated by the WNT proteins: one β-catenin-dependent pathway (named canonical WNT pathway) and two β-catenin-independent pathways (named non-canonical WNT pathways), the planar cell polarity and the Ca^{2+} pathways [5]. Usually, WNTs are classified according to their ability to signal through either the canonical or non-canonical pathways. While WNT1 is mainly associated to the activation of the canonical pathway [23] and WNT5a to the non-canonical ones [23], WNT6 has been linked to all [24-40]. Indeed, while WNT6-mediated regulation of the epithelium formation, adhesion, cell-cell communication, and development of several tissues (e.g., heart muscle) was associated to the canonical pathway [25-27, 30-32, 36-39, 41], WNT6 signals through the non-canonical pathway in the neural crest induction, and in differentiation of the skeletal muscle, dental pulp, and macrophages [22, 24, 33-35]. In cancer, the mechanism of action of WNT6 via the canonical or non-canonical pathways were still unknown. Here, we showed that WNT6 is an activator of the canonical WNT pathway in GBM, as assessed by the TCF/LEF reporter assay (Chapter 2). Interestingly, TCF/LEF binding sites were also identified in our *in silico* analysis (MatInspector tool, Genomatix; Figure 3.3) with significant matrix similarities within the *WNT6* promoter region, suggesting the existence of a positive regulatory feedback loop. Regarding the non-canonical pathways, the capacity of WNT6 to activate them can be evaluated through the assessment of p-JNK and p-PKC by western blot or *ROR1/2* and *RYK* expression by qRT-PCR. Of note, p-JNK levels were already assessed in our WNT6-silencing models using the phospho-kinase arrays, but without concordant results between the two cell lines evaluated (Figure 2.6). Further studies are thus required to elucidate on WNT6 role on the WNT non-canonical pathways. Moreover, we already identified SFK and STAT3 pathways as other potential effectors of WNT6 aggressiveness in GBM. Our data, together with some reports associating the WNT pathway with the SFK and STAT3 pathways [42-45], strongly support a role of WNT6 in the regulation of these pathways in GBM, which might be performed through canonical or non-canonical signals.

Regarding the upstream regulators of *WNT6* expression in gliomas, in Chapter 3 we showed that *WNT6* might be regulated by DNA methylation, similarly to what was previously described for other WNT pathway components [46-63]. Specifically, we showed that the DNA methylation levels of *WNT6* promoter region are inversely correlated with its expression, while *WNT6* gene body DNA methylation levels are positively correlated. Moreover, we demonstrated that 5-Aza treatment successfully decreased the DNA methylation of the promoter region of GBM cells, which correlated with increased *WNT6* expression.

However, 5-Aza, which has been used for cancer treatment, is a genome-wide DNA demethylating agent, having off-target effects that hinder one from taking conclusions about the correlation between specific CpG sites and its effects on gene expression [64]. Indeed, the possibility of epigenome editing at specific CpG sites is an attractive method to be use not only for mechanistic studies, but also as a therapeutic option, with CRISPR/Cas9 technology having emerged as a successful method for this site-specific DNA targeting. Cas9 nuclease uses small base pairing guide RNAs (gRNAs) to target and cleave foreign DNA elements in a sequence-specific manner. Interestingly, the use of Cas9 mutant nucleases (e.g., D10A and H840A), named as death Cas9 (dCas9), lacks the endonuclease activity but can still be recruited by gRNA [65]. This catalytically inactive dCas9 may be coupled/fused to DNA methyltransferases (DNMTs) or TET enzymes to artificially alter the DNA methylation in a site-specific manner [65]. While DNMTs are responsible for introducing and maintaining the DNA methylation, TET enzymes catalyzes its removal [65]. Of note, *de novo* DNA methylation is controlled by DNMT3a and DNMT3b, while DNMT1 is responsible by its maintenance [65]. Over the last years, this technology has boosted the field of gene-targeted epigenome reprogramming [66-71]. Moreover, inducible promoters, e.g. using tetracycline-induced transcriptional activation, may be used to achieve timely precise modulation of the DNA methylation levels. Thus, using this technology, we would be able to evaluate the impact of the DNA methylation levels of specific regions on the *WNT6* expression in GBM cells, which might be further translated into a therapeutic tool.

Furthermore, we also demonstrated in Chapter 3 that HOXA9, which we previously showed to be an important oncogenic transcription factor in GBM [72-74], is a direct regulator of WNT6 expression in GBM cells, identifying WNT6 as a direct effector of HOXA9-mediated aggressiveness. Although we observed that i) HOXA9 binds to the promoter region of *WNT6*; ii) *WNT6* and *HOXA9* are co-expressed in glioma patients from independent cohorts; and iii) *WNT6* expression follows *HOXA9* expression when the latter is modulated in GBM cells, it would be important to confirm that there is a direct link between HOXA9 binding to the *WNT6* promoter region and the activation of its transcription. To this end, a luciferase or GFP reporter assay, i.e. where the expression of the luciferase/GFP protein is under the control of the promoter of *WNT6* (containing wildtype or mutant HOXA9-binding sites), might be employed in the GBM cell lines with modulated levels of HOXA9. This assay allows to evaluate if the binding of HOXA9 to the promoter region of *WNT6* will activate its expression by the indirect measure of the luciferase activity or GFP expression. Moreover, as *WNT6* DNA methylation may interfere with the binding of transcription factors, including with HOXA9 binding, it would be interesting to understand if these mechanisms are co-occurring in GBM cells and patients, or if they are mutually exclusive.

4.2. Future perspectives

Although, in Chapter 2 and Chapter 3, we identified *WNT6* as a novel oncogene in human glioma, and identified some of its upstream regulatory and downstream effector mechanisms, some questions of paramount importance remain:

4.2.1. Is WNT6 a tumorigenic factor in GBM?

With the advances of high-throughput technologies, the origin of some cancer entities began to be partially understood [75-79]. This understanding is crucial to allow the application of preventive measures and/or the early detection of malignant lesions. Some works have already tried to identify the cell of origin and the (epi)genetic alterations underlying GBM (reviewed in [80]). Although no striking results were obtained so far, it is more than consensual that malignant glioma may arise due to the accumulation of multiple genetic alterations [80, 81]. In this context, Sonoda *et al.* (2001) used human immortalized astrocytes (with the overexpression of the human oncoprotein telomerase [hTERT], E6-mediated inactivation of the tumor suppressor p53, and E7-mediated inactivation of the tumor suppressor RB), which *per se* are not tumorigenic in immunocompromised mice [73, 82], and introduced serial genetic alterations, such as EGFR (wildtype or mutant), AKT, and RAS [82]. Only the addition of RAS to hTERT/E6/E7 genetic alterations showed to be tumorigenic when injected subcutaneously in mice, while other combinations (e.g., E6/E7/RAS or E7/hTERT/RAS) were not [82]. Taking into consideration that hTERT/E6/E7 cells do not express WNT6 (data not shown), they might be an interesting tool to further understand the impact of WNT6 in glioma initiation, as WNT6 might be straightforwardly overexpressed in these cells, and its potential tumorigenic value might be evaluated upon implantation of these cells in the brain of immunocompromised mice. In addition, more sophisticated results might be obtained using i) genetically engineered mice models with low glioma penetrance (e.g., Ink4a-Arf$^{-/-}$;Kras^{-};Akt^{-} mice with 42 to 49% of GBM formation by 12 weeks [83]) – by conditionally knock-in *WNT6* (which might be expressed constitutively or under the promoter of specific relevant brain cells in the context of glioma, like astrocytes, neural stem cells, among others) and by the observation of its effects in glioma penetrance and mice survival; ii) genetically engineered mice models with predominance of lower-grade gliomas (e.g., Pten$^{f/f}$;hGFAP-Cre;EGFRvIII mice with grade II to IV tumors in 93% of the mice by 6 to 15 weeks [84]) – by conditionally knock-in *WNT6* and by the observation of the percentage of gliomas formed and their grading; and iii) genetically engineered mice models with no spontaneous formation of gliomas (e.g., Ink4a-Arf$^{-/-}$ mice) – by conditionally knock-in *WNT6* and by the evaluation of mice spontaneous tumor formation and survival. The second proposed model would allow to better study the association between WNT6 expression and glioma grading observed in Chapter 2 and Chapter 3. Good reviews and summaries

of the available glioma mice models can be found in [85, 86].

4.2.2. Is WNT6 a biomarker of tumor recurrence in GBM?

Considering the data obtained in Chapter 2, where WNT6 was associated with characteristics linked to tumor recurrence, like resistance to chemotherapy, increased cell motility and stem cell capacity, the value of WNT6 as a biomarker of glioma recurrence should be further evaluated. To this end, using large and independent cohorts of glioma patients with information about tumor recurrence for each, statistical analyses might be performed to understand if WNT6-high primary tumors are more prone to recur than WNT6-low primary tumors.

In clinical practice, a significant percentage of GBM patients show a pseudoprogression upon brain magnetic resonance imaging (MRI), a phenomenon that occurs due to contrast enhancement changes surrounding the tumor site in result of the radiochemotherapy [87-89]. In this context, one of the main challenges in the management of GBM patients is the determination of when the tumor is effectively progressing. WNT proteins are considered morphogens as they can spread over a distance to induce patterning during development [90]. Moreover, they are hydrophobic, and thus require a special mechanism to be transported across tissues. As described in Chapter 1, although WNT proteins were found to bind to lipoprotein particles, recently it was observed that a substantial amount of active WNT proteins are found in exosomes [90]. Both lipoprotein particles and exosomes have been found in several body fluids, such as blood plasma, aqueous humor, saliva, cerebral spinal fluid, etc., some of which are of easy access [91-100]. Thus, the identification of biomarkers of recurrence in lipoproteins/exosomes (e.g., WNT6) may help in the differentiation between pseudoprogression and a true progression. In fact, these biomarkers may identify progressions earlier and easier than possible by imaging, and may spare patients with pseudoprogression from unnecessary treatments and allow faster treatment decisions for those in which progression is detected earlier.

4.2.3. Is WNT6 involved in glioma immune evasion?

In the last years, immunotherapy has emerged as an attractive therapeutic option for cancer treatment [101]. However, despite the excellent results obtained with certain immune checkpoint inhibitors (ICI), namely in melanoma, non-small cell lung, and head and neck cancers [102-104], most patients still do not respond to immunotherapy, likely due to tumor intrinsic mechanisms of resistance [105]. One of such mechanisms has been hypothesized to be aberrant WNT/β-catenin signaling, which has been described to control the tumor-immune interaction at several levels [106-116]. Indeed, it is well recognized that this pathway affects critical regulators implicated in the antitumor activity of dendritic, T,

and microglial cells (as reviewed in [112]). In Chapter 2, we found that WNT6 is an activator of the WNT/β-catenin signaling in gliomas, and WNT6 alone was also reported to be involved in the regulation of immune cells [34]. Together, these data raise the hypothesis that WNT6 may be involved in glioma immune evasion. In our study, we used NSG immunocompromised mice, which have absent T, B and NK cells, and defective dendritic cells and macrophages [117]. Thus, this model is not ideal to evaluate the potential effect of WNT6 on tumor immune evasion. To address this question, two types of mice models might be used: immunocompetent mice implanted with mice glioma cells (e.g., GL261 or KR158B with silencing or overexpression of WNT6, and their respective controls, implanted in C57BL/6J mice), or humanized immunocompromised mice that allow the implantation of human glioma cells (e.g., U373 with silencing of WNT6 or U87 with overexpression of WNT6, and their respective controls, implanted in NSG mice previously humanized with human peripheral blood mononuclear cells [PBMCs]). In both contexts, the effect on i) the immune system, namely analyzing the immune cell infiltration in the tumor (e.g., using flow cytometry and/or immunofluorescence to characterize the presence of CD3, CD4, CD8, CD19, CD11b, FoxP3, NK1.1, and CD86 immune cells) and their activation profile (e.g., through flow cytometry and/or immunofluorescence to quantify their profile of activation [IL2, IFNγ, TNF, CD40, CD69, CD80, CD86 and CD25], proliferation [CFSE], senescence [KLRG1], and exhaustion [PD1, LAG3 and TIM3]), and ii) the tumor itself (e.g., tumor volume, and expression of proliferation [Ki-67, Cyclin D1], stem [NESTIN, SOX2] and apoptosis [BCL2] markers) should be further explored. Moreover, in the context of tumor shrinkage, contralateral re-challenging should be considered to study immune cell memory against the tumor. This experiment would allow to understand if WNT6 may have a role in avoiding immune cell infiltration and/or activation. These findings might help identifying a potential mechanism of immune modulation in tumors non-responsive to immunotherapy, which might be specifically targeted (as discussed abaixo) to improve the response to immunotherapies and, consequently, the prognosis of patients. Indeed, if WNT6/WNT pathway proves to have an important role in glioma immune evasion, the combination of WNT6/WNT inhibition and ICIs should be further investigated.

4.2.4. May WNT6 be used as a therapeutic target?

We are fast entering the era of precision medicine. The era where scientists and clinicians, despite knowing that tumors are highly complex and heterogeneous, are technically more able to obtain genomic and epigenomic information from tumors, and acknowledge the limitations of unsatisfactory “one serves all” therapies. But how can the treatment of GBM be optimized for the benefit of individual patients? To reach this goal, several scientists have been focusing their research on the identification of molecular target(s) that may help in the prevention, diagnosis, prognosis, treatment, or recurrence of GBM patients.

Our data from Chapter 2 and Chapter 3 identifies WNT6 as a biomarker of poor prognosis in GBM patients, independently of other potential markers, such as age, KPS, therapy, or even *HOXA9* expression, one of its upstream regulators. Moreover, WNT6 silencing through shRNA in GBM cells was sufficient to significantly prolong the survival of mice, while WNT6 overexpression in GBM cells accelerated their death. Integrating these concordant *in vivo* data with the functional oncogenic roles pinpointed *in vitro*, it is reasonable to hypothesize that WNT6 may be an attractive therapeutic target in GBM. Presently, there are no direct WNT6 inhibitors available. However, since we found WNT6 was associated with WNT, SFK and STAT pathways activation (Chapter 2), for which several inhibitors have been designed and some are even in clinical trials (e.g., WNT974, dasatinib, and C188-9, respectively; see *www.clinicaltrials.gov* for more details) [23, 118, 119], it may be useful to combine current standard-of-care therapies with those targeted drugs that may revert WNT6-mediated effects in GBM. On the other hand, we showed that HOXA9 is an upstream regulator of WNT6 in gliomas (Chapter 3), for which there are also indirect inhibitors available (e.g., HXR9 peptide or PI3K pathway inhibitors) [120, 121]. In this sense, HOXA9 inhibitors might also be a viable alternative to decrease the WNT6-associated aggressive phenotype in GBM.

After a long and tortuous journey, gene therapy is finally being accepted and used in clinical trials as a treatment option for several incurable diseases [122]. Indeed, in the last year, they were almost 2600 completed, ongoing or approved gene therapy clinical trials worldwide [122]. Gene therapies allow the manipulation of gene expression *in vivo* (through siRNA, antisense oligonucleotides, or CRISPR/Cas9, for example) in specific organs, being very attractive in several contexts, including brain cancer where some pre-clinical studies have already shown interesting results [123-125]. In that sense, the current lack of direct inhibitors for WNT6 may also be overcome using gene therapy technologies in the future.

On the other hand, in the last three decades, over 60 monoclonal antibodies have been approved for clinical use [126, 127], half of them for oncology use. In addition, the interest in bispecific antibodies, which present the advantage of simultaneously targeting two different epitopes (either in the same or in different antigens), have significantly increased [128]. Indeed, it was recently approved the use of two bispecific antibodies, one of them for oncological treatment purposes [128]. Considering the success of antibody therapies, cancer immunotherapy emerged as a fourth modality of treatment (after the conventional surgery, radio- and chemotherapy) [128]. However, regarding brain disease, there is an extra challenge in the design of antibody therapies: the transport through the blood brain barrier (BBB) into the brain. This problem implies the use of higher doses of antibody to increase its concentration near the targeted brain cells. Although high doses are still safe and effective, the supply of sufficient drug with acceptable cost-of-goods are unacceptable for successful commercialization. In this context, antibodies

are being engineered to co-opt a natural molecular transportation system, called the "Trojan horse" (e.g., transferrin, insulin, LRP1 or GLUT1 receptors), with the goal of improving their transport through the BBB into the brain [129-136]. Although still in optimizations, antibodies may also be engineered to easily reach intracellular targets (as reviewed in [137]). Since WNT6 may be found intra- and extra-cellularly, and considering its potential therapeutic role in GBM (Chapter 2), the construction of T-cell engaging bispecific antibodies targeting both WNT6 and CD3 proteins might be interesting. With this type of approach, we would be able to simultaneously neutralize WNT6, and thus its mediated aggressive phenotype, and recruit CD3-positive immune cells (i.e. CD8, CD4, NK, and γδ T cells) to the tumor milieu, fostering the immune response against the tumor cells. Future efforts should be made to create therapies specifically targeting WNT6, as not only GBM patients may benefit from these therapies, but also those with other tumors, namely gastric, esophageal, colorectal, osteosarcoma and bladder cancer patients, where WNT6 presented oncogenic functions and/or was predictive of prognosis, recurrence and response to therapy [11-13, 21, 22, 57].

4.3. Conclusion

Gliomas are a devastating disease [4]. Despite the recent advances in their classification, it seems that we are still far from obtaining true therapeutic value from it [1]. Some reasons for that may include the fact that: i) gliomas are localized in the brain, the most complex organ, with almost no capacity to self-repair after damage; ii) patients with glioma have a short clinical history (less than 3 months); iii) gliomas are invasive, hampering total resection and thus with high probability of recurrence; iv) the brain is protected by the BBB, hindering drug penetration into the tumor; and v) current therapies are mostly palliative [4]. In this context, there is an increasing need to identify new biomarkers of aggressiveness, and to understand their associated molecular mechanisms to try to improve the treatment of this deadly disease. In this thesis, we dissect for the first time the functional roles and underlying upstream and downstream molecular mechanisms associated with WNT6 in human GBM, establishing it as a clinically relevant prognostic biomarker. Concretely, we showed that WNT6 expression is associated with increased glioma malignancy, independently of IDH mutation and 1p/19q co-deletion status. In GBM, WNT6 was associated with increased tumor aggressiveness *in vitro* and *in vivo*, implicating it as an important oncogenic factor in glioma. Most importantly, in several independent cohorts of GBM patients, WNT6 was a biomarker of shorter overall survival. Since almost all GBM will sooner or later recur, further studies evaluating the potential value of WNT6 as a biomarker of GBM recurrence might be important to try to improve the management of these patients. Both WNT6 mRNA and protein levels can be easily assessed

with routine molecular biology techniques, which may facilitate its widespread implementation in the clinical setting as a biomarker.

In this thesis, we also investigated for the first time the signaling mechanisms mediated by WNT6 in GBM, as well as its upstream regulatory mechanisms. We identified the WNT, SFK and STAT pathways as downstream mediators of WNT6 aggressiveness in GBM. Specifically, we showed that WNT6 is a canonical WNT ligand in GBM, leading to the activation of the WNT/β-catenin signaling pathway. Additionally, both *WNT6* DNA methylation and HOXA9, an oncogenic transcription factor in GBM, were identified as WNT6 regulators in glioma. In fact, HOXA9 also led to the activation of the β-catenin-dependent WNT signaling pathway in GBM. In this context, future work should focus on the development and application of drugs targeting WNT6 (e.g., with antisense oligonucleotides or neutralizing antibodies), its downstream effectors (e.g., using WNT and SRC inhibitors) or its upstream regulators (e.g., using HOXA9-targeting HXR9 peptide or PI3K inhibitors), which may prove useful to improve the treatment of GBM patients, either when applied as single treatment regimens or in combination with TMZ (the gold-standard chemotherapy for GBM) or ICIs (which recently emerged as a potential therapy for cancer).

Overall, the work presented throughout this thesis has an important impact in: i) the neuro-oncology field, by the identification of a new molecular prognostic marker in GBM and by the characterization of its functional role; ii) the WNT signaling field in general, by the identification of HOXA9 and *WNT6* DNA methylation as upstream regulators of WNT6 in GBM, as well as from the characterization of the signaling pathway mediated by WNT6; and iii) the oncology field, profiting from our data showing that WNT6 silencing significantly prolongs the survival of GBM orthotopic mice models, which will undeniably inspire the creation of WNT6-directed therapies that may not only be used for GBM patients, but also for gastric, bladder, esophageal, colon, and osteosarcoma patients, where WNT6 overexpression has also been reported [10-13, 21, 57]. Not less important, we feel the discoveries of this thesis raise interesting scientific and clinical questions, and open new exciting avenues of research for the (near) future.

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