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Investigating the role of TWIST1 in medulloblastoma metastasis

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Abstract

Introduction

Medulloblastoma is the most common malignant paediatric brain tumour. Many patients present with metastatic disease and sadly this remains a powerful predictor for poor outcome. Metastasis is a complex multi-step process and the underlying molecular mechanisms behind medulloblastoma metastasis are poorly understood. One of the initial stages of this process involves the invasion of tumour cells through the basement membrane and into the leptomeningeal space. The transcription factor TWIST1 has been extensively studied in epithelial tumours and is considered as one of the master regulators initiating the first stage of this process. An in-depth analysis of the functional role of TWIST1 in initiating medulloblastoma metastasis remains to be explored. This project therefore aimed to assess the functional role of TWIST1 and whether it could play an essential part in driving medulloblastoma metastasis.

Methods

TWIST1 gene and protein expression was analysed in medulloblastoma patient samples to investigate if expression correlated with metastatic disease and patient outcome. Metastatic cell lines representing two out of the four medulloblastoma molecular subgroups (SHH and Group 3) were employed for *in vitro* analysis of TWIST1 function. A 3D transwell assay was used to quantify cellular migration and invasion through a simple basement membrane. Inhibition of TWIST1 using genetic (shRNA) and chemical (harmine, a beta-carboline alkaloid) methods was investigated. We then explored the presence of a TWIST1-ABCB1 axis, using chromatin immunoprecipitation (ChIP) and the specific ABCB1 inhibitor vardenafil. Finally, ChIP-Sequencing was carried out to define additional members of TWIST1's transcriptional regulatory network.

Results

TWIST1 was highly expressed across the four medulloblastoma molecular subgroups compared to the normal cerebellum. Analysis of patient tissue microarrays revealed that high expression was a strong indicator for worse event-free survival and for driving metastatic disease in medulloblastoma patients. Reduced TWIST1 expression significantly decreased medulloblastoma cell migration in the 3D transwell migration and invasion assay. ChIP analysis confirmed TWIST1 bound to the ABCB1 promoter and inhibition of ABCB1 with vardenafil reduced cell migration. ChIP-Sequencing identified many TWIST1 downstream target genes and pathways that are important in initiating the metastatic process. This included pathways and genes that are involved in interacting with cell adhesion molecules, focal adhesions and the extracellular matrix.

Conclusions

This thesis has shown that TWIST1 expression is crucial for initiating medulloblastoma metastasis through its ability to target pathways and genes that are essential in initiating the metastatic process. Therapeutically targeting TWIST1 in the primary tumour offers a novel approach to prevent primary tumour cells from invading the extracellular matrix and ultimately halt metastatic dissemination and, thereby greatly improving patient outcome.

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List of Common Abbreviations

2D	Two-dimensional
3D	Three-dimensional
ABCB1	ATP Binding Cassette Subfamily B Member 1
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
bHLH	basic Helix-Loop-Helix
BME	Basement membrane extract
BSG	Basigin
ChIP	Chromatin Immunoprecipitation
ChIP-Seq	Chromatin Immunoprecipitation Sequencing
CNS	Central nervous system
CSC	Cancer stem cell
CSF	Cerebrospinal fluid
CTC	Circulating tumour cell
ECM	Extracellular matrix
EGF	Epidermal growth factor
EMT	Epithelial-mesenchymal transition
FBXL17	F-box and Leucine Rich Repeat Protein 17
FDR	False discovery rate
FGF	Fibroblast growth factor
GO	Gene ontology
GSEA	Gene set enrichment analysis
H3K4me3	Tri-methylation at Lysine 4 of the Histone 3 protein
HA	Hyaluronic acid
HYAL2	Hyaluronidase-2
IGF1	Insulin-like growth factor 1
IGF1R	Insulin-like growth factor 1 receptor
IHC	Immunohistochemistry
IP	Immunoprecipitation

KD	Knockdown
LCA	Large cell anaplastic
MBEN	Medulloblastoma with extensive nodularity
MMP	Matrix Metalloproteinases
MRI	Magnetic resonance image
mRNA	messenger RNA
MSigDB	Molecular signatures database
NSCLC	Non-small cell lung cancer
PCR	Chain Reaction
PTCH	Patched
RFU	Relative fluorescence units
SEM	Standard error of the mean
shRNA	small hairpin RNA
SHH	Sonic Hedgehog
SMO	Smoothened
SORCS2	Sortilin Related VPS10 domain containing receptor 2
SUFU	Suppressor of fused homolog
TMA	Tissue microarray
TP53	Tumour Protein 53
TSS	Transcriptions start site
WHO	World Health Organisation
WNT	Wingless

Chapter 1. Introduction

Childhood cancer is extremely rare, with around 1,900 new cases diagnosed every year in the UK, accounting for less than 1 % of all new cancer cases (Cancer Research, UK). Tumours of the brain and central nervous system (CNS) are the second most common group of cancers and the most common group of solid tumours in children, accounting for approximately 26 % of all UK childhood cancers. Malignant brain tumours are the leading cause of all childhood cancer deaths (Children with Cancer, UK). Medulloblastoma is the most common malignant paediatric brain tumour and is the focus of this thesis.

1.1 Paediatric medulloblastoma

1.1.1 Epidemiology

Medulloblastoma accounts for approximately 20 % of all childhood brain tumours, with an annual incidence rate of approximately 5 cases per 1 million children (Northcott et al., 2019). They can arise throughout childhood and into adulthood, although 70 % of cases are diagnosed in children under the age of 10. The incidence rate peaks between the age of 4 and 9 years, with a median age at diagnosis of 6 years (Kool et al., 2012, Juraschka and Taylor, 2019). Medulloblastoma is more common in males than females, with a 1.8:1 male:female ratio. Currently, there do not appear to be any substantial differences in ethnicities or geographical regions (Northcott et al., 2019).

1.1.2 Clinical presentation

Patients can initially present with nonspecific symptoms such as headaches, nausea, fatigue, clumsiness or poor school performance as a result of increased intracranial pressure due to obstructive hydrocephalus.

Medulloblastoma are neuroectodermal tumours that arise in the cerebellum. Symptoms specific to medulloblastoma tumours include ataxia, complex motor skills and problems with vision, with symptoms evolving over a period of weeks to a few months (Polkinghorn and Tarbell, 2007, Millard and De Braganca, 2016). Symptoms such as back pain and gait could be an indication of spinal metastasis, however, 'sugar coating' spinal metastasis is often asymptomatic (Northcott et al., 2019).

1.1.3 Diagnosing medulloblastoma

Medulloblastoma is diagnosed based on a number of factors. These include clinical symptoms, computerised tomography (CT) scan and magnetic resonance image (MRI) imaging of the brain and spine to identify if the patient has a mass occupying the posterior fossa region, cerebral spinal fluid (CSF) cytology to detect microscopic metastasis, and histopathological and molecular analysis (Northcott et al., 2019, Szalontay and Khakoo, 2020).

Whilst a CT scan can be performed safely and quickly on critically ill patients, an MRI image is mandatory to provide further detail of the primary tumour and identify any macroscopic metastasis (Massimino et al., 2016).

1.2 Classification of medulloblastoma

Historically, medulloblastoma have been defined into four histological subgroups: classic, large cell/ anaplastic (LCA), desmoplastic/ nodular and medulloblastoma with extensive nodularity (MBEN). However, more recent advancements in the molecular understanding of medulloblastoma has meant that histopathological *and* molecular analysis are essential for diagnosing medulloblastoma. In 2012, a consensus was reached on four distinct molecular

subgroups; WNT, SHH, Group 3 and Group 4 (Cho et al., 2011, Northcott et al., 2011, Kool et al., 2012). The histological and molecular subgroups were integrated into the 2016 World Health Organisation (WHO) classification guidelines for medulloblastoma tumours, which are summarised in Table 1.1 (Louis et al., 2016). These guidelines are a practical advance over the previous 2007 guidelines because it allows patients to be diagnosed according to both the molecular and histological defined features of the tumour.

Table 1.1 Summary of the most common integrated medulloblastoma diagnoses from the 2016 WHO classification. (Louis et al., 2016)

Molecular Subgroup	Histology	Prognosis
WNT	Classic	Low risk
	Large cell/ anaplastic	Uncertain of clinicopathological significance
SHH TP53-mutant	Classic	Uncommon high-risk tumour
	Large cell/ anaplastic	High risk tumour
	Desmoplastic/ nodular	Uncertain of clinicopathological significance
SHH TP53-wildtype	Classic	Standard risk
	Large cell/ anaplastic	Uncertain of clinicopathological significance
	Desmoplastic/ nodular	Low risk
	Extensive nodularity	Low risk
Group 3	Classic	Standard risk
	Large cell/ anaplastic	High risk
Group 4	Classic	Standard risk
	Large cell/ anaplastic	Uncertain of clinicopathological significance

1.2.1 Histological classification of medulloblastoma

The diagnosis of medulloblastoma is impossible without histological analysis of a surgical specimen, especially as the posterior fossa is a common location for other paediatric brain tumours such as ependymoma and pilocytic astrocytoma (Northcott et al., 2019). Figure 1.1 demonstrates the four medulloblastoma histological variants.

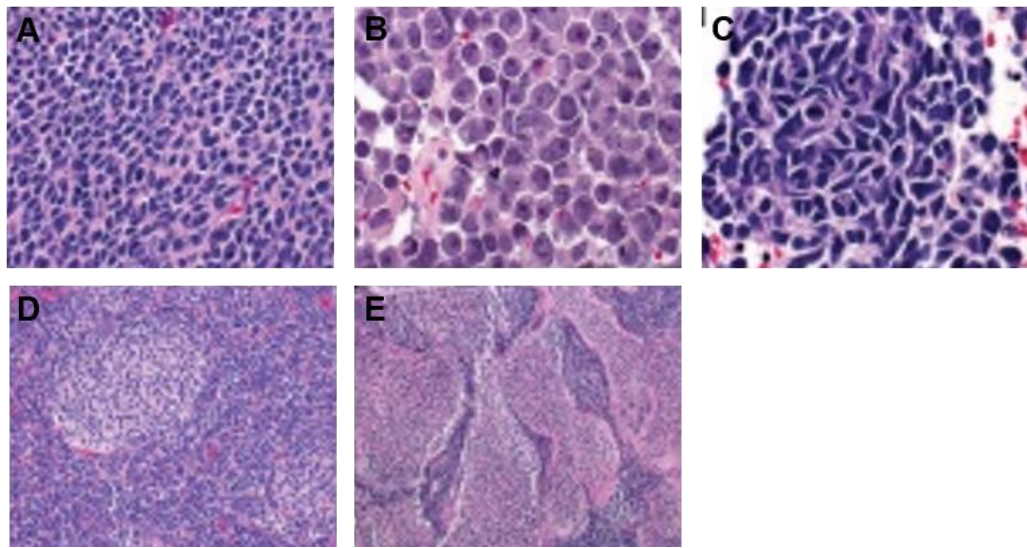


Figure 1.1 Medulloblastoma histologically defined subgroups.

Medulloblastoma tumour samples demonstrating the four histological subgroups. The classical variant (A) demonstrating small cells with round to ovoid nuclei. Large cell/ anaplastic variant (B and C) with large discohesive cells with a prominent nucleoli (B), and the anaplastic cells characterised by nuclear pleomorphism, frequent mitotic activity and apoptotic bodies (C). The desmoplastic/ nodular variant (D) is characterised by nodules of neurocytic differentiation surrounded by inter-nodular areas. MBEN (E) is characterised by a high proportion of nodules in a linear streaming pattern. Images are from (Orr, 2020).

1.2.1.1 Classic histology

The classic variant is the most frequently encountered variant in medulloblastoma tumours, accounting for approximately 72 % of all cases. Most classic tumours are characterised as sheets of small densely packed round cells with little cytoplasm and round nuclei (Figure 1.1A). Rosettes of cells are present in a small subset of classical medulloblastoma tumours. These are a sign of neuronal differentiation but are not necessarily a prognostic marker (Polkinghorn and Tarbell, 2007, Ellison, 2010).

1.2.1.2 Large cell/ anaplastic histology

The large cell/ anaplastic (LCA) subtype is the least frequently observed subtype, accounting for approximately 5 % of medulloblastoma tumours. This subtype is associated with aggressive tumours, such as SHH *TP53*-mutant and Group 3 subgroup tumours, and has the least favourable outcome. Large cell anaplastic and anaplastic cells show cytological overlap and hence fall under one group. Cells are often characterised as being large and round with a large nuclei and a prominent single nucleoli, surrounded by abundant cytoplasm (Figure 1.1B). Anaplastic cells are marked by nuclear pleomorphism, a high mitotic count and abundant apoptotic bodies (Figure 1.1C) (Ellison et al., 2011).

1.2.1.3 Desmoplastic/ nodular histology

Desmoplastic/ nodular histology are the second most common subtype and are observed in approximately 20 % of medulloblastoma tumours. They are normally observed in children below the age of 3 as well as adults and are associated with a better outcome in infants. These cell types commonly show zones of reticulin-free nodules that represent neuronal maturation and differentiated neurocytic cells. Inter-nodular regions are characterised by

undifferentiated embryonal cells surrounded by collagen strands (Figure 1.1D) (Polkinghorn and Tarbell, 2007, Borowska and Józwiak, 2016).

1.2.1.4 Medulloblastoma with extensive nodularity histology

Medulloblastoma with extensive nodularity (MBEN) occurs almost exclusively in infants and is associated with a good prognosis (Massimino et al., 2016). This subtype accounts for approximately 5 % of medulloblastoma tumours. Similar to the desmoplastic/ nodular tumours, MBEN are characterised by nodules, but these are more abundant and are typically large and irregular shaped in linear streaming patterns (Figure 1.1E) (Polkinghorn and Tarbell, 2007, Ellison, 2010).

1.2.2 Molecular classification of medulloblastoma

Medulloblastoma is widely recognised as a heterogeneous disease. Over the past years, the molecular characterisation of larger medulloblastoma cohorts has resulted in further classification of the four subgroups to include subtypes. The four subgroups and subtypes have distinct clinical, genetic and transcriptional characteristics that are summarised in Table 1.2 and Figure 1.2 and discussed in detail in the following sections.

Table 1.2 Medulloblastoma molecular subgroups and subtypes

The molecular subgroups and subtypes of medulloblastoma. The table is based on information from Northcott *et al.*, 2011 and Taylor *et al.*, 2012.

		WNT	SHH	Group 3	Group 4
Prevalence (%)		10	30	25	35
Age group		Children and adults	Infants, children and adults	Infants and young children	Children
Gender ratio (M:F)		1:1	1:1	2:1	3:1
Histology		Classis, Rarely LCA	Desmoplastic, Classic, LCA	Classic, LCA	Classic, LCA
Metastasis at diagnosis (%)		5 – 10	15 - 20	40 - 45	35 - 40
Prognosis		Very good	Infants good, others intermediate	Poor	Intermediate
Overall survival (5 years)		~ 95 %	~75 %	< 60 %	~ 75 %
Proposed cell of origin		Progenitor cells in lower rhombic lip	Granule precursors of the external granule layer	Neural stem cells	Unipolar brush cells
Recurrent gene amplifications		-	MYCN GLI1 or GLI2	MYC MYCN OTX2	SNCAIP MYCN OTX2 CDK6
Cytogenetic Events Gain Loss		6	3q, 9p 9q, 10q, 17p	1q, 7, 18 8, 10q, 11, 16q i17q	7, 18q 8, 11p, 17p, X i17q
Medulloblastoma subtypes	Cavalli <i>et al.</i> , 2017	WNT α WNT β	SHH α SHH β SHH δ SHH γ	Group 3 α Group 3 β Group 3 γ	Group 4 α Group 4 β Group 4 γ
	Schwalbe <i>et al.</i> , 2017	WNT	SHH-infant SHH-child	Group 3 low-risk Group 3 high-risk	Group 4 low-risk Group 4 high-risk
	Robinson <i>et al.</i> , 2018		iSHH-I iSHH-II		

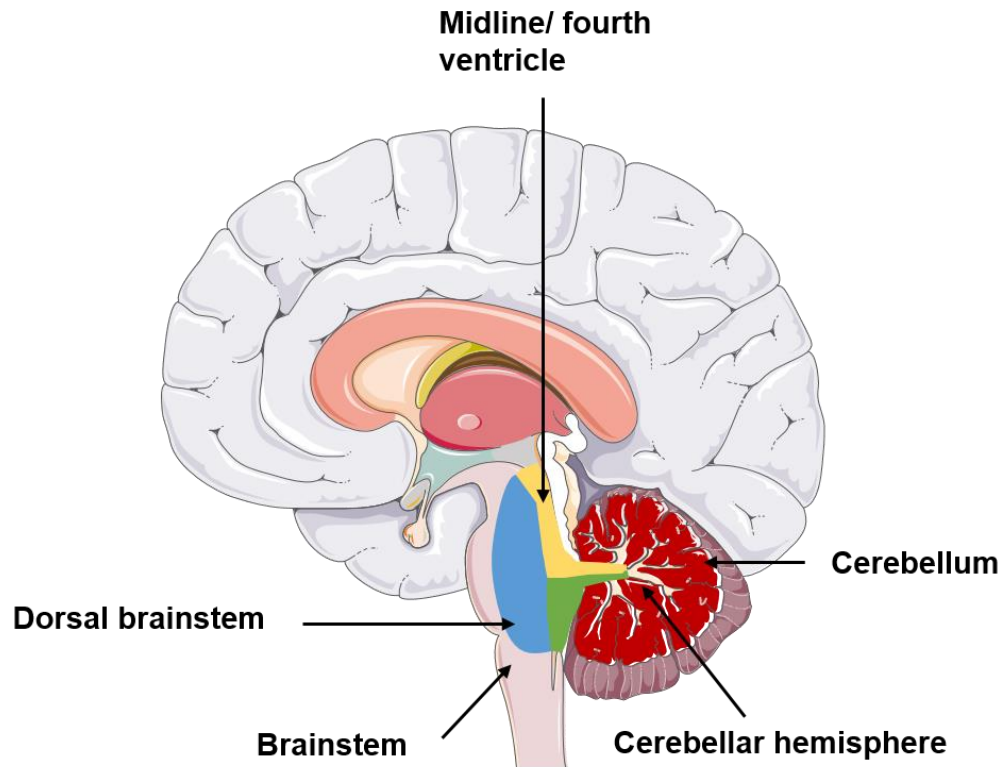


Figure 1.2 Location of medulloblastoma tumours

Location of the four medulloblastoma subgroups: WNT (blue); SHH (red); Group 3 (yellow); Group 4 (green). Image adapted from (Northcott et al., 2019).

1.2.2.1 WNT

WNT medulloblastoma tumours are the least common of the molecular subgroups, accounting for 10 % of all medulloblastoma diagnoses. They primarily occur in children over the age of 4 and adolescents, with a median age of diagnosis of 11 years and have a balanced male:female ratio (Ramaswamy and Taylor, 2017, Hovestadt et al., 2020). WNT tumours are infrequently metastatic at diagnosis and patients younger than 16 years of age have a favourable outcome with a 5 year survival rate of > 95 % (Table 1.2). The reason for this is unknown, but is thought it could be related to increased sensitivity to radiotherapy, or due to the lack of a blood brain barrier observed in WNT tumours that increases the exposure to systemic chemotherapy (Salaroli et al.,

2015, Phoenix et al., 2016). On the other hand, adults diagnosed with WNT tumours have an intermediate outcome (Remke et al., 2011).

The majority of WNT tumours are of the classic type, however, rare cases of LCA histology have been documented. They can be identified by immunohistochemistry (IHC) expression of DKK1, Filamin-A, YAP-1 and β -catenin. WNT tumours are typically located within midline of the brain, occupying the fourth ventricle and infiltrating the brainstem (Figure 1.2), and are thought to arise from progenitor cells in the lower rhombic lip of the developing brainstem (Massimino et al., 2016).

WNT tumours have activated WNT signalling. In normal cells, the absence of WNT keeps the concentration of the downstream target β -catenin low and the pathway silent. Normally, WNT binds to its cell surface receptor frizzled (FRZ), resulting in dishevelled (DSH) protein blocking targeted degradation of β -catenin by the APC/Axin/GSK-3 β complex (Figure 1.3) (Komiya and Habas, 2008). In WNT tumours the pathway is constitutively activated through mutation. Approximately 85 - 90 % of WNT tumours have highly recurrent, activating somatic mutations in exon 3 of β -catenin (*CTNNB1*), rendering it resistant to degradation and allowing it to upregulate target genes involved in cell growth and proliferation, such as *Cyclin D1* and *c-Myc* (Gilbertson, 2004). Those that lack *CTNNB1* mutations, tend to have germline mutations in the adenomatous polyposis coli (*APC*) tumour suppressor gene (Taylor et al., 2012). Patients with WNT medulloblastoma have the second highest burden of somatic genome-wide single-nucleotide variants (SNVs). In addition to *CTNNB1*, several other somatic mutations have been identified; *DDX3X* (36 % of patients), *SMARCA4* (19 % of patients) and *TP53* (14 % of patients) (Juraschka and Taylor, 2019, Northcott et al., 2019). Cytogenetically, 70 % to

80 % of WNT tumours are characterised by deletion of one copy of chromosome 6 (monosomy 6) (Northcott et al., 2017).

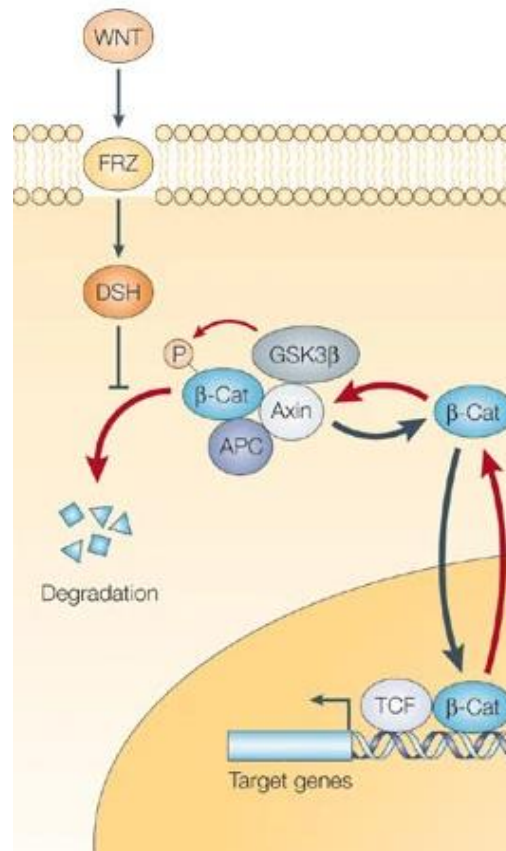


Figure 1.3 Schematic describing the WNT signalling pathway

WNT binds to the cell-surface receptor frizzled, consequently blocking the phosphorylated-activated degradation of β -catenin. This allows β -catenin to translocate to the nucleus and activate transcriptional events. Black steps are activating, and red steps are inhibitory. Image from (Scotting et al., 2005).

Two molecular WNT subtypes have been suggested by Cavalli *et al*; WNT α and WNT β , which differ in age at diagnosis (median age of 10 and 20 years, respectively). 98 % of WNT α patients had monosomy 6, compared to only 29 % of WNT β patients (Cavalli et al., 2017).

1.2.2.2 SHH

SHH tumours account for about 30 % of medulloblastoma tumours and have a bimodal age distribution occurring in infants (< 3 years), and adults (> 16 years),

accounting for two-thirds of medulloblastoma cases in these age groups. They occur less frequently in patients aged between 3 to 16 years and are slightly more common in male than female infants (Table 1.2) (Massimino et al., 2016). Prognosis strongly depends on patient age, metastatic status, histological and molecular characteristics. Infants have a better outcome compared to children and adults (5 year survival; 77 %, 68 % and 75 %, respectively, and 10 year survival; 77 %, 51 % and 34 % respectively) (Kool et al., 2012, Ramaswamy et al., 2016b).

Typically, SHH tumours are desmoplastic/ nodular, with MBEN being exclusively classified in this subgroup. Classic or LCA histological variants are observed in this subgroup too. Infants tend to have a higher frequency of desmoplastic histology which is associated with a favourable prognosis in this age group (5 year survival; 84 %) (Kool et al., 2012, Northcott et al., 2012a). They can be identified by IHC expression of GAB1, SFRP and GLI1 protein (Massimino et al., 2016). SHH tumours are almost exclusively located within the hemispheric location in the cerebellum, although some can occur in the midline vermis (Figure 1.2). They are thought to arise from granule cell precursors of the external granule layer (Juraschka and Taylor, 2019).

Approximately 15 - 20 % of patients present with metastatic disease, with metastatic dissemination occurring locally (in the tumour bed) (Ramaswamy et al., 2013, Ramaswamy and Taylor, 2017). Metastatic disease is primarily diagnosed in infants and children rather than adults (Kool et al., 2012).

The SHH subgroup is named after the Sonic Hedgehog signalling pathway, and genetically, is the best understood out of all the medulloblastoma subgroups. The pathway plays a key role in normal cerebellar development whereby Purkinje cells release SHH glycoproteins and stimulate the proliferation of granule neuronal precursor cells in the developing cerebellum, allowing the

migration of granule cells into the internal granule cell layer (Skowron et al., 2015). The activation of the pathway by SHH ligands binding to patched (PTCH), results in the suppression of the inhibition of smoothened (SMO). SMO can then release GLI from kinase suppression, allowing it to translocate to the nucleus and activate target genes (Figure 1.4) (Borowska and Jóźwiak, 2016). Suppressor of fused homolog (SUFU) normally suppresses GLI by directly repressing its transcriptional activity. The majority of SHH tumours contain germline or somatic mutations or copy number alterations in the SHH signalling pathway, leading to the over-activation of the pathway, and driving tumour growth and proliferation (Juraschka and Taylor, 2019).

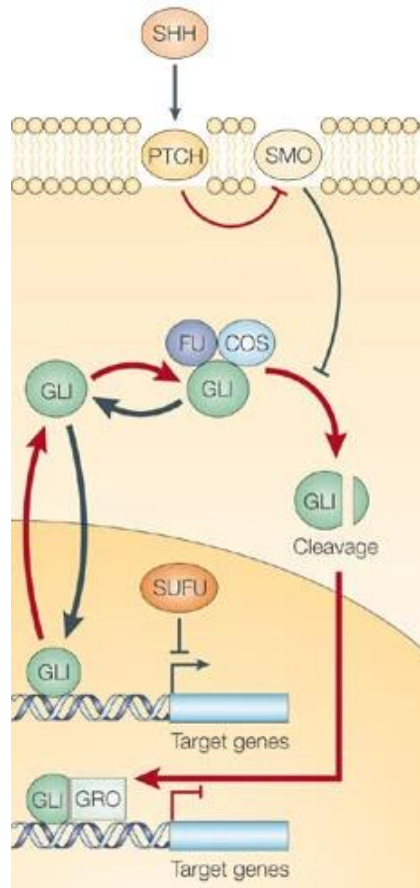


Figure 1.4 Schematic describing the SHH signalling pathway

SHH binds to the cell-surface receptor patched (PTCH), inhibiting PTCH function and causing the release of smoothened (SMO) to activate intracellular events. SMO represses the cleavage of GLI allowing its translocation to the nucleus and activating target genes. Black steps are activating, and red steps are inhibitory. Image from (Scotting et al., 2005).

Loss-of-function mutations or deletions in *PTCH1* or *SUFU* is observed in 43 % and 10 % of patients, respectively. *PTCH1* mutations have an equal frequency across infants, children and adults, whereas, *SUFU* mutations are nearly exclusively found in infants (Kool et al., 2014). Infants with *PTCH1* mutations also present with Gorlin syndrome (nevoid basal-cell carcinoma syndrome). Activating mutation of *SMO* and amplification in *GLI1* or *GLI2* and *MYCN* are frequently observed in SHH medulloblastoma (Northcott et al., 2019).

Germline and somatic *TP53* mutations are often seen in older children and adolescents (3 to 17 years of age) and are rarely found in infants or adults. *TP53* mutations are observed with *MYCN* and/or *GLI2* amplification, LCA histology, and loss of chromosome 17p (Kool et al., 2014). Unfortunately, those harbouring *TP53* mutations have a very poor prognosis, which has been recognised by the WHO in their 2016 review, and SHH-*TP53* mutations are now considered very-high risk groups (Louis et al., 2016, Ramaswamy et al., 2016b).

Cytogenetic events include the losses of the long arms of chromosome 9 (9q) and 10 (10q), which subsequently leads to the loss of heterozygosity of tumour suppressor genes, such as *PTCH1* and *SUFU*, respectively (Northcott et al., 2019).

SHH tumours have been further divided into several subtypes. Schwalbe *et al*, described two SHH subgroups that were split into age-dependent subgroups; SHH-infant (<4.3 years) and SHH-child (≥4.3 years) (Schwalbe et al., 2017). In younger children (≤ 5 years), this subtype could be further divided into two distinct subtypes; iSHH-I and iSHH-II. The first subtype is enriched for germline or somatic *SUFU* mutations and chromosome 2 gain with a progression free survival (PFS) rate of 27.8 %, whereas, the latter is enriched for activating *SMO* mutations and mutations in chromatin-modifying genes such as *KM2D* and

BCOR, with a more favourable 5 year PFS rate of 75.4 % (Robinson et al., 2018).

In addition, Cavalli *et al* described four molecular subtypes; SHH α , SHH β , SHH γ and SHH δ . Interestingly, the SHH β and SHH γ corresponded with the two infant groups (iSHH-I and iSHH-II, respectively), in terms of age and prognosis, whereas, SHH α and SHH δ corresponded with childhood/ adolescent and adult subtypes, respectively. As expected, SHH α subtype is characterised by the *TP53* mutation and *MYCN/ GLI2* amplifications, and have inferior outcomes. SHH β subtype is frequently metastatic and associated with a poor outcome. SHH δ have a favourable prognosis and are strongly enriched for *TERT* promoter mutations (Cavalli et al., 2017).

1.2.2.3 Group 3

Group 3 tumours account for approximately 25 % of medulloblastoma tumours, occur almost exclusively in infants and young children and have a male predominance (Table 1.2) (Taylor et al., 2012). They have the worst prognosis out of all of the medulloblastoma subgroups, with a 5 year survival rate of < 60 %, which strongly correlates with the presence or absence of different molecular, genetic and clinical features (Northcott et al., 2011, Northcott et al., 2019).

The histology of Group 3 tumours is commonly classic or LCA, with a higher incidence of LCA histology in Group 3 tumours compared to any of the other subgroups. LCA histology is associated with aggressive tumours and a poor outcome, which is in agreement with the poor outcome associated with this subgroup (Northcott et al., 2012a). IHC positivity of natriuretic peptide receptor 3 (NPR3) has been reported as a marker to confirm this subgroup, however, others have questioned its validity (Taylor et al., 2012, Massimino et al., 2016).

The current gold standard for diagnosis of Group 3 tumours is a transcriptional profile that clusters with other Group 3 tumours (Northcott et al., 2011). They are located within the fourth ventricle up against the brainstem and likely arise from a neural stem cell population (Figure 1.2) (Huang et al., 2016).

Group 3 tumours present with the highest rates of metastasis (40 to 45 %), resulting in an even poorer outcome for these patients. Recurrence is almost exclusively with metastatic dissemination (Skowron et al., 2015).

The genetic basis for Group 3 tumours is poorly understood. *MYC* amplification is the most common genetic feature (observed in 10 – 20 % of Group 3 tumours), and frequently co-occurs with *PVT1-MYC* fusions (Ramaswamy and Taylor, 2017). *MYC* expression is also associated with the LCA histological subtype, resulting in a poor prognosis. Recurrent somatic mutations are rare in Group 3 tumours and primarily occur in *SMARCA4* (9 %), *KBTBD4* (6 %), *CTDNEP1* (5 %) and *KMT2D* (5 %). A small subset of tumours are driven by amplification in *MYCN* (5 %) and *OTX2* (3 %) (Northcott et al., 2017, Juraschka and Taylor, 2019, Northcott et al., 2019).

Enhancer hijacking occurs in one third of Group 3 medulloblastoma tumours and results in the activation of the oncogenes *GFI1* and *GFI1B*, whereby regulatory elements are translocated close to coding genomic regions resulting in gene overexpression and tumour formation (Northcott et al., 2014, Szalontay and Khakoo, 2020).

Cytogenetic features include the presence of isochromosome 17q (i17q) (40-50 % of cases; whereby the q arm is duplicated and the p arm is lost), gain of chromosome 1q, 7 and 18, and depletion of 8, 10q, 11, and 16q. Shih *et al* showed that i17q, *MYC* amplification and the presence of metastatic disease

conferred a worse prognosis in this subgroup, whereas, those without these markers fared relatively better (Shih et al., 2014).

Many studies have described varying subtypes in Group 3 tumours. Cho *et al* and Schwalbe *et al* subdivided Group 3 tumours into two subtypes; c1 and c5, and Group 3 low-risk and Group 3 high-risk, respectively. C1 and Group 3 high-risk were both associated with frequent *MYC* amplifications and a poor outcome (Cho et al., 2011, Schwalbe et al., 2017). Cavalli *et al* identified three Group 3 subtypes; Group 3 α , Group 3 β and Group 3 γ . Group 3 α subtype occurred in infants and frequently metastatic but were associated with a better outcome. Group 3 β were associated with older children and a high frequency of *GFI1* and *GFI1B* amplification and were less likely to be metastatic, whereas Group 3 γ occurred mainly in infants and were associated with high rates of *MYC* amplification and subsequently metastatic disease and poor outcome (Cavalli et al., 2017).

1.2.2.4 Group 4

Group 4 tumours are the most prevalent subgroup accounting for approximately 35 % of all medulloblastoma cases. They typically occur in childhood and adolescence and are far more frequent in males than females, with a 3:1 male:female ratio (Table 1.2). Survival outcomes are intermediate in this subgroup, with a 5 year survival rate of 75 %, although, several different risk groups have been proposed on the basis of cytogenetics and molecular subtypes (Northcott et al., 2019).

Histologically, the vast majority of Group 4 tumours display a classic histology, although LCA histology have been observed in some cases. IHC staining of KCNA1 has been suggested as a marker for Group 4 tumours, but this is awaiting validation (Massimino et al., 2016). This subgroup is located in the

midline/ fourth ventricle (Figure 1.2), but a cell of origin has not been definitively established. They appear to have transcriptional similarities to unipolar brush cells that are derived from the upper rhombic lip (Vladoiu et al., 2019).

Approximately one third of patients with Group 4 medulloblastoma present with metastatic disease and these patients are considered high-risk. Recurrences tend to be at distant sites and occur late in this subgroup (> 5 years after diagnosis) (Ramaswamy et al., 2013, Juraschka and Taylor, 2019).

Group 4 tumours are the least genetically understood subgroup. Somatic gene mutations are rare in Group 4, with no single gene found to be mutated in > 10 % of cases. Somatic mutations involve histone-modifying genes such as *KDM6A* (9 %), *ZMYM3* (3 %), *KMT2C* (6 %) and *KBTBD4* (6 %). Amplifications are also observed in *MYCN*, *CDK6*, *SNCAIP* and *OTX2*, and enhancer hijacking occurs in 17 % of patients resulting in the activation of *PRDM6* (Northcott et al., 2012b, Northcott et al., 2017).

Similar to Group 3, isochromosome 17q is also observed in this subgroup, but is found in over 80 % of cases (Kool et al., 2012). Other chromosome aberrations include gain of chromosomes 7 and 18q, and loss of chromosomes 8q, 8p, 11p, 17p and X, with tumours considered low-risk (> 90 % survival) if they are non-metastatic and have loss of chromosome 11 (Taylor et al., 2012, Northcott et al., 2017). Interestingly, the loss of one copy of chromosome X is seen in 80 % of females with Group 4 tumours (Northcott et al., 2011).

Group 4 medulloblastoma subtypes have also been identified. Schwalbe et al/ characterised Group 4 tumours into two subtypes; Group 4 low-risk and Group 4 high-risk. Patients in the low-risk subtype had an 80 % 5 year survival rate and loss of chromosome 11, whereas those categorised in the high-risk subtype had 69 % 5 year survival and was enriched for isochromosome 17 q (Schwalbe

et al., 2017). Cavalli *et al* subdivided Group 4 tumours into three subtypes; Group 4 α , Group 4 β and Group 4 γ . Whilst there was no significant difference in overall survival or rate of metastatic dissemination at diagnosis, there was a slight difference for the median age of a patient (8.22 10 and 7 years for α , β , and γ respectively). *MYCN* was strongly enriched in the Group 4 α subtype, *CDK6* amplification was observed in Group 4 α and Group 4 γ , and *SNCAIP* duplications were strongly enriched in the Group 4 β subtype (Cavalli et al., 2017).

1.3 Medulloblastoma treatment and risk stratification

1.3.1 Treatment

Current treatment strategies are based upon patient risk, which takes into account factors such as patient age at diagnosis, metastatic stage, extent of resection, and histological subtypes. These are categorised into three groups. The first includes infants under the age of 3. The second is a standard risk group and includes patients who are older than 3 years of age with no evidence of metastatic disease, with total or near-total resection (NTR) of the tumour (≤ 1.5 cm²) and have non-anaplastic histology. The third is a high risk group and includes patients who present with any of the following: metastatic dissemination, have post-surgical residual disease > 1.5 cm², or have LCA histology (Pizer and Clifford, 2009, Ramaswamy and Taylor, 2017, Khatua et al., 2018).

1.3.1.1 Surgery

The first line of treatment for medulloblastoma patients is complete or near complete surgical resection of the primary tumour whilst trying to achieve minimal damage to neurological functions. The extent of surgical resection

remains a prognostic factor. Sub-total resection (STR; $> 1.5 \text{ cm}^2$) has been continuously associated with worse PFS, compared to gross-total resection (GTR) or NTR ($\leq 1.5 \text{ cm}^2$). Patients with $> 1.5 \text{ cm}^2$ of residual disease often have a second look surgery to achieve GTR, or are treated with higher doses of craniospinal radiotherapy (CSRT) or more intense chemotherapy. A study on 787 medulloblastoma patients, however, revealed that there was no observed PFS or overall survival (OS) benefit of GTR over NTR (Thompson et al., 2016), indicating that overambitious resection of small residual tumours covering the brainstem should be avoided to minimise the risk of neurological morbidity (Schreiber et al., 2017).

Following surgical resection patients receive chemotherapy or a combination of chemotherapy and radiotherapy that is tailored to the patient's age and risk category of the tumour.

1.3.1.2 Radiation

Infants

In the past, the survival of infants with medulloblastoma was inferior compared to older children. This could have been for many reasons such as, late diagnosis, increased surgical risk, more aggressive tumours, or the increased toxicity of radiotherapy leading to devastating long-term neuropsychological sequelae (Pizer and Clifford, 2009, Taylor et al., 2012). In the mid-1980s it was decided where possible to avoid or delay the use of CSRT in infants, although, the age below which national groups avoid CSRT varies between 3 and 6 years (Duffner et al., 1993).

Standard-risk

Patients older than 3 – 5 years of age at diagnosis are treated with a reduced dose of radiotherapy. This consists of a total dose of 24.3-Gy CSRT, which

covers the entire craniospinal axis (whole brain and spinal cord), and a localised boost to the posterior fossa up to a total dose of 55.0-Gy (Ramaswamy and Taylor, 2017). Prolonged delay in CSRT is typically associated with inferior outcomes (Rieken et al., 2011).

The SIOP-PNET4 clinical trial compared the use of conventional radiotherapy (one dose per day) against hyperfractionated radiotherapy (HFRT; two doses per day) in standard-risk patients, which revealed no difference in event free survival (EFS) and OS. The use of standard fractionated radiotherapy is considered gold-standard for treatment in standard-risk patients, because HFRT was more time consuming and had significant implications for patient care (Lannering et al., 2012).

Proton radiotherapy has recently been trialled as an alternative to conventional radiotherapy, as it irradiates less normal tissue and could improve neurocognitive outcomes commonly observed with photon radiotherapy. In a phase II clinical trial by Yock *et al*, they used proton radiotherapy and chemotherapy, resulting in acceptable toxicity and similar survival outcomes to those noted with conventional radiotherapy, suggesting that this could be an alternative approach to photon-based treatments (Yock et al., 2016).

High-risk

The prognosis for high-risk patients still remains unsatisfactory. In North America, conventional treatment consists of surgical resection followed by CSRT of 36.0-Gy with a 54.0-Gy dose to the tumour bed and a 50.0-54.0-Gy boost to nodular metastatic deposits. In most European regimens the use of upfront chemotherapy followed by either conventional CSRT (1.6 - 1.8-Gy per day) or hyperfractionated radiotherapy (HFRT; two doses of 1.3-Gy per day) is used (Gandola et al., 2008).

1.3.1.3 Chemotherapy

Infants

As mentioned above, CSRT is avoided or delayed for as long as possible in infants due to devastating neurocognitive impairments, and so surgical resection of the tumour followed by chemotherapy is used with the goal of improving survival whilst decreasing neurologic sequelae, but survival still remains inferior to that of older children treated with CSRT.

There is little agreement on the best management strategy for these patients. Rutkowski *et al* described treating infants with an intensive course of postoperative chemotherapy consisting of three cycles of intravenous chemotherapy (cyclophosphamide, vincristine, methotrexate, carboplatin and etoposide) and intraventricular methotrexate. The results were particularly promising for patients without initial metastasis or postoperative residual tumours, with a 5-year PFS rate of 82 %. The outcome for patients without macroscopic metastasis with or without the presence of postoperative residual tumours was also encouraging, with a 5 year PFS rate reaching 68 % (Rutkowski *et al.*, 2005).

A multicentre phase II trial (SJYC07) was designed to evaluate risk-adapted multi-modality therapies in young children (< 3 years of age). They were postoperatively risk-stratified into three groups (low, intermediate and high) based on clinical and histological criteria. Unfortunately, the trial was not a therapeutic success, with the 5-year EFS not improving upon previously reported EFS rates. However, they did identify a subset of SHH tumours (iSHH-II) that could be treated with reduced-intensity chemotherapy and another subset (iSHH-I) that would require more intensive and/ or novel therapy (Robinson *et al.*, 2018).

Standard-risk

Following surgical resection of the tumour, standard-risk patients receive a combination of radiotherapy and multi-agent chemotherapy. The addition of chemotherapy to treatment regimens has further increased the survival rates of patients by 10 – 15 % (Gerber et al., 2014). Multi-agent chemotherapy was added to medulloblastoma management to increase patient survival. In children over 3 to 5 years of age, radiation therapy is followed with maintenance chemotherapy of 4 to 9 cycles of vincristine, cisplatin and either cyclophosphamide or lomustine, known as the 'Packer regimen', with a 5-year PFS rate of around 80 % (Packer et al., 2006).

High-risk

The optimal chemotherapy regimen in high-risk patients has yet to be categorised, resulting in variability in chosen agents and number of cycles. The 5-year survival rates of patients who are treated with high-dose CSRT and adjuvant chemotherapy regimens is around 60 %. Adjuvant chemotherapy regimens typically include 4 to 9 cycles of vincristine, cisplatin, etoposide, carboplatin or cyclophosphamide (Northcott et al., 2019).

Gajjar *et al* showed that high-risk patients who received maximum resection and risk-adapted CSRT followed by four cycles of cyclophosphamide-based dose-intensive chemotherapy resulted in a 5 year EFS of around 70 % (Gajjar et al., 2006). The HIT 2000 trial used a regimen of neoadjuvant chemotherapy followed by hyperfractionated CSRT and maintenance chemotherapy, with a 5-year EFS and OS rate of 60 % and 70 %, respectively (von Bueren et al., 2016).

1.3.1.4 Treatment impacts on quality of life

The close relationship of medulloblastoma to the fourth ventricle and sometimes to the brainstem is associated with post-surgical neurological morbidity and

sadly many survivors are incapable of living an independent life. Regardless of the extent of residual tumour, post-surgical neurological morbidity can occur in up to 24 % of patients and can increase to 44 % after GTR. There is a high risk of cerebellar mutism following surgical resection, which occurs in up to 29 % of patients, resulting in diminished speech or mutism and can be accompanied by ataxia and other neurobehavioral abnormalities (Schreiber et al., 2017, Thompson et al., 2018).

Radiotherapy and cytotoxic chemotherapy are associated with impaired quality of life in medulloblastoma survivors. Treatment can lead to neurocognitive impairment, hearing loss, reduced growth, impaired endocrine function, and secondary malignancies. Studies on the avoidance of radiation for infants has shown long-term functional outcomes that are significantly better than older irradiated children (Massimino et al., 2016, Juraschka and Taylor, 2019). Therefore, it is vital that more effective therapies are identified to improve survival rates and reduce the long-term sequelae of current treatments.

1.3.2 Clinical trials

The advancements made in our understanding of the molecular targets for medulloblastoma tumours has meant that many clinical trials are currently underway to improve patient survival and reduce the long-term side effects of treatment-related toxicities.

Numerous trials are investigating treatment de-escalation in children who harbour low-risk WNT tumours by investigating reduced-dose craniospinal radiation and primary tumour bed boost in combination with reduced-dose chemotherapy, with the goal of improving the quality of life whilst still maintaining excellent survival rates in these patients (Juraschka and Taylor, 2019).

SHH tumours are the best understood medulloblastoma subgroup, which means that many studies have targeted patients who have genetic alterations in the SHH signalling pathway. This has included the use of vismodegib and sonidegib that targeted the SMO protein, blocking upstream pathway activation and resulting in improved PFS. However, many patients treated with SMO inhibitors have developed treatment resistance over time, suggesting monotherapy may be insufficient to prevent remission (LoRusso et al., 2011, Robinson et al., 2015, Bautista et al., 2017).

MYC amplification in Group 3 tumours is associated with highly aggressive tumours and a dismal outcome. Pre-clinical trials using JQ1 to inhibit BET bromodomain was shown to suppress *MYC*-associated transcriptional activity and decrease overall cell viability. These findings highlight promise in targeting *MYC*-amplified medulloblastoma (Bandopadhyay et al., 2014).

Moving forwards, clinical trials should incorporate subgroup information, genetic, cytogenetic, and epigenetic signatures that can predict outcome. Given that metastatic disease is a key risk factor for treatment failure, it is important to also consider the addition of novel agents to current first-line treatments to prevent metastatic disease, rather than initiating these treatments after disease progression or recurrence.

1.3.3 Improving risk stratification

Medulloblastoma tumours are stratified into standard-risk or high-risk for the purposes of treatment and clinical trial design. As mentioned above, the system is based upon a patient's age at diagnosis, metastatic stage, extent of resection, and histological subtypes. However, several studies have now identified genetic and cytogenetic aberrations to more accurately predict patient outcome. This has led to an updated risk stratification proposal for medulloblastoma patients

aged between 3 to 17 years; low risk, standard risk, high risk, and very high risk which are summarised in Table 1.3 and detailed below.

Table 1.3 Risk stratification for non-infant childhood medulloblastoma.

Proposed risk-stratification for non-infant medulloblastoma based on molecular markers and subgroups (Ramaswamy et al., 2016b).

	WNT	SHH	Group 3	Group 4
Low risk > 90 % survival	Under 16 years of age			All of the following: Non-metastatic Chromosome 11 loss
Standard risk 75 – 90 % survival		<i>TP53</i> -wildtype (somatic or germline) No <i>MYCN</i> amplification Non-metastatic	All of the following: Non-metastatic No <i>MYC</i> amplification	All of the following: Non-metastatic No chromosome 11 loss
High risk 50 – 75 % survival		One or both: Metastatic <i>MYCN</i> amplification		Metastatic
Very high risk < 50 % survival		<i>TP53</i> mutation (metastatic or non-metastatic)	Metastatic	
Unknown	Metastatic		Non-metastatic with <i>MYC</i> amplification Significance of anaplasia i17q	Significance of anaplasia

Low-risk medulloblastoma

Many global studies have identified non-metastatic WNT patients under the age of 16 to have an excellent prognosis independent of their treatment regimen, meaning that these patients are categorised as low-risk (Ellison et al., 2005, Clifford et al., 2015). The loss of chromosome 11 and/or whole gain of chromosome 17 in non-metastatic Group 4 tumours also harbours excellent

prognosis, with the loss of chromosome 11 observed in one-third of Group 4 patients (Shih et al., 2014).

Standard-risk medulloblastoma

Non-metastatic SHH tumours without *TP53* mutations fall into the standard-risk category, with clinical trials from the SIOP (International society of Paediatric Oncology), COG (Children's Oncology Group) and St Jude's groups revealing that the 5-year survival for these patients exceeds 80 % (Gajjar et al., 2006, Packer et al., 2006, Lannering et al., 2012). Whilst most Group 3 patients are identified with a poor outcome, a subset of patients in clinical trials with non-metastatic Group 3 tumours who received CSRT did not have an inferior prognosis to other standard-risk patients (Ramaswamy et al., 2016a). Non-metastatic Group 4 tumours without the loss of chromosome 11 have also been identified as standard-risk tumours (Shih et al., 2014).

High-risk medulloblastoma

Current high-risk treatment protocols result in survival rates between 50 to 65 %. Metastatic dissemination remains a marker for poor outcome with these patients, and due to the very low rates of metastatic disease observed in WNT tumours, the vast majority of high-risk patients present with non-WNT tumours. However, *MYCN* amplification in SHH tumours with or without metastatic disease are still considered high-risk tumours (Korshunov et al., 2012).

Very high-risk medulloblastoma

Patients classified with very high-risk tumours have < 50 % survival rates. Two groups have been identified as very-high risk, those that harbour *TP53* mutated SHH tumours, and those with *MYC* amplified, metastatic Group 3 tumours. *TP53* mutated SHH tumours are almost always characterised by anaplastic morphology, and as mentioned previously, this histological subtype is

associated with aggressive tumours and the least favourable outcome. Germline *TP53* mutated SHH patients almost always fail therapy and sadly have a fatal outcome, studies have shown that a subset of patients who do survive long-term eventually died of secondary tumours (Zhukova et al., 2013, Ramaswamy et al., 2015).

1.4 Metastasis

The presentation of metastatic disease in any malignant tumour is largely incurable and constitutes as the primary cause of death for > 90 % of cancer patients. This is mainly due to disseminated tumour cells being resistant to existing therapeutic agents, but the molecular mechanisms of how the tumour cells metastasise remains poorly understood (Gupta and Massague, 2006, Steeg, 2006). Hanahan and Weinberg defined six hallmarks of malignant growth, detailing tissue invasion and metastasis as one of these hallmarks (Hanahan and Weinberg, 2011). Metastasis is a complex multi-step process termed the metastatic cascade, whereby, a subset of cells escape the primary tumour, invade adjacent tissue and travel to near or distant sites where they can form secondary tumours. This process is widely documented in epithelial cells and will be discussed in the following sections (Singh et al., 2018).

1.4.1 Metastatic cascade

The metastatic cascade has been described as a complex succession of cell-biological events (Valastyan and Weinberg, 2011) which can be divided into several steps and is outlined in Figure 1.5 :

1. Invasion of cells through the surrounding extracellular matrix

The first step that initiates this complex process is the invasion of tumour cells through the basement membrane. The basement membrane is a specialised extracellular matrix (ECM) that covers the basal layer of all endothelial and epithelial cells (Valastyan and Weinberg, 2011). Tumours cells invade through the surrounding ECM via the epithelial-mesenchymal transition (EMT) pathway, which will be discussed in further detail below. Briefly, epithelial cells lose their cell-cell junctions and apical-basal cell polarity, causing the cells to change phenotype to a spindle-like cell shape, resulting in increased migratory and invasive capabilities (Gupta and Massague, 2006, Diepenbruck and Christofori, 2016).

2. Intravasation

After local invasion of the ECM, tumour cells migrate between endothelial cells and extend filopodia into the lumen allowing entry into the lymphatic and blood systems (Welch and Hurst, 2019).

3. Transport through the bloodstream

Once the tumour cells have intravasated into the lumina of blood vessels they can disseminate widely throughout the bloodstream. Circulating tumour cells (CTCs) must survive a variety of stresses in order to reach distant sites. Normally, epithelial cells adhere to ECM components via integrins, which is thought to be essential for their survival. Without these interactions, epithelial cells undergo anoikis, a form of apoptosis triggered by the loss of anchorage to the ECM. It is thought that tumour cells might only spend brief periods of time in the bloodstream to prevent anoikis from occurring (Steeg, 2006, Valastyan and Weinberg, 2011).

4. Extravasation into the parenchyma of distant tissues

Once the CTCs have arrived at a secondary site the metastatic cells must escape out of the endothelial vasculature into a target tissue by a process called extravasation. The tumour cells can penetrate the endothelial cells, leading to the attachment of cells to the sub-endothelial ECM (Gupta and Massague, 2006).

5. Colonisation at the distant site

Now that the tumour cells have left the circulatory system they must survive in their new environment. The new environment normally differs greatly from that of the primary tumour, suggesting that tumour cells are poorly adapted to their new-found homes. However, it has been proposed that tumour cells can establish a pre-metastatic niche via exosomes which are extracellular vesicles that communicate signals to local and remote cells, and tissues in order to form micrometastatic lesions and eventually macroscopic tumours (Guo et al., 2019, Fares et al., 2020).

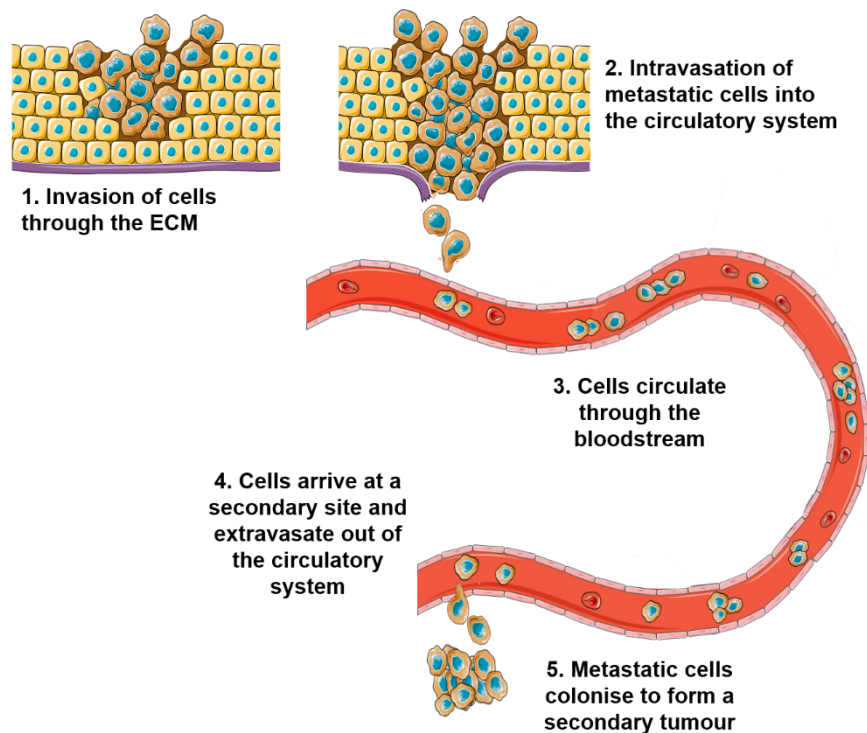


Figure 1.5 Schematic describing the metastatic cascade

Schematic representing the multiple stages of the metastatic cascade. Metastatic cells invade through the surrounding ECM (1), intravasate into the circulatory system (2), and disseminate through the vascular system (3). Tumour cells then extravasate out of the circulatory system (4) where they colonise to form a secondary tumour (5).

1.4.2 Local invasion via the epithelial-to-mesenchymal transition

Normally, EMT is a tightly regulated process of cellular remodelling that is critical during early stages of embryonic development and organogenesis (Diepenbruck and Christofori, 2016). However, in cancer cells EMT is an aggressive and uncontrolled program that occurs as one of the initial steps during the metastatic cascade. It is activated in a distinct set of tumour cells, often referred to as tumour initiating cells or cancer stem cells (CSCs), by signals from cells within the tumour microenvironment (Chaffer et al., 2016).

The EMT pathway is orchestrated by a small group of master regulators, specifically EMT-inducing transcription factors; SNAIL, SLUG, TWIST1 and

ZEB, that are regulated via signalling pathways. Extracellular stimuli can activate the signalling pathway cascades that mediate EMT tumour progression, such as the WNT, Notch, Hedgehog, NF- κ B and Hippo signalling pathways (Thiery et al., 2009, Diepenbruck and Christofori, 2016).

A central hallmark of EMT is the ‘cadherin switch’, whereby *E-cadherin*, a key cell-to-cell adhesion molecule, is downregulated or degraded causing the destabilisation of adherens junctions between cells, and *N-cadherin* is upregulated causing the transitioning cell to change into a mesenchymal phenotype. Some transcription factors, such as SNAIL and ZEB, can repress the expression of *E-cadherin* by binding directly to the E-box sequence located in the promoter region, whilst other transcription factors, such as TWIST1 and FOXC2, repress *E-cadherin* transcription indirectly through the association with other proteins. Other cell-cell junctions that are lost upon the initiation of EMT include tight junctions, gap junctions and desmosomes (Lamouille et al., 2014).

The loss of cell polarity is another crucial step for EMT. In epithelial cells, three protein complexes participate in maintaining apical-basal polarity; Par, Crumbs and Scribble. These complexes are disassembled during EMT by SNAIL1 and ZEB1 causing front-rear polarity of the cell (Thiery et al., 2009, Chatterjee et al., 2016).

As mentioned previously, invading epithelial cells change their phenotype into one that enables dynamic cell elongation and directional motility. This is done by the remodelling of the cellular cytoskeleton. The cells form lamellipodia, filopodia and invadopodia projections that facilitate cell movement and act as sensory extensions of the cytoskeleton (Lamouille et al., 2014, Jacquemet et al., 2015). Lamellipodia can extend long distances through the ECM and pull the cells through this matrix (Friedl and Gilmour, 2009). Filopodia explore the

cell's surroundings (Eilken and Adams, 2010), and actin-rich invadopodia allow for focal degradation of the ECM (Ridley, 2011).

After the loss of cell-cell junctions and cellular remodelling, the final stage of this particular process is the invasion of tumour cells through the ECM. This is achieved by altering the expression of genes that encode cell-to-ECM adhesion molecules and matrix-degrading enzymes. Tumour cell movement through the ECM is a dynamic process, involving the formation of adhesions to the ECM at the leading edge of the cell, and detachment from the ECM at the trailing edge. Adherence to ECM components, such as laminin, collagen, and fibronectin, is mediated by integrins and other tumour cell receptors for ECM proteins such as CD44. The proteolytic degradation of the ECM is mediated by matrix metalloproteinases (MMPs) cathepsins, and heparanases, with MMPs able to directly bind to integrins and degrade the ECM matrix (Friedl and Wolf, 2003, Steeg, 2006).

1.5 Medulloblastoma metastasis

Metastasis has remained a powerful predictor for poor outcome in nearly all medulloblastoma patients. Unfortunately, little is known about the underlying molecular mechanisms that are driving medulloblastoma metastatic dissemination. Medulloblastoma tumour cells almost exclusively disseminate to the leptomeninges, which distinguishes them from other solid tumours, which spread to distant organs through the bloodstream or lymphatic channels (as mentioned above) (Fults et al., 2019). Understanding the dynamics of this process will help identify targets for molecular therapies that may halt or reverse cancer growth and metastasis, and greatly improve patient outcome.

1.5.1 Medulloblastoma metastatic staging

Medulloblastomas are characterised according to the Chang staging system. It was initially devised in 1969 as a means for staging medulloblastoma patients according to metastatic dissemination (M-stage), which is summarised in Table 1.4. The system defines 5 M-stages (M0 – M4) based on MRI and CSF cytology (Chang et al., 1969). M0 is described as no metastatic dissemination, whereas, M4 is associated with metastasis to organs outside the CNS.

Table 1.4 Clinical staging criteria for medulloblastoma metastasis.

Adaptation of Chang staging system from 1969 (Chang et al., 1969).

M-Stage	Criteria
M0	No evidence of metastasis
M1	Microscopic tumour cells in CSF and no radiographic abnormality
M2	Tumour extension beyond primary site to subarachnoid spaces in cerebellum or dissemination to cerebrum, lateral ventricles, or third ventricles (intracranial LMD)
M3	Nodular seeding of spinal subarachnoid spaces (spinal LMD)
M4	Metastasis to organ sites outside CNS

1.5.2 Mechanisms of medulloblastoma metastasis

Leptomeningeal dissemination is the predominant pattern of medulloblastoma metastasis with the spread of tumour cells outside the CNS rare. The section above described epithelial derived tumour metastasis as a multi-step process whereby cells invade the ECM via EMT, circulate through the bloodstream and extravasate at a distant site where the metastatic cells can proliferate into macrometastases. However, medulloblastoma have a neuronal cell of origin

rather than epithelial and so the above metastatic cascade which incorporates the haematogenous route, must be amended to build an accurate model of leptomeningeal dissemination. Fults *et al*, proposed a 3-stage process;

1. Initiation

Medulloblastoma cells escape from the primary tumour and enter the CSF.

2. Dispersal

Surviving tumour cells spread through the CSF channels.

3. Colonisation

Disseminated cells or cell aggregates implant on leptomeningeal surfaces of the spinal cord to establish a proliferating metastatic niche.

Medulloblastomas arise in the cerebellum allowing them to be in close proximity to the CSF-filled fourth ventricle. To be able to metastasise, medulloblastoma cells must acquire enhanced migration and invasive properties and be able to weaken cell-cell adhesions to loosen their attachment from neighbouring tumour cells, invade the ECM, and gain access into the CSF (Fults *et al.*, 2019).

The genetic landscape of medulloblastoma leptomeningeal dissemination must be investigated in order to understand the complex processes underpinning medulloblastoma metastasis. A study by Wu *et al*, used a Sleeping Beauty transposon system, to identify candidate genes that were driving leptomeningeal dissemination in SHH tumours. The system worked by a transposase excising the Sleeping Beauty transposon from its chromosomal locus and inserting itself widely throughout the genome to activate the transcription of proto-oncogenes or suppress transcription of tumour suppressor genes that were located at the insertion sites. The DNA adjacent to the transposon insertion sites were sequenced, identifying 584 candidate genes in

a subpopulation of cells that seeded the leptomeninges and subsequently underwent clonal expansion (Wu et al., 2012).

Pathway analysis comparing the genes affected by metastasis from the above study, identified only the insulin signalling pathway, which is important in the formation of medulloblastoma (Hahn et al., 2000). In addition, one of the genes identified was *HGF* (hepatocyte growth factor), which has emerged as an inducer of EMT. *HGF* can stimulate cell motility and invasiveness, and drive cell cycle progression, with its effects mediated by its cell surface receptor c-Met. Ectopic expression of HGF has been shown to cooperate with SHH to induce aggressive medulloblastoma tumours *in vivo* (Binning et al., 2008).

The PI3K signalling pathway has been identified as a putative contributor of leptomeningeal dissemination. *In vivo* studies have shown PI3K signalling enhanced both primary tumour formation and leptomeningeal dissemination in SHH tumours (Mumert et al., 2012). In Group 3 tumours enrichment of genes encoding PI3K signalling proteins were identified, with PI3K inhibition suppressing the growth of *MYC* stimulated tumour cells (Pei et al., 2012).

Another recent study by Garzia *et al* identified that medulloblastoma cells could spread via a haematogenous route. Medulloblastoma CTCs were identified in therapy-naïve patients which lead them to investigate the connection between CTCs and leptomeningeal metastasis. In a parabiosis model, in which pairs of mice were surgically joined together to create a shared circulatory system, medulloblastoma cells were able to metastasise to the spine of the donor and recipient mouse, even though they did not share common CSF channels. This indicated that the tumour cells shed from the donor mouse and disseminated via a haematogenous system. They suggested that dissemination occurred via a CCL2/CCR2 signalling axis, highlighting a biological target for medulloblastoma metastasis (Garzia et al., 2018).

The fact that medulloblastoma cells can metastasise via haematogenous and CSF routes highlights the crossover in traits between the epithelial metastatic cascade and the leptomeningeal metastatic cascade. HGF was identified as a key inducer of the EMT pathway and could induce aggressive medulloblastoma tumours to metastasise. This suggests that other transcription factors involved in initiating the EMT in epithelial tumours could also be relevant during medulloblastoma metastasis.

1.5.3 Invading the brain extracellular matrix

As mentioned above the main method of medulloblastoma metastasis is via leptomeningeal dissemination. The leptomeninges surrounds the brain and spinal cord and consists of two main layers; the arachnoid mater and the pia mater. Between these layers lies the subarachnoid space, which is where the CSF flows (Barshes et al., 2005).

Medulloblastoma tumour cells in the primary tumour extensively interact with unique components of the brain ECM. However, the ECM that comes into contact with the primary tumour is different from the ECM that leptomeningeal metastatic cells come into contact with. The interstitial matrix (the primary tumour ECM) is mainly composed of hyaluronan, tenascin C and R, with less abundant proteins such as collagen and adhesive glycoproteins such as laminin and fibronectin present (Zimmermann and Dours-Zimmermann, 2008). The tumour cells modify this ECM by the active secretion of ECM proteins such as collagen, allowing for the migration and invasion of cells. In contrast, the basal lamina, which covers the endothelial cells of parenchyma blood vessels and the surface of the pia mater, is mainly composed of a dense network of collagen, laminin and fibronectin, and cell movement is mediated by cell adhesion molecules and integrins (Van Ommeren et al., 2020).

1.5.4 Subgroup specific metastasis

Metastatic disease is present in 20 – 30 % of all medulloblastoma patients at the time of diagnosis. The rate of metastasis differs between medulloblastoma subgroups. A study by Ramaswamy *et al*, revealed that the recurrence patterns across medulloblastoma patients was highly subgroup specific. SHH medulloblastomas recurred locally at the previously treated resection cavity, whereas, Group 3 and 4 tumours recurred almost exclusively with metastatic dissemination. Group 4 tumours recurred significantly later than Group 3 and SHH tumours and these patients had a significantly longer survival post-recurrence. Interestingly, the tumours retained the same subgroup at the time of recurrence, highlighting the stability of each of the four subgroups (Ramaswamy et al., 2013).

A study by Zapotocky *et al*, investigated the pattern of metastatic dissemination at diagnosis, and found it to be highly subgroup specific. Laminar metastasis (non-measurable thin coating of cells) was observed in Group 3 tumours, compared to nodular metastasis (measurable deposits of cells) being more common in SHH tumours, with Group 4 tumours exhibiting both of these patterns. Group 3 primary tumours were frequently smaller in the presence of metastasis compared to SHH and Group 4 tumours, which could be due to an early metastatic event leading to a more diffuse presentation (Zapotocky et al., 2018).

1.6 TWIST1

TWIST1 is a highly conserved transcription factor that belongs to the basic helix-loop-helix (bHLH) family. It was originally identified in *Drosophila* as one of the zygotic genes essential for mesoderm specification and subdivision into

different tissue types. In humans, it has also been identified in mesoderm development during development. Mutations in the *TWIST1* gene cause Saethre-Chotzen syndrome (SCS), an autosomal dominant disorder that gives rise to craniofacial and limb abnormalities (Howard et al., 1997, Qin et al., 2012).

1.6.1 *TWIST1* expression in normal tissue

Molecular structure

TWIST1 maps to chromosome 7q21.2 and consists of two exons and one intron, with only exon 1 coding for protein. Structurally, bHLH proteins are characterised by the presence of a conserved domain containing a stretch of basic amino acids adjacent to two amphipathic α -helices separated by an inter-helical loop (Figure 1.6). The Loop-Helix II junction is involved in DNA binding and the α -helices mediate the interaction of *TWIST1* with a second bHLH factor, leading to the formation of a dimer that can bind to regulatory E-boxes (which will be discussed later on). Mutations in the Loop-Helix II junction have been shown to prevent *TWIST1* efficiently binding to E-boxes, and is one of the underlying causes of SCS (El Ghouzzi et al., 2001).

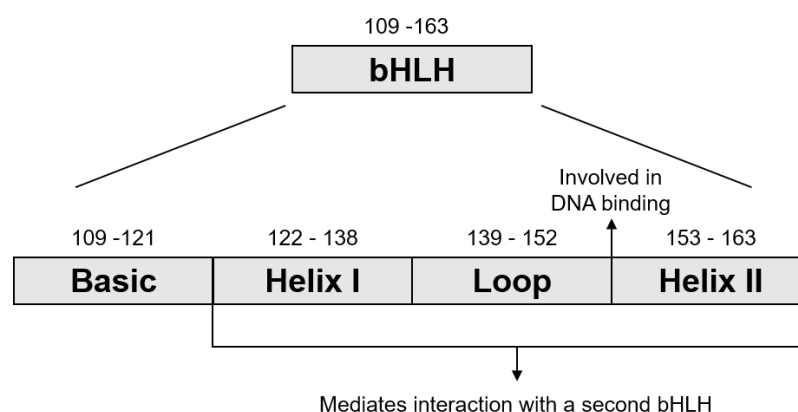


Figure 1.6 Molecular structure of the human *TWIST1* protein

Structure of the *TWIST1* protein. The number of amino-acid residues for each structural domain is indicated. Figure is adapted from Qin *et al.*, 2012.

TWIST1 Expression

TWIST1 is primarily expressed in mesoderm-derived tissue. Its expression patterns have been well studied in *Drosophila* and mouse, but information regarding TWIST1's expression profile in humans during embryogenesis is lacking.

In *Drosophila*, TWIST1 is expressed during embryogenesis. It is first detected in the cellular blastoderm stage in cells that are destined for mesoderm and mesectoderm formation. TWIST1 accumulates at the beginning of cellular blastoderm to the late stages of gastrulation in all cells of the presumptive mesodermal layer. TWIST1 expression decreases to relatively low levels in the mesodermal layer cells during later stages of embryogenesis, and after birth, *TWIST1* is expressed in adult mesenchymal cells, such as muscle stem cells (Bate et al., 1991, Figeac et al., 2007).

Wang *et al.*, analysed various adult human tissues, as well as several cell lines originating from different normal human tissues, and found the strongest signals for *TWIST1* mRNA were observed in the placenta, in particular the foetal portion of the placenta developed from the chorionic sac (mostly derived from the mesoderm). Intermediate signals were seen in the adult heart and skeletal muscle and weak signals were found in the kidney and pancreas, whilst no signals were observed in the ectoderm-derived cells in the brain, or in human epithelial cells (Wang et al., 1997).

TWIST1 Regulation

Regulation of TWIST1 protein is an important way to control its function. TWIST1 proteins function by forming dimers with another bHLH member and subsequently binding to *cis*-regulatory elements called hexanucleotide

sequence E-boxes (5'-CANNTG-3'). E-boxes are present in the regulatory elements of many genes that are essential for organogenesis (Zhu et al., 2016).

Traditionally, bHLH proteins are categorised into three subfamilies: class A, class B and class C. Class A proteins include the E2A proteins E12 and E47, HEB, and E2-2, which are ubiquitously expressed in mammalian cells and are involved in B cell, muscle- and pancreatic-specific gene regulation (Murre et al., 1994). Class B proteins, which includes TWIST1, are tissue-specific, and class C molecules consist of MYC proteins. The different dimer forms of the bHLH proteins can act as either positive or negative transcriptional regulators. Class B can form homodimers or heterodimers with class A proteins, whereas class C proteins can only form dimers with other class C proteins (Castanon and Baylies, 2002).

TWIST1 homodimers are thought to enhance the FGF pathway during the formation of cranial sutures, thereby promoting suture closure (Connerney et al., 2008). TWIST1 proteins tend to form heterodimers with the E proteins (E2A). Formation of TWIST1/E47 heterodimers are said to stabilise TWIST1 proteins. The Id (inhibitor of DNA) family of bHLH proteins, which lack a functional domain and cannot bind to DNA, negatively regulate TWIST1 by forming heterodimers and destabilising the TWIST1 protein through the induction of its degradation (Hayashi et al., 2007). Id proteins can also regulate TWIST1 by binding to E proteins (preferentially E12), and prevent E proteins forming functional dimers with TWIST1 (Qin et al., 2012, Zhu et al., 2016). Recently, it was reported that TWIST1 protein stability could be regulated by mitogen-activated protein kinase (MAPK)-mediated phosphorylation on Serine 68 (S68). S68 in TWIST1 can be phosphorylated, preventing ubiquitination-mediated degradation. Mutation of S68 to Alanine (S68A), or inhibition of MAPK

activities, accelerates TWIST1 ubiquitination and degradation, suggesting a positive correlation for MAPKs and TWIST1 protein levels (Hong et al., 2011).

TWIST1 Function

TWIST1 functions as a transcription factor in the cell nucleus. It is essential for mesoderm formation and important to the morphology and behaviours of head mesenchyme cells that support morphogenesis of the cranial neural tube (Chen and Behringer, 1995). TWIST1 is believed to act upon a set of downstream target genes and control a variety of cellular events for the formation of mesenchyme derivatives (Qin et al., 2012). It can maintain mesenchymal cells in an undifferentiated state by negatively regulating *Runx2*. *Runx2* is a critical osteoblast differentiation transcription factor essential for bone formation. Expression of *Runx2* occurs only after *TWIST1* expression decreases (Bialek et al., 2004).

As previously mentioned, TWIST1 is involved in FGF signalling but can also activate genes in the SHH signalling pathway. Its deletion in the limb bud (precursor structures of the limbs) has been associated with reduced activity of genes involved in this pathway; *SHH*, *GLI1*, 2 and 3, and *PTCH* (O'Rourke et al., 2002). TWIST1 can also regulate cytokine signalling through a negative feedback loop that represses NF- κ B activity via interactions with the p65 subunit of NF- κ B (Šošić et al., 2003).

1.6.2 Biological functions of TWIST1 in cancer

TWIST1 is expressed in many types of aggressive tumours including breast, bladder, prostate, gastric and pancreatic cancer and over expression is associated with an inferior prognosis and progression-free survival. TWIST1 can exert multiple biological effects via various downstream pathways as a

transcriptional regulator of pathway target genes (Qin et al., 2012, Zhao et al., 2017).

Facilitate cell invasion and metastasis

The most critical pathological function of TWIST1 in cancer is facilitating cancer invasion and metastasis by promoting EMT. The process of how TWIST1 promotes EMT will be discussed in more detail later on. Studies have demonstrated that TWIST1 is involved in other steps of the tumour invasion and metastatic cascade. TWIST1 is capable of promoting the formation of invadopodia to aid in the degradation of the ECM and promote tumour metastasis (Eckert et al., 2011). During extravasation, *TWIST1* expression can cause tumour cells to extravasate through the vessel wall and cause cells to switch to a $\beta 1$ integrin independent mode to facilitate in the protrusion of cells out of the blood circulation to form secondary tumours (Stoletov et al., 2010)

Override oncogene-induced cell senescence and apoptosis

Cellular senescence and apoptosis are well known defence mechanisms against tumour initiation (Hanahan and Weinberg, 2011). TWIST1 has been shown to inhibit oncogene-induced and p53-dependent cell death by interfering with the activation of the p53-dependent reporter *ARF*, causing impaired induction of *p53* target genes in response to DNA damage (Maestro et al., 1999). Kwok *et al*, demonstrated that inactivation of TWIST1 led to the promotion of cellular senescence and growth arrest, suggesting that TWIST1 plays a key role in the continuous proliferation of immortalised cells (Kwok et al., 2007)

Increase cell resistance to chemotherapy

Chemoresistance is a major problem in many cancers resulting in treatment failure. Multidrug resistance is associated with increased expression of ATP

binding cassette transporters (ABC transporters) including ABCB1, ABCC1 and ABCC4. Many studies have highlighted the association with TWIST1 and multidrug resistant cancer cells. Saxena *et al* demonstrated that the promoters of ABC transporters in invasive breast cancer cells carry several E-box binding sites, and overexpression of TWIST1 increased the promoter activity of the ABC transporters. Furthermore, chromatin immunoprecipitation analysis revealed TWIST1 could bind directly to the E-boxes on *ABCC4* and *ABCC5* (Saxena et al., 2011). Liu *et al*, showed that expression of TWIST1 increased expression levels and promoter activity of *ABCB1* and *ABCC1* in colon cancer cells, and TWIST1 silencing enhanced the chemosensitivity of the cells and downregulated the expression of *ABCB1* and *ABCC1* (Liu et al., 2017).

Enhance CSC population

Cancer stem cells are a subpopulation of cells that are responsible for cancer recurrence and treatment failure by sustaining tumour cell growth and disseminating and migrating to distant organs to give rise to secondary tumours. Multiple studies have demonstrated a link between TWIST1-induced EMT and stem-like properties. Yang *et al* showed that TWIST1 could directly regulate BMI1 and both played a cooperative role in promoting EMT and cancer stemness (Yang et al., 2010). Vesuna *et al* demonstrated the association between TWIST1 and breast cancer stem cells, whereby TWIST1 expression promoted the generation of a breast cancer stem cell phenotype characterised by the high expression of CD44, low expression of CD24, and increased expression and activity of *ABCC1* (Vesuna et al., 2009a).

1.6.3 TWIST1's role in metastasis via EMT

During embryogenesis the EMT program has been observed as underlying a variety of tissue remodelling events, including mesoderm formation and neural

crest development, both of which are facilitated by TWIST1. EMT occurs in response to injury to promote tissue repair, or during organ fibrosis of the lung, liver, and heart (Yang and Weinberg, 2008). Many studies have also highlighted the role of TWIST1 in promoting cancer cell EMT and metastasis.

The EMT process during cancer metastasis has been described in previous sections. Here, we describe how TWIST1 facilitates this process. TWIST1 is a transcription factor, and its overexpression is associated with more invasive cancer types. TWIST1 has been proposed as one of the main transcription factors involved in initiating the EMT process and the dissemination of cancer cells (Zhu et al., 2016).

Loss of E-cadherin is a hallmark of EMT. It is believed that whilst TWIST1 doesn't directly bind to *E-cadherin*, it can activate the transcription of *BMI1*, a chromatin modifier, which interacts with BMI1-containing polycomb-repressive complex 1 (PRC1), to suppress *E-cadherin* expression (Yang et al., 2010). Expression of microRNAs (miRNAs) have been implicated in regulating TWIST1 during EMT, such as miR-720 in breast cancer cells, which downregulated TWIST1 expression leading to the inhibition of cell invasion and migration (Li et al., 2013). Several signalling pathways have been noted to activate TWIST1 in different types of cancer cells, resulting in the induction of EMT. This includes the WNT, STAT3, NOTCH1 and TGF- β signalling pathways (Howe et al., 2003, Cheng et al., 2008, Hsu et al., 2012).

The combination of TWIST1 dimerisation partner determines which genes and pathways TWIST1 regulates. Whilst we mentioned earlier that during embryogenesis TWIST1 can form hetero or homodimers, during EMT and metastasis TWIST1 preferentially forms heterodimers with E-proteins (E2A; *TCF3*, HEB; *TCF12* and E2-2; *TCF4*). Fan *et al* showed that *TCF12* expressing cells activated more genes associated with EMT signalling, and the

TWIST1/HEB heterodimer was a potent driver of EMT and mesenchymal differentiation compared to TWIST1 homodimers (Fan et al., 2020). TWIST1/E2A dimers have been shown to bind more effectively to the E-box sequence CATCTG (Firulli et al., 2007), and Yochum *et al* revealed that this dimerisation was necessary for TWIST1-mediated functions in non-small cell lung cancer (Yochum et al., 2017).

1.6.4 TWIST1's function in non-epithelial tumours

Whilst the EMT pathway is associated with epithelial tumours, it is believed that non-epithelial tumours can still undergo an EMT-like pathway, whereby cells detach from the local tumour and acquire an invasive phenotype to invade through the ECM. Haematogenous dissemination has recently been identified in medulloblastoma tumours, supporting the idea that non-epithelial tumour cells can disseminate via an EMT-like pathway (Kahlert et al., 2017, Garzia et al., 2018).

Elias *et al* revealed high *TWIST1* expression was associated with the highest grade gliomas and promoted cell invasion through matrigel extracellular matrices (Elias et al., 2005). Furthermore, Mikheeva *et al* found that TWIST1 increased the expression of genes associated with adhesion, ECM proteins, cell migration and actin cytoskeleton organisation in glioblastoma (GBM) cells, and that TWIST1 still promoted an invasive mesenchymal phenotype in GBM cells even though it did not generate the canonical cadherin switch (Mikheeva et al., 2010). More recently, Kahn *et al* identified a NOTCH1-TWIST1-BMI1 signalling axis in Group 3 medulloblastoma that regulated the initiation of metastasis (Kahn et al., 2018). Overall, these studies indicate that non-epithelial tumours display traits of the EMT pathway and that TWIST1 expression could be important in initiating the metastatic cascade.

1.7 ABCB1

The previous section highlighted the importance of ABCB1 in multidrug resistance via the association with TWIST1. However, increasing evidence supports a metastatic role for ABCB1. The following sections will discuss in further detail ABCB1's structure, function, regulation, and role in metastasis.

1.7.1 ABCB1's Structure

ABCB1, also known as multidrug resistance 1 (*MDR1*), is a transmembrane glycoprotein. It is located on chromosome 7q21.1 and consists of 28 exons and 28 introns. ABCB1 protein has four core structural domains: two symmetrical cytoplasmic nucleotide-binding domains (NBD) and two transmembrane domains (TMD) consisting of six α -helical membrane-spanning domains (Katayama et al., 2014) (Figure 1.7). The two NBDs bind and hydrolyse ATP to power the transportation process, and the two TMDs recognise and transport substrates. The structure and function of NBDs are similar throughout the ABC subfamilies, whereas, TMDs are highly heterogeneous and recognise a diverse range of substrates (Robey et al., 2018, Kodan et al., 2021).

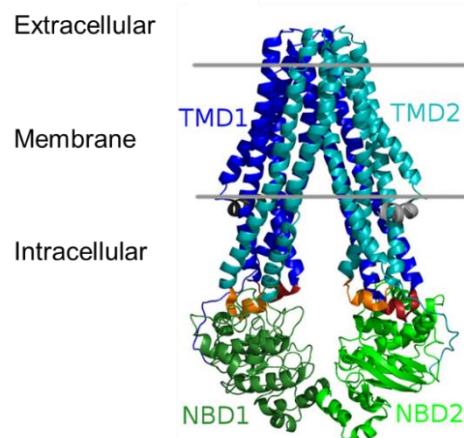


Figure 1.7 Crystal structure of the ABCB1 protein

Crystal structure detailing ABCB1's four core domains: two transmembrane domains and two nucleotide-binding domains. The structure represents an inward open structure with separated NBDs. Image is from (Szöllősi et al., 2018).

1.7.2 ABCB1's Function

ABCB1 exports various hydrophobic compounds, including therapeutic drugs, alkaloids, flavonoids, and other natural toxic compounds, across biological membranes. The process in which the transportation of substrates across the membrane occurs is described in Figure 1.8. A specific substrate binds to the TMDs and ATP binds to the binding sites located in the NBDs. Hydrolysis of ATP generates a conformational change in the ABCB1 protein, allowing for the substrate to be released and transported across the membrane (Robey et al., 2018, Kodan et al., 2021).

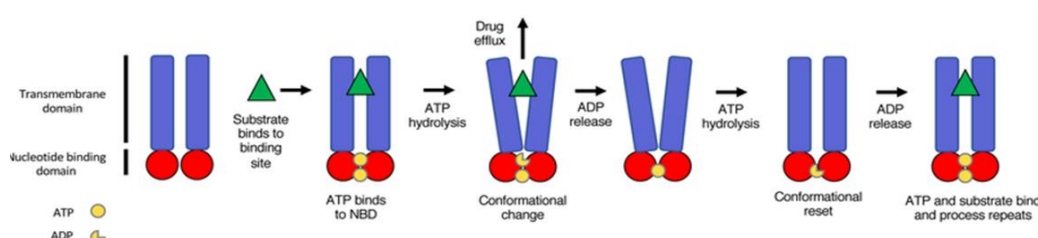


Figure 1.8 Schematic representation of ABCB1's function

Schematic detailing the pumping action of ABCB1 which allows for the transportation of substrates across a biological membrane. ABCB1 undergoes conformational change, transitioning from an inward facing state to an outward facing state, to allow for the efflux of substrates. Image is from (Robey et al., 2018).

Under physiological conditions, the transportation of drugs occurs in a single direction, with ABCB1 acting as an essential protective barrier in many organs, including the brain by preventing drugs crossing the blood-brain barrier and into the CNS. However, overexpression of ABCB1 in tumour cells results in treatment failure due to the transportation of chemotherapeutic drugs, such as anthracyclines, vinca alkaloids, and epipodophyllotoxins, out of cancerous cells. This reduces intracellular drug concentrations, and subsequently results in multidrug resistance (Katayama et al., 2014).

Many studies have focussed on overcoming multidrug resistance via ABCB1 inhibition. Although, clinical trials investigating the use of ABCB1 inhibitors have not been very promising. To date, three generations of ABCB1 inhibitors have been reported, with some trials utilising compounds that have already been approved for clinical use, such as verapamil and cyclosporine A. Unfortunately, many of these inhibitors were non-selective since they competed with other substrates and had unacceptable levels of toxicity (Gottesman et al., 2002).

Moving forwards, vardenafil, a Food and Drug Administration (FDA) approved phosphodiesterase 5 (PDE-5) inhibitor, has shown promising results both *in vitro* and *in vivo*. It is currently used to treat erectile dysfunction in men and pulmonary arterial and portal hypertension in children (Figure 1.9). Ding *et al*, demonstrated that vardenafil could reverse ABCB1-mediated MDR by directly blocking the drug efflux function and was non-toxic when used alone in human epidermoid carcinoma cell lines (Ding et al., 2011). In an *in vivo* setting vardenafil is well tolerated and has been shown to enhance delivery of ABCB1 substrates Herceptin and Adriamycin by increasing blood-brain and blood-brain tumour permeability in rodents bearing primary brain tumours (Black et al., 2008, Ding et al., 2011).

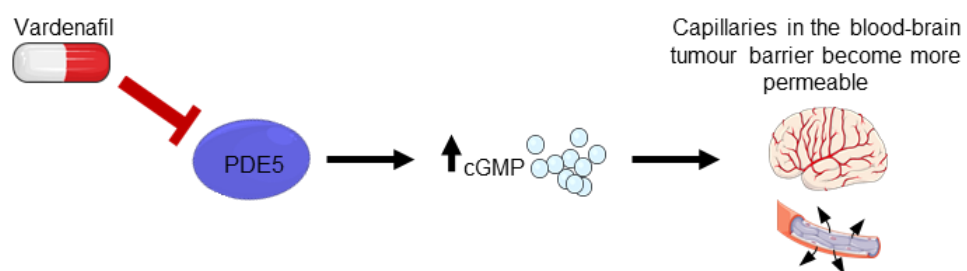


Figure 1.9 Schematic illustrating vardenafil's mode of action

Vardenafil inhibits PDE5, causing an increase in cyclic guanine monophosphate (cGMP) accumulation, allowing capillaries to be more permeable, including those in the blood-brain tumour barrier.

1.7.3 ABCB1's Regulation

Transcriptional regulation of *ABCB1* can occur via the binding of transcription factors, such as NF- κ B, AP-1 and FOXO1, to the *ABCB1* promoter. Signalling pathways such as the WNT signalling pathway has also been shown to regulate the expression of *ABCB1*. Corrêa *et al* demonstrated that β -catenin activated the transcription of TCF/LEF resulting in the upregulation of *ABCB1* in chronic myeloid leukaemia (Corrêa *et al.*, 2012, Katayama *et al.*, 2014)

Several mRNAs have been reported to modulate the expression of *ABCB1*. Zhu *et al* showed that upregulation of miR-27a and miR-451 activated *ABCB1* expression in multidrug resistant cancer cells (Zhu *et al.*, 2008). Epigenetic mechanisms have also been demonstrated as way of controlling *ABCB1* expression. Methylation of the *ABCB1* promoter in leukaemia and bladder cancer resulted in activation of *ABCB1* expression (Tada *et al.*, 2000, Garcia-Manero *et al.*, 2002).

1.7.4 ABCB1's role in metastasis

ABCB1 is commonly known for its role in multidrug resistance. However, emerging evidence has started to indicate that it could be linked to tumour metastasis. Milette-González *et al* found a correlation between the expression of *ABCB1* and the adhesion molecule CD44 in breast cancer cells and that both proteins co-localised within the cell membrane resulting in a multidrug resistant phenotype and increased cell migration and invasion (Milette-González *et al.*, 2005). Furthermore, Colone *et al* also showed that *ABCB1* co-localised with CD44 in the plasma membrane of melanoma cells through the activation of the ERK1/2 and p38 MAPK pathways. This interaction led to increased invasive behaviour, associated with increased MMP2, 3 and 9 expression (Colone *et al.*, 2008).

1.8 Hypothesis and aims

The underlying molecular mechanisms of medulloblastoma metastasis remains poorly understood. Whilst the role of TWIST1 has been extensively studied in many epithelial tumours, an in-depth functional analysis of the potential underlying molecular mechanisms that TWIST1 could be regulating during medulloblastoma metastasis remains to be explored.

We hypothesised that TWIST1 plays a pivotal role in initiating the medulloblastoma metastatic cascade.

The aims and objectives of this study were to:

1. Investigate TWIST1 expression in medulloblastoma tumours across the four molecular subgroups and assess if expression correlated with patient outcome and metastatic disease. This was performed using online medulloblastoma patient datasets, medulloblastoma cell lines and patient tissue microarrays.
2. Assess if TWIST1 is playing an essential role in medulloblastoma cell migration by chemically and genetically inhibiting TWIST1 using harmine, a beta-carboline alkaloid, and shRNA respectively. A 3D spheroid model and a 3D transwell migration and invasion assay were utilised to investigate the effect TWIST1 inhibition had upon cell migration.
3. Investigate the potential of a TWIST1-ABCB1 axis using chromatin immunoprecipitation (ChIP) and understand if both are contributing towards medulloblastoma metastasis. Functional experiments utilising the 3D transwell migration and invasion assay was explored following inhibition of ABCB1 with vardenafil and TWIST1 with harmine or shRNA.

4. Unravel TWIST1's transcriptional regulatory network using ChIP-Sequencing to understand the functional mechanisms of TWIST1 and if these mechanisms drive medulloblastoma metastasis. This was explored using metastatic medulloblastoma cell lines from the SHH and Group 3 subgroups.

Understanding the mechanisms of medulloblastoma metastasis will ultimately help identify biological relevant therapeutic targets.

Chapter 2. Methods and Materials

2.1 Cell Culture

2.1.1 Standard culture media

One non-tumourigenic cell line and eight medulloblastoma cell lines were used for this study. Clinical information for each medulloblastoma cell line is summarised in (Table 2.1). DAOY, D283, D425, D458 and FB83 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Fisher Scientific, 11574446) supplemented with 10 % foetal bovine serum (FBS; Hyclone, SH30541.03). ONS-76 and HD-MB03 cells were cultured in RPMI 1640 (Sigma-Aldrich, R8758) supplemented with 10 % FBS. CHLA01 and CHLA01R were cultured in DMEM/F-12 (Fisher Scientific, 11520396) supplemented with 2 % B-27 (Gibco, 17504044) 20 ng/mL recombinant human epidermal growth factor (EGF; Gibco, PHG0315), and 20 ng/mL recombinant human basic fibroblast growth factor (FGF; Gibco, PHG0266).

2.1.2 Recovering cell lines

Cryo-vials of cells were thawed quickly in a 37°C water bath. The cells were transferred to a 15 mL tube (Greiner Bio-One, 188261), diluted in 5 mL standard culture medium and centrifuged for 5 minutes at 100 x *g*. The supernatant was discarded and cell pellets were resuspended in pre-warmed standard culture medium and transferred to a cell culture flask. All cell lines were grown in a 37°C incubator with 5% CO₂ (New Brunswick, Galaxy 170R).

Table 2.1 Clinical information for medulloblastoma cell lines used in this study

Eight medulloblastoma cell lines were used throughout this study, representing three of the four molecular subgroups.

*^Cell lines with matched primary and metastatic pairs.

Subgroup	Cell line	Clinical information	Metastatic stage	Source	References
SHH	ONS-76	2 year old female. Large (3 cm) midline cerebellar tumour	Metastatic tumours at the right prepontine cistern (M2)	Annette Künkele (Charité Universitätsmedizin, Berlin)	Tamura <i>et al.</i> , 1989
	DAOY	4 year old male. Cerebellar medulloblastoma of the posterior fossa with a <i>TP53</i> mutation	Non-metastatic (M0)	ATCC® HTB-186TM	Jacobsen <i>et al.</i> , 1985 Raffel <i>et al.</i> , 1993
Group 3	D283	6 year old male. Mass (3 cm) in the cerebellar vermis	Peritoneal metastasis and malignant ascites (M2)	ATCC® HTB-185™	Friedman <i>et al.</i> , 1985
	HD-MB03	3 year old male. Large cell mass in midline (fourth ventricle and vermis)	Spinal metastasis at diagnosis (M3)	Till Milde (DKFZ, Heidelberg)	Milde <i>et al.</i> , 2012
	D425*	5 year old male. Primary cerebellar tumour	Non-metastatic (M0)	Marcel Kool (DKFZ, Heidelberg)	He <i>et al.</i> , 1991
	D458*	5 year old male. Matched metastatic recurrence	Metastatic (M+)	John R. Silber (University of Washington, Seattle)	
Group 4	CHLA01^	8 year old male. Primary tumour: cerebellar mass with diffuse leptomeningeal disease	Leptomeningeal metastasis at diagnosis (M3)	ATCC® CRL-3021™	Xu <i>et al.</i> , 2015
	CHLA01R^	8 year old male. Metastatic recurrence (8 months after diagnosis): tumour cells from pleural fluid	Recurrent metastatic cells from pleural fluid (M2)	ATCC® CRL-3034™	

2.1.3 Preparation of cell pellets

Cells were collected into a 50 mL falcon and centrifuged at 100 x *g* for 5 minutes. The supernatant was discarded, and cells resuspended in 1 mL of HBSS and transferred to a 1.5 mL microcentrifuge tube (Eppendorf, 0030120086). The cell suspension was centrifuged again for 5 minutes at 1000 rpm in a microcentrifuge and the supernatant was carefully removed and the pellet was snap frozen in liquid nitrogen and immediately transferred to a - 80°C freezer.

2.1.4 Mycoplasma testing and cell line authentication

Mycoplasma testing was performed after recovering cells and on a monthly basis using a sample of medium that had been in contact with the cells for at least 3 days in culture. Screening for Mycoplasma was done using the MycoAlert Mycoplasma Detection Kit (Lonza, LT07-118).

Whilst cell lines were not authenticated during the course of this project, RNA-sequencing was performed on six of the medulloblastoma cell lines (ONS-76, DAOY, HD-MB03, D458, CHLA01 and CHLA01R). Gene expression analysis correlated with the publicly available dataset from Cavalli *et al.* Principal component analysis (PCA) showed ONS-76 and DAOY cells clustered with the SHH patient's cohort, HD-MB03 and D458 cells clustered with Group 3 and CHLA01 and CHLA01R clustered with Group 4 patients (Cavalli *et al.*, 2017, Linke *et al.*, 2021).

2.1.5 Subculturing cell lines

2.1.5.1 Adherent cells

The SHH medulloblastoma cell lines and the non-tumourigenic FB83 cell line grew adherently in standard culture. Cell monolayers were sub-cultured when they had reach 70 % confluency. To subculture cells, the medium was

discarded and cells washed with 10 mL Hanks Balanced Salt solution (HBSS; Gibco, 14170). To detach cells, 1.5 mL of pre-warmed 1x Trypsin-EDTA (Sigma, LH-SIG2044) was added to the cell culture flask and incubated for 5 minutes at 37°C. Trypsinisation was inhibited by the addition of fresh media and the cells were then seeded into a T75 tissue cultured treated flask (Eppendorf, 0030711114) at a ratio of 1:10 and incubated at 37°C in 5 % CO₂.

2.1.5.2 Semi-adherent cells

The Group 3 medulloblastoma cell lines HD-MB03, D283, D425 and D458 grew semi-adherently in standard culture, consisting of adherent cells, semi-adherent cells and suspension single cells or as small cell clusters. The cells were sub-cultured once they had reach approximately 70 % confluency. The media was removed and collected into a 50 mL tube. Cells were washed with 10 mL HBSS and transferred to the same tube. Pre-warmed 1xTrypsin-EDTA was added to the adherent population of cells and incubated for 5 minutes at 37°C to promote detachment. Detached cells were resuspended in pre-warmed media and collected with the suspension cells and then centrifuged for 5 minutes at 100 x *g*. Following centrifugation, the supernatant was discarded and the cell pellet resuspended in their standard culture media and seeded into tissue culture treated T75 flasks at a ratio of 1:3 (D283 cells) to 1:10 (HD-MB, D425 and D458 cells) and incubated at 37°C in 5 % CO₂.

2.1.5.3 Suspension cells

The Group 4 medulloblastoma cell lines CHLA01 and CHLA01R grew as clusters of cells and single cells in suspension. To maintain cells, fresh medium was added every 3 – 4 days. To subculture the cells, once a week the cell suspension was transferred into a 50 mL tube and centrifuged for 5 minutes at 100 x *g*. Cell pellets were resuspended in a ¼ of conditioned media and ¾ of

fresh media and seeded into non-treated T25 flask (Eppendorf, 0030711017) at a ratio of 1:2 to 1:4 and incubated at 37°C in 5 % CO₂.

2.1.6 Preparation of cell line stocks

Cells were collected into a 50 mL falcon and centrifuged at 100 x g for 5 minutes. The supernatant was removed, and cell pellets were resuspended in Cellbanker® freezing medium (AMSBIO Ltd, 11891). Cells were frozen at a concentration of 1 x 10⁶ cells/mL as 0.5 mL aliquots in FluidX tubes (Brooks Life Sciences, 68-0703). Tubes were placed directly at - 80°C for 24 hours before being transferred to liquid nitrogen for long-term storage.

2.1.7 Cell Counting

Cells were counted using a haemocytometer. Once harvested, 10 µL of cell suspension was combined with 10 µL of Trypan blue (Fisher, 15393661), which coloured dead cells blue. 10 µL of suspension was pipetted onto a glass counting haemocytometer (Hawksley) and counted on a brightfield microscope (Olympus CK2). The number of cells/ mL was calculated using the following formula:

$$\text{Cells per mL} = (\text{number of live cells}/4) \times (\text{dilution factor } 2) \times (1 \times 10^4)$$

2.2 3D spheroid assays

2.2.1 Neurosphere medium

Neurosphere medium for spheroid culture was made using DMEM/F-12, 2 % B-27 supplement, 1 % N-2 supplement (Gibco, 17502048), 2 µg/mL Heparin (Stem Cell Technologies, 07980), 20 ng/ mL EGF, 10 ng/mL FGF, and 0.24 %

methyl cellulose. Fresh Neurosphere medium was made weekly and stored at 4°C.

Methyl cellulose was required to promote tighter spheroid formation. To prepare a 1.2 % stock, 600 mg of methyl cellulose (Sigma, M7027) was autoclaved and diluted in 50 mL in DMEM/F-12 that had been heated to 60°C. The solution was stirred for 30 minutes at room temperature, then for an additional 2 hours at 4°C. The solution was then centrifuged for 2 hours at 5000 x *g*, and the clear viscous supernatant was removed. The methyl cellulose solution was stored at 4°C for up to 3 months.

2.2.2 Spheroid formation

The HD-MB03 cell line was the only cell line used for spheroid assays. Cells were grown in their standard culture conditions until they reached approximately 70 % confluency. The cells were harvested, centrifuged at 100 x *g* for 5 minutes and resuspended in neurosphere medium. 250 cells/well were seeded in ultra-low attachment (ULA) round-bottom 96-well plates (Corning, 7007) in a volume of 200 µL/well. The outer wells of the ULA plate were filled with HBSS to reduce the effects of evaporation. After seeding, plates were centrifuged at 100 x *g* for 5 minutes to encourage spheroid compactness. Plates were incubated at 37°C in 5 % CO₂. Spheroids were maintained by replenishing 50 % of the medium every 3 – 4 days.

2.2.3 3D Spheroid drug treatment

Spheroids were generated as described in section 2.2.2 in black-walled 96-well ULA plate (Corning, 4520) to avoid transfer after drug treatment. Spheroids were drug treated on day 4 of growth by removing 50 % of medium from each well and replacing with harmine (abcam, ab120225) at 2x concentration of increasing concentrations. All conditions were performed in triplicate. Images

were taken on day 4 (pre-treatment) and then daily until day 7 to monitor spheroid growth (Figure 2.1 and Section 2.2.5).

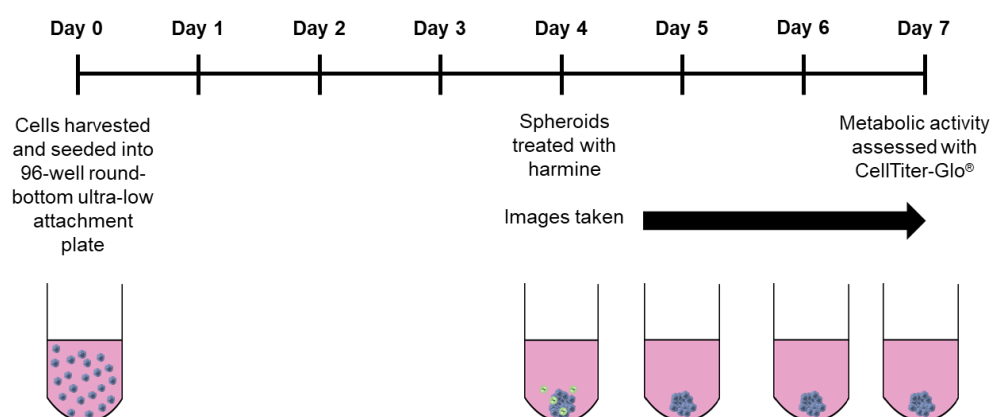


Figure 2.1 Schematic illustrating the 3D spheroid drug treatment assay

On day 0 a dissociated cell suspension was seeded into a well of a 96-well round bottom ultra-low attachment plate. A single spheroid formed by day 4 and was subjected to treatment with harmine. To assess spheroid growth, images were taken daily until day 7. Metabolic activity was assessed on day 7 using the CellTiter-Glo® assay.

Following 72 hours of drug treatment, CellTiter-Glo® 3D reagent (Promega, G9683; thawed to room temperature) was added to each well at a 1:1 ratio to assess metabolic activity. The luciferase-based assay lysed the cells which generated a luminescent signal proportional to the amount of adenosine triphosphate (ATP) present in the cells, indicative of the number of metabolically active cells within the spheroid. The plates were shaken at 250 rpm for 5 minutes in an orbital incubator (Stuart®, S1500) to promote cell lysis, followed by an additional 25-minute incubation at room temperature. Luminescence was measured on a FLUOstar Omega plate reader (BMG Labtech Ltd). Data was exported to Microsoft excel using the Omega software. The following equations were used to calculate relative metabolic activity:

1. *Average luminescent reading of either the blank (medium only),
vehicle or treatment*
2. *Vehicle or treatment – blank*
3. $\left(\frac{\text{Treatment}}{\text{vehicle}} \right) \times 100$

2.2.4 3D spheroid migration assay

Collagen IV (Cultrex, 3410-010-01) was thawed on ice for 2 hours and diluted in cold, sterile water to a concentration of 10 µg/mL. 50 µL of collagen IV was added to each well of a tissue-culture treated 96-well plate (Eppendorf, 0030730119) and the solution was spread out to ensure the entire surface of the well was covered. The collagen IV-coated plates were left to dry for 2 hours at room temperature before wells were washed with cold culture media. Day 4 spheroids (grown as per section 2.2.2) were transferred from the 96-well ULA plate in 100 µL of neurosphere medium. 100 µL of neurosphere medium with harmine at 2x concentration was then added to each well. All conditions were performed in triplicate. Plates were incubated at 37°C for 1 hour to allow the spheroids to settle on the collagen IV matrix. Spheroid migration across the collagen IV coating was then assessed every 24 hours over a 72-hour period by manual imaging and counting cells that had migrated away from the spheroid body (Figure 2.2).

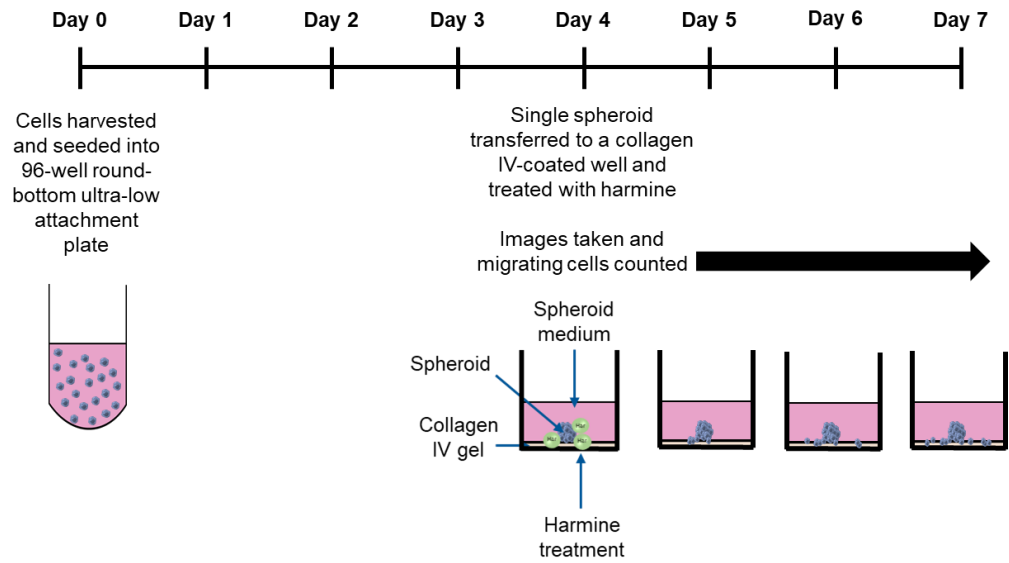


Figure 2.2 Schematic illustrating the 3D spheroid migration assay

On day 0 a dissociated cell suspension was seeded into a well of a 96-well round bottom ultra-low attachment plate. A single spheroid formed by day 4 and was transferred to a collagen-IV coated plate and subjected to treatment with harmine. To assess cell migration, images were taken daily until day 7 and migrating cells counted.

2.2.5 Imaging of spheroids

Spheroid images were taken on the Brunel Multiple Interface camera attached to a brightfield inverted microscope at 10x magnification. The scale of images was determined using a calibration slide. A macro developed by Ivanov *et al* compatible with the Fiji imaging software was used to analyse spheroid size (Ivanov et al., 2014). The macro calculated the spheroid area by drawing a blue line around the edge of the detected spheroid. If the macro failed to detect the spheroid then the circumference was outlined manually. The spheroids were not completely spherical so the circumference of each spheroid was calculated using the following equations:

$$\text{Radius } (r) = \sqrt{\frac{A}{\pi}} \quad \text{Circumference} = 2\pi r$$

2.3 3D Migration and invasion assay

2.3.1 2D drug treatment

Cells were harvested and seeded according to the seeding densities listed in Table 2.2 into a black-walled 96-well plate (Greiner, 655090) in a volume of 100 μL /well and incubated for 24 hours at 37°C at 5 % CO_2 to allow for cell attachment. The cells were treated with 50 μL of harmine at 3x concentration at increasing concentrations and incubated for 72 hours at 37°C in 5 % CO_2 .

To measure cell metabolic activity, PrestoBlue™ reagent (Thermo Fisher Scientific, A13262) was diluted 1:2.5 in cell culture media and 50 μL added to each well, creating a final 1:10 dilution. PrestoBlue™ measures metabolic activity through the reduction of resazurin to resorufin by metabolically active cells, producing a fluorescent colour change from blue to red. Plates were incubated at 37°C in the dark for either 45 minutes or 60 minutes (Table 2.2) and the fluorescent activity measured at excitation: 560 nm and emission: 590 nm using the FLUOstar Omega plate reader. Data was exported to excel using the Omega software. The following equations were used to calculate relative metabolic activity:

1. *Average absorbance reading for either the blank (medium only), vehicle or treatment*
2. *Vehicle or treatment – blank*
3. $\left(\frac{\text{Treatment}}{\text{vehicle}}\right) \times 100$

Table 2.2 Seeding densities and PrestoBlue™ incubation time for 2D drug treatment assays.

Cell line	Number of cells	PrestoBlue™ incubation time (min)
ONS-76	1000	45
D283	2000	60
HD-MB03	1000	45

2.3.2 Setting up the 3D migration and invasion assay

Cell migration and invasion was quantified in a modified Boyden chamber assay using transwell inserts with 8 µm pores (Greiner Bio-one Thincert, 662638) in 24-well plates. 24 hours prior to seeding, the cells were starved in medium supplemented with 2 % FBS and incubated at 37°C and 5 % CO₂. Transwells were pre-coated with 50 µL of collagen IV (10 µg/mL) and dried overnight at room temperature in a tissue culture hood. The following day, laminin 111 (Cultrex, 3400-010-02) was diluted to 100 µg/mL in serum-free medium and 50 µL was added to each transwell insert and allowed to partially dry for 1 hour at room temperature in a tissue culture hood, any remaining liquid was aspirated. Cells were harvested and 1x 10⁵ viable cells were seeded onto the coated transwell inserts in 100 µL of starvation medium. 600 µL of standard culture medium was pipetted into the bottom chamber of the transwell plate and the plate incubated at 37°C and 5 % CO₂ for 48 hours to allow for migration. The number of migrated cells in the lower chamber was quantified relative to a standard curve and cell migration normalised relative to the vehicle control.

For harmine treatment, cells were pre-treated 72 hours prior to the seeding of cells onto the coated inserts in standard culture medium. 48 hours later the cells were re-treated with harmine in starvation medium for 24 hours. For ABCB1

inhibition, cells were treated with 10 μ M vardenafil (Selleckchem, S2515) upon seeding the cells onto transwell-coated inserts.

2.3.3 Quantification of migrated cells

To quantify cell migration, a standard curve of known cell numbers was set up for every biological replicate, ranging from 0 to 1×10^5 cells in 1 x cell dissociation solution (AMSBIO, 3455-05-03). 50 μ L of cells were seeded into a black-walled clear bottom 96-well plate (Brandtech). PrestoBlue™ was diluted with 1 x cell dissociation solution to make a 1:5 dilution and 50 μ L added to each well of the standard curve to make a final 1:10 dilution. The plate was incubated for 1 hour at 37°C and 5 % CO₂ in the dark. After incubation the fluorescent activity was measured at excitation: 560 nm and emission: 590 nm using the FLUOstar Omega plate reader.

2.4 Molecular Techniques

2.4.1 RNA extraction

RNA isolation from cell pellets was performed for qRT-PCR analysis using the NucleoSpin® RNA plus kit (Macherey-Nagel, 740984.50). 350 μ L of lysis buffer was added to each tube and samples were carefully resuspended to disrupt the cell pellet. The lysate was transferred to a NucleoSpin gDNA removal column and centrifuged at 11,000 x *g* for 30 seconds. Following centrifugation, the column was discarded and 100 μ L of binding solution was added to the flow-through to adjust the RNA binding conditions. The lysate was transferred to a NucleoSpin RNA Plus column and centrifuged at 11,000 x *g* for 15 seconds. The membrane was washed with 200 μ L of wash buffer 1, followed by 600 μ L of wash buffer 2 and both centrifuged at 11,000 x *g* for 15 seconds. A final wash

with 250 µL of wash buffer 2 was performed and samples were centrifuged at 11,000 x *g* for 2 minutes to dry the membrane completely. RNA was eluted twice in 30 µL of RNase-free water and centrifuged at 11,000 x *g* for 1 minute. RNA was stored at -80°C.

2.4.1.1 Measuring RNA concentration and quality

RNA concentration and purity were assessed on a Nanodrop 2000C spectrophotometer (Thermo Scientific). RNA concentration was expressed as ng/µl. The 260/280 values were ~2.0 and the 260/230 values were in the range of 2.0 to 2.2.

2.4.2 Complementary DNA (cDNA) synthesis

All sample preparation steps were performed on ice. RNA was diluted in RNase-free water (Stratech PCR-258L-JEN) to 2 µg in a volume of 11 µl and added to a random primers and dNTPs mix (Table 2.3). Tubes were heated to 65°C for 5 minutes and then put on ice for 1 minute. Following this, a master mix consisting of 5X First Strand Buffer, DTT, RNaseOUT and Superscript III Reverse Transcriptase was added to each sample. A no reverse transcriptase control (NRT) was ran in parallel, replacing Superscript III Reverse Transcriptase with RNase-free water.

Table 2.3 cDNA synthesis master mix.

Reagent	For 1 reaction (µl)
Random primers 250 ng/µl (Invitrogen, 48190-011)	1
dNTPs 10mM (Bioline, BIO-39044)	1
5X First Strand Buffer (Invitrogen, 18080093)	4
DTT 0.1M (Invitrogen, 18080093)	1
RNaseOUT (Thermo Fisher Scientific, 10777019)	1
SuperScript III Reverse Transcriptase (Invitrogen, 18080093)	1

The samples were placed in a thermal cycler with the following programme:

- 25°C for 5 minutes
- 50°C for 45 minutes
- 70°C for 15 minutes

cDNA samples (100 ng/µl) were stored at -20°C.

2.4.3 Primer Design

2.4.3.1 Gene expression primers

Primers for gene expression analysis were designed using NCBI Primer Blast: (www.ncbi.nlm.nih.gov/tools/primer-blast/). The NM number, which refers to the transcript for the gene of interest, was copied into NCBI Primer Blast. PCR products were between 50 – 150 bp and primers were between 20 - 25 bp long. The following online tools were used to predict the formation of primer dimers and hairpin structures:

- Oligonucleotide properties calculator
(biotools.nubic.northwestern.edu/OligoCalc.html)
- OligoAnalyzer
(eu.idtdna.com/pages/tools/oligoanalyzer)

2.4.3.2 Chromatin Immunoprecipitation (ChIP) primers

Manual identification of E-boxes

The entire transcript of a gene was identified on NCBI using the FASTA tool. The sequence was downloaded and exons and introns identified using Ensembl (www.ensembl.org). The sequence was manually searched for putative TWIST1 E-boxes (CANNTG) within promoter regions. A specific area containing an E-box was selected and primers designed flanking the E box using NCBI Primer blast.

Genomatix identification of E-boxes

The bioinformatics software 'Genomatix' (www.genomatix.de) was also used to identify putative TWIST1 E-boxes. The 'MatInspector' tool was used to search for transcription factor binding sites within the promoter regions of a given gene. The software utilises a large library of transcription factor binding sites to locate matches in DNA sequences and details the position and sequence of a transcription factor binding site. The sequences were mapped onto a gene's transcript and primers designed within this area using NCBI Primer blast.

2.4.4 Primer optimisation

Once primers had been designed, a gradient Polymerase Chain Reaction (PCR) was performed to identify the optimum annealing temperature. Primers were diluted to 1 μ M in RNase-free water, and 1 μ L of control cDNA (10 ng/ μ l) was added to SYBR Green, forward and reverse primer, and RNase-free water

mix in 0.2 mL sterile Eppendorfs (Table 2.4). A no template control (NTC) was set up, replacing cDNA with RNase-free water.

Table 2.4 PCR gradient master mix.

Reagent	Volume for 1 reaction (µl)
iQ™ SYBR® Green Supermix (Bio-Rad, 1708884)	7.5
Forward primer (150 nM)	2.25
Reverse primer (150 nM)	2.25
RNase-free water	2

The samples were run on a thermal cycler (Primer Thermal Cycler) with the following programme:

- Initial denaturation: 95°C for 3 minutes
- 40 cycles of:
 - Denaturation: 95°C for 10 seconds
 - Annealing: 56-64°C for 60 seconds
 - Extension: 72°C for 30 seconds
- Final extension: 72°C for 5 minutes

The PCR product was visualised by agarose gel electrophoresis alongside a molecular weight marker Quick-Load® Purple 100 bp DNA ladder (NEB, N0551S). The annealing temperature which produced the strongest intensity without the presence of primer dimers was selected and the efficiency of the primers calculated by quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR; 2.4.5) using a dilution series of control RNA (1, 0.5, 0.25, 0.125 and 0.0625). Accepted efficiency values were between 90 and 110 %, with 100 % indicating doubling of target after each PCR cycle, and a slope value between -3.0 and -3.6 (with -3.3 indicating 100 % efficiency). A summary of the

primers used for qRT-PCR and ChIP qRT-PCR are described in Table 2.5 and Table 2.6 respectively. *GAPDH* was used as a house-keeping gene for experiments where TWIST1 had not been inhibited. *HPRT* was used as a house-keeping gene when analysing TWIST1 inhibition experiments due to *GAPDH* being a putative TWIST1 downstream target.

Table 2.5 Sequence of primers used for qRT-PCR analysis.

Gene	Forward primer 5' to 3'	Reverse primer 5' to 3'
<i>ABCB1</i>	CCCATCATTGCAATAGCAGG	GTTCAAACCTTCTGCTCCTGA
<i>BSG</i>	GTTCTTGCCTTTGTCATTCTG	TCACCATCATCTTCATCTACGA
<i>FBXL17</i>	ACAGATTATGCACTGATAGCC	ACTTGCTGCTGTGTGCAAT
<i>GAPDH</i>	ATGTTTCGTCATGGGTGTGAA	GTCTTCTGGGTGGCAGTGAT
<i>HPRT</i>	ATTATGCTGAGGATTTGGAAAGGG	GCCTCCCATCTCCTTCATCAC
<i>HYAL2</i>	GATTACCTGACACGGCTG	GAAACTGTTGGTGCTGAGA
<i>IGF1</i>	ATCAGCAGTCTTCCAACCCA	TGGTGTGCATCTTCACCTTCA
<i>IGF1R</i>	TGTCCAGGCCAAAACAGGAT	CATTCCCCAGCCTGCTGTTA
<i>SORCS2</i>	CCTGGCCGTTTCCTTTTGAAC	TGGTGCTGTTTGTCATCGGG
<i>TWIST1</i>	CAAGCTGAGCAAGATTCAGACCC	AGACCGAGAAGGCGTAGCTGA

Table 2.6 Sequence of primers used for ChIP qRT-PCR analysis.

Gene and E- box sequence	Forward primer 5' to 3'	Reverse primer 5' to 3'
<i>ABCB1</i> CATCTG	GTCATCTGTGGTGAGGCTGA	AGGCTTCCTGTGGCAAAGAG
<i>ABCB1</i> CAGATG	TTGTACCTTGAAATACCTGTCTCTA	TGCTTGACAGTTTCTGAGTTACA
<i>GAPDH</i> CATCTG	TCTTTGCAGTCGTATGGGGG	GGGAGTAGGGACCTCCTGTT
<i>GAPDH</i> CAGGTG	ACAGTCAGCCGCATCTTCTTT	TTTCTCTCCGCCCGTCTTCA
<i>SNAI2</i> CAGATG	CAAACCTCTCCAGATGCCACT	TGTCCGCCAGGAGAAGGAAG

2.4.5 qRT-PCR

All preparation steps were performed on ice. Reactions were set up in 384 well plates (Bio-Rad, HSP3805). A mastermix containing SYBR Green, forward and reverse primers was created (Table 2.7) and dispensed into the 384 well plate. 2 µl of cDNA (5 ng/µl) was added to each well in triplicate and a NRT and NTC control was also added in triplicate.

Table 2.7 qRT-PCR mastermix.

Reagent	Volume for 1 reaction (µl)
iQ™ SYBR® Green Supermix	5
Forward primer (150 nM)	1.5
Reverse primer (150 nM)	1.5

The plates were sealed and briefly centrifuged in a mini plate spinner (MPS 1000™; Biomerieux) and samples ran on a Bio-Rad CFX384 C1000 Touch Real-Time PCR detection System with the following programme:

- Initial denaturation: 95°C for 3 minutes
- 40 cycles of:
 - Denaturation: 95°C for 10 seconds
 - Annealing: 60°C for 60 seconds
- Extension: 72°C for 5 minutes
- Melt curve: Increments of 0.5°C (65 -95°C) every 5 seconds

2.4.6 Gene expression analysis

qRT-PCR data was analysed using CFX Maestro™ software. Gene expression was calculated using the following equations:

$$\text{Gene expression } (\Delta Ct) = 2^{-(\text{average Ct of target gene}) - (\text{average Ct of housekeeping gene})}$$

$$\text{Fold gene expression } (2^{-\Delta\Delta Ct}) = 2^{-(\Delta Ct \text{ treated sample}) - (\Delta Ct \text{ untreated sample})}$$

2.5 Chromatin Immunoprecipitation

ChIP was performed using the Magna ChIP kit (Millipore, 17-611) according to the manufacturer's instructions.

2.5.1 Optimising cross-linking conditions

2.5.1.1 Cross-linking

Cell lines were treated with nine different cross-linking conditions (Table 2.8) to identify the optimum condition that produced chromatin fragments between 200 – 500 bp.

Table 2.8 Cross-linking conditions

Paraformaldehyde concentration (%)	Time (minutes)
1	7, 10, 15
0.75	7, 10, 15
0.5	7, 10, 15

Cells were harvested and 5×10^6 cells were dispensed into 15 mL falcons. Cells were pelleted by centrifugation for 5 minutes at $100 \times g$, the supernatant discarded, and the cells resuspended in 4 mL phosphate buffer saline (PBS). 4 mL of paraformaldehyde (PFA) diluted in PBS was added at a 2x concentration to each tube for either 7, 10 or 15 minutes and rotated on a roller table at room temperature. Unreacted formaldehyde was quenched by adding glycine for 5 minutes. Cross-linked cells were then washed twice with 1 mL of ice-cold PBS and both centrifuged at $300 \times g$ for 5 minutes at 4°C with the supernatant removed following each centrifugation. Cell pellets were resuspended in 1 mL of ice-cold PBS containing Protease Inhibitor Cocktail II (PIC), transferred to a 1.5 mL eppendorf and centrifuged at $800 \times g$ for 5 minutes at 4°C . The supernatant was discarded and cell pellets resuspended in 500 μL of cell lysis buffer containing PIC. The suspension was incubated on ice for 15 minutes and briefly vortexed every 5 minutes. The cell suspension was centrifuged at $800 \times g$ for 5 minutes at 4°C , and the supernatant discarded and cell pellets resuspended in 250 μL nuclear lysis buffer containing PIC.

2.5.1.2 Sonication

To shear the DNA, cell lysates were sonicated in a Bioruptor (Diagenode) at high power for 3 x 10 minute intervals with a 10 second on/off duty time. Fresh ice-water changes occurred after each 10 minute sonication. Following sonication, samples were centrifuged at $13,000 \times g$ for 10 minutes at 4°C to

pellet insoluble material. A 10 μ L aliquot was removed to analyse the sheared DNA and the remaining sample was snap frozen in liquid nitrogen and stored at - 80°C

2.5.1.3 Determining DNA fragment size

Elution buffer was added to each sample of sheared DNA to create a final volume of 50 μ L. 1 μ L of RNase A (10 mg/mL) was added to each tube and incubated for 30 minutes at 37 °C. Following this, 1 μ L of proteinase K (20 mg/mL) was added and samples incubated at 62 °C for 4 hours. To determine the fragment length, 5 μ L of loading buffer was added to each sample and 8 μ L of sample and a 100 bp DNA ladder ran on a 1.5 % agarose gel at 120 V.

2.5.2 Immunoprecipitation of cross-linked protein/ DNA

Once the optimum cross-linking condition had been decided for each cell line, 50 μ L of sonicated cross-linked chromatin (approximately 1×10^6 cells) was used for each immunoprecipitation. Cells were diluted in 350 μ L dilution buffer containing 1.35 μ L of PIC. 1 % of the supernatant was reserved for the input and stored at - 20°C. 5 μ g of antibody (Table 2.9) and 20 μ L of fully suspended protein G magnetic beads was used for each immunoprecipitation (IP) and added to the diluted cross-linked chromatin. The antibody-bead-chromatin suspension was incubated overnight at 4°C whilst rotating.

Table 2.9 Antibodies used for ChIP

Antibody
TWIST1 (TWIST2C1a; Abcam, ab50887)
IgG1 (G3A1; Cell Signalling Technology, 5415)
Histone (H3K4Me3; Active Motif, 61979)

Following incubation, a magnetic separator (Promega) was used to pellet the antibody-bead-chromatin complex and the supernatant carefully removed. The complexes were washed four time for 5 minutes whilst rotating in 500 μ L of four different cold wash buffers of increasing salt concentrations to remove binding of non-specific chromatin and protein and reduce background signals (1. Low salt immune complex wash buffer, 2. High salt immune complex wash buffer, 3. Lithium chloride wash buffer, and 4. TE buffer). Following each wash the complexes were pelleted using the magnetic separator and the supernatant carefully removed.

2.5.3 Cross-link reversal and purification of DNA

The protein-DNA complexes were eluted from the magnetic beads and the cross-links reversed (including the input sample). 100 μ L of elution buffer and 1 μ L of proteinase K was added to each sample and incubated at 62°C for 2 hours whilst shaking, followed by a 10 minute incubation at 95°C. The samples were cooled down to room temperature and the beads separated from the complex via the magnetic separator, and the supernatant (containing the immunoprecipitated DNA or input) was transferred to a new tube.

Spin filter columns were used to purify the DNA. 500 μ L of binding reagent A was added to each tube, transferred to a spin filter column in a collection tube, and centrifuged for 30 seconds at 10,000 x *g*. The liquid was removed and the membrane was washed with 500 μ L of wash reagent B and centrifuged twice for 30 seconds at 10,000 x *g* to dry the membrane. The spin filter column was placed in a clean collection tube and the DNA eluted using 50 μ L of elution buffer C and centrifuged for 30 seconds at 10,000 x *g*. The purified DNA was stored at -20°C for either qRT-PCR analysis or ChIP-Sequencing.

2.5.4 ChIP qRT-PCR analysis

Samples were analysed by qRT-PCR following section 2.5.4 to quantify the percentage of chromatin that was immunoprecipitated relative to the input. The following method was used:

1. Adjust input to 100 %: (1 % of input was initially used which equates to a dilution factor of 100 (6.644 cycles): $Ct \text{ of input} - 6.644$)
2. Percentage input: $100 * 2^{(adjusted \text{ input} - Ct(IP))}$

2.6 ChIP-Sequencing

2.6.1 Library preparation

Library preparation was performed by Deep Sequencing (an in-house sequencing facility). For each cell line (ONS-76, D283 and HD-MB03), libraries were prepared from one input and two IP samples. Sample concentrations were first measured using the Qubit Fluorometer and the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Q32854). ChIP-Seq libraries were prepared with 1 μ L of input and 25 μ L of IP samples using the NEBNext Ultra II DNA library Prep kit for Illumina (NEB, E7645) and NEBNext Multiplex Oligos for Illumina (NEB, E7500, E7710, and E7730). During this step, each sample was tagged with a unique dual index (UDI) barcode, allowing for the multiplexing of libraries on a single sequencing run. Libraries were quantified using the Qubit Fluorometer and the Qubit dsDNA HS Assay kit, and library fragment-size distribution was assessed using the Agilent TapeStation 4200 and the High Sensitivity D1000 ScreenTape Assay (Agilent, 5067-5584, 5067-5585).

2.6.2 Sequencing

Libraries were sequenced on the Illumina NextSeq 500 using a NextSeq 500 High Output v2.5 150 cycle kit (Illumina; 20024907) to generate over 30 million pairs of 75 bp paired-end reads per input sample and over 20 million pairs of 75 bp paired-end reads per IP sample.

2.6.3 Mapping

After the libraries were sequenced the quality of reads were examined by *FastQC* and the adapters trimmed from the raw reads using the *skewer* tool (Jiang et al., 2014). Trimmed reads were mapped onto the human genome GRCh38 (GCA_000001405.28) by *hisat2* (Kim et al., 2015), and uniquely and correctly aligned reads were extracted and duplicate reads were removed by *picard-tools*.

2.6.4 Peak calling

The binding sites of the transcription factor that had aligned to the reference genome were identified using the *Model-based analysis of ChIP-Seq* (MACS2) software (Zhang et al., 2008). The distribution of peaks was assessed using statistical measures and enriched regions were compared against background (input). Statistical significance of a peak was estimated using a Poisson distribution. To annotate the binding sites, *ChIPpeakAnno* using the Ensembl R database (version 86) was used and shared peaks between replicates and subgroup specific samples were identified by the *findOverlapsOfPeaks* tool (Zhu et al., 2010) with a maximum of 10 bp gaps allowed between peaks.

2.6.5 Gene set enrichment analysis

Gene set enrichment analysis (GSEA) was performed using publicly available software from the University of California San Diego and the Broad Institute

(software.broadinstitute.org/gsea). GSEA was used to determine if the genes identified through TWIST1 ChIP-Sequencing analysis overlapped with annotated gene sets from the Molecular Signature Database (MSigDB) (Subramanian et al., 2005, Liberzon et al., 2011). Two gene set collections were used in this study: Gene Ontology (GO) (biological processes and molecular function) (Ashburner et al., 2000, The Gene Ontology Consortium, 2018), and Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway (Kanehisa and Goto, 2000). Significance in overlap with the GO and KEGG gene sets was determined using an uncorrected p-value and a p-value adjusted using the Benjamini-Hochberg False Discovery Rate (FDR) method (FDR q-value) to correct for multiple testing.

2.7 Genetic knockdown

2.7.1 Transduction efficiency

To knockdown TWIST1 in the medulloblastoma cell lines, cells were transduced with TWIST1-small hairpin RNA (shRNA) GIPZ Lentiviral clones (Dharmacon; either V2LHS_231097, V3LHS_329863, or V3LHS_329861). A non-targeting GIPZ lentiviral shRNA clone was used as a negative control (V19090501). The GIPZ lentiviral vector contained a TurboGFP reporter for visual tracking of transduction, and a puromycin resistance element to permit antibiotic-selective pressure. To determine the transduction efficiency of each cell line, cells were transduced with three different multiplicity of infections (MOI; 1, 0.5 and 0.3). 24 hours prior to transducing, cells were seeded into 12 well plates in standard culture medium according to the seeding densities listed in Table 2.10, and an extra well was seeded to allow for cell counting.

Table 2.10 Seeding densities for shRNA transduction

Cell line	Seeding density
ONS-76	3×10^4
D283	9×10^4
HD-MB03	4×10^4

On the day of transduction, medium was removed from each well and the cells were washed with 1 mL HBSS. The cells that had been seeded into the extra well were counted and the volume of virus required for each MOI was calculated using the following equation:

$$\text{Volume} = \frac{\text{Number of cells} \times \text{MOI}}{\text{virus transduction efficiency (TU/mL)}} \times 1000$$

500 μ L of virus in serum-free medium was added dropwise to each well and the plate gently swirled to make sure the virus was evenly distributed. To increase efficiency in the D283 cell line, the plate was centrifuged for 60 minutes at 1000 rpm. The plates were incubated at 37°C at 5 % CO₂ for 6 hours. Following incubation, 500 μ L of standard culture medium supplemented with 2x FBS concentration was added to the cells and incubated for 48 hours at 37°C at 5 % CO₂.

Following transduction, cells were examined for the presence of GFP using a fluorescent microscope (Nikon). To select for transduced cells, transduction medium was removed and replaced with standard culture medium supplemented with a concentration of puromycin that would kill non-transduced cells. The medium was changed every 2-3 days and the cells expanded into a T75 tissue culture flask. Once confluent, cell pellets were snap frozen to test the knockdown efficiencies of each clone and MOI.

2.7.2 Puromycin kill curves

Puromycin treatment was used to select for transduced cells. To determine a concentration that would kill non-transduced cells, the method mentioned in section 2.3.1 was followed except 2000 cells were seeded per well and treated with increasing concentrations of puromycin dihydrochloride (Sigma, P8833) for 48 hours before analysing metabolic activity with PrestoBlue™.

2.7.3 Determining knockdown efficiency

Knockdown efficiency was assessed by analysing gene and protein expression of the target knocked down gene for each shRNA clone at the three different MOIs. Gene expression was quantified by qRT-PCR (2.4.5) to determine the % knockdown relative to the non-targeting control, and protein expression was assessed by western blotting.

2.8 Protein assays

2.8.1 Protein isolation

Protein was isolated from snap frozen cell pellets using NP-40 lysis buffer, which was prepared and stored at -20°C for up to 1 year:

- 150 mM sodium chloride
- 0.5 % NP-40
- 50 mM Tris pH 7.4
- 1 mM EDTA

Fresh aliquots of NP-40 were thawed for each protein extraction, and cComplete™ Mini EDTA-free protease inhibitor cocktail (Sigma, 11836170001) was added to a final 1x concentration. Cell pellets were lysed in 100 µL of NP-

40 lysis buffer and incubated on ice for 30 minutes, vortexing briefly every 10 minutes. The lysates were then centrifuged at 14,000 x *g* for 15 minutes at 4°C and the supernatant transferred to a fresh eppendorf.

2.8.2 Measuring protein concentration

Protein concentration of the cell lysates was determined using a Bradford assay. Bovine serum albumin (BSA) stock solution (100 µg/mL) was stored at -20°C. A BSA standard dilution series was prepared by diluting BSA in ddH₂O (0-100 µg/mL) to quantify protein concentration. Cell lysates were diluted 1:100 with ddH₂O, and 50 µL of each diluted lysate and BSA standard dilution was pipetted in triplicate into a 96-well plate. Bradford reagent (Bio-Rad, 5000006) was diluted 1:5 with ddH₂O and 200 µL added to each sample. The plate was incubated in the dark for 5 minutes at room temperature and the optical density measured at 595nm using the FLUOstar Omega plate reader. Protein concentration was calculated relative to the BSA standard curve.

2.8.3 Analysing protein expression

2.8.3.1 Protein sample preparation

Protein lysates of known concentrations were diluted to 2.5 µg/µL in lysis buffer and 4x Laemmli sample buffer containing β-mercaptoethanol to create a final 1x concentration of sample buffer. Samples were heated to 95°C or 70°C (for ABCB1 protein expression) for 10 minutes, and then cooled down on ice and stored at -20°C.

2.8.3.2 SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to separate proteins in a given sample. Separating gels were prepared according to the target protein size (ABCB1; 10 % and TWIST1; 12 %).

The reagents for the separating gel were prepared in a 50 mL falcon and carefully pipetted into a 1.5 mm gel casket (Table 2.11). Isopropanol was pipetted on top of the gel to prevent the formation of air bubbles and the gel left to polymerise for 20 to 30 minutes. Once set, the isopropanol was poured away and the stacking gel was prepared and pipetted on top of the separating gel (Table 2.11). A 1.5 mm comb was inserted slowly into the stacking gel and left to polymerise for 1 hour. The gel was either used directly or wrapped in wet tissue paper and aluminium and stored at 4°C for 1 week.

Table 2.11 Gel composition

Reagent	Separating Gel		Stacking Gel
	10 %	12 %	
1M Tris (Sigma, T1503)	3.9 mL (pH 8.8)	3.9 mL (pH 8.8)	650 µL (pH 6.8)
ddH ₂ O	2.5 mL	1.9 mL	3.58 mL
30 % Acrylamide (Sigma, A3699)	3.4 mL	4 mL	670 µL
10 % Sodium dodecyl sulfate (SDS; Merck, L5750)	100 µL	100 µL	50 µL
Ammonium Persulfate 10 % (Merck, A3678)	100 µL	100 µL	50 µL
Tetramethylethylenediamine (TEMED; Thermo Fisher Scientific, 17919)	10 µL	10 µL	5 µL

Gels were put into a chamber with 1x running buffer (diluted in ddH₂O; Table 2.12) and the comb carefully removed. 20 µL of protein was loaded into each well of the gel, 5 µL of Amersham ECL Rainbow Marker (Sigma, RPN800E) was run in parallel as a molecular weight marker, and all free wells were filled with water and sample buffer. Samples were run in a vertical electrophoresis tank (Bio-Rad) at 20 mA through the stacking gel, then increased to 40 mA through the separating gel.

Table 2.12 10x Running buffer recipe

Reagent	Quantity for 1L
Tris base	30.275 g
Glycine	144 g
20 % SDS	50 mL
ddH ₂ O	Make up to 1L

2.8.3.3 Transfer

The separated proteins were transferred onto Amersham Hybond 0.45 μ m western blotting PVDF membrane (Scientific Laboratory Supplies; 10600023). The PVDF membrane was activated for 30 seconds in methanol, 3 minutes in water, and 10 minutes in 1x transfer buffer (700 mL ddH₂O, 200 mL methanol, and 100 mL 10x transfer buffer; Table 2.13). The membrane was assembled in a plastic grid following the steps below:

1. Plastic grid (black side down)
2. Sponge
3. Filter paper
4. Gel
5. PVDF membrane
6. Filter paper
7. Sponge
8. Plastic grid (white side up)

The proteins were transferred onto the PVDF membrane in 1x transfer buffer for 1 hour at 4°C at 100V.

Table 2.13 10x Transfer buffer recipe

Reagent	Quantity for 1L
Tris base	30.275 g
Glycine	144 g
ddH ₂ O	Make up to 1L

Following transfer, the binding capacity was increased by air drying the membrane for 10 minutes and rehydrating with TBS-Tween wash buffer (TBS-T; Table 2.14 and Table 2.15) until the membrane was transparent again.

Table 2.14 10x TBS recipe

Reagent	Quantity for 1L
Tris base	24.2 g
Sodium Chloride	80 g
ddH ₂ O	700 mL

Table 2.15 1x TBS-Tween

Reagent	Quantity for 1L
10x TBS	100 mL
Tween-20	1 mL
ddH ₂ O	899 mL

2.8.3.4 Western blotting

Membranes were carefully placed in 15 mL or 50 mL falcons depending on the size of the membrane, and blocked in 5 mL of 5 % milk in TBST-T for 1 hour at room temperature. Membranes were then incubated with the primary antibody in 5 % milk or BSA diluted in TBS-T (Table 2.16) overnight at 4°C whilst falcons were continuously rolling. The following day, the membranes were washed

three times in TBS-T for 10 minutes and then incubated in 5 mL of secondary antibody (Table 2.17) for 1 hour at room temperature whilst rolling continuously. The membranes were then washed three more times in TBST-T for 10 minutes before chemiluminescence measurement.

Table 2.16 Primary antibodies for western blotting

Protein	Species	Concentration
ABCB1 (Insight Biotechnology, GTX23364)	Mouse	1:50 in milk
TWIST1 (TWIST2C1a)	Mouse	1:250 in BSA
GAPDH (Cell Signalling Technology, 2118S)	Rabbit	1:1000 in milk
β -actin (Cell Signalling Technology, 13E5)	Rabbit	1:1000 in milk

Table 2.17 Secondary antibodies for western blotting

Antibody	Concentration
anti-mouse IgG HRP-conjugated (Cell Signalling Technology, 7076S)	1:2000
anti-rabbit IgG HRP-conjugated (Cell Signalling Technology, 7074S)	1:2000

2.8.3.5 Western blot chemiluminescence measurement

The membranes were incubated in the dark with SignalFire™ ECL solution (Cell Signalling Technology, 12757S) by combining equal parts of luminol solution and peroxide solution for 1 to 3 minutes. Excess solution was removed and the membranes imaged using a LAS 4000 mini Biomolecular imager (GE Healthcare).

2.8.3.6 Western blot quantification

Protein concentration was quantified using Fiji imaging software (ImageJ). The software creates peaks that represents the relative intensity of bands. The size of the peak was measured, generating a numerical value that represents the area of the peak. The values for the protein of interest were calculated relative to the house-keeping protein.

2.8.4 Patient tissue microarrays

TWIST1 protein expression was analysed in medulloblastoma patient tissue microarrays (TMA) using immunohistochemistry (IHC). The TMAs represented patients treated at Birmingham and Nottingham University hospitals between 1985 and 2012. Clinical and histological patient data for the primary tumour samples obtained from the Children's Brain Tumour Research Centre (CBTRC), Birmingham and Nottingham cohorts were previously published by (Othman et al., 2014, Nasir and Cardall et al., 2021). These studies, and the experimental protocols required, were reviewed and approved by the National Research Ethics Service Committee East Midlands – Nottingham 2 and the Ethics Committee of the Burdenko Neurosurgical Institute (Ethical vote number 563/6-16). The study was in accordance with the ethical standards laid down in an appropriate version of the 1975 Declaration of Helsinki, as revised in 1983. For all patients, informed consent was obtained from the patient, or a parent and/or legal guardian where the patient was under 18 years of age, prior to their inclusion in the study.

2.8.5 Immunohistochemistry

Slides were placed in a rack and deparaffinised in xylene for 15 minutes and rehydrated in a series of ethanol baths (100 % 2 x 5 minutes, 95 % 2 x 5 minutes, and 80 % and 70 % 2 minutes each) before being washed in running

tap water. Heat-induced antigen retrieval was performed by placing the rack in a pre-heated steamer containing sodium citrate buffer (Table 2.18) for 40 minutes. Slides were then washed in PBS for 2 minutes and placed in a humidified chamber.

Table 2.18 Sodium citrate buffer recipe

Reagent	Quantity for 1 L
Citric acid monohydrate	2.1 g
Sodium hydroxide (pH 6.0)	~ 13 mL
Tween 20	0.5 mL
ddH ₂ O	Make up to 1 L

Endogenous peroxidase activity was suppressed to reduce background signal by incubating slides in peroxidase suppressor (Thermo Fisher Scientific, 35000) for 30 minutes, followed by two 3 minute washes in PBS. Next, Universal Blocker™ blocking buffer (Thermo Fisher Scientific, 1859332) was added to the slides for 30 minutes. Excess blocking buffer was removed from the slides, and primary antibody (anti-TWIST1; Millipore, ABD29) diluted in blocking buffer (1:250) was then added and slides incubated for 1 hour at room temperature. A control tissue, which the protein of interest was known to be expressed in, was included as a positive (with primary antibody) and negative (with blocking buffer only) control.

Slides were then washed twice for 3 minutes in PBS, before a 1 hour incubation in a horseradish peroxidase (HRP)-tagged secondary antibody (goat anti-rabbit; Abcam, ab214879), then washed twice for 3 minutes in PBS. Antibody detection was performed using diaminobenzidine (DAB) chromogen, by diluting 30 µL of SignalStain® DAB chromogen concentrate (Cell Signalling Technology, 11725) in 1 mL of SignalStain® DAB chromogen diluent (Cell Signalling Technology,

11724) for 10 minutes. Slides were washed twice for 3 minutes in PBS and rinsed in water. Harris Modified Haematoxylin (Thermo Fisher Scientific, 1859352) was added to the slides for 2 minutes and washed in water. Slides were dehydrated in ethanol (95 %, then 100 %) followed by xylene, before mounting with medium, applying a coverslip and leaving to set at room temperature.

2.8.6 TMA imaging and scoring

TMA slides were imaged by the Nottingham University Hospitals Trust Imaging Unit at the Queen's Medical Centre on a Nanozoomer instrument (Hamatsu). The TMA cores were scored independently and blindly by two people. For each core, 50 cells in 5 different areas were analysed, totalling 250 cells. The number of positively stained TWIST1 nuclei was recorded and converted to a percentage. The scores across a patient's tumour cores were then averaged to obtain a final percentage of TWIST1 expression in a patient's tumour sample. The percentage was then categorised as either low TWIST1 expression (<30% nuclear positivity) or high TWIST1 expression ($\geq$ 30% nuclear positivity). Any differences in scoring were re-evaluated together to reach a consensus in scores.

2.9 Patient dataset analysis

The R2 Genomics Analysis and Visualization Platform (r2platform.com) was used to analyse gene expression in publicly available patient datasets. The datasets used in this study are detailed in Table 2.19.

The datasets were used to assess gene expression across the medulloblastoma subgroups, in metastatic and non-metastatic patient tumours and to generate Kaplan Meier survival curves. Distribution graphs for the Pfister and Cavalli datasets are highlighted in Appendix 1, Appendix Figure 1 and Figure 2 respectively.

Table 2.19 Patient datasets analysed on the R2 Genomics Analysis and Visualisation Platform.

Dataset	Number of samples	Sample Type	Reference
Roth	9	Normal cerebellum	Roth <i>et al.</i> , 2006
Pfister	223	Primary medulloblastoma	Northcott <i>et al.</i> , 2017
Cavalli	763	Primary medulloblastoma	Cavalli <i>et al.</i> , 2017
Wang	9	Matched medulloblastoma	Wang <i>et al.</i> , 2015

2.10 Statistics

Patient TMA data was analysed in SPSS version 24 statistical software (IBM) using either a Fisher's exact test or a Pearson Chi-Squared test. Kaplan Meier survival curves were analysed using a logrank test. All remaining statistical differences were calculated using GraphPad Prism Version 9.0. Results were presented as mean $\pm$ standard error of the mean. D'Agostino-Pearson normality test was performed on all patient datasets to determine if they fit a Gaussian distribution. Statistical differences for datasets that did not fit a Gaussian distribution were calculated using a Kruskal-Wallis test with Dunn's multiple comparison post-hoc test or a Mann-Whitney test. Statistical differences for datasets that did fit a Gaussian distribution were calculated using one-way ANOVA with Tukey's or Dunnett's multiple comparisons post-hoc test, or a paired or unpaired Student's t-test as specified in each figure legend. Some of

the analysed data had been normalised to a constant value. In such instances, considerations during statistical tests had been taken into account when comparisons had been performed against a constant value. p values ≤ 0.05 were considered significant.

Chapter 3. Investigating TWIST1 expression in medulloblastomas

3.1 Introduction

Medulloblastoma is a highly aggressive malignant brain tumour. Advances in the classification of medulloblastomas have revealed four unique molecular subgroups; wingless (WNT), sonic hedgehog (SHH), Group 3 and Group 4, each of which have distinct clinical outcomes and molecular characteristics (Ramaswamy and Taylor, 2017).

WNT tumours are associated with the most favourable prognosis and rarely metastasise or reoccur. SHH tumours vary primarily according to p53 status; SHH-*TP53* mutations result in a poor prognosis and frequently reoccur, whereas, *TP53* wild-type tumours have a much better overall survival rate. Group 3 tumours have the worst survival outcomes and are associated with the highest rates of metastasis at diagnosis. Similar to Group 3, Group 4 tumours also present with high rates of metastasis but survival outcomes are intermediate and recurrences occurring later on (Archer et al., 2017, Juraschka and Taylor, 2019).

Metastatic disease has been continuously linked to cancer-related deaths. The high percentage of patients exhibiting metastatic dissemination at diagnosis is such a poor indicator of prognosis (Northcott et al., 2019) that factors which mediate the early steps of the metastatic cascade in medulloblastoma are of interest.

The transcription factor TWIST1 has been characterised as one of the master regulators that initiates the first event in the metastatic cascade, the EMT pathway (Xu et al., 2017, Mittal, 2018). In normal tissue it has been identified

as an essential regulator during embryonic morphogenesis, especially in mesoderm formation, specification and differentiation, but its expression decreases to extremely low levels in most cell types after embryogenesis (Qin et al., 2012). However, in many cancers expression of TWIST1 is upregulated, with numerous studies demonstrating that it is implicated in tumour initiation, stemness, dissemination and chemo-resistance, suggesting it could be an attractive biomarker for cancer progression (Zhao et al., 2017, Georgakopoulos-Soares et al., 2020).

To date, only one study has alluded to the role of TWIST1 in medulloblastoma metastasis. Kahn *et al* revealed a NOTCH1-TWIST1-BMI1 axis that regulates medulloblastoma metastasis, and showed that the loss of TWIST1 resulted in reduced BMI1 expression and inhibition of Group 3 medulloblastoma metastasis (Kahn et al., 2018). Notably, they only focussed on Group 3 medulloblastoma metastasis and also did not investigate TWIST1 as an independent predictor of prognosis or consider other TWIST1 targets as mediators of the poor prognosis of Group 3 tumours.

Therefore, the aim of this chapter was to begin exploring the role of TWIST1 in medulloblastoma, and to see if it played a role in initiating metastatic disease. TWIST1 expression was analysed in a combination of online patient datasets, tissue microarrays (TMA) and medulloblastoma cell lines, with the analysis covering all four subgroups, survival outcomes and metastatic disease.

3.2 Results

3.2.1 TWIST1 gene expression in publicly available medulloblastoma patient datasets

At the outset of the project there was little evidence supporting the role of TWIST1 in medulloblastoma. To begin addressing this gap in knowledge, an online bioinformatics platform; R2 Genomics Analysis and Visualization Platform (r2.amc.nl), was used to access publicly available medulloblastoma datasets.

Firstly, *TWIST1* and *TCF3* gene expression were evaluated across all four medulloblastoma subgroups and compared against the normal cerebellum using the Roth and Pfister patient datasets. It should be noted, for the purpose of this thesis gene expression analysis refers to transcript expression rather than transcriptome expression.

Briefly, the Roth dataset comprises of 9 normal cerebellum samples and the Pfister dataset comprises of 223 samples spanning all subgroups (WNT n = 17, SHH n = 59, Group 3 n = 56 and Group 4 n = 91). Gene analysis was compared between these patient datasets as the same microarray platform and normalisation technique had been applied. (Roth et al., 2006, Northcott et al., 2017). (Figure 3.1). It was of interest to compare *TWIST1* and *TCF3* gene expression, as TCF3 is reported to be TWIST1's preferential binding partner (Fan et al., 2020). Interestingly, *TWIST1* and *TCF3* had similar expression patterns and the expression appeared higher in each of the four subgroups compared to the normal cerebellum. Significant differences were observed for all subgroups for *TCF3* expression analysis and in WNT, Group 3 and Group 4 for *TWIST1* expression analysis. Surprisingly, WNT, the subgroup with the lowest rates of metastasis, had the highest median *TWIST1* levels, however,

this could be because *TWIST1* is a target of the WNT signalling pathway (Goodnough et al., 2016). Also, a large range in *TWIST1* expression levels was observed in the WNT dataset and upon closer analysis this revealed one data point with a minimum value of 0.

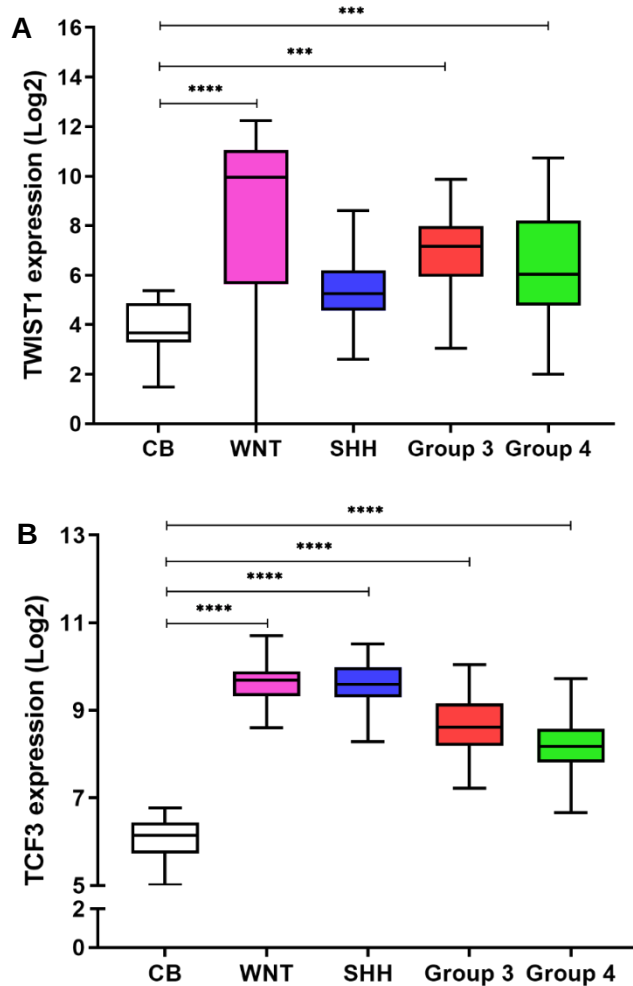


Figure 3.1 *TWIST1* and *TCF3* gene expression in medulloblastoma subgroups compared to the normal cerebellum.

TWIST1 (A) and *TCF3* (B) gene expression (Log2) were assessed using R2 Genomics Analysis and Visualization Platform. The MegaSampler function was used to assess gene expression in two datasets: Roth (normal cerebellum (CB) $n = 9$) and Pfister (WNT $n = 17$, SHH $n = 59$, Group 3 $n = 56$ and Group 4 $n = 91$). The box plot diagram represents the minimum and maximum values, the median and the upper and lower quartiles. Significant differences in gene expression compared to the normal cerebellum were calculated using a one-way ANOVA with Dunnett's multiple comparison post-hoc test ($***p < 0.001$, $****p < 0.0001$).

As mentioned earlier, Group 3 medulloblastoma tumours have the highest rates of metastasis, whereas WNT tumours have the lowest rates. Despite some recent advances, we still do not understand the molecular pathways necessary for medulloblastoma metastasis (Juraschka and Taylor, 2019). The Cavalli dataset (Cavalli et al., 2017), which is the largest publicly available online dataset of primary medulloblastoma tumours, was next investigated to see if *TWIST1* expression correlated with medulloblastoma metastasis, as this dataset provides information on the metastatic status of patients. This was achieved by comparing *TWIST1* expression levels between non-metastatic (M0) and metastatic (M+) samples, with samples classified as metastatic if the patient presented with metastatic dissemination at diagnosis (Figure 3.2).

There was no significant difference in *TWIST1* expression between non-metastatic and metastatic patients when analysing all of the subgroups combined or when separated into the respective medulloblastoma subgroups. The mean *TWIST1* expression was only slightly higher in metastatic tumours compared to non-metastatic tumours when all subgroups were combined and in the Group 4 subgroup. It should be noted, however, that the Cavalli dataset does not include matched tumour samples (i.e. primary and recurrent pairs) and does not provide follow-up information on whether patients with non-metastatic disease later relapsed. This information would be beneficial in order to know if *TWIST1* expression in the primary tumour predicted recurrent disease, or if *TWIST1* expression was higher in primary tumours compared to matched tumour samples. Therefore, based on this data alone it is difficult to conclude if and how *TWIST1* is contributing to metastatic disease.

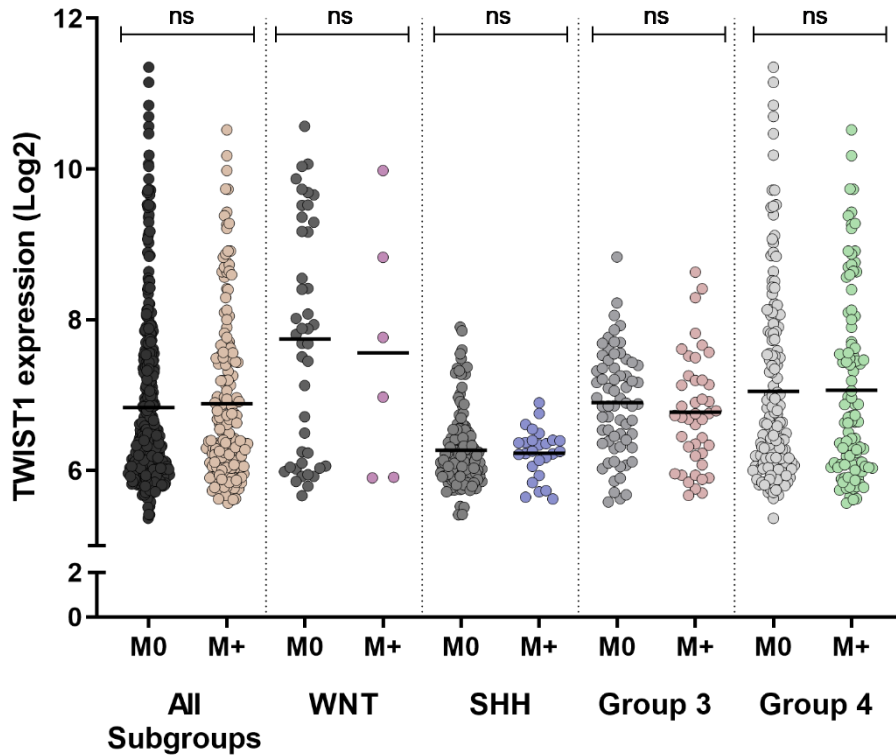


Figure 3.2 *TWIST1* gene expression in non-metastatic (M0) and metastatic (M+) medulloblastoma patient samples.

TWIST1 gene expression (Log2) was assessed using R2 Genomics Analysis and Visualization Platform. Gene expression for all molecular subgroups (M0 = 397 and M+ = 176), WNT subgroup (M0 = 43 and M+ = 6), SHH subgroup (M0 = 134 and M+ = 26), Group 3 subgroup (M0 = 66 and M+ = 43) and Group 4 (M0 = 154 and M+ = 101) were assessed using the Cavalli dataset. Significant differences in gene expression were calculated using a Mann-Whitney test (ns = not significant).

Although a very small dataset, the Wang patient dataset (Wang et al., 2015b), has the advantage of comprising nine matched tumour samples from medulloblastoma patients. Whilst analysis comparing *TWIST1* expression between primary and metastatic tumours using a Student's paired t-test revealed no significant differences (Figure 3.3), a general trend was present; *TWIST1* expression was higher in the primary tumour compared to the metastatic tumour, with 6 out of the 9 matched tumour samples displaying this

trait, and only 3 out of the 9 matched samples expressed lower *TWIST1* levels in the primary tumour compared to the metastatic tumour, although, there was no cases where expression was different by more than 2-fold. The results presented indicated that high *TWIST1* expression in the primary tumour could be initiating medulloblastoma metastasis.

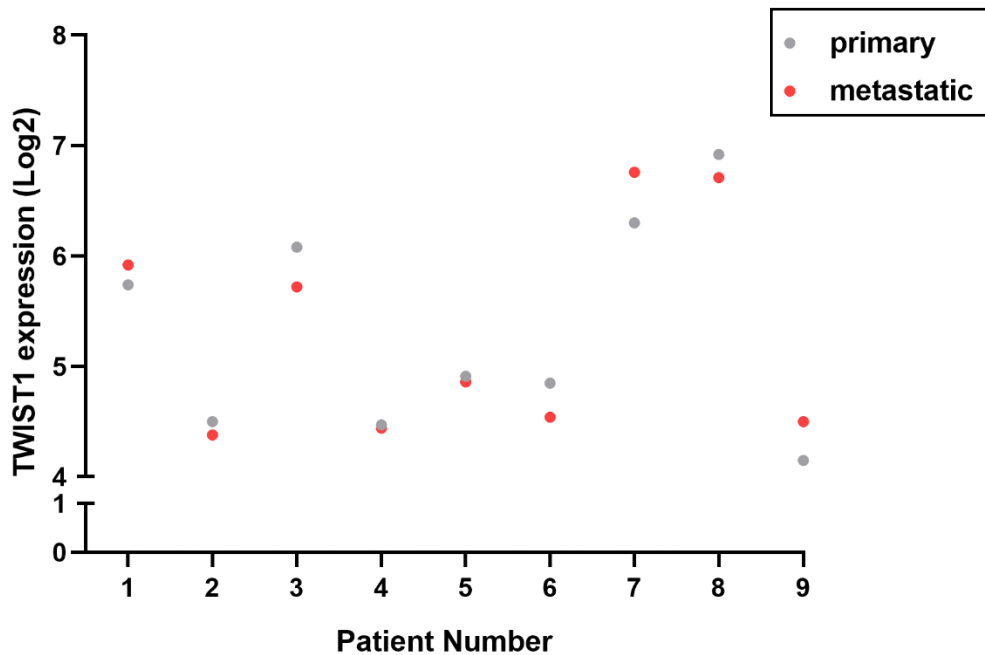


Figure 3.3 *TWIST1* expression in patient matched tumours samples from medulloblastoma patients.

TWIST1 gene expression (Log2) was assessed in patients with metastatic medulloblastoma from primary medulloblastoma samples (grey) and the matched metastatic tumour samples (red) using the Wang dataset (n = 9).

Survival rates for medulloblastoma patients vary greatly depending on age, the presence of metastasis, extent of residual disease after surgery and molecular characteristics (Thomas and Noël, 2019). Continuing with the R2 online platform, *TWIST1* gene expression (high or low) and metastatic status (M0 or M+) were analysed to see if these factors correlated with patient survival. Samples were categorised into either high or low *TWIST1* expression using the 'Kaplan Scan' feature available on the R2 platform. This feature finds an optimum survival cut-off value for the two groups by establishing the most significant p value in a logrank test. Patient overall survival was assessed using the Cavalli patient dataset over a 120 month follow-up period for all subgroups combined, SHH, Group 3 and Group 4 (Figure 3.4). Unfortunately, there were insufficient numbers of metastatic WNT patients and analysis for this subgroup could not be performed.

When analysing all of the subgroups combined, patients who were diagnosed with metastatic disease but had low *TWIST1* expression had worse overall survival, whereas patients who presented with non-metastatic disease and had high *TWIST1* expression had the best overall survival. This suggests that high *TWIST1* expression might not result in a poor outcome. However, as mentioned earlier, the prognosis of a patient can vary greatly depending on the subgroup classification of the tumour. Analysing the overall survival probability for each subgroup gave a different perspective. Patients diagnosed with metastatic SHH tumours and high *TWIST1* expression had a significantly worse overall survival compared to those diagnosed with non-metastatic disease and high *TWIST1* expression, who had a better overall survival.

Interestingly, patients diagnosed with non-metastatic Group 3 and Group 4 tumours and low *TWIST1* expression had the best overall survival probability, suggesting *TWIST1* expression could be used as a prognostic biomarker in

non-metastatic disease for these two subgroups. In addition, Group 3 metastatic and non-metastatic tumours with high *TWIST1* expression were associated with the worst overall survival. This suggests that, irrespective of the metastatic status at diagnosis, high *TWIST1* expression in Group 3 tumours could be predictive of a poor outcome.

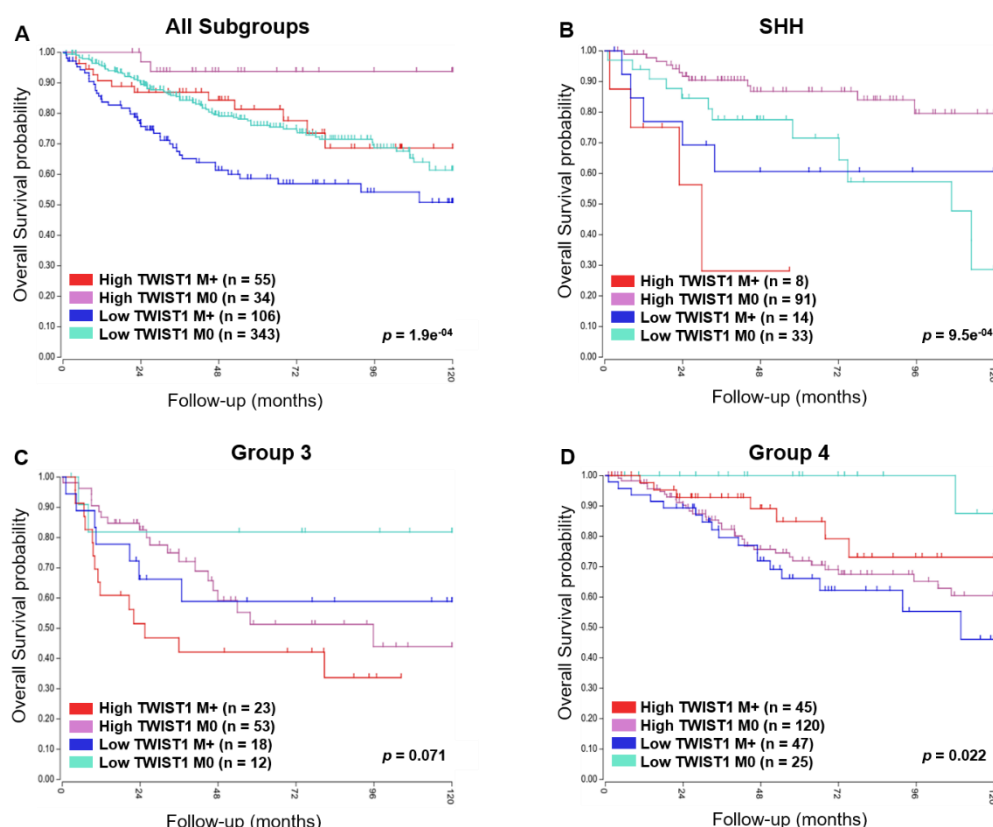


Figure 3.4 Kaplan Meier survival curves of the overall survival probability for high or low *TWIST1* gene expression in non-metastatic and metastatic patients.

Kaplan Meier survival curves of the overall survival probability over a 120 month follow-up period based on high or low *TWIST1* gene expression and non-metastatic (M0) or metastatic (M+) disease. Overall survival probability for all molecular subgroups (A), SHH (B), Group 3 (C) and Group 4 (D) subgroups were assessed using the Cavalli dataset. Statistical significances were calculated using the logrank test, comparing the overall survival between *TWIST1* gene expression and metastatic disease.

3.2.2 TWIST1 expression in patient tissue microarrays

Whilst *TWIST1* gene expression has been investigated in metastatic disease, studies into protein expression using medulloblastoma patient tissue microarrays (TMAs) remains to be performed. This thesis presents a sizeable analysis of TWIST1 protein expression in TMAs constructed from Birmingham and Nottingham medulloblastoma patient cohorts, to elucidate if TWIST1 expression correlated with medulloblastoma metastasis and/or patient survival. The clinical and histological data of the Birmingham and Nottingham patients were previously published by Nasir and Cardall *et al* and Othman *et al* respectively (Othman et al., 2014, Nasir and Cardall et al., 2021). The combined cohorts consisted of 100 patient primary tumour samples, however, for some patients no clinical or very limited clinical data was available.

The samples were first validated using Kaplan Meier analysis to see if the patient clinical data from both cohorts combined were representative of the survival outcomes for each subgroup as identified within the wider medulloblastoma datasets (Figure 3.5) (Juraschka and Taylor, 2019). The analysis revealed patients who were diagnosed with WNT tumours had the most favourable overall survival probability, whereas, patients with Group 3 tumours presented with the sharpest decrease in survival within the first 24 months followed by Group 4 and SHH tumours. This suggests that the clinical data was in agreement with the literature and the TMAs were a strong representation of medulloblastoma clinical characteristics.

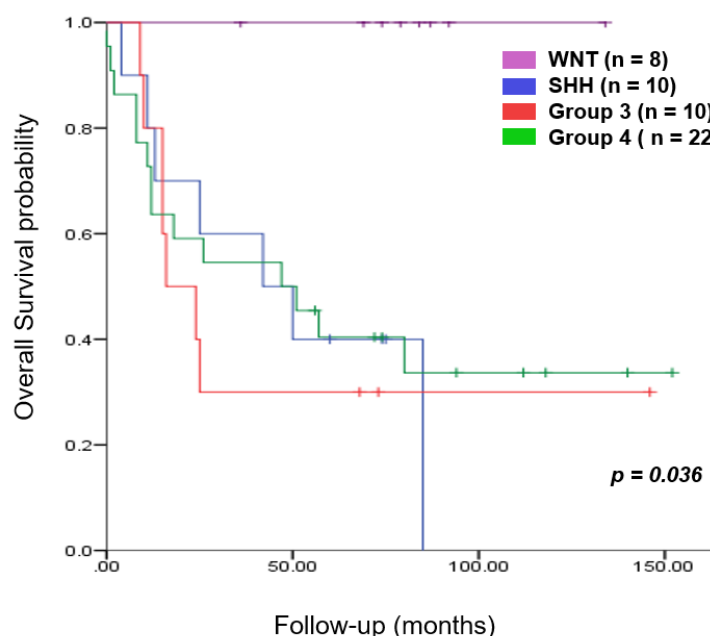


Figure 3.5 Kaplan Meier survival curves of the overall survival probability between each subgroup using the TMAs of medulloblastoma patients.

Kaplan Meier survival curves of the overall survival probability of medulloblastoma patients for the four subgroups; WNT, SHH, Group 3 and Group 4, using the Birmingham and Nottingham patient TMAs. Statistical significance was calculated using the logrank test, comparing the overall survival between the four medulloblastoma subgroups.

The TMAs from both cohorts were stained with a TWIST1 antibody by immunohistochemistry (IHC). Normal tonsil tissue was used as a positive control, and the same tissue, stained with only the secondary antibody was used as a negative control. At least three cores had been taken from a patient's tumour, but due to depletion of some of these cores, only 83 patient samples were successfully stained. Samples were scored independently and blindly by two people and the percentage of positively stained TWIST1 nuclei for each core was recorded and the scores across each core were averaged to obtain a final percentage of TWIST1 expression in a patient's tumour sample. The

percentage was categorised as either low TWIST1 expression (<30% nuclear positivity) or high TWIST1 expression ($\geq$ 30% nuclear positivity) (Figure 3.6). Any differences in scoring were re-evaluated together to reach a consensus in scores. The cut-off value was decided firstly, because of the small sample size; sub-dividing the staining into low, medium, and high categories would result in smaller subsets of groups resulting in less meaningful data. Secondly, TWIST1 expression was lower in the normal cerebellum compared to the four medulloblastoma subgroups (Figure 3.1) and as noted remains extremely low after embryogenesis. Therefore, any positive staining of TWIST1 within a tumour core was considered important in medulloblastoma tumourigenesis and patient outcome.

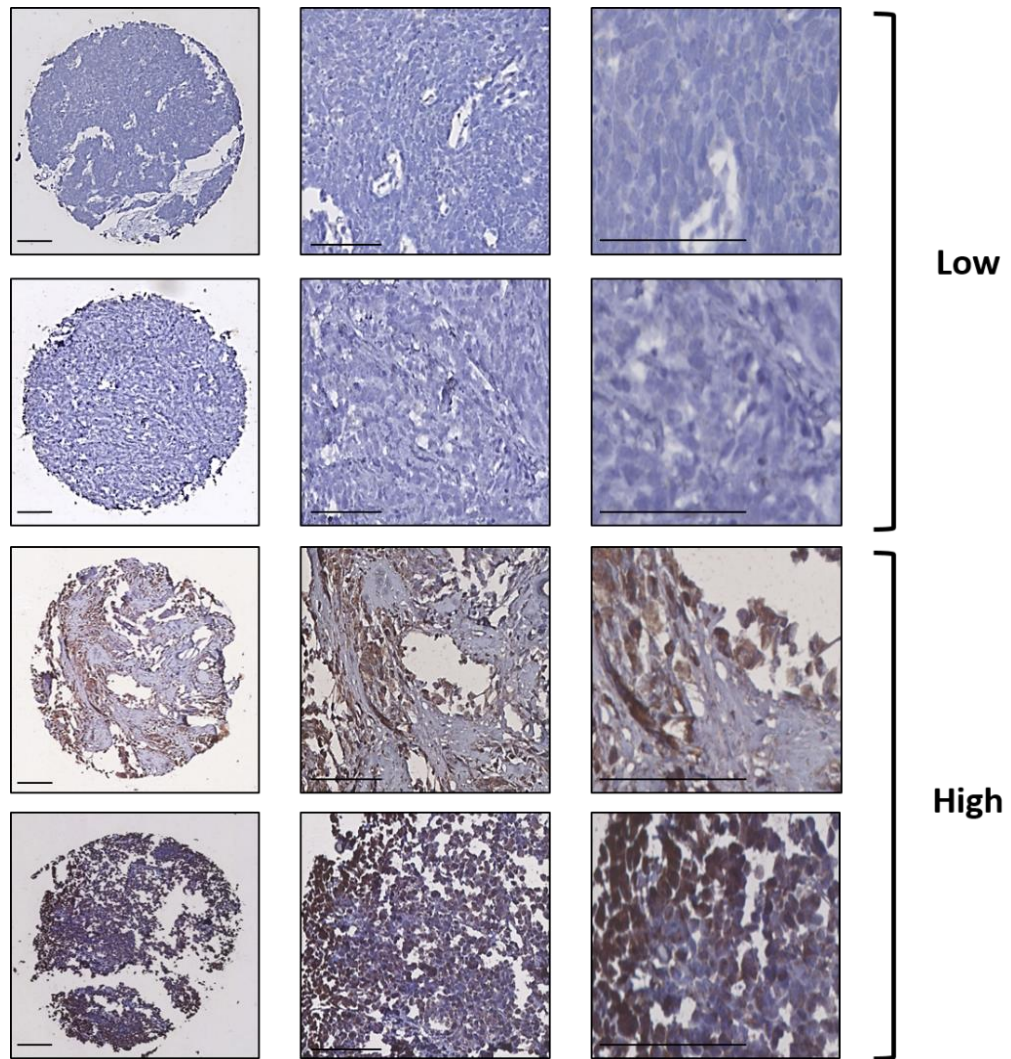


Figure 3.6 Nuclear TWIST1 IHC staining of medulloblastoma patient TMAs.

Birmingham and Nottingham patient TMAs were stained with anti-TWIST1 polyclonal antibody by IHC. Each core was scored either as low TWIST1 (<30% nuclear positivity) or high TWIST1 (≥30% nuclear positivity). Scale bars represent 100 μ m.

The TMAs enabled a novel analysis into TWIST1 protein expression in a large cohort of medulloblastoma patients. The TMAs were analysed to compare if TWIST1 expression (high or low) correlated with subgroup classification. The subgroups were only known for 53 out of the 83 patient samples. Notably, this number is different to Figure 3.5, because the clinical data available for each patient was not always consistent, and the outcome of a patient was not always known. A Fisher's exact test revealed that TWIST1 expression levels were not significantly different between the subgroups (Table 3.1). However, the Group 3 tumours, which are normally associated with the worst overall survival and highest rates of metastasis, had the highest percentage of samples expressing high TWIST1 (89 %) compared to low TWIST1 levels (11 %).

Table 3.1 TWIST1 expression across the four subgroups using the medulloblastoma patient TMAs.

		Low TWIST1		High TWIST1		Fisher's Exact Test
Subgroup	Total No of Patients	No of Patients	%	No of Patients	%	$p = 0.375$
WNT	8	3	38	5	62	
SHH	13	6	46	7	54	
Group 3	9	1	11	8	89	
Group 4	23	9	39	15	61	

Next, the patient TMAs from both cohorts were analysed to determine if TWIST1 expression correlated with metastatic disease. Metastatic status was known for 70 out of the 83 patients. Remarkably, 81 % of patients with metastatic disease (M+) showed high TWIST1 expression in the primary tumour compared to just 54% of patients with no metastatic disease at diagnosis (M0). A chi-squared test revealed that TWIST1 expression significantly correlated with medulloblastoma metastasis (Table 3.2).

Table 3.2 TWIST1 expression correlates with medulloblastoma metastasis.

		Low TWIST1		High TWIST1		Pearson Chi – Squared Test
Metastatic Status	Total No of Patients	No of Patients	%	No of Patients	%	$p = 0.027$
M0	44	20	46	24	54	
M+	26	5	19	21	81	

One of the limitations of the gene expression datasets that had been mentioned in this chapter was the absence of follow-up patient information to conclude if TWIST1 expression was associated with metastatic disease. The patient TMA data provided us with this valuable information, allowing for further analysis into the role of TWIST1 in medulloblastoma metastasis (Table 3.3). Follow-up information on 36 out of the 44 patients who were diagnosed with non-metastatic (M0) disease revealed that patients were significantly more likely to develop metastatic disease if the primary tumour expressed high levels of TWIST1. Only 23 % of M0 patients with low TWIST1 expression developed metastatic disease, whereas 58 % of M0 patients who had high TWIST1 expression later developed metastatic disease ($p = 0.037$). This finding that high TWIST1 expression significantly correlated with metastatic disease, further emphasises the potential importance of TWIST1 expression as a prognostic biomarker for metastatic disease in medulloblastoma patients.

Table 3.3 TWIST1 expression in non-metastatic medulloblastoma tumours.

		Original sample Low TWIST1		Original sample High TWIST1		Pearson Chi-Squared Test
Original M0 – Relapsed?	Total No of Patients	No of Patients	%	No of Patients	%	$p = 0.037$
No	21	13	62	8	38	
Yes	15	4	27	11	73	

Long term survival rates for medulloblastoma patients have improved over the years, however, the presence of metastatic disease still remains a poor prognostic factor (Thomas and Noël, 2019). Therefore, the patient TMAs were analysed to investigate if TWIST1 expression had an effect on overall survival (OS) and event free survival (EFS). Due to a limited number of patient TMA samples in each subgroup the effect of TWIST1 expression on OS and EFS could only be investigated on all subgroups combined rather than the four individual subgroups (Figure 3.7). Kaplan Meier analysis showed that high expression of TWIST1 trended towards worse overall survival, and was significantly correlated with EFS. This data supports the above information, whereby patients were more likely to relapse if the primary tumour expressed high levels of TWIST1.

In conclusion, the analysis from the TMA data revealed a significant role for TWIST1 in medulloblastoma metastasis with a particular emphasis on the Group 3 subgroup (which has the poorest overall patient survival rates).

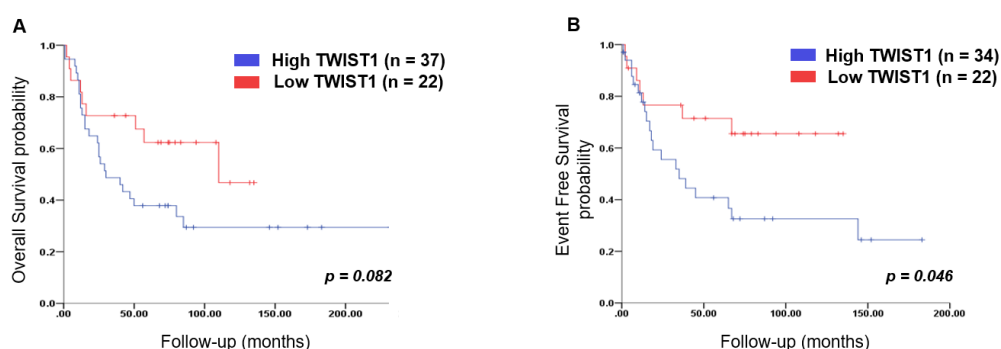


Figure 3.7 Kaplan Meier overall and event free survival curves using medulloblastoma TMAs.

Kaplan Meier overall (A) and event free (B) survival curves using the Nottingham and Birmingham cohort TMAs. Curves show low and high TWIST1 protein expression with statistical significances calculated using the logrank test.

3.2.3 TWIST1 expression *in vitro*

So far, we have shown that the expression level of TWIST1 in primary tumours could be contributing towards metastatic disease and that *TWIST1* gene expression in patient datasets is higher for each subgroup compared to the normal cerebellum. The final part of this chapter utilised the medulloblastoma cell lines available within our research group to assess TWIST1 gene and protein expression across the SHH, Group 3 and Group 4 subgroups. These data will not only expand our understanding of how TWIST1 levels differ within the medulloblastoma subgroups, but will also assess if expression levels are comparable to those observed in patient datasets and therefore validating their applicability for downstream functional analysis.

TWIST1 gene expression was analysed across the medulloblastoma cell lines (DAOY, ONS-76, **D283**, **HD-MB03**, **D425**, **D458**, *CHLA01* and *CHLA01R*), which incorporated the three subgroups (SHH, **Group 3** and *Group 4* respectively) and the non-tumorigenic human foetal neural stem cell line (FB83), by qRT-PCR analysis (Figure 3.8). The SHH and Group 3 medulloblastoma subgroups had higher *TWIST1* expression compared to the non-tumorigenic cell line FB83, and these two subgroups revealed a similar pattern to the Roth and Pfister dataset (Figure 3.1). However, the two Group 4 cell lines (from a matched patient pair) had much lower *TWIST1* expression compared to the FB83 cell line, which was the opposite result to what was observed in Figure 3.1.

Interestingly, when comparing *TWIST1* gene expression between the Group 3 and Group 4 matched cell lines, both non-metastatic primary cell lines (D425 and CHLA-01) had higher *TWIST1* expression compared to the matched metastatic cell lines (D458 and CHLA01R), which was similar to the result observed in the Wang dataset (Figure 3.3). Thus suggesting that the

upregulation of TWIST1 in the primary tumour could be initiating the metastatic cascade.

Next, TWIST1 protein expression was analysed across the same medulloblastoma cell lines by western blotting. Images were quantified by densitometry using the Fiji imaging software and TWIST1 protein expression calculated relative to a house-keeping protein GAPDH (Figure 3.9). Whilst expression was not analysed in the FB83 cell line, TWIST1 protein expression in the medulloblastoma cell lines revealed a similar trend to *TWIST1* gene expression. SHH and Group 3 had much higher protein expression compared to Group 4 cell lines. Although, the expression levels in the CHLA01 Group 4 cell line was negligible, with significant differences observed between SHH and Group 4. When comparing TWIST1 protein expression between the matched cell lines, the same pattern was observed; TWIST1 expression was higher in the non-metastatic primary cell lines (D425 and CHLA01) compared to the matched metastatic cell lines (D458 and CHLA01R), further confirming that upregulation of TWIST1 in primary tumours could be initiating the metastatic dissemination of tumour cells.

Taking into consideration the entirety of the data presented on TWIST1 expression across the four medulloblastoma subgroups and the significance of high TWIST1 protein expression correlating with metastatic disease, the remainder of this thesis will focus on investigating the high TWIST1 expressing metastatic SHH (ONS-76) and Group 3 (D283 and HD-MB03) medulloblastoma cell lines, to gain an understanding of how TWIST1's function, and its downstream targets, are driving medulloblastoma metastasis.

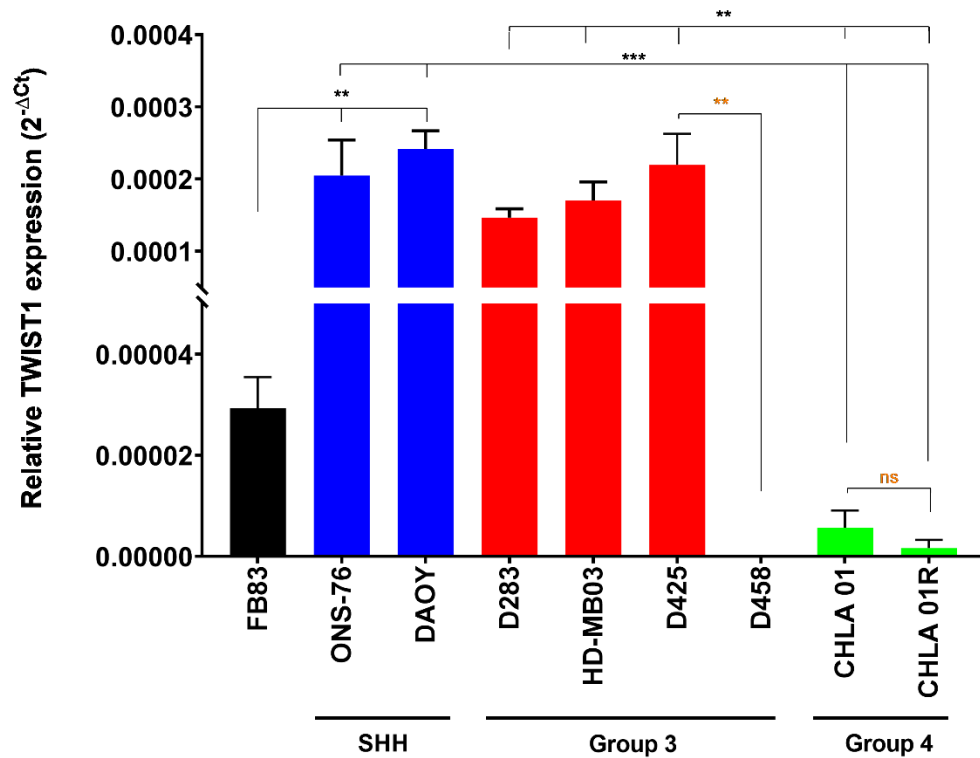


Figure 3.8 TWIST1 gene expression in medulloblastoma cell lines.

TWIST1 gene expression across the three medulloblastoma subgroups: SHH, Group 3 and Group 4, and the non-tumourigenic FB83 cell line. Gene expression was calculated relative to the house-keeping gene *GAPDH* using the $2^{-\Delta Ct}$ method. Error bars represent the mean $\pm$ SEM of $n = 3$ experiments each containing three replicates. Significant differences between subgroups and the matched paired cell lines were calculated using one-way ANOVA analysis with Tukey's multiple comparison post-hoc test, or an unpaired Student's t-test highlighted with orange text, respectively (ns = not significant, $**p \leq 0.01$, $***p \leq 0.001$).

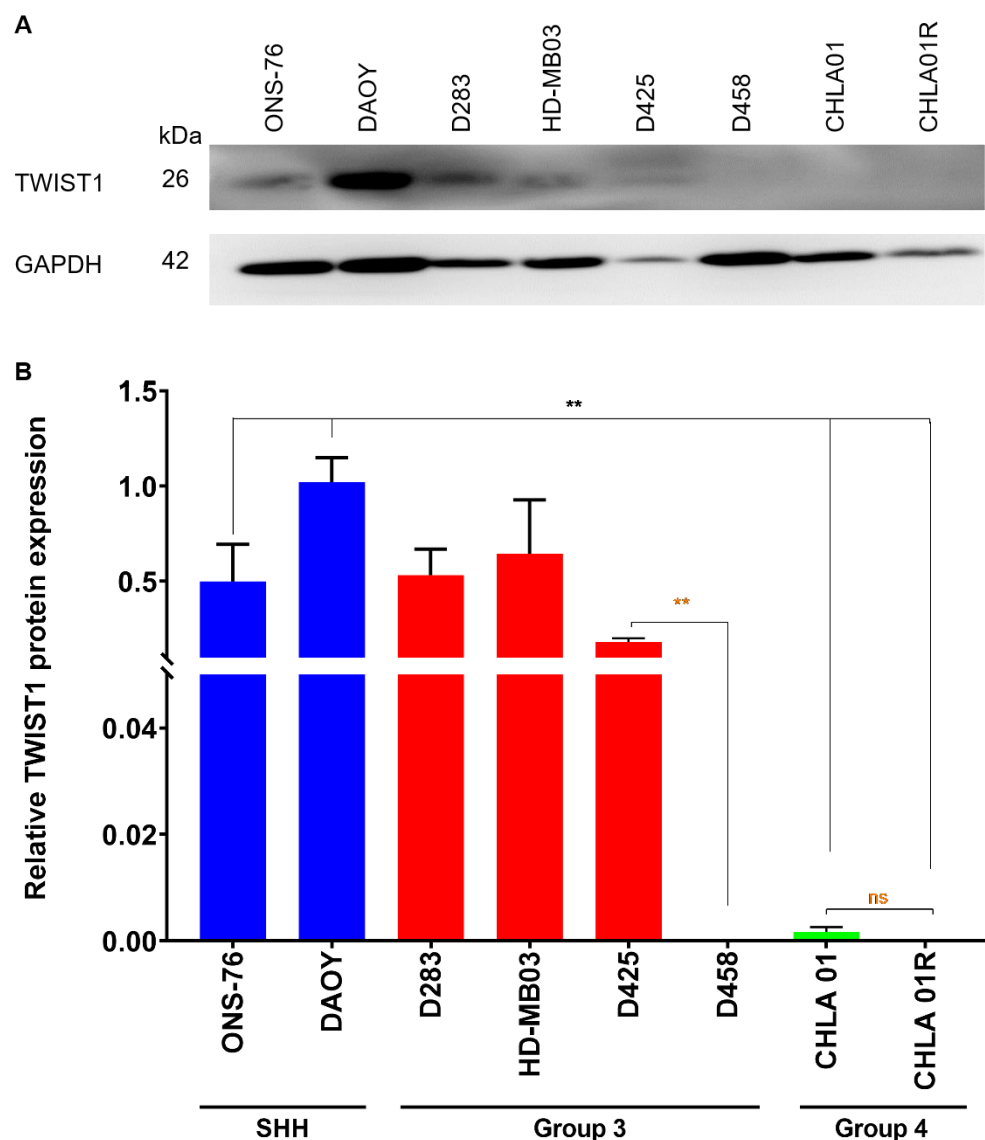


Figure 3.9 TWIST1 protein expression in medulloblastoma cell lines.

TWIST1 protein expression in the medulloblastoma cell lines. 50 μ g of total protein was resolved on a 12 % acrylamide gel by western blotting (A). Protein expression was quantified using Fiji imaging software and calculated relative to a house-keeping protein GAPDH to analyse expression in the three medulloblastoma subgroups: SHH, Group 3 and Group 4 and between matched cell lines. Error bars represent the mean $\pm$ SEM of $n \geq 2$ experiments. Significant differences between subgroups and the matched paired cell lines were calculated using one-way ANOVA analysis with Tukey's multiple comparison post-hoc test, or an unpaired Student's t-test highlighted with orange text, respectively (ns = not significant, $**p \leq 0.01$).

3.3 Summary

- The TMA clinical data indicated that patients diagnosed with Group 3 medulloblastomas were associated with worse overall survival, in accordance with this subgroup being the most aggressive.
- TWIST1 expression analysis in the patient TMAs revealed that high expression significantly correlated with metastatic disease ($p = 0.027$) and worse event-free survival ($p = 0.046$).
- The patient follow-up information from the TMAs analysis, indicated patients diagnosed with non- metastatic disease were significantly more likely to relapse and develop metastatic disease if the tumour expressed high levels of TWIST1 ($p = 0.037$).
- TWIST1 gene and protein expression in the medulloblastoma cell lines was highest in the SHH and Group 3 subgroups. Analyses of the two matched medulloblastoma cell lines (Group 3; D425 and D458 and Group 4; CHLA01 and CHLA01R) revealed higher TWIST1 levels in the primary cell lines compared to the metastatic cell line (Group 3; $p \leq 0.01$). Taken together with the TMA data mentioned above, this data begins to suggest that TWIST1 could be initiating medulloblastoma metastasis.
- Overall, the patient datasets started to highlight an important role for TWIST1 in medulloblastoma metastasis; high levels in the primary tumour appeared to be linked with metastatic disease and a predictor of worse event-free survival. The next chapter will explore the functional role of TWIST1 in the highly expressing metastatic TWIST1 cell lines.

Chapter 4. Inhibiting TWIST1 to investigate its role in medulloblastoma metastasis.

4.1 Introduction

Metastasis is a multistep process, termed the metastatic cascade. It involves the spread of cancerous cells to tissues and organs beyond where the tumour originated from leading to the formation of secondary tumours (Valastyan and Weinberg, 2011, Martin et al., 2013). The initial steps of this complex cascade involve tumour cells acquiring the ability to penetrate and invade surrounding tissues via the epithelial-mesenchymal transition (EMT) pathway. The EMT pathway is a migratory process driven by a set of specific transcription factors including TWIST1 (Cebrià-Costa et al., 2014, Lamouille et al., 2014), and as mentioned in Chapter 3, TWIST1 plays a critical pathological function in cancer progression (Zhao et al., 2017).

Unfortunately, little is known about the mechanisms involved in medulloblastoma metastasis. Medulloblastomas have a neuronal cell of origin and frequently disseminate to the leptomeningeal space via the cerebrospinal fluid (CSF) in a non-EMT like process (Dufour et al., 2012). However, even though medulloblastomas do not originate from a classical epithelial background, emerging evidence suggests that EMT-like processes can occur in non-epithelial tumours (Kahlert et al., 2017). Garzia *et al* showed that circulating medulloblastoma cells could disseminate via a haematogenous route, a metastatic process observed in epithelial tumours (Garzia et al., 2018). In addition, data from our research group revealed that medulloblastoma cells can metastasise via an EMT-like pathway, whereby cells over-expressing TWIST1 altered phenotypically and acquired stem cell-like properties to form viable cell aggregates in a three dimensional (3D) basement membrane extract

(BME) model (Nasir, 2016). This suggests that pathways of metastasis identified in epithelial tumours, including the EMT pathway, could be relevant during medulloblastoma metastasis.

The data presented in this thesis has started to highlight the importance of TWIST1 in medulloblastoma metastasis. TWIST1 expression analyses in the Group 3 and 4 medulloblastoma matched paired cell lines D425 (M0) and D458 (M+), and CHLA01 (M0) and CHLA01R (M+), respectively, showed that TWIST1 expression was higher in the non-metastatic cell lines compared to the metastatic cell lines. In addition, when analysing patient tissue microarrays (TMAs), follow-up information revealed that patients who were diagnosed with non-metastatic disease *and* high TWIST1 expression were significantly more likely to develop metastatic disease.

Together this data begins to indicate that TWIST1 expression could be driving medulloblastoma cells to metastasise in an EMT-like process. In this chapter, the impact of chemical inhibition and degradation of TWIST1, and of shRNA mediated knockdown of *TWIST1* gene expression is investigated in a relevant 3D model of medulloblastoma metastasis.

4.2 Results

4.2.1 Inhibiting the transcription factor TWIST1

4.2.1.1 Chemically inhibiting TWIST1 with harmine

To begin investigating the functional role of TWIST1 during medulloblastoma metastasis, inhibition of TWIST1, in the previously identified metastatic cell lines (SHH; ONS-76 and Group 3; D283 and HD-MB03), was carried out using two different methods:

1. Chemical inhibition of TWIST1 with harmine (a beta-carboline alkaloid) (Yochum et al., 2017).
2. Genetic inhibition of TWIST1 using small hairpin RNA (shRNA)

The first part of this chapter focused on using harmine to inhibit TWIST1. Harmine is normally used to inhibit the activity of dual-specificity tyrosine phosphorylation-regulated kinase (DYRK1A) by inhibiting substrate phosphorylation (Göckler et al., 2009). However, recently harmine has been shown to inhibit multiple cellular events linked to TWIST1 function in non-small cell lung cancer (NSCLC) cells including, single-cell dissemination, proliferation and the evasion of oncogene-induced senescence. The exact mechanism of how harmine inhibits TWIST1 function is not completely understood but it is thought that it induces TWIST1 protein degradation (Yochum et al., 2017).

Based on this information, harmine cannot be classed as a pharmacological inhibitor because it does not prevent TWIST1's function but rather acts as a negative regulator of its function by causing targeted protein degradation. For simplicity though harmine will be referred to as a TWIST1 inhibitor. In addition, there are limitations with using harmine due to the mode of action on TWIST1 protein expression not being completely understood. Harmine might cause off-

target effects which could contribute positively or negatively to any observed effects.

Initially, cells were treated with increasing concentrations of harmine (0 - 50 μ M) for 72 hours and metabolic activity was assessed using the PrestoBlue™ assay. However, high concentrations of harmine (> 20 μ M) resulted in a complete loss of cellular metabolic activity. Therefore, harmine concentrations were adjusted for each cell line and the metabolic activity was analysed relative to the vehicle control. Images were also taken of the HD-MB03 cell line 72 hours post harmine treatment to assess if treatment resulted in visible cell death or changed the cells growth patterns and/ or morphology (Figure 4.1).

All cell lines showed a decrease in metabolic activity in a dose-dependent manner (Figure 4.1A). Interestingly, harmine had no effect on the morphology of the HD-MB03 cells. The cells continued to grow in a semi-adherent manner, with cells appearing both adhered to the bottom of the well (red arrows) and in suspension as small tight clumps (black arrows) (Figure 4.1B). Most of the cells that were treated with high concentrations of harmine (10 μ M and 20 μ M) appeared to be the same size as the vehicle control and were still attached to the bottom of the well (red arrow), suggesting little cell death had occurred. When the concentration of harmine increased to 10 μ M and 20 μ M, the cells were not as confluent as the vehicle control and did not form many large adherent aggregates, instead the cells remained in smaller clumps. This data suggests that harmine could be acting as a cytostatic drug by reducing cell proliferation, and the cells might be entering into a senescent state supporting previous findings that TWIST1 plays a crucial role in evading oncogene-induced senescence (Zhao et al., 2017).

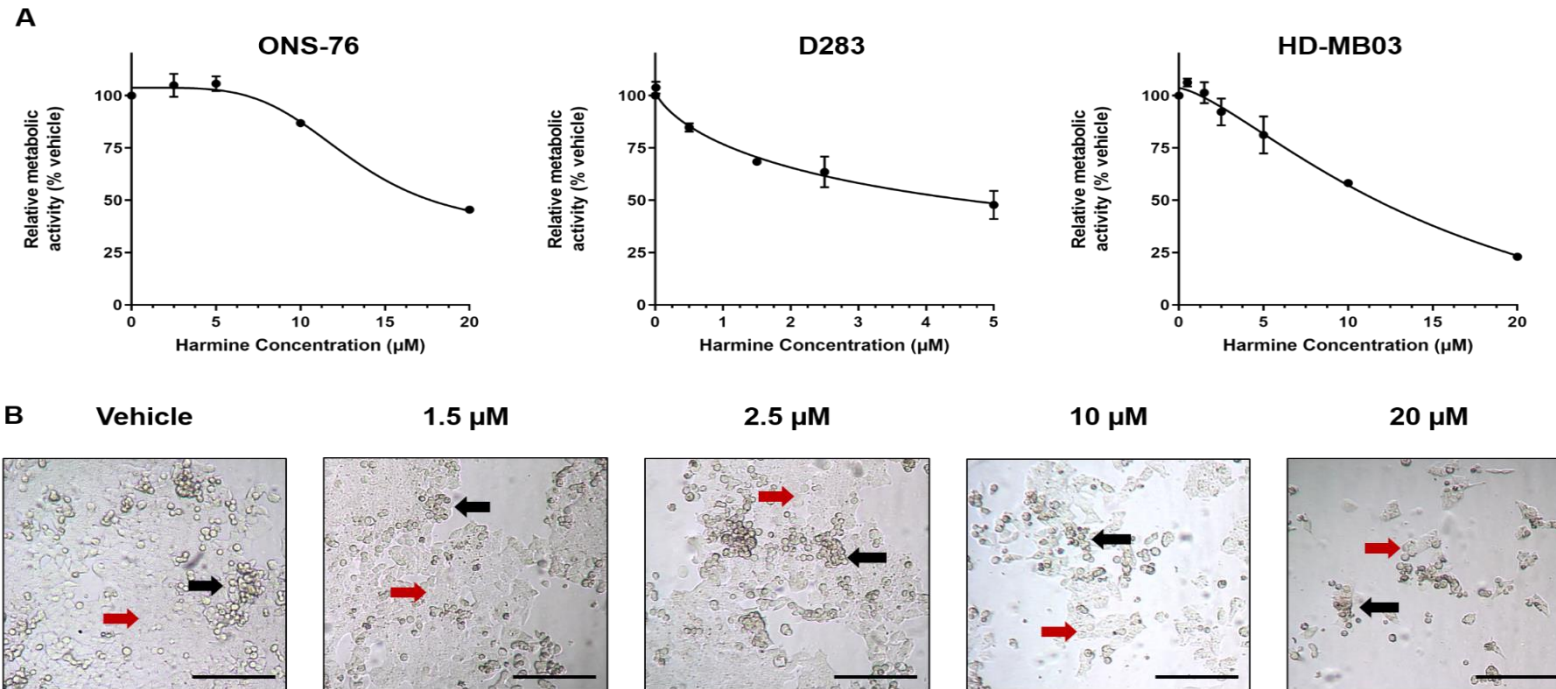


Figure 4.1 Relative metabolic activity and cell morphology of medulloblastoma cell lines after treatment with harmine for 72 hours.

ONS-76, D283 and HD-MB03 cell lines were treated with increasing concentrations of harmine for 72 hours (A). The PrestoBlue™ assay was performed to assess metabolic activity, which was calculated as a percentage relative to the vehicle control. Dose response curves were generated using non-linear regression analysis and error bars represent the mean $\pm$ SEM of $n = 2$ experiments each containing 3 replicates. Cell images of the HD-MB03 cells (B) were taken on The Brunel Multiple Interface camera attached to the brightfield inverted microscope at 10x magnification. Cells continued to grow either adhered to the bottom of the well (red arrows) or in suspension (black arrows) (scale bar: 100 μ m).

Moving forward, a range of concentrations were chosen for each cell line, representing cytostatic and non-cytostatic concentrations (Table 4.1), to investigate what affect this had on TWIST1 protein expression and assess if the observed reduction in metabolic activity and cell proliferation (Figure 4.1) correlated with a reduction in TWIST1 protein expression. The cells were treated for 72 hours and protein expression was analysed by western blotting. Western blot images were quantified by densitometry using the Fiji imaging software and TWIST1 protein expression was calculated relative to a house-keeping protein β -Actin. The data were normalised to the vehicle control (Figure 4.2).

Table 4.1 Harmine concentrations chosen to treat medulloblastoma cell lines.

Cell Line	Harmine Concentration (μ M)
ONS-76	2.5 and 20
D283	0.01, 0.1, 1.5 and 5
HD-MB03	1.5, 2.5, 10 and 20

When the cells were treated with non-cytostatic concentrations of harmine, TWIST1 protein expression was unchanged compared to the vehicle control. However, treatment with cytostatic concentrations of harmine (ONS-76; 20 μ M, D283; 5 μ M and HD-MB03; 20 μ M), resulted in a 70 %, 77 % and 93 % reduction in TWIST1 protein expression, respectively, mirroring the reduction in metabolic activity and cell the growth patterns seen in Figure 4.1.

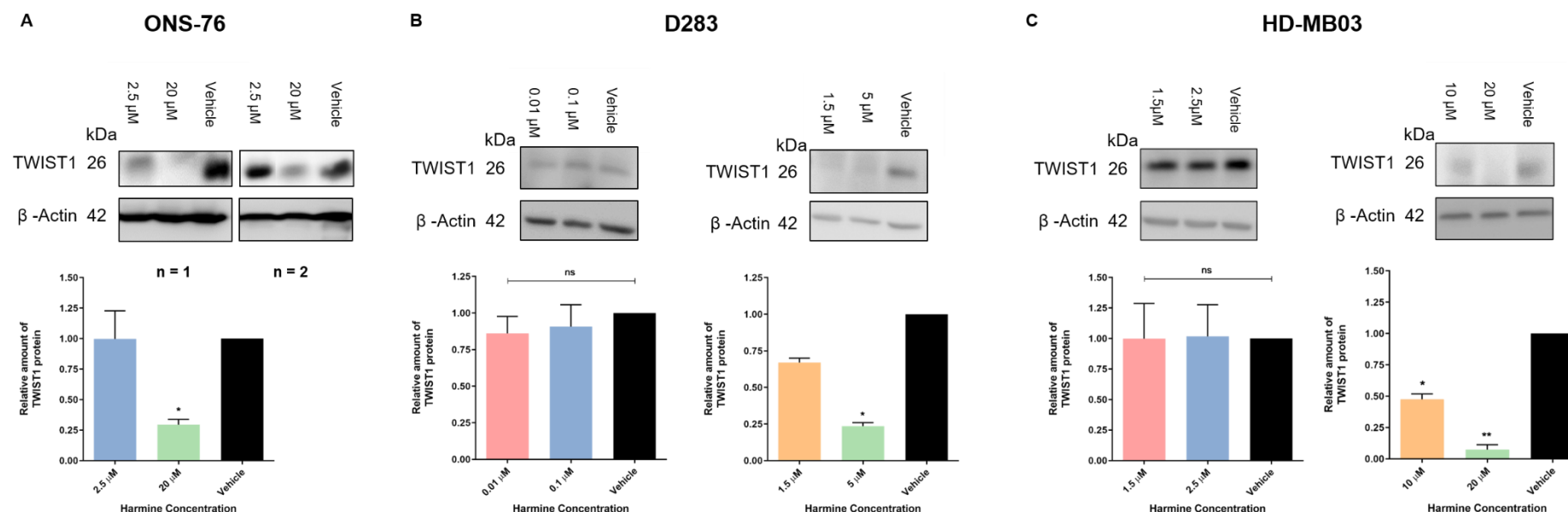


Figure 4.2 TWIST1 protein expression in ONS-76, D283 and HD-MB03 following harmine treatment.

Twist1 protein expression after ONS-76 (A), D283 (B) and HD-MB03 (C) cells were treated with increasing concentrations of harmine for 72 hours. 50 μg of total protein was resolved on 12% acrylamide gels by western blotting. Western blots are shown as split panels as experiments were performed at separate times and the nitrocellulose was cut prior to incubation with anti-TWIST1 or anti-β-Actin antibodies. Protein expression was quantified using Fiji imaging software and normalised to the vehicle control. Error bars represent the mean ± SEM of n = 2 (A and B) or n = 3 (C) experiments. Statistical significance was calculated using a one-way ANOVA analysis with Dunnett's multiple comparison post-hoc test comparing to the vehicle control (ns = not significant, *p ≤ 0.05, **p ≤ 0.01).

In addition to analysing TWIST1 protein expression after harmine treatment, *TWIST1* gene expression was also assessed to confirm that the effect of harmine occurred through a post-translational mechanism (Yochum et al., 2017). For each cell line, the concentration of harmine which caused the greatest reduction in TWIST1 protein expression was used to analyse gene expression (Figure 4.3). There were no significant differences in TWIST1 gene expression in any of the cell lines after harmine treatment when compared to the vehicle control, confirming that harmine did not affect *TWIST1* gene expression and instead degraded TWIST1 protein.

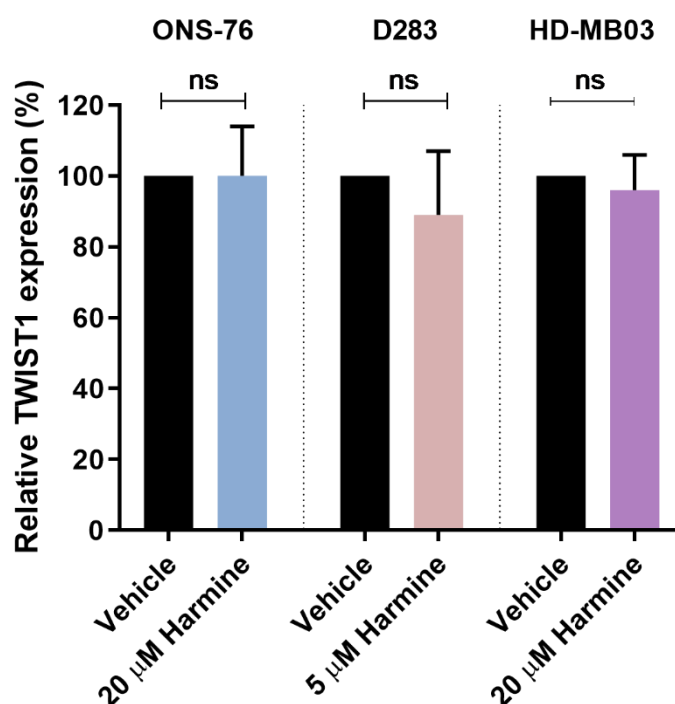


Figure 4.3 TWIST1 gene expression in medulloblastoma cell lines after treatment with harmine.

TWIST1 gene expression after 72 hour treatment with 20 µM (ONS-76 and HD-MB03) or 5 µM (D283) harmine. Gene expression was calculated relative to a house-keeping gene *GAPDH* and normalised relative to the vehicle control using the $2^{-\Delta\Delta Ct}$ method. Error bars represent the mean $\pm$ SEM of $n = 2$ experiments each containing three replicates. Statistical significance was calculated using a paired Student's t-test (ns = not significant).

4.2.1.2 Knockdown of TWIST1 using shRNA

The second method used to inhibit TWIST1's expression was by shRNA. The three metastatic cell lines were transduced with TWIST1-shRNA GIPZ Lentiviral clones. The cloning vectors contained a TurboGFP reporter element to visualise and track the transduction, and a puromycin resistance marker to allow for antibiotic-selection of transduced cells. Before transducing the cells, the concentration of puromycin required to kill non-transduced cells was determined. The three cell lines were treated with a range of puromycin concentrations (0 – 1 µg/ ml) for 48 hours and the metabolic activity was assessed using the PrestoBlue™ assay (Figure 4.4). The ONS-76 cell line required 1 µg/ml of puromycin and the D283 and HD-MB03 cell lines required 0.6 µg/ml of puromycin to effectively kill the cells.

The next stage was to determine the number of lentiviral vector particles required for the optimal silencing of TWIST1. This was done by varying the multiplicity of infection (MOI) of each cell line. The MOI refers to the number of virus particles that can infect individual cells. If the MOI is too high then this can cause cell toxicity or off-target effects due to more than one copy of DNA being integrated into the target cell.

The cell lines were transduced with three different TWIST1 clones that targeted different regions of the TWIST1 transcript (TWIST1.1, TWIST1.2 and TWIST1.3) and a non-silencing negative control at three MOIs (0.3, 0.5 and 1). After transduction, the cells were selected using puromycin and examined on a fluorescent microscope for the presence of the TurboGFP reporter to see if the transduction was successful (Figure 4.5).

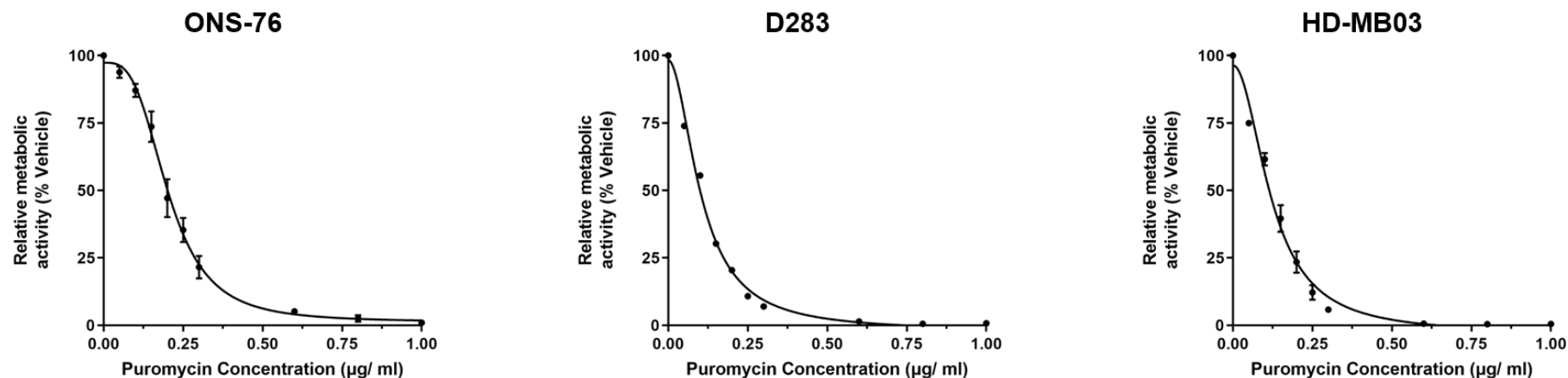


Figure 4.4 Determination of puromycin concentration for selection of transduced cell lines.

ONS-76, D283 and HD-MB03 cell lines were treated with increasing concentrations of puromycin for 48 hours. The PrestoBlue™ assay was performed to assess metabolic activity which was calculated as a percentage relative to the vehicle control. Dose response curves were generated using non-linear regression analysis and error bars represent the mean $\pm$ SEM of $n \geq 1$ experiments each containing 3 replicates.

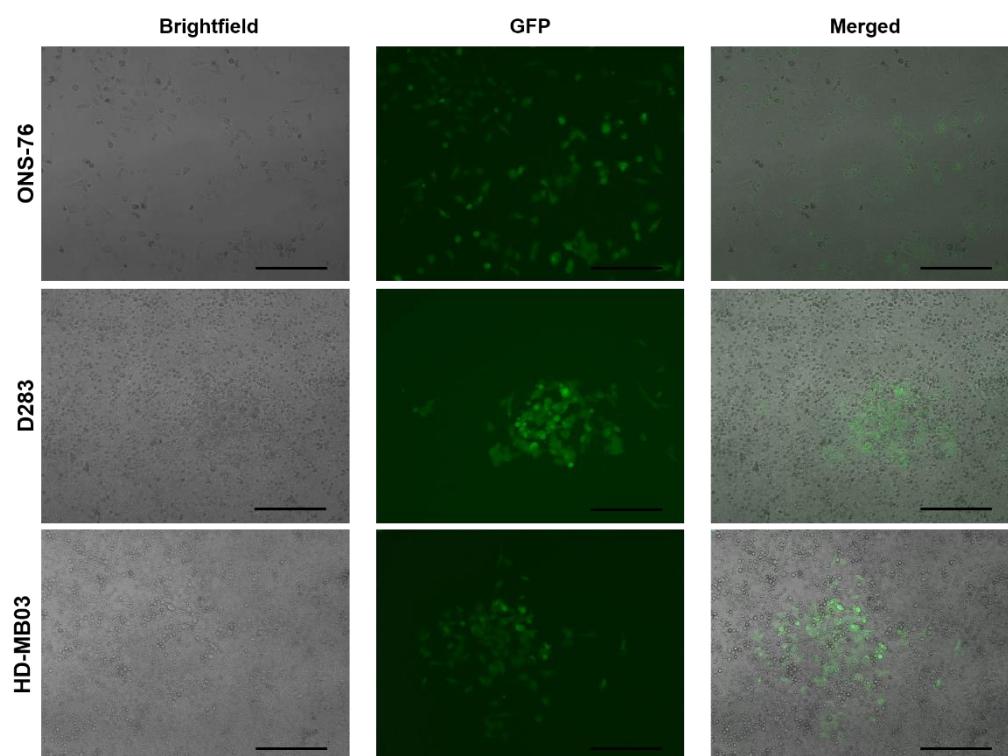


Figure 4.5 Lentiviral transduction of metastatic medulloblastoma cell lines with shRNA constructs.

ONS-76, D283 and HD-MB03 cell lines were transduced with lentiviral vectors to express shTWIST1. Brightfield and GFP images were taken 6 days post transduction to visualise transduced cells. The above images are of cells transduced at an MOI of 0.5. Cells were imaged on a Nikon Eclipse Ti inverted widefield microscope using the GFP filter at 10X magnification (scale bar: 100 μ m).

After confirming that the transduction had worked by the presence of green fluorescent cells after puromycin selection, the cell lines were expanded and the knockdown (KD) efficiencies were analysed by qRT-PCR using consecutive cell passages and by western blotting (ONS-76; Figure 4.6, D283; Figure 4.7 and HD-MB03; Figure 4.8).

Analysing each cell line revealed that some of the clones were more efficient at knocking down TWIST1 than others. It was important to choose a clonal population of cells and an MOI that resulted in a decrease in both the TWIST1

gene and protein expression, with the selected clones highlighted in each western blot image. The TWIST1.2 and TWIST1.3 clones at an MOI of 0.5 resulted in the biggest reduction in TWIST1 gene and protein expression when compared to the non-silencing controls in the ONS-76 and D283 cell lines, and therefore, were selected for subsequent experiments. However, when analysing the HD-MB03 KD efficiencies, large variabilities in the MOI 1 and 0.5 datasets were observed, (see error bars of Figure 4.8, panel A). Further investigation, revealed that *TWIST1* gene expression started to increase again after two cell passages even with continued puromycin selection. The increased error bars are witness to the loss of repressed gene expression seen in later passages. In order to afford confidence in functional experiments cells were recovered from 1-2 passages after selection when the reduction in gene expression was at its greatest, with an MOI of 1 being selected for these experiments.

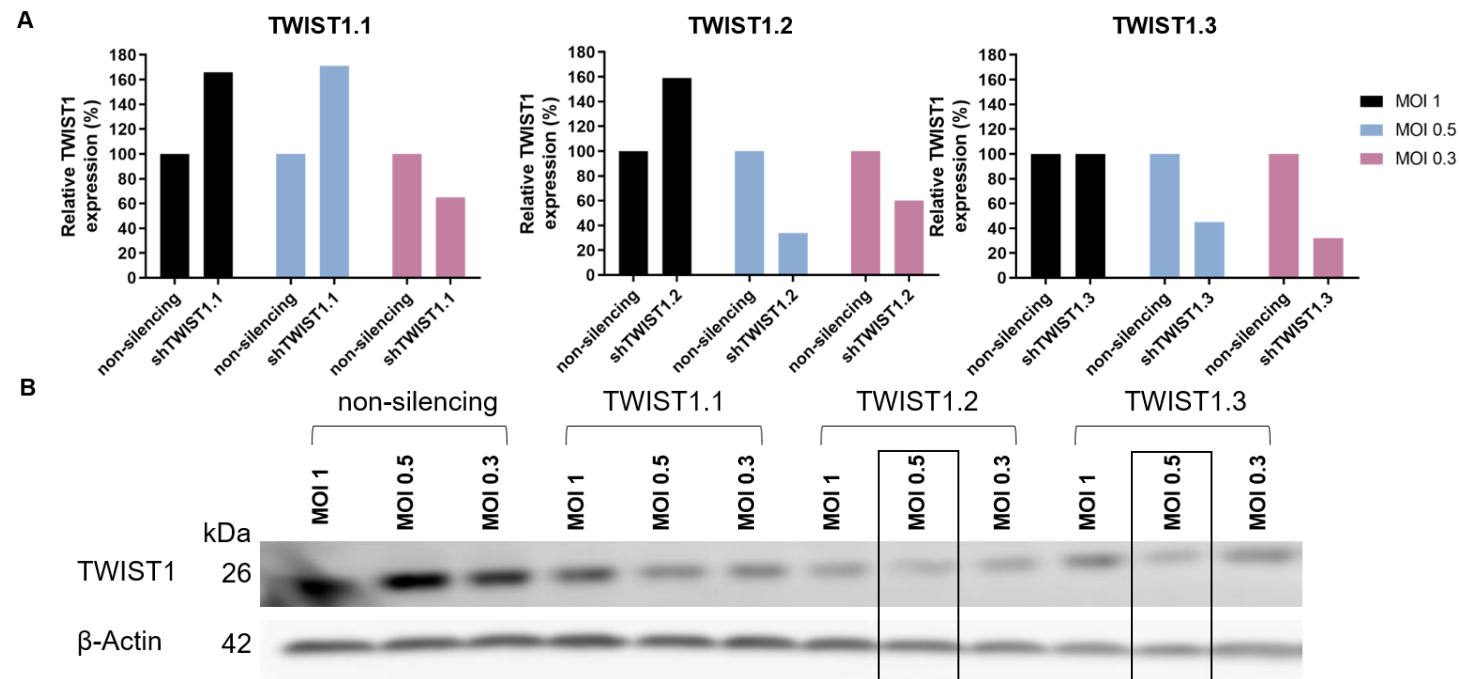


Figure 4.6 TWIST1 gene and protein expression after treatment with shTWIST1 lentiviral clones in the ONS-76 cell line.

TWIST1 gene (A) and protein (B) expression in the ONS-76 cell line after treatment with three shTWIST1 clones at three MOI's. Gene expression was calculated relative to the house-keeping gene *HPRT* and normalised to the non-silencing control using the $2^{-\Delta\Delta ct}$ method. Cells were transduced once and the data represents analysis from one cell pellet with the mean of three qRT-PCR replicates. 50 μ g of total protein was resolved on 12% acrylamide gels by western blotting. Western blots are shown as split panels as the nitrocellulose was cut prior to incubation with anti-TWIST1 or anti- β -Actin antibodies.

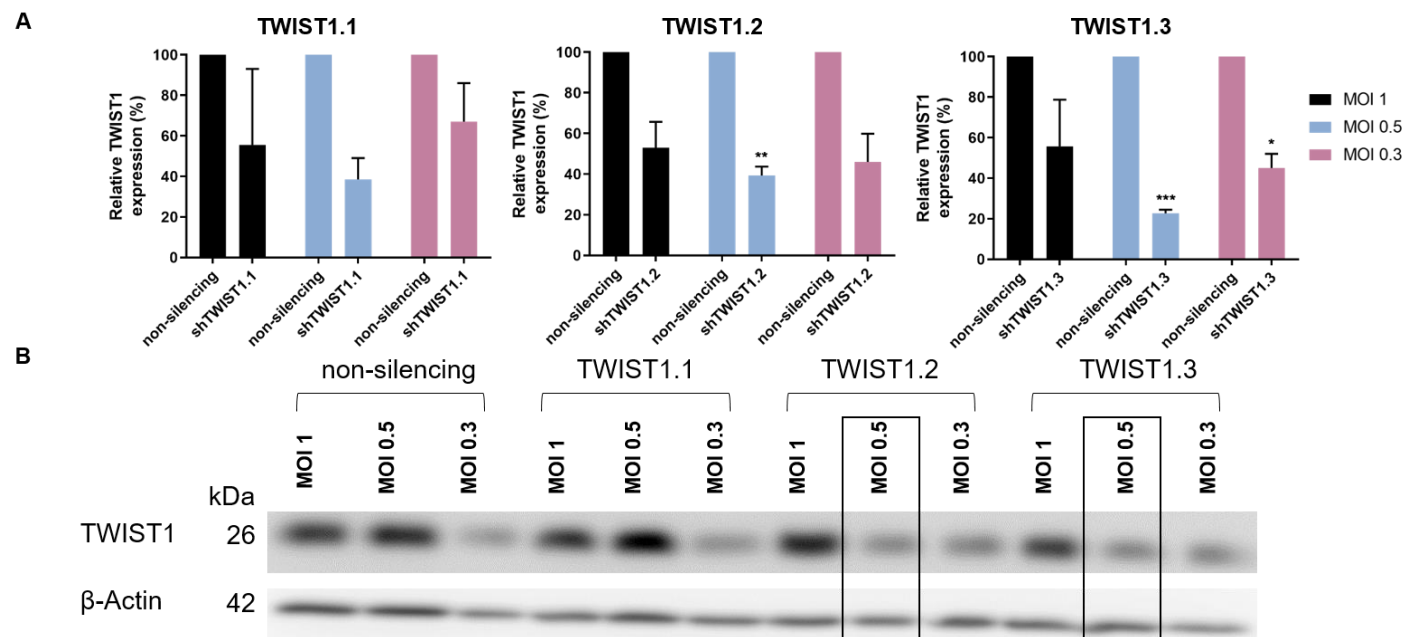


Figure 4.7 TWIST1 gene and protein expression after treatment with shTWIST1 lentiviral clones in the D283 cell line.

TWIST1 gene (A) and protein (B) expression in the D283 cell line after treatment with three different shTWIST1 clones at three MOI's.

Gene expression for each MOI was calculated relative to the house-keeping gene *HPRT* and normalised to the non-silencing control using the $2^{-\Delta\Delta Ct}$ method. Cells were transduced once and error bars represent the mean $\pm$ SEM of $n \geq 2$ experiments from subsequent passages, with each qRT-PCR experiment containing three replicates. Significant differences were calculated using a paired Student's t-test comparing to the non-silencing control ($*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$). 50 μ g of total protein was resolved on 12% acrylamide gels by western blotting. Western blots are shown as split panels as the nitrocellulose was cut prior to incubation with anti-TWIST1 or anti- β -Actin antibodies.

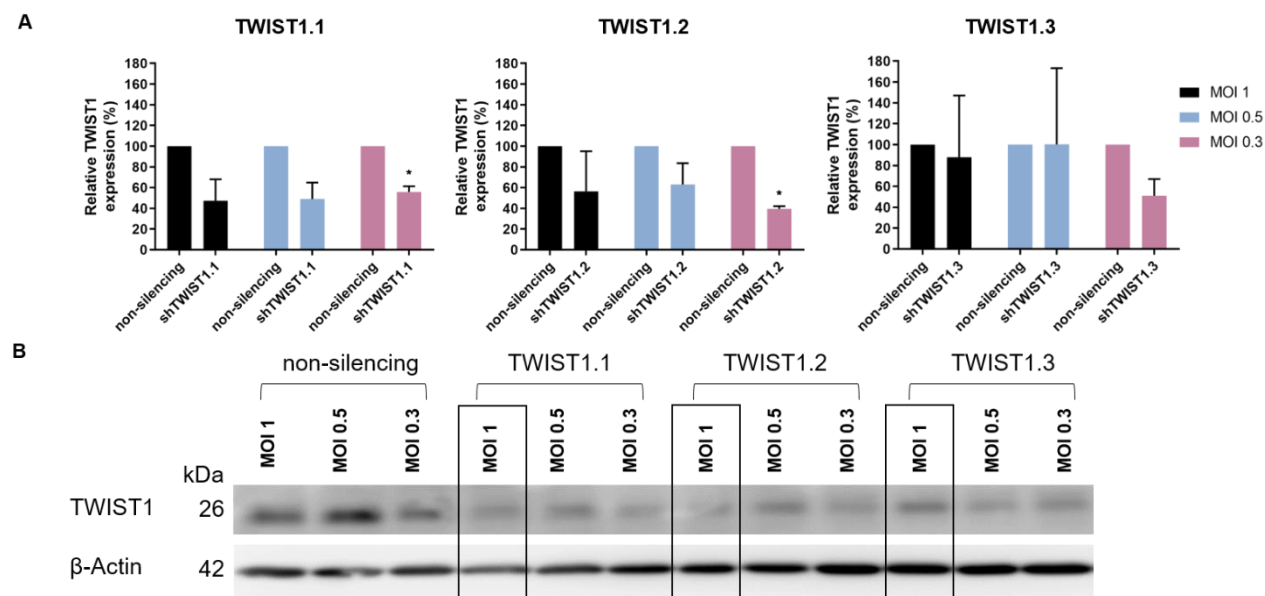


Figure 4.8 TWIST1 gene and protein expression after treatment with shTWIST1 lentiviral clones in the HD-MB03 cell line.

TWIST1 gene (A) and protein (B) expression in the HD-MB03 cell line after treatment with three different shTWIST1 clones at three MOI's. Gene expression for each MOI was calculated relative to the house-keeping gene *HPRT* and normalised to the non-silencing control using the $2^{-\Delta\Delta ct}$ method. Cells were transduced once and error bars represent the mean $\pm$ SEM of $n \geq 2$ experiments from subsequent passages, with each qRT-PCR experiment containing three replicates. The large error bars were due to a degree of recovery of expression in higher passages. Significant differences were calculated using a paired Student's t-test comparing to the non-silencing control (* $p \leq 0.05$). 50 μ g of total protein was resolved on 12% acrylamide gels by western blotting. Western blots are shown as split panels as the nitrocellulose was cut prior to incubation with anti-TWIST1 or anti- β -Actin antibodies.

4.2.2 Inhibiting TWIST1 in 3D migration models

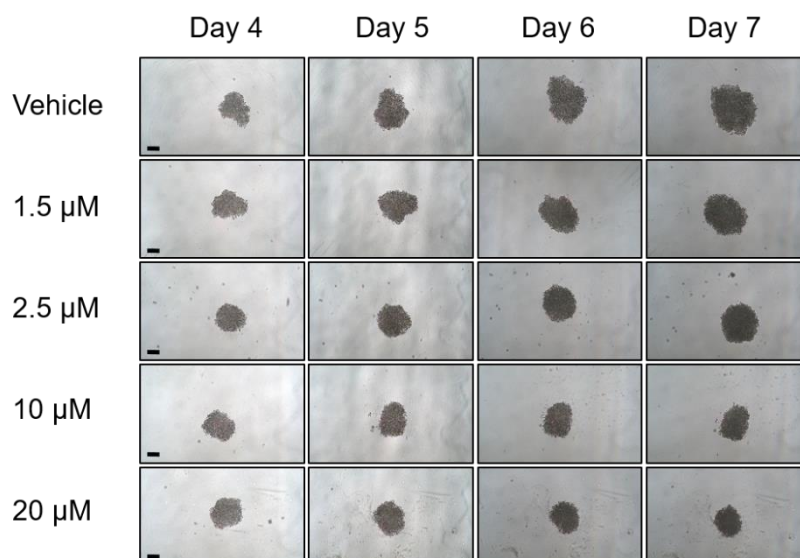
Having established the clinical correlation of TWIST1 with metastatic potential, and identified conditions for either pharmacological or genetic reduction in TWIST1 expression, it was of interest to explore the impact of this reduced expression in 3D models of cellular invasion and migration. Two different 3D models were compared for their ability to recapitulate medulloblastoma migration; a spheroid model and a transwell migration and invasion assay.

The first assay tested was a 3D spheroid model optimised by Dr Sophie Roper, a previous PhD student in our research group (Roper, 2019). Spheroid models are commonly used due to their relative ease of assembly and reproducibility in terms of shape and size (Ferreira et al., 2018). This spheroid model allowed for the seeding of a dissociated cell suspension to form a single spheroid by day 4 (Vinci et al., 2013, Roper et al., 2021). The spheroids could then be used for drug response analysis and migration assays. The HD-MB03 cell line was initially tested in the 3D spheroid model. A 3D spheroid cell viability assay using increasing concentrations of harmine (0 – 50 μ M) confirmed that the effect of harmine on spheroid culture was similar to that observed in 2D culture (Appendix 2, Appendix Figure 3).

The HD-MB03 spheroids were treated with the same concentrations of harmine identified in Table 4.1 to investigate TWIST1's function during cell migration across a collagen IV gel. Daily images of the spheroids revealed that they failed to increase in size at the same rate as the vehicle control, suggesting that harmine prevented cell proliferation even when used at low concentrations (Figure 4.9). Whilst all conditions showed some cell migration, it was difficult to conclude what effect TWIST1 was having, not only because of the low numbers of migrating cells but also because of the cytostatic effect of harmine (Figure

4.10). Therefore, based on this data it was decided that the 3D spheroid model was not the correct assay to investigate medulloblastoma metastasis.

A



B

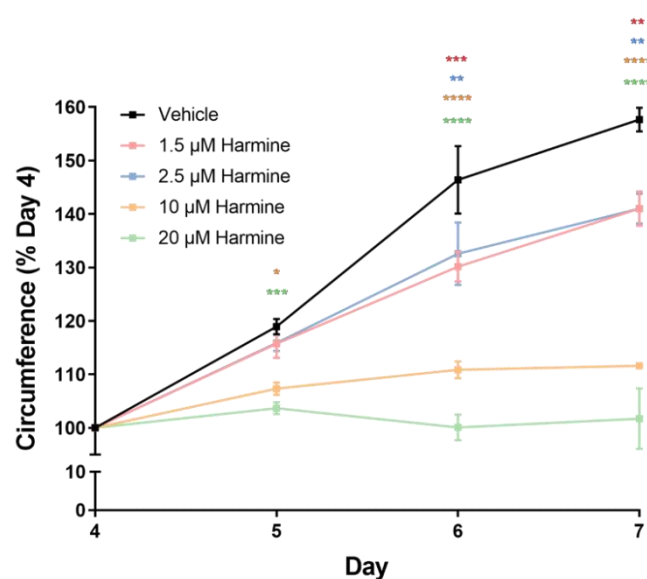
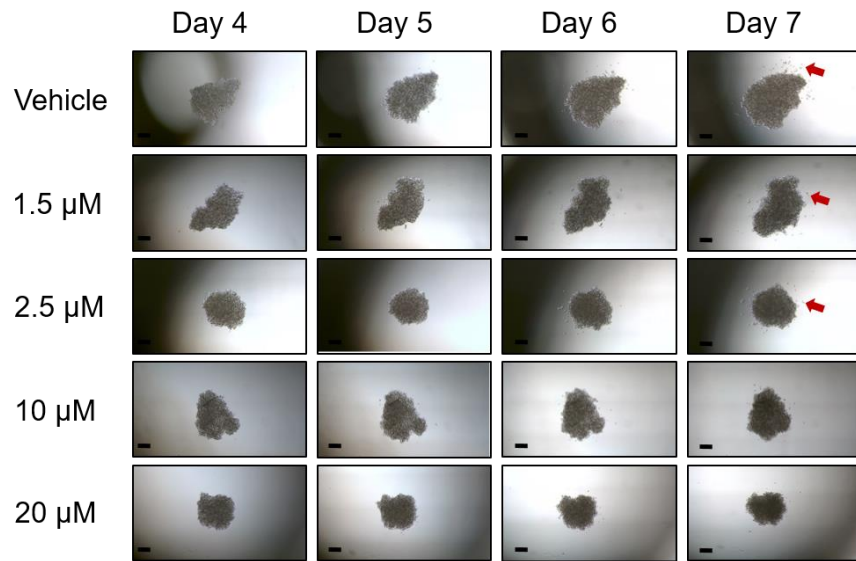


Figure 4.9 Growth rate of HD-MB03 3D spheroids after treatment with harmine.

HD-MB03 spheroids were treated with increasing concentrations of harmine (1.5 μM – 20 μM) and images were taken daily at 5X magnification (Scale bar: 100 μm) (A) to calculate the circumference of each spheroid relative to day 4 of growth (%) (B). Error bars represent the mean ± SEM of n = 3 experiments each containing 3 replicates. Statistical significance was calculated using a two-way ANOVA analysis with Dunnett's multiple comparisons post hoc test.

A



B

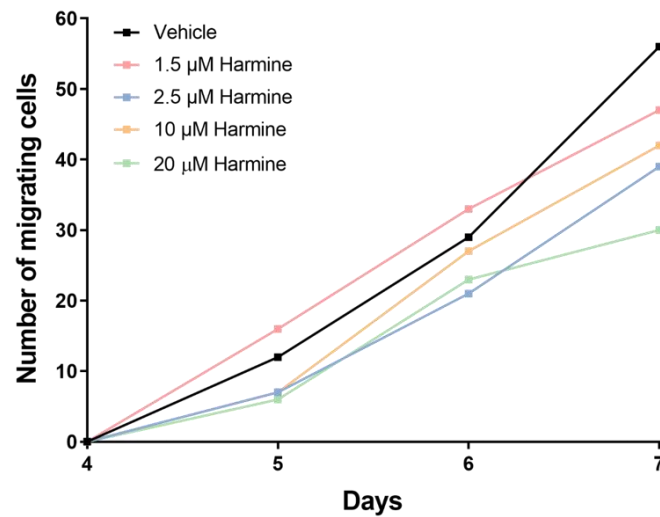


Figure 4.10 Number of HD-MB03 cells migrating after harmine treatment.

Single HD-MB03 spheroids were transferred onto a collagen IV coated well in medium containing increasing concentrations of harmine (1.5 μ M – 20 μ M) for 3 days. Images were taken daily at 5X magnification to visualise cell migration (red arrows) (Scale bar: 100 μ m) (A) and to quantify single cell migration across the collagen IV matrix (B). Data represents a single experiment containing 3 replicates.

The second 3D model to be tested was a 3D transwell migration and invasion model. A transwell assay is an effective way to measure tumour cell migration *in vitro*. The addition of a simple matrix coated on top of the transwell insert can provide cells with a mimetic of the physiological basement membrane, thus, creating an assay that can mimic both tumour metastasis and cell invasion (Rommerswinkel et al., 2014).

The 3D transwell migration and invasion assay had been optimised by Dr Macha Aldighieri, a PhD student in the group (Aldighieri, M., 2020). The assay was used to investigate the effect of inhibiting TWIST1 with harmine or shRNA on medulloblastoma metastasis. To ensure that the cytostatic effect of harmine did not bias the results, the cells were starved in medium supplemented with 2 % FBS 24 hours before seeding and upon seeding cells onto the transwell inserts to prevent cell proliferation. This meant that only the functional effect of TWIST1 on cell migration was being investigated. A preliminary experiment using the HD-MB03 cell line was performed to ensure that the starvation medium did not result in loss of TWIST1 protein expression (Figure 4.11).

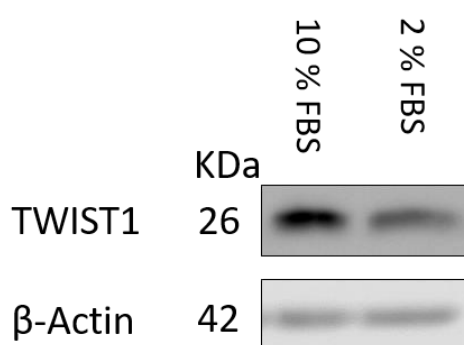


Figure 4.11 TWIST1 protein expression after culture in medium supplemented with 10 % or 2 % FBS.

TWIST1 protein expression in the HD-MB03 cell line after cells were cultured in 10 % or 2 % FBS for 24 hours. 50 µg of total protein was resolved on 12% acrylamide gels and western blots following incubation with anti-TWIST1 or anti-β-Actin antibodies are shown.

Even though a modest reduction in TWIST1 protein expression was observed, it did not completely reduce its expression, and therefore, the 3D transwell assay was set up according to Figure 4.12. The ONS-76 and D283 cell lines were treated with a concentration of harmine that caused the greatest reduction in TWIST1 protein expression (20 μ M and 5 μ M respectively), and the HD-MB03 cell line was treated with increasing concentrations of harmine (1.5 μ M – 20 μ M). Cell viability was analysed using trypan blue before seeding viable cells onto the coated transwell inserts. Cell migration was assessed following (2.3.3) and the number of migrating cells was calculated relative to a standard curve of known cell numbers and the data normalised relative to the vehicle control (Figure 4.13).

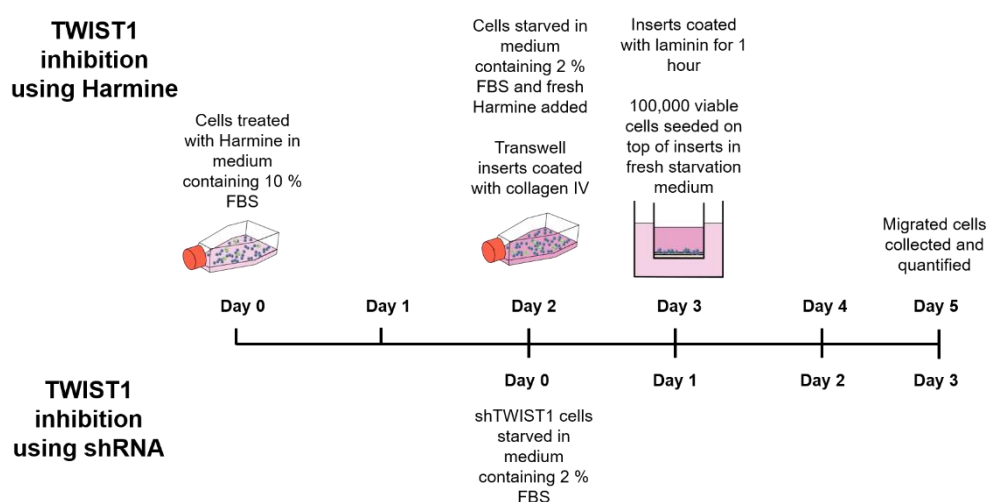


Figure 4.12 Schematic of the 3D migration and invasion model.

Schematic illustrating the 3D transwell model. TWIST1 was inhibited with either shRNA or harmine. 1 day before seeding, cells were starved in medium containing 2 % FBS. Viable cells were seeded onto a collagen IV and laminin gel in starvation medium and migrated cells were quantified 2 days later.

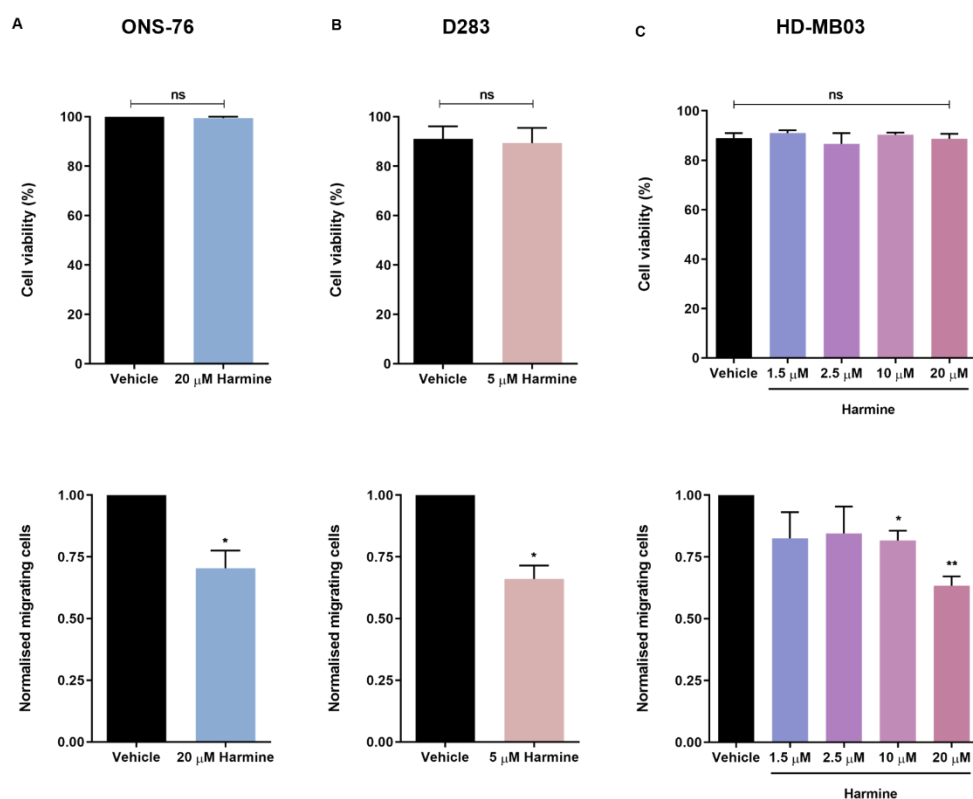


Figure 4.13 Cell viability and migration after TWIST1 inhibition with harmine using the 3D transwell model.

Cells were treated with harmine (ONS-76; 20 μ M (A), D283; 5 μ M (B) and HD-MB03; 1.5 μ M – 20 μ M (C)) and cell viability was assessed relative to the vehicle control before seeding viable cells onto a collagen IV and laminin coated transwell insert. Migrated cells were subjected to a PrestoBlue™ assay to determine the relative fluorescence units (RFU) and migrated cell numbers calculated against a standard curve of known numbers and normalised relative to the vehicle control. Error bars represent the mean $\pm$ SEM of $n \geq 2$ experiments each containing three replicates. Significant differences were calculated using a paired Student's t-test (ONS-76 and D283) or one-way ANOVA with Dunnett's multiple comparison post-hoc test (HD-MB03) comparing to the vehicle control (ns = not significant, * $p < 0.05$, ** $p < 0.01$).

Figure 4.13 revealed that incubation of cells with harmine at concentrations that were not cytotoxic (upper panels) but which were able to reduce TWIST1 protein expression (Figure 4.2) caused a significant reduction in cell migration compared to the vehicle control (lower panels).

Cell migration was also assessed in the TWIST1 knocked-down cell lines using the 3D migration model (ONS-76 and D283; Figure 4.14 and HD-MB03; Figure 4.15). For the TWIST1 clones that provided a consistent knockdown (shTWIST1.2 for ONS-76 and D283), the experiments clearly showed a consistent reduction in cell migration in tandem with *TWIST1* gene expression knockdown. For the clones where a transient TWIST1 knock-down only was observed (Figure 4.8 and, Appendix 2, Appendix Figure 4) experiments were repeated multiple times but each dataset is shown as the variation in knockdown of gene expression was highly variable as previously described. For these datasets it was clear that there was a strong correlation between the degree of knockdown and the degree of cell migration.

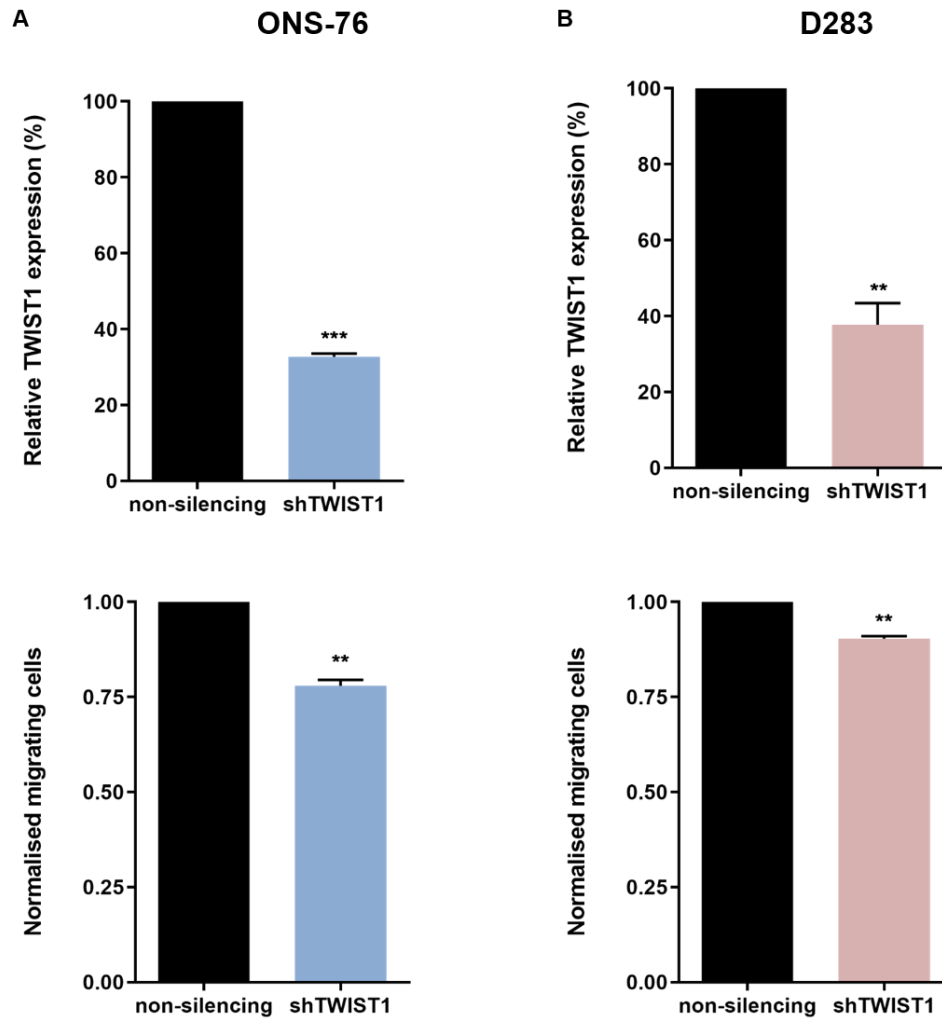


Figure 4.14 ONS-76 and D283 TWIST1 expression and cell migration after shTWIST1 using the 3D migration assay.

TWIST1 gene expression in the ONS-76 (A) and D283 (B) cell lines after transduction with the shTWIST1.2 clones at an MOI 0.5. Gene expression was calculated relative to a house-keeping gene *HPRT* and normalised to the non-silencing control using the $2^{-\Delta\Delta Ct}$ method. Cell numbers were calculated against a standard curve of known numbers and normalised relative to the non-silencing control. Error bars represent the mean $\pm$ SEM of $n = 3$ experiments each containing three replicates. Significant differences were calculated using a paired Student's t-test (** $p < 0.01$, *** $p < 0.001$).

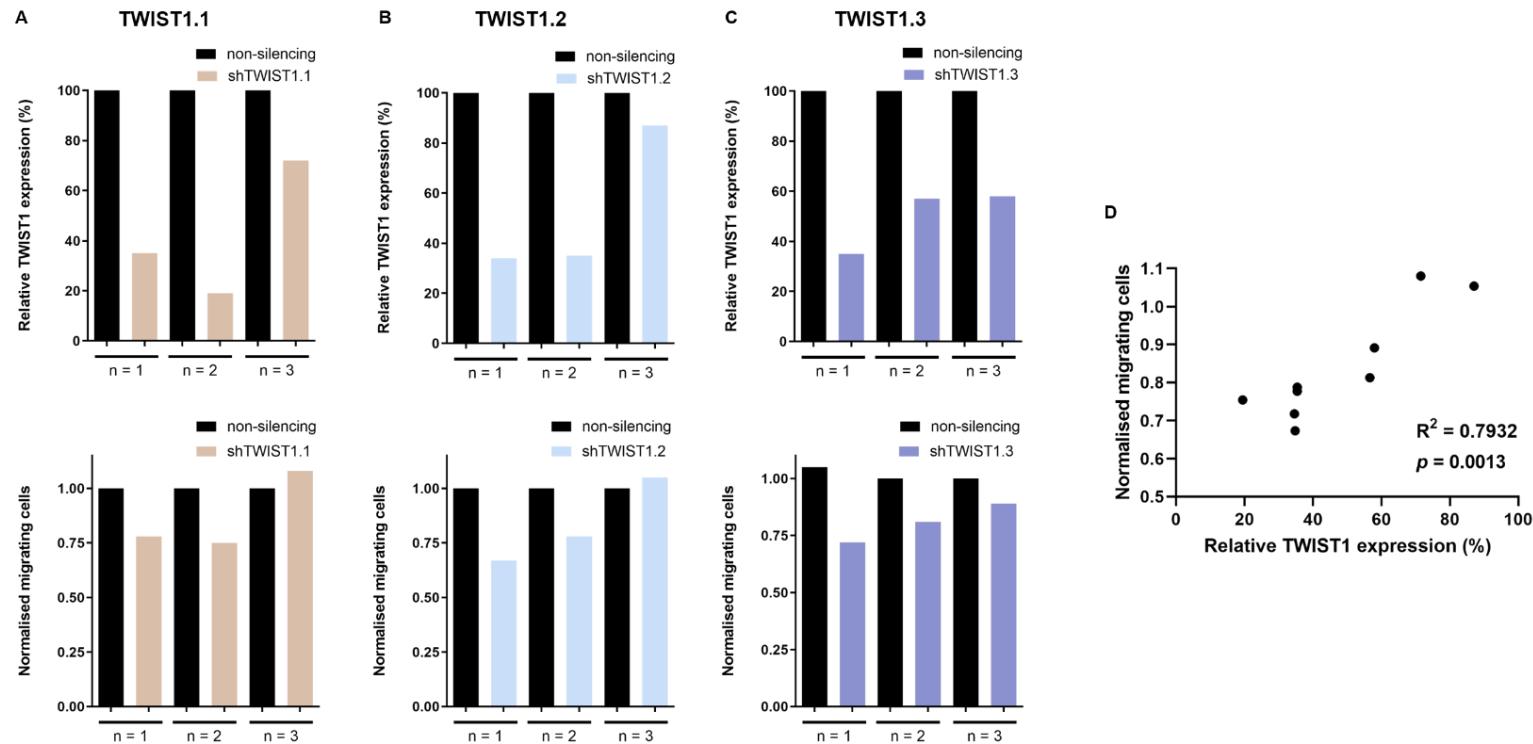


Figure 4.15 HD-MB03 TWIST1 expression and cell migration after shTWIST1 using the 3D migration assay.

TWIST1 gene expression in the HD-MB03 cell line after transduction with the three sh*TWIST1* clones at an MOI 1. Gene expression was calculated relative to a house-keeping gene *HPRT* and normalised to the non-silencing control using the $2^{-\Delta\Delta Ct}$ method. Cell numbers were calculated against a standard curve of known numbers and normalised relative to the non-silencing control (A – C). Individual values from graphs A – C were used to analyse the correlation between *TWIST1* expression and relative cell migration (D).

4.3 Summary

- Harmine reduced the TWIST1 protein in the ONS-76, D283 and HD-MB03 cell lines and did not have any effect on gene expression, suggesting its effect occurs through a post-translational mechanism.
- Inhibition of TWIST1 using shRNA was successful in the ONS-76 and D283 cell lines with the shTWIST1.2 clone. All three clones failed to produce a persistent knock-down in the HD-MB03 cell line.
- The 3D transwell migration and invasion model was the most appropriate model for investigating the functional role of TWIST1 in medulloblastoma metastasis.
- Inhibition of TWIST1 either chemically or by shRNA significantly reduced cell migration in all three metastatic medulloblastoma cell lines, suggesting TWIST1 could be contributing to medulloblastoma metastasis.
- The next chapter will utilise the 3D migration and invasion model to investigate the relationship between TWIST1 and the multidrug transporter *ABCB1* to determine if both are contributing towards medulloblastoma metastasis.

Chapter 5. Investigating the relationship between TWIST1 and ABCB1 in medulloblastoma metastasis.

5.1 Introduction

The multidrug efflux pump ATP-binding cassette subfamily B member 1 (ABCB1) is expressed in many cancers (Christie et al., 2019, Wang et al., 2020a). Overexpression has been associated with multidrug resistance in cancer patients through ABCB1's ability to actively efflux chemotherapeutic agents out of cancerous cells, thus leading to treatment failure (Callaghan et al., 2008, Gottesman et al., 2016, Robey et al., 2018). In medulloblastoma patient tumour samples, ABCB1 was more likely to be expressed in high risk samples compared to standard risk samples ($p \leq 0.05$) (Othman et al., 2014).

Even though ABCB1 expression is associated with multidrug resistance, increasing evidence suggests that it is also involved in tumour migration and invasion. The mechanism of how ABCB1 contributes to tumour migration is not fully understood. Studies in breast cancer and melanoma have highlighted a correlation between the expression of ABCB1 and the adhesion molecule CD44, with both proteins co-localising within the cell membrane and resulting in increased invasive behaviour (Miletti-González et al., 2005, Colone et al., 2008). Analysis by our research group demonstrated that ABCB1 expression in a sub-population of ependymoma tumour cells correlated with a poorer event-free survival across all patients who received chemotherapy, and inhibition reduced cell migration ($p \leq 0.05$) (Sabnis et al., 2019). Furthermore, ABCB1 protein expression significantly correlated with metastasis in medulloblastoma patient tissue microarrays (TMAs), and inhibition with vardenafil in highly

expressing ABCB1 medulloblastoma cell lines significantly attenuated cell aggregation ($p \leq 0.05$) (Nasir and Cardall et al., 2021).

Several studies have investigated the relationship between the transcription factor TWIST1 and the multidrug transporter ABCB1 in drug resistance and metastasis. In colon cancer, TWIST1 expression was significantly increased in vincristine resistant cells, causing an increase in the promoter activity and expression levels of ABCB1, and silencing TWIST1 resulted in a decrease in ABCB1 expression (Liu et al., 2017). Moreover, in invasive breast cancer cells, Saxena *et al* reported three TWIST1 E-box binding sites in the promoter region of *ABCB1*, and reported that downregulating TWIST1 led to reduced ABCB1 expression (Saxena et al., 2011).

This thesis has started to reveal a role for TWIST1 in medulloblastoma metastasis. Therefore, this chapter focussed on investigating the physical and functional relationship between TWIST1 and ABCB1 in medulloblastoma metastasis. Chromatin immunoprecipitation (ChIP) analysis was performed to investigate the physical interaction between TWIST1 and *ABCB1*, and functional experiments were carried out using the 3D migration and invasion model utilised in Chapter 4 (4.2.2).

5.2 Results

5.2.1 ABCB1 expression in medulloblastoma cell lines

ABCB1 gene expression status was first analysed using the Cavalli dataset (Cavalli et al., 2017), and across a panel of medulloblastoma cell lines (DAOY, ONS-76, HD-MB03, D283, D425, D458, CHLA01 and CHLA01R), firstly, to assess if *ABCB1* expression in our cell lines were comparable to other patient datasets, and secondly, to see if there were any differences in expression between and within the subgroups. (Figure 5.1).

ABCB1 expression was observed in all of the medulloblastoma subgroups and cell lines. When comparing *ABCB1* expression between the subgroups (Figure 5.1A), the WNT subgroup displayed the lowest levels of *ABCB1*, and the SHH subgroup had the highest expression levels compared to the other subgroups ($p \leq 0.0001$), with no significant difference observed between Group 3 and Group 4. Remarkably, analysis with the available medulloblastoma cell lines revealed the exact same pattern as the Cavalli dataset (Figure 5.1B). Comparing *ABCB1* expression within the subgroups, revealed that subgroup specific cell lines showed similar expression levels and no significant differences were observed.

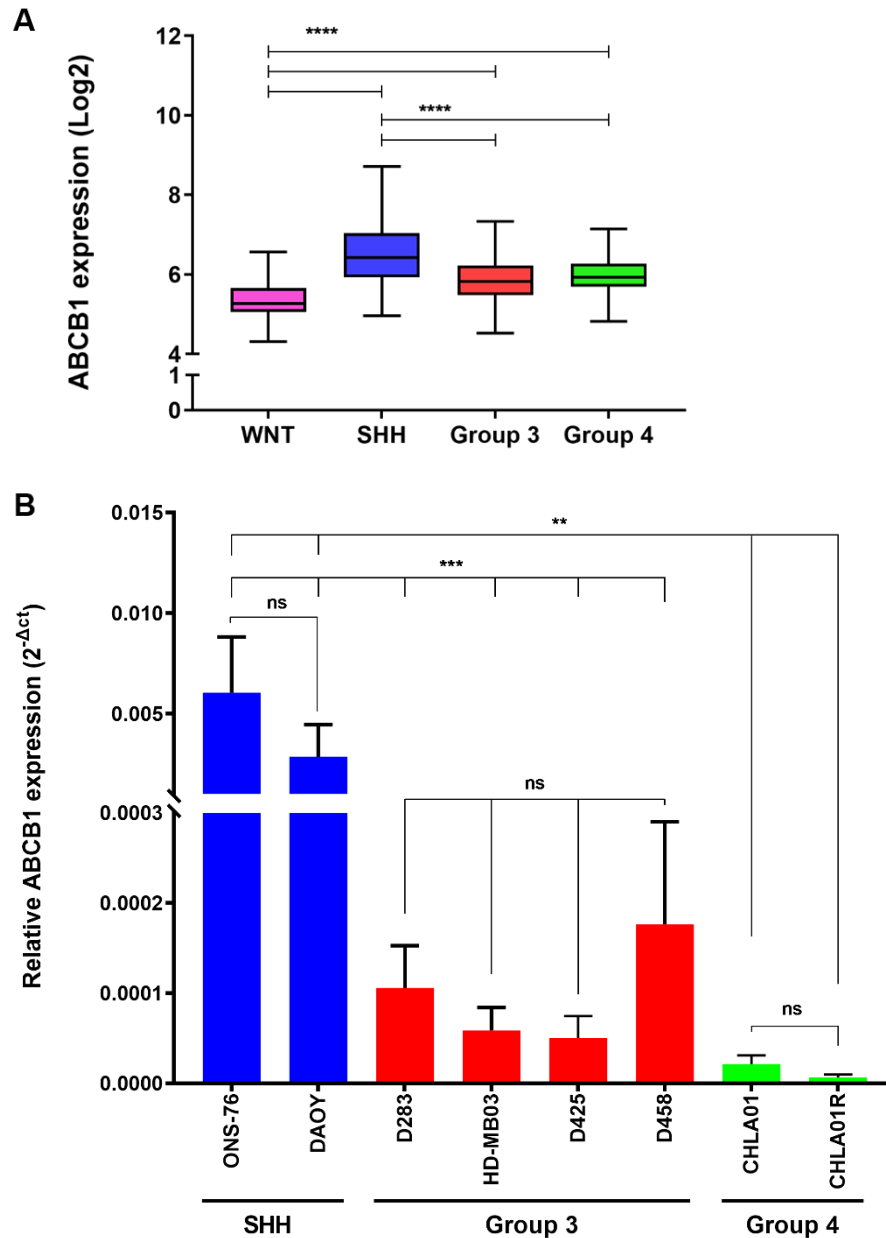


Figure 5.1 *ABCB1* gene expression in medulloblastoma

ABCB1 gene expression was assessed using the Cavalli dataset (A) and across a panel of medulloblastoma cell lines (B). The box plot diagram represents the minimum and maximum values, the median and the upper and lower quartiles (A). Gene expression was calculated relative to GAPDH using the $2^{-\Delta Ct}$ method. Error bars represent the mean $\pm$ SEM of $n = 3$ experiments each containing three replicates (B). Significant differences between and within subgroups were calculated using Kruskal-Wallis with Dunn's multiple comparison post-hoc test (A), one-way ANOVA analysis with Tukey's multiple comparison post-hoc test, or a Student's t-test (SHH and Group 4 (B)) (ns = not significant, $**p \leq 0.01$, $***p \leq 0.001$, $****p \leq 0.0001$).

5.2.2 Optimising ChIP cross-linking conditions in metastatic medulloblastoma cell lines

Three metastatic medulloblastoma cell lines were selected representing SHH (ONS-76) and Group 3 (D283 and HD-MB03) subgroups to investigate the relationship between TWIST1 and *ABCB1* in medulloblastoma metastasis. To determine if TWIST1 was controlling *ABCB1*, ChIP analysis was first performed to see if the TWIST1 protein could bind to the *ABCB1* promoter in the metastatic cell lines, using the method outlined in Figure 5.2. ChIP is a powerful tool that can capture specific protein-DNA interactions which naturally occur within a cell by immunoprecipitation with an antibody specific to a target protein (Orlando, 2000). The DNA fragments isolated in the complex with the target protein can be identified using a variety of techniques including PCR, DNA microarray and ChIP- sequencing (ChIP-Seq).

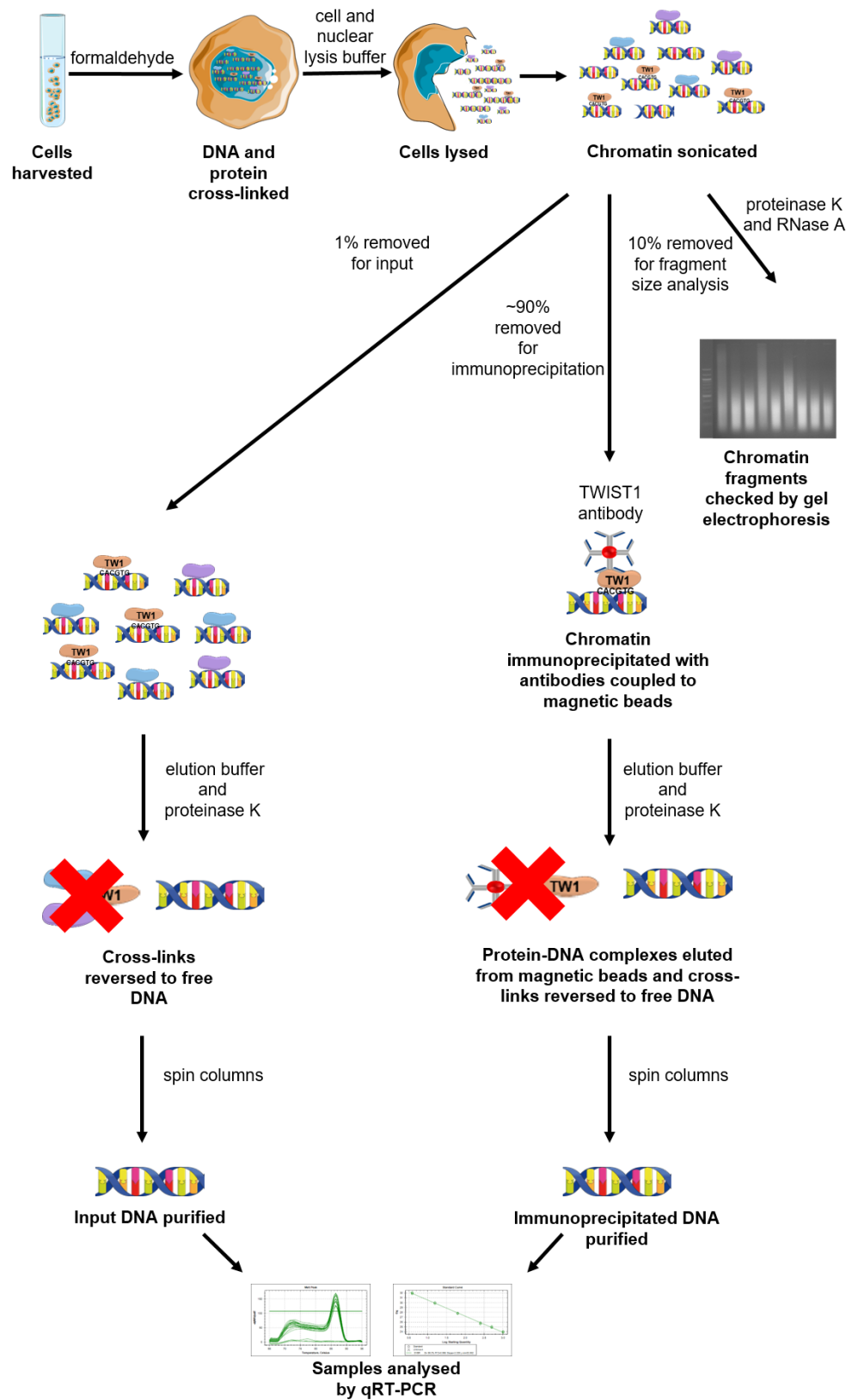
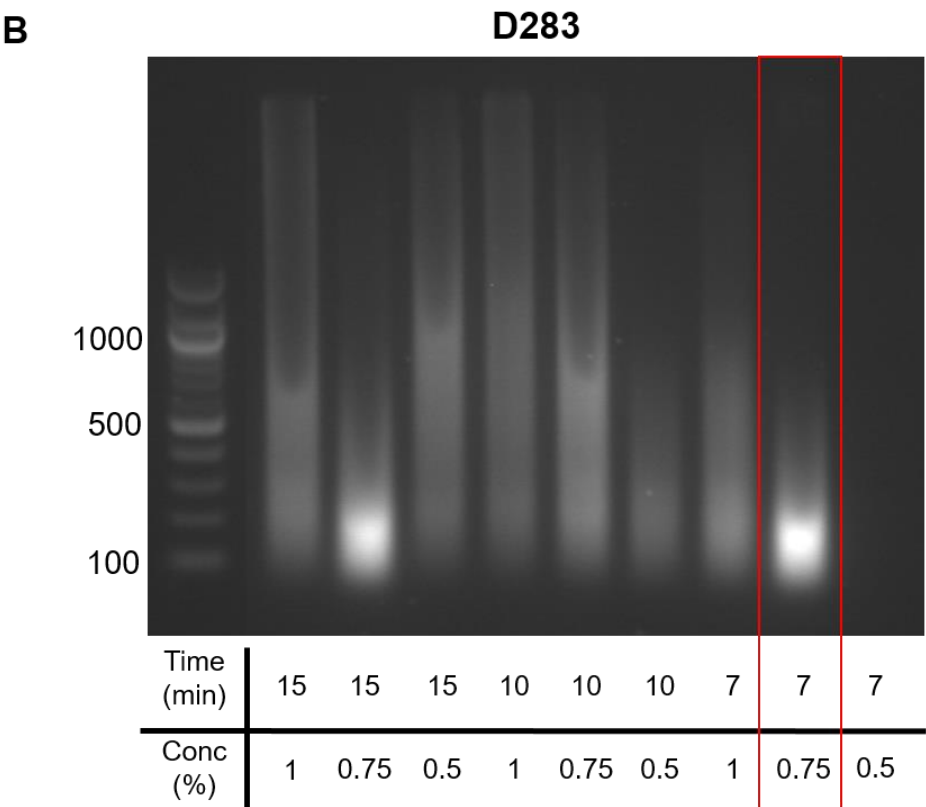
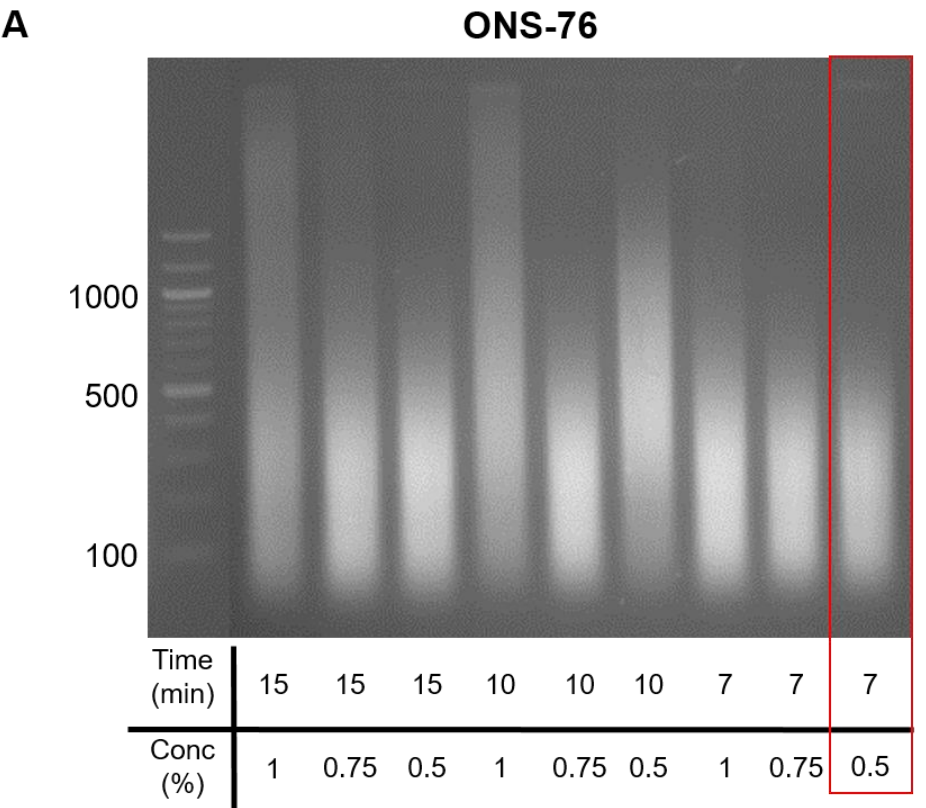


Figure 5.2 Schematic diagram describing ChIP.

Schematic describing the steps involved in ChIP. Cells were harvested and DNA and proteins were cross-linked with formaldehyde for a set time period. Cells were lysed to release nuclear contents and then sonicated. To check sonicated chromatin fragment sizes, proteins and RNA were removed and the DNA electrophoresed on an agarose gel. To immunoprecipitate the chromatin, the cross linked protein-DNA complexes were added to magnetic beads coupled with antibodies to TWIST1 (target protein), H3K4me3 (positive control) or IgG (negative control). The protein-DNA complexes were next eluted from the magnetic beads, cross-links were reversed and the DNA purified using spin columns. For the input, cross-linked protein-DNA were subjected to elution buffer and proteinase K to reverse the cross-links and remove proteins. The DNA was then purified and both the input and immunoprecipitations (IPs) were analysed by qRT-PCR.

To allow for efficient immunoprecipitation of the protein-DNA complexes with the TWIST1 antibody, cross-linking conditions needed to be optimised for all three cell lines to determine a condition which produced chromatin fragments with an optimal size of 200 - 500 base pairs (bp) and not exceeding 1000 bp (Arrigoni et al., 2015). Long periods of cross-linking could decrease sonication efficiency, destroy the epitope of the target protein and hence decrease antibody binding efficiency. However, inadequate cross-linking could result in loss of protein-DNA interactions during subsequent steps (Asp, 2018). Therefore, a time course experiment was carried out (7, 10 and 15 minutes) using a range of formaldehyde concentrations (0.5, 0.75 and 1 %). Agarose gel analysis was used to visualise the size of the chromatin fragments for each cross-linking condition (Figure 5.3). When deciding the optimal cross-linking condition it was important to choose a condition that did not show a smearing effect on the agarose gel as this was indicative of a greater distribution of larger fragment lengths, and instead to select a condition whereby the majority of the

chromatin fragments were concentrated within the optimal size ranges. The optimal cross-linking condition is highlighted with a red box in each figure.



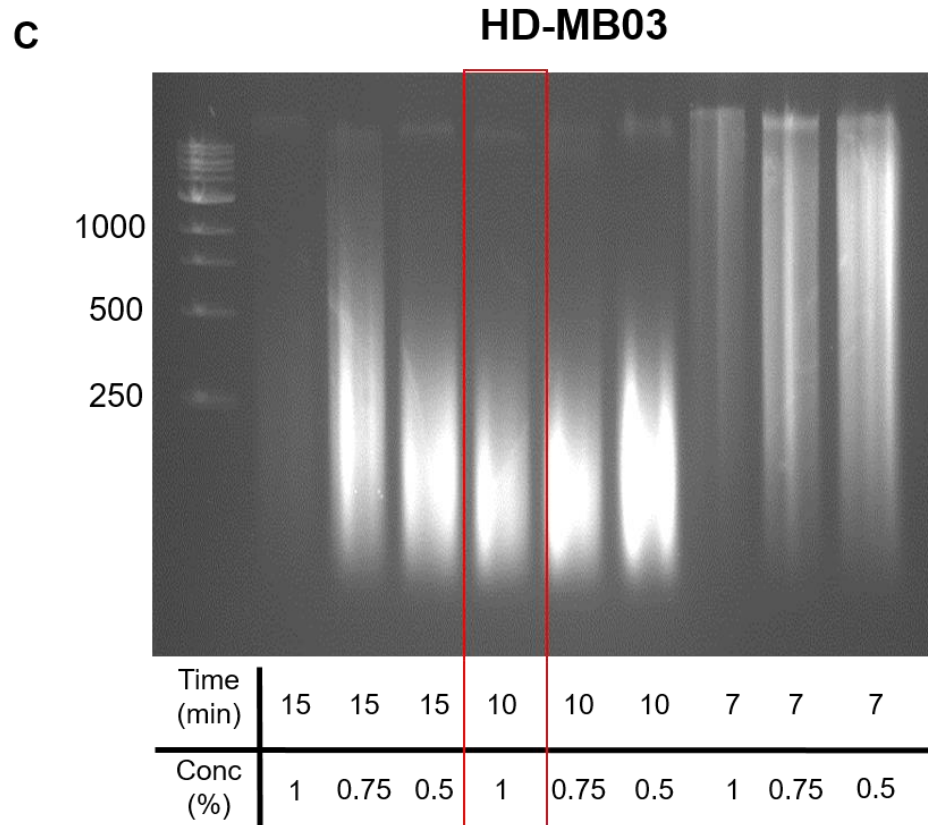


Figure 5.3 DNA sonication of ONS-76, D283 and HD-MB03 cell lines.

Sheared chromatin from cross-linked medulloblastoma cells were loaded onto a 1.5% agarose gel and electrophoresed at 120 V with a 100 bp ladder. ONS-76 (A), D283 (B) and HD-MB03 (C) cell lines.

5.2.3 Investigating functional TWIST1 binding sites in the *ABCB1* promoter.

TWIST1 is able to bind to the E-box motif CANNTG where 'N' represents any base. To find putative TWIST1 binding sites, the *ABCB1* promoter region (located within exon 3) was manually searched and four E-boxes were identified (Appendix 3, Appendix Figure 5A). To determine whether these four E-boxes were significant for TWIST1 binding a bioinformatics software tool 'Genomatix' was used (www.genomatix.de/), which utilises a large library of transcription factor binding sites to locate matches in DNA sequences and provides evidence of the binding sites using ChIP-Seq datasets. Genomatix only identified two of the four TWIST1 binding sites in the *ABCB1* promoter (Table 5.1; Appendix 3, Appendix Figure 5A). Primer design issues restricted research to the two shared CAGATG and CATCTG E-boxes to see which was crucial for TWIST1 binding.

Genomatix also identified two TWIST1 binding sites in the house-keeping gene *GAPDH*, with both sites recognised in ChIP-Seq data, and therefore *GAPDH* was used as a positive control. Manual searching of the *GAPDH* promoter region identified three E-boxes were (CAGGTG, CATCTG and CACGTG) (Table 5.1; Appendix 3, Appendix Figure 5B). Primers which covered all three binding sites were designed, however upon testing the efficiency of the primers, one set (covering the E-box CACGTG) had a low efficiency and was disregarded (Appendix 3, Appendix Figure 6). The primers covering the E-boxes CAGGTG and CATCTG were carried forward to determine which E-boxes were functional TWIST1 binding sites.

Previous work within the research group had identified that *SNAI2* gene expression was not observed or was below the limit of detection in some of the

medulloblastoma cell lines, despite predictions that TWIST1 binds to its promoter (Casas et al., 2011, Nasir and Cardall et al., 2021). Manual searching of the *SNAI2* promoter revealed one E-box (CAGATG) and primers were designed covering this E-box to see if *SNAI2* also could be used as a positive control.

Table 5.1 Putative TWIST1 E-box binding sites in the *ABCB1*, *GAPDH* and *SNAI2* promoters.

Gene	TWIST1 E-box binding motif location	Manually predicted	Genomatix predicted	Investigated using ChIP
<i>ABCB1</i>	-1267 CATTG -1261	Yes	No	No
	-1181 CAGATG -1175	Yes	Yes	Yes
	-929 CAGCTG -923	Yes	No	No
	-796 CATCTG -790	Yes	Yes	Yes
<i>GAPDH</i>	-266 CAGGTG -260	Yes	Yes	Yes
	231 CATCTG 237	Yes	No	Yes
	351 CACGTG 357	Yes	Yes	No
<i>SNAI2</i>	-594 CAGATG -588	Yes	No	Yes

The D283 cell line was used to determine which E-boxes were functionally active for each gene. Sheared chromatin was precipitated with either a TWIST1, H3K4me3 (Tri-methylation at Lysine 4 of the Histone 3 protein) IgG antibody. The H3Kme3 antibody was used as a positive control to check if the promoter region was active or not. The IgG antibody matched the species and class of the primary antibody (TWIST1) and was used as a negative control to determine non-specific binding. Once the chromatin had been immunoprecipitated the complexes were eluted (including the input), the cross-links reversed and the DNA purified. The IPs (TWIST1, H3K4me3 and IgG1) were analysed by qRT-PCR using the primer pairs covering the E-boxes identified in Table 5.1. The signal obtained for each IP was calculated against the total signal from the input sample and values expressed as a percentage (Figure 5.4).

Upon immunoprecipitation of chromatin with the H3K4me3 antibody, the resulting DNA was enriched when compared against the IgG negative control for all the three genes (*ABCB1*, *GAPDH* and *SNAI2*), demonstrating that the promoter regions were active. Interestingly, TWIST1 significantly bound to only one of the E-box binding sites (CATCTG) in the *ABCB1* promoter compared to the IgG negative control, and failed to show any binding to the other E-box binding site (CAGATG). TWIST1 bound to both E-boxes in the *GAPDH* promoter, with a greater enrichment observed with the CATCTG E-box. Notably, this E-box was not predicted by the Genomatix software. Enrichment of immunoprecipitated chromatin with the TWIST1 IP sample was equivalent to the levels of the IgG negative control for *SNAI2*, confirming that TWIST1 did not bind to the *SNAI2* E-box.

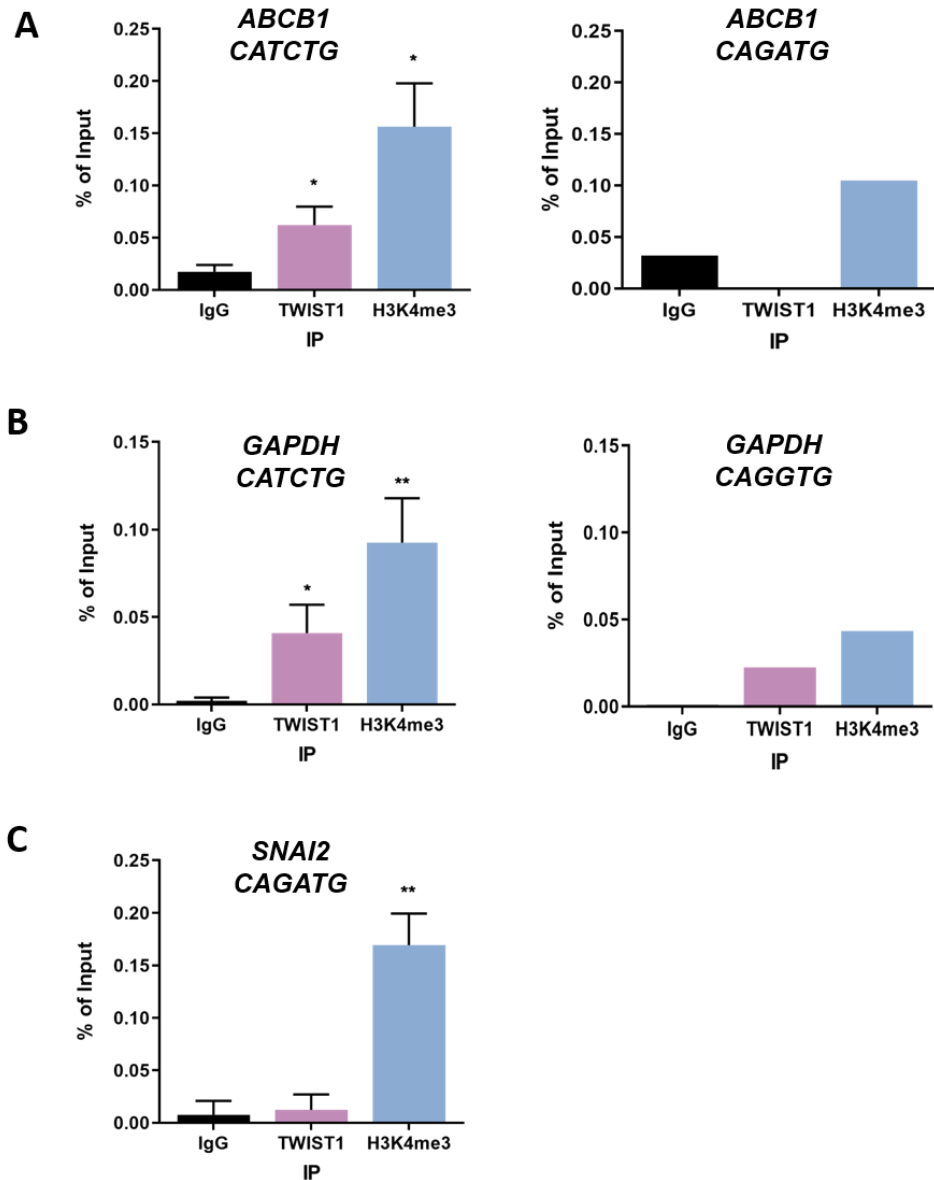


Figure 5.4 ChIP qRT-PCR analysis of the TWIST1 binding sites in the *ABCB1*, *GAPDH* and *SNAI2* promoters using the D283 cell line.

ChIP qRT-PCR analysis of TWIST1 binding to the E-boxes in the *ABCB1* promoter (A), the *GAPDH* promoter (B) and the *SNAI2* promoter (C) to determine which E-boxes were functionally active. Data was calculated and expressed as a % of the input. Error bars represent the mean $\pm$ SEM of $n \geq 1$ experiments each containing three qRT-PCR replicates. Significance differences were calculated only for experiments with more than one biological replicate using a paired Students t-test, comparing either the TWIST1 or H3K4me3 ChIP samples against the negative control (IgG1). (* $p \leq 0.05$, ** $p \leq 0.01$).

5.2.4 Investigating if TWIST1 binds to the ABCB1 promoter in metastatic medulloblastoma cell lines.

Having optimised the cross-linking and sonication conditions for the three cell lines, and established a functional TWIST1 binding site for *ABCB1* (Figure 5.5) and *GAPDH*, the final stage of the ChIP assay was to confirm if TWIST1 bound to the *ABCB1* promoter in all three cell lines. The method outlined in Figure 5.2 was followed using the relevant primers that covered the functional TWIST1 E-boxes in the *ABCB1* (CATCTG) and *GAPDH* (CATCTG) promoters, and the amount of DNA immunoprecipitated was calculated as a % of the input (Figure 5.6).

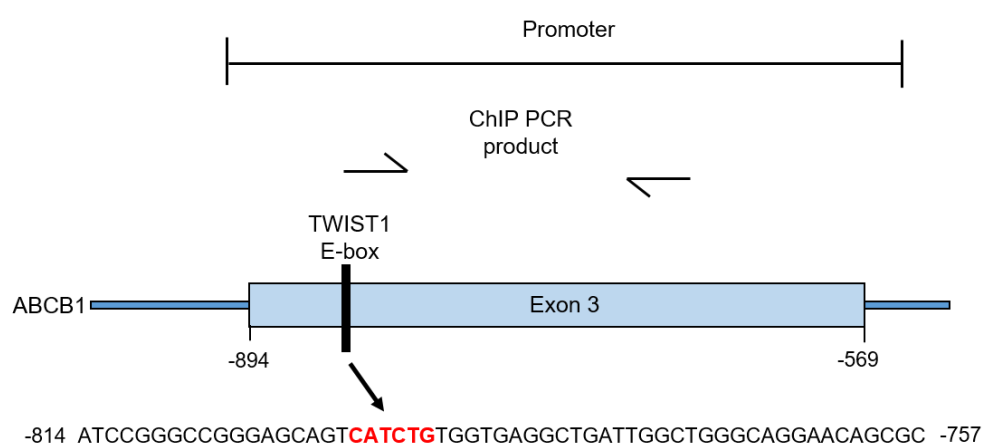


Figure 5.5 TWIST1 E-box binding site in the *ABCB1* promoter.

Schematic of the functional TWIST1 E-box binding site in the promoter region within exon 3 of the human *ABCB1* gene, detailing where the ChIP primers were designed.

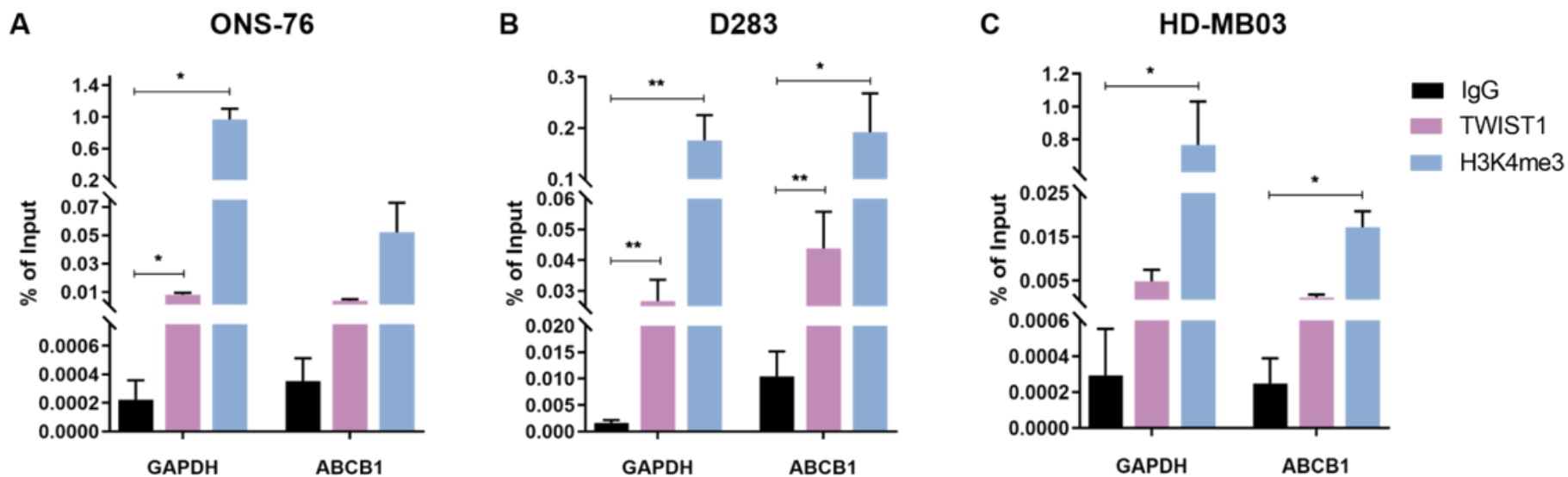


Figure 5.6 ChIP qRT-PCR analysis of TWIST1 binding sites in the *ABCB1* and *GAPDH* promoters using the ONS-76, D283 and HD-MB03 medulloblastoma cell lines.

ChIP qRT-PCR analysis to investigate TWIST1 binding to the E-boxes in the promoter regions of *ABCB1* and the positive control *GAPDH* with the metastatic medulloblastoma cell lines ONS-76 (A), D283 (B) and HD-MB03 (C). Data is calculated and expressed as a % of the input. Error bars represent the mean $\pm$ SEM of $n = 3$ experiments each containing three qRT-PCR replicates. Significant differences between the negative control IgG and either the TWIST1 or H3K4me3 IPs were calculated using a paired Student's t-test ($*p \leq 0.05$, $**p \leq 0.01$).

After analysing the ChIP qRT-PCR, enrichment of the immunoprecipitated chromatin using the TWIST1 antibody was observed in all three cells lines compared to IgG for both *ABCB1* and *GAPDH* (positive control). However, significant enrichment was only observed in the D283 cell line for both genes and in the ONS-76 cell line for *GAPDH*. Even though the ONS-76 and HD-MB03 cell lines did not show significant enrichment with the TWIST1 antibody, an 11 fold and 5 fold mean increase, respectively, was still observed. Active promoter regions were again observed with the H3K4me3 IP samples in both genes across all cell lines.

5.2.5 Inhibiting TWIST1 and ABCB1 in a 3D transwell migration and invasion model

The data provided supports a hypothesis that TWIST1 could activate and regulate *ABCB1* in metastatic medulloblastoma cells and that both could be key drivers in medulloblastoma metastasis. To further explore this hypothesis functional experiments were carried out:

1. Inhibiting TWIST1 using shRNA or harmine (a beta-carboline alkaloid) to see if *ABCB1* expression was affected.
2. Inhibiting *both* TWIST1 and *ABCB1* to see what effect this had on cell migration in the 3D transwell migration and invasion model used in Chapter 4.

ABCB1 gene expression was assessed in the TWIST1 knocked-down cell lines that had been described in Chapter 4 (4.2.1.2). As a transient TWIST1 KD was observed in the HD-MB03 cell line, with expression returning after two passages, it was decided to analyse *ABCB1* expression using cells stocks from early passages where the TWIST1 KD had been confirmed by qRT-PCR. *ABCB1* gene expression was also analysed in harmine treated cells. The ONS-76 and D283 cell lines were treated with a concentration of harmine (that had been identified in Chapter 4, Figure 4.2) that significantly reduced TWIST1 protein expression (20 μ M and 5 μ M, respectively). Whilst the HD-MB03 cell line was treated with increasing concentrations of harmine (1.5 μ M – 20 μ M). *ABCB1* gene expression was analysed relative to a house-keeping gene *HPRT* and normalised to the non-silencing or vehicle control using the $2^{-\Delta\Delta Ct}$ method (Figure 5.7A – C). *ABCB1* protein expression in the HD-MB03 cell line was also analysed by western blotting following treatment with harmine. Western blot images were quantified and *ABCB1* protein expression was calculated relative

to a house-keeping protein β -Actin and normalised to the vehicle control (Figure 5.7D and E).

ABCB1 gene and protein expression decreased in a dose-dependent manner in the harmine treated HD-MB03 cells, and when treating the cells with lower concentrations of harmine (1.5 μ M and 2.5 μ M) this still caused a small decrease in ABCB1 expression (Figure 5.7C-E). This is consistent with data from Figure 4.10 showing that concentrations of harmine that did not manifest as visibly reduced protein expression were still indicative of impaired TWIST1 function leading to a decrease in ABCB1 expression.

Furthermore, *ABCB1* gene expression decreased when TWIST1 was inhibited by shRNA or harmine, but significant differences were only observed with the ONS-76 cell line and with the D283 and HD-MB03 cells that were treated with a concentration of harmine that resulted in a significant reduction in TWIST1 protein expression. Interestingly, a greater reduction in *ABCB1* expression was observed in the Group 3 cell lines (D283 and HD-MB03) compared to the SHH cell line (ONS-76) following harmine treatment. This could be linked to the magnitude of reduction in TWIST1 protein levels after harmine treatment (ONS-76; 70 %, D283; 77% and HD-MB03; 93%), or *ABCB1* could be more dependent on TWIST1 in the Group 3 cell lines.

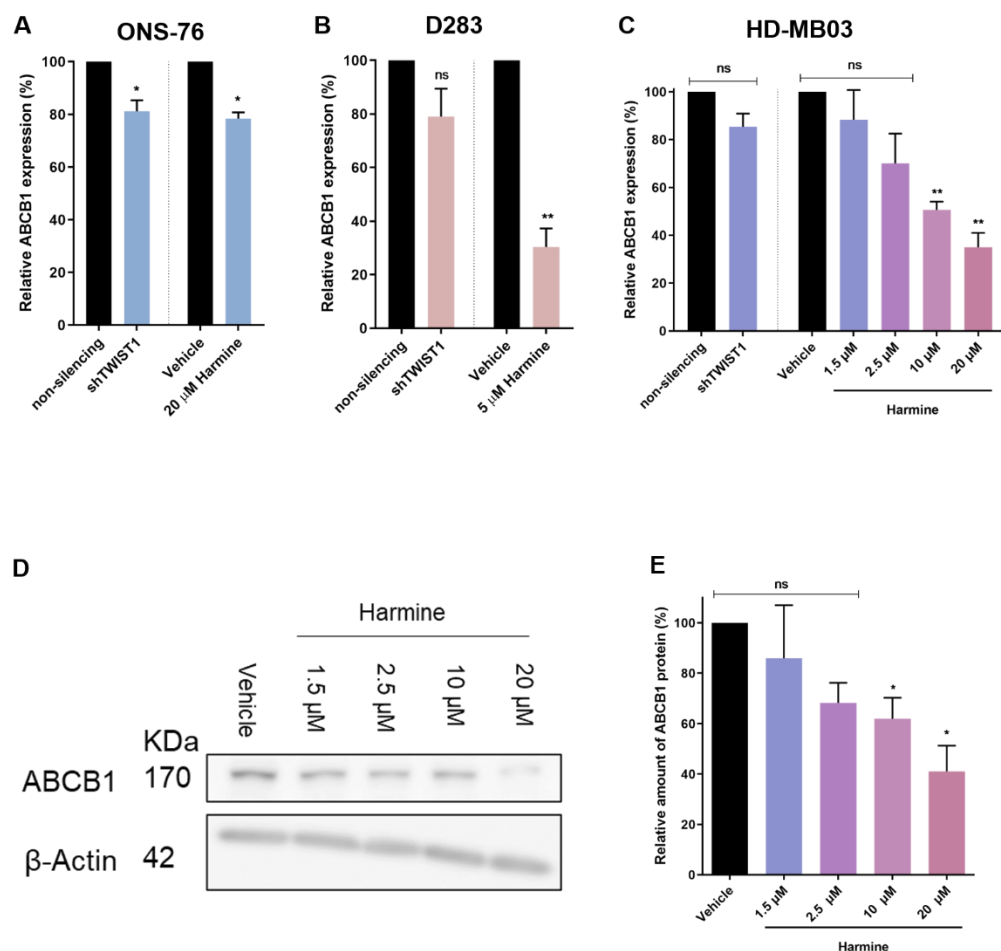


Figure 5.7 ABCB1 expression in the ONS-76, D283 and HD-MB03 cell lines following TWIST1 inhibition with either shRNA or harmine.

TWIST1 was inhibited with shRNA or treatment with harmine (ONS-76; 20 μ M (A), D283; 5 μ M (B) and HD-MB03; 1.5 μ M – 20 μ M (C)) for 72 hours. *ABCB1* gene expression was calculated relative to the house-keeping gene *HPRT* and normalised to the non-silencing or vehicle control using the $2^{-\Delta\Delta Ct}$ method and expressed as a percentage. ABCB1 protein expression was analysed after HD-MB03 cells were treated with increasing concentrations of harmine for 72 hours. 50 μ g of total protein was resolved on 12 % acrylamide gels by western blotting (D). Western blots are shown as split panels as the nitrocellulose was cut prior to incubation with anti-ABCB1 or anti- β -Actin antibodies. Protein expression was quantified using Fiji imaging software and normalised to the vehicle control (E). Error bars represent the mean $\pm$ SEM of $n = 3$ experiments with A - C containing three qRT-PCR replicates. Significant differences were calculated using either a paired Student's t-test (A and B) or one-way ANOVA analysis with Dunnett's multiple comparison post-hoc test comparing to the vehicle control (C and E) (* $p \leq 0.05$, ** $p \leq 0.01$).

ChIP analysis confirmed that TWIST1 could physically bind to the E-box located in the *ABCB1* promoter for the D283 cell line. Although not significant, the HD-MB03 and ONS-76 cell lines did show an enrichment in the amount of chromatin immunoprecipitated with the TWIST1 antibody. However, due to the suitability of the ONS-76 and HD-MB03 cell line for migration assays, these cell lines were used in the following section.

Vardenafil is known to specifically inhibit ABCB1's function without affecting gene or protein expression (Appendix 3, Appendix Figure 7). The 3D transwell migration and invasion assay that was investigated in Chapter 4 was used to see what effect inhibiting *both* TWIST1 and ABCB1 would have on medulloblastoma cell migration. Harmine and vardenafil were used independently and in combination to inhibit TWIST1 and ABCB1's function in the ONS-76 and HD-MB03 cell lines. The cells were pre-treated with harmine (ONS-76; 20 μ M and HD-MB03; 1.5 μ M or 20 μ M) for 72 hours as this time period allowed for harmine to impact TWIST1 protein and function. When the cells were seeded onto the transwell inserts, fresh medium containing 10 μ M of vardenafil was added as vardenafil is a reversible inhibitor. Cell migration was analysed using the PrestoBlue™ assay and the number of migrating cells was calculated relative to a standard curve of known cell numbers. The data was normalised to the vehicle control (Figure 5.8).

Neither harmine nor vardenafil, alone or in combination, caused toxicity to the cells. Inhibition of TWIST1 or ABCB1 with harmine or vardenafil respectively, reduced cell migration compared to the vehicle control, indicating that they both could be playing a role in medulloblastoma cell migration. Whilst similar reductions in cell migration were observed with harmine treatment (20 μ M) for both cell lines (30 % and 32 %), vardenafil treatment resulted in only a 14 % and 22 % reduction. In addition, the greater reduction in cell migration with

harmine treatment (20 μ M) compared to vardenafil treatment for both cell lines suggests that ABCB1 might not be the only gene TWIST1 is controlling during cell migration.

Interestingly, when vardenafil was used in combination with 20 μ M of harmine (a concentration which causes degradation of TWIST1 protein), an additive effect was observed in the HD-MB03 cell line but not with the ONS-76 cell line. Similar to Figure 5.7 analysis, this could indicate that ABCB1 regulation is less dependent on TWIST1 in the ONS-76 cell line. Overall the data presented demonstrates that TWIST1 is effecting ABCB1 expression and both are contributing to medulloblastoma metastasis.

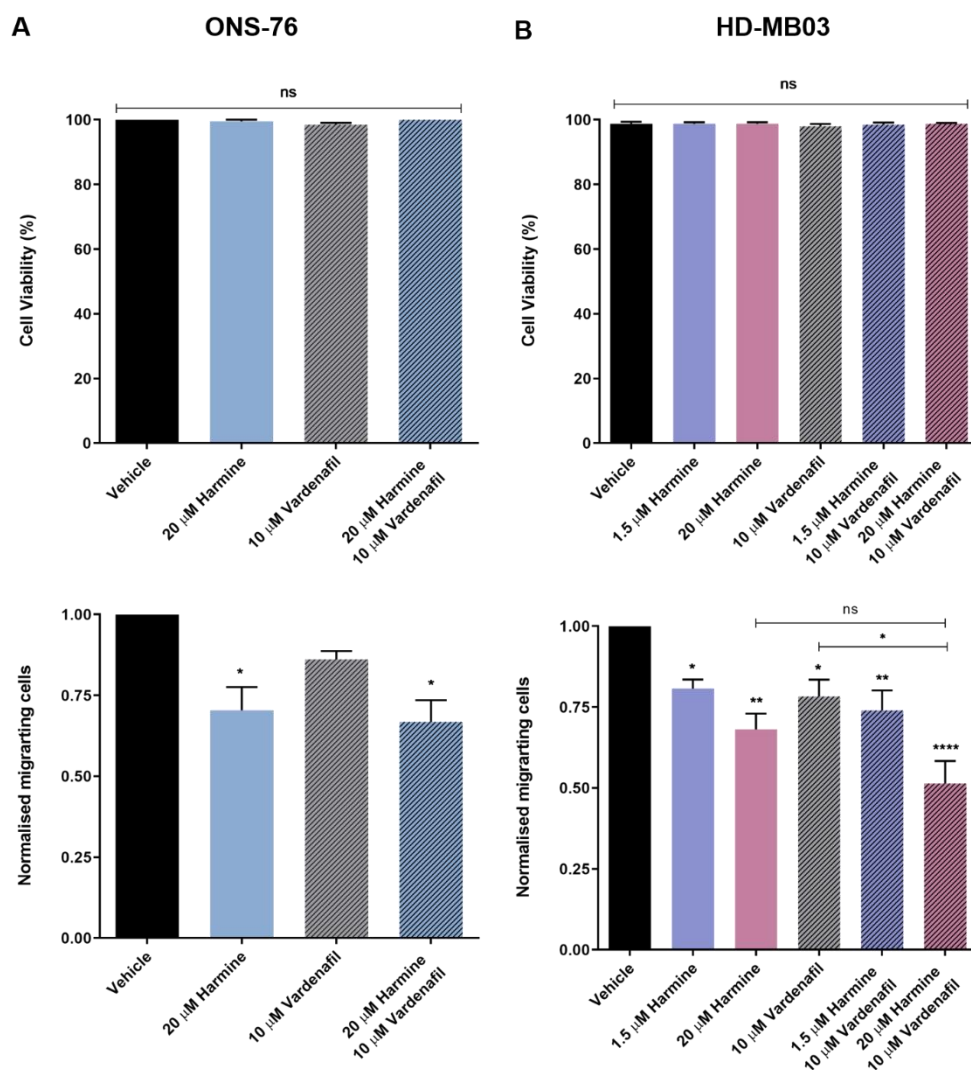


Figure 5.8 Cell viability and migration of ONS-76 and HD-MB03 cells following TWIST1 and ABCB1 inhibition using a 3D transwell model.

Cells were treated with harmine (ONS-76; 20 μ M (A) and HD-MB03; 1.5 μ M or 20 μ M (B)) and/ or vardenafil (10 μ M) and cell viability was assessed before seeding cells onto a collagen IV and laminin coated transwell insert for 48 hours. Migrated cells were subjected to a PrestoBlue™ assay to determine the relative fluorescence units (RFU) and migrated cell numbers calculated against a standard curve of known numbers and normalised relative to the vehicle control. Error bars represent the mean $\pm$ SEM of $n = 2$ (ONS-76) and $n = 4$ (HD-MB03) experiments each containing three replicates. Significant differences were calculated using a one-way ANOVA with Dunnett's multiple comparison post-hoc test comparing to the vehicle control and between inhibition treatment (* $p < 0.05$, ** $p < 0.01$ and **** $p < 0.0001$).

5.3 Summary

- *ABCB1* is expressed across all medulloblastoma cell lines and expression patterns are comparable to the Cavalli dataset. Expression is significantly increased in the SHH subgroup compared to the other subgroups.
- Functional TWIST1 E-boxes are located within the promoter regions of *ABCB1* and *GAPDH*, but a functional E-box was not observed in the *SNAI2* promoter.
- TWIST1 could bind to the E-boxes located in the *ABCB1* and *GAPDH* promoters in the three metastatic cell lines (ONS-76, D283 and HD-MB03), but this was only significant in the D283 cell line.
- Inhibiting TWIST1 with shRNA or harmine caused a reduction in *ABCB1* expression suggesting TWIST1 could regulate *ABCB1*.
- Inhibition of TWIST1 and/ or *ABCB1* with harmine and/ or vardenafil, reduced cell migration in the 3D migration and invasion model, indicating that both might be involved in medulloblastoma metastasis.
- The next chapter will investigate other TWIST1 downstream targets to gain an understanding of the TWIST1 regulatory network, and to see gene targets and pathways differ or are the same for the SHH and Group 3 subgroups.

Chapter 6. Identifying TWIST1 downstream target genes.

6.1 Introduction

TWIST1 is a major EMT transcription factor and is known to orchestrate the EMT process during tumour progression by directly repressing and activating genes involved in cellular adhesion, polarity and cytoskeleton reorganisation (Zhu et al., 2016, Goossens et al., 2017, Huilgol et al., 2019). However, as mentioned previously, medulloblastoma tumours do not originate from an epithelial cell of origin, and our studies instead points towards an EMT-like pathway (Nasir and Cardall et al., 2021).

We have so far that TWIST1 plays a role in driving medulloblastoma metastasis, in a manner that is partially mediated through regulation of expression of the multidrug transporter *ABCB1*. Target-specific ChIP analysis confirmed TWIST1 binding to the *ABCB1* promoter region in medulloblastoma metastatic cell lines (ONS-76, D283 and HD-MB03). Whilst ChIP allowed for the study of a single target, the wider targets of TWIST1 in regulation of medulloblastoma invasion and metastasis remain unknown.

To address this knowledge gap, ChIP-Sequencing (ChIP-Seq) analysis was performed to identify putative TWIST1 downstream target genes in the three medulloblastoma metastatic cell lines across two different subgroups (SHH; ONS-76 and Group 3; D283 and HD-MB03). The genes could then be used to advance our understanding of the TWIST1 transcriptional regulatory network, and identify TWIST1 regulated pathways that are associated with SHH and Group 3 medulloblastoma metastasis.

6.2 Results

6.2.1 ChIP sample preparation

ChIP-Seq analysis involves many steps as described in Figure 6.1. To begin this process, two biological replicates (TWIST1 IPs) and one control (input) sample were prepared for each cell line (ONS-76, D283 and HD-MB03) following the ChIP method described in section 2.5.2. Samples were then sent to DeepSeq (an in-house sequencing facility) for library preparation, sequencing and bioinformatics analysis.

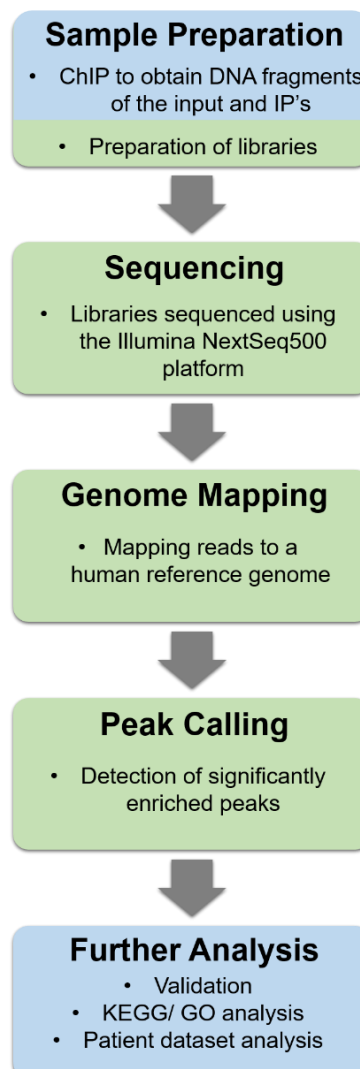


Figure 6.1 ChIP-Seq work flow.

Workflow describing the steps involved in ChIP-Seq analysis. Blue boxes represent the work carried out by myself, whilst green boxes represent the work performed by DeepSeq.

After library preparation they were quantified, whereby the fragment-size distribution and concentration of each library was assessed using an Agilent TapeStation to determine the volume required to sequence the libraries (Table 6.1 and Figure 6.2).

Table 6.1 Quantification of prepared input and TWIST1 IP libraries.

The molarity (nM) of each library was calculated using the following equation: $(1000 * \text{library concentration ng/}\mu\text{L}) / (0.66 (\text{approx. MW of a nucleotide pair}) * \text{mean fragment size})$.

Sample		Library Concentration (ng/ μ L)	Mean Fragment size (bp)	Molarity of Library (nM)
ONS-76	Input	11	516	32.3
	IP 1	1.13	506	3.38
	IP 2	2.62	442	8.98
D283	Input	27.6	442	94.6
	IP 1	23.4	462	76.74
	IP 2	9.62	535	13.76
HD-MB03	Input	33.4	493	71.9
	IP 1	0.596	411	2.19
	IP 2	6.78	424	24.23

As mentioned in Chapter 5, the optimal size of DNA fragments for ChIP analysis should be between 200 – 500 base pairs (bp) and not exceeding 1000 bp. The mean size of the DNA-fragments across all samples ranged from 411 to 535 bp (Table 6.1), however, adapters (which are approximately 120 bp long) were added to each DNA-fragment during library preparation. With these subtracted from the above values, the mean fragment size of each sample fell within the optimal range. When assessing the DNA-fragment peaks (Figure 6.2), the height of the ONS-76 and HD-MB03 replicate 1 libraries relative to the electronic marker were much lower compared to the other libraries, which is reflected in the low library concentrations observed in Table 6.1.

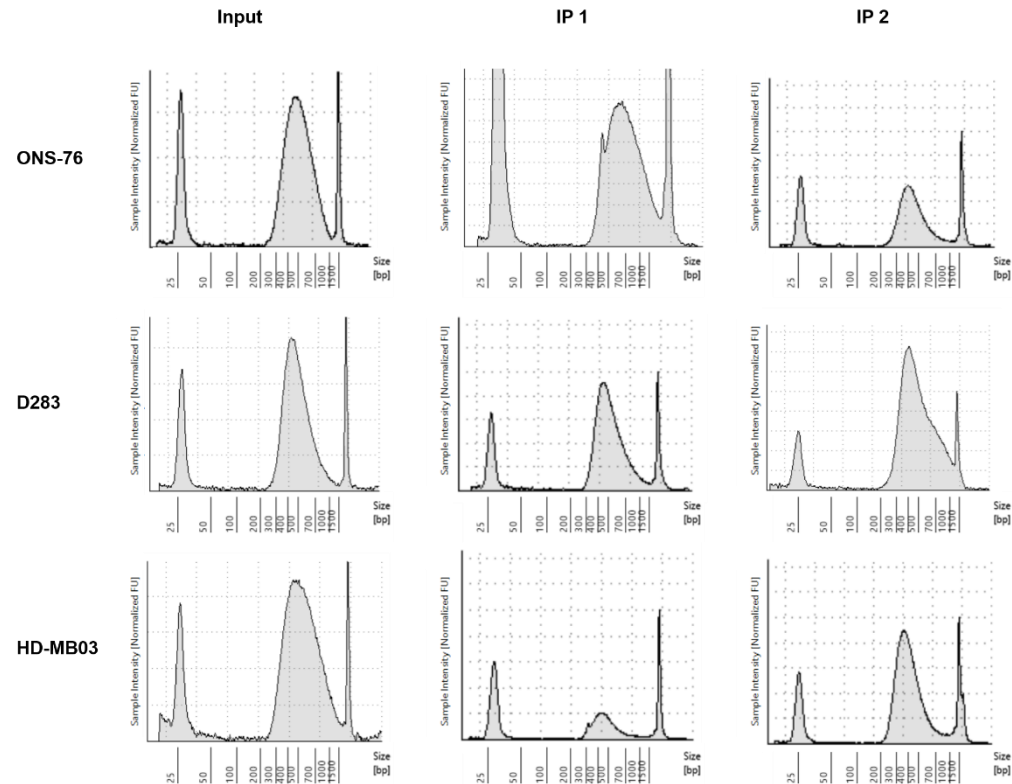


Figure 6.2 DNA fragment profiles of input and IP libraries.

Fragment-size distribution (bp) of the libraries prepared from the input and IPs of the three medulloblastoma cell lines (ONS-76, D283 and HD-MB03). Libraries were assessed using the Agilent TapeStation, with the lower (25 bp) and upper (1500 bp) markers of the electronic ladder included on each graph. Final libraries include the sample and dual indexed adapters.

6.2.2 ChIP-Sequencing and mapping

The libraries were sequenced to generate over 30 million pairs of 75bp paired-end reads per input sample and over 20 million pairs of 75bp paired-end reads per IP sample. After the sequencing, the adaptors were trimmed from the raw reads which were then mapped onto the human genome GRCh38 (GCA_000001405.28). The uniquely and correctly aligned reads were extracted and duplicate reads were removed. Duplicate reads refer to the reads that have been mapped more than once to the same genomic location and strand. It is common for ChIP-Seq data to contain duplicates due to the number of PCR cycles performed during library amplification. An overview of the number and percentage of paired reads after each step is detailed in Table 6.2 and the percentage of duplicate reads is summarised in Figure 6.3.

After initial sequencing, some of the samples had a low number of trimmed reads, and therefore, it was decided to increase the number of reads (samples highlighted in blue in Table 6.2). The percentage of uniquely mapped reads to the reference genome ranged from 38 % to 85 %. Even though for some of the samples this percentage might seem low, on average 40 % of reads fail to align to the corresponding genome, with some datasets seeing as much as 80 % of reads failing to align (Ouma et al., 2015). After removing duplicates the percentage of total reads remaining ranged from 25 % to 77 % (Table 6.2). All three sequenced input libraries had a small percentage of duplicates (approximately less than 5 %), whereas, the ONS-76 and HD-MB03 replicate 1 samples contained the highest percentage of duplicates (Figure 6.3). This could be due to a low concentration of starting material leading to over amplification of the sample before sequencing. This was evident after the library preparation, as both samples had the lowest library concentrations (Table 6.1).

Table 6.2 Overview of the number and percentage of paired reads remaining after raw reads were trimmed, mapped onto the human genome and duplicates removed.

Samples which underwent additional sequencing are highlighted in blue.

Sample		Trimmed Reads	Mapped Reads		Uniquely Mapped Reads		Reads after removing duplicates		
			Number	Percentage (%)	Number	Percentage (%)	Duplicates	Number	Percentage (%)
ONS-76	Input	35,756,430	22,891,266	64.02	13,925,718	38.95	229,948	13,695,770	38.3
	Replicate 1	22,095,783	19,813,289	89.67	14,130,315	63.95	8,832,431	5,597,881	25.33
	Replicate 2	28,786,344	24,333,097	84.53	13,505,147	46.92	2,519,309	10,985,838	38.16
D283	Input	32,121,805	28,353,917	88.27	20,839,650	64.88	636,018	20,203,632	62.9
	Replicate 1	29,813,772	26,474,630	88.8	15,810,324	53.03	575,539	15,234,785	51.1
	Replicate 2	17,895,997	15,889,856	88.79	8,361,122	46.72	1,012,507	7,348,615	41.06
HD-MB03	Input	34,387,801	31,131,276	90.53	21,959,986	63.86	655,465	21,304,521	61.95
	Replicate 1	24,865,161	23,064,923	92.76	19,001,199	76.42	9,855,449	9,145,750	36.78
	Replicate 2	33,985,993	32,303,686	95.05	29,118,584	85.68	2,616,890	26,501,694	77.98

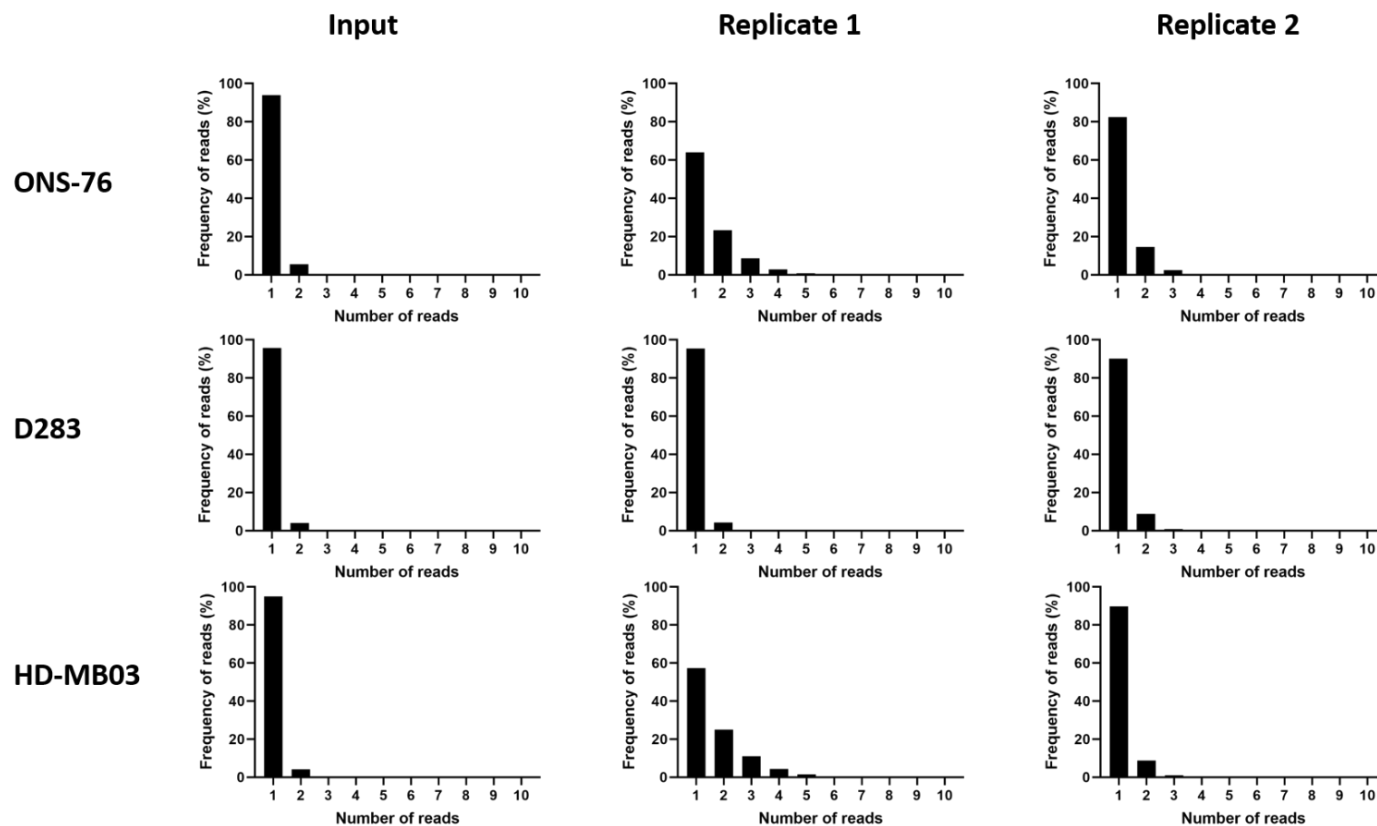


Figure 6.3 Frequency (%) of the number of reads from each sequenced library.

Frequency (%) of the number of reads obtained during ChIP sequencing for the input and IP's (biological replicates) for the three medulloblastoma cell lines (ONS-76, D283 and HD-MB03).

6.2.3 Peak calling

Next, peak calling was performed to identify TWIST1 binding sites that had aligned to the reference genome. These were identified using the *MACS2* (Model-based Analysis of ChIP-Seq) software. All samples were scanned to find highly significant enriched peaks. The peaks on the 5' ends of a selected fragment were separated into positive and negative reads (centred around the binding site) and aligned to the reference genome by the midpoint between their centres (Figure 6.4). The distribution of the peaks was assessed using statistical measures and enriched regions were compared against background (input) to determine if the site of enrichment was likely to be a true binding site.

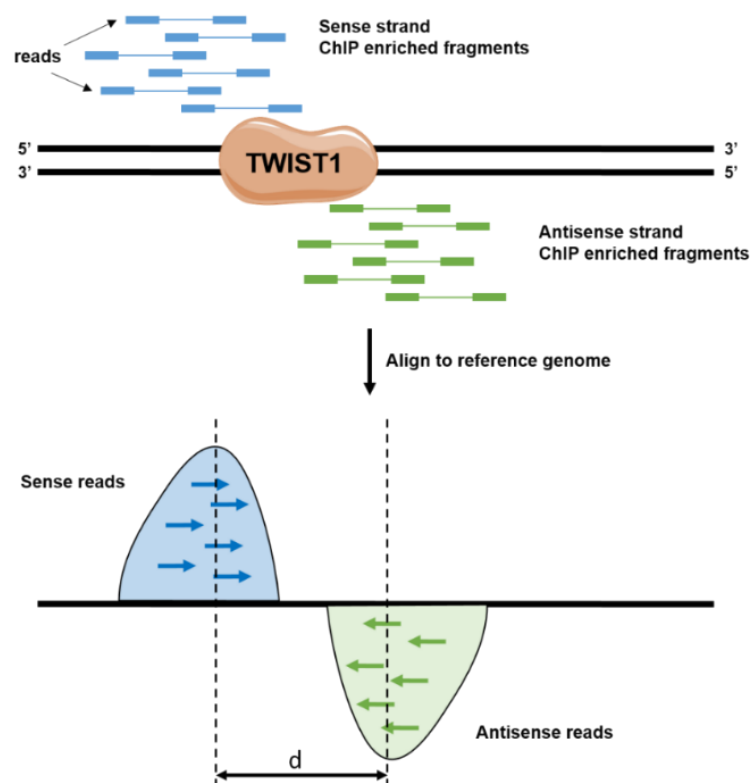


Figure 6.4 Schematic describing the peak calling.

Schematic describing the peak calling process. Peaks are formed from sequenced reads on the 5' positive (sense) and negative (antisense) strands, which are centred around the TWIST1 transcription factor. Sequenced reads are aligned to a referenced genome. The separation between the peaks (d) corresponds to the average sequenced fragment length.

The next stage was to annotate the TWIST1 binding sites and identify shared peaks amongst biological replicates to see if these peaks overlapped between the two subgroups (Figure 6.5). The proportion of shared peaks between the HD-MB03 biological replicates was vastly smaller (2 % and 7 % for replicate 1 and 2, respectively) compared to the ONS-76 (36 % and 76 %, respectively) and D283 replicates (25 % and 92 %, respectively). This could be because of a couple of factors: firstly, the low starting concentration of the HD-MB03 replicate 1 sample resulted in the over amplification of DNA fragments during library preparation, or secondly, the increased amount of sequencing with the HD-MB03 replicate 2 sample, with both factors reducing the complexity of the libraries. The overall number of peaks in the HD-MB03 replicates were lower compared to the other cell line replicates. A similar study on the binding regions of five transcriptions factors (that used the same *MACS* software mentioned above) identified 60,000 - 120,000 ChIP-Seq peaks (Matsuda et al., 2017), suggesting that the HD-MB03 data could provide less meaningful information.

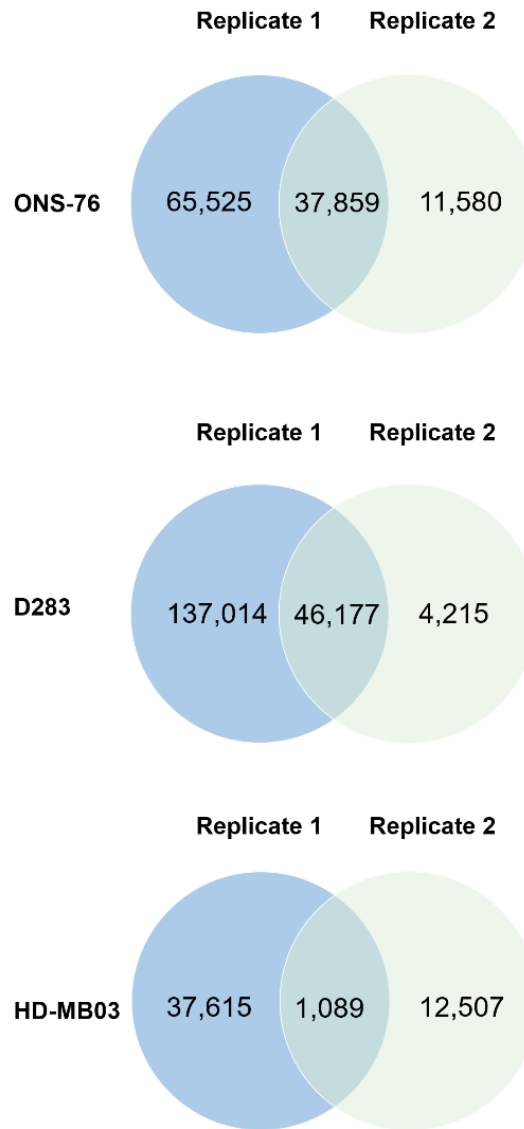


Figure 6.5 Number of shared peaks between biological replicates for all three cell lines.

The Venn diagrams represent the number of shared peaks (TWIST1 binding sites) between the two biological replicates in the three medulloblastoma cell lines (ONS-76, D283 and HD-MB03).

As mentioned at the start of this chapter, the aim was to identify similar and unique pathways between the two subgroups. To achieve this the shared peaks, and subsequent target genes, between the two subgroups (SHH; ONS-76 and Group 3; D283 and HD-MB03) needed to be identified. However, the number of shared peaks in the HD-MB03 replicates (1,089) was a lot lower

compared to the D283 replicates (46,177) and when combined to obtain the Group 3 shared peaks it resulted in a very small number of peaks (619). Even though the HD-MB03 cell line did share over 50 % of those peaks with the other Group 3 cell line (D283), it was decided to concentrate the comparison on the ONS-76 (SHH) and D283 (Group 3) cell lines (Figure 6.6).

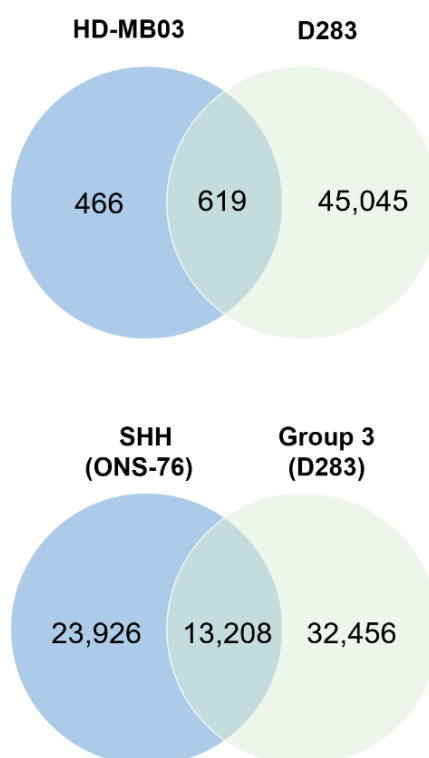


Figure 6.6 Number of shared peaks between the different cell lines.

Venn diagrams represent the number of shared peaks between the different Group 3 cell lines (HD-MB03 and D283) and subgroups (SHH; ONS-76 and Group 3; D283).

ChIP-Seq of transcription factors allows for the mapping of target regions in both the promoter region (located near the transcription start site (TSS)) and at gene-distal regions, including enhancers (which can be located thousands of base pairs upstream or downstream of the TSS) (Devailly and Joshi, 2018, Lambert et al., 2018). The location of the TWIST1 binding peaks relative to the TSS and the nearest genomic feature were analysed in the ONS-76 (SHH) and D283 (Group 3) cell lines and for the shared peaks between both subgroups (Figure 6.7).

The analysis revealed that for the ONS-76 cell line there was an enrichment of TWIST1 binding sites located downstream of the TSS rather than upstream, whereas, binding sites for the D283 cell line were mainly enriched around the TSS. The shared TWIST1 binding sites for both subgroups revealed enrichment downstream of the TSS. This suggests, that TWIST1 tends to bind downstream of gene regulatory sequences. This was supported by the distribution of the peaks over the different genomic features, which were mainly located at intronic and distal intergenic regions, with smaller proportions observed in the 5' and 3' UTR and promoter regions. This data is comparable with other TWIST1 ChIP-Seq datasets, which demonstrated that only 5 % of binding occurred within the promoter region and TWIST1-binding peaks were enriched at distal intergenic and intronic regions (Dozmorov et al., 2015, Bildsoe et al., 2016).

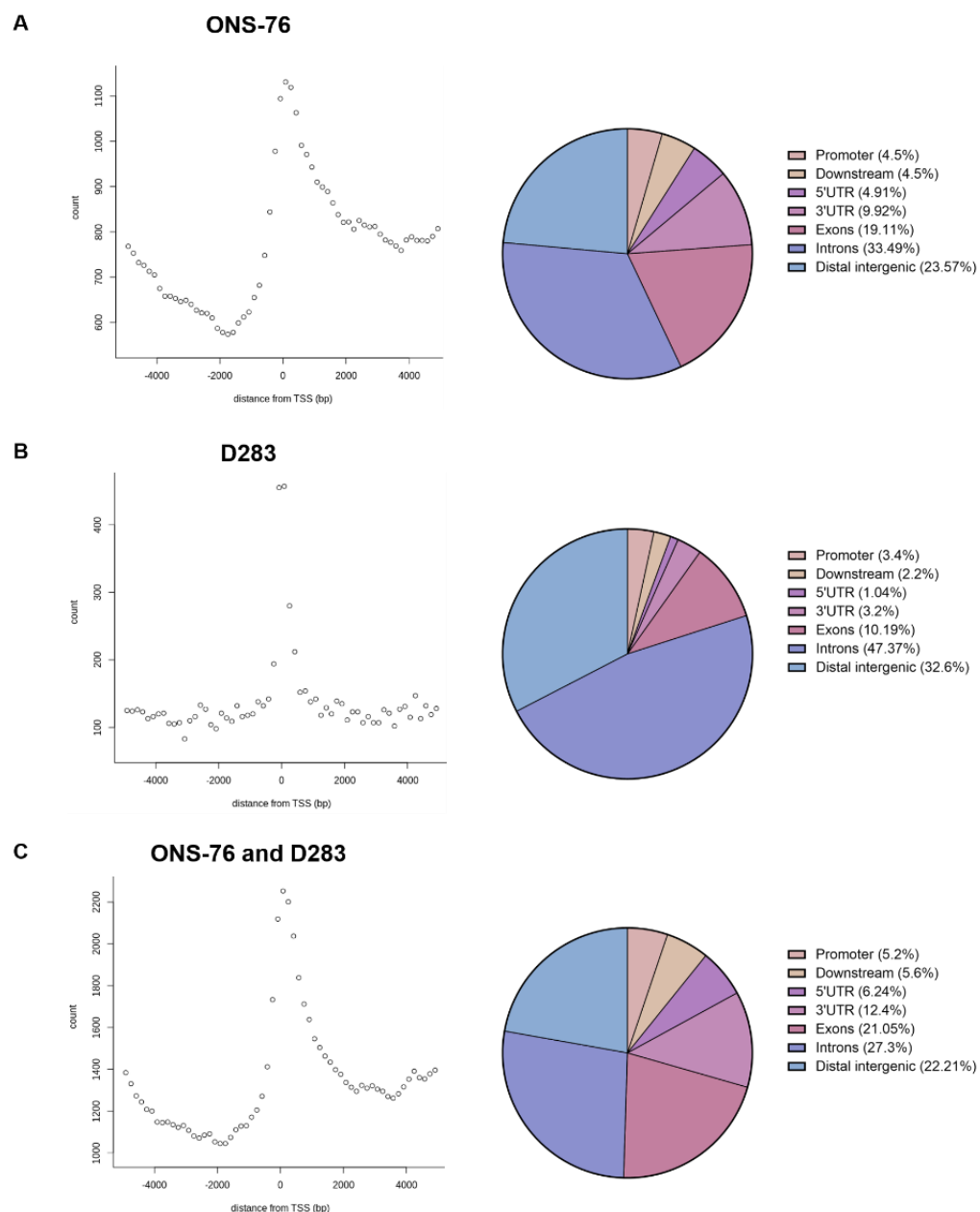


Figure 6.7 Distribution of ONS-76 and D283 peaks over different genomic features.

The distribution of TWIST1 binding peaks within 5 kb of the TSS and relative to the nearest genomic features for the ONS-76 (A), D283 (B) and shared peaks in both cell lines (C).

6.2.4 ChIP-Seq Validation

6.2.4.1 TWIST1 downstream targets

The analysis so far had revealed that the ChIP-Seq data was comparable with other datasets, in particular, with the expected TWIST1 genomic binding regions. To begin validating the dataset, TWIST1 downstream targets were selected and their expression was analysed in the ONS-76 and D283 medulloblastoma cell lines by comparing gene expression levels in cells where TWIST1 had been inhibited by shRNA or harmine (using methods optimised in Chapter 4) against control cells. If TWIST1 directly regulated the expression of the target genes, as predicted by ChIP-Seq, then upon reduction of TWIST1 gene or protein expression the expression of the target should have reduced.

Due to time constraints and the large number of TWIST1 target genes, we elected to investigate genes where PCR primers had already been designed and optimised within our research group. Table 6.3 outlined the genes selected for ChIP-Seq validation and the corresponding subgroup(s) they had been identified in during the peak calling process. *IGF1R* was the only gene to be identified solely within the Group 3 subgroup, all remaining genes were common across both subgroups.

Cell pellets were collected from TWIST1 KD cells and harmine treated cells, RNA was extracted and cDNA synthesised for subsequent qRT-PCR analysis. Gene expression was calculated relative to the house-keeping gene *HPRT* and normalised to either the non-targeting or vehicle control for the TWIST1 KD cells or harmine treated cells, respectively, using the $2^{-\Delta\Delta Ct}$ method (Figure 6.8).

Table 6.3 Genes selected for ChIP-Seq validation

Gene	SHH only	Group 3 only	Both
Insulin-like growth factor 1 receptor (<i>IGF1R</i>)	x	✓	x
Basigin (<i>BSG</i>)	x	x	✓
F-Box and Leucine Rich Repeat Protein 17 (<i>FBXL17</i>)	x	x	✓
Hyaluronidase-2 (<i>HYAL2</i>)	x	x	✓
Insulin-like growth factor 1 (<i>IGF1</i>)	x	x	✓
Sortilin Related VPS10 domain containing receptor 2 (<i>SORCS2</i>)	x	x	✓

The data presented in Figure 6.8 revealed that the decrease in gene expression of the target genes after TWIST1 inhibition was in agreement with Table 6.3. *IGF1R* was identified in Group 3 and its gene expression was significantly decreased only in the Group 3 cell line upon TWIST1 inhibition. The remaining genes that were analysed appeared in both subgroups and remarkably all showed a decrease in gene expression, with the biggest decreases observed with *IGF1* and *IGF1R* for the ONS-76 and D283 cell lines, respectively. Interestingly, for the majority of genes, treatment with harmine resulted in a bigger decrease in gene expression compared to TWIST1 KD cells. This could be because harmine reduced TWIST1 expression to a greater extent than shRNA (ONS-76; 70 % and D283; 77 % compared to ONS-76; 65 % and D283; 60 %).

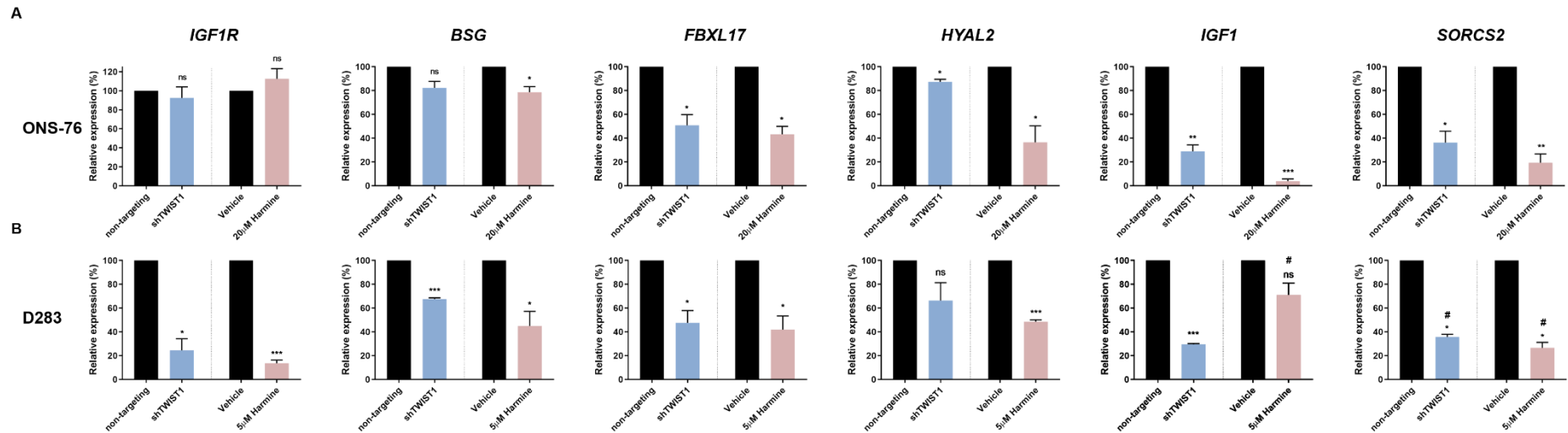


Figure 6.8 Gene expression of TWIST1 target genes following TWIST1 inhibition with either shRNA or harmine in the ONS-76 and D283 cell lines.

Gene expression of selected TWIST1 target genes, identified by CHIP-Seq, was performed after targeted shRNA or 72 hour treatment with 20 μ M or 5 μ M of harmine in the ONS-76 (A) and D283 (B) cell lines, respectively. Gene expression was calculated relative to the house-keeping gene *HPRT* and normalised to the non-targeting or vehicle control using the $2^{-\Delta\Delta Ct}$ method and expressed as a percentage. Error bars represent the mean $\pm$ SEM of $n = 3$ (# representing $n = 2$) experiments each containing three replicates. Statistical significance was calculated using a paired Student's t-test comparing to the non-targeting or vehicle control (ns = not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

6.2.4.2 Patient dataset analysis

Having confirmed that inhibition of TWIST1 was associated with reduced gene expression of the candidate genes in our representative cell lines, we next sought to determine if their expression correlated with TWIST1 expression and outcome in medulloblastoma patient datasets using the R2 online platform (previously used in Chapter 3). Firstly, SHH and Group 3 patient tumour samples from the Cavalli dataset (Cavalli et al., 2017) were used to investigate if gene expression correlated with *TWIST1* expression. Impressively, positive correlation was observed for five out of the six genes, with *BSG* and *FBXL17* showing the strongest correlation. Only *IGF1R* (which did not appear as a shared gene in both subgroups) showed no correlation with TWIST1 (Figure 6.9). This data agrees with the qRT-PCR analysis and demonstrates that TWIST1 is regulating these genes, and further supports the ChIP-Seq analysis and the subgroup specific TWIST1 target genes.

To investigate patient outcome, Kaplan Meier analysis of the Cavalli patient dataset was performed within a 120 month follow-up period for all the subgroups combined, SHH and Group 3 subgroups (Figure 6.10 and Appendix 4, Appendix Figure 8). Whilst all six genes were assessed, *BSG*, *HYAL2* and *SORCS2* revealed the most striking results and therefore this analysis was presented in the remainder of this section (data for *IGF1R*, *FBXL17* and *IFG1* can be found in Appendix 4, Appendix Figures 8, 9 and 10). High expression levels of all three genes showed a clear trend towards worse overall survival when analysing all subgroups and Group 3. Only low *HYAL2* expression in the SHH subgroup resulted in a worse overall survival.

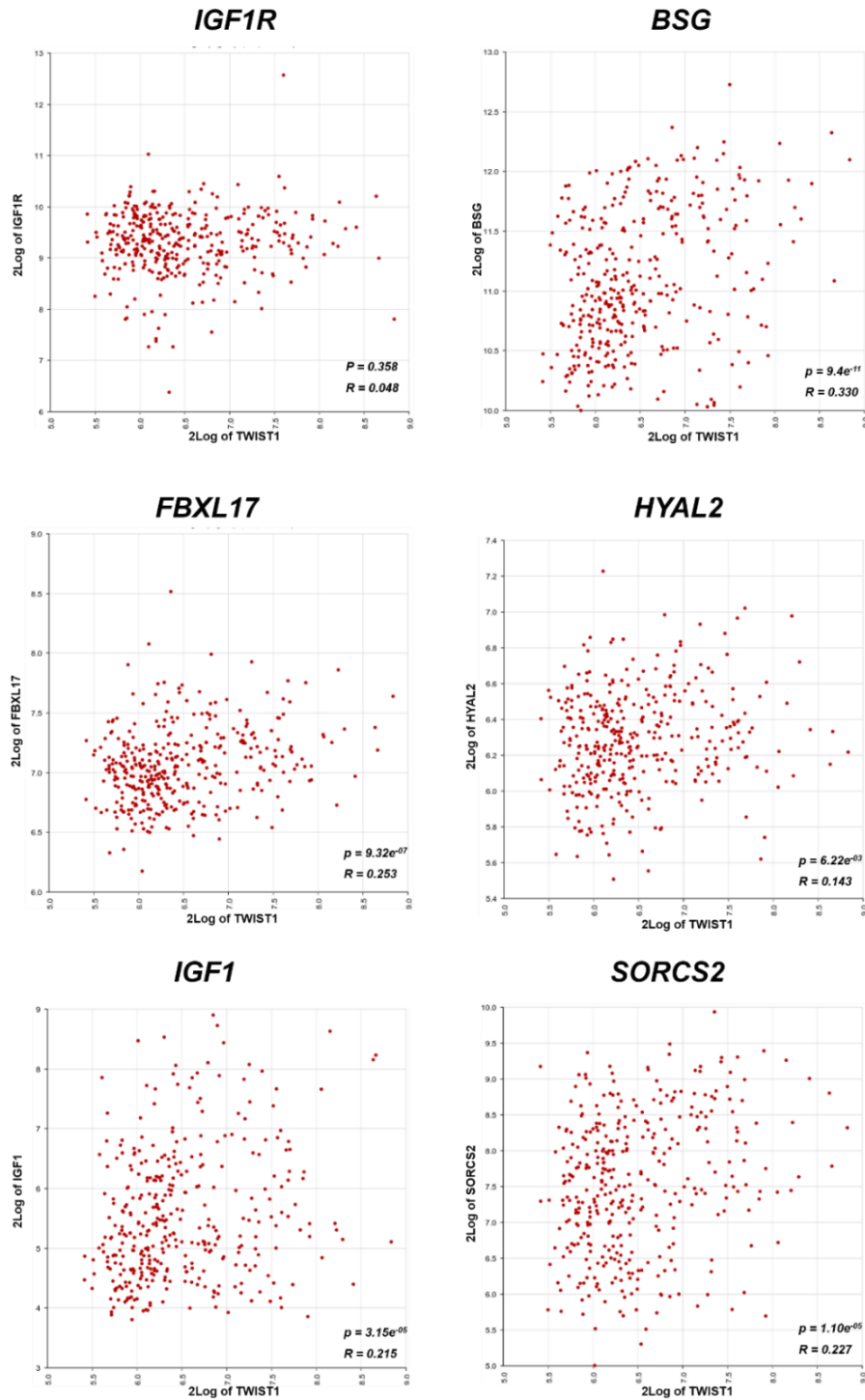


Figure 6.9 Correlation of TWIST1 expression with the selected gene targets in SHH and Group 3 tumour samples from the Cavalli dataset.

Correlation graphs of *TWIST1* expression with downstream target genes identified using ChIP-Seq analysis. Correlation was assessed in SHH and Group 3 medulloblastoma samples using the Cavalli dataset. The *R* value represents the degree of correlation between genes, whilst *p* represents the probability that correlation occurred by chance.

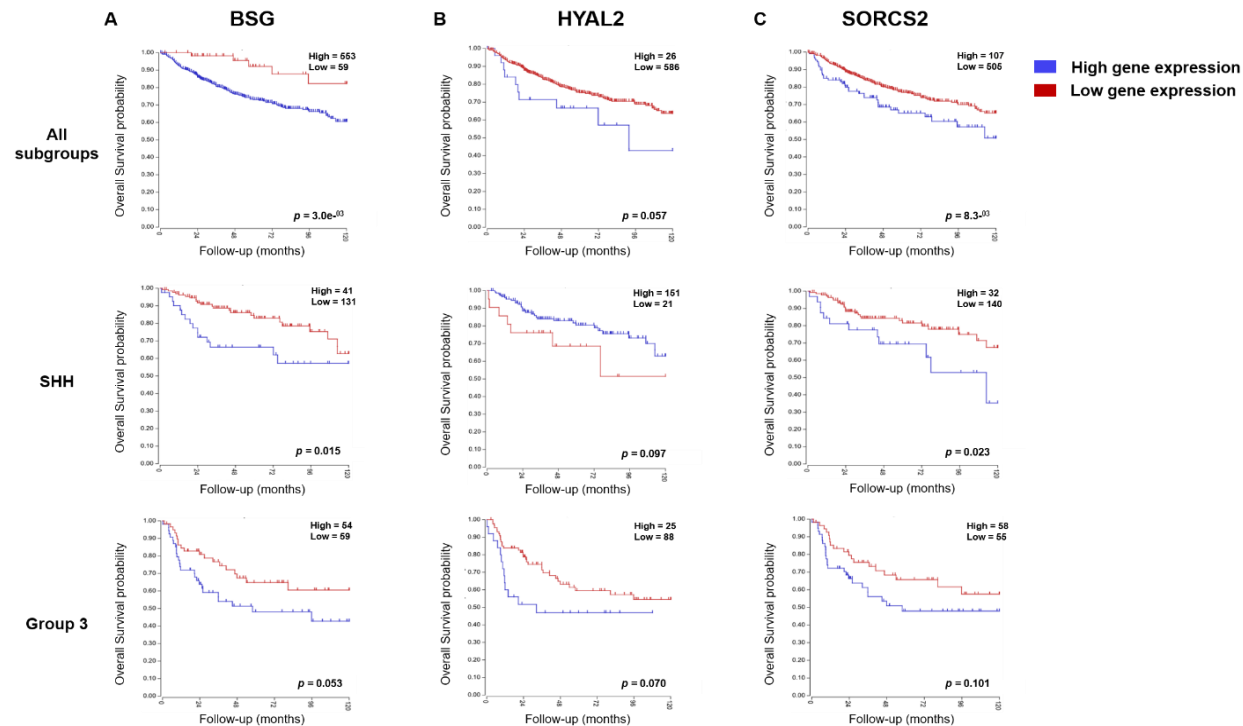


Figure 6.10 Kaplan Meier survival curves of the overall survival probability for high and low gene expression of *BSG*, *HYAL2* and *SORCS2* in medulloblastoma patient samples.

Kaplan Meier survival curves of the overall survival probability in a 120 month follow-up period based on high or low gene expression for *BSG* (A), *HYAL2* (B), and *SORCS2* (C). Overall survival probability for all molecular subgroups (n = 612), SHH subgroup (n = 172), and Group 3 subgroup (n = 113) was assessed using the Cavalli dataset. Statistical significance was calculated using the logrank test.

Next, gene expression across the medulloblastoma subgroups was compared against the normal cerebellum, using the Roth and Pfister patient datasets, to investigate if expression was higher in the SHH and Group 3 subgroups. (Figure 6.11 and Appendix 4, Appendix Figure 9) (Roth et al., 2006, Northcott et al., 2017). These two datasets were selected because they allowed for gene expression analysis across multiple datasets due to both using the same microarray platform and normalisation technique. Only *HYAL2* gene expression was significantly higher in the SHH and Group 3 patient samples compared to the normal cerebellum, whereas *BSG* and *SORCS2* expression was lower in the SHH patient samples. Expression was also higher in Group 3 samples compared to SHH samples for all three genes.

The data throughout this thesis demonstrates that *TWIST1* is a major factor driving medulloblastoma metastasis. The Cavalli dataset was used to investigate the gene expression of the same genes in non-metastatic (M0) and metastatic (M+) patient samples to see if expression was higher in the tumours that had metastasised. Gene expression was analysed for all subgroups combined and for the SHH and Group 3 patient samples (Figure 6.12 and Appendix 4, Appendix Figure 10). There was no significant differences in expression for any of the genes between non-metastatic and metastatic samples when analysing all of the subgroups combined and the SHH subgroup. However, the mean expression of all three genes was higher for the Group 3 metastatic samples compared to the non-metastatic samples, with significant differences observed in the *BSG* and *SORCS2* genes.

Metastatic disease is often associated with a poor prognosis in medulloblastoma patients, and the results gathered from the patient datasets showed that *TWIST1* in Group 3 medulloblastoma tumours is regulating pathways linked to metastatic pathways and outcome. Overall, the data

presented validates the ChIP-Seq analysis of TWIST1 targets that were identified in the SHH and Group 3 metastatic cell lines. Whilst *BSG*, *HYAL2* and *SORCS2* are possible follow-up targets, the wider ChIP-Seq data might also harbour stronger genes as predictors of outcome and metastasis.

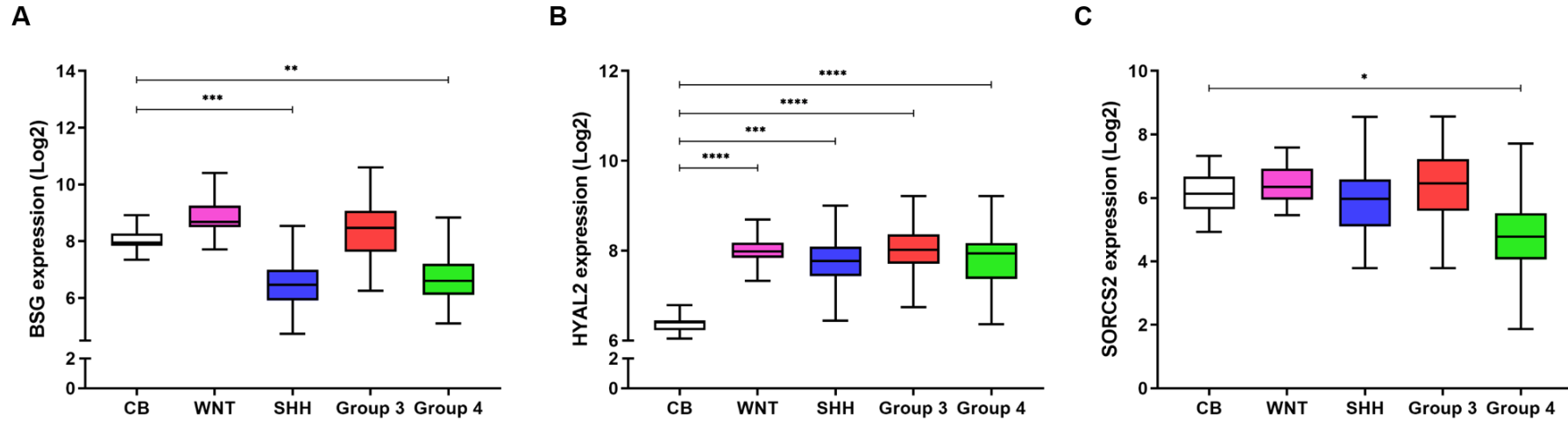


Figure 6.11 Gene expression of *BSG*, *HYAL2* and *SORCS2* in medulloblastoma subgroups compared to the normal cerebellum.

Gene expression (Log2) of *BSG* (A), *HYAL2* (B), and *SORCS2* (C) was assessed using R2 Genomics Analysis and Visualization Platform. The MegaSampler function was used to assess gene expression in two datasets: Roth (normal cerebellum (CB) n=9) and Pfister (WNT n = 17, SHH n = 59, Group 3 n = 56 and Group 4 n = 91). Significant differences in gene expression compared to the normal cerebellum were calculated using Kruskal-Wallis test with Dunn's multiple comparison post-hoc test (* $p < 0.05$, ** $p < 0.001$, *** $p < 0.001$ and **** $p < 0.0001$).

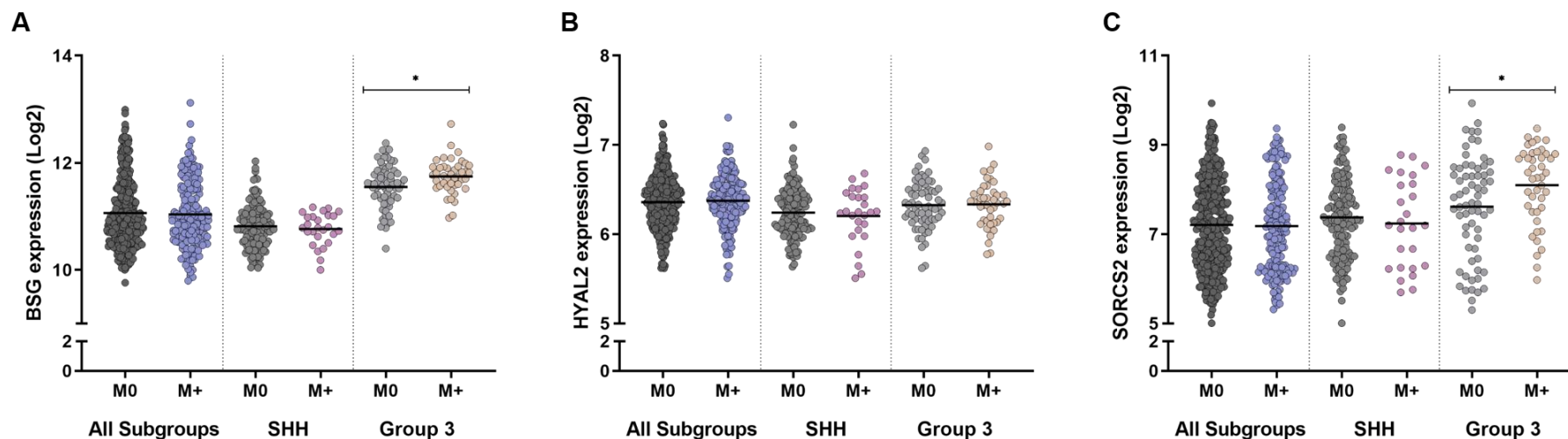


Figure 6.12 Gene expression of *BSG*, *HYAL2* and *SORCS2* in non-metastatic (M0) and metastatic (M+) medulloblastoma patient samples.

Gene expression (Log2) of *BSG* (A), *HYAL2* (B), and *SORCS2* (C) was assessed using R2 Genomics Analysis and Visualization Platform. Gene expression for all molecular subgroups (M0 = 397 and M+ = 176), SHH subgroup (M0 = 134 and M+ = 26) and Group 3 subgroup (M0 = 66 and M+ = 43) was assessed using the Cavalli dataset. Significant differences in gene expression were calculated using either a Mann-Whitney test (*BSG* and *SORCS2*) or an unpaired Student's t-test (*HYAL2*) (* $p < 0.05$ and ** $p < 0.001$).

6.2.5 ChIP-Seq Functional Analysis

Having started to establish the importance of the selected TWIST1 target genes in medulloblastoma metastasis, we next carried out functional enrichment analysis using the gene annotations for the significant TWIST1 binding sites in order to identify TWIST1 downstream pathways amongst the identified genes. Gene Set Enrichment Analysis (GSEA; www.gsea-msigdb.org/gsea/index.jsp) was used to determine which TWIST1 targets overlapped with annotated gene sets from the Molecular Signature Database (MSigDB). Two different gene set collections were used: Gene Ontology (GO) and Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway with analysis limited to the top 20 significant gene sets. Significance between overlapping genes with the KEGG and GO gene sets were determined using an uncorrected p-value and a p-value adjusted using the Benjamini-Hochberg False Discovery Rate (FDR) method (FDR q-value), indicating a degree of mathematical significance that TWIST1 binds to genes implicated in the named biological pathway.

For the purpose of this chapter, only protein coding-genes were analysed using the MSigDB. Duplicated gene names (where TWIST1 bound to more than one region of a gene), pseudogenes, non-coding RNA transcripts such as microRNA (miRNA), long non-coding RNA (lncRNA) and long intergenic non-coding RNA (lincRNA) were reserved for later analysis by incoming PhD students. Analysis was performed using the list of genes identified in the SHH subgroup (ONS-76 cell line; 241 genes), the Group 3 subgroup (D283 cell line; 876 genes), and those shared between both subgroups (SHH and group 3; 1893 genes).

Firstly, Gene Ontology (GO) analysis was performed using the genes identified in the SHH subgroup, Group 3 subgroup and the genes shared in both

subgroups using the molecular function and biological process domains (Table 6.4, Table 6.5 and Table 6.6 respectively). The domains provide information about how a gene functions at the molecular level and what biological process it helps to carry out. The functions and processes that the TWIST1 target genes regulated in both subgroups included cell signalling, movement and adhesion, and transport activity. Those that were unique to SHH tumours were centred around the positive regulation of nervous system development, transportation, and cell projection and assembly, whereas, the Group 3 functions and processes were mainly associated with the initiation of cell migration, regulating cell adhesion, morphogenesis and interaction with DNA regions. The Group 3 functions support the analysis performed in the ChIP-Seq validation section, which indicated TWIST1 in Group 3 medulloblastoma tumours was regulating pathways linked to metastasis.

Table 6.4 GSEA using GO to identify TWIST1 target gene sets in the SHH subgroup.

Gene sets highlighted in blue represented GO gene sets shared between all three analyses, those not highlighted appeared in two analysis, and green highlights those specific to the SHH subgroup.

Gene Set Name (GO Pathway)	Number of genes in overlap	p-value	FDR q-value
Positive regulation of cell projection organisation (GO:0031346)	10	7.05e ⁻⁷	3.85e ⁻³
Cell projection organisation (GO:0030030)	19	9.49e ⁻⁷	3.85e ⁻³
Regulation of cell differentiation (GO:0045595)	21	1.24e ⁻⁶	3.85e ⁻³
Positive T cell selection (GO:0043368)	4	5.75e ⁻⁶	1.17e ⁻²
Cell-cell signalling (GO:0007267)	19	6.29e ⁻⁶	1.17e ⁻²
Central nervous system development (GO:0007417)	14	7.92e ⁻⁶	1.2e ⁻²
Regulation of transport (GO:0051049)	19	9.04e ⁻⁶	1.2e ⁻²
Neurotransmitter transport (GO:0006836)	7	1.06e ⁻⁵	1.23e ⁻²
Head development (GO:0060322)	12	1.29e ⁻⁵	1.33e ⁻²
T cell selection (GO:0045058)	4	2e ⁻⁵	1.83e ⁻²
Neuron development (GO:0048666)	14	2.21e ⁻⁵	1.83e ⁻²
Positive regulation of plasma membrane bounded cell projection assembly (GO:0120034)	5	2.37e ⁻⁵	1.83e ⁻²
Neurogenesis (GO:0022008)	17	3.1e ⁻⁵	2.21e ⁻²
Synaptic signalling (GO:0099536)	11	3.6e ⁻⁵	2.38e ⁻²
Small GTPase mediated signal transduction (GO:0007264)	9	4.54e ⁻⁵	2.81e ⁻²
Neuron differentiation (GO:0030182)	15	5.41e ⁻⁵	3.14e ⁻²
Regulation of nervous system development (GO:0051960)	12	7.04e ⁻⁵	3.5e ⁻²

Regulation of neuron differentiation (GO:0045664)	10	7.5^{-5}	$3.5e^{-2}$
Skin development (GO:0043588)	8	7.89^{-5}	$3.5e^{-2}$
Positive regulation of nervous system development (GO:0051962)	9	8.43^{-5}	$3.5e^{-2}$

Table 6.5 GSEA using GO to identify TWIST1 target gene sets in the Group 3 subgroup.

Gene sets highlighted in blue represented GO gene sets shared between all three analyses, those not highlighted appeared in two analysis, and green highlights those specific to the Group 3 subgroup.

Gene Set Name (GO Pathway)	Number of genes in overlap	p-value	FDR q-value
Neurogenesis (GO:0022008)	106	1.43e ⁻²⁶	8.11e ⁻²³
Neuron differentiation (GO:0030182)	96	1.75e ⁻²⁶	8.11e ⁻²³
Neuron development (GO:0048666)	81	1.95e ⁻²³	6.02e ⁻²⁰
Biological adhesion (GO:0022610)	91	5.84e ⁻²²	1.35e ⁻¹⁸
Positive regulation of multicellular organismal process (GO:0051240)	101	1.56e ⁻²⁰	2.9e ⁻¹⁷
Cell projection organisation (GO:0030030)	88	2.12e ⁻¹⁸	3.28e ⁻¹⁵
Regulation of cell differentiation (GO:0045595)	99	4.17e ⁻¹⁸	5.52e ⁻¹⁵
Cell junction organisation (GO:0034330)	53	1.55e ⁻¹⁶	1.79e ⁻¹³
Positive regulation of developmental process (GO:0051094)	79	6.31e ⁻¹⁶	6.5e ⁻¹³
Homeostatic process (GO:0042592)	94	1.43e ⁻¹⁵	1.33e ⁻¹²
Regulatory region nucleic acid binding (GO:0001067)	78	2.42e ⁻¹⁵	1.93e ⁻¹²
Sequence specific DNA binding (GO:0043565)	85	2.5e ⁻¹⁵	1.93e ⁻¹²
Regulation of nervous system development (GO:0051960)	60	3.64e ⁻¹⁵	2.59e ⁻¹²
Cellular component morphogenesis (GO:0032989)	54	4.57e ⁻¹⁵	3.02e ⁻¹²
Cell-cell adhesion (GO:0060322)	57	5.56e ⁻¹⁵	3.43e ⁻¹²
Head development (GO:0060322)	52	7.63e ⁻¹⁴	4.42e ⁻¹¹

Regulation of cell development (GO:0060284)	58	1.34e ⁻¹³	7.31e ⁻¹¹
Cell morphogenesis (GO:0000902)	60	1.43e ⁻¹³	7.35e ⁻¹¹
Anatomical structure formation involved in morphogenesis (GO:0048646)	65	2.39e ⁻¹³	1.17e ⁻¹⁰
Cell morphogenesis involved in differentiation (GO:0000904)	49	3.41 ⁻¹³	1.58e ⁻¹⁰

Table 6.6 GSEA using GO to identify TWIST1 target gene sets between the two subgroups.

Gene sets highlighted in blue represented GO gene sets shared between all three analyses, those not highlighted appeared in two analysis, and green highlights those specific to both subgroups.

Gene Set Name (GO Pathway)	Number of genes in overlap	p-value	FDR q-value
Regulation of cell differentiation (GO:0045595)	253	4.06e ⁻⁵⁰	3.76e ⁻⁴⁶
Cell-cell signalling (GO:0007267)	241	1.63e ⁻⁴⁹	5.17e ⁻⁴⁶
Neurogenesis (GO:0022008)	230	1.67e ⁻⁴⁹	5.17e ⁻⁴⁶
Ion transport (GO:0006811)	227	6.63e ⁻⁴⁷	1.54e ⁻⁴³
Positive regulation of multicellular organismal process (GO:0051240)	235	1.46e ⁻⁴⁴	2.7e ⁻⁴¹
Neuron differentiation (GO:0030182)	195	1.89e ⁻⁴²	2.91e ⁻³⁹
Homeostatic process (GO:0042592)	238	7.84e ⁻⁴²	1.04e ⁻³⁸
Transmembrane transport (GO:0055085)	209	3.71e ⁻⁴¹	4.3e ⁻³⁸
Transporter activity (GO:0005215)	175	4.81e ⁻⁴¹	4.96e ⁻³⁸
Biological adhesion (GO:0022610)	197	3.2e ⁻⁴⁰	2.97e ⁻³⁷
Signalling receptor binding (GO:0005102)	207	3.2e ⁻⁴⁰	2.97e ⁻³⁷
Positive regulation of signalling (GO:0023056)	225	4.88e ⁻³⁸	3.77e ⁻³⁵
Neuron development (GO:0048666)	160	2.55e ⁻³⁵	1.82e ⁻³²
Cell projection organisation (GO:0030030)	195	6.25e ⁻³⁵	4.14e ⁻³²
Ion transmembrane transport (GO:0034220)	161	9.95e ⁻³⁵	6.15e ⁻³²
Cell motility (GO:0048870)	211	3.29e ⁻³⁴	1.91e ⁻³¹
Cell activation (GO:0001775)	184	7.59e ⁻³⁴	4.14e ⁻³¹
Cell morphogenesis (GO:0000902)	148	1.85e ⁻³³	9.54e ⁻³¹
Animal organ morphogenesis (GO:0009887)	151	4.09e ⁻³³	2e ⁻³⁰
Synaptic signalling (GO:0099536)	120	5.04e ⁻³²	2.33e ⁻²⁹

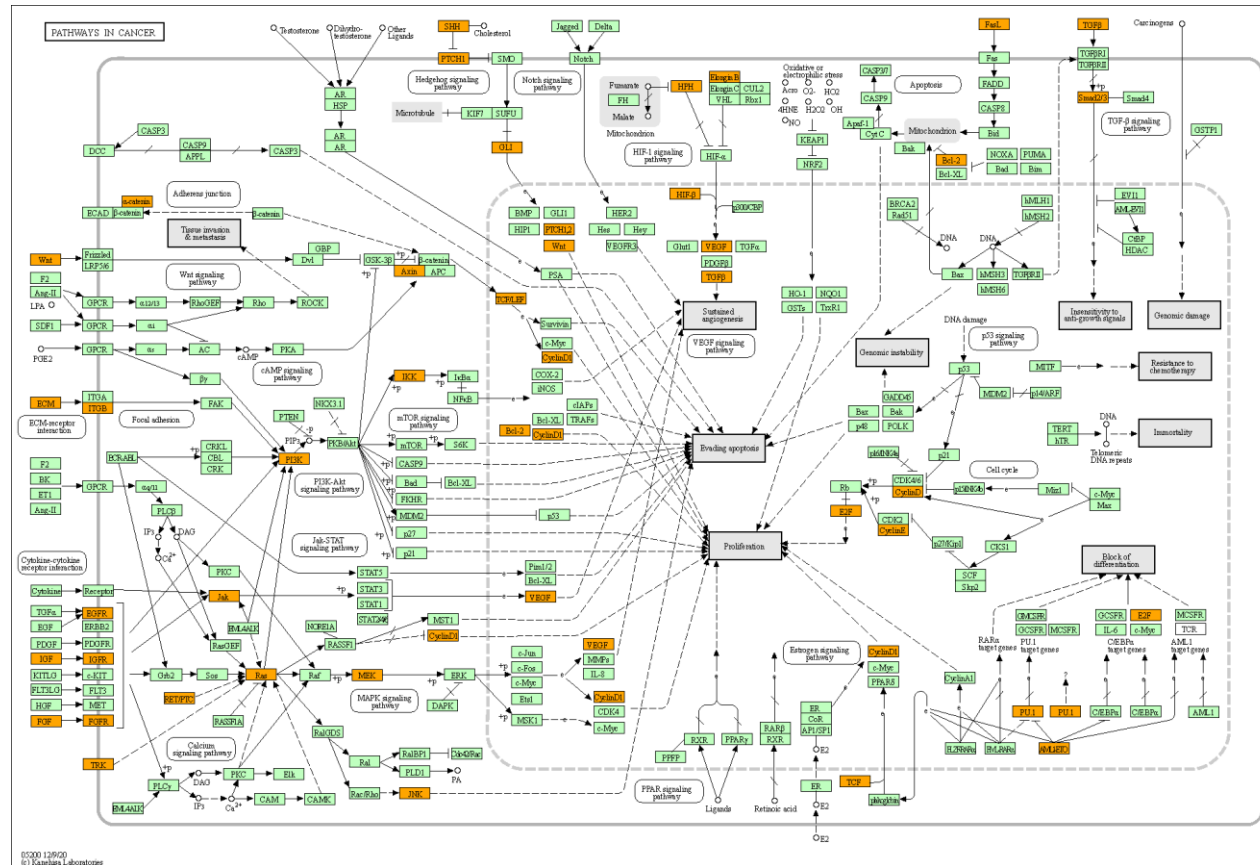


Figure 6.15 Pathways in cancer KEGG pathway, highlighting TWIST1 target genes

The KEGG 'Pathways in cancer (hsa05200)' pathway. TWIST1 target genes identified by ChIP-Seq analysis are highlighted in orange. The black arrows represent molecular interactions and activations with downstream targets associated with this KEGG pathway.

Utilising the identified GO gene sets and KEGG pathways, Figure 6.16 provides an overview of TWIST1's transcriptional network in medulloblastoma metastasis, and highlights some of the key genes involved in these pathways. The following sections will discuss in further detail the function of TWIST1 in regulating the genes associated with these pathways, including some of the genes used in the ChIP-Seq validation section.

Focal Adhesion pathway

IGF1R expression has been observed in high-risk medulloblastoma primary tumours and leptomeningeal metastases, and has been shown to be a marker for poor prognosis in Group 3 medulloblastoma patients. In addition, its expression is linked with increased migration and adhesion to ECM components and cell types associated with the leptomeninges (Svalina et al., 2016, Park et al., 2019). *IGF1* along with *IGF1R* was identified in the focal adhesion pathway. Focal adhesions (FAs) are specialised structures that are formed to mediate the regulatory effects of a cell in response to ECM adhesion. Integrins are a major component of this process as they facilitate a cells adhesion to components of the ECM. A high turnover of FAs is required at the posterior of a cell during migration in order to create an unstable adhesion and release the cells from the ECM (Nagano et al., 2012). FAs are regulated dynamically during cell migration by FAK (focal adhesion kinase), a tyrosine kinase which is phosphorylated by IGF1R in response to clustering of integrins. (Zheng et al., 2010). *IGF1R* was identified only in the Group 3 subgroup, suggesting that the FA pathway might be of particular importance during cell migration in Group 3 tumours.

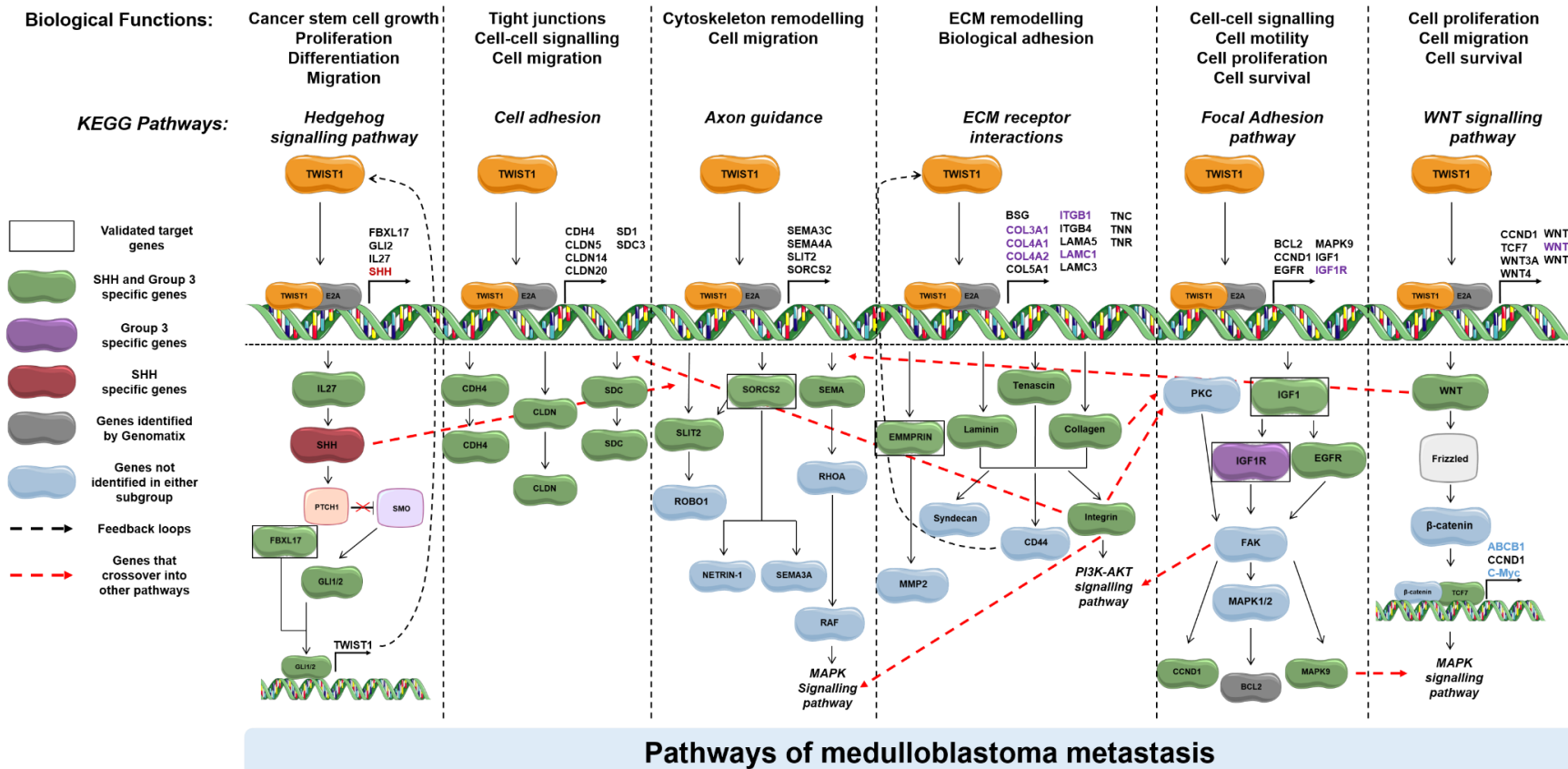


Figure 6.16 Schematic illustrating the TWIST1 transcriptional network involved in medulloblastoma metastasis.

Schematic illustrating the TWIST1 genes and pathways involved during medulloblastoma metastasis in SHH and Group 3 tumours. The pathways that the genes are involved in are based upon KEGG pathway analysis and information from the literature.

ECM receptor interactions

As mentioned above, integrins are a crucial component of the focal adhesion pathway because they facilitate the binding of cells to the ECM. Integrins consist of one α and one β subunit that form a transmembrane heterodimer (Cox, 2021). Our CHIP-Seq data identified three β subunits; *ITGB1* (Group 3 specific), *ITGB2* and *ITGB4* (SHH and Group 3), which upon forming heterodimers, can interact with components of the basement membrane including collagen (integrins $\alpha2\beta1$) and laminin (integrins $\alpha3\beta1$, $\alpha6\beta1$ or $\alpha6\beta4$) (Quaranta and Plopper, 1997, Gullberg and Lundgren-Akerlund, 2002, Yazlovitskaya et al., 2019, Romagnoli et al., 2020).

COL4A1 and *COL4A2* were identified as TWIST1 targets in the Group 3 subgroup, but many other collagen subtypes were common between both subgroups. Collagen IV consists of three α subunits, which can make up three isoforms; $\alpha1\alpha1\alpha2$, $\alpha3\alpha4\alpha5$ and $\alpha5\alpha5\alpha6$. The first isoform, which consists of the two subunits identified in the Group 3 subgroup, is ubiquitously expressed and has been shown to be important in promoting proliferation, migration and invasion in hepatocellular cancer (Wang et al., 2020b). Collagen and laminin are also able to bind to proteoglycans, such as CD44 and Syndecan 1 and 2 (as shown in the ECM-interaction KEGG pathway). CD44 is overexpressed in many cells, including cancer stem cells and has been shown to play an important role in cell migration through the EMT pathway by binding to extracellular hyaluronic acid (HA) resulting in weakening a cell's ability to anchor itself to the ECM (Chen et al., 2018, Dovedytis et al., 2020). Moreover, co-expression of CD44 and TWIST1 have been implicated as diagnostic and prognostic biomarkers in patients with gastric and breast cancer, demonstrating their importance in cancer progression (El Kholy et al., 2018, Strati et al., 2019). When we inhibited TWIST1 in chapter 4, a reduction in cells migrating through

a collagen IV and laminin coating was observed. This could be because the cells were unable to express the relevant integrins required to bind to the ECM components; collagen IV and laminin, subsequently inhibiting the binding to extracellular HA, therefore, preventing invasion through the matrix coating.

In addition to collagen and laminin, *BSG*, also known as extracellular matrix metalloproteinase inducer (Emmprin), is enriched on the surface of many types of tumour cells and can regulate tumour cell invasion by its association with matrix metalloproteinase 2 (MMP2) (Xin et al., 2016). MMPs are enzymes that are responsible for the degradation of collagen in the ECM. MMP2 and MT1-MMP (membrane-type matrix metalloproteinase-1) can co-localise with integrins $\beta 1$ and $\beta 3$ as they adhere to collagen fibres (Friedl and Wolf, 2003, Jabłońska-Trypuć et al., 2016), therefore, making Emmprin an ideal TWIST1 downstream target in facilitating cell migration. Furthermore, studies in our research group have highlighted the importance of Emmprin and MMP2 and MMP9 expression in facilitating medulloblastoma cell migration (Mitoko, 2021; Jackson, 2021 (unpublished data). The ChIP-Seq data identified Emmprin in both subgroups, and high expression has been associated with a poor prognosis in medulloblastoma patients (Chu et al., 2011). Overall, the data suggests that targeting TWIST1 and the proteins involved in ECM degradation or interactions, such as Emmprin or specific collagen and laminin subtypes, would be pivotal in preventing cell migration in SHH and Group 3 medulloblastoma cells.

Axon guidance pathway

Another pathway that was identified during GSEA was the axon guidance pathway. Slits, semaphorins and netrins are three families of proteins that have been identified in this pathway and were genes that appeared in both subgroups

in our ChIP-Seq data. Molecules of the axon guidance pathway can attract or repel growing axons and migrating neurons in the developing nervous system, but can also regulate cell migration and apoptosis in tumourigenic tissues. Semaphorins (in particular SEMA3C) have been shown to drive the EMT pathway in prostate cancer cells by upregulating EMT markers and promoting cell migration and invasion using *in vitro* models (Tam et al., 2017). The growth cone, which is found at the growing end of a developing axon, is able to facilitate the above processes through the binding of filopodia and adhesion receptors to ECM components (such as collagen) or ligands, allowing for the remodelling of the cytoskeleton (Santiago-Medina et al., 2015, Roumazeilles et al., 2018).

Recently, *SORCS2* was identified by Dr Macha Aldighieri (a previous PhD student in our research group) as being expressed in the Group 3 metastatic cells during migration through a collagen IV and laminin matrix (Aldighieri M., 2020). We found *SORCS2* to be a *TWIST1* downstream target in both SHH and Group 3 cell lines, and its expression could be important in influencing axon guidance via Netrin-1, Slit1 and 2, and semaphorins (Boggild et al., 2018). Of interest, Slit-2 and Robo1 signalling have been shown to degrade E-cadherin (*CDH1*) through the recruitment of an ubiquitin ligase, and induce EMT in colorectal cancer cells (Zhou et al., 2011, Koohini et al., 2019), which may explain why we didn't observe E-cadherin as a *TWIST1* downstream target. This data suggests that a *SORCS2*-*SLIT2*-*ROBO1* signalling axis could be important in regulating levels of E-cadherin and allowing the cellular invasion of medulloblastoma cells through the basement membrane.

Cell adhesion molecules

Cell adhesion molecules play an important role in mediating cell-cell or cell-ECM interactions and are classified into four families; immunoglobulin

superfamily, integrins, cadherins and selectins (Moh and Shen, 2009, Janiszewska et al., 2020). They contain an intercellular domain that interacts with the cytoskeleton and is responsible for signalling, and an extracellular domain that interacts with either cell adhesion molecules and/or the ECM. As mentioned above, the proposed SORCS2-SLIT2-ROBO1 signalling axis regulated by TWIST1, might be of importance in regulating E-cadherin in medulloblastoma tumour progression and metastasis. Other cadherins that were identified in both subgroups included *CDH4*, *CDH10* and *CDH12*. In GBM patients, *CDH4* expression correlated with a shorter survival time, and down regulation reduced tumour infiltration and proliferation (Ceresa et al., 2019).

The functional importance of the identified integrins in our ChIP-Seq data (*ITGB1*; Group 3 and *ITGB2* and *ITGB4*; SHH and Group 3) have already been discussed in some of the other signalling pathways. This section focusses on their importance in the cell adhesion pathway. A study by Fiorilli *et al*, investigating molecules that mediate medulloblastoma adhesion to the leptomeninges, used the D283 Group 3 medulloblastoma cell line, and revealed that D283 cells preferentially expressed the $\alpha 9$ and $\beta 1$ integrin subunits. This heterodimer complex formed adhesions with an ECM matrix rich in tenascin (TNC), as well as activating the MAPK pathway, which was necessary for both cell survival and cell proliferation (Fiorilli et al., 2008). *ITGB1* was solely expressed in our Group 3 (D283) ChIP-Seq dataset, but *TNC* was identified in both subgroups. This data suggests that *ITGB1* is playing an important role in Group 3 medulloblastoma cell migration, by facilitating the adherence of cells to the ECM, in particular to TNC, but there might be other integrins that are activated in the SHH group that are facilitating the binding to TNC.

WNT signalling pathway

In Chapter 3, we identified that TWIST1 was highly expressed in WNT medulloblastoma tumours, which could have been linked to TWIST1 playing a fundamental role in the WNT signalling pathway. Functional enrichment analysis of our ChIP-Seq dataset also identified WNT signalling as a pathway that was common across both SHH and Group 3 subgroups. WNT signalling is a known pathway involved in EMT. It has been described that the loss of E-cadherin promotes WNT signalling, causing β -catenin to translocate to the nucleus, where it forms a complex with *TCF* (including *TCF7*) and *LEF* (lymphoid enhancer-binding factor 1) to activate the transcription of genes promoting proliferation and migration, including *ABCB1*, *c-Myc*, and *CCND1* (Cyclin D1) (Corrêa et al., 2012, Gougelet and Colnot, 2012, Lamouille et al., 2014, Colella et al., 2019).

Geron *et al.*, investigated the activation of the WNT pathway in ONS-76 (SHH), UW402 (Group 3) and UW473 (Group 4) medulloblastoma cell lines. They observed low expression of *CTNNB1* (β -catenin) but up-regulation of *c-Myc* and *Cyclin D1*, indicating that the WNT pathway was not activated in these cell lines, and instead *c-Myc* and *Cyclin D1* might be expressed independently of β -catenin (Geron et al., 2018). *Cyclin D1* was identified as a TWIST1 downstream target in both subgroups, and it has been noted that WNT can regulate Cyclin D1 protein stability independent of β -catenin (Qie and Diehl, 2016). Cyclin D1 plays an essential role in cell proliferation through the regulation of cells through the G1 phase of the cell cycle, and high levels have been shown to promote GBM cell dissemination via FAK (Yang et al., 2006, Montalto and De Amicis, 2020). A reason why WNT signalling is activated by TWIST1 during SHH and Group 3 metastasis, could be that the cells that have the potential to metastasise increase TWIST1 expression in order to upregulate downstream

genes (such as *c-Myc* and *Cyclin D1*) and pathways that are vital in cell migration, cell survival and tumour progression.

SHH signalling pathway

SHH signalling was another pathway common between both subgroups. Somatic mutations or copy number alterations often lead to the activation of this pathway in SHH subgroup tumours (Juraschka and Taylor, 2019). *SHH*, the main ligand involved in initiating the pathway, was identified only in the ONS-76 (SHH) cell line, indicating the importance of this pathway in SHH tumours. The signalling pathway can play a role in CSCs through its ability to drive tumour development, promote tumour growth, and regulate residual cancer cells after therapy, with expression levels of components from the pathway high in CSCs. Targeting the SHH pathway would be extremely beneficial in preventing TWIST1 mediated tumour progression in SHH tumours. Whilst studies have already investigated the use of competitive antagonists of the smoothened (SMO) receptor, patients have developed treatment resistance over time. Some of the gene sets identified in the SHH subgroup from the GSEA analysis were associated with transport activity, which could explain why treatment failure occurs in these patients. Therefore, it might be of interest to therapeutically target more than one gene in the SHH signalling pathway to try and prevent treatment resistance and improve patient outcome.

FBXL17 (F-box and leucine-rich repeat protein 17), a TWIST1 downstream target gene identified in both subgroups, has been implicated in contributing to cancer stem cell growth via the SHH pathway. *FBXL17* can target Sufu for proteolysis in the nucleus, thus, leading to the release of GLI from Sufu and allowing GLI to translocate to the nucleus and activate the transcription of target genes, including TWIST1. Interestingly, GLI2 has dual functions in activating

and inhibiting the transcription of target genes for cell growth, proliferation and differentiation, and was common amongst both the SHH and Group 3 subgroups (Raducu et al., 2016, Yang et al., 2020). Therapeutic approaches to inhibit TWIST1, FBXL17 and GLI could prevent the transcription of GLI target genes activated via the SHH signalling pathway, and ultimately target medulloblastoma cancer stems and reduce tumour development and growth.

6.3 Summary

- ChIP-Seq revealed that the majority of TWIST1 binding sites were located within intron and distal intergenic regions, with approximately 5 % of binding sites occurring in the promoter region, which was in agreement with other TWIST1 ChIP-Seq datasets.
- Bioinformatics analysis of the ChIP-Seq dataset identified TWIST1 target genes that were specific and shared between the two subgroups.
- Six target genes were validated by qRT-PCR. Inhibition of TWIST1 either chemically or genetically using harmine or shRNA, respectively, resulted in a reduction in gene expression of the selected target genes when compared to control cells.
- Further validation using a large patient dataset revealed a positive correlation in TWIST1 gene expression with the selected target genes. In addition, the analysis highlighted that TWIST1 could be regulating pathways linked to metastasis in Group 3 tumours.
- Functional enrichment analysis of the wider ChIP-Seq dataset further supported a key role for TWIST1 in the initiation of medulloblastoma metastasis in Group 3 tumours, through the identification of GO and KEGG pathways involved in ECM interactions.

Chapter 7. Discussion

7.1 Overview

Transcriptional profiling of medulloblastoma cohorts has consistently identified four distinct molecular subgroups; WNT, SHH, Group 3 and Group 4 (Ramaswamy and Taylor, 2017). Each subgroup is linked to different clinical outcomes that greatly depend on factors including molecular abnormalities and the presence of metastasis. Metastatic disease has been continuously linked to a poor prognosis. Approximately one third of medulloblastoma patients present with metastatic disease, with frequencies varying greatly amongst the subgroups. Little is known about the molecular programme necessary for medulloblastoma metastasis (Juraschka and Taylor, 2019, Northcott et al., 2019). Therefore, this thesis investigated factors and pathways that mediate the initial steps of the metastatic cascade in medulloblastoma, with a focus on the transcription factor TWIST1 and whether it could play an essential role in medulloblastoma metastasis. Understanding the metastatic mechanisms driving these tumour cells will open the door to developing novel therapies that prevent metastasis and improve long-term outcomes for patients.

TWIST1 expression was identified as a strong indicator for worse event-free survival (EFS) and driving metastatic disease in medulloblastoma patient tissue microarrays (TMAs; $p < 0.05$). Functional studies in highly expressing TWIST1 metastatic SHH and Group 3 cell lines, revealed that reduced TWIST1 expression significantly decreased cell migration in a 3D transwell migration and invasion assay. In addition, functional experiments showed that TWIST1 could be regulating the multidrug efflux pump *ABCB1*, which was also involved in medulloblastoma cell migration. Further analysis into TWIST1 downstream targets using ChIP-Seq analysis, allowed for a wider investigation into

TWIST1's regulatory network, which highlighted a key emphasis on ECM-receptor interactions and cell adhesion molecule pathways.

7.2 TWIST1 expression in medulloblastoma

A review by Zhao *et al*/ highlighted the involvement of TWIST1 in many biological functions and molecular pathways in tumourigenesis (Zhao *et al.*, 2017). TWIST1 plays multiple roles in cancer initiation, progression and metastasis, and more specifically can override oncogene-induced cell senescence, enhance cancer stem cell (CSC) population, increase drug resistance, and facilitate cancer cell invasion and metastasis (Ansieau *et al.*, 2008, Li *et al.*, 2009, Vesuna *et al.*, 2009b). Moreover, TWIST1 had been linked to inducing the EMT pathway, the first event in the metastatic cascade, in numerous tumour types (Zhao *et al.*, 2017). However, TWIST1's expression, functions and molecular mechanisms can vary between different types of cancers (Qin *et al.*, 2012).

To date, only one study has alluded the role of TWIST1 in medulloblastoma metastasis, and this arose during this thesis. Khan *et al*/ identified a NOTCH1-TWIST1-BMI1 axis in regulating Group 3 medulloblastoma metastasis, but failed to investigate TWIST1 as an independent predictor of prognosis or consider the role of TWIST1 and other downstream targets across other medulloblastoma subgroups (Kahn *et al.*, 2018). Therefore, Chapter 3 explored TWIST1 expression across all four medulloblastoma subgroups to assess its role in medulloblastoma outcome and metastatic disease.

7.2.1 TWIST1 is highly expressed in medulloblastoma tumours

The R2 Genomics Analysis and Visualization online platform was used to access medulloblastoma patient datasets. It provides a wealth of information to analyse gene expression across multiple patient datasets, although, only if the same microarray platform has been used, so limiting the number of datasets that can be combined and assessed. In addition, patient datasets do not always include the same clinical and pathological information (e.g. subgroup, metastatic status, and age groups), again restricting the datasets that can be used for a particular analysis. Due to these limitations, only 4 datasets were assessed throughout this thesis to explore the role of TWIST1 in medulloblastoma.

TWIST1 expression levels are high during embryogenesis but fall to extremely low levels in most cell types following this process. In many cancers, aberrant upregulation of TWIST1 is observed, making it a candidate biomarker for cancer progression (Qin et al., 2012). Analysis of *TWIST1* gene expression using the Roth (normal cerebellum samples) and Pfister (medulloblastoma tumour samples) datasets revealed that on average *TWIST1* levels were higher across all four subgroups compared to the normal cerebellum, with WNT tumours displaying the highest expression levels (Roth et al., 2006, Northcott et al., 2017). Whilst this result may seem unusual, that the subgroup which has the lowest rates of metastasis had the highest levels of TWIST1, this could be due to TWIST1 playing a fundamental role in the WNT signalling pathway, which could result in TWIST1 being continuously expressed in WNT tumours when the pathway is constitutively activated through mutations (He et al., 2009, Goodnough et al., 2016).

TWIST1 expression levels in the cell lines available within our research group (covering the three subgroups; SHH, Group 3 and Group 4) were comparable

to the Roth and Pfister datasets, with the exception of the Group 4 cell lines, which expressed lower levels of *TWIST1* compared to the non-tumourigenic FB83 cell line. However, closer analysis of the Roth and Pfister datasets showed that whilst the median *TWIST1* level was higher in Group 4 compared to the normal cerebellum, the minimum *TWIST1* values for Group 4 and the normal cerebellum were comparable to each other. The Cavalli dataset identified three Group 4 subtypes (Group 4 α , β and γ), suggesting that maybe a Group 4 subtype harboured low *TWIST1* expression (Cavalli et al., 2017). This data not only demonstrates that the cell lines within our research group are representative of medulloblastoma subgroups but also agrees with the literature that *TWIST1* is upregulated in many tumour types compared to normal tissue, making it a candidate biomarker in tumourigenesis.

7.2.2 High *TWIST1* protein expression is an indicator of worse event-free survival

High *TWIST1* levels have been shown as a poor prognostic factor in many cancers including, cervical, breast and colon cancers (Shibata et al., 2008, Riaz et al., 2012, Kim et al., 2014). As mentioned previously, the subgroup classification of the tumour and the presence of metastatic disease is a strong indicator of poor overall survival in medulloblastoma patients.

Analysis of *TWIST1* gene expression (high or low) and metastatic status (M0 or M+) with the Cavalli dataset revealed that high *TWIST1* expression in non-metastatic tumours had a more favourable outcome when assessing all subgroups combined. This data doesn't agree with the above literature. However, this could be linked to the high expression of *TWIST1* observed in the WNT tumours, as this subgroup is known to be an indication of a favourable prognosis with the lowest rates of metastasis. This highlighted the importance

of a subgroup-specific analysis, which revealed different trends amongst each subgroup. Patients diagnosed with high *TWIST1* expression and metastatic SHH tumours resulted in a sharp decrease in survival within 24 months of diagnosis, suggesting that patients who present with these two factors have an inferior outcome. Low *TWIST1* expression in non-metastatic Group 3 and Group 4 tumours had a better overall prognosis, whereas high *TWIST1* expression in Group 3 tumours, regardless of the patient's metastatic status, was associated with a worse overall survival. This data indicated that high *TWIST1* expression in Group 3 medulloblastoma tumours could be predictive of a poor outcome.

Gene expression levels do not always correlate well with protein expression levels, and the analysis of gene expression alone makes it difficult to make conclusions about the biological significance (Timp and Timp, 2020). In a recent review by Hovestadt *et al*, Group 3 and Group 4 medulloblastomas exhibited the lowest correlation between mRNA and protein expression, with only 45 % of genes having a significant positive mRNA-protein correlation, empathising the importance of proteomic analysis in medulloblastoma tumours (Hovestadt *et al.*, 2020). Therefore, in this thesis a novel analysis of *TWIST1* protein expression in primary medulloblastoma TMAs was performed and revealed that high *TWIST1* expression was indicative of a worse overall survival (OS) and EFS ($p = 0.082$ and $p = 0.046$ respectively). This is in agreement with other studies that have assessed patient TMAs and highlighted the importance of *TWIST1* as a prognostic marker for poor OS and EFS (Wang *et al.*, 2015a, Zhu *et al.*, 2015, Rasti *et al.*, 2018).

7.2.3 TWIST1 protein expression is linked to medulloblastoma metastatic disease

The upregulation of *TWIST1* in primary tumours promotes the EMT pathway and enhances the ability of tumour cells to migrate and invade the extracellular matrix (ECM) (Wang et al., 2018). When we compared the *TWIST1* gene expression levels in the primary tumours of patients who presented with non-metastatic disease against those with metastatic disease using the Cavalli dataset (Cavalli et al., 2017), no significant difference was observed. However, the Wang dataset (Wang et al., 2015b), which comprises of matched tumour samples, showed that two thirds of samples had slightly higher *TWIST1* expression levels in the primary tumour compared to the metastatic tumour (Figure 3.3). Furthermore, our matched paired cell lines revealed higher *TWIST1* levels in the primary cell lines compared to the matched metastatic cell lines, which begins to demonstrate the potential of upregulation of *TWIST1* in initiating medulloblastoma metastasis.

Whilst this initial gene expression data does not provide a definitive answer on the difference in *TWIST1* expression levels between non-metastatic and metastatic tumours, analysis into protein expression (which is a better indication of protein function) using medulloblastoma patient TMAs revealed that *TWIST1* expression was associated with medulloblastoma metastasis ($p = 0.027$), with 81 % of patients who were diagnosed with metastatic disease expressing high levels of *TWIST1* in the primary tumour. Unfortunately, due to the limited amount of clinical information available and the small sample sizes, subgroup-specific analysis could not be performed.

In addition to metastatic disease, recurrent medulloblastoma is another significant morbidity factor influencing patient outcomes and is nearly always

fatal in previously irradiated patients (Ramaswamy et al., 2013). Sadly, 75 % of paediatric patients relapse within 2 years of diagnosis, with Group 3 and Group 4 tumours recurring almost exclusively with metastatic dissemination (Massimino et al., 2016, Ramaswamy and Taylor, 2017). It is crucial to identify biomarkers that are expressed in the primary tumour that can be therapeutically targeted in order to prevent metastatic dissemination and recurrent disease. Analysing the TMA patient follow-up information, we showed that if the primary tumour expressed high levels of TWIST1 then the patient was significantly more likely to develop metastatic disease ($p = 0.037$).

Therefore, if the levels of TWIST1 in the primary tumour are causative of metastasis, TWIST1 should be therapeutically targeted in the primary tumour to prevent metastatic dissemination. The data presented in Chapter 3 demonstrated the necessity to analyse protein expression data in order to gain a more thorough and accurate understanding of TWIST1 in medulloblastoma metastasis. Our analysis indicated that high levels of TWIST1 in the primary tumour are predictive of EFS and metastatic disease. However, further functional studies are needed to determine how TWIST1 is contributing to metastatic disease.

7.3 TWIST1: A driver for medulloblastoma metastasis

Activation of the EMT pathway is described as one of the initial events in the metastatic cascade, driven by specific transcription factors including TWIST1 (Lamouille et al., 2014, Dongre and Weinberg, 2019). Whilst medulloblastomas do not originate from a classical epithelial background, and instead have a neuronal cell of origin, increasing evidence suggests that pathways of metastasis identified in epithelial tumours, including the EMT pathway, could be

relevant during medulloblastoma metastasis (Garzia et al., 2018, Nasir and Cardall et al., 2021). To advance our knowledge on the importance of TWIST1 in medulloblastoma metastasis, Chapter 4 investigated the functional potential of TWIST1 through inhibition studies in 3D models of medulloblastoma metastasis.

7.3.1 Chemical inhibition of TWIST1 decreases migration in metastatic medulloblastoma cell lines

We inhibited TWIST1 using two different techniques; chemical inhibition using harmine and genetic inhibition using shRNA in metastatic medulloblastoma cell lines (SHH; ONS-76 and Group 3; D283 and HD-MB03).

Until recently, a pharmacological TWIST1 inhibitor had not been identified. Yochum *et al* used a chemical bioinformatics approach to identify TWIST1 inhibitors, and identified the harmala alkaloid, harmine. It was able to inhibit cellular events linked to TWIST1 function in non-small cell lung cancer, including single cell dissemination and proliferation, and inhibit cell growth through the induction of oncogene-induced senescence by degrading the TWIST1 protein (Yochum et al., 2017). However, harmine cannot be classed as a true pharmacological inhibitor because it prevented the function of TWIST1 through targeted protein degradation.

The effect of harmine treatment on the medulloblastoma cells was investigated in 2D and 3D culture (spheroids). The data revealed that harmine treatment reduced cell metabolic activity in a dose-dependent manner. High concentrations of harmine in 2D culture (20 μ M; ONS-76 and HD-MB03, and 5 μ M; D283) resulted in TWIST1 protein degradation (Figure 4.2, $p < 0.05$), but had no effect on *TWIST1* gene expression, a result comparable to Yochum *et al*, suggesting that the effect of harmine on TWIST1 occurred through a post-

transcriptional mechanism. A more recent study by Nafie *et al* used harmine to inhibit TWIST1 in breast cancer cells and revealed that harmine-induced TWIST1 degradation was proteasome-dependent (Nafie et al., 2021).

When assessing cell morphology in the HD-MB03 cells following treatment with concentrations of harmine that caused a significant reduction in TWIST1 protein expression (10 μ M and 20 μ M), we observed no morphological change, and instead an apparent reduction in cell proliferation in comparison to the vehicle control in both 2D and 3D culture. Assessment of the viability of harmine treated cells revealed no significant difference compared to the vehicle control. This demonstrated that harmine was not toxic to the cells at the tested concentrations but instead had a cytostatic effect. Nafie *et al* observed a similar trend, whereby harmine treatment on breast cancer cells degraded TWIST1 protein but did not cause significant changes in cell viability (Nafie et al., 2021). Due to the reduction in cell proliferation, our data suggests that when TWIST1 protein was degraded the cells might have entered into a senescent state, further agreeing with the findings from Yochum *et al* and the known cellular events linked to TWIST1 function in evading oncogene-induced senescence (Smit and Peeper, 2008, Burns et al., 2013). To confirm that the cells had entered a senescence state β -Galactosidase activation could be measured.

To assess TWIST1's function, we investigated two 3D models of cellular invasion and migration for their ability to recapitulate medulloblastoma migration. *In vitro* 3D tumour models offer a more accurate representation of the tumour microenvironment within a laboratory setting. They offer the opportunity to recapitulate the complexity of tumours *in vivo*, including the cell architecture to mimic cell-cell and cell-matrix interactions and also physiological gradients (Monteiro et al., 2020).

The first model was a 3D spheroid model, which placed 3D spheroids on a collagen IV-coated matrix (one of the major components of the basement membrane), to assess cell migration after harmine treatment (Rowe and Weiss, 2008, Cox, 2021). Unfortunately, cells failed to migrate across the collagen IV matrix. Vehicle control experiments suggest that this was not due to the cytostatic effect of harmine but more likely a matrix suitability issue. Our laboratory group has recently developed two other 3D models to recapitulate cell migration. The first utilised 3D spheroids on a hyaluronan (HA) matrix which resulted in cells migrating across the matrix and away from the main spheroid body (Roper et al., 2021). The second study encapsulated single cells on 3D hydrogels composed of HA, and identified subgroup-specific ECM components that supported the nodular growth of SHH tumours (Laminin) and the laminar growth of Group 3 tumours (Vitronectin) (Linke et al., 2021). Both of these models offer alternative methods to investigate TWIST1 function in cell migration across a more complex matrix.

A modified Boyden chamber assay using collagen IV and laminin coated transwell inserts was used as an alternative approach to investigate cell migration and invasion following harmine treatment. To assess cell migration and invasion, cells were starved in 2 % FBS 24 hours prior to seeding *and* upon seeding onto the coated transwell inserts. The reduction in the percentage of FBS from the cells standard medium ensured that both the vehicle and harmine treated cells did proliferate before migrating, allowing for only the functional effect of TWIST1 in cell migration to be investigated and mitigating the cytostatic effect of harmine. Cell migration was reduced in all three cell lines ($p < 0.05$) upon a significant reduction in TWIST1 protein. Even when TWIST1 protein expression was not reduced, a small reduction in cell migration was still observed in the HD-MB03 cell line, demonstrating that TWIST1 protein

degradation was not the sole cause of impaired TWIST1 function. This result was also in agreement with the study performed by Yochum *et al*, whereby cells treated with 1 μ M and 5 μ M of harmine still caused a modest reduction in cell dissemination, with TWIST1 protein being degraded at much higher concentrations (> 20 μ M) (Yochum et al., 2017).

7.3.2 TWIST1 expression correlates with medulloblastoma cell migration

The second approach to inhibit TWIST1 was by RNA interference (RNAi). RNAi is a natural process occurring within cells to regulate gene expression via the degradation of mRNA. There are many techniques available to selectively knockdown genes. siRNA offers a simple, transient approach compared to shRNA which allows for a stable genetic knockdown (Rao et al., 2009). In this chapter, TWIST1-knockdown was performed in the three metastatic cell lines via lentiviral transduction using three different shTWIST1 clones. Combinations of lentivirus and different multiplicity of infections (MOIs; 0.3, 0.5, and 1) were employed to determine the most effective knockdown. Only one clone produced a stable knockdown in 2 out of the 3 cell lines (ONS-76 and D283). For the HD-MB03 cell line, TWIST1 expression started to increase again, even after selection with puromycin. Unless genome sequencing was performed, it is impossible to know where the shTWIST1 clones had been inserted and it might have been in areas which were not highly transcribed, therefore, preventing genetic knockdown. Another reason for this effect could be that knockdown of TWIST1 might be toxic to the HD-MB03 cells and only cells which produced an insufficient knockdown were selected.

When the ONS-76 and D283 TWIST1 knocked-down cell lines were used in the 3D transwell migration and invasion assay a significant reduction in cell

migration compared to the non-silencing control was observed (Figure 4.14, $p < 0.01$). Even though there was a transient TWIST1 knock-down for the HD-MB03 cell line, the magnitude of the knockdown significantly correlated with reduced cell migration ($p = 0.0013$). This data agrees with the results obtained from the TMA study, whereby high levels of TWIST1 in the primary tumour resulted in metastatic disease, and highlights the importance of high TWIST1 expression as a significant factor in driving medulloblastoma metastasis.

Whilst the 3D transwell migration and invasion model offered a simple, inexpensive approach to explore the functional role of TWIST1 in medulloblastoma metastasis, the development of more complex and accurate 3D models that better represent the brain ECM will be required to capture the specific interactions and functions of TWIST1 in medulloblastoma metastasis. This will help translate these findings into more relevant therapeutics for medulloblastoma patients. Future work should also try to overcome the transient TWIST1 expression observed in the HD-MB03 cell line or eliminate the residual protein remaining after chemical and genetic inhibition by using techniques that can induce TWIST1 expression or completely remove TWIST1 expression through CRISPR-Cas 9 technology.

7.4 TWIST1 and ABCB1; a partnership in medulloblastoma cell migration?

Patients with recurrent medulloblastoma often have dismal outcomes due to treatment failure even after receiving aggressive treatment approaches, including the use of high-dose chemotherapy regimens. Metastatic disease is a key risk factor for treatment failure. It is believed that a distinct set of sub-clones from the primary tumour are observed in the metastatic tumour, with this clonal

selection driving drug resistance and treatment failure in metastatic medulloblastoma tumours (Wu et al., 2012, Juraschka and Taylor, 2019). The multidrug transporter ABCB1 has been linked to chemo-resistance in many cancers (Gottesman et al., 2016), and increasing evidence suggests that it is also involved in tumour migration and invasion. Data from our research group have identified an important role for ABCB1 during metastasis, with positive expression associated with high-risk medulloblastoma tumours (Othman et al., 2014) and metastatic disease (Nasir and Cardall et al., 2021) ($p = 0.035$ and $p = 0.04$ respectively). Functional inhibition with the known ABCB1 inhibitor vardenafil (Ding et al., 2011), decreased cell migration in ependymoma tumours and attenuated cell aggregation in medulloblastoma cells (Sabnis et al., 2019, Nasir and Cardall et al., 2021). Furthermore, studies by Saxena *et al* and Lui *et al*, have both shown a correlation between TWIST1 and ABCB1 expression in breast and colon cancer, respectively (Saxena et al., 2011, Liu et al., 2017), leading us to investigate TWIST1 and ABCB1 in medulloblastoma metastasis

7.4.1 Does TWIST1 bind to ABCB1?

Associations between proteins and DNA are vital for many cellular functions including gene transcription, DNA replication and cell cycle progression (Das et al., 2004). Chromatin immunoprecipitation (ChIP) is a widely used technique that allows for the study of protein-DNA interactions. We used ChIP to determine if TWIST1 could bind to the promoter region of ABCB1 in metastatic medulloblastoma cell lines. Initial ChIP analysis was performed to determine which predicted E-boxes were functionally active for all three genes (ABCB1, GAPDH and SNAI2). Interestingly, the E-box sequence 'CATCTG' only showed enrichment with the TWIST1 antibody compared to the IgG negative control (which detects non-specific binding) for both ABCB1 and GAPDH in the D283

cell line ($p < 0.05$). TWIST1 ChIP-Seq analysis by Bildsoe *et al* also identified that TWIST1 preferred to bind to the CATCTG sequence (Bildsoe *et al.*, 2016).

ChIP analysis revealed that significant differences for TWIST1 immunoprecipitation were only observed for the D283 cell line. *ABCB1* consists of 28 exons and 2 reported promoters (Reed *et al.*, 2008, Fung and Gottesman, 2009), and searching the transcript revealed numerous TWIST1 E-boxes. We initially took the approach to design and test primers covering the E-boxes located within both promoter regions. Unfortunately, we were unable to design primers that were efficient enough for qRT-PCR analysis of the E-boxes located in the first promoter.

7.4.2 A regulatory relationship between TWIST1 and ABCB1?

There are many studies indicating a functional relationship between TWIST1 and ABCB1. Zhu *et al* knocked down TWIST1 using shRNA in cervical cancer cells and revealed a significant decrease in ABCB1 protein expression leading to a reduction of its function in drug efflux (Zhu *et al.*, 2012). Li *et al* demonstrated a positive correlation between TWIST1 and ABCB1 expression in liver cancer samples, and knocking down TWIST1 also decreased ABCB1 expression, leading to an increase in cytotoxicity of chemotherapeutic drugs and inhibited the migration of liver cancer cells by reversing the EMT to MET (mesenchymal-epithelial transition) (Li *et al.*, 2018a). To further explore the functional relationship between TWIST1 and *ABCB1* during medulloblastoma metastasis, we inhibited TWIST1 and ABCB1 using chemical (harmine) and genetic (shRNA) methods optimised in Chapter 4, and with vardenafil, respectively. We analysed the effects this had on ABCB1 gene and protein expression, and medulloblastoma cell migration. When we knocked-down TWIST1 with shRNA we observed a decrease in *ABCB1* gene expression, and a dose-dependent decrease in ABCB1 gene and protein expression following

harmine treatment in the HD-MB03 cell line (Figure 5.7). This result also echoes the effect of harmine treatment on TWIST1's function in Chapter 4; that even without a visible reduction in TWIST1 protein expression, it could still impair TWIST1's functional effect on downstream targets.

Increasing evidence from our research group has indicated a role for ABCB1 in medulloblastoma metastasis. Investigations using a basement membrane extract (BME) model showed increased cell aggregation in highly expressing *ABCB1* cells which could be attenuated by vardenafil (Nasir and Cardall et al., 2021). Whilst the BME model showed cell movement, it did not represent cellular invasion from one area to another, a characteristic of metastatic disease (Turajlic and Swanton, 2016).

We used the previously optimised 3D migration and invasion transwell model, which allowed for the invasion of cells through a simple matrix from one compartment to another. When we inhibited ABCB1 with vardenafil or TWIST1 with harmine in the metastatic ONS-76 and HD-MB03 cell lines, cell migration decreased. Although, a greater reduction in cell migration was seen for both cell lines following harmine treatment compared to vardenafil treatment, indicating that TWIST1 is regulating other downstream targets that are involved in medulloblastoma metastasis. When harmine and vardenafil were used in combination an additive effect was observed only for the HD-MB03 cell line and not for the ONS-76 cells. Furthermore, harmine treatment resulted in a much greater decrease of ABCB1 expression for the Group 3 cell lines (D283 and HD-MB03) compared to the SHH cell line (ONS-76), suggesting that *ABCB1* regulation could be more reliant on TWIST1 mediated transcription in Group 3 tumours compared to SHH tumours, which also agrees with the ChIP qRT-PCR data that shows a greater enrichment with the TWIST1 antibody for the D283 (Group 3) cell line compared to the ONS-76 (SHH) cell line (Figure 5.6). Whilst

ChIP analysis did not conclude a definitive answer for TWIST1 binding to *ABCB1* for two out of the three cell lines, the decrease in *ABCB1* expression levels following TWIST1 inhibition does indicate that TWIST1 could be regulating *ABCB1*.

Interestingly, *ABCB1* gene expression was significantly lower in the least metastatic subgroup, WNT, compared to the other three subgroups, and subgroup-specific analysis revealed that the medulloblastoma cell lines had comparable *ABCB1* levels regardless of metastatic status. Our data supports a regulatory relationship between TWIST1 and *ABCB1* and both appear to play a functional role in medulloblastoma metastasis. As *ABCB1* was expressed in both metastatic and non-metastatic cell lines, it highlights the importance of *ABCB1* expression as an indicator of metastatic disease and chemo-resistance, driven by TWIST1. Othman *et al* observed a subpopulation of cells with membranous *ABCB1* positive expression in medulloblastoma tumour samples, and Nasir and Cardall *et al* found TWIST1 to be expressed in cells at the migratory edge of an *in vivo* metastatic medulloblastoma model (Othman *et al.*, 2014, Nasir and Cardall *et al.*, 2021). It would be of interest to perform co-staining of TWIST1 and *ABCB1* in medulloblastoma tumour samples, by immunofluorescence, to determine if they are expressed in the same subpopulation of cells that are responsible for medulloblastoma metastasis.

7.5 Uncovering TWIST1's regulatory network during medulloblastoma metastasis

So far, we have confirmed that TWIST1 expression correlates with metastatic disease and plays a crucial role in driving medulloblastoma metastasis. However, the overall aim of this thesis was to understand the functional

mechanisms defining TWIST1 in medulloblastoma metastasis in order to gain a deeper understanding of the TWIST1 regulated genes and pathways that are involved in the migratory process. To investigate this, ChIP-Sequencing was performed using the three metastatic medulloblastoma cell lines from two subgroups (SHH; ONS-76 and Group 3; D283 and HD-MB03), that had been shown to express high level of TWIST1. Whilst we know TWIST1 is expressed across all four subgroups, protein levels were barely detectable in the Group 4 cell lines, and WNT tumours rarely metastasise, therefore, analysis was not performed for these subgroups. Due to the heterogeneity of the medulloblastoma subgroups, we took the approach to identify any differences and similarities of the TWIST1 regulated pathways for these two subgroups (Khatua et al., 2018, Juraschka and Taylor, 2019).

7.5.1 TWIST1 ChIP-Sequencing

ChIP-Seq allows for the characterisation and mapping of protein-DNA interactions at genomic locations (Das et al., 2004, Mundade et al., 2014). The technique is not limited to identifying genes that are regulated by transcription factors, but can be used to study the effect of histone modifications, such as acetylation and methylation, on gene regulation (O'Geen et al., 2011).

We initially set out to analyse TWIST1's transcriptional network across the mentioned SHH and Group 3 cell lines. Unfortunately, after peak calling, the proportion of shared peaks between the HD-MB03 biological replicates was vastly smaller compared to the other two cell lines and compared to studies that have used the same peak calling software; *MACS2* (Matsuda et al., 2017). The reason for this could have been the low starting concentration of the sample, leading to over amplification of DNA fragments during library preparation, and hence resulting in a high proportion of duplicates after sequencing and reducing the complexity of the library. We therefore decided to only use the D283 cell

line to represent the Group 3 subgroup. Many protocols suggest that 1×10^5 – 1×10^6 cells are required for each immunoprecipitation. We used 1×10^6 cells, but due to the low concentrations of some of the libraries, it would be beneficial to increase the number of cells for future ChIP work.

The distribution of TWIST1 binding peaks was analysed relative to the transcription start site (TSS) and the nearest genomic feature. Our analysis revealed that many of the binding sites were enriched around the TSS and slightly downstream of this feature, with the highest proportion of peaks located at intronic and distal intergenic regions. Only a small percentage of peaks were observed within the promoter region. Whilst it may be expected that a transcription factor would predominately bind at a promoter region, this data is consistent with other published studies on TWIST1 ChIP-Seq data (Dozmorov et al., 2015, Bildsoe et al., 2016). In addition, when we analysed the *ABCB1* transcript for E-boxes, the order of the genomic features did not follow a typical order, i.e. exon 1 did not follow the second promoter, and instead the second promoter was located in exon 3, with the TSS located further downstream. A study by Devailly and Joshi investigated 1735 ChIP-Seq datasets from mouse and human cell types to unravel the diverse features of transcription control. They found that transcription factors tend to have a preferred genomic location (i.e. exons, introns, promoters etc), which was evident when comparing our ChIP-Seq data to other TWIST1 datasets showing that TWIST1 binding locations were clustered around intronic and distal regions (Devailly and Joshi, 2018).

Overall, the ChIP-Seq data suggests that TWIST1 is not only initiating the transcription of some of these genes (due to binding at regions located near the TSS), but because of the enrichment observed downstream of the TSS,

TWIST1 could also be controlling gene transcription by binding to regulatory elements that are located far from the gene start site.

7.5.2 TWIST1 downstream targets and pathways

Bioinformatics analysis of the ChIP-Seq dataset revealed many shared TWIST1 target genes across the two subgroups, but also subgroup-specific downstream targets. The following sections will discuss the validation of the ChIP-Seq dataset and also the importance of TWIST1's binding partners, and the potential functional mechanism of TWIST1 and ABCB1 in medulloblastoma metastasis.

ChIP-Seq validation

Six TWIST1 downstream target genes (*BSG*, *FBXL17*, *HYAL2*, *IGF1*, *IGF1R* and *SORCS2*) were identified for validation for the ChIP-Seq data. Of these genes, *IGF1R* was the only gene to be identified solely in Group 3, the remaining genes were common across both subgroups.

We utilised previous genetic and chemical repression of TWIST1 to assess gene expression of the target genes for the D283 and ONS-76 cell lines. We observed a reduction in gene expression in both cell lines for all of the genes that were common between both subgroups, and reassuringly, a reduction in *IGF1R* expression only with the D283 (Group 3) cell line. For most of the genes, the reduction in gene expression was greater following harmine treatment compared to knock-down of TWIST1 (Figure 6.8). This could be because of a greater reduction in TWIST1 protein expression with harmine compared to shTWIST1 treatment, or it might be due to harmine targeting genes upstream of TWIST1. As well as a TWIST1 inhibitor, harmine is known to inhibit DYRK1A (dual specificity tyrosine phosphorylation regulated kinase). Yang-Ling *et al*, inhibited DYRK1A with harmine in NSCLC cells which led to the inactivation of the STAT3 (signal transducers and activator of transcription) signalling pathway

(Li et al., 2019). STAT3 has been shown to induce the transcription of TWIST1 (Cheng et al., 2008), leading to the activation of *TPPP3* (tubulin polymerization promoting protein family member 3) in NSCLC (Li et al., 2018b) and the induction of the EMT program in breast cancer cells (Kim et al., 2019). Further studies could be performed to investigate the protein expression of STAT3 after harmine treatment, and ChIP analysis to confirm if it bound to TWIST1. However, if a STAT3-TWIST1 axis was confirmed, it wouldn't detract from the qRT-PCR results, that demonstrates the effect of TWIST1 inhibition on the target genes. Instead it might offer the opportunity to investigate the STAT3 signalling pathway, especially as *TPPP3* was identified as a TWIST1 downstream target in our ChIP-Seq data.

We then analysed the six genes using R2 analysis of the Cavalli patient dataset to see how their expression levels correlated with TWIST1 expression, patient survival and metastatic disease. Impressively, five out of the six genes showed a positive correlation with *TWIST1* expression when analysing SHH and Group 3 samples, suggesting that TWIST1 could be regulating the expression of these genes. High expression of *IGF1R*, *BSG*, *HYAL* and *SORCS2* was associated with worse overall survival in Group 3 patients, with a sharp drop off in patient survival in the first 24 months, which corresponds to the time when patients relapse (Massimino et al., 2016). Furthermore, expression levels of these genes were higher in patients with Group 3 metastatic disease (M+) compared to non-metastatic disease (M0), indicating that the sharp decrease in survival could be related to metastatic disease.

Whilst the analysis presented a strong validation for the ChIP-Seq dataset, Figure 6.16 details many more pathways and genes that can be further explored to understand and appreciate the complexity of TWIST1's involvement in medulloblastoma metastasis. In particular, it would be interesting to understand

more about the regulation of the ECM and how TWIST1 could be orchestrating the movement of cells through this matrix via interactions with specific integrins, collagen, laminin, and the some of the genes used for the validation of the ChIP-Seq dataset, including *BSG*, *SORCS2* and *HYAL2*.

TWIST1's binding partner

In order for TWIST1 to function it must form homo- or hetero-dimers with another protein. Many studies have shown that dimerisation with the E2A proteins is critical for the transcriptional activation of TWIST1's target genes in tumorigenesis and EMT (Fan et al., 2020). Interestingly, *TCF3*, which codes for the E2A proteins (E12 and E47), was identified in both subgroups. Harmine has been shown to target and degrade both TWIST1 and the E2A proteins, with both regulating the other's stability (Yochum et al., 2017). In addition, *TCF3* gene expression was analysed in Chapter 3 using the Cavalli dataset (Figure 3.1), and expression levels were comparable to *TWIST1* for all four subgroups.

The data presented indicates that the expression of *TCF3* could be important in regulating TWIST1's function by controlling TWIST1's dimerisation partner, especially as *TCF3* was identified in both subgroups in the ChIP-Seq data. These results open up the possibility that *TCF3* could pose as a novel target to regulate TWIST1 and prevent it exerting its functions during medulloblastoma metastasis. One hypothesis could be that if there was limited *TCF3* protein available, then this could allow Id proteins to dimerise with TWIST1, resulting in its destabilisation and ultimately degradation. Future studies into the regulation of TWIST1 during medulloblastoma metastasis would be required to understand more about which proteins TWIST1 dimerises with, which ones are essential during metastasis and the impact of inhibiting TWIST1's binding partner on medulloblastoma metastasis.

ABCB1 and metastasis

In Chapter 5 we investigated the functional relationship between TWIST1 and ABCB1. Our ChIP-Seq analysis did not identify *ABCB1* as a TWIST1 downstream target. This could be because the ChIP conditions were not optimal, or a significant peak was not observed following ChIP-Seq analysis. The latter point also correlates with the ONS-76 ChIP qRT-PCR analysis, whereby the observed enrichment using the TWIST1 antibody was not significant when compared against the IgG negative control. However, even though it was not identified, we still observed a reduction in ABCB1 expression after TWIST1 inhibition, suggesting that it might indirectly bind to *ABCB1* and there could be other intermediate genes involved in *ABCB1* regulation as highlighted in Figure 6.16.

Whilst we have shown that TWIST1 and ABCB1 reduce medulloblastoma cell migration, the exact mechanism of how ABCB1 is contributing towards medulloblastoma metastasis remains to be explored. Studies have found that ABCB1 is co-expressed on the cell surface of human foetal neural stem/progenitor cells (NSPC) along with Nestin, a NSPC marker (Yamamoto et al., 2009). Medulloblastoma tumours, in particular Group 3 medulloblastomas, are thought to arise from neural stem cells. Group 3 tumours are the most aggressive tumours out of all four subgroups and present with the highest rates of metastasis. Nestin has been implicated in regulating tumour migration and invasion in lung adenocarcinoma and pancreatic ductal adenocarcinoma, with knockdown studies inducing tight cell-cell adhesions and resulting in attenuated cell migration (Matsuda et al., 2011, Narita et al., 2014). One hypothesis could be that in Group 3 medulloblastoma tumours, the co-expression of Nestin and ABCB1 influence factors that are linked to the regulation of cells moving through

the ECM, such as integrins and cadherins, resulting in the loss of tight junctions and increasing cell migration.

Another potential mechanism could be via ABCB1's interaction with CD44. As mentioned earlier, Colone *et al*/ demonstrated a correlation between ABCB1 and CD44 in melanoma leading to increased cell migration and an increase in MMP2 (Colone et al., 2008). Figure 6.16 illustrates CD44 as a positive regulator and downstream target of TWIST1. CD44 can bind to extracellular hyaluronic acid and weaken the ability of a cell to anchor itself to the ECM (Dovedytis et al., 2020). Therefore, another hypothesis could be that TWIST1 regulates the expression of CD44 and ABCB1, which in turn could enable CD44 to aid in the destabilisation of tumour cells to the ECM, and ABCB1 to facilitate in the upregulation of MMP2, resulting in the degradation of the ECM and providing a pathway for tumour cells to migrate through.

7.6 Conclusion

This thesis has demonstrated the importance of TWIST1 expression in driving medulloblastoma metastasis. Analysis into TWIST1's transcriptional network has unravelled its functional capabilities by identifying many key pathways and genes that are contributing towards this complex process. We have shown that interactions with the ECM via cell adhesion molecules and focal adhesions are vital in facilitating medulloblastoma cell migration in both the SHH and Group 3 subgroups.

The results from the ChIP-Seq analysis will allow for more in-depth investigations to explore the many different pathways regulated by TWIST1 during medulloblastoma metastasis. It would be of interest to analyse TWIST1's preferential binding partner during dimerisation as this would be pivotal in understanding which partners are important in promoting tumourigenesis in medulloblastoma. In addition, the incorporation of more specialised 3D models would facilitate in exploring TWIST1's interactions with the ECM, especially by inhibiting some of the key genes identified in the Group 3 subgroup such as *ITGB1*, *COL4A1* and *COL4A2*.

Overall, TWIST1 expression is crucial in initiating medulloblastoma metastasis and therapeutically targeting TWIST1 could be key to preventing metastatic disease and improving patient overall survival.

PhD project outputs

This project was funded by the Biotechnology and Biological Sciences Research Council (BBSRC) Doctoral Training Partnership (DTP). It was a four-year PhD programme consisting of three seven-week research projects completed before starting the PhD project. During the programme, a three-month professional internship is also undertaken.

Laboratory rotations

October – December 2016: ‘Regulation of proliferation in stem cells: a pivotal role for Elk-1 ubiquitylation’ supervised by Professor Peter Shaw.

January – March 2017: ‘ABC transporter: a unifying force in tumour invasion and drug resistance?’ supervised by Dr Beth Coyle and Dr Ian Kerr.

March – May 2017: ‘Galectin-3 and Laminin receptor as a platform for pathogenic bacteria’ supervised by Dr Karl Wooldridge.

Publications

- Nasir, A*, Cardall, A*, Othman, R. T., Nicolaou, N., Lourdasamy, A., Linke, F., Onion, D., Ryzhova, M., Cameron, H., Valente, C., Korshunov, A., Pfister, S.M., Grabowska, A.M., Kerr, I.D., Coyle, B; “ABCB1 inhibition provides a novel therapeutic target to block TWIST1 induced migration in medulloblastoma”. *Neuro-Oncology Advances*, 2021 (in press).
- Thompson, J.L*, Downie Ruiz Velasco, A*, Cardall, A*, Tarbox, R*, Richardson, J*, Clarke. G., Lister. M., Howson-Wells, H.C., Fleming,

V.M., Khakh, M., Sloan, T., Duckworth, N., Walsh, S., Denning, C., McClure, C.P., Benest, A.V., Seedhouse, C.H; “Comparative effects of viral transport medium heat inactivation upon downstream SARS-CoV-2 detection in patient samples”. *Journal of Medical Microbiology*, 2020 (in press). <https://doi.org/10.1101/2020.07.30.20164988>

*Joint first authors

Conference Papers

- Cardall, A., Linke, F., Kerr, I.D., Coyle, B: MBRS-45. TWIST1 and ABCB1 are functional determinants of metastasis in medulloblastoma, *Neuro-Oncology*, Volume 22, Issue Supplement_3, December 2020, Page iii406, <https://doi.org/10.1093/neuonc/noaa222.554>
- Cardall, A., Nasir, A., Linke, F., Grabowska, A.M., Kerr, I.D., Coyle, B; MBRS-39. TWIST1 plays a regulatory role in medulloblastoma metastasis, *Neuro-Oncology*, Volume 20, Issue suppl_2, June 2018, Pages i136–i137, <https://doi.org/10.1093/neuonc/nyy059.484>

Oral presentations

- June 2019: Medicine and Health Sciences Faculty Postgraduate Research Forum
- November 2018: Sue Watson School of Medicine Postgraduate Research Forum
- September 2018: Children’s Brain Tumour Research Centre Research Day
- April 2017 – 2019: Annual BBSRC DTP Spring School presentations

Poster presentations

- December 2020: International Symposium on Paediatric Neuro-Oncology (Nagano, Japan. Virtual meeting)
- June 2019: Medicine and Health Sciences Faculty Postgraduate Research Forum
- September 2018: Cancer Research Nottingham Symposium
- July 2018: International Symposium on Paediatric Neuro-Oncology (Denver, United States)
- April 2018: BBSRC DTP Spring School

Awards and Achievements

- March 2020 – March 2021: Chair of the Biodiscovery Institute ECR committee
- April 2019: Received the UNICAS Graduate School Sandpit grant (£5,000)
- June 2018: Received the University of Nottingham Graduate School Travel Prize (£600 to attend the International Symposium on Paediatric Neuro-Oncology conference in Denver, USA)
- June 2018: Received the University of Nottingham School of Medicine Postgraduate Research Development Fund (£600 to attend the International Symposium on Paediatric Neuro-Oncology conference in Denver, USA)

Professional Internship

Note to examiners: This statement is included as an appendix to the thesis in order that the thesis accurately captures the PhD training experienced as a BBSRC Doctoral Training Partnership student. The Professional Internship for PhD Students (PIPS) is a compulsory three-month placement which must be undertaken by DTP students. It is usually centred on a specific project and must not be related to the PhD project. This reflective statement is designed to capture the skills development which has taken place during the student's placement and the impact on their career plans it has had.

September 2019 – December 2019: 'Communicating the impact of science research' with Cancer Council NSW, Australia.

Having the opportunity to undertake a three-month internship that was unrelated to my PhD was an exciting experience that allowed me to explore a different career path. I decided that I wanted to gain experience outside of a laboratory environment, but still within cancer research. I also wanted to take this opportunity to complete my internship in a different country and therefore contacted Cancer Council NSW, Australia's leading cancer charity.

Cancer Council NSW, is a not-for-profit organisation, working across every area of every cancer. The organisation provides support to families affected by cancer and invests in cancer research. During my time at Cancer Council NSW, I worked with the Research Communications Specialist Division to complete several writing projects to communicate the compelling impacts of scientific research that the organisation had funded. These projects required interviewing researchers, translating research outcomes and applications into lay language, and sourcing up-to-date statistics on cancer in Australia.

The outcomes of these writing projects were in the form of blog posts, articles and project outcome summaries that were posted on the organisations website. One of these projects was entitled 'Five-year look back stories', which involved interviewing researchers whose funded had finished five years ago, and discussing the outcomes and impacts that funding from Cancer Council NSW had made towards cancer research.

I also supported my team more broadly on other projects including:

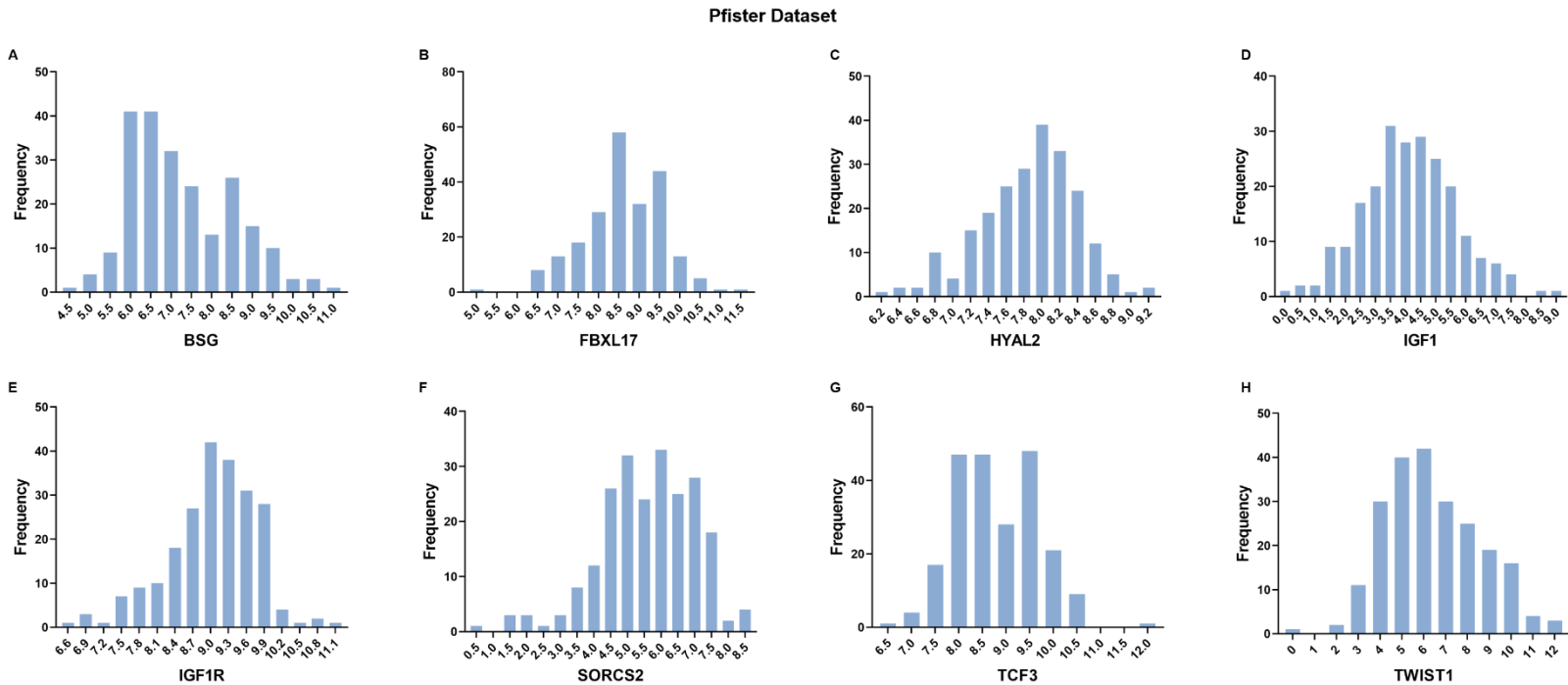
- Developing the annual research highlights flyer
- Supporting preparations for their annual consumer review panel
- Developing social media content related to Cancer Council research
- Updating researchers biographies

In addition, I had the opportunity to learn about the work carried out by the other divisions, including Cancer Prevention and Advocacy, Cancer Information and Support Services, and Marketing and Fundraising.

Overall, I really enjoyed my time at Cancer Council NSW. It was a great opportunity to learn about how a cancer organisation works and the role of each of the different divisions. I particularly enjoyed interviewing scientists to discover the amazing research they had done and communicate their research outcomes to the wider public.

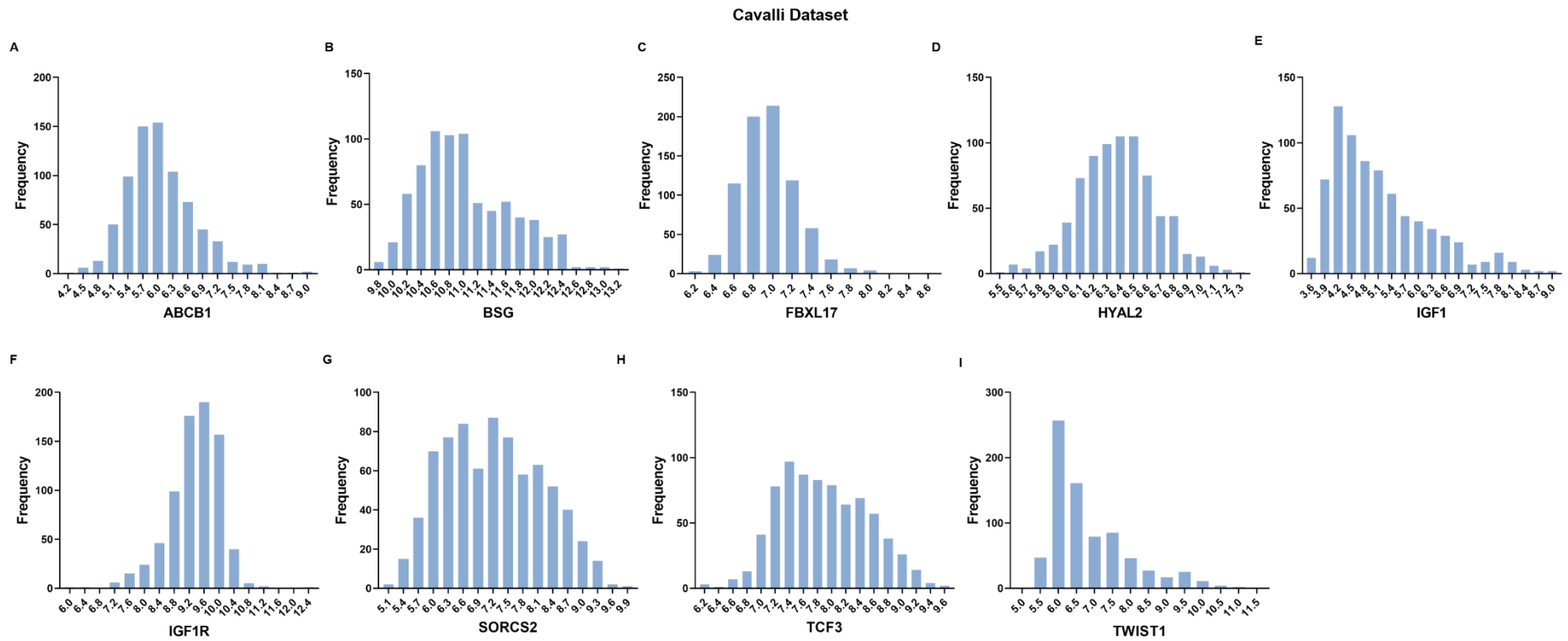
Appendices

Appendix 1



Appendix Figure 1. Distribution graphs for the genes analysed in the Pfister dataset

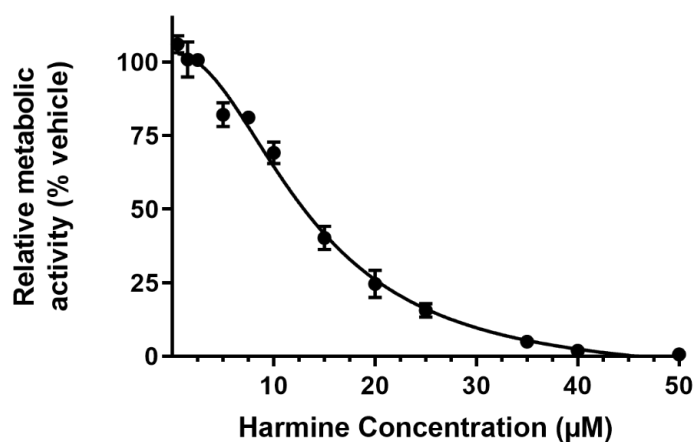
Histograms of *BSG* (A), *FBXL17* (B), *HYAL2* (C), *IGF1* (D), *IGF1R* (E), *SORCS2* (F), *TCF3* (G), and *TWIST1* (H) gene expression for the Pfister patient dataset.



Appendix Figure 2. Distribution graphs for the genes analysed in the Cavalli dataset

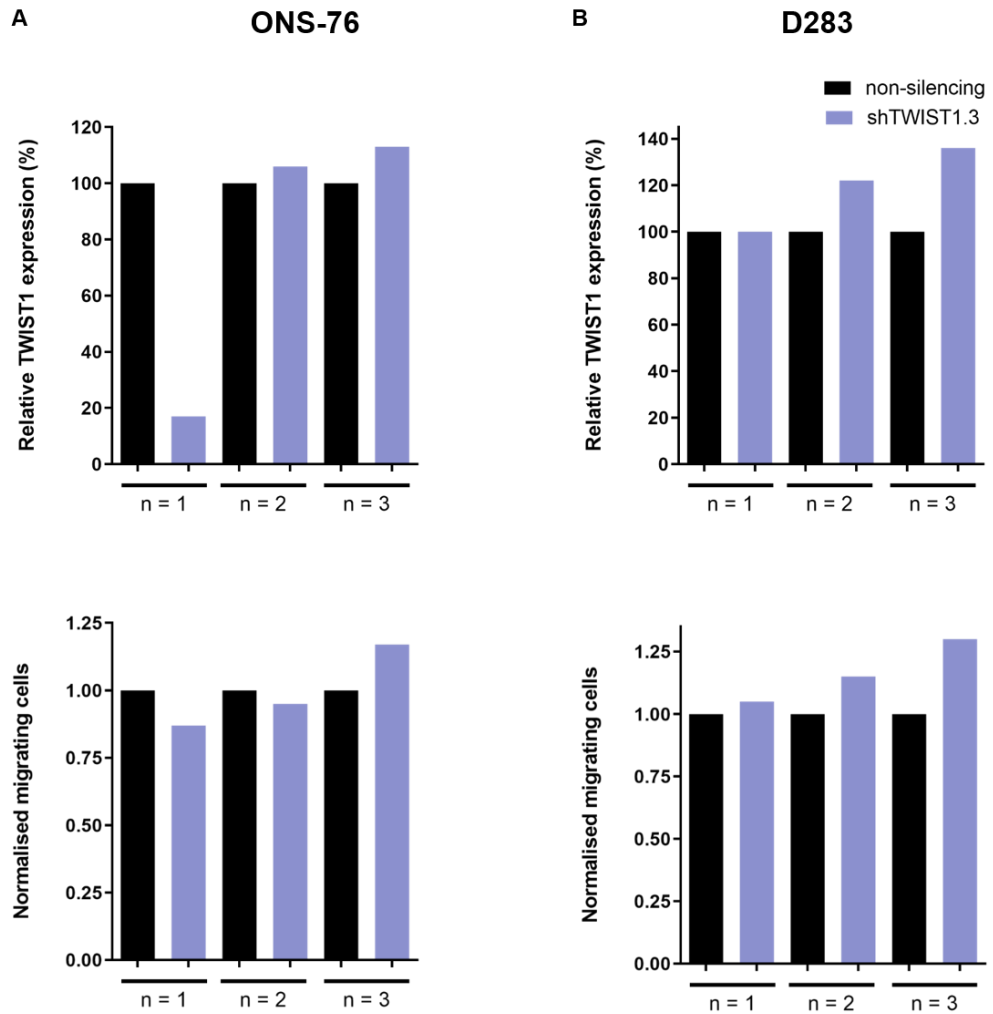
Histograms of *ABCB1* (A), *BSG* (B), *FBXL17* (C), *HYAL2* (D), *IGF1* (E), *IGF1R* (F), *SORCS2* (G), *TCF3* (H), and *TWIST1* (I) gene expression for the Cavalli patient dataset.

Appendix 2



Appendix Figure 3. Relative metabolic activity of HD-MB03 medulloblastoma 3D spheroids after treatment with harmine.

HD-MB03 spheroids were treated with increasing concentrations of harmine for 72 hours. The CellTiter-Glo® assay was performed to analyse the metabolic activity of cells which was calculated as a percentage of the vehicle treated control. Dose response curves were generated using non-linear regression analysis and error bars represent the mean $\pm$ SEM of $n = 3$ experiments each containing 3 replicates.



Appendix Figure 4. ONS-76 and D283 TWIST1 expression and cell migration after shTWIST1 using the 3D migration assay

TWIST1 gene expression increases in the ONS-76 (A) and D283 (B) cell line after transduction with the shTWIST1.3 clone at an MOI 0.5. Gene expression was calculated relative to a house-keeping gene *HPRT* and normalised to the non-silencing control using the $2^{-\Delta\Delta Ct}$ method. Cell numbers were calculated against a standard curve of known numbers and normalised relative to the non-silencing control.

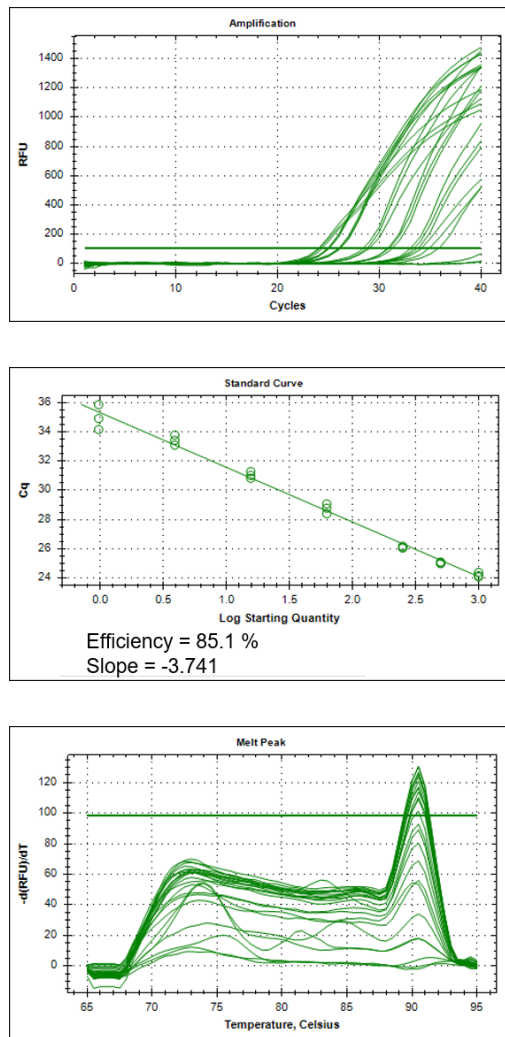
Appendix 3

A -1272 AATAGCATTGTACCTTGAAATACCTGTCTCTATATATTTTTAAACTTCCTTTTTCTTTCATTCCATTTATCATC
-1195 AAATAAAGGATGAACAGATGTAACTCAGAAACTGTCAAGCATGCTGAAGAAAGACCACTGCAGAAAAATTTCTC
-1121 CTAGCCTTTTCAAAGGTGTTAGGAAGCAGAAAGGTGATACAGAATTGGAGAGGTCGGAGTTTTTGTATTAAGTG
-1047 TATTAATGCGAATCCCGAGAAAAATTTCCCTTAACACGTCCTGTAGTTATATGGATATGAAGACTTATGTGAAC
-972 TTTGAAAGACGTGTCTACATAAGTTGAAATGTCCCCAATGATTCAGCTGATGCGCGTTTCTCTACTTGCCCTTTC
-897 TAGAGAGGTGCAACGGAAGCCAGAACATTCCCTCGGAAATTC AACCTGTTTCGCAGTTTCTCGAGGAATCAG
-824 CATTCACTCAATCCGGGCCGGGAGCAGTCATCTGTGGTGAGGCTGATTGGCTGGGCAGGAACAGCGCCGGG
-752 GCGTGGGCTGAGCACAGCCGCTTCGCTCTCTTTGCCACAGGAAGCCTGAGGTCATTTCGAGTAGCGGCTCTTC

B -421 CCTGTTCGACAGTCAGCCGCATCTTCTTTTGCCTCGCCAGGTGAAGACGGGCGGAGAGAAACCGGGAGGC
-351 TAGGGACGGCCTGAAGGCGGCAGGGGCGGGCGCAGGCCGGATGTGTTTCGCGCCGCTGCGGGGTGGGCCC
-282 GGGCGGCCTCCGCATTGCAGGGGCGGGCGGAGGACGTGATGCGGCGCGGGCTGGGCATGGAGGCCCTGGT
-213 GGGGAGGGGAGGGGAGGCGTGTGTGTCGGCCGGGGCCACTAGCGCTCACTGTTCTCTCCCTCCGCGCA
-143 GCCGAGCCACATCGCTCAGACACCATGGGGAAGGTGAAGGTGCGAGTCAACGGGTGAGTTTCGCGGGTGGCT
-72 GGGGGGCCCTGGGCTGCGACCGCCCCGAACCGCGTCTACGAGCCTTGCGGGTCCGGGTCTTTGCAGTC
-2 GTATGGGGGCAGGGTAGCTGTTCCCGCAAGGAGAGCTCAAGGTGAGCGCTCGGACCTGGCGAGCCCCG
69 CACCCAGGCTGTGGCGCCCTGTGCAGTCCGCCCTTGCGGCGCCATCTGCCCCGAGCCTCCTTCCCTAGT
140 CCCCAGAAACAGGAGGTCCCTACTCCCGCCCGAGATCCCGACCCGGACCCCTAGGTGGGGGACGCTTTCTTT
212 CCTTTCGCGCTCTGCGGGGTCAGCTGTCGCAGAGGAGCCCTCCCCACGGCCTCCGGCACGCGAGGCCCC

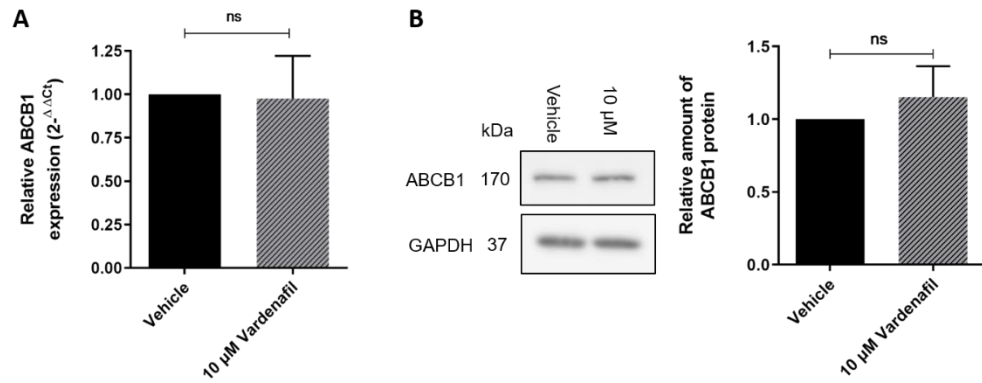
Appendix Figure 5. Putative TWIST1 binding sites located on *ABCB1* and *GAPDH*

Putative TWIST1 binding sites located on *ABCB1* (A) and *GAPDH* (B) promoter regions. All red text indicated E-boxes identified by manual searching of the genes promoter regions, red underlined text indicates E-boxes identified by Genomatix, and blue text highlights the start codon.



Appendix Figure 6. GAPDH primer efficiency testing

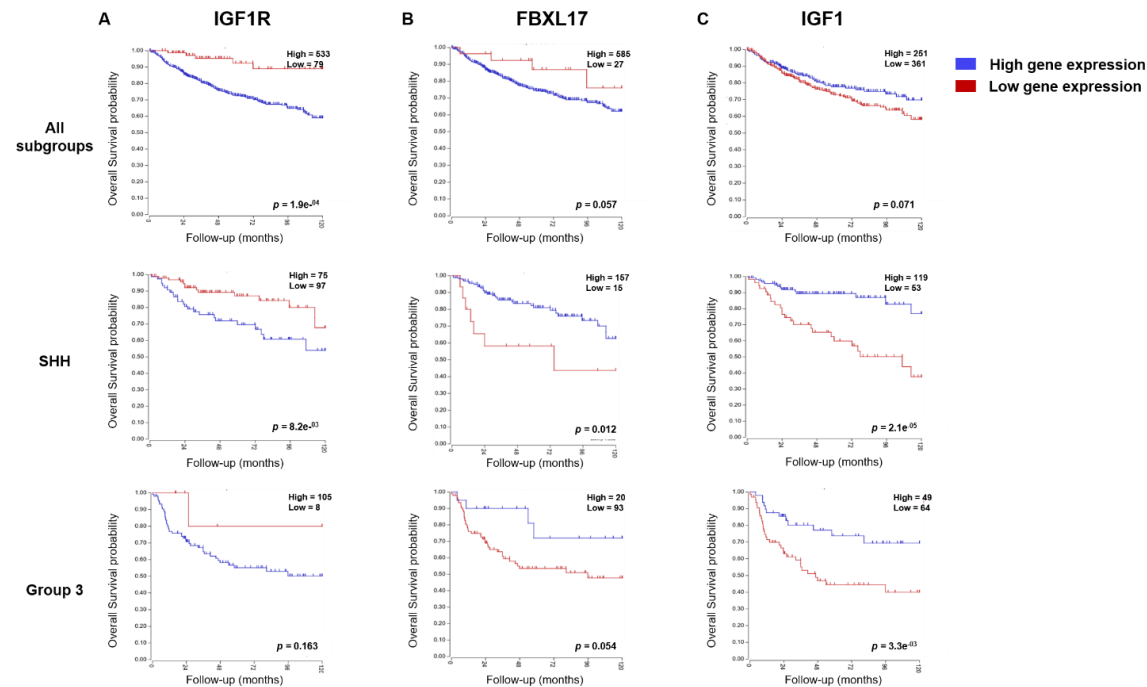
The primer efficiency of the *GAPDH* primers covering the 'CACTTG' E-box. The efficiency = 85.1% and the slope = -3.741, both of which failing to fall within the parameters identified in



Appendix Figure 7. ABCB1 gene and protein expression following vardenafil treatment

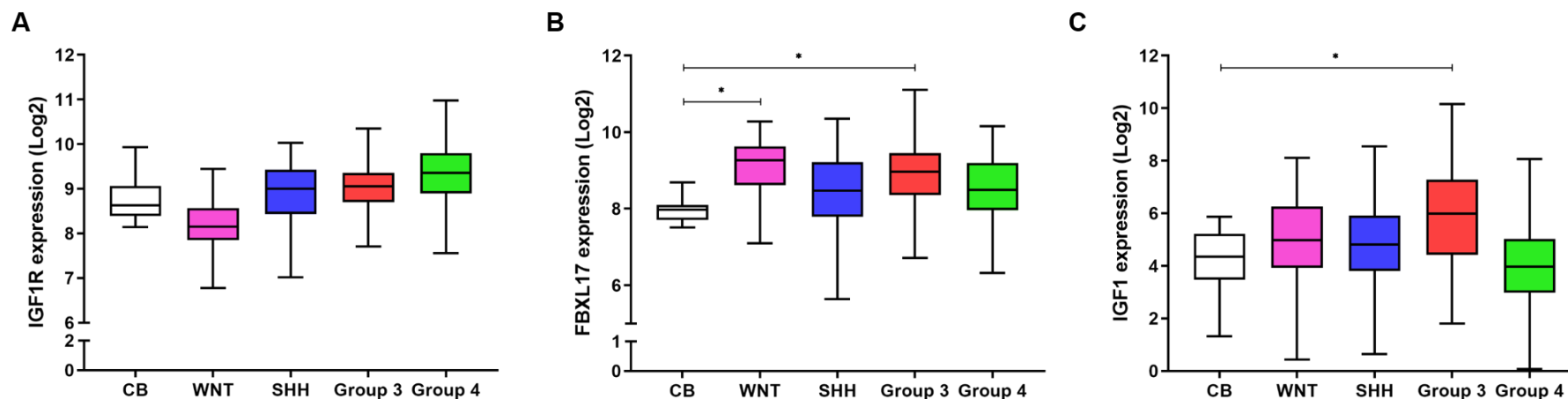
Relative *ABCB1* gene expression in the HD-MB03 cell line, following treatment with 10 μ M vardenafil for 72 hours. *ABCB1* gene expression was calculated relative to the house-keeping gene *GAPDH* and normalised to the vehicle control using the $2^{-\Delta\Delta ct}$ method. ABCB1 protein expression was analysed after HD-MB03 cells were treated 10 μ M vardenafil for 72 hours. 50 μ g of total protein was resolved on 10 % acrylamide gels by western blotting (B). Western blots are shown as split panels as the nitrocellulose was cut prior to incubation with anti-ABCB1 or anti-GAPDH antibodies. Protein expression was quantified using Fiji imaging software and normalised to the vehicle control (B). Error bars represent the mean $\pm$ SEM of $n = 3$ experiments. Statistical significance was calculated using a paired Student's t-test comparing to the vehicle control (ns = not significant).

Appendix 4



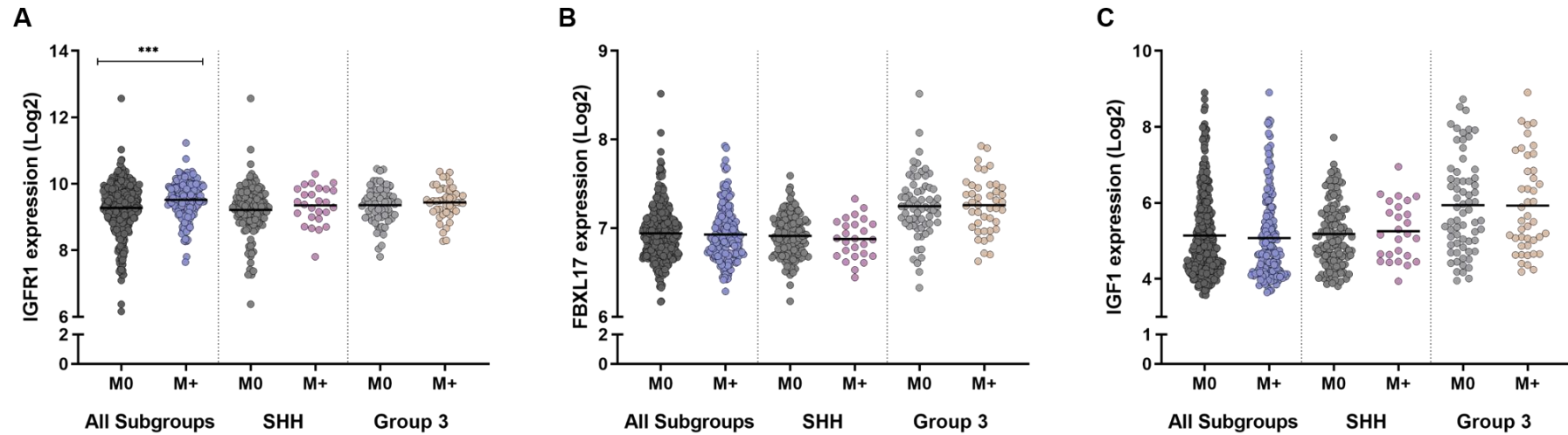
Appendix Figure 8. Kaplan Meier survival curves of the overall survival probability for high and low gene expression of *IGF1R*, *FBXL17* and *IGF1* in medulloblastoma patient samples.

Kaplan Meier survival curves of the overall survival probability in a 120 month follow-up period based on high or low gene expression for *IGF1R* (A), *FBXL17* (B), and *IGF1* (C). Overall survival probability for all molecular subgroups (n = 612), SHH subgroup (n = 172), and Group 3 subgroup (n = 113) was assessed using the Cavalli dataset. Statistical significance was calculated using the logrank test.



Appendix Figure 9. Gene expression of *IGF1R*, *FBXL17* and *IGF1* in medulloblastoma subgroups compared to the normal cerebellum.

Gene expression (Log2) of *IGF1R* (A), *FBXL17* (B), and *IGF1* (C) was assessed using R2 Genomics Analysis and Visualization Platform. The MegaSampler function was used to assess gene expression in two datasets: Roth (normal cerebellum (CB) n=9) and Pfister (WNT n = 17, SHH n = 59, Group 3 n = 56 and Group 4 n = 91). Significant differences in gene expression compared to the normal cerebellum were calculated using either Kruskal-Wallis test with Dunn's multiple comparison post-hoc test (*IGF1R*) or a one-way ANOVA with Dunnett's multiple comparison post-hoc test (*FBXL17* and *IGF1*) (* $p < 0.05$).



Appendix Figure 10. Gene expression of *IGF1R*, *FBXL17* and *IGF1* in non-metastatic (M0) and metastatic (M+) medulloblastoma patient samples.

Gene expression (Log2) of *IGF1R* (A), *FBXL17* (B), and *IGF1* (C) was assessed using R2 Genomics Analysis and Visualization Platform. Gene expression for all molecular subgroups (M0 = 397 and M+ = 176), SHH subgroup (M0 = 134 and M+ = 26) and Group 3 subgroup (M0 = 66 and M+ = 43) was assessed using the Cavalli dataset. Significant differences in gene expression were calculated using a Mann-Whitney test ($***p < 0.0001$).

Appendix Tables

Appendix Table 1. GSEA using KEGG to identify TWIST1 target gene sets between the two subgroups.

Gene sets highlighted in blue represented KEGG pathways shared between both subgroups and Group 3, gene sets not highlighted were unique to both subgroups.

Gene Set Name (KEGG Pathway)	Number of genes in overlap	p-value	FDR q-value
Axon guidance (hsa04360)	26	3.07e ⁻¹⁰	5.71e ⁻⁸
MAPK signalling pathway (hsa04010)	37	4.78e ⁻⁹	4.45e ⁻⁷
Neuroactive ligand-receptor interaction (hsa04080)	36	2.58e ⁻⁸	1.6e ⁻⁶
Focal adhesion (hsa04510)	29	7.03e ⁻⁸	3.27e ⁻⁶
Pathways in cancer (hsa05200)	37	7.86e ⁻⁷	2.92e ⁻⁵
Regulation of actin cytoskeleton (hsa04810)	28	1.01e ⁻⁶	3.05e ⁻⁵
Cell Adhesion Molecules (CAMs) (hsa04514)	21	1.15e ⁻⁶	3.05e ⁻⁵
Fc epsilon RI signalling pathway (hsa04664)	15	3.72e ⁻⁶	8.64e ⁻⁵
T cell receptor signalling pathway (hsa04660)	17	1.22e ⁻⁵	2.52e ⁻⁴
ECM-receptor interaction (hsa04512)	13	3.64e ⁻⁵	6.77e ⁻⁴
Hedgehog signalling pathway (hsa04340)	11	5.2e ⁻⁵	8.79e ⁻⁴
Proximal tubule bicarbonate reclamation (hsa04964)	7	6.43e ⁻⁵	9.97e ⁻⁴
Melanoma (hsa05218)	12	1.12e ⁻⁴	1.61e ⁻³
B cell receptor signalling pathway (hsa04662)	12	1.92e ⁻⁴	2.56e ⁻³
Basal cell carcinoma (hsa05217)	10	2.22e ⁻⁴	2.75e ⁻³
Insulin signalling pathway (hsa04910)	17	2.57e ⁻⁴	2.97e ⁻³
Wnt signalling pathway (hsa04310)	18	2.84e ⁻⁴	2.97e ⁻³

Toll-like receptor signalling pathway (hsa04620)	14	3.1e ⁻⁴	2.97e ⁻³
p53 signalling pathway (hsa04115)	11	3.19e ⁻⁴	2.97e ⁻³
Histidine metabolism (hsa00340)	7	3.2e ⁻⁴	2.97e ⁻³

Appendix Table 2. GSEA using KEGG to identify TWIST1 target gene sets in the Group 3 subgroup.

Gene sets highlighted in blue represented KEGG pathways shared between both subgroups and Group 3, and gene sets not highlighted were unique to the Group 3 subgroups.

Gene Set Name (KEGG Pathway)	Number of genes in overlap	p-value	FDR q-value
Pathways in cancer (hsa05200)	21	2.22e ⁻⁶	4.12e ⁻⁴
Cell adhesion molecules (CAMs) (hsa04514)	11	6.52e ⁻⁵	6.06e ⁻³
ECM-receptor interaction (hsa04512)	8	2.44e ⁻⁴	1.51e ⁻²
Pathogenic Escherichia coli infection (hsa05130)	6	7.78e ⁻⁴	3.62e ⁻²

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