
# CHARACTERISATION OF WNT SIGNALLING PATHWAY IN RHABDOMYOSARCOMA

Thesis submitted in accordance with
the requirements of the University of Liverpool for
the degree of
Doctor in Medicine (M.D.)
by

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# THESIS DECLARATION

- I, Dr. Srinivas Rao Annavarapu, declare that the contents of the thesis submitted the University of Liverpool towards MD is my original work and has not been submitted elsewhere.
- I conceived the overall structure of this research project under the able guidance of Prof. Tim Helliwell (main supervisor), Dr. Heather McDowell (co-supervisor) and Prof. Carlo Dominici my collaborator at La Sapienza University, Rome. They also supported me in procuring research grant to support this project.
- I carried out the experiments (construction of tissue microarray, immunohistochemical expressional analysis, western blot analysis, immunofluorescence analysis, reporter gene assay, proliferation assay, apoptosis assay) at the University of Liverpool. Some of the experiments (functional immunoblot analysis, confocal analysis and proliferation assay) were performed by Samantha Cialfi, a research assistant who works at La Sapienza University, Rome with Prof Dominici.
- I have written the manuscript for the MD myself and have been guided by my main supervisor Prof. Helliwell.

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# CHARACTERISATION OF WNT SIGNALLING PATHWAY IN RHABDOMYOSARCOMA

## Dr Srinivas Rao Annavarapu

### Introduction

Rhabdomyosarcoma (RMS) remains one of the most challenging tumours in paediatric oncology, accounting for around 5% of all malignant paediatric tumours. Of the two major subtypes of RMS, embryonal and alveolar, the latter portends a poorer clinical outcome. Canonical Wnt signalling pathway is an important evolutionarily conserved signalling pathway that is required for muscle development and embryonal somite patterning. β-Catenin is a potent nuclear transcriptional activator and is the central effector of the canonical Wnt signalling pathway. Interestingly, constitutional activation of Wnt signalling also promotes tissue invasion and metastasis in various tumours.

### Aims

This study aims to characterise the canonical Wnt/β-catenin signalling in paediatric RMS to assess its functional relevance to the tumourigenesis of RMS and to investigate if modulation of this pathway could provide a therapeutic target for RMS.

### Results

When we evaluated the immunohistochemical expression of β-catenin in the paraffin-embedded tissues derived from 44 RMS patients, we found positive expression in 26 cases. There was positive expression of β-catenin in the cytoplasm or the cell membrane of alveolar (9/14) and embryonal RMS (15/30); nuclear staining was only seen in two embryonal RMS cases. Next, we assessed β-catenin expression by immunoblot analysis in four RMS cell lines – RD and RD18 (embryonal); Rh4 and Rh30 (alveolar). We were able to demonstrate expression of major canonical Wnt proteins in all cell lines that included: β-catenin, glycogen synthase kinase-3β, disheveled, axin-1, naked and LRP-6. To assess the functional significance of these proteins, we incubated the RMS cell lines with human recombinant Wnt3a to stimulate the Wnt signalling pathway. Thereafter, by using cellular fractionation and immunofluorescence experiments, we demonstrated change in the phosphorylation status of β-catenin, stabilisation of its active form and its nuclear translocation. By employing a TOP/FOP flash reporter gene assay, we showed a T-cell factor/lymphoid-enhancing factor (TCF/LEF)-mediated transactivation. In addition, we found a significant decrease in the proliferation rate of the alveolar RMS cells after Wnt3a stimulation. This decrease in proliferation rate was thought to be due to the concomitant activation of myodifferentiation as seen by the immunoblot expression of myogenin, MyoD1 and myf5. Our data indicates that the major regulatory proteins of the canonical Wnt/β-catenin signalling are expressed in RMS and that functional activation of this pathway, at least in a subset of RMS, may represent a novel therapeutic target.

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| Akt | Protein Kinase B |
| APC | Adenomatous polyposis coli |
| APS | Ammonium persulphate |
| ARM | Armadillo |
| ARMS | Alveolar rhabdomyosarcoma |
| bHLH | basic helix-loop-helix |
| β-TrCP | β-transducin repeat containing protein |
| BCL9 | B-cell CLL/lymphoma-9 |
| BCL-XL | B-cell lymphoma-extra large |
| BMP | Bone morphogenetic protein |
| BRG-1 | BRM/SWI2-related gene-1 |
| BSA | Bovine serum albumin |
| CBP | CREB binding protein |
| CBY | Chibby |
| CCLG | Children's Cancer and Leukemia Group |
| CER | Cerberus |
| CK1 | Casein Kinase 1 |
| c-MET | c-Epithelial mesenchymal transition |
| CREB | Cyclic adenosine monophosphate (cAMP)-response element-binding protein |
| DAPI | 4',6-diamidino-2-phenylindole |
| $dH_2O$ | distilled water |
| $ddH_2O$ | double distilled water |
| DKK | Dickkopf |
| DMSO | Dimethyl sulphoxide |
| DVL | Dishevelled |
| EDTA | Ethylenediaminetetraacetic acid |
| EMT | Epithelial-to-mesenchymal transition |
| ERK | Extracellular signal regulated kinase |
| ERMS | Embryonal rhabdomyosarcoma |
| FAP | Familial adenomatous polyposis syndrome |
| FCS | Foetal calf serum |
| FGFR | Fibroblast growth factor receptor |
| FISH | Fluorescence in-situ hybridisation |
| FRAT-1 | Frequently reaaranged in advanced T-cell lymphoma |
| FZD | Frizzled |
| GBP | Glycogen synthase kinase binding protein |
| GSK3β | Glycogen synthase kinase 3β |

| | |
|---|---|
| HCl | Hydrochloric acid |
| HDAC | Histone deacetylase |
| HGF | Hepatocyte growth factor |
| HRP | Horseradish peroxidase |
| ICAT | Inhibitor of β-catenin and TCF |
| IGF1R | Insulin-like growth factor-1 receptor |
| ILK | Integrin-linked kinase |
| JNK | c-Jun N-terminal kinase |
| LBX1 | Ladybird Homeobox-1 |
| LEF | Lymphoid enhancer factor |
| LGS | Legless |
| LOH | Loss of heterozygosity |
| LRP-5/6 | Low-density lipoprotein receptor-related proteins 5/6 |
| MAPK | Mitogen activated protein kinases |
| MEF | Myogenic enhancer factor |
| miR-206 | MicroRNA-206 |
| MMP-7 | Matrix metalloproteinase-7 |
| MOX2 | Mesenchyme Homeobox-2 |
| MPC | Myogenic precursor cells |
| MRF | Myogenic regulatory proteins |
| MSX-1 | Muscle Segment Homolog of Drosophila-1 |
| MTT | MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| MW | Molecular weight |
| NaCl | Sodium chloride |
| $NaVO_3$ | Sodium metavanadate |
| NF-1 | Neurofibromatosis 1 |
| NLK | NEMO-like kinase |
| NP40 | Nonyl phenoxypolyethoxylethanol |
| p300 | E1A binding protein p300 |
| PAGE | Poly-acrilamide gel electrophoresis |
| PAR-1 | Protease-activated receptor-1 |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| PI | Propidium iodide |
| PI3K | Phosphatidylinositide 3-kinase |
| PKA | Protein kinase A |
| PP1 | Protein phosphatase-1 |
| PP2A | Protein phosphatase-2A |
| PVDF | Polyvinylidene difluoride |
| PYGO | Pygopus |
| RIPA | Radioimmunoprecipitation assay buffer |
| RMS | Rhabdomyosarcoma |

| | |
|---|---|
| SEM | Standard error around mean |
| SD | Standard devation |
| SDS | Sodium dodecyl sulphate |
| SIAH-1 | Seven in absentia homolog-1 |
| Ser | Serine |
| sFRPs | secreted Frizzled related proteins |
| SHH | Sonic Hedgehog |
| SOST | Sclerostin |
| SRF | Serum response factor |
| STS | Soft tissue sarcoma |
| TBS-T | Tris-buffered saline with Tween-20 |
| TCF | T-cell factor |
| TEMED | Tetramethylethylenediamine |
| TGF-β | Transforming growth factor-β |
| TMA | Tissue Microarray |
| Thr | Threonine |
| TP53 | Tumour protein 53 |
| TKIs | Tyrosine kinase inhibitors |
| Tyr | Tyrosine |
| UKCCSG | United Kingdom Children's Cancer Study Group |
| WHO | World Health Organisation |
| WIF | Wnt inhibitory protein |
| Wnt | Canonical Wnt signalling pathway |

# CHAPTER 1 –

# INTRODUCTION

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## 1.1 Soft tissue tumours

Sarcomas are a heterogeneous group of malignancies arising from mesodermal structures and show a myriad range of differentiation. A subset of these, are called soft tissue sarcomas (STSs) that commonly arise from the connective tissues of the body that includes muscle, nerve, fat and blood vessels [1].

Most soft tissue tumours are benign in behaviour with good prognosis after complete surgical excision. Malignant soft tissue tumours are rare (<1% of all malignant tumours) but may present with diagnostic therapeutic challenges since greater than fifty histological subtypes are recognized, each having their own distinctive clinicopathological and therapeutic attributes. Histologic classification is based upon morphologic demonstration of a specific line of differentiation. With the advances in genetics, our understanding of the biological behaviour of the soft tissue tumours has increased remarkably over the past two decades as ancillary diagnostic techniques like immunohistochemistry, fluorescent in-situ hybridisation (FISH) and electron microscopy has become routine. The STTs are stratified according to their lineage of origin by taking into account their morphologic features and novel genetic findings [2].

STTs occur in both children and adults, however the incidence is much higher in children, where it accounts for around 20% of all malignancies as compared to adults where it makes up for around 1% of cases [3]. The annual incidence of paediatric tumours in the UK is around 1700 with about 257 deaths every year [4]. Therefore, continued research towards identifying novel effective management strategies is crucial.

### 1.2 Paediatric Soft tissue tumours

STSs embrace features of various differentiation stages seen during the maturation of mesenchymal stem cells to mature mesodermal structures such as skeletal muscle (rhabdomyosarcoma), smooth muscle (leiomyosarcoma), Schwann cells (malignant peripheral nerve sheath tumors), fibrous tissue (fibrosarcoma), adipose tissue (liposarcoma) and blood vessels (angiosarcoma, hemangiopericytoma) [1].

In a subset of STSs, the definitive origin remains unclear (e.g., synovial sarcoma, malignant fibrous histiocytoma, alveolar soft part sarcoma). In some cases, the STSs show very primitive mesenchymal phenotype similar to the mesenchymal stem cells (Ewing sarcoma, undifferentiated sarcomas) [1].

In 2013, the World Health Organization (WHO) classified soft tissue sarcomas on the basis of their putative phenotype (tissue they resemble/likely cell of origin) and biological behavior [5]. In some

cases, the origin of the sarcoma is unclear and these are believed to have mesenchymal stem cell phenotype. Thus, the soft tissue sarcomas encompass a heterogeneous group of entities, which show diverse phenotypes and have variable biological behaviour depending upon the underlying genetic abnormality. Sarcomas are graded histologically in benign, borderline and malignant tumours using morphological criteria like tumour differentiation score, mitotic count and tumour necrosis (French Federation of Cancer Centers Sarcoma Group system) [6]. This allows predicting the clinical behaviour of the tumour and helps to correlate with its underlying genetic abnormality. The current study focusses on the molecular pathology of the most common paediatric STS – rhabdomyosarcoma.

### 1.3 Rhabdomyosarcoma

Rhabdomyosarcoma (RMS), accounts for around 4-5% of paediatric malignancies and is the most common soft-tissue sarcoma of childhood and adolescence [7, 8]. The incidence of RMS in the UK is around 60 cases per annum [9], whereas in the United States, a diagnosis of RMS is made in around 350 children and adolescents [10, 11]. Most cases are sporadic in nature, although familial cases (around 5%) may be associated with germ line mutation of Tumour protein 53 (TP53) in Li-Fraumeni syndrome [12] and with germ line mutations in NF-1 in Neurofibromatosis [13]. It is reported to be more common in males than

in females [7]. RMS shows a bimodal age distribution depending upon its subtype, though most children are less than 10 years of age [7].

### 1.4 Classification of RMS

Histogenetically, RMS as a group demonstrates skeletal muscle differentiation but histo-morphologically they can show diverse array of patterns.

The WHO classification (2013) recognizes the following RMS subtypes: embryonal, alveolar, spindle cell/sclerosing and pleomorphic [8, 14, 15]. Of these, the alveolar and the embryonal RMS, are the two major clinicopathological subtypes that need to be separated as they have important prognostic and therapeutic implications.

Embryonal RMS (ERMS) is more common subtype (70% of cases) and has a better prognosis. On the other hand, the alveolar RMS (ARMS) subtype (30% of cases) has a more aggressive course and has poorer prognosis with limited therapeutic options [9, 16, 17].

The International Classification of Rhabdomyosarcoma stratifies RMS subtypes into prognostic categories (See Table 1.1) where botryoid and spindle-cell RMS subtypes are considered in the superior-risk group category. Embryonal RMS and alveolar RMS fall into the intermediate-risk and the unfavorable-risk group respectively [7, 15, 18, 19]

**Table 1.1 - International Classification of Rhabdomyosarcoma [14]**

| | |
|---|---|
| **Superior Prognosis** | Botryoid RMS<br>Spindle cell RMS |
| **Intermediate Prognosis** | Embryonal RMS |
| **Poor Prognosis** | Alveolar RMS<br>Undifferentiated RMS |
| **Unknown Prognosis** | RMS with rhabdoid features |

In contrast to embryonal RMS which presents in early childhood, the alveolar RMS occurs more frequently in older children, has a more aggressive behaviour, portends a poorer prognosis and more commonly present with metastasis to bone marrow [20].

Botryoid and spindle cell RMS subtypes have very characteristic presentations, have very good prognosis and most likely represent variants of embryonal RMS [7].

As a group, both ERMS and ARMS are diagnosed by nuclear immunostaining for the myogenic nuclear transcription factors – Myogenin and MyoD1 and structural proteins markers like desmin, myosin heavy chain, myoglobin and skeletal α-actin [21-24] (See Fig1.1).

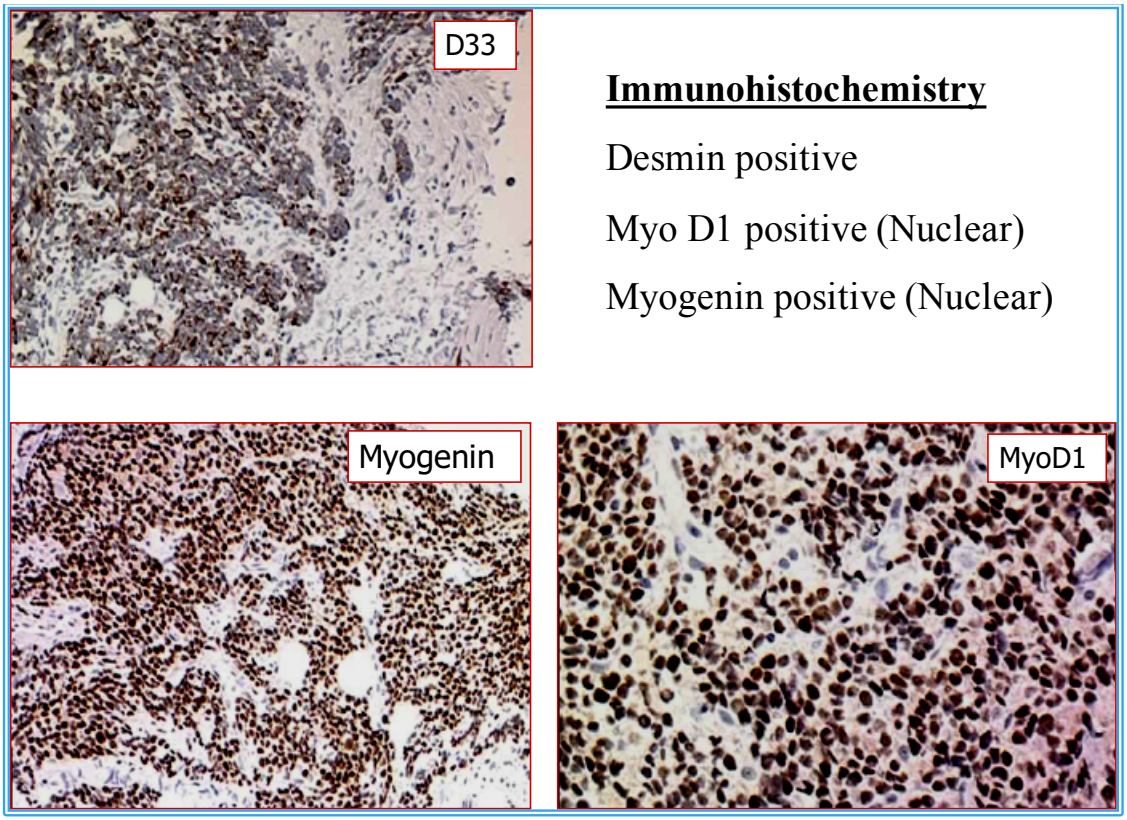



**Figure 1.1**- Immunohistochemical panel for the diagnosis of RMS

Although it is tempting to conclude that these markers indicate a skeletal muscle differentiation from mesenchymal stem cells, non-myogenic lineage of the tumor cells has also been demonstrated [25]. RMS most commonly arises from skeletal muscle but can also be seen developing from non-myogenic sources like salivary glands, biliary/genitourinary tract and para-meninges [24, 25]. However, from a diagnostic standpoint, immunohistochemical demonstration of nuclear MyoD1 and Myogenin with/without cytoplasmic desmin is central to establish a diagnosis of RMS [22, 26] (Fig 1.1). Reticulin stain is useful as an adjunct to differentiate ARMS from ERMS; whereas the distribution is peri-cellular in ERMS, reticulin outlines cellular aggregates of the

tumour cells in ARMS [27]. Recently AP2β and β-catenin have been shown to be potent markers of fusion-positive ARMS [28].

### 1.5 Embryonal RMS

ERMS is a malignant soft tissue tumour that shows differentiation towards skeletal muscle differentiation of up to 10-12 weeks in embryonic development. They are thought to be arising from primitive mesenchymal stem cells showing variable myogenic differentiation [29]. Most children are less than 5 years old. They can occur at any site though their favored locations are genitourinary tract, where they can present as urinary obstruction or in the head/neck region presenting as retro-orbital mass. They also can occur as visceral masses (especially biliary), prostatic lesions or limbs [7, 8].

The pathology of ERMS recapitulates embryonic myogenesis and shows primitive mesenchymal cells set in a hypocellular myxoid background with hyperchromatic nuclei and variable amount of amphophilic cytoplasm [29]. Focally, the tumour shows elongated, eosinophilic strap cells with cross-striations – rhabdomyoblasts, providing histological evidence of skeletal muscle differentiation [29]. Immunohistochemistry is crucial to ascertain the myogenic lineage – nuclear MyoD1 and Myogenin with cytoplasmic Desmin [26].

The botryoid subtype presents clinically with distinctive grape-like polyps projecting from mucosal surfaces. The sub-epithelial regions show distinctive zones of cellular condensation, the so-called the Cambium layer (See Fig 1.2). The tumour cells show nuclear staining for MyoD1 and Myogenin with variable cytoplasmic positivity for Desmin [7].

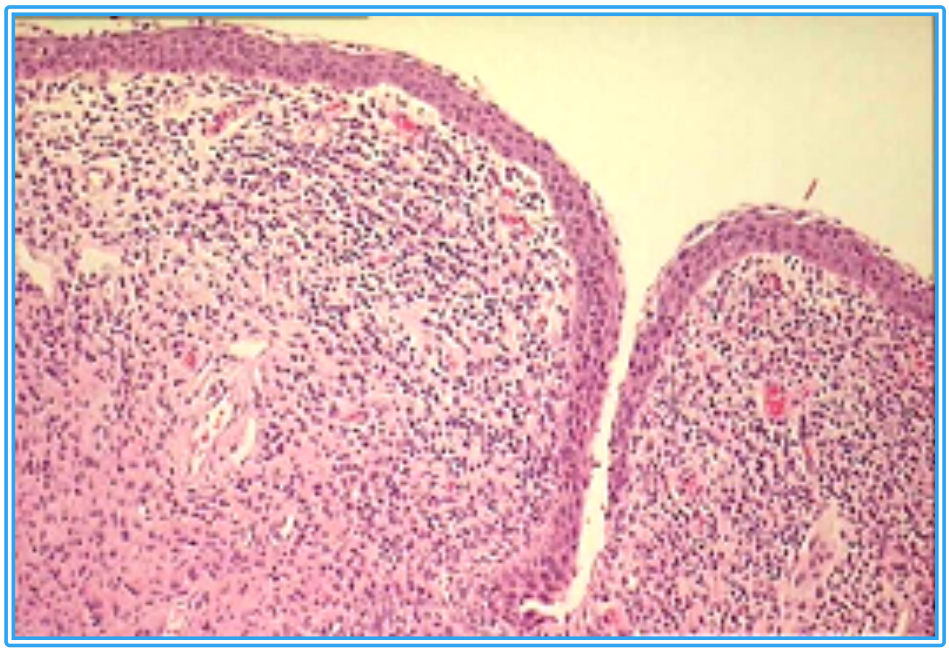


**Figure 1.2** - Histology of botryoid type RMS showing characteristic sub-epithelial Cambium layer.

The spindle cell subtype presents mostly as a paratesticular or a head/neck mass with dense fascicles of spindle cells forming a whorled/ herring-bone pattern. They show minimal cellular pleomorphism with little or no nuclear atypia and low mitoses [30]. Focally, rhabdomyoblasts are present. The tumour cells also show nuclear

staining for MyoD1 and Myogenin. Spindle cell RMS and botryoid RMS may also co-exist [29].

Anaplastic variant of ERMS is characterised by tumour cells having enlarged, hyperchromatic nuclei and multipolar mitoses [29]. Analogous to that defined for Wilms' tumour, anaplasia in RMS can be divided into focal and diffuse. Diffuse anaplasia when occurring in children with intermediate-risk embryonal rhabdomyosarcoma may have a poor prognosis [31]. Similar to the other ERMS subtypes, the tumour cells show nuclear staining for MyoD1 and Myogenin. Spindle cell RMS with anaplasia can resemble pleomorphic RMS seen in adults, but this is extremely rare [29]. Prognostically, the low-risk patients have a 5-year survival rate of 80-90%, whereas patients in the intermediate and the high-risk groups have 4-year survival rates of 70% and 23% respectively [32-34].

**1.6 Alveolar RMS**

Alveolar RMS is an aggressive soft tissue tumour with myogenic differentiation that is characterised by small round blue cells. Most tumours show an alveolar pattern but solid and anaplastic variants are also known [29]. In contrast to ERMS, these tumours mostly arise from the extremities, perineum and the sinonasal region as painless soft tissue mass. Another distinguishing feature is the wider age range of presentation as it is relatively more common in older children and

adolescents. The tumour grows rapidly as an expansive mass that has the propensity to invade adjacent structures and metastasize early to bone marrow, lymph nodes and lungs [29].

Histologically, there are two main types of patterns – classic alveolar and solid variants. The alveolar pattern shows delicate fibrovascular septae lined by small round blue cells, which have a tendency for central dyscohesion (See Fig 1.3). There is scant evidence of myogenesis, although some multinucleated giant cells analogous to embryonic fused myoblasts may be seen [7, 29].

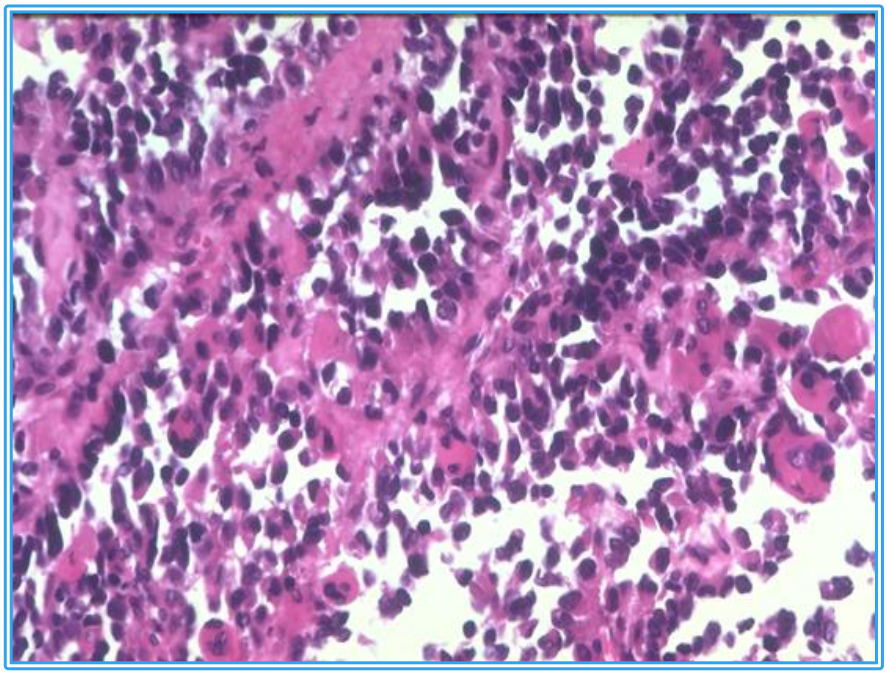


**Figure 1.3** - Histology of classic alveolar RMS showing small round blue cells with central dyscohesion.

## 1.7Genetics of RMS

Alveolar RMS shows distinctive t(2;13)(q35;q14) and t(1;13)(q36;q14) translocations, involving the PAX3–FOXO1(Forkhead box protein O1; formerly known as Forkhead receptor/FKHR) and PAX7–FOXO1 fusion genes, respectively [35, 36]. *Pax7* (chromosome 1) and *Pax3* (chromosome 2) genes belong to the Pax family of transcription factors that contain a C-terminal transactivation domain and an N-terminal DNA-binding domain composed of homeobox motifs and paired box. *FOXO1* also contains C-terminal transactivation domain and an N-terminal DNA-binding site [35, 36]. The breakpoint in the reciprocal translocation occurs at the site of intron 7 for Pax genes and intron 1 for FOXO1; after fusion, this encodes a chimeric proteins Pax3–FOXO1 and Pax7–FOXO1, which contains 5′ DNA-binding domain of Pax and the 3′ transactivation domain of FOXO1 [24,] (See Fig. 1.4).

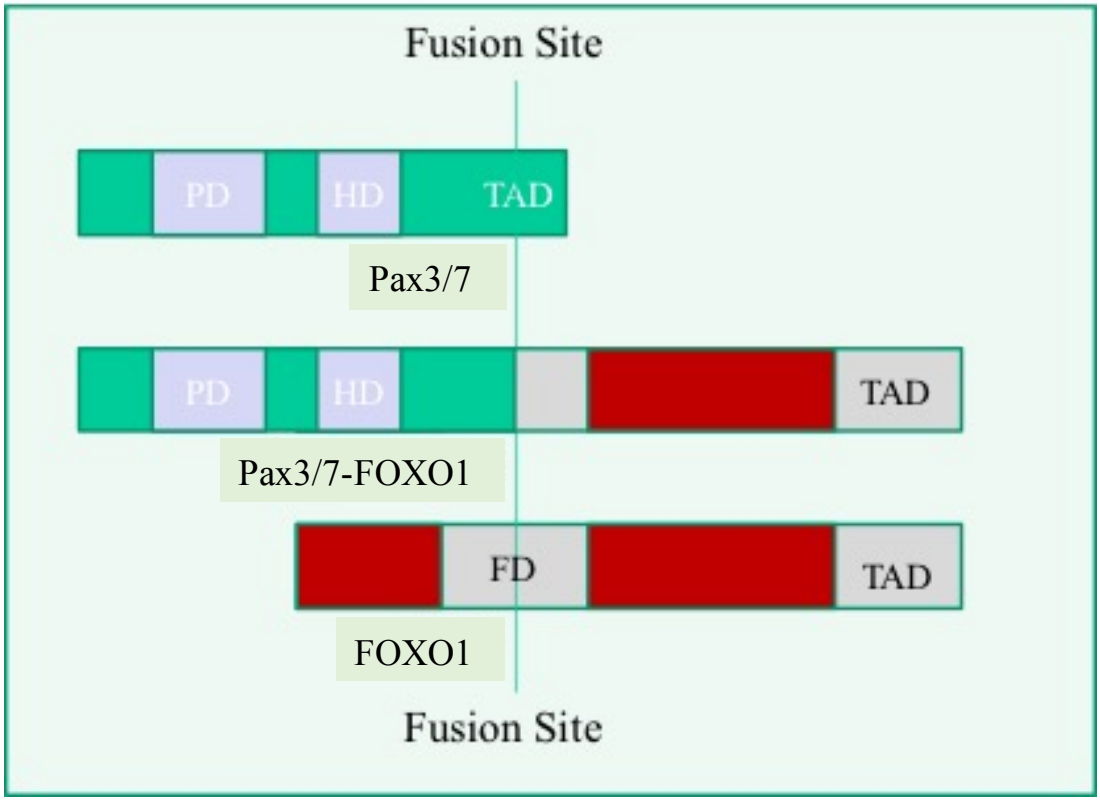



**Figure 1.4** - Schematic diagram showing the characteristic Pax3/7-FOXO1 fusion gene found in ARMS. PD = Pairing domain; HD = Homeodomain; TAD = transactivation domain.

The mechanisms of how this fusion gene product, detectable by fluorescent in situ hybridization (FISH), causes dysregulation of differentiation of myogenic progenitor stem cells, is not fully understood. PAX3-FOXO1-positive ARMS also frequently show gene amplification of other critical genes including MDM2, CDK4, MYC-N and GLI proto-oncogenes. Some ARMS show alternate fusion transcripts to FOXO1 by incorporating NCOA1, NCOA2 or AFX. Rarely, PAX gene may be replaced by FGFR [20].

Some tumours with otherwise typical ARMS histology are negative for PAX-FOXO1 defects but have prognosis similar to ERMS [37]. Around one-fifth of ARMS tumors do not express PAX-FOXO1 rearrangement; these “fusion negative” ARMS cases have clinical outcomes comparable to those with ERMS [38, 39]. Since the prognosis of translocation-negative ARMS is largely similar to that of ERMS, Lupo (2015) recommends that these two entities should be clubbed in future epidemiologic investigations as they may represent the same entity [40].

Unlike ARMS, ERMS show a diverse range of non-specific genetic abnormalities, possibly related to the morphological variations in myodifferentiation in ERMS [41]. However, epigenetic changes such as loss of heterozygosity (LOH) for parts of Chromosome 11p15.5 alterations are consistently seen [42]. This has been postulated primarily to be a consequence of uniparental disomy [43].

**1.8 Molecular biology of RMS**

Pax3 is physiologically required in muscle development but transfection of Pax3 in cultured fibroblasts does not transform them. However, when co-transfected with Pax3–FOXO1, the fibroblasts were transformed [44, 45]. Similarly, transfecting ERMS cell line with Pax3–FOXO1 resulted in increased cell proliferation and tumor growth [36].

The underpinning mechanisms of how Pax–FOXO1 effects oncogenesis are not completely understood. However, it has been shown that Pax–FOXO1 chimeric proteins is capable of a 100-fold increase in the transcriptional activity as compared wild-type Pax3/7. Thus, the dosage of the Pax3–FOXO1/Pax7–FOXO1 chimeric proteins is critical [46, 47]. There are subtle differences in the Pax3–FOXO1 and Pax7–FOXO1 translocations, whereas the former results in a hectic increase in gene transcription [48], overexpression of the latter causes gene amplification [49]. In addition, Pax3–FOXO1 has a significantly longer half-life as compared to Pax3 [50]. Pax–FOXO1 proteins also induces expression of *B-cell lymphoma-extra large* (BCL-XL) gene, an anti-apoptotic gene [51, 52]. In addition, Pax–FOXO1 suppresses terminal muscle differentiation [53].

On microarray analysis, Pax–FOXO1 proteins shows an explicit molecular signature of ARMS consistent with its action of promoting proliferation and suppressing terminal muscle differentiation [41]. One such target is fibroblast growth factor receptor-4 (FGFR4) that is known to promote tumour growth and survival [54]. Pax3 also activates FGFR signalling, which promotes growth of myoblasts [55]. It is thought that Pax3–FOXO1 promotes cell proliferation through the FGFR4 pathway. Insulin-like growth factor-1 receptor (IGF1R) is another downstream target of Pax3–FOXO1 that may promote myoblast growth [56]. Since

both FGFR and IGF1R promote differentiation of myoblasts, it is plausible that Pax3–FOXO1 signals through these receptors keep the RMS cells in a state of proliferation whilst retaining some differentiation markers [24].

Hepatocyte Growth Factor (HGF)/c-mesenchymal-epithelial transition (c-MET) receptor is another important growth-promoting gene that is overexpressed in fusion-positive ARMS and appears to be a direct target of Pax3–FOXO1 [57, 58]. c-Met receptor signalling activates the quiescent myogenic progenitor cells to proliferate after muscular damage [24, 59]. c-Met appears to regulate dichotomy of cell fates in ARMS. Tumours with decreased c-Met expression show a more differentiated phenotype (See Fig 1.5). It has recently been shown that microRNA-206 (miR-206) induces ARMS differentiation by targeting c-Met [24, 60].

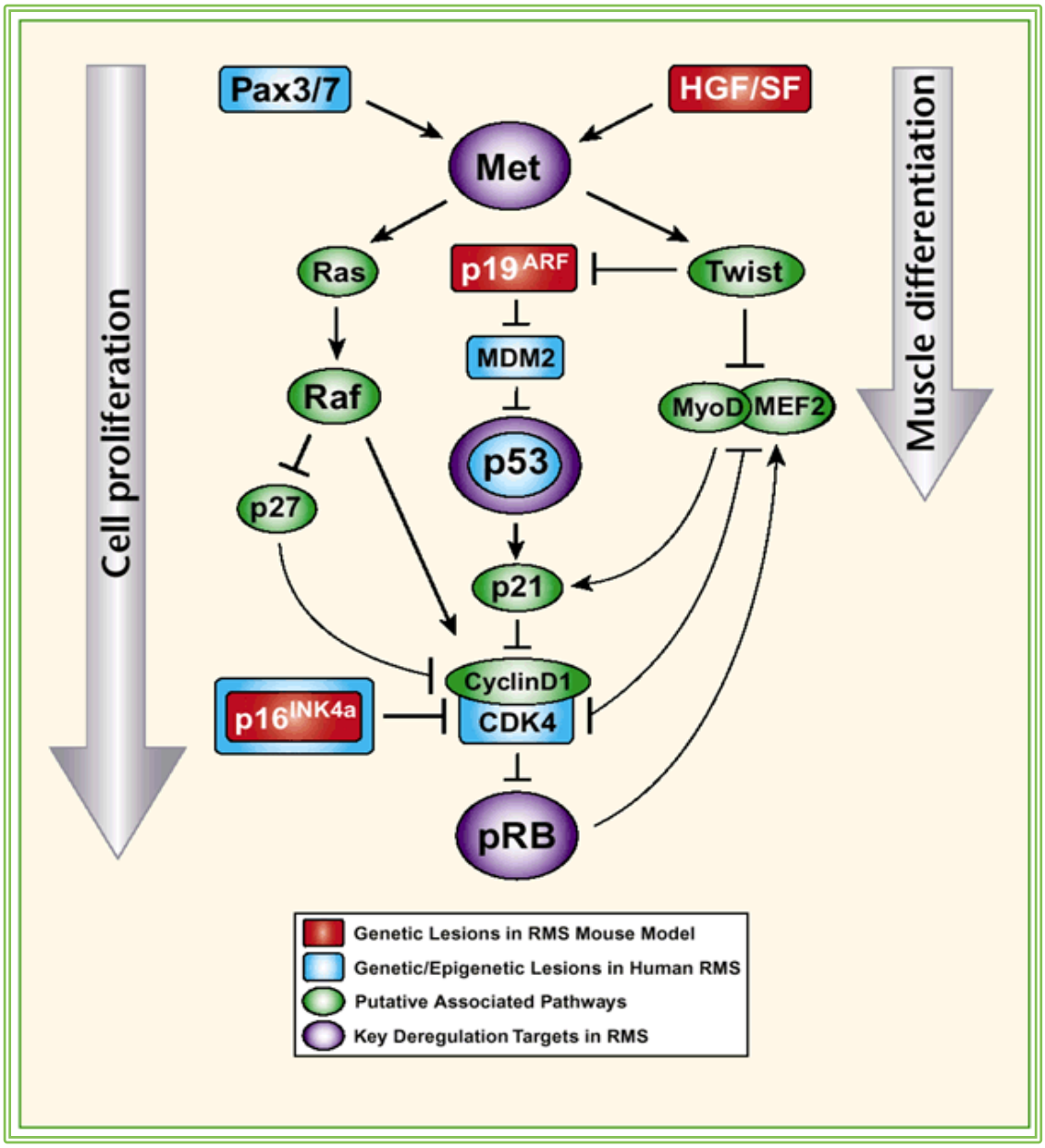



Fig 1.5 - Schematic diagram showing dichotomy of fates effected by c-Met, a downstream target of Pax3/7. [61].

Pax–FOXO1 inhibits muscle differentiation by effecting strong epigenetic inhibition of MyoD transcriptional activity [24]. It inhibits MyoD (and its downstream Myogenin) by preventing chromatin remodeling by reducing acetylation of histone H4 and RNA polymerase II [62]. This may maintain RMS cells in a less differentiated state since Myogenin is required for myogenic differentiation [24]. MyoD also binds with histone acetyltransferases (e.g. p300/CREB-binding protein) and histone deacetylases to regulate expression of myogenic genes. Myostatin, part of the transforming growth factor-β (TGF-β) superfamily, is another mediator of Pax–FOXO1 signals that repress myogenic differentiation by acting through activin receptor type IIb to activate Smad proteins [24].

Recent studies have shown that Wnt signalling pathway, crucial in embryonal development and myogenesis, may be linked to rhabdomyosarcoma [63, 64]. Moreover, Wnt also regulates the expression of MyoD and Myogenin and may be important in the regulation of myogenic differentiation [65, 66].

The following section examines the Wnt signalling pathway and highlights how this pathway could be important in the pathogenesis of RMS.

## 1.9 Wnt Signalling Pathway

Wnt signalling pathway is conserved through the evolution across invertebrates and vertebrates and is key in regulating key cellular interactions during embryonic development [67, 68]. There are three recognised arms of Wnt signalling pathway: canonical Wnt pathway, Wnt/calcium pathway and the planar cell polarity pathway [68]. As a group, the Wnt pathways become active when an appropriate ligand binds to a Frizzled family of receptors (Fzd), which activates downstream signalling and relays the biological signal to the nuclear machinery. The canonical Wnt pathway (main focus of the study) regulates gene transcription of the downstream targets; the non-canonical planar cell polarity pathway regulates the cell cytoskeleton and the Wnt/calcium pathway regulates intracellular levels of calcium [69]. Wnt signalling pathways may act in a paracrine or autocrine manner [68].

### 1.9.1 Regulation of Wnt signalling

β-catenin, the key player of the canonical Wnt signalling pathway, is a nuclear transcription factor, that executes the Wnt functions in cellular interactions during development [67]. Within the cell, β-catenin exists in two major pools – bound to the Cadherins as a part of the adherens junction [70] and as free β-catenin that is actively degraded in the

cytoplasm and the amount of free intracellular β-catenin is tightly regulated [68].

In brief, when the Wnt pathway is switched off, β-catenin is retained in the cytoplasm by a degradation complex containing CK1α, GSK3β, Axin and APC protein [68]. This complex sequentially phosphorylates β-catenin (inactive form) at various sites that causes ubiquitination of β-catenin and subsequent degradation by the proteasome [67, 69] (See Fig 1.6).

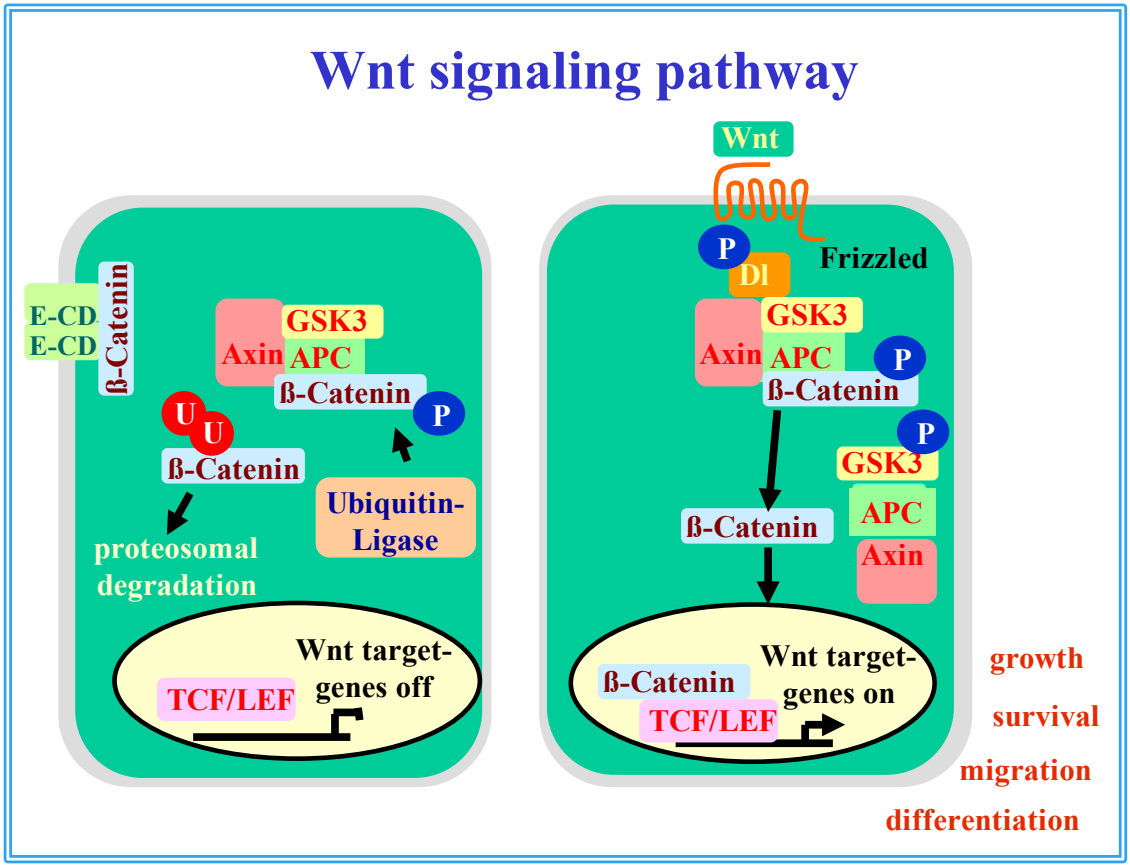



**Figure 1.6** - Schematic diagram of Wnt signalling pathway. Under basal conditions β-catenin is sequestered by the degradation complex and marked for proteasome destruction. In the presence of Wnt ligand, Disheveled rescues β-catenin from destruction. Free β-catenin translocates to the nucleus and activates Wnt target genes.

In the active state, the Frizzled receptors interact with Wnt ligands and the Low-density lipoprotein receptor/LRP-group of co-receptors on the cell surface. This binding triggers a relay of cytosolic signals that activates a downstream protein Dishevelled (Dvl) that consequently destabilizes the degradation assembly by inhibiting GSK3β via phosphorylation and rescues β-catenin from proteosomal degradation [68]. The unphosphorylated β-catenin (active form) can now translocate to the nuclear and form a complex with T-cell factor/lymphoid enhancer-binding factor (TCF/LEF) to activate downstream target genes like Axin-2, c-Myc, c-Jun, Cyclin D1, Slug, Matrix metalloproteinase-7 (MMP-7) etc. that promote cellular proliferation, migration, invasion, survival and metastasis [68]. In addition, Wnt pathway may cross talk with other signalling pathways to modulate cellular responses to various stimuli [69].

### 1.9.2 Wnt signalling in normal development

Developmental studies in Xenopus embryos, Drosophila and C. elegans have shed valuable information on the mechanisms operative in Wnt signalling pathway [68]. Wnt signalling is important in the regulation of body axis patterning, cell fate specification, cell proliferation and cell migration [71]. It is required for various developmental processes including stem cell renewal [71-73]; haematopoiesis [74, 75]; development of hair follicle [76]; testicular development and

spermatogenesis [77, 78], proliferation of gastric [79, 80] and intestinal epithelium; craniofacial development [81], bone development [82, 83]; fracture healing [84] and development of teeth [85, 86]. The differential gradients created by the Wnt proteins in relation to Sonic hedgehog (Shh), Notch and Bone morphogenetic protein (BMP) signalling is crucial in axis patterning as shown in Fig 1.7 [87].

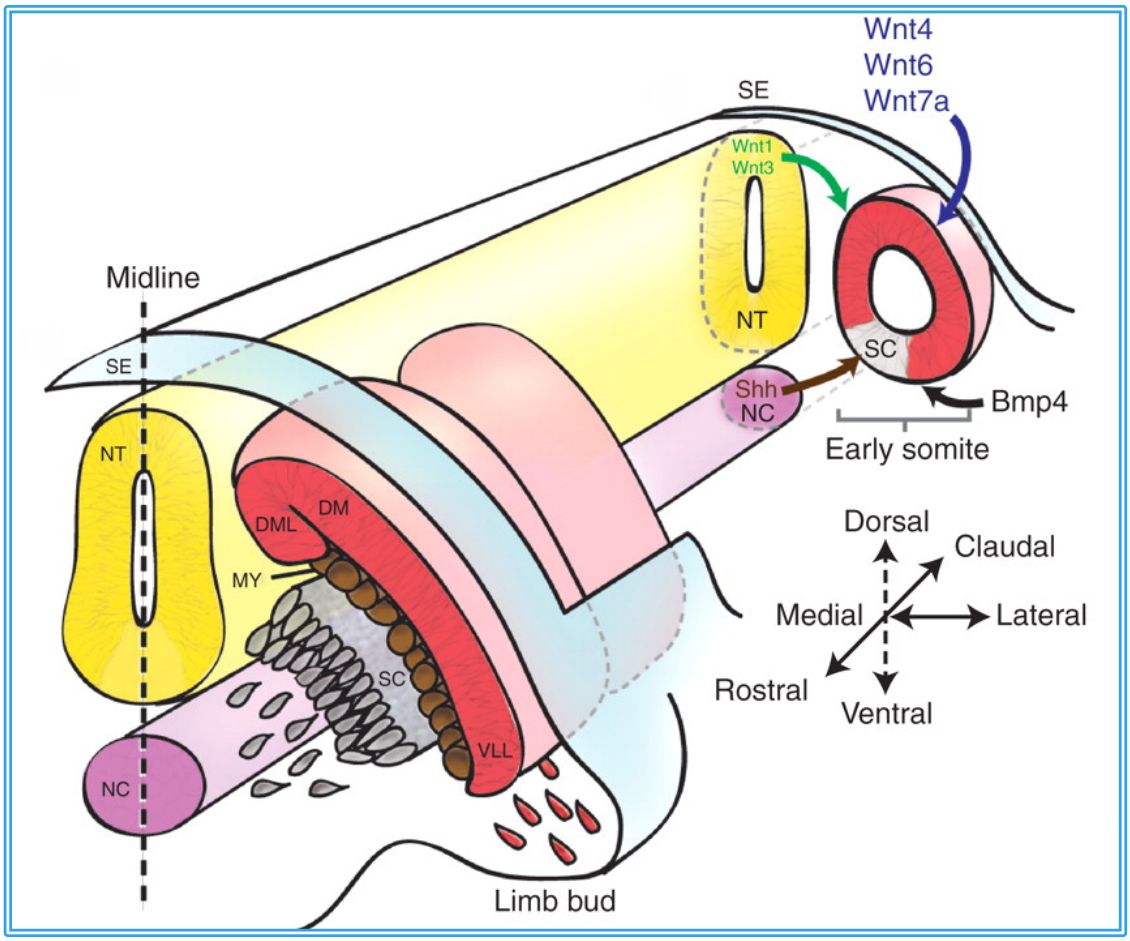



**Figure 1.7** – Role of Wnt signalling in somite patterning and myogenesis. Role and Function of Wnts in the Regulation of Myogenesis: When Wnt Meets Myostatin in "Skeletal Muscle [87]

Previous and recent studies confirm that Wnt signalling is crucial in myogenesis [83, 87-91]. In particular, dysregulation of Wnt pathway can lead to a plethora of skeletal developmental anomalies [69].

### 1.9.3 Wnt signalling in Cancer

Deregulation of Wnt signalling, caused by germ line and/or somatic mutations in Wnt pathway components, causes many types of human tumours [93-95]) (See Fig 1.8).

Activating β-catenin mutations, that alter specific β-catenin residues required for GSK3β phosphorylation and its subsequent degradation, have been reported in human colon cancer [96] and melanoma [97]. Since mutated β-catenin may constitutively activate downstream target genes, many of which may promote cellular proliferation, migration, invasion, survival and metastasis, it is easy to see how dysregulation of Wnt/β-catenin signalling can facilitate tumourigenesis in various organ systems where Wnt signalling is active [68, 69, 98].

Deregulation of Wnt pathway causes malignancies in a wide variety of organs such as colon [99, 100], skin [101, 102], liver [103, 104], lung [105], pancreas [106], thyroid [107-109], endometrium [110, 111], prostate [113] and brain [113-114]. Mutations in other Wnt proteins such as APC were identified in colorectal carcinomas [115];

Axin1 gene mutations have been reported in hepatocellular carcinomas [116].

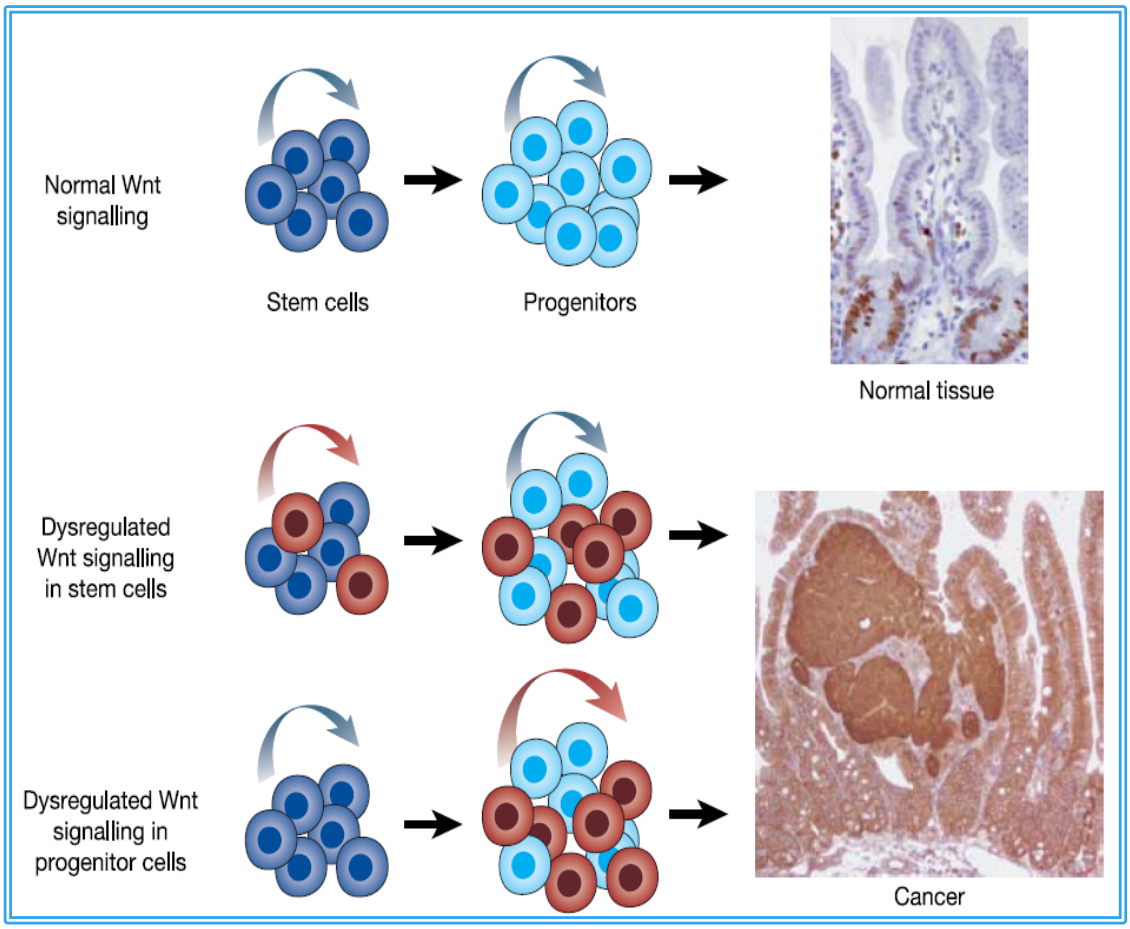



**Figure 1.8** – Dysregulation of Wnt signalling (red arrow) results in persistent renewal and abnormal expansion of the stem cells that can cause colorectal cancer [93].

### 1.10 RMS and Wnt – Is it relevant?

There are several compelling arguments in favour of why Wnt signalling pathway could be relevant and important in rhabdomyosarcoma.

1. Wnt signalling is an important developmental pathway required for somite patterning and limb development (as described in section 1.6). The Wnt signalling pathway is also actively involved

in myogenesis [117-119] and embryonic somite patterning [120, 121]. Petropoulos (2002) showed that β-catenin was essential and sufficient for skeletal myogenesis in P19 cell lines. They used P19 cell lines with stably expressed Wnt3a or with mutated β-catenin and found evidence of myogenesis in the test P19 cell lines as compared to the control cells. Prior to MyoD expression, other myogenic markers Pax3, Gli2, Mox1, and Six1 were also expressed upon Wnt3a/β-catenin-induced differentiation [122].

2. Diagnosis of RMS is based on the demonstration of immunohistochemical expression of nuclear transcription factors, Myogenin and MyoD1 [26], both of which are capable of inducing myogenic differentiation [91] and appear to be downstream of canonical Wnt signalling pathway [123] (See Fig 1.9).

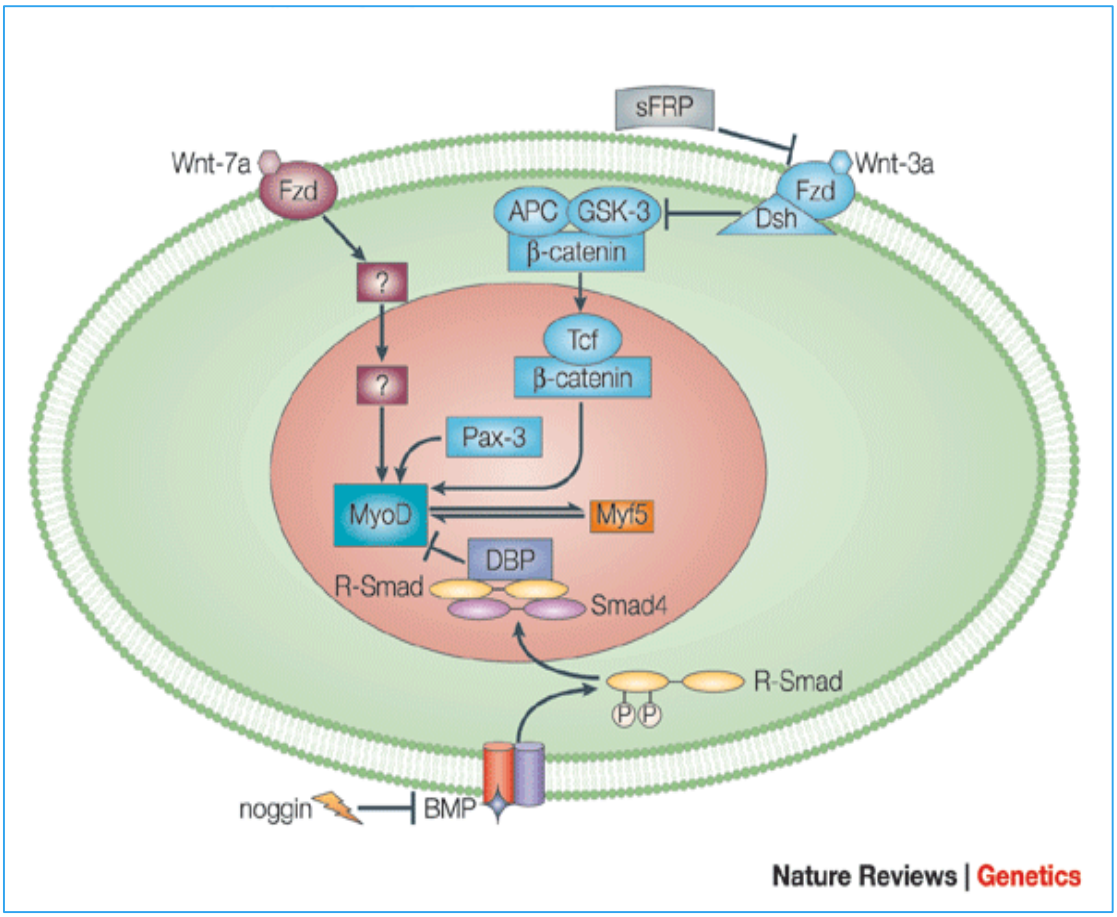



**Figure 1.9** – Myogenic differentiation markers MyoD and Myf5 appear to be downstream of canonical Wnt signalling pathway and are modulated by BMP via SMAD [123].

3. ARMS show distinctive translocations, involving the PAX3–FOXO1 and PAX7–FOXO1 fusion genes [36]. Interestingly, Pax3 and Pax7 genes are required for myogenesis and have been demonstrated to be downstream of Wnt signalling in muscle development [118]. Furthermore, it has been shown that Wnt pathway cooperates with both Pax3 and Pax7 genes to promote myotubule formation [124].

4. Wnt pathway also co-operates closely with the other embryonal pathways like Shh [125] and Notch [126] during skeletal development. Further, there is evidence that Wnt pathway may crosstalk with Shh and TGF-β pathways in the process of epithelial-to-mesenchymal transition (EMT), which is important in embryonic development, wound healing, and tumour formation [127].

Taken together, it appears that there could be important links between the Wnt signalling pathway and rhabdomyosarcoma that warrants further investigation. Hence, I found it justifiable to ask the question if Wnt signalling could be relevant in the pathogenesis of RMS.

## 1.11 Aims

Given its role in myogenesis and tumourigenesis, the main purpose of the project is to characterise Wnt signalling pathway in RMS with an aim to elucidate whether canonical Wnt pathway plays any role in the tumourigenesis of RMS and whether manipulation of this pathway could provide any therapeutic target for RMS.

- To verify the expression of the central Wnt regulator β-catenin in tissue samples of RMS patients to establish clinical relevance of Wnt signalling pathway to RMS.
- To investigate whether the major Wnt pathway proteins and their downstream targets are expressed in the RMS cell lines in-vitro.
- To investigate whether Wnt signalling pathway is functionally active in RMS cell lines.
- To investigate whether modulation of Wnt signalling pathway in RMS cell lines could have any therapeutic implications.

## 1.12 Objectives

- To assess the expressional analysis of β-catenin by the means of a Tissue Microarray (TMA) on archival clinical RMS tissue samples from two major Children's hospitals.
- To assess the expressional analysis of Wnt components (including beta-catenin, components of the degradation complex, Wnt co-receptors and downstream targets) in the RMS cell lines by Western blot analysis.
- To assess the phosphorylation status of β-catenin after Wnt3a activation in-vitro in the RMS cell lines using different β-catenin phospho-antibodies.
- To assess the localisation of β-catenin in cellular compartments (cell membrane, cytoplasm and nucleus) after Wnt3a activation in-vitro in RMS cell lines by confocal immunofluorescence microscopy and immunoblot assays using nuclear/cytoplasmic cell extracts.
- To assess functional status of β-catenin/LEF-TCF interaction after Wnt3a activation in-vitro by Report gene assay (TOP/FOP flash using Gaussia Luciferase assay) and by immunoblot expression analysis of the downstream targets.

- To assess the effect of Wnt3a activation (in-vitro) on the proliferation activity of RMS cell lines using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) proliferation assay.
- To assess the effect of Wnt3a activation (in-vitro) on apoptosis in RMS cell lines using Caspase 3/7 assay.
- To assess the effect of Wnt3a activation on the morphology of RMS cells lines using confocal immunofluorescence microscopy.

# CHAPTER 2 –

# REVIEW OF LITERATURE

# CHAPTER 2 – REVIEW OF LITERATURE

## 2.1 What is Wnt signalling pathway?

Wnt signalling is a remarkably conserved pathway across invertebrates and vertebrates that plays a vital role in the skeletal development [68]. The pathway comprises three arms, canonical Wnt/β-catenin pathway, the planar cell polarity pathway and the Wnt/$Ca^{2+}$ pathway. These pathways are involved in intracellular signalling following Wnt-ligand interactions with cell surface receptors [68, 69, 128].

Canonical Wnt signalling pathway comprises a family of secreted glycolipoproteins that regulate cell fate determination, cell polarity and cell proliferation during embryonic development [113]. It cross talks with other embryonal pathways like Shh and Notch in formation of the body axis, somite patterning and limb development [64]. Since the Wnt pathway directs many of the steps related to tissue homeostasis, dysregulation of this pathway results in congenital skeletal defects and tumours [69]. Canonical Wnt pathway regulates the amount of intracellular β-catenin, a powerful nuclear transcriptional activator that controls the genes expression of downstream targets [128, 129] (See Fig 2.1).

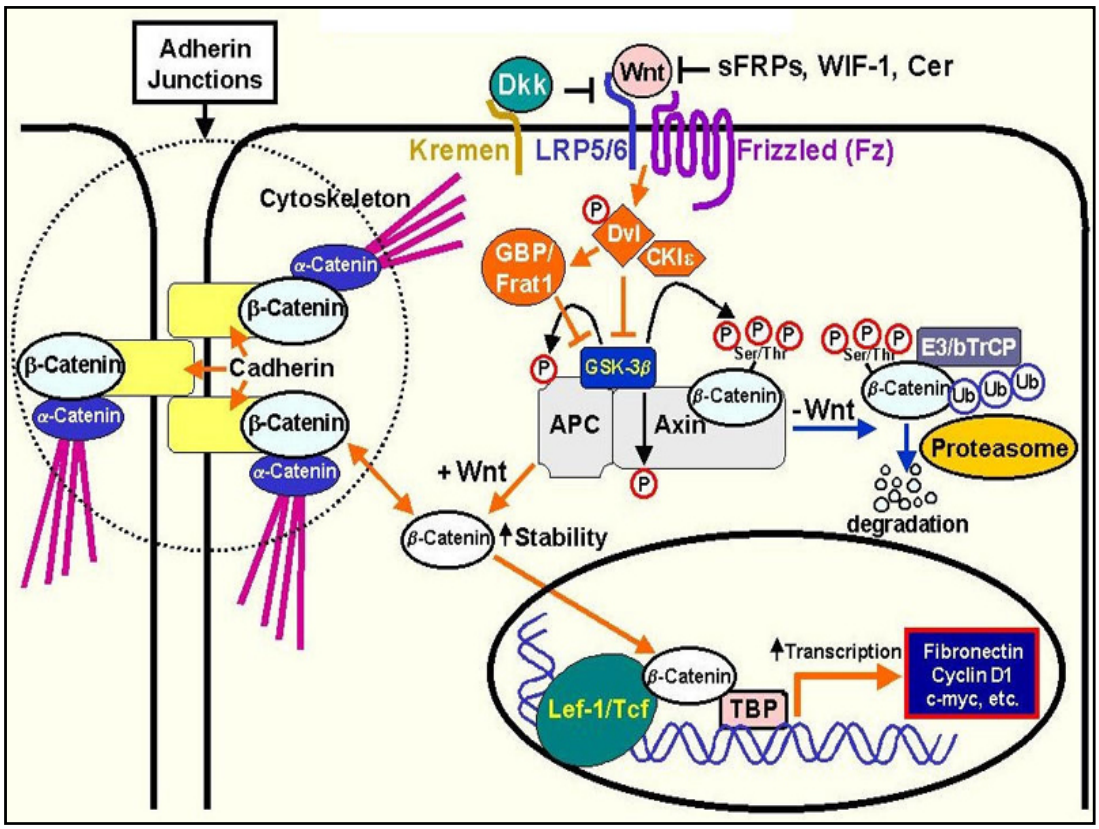



**Figure 2.1** - β-catenin exists within the cell in two major pools – as a part of the adherens junction in association with Cadherin and other catenins, and as a that is tightly regulated nuclear transcription factor, central to the canonical Wnt signalling pathway [130].

Historically, Wnt signalling was first described for its role in cancer and its role in embryonic development was discovered later [68]. In 1982, Roel Nusse and Harold Varmus infected mice models with oncogenic retroviruses in an attempt to mutate mouse genes to see which transformed genes could cause mammary tumours. A new mouse proto-oncogene was identified that was named int1 (integration 1) [68, 131]. Int1 was found to be highly conserved across vertebrates and invertebrates including humans and Drosophila. It was soon realized however that the int1 gene described in Drosophila had a homologous

gene in Wingless (Wg) family of genes [132] that had been characterized previously in Drosophila [133]. Wg was implicated in segment polarity during the formation of body axis during embryonic development [68, 134]. Hence, the int and the Wg family was renamed as the Wnt family reflecting "Wingless-related integration site" [134].

### 2.1.1 Activation of the canonical Wnt signalling pathway

Wnt/β-catenin signalling pathway is activated when Wnt ligands, a family of secreted glycolipoproteins, bind to the transmembrane Frizzled group of receptors [69]. There are 19 different members in the humans [128]. Wnt ligands are actively secreted and they interact with two co-receptors on the cell surface – seven transmembrane Frizzled (Fzd) receptors that directly bind with the Wnt ligand through cysteine-rich domains at N-terminus [113, 135] and single-pass transmembrane low-density lipoprotein receptor-related proteins 5/6 (LRP-5/6) [136, 137] (See fig 2.2).

The Fzd receptors once activated by Wnt ligand binding, relay signals intracellularly as they are coupled with heterotrimeric G proteins [135]. LRP-5/6 is an important co-receptor that can modulate the Fzd-Wnt interaction by the binding to extracellular inhibitors WISE and Dickkopf (Dkk) [137]. Several versions of DKK genes exist in humans, however, DKK1 is the most important inhibitor of the Wnt/β-catenin signalling [138]. DKK1 binds with LRP-5/6 and Kremen that causes

internalization of LRP-5/6, consequently inhibiting Wnt/β-catenin signalling. The type of internalization of LRP-5/6 determines whether the Wnt pathway will be activated or inhibited. DKK-induced-Clathrin mediated internalization, causes Wnt pathway inhibition whereas LRP-5/6 internalization via Caveolin activates Wnt/β-catenin signalling [139, 140].

On the other hand, Wise and Sclerostin (SOST) proteins interfere with the association between Wnt ligands and LRP5/6 [141]. Inhibitors like Cerberus (CER) and FrzB-1 sequester Wnt ligands and prevent their interaction with the Fzd receptor [142]. Wnt inhibitory factor (WIF) directly binds to the Wnt ligands and prevent their availability [128]. Secreted Frizzled related proteins (sFRPs) compete with Fzd receptors as structurally they share homologous cysteine rich domains [143]. Shisa protein inhibits maturation of the Fzd receptors and prevents them from reaching the cell surface and isolating them in the endoplasmic reticulum [144]. Recently, it has been shown that post-translational fatty acylation is a key step in Wnt secretion and receptor recognition [129].

When Wnt ligands interact with Fzd and LRP5/6, the receptors coupled to G proteins on the inner side of the plasma membrane transduce signal to Dishevelled (Dvl) and Axin, and bind with β-catenin through their DIX domain [145]. Protease-activeated receptor-1 (PAR1) protein phosphorylates Dvl which then binds to Fzd intracellularly and

rescues β-catenin from degradation by recruiting glycogen synthase kinase binding protein (GBP)/Frequently rearranged in advanced T-cell lymphoma-1 (FRAT-1) [142] (See Fig 2.2).

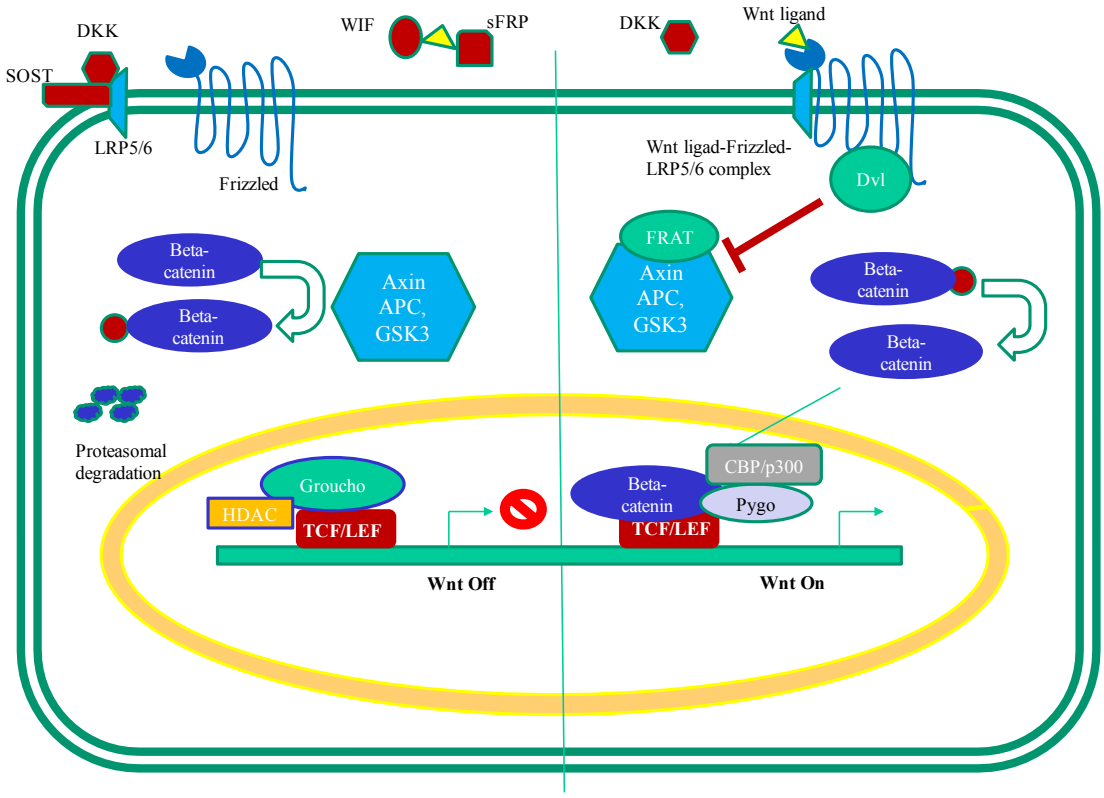



**Figure. 2.2** – Schematic diagram depicting regulation of Fzl-LRP5/6, APC-Axin-GSK3 complex and TCF/LEF repressors and activators during Wnt off and Wnt on stages. In the Wnt off state, TCF/LEF transcription is blocked is by Groucho and histone deacetylase (HDAC). β-catenin accumulates in the cytoplasm and translocates to the nucleus once it is rescued by the action of Dvl upon Wnt ligand binding association with Fzl and LRP5/6. β-catenin displaces the TCF/LEF repressors and with the help of TCF/LEF activators, swtiches on the Wnt target gene transcription.

GBP/FRAT-1 mediates interaction between Dvl and the 'β-catenin destruction complex', enabling them to interact. The destruction complex comprises scaffold protein Axin, tumour suppressor APC and

serine/threonine kinases GSK3β and Casein Kinase 1α (CK1α). The degradation complex captures free β-catenin, sequentially phosphorylates it and marks it for subsequent ubiquitination and proteasomal degradation [146, 147]. Upon Wnt activation however, Dvl inhibits the destruction complex causing β-catenin to be unphosphorylated enabling its nuclear translocation. FRAT-1 causes dissociation of GSK3β from the degradation complex, interfering with GSK3β mediated β-catenin phosphorylation, subsequently preventing its degradation [148]. At the same time, LRP-5/6 facilitates degradation of Axin. The cytoplasmic tail of LRP-5/6 contains residues that become phosphorylated upon Wnt ligand binding, effected by GSK3β and CK1 which in turn creates a binding site for Axin on LRP-5/6 enabling recruitment of Axin to the plasma membrane and facilitating its degradation [149]. Overall, Wnt activation causes disassembly of the destruction complex, thus rescuing β-catenin from degradation [140, 150].

Many other co-factors modulate the subcellular location of β-catenin. Kramps et al. (2002) identified two segment polarity genes in Drosophila – legless (lgs)/homologous to human B-cell CLL/lymphoma-9 (BCL9) and pygopus (pygo), that were required for nuclear signal transduction of Wnt pathway [151] (See Fig 2.2). Their results suggested that recruitment of Pygo permitted nuclear β-catenin to activate gene

transcription of downstream Wnt target genes [151]. Independently, Axin and APC act as nucleo-cytoplasmic shuttles and control subcellular localization of β-catenin [152, 153]. Chibby (CBY) inhibits β-catenin-mediated TCF/LEF transcriptional activation [154]. 14-3-3, which when bound to CBY results in its cytoplasmic sequestration, regulates it. Moreover, β-catenin, CBY and 14-3-3 form a stable complex that causes cytoplasmic partition of β-catenin [155].

### 2.1.2 Regulation of transcriptional activation

When β-catenin is rescued from degradation, it accumulates in the cytoplasm and subsequently enters the nucleus where it binds to the transcription factors T-cell factor (TCF) and lymphoid enhancer factor (LEF) to activate downstream genes [68]. Transcriptional repressors that mask the activation site normally block the TCF/LEF. Nuclear β-catenin displaces these repressors and other transcriptional co-activators are recruited [140]. Transcriptional co-activators include BRM/SWI2-related gene 1 (BRG-1) which is involved in chromatin remodeling [156] and histone acetylase Cyclic adenosine monophosphate (cAMP)-response element-binding (CREB)-binding protein (CBP)/p300 that binds to the β-catenin at its carboxyl domain [157]. In addition, BCL9/Legless and Pygopus function to sequester β-catenin in the nucleus and modulate its association with TCF [151]. Inhibitor of β-catenin and TCF (ICAT) inhibits Wnt target gene transcription by

preventing the binding of β-catenin with TCF [158] and dissociating the β-catenin-LEF-CBP/p300 complex [159]. The TCF function is context dependent. TCF complexes with Groucho and acts a Wnt target gene repressor when the Wnt signaling is off [159]. However, when the Wnt ligand activates Wnt/β-catenin signalling, nuclear β-catenin disrupts the Groucho-TCF complex to activate Wnt target gene transcription [159].

### 2.1.3 Inhibition of the Wnt/β-catenin signalling pathway

β-catenin is a powerful nuclear transcription factor that is actively degrades in the cytoplasm so that it is prevented from reaching the nucleus to transactivate the downstream Wnt target genes [69].

CK1α and GSK3β operate like a dual-kinase mechanism and sequentially phosphorylate β-catenin at its the N-terminal end. Initial phosphorylation at Ser45 by CK1α primes β-catenin for subsequent phosphorylation at Thr41, Ser37 and Ser33 by GSK3β [147, 160]. β-catenin phosphorylation at Ser33 and Ser37 generates a docking site for the E3-ubiquitin ligase, β-transducin repeat containing protein (β-TrCP). This marks β-catenin for subsequent ubiquitination and proteasomal degradation [137, 161] (See Fig 2.3).

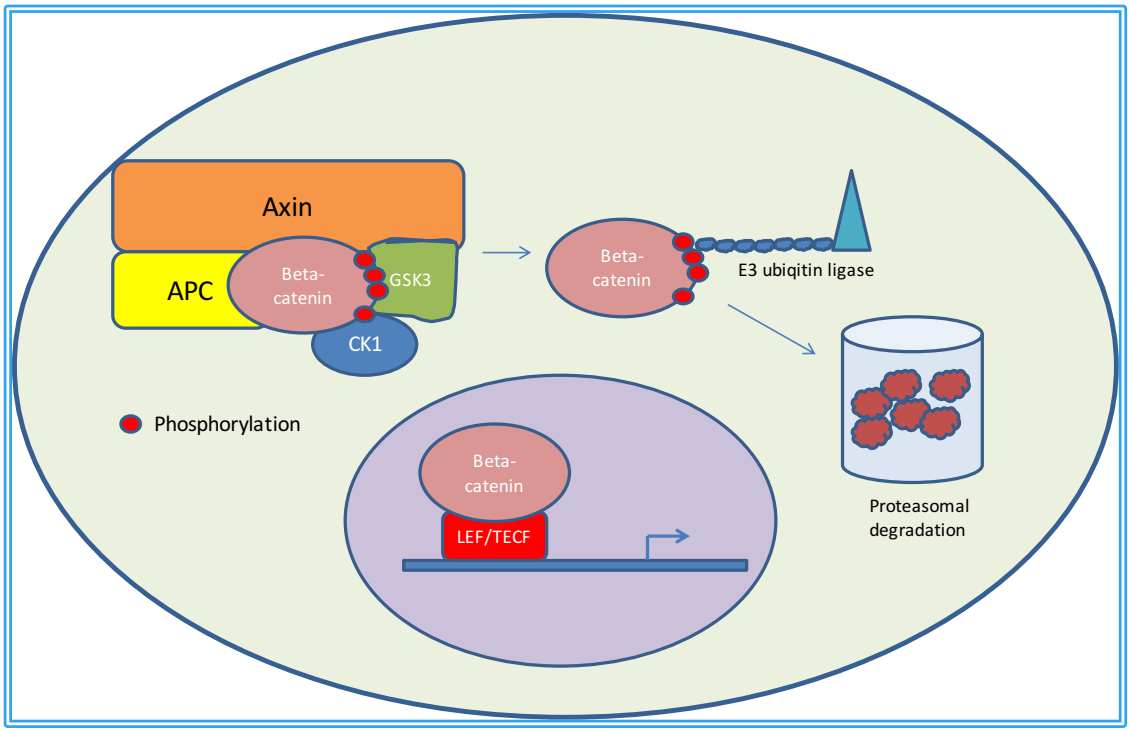



**Figure. 2.3** – Wnt 'off' state: β-catenin is actively degraded within the cytoplasm by in the nucleus or cytoplasm by CK1α and GSK3β in a dual-kinase mechanism and mark it for proteasomal degradation in the Wnt 'off' state.

CK1α and GSK3β also phosphorylate APC and Axin to increase their affinity for β-catenin ensuring its phosphorylation and degradation [135, 147]. Additionally, protein phosphatase-1 (PP1) and protein phosphatase-2A (PP2A) are two serine/threonine phosphatases that bind with Axin and APC to counteract the actions of CK1α and GSK3β in the β-catenin degradation complex. PP1 causes de-phosphorylation of Axin and promotes disassembly of the β-catenin degradation complex [162]. PP2A on the other hand reduces β-catenin degradation by dephosphorylating β-catenin [163].

Lee (2003) found intracellular Axin levels to be rate limiting in Wnt signalling as there were lower quantities of Axin as compared to the quantities of the other members of the degradation complex [164]. Their study showed that the half-life of β-catenin halved by increasing the concentration of Axin. Such a remarkable effect was not seen in varying the amounts of any other component of the degradation complex [164]. In the nucleus, the Wnt target genes are maintained in repression by TCF/LEF family of proteins [68].

### 2.1.4 Beta-Catenin

β-catenin is a powerful nuclear transcription factor that is the central effector of the canonical Wnt signalling pathway [128]. When the Wnt signalling is inactive (absence of Wnt ligand-Fzd interaction), cytoplasmic β-catenin is continually degraded by the action of the 'degradation complex' that comprises scaffolding protein Axin, the tumor suppressor APC, casein kinase 1α (CK1α), and glycogen synthase kinase 3β (GSK3β) [69, 165]. Axin has three separate domains to interact respectively with β-catenin, GSK3β and CK1α. In addition, Axin interacts with APC through an RGS domain [69].

#### *Structure of β-catenin*

Beta-catenin, encoded in the humans by the CTNNB1 gene, is a dual function protein (781 amino acids) that plays a central role in Wnt signalling and cell-to-cell adhesion [128]. It is homologous in

Drosophila to the protein armadillo. It also regulates cell adhesion by associating with Cadherins and other catenin family of proteins as a part of the adherens junction [166]. β-catenin anchors the cadherin complex to the actin cytoskeleton by binding to the cytoplasmic domain of E-cadherin and by forming a complex with α-catenin, [167]. β-catenin is a key mediator of Wnt signalling homologous with Armadillo (ARM) of Drosophila and β-catenin in Xenopus [168].

Different parts of the β-catenin domains play important roles in their function (See Fig 2.4). The amino-terminus of β-catenin regulates its stability by providing phosphorylation sites for GSK3β and CK1α to act on; on the other hand, the carboxyl terminus functions as the transcriptional activator domain [68].

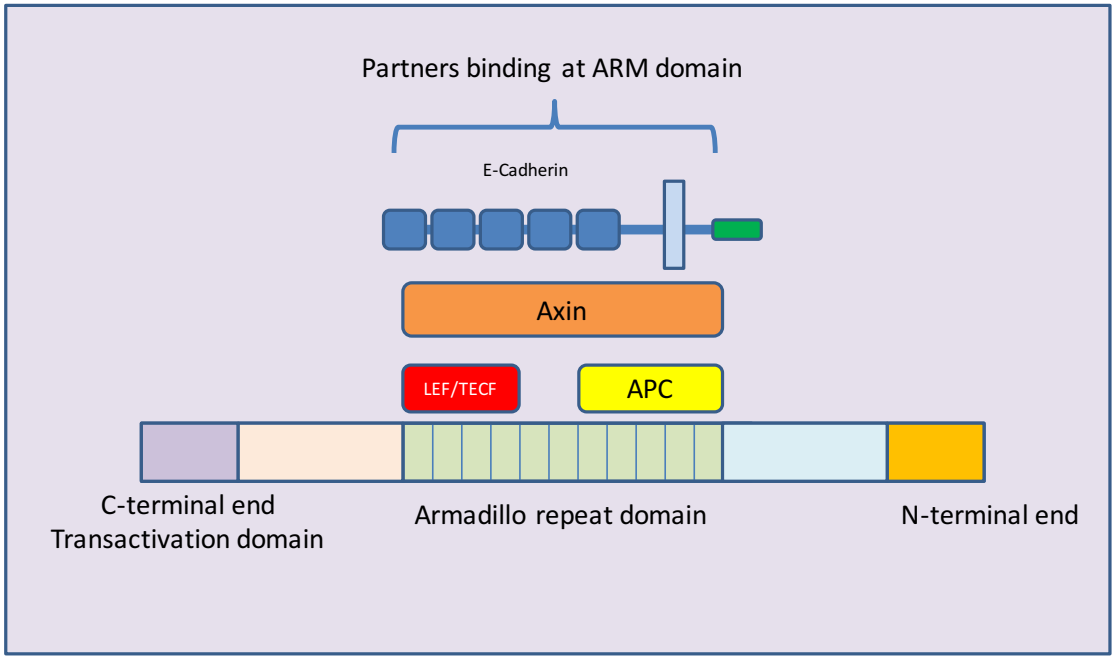



**Figure 2.4** – Schematic diagram of the structure of β-catenin with its various domains and binding partners.

A core of 12 ARM repeats is present at the centre of β-catenin, which forms a positively charged domain allowing association of multiple β-catenin binding partners. The N-terminus is vital in determining the fate of β-catenin since after phosphorylation by GSK3β and CK1α it gets recognized by the β-TrCP ubiquitin ligase which ubiquitinates it and destines it for consequent proteasomal degradation [169].

***β-catenin Phosphorylation***

Under basal conditions phosphorylation status of β-catenin determines whether or not it will be degraded by the proteasome. In the Wnt 'off' state, β-catenin is first phosphorylated by CK1α on the N-terminus at Ser45, which primes it and facilitates further phosphorylation of Thr41, Ser37 and Ser33 by GSK3β [147, 160] (See Fig 2.5). Phosphorylation at Ser37 and Ser33 on β-catenin directs ubiquitination of β-catenin by β-TrCP causing it to be degraded by the proteasome [161, 165] (See Fig 2.5). The nuclear import of β-catenin however, requires Rac-JNK-mediated phosphorylation of β-catenin at Ser191 and Ser605 residues [170].

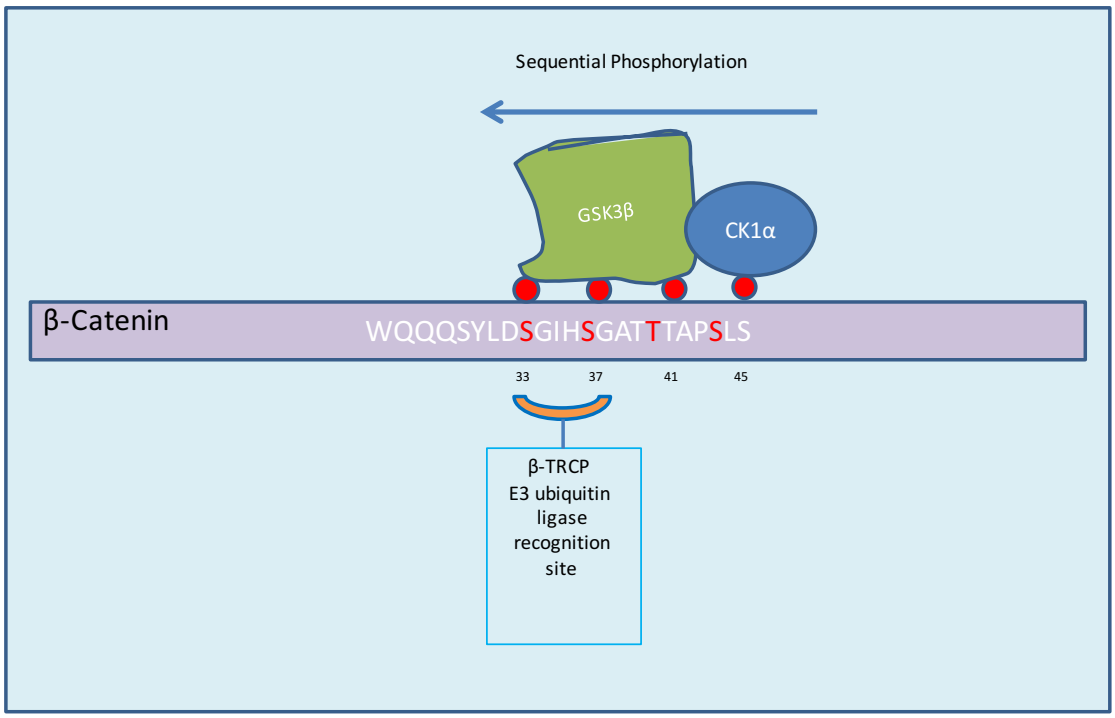



**Figure 2.5** – Sequential phosphorylation of β-catenin by CK1α and GSK3β

In the Wnt 'on' state, phosphorylation at Ser552 promotes nuclear accumulation of β-catenin accumulation and increases its transcriptional activity [171, 172]. Phosphorylation of β-catenin at Ser552 can be facilitated by either Protein Kinase B (Akt) or Protein Kinase A (PKA) [173].

There are two pathways through which β-catenin can be degraded. β-TrCP and Ebi are two F-box proteins that recognise and bind to the same N-terminal domain of β-catenin [174]. Whereas β-TrCP requires prior phosphorylation of β-catenin, Ebi is independent of its phosphorylation status. Ebi cooperates with Seven in absentia homolog-1 (SIAH-1), which itself is induced by TP53 [175, 176]. However, intact APC

protein is essential for both pathways [174]. The stabilized cytosolic β-catenin translocates into the nucleus by a yet not fully understood mechanism. Nuclear β-catenin interacts and binds to the TCF/LEF family of transcription factors, and activates expression of downstream Wnt genes [68].

### 2.1.5 Glycogen synthase kinase 3β (GSK3β)

GSK3β is a serine/threonine kinase that plays a crucial role in the stabilization of β-catenin and is a key component of the degradation complex. GSK3β phosphorylates β-catenin at Ser33, Ser37 and Thr41 after a priming phosphorylation at Ser45 by CK1. Phosphorylation at Ser33 and Ser37 creates a recognition site for β-TrCP E3-ubiquitin ligase that targets β-catenin for proteasomal degradation [160]. It is an 832 amino acid protein that contains binding sites for β-catenin, Axin and Dvl allowing it to form a multi-protein β-catenin degradation complex [177-178].

The stability of GSK3β depends upon its phosphorylation status where phosphorylation at Ser9 inactivates it [179]. Phosphorylation at Ser9 creates a pseudo-substrate that interferes with its active site thus making it incapable to phosphorylate its target proteins, primarily β-catenin [180]. Numerous other kinases can phosphorylate GSK3β at Ser9, and thus regulates its function.

Although GSK3β can be phosphorylated at Ser9 by PKA, p90Rsk and integrin-linked kinase (ILK) amongst others, the main regulator of GSK3β is Akt that inhibits it through the phosphatidylinositide 3-kinase (PI3K) pathway [181]. This places GSK3β as a converging point in the crosstalk of various signal transduction pathways bringing about complex and intricate control of cellular processes. Phosphorylation on Ser9 is further enhanced by two further phosphorylation events – phosphorylation at Thr43 mediated by extracellular signal regulated kinase (ERK); and phosphorylations at Ser389 and Thr390 by p38 mitogen activated protein kinase (MAPK). Both of these phosphorylations further increase Ser9 phosphorylation of GSK3β and thus facilitate its inhibition [179].

Although GSK3β is constitutively active, phosphorylation at Tyr216 makes it more active by changing the conformation of its active site. If there is no phosphorylation at Tyr216, the base of its active site is blocked by a side-chain, which diminishes its ability to bind to its substrate. The active site is freed up when the Tyr216 residue is phosphorylated, allowing easier access by substrates to GSK3β [180, 182].

GSK3β is a multifunctional protein and besides β-catenin also regulates the intracellular levels of c-myc through its phosphorylation on Thr58 that marks it for degradation [180]. Before this can happen, c-myc

requires phosphorylation on Ser62 by ERK for its stabilisation. After the GSK3β and ERK-mediated phosphorylations, PP2A dephosphorylates at Ser62 to allow for subsequent ubiquitination and proteasomal degradation [183].

### 2.1.6 TCF/LEF Transcription Factors

The TCF/LEF are a family of nuclear transcription factors that has four members: TCF1, TCF3, TCF4, and LEF1 [184]. They are characterised by a *β-catenin binding domain* on the N-terminal and a high mobility group *DNA binding domain* on the C-terminal that associates with the nuclear transcription regulating Wnt-responsive elements which in turn activates the promoter regions of the target genes such as Cyclin D1 and c-myc, thus upregulating their transcription [184-186]. Downstream activation of Wnt target genes such as CyclinD1 and c-Myc, may regulate many cellular processes including survival, growth, proliferation and differentiation. Not surprisingly therefore, constitutional activation of Wnt signaling is seen in many different types of tumours [69].

If the β-catenin is not phosphorylated, it escapes ubiquitination by β-TrCP and subsequent proteasomal degradation and accumulates in the cytoplasm, referred to as stabilization of β-catenin. Stabilized β-catenin then translocates into the nucleus and associates with TCF/LEF transcription factors [68]. For this interaction to take place, it first has to

displace the transcriptional repressors, which mask the β-catenin binding site. This process also requires the co-operation of other transcriptional co-activators [140].

The activity of TCF/LEF is modulated by a set of transcriptional co-activators (e.g. CBP/p300) and transcriptional repressors (e.g. Groucho). After translocating into the nucleus, β-catenin displaces transcriptional co-repressor Groucho from the TCF/LEF complex and itself binds to the transcriptional activation domain of TCF/LEF [186] (Fig 2.2). Transcriptional co-activator CBP/p300 facilitates TCF/LEF-β-catenin interaction. On the other hand, Nemo-like kinase (NLK)/Nemo acts like a Wnt repressor. It phosphorylates TCF/LEF that in turn decreases its affinity to form the TCF/LEF-β-catenin complex with DNA [128, 140, 187]. Many post-translational modifications are crucial in regulating the binding of TCF/LEF transcription factors with their transcriptional co-activators or repressors. These include phosphorylation, ubiquitination, acetylation and sumoylation [140]. Thus, downstream activation of Wnt targets is tightly regulated, given their influence on the various growth processes that may promote tumour formation if deregulated.

### 2.1.7 Wnt Target Genes

When the Wnt signaling is activated, β-catenin stabilizes in the cytoplasm and subsequently translocates to the nucleus, where it

engages DNA-bound TCF/LEF transcription factors [188, 189]. The TCF/LEF-β-catenin complex then drives the activation of the many downstream Wnt target genes that have a wide variety of cellular functions [190]. Some of the key target genes are shown in Table 2.1.

**Table 2.1:** Wnt target genes, adapted from Nusse's Wnt Homepage [68]

| Gene | up/down | References |
|---|---|---|
| c-myc | up | He, 1998 [191] |
| Cyclin D | up | Tetsu, 1999 [192]; Shtutman, 1999 [193] |
| Tcf-1 | up | Roose, 1999 [194] |
| LEF1 | up | Hovanes, 2001 [195] Filali, 2002 [196] |
| c-jun | up | Mann, 1999 [197] |
| Matrix metalloproteinase MMP-7 | up | Brabletz, 1999 [198] Crawford, 1999 [199] |
| Axin-2 | up, feedback inhibitor of Wnt pathway | Yan, 2001 [200] Lustig, 2002 [201] Jho, 2002 [202] |

Many of the Wnt target genes are specific to a particular tissue or to the stage of development; however, Axin2 gene is considered to be a general indicator of the Wnt pathway activity [201]. The mechanism of the nuclear importation of β-catenin remains unclear, although recently it has been suggested that it may be related to microtubules and active transport [203]. Interestingly, it has been shown that active Wnt signalling may not need an overall demonstrable increase in the level of nuclear β-catenin. It has been suggested that fold change rather than

absolute levels of nuclear β-catenin may be crucial. This implies that nuclear β-catenin even at low levels may be sufficient for transcriptional activity without having any constitutionally activating β-catenin mutations [204].

To conclude, Wnt signalling is crucially involved not only in development but also in cell fate determination, cell proliferation, survival, migration, cell polarity and death; furthermore, dysregulation of this pathway may cause cell transformation and tumour formation.

### 2.1.8 Role of Wnt signalling pathway in cancer

Wnt signalling is required for stem cell renewal. Hence, it is not entirely unexpected that mutations within the major Wnt proteins like β-catenin, APC and Axin frequently lead to cancer, in tissues that require Wnt signaling for self-renewal or repair [69]. It is known that deregulation of Wnt signalling promotes stromal invasion and metastasis in various tumours. This is explained by the fact that the downstream Wnt targets regulate a myriad of cellular processes including growth and proliferation [69]. In colon carcinomas, APC mutations, a key component of the β-catenin degradation complex, results in stabilization of β-catenin allowing it to initiate oncogenic transformation [115, 205].

Anaplastic thyroid cancer shows a high frequency of β-catenin mutations with consequent nuclear β-catenin accumulation, which supports the role of Wnt activation in its pathogenesis [206].

Additionally, papillary thyroid carcinomas associated with familial adenomatous polyposis, a hereditary disorder in which the APC gene is mutated, display nuclear β-catenin immunostaining [207]. Previously, we have provided evidence for the relevance of the Wnt pathway in the regulation of thyrocyte proliferation [208, 209] Inactivation of E-cadherin appears to be another mechanism by which the unphosphorylated cytosolic β-catenin can be stabilized [210].

Germ line APC gene mutations cause a hereditary cancer syndrome known as familiar adenomatous polyposis (FAP) syndrome. FAP patients are heterozygous for APC mutations. The loss of the second allele in the cells results in colorectal adenomas or polyps, in these patients. The adenoma-carcinoma sequence progresses as additional mutations accrue in genes like K-ras, p53, and Smad4 [211]. Moreover, APC and Axin-2 mutation results in stabilisation of β-catenin and resultant constitutively active complexes with Tcf4 [212, 213]. Recently, exome sequencing has shown that many of the colorectal cancers harbour inactivating APC mutations [69].

### 2.1.9 Role of Wnt signalling pathway in normal muscle development

Embryonic myogenesis is regulated spatio-temporally by a complex orchestration of embryonic signalling pathways, which include Wnt pathway, Shh pathway and BMP. Wnt signalling is vital during development of musculature in the embryo [91]. Wnt pathway controls

the expression of certain myogenic regulatory factors (MRFs) like MyoD and Myf5, which are essential for myogenic lineage progression in the embryonal stage of muscle development [91]. The regulation of the myogenic stem cells, also known as the satellite cells, is under the influence of canonical Wnt signalling [91]. Many studies using genetic knockout models have confirmed the need for several Wnt proteins in the normal myogenesis [214, 215].

Studies on muscle development on the mice have provided insights into the mechanism of muscle development. The mesodermal primary germ layer gives rise to the vertebrate skeletal muscle [216]. The paraxial mesodermal cells mature to form paired segmental somites along the embryonal anterior/posterior axis, which later forms the majority of skeletal muscles [217]. The somite gradually patterns into the dorsal epithelial dermomyotome and the ventral mesenchymal sclerotome. The latter forms cartilages and bones whilst the former gives rise to skin and skeletal muscles of the body and extremity [123].

The expression of Pax3/Pax7 transcription factors direct the formation of myogenic precursor cells (MPCs), within the dermomyotomes [218]. Myogenic regulatory factors are expressed by the delaminating cells of the dermomyotome that repress Pax3/Pax7 and promote myotome formation, from which muscles of the limbs develop

[219]. During embryonic development, the skeletal muscles of the trunk and limb are derived from somites [123, 220-222].

### 2.2 Skeletal Muscle Myogenesis

During embryonal development, the muscle progenitor cells that differentiate to form the future skeletal muscles are located in the dermomyotomes within the somites [223]. A regulatory network of genes including Tbx6, Mesp-b and Ripply1, are involved in the determination of dermomyotome and myotome [224]. Skeletal myogenesis crucially rests upon the tight regulation of gene sets that help the muscle progenitor cells differentiate into myofibres.

#### 2.2.1 Genetics of muscle development

Myogenic basic helix-loop-helix (bHLH) transcription factors are a group of genes that include Myogenin, MyoD, Myf5 and MRF4. These are critical for the formation of skeletal muscle. MyoD and Myf5 are initially required for the myogenic progenitors to differentiate into myoblasts. Following this, Myogenin helps in the differentiation of the myoblast into myotubes [223]. MRF4 is a very important factor that ensures the growth and proliferation of myogenic progenitor cells before they can differentiate by blocking the transcription of muscle-specific promoters [223].

The myoblast differentiation occurs in three defined stages. In the first stage, the myoblasts exit the cell cycles express regulatory genes that drive the second stage differentiation where the myoblasts align with each other. In the third stage the myoblasts actually fuse with each other to form the myotubes; actin is actively recruited at the plasma membrane, which appose closely to form a pore that gradually widens. Calcium and certain metalloproteinases are essential for the last stage and their regulation may serve as another control of myogenesis. A number of genes expressed during myogenesis are being actively investigated [223].

The genes fall into the following groups:

1. Myocyte enhancer factor-2 (Mef-2) are transcription factors, which promote myogenesis. There are 4 versions of Mef-2 genes in the vertebrates; human Mef-2 genes are denoted as MEF2A, MEF2B, MEF2C and MEF2D [225].

2. Serum Response Factor (SRF) induces the expression of striated alpha-actin genes [226] through recruitment of androgen receptors [227]. Thus, androgens can regulate myogenesis via regulation of the expression of striated alpha-actin genes.

3. Myogenic regulatory factors (MRFs) that include – Myogenin, MyoD (Myf3), Myf5 and Myf6, are critical determinants of muscle identity in development, growth and regeneration [228]. MyoD has provided a

deeper insight into our understanding of the processes of myo-differentiation [229] when it was shown that transfection of a single cDNA could convert fibroblasts into myoblasts [230]. This established MyoD as the master regulator of cell fate in myogenesis. Soon the other members of the MRFs were identified including Myf5 [231], Myogenin [232] and MRF4 [233]. These factors also shared the ability of MyoD to convert fibroblasts into myoblasts and activate muscle gene expression. Together, these genes are expressed in the myogenic cells during embryonic myogenesis [234] albeit at varying stages of myogenesis, reflecting subtle differences in the roles during myo-differentiation [235, 236]. Pestronk described various stages of myogenesis, each of which is controlled by a set of defined genes that are critical [237]. The different stages with their associated genes are described in Table 2.2.

**Table 2.2: Genes associated with muscle development [223]**

| Stage | Associated Genetic Factors |
|---|---|
| Delamination | PAX3, c-Met |
| Migration | c-met/HGF, LBX1 |
| Proliferation | PAX3, c-Met, Mox2, MSX1, Six1/4, Myf5, MyoD |
| Determination | Myf5 and MyoD |
| Differentiation | Myogenin, MCF2, Six1/4, MyoD, Myf6 |
| Specific Muscle Formation | Lbx1, Meox2 |
| Satellite Cells | PAX7 |

### 2.2.2 Stages of Myogenesis

#### 2.2.2.1 Delamination

The main associated genetic factors in this stage are PAX3 and c-Met. Pax3 is highly expressed during embryonic stage of development and to some extent around the foetal stage. Pax3 is differentially expressed in the migrating hypaxial cells and within the cells of dermomyotome, but never during facial musculature development [237]. Pax 3 gene is needed for the expression of c-Met, which is in turn needed for lateral migration. Any mutations in Pax3 may cause aberrant c-Met expression resulting in lateral migration defects. PAX3 also mediates MyoD

expression, which is one of the master regulators of myoblast cell fate as described previously [237].

#### 2.2.2.2 Migration

The associated genetic factors in this stage are c-MET and Ladybird Homeobox-1 (LBX1). Mutations in c-MET and/or LBX1 can cause migration arrest. Since LBX1 is essential for the structural development of dorsal forelimb muscles as well as the migration of dorsal muscles into the limb [237], any absence of LBX1 results in failure of the limb for develop normally. Deletion of LBX1 results in the hind limb muscles to be affected severely. As a result, only the flexor compartment muscles develop in the forelimb because of the ventral muscle migration [237]. c-Met on the other hand, is a receptor tyrosine kinase that is essential for the migrating myoblasts to proliferate and survive and requires PAX3 for its transcription. c-Met deletion curtails secondary myogenesis and prevents the formation of muscle in the limbs [237].

#### 2.2.2.3 Proliferation

This is an important cell fate of the myoblasts and need the cooperation of many genetic factors including Pax3, c-Met, Mox 2, Muscle Segment Homolog of Drosophila-1 (MSX1), Six, mesoderm and regional specification [237]. Myf5 is required for proper myoblast proliferation and is the first myogenic regulator to be expressed. Deletion of Myf-5 and MyoD results in amyoplasia [237].

#### 2.2.2.4 Determination

Myf5 and MyoD are myogenic bHLH proteins and their expression is tantamount for myogenesis since they make the cell committed to muscle development [238]. Co-deletion of Myf5 and MyoD results in amyoplasia [239]. Likewise, mutation in either of the two causes cells to adopt non-muscular phenotypes [237]. MyoD auto-regulates its own gene; the gene product binds to the MyoD gene and induces its own production [239]. Myf5 expression is under the control of other embryonic developmental pathways such as regulated by Shh, Wnt1 and MyoD itself [237]. The correlation of MyoD in the regulation of Myf5 shows how the two genetic factors are connected [237].

#### 2.2.2.5 Differentiation

This is regulated by Myogenin, Mcf2, MyoD, Myf6 and Six. Myogenin (Myf4) is required for the fusion of myoblasts. Deletion of Myogenin causes a near total loss of expression of differentiated muscle fibers severe skeletal muscle hypoplasia in the ventral body wall [237]. Myf-6 (MRF4) is required for differentiation of myotubes; Myf-6 loss causes centronuclear myopathy and Becker muscular dystrophy [237].

#### 2.2.2.6 Specific muscle formation

Mutations in LBX1 and/or Mesenchyme Homeobox 2 (Mox2) may affect specific regions of muscular development. Since LBX1 is required for the proper formation of the dorsal limb muscles, any disruption of

LBX1 gene results in congenital hypoplasia of the musculature of extensor compartment of the hind limbs [237]. Mox2 loss on the other hand gives rise to abnormal patterning of the limb musculature resulting in hypoplasia of the hind limbs and its musculature [237].

#### 2.2.2.7 Satellite cells

Satellite cells are under the regulation of Pax7. Loss of Pax7 function would prevent satellite cells formation and consequently cause inhibition of postnatal growth of muscle. Satellite cells are hypothesized to be quiescent myoblasts that are required for muscle repair but have a very limited ability to multiply [237]. Satellite cells can also differentiate into metaplastic osseous or adipose tissue [237].

The muscle differentiation is chiefly under the controls of two tightly regulated signal transduction pathways: the PI3K/Akt pathway and the Notch/Hes pathway. These signaling pathways cooperate with each other to inhibit the MyoD transcription, the master regulator of myogenic differentiation [238]. By stabilizing Notch/Hes binding, the O subfamily of Forkhead proteins (FOXO) plays a crucial role in exercising control over of myogenic differentiation [238].

## 2.3 Does Wnt signalling play a role in RMS?

β-catenin is a potent nuclear transcription factor that plays a central role in the canonical Wnt signalling pathway, which is actively involved in muscle development and embryonal somite patterning. Muscle development and regulation of its proliferation is precisely controlled. Under basal conditions, muscle cells are highly differentiated and rarely divide. The mechanisms that regulate this dichotomous fate of muscle cell proliferation and differentiation is incompletely understood although in recent years new molecular data are emerging which may lead to a better understanding of the molecular complexity of this process.

### 2.3.1 Wnt genes involved in myogenesis

Wnt signalling is very important for the embryonic development of limb and trunk musculature. Both canonical and non-canonical Wnt signalling pathways play essential roles in embryonic myogenesis [222]. Myogenesis is influenced by differential Wnt signals expressed spatiotemporally within the tissues surrounding the developing muscle. Dorsal region of the neural tube expresses Wnt1, Wnt3a and Wnt4 whereas Wnt4, Wnt6 and Wnt7a are expressed by the dorsal ectoderm and Wnt11 within the epaxial dermomyotome [91]. Somitic myogenesis is induced by Wnt signals (Wnt1, Wnt3a and Wnt4) that are expressed in the dorsal regions of the neural tube in cooperation with Shh signalling from the notochord [240]. Brack (2009) demonstrated that for normal

myogenesis, a temporal switch is required in myogenic progenitor cells from Notch to Wnt signaling [241].

### 2.3.2 Muscle specific genes and Wnt pathway

Crucially, Wnt also regulate the expression of myogenic regulatory proteins Pax3/7, MyoD and Myf5, which are essential for myogenesis [219, 242, 243]. In mouse model, Wnt1 induces expression of the Myf5 via the canonical β-catenin pathway [242]. On the other hand, MyoD can be also be activated by Wnt7a through a PKC-dependent non-canonical pathway under the influence on Pax3 that is β-catenin-independent [244, 245]. Previously, Borello (1999) demonstrated in a mouse model that transplacental delivery of Wnt antagonist Frzb1 inhibits the development of skeletal myogenesis [246]. Furthermore, using conditional deletion of β-catenin driven by Pax3-Cre/Pax7-Cre, Hutcheson (2009) showed that β-catenin is essential for the formation of the myotome [247]. Gros (2009) studied chick embryos and found that Wnt11expression in the epaxial dermomyotome was required for the development of the myotome through the non-canonical PCP pathway. But interestingly, Wnt11 expression itself was induced through a β-catenin-dependent mechanism involving Wnt1 and Wnt3a from the dorsal neural tube [216].

### 2.3.3 Rationale for investigating Wnt Pathway in RMS

There is emerging data that dysregulation of embryonal pathways may be central in paediatric tumours. The literature on Wnt pathway in RMS is patchy and there is conflicting data as to its therapeutic and prognostic significance. However, given the role that Wnt signalling plays in embryonal myogenesis and tumourigenesis, this study aims to characterise the Wnt pathway in RMS using clinical material and by in-vitro studies using RMS cell lines in order to improve our understanding of the mechanisms underpinning tumourigenesis in RMS. We also intend to investigate whether modulation of Wnt pathway could be exploited as a viable therapeutic option.

# CHAPTER 3 –

# MATERIAL AND METHODS

# CHAPTER 3 – MATERIAL & METHODS

This collaborative study between groups in Liverpool and Carlo Dominici's group at the University of Rome aims to characterise Wnt/β-catenin signalling in RMS in order to improve our understanding of the mechanisms underpinning tumourigenesis in RMS. The results have been published in a journal article in 2013 [248].

## 3.1 Clinical Material

The sample for the study comprised 54 RMS cases obtained from the histopathology archives of Alder Hey Children's NHS Foundation Trust (n=40, 1991–2009) and 'Policlinico Umberto I' Hospital, Sapienza University, Rome, Italy (n=14; 2005–2009) [248]. Ethical approval was obtained from research committees of Alder Hey Children's NHS Foundation Trust and 'Policlinico Umberto I' Hospital. Informed consent was obtained from all patients or their parents in accordance with the guidelines of Children's Cancer and Leukemia Group (CCLG) and United Kingdom Children's Cancer Study Group (UKCCSG). The minimum follow-up period of the patients was 5 years. Forty-four cases out of 54 had sufficient tissue to be included [248]. Patient data and tumour characteristics are presented in Table 4.1.

RMS was diagnosed in both centres using standard diagnostic criteria and was stratified histomorphologically (embryonal, alveolar, not otherwise specified); nuclear expression of Myogenin and MyoD1

was required for the diagnosis of RMS (Figure 1.1). Pax3/7–FOXO1 translocation status was assessed using standard FISH analysis, in all cases. We used normal skeletal muscle tissue (n=8) obtained at surgery for benign conditions from age-matched controls [248].

**3.2 Cell Culture**

RMS cell lines were used for the in-vitrp studies. We used four RMS cells, two from embryonal RMS (RD and RD18) and two from alveolar RMS (Rh4 and Rh30). RMS embryonal cell lines were kindly donated by Dr P.L. Lollini (from the Department of Experimental Pathology, Cancer Research Section, University of Bologna, Italy).

RD cells are one of the most commonly used cell lines in RMS research. RD cell line was established from biopsy material of a 7-year-old girl with a pelvic RMS that wad previously treated with chemo/radiotherapy but was refractory to it [249]. Rh4 (RH41) cell line was was established by Dr. Peter Houghton's laboratory at St. Judes Children's Research Hospital (Memphis, Tennesse, USA), from a 7-year-old girl with alveolar RMS. Rh4 harbors the t(2;13)(q35;q14)translocation, representing ARMS [249]. RH30 cell line was derived from the culture of bone marrow obtained from a 16-year-old male with untreated metastatic alveolar RMS. It harbours t(2;13) translocation [249]. Details of the RMS cell lines are tabulated in Table 3.1.

## Table 3.1: Characteristics of RMS cell lines, their origin and karyotype/translocation status [249]

| Cell Line | RMS subtype | Origin | Karyotype/Gene fusion status | Ploidy and doubling time | Source |
|---|---|---|---|---|---|
| **RD** | ERMS | Derived from pelvic tumour in a 7yr old girl | • MYC (8q24.21) amplified and overexpressed<br>• homozygous p53 mutation (R248W)<br>• heterozygous NRAS mutation (Q61H)<br>• p53 OFF, Rb1 OFF and Ras ON sigatures | 51-hyperdiploid | #4241949<br>ATCC (cat# CCL-136) |
| **RD18** | ERMS | Same patient above (as RD) | • Same as above | Same as above | P. Nanni (Università di Bologna, Italy) |
| **Rh4 (RH41)** | ARMS | Derived from ARMS in a 7yr old girl with progress-ive disease | • Pax3:FOXO1a t(2;13) (q35;ql4)<br>• homozygous p53 (exon 5-13 bp deletion of amino acids 150-154) | Diploid<br>doubling time – 60hrs | #6950170<br>D.N. Shapiro/ P. Houghton (St. Jude); www.cogcell.org |
| **Rh30** | ARMS | Derived from untreated ARMS in a 16 yr old boy; bone marrow metastasis | • Pax3:FOXO1a t(2;13) (q35;q14)<br>• heterozygous p53 (R273C and R280S)<br>• c-MET, GLI, CDK4 and MDR1 amplification | near triploid with chromosomes between 51-87<br>doubling time – 35hrs | #3691179<br>P. Houghton/ T. Look (St. Jude); www.cogcell.org |

The cell lines were maintained at 37 °C in 5% $CO_2$ in high glucose DMEM medium supplemented with 10% foetal calf serum (Sigma, Dorset, UK), 1% L-glutamine, 100 µg/ml streptomycin and 100 U/ml penicillin. The RMS cells were passaged in T-25 flasks at an approximate starting density of $7x10^5$/ml in 3-5mls of DMEM culture medium every 3-4 days once they reached a confluence of around 80-90%. The cell density was counted using a haemocyometer. The cell lines were re-suspended in fresh culture 24 hours before the in-vitro experiments to ensure optimal exponential growth prior to use [248].

**3.3 Tissue Microarray and Immunohistochemistry**

Tissue microarrays (TMA) using multi-tissue blocks were assembled according to tissue array technology developed in the Department of Cellular Pathology, Royal Liverpool University Hospital. The TMA contained 136 cores from 34 RMS cases. Four cores (1mm deep) were selected from the each tumour after reviewing the H&E slides. Sixteen cores from eight cases containing normal muscle tissue served as controls. Those tumour blocks that were less than 1mm were not included for TMA (n=10); instead, full-block sections were used for immunohistochemistry [248].

Briefly, H&E slides were reviewed for each case and different areas of the tumour were marked to account for tumour heterogeneity. Corresponding marks were made of the tumour blocks from which the

TMA was constructed. Four 1mm cores were punched out from the blocks using the TMA punching machine and these cores were assembled in a single paraffin block to create a homogenous TMA multi-block. The blocks were incubated overnight to set. The blocks were then cut on to a microtome to generate 4μm sections after they were dewaxed in xylene (10min twice) and rehydrated by graded alcohol (20min). Antigen retrieval was done in a microwave oven (20 minutes C) in 10mM sodium citrate; pH 6.0). [248]

Anti-β-catenin primary antibody (1:200, monoclonal mouse anti-human β-catenin, DAKO, UK) was applied to the sections and biotinylated secondary antibodies used for signal amplification (Amersham, UK). Endogenous tissue peroxidase activity was blocked by incubation with 3% hydrogen peroxide for 8 min. Expressional analysis of β-catenin was performed by two independent pathologists (blind). [248].

The criteria for positivity was defined as at least 10% of the tumour cells expressing β-catenin in four representative cores. The intensity of β-catenin expression was scored: 0=negative, 1=weak, 2=moderate, 3=strong [248]. The localisation of β-catenin was defined as cytoplasmic/membranous or nuclear. Cases where all four cores were not positive for β-catenin expression, only those cases were deemed positive for β-catenin where at least three cores showed 2+ expression

[248]. The case was considered to be negative if the expression was weak (1+). Those cases that were unsuitable for TMA, full-block sections (n=10) were examined for β-catenin expression and were only regarded as positive if at least 10% cells showed moderate or strong expression [248].

### 3.4 Protein Extraction

All RMS cell lines were grown in 100mm culture dishes or T-25 culture flasks (Falcon, UK). Once the RMS cells reached 80-90% confluence, the growth medium was discarded and cells were washed with sterile PBS (8g NaCl, 0.2g of KCl, 1.44g $Na_2HPO$ and 0.24g $KH_2PO_4$ in 1L distilled water, pH 7.4). The RMS cells were detached from the flask by incubation with Trypsin (50 μg/ml, Sigma) for 5-10 minutes at 37°C. The action of Trypsin was then blocked by adding complete DMEM media. Cells were collected by centrifugation at 1000 rpm at 4°C for 5 minutes. The supernatant was discarded and cell pellets were resuspended in one ml PBS, then transferred to 1.5ml eppendorf tubes and centrifuged at 3000 RPM at 4°C for 5 minutes.

Cell scraping (~5 x $10^6$ cells) were obtained from culture flasks after trypsinisation and were washed twice with cold PBS. Standard cell lysis buffer was used to obtain cellular protein extracts. Cells were lysed in 100μl lysis buffer [50mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid/HEPES (pH 7.4), 150mM Sodium

Chloride (NaCl), 10% glycerol, 1mM Ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, 1mM Sodium metavanadate ($NaVO_3$), 100mM Sodium fluoride (NaF), 1mM phenylmethysufonyl fluoride (PMSF) and protease inhibitor cocktail (Roche)] [248]. Protease inhibitors were added fresh: aprotinin 2µg/ml, leupeptin 2µg/ml and 2mmol PMSF) at 4°C for 30min. Extracted proteins were stored in lysis buffer at -80°C. For in-vitro drug studies, 2 x $10^6$ cells/ml were cultured in DMEM supplemented media at 37°C for 24 hours with or without 200ng/ml of human recombinant Wnt3a or SDF-1 (R&D System).

### 3.5 Determination of Protein Concentration

Protein determination was performed using Bradford colorimetric method according to manufacturer's recommendations (Bio-Rad, Hemel Hempstead, Hertfordshire). This method involves the binding of Coomassie Brilliant Blue G-250 to amino acids. The binding of the dye to proteins causes a shift in the absorption spectrum of the dye from 465 to 595 nm, and it is the increase in absorption at 595 nm which is monitored.

To determine the unknown quantity of protein in the lysates, a standard curve was made from a series of known concentrations of Bovine Serum Albumin (BSA) solution (1mg/ml ddH2O) as shown below in Table 3.2. The protein sample being tested was made from 5µl whole cell protein lysate and 995µl ddH2O. Thereafter, 200µl of the

Bradford reagent was added to each of the BSA and the protein samples, mixed thoroughly and kept in the dark for 15minutes.

**Table 3.2: Concentration of reagents for determine the unknown quantity of protein in the lysates**

| Final conc. (µg/ml) | BSA Stock (10µg/ml) | 1% Lysis Buffer |
|---|---|---|
| 0 | 1000µl $ddH_20$ | - |
| 0.5 | 50µl | 950µl |
| 1 | 100µl | 900µl |
| 1.5 | 150µl | 850µl |
| 2 | 200µl | 800µl |
| 2.5 | 250µl | 750µl |
| 3.0 | 300 µl | 700 µl |

To quantify the proteins in the samples 200µl from each of the standard and the protein samples were loaded onto a 96-well plate and absorbance was read at 595nm in a micro-plate reader using XRead Plus v4.30 software. Once plotted against the standard curve, results from the protein samples represented the amount of total protein present in 5µl of the original extracted protein sample.

### 3.6 Western Blot Analysis

For western blot analysis, proteins (10µg/lane) were resolved in 7.5% SDS-PAGE; 10µg proteins in 20µl of radioimmunoprecipitation assay (RIPA) lysis buffer (50 mM Tris HCl, (pH 8.0), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS) were used per lane. Each sample was mixed with 5 µl sample buffer (2% SDS, 0.125 M Tris-base HCl pH 6.8, 0.001% w/v bromophenol blue, 10% v/v glycerol) and β-mercaptoethanol (one quarter volume of loading sample buffer), vortexed briefly and heated at 100°C for 2 minutes. The samples were centrifuged briefly before loading them onto the gel. The denatured protein samples were separated by 7.5% Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis/SDS-PAGE (4.2ml of ddH2O, 3.3 ml polyacrylamide (30%, Sigma), 2.5ml 1.5M Tris-base HCl pH 8.8, 100µl of 10% w/v SDS, 50µl of 10% w/v APS (ammonium persulphate) and 5µl TEMED (Sigma). TEMED was added just before loading the mixture into the gel cassette. The gel was allowed to set for about 45 minutes. A 4% stacking gel (10ml final volume) was made up of 6.6 ml ddH2O, 1.3ml 30% acrylamide (Sigma), 2.5 ml of 0.5M Tris-base HCl pH 6.8, 100µl of 10% w/v SDS, 50µl of 10% w/v APS (ammonium persulphate) and 10µl TEMED (Sigma). The mixture was poured immediately over the resolving gel. A multiwell comb was inserted carefully and the gel was allowed to set for 20-30 minutes. The comb

was removed and the protein samples were carefully loaded into the wells, avoiding any loss or spillage into adjacent lanes. The proteins were first allowed to penetrate through the stacking gel by running at a low voltage (50 V) for 30 minutes in running buffer (14.4 g glycine (Sigma), 3.03 g Tris-base (Sigma) and 10 ml 10% w/v SDS (Sigma) in one litre ddH2O). Thereafter, the running voltage was increased to 100-150V in order to separate the proteins according to their molecular weight. The separated proteins were transferred to a polyvinylidene fluoride/PVDF membrane (Millipore, Billerica, MA, USA) using standard procedures [248]. The protein transfer was done in a transfer buffer (14.4 g glycine, Sigma, 3.03 g Tris-base, Sigma and 200 ml MeOH in one litre ddH2O) using a 100V current for one hour. After blocking with 5% non-fat dry milk in TBS-T buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween-20) for 20-30 minutes with constant agitation on a roller, the blots were incubated with the following primary antibodies (See Table 3.3 for details of primary antibodies) in the blocking solution for either 2 hours at room temperature or overnight at 4°C with constant agitation. Successful protein transfer was tested by soaking the membranes in Ponceau S solution (5% w/v in glacial acetic acid, Sigma) for few minutes. The membranes were then washed with PBS. As a loading control, membranes were re-probed with 1:1000

mouse monoclonal anti-beta-tubulin antibody in the blocking solution for one hour at room temperature.

**Table 3.3: Details of the primary antibodies used in western blot experiments**

| Specificity of Primary Antibody | Company | Catalog Number | Species and Isotype | Molecular Weight (kDa) | Dilution |
|---|---|---|---|---|---|
| Anti-β-catenin (L87A12) | Cell Signalling Technology, Beverly, USA | #2698, #2698S | mouse monoclonal | 92 | 1:5000 |
| Anti-phospho- -β-catenin (Ser552) | Cell Signalling Technology, Beverly, USA | #9566 | rabbit polyclonal | 92 | 1:1000 |
| Anti-phospho- -β-catenin (Ser33/37Thr41) | Cell Signalling Technology, Beverly, USA | #9651 | rabbit polyclonal | 92 | 1:1000 |
| Anti-phospho- -β-catenin (Ser33/37Thr41) | Cell Signalling Technology, Beverly, USA | #9651S | rabbit polyclonal | 92 | 1:1000 |
| Anti-phospho-Ser9-GSK3β | Cell Signalling Technology, Beverly, USA | #9336 | rabbit polyclonal | 46 | 1:1000 |
| Anti-GSK3β | Cell Signalling Technology, Beverly, USA | #9315 | rabbit monoclonal | 46 | 1:5000 |
| Anti-phospho-Akt (Ser473) | Cell Signalling Technology, Beverly, USA | #9271S | rabbit polyclonal | 60 | 1:1000 |
| Anti-Akt | Cell Signalling Technology, Beverly, USA | #9272 | rabbit polyclonal | 60 | 1:1000 |
| Anti-Slug | Cell Signalling Technology, Beverly, USA | #9585 | rabbit monoclonal | 30 | 1:1000 |
| Anti-c-jun | MBL | EP-7186 | mouse monoclonal | 40 | 1:1000 |
| Anti-c-myc | MBL | M0473 | mouse monoclonal | 62 | 1:1000 |
| Anti-cyclinD1 | MBL | 553 | rabbit polyclonal | 36 | 1:1000 |
| Anti-Lamin B | Abcam, Cambridge, UK | ab102036 | rabbit polyclonal | 71 | 1:1000 |
| Anti-Myogenin | Merck Millipore, | MAB3876 | mouse monoclonal | 34 | 1:1000 |

| | | | | | |
|---|---|---|---|---|---|
| | Germany | | | | |
| Anti-myoD1 | Merck Millipore, Germany | MAB3878 | mouse monoclonal | 45 | 1:1000 |
| Anti-myf5 | Merck Millipore Germany | ABD19 | rabbit polyclonal | 29 | 1:2000 |
| Anti-desmin | Merck Millipore Germany | DE-B-5 | mouse monoclonal | 53 | 1:2000 |
| Anti-Axin-1 | Cell Signalling Technology, Beverly, USA | #2087S | rabbit monoclonal | 110 | 1:1000 |
| Anti-Axin2 | Cell Signalling Technology, Beverly, USA | #2151S | rabbit monoclonal | 96 | 1:1000 |
| Anti-Naked 1 | Cell Signalling Technology, Beverly, USA | 2201 | rabbit monoclonal | 59-61 | 1:2500 |
| Anti-LRP6 | Cell Signalling Technology, Beverly, USA | #3395 | mouse monoclonal | 180-210 | 1:2500 |
| Anti-Dvl-3 | Cell Signalling Technology, Beverly, USA | #3218 | rabbit monoclonal | 88-93 | 1:2000 |
| Anti-β-tubulin | Santa Cruz Biotechnology, Santa Cruz, CA, USA | Sc-55529 | mouse monoclonal | 51 | 1:1000 |
| Anti-Pan Cadherin | Cell Signalling Technology, Beverly, USA | #4068 | Rabbit polyclonal | 130-150 | 1:1000 |

The membranes were then washed three times, for 5 minutes each in 0.1% v/v Tween20 in PBS with constant agitation. Then they were incubated with the appropriate HRPconjugated secondary antibody (Dako) at a dilution of 1:5000 in the blocking solution for one hour at

room temperature again with constant agitation. The membranes were then washed three times, for 5 minutes each with agitation.

After incubation with horseradish peroxidase-labeled goat-anti-rabbit or goat-anti-mouse (1:5000) (GE Healthcare Life Sciences, USA) the membranes were developed with enhanced chemiluminescent reagent (Pierce Biotechnology, Rockford, USA Amersham, UK). Finally, the blots were reprobed using antibodies against ∝-tubulin (1:1000); Santa Cruz Biotechnology, Santa Cruz, CA, USA) to ensure equal loading and transfer of proteins. All experiments were carried out at least three times [248].

**3.7 Dose-response and time-course experiments to see the phosphorylation status of β-catenin after human recombinant Wnt3a activation:**

Wnt3a is a 44kDa secreted hydrophobic glycoprotein containing a conserved pattern of 24 cystience residues [94]. It is a natural agonist of the canonical Wnt pathway [134]. It inactivates GSK-3β by disrupting the degradation complex allowing free β-catenin to stabilise within the cytoplasm and subsequently translocate to the nucleus to activate its downstream targets [94, 134]. We prepared a stock solution of human recombinant (hr) Wnt3a (R&D Systems, catalog #5036-WN-010) by reconstituting 200μg/ml in sterile PBS containing 0.1% BSA. We then tried various dilutions of hrWnt3a as recommended by the manufacturer

($ED_{50}$ <500ng/ml), to activate Wnt signalling in the RMS cells. Western blot experiments were designed done to look at the density of β-catenin expression ranging from 25-500ng/ml Wnt3a for 24 hrs in RD and Rh30 cells lines. The lowest concentration giving the maximal response, assessed by beta-catenin expression on western blot analysis, was 200ng/ml and this concentration was then used in all subsequent investigations. All experiments were carried out in triplicate.

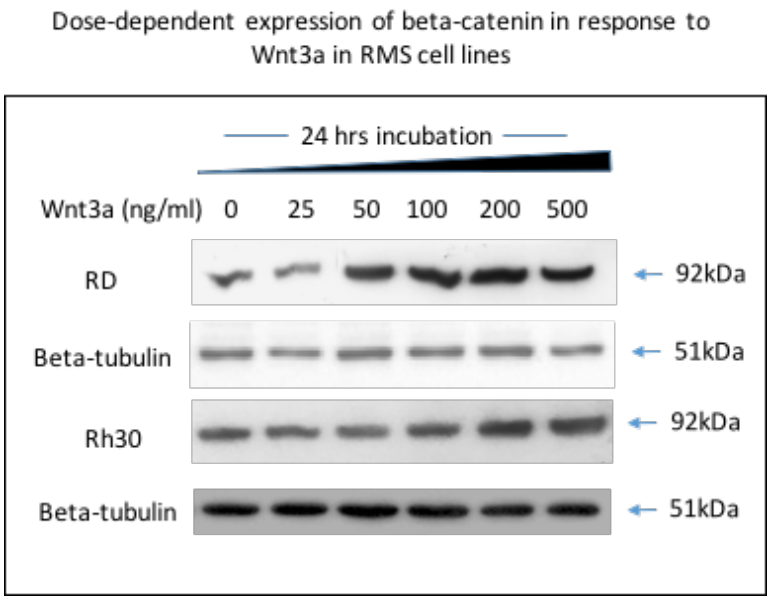



Figure 3.1: RD and Rh30 cells were grown in 6-well culure dishes at 70-80% confluence (0.8-1 x $10^6$ cells) and were incubated for 24 hours with increasing doses of human recombinant Wnt3a (0, 25, 50, 100, 200 and 500ng/ml) in complete DMEM medium supplemented with 10% FCS. After 24hrs the cells were lysed and 7.5% SDS-PAGE was performed to estimate the total amount of β-catenin using mouse monoclonal antibody (1:5000; Cell Signalling). There was a dose-dependent increase in β-catenin levels after 50ng/ml in RD cells steadily increasing from 100-200ng/ml after which is remained relatively constant at higher dosage (500ng/ml). Similar effect was seen in Rh30 cells; there was only a mild increase in the amount of β-catenin at 500ng/ml as compared to 200ng/ml. The lowest dose showing maximal effect in both cell lines by visial inspection was 200ng/ml, hence we used this concentration for all further experiments. Beta-tubulin was used as the loading control. All experiments were repeated three times.

Using a time-course experiment, we assessed the phosphorylation status of β-catenin after treatment of the RMS cell lines with human recombinant Wnt3a. Rh30 and RD18 cells were stimulated with 200ng/ml Wnt3a for 0′–10′–30′–60′–180′–300′–12 h–24h after overnight serum starvation; untreated Rh30 and RD18 cells served as control. Protein extraction and western blotting was performed as described above. All experiments were carried out in triplicate [248].

**3.8 Cellular and Nuclear Extracts**

Cellular and nuclear were done as described previously [209]. For nuclear and cytoplasmic fractions, three buffers were prepared. Buffer A [10mM HEPES pH 7.4, 50mM NaCl, 1mM EDTA, 0.2% Triton X-100, 500μM Sucrose, 250μM PMSF and protease inhibitor cocktail] was used to release nuclei and to recover the supernatant retained as cytoplasmic fraction; Buffer B [10mM HEPES pH 7.4, 50mM NaCl, 0.1mM EDTA and 25% glycerol] was used to rinse nuclei; and Buffer C [10mM HEPES pH 7.4, 350mM NaCl, 0.1mM EDTA, 25% glycerol, 250μM PMSF and protease inhibitor cocktail] was used to re-suspend clean nuclear pellets [248].

RMS cells were pre-incubated with or without Wnt3a (200ng/ml) in culture flasks. Cell scraping were obtained from T-25 culture flasks after trypsinisation and were washed twice with cold PBS. The

supernatant was discarded and the cell pellet was collected in a pre-chilled microcentrifuge tube. The cell pellet was gently resuspend cells in 500µl of Buffer A (hypotonic buffer) and was incubated on ice for 15 minutes. Thereafter, 25 µl detergent (10% NP40) was added and the mixture vortexed for 10 seconds. The homogenate was then centrifuged for 10 minutes at 3,000 rpm at 4°C. The supernatant constituted the cytoplasmic fraction. The pelleted nuclei were rinsed with Buffer B three times. They were then resuspended in in 50µl of Buffer C (with 1 mM PMSF with protease inhibitor cocktail) and incubated in ice for 30 min. Nuclear extract was collected by centrifugation at 12,000g for 15 min at 4°C. Protein concentration of the nuclear extract was estimated using as described in **Section 3.5**. The extract was either immediately used or stored at -70°C till further use. Aliquots of these samples were fractionated by 7.5% SDS-PAGE, electrotransferred to nitrocellulose membranes, and analyzed by Western blotting [248].

### 3.9 Immunofluorescence

RD18 and Rh30 RMS cells were seeded and grown to a confluence of 70–90% on 12-mm glass coverslips placed within 6-well plates. The cells were serum-starved overnight and were then treated with 200 ng/ml Wnt3a-treated cells (for 6 hrs). Untreated RD18 and Rh30 RMS cells served as controls. The cells were then fixed in 4% paraformaldehyde for 30 min and permeabilised with 0.1% Triton X-100 in PBS for 5 min

at room temperature. The cells were then incubated with: primary rabbit polyclonal anti-phospho-S33S37T41-β-catenin antibody (1:1000), primary rabbit polyclonal anti-phospho-S552-β-catenin antibody (1:1000), primary mouse monoclonal anti-β-catenin antibody (1:1000) (Cell Signalling Technology, Beverly, MA, USA); primary mouse monoclonal MyHC antibody (Millipore) or TRITC-conjugated phalloidin (1:2000) (Sigma-Aldrich), at room temperature for 1 h. After washing with PBS, cover slips were incubated with FITC or Texas red-conjugated goat anti-rabbit secondary antibody (1:2000) (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 30 min in the dark. After final washing, nuclei were counterstained with Propium iodide (PI) or 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich). The slides were then mounted in Moviol or Vecta Shield and examined by immunofluorescence microscopy. All experiments were carried out at least three times [248].

**3.10 Cell Proliferation Assay**

Aliquots of RD18 (4 x$10^3$ cells) and Rh30 (3 x $10^3$ cells) RMS cells were seeded in triplicate on 96-well plates and incubated with complete DMEM medium (with FCS) for 24 h and then starved with serum-free medium for 24 h. Quiescent cultures were treated with daily supplemented medium with or without 200ng/ml human recombinant Wnt3a for 24 and 48 hours. The effect of Wnt3a on cell proliferation

was determined by cleavage of tetrazolium salt WST-1 to formazan (Roche Applied Science, USA), by reading the absorbance of treated and untreated cells at 440 nm. The value for untreated cells was considered as 100% cell proliferation. Each point represents a mean value and S.D. of three experiments with three replicates [248].

### 3.11 Transfection and Luciferase Reporter Gene Assay

For the detection of β-catenin-driven Wnt-transcriptional activity, Gaussia luciferase assay kit (New England Biosciences, UK) based on TOPflash/FOPflash reporter plasmid system was used. Gaussia luciferase assay kit (New England Biosciences, UK) based on TOPflash/FOPflash reporter plasmid system was used for the detection of β-catenin driven Wnt-transcriptional activity [248]. The TOP Flash reporter construct contains three optimal copies of TCF/LEF sites upstream of a thymidine kinase minimal promoter that, when bound by β-catenin induces transcription of luciferase reporter gene. Hence if the canonical Wnt signaling is activated, β-catenin will translocate to the nucleus to associate with TCF/LEF transcription factors to activate transcription of Wnt target genes. Since this is a luciferase plasmid, one would see an increase in relative luciferase activity when substrate is added. If Wnt signaling is inhibited, one would see loss of luciferase expression. The FOPflash is used as a negative control because the TCF binding regions upstream of the luciferase gene is mutated so even if β-

catenin translocates to the nucleus to activate TCF-mediated transcription, there will not be any luciferase activity.

Luciferase reporter gene analysis was performed in control and Wnt3a-treated (200ng/ml) RMS cell lines (RD18 and Rh30). A β-galactosidase vector was co-transfected for standardization. One day before transfection, $10^5$ cells/well were seeded per well into 24-well plate. After overnight incubation, the cells (90% confluence) were transiently co-transfected with 1μg DNA of reporter constructs (TOP/FOP Flash) and control plasmid (pCMV-GLuc) using 2μL Lipofectamine 2000$^{TM}$ (Invitrogen) in 50μL OptiMEM$^{R}$ I reduced serum media, achieving DNA/Lipofectamine 2000$^{TM}$ ratio of 1:2. (Invitrogen). Thereafter, the test cells were stimulated with human recombinant Wnt3a (200ng/ml) for 48 hours; control cells were untreated.

Gaussia luciferase has a secretory signal that is secreted into the cell medium. Medium from the treated and control cells were collected for Gaussia luciferase assay (New England Biosciences, UK) according to manufacturer's instructions. Each assay was performed in triplicate and the reporter activity was expressed as mean ± SD [248].

**3.12 Caspase-3/7 Apoptosis Assay**

Apoptosis was measured by Apo-ONE homogeneous caspase-3/7 assay according to the manufacturer's protocol (Promega, UK). Apo-ONE

caspase-3/7 reagent was added to serum-starved and Wnt3a-treated (200ng/ml) and Rh30 and RD18 cell lines in 96-well plates in a ratio of 1:1 and incubated for 90 minutes. Appropriate negative controls and blank were taken. The fluorescence of each well was measured at an excitation wavelength of 485 ± 20nm and an emission wavelength of 530 ± 25nm. Each assay was performed in triplicate and the reporter activity was expressed as mean ± SD [248].

### 3.13 Statistical Analysis

The experimental results are expressed as mean ± S.D. from at least three separate experiments performed in triplicate. Statistical analysis was done using Student's t-test and p-value $<0.05$ was considered as statistically significant [248]. Student's t-test was done by hand.

# CHAPTER 4 –

# RESULTS

## 4.1 Is β-catenin, the central effector of Wnt signalling pathway, expressed in the clinical RMS samples?

In order to characterise Wnt signalling pathway in rhabdomyosarcoma, we set out first by investigating if β-catenin, the main effector of Wnt signalling pathway, is expressed in the clinical samples (n=44). In order to do this, we assembled Tissue microarrays (TMA) containing 136 samples (four 1mm deep cores per case) from 34 RMS cases from the histopathology archives of two major children's hospital in the UK and Italy respectively. Some cases where the thickness was <1mm were not included in the TMA; these cases has their full-face sections examined (n=10). Positive cases showed at least 10% β-catenin immunopositivity.

We attempted to separate β-catenin into 3 subcellular compartments – membranous, cytoplasmic and nuclear, but the distinction between membranous and cytoplasmic compartments was not clear in some cases hence for the purpose of for analysis, it was considered more pragmatic to group them into one category. The membranous/cytoplasmic location represented the inactive form of β-catenin whereas nuclear β-catenin indicated the active form. Patient demographics with details of the tumour subtype, location and the tumour genetics is shown in Table 4.1. The sequential steps are shown in Figure 4.1. Figs 4.2 and 4.3 show TMA assembly and design plan with β-catenin score sheet respectively.

**Table 4.1:** Demographic details of the 44 patients on the TMA

| | Age | Sex | Survival | DOD/Last visit | Site | Diagnosis | Cytogenetics |
|---|---|---|---|---|---|---|---|
| **1** | 14y | M | Dead | 08.02.99 | Head and neck, Right Zygoma | ERMS | |
| **2** | 1y 5m | M | Dead | 15.08.98 | Perineal | ARMS | Pax 3/7-FKHR positive |
| **3** | 6y | M | Dead | 28.04.02 | Head and neck, Nose | Botryoid ERMS | |
| **4** | 12y | F | Alive | 20.01.09 | Retroperitoneum, Adrenal region | ERMS | |
| **5** | 1y 8m | M | Dead | 06.02.00 | Pelvic region, near UB | ERMS | |
| **6** | 18y | M | Dead | 09.04.99 | Pelvis/Sarum | ERMS | |
| **7** | 2y 11m | M | Alive | 27.05.08 | Paratesticular | Spindle ERMS | |
| **8** | 21y | M | Alive | 30.08.05 | Paratesticular | ERMS | |
| **9** | 4y | M | Alive | 18.11.08 | Paratesticular | ERMS | |
| **10** | 3y 1m | F | Alive | 27.02.09 | Vagina | ERMS | |
| **11** | 4y 10m | F | Alive | 16.12.08 | Chest wall | ARMS | Pax 3/7-FKHR negative |
| **12** | 1y 10m | M | Alive | 02.03.09 | Parotid | ERMS | |
| **13** | 13y | F | Alive | 23.10.08 | Nose | ERMS | |
| **14** | 7y | M | Dead | 18.09.08 | Nasopharynx | ERMS | |
| **15** | 2y 10m | M | Alive | 17.02.09 | Bladder/Prostate | ERMS | |
| **16** | 8y | F | Dead | 14.05.05 | Infratemporal region | ARMS | Pax 3/7-FKHR positive |
| **17** | 7y | F | Alive | 10.02.09 | Orbit | ARMS | Pax 3/7-FKHR negative |
| **18** | 3y 11m | F | Alive | 16.01.09 | Zygomatic region | ARMS | Ampl of Pax and FKHR, Pax 3/7-FKHR negative |
| **19** | 5y 1m | F | Dead | 15.05.08 | Ext. ear | ERMS | |
| **20** | 16y | F | Dead | 12.08.06 | Forearm | ARMS | Pax3/FKHR positive |
| **21** | 8y | M | Dead | 10.08.07 | Calf | ARMS | Pax7/FKHR positive |
| **22** | 7y | F | Alive | 06.01.09 | Pelvis/Sarum | ARMS | Pax 3/7-FKHR negative |
| **23** | 8y | M | Alive | 12.01.09 | Maxilla | ERMS | |
| **24** | 2y 6m | M | Alive | 13.05.08 | Mandible | ERMS | |
| **25** | 10y | M | Dead | 23.01.09 | Prostate | ERMS | |
| **26** | 17y | M | Dead | 15.08.03 | Paraspinal mass | ARMS | Pax 3/7-FKHR positive |
| **27** | 17y | M | Alive | 16.08.05 | Nasopharynx | ARMS | Pax 3/7-FKHR negative |
| **28** | 4y 5m | M | Alive | 02.12.08 | Orbit | ERMS | |
| **29** | 19y | F | Alive | 10.07.06 | Pelvis | ERMS | |
| **30** | 24y | M | Alive | 12.08.03 | Paratesticular | ERMS | |
| **31** | 5y 10m | F | Dead | 13.01.98 | Post nasal space | ARMS | Pax 3/7-FKHR positive |
| **32** | 3y 9m | M | Dead | 03.06.96 | Nasal space | ERMS | |
| **33** | 14y | F | Alive | 10.06.97 | Thigh | Anaplastic ERMS | |
| **34** | 16y | M | Alive | 03.11.08 | Paratesticular | Spindle ERMS | |
| **35** | 4y 11m | F | Dead | 11.11.95 | Orbit | ERMS | |
| **36** | 17y | F | Alive | 04.11.08 | Vagina | ERMS | |
| **37** | 14y | F | Dead | 02.02.95 | Foot | ARMS | Pax 3/7-FKHR positive |
| **38** | 17y | M | Dead | 26.08.96 | Neck | ERMS | |
| **39** | 20y | M | Alive | 27.07.03 | Perirectal polyp | ERMS | |
| **40** | 29y | F | Alive | 28.01.00 | Axillary Mass | ARMS | Pax 3/7-FKHR negative |
| **41** | 17y | F | Alive | 14.04.08 | Vagina | Botryoid ERMS | |
| **42** | 9y | M | Dead | 19.03.03 | Palate | ERMS | |
| **43** | 23y | F | Alive | 28.10.02 | Calf | ARMS | Pax 3/7-FKHR negative |
| **44** | 16y | M | Dead | 28.08.04 | Pelvis | Anaplastic ERMS | |

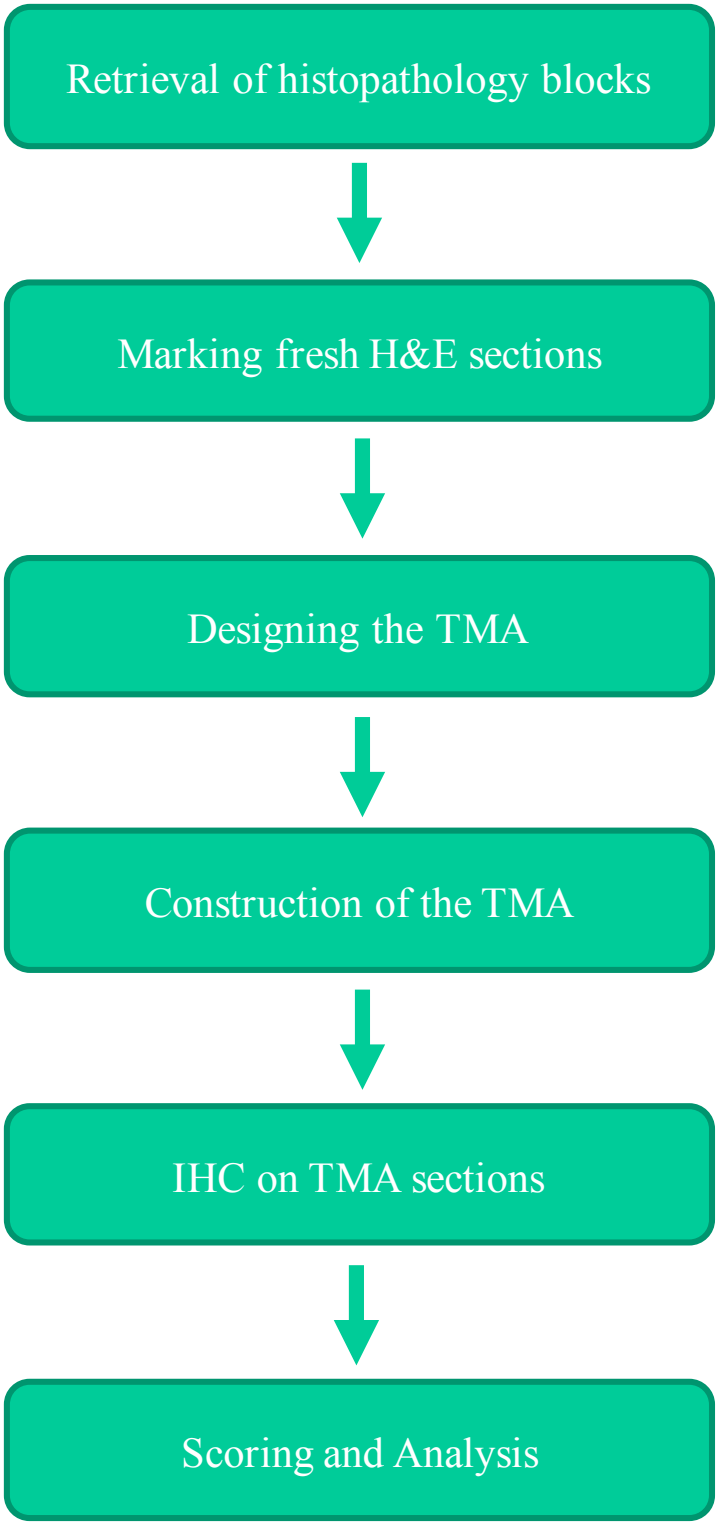
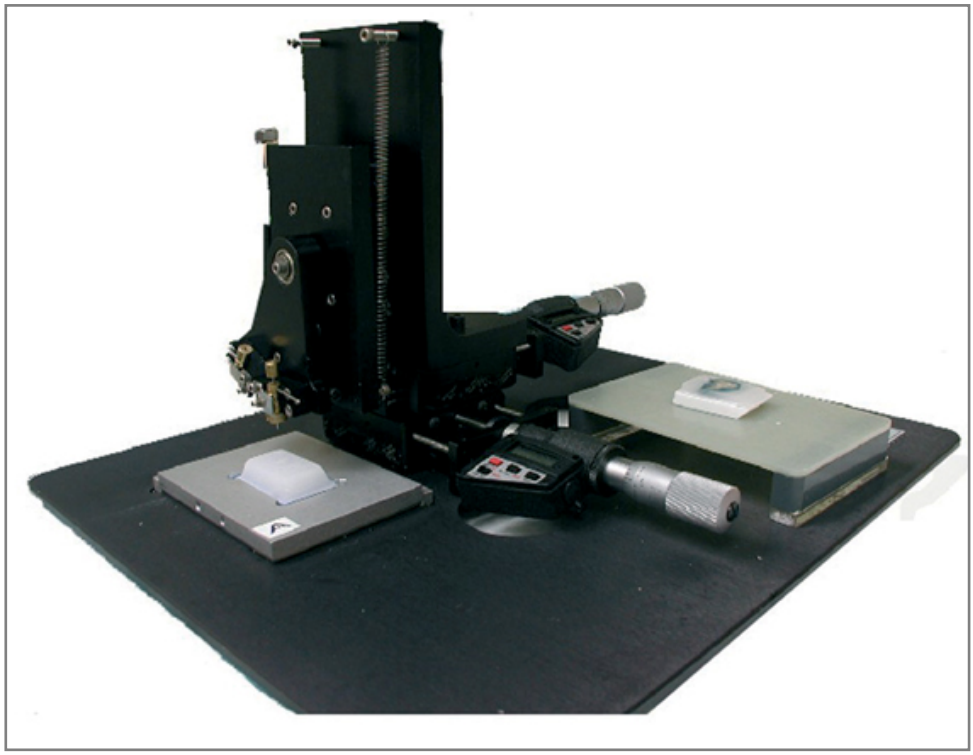



**Figure 4.1**: Steps in designing of the TMA block cores.



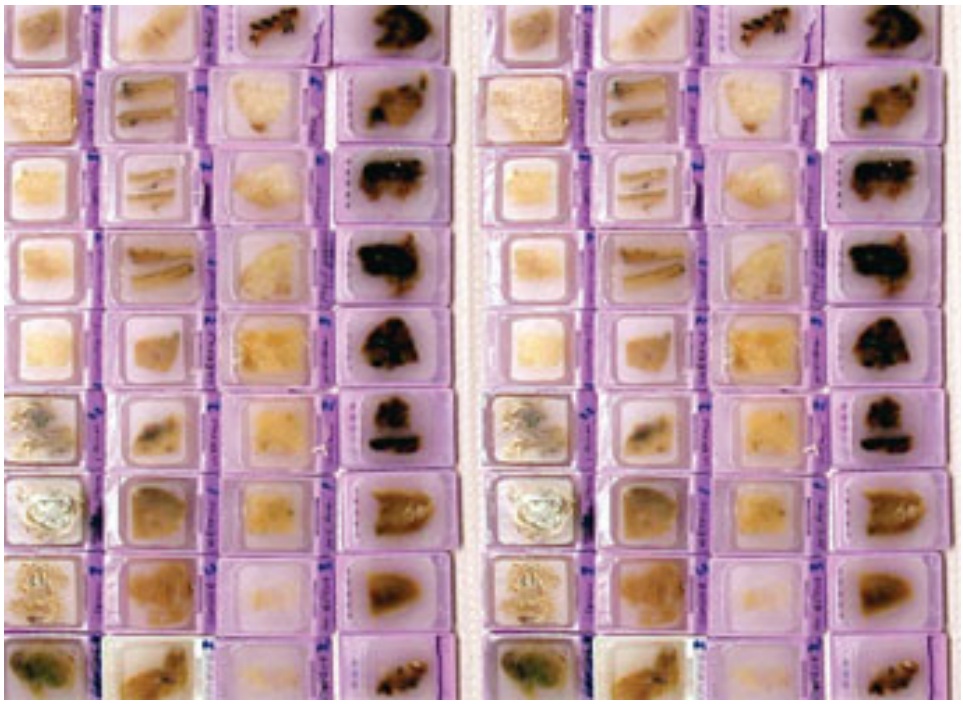


**Figure 4.2:** Tissue microarray machine (top); paraffin blocks from the RMS cases used in the designing of the TMA block cores.

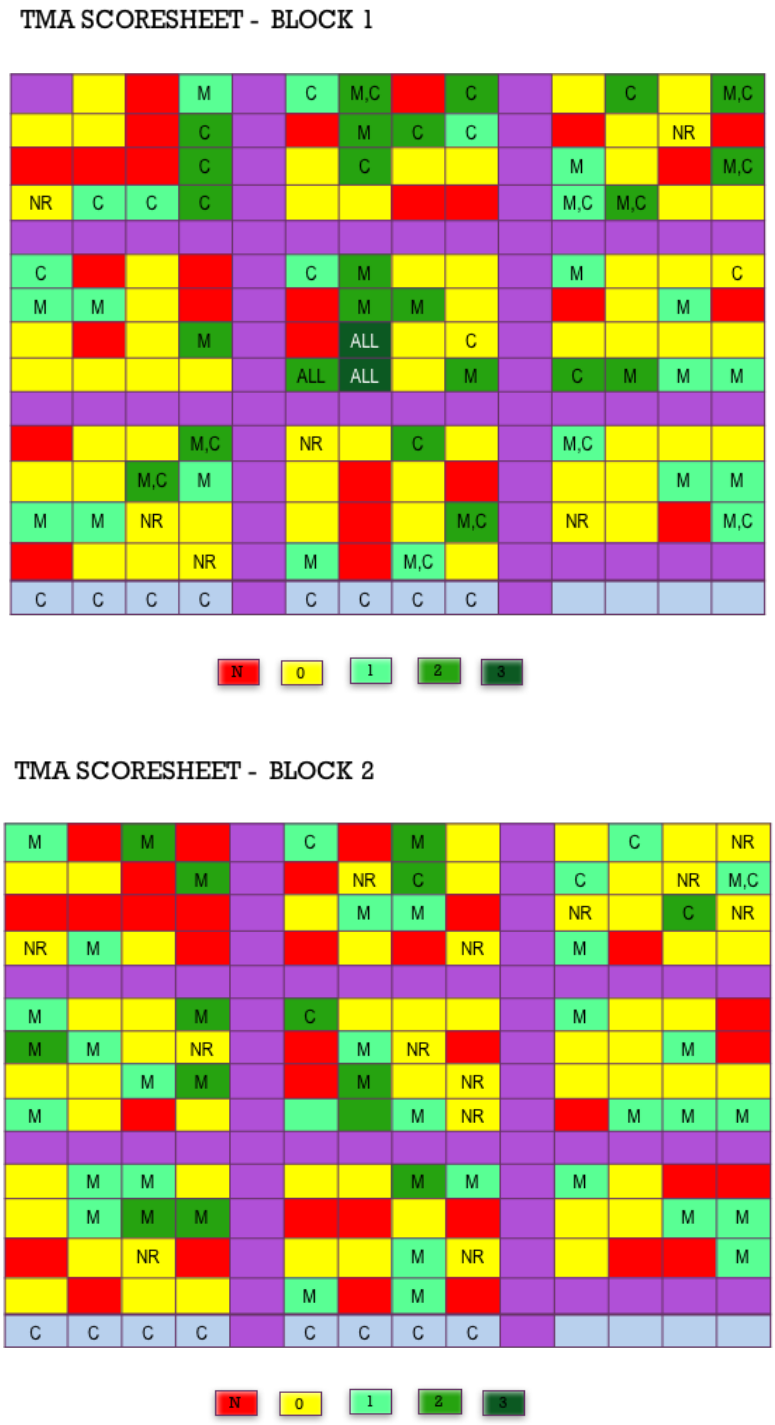



**Figure 4.3:** Design of the TMA block in duplicate (Block 1 above and Block 2 below) with score sheet for β-catenin expression and its localization within the cell. Colour code for pattern of staining: light Green – 1+ staining; green – 2+ staining; dark olive green – 3+ staining; yellow – negative; red – no cores. The letters NR, M and C indicate β-catenin localisation in nucleus, cell membrane and cytoplasm respectively.

### 4.1.1 Immunohistochemical expression of β-catenin in TMA and full-face sections

β-catenin expression was evaluated by immunohistochemistry in a total of 44 RMS cases; 34 cases were assembled into the TMA multi-block and for the remaining 10 cases, full-block sections were assessed. The localisation and the intensity of β-catenin expression were recorded according to the criteria described in Material and Methods section. In all, there were 30 cases of embryonal RMS and 14 cases of alveolar RMS.

Overall, β-catenin immunostaining was seen in 59% of all RMS samples (26/44 cases). When assessed for subtype, however, β-catenin expression was higher in alveolar RMS (64%, 9/14 cases) as compared to embryonal RMS (56%; 17/30 cases). When assessed for localisation, 18 out of the 34 RMS samples showed cytosolic/membranous staining for β-catenin (9 ARMS; 9 ERMS); nuclear localisation was only seen in 2 cases, both of which were ERMS cases. Of the 10 cases studied using full-block sections (all ERMS cases), 6 showed membranous/cytoplasmic β-catenin expression; none showed nuclear staining (Figure 2; Table 2).

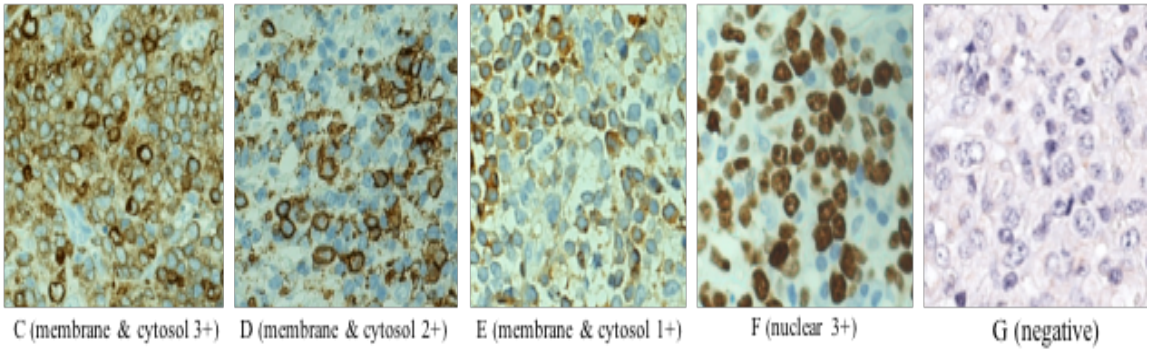



**Figure 4.4**: IHC scoring for β-catenin expression. Membranous/cytoplasmic and nuclear expression patterns and scoring for β-catenin.

PAX–FOXO1 translocation status was known in all ARMS cases (n=14) and was carried out by standard FISH analysis as a part of routine diagnostic work up of the cases. Half of the alveolar RMS cases (7/14) had PAX–FOXO1 translocation (six had Pax3-FOXO1; one had Pax7-FOXO1). Next, we wanted to see if there was any differential expression of β-catenin in the translocation positive and negative ARMS cases. β-catenin was expressed in five of the seven ARMS cases carrying PAX–FOXO1 translocations (four in Pax3-FOXO1; one in Pax7-FOXO1) and in four of the seven fusion negative-ARMS cases. The results were not considered to significant in the small sample studied.

RMS does not have any activating mutations in Wnt pathway, unlike cancers like endometrial carcinoma, colorectal carcinoma, gastric carcinoma, anaplastic thyroid carcinoma, Wilms' tumour or desmoid tumours, where there is a dysregulation of Wnt pathway. In these tumours, β-catenin is activated constitutively whereby it escapes cytosolic

degradation and translocates to the nucleus (showing predominant nuclear expression) affecting cellular proliferation and invasion, rather than cell differentiation. RMS expectedly does not show nuclear β-catenin as shown by Bouron-Dal Soglio et al. [250], who found no mutations of β-catenin in their RMS cases (n =13). In our study, there were only 2 ERMS cases with nuclear β-catenin expression.

**Table 4.2. Immunohistochemical expression of β-catenin in alveolar and embryonal RMSs**

| | Alveolar RMS (n=14) | Embryonal RMS (n=30) |
|---|---|---|
| Total number of RMS cases (n=44) | | |
| TMA cases (n=34) | 14 | 20 |
| Non-TMA cases (n=10) | 0 | 10 |
| | | |
| Membranous/cytoplasmic expression | | |
| TMA | 9 | 9 |
| Full section cases | 0 | 6 |
| | | |
| Nuclear expression | | |
| TMA | 0 | 2 |
| Full section cases | 0 | 0 |
| Total β-catenin expression (26/44) | 9/14 | 17/30 |

In summary, our results indicate that β-catenin, the key component of Wnt signalling pathway, is expressed in around 59% (26/44) of the 44 cases tested by immunohistochemistry (n=44; 34 TMA cases and 10 full face slide sections) (Table 4.2) [248].

## 4.2 Are Wnt signalling proteins expressed in the RMS cell lines?

Next, we wanted to see if the major proteins of the non-canonical pathway are expressed in RMS cell lines. To this end, we wanted to see if the other Wnt proteins besides β-catenin, including the members of the degradation complex and Wnt co-receptors, are expressed in the RMS cell lines.

### 4.2.1 Wnt/β-Catenin Signalling proteins are expressed in the RMS cell lines

The expression of the main components of Wnt/β-catenin pathway was tested using immunoblot analysis in four cell lines derived from alveolar (Rh4 and Rh30) and embryonal (RD and RD18). Western blot experiments demonstrated that β-catenin and its main regulator GSK-3β was expressed in all RMS cell lines (Figure 4.5). We further wanted to check if cadherin, the main binding partner of β-catenin at the adherens junction at the cell membranes, was expressed or not. We also included Protein Kinase B/Akt, which itself regulates GSK-3β, in the study to see possible interactions with other pathways like IGFR, MAPK and CXCR signalling.

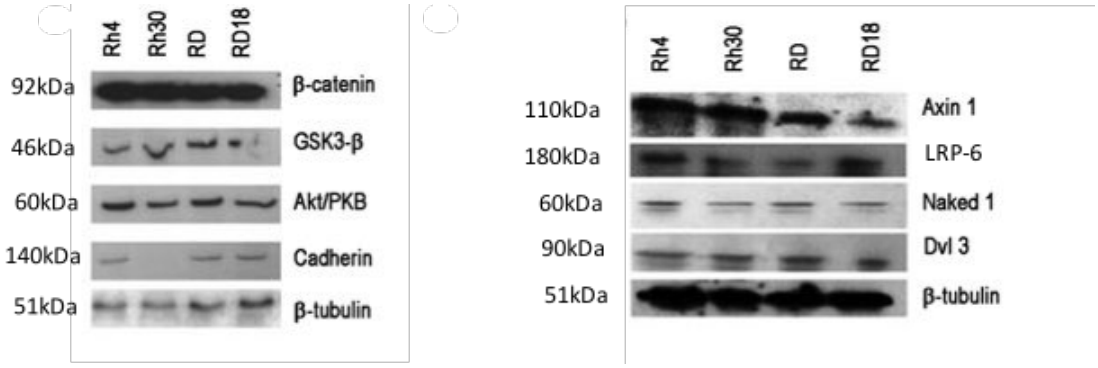



**Figure 4.5:** Western blot analysis on the RMS cell lines to demonstrate expression of central Wnt regulatory proteins and other Wnt signal-related proteins. Fig 4.5 (A) Immunoblot analysis demonstrated the major Wnt signalling proteins, β-catenin and its inhibitor GSK3β, in all RMS cell lines. In addition, Akt was expression in all RMS cell lines. Cadherin, the main binding partner of β-catenin in the cell membranes, was also expression in all RMS cell lines, albeit at a very low signal Rh30 ARMS cell line. (B) Further, other Wnt signal-related proteins (Axin1, LRP6, Naked1 and Dvl3) could also be demonstrated in all cell lines [B]. Equal amout of protein was loaded in all lanes (10µg/lane); β-tubulin served a sthe loading control. The western blot was repeated three times.

Cadherin was expressed in all four RMS cell lines, albeit in Rh30 cell line it was expressed at a lower concentration than the others. But on higher exposure, cadherin was detected in Rh30 cells as well (Figure 4.5a). Other regulatory components of Wnt pathway proteins such as Axin-1, Naked-1, LPR6 and Dsh3, were also expressed in all RMS cell lines (Figure 4.5b). Thus, the immunoblot analysis confirmed that the main Wnt pathway proteins were expressed in human RMS cells.

Now that we knew that the Wnt proteins were expressed in the RMS cell lines, we wanted to investigate further if these constituted a functional apparatus for active Wnt signalling to be operative in these cell lines. To test this, we used human recombinant Wnt3a, a natural agonist of the Wnt pathway, to see if RMS cell Wnt signalling pathway could be

activated in-vitro. For all further experiments, we chose Rh30 and RD18 cell lines representing alveolar and embryonal RMS respectively.

After standardisation with various dosages ranging from 25-500ng/ml and timing (0-48 hours), the human recombinant Wnt3a was optimised for immunoblot analysis at a dosage of 200ng/ml. We used this concentration throughout for the rest of the experiments. The RMS cell lines were incubated in serum-starved conditions with 200ng/ml of human recombinant Wnt3a to activate Wnt signalling in-vitro.

### 4.3 Are Wnt signalling proteins functionally active in the RMS cell lines?

To check for Wnt activation first we investigated the phosphorylation status of β-catenin and also its localization in ERMS and ARMS. It is expected that if Wnt3a activates the signalling, β-catenin should be rescued from degradation and the active form of β-catenin should translocate to the nucleus. We exploited the use of specific phsopho-β-catenin antibodies to demonstrate this.

#### 4.3.1 Phosphorylation Status of β-catenin in response to Wnt3a stimulation

When the Wnt signalling is switched 'off', the inactive cytosolic β-catenin is actively phosphorylated on residues Ser 33, Ser 37 and Thr 41 by GSK-3β and is targeted for proteasomal degradation [94]. In the presence of the Wnt3a ligand, GSK-3β is inactivated that allows

unphosphorylated active β-catenin to stabilise and accumulate in the cytoplasm. Further, phosphorylation on Ser552 residue, enables β-catenin to translocate into nucleus. This is vital for it to bind to TCF/LEF and transactivate its downstream targets [172].

By designing time-course immunoblot analyses, we checked the phosphorylation status of the β-catenin after Wnt3a activation to test the activation status of Wnt pathway. We found that in the control Rh30 cells (0′) there was a pronounced expression of phospho-S33-S37-T41-β-catenin that gradually decreased between 10′ and 180′. The level was very faint at 60′ (Figure 4.6). The values gradually increased between 180’ and 24hrs. The phospho-S552-β-catenin showed a minimal increase in the Rh30 cells between 180’-300’. The total β-catenin remained relatively constant. It was expected that in response to Wnt3a, the inactive phosphorylated Ser9-GSK-3β levels would increase, however, its level decreased. Total GSK-3β levels remained relatively constant (Figures 4.6). There was steady reduction in the levels of Akt and phospho-Akt up to 60′, after which the levels fluctuated over the 24hrs. Comparable changes were seen in the phosphorylated forms of β-catenin, GSK-3β and Akt in RD18 cells, albeit with slightly different kinetics (Figure 4.6). Phospho-S33S37T41-β-catenin reduced significantly at 30’ and gradually returned back over 24hr period. The phospho-S552-β-catenin showed a very faint increase in the RD18 cells over 24hrs

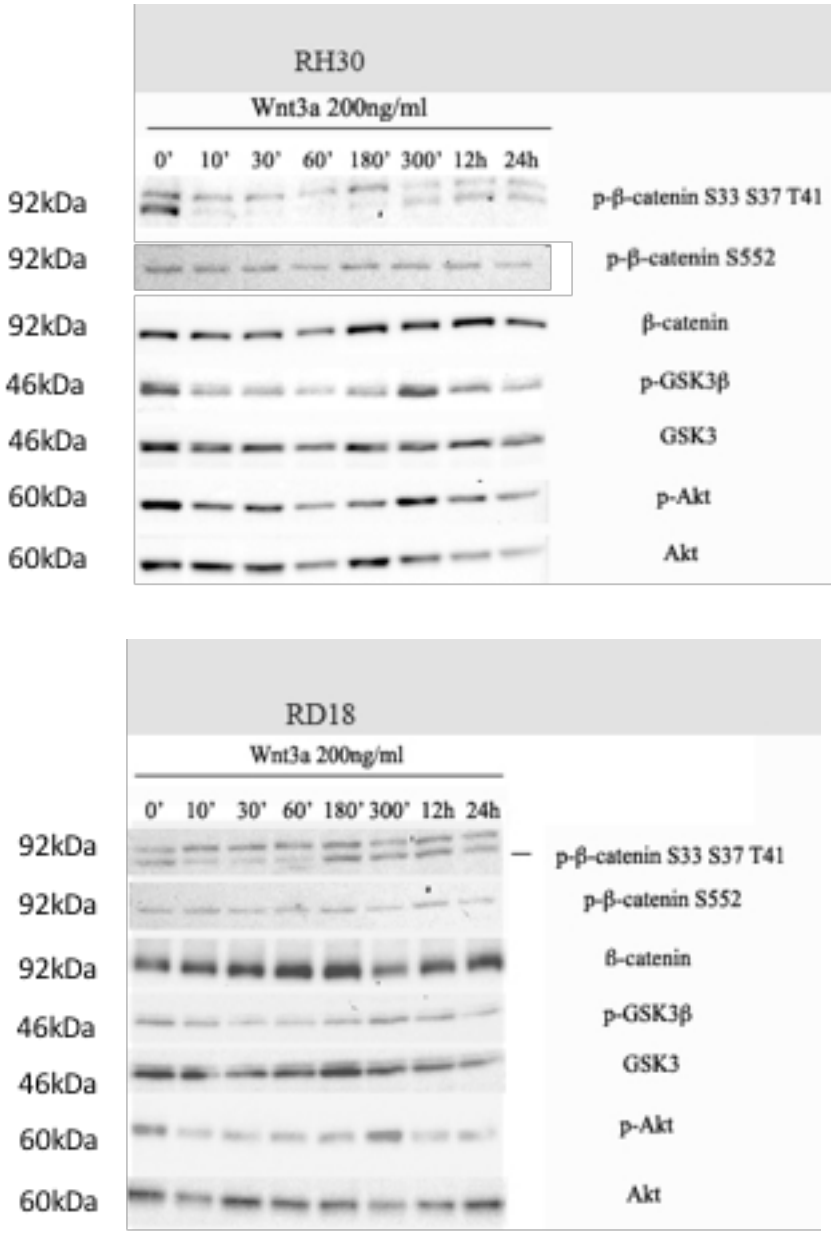



**Figure 4.6:** Western blot analysis on the RMS cell lines (RD18 and Rh30) to show time-course fluctuations in the levels of total β-catenin, phospho-β-catenin-S33S37T41 (inactive form), phospho-β-cateninS552 (active form); GSK3β; phospho-GSK3β (inactive form); Protein kinase B/Akt and its phosphorylated form (active), in response to Wnt3a (200ng/ml) at different time periods over 24 hours. After treatment with Wnt3a, there was a decrease in the level of phospho-β-catenin-S33S37T41 in Rh30 and RD18 cell lines. On the other hand, phospho-β-catenin-S552 showed a faint increase in Rh18 and RD18 cells. The level of total β-catenin remained relatively constant throughout. Equal amount of protein was loaded in all lanes (10µg/lane). The experiment was repeated three times.

Taken together, the results indicate that is appreciable change in the phosphorylation status of β-catenin indicating in response to human recombinant Wnt3a in both RMS cell lines.

### 4.3.2 Effect of Wnt-3a on the subcellular localisation of β-Catenin

The sub-cellular localisation of β-catenin reflects its activity within the cells (Klaus, 2008; Lu, 2011). We performed immunofluorescence experiments to assess the sub-cellular location of β-catenin after activation of Wnt signalling through Wnt-3a treatment. There was a convincing redistribution of β-catenin within cells upon Wnt3a stimulation. β-catenin translocated from the membranous/cytolsolic to perinuclear/nuclear location in Rh30 cells (Figure 4.7). This shift in the localisation of β-catenin was supported by immunoblot analysis using nuclear and cytosolic extracts. (Figure 4.8) This proved that not only is there a change in the phosphorylation state of β-catenin, there also is a change in its intracellular localisation.

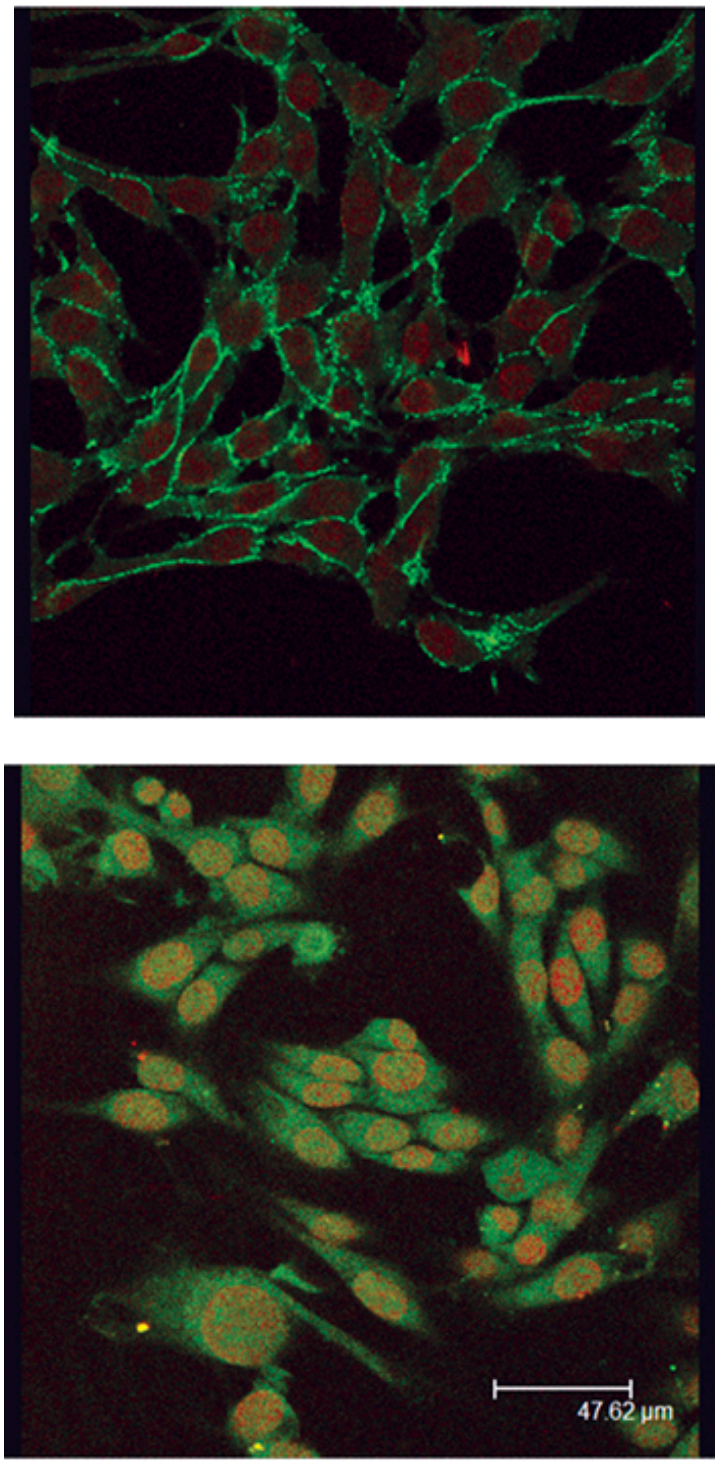



**Figure 4.7:** Immunofluorescence experiment to demonstrate changes in the sub-cellular localisation of β-catenin in Rh30 cells in response to Wnt3a. The image on top (Rh30 control; without Wnt3a) shows a predominantly membranous expression for β-catenin. The image in the bottom shows Rh30 treated with Wnt3a (200 ng/ml for 6hrs) show nuclear localisation of β-catenin, indicating active tranlocation of β-catenin in response to Wnt3a. Mouse monoclonal primary anti-β-catenin antibody (1:1000) and FITC-conjugated goat anti-mouse secondary antibody (1:2000) was used for localisation of β-catenin. Propium iodide was used for nuclear counterstaining. The experiment was repeated three times.

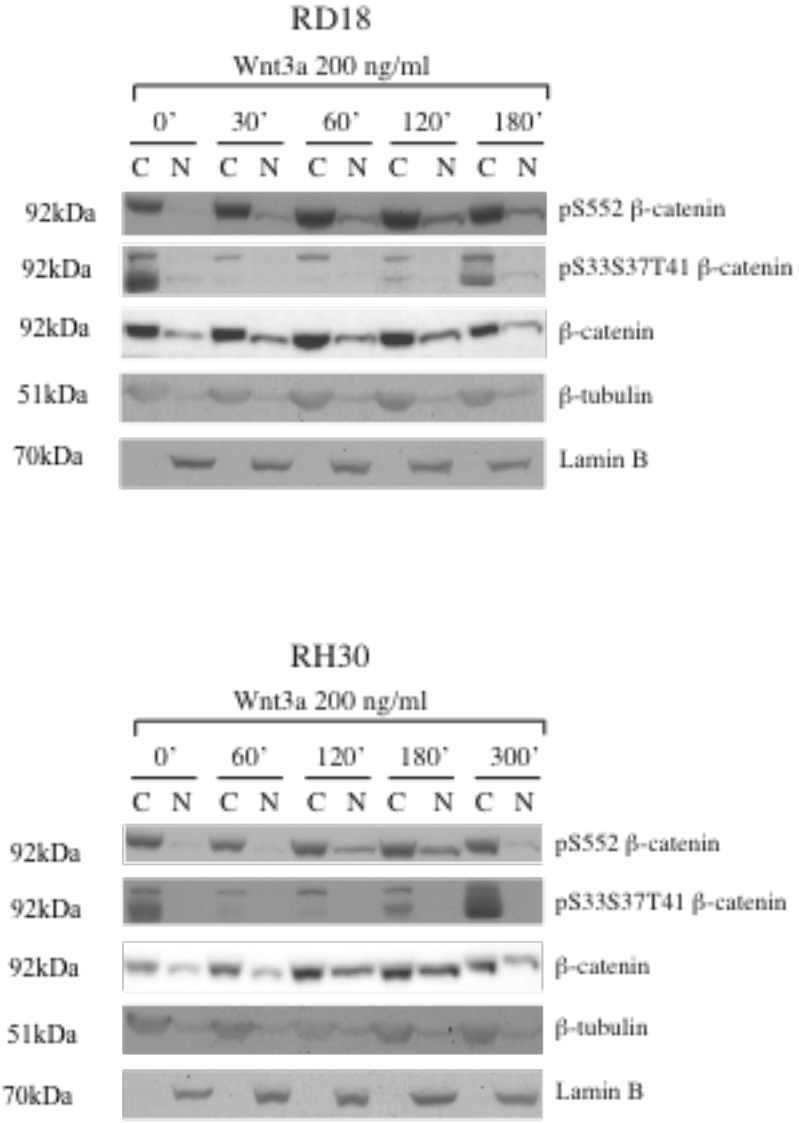



**Figure 4.8:** Immunoblot analysis of total and phosphorylated forms of β-catenin protein in the cytoplasmic (C) and nuclear (N) fractions of RD18 and Rh30 cells in response to Wnt3a. Total β-catenin and phospho-β-catenin proteins (S552 and S33S37T41) were investigated in response to Wnt3a (200ng/ml) in RD18 (top; 0-180 mins) and Rh30 (bottom; 0-300 mins) by immunoblotting. There were appreciable changes in the sub-cellular localisation of β-catenin in both cell lines. There was a predominantly membranous expression for β-catenin in untreated control cells. After treatment with Wnt3a, the cells showed nuclear localisation of β-catenin, in a time-dependent manner. There were demonstrable changes in β-catenin phosphorylation/de-phosphorylation status in response to Wnt3a, albeit at different times in both cell lines. Equal amout of protein was loaded in all lanes (10µg/lane); β-tubulin and Lamin B served as the loading controls for the cytoplasmic and nuclear fractions respectively. The experiment was repeated three times.

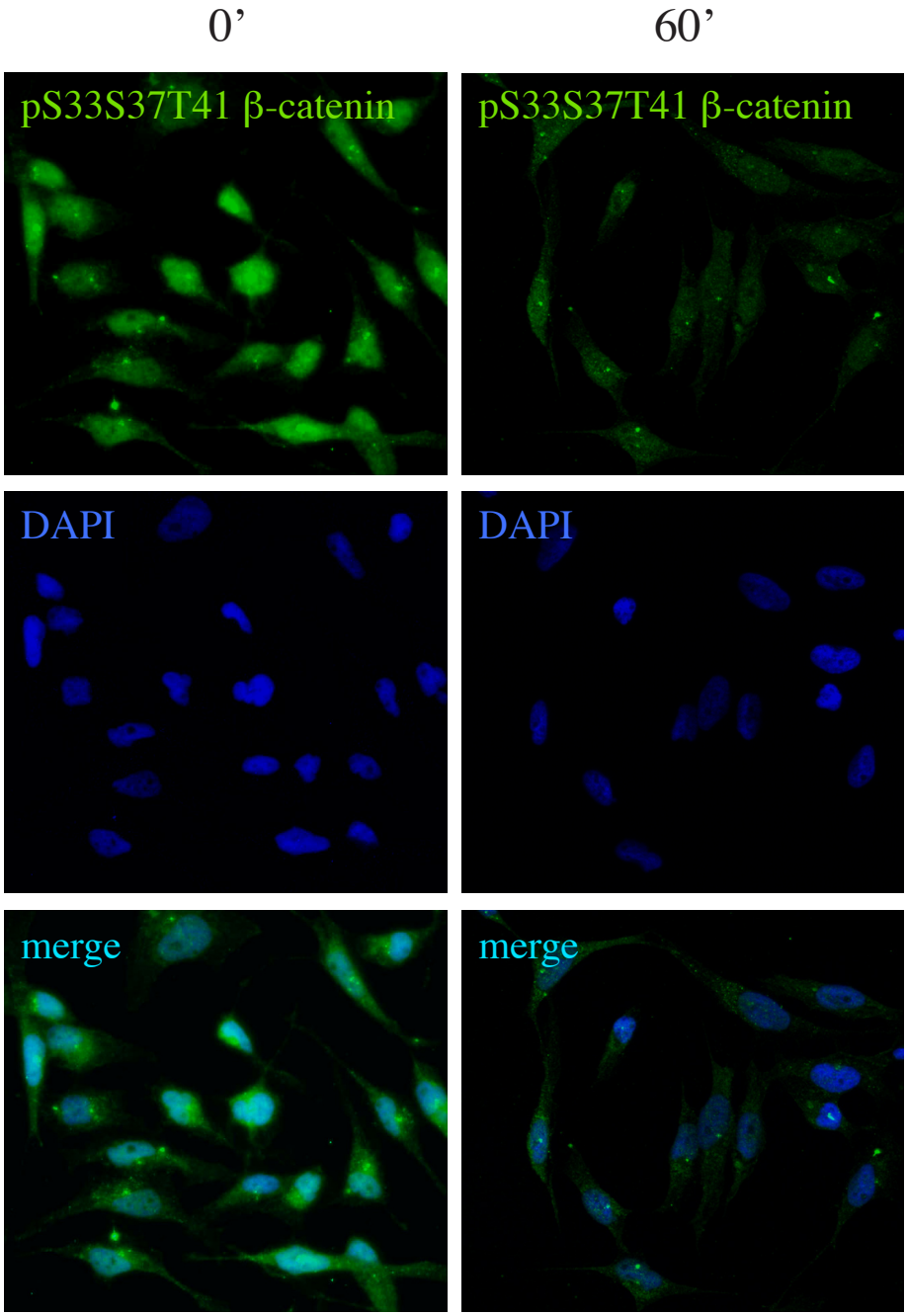



**Figure 4.9:** Expressional analysis of phospho-β-catenin-Ser33Ser37Thr41 (inactive form of β-catenin) in Rh30 cells in response to Wnt3a by immunofluorescence. There was a reduction in the amount of cytosolic S33S37T41-phospho-β-catenin in response to Wnt3a. The image on left (Rh30 control; without Wnt3a) showed a higher cytosolic expression of phospho-β-catenin-Ser33Ser37Thr41 as compared to Rh30 cells treated with Wnt3a (200ng/ml for 60 mins; image on the right). Changes in the phosphorylation status of β-catenin suggest an active Wnt signalling in Rh30 cell line. Rabbit polyclonal primary anti-phospho-β-catenin-Ser33Ser37Thr41 antibody (1:1000) and FITC-conjugated goat anti-rabbit secondary antibody (1:2000) was used for localisation of phospho-β-catenin-Ser33Ser37Thr41. DAPI was used for nuclear counterstaining. The experiment was repeated three times.

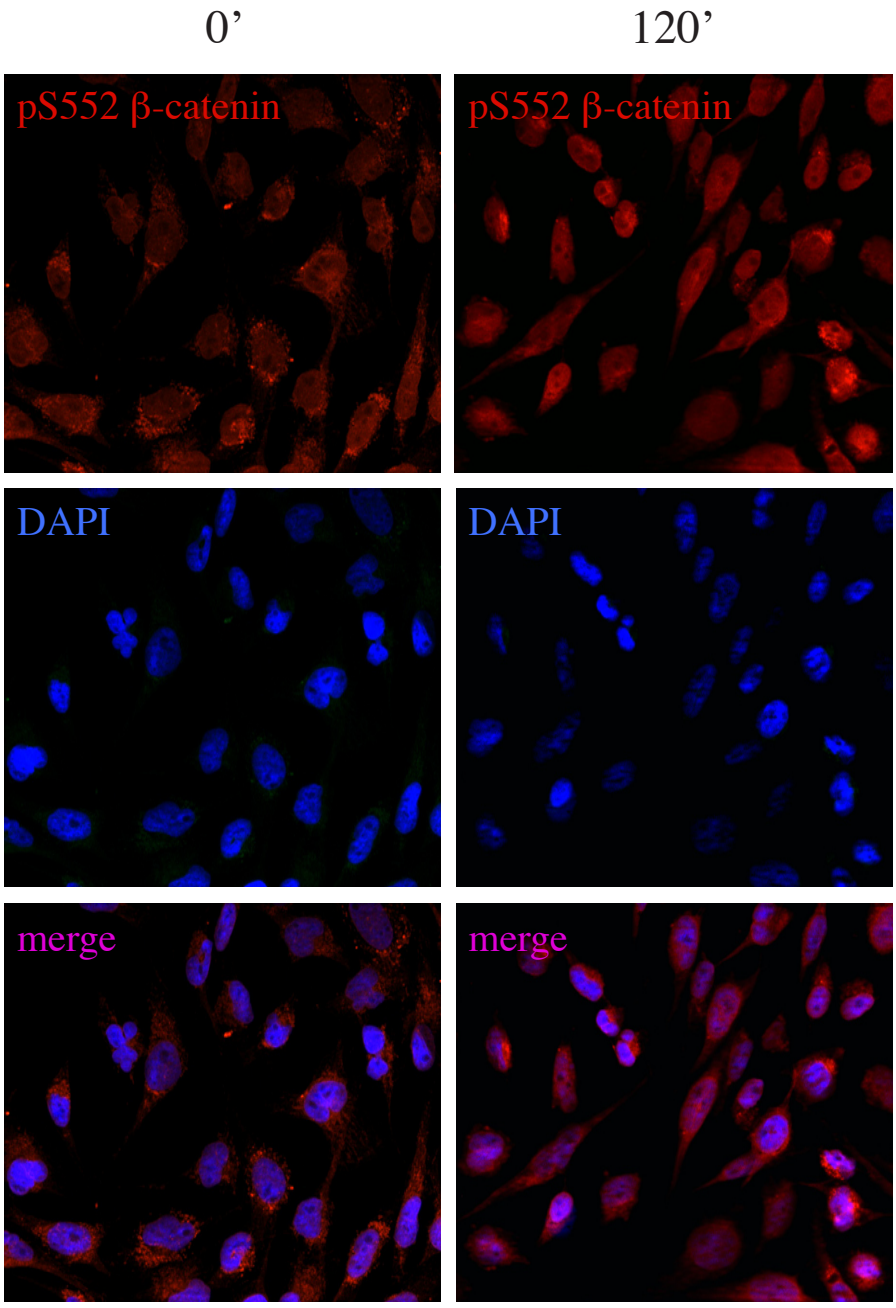



**Figure 4.10:** Expressional analysis of phospho-β-catenin-Ser552 (active form of β-catenin) in Rh30 cells in response to Wnt3a by immunofluorescence. There was an increased in the amount of nuclear phospho-β-catenin-Ser552 in response to Wnt3a. The image on left (Rh30 control; without Wnt3a) showed a lower nucler expression of phospho-β-catenin-Ser552 as compared to Rh30 cells treated with Wnt3a (200ng/ml for 120 mins; image on the right). Changes in the phosphorylation status of β-catenin suggest an active Wnt signalling in Rh30 cell line. Rabbit polyclonal primary anti- phospho-β-catenin-Ser552 antibody (1:1000) and TRITC-conjugated goat anti-rabbit secondary antibody (1:2000) was used for localisation of phospho-phospho-β-catenin-Ser552. DAPI was used for nuclear counterstaining. The experiment was repeated three times.

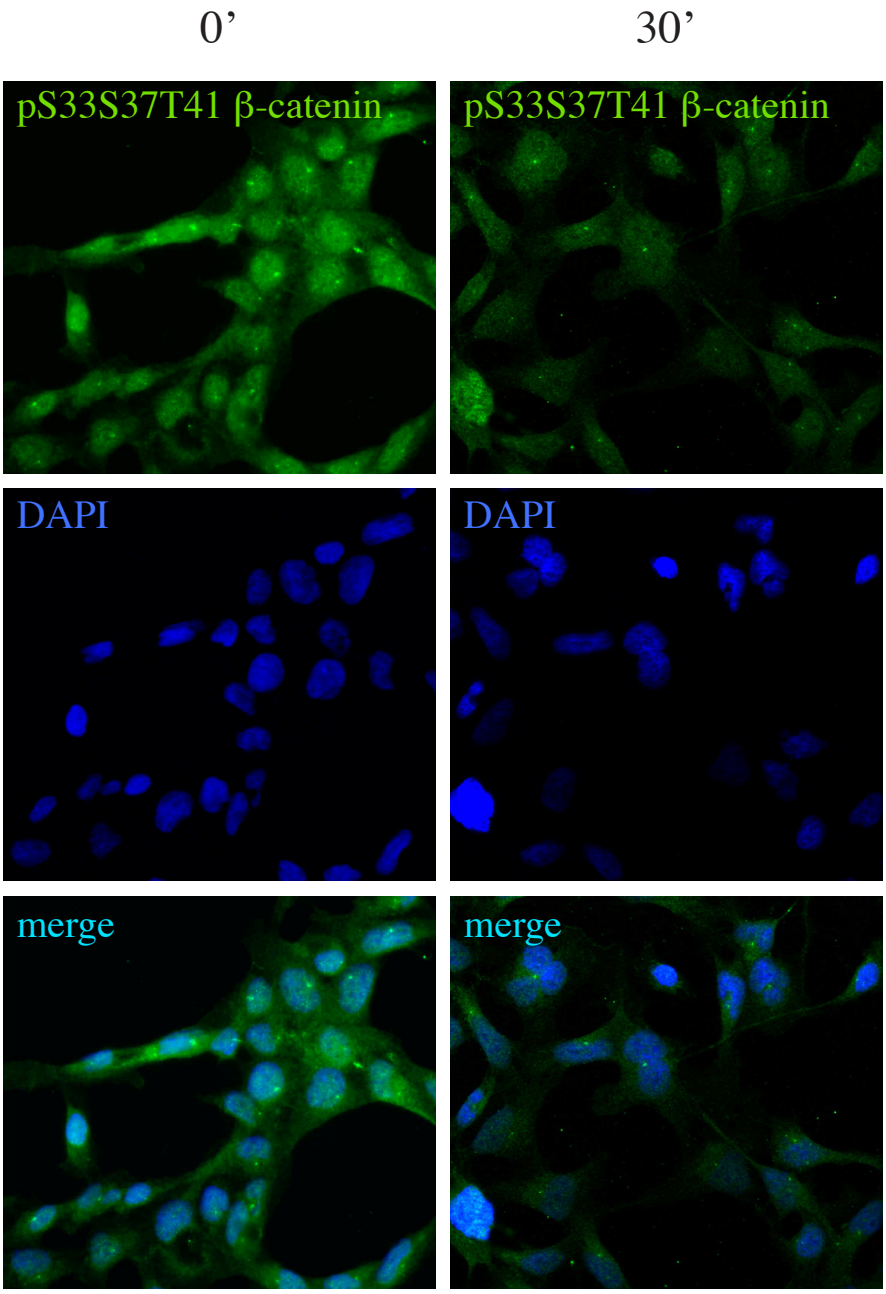



**Figure 4.11:** Expressional analysis of phospho-β-catenin-Ser33Ser37Thr41 (inactive form of β-catenin) in RD18 cells in response to Wnt3a by immunofluorescence. There was a reduction in the amount of cytosolic S33S37T41-phospho-β-catenin in response to Wnt3a. The image on left (RD18 control; without Wnt3a) showed a higher cytosolic expression of phospho-β-catenin-Ser33Ser37Thr41 as compared to RD18 cells treated with Wnt3a (200ng/ml for 30 mins; image on the right). Changes in the phosphorylation status of β-catenin suggest an active Wnt signalling in RD18 cell line. Rabbit polyclonal primary anti-phospho-β-catenin-Ser33Ser37Thr41 antibody (1:1000) and FITC-conjugated goat anti-rabbit secondary antibody (1:2000) was used for localisation of phospho-β-catenin-Ser33Ser37Thr41. DAPI was used for nuclear counterstaining. The experiment was repeated three times.

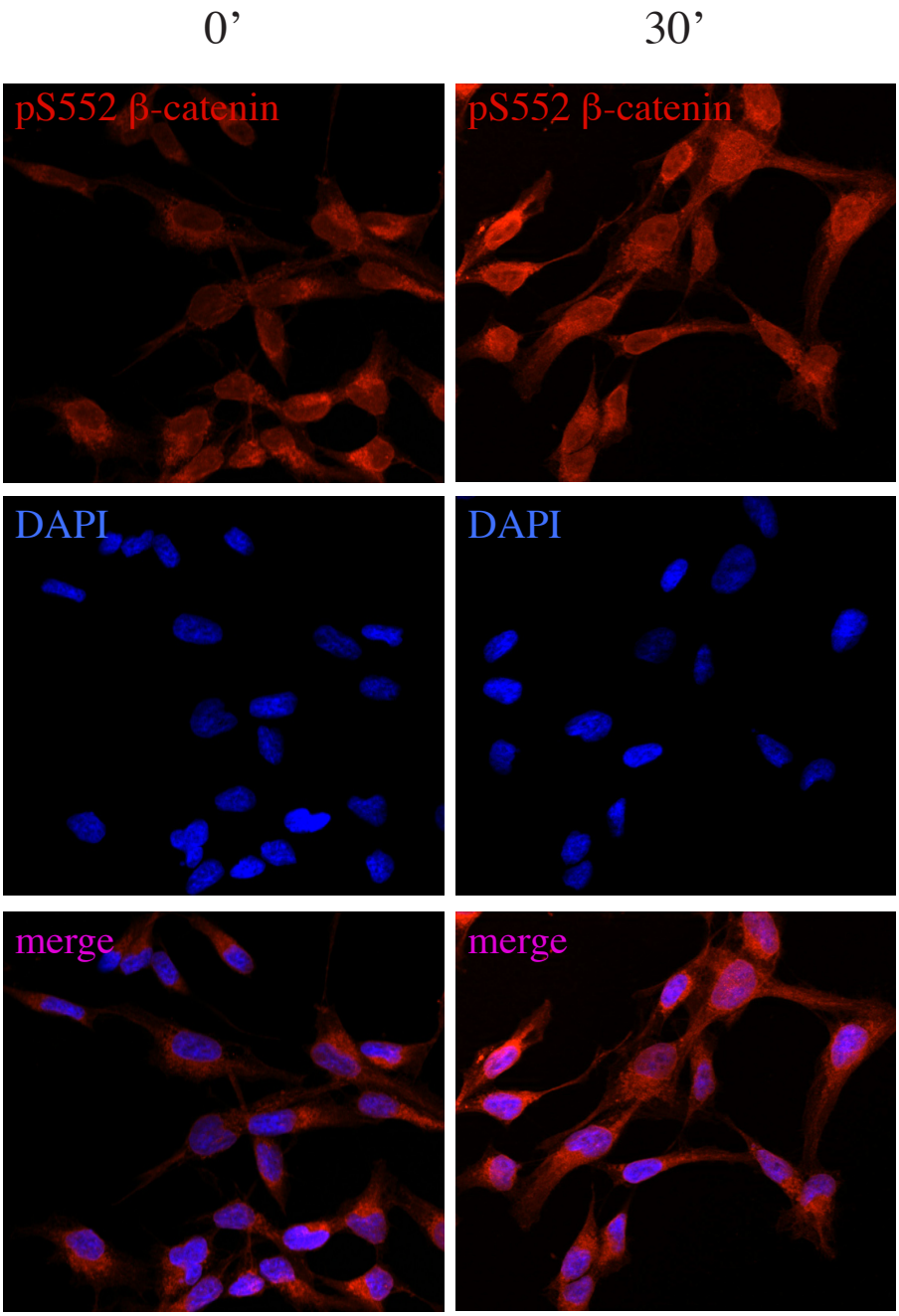



**Figure 4.12:** Expressional analysis of phospho-β-catenin-Ser552 (active form of β-catenin) in RD18 cells in response to Wnt3a by immunofluorescence. There was an increased in the amount of nuclear phospho-β-catenin-Ser552 in response to Wnt3a. The image on left (Rh30 control; without Wnt3a) showed a lower nucler expression of phospho-β-catenin-Ser552 as compared to Rh30 cells treated with Wnt3a (200ng/ml for 30 mins; image on the right). Changes in the phosphorylation status of β-catenin suggest an active Wnt signalling in RD18 cell line. Rabbit polyclonal primary anti-phospho-β-catenin-Ser552 antibody (1:1000) and TRITC-conjugated goat anti-rabbit secondary antibody (1:2000) was used for localisation of phospho-phospho-β-catenin-Ser552. DAPI was used for nuclear counterstaining. The experiment was repeated three times.

We also looked at the localisation of the phosphorylated forms of β-catenin using immunofluorescence experiments. Wnt3a stimulation increased perinuclear localisation of phospho-S552-β-catenin; at the same time, it decreased the level of cytosolic phospho-S33S37T41-β-catenin in both alveolar and embryonal cell lines, whereas the amount of control nuclear protein Lamin B remained unchanged (Figure 4.9-4.12). Wnt3a was added to the cell medium of the RMS cells for 60', as this was the most responsive time period as inferred from the previous time course experiments.

The results from the immunoblot analysis indicate that not only are the Wnt proteins expressed in the RMS cells lines, they also show change in functional phosphorylation status in response to the natural Wnt agonist Wnt3a at a concentration of 200ng/ml. Whilst there was an increase in the inactive phospho-S33S37T41-β-catenin in the control (Wnt off) state, in the activated state (Wnt on) there was an appreciable decrease in the cytosolic levels. Further, there was a physical change in the intracellular location of β-catenin in response to Wnt3a as confirmed by the cell fractionation analysis and confocal analysis.

After establishing that the central Wnt proteins are expressed in RMS cell lines; that they show change in their phosphorylation status and

that there is a shift in their subcellular locations upon Wnt3a activation, we wanted to see next if β-catenin was functionally active in the nucleus.

### 4.4 Does β-catenin bind to the TCF/LEF in the nucleus and activate downstream Wnt targets in the RMS cell lines?

To evaluate the functional significance of nuclear β-catenin in RMS cell lines after Wnt3a-mediated activation, we employed luciferase-based TOP/FOP Flash reporter gene assay. *Gaussia* Luciferase (GLuc) is a naturally secreted protein produced by *Gaussia princeps*. The assay principally employs two reporter plasmids – the TOPflash (which contains two sets of 3 copies of the wild-type TCF binding regions; acts as the positive control) and the FOPflash (that contains truncated TCF incapable of binding to the transactivation domain of β-catenin; acts as the negative control). If the canonical Wnt signaling is activated, β-catenin would translocate to the nucleus to associate with TCF/LEF transcription factors and would activate downstream Wnt target genes, resulting in an increased luciferase activity when the substrate is added. On the other hand, there would be a loss of luciferase expression, if Wnt signaling is inhibited. Increased FOP flash activity indicates non-specific activation of the TCF/LEF under experimental conditions.

#### 4.4.1 Regulation of TCF/LEF Transcriptional Activation

Luciferase reporter gene analysis was performed in control and Wnt3a-treated-RMS cell lines (RD18 and Rh30); β-galactosidase vector was co-

transfected for standardisation. Rh30 and RD18 cells were stimulated by Wnt3a (200ng/ml for 48 hours) after TOP flash-transfection to see if there was any change in the reporter gene activity. The control RMS cells were untreated. Gaussia luciferase has a secretory signal that is secreted into the cell medium. Medium from the treated and control cells were collected for Gaussia luciferase assay (New England Biosciences, UK) according to manufacturer's instructions. Each assay was performed in triplicate and the reporter activity was expressed as mean ± SD [248].

We found a 7-fold increase in the reported gene activity in test cells as compared to the untreated control cells in both cell lines. On the other hand, there was no significant difference in the reported gene activity after the transfection of FOP flash control vector. This shows the specificity of β-catenin-driven nuclear transcription (Figure 4.13a) [248].

Immunoblot analysis for Wnt targets showed minor transient changes in the levels of c-Jun, cyclin D1, slug and c-myc expression over the first 30 minutes (Figure 4.13b). However, the expression of Axin-2 (Wnt inhibitor) was remarkably increased in Rh30 and RD18 cell lines following Wnt3a stimulation on immunoblot analysis, indicating a specific downstream effect (Figure 4.13c) [248].

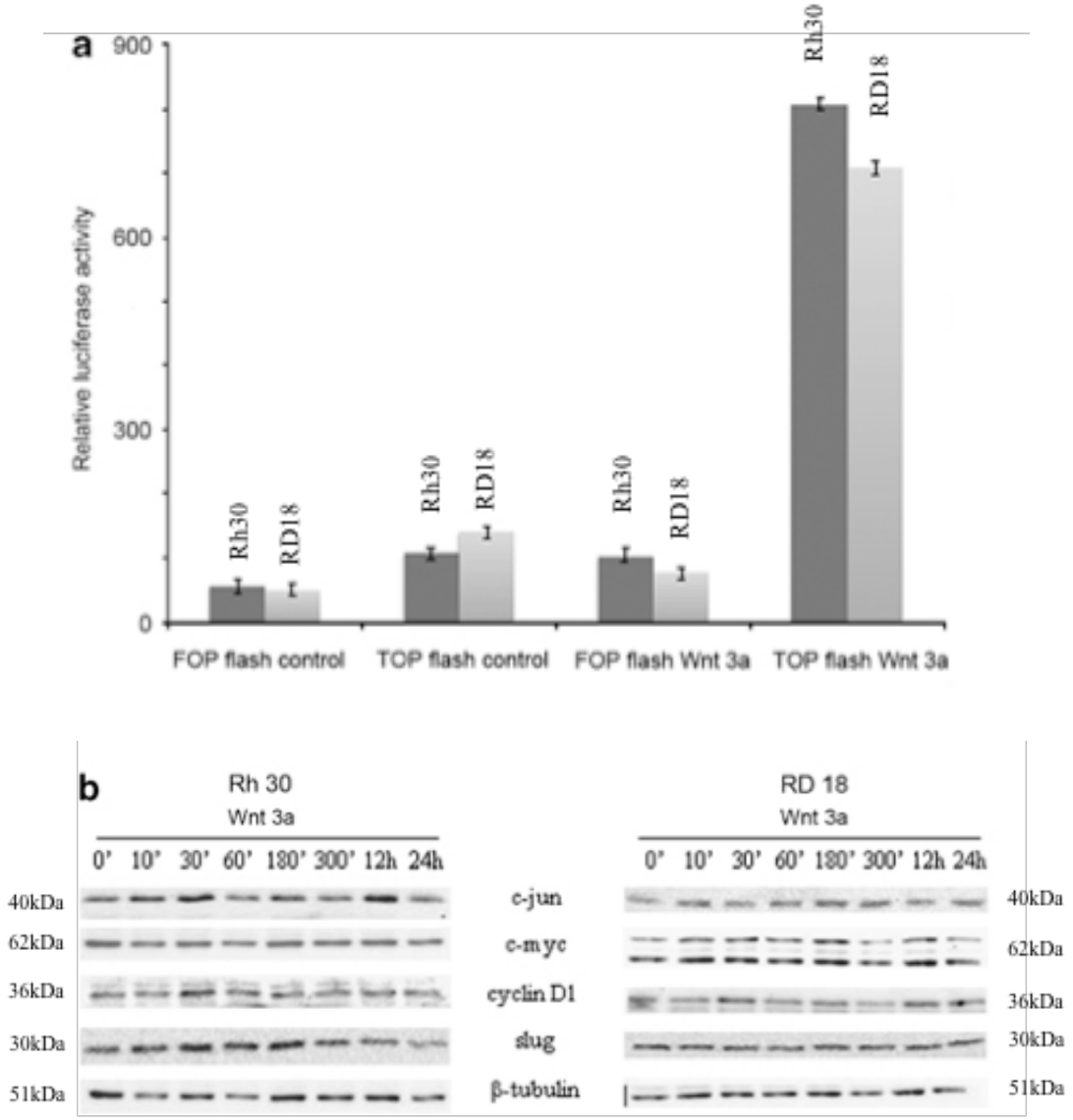



Fig 4.13 (**a**) Functional activation of Wnt signaling in control (untreated) and treated (Wnt3a 200 ng/ml for 48hrs) Rh30 (dark grey) and RD18 (light grey) cell lines. Rh30 and RD18 cells were transfected with TOPflash (positive control) and FOPflash reporter (negative control) plasmids ($n$=3), according to the manufactuer's instructions, and were grown for 48hrs without or with Wnt3a. After Wnt3a stimulation, TOP flash-transfected Rh30 and RD18 cells, showed approximately seven-fold significant induction of $\beta$-catenin-driven TCF/LEF luciferase activity compared with untreated control cells; this effect appears to be specific because Wnt3a treatment did not affect luciferase activity of FOPflash control vector in both cell lines. Data shown are mean ± SD of 3 independent experiments. (b) Immunoblot analyses of Wnt target proteins (c-jun, c-myc, cyclinD1 and slug) in Rh30 and RD18 cell lines, in response to Wnt3a stimulation over time (0-24 hrs). There were minor transient fluctuations over 30 mins but there was no sustained effect. Equal amount of protein was loaded in all lanes (10μg/lane). $\beta$-tubulin served as the loading control. The experiment was repeated three times.

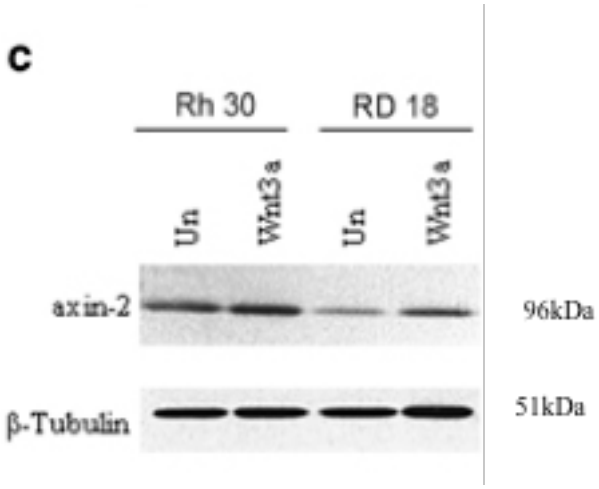



Fig.14.3 c: Expression of Wnt-feedback inhibitor Axin-2 in Wnt3a stimulated Rh30 and RD18 cells as compared to untreated controls. Increased expression of Wnt-feedback inhibitor Axin-2 in Rh30 and RD18 cells treated with Wnt3a (200 ng/ml for 24 hrs) indicate functional activation of downstream Wnt target genes. Equal amount of protein was loaded in all lanes (10µg/lane). *β*-tubulin served as the loading control. The experiment was repeated three times.

## 4.5 What effect does activation of Wnt signalling pathway have on the proliferation of RMS cell lines?

Wnt signalling is an important cell fate determinant. Since Wnt pathway drives many tumours, next we set out to see what impact it has on the proliferation rate of RMS cell lines. It was expected that Wnt3a treated RMS cells may activate proliferation as compared to the control cells and may shed important insights into the pathogenesis of RMS.

### 4.5.1 Effect of Recombinant Wnt-3a on Proliferation and Apoptosis in RMS Cell Lines

We checked for proliferation rate in RMS cell lines after treating them treated with Wnt3a over a period of 24-72hrs. Since the reported gene mediated nuclear transactivation was maximum after 48hrs, we expected to see the maximum change in proliferation rate during this period. However, there was approximately 20% decrease in proliferation rates of both alveolar RMS cell lines, (Rh4 cells $P<0.02$ and Rh30 $P<0.05$; Figure 4.14). There was no significant decrease in proliferation after Wnt3a treatment in the embryonal RMS cell lines (RD18 and RD; Figure 4.14).

Since the results were unexpected, the experiment was repeated thrice at both University of Liverpool and at La Sapienza University Rome, given the implications of such a finding. To ensure that the decrease in proliferation was not due to cell toxicity or due to increased

apoptosis, we did morphological analysis by phase contrast microscopy and the Caspase 3/7 assay to address these issues.

To further characterise the mechanism(s) by which Wnt activation may have brought about a decrease in the proliferation rate in the alveolar RMS cell lines, we performed apoptosis assay to exclude any toxic or cytostatic effects on the cells. There was no significant difference in the Caspase3/7 activity after Wnt3a stimulation in any of the RMS cell lines suggesting that Wnt3a could have had an effect on the cell differentiation. To prove this, we looked at the myogenic differentiation markers in the RMS cell lines before and after Wnt3a treatment. To our surprise, we found that, after Wnt3a treatment over a period of 24-48 hours, there is a definite increase in the expression of early myogenic differentiation markers (MyoD1, myf5 and Myogenin), where a late differentiation marker desmin showed variable results in both cell lines (Figure 4.15).

Next, we looked at the myosin and actin arrangement using immunofluoresence experiments using MyHC antibody and phalloidin TRITC-conjugated after 96 h Wnt3a treatment. Treated cells were MyHC positive compared with untreated cells (data not shown) and, after phalloidin staining, areas characterised by a more concentrated assembly of actin bundles appeared (Figure 4.16a). Cells treated with Wnt3a revealed morphological features typical of differentiating muscle cells, progressively changing from fusiform or star shaped into elongated

confluent cells as shown by confocal microscopy (Figure 4.16b). Therefore, increased differentiation appears to be the most plausible explanation for the decline in proliferation in alveolar RMS cell lines. Overall, We found a 20% decrease in the proliferation rate in the ARMS cells treated with Wnt3a (200ng/ml) for 48-72 hours as compared to the untreated control ARMS cells. There was an increase in the muscle differentiation markers during this period; there was no significant increase in the rate of apoptosis in test or control cells (Fig 4.17).

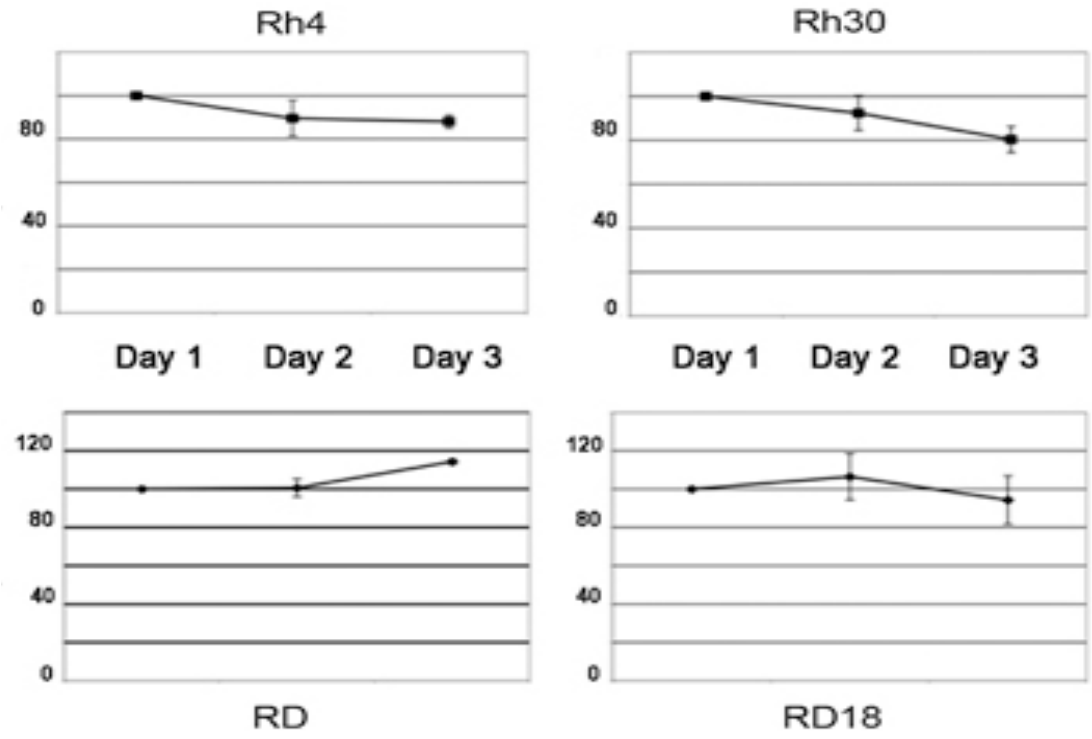



**Figure 4.14**: Effect of Wnt3a (200 ng/ml) on the proliferation of alveolar (Rh4 and Rh30) and embryonal (RD and RD18) RMS cell lines expressed as % inhibition. There was 15–20% decrease in proliferation rate of alveolar RMS cells (Rh4 $P<0.02$ and Rh30 $P<0.05$) and no significant change in embryonal RMS cells (RD $P>0.05$ and RD18 $P>0.05$; n=3). Data shown are mean ± SEM of 3 independent experiments.

### 4.5.2 Expression of muscles-specific markers in response to Wnt activation

We also performed IF experiments using MyHC antibody and phalloidin-conjugated to show actin and myosin arrangement. 96h Wnt3a treated cells showed induction of MyHC protein and reorganization of actin fibers typical of myo-differentiation (Figure 4.15). Based on these data, the decrease in proliferation rate appears to be related in ARMS to an increase in cell differentiation rather than toxic cell death or apoptosis. Thus, it appears possible that Wnt pathway expression in RMS may be in a functionally repressed state (possibly from antagonistic activity of other pathways active in RMS like MAPKinase/Ras pathway or CXCR pathways etc.)

Our results in RMS cell lines indicated that activation of Wnt pathway (by Wnt agonist Wnt3a) can increase muscle differentiation in vitro and can decrease the cell proliferation; there was no increase in the rate of apoptosis between control and test cells after 200ng/ml of Wnt3a for 48 hours.

Thus, functional activation of Wnt pathway by the Wnt agonists (like LiCl, BIOS, Sodium Valproate etc.), may have a therapeutic role in RMS in the future.

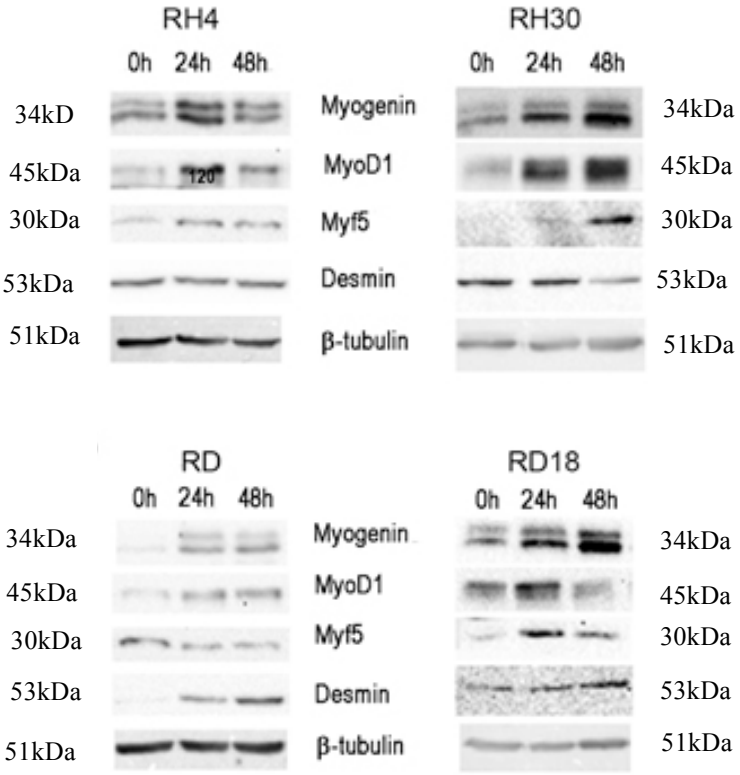



**Figure 4.15**: Increased expression of myogenic markers (Myogenin, Myf-5 and MyoD1 and desmin) after Wnt3a (200 ng/ml for 24 and 48hrs) stimulation in rhabdomyosarcoma (RMS) cells as compared to the untreated control cells (at 0 hrs). Equal amount of protein was loaded in all lanes (10µg/lane). *β*-tubulin served as the loading control. The experiment was repeated three times.

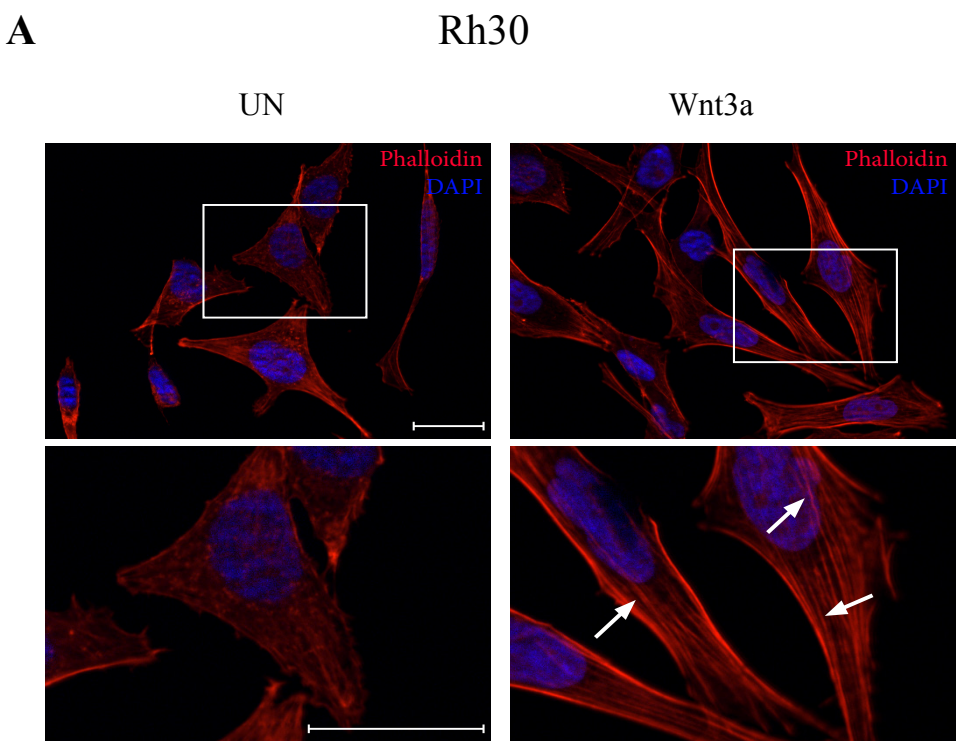



**Figure 4.16a**: The effect of Wnt3a stimulation (200ng/ml for 96hrs) on Rh30 cells. Actin rearrangement to form condensed filamentous bundles (arrows) is shown by immunofluorescence with TRITC-conjugated phalloidin. The experiment was repeated three times.

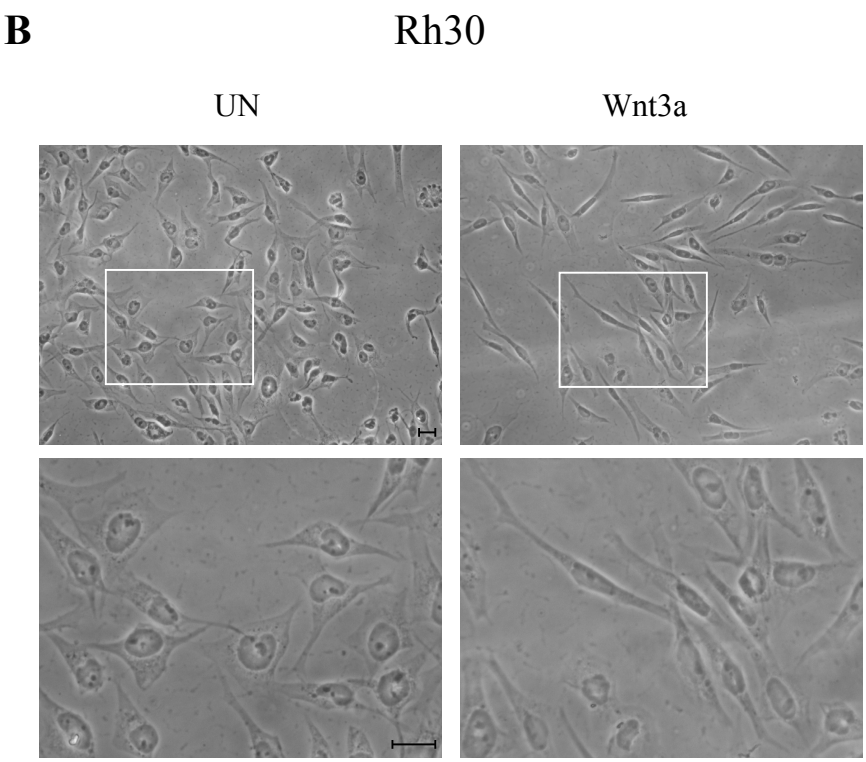



**Figure 4.16b**: The effect of Wnt3a stimulation (200ng/ml for 96hrs) on Rh30 cells. Phase contrast microscopy shows a change in morphology from stellate to elongated cells. Representative images for each treatment group are shown with the areas in the upper panels enlarged in the lower bottom panels. Scale bars 20μm. The experiment was repeated three times.

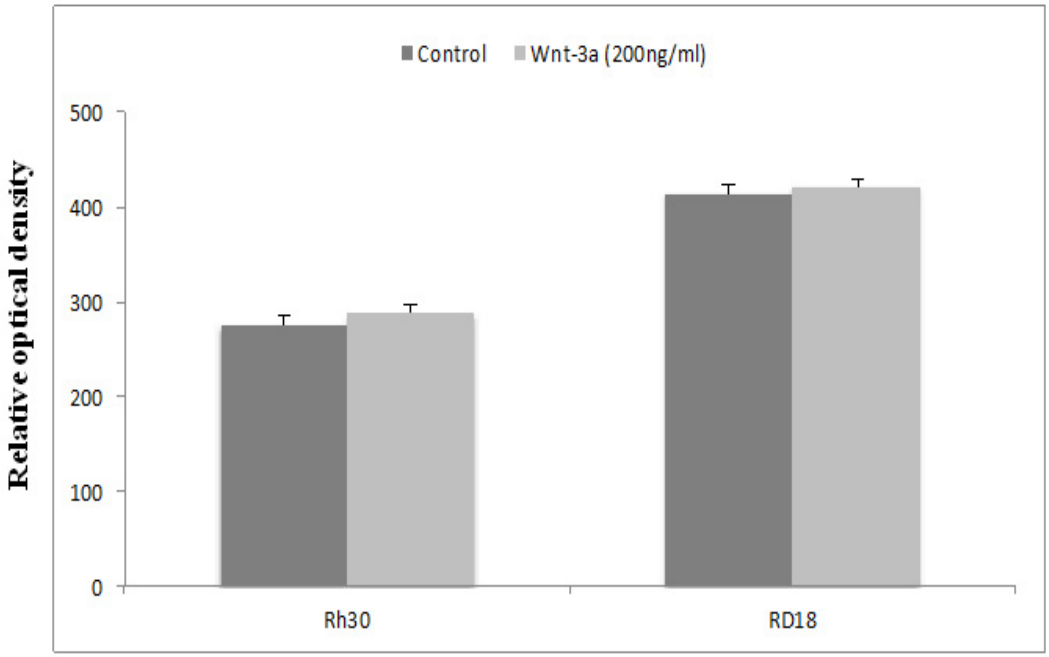



**Figure 4.17**: The effect of Wnt3a stimulation (200ng/ml for 48 hrs) on Rh30 and RD18 cells. Caspase3/7 assay does not show any significant increase in the apoptosis to account for the decrease in the proliferation rate seen in the both RMS cell lines. Data shown are mean ± SEM of 3 independent experiments.

## 4.6 Does Wnt signalling pathway cross-react with CXCR pathway in RMS cell lines?

Chemokines are small pro-inflammatory cytokines that regulate cell trafficking and adhesion by binding to specific G-protein coupled transmembrane receptors [251]. The chemokine CXCL12 or SDF-1 (stromal-derived factor-1) is an important α-chemokine that is secreted by bone marrow stromal cells [252]. It binds to its receptor CXCR4 and is believed to play an important role in bone marrow metastasis [253, 254]. By induction of VLA-4 and LFA-1 integrins on the surface of CD34 cells, SDF-1 promotes these cells to migrate and home in to the bone marrow endothelium [255].

It has been proposed that SDF-1/CXCR4 axis may facilitate multiple stages of metastasis, including adherence to endothelium, blood vessel extravasation, angiogenesis, proliferation and evasion of host defence mechanism [251, 256]. CXCR4–SDF-1 axis has been implicated in the metastasis of breast cancer and RMS to bone marrow and lymph nodes that naturally express high levels of SDF-1 [257-259]. Interestingly, there are some studies in the literature that link SDF-1/CXCR4 axis with Wnt/β-catenin signalling. SDF-1/CXCR4 signalling has been shown to stimulate the activity of β-catenin in the neural progenitor cells of rat [260]. Wang (2007) showed the relevance of SDF-1/CXCR4 signalling in metastasis of pancreatic cancer [261, 262].

The downstream effectors of CXCR4 in RMS have not been clarified completely and have mainly been linked to the stimulation of survival pathways such as ERK/MAPK, PI-3K/Akt/mTOR, and/or Jak/STAT with activation of downstream genes like c-Myc and cyclin D1 [251, 263, 264]. Many of these pathways cross talk with Wnt/β-catenin signalling. One would expect that if the PI3K/Akt pathway is activated, it would inhibit GSK3β (a protein which promotes cytosolic β-catenin degradation) and would allow nuclear translocation of β-catenin that stimulates downstream genes such as c-Myc and cyclin D1.

Miekus (2013) demonstrated induction of myodifferentiation with reduced MyoD expression upon down regulation of the c-MET receptor [59]. He also showed reduced metastatic potential of ARMS cells to the bone marrow with down regulation of CXCR4 receptor expression [59]. He proposed the clinical utility of c-MET receptor inhibition and activators of myodifferentiation in RMS. Since β-Catenin is known to regulate MyoD transcription, and since CXCR4 expression in ARMS is related to bone metastasis, we wished to exclude any potential cross talk between the Wnt and SDF-1/CXCR4 pathways.

It is expected that if a cross talk exists between Wnt/β-catenin and SDF-1/CXCR4 axis and that if the SDF-1 mediates its effects in RMS via the PI3K/Akt pathway, there should be appropriate changes in the

phosphorylation status of β-catenin, GSK3β, Akt and Erk. Also there should be increased expression of the downstream Wnt targets, c-Myc and Cyclin D1.

### 4.6.1 Time-dependent expression of SDF-1 in RMS cell lines after 24 hours of Wnt3a stimulation in vitro.

Since Wnt pathway is associated with myogenesis and also with increased myogenic differentiation we wished to investigate if CXCR4–SDF-1 axis cross talks with Wnt signalling in RMS. To this end, ARMS cell line RH30 and ERMS cell line RD18 were treated with human recombinant SDF-1 (200ng/ml) for 24 hours and cell lysates were prepared at 0', 10', 30', 60', 180', 300', 12hrs and 24hrs to see time dependent changes in the various Wnt proteins by western blotting (beta-catenin, pS33S37T41phospho-beta-catenin, pS552phospho-beta-catenin, GSK3beta, S9phospho-GSK3beta, CyclinD1 and c-Myc), Protein Kinase B/Akt, phospho-Akt, ERK1/2 and phospho-ERK1/2.Beta-tubulin served as the loading control.

Results did not indicate any significant change in any of the Wnt proteins or their phosphorylated forms in response to SDF-1 in-vitro in ARMS cell line RH30. Total β-catenin and its phosphorylated forms, pS33S37T41phospho-β-catenin and pS552phospho-β-catenin did not show any appreciable change over time with SDF-1 treatment. Similarly,

protein levels of total GSK3β and its phosphorylated form S9phospho-GSK3β remained constant. Total ERK and phopsho-ERK1/2 however, increased strongly at 10’ until 30’. Whereas phospho-ERK reduced sharply after an hour and disappeared over 24 hours, total Erk reduced gradually returned to basal level over 24 hours. Downstream Wnt target Cyclin D1 expressed sharply around 180’, peaked at 300’ and then started falling after 12 hours to disappear by 24 hours of SDF-1 treatment. C-Myc levels were difficult to assess; the expression dropped between 60’ and 300’ and returned to basal levels by 24 hours. β-tubulin served as loading control.

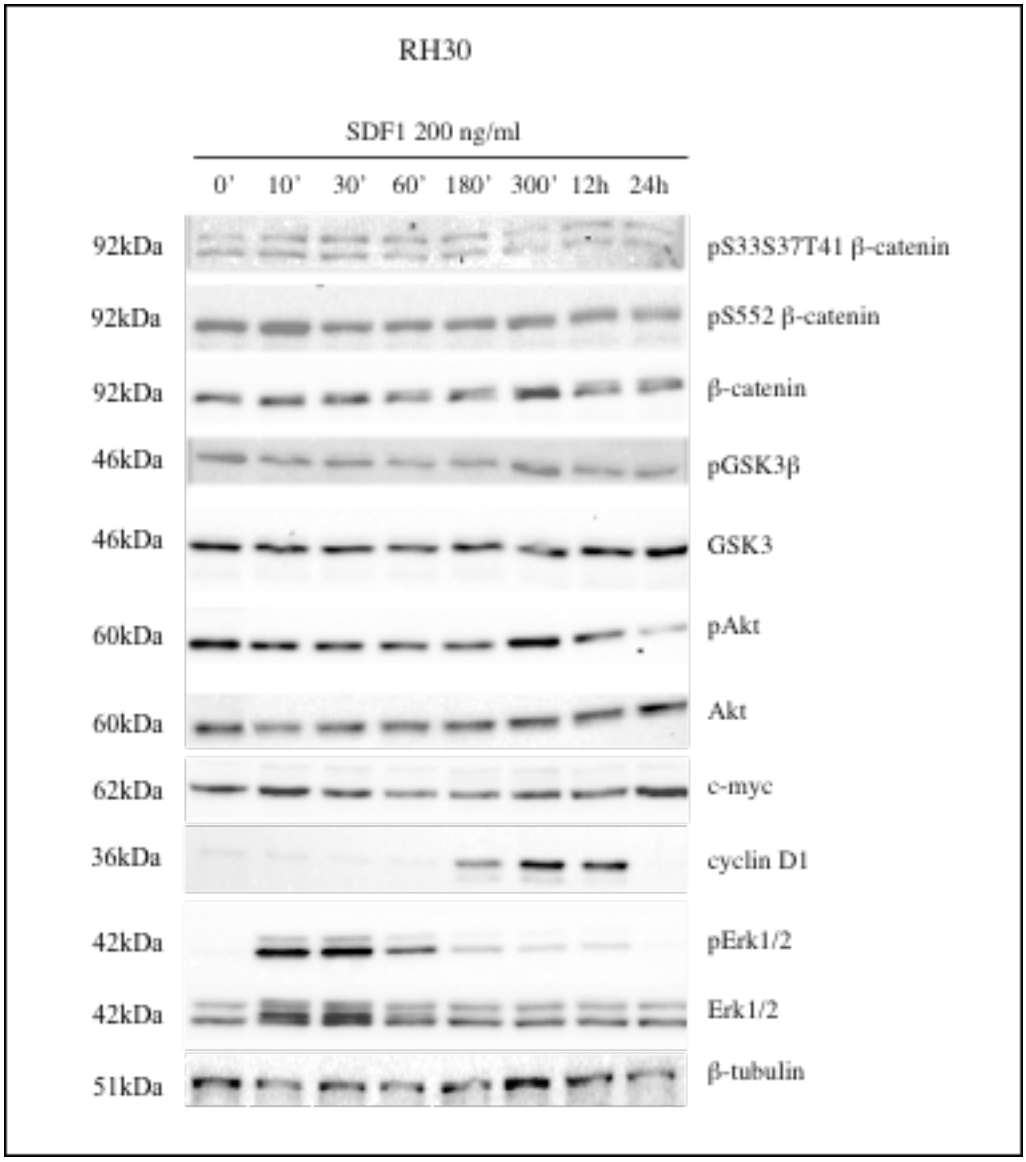



**Figure 4.18:** Western blot analysis on the RMS cell lines. Levels of β-catenin, phospho-β-cateninS33S37T41 (inactive form), phospho-β-cateninS552 (active form); GSK3β; phospho-GSK3β (inactive form); Protein kinase B/Akt and its phosphorylated form (active); Erk and its phosphor form and downstream Wnt targets (cyclin D1 and c-myc) are seen in response to Wnt3a (200ng/ml for 24hrs) in Rh30 cell line. Equal amount of protein was loaded in all lanes (10µg/lane). *β*-tubulin served as the loading control. The experiment was repeated three times.

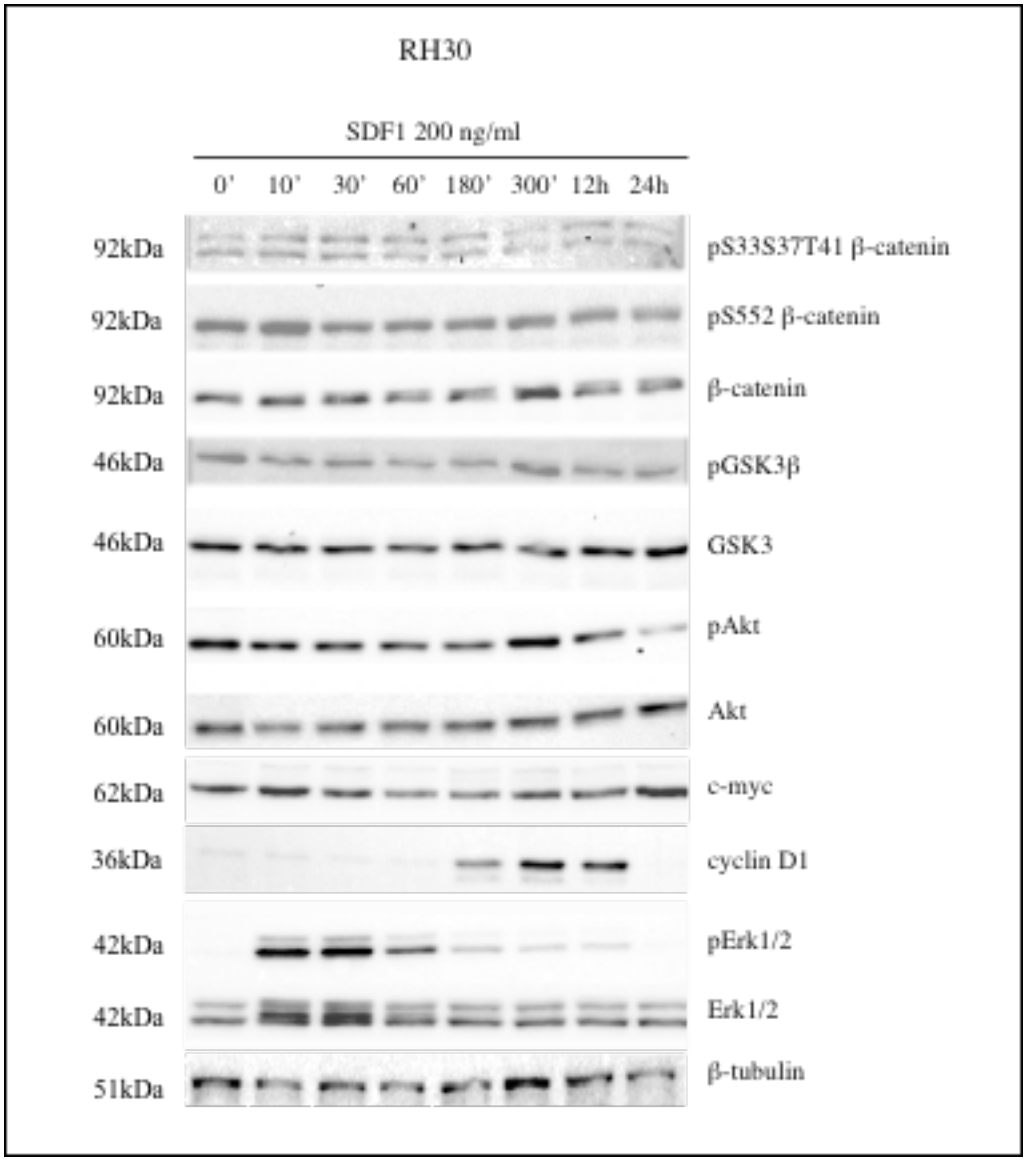



**Figure 4.19:** Western blot analysis on the RMS cell lines. Levels of β-catenin, phospho-β-cateninS33S37T41 (inactive form), phospho-β-cateninS552 (active form); GSK3β; phospho-GSK3β (inactive form); Protein kinase B/Akt and its phosphorylated form (active); Erk and its phosphor form and downstream Wnt targets (cyclin D1 and c-myc) are seen in response to Wnt3a (200ng/ml for 24hrs) in RD18 cell line. Equal amount of protein was loaded in all lanes (10µg/lane). *β*-tubulin served as the loading control. The experiment was repeated three times.

In the RD18 cell line, apart from Ser9-phospho-GSK3β, which showed time dependent decrease over 24 hours, there were no significant changes in any of the Wnt proteins or their phosphorylated forms. Phospho-Akt and phopsho-ERK1/2 levels remained unchanged.

Hence, we did not find any evidence of cross talk between CXCR4 and Wnt signalling pathway.

# CHAPTER 5 –

# DISCUSSION

# CHAPTER 5 – DISCUSSION

β-catenin is a potent nuclear transcription factor that mediates the canonical Wnt signalling pathway, which is actively involved in muscle development during embryonal stage [119, 265]. Wnt signalling is a pathway crucially involved in development of tissue and also in cell fate, proliferation, migration, polarity and death [68]. Deregulation of Wnt signalling facilitates cell transformation, tumour invasion and metastasis [59, 249].

When Wnt signalling is off, β-catenin is degraded by a degradation complex comprising CK1-α, GSK3β, Axin and APC protein. This degradation complex causes β-catenin phosphorylation, which in turn facilitates ubiquitination and proteasomal degradation [68]. When Wnt signalling is on, the signal cascade activates Dsh protein, which in turn enhances the phosphorylation of GSK3β in the cytoplasm and disassembles the degradation complex thereby rescuing β-catenin from degradation. β-catenin then shifts to the nucleus where it associates with TCF/LEF complex with sequence specificity promoting changes in transcriptional machinery that lead to activation of several downstream target genes [172].

There are many downstream Wnt target genes, which play crucial roles in various cell processes like proliferation, survival and

differentiation. Targets include: MMP7, CD44, c-Myc, c-Jun, Cyclin D1, Slug and Cox etc. Deregulation of Cyclin D1 and c-myc can lead to cell transformation and cancer. We have undertaken this work to characterise Wnt/beta-catenin signalling in rhabdomyosarcoma (RMS) and to examine if it plays a role in pathogenesis of RMS.

### 5.1 Does Wnt/ β-catenin signalling have a role in the pathogenesis of RMS?

It is well known that Wnt signalling plays a central role in myogenic differentiation and cell proliferation [172, 265]. However, there is no convincing evidence to its relevance in the pathogenesis of RMS. Bouron-Dal Soglio et al. (2009) found no activating mutations of β-catenin in their study and proposed that Wnt signalling may not be relevant in RMS [265]. Wnt2 overexpression was demonstrated in embryonal RMS cells derived from p53/c-fos double mutant mice by Singh et al. (Singh, 2010). They concluded that there might be a role for impaired Wnt signalling in embryonal RMS cells [63].

We undertook this study to characterise the role of Wnt/β-catenin signalling pathway in RMS and to see if this pathway had any relevance in the pathogenesis of RMS and whether this pathway could be targeted therapeutically in alveolar RMS where there are limited treatment options.

To see whether β-catenin is expressed in clinical samples of RMS, we did an immunohistochemical expressional analysis in 44 RMS cases. We constructed a TMA multiblock incorporating 34 cases and the rest were examined as full-face sections. Results indicated that β-catenin is expressed in around 60% of the cases, mainly in the cytoplasm/membrane. Those cases where there was a distinct perinuclear expression, we counted them as nuclear staining. Only 2/44 RMS cases, both embryonal RMS, showed nuclear expression of β-catenin, which is in agreement with the fact that there is no activating β-catenin mutation in RMS [265]. We did not find any statistically significant differences in the immunohistochemical expression of β-catenin in alveolar and embryonal RMS (64% vs. 56%). There also was no particular correlation between the expression of β-catenin with site, age or clinical outcome (data not shown). β-catenin expression also did not show any specific association when compared between the translocation-positive and negative alveolar RMS (5/7 vs. 4/7).

In-vitro immunoblot experiments were designed next to see if the similar β-catenin expression could be elucidated in human RMS cell lines derived from alveolar and embryonal RMS. We not only found a high expression of β-catenin in all cell lines, but also other major structural and regulatory components of Wnt pathway. The cadherin family of proteins, which bind with β-catenin at cell membranes, was also present

in all cases. With the help of dosage and time-course immunoblot analyses, we showed that upon activation of the Wnt signalling pathway by its natural agonist, human recombinant Wnt3a, β-catenin could be stabilized and rescued from proteasomal degradation. This was comprehensively shown by the change in the phosphorylation status of β-catenin before and after application of Wnt3a to the RMS cell lines. Changes in β-catenin phosphorylation were also mirrored in our immunofluorescence experiments where we could also demonstrate a time-dependent redistribution of β-catenin from the membranes to the perinuclear/nuclear region [172].

PI3K/Akt pathway is a point of convergence of many signalling pathways (including EGFR, insulin, insulin-like growth factor etc.) that induce GSK-3β inactivation of by Ser9 phosphorylation. Since Akt and phospho-Akt remained unchanged after Wnt3a-mediated Wnt activation, it appeared that GSK-3β inactivation (by Ser9-phosphorylation) was independent of PI3K/Akt pathway. However, it was expected that Wnt3a activation would mediate GSK-3β inactivation by Ser9-phosphorylation; however, we saw a decrease in the levels of phospho-GSK-3β levels after Wnt3a stimulation (Figures 2c and d). This unveils the complexity of the regulation of central regulatory proteins at multiple levels by different effectors. Thus, there are likely to alternative mechanisms for GSK-3β inactivation following Wnt stimulation. One possible explanation could

be physical sequestration of GSK-3β by Disheveled, rather than inhibitory Ser9 phosphorylation, as shown by Aschenbach et al. in rat skeletal muscle [266]. In another study, Rao et al. (2008) suggested that interaction of β-catenin and APC does not need GSK-3β phosphorylation at Ser9 [267].

By using nuclear and cytoplasmic cell extracts, we also showed that there was an increase in nuclear β-catenin fraction and a concomitant decrease in the cytosolic fraction after Wnt3a-mediated activation of Wnt signalling. Further, using specific phopho-antibodies we showed that nuclear β-catenin was phosphorylated at Ser552, phosphorylation that promotes nuclear import once β-catenin is rescued from degradation. There also was a simultaneous decrease in the level of phospho-S33S37T41-β-catenin in the cytoplasm of both cell lines after Wnt3a stimulation. These results were supported by similar findings on immunofluorescence experiments.

Once it was established that β-catenin was stabilised and translocated to the nucleus, next we asked whether the nuclear β-catenin was functionally active. To test this, we employed a Wnt-specific luciferase based reported gene assay. We also looked at the expression pattern of downstream Wnt targets. Axin-2 is widely considered to be a specific Wnt target gene that is also a feedback inhibitor of the pathway.

We found a seven-fold increase in the transcriptional activity of the TOP flash reporter gene as compared to the FOP flash negative control after Wnt3a activation of RMS cells. Immunoblot assay was performed to look at the expression of Axin-2 after Wnt3a activation. Results indicated an increased expression of Axin-2 in both RMS cell lines. Previously, Lustig et al. (2002) have shown that Axin-2 is a feedback inhibitor of the Wnt pathway and limits it after activation [201]. Thus, our results indicate that transient Wnt signalling activation may be sufficient to redistribute β-catenin to the nucleus to induce TCF/LEF transactivation resulting in activation of its downstream targets.

### 5.2 Could Wnt/ β-catenin signalling be a potential therapeutic target for RMS?

Given the role of Wnt signalling in the various cell processes including cell growth, proliferation and survival, we now wanted to see the impact of Wnt signalling activation on cell proliferation in RMS cell lines. Upon Wnt3a-mediated Wnt signalling activation (over 72hrs), we found a 20% fall in the proliferation rate of alveolar, but not embryonal RMS cell lines. Two independent researchers in different labs who did not know the previous results confirmed the results. It was clear that Wnt signalling activation somehow retards cell proliferation alveolar RMS cell lines. To elucidate the mechanism underpinning this effect, we did apoptosis and cell differentiation assays. None of the RMS cell lines showed any

appreciable increase in apoptosis after Wnt3a activation. However, Wnt signalling activation was able to induce myodifferentiation in both alveolar and embryonal RMS cell lines. This was inferred from an increased expression of specific early muscle differentiation markers such as Myogenin, MyoD1 and Myf5, highlighting that Wnt signalling may reduce the proliferative ability of alveolar RMS cell lines by in promoting myogenic differentiation in RMS [266].

It remains unclear as to why even after the Wnt3a-induced expression of early myogenic differentiation markers, there was no significant reduction in the proliferative potential of embryonal RMS cells. It is possible that alveolar RMS, driven by Pax–FKHR fusion gene protein, could be inhibited by activation of Wnt signalling, at least in a subset of alveolar RMS cases. It is also possible that there may be other genetic/epigenetic mechanisms that may uncouple proliferation and differentiation in embryonal RMS cells. Further research is needed to understand these mechanisms.

Our results indicate that activation of Wnt/β-catenin pathway may provide a therapeutic tool in treating alveolar RMS patients as it promotes myogenic differentiation and retards cell proliferation. In many ways, activation of Wnt signalling, which regulates a myriad of cellular functions is much easier than trying to inhibit it without having significant side effects, since it is also required for renewal for stem cells.

Lithium chloride, an FDA-approved drug used for bipolar mania patients, could have a potential role in the treatment of a subset of RMS patients as it inhibits GSK-3β and activates Wnt signaling [209]. There are many new Wnt agonists that are safe for patient use and could be used for clinical trials [64].

To conclude, Wnt proteins are expressed in tissue samples and in the human RMS cell lines. It appears that this pathway is present in a repressed state in RMS and can be activated to provide a therapeutic target. Wnt signalling is relevant in the pathogenesis of alveolar RMS and we believe that it may have a tumour-suppressive role in RMS. There is potential to modulate the Wnt signalling pathway thereby providing additional therapeutic targets in alveolar RMS patients who, currently have a poor clinical outcome and limited treatment options.

# CHAPTER 6 –

# FUTURE RESEARCH

### 6.1 Wnt activators as therapeutic option

Our results indicate that Wnt signalling may have a tumour suppressor role in at least a subset of alveolar RMS. We have shown in-vitro that activation of Wnt signalling pathway using natural Wnt agonists can stimulate muscle differentiation and decrease proliferation rates in alveolar RMS cell lines. Thus, it might be expected that FDA-approved drugs like Lithium Chloride (LiCl) could be safely used in clinical trials and could have a therapeutic use in the treatment of alveolar RMS [268]. Since Wnt signalling is vital for many cellular processes including stem cell regulation, it can be envisaged that activation of a repressed Wnt pathway using Wnt agonists would be much easier than inhibition of a deregulated Wnt pathways (e.g. colorectal carcinoma).

1. LiCl is a drug used in the treatment of bipolar maniac illness and has the potential to be used in therapeutic Clinical Trials for alveolar RMS, as it inhibits GSK3β and activates the Wnt signalling pathway [269].
2. Bromoindirubin-30-Oxime (6-BIO), is another inhibitor of GSK3β, that is neuroprotective in Alzheimer's disease and has the potential as a potential therapeutic agent in alveolar RMS [270].
3. Valproic acid and Staurosporine are other drugs that can be used to activate Wnt signalling by targeting GSK3β.
4. Simvastatin synergises with Wnt signalling both in vivo and in vitro [268].

5. Curcumin is an agonist of Wnt signalling pathway and is neuroprotective for brain. Possible mechanisms of curcumin action are thought to be activation of Wnt antagonists like Wif-1, Dickkopf and GSK3β [271].

Thus, using a range of safe, approved drugs one could target the Wnt signalling pathway to activate muscle differentiation in a subset of ARMS which may provide alternative therapeutic options.

### 6.2 Animal experiments in athymic nude mice with/without control to see if Wnt agonists rescue the cohort with Wnt3a activation?

We also plan to take this further into in-vivo experiments whereby we will administer Wnt agonists and placebo respectively to test and control athymic nude mice after inoculating them with RMS cells to investigate any differential pathological effects of Wnt signalling pathway on tumour characteristics – size, degree of differentiation, number of mitoses and apoptosis and micro-vessel density etc. We may expand this further to include experiments on control and transgenic Wnt -/- mice.

**6.3 To see the effect of miR-28 as the master switch regulating the Wnt/β-catenin signalling in RMS.**

To further characterise the role of Wnt in muscle differentiation in RMS, we are investigating if any in-vitro interaction exists between Wnt signalling pathway and other muscle repressor proteins (like Id family) upon activation by Wnt3a.

Inhibitor of DNA binding proteins (Id) is a family of basic helix-loop-helix (bHLH) transcription factors that regulate differentiation [272]. Id gene family (Id1-4) recognised in humans, and the proteins they encode share homology primarily in their HLH domain. They negatively regulate differentiation by forming inactive heterodimers with other bHLH proteins, and their basic domain binds to a DNA sequence element, the E-box, activating transcription. E proteins normally from hetero dimers with tissue restricted bHLH proteins such as MyoD1, NeuroD, etc. and forms active transcription complexes. Thus, Id negatively regulates differentiation by sequestering E-proteins [272]. Wnt signalling has been shown to induce Id3, which in turn stimulates myoblastic differentiation [273]. In addition, Id-1 inhibits the transcription of both p53 and PTEN to activate the Akt pathway that phosphorylates GSK-3β (at Se9) and stimulates Wnt signalling [274].

Recently, Macquarrie et al. (2012) have shown that both replicating myoblasts and RMS cells express MyoD. However, unlike myoblasts, the RMS cells cannot transit from the growth phase into terminal differentiation phase because they cannot dimerise with E-protein. If E-protein dimerization is forced

in the RMS cells (by RUNX1 and ZNF238), myodifferentiation could be achieved. miR-206, is thought to act like a 'genetic switch' that controls the transition from growth to differentiation [275].

Since Wnt3a-mediated activation of Wnt signaling path increases myo-differentiation in RMS cell lines by increasing muscle specific differentiation markers including MyoD1 [248], a known E-box interacting bHLH protein, we would like to investigate whether an upstream interaction between Wnt signaling and Id (Id1-4) exists in RMS cells. If it does, we will look in to whether this 'Wnt switch' is mediated by miR-206. This will enable us to understand better how muscle differentiation is regulated in RMS so that better targeted therapeutic strategies could be developed.

**6.4 Final Conclusion**

Rhabdomyosarcoma (RMS) remains one of the most challenging tumours in paediatric oncology, accounting for around 5% of all malignant paediatric tumours [7-9, 276]. Of the two major subtypes of RMS, embryonal and alveolar, the latter portends a poorer clinical outcome [9].

Canonical Wnt signalling pathway is an important evolutionarily conserved signalling pathway that is required for muscle development and embryonal somite patterning [118, 265]. β-catenin is a potent nuclear transcriptional activator and is the central effector of the canonical Wnt signalling pathway. Interestingly, constitutional activation of Wnt signalling also promotes tissue invasion and metastasis in various tumours [69].

We have shown that key regulatory proteins of the Wnt signalling pathway are expressed and are functional in rhabdomyosarcoma. β-catenin, the central regulatory protein of Wnt signaling pathway was expressed in over half of the RMS samples examined by IHC (n=44). Beta-catenin was also up-regulated in the human RMS cell lines derived from alveolar (Rh4, Rh30) and embryonal (RD, RD18) subtypes in-vitro after activation by its natural ligand, human recombinant Wnt3a (200ng/ml) [248].

Following Wnt3a activation, we showed that beta-catenin was rescued from degradation, accumulated in the cytoplasm (active form) and translocated into the nucleus where it transactivated TCF/LEF to activate downstream targets like Axin 2. TCF/LEF nuclear transcription was confirmed by TOP/FOP Flash reporter gene assay, which showed 7-fold increase in activity following Wnt3a stimulation [248]. Furthermore, we demonstrated that activation of Wnt signaling decreased the proliferation rate of RMS cells by increasing myo-differentiation. This was shown by increased expression of MyoD1, Myf5 and Myogenin in RMS cells by western blot experiments following Wnt3a stimulation [248]. Thus, we believe that Wnt signaling may confer tumour suppressive activity in RMS and is a therapeutic target in a subset of ARMS cases.

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