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Molecular mechanisms of the muscle differentiation blockade in Rhabdomyosarcoma

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CONTENT

LIST OF ABBREVIATIONS

4-1BBL	4-1BB Ligand
Ab	Antibody
ACT	adoptive T cell therapy
APC	adenomatous polyposis coli gene product
APC conjugated	Allophycocyanin conjugated
APS	Ammonium Persulfate
AXIN2	Axis Inhibition Protein 2
bHLH	basic helix–loop–helix
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
Ca^{2+}	Calcium
CaMKII	Calcium/Calmodulin Dependent Protein Kinase II
CAR-T	Chimeric antigen receptor T
CCND1	CyclinD1
CHRNA	Cholinergic Receptor Nicotinic Alpha Subunit
CHRNG/ fAChR	Cholinergic Receptor Nicotinic Gamma Subunit/ The fetal form of the acetylcholine receptor
CK1	casein kinase 1
CO2	Carbon dioxide
CTNNB1	β –Catenin, Beta-Catenin, Cadherin-Associated Protein, Beta 1
CTR	Control
ddH2O	Double-distilled water
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco's phosphate-buffered saline
Dvl	dishevelled
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic Reticulum
FACS	Fluorescence-activated cell sorting
FAP	fibroblast activation protein
FBS	Fetal Bovine Serum
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
FOXO1	Forkhead box protein O1
Fwd	Forward
FZD	Frizzled
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GD2	disialoganglioside
GSK 3	glycogen synthase kinase 3
HDAC2	Histone Deacetylase 2
HEK cells	Human embryonal kidney cells
HER2	human epidermal growth factor receptor 2
HGF	Hepatocyte growth factor
HRP	Horseradish peroxidase
IGF-1	Insulin-like growth factor
IGF2	Insulin-like Growth Factor II
JNK	JUN-N-terminal kinase

KD	Knock down
LE Agarose	Low Electroendosmosis Agarose
LEF1	lymphoid enhancer-binding factor
LIF	Leukemia inhibitory factor
LOH	loss of heterozygosity
LRP	low-density lipoprotein-related proteins
MRFs	myogenic regulatory factors
MTT	3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide
Na	Sodium
NFAT	nuclear factor of activated T cells
NLK	nemo-like kinase
OE	Overexpression
PAX3/7	Paired box gene 3/7
PBMCs	Peripheral blood mononuclear cells
PCP	Planar Cell Polarity
PCR	Polymerase chain reaction
PE-Cy 7	Phycoerythrin-Cyanin 7
PFmin RMA	Translocation negative RMA
PFplus RMA	Translocation positive RMA
PKC	protein kinase C
Porcn	O-acyltransferase Porcupine
Rev	Reverse
RFU	Relative fluorescence units
rhEGF	Recombinant human Epidermal Growth Factor
rhFGF basic	Recombinant human Fibroblast Growth Factor basic
RMA	Alveolar Rhabdomyosarcoma
RME	Embryonal Rhabdomyosarcoma
RMS	Rhabdomyosarcoma
ROR 1/2	Receptor Tyrosine Kinase Like Orphan Receptor 1/2
RPMI1640	Roswell Park Memorial Institute
RT	Reverse transcriptase
rWNT3A	Recombinant WNT3A
rWNT5A	Recombinant WNT3A
RYK	Receptor Like Tyrosine Kinase
scFv	Single-chain variable fragment
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SOX2	SRY-Box Transcription Factor 2
SP1	Specificity Protein 1
SURVIVIN/ BIRC5	Baculoviral IAP Repeat Containing 5
TAK 1	Transforming Growth Factor-Beta-Activated Kinase 1
TBS	Tris-buffered saline
TCF	T cell factor
TEMED	Tetramethylethylenediamine
TRIS	Trisaminomethane
TTA	tumor associated antigen
v/v	volume/volume
VC	Vector control
WLS	Wntless

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1 INTRODUCTION

1.1 Rhabdomyosarcoma (RMS)

Rhabdomyosarcoma is the most common pediatric sarcoma (Arndt, Bisogno, & Koscielniak, 2018; Dasgupta, Fuchs, & Rodeberg, 2016), which accounts for 4.5% of childhood malignancies with an incidence rate of 4.5 cases for each 1 million children per year. It is the third most common extracranial solid tumors in children after Wilms tumor and neuroblastoma (C. Chen, Dorado Garcia, Scheer, & Henssen, 2019; Dasgupta et al., 2016). The pathogenesis of RMS is still unclear but is thought to be originating from skeletal muscle progenitor cells or embryonal mesenchyme (Drummond & Hatley, 2018; Keller & Guttridge, 2013). This explains why RMS can occur anywhere in the body including non-muscle sites, such as the salivary glands, skull base, biliary tree and genitourinary tract (Hatley et al., 2012). RMS show a skeletal muscle differentiation phenotype. Therefore, diagnosis is based on the immunohistochemical expression of myogenic regulatory factors (MRFs), such as Myogenin and MyoD1 as well as the structural proteins Desmin (Dasgupta et al., 2016; Keller & Guttridge, 2013; Sebire & Malone, 2003). In addition, RMS is subdivided into two major subtypes, embryonal RMS (RME) and alveolar RMS (RMA) (C. Chen et al., 2019; Perez et al., 2011; C. Wang, 2012), which show distinct histology, clinical behavior, biological mechanism and prognosis (Davicioni et al., 2009). However, both show the above-mentioned skeletal muscle differentiation and were therefore classified as RMS. 67% of RMS cases are of the embryonal subtype. This subtype shows a better prognosis compared to the alveolar RMS which consists of 30% of the cases and is associated with higher metastasis and recurrence rates (Perez et al., 2011; Skapek et al., 2013). RME affect mainly younger children (≤10 years old) and arise in head, neck and genitourinary tract. In contrast, RMA are more common in older children and adolescents and arise in trunk and extremities (Dasgupta et al., 2016; Perez et al., 2011).

RME are genetically imbalanced tumors, which are characterized by numerous gains of functions, mostly affecting chromosomes 2, 8, 12 and 13 or, as in 80% of RME tumors, loss of heterozygosity (LOH) at the short arm of chromosome 11 (11p15.5) (Dasgupta et al., 2016; C. Wang, 2012), where the Insulin-like Growth Factor II (IGF2) gene is located (F. Huang et al., 2010; C. Wang, 2012). It was shown before that overexpression of IGF2 is strongly associated with the development of tumors in general and with RME tumors in particular (C. Wang, 2012; Xia, Pressey, & Barr, 2002). In contrast, RMA tumors are characterized by a specific translocation of FOXO1 and PAX3 or PAX7 and result in PAX3-FOXO1 or PAX7-

FOXO1 fusion proteins, respectively, which were found in 80% of RMA tumors (Marshall & Grosveld, 2012; Olanich & Barr, 2013; C. Wang, 2012). The resulting fusion proteins affect differentiation, proliferation and apoptosis pathways (Davicioni et al., 2009; Xia et al., 2002). In addition to the highly aggressive translocation positive RMA, 20% of the histologically defined RMA tumors miss any translocation. These translocation negative RMA are histologically indistinguishable from RME with respect to prognosis and molecular genetics (Marshall & Grosveld, 2012; Wachtel et al., 2004). It was found that, despite the presence of unrepairable DNA breaks, induced by chemotherapy or radiation, cells with high PAX3-FOXO1 expression during G2 cell cycle can still divide and survive, which leads to chemotherapy resistance, disease progression and relapse in translocation positive RMA (Kikuchi et al., 2014).

The 5-year overall survival is approximately 80% for RME compared to 52% for RMA (Belyea, Kephart, Blum, Kirsch, & Linardic, 2012). Nevertheless, the survival rate for metastatic disease is low for both groups and is only 43% for RME (Ricker et al., 2020) and 10–30% for RMA (De Giovanni, Landuzzi, Nicoletti, Lollini, & Nanni, 2009).

Considering that RMS is showing abortive skeletal muscle differentiation, dysregulated myogenesis pathways are thought to contributed to the tumorigenesis of RMS (E. Chen, 2016). One of the key pathways that regulates myogenesis is the WNT signaling pathway. WNT signaling also contributes to many types of cancers such as colorectal, breast and skin cancers. The role of WNT pathway and its functional activity starts to be appreciated in the pathogenesis of RMS (E. Chen, 2016; Klaus & Birchmeier, 2008; Logan & Nusse, 2004).

1.2 WNT signaling pathway

Various signaling pathways are involved in controlling developmental process including the WNT pathway, which got a lot of attention in order to understand its molecular mechanisms and role in tumorigenesis (Adhikari & Davie, 2019). The WNT signaling pathway plays a role in cell proliferation, polarity, migration and apoptosis (Komiya & Habas, 2008; Nusse & Clevers, 2017) and is known to be essential during myogenesis, as it regulates the myogenic regulatory factors (MRFs) (Jones et al., 2015; von Maltzahn, Chang, Bentzinger, & Rudnicki, 2012). This crucial role is not only conserved during embryogenesis but also essential for the growth and maintenance of different tissues. The consequences of WNT pathway dysregulation is catastrophic ranging from birth defects such as spina bifida, various myogenic and dermatological diseases to cancers (Adhikari & Davie, 2019; Ring, Kim, & Kahn, 2014).

The regulatory role of the WNT pathway is achieved through secretory WNT proteins, which were first discovered in Drosophila and found to activate different pathways (Komiya & Habas, 2008). 19 WNT proteins are known in mammals until now. After translation they have to undergo posttranslational modification and become lipid-bound in order to be secreted and functional (Routledge & Scholpp, 2019). The posttranslational modification of WNT proteins happens in the endoplasmic reticulum (ER) by O-acyltransferase Porcupine (Porcn), which catalyses the transfer of palmitoleic acid onto WNT proteins (MacDonald et al., 2014; Willert et al., 2003). Inhibition of Porcn blocks the secretion and activity of all WNT ligands (Najdi et al., 2012; Proffitt & Virshup, 2012). After modification, the WNT proteins are secreted through the Golgi apparatus to the plasma membrane with the aid of Wntless (WLS) (Figure 1.2-1) (Langton, Kakugawa, & Vincent, 2016).

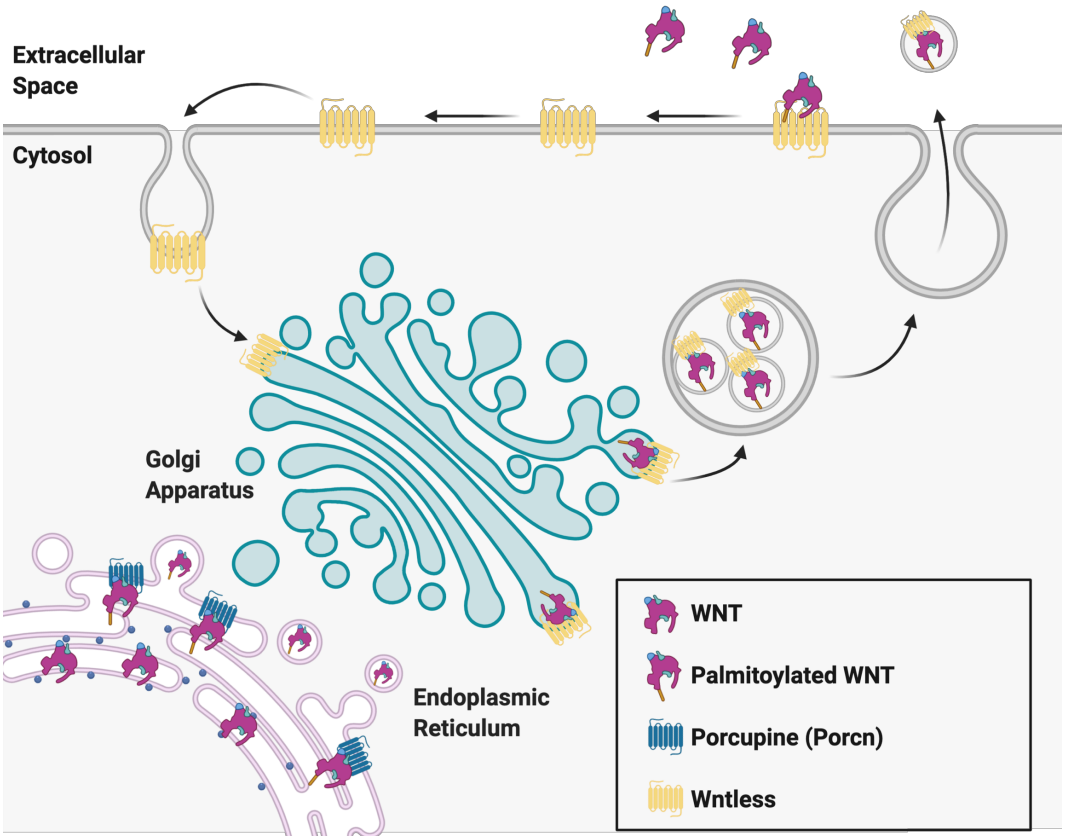

Figure 1.2-1: Posttranslational modification and secretion of WNT proteins
Following synthesis, WNT proteins undergo posttranslational palmitoylation by O-acyltransferase Porcupine (Porcn), which is required for WNT proteins secretion and function. WNT proteins are then transported to the plasma membrane through the Golgi apparatus by the aid of Wntless (WLS). Figure was created with BioRender.com.

WNT ligands stimulate several intracellular signaling pathways including the canonical β-catenin-dependent pathway and the non-canonical β-catenin-independent pathways. Disheveled is the main component that mediate the stimulation of both canonical and non-canonical pathways (Komiya & Habas, 2008; Sugimura & Li, 2010). The non-canonical pathway is further divided into WNT/Ca^{2+} pathway and WNT/PCP pathway (Habas & Dawid, 2005; Niehrs, 2012; Nusse & Clevers, 2017). However, WNT ligands cannot be classified according to the pathway they stimulate, as the activation of specific WNT pathways does not only depend on the WNT ligands but also on the receptor context. In order to stimulate different WNT pathways, WNT proteins have to bind to cell surface transmembrane receptors, Frizzled (FZD). Ten of them have been discovered in humans (Strutt, Madder, Chaudhary, & Artymiuk, 2012). In addition, the coreceptors, low-density lipoprotein-related proteins (LRP5 and LRP6), as well as receptor tyrosine kinases, RYK and ROR1/2 (Niehrs, 2012) play a crucial role in the activation of the various pathways. For example WNT1, WNT3A and WNT8 commonly are canonical pathway activators by binding to FZD receptors in the presence of LRP5/6, while WNT5A and WNT11 are involved in the activation of the non-canonical pathway after binding to FZD receptors in the presence of ROR1/2 coreceptors (Niehrs, 2012).

1.2.1 Canonical (β-catenin-dependent) pathway

Canonical (β-catenin-dependent) pathway is the most investigated WNT pathway, which was first identified by the genetic screening of Drosophila (Komiya & Habas, 2008). In the absence of WNT ligands, the destruction complex composed of two kinases, glycogen synthase kinase 3β (GSK 3β) and casein kinase 1 (CK1), along with the scaffolding proteins AXIN and adenomatous polyposis coli gene product (APC) is assembled. This complex is responsible for the phosphorylation of cytoplasmic β-catenin, which promotes β-catenin ubiquitination by ß-Trcp and E3 ubiquitin ligase. β-catenin ubiquitination leads to its proteasomal degradation (Flack, Mieszczanek, Novcic, & Bienz, 2017; Katoh, 2017; Niehrs, 2012). Activating the canonical pathway by WNT factors leads to the recruitment of the scaffolding protein dishevelled (Dvl), which in turn inhibits GSK3 leading to the disassembly of the destruction complex. This promotes the accumulation of β-catenin in the cytoplasm from where β-catenin is subsequent translocated to the nucleus, where it forms a complex with the DNA bound T cell factor/ lymphoid enhancer-binding factor (TCF/LEF1) (Figure 1.2-2). They belong to a family of transcription factors that activate the expression of WNT target genes, including CyclinD1 (CCND1) and c-MYC (MYC) (Ljungberg, Kling, Tran, & Blumenthal, 2019; Niehrs, 2012; Steinhart & Angers, 2018). The WNT canonical pathway plays a crucial role in cell

proliferation and differentiation (Niehrs, 2012; Roma, Almazan-Moga, Sanchez de Toledo, & Gallego, 2012; van Amerongen & Nusse, 2009).

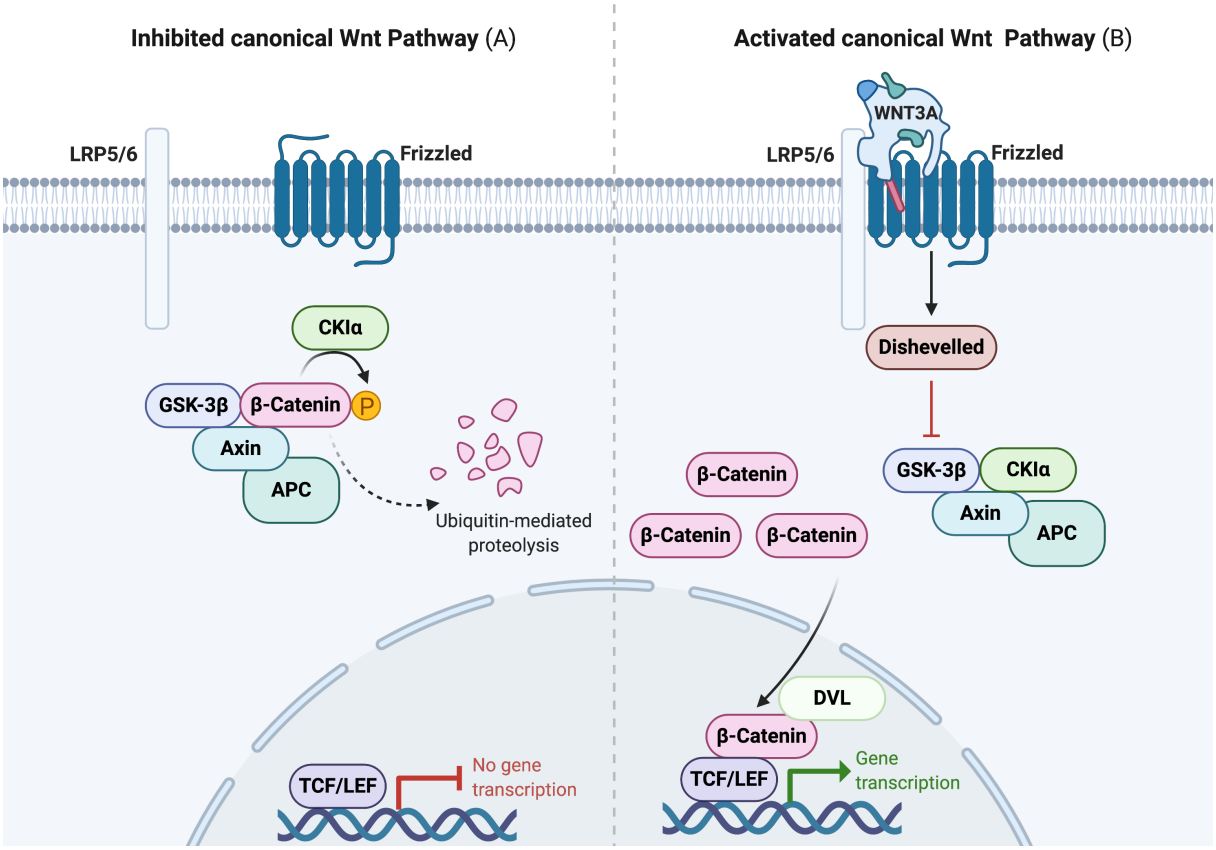

Figure 1.2-2: Canonical WNT signaling pathway
Depending on WNT molecules, receptors and co-receptors, the canonical or the non-canonical pathway is activated. **(A)** In the absence of WNT ligands, the destruction complex (composed of GSK 3β, CK1, AXIN and APC) is assembled leading to the phosphorylation of cytoplasmic β-catenin and its subsequent proteasomal degradation. **(B)** Activating the canonical pathway by WNT3A leads to the recruitment of Dvl which results in the disassembly of the destruction complex. This leads to the accumulation β-catenin and its subsequent translocation to the nucleus, where it forms a complex with TCF/LEF1 mediating WNT target gene expression. Figure was created with BioRender.com.

1.2.2 Non-canonical signaling pathway

The non-canonical pathway is known as β-catenin independent. This pathway is subdivided into two major pathways WNT/Ca^{2+} and WNT/PCP. WNT5A is one of the main ligands of the non-canonical pathway (Ljungberg et al., 2019; Roma et al., 2012; Sugimura & Li, 2010). WNT/Ca^{2+} pathway stimulation leads to the activation of phospholipase C (PLC) which in turn mediates cytosolic calcium (Ca^{2+}) release. The enhanced cytosolic Ca^{2+} level activates two Ca^{2+} sensitive enzymes leading to the activation of two branches of the WNT/Ca^{2+} pathway, the CaMKII-TK1-NLK pathway (Ishitani et al., 2003) and the Calcineurin-NFAT pathway (Saneyoshi, Kume, Amasaki, & Mikoshiba, 2002). In the CaMKII-TK1-NLK pathway, the enhanced intracellular Ca2+ level activates protein kinase C (PKC) and Ca^{2+}/calmodulin-

dependent kinase II (CaMKII) leading to the stimulation of the TAK1-NLK MAPK pathway, where TAK1 stimulates the WNT/Ca^{2+} effector protein nemo-like kinase (NLK). In the second Ca2+ mediated pathway, the Calcineurin-NFAT pathway, nuclear factor of activated T cells (NFAT) is the effector protein. A calmodulin-mediated activation of calcineurin leads to NFAT transcription factor dephosphorylation and its subsequent translocation into the nucleus (Figure 1.2-3A)(Ljungberg et al., 2019; Niehrs, 2012; Roma et al., 2012). Five NFAT isoforms are present in human: NFAT 1-5. NFATs 1-4 are Ca^{2+} regulated isoforms (T. Huang et al., 2011).
In contrast, the planar cell polarity pathway has emerged from genetic studies in Drosophila, where mutations in this pathway components led to disruption in the orientation of epithelial structures including cuticle hairs and sensory bristles (Komiya & Habas, 2008). WNT/PCP stimulation leads to the activation of small G proteins (RhoA, Rhou, Rac and CDC42), which reorganizes the cytoskeleton, thereby influencing cell shape and polarization. Small GTPases mediate the stimulation of JUN-N-terminal kinase (JNK) (Komiya & Habas, 2008; Ljungberg et al., 2019; Sugimura & Li, 2010), which leads to the phosphorylation of c-JUN and its translocation to the nucleus (Figure 1.2-3B)(Ljungberg et al., 2019). However, the transcriptional regulation induced by this pathway is not clear (Komiya & Habas, 2008).
There is a crosstalk between the canonical and the non-canonical WNT signaling pathway, however this interaction is complex and not fully understood. It is found that the non-canonical pathway antagonizes the β-catenin dependent pathway through two branches. In the CaMKII-TK1-NLK pathway, the active NLK phosphorylates TCF and thus prevents the β-catenin-TCF complex from binding DNA, thereby inhibiting the ability of β-catenin-TCF to activate transcription of β-catenin target genes. Thus, TAK1 and NLK appear to act in a pathway parallel to the WNT/β-catenin pathway (Ishitani et al., 2003).
It has been reported that DVL is localized in the nucleus and plays a role in β-catenin dependent transcription. Additionally, its role in β-catenin accumulation is associated with β-catenin-LEF1/TCF transcription complex by interacting with β-catenin resulting in β-catenin-LEF1/TCF transcription complex stabilization (Gan et al., 2008; Itoh, Brott, Bae, Ratcliffe, & Sokol, 2005; Torres & Nelson, 2000). NFAT, in contrast, mediates the inhibition of the β-catenin dependent pathway by interacting with DVL in the nucleus, preventing its recruitment to the β-catenin transcription complex (T. Huang et al., 2011; Saneyoshi et al., 2002) . Additionally, JNK was found to antagonize the canonical pathway by regulating the nucleocytoplasmic transport of β-catenin (Liao et al., 2006).

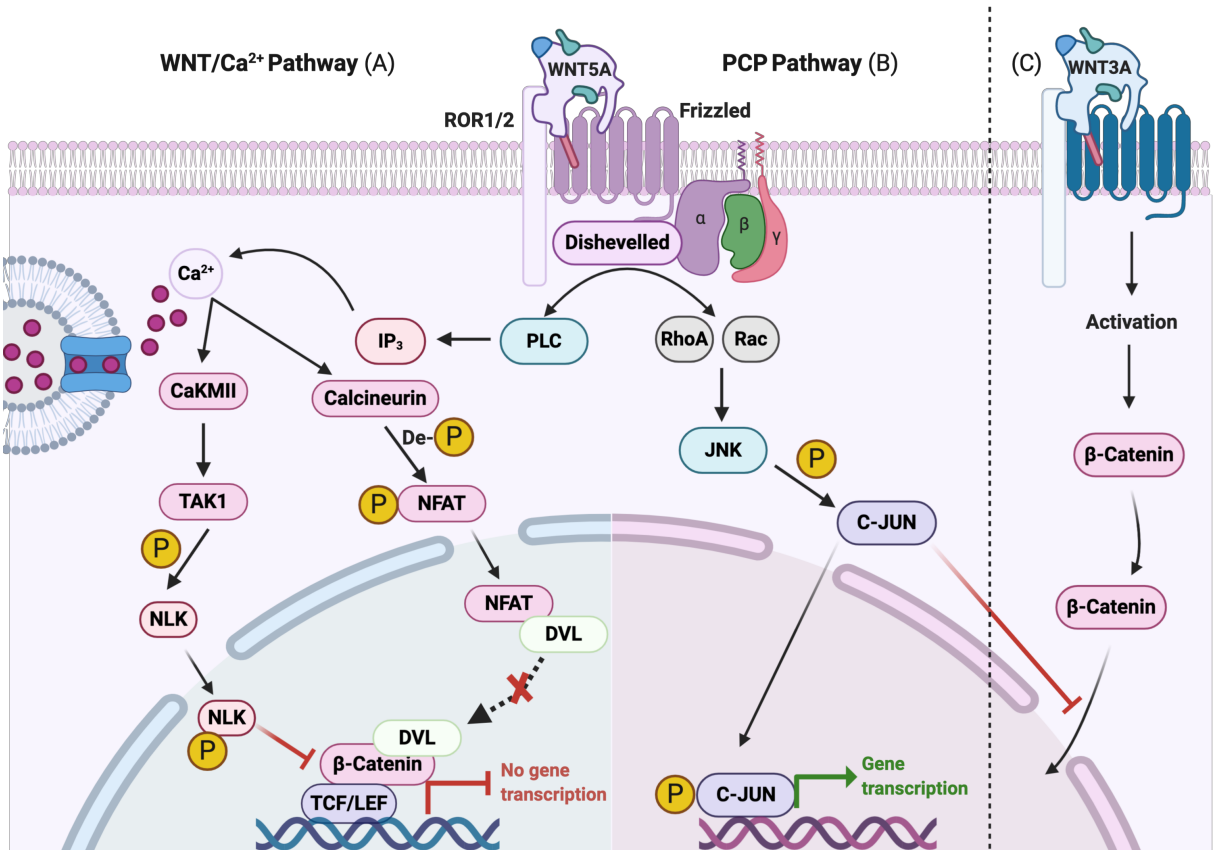

Figure 1.2-3: Non-canonical WNT signaling pathway
The activated non-canonical pathway is implicated in the cytoskeletal organization in case of the planar cell polarity (PCP) pathway **(B)**, which was also found to antagonize the canonical pathway by regulating the nucleocytoplasmic transport of β-catenin. Additionally, the canonical pathway is inhibited by the WNT/Ca^{2+} pathway **(A)**. The ß-catenin dependent pathway is antagonized by CaMKII-TAK1-NLK pathway that inhibits ß-catenin-TCF dependent transcription due to TCF phosphorylation and/or Calcineurin-NFAT pathway that suppresses ß-catenin-dependent transcription. **(C)** Simplified canonical WNT pathway. Figure was created with BioRender.com.

1.3 Myogenesis

Myogenesis is a process of progenitor proliferation followed by differentiation of myoblasts into mature myotubules (Arrighi, 2018; Murphy & Kardon, 2011). This is regulated by four basic helix–loop–helix (bHLH) transcription myogenic regulatory factors (MRFs; MYF5, MRF4, MYOD, Myogenin and PAX3/7) (Arrighi, 2018; Sher, Cox, & Ackert-Bicknell, 2012). The myogenic regulatory factors are spatiotemporally expressed or repressed during myogenesis (Bentzinger, Wang, & Rudnicki, 2012). Following paracrine signals, muscle progenitor cells activate PAX3/7, which control the expression of MYF5, MYOD in order to commit precursors to enter myogenesis. The role of Myogenin and Mrf4 is reserved in the later step of muscle differentiation, where myogenin is required for mononucleated myoblast fusion into myotubes (Arrighi, 2018; Sher et al., 2012).

In adult muscle, growth and regeneration are mediated by a precursor population of muscle stem cells, which are called satellite cells (Zuk, Benhaim, & Hedrick, 2004). Growth factors such as; insulin-like growth factor (IGF-1), Leukemia inhibitory factor (LIF), hepatocyte growth factor (HGF) and fibroblast growth factor (FGF) influence the lifecycle of satellite cells and their participation in myogenesis (Arrighi, 2018). In order to prevent excess muscle growth, Myostatin and transforming growth factor β limit the proliferation of PAX-positive progenitor cells during embryonic and fetal development (Sher et al., 2012). Myogenesis is instructed by a multitude of signaling pathways during embryonic development and in postnatal life (Bentzinger et al., 2012; Murphy & Kardon, 2011). The failure of these pathways to orchestrate together leads to the development of various skeletal muscle disorders (Murphy & Kardon, 2011). One of these relevant pathways is the WNT pathway, which induces the expression of MRFs and hence the formation of functional myotubes (Cisternas, Henriquez, Brandan, & Inestrosa, 2014).

The canonical and non-canonical WNT pathway work synergistically in order to maintain skeletal muscle hemostasis. The canonical WNT pathway regulates satellite cell differentiation, as WNT1 and WNT3A mediated activation of the canonical WNT pathway induces Myf5 and MyoD expression during embryogenesis (Bentzinger et al., 2012; von Maltzahn et al., 2012). In contrast, myogenesis in adult muscle stem cells, that become active following injuries, is more regulated by the non-canonical WNT pathway, and it was found that WNT5A is upregulated in the early stages of muscle regeneration (von Maltzahn et al., 2012). The WNT/PCP pathway acts on multiple levels to regulate muscle differentiation, as it promotes the expansion of satellite stem cells which in turn accelerates and enhances muscle regeneration. Additionally, activation of the WNT/PCP pathway promotes the polarity and directional migration of satellite cells as well as myogenic progenitor cells (Rudnicki & Williams, 2015). However, the role of the calcineurin-NFAT pathway in regulating myogenesis is complex, as NFAT proteins have different functions in the different stages of myogenesis (Bailey, Holowacz, & Lassar, 2001).

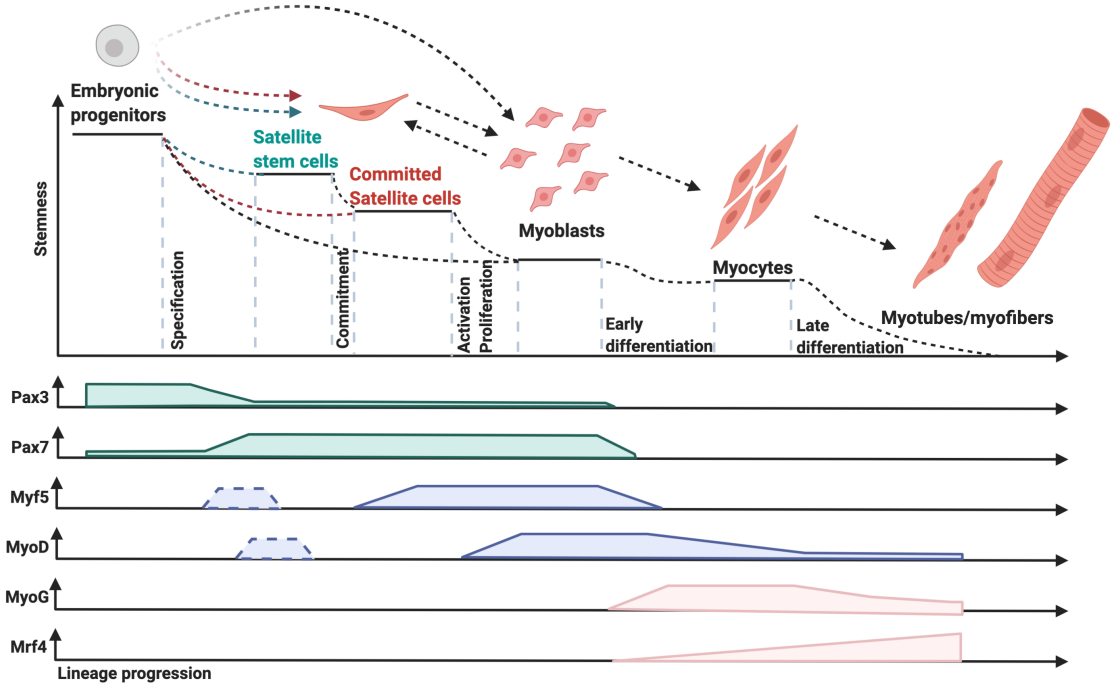

Figure 1.3-1: Myogenesis
The role of different molecular markers during muscle differentiation. Activation of PAX3/7 by muscle progenitor cells leads to the proliferation of satellite cells to myoblasts (unclear sentence) followed by their differentiation and fusion into myotubes, which then maturated into myofibers. Figure was created with BioRender.com.

1.4 WNT signaling pathway in rhabdomyosarcoma

As mentioned above, RMS shows abortive skeletal muscle differentiation. Since WNT pathway is important for myogenesis, its dysregulation in RMS is likely. Most of the studies that were done in order to investigate the role of WNT signaling pathway in the pathogenesis of RMS were focused on the canonical WNT pathway, while little is known about the role of the non-canonical pathway. However, the exact role of the canonical WNT pathway is not fully understood, as previous studies were controversial. Soglio et al. claimed to have excluded a role of β-catenin mutations in RMS pathogenesis, after they had analysed 8 RME and 3 RMA with no evidence of any β-catenin mutation (Soglio et al., 2009). This finding was supported by a study done by Singh et al., who also concluded that the tumorigenesis of RME is not attributed to β-catenin mutations. In the same study they found that the canonical WNT pathway was downregulated in the RME cells from p53/c-fos double mutant mice compared to normal myoblasts, which is related to c-JUN contained AP1 activity. Furthermore, they showed that activating the WNT/β-catenin pathway by LiCl induced myogenic differentiation (Singh et al., 2010). Aiming to characterize the role of WNT/ β-catenin signaling pathway in RMS, Annavarapu et al. showed that the main pathway components are expressed and the pathway is functionally active in RMA and RME tumor cells. Additionally, they found that activating the WNT/β-catenin pathway by recombinant WNT3A promoted β-catenin stabilization, which

induced expression of myogenic differentiation markers (Myogenin, MyoD and myf5) in RMA as well as RME cell lines, while inhibiting the proliferation exclusively of RMA cell lines (Annavarapu et al., 2013).

1.5 Treatment of RMS

RMS treatment is very aggressive including; surgical tumor resection, irradiation and long periods of chemotherapy (Meyer & Spunt, 2004). Improvement of the multimodal therapy for RMS has proved success in 90% of patients with low risk localized tumor. However, this success was only limited to the low risk patients and failed to enhance the survival rates of patients with metastatic or recurrent tumor where they remain decimal at 21% and 30%, respectively for more than 4 decades (C. Chen et al., 2019; Crawford et al., 2019). Better understanding of the molecular mechanisms underlying RMS tumorgenesis is critical to develop new therapeutic strategies (Shern et al., 2014). One of the novel strategies which was approved by the U.S. Food and Drug Administration (FDA) and showed efficiency against refractory CD19 positive B cell acute lymphoblastic leukemia is an immunotherapy using chimeric antigen receptor (CAR) T cells (June, O'Connor, Kawalekar, Ghassemi, & Milone, 2018; McHayleh, Bedi, Sehgal, & Solh, 2019). Recently, several CAR T cells directed to different sarcomas have emerged and on targeting the human epidermal growth factor receptor 2 (HER2), disialoganglioside (GD2), fibroblast activation protein (FAP), B7-H3 and tyrosine kinase orphan-like receptor 1 (ROR1) showed promising efficiency in clinical trials (Thanindratarn, Dean, Nelson, Hornicek, & Duan, 2020).

CAR T cell therapy is an adoptive T cell therapy (ACT) which is one of the main categories of immunotherapy among others, including tumor vaccinations and immune checkpoint regulators. CAR T cells are engineered T cells expressing CARs on their surface. The receptors are modified surface antibodies that give T cells specificity against specific tumor associated antigen (TTA). CARs are composed of an extracellular domain of a single chain fragment (scFv) of a specific antibody linked through a transmembrane domain to an intracellular CD3ζ signaling domain either alone or in combination with a costimulatory domain (e.g. CD28 or 4-1BB, depending on the CAR T cell generation).

First-generation CAR T cells were only composed of CD3ζ as a signaling domain, but these engineered T cells did not pass clinical trials as they failed to induce resting T cells proliferation or promote optimal cytokines production (Brocker, 2000; Jensen et al., 2010). This led to the development of second- and third-generation CAR T cells, which have as costimulatory domain

either CD28 or/and 4-1BB, respectively, linked to CD3ζ. This enhances cytokins production and simultaneously increases the proliferation and survival of such T cells. In order to defeat the inhibitory tumor microenvironment, fourth-generation CAR T cells have been developed, which secrete cytokines such as IL-2 and IL-12 after antigen recognition, which improve CAR T cells efficacy and persistence (McHayleh et al., 2019).
The CAR T cells production procedure involves multiple steps, including the separation of patients' T cells by leukapheresis, their selection, activation, lentiviral or retroviral transduction to develop CAR T cells, expansion and finally injection of the modified T cells back to the patients (Roberts, Better, Bot, Roberts, & Ribas, 2018; Zhang, Liu, Zhong, & Zhang, 2017).

The fetal form of the acetylcholine receptor (AChR) is an oligomeric membrane protein composed of alpha, 2beta, delta and gamma (α2βγδ) subunits. In the adult form, gamma subunit is replaced by an epsilon subunit, where the expression of the fAChR is limited to thymic myeloid cells and some extraocular muscles (Gattenloehner et al., 1998; Thanindratarn et al., 2020). fAChR are highly expressed on both alveolar and embryonal rhabdomyosarcoma tumor cells but not on other nonrhabdomyomatous tumors or normal cells, which led to the development of fAChR specific CAR T cell therapy (Katja Simon-Keller, Stefan Barth, Angela Vincent, & Alexander Marx, 2013; Thanindratarn et al., 2020). First-generation fAChR CAR T cells, showed efficiency in killing RMS cells in vitro as they led to INF-γ secretion and delayed lysis of RMS tumor cells. In the same study, an induced fAChR expression on the residual RMS tumor cells after chemotherapy was reported in vivo, suggesting that CAR T cell therapy might be promising either as primary treatment or complementary treatment after chemotherapy, in order to eradicate residual RMS tumor cells (Gattenlöhner et al., 2006). In contrast, a study done by Simon-Keller et al. reported a resistance of RMS cells towards second generation fAChR CAR T cells despite full T-cell activation, suggesting that inhibiting anti-apoptotic pathways in RMS cells might improve the killing efficiency of CAR T cells (Simon-Keller et al., 2010). Better understanding of biological mechanism which induce target expression and resistance mechanism is needed in order to improve immunotherapy approach in RMS patients.

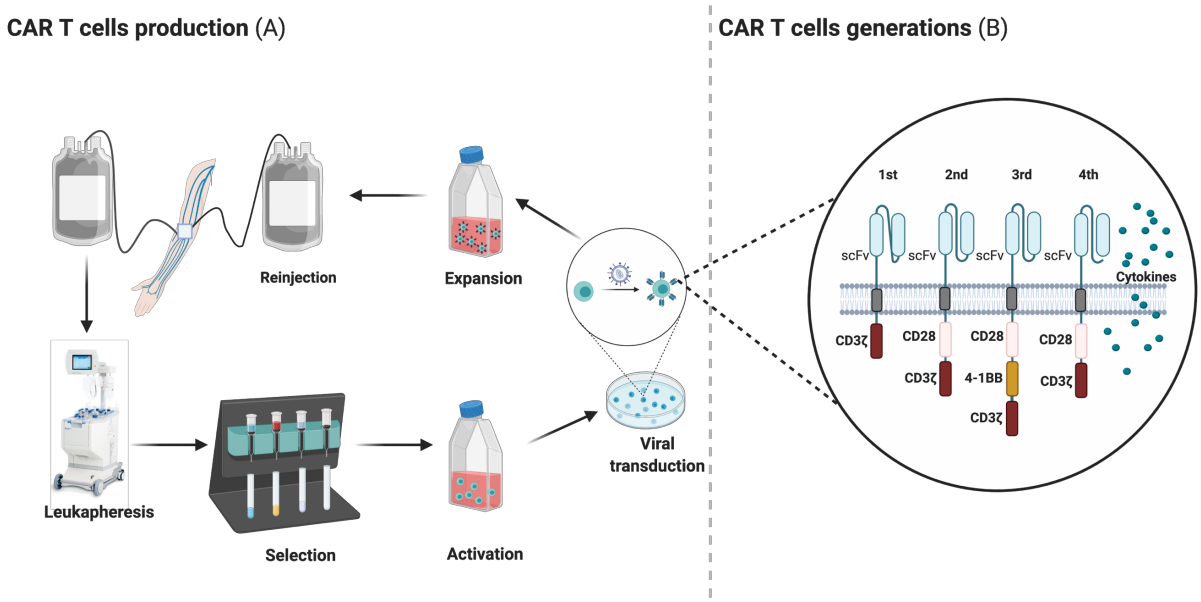

Figure 1.5-1: CAR T cells production
(A) CAR T cell production is a multi-step process, which includes the separation of patients' T cells by leukapheresis, selection, activation, lentiviral or retroviral transduction to develop CAR T cells, expansion and finally injecting the modified T cells back to the patients. **(B)** CARs are composed of an extracellular domain derived from an antibody linked to an intracellular signaling domain. First generation CARs are composed of CD3ζ as signaling domain whereas second-generation CARs possess a costimulatory domain (CD28 or 4-1BB) fused to CD3ζ. Third-generation CARs consist of two costimulatory domains linked to CD3ζ. Whereas, fourth generation CARs have one costimulatory domain and secrete cytokines such as IL-2 and IL-12 after antigen recognition, which improve CAR T cells efficacy and persistence. Figure was created with BioRender.com.

1.6 Aim of the study

Based on our preliminary data, which showed over-expression of WNT5A in the less aggressive RME, we hypothesized that WNT5A signaling as well as other WNT related molecules are important for RMS tumorigenesis. We aimed to uncover the impact of WNT5A on RMS aggressiveness and the pathways involved in this by answering the following questions:

1. What is the impact of WNT5A on functional features of RMS by establishing stable WNT5A KD and OE RMS cell lines?
2. Is there a crosstalk between the canonical and the non-canonical pathway in RMS, and which impact does it have on RMS features, particularly on skeletal muscle differentiation *in vitro*?
3. Which non-canonical pathway plays a role in RMS tumorigenesis?
4. Which impact does overexpressed or downregulated non-canonical WNT signaling in RMS xenografts have on gene expression profiles, particularly on the expression of the fetal acetylcholine receptor (i.e. the target of an established, RMS-specific adoptive, immune therapy)?
5. Does WNT5A-mediated signaling affect the susceptibility of RMS towards an established, fAChR CAR T cell based immune therapy?

2 MATERIAL AND METHODS

2.1 Material

2.1.1 Consumables

12 well plate	Greiner Bio-One, Kremsmünster, Österreich
24 well plate	Greiner Bio-One, Kremsmünster, Österreich
6 well plate	Greiner Bio-One, Kremsmünster, Österreich
96 well flat bottom plate	Greiner Bio-One, Kremsmünster, Österreich
96 well round bottom plate	Greiner Bio-One, Kremsmünster, Österreich
96-wells black walled plate	Greiner Bio-One, Kremsmünster, Österreich
Amersham Protran 0.2 Nitrocellulose membrane	GE Healthcare, Fairfield, CT, USA
Costar® 96 well plate, Ultra-Low attachment surface	CORNING Life Sciences, New York, United States
Falcon™ tubes (10, 50 ml)	Corning Life Sciences, Tewksbury, MA, USA
Glass Wool	Sigma-Aldrich, St. Louis, Missouri, USA
Mr. Frosty™	Thermo Fisher Scientific, Waltham, Massachusetts, USA
Multichannel pipette	Eppendorf, Hamburg, Geramny
Neubauer Improved Neubauer Cell Counting Chamber	BRAND GMBH, Wertheim, Germany
Pipette boy	BRAND GMBH, Wertheim, Germany
Pipette TIPs (0.5, 1, 2, 5 ml)	Eppendorf, Hamburg, Geramny
Pipette TIPs (10, 200, 1000 µl)	Eppendorf, Hamburg, Geramny
Pipettes (10, 100, 200, 1000 µl)	Eppendorf, Hamburg, Geramny
Serological pipettes (1, 2, 5, 10, 25, 50 ml)	Corning Life Sciences, Tewksbury, MA, USA
Cell culture T-75 Flasks, red filter cap	Corning Life Sciences, Tewksbury, MA, USA
Cell culture T-25 Flasks, white filter cap	Corning Life Sciences, Tewksbury, MA, USA
Cryopreservation tubes (CRYO.S™)	Greiner Bio-One, Kremsmünster, Österreich

2.1.2 Equipments

Balance (BP 301 S)	Sartorius group, Göttingen, Germany
Electrophoresis power supplies	Biorad, Hercules, California, United States
Centrifuge (5810 r)	Eppendorf, Hamburg, Geramny
Microcentrifuge (Biofuge*pico*)	Heraeus, Hanau, Hesse, Germany

FACSLYRIC™	BD Biosciences, Franklin Lakes, New Jersey, USA
Fusion SL Imaging system	Peqlab, Erlangen, Germany
GEL iX 20 imaging system	Intas Science Imaging, Göttingen, Germany
Heracell CO_2 Incubator	Heraeus, Hanau, Hesse, Germany
Herasafe Biological safety cabinets	Kendro, Hanau, Hesse, Germany
Inverse microscope (DFC 450 camera)	Leica, Wetzlar, Germany
Microscope (DMIL)	Leica, Wetzlar, Germany
Neon® Transfection system	Thermo Fisher Scientific, Waltham, Massachusetts, USA
peqSTAR 2X Thermocycler	Peqlab, Erlangen, Germany
REAX top Vortex	Heidolph, Schwabach, Geramny
StepOnePlus Real-Time PCR system	Thermo Fisher Scientific, Waltham, Massachusetts, USA
Mini-PRPTEAN® Tetra Handcast Systems	Biorad, Hercules, California, United States
TECAN Spark microtiter plate reader	TECAN, Männerdorf, Switzerland
ThermoMixer	Eppendorf, Hamburg, Geramny
Tumbling table (Biometra WT12)	Biometra GmbH, Göttingen, Lower Saxony, Germany
pH Meter (WTW pH 320)	WTW, Weilheim, Oberbayern, Germany
-80 °C Freezer	Heraeus, Hanau, Hesse, Germany
Autoclave (Systec VX-150)	Systec GmbH, Linden, Hessen, Germany

2.1.3 Software

bioRender	www.biorender.com
FlowJo™ v10 software	BD Biosciences, Franklin Lakes, New Jersey, USA
Fusion-Capt-Software	Peqlab, Erlangen, Germany
Graphpad Prism 8.0 mac	GraphPad Software, La Jolla California USA (www.graphpad.com)
Image J	https://imagej.net
Intas-Capture-Software	Intas Science Imaging, Göttingen, Lower Saxony, Germany
Microsoft PowerPoint software	Microsoft, Redmond, Washington, United States
Microsoft Word software	Microsoft, Redmond, Washington, United States
StepOne™ software v2.3	Thermo Fisher Scientific, Waltham, Massachusetts, USA

2.1.4 Media and buffers

10x TG Transfer buffer	Biorad, Hercules, California, United States
10x TGS Running buffer	Biorad, Hercules, California, United States
4x Laemmli sample buffer	Biorad, Hercules, California, United States
Dulbecco's Modified Eagle Medium (DMEM)	Gibco, Carlsbad, CA, USA
Dulbecco's phosphate-buffered saline (DPBS)	Gibco, Carlsbad, CA, USA
Fetal Bovine Serum (FBS)	Sigma Aldrich, St. Louis, MO, USA
HEPES	Gibco, Carlsbad, CA, USA
Neurobasal Medium	Gibco, Carlsbad, CA, USA
Pen Strep	Gibco, Carlsbad, CA, USA
Pierce RIPA Buffer	Thermo Fisher Scientific, Waltham, Massachusetts, USA
Re-Blot strong sloution 10x	Merck, Darmstadt, Germany
Roswell Park Memorial Institute (RPMI1640)	Gibco, Carlsbad, CA, USA
Trypsin-EDTA (0.05%)	Gibco, Carlsbad, CA, USA

2.1.5 Chemicals and reagents

2-(p-Chlorophenoxy)-2-methyl-propionic acid (Clofibric acid)	Sigma-Aldrich, St. Louis, Missouri, USA
Acetone	Carl Roth, Karlsruhe, Germany
Ammonium Persulfate (APS)	Sigma-Aldrich, St. Louis, Missouri, USA
Ampicilline sodium salt	Merck, Darmstadt, Germany
Anti-human CD28 antibody	BioLegend, San Diego, CA, USA
Anti-human CD3 (OKT3) antibody	BioLegend, San Diego, CA, USA
B-27 supplement without Vitamin A	Gibco, Carlsbad, CA, USA
BamH I	New England Biolabs, Ipswich, Massachusetts, United States
Boric acid	Sigma-Aldrich, St. Louis, Missouri, USA
Chloroform	Carl Roth, Karlsruhe, Germany
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, St. Louis, Missouri, USA
DNase I recombinant	Sigma-Aldrich, St. Louis, Missouri, USA
EcoR I	New England Biolabs, Ipswich, Massachusetts, United States
EDTA Na_2	Carl Roth, Karlsruhe, Germany
Ethanol	Carl Roth, Karlsruhe, Germany

Ficoll-Paque™ PREMIUM	GE Healthcare, Fairfield, CT, USA
Hexadimethrine bromide	Sigma-Aldrich, St. Louis, Missouri, USA
Isopropanol	Carl Roth, Karlsruhe, Germany
Kanamycin Solution	Sigma-Aldrich, St. Louis, Missouri, USA
LE Agarose	Biozym, Hessisch Oldendorf, Germany
Midori Green Advance	NIPPON Genetics EUROPE, Düren, Germany
Milk Powder fat-free	Carl Roth, Karlsruhe, Germany
MTT (Thiazolyl blue TetrazoliumBromid)	Sigma-Aldrich, St. Louis, Missouri, USA
Nuclease free H_2O	Sigma-Aldrich, St. Louis, Missouri, USA
Pertussis toxin	Cayman, Ann Arbor, Michigan, United States
Prestained protein ladder	Thermo Fisher Scientific, Waltham, Massachusetts, USA
Proleukin (Interleukin-2)	CHIRON NOVARTIS, Emeryville, California, USA
Protease inhibitor cocktail tablet	Sigma-Aldrich, St. Louis, Missouri, USA
Puromycin	Sigma-Aldrich, St. Louis, Missouri, USA
Recombinant human WNT3A	R&D systems, Minneapolis, USA
Recombinant human WNT5A	R&D systems, Minneapolis, USA
rhEGF	R&D systems, Minneapolis, USA
rhFGF basic	R&D systems, Minneapolis, USA
Rotiphorese® Acrylamide 30%	Carl Roth, Karlsruhe, Germany
SDS, ultra pure	Carl Roth, Karlsruhe, Germany
Sodium Chloride	Carl Roth, Karlsruhe, Germany
SuperSignal West Dura Extended Duration Substrate	Thermo Fisher Scientific, Waltham, Massachusetts, USA
SYBR Premix Ex Taq™II (Tli RNase H Plus), ROX plus	TAKARA, Kusatsu, Shiga, Japan
T4 DNA ligase	New England Biolabs, Ipswich, Massachusetts, United States
TEMED	Carl Roth, Karlsruhe, Germany
Trichloroacetic acid	Carl Roth, Karlsruhe, Germany
TRIS base	Carl Roth, Karlsruhe, Germany
TRIS base	Carl Roth, Karlsruhe, Germany
TRIzol® Reagent	Invitrogen, Carlsbad, CA, USA
TWEEN® 20	Carl Roth, Karlsruhe, Germany

2.1.6 Kits

APC BrdU Flow Kit	BD Bioscience, Franklin Lakes, New Jersey, USA
Calcium Assay Kit	BD Bioscience, Franklin Lakes, New Jersey, USA
Calcium phosphate transfection kit	Sigma-Aldrich, St. Louis, Missouri, USA
CytoSelect™ Cell Migration Assay Kit	Cell Biolabs, San Diego, California, United States
EndoFree® Plasmid Maxi Kit	Qiagen, Hilden, Germany
NE-PER™ Nuclear and Cytoplasmic Extraction kit	Thermo Fisher Scientific, Waltham, Massachusetts, USA
PrimeScriptTM RT Reagent Kit (Perfect Real Time)	TAKARA, Kusatsu, Shiga, Japan
Protease inhibitor cocktail tablet	Sigma-Aldrich, St. Louis, Missouri, USA
Neon® Transfection Kit	Thermo Fisher Scientific, Waltham, Massachusetts, USA
QIAprep Spin Miniprep Kit	Qiagen, Hilden, Germany

2.1.7 Cell lines

Cell line	Cell type	Source
CRL2061, RH30, Rh41	Translocation positive ARMS	American Type Culture Collection (ATCC), Menasses, VA, USA
FLOH1	Translocation negative ARMS	Kind gift from Ewa Koscielniak, Stuttgart, Germany
RD, TE671	ERMS	ATCC, Menasses, VA, USA
293 T cells	Human embryonal Kidney Cells (HEK cells)	ATCC, Menasses, VA, USA

2.1.8 RMS biopsy specimens

20 RNA samples from the Cooperative Weichteilsarkom Studiengruppe (CWS) tissue bank (Stuttgart, Germany) (S1 to S20) were studied (patient characteristics were summarized in Table 7.1) (K. Simon-Keller, A. Paschen, et al., 2013). The histopathological features of all cases were reviewed by Prof. I. Leuschner (Pediatric Tumor Registry, Kiel, Germany; deceased). All patients were treated according to CWS protocols (CWS-96 or CWS-2002P).

All samples were isolated and provided by the CWS study group in 2010 [approval 158/2009/b02; University of Tübingen, Tübingen, Germany; April 2, 2009). The present study was also approved by the ethical and review committee Mannheim (2012-257N-MA and 2017-802R-MA (University of Heidelberg, University Medical Centre Mannheim, Mannheim)]. All cases were studied by RT-PCR for fAChR expression and for PAX-FKHR fusion transcript status by our lab (Simon-Keller; 2013)

2.1.9 Primers

All the primers used in this work were ordered from Sigma-Aldrich, St. Louis, Missouri, USA.

2.1.9.1 Primers used for WNT5A amplification

Primers	Sequence	Restriction site
WNT5A_Fwd	GCGAT**GGATCC**ATGAAGAAGTCC	**GG**ATCC (**BamH I)**
WNT5A_Rev	CGCTA**GAATTC**CTTGCACACAAAC	**GA**ATTC (**EcoR I)**

2.1.9.2 Primers used for qRT-PCR

Name	Orientation	Sequence
AXIN2	Fwd.	GCCAACGACAGTGAGATATCC
	Rev.	CTCGAGATCAGCTCAGCTGCA
CD133	Fwd.	GCTGACCCTCATCGTGATTT
	Rev.	TTCCACATTTGCACCAAAGA
CHRNA	Fwd.	TGCGGAAGGTTTTTATCGAC
	Rev.	TGATCAGGGGAGAGTGGAAG
CHRNG	Fwd.	GCGCTGGAGAAGCTAGAGAA
	Rev.	CACCAGGAACCACTCCTCAT
cMYC	Fwd.	GTAGTGGAAAACCAGCAGCC
	Rev.	CCTCCTCGTCGCAGTAGAAA
CTNNB1	Fwd.	GAAACGGCTTTCAGTTGAGC
	Rev.	CTGGCCATATCCACCAGAGT
DESMIN	Fwd.	CATCGCGGCTAAGAACATTT
	Rev.	GCCTCATCAGGGAATCGTTA
FZD1	Fwd.	CATCTTCTTGTCCGGCTGTT
	Rev.	GACAGGATCACCCACCAGAT
FZD2	Fwd.	CATCATGCCCAACCTTCT
	Rev.	GGCTTCGCAGCCCT

FZD4	Fwd.	CGAGCTGCAGCTGACAACTT
	Rev.	TGGTCGTTCTGTGGTGGGAA
FZD5	Fwd.	CCCAGCGTCAAGTCCAT
	Rev.	TTGATGACGCTGCGGAT
FZD6	Fwd.	CTGGGTTGGAAGCAAAAAGA
	Rev.	CCATGGATTTGGAAATGACC
FZD7	Fwd.	TCGTGTCCCTCTTCCGTATC
	Rev.	CATAGCTCTTGCACGTCTGC
GAPDH	Fwd.	TGCACCACCAACTGCTTAGC
	Rev.	GGCATGGACTGTGGTCATGAG
LEF1	Fwd.	CGGGTACATAATGATGCCAA
	Rev.	CGTCACTGTAAGTGATGAGGG
MYOGENIN	Fwd.	CCAGGGGATCATCTGCTCAC
	Rev.	CTGTGATGCTGTCCACGATG
ROR1	Fwd.	AATGATGCTCCTGTGGTCCAG
	Rev.	GCACATGCAATCCCTCTGTAT
ROR2	Fwd.	AACCGGACCATTTATGTGGA
	Rev.	ACACGAAGTGGCAGAAGGAT
RYK	Fwd.	AGTTGTAAGCTGCGAGGTCT
	Rev.	CTTCTGGCCAGGTAGCTCAT
SOX2	Fwd.	AGCTCGAGACCTACATGAA
	Rev.	TGGAGTGGGAGGAAGAGGTA
SURVIVIN	Fwd.	AGCATTCGTCCGGTTGCGCT
	Rev.	TCGATGGCACGGCGCACTTT
TCF1	Fwd.	GCAACCTGAAGACACAAGCA
	Rev.	GCAATGACCTTGGCTCTCAT
TCF3	Fwd.	GAGTCGGAGAACCAGAGCAG
	Rev.	CTGTCCTGAGGCCTTCTCAC
TCF4	Fwd.	ATGCTTCCATGTCCAGGTTC
	Rev.	CACTCTGGGACGATTCCTGT
WNT5A	Fwd.	TGGCTTTGGCCATATTTTTC
	Rev.	CCGATGTACTGCATGTGGTC

2.1.10 Antibodies

2.1.10.1 Western Blot Primary antibodies

Name and MW (kDa)	Dilution	Biological source	Company
Frizzled 1 (73)	1:1000	Rabbit	Thermo Fisher Scientific
Frizzled 2 (85)	1:1000	Rabbit	Thermo Fisher Scientific
Frizzled 5 (50-65)	1:1000	Rabbit	Cell signaling technology, Danvers, Massachusetts, USA
Frizzled 6 (75-85)	1:1000	Rabbit	Cell signaling technology
GAPDH (37)	1:1000	Mouse	Cell signaling technology
HDAC2 (60)	1:1000	Mouse	Cell signaling technology
NFATc1(90-140)	1:1000	Mouse	Santa Cruz, Dallas, Texas, USA
NLK (58)	1:1000	Rabbit	Cell signaling technology
P16(16)	1:1000	Mouse	Dako, Jena, Thuringia, Germany
P27(22)	1:1000	Mouse	Dako
Phospho-c-Jun (95)	1:1000	Rabbit	Abcam
Non-phospho-β – Catenin (92)	1:1000	Rabbit	Cell signaling technology
ROR1(135)	1:1000	Rabbit	Cell signaling technology
ROR2(120)	1:1000	Rabbit	Thermo Fisher Scientific
SP1 (90)	1:1000	Rabbit	Cell signaling technology
Wnt5A (45)	1:1000	Rabbit	Cell signaling technology
β –Actin (45)	1:1000	Mouse	Santa Cruz
Desmin (53)	1:1000	Mouse	Leica
Myogenin (34)	1:1000	Mouse	Bio SB, Goleta, CA, USA

2.1.10.2 Western Blot secondary antibodies

Name	Dilution	Biological source	Company
Anti-mouse Ab	1:1000	Horse	Cell signaling technology
Anti-rabbit Ab	1:1000	Horse	Cell signaling technology

2.1.10.3 FACS antibodies

Name	Company
APC conjugated anti-fAChR antibody	US Biological, Salem, Massachusetts, USA
PE-Cy™ 7 CD3	BD Bioscience, Franklin Lakes, New Jersey, USA
FITC AffiniPure Goat Anti-Human IgG + IgM	Jackson ImmunoResearch, West Grove, Pennsylvania, United States

2.2 Methods

2.2.1 Cultivation of Human rhabdomyosarcoma (RMS) cell lines

The ARMS/RMA cell lines (CRL2061, RH30, RH41 and FLOH1) were cultivated in RPMI1640 supplemented with 10% (v/v) FCS, while the ERMS/RMERD, RH36 and TE671in addition to 293 T HEK cells were cultivated in DMEM supplemented with 10% (v/v) FCS. The cells were incubated in 5% CO2 at 37°C and used for experiments during the logarithmic growth phase.

2.2.2 Sub-cultivation

For a 75 cm^2 cell culture flask, 10 ml of growth medium was used per flask. When the cells were approximately 80-90% confluent, culture medium in the flask was discarded and the cell layer was briefly washed with 5 ml of PBS. After discarding the PBS, 1.0 ml of Trypsin-EDTA was added and cells were observed under an inverted microscope until cell layer is dispersed (digesting time depends on cell lines). To avoid clumping, do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. When cells were detached, 9.0 ml of complete growth medium was added and cells were aspirated by gentle pipetting. Cell suspension was transferred into a 15 ml falcon tube and can be stored at 4 °C for 3 days, leaving the required volume in the flask depending on the splitting ratio (1:8 to 1:10). Culture flasks were incubated in a cell culture incubator at 37°C, 95% air, 5% carbon dioxide (CO_2).

2.2.3 Cells cryopreservation and thawing

For suitable cell growth after thawing, cells need to be in the log phase immediately before freezing. Cells were counted and cell density was adjusted to a number of 5×10^5 cells / 900 µl. Freezing solution was prepared immediately before use, containing 30% growth medium (with FCS), 20% DMSO and 50% FCS. 900 µl of freezing solution and 900 µl of cell suspension were added into each freezing tube. After mixing the freezing tubes by inverting them for

several times, tubes were transferred to a cell freezing container (Mr. Frosty™, Thermo Fisher Scientific) (containing Isopropanol), and stored at -80°C for 24 h. For long term storage freezing tubes should be transferred to liquid nitrogen. To thaw the cells, freezing tubes containing the cells were removed from the liquid nitrogen and kept directly on ice. The tubes then were placed in a 37°C water bath to thaw. The cells were washed with growth medium, centrifuged at speed 300 g for 5 mins, resuspended and transferred into T75 cm^2 flasks and were incubated in a cell culture incubator at 37°C, 95% air, 5% carbon dioxide (CO_2).

2.2.4 Human peripheral blood mononuclear cells (PBMCs) isolation

PBMCs were isolated from the human peripheral blood using Ficoll. Around 30 ml human peripheral blood was placed in 50 ml falcon tube and diluted with HBSS buffer to 50 ml. 25 ml Ficoll were added into two 50 ml falcon tubes, 25 ml human peripheral blood with HBSS were pipetted slowly on the wall of the 50 ml falcon tubes containing Ficoll while tilting the tubes to not disturb the Ficoll layer. The 50 ml falcon tubes were then centrifuged at speed of 800 g for 25 mins without break. The upper layer was discarded and the interface containing the lymphocytes was transferred to 15 ml falcon tubes (4 tubes) and PBS was added to 15 ml. The 15 ml falcon tubes were centrifuged at speed of 150 g for 10 mins. The supernatant was discarded leaving the lymphocytes' pellets which were pooled together in one 15 ml falcon tube and washed with PBS at speed of 300 g for 5 mins. The lymphocytes were resuspended in RPMI medium supplemented with 10 % heat-inactivated FCS (HI-FCS), transferred to T25 suspension flask and activated with 0.5 µg/ml OKT-3 antibody and 0.5µl/ml anti-CD28 antibody. In the next day the medium was refreshed with complete RPMI1640 containing 400 U/ml interleukin-2.

2.2.4.1 Production of the 2nd generation fACh-CART cells

A chimeric antibody receptor (CAR) was developed to recognize RMS tumor cells via the fetal acetylcholine receptor on its surface. A recombinant Fab Antibody fragment with specificity against γAChR was generated from a phage display library of a patient whose fetus suffered from arthrogryposis multiplex congenita due to the transplacently transferred anti-fAChR autoantibodies(Matthews et al., 1998; Matthews et al., 2002; Palace, Vincent, & Beeson, 2001). A single-chain VH-VL fragment was in turn cloned from this Fab fragment by PCR. To produce second generation CAR Ts, the single chain fragment was ligated with a CD28-CD3ζ signal transduction domain.

For Transfection, $2x10^5$ cells/well 293 T cells were seeded out in a 6-well plate and cultivated until the next day. Cells were transfected by calcium phosphate transfection kit with 3:2:1, transfer: packaging: envelope, ratio.

To prepare the cells for transfection, 2 ml fresh complete DMEM medium containing 25 µM chloroquine were added to each well. After 2 h, 2 tubes with transfection reagents were prepared according to table 2.2.4.1-1. Tube A's reagents were mixed gently and its content were transferred to tube B with pipetting up and down to produce bubbles in order to have small precipitates for better transfection efficiency. The tube was then vortexed for 5 sec and was allowed to sit for 20 mins. 115 µl of tube B's content were evenly dropped on each well. Cells were cultivated until the next day to allow virus production. Medium was changed to complete RPMI medium containing 400 U/ml and$1x10^6$ cells/well PBMCs were transduced by adding them to the virus producing 293 T cells for 48 h. After cocultivation, PBMCs were collected and maintained in RPMI1640 medium supplemented with 10% HI-FCS and 400 U/ml interleukin-2. After 3 days of cultivation the expression of the chimeric receptors was verified by flow cytometry analysis using anti-human IgG antibody and anti-human CD3 antibody. After the expansion of the cells, they were used for further assays.

Table 2.2.4.1-1: Transfection protocol

	Tube A			**Tube B**
Components	$CaCl_2$ (µl)	H_2O (µl)	DNA (µg)	HeBS (µl)
/ well	6	49	5	60
/ 6-wells	36	294	30	360

2.2.5 Cloning of WNT5A shRNA and WNT5A in a retroviral vector system

To knock down and over-express WNT5a in RMS cells, pGIPZ-shRNA and pBABE-WNT5a constructs were generated respectively.

Knock down

Cloning of WNT5a shRNA in pGIPZ was done in the lab of Professor Heidi Hahn in Göttingen

Overexpression

To establish a stable over expression of WNT5a in RMS cells, WNT5a fragment was cloned in pBABE vector according to the following steps:

2.2.5.1 WNT5a amplification and isolation

WNT5a gene was synthesized by GeneWIZ. A pUC vector was used as vector backbone with Ampicillin resistance gene as selection markerWNT5a was amplified from the plasmid using the primers listed in section 2.1.8.1 containing the desired restriction sites. a Taq DNA polymerase master mix including dNTPs and buffer components was used (table 2.2.5.1-1,2) for amplification. In order to check if the right product is amplified and for further purification, the PCR product were run on 1% agarose gel (table 2.2.5.1-3) containing Midori green in 1x TBE buffer (table 2.2.5.1-3) to detect DNA. The bands were visualized using a GEL iX 20 imaging system (Intas Science Imaging Systems) and Intas-Capture-Software to analyze results.

Table 2.2.5.1-1: RT-PCR protocol

Components	**Volume (µl)**
WNT5A DNA	1
Fwd primer	1.5
Rev primer	1.5
Taq DNA polymerase master mix	12.5
Add H2O to 25µl	8.5

Table 2.2.5.1-2: RT-PCR conditions

Stage		**Temperature**	**Time**
Initial Denaturation		95 °C	5 min
Cycling stage (35x)	Denaturation	95 °C	30 sec/cycle
	Annealing	55 °C	
	Elongation	72 °C	
Final Elongation		72 °C	10 min
Storage		8 °C	Forever

Table 2.2.5.1-3: 1% agarose gel

Components		**Quantity**
Agarose		1.5 g
1x TBE		150 ml
TRIS base	10.8 g	
Boric acid	5.5 g	
0.5 M EDTA Na_2	0.75 g	
ddH_2O	Add to 1 L	
Midori green		7 µl

Glass wool extraction was used to isolate WNT5a from the agarose gel. WNT5a band was cut and placed in 0.5 ml microcentrifuge tube, which was poked with a hot needle to make a hole

on the bottom and partially filled with glass wool. The 0.5 ml microcentrifuge tube containing the agarose gel were then placed in a 1.5 ml microcentrifuge tube and centrifuged at a speed of 800 rcf at 4 °C for 10 mins. The volume in the 1.5 ml tube was then determined and the DNA was precipitated using sodium acetate/ ethanol according to table 2.2.5.1-4.

Table 2.2.5.1-4: DNA precipitation protocol

Components	Volume
DNA	Determined volume
Sodium Acetate	10% DNA volume
100% Ethanol	2.5-fold DNA volume

The reaction tube was then centrifuged at > 12.000 rcf at 4 °C for 30 mins. The supernatant was discarded and the DNA pellet was washed with 500 µl 70% Ethanol, the tube was briefly vortexed and centrifuged at 16.000 rcf at 4 °C for 10 mins. The supernatant was discarded and the pellet was left to air dry for 5-10 mins and resuspended in 10 µl nuclease free H2O.

2.2.5.2 Cloning of WNT5A into pBABE vector

A double digestion was done to open pBABE vector (Figure 2.2-1) and produce sticky ends on both pBABE vector and WNT5a fragment. BamH I and EcoR I restriction enzymes were used according to table 2.2.5.2-1.

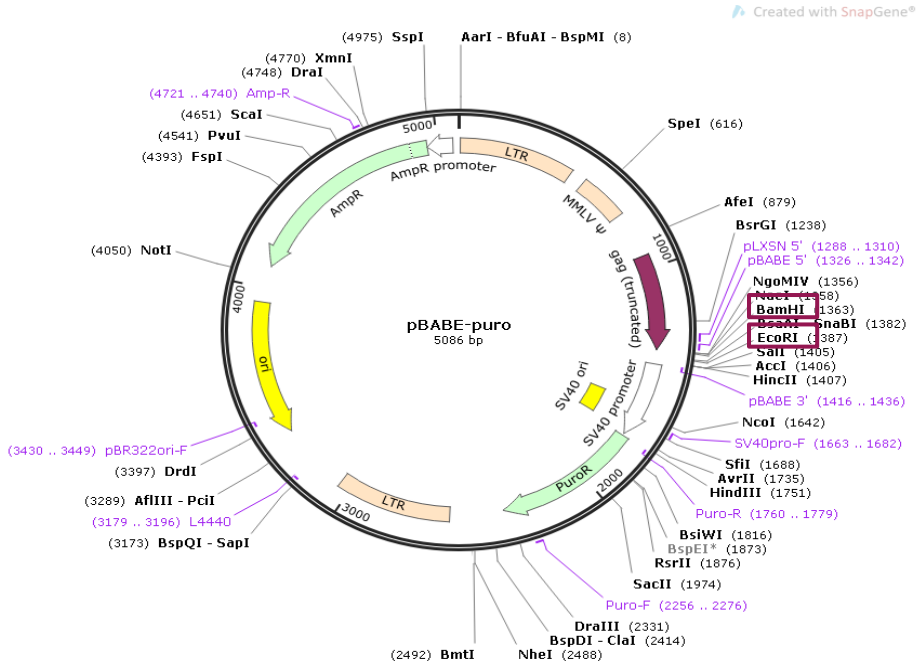

Figure 2.2-1: pBABE vector map

Table 2.2.5.2-1: double digest protocol

Components	Negative control	Single digest		Double digest	
		BamH I	EcoR I	pBABE	WNT5A
pBABE DNA	--------	500 ng	500ng	2 µg	--------
WNT5A DNA	--------	--------	--------	--------	10 µl
BamH I	1 µl	1 µl	--------	1 µl	1 µl
EcoR I	1 µl	--------	1 µl	1 µl	1 µl
NEB 2.1 buffer	2 µl	2 µl	2 µl	2 µl	2 µl
Nuclease free H_2O	Add to 20 µl	Add to 20 µl	Add to 20 µl	Add to 20 µl	Add to 20 µl

To control for unspecific restriction sites, single digests were included in the experiment. The double digested pBABE vector and WNT5a were run on 1% agarose gel and the DNA were extracted from the gel as mentioned before (section *1.2.4.1*). Double digested pBABE vector pellet were resuspended in 10 µl nuclease free H_2O while the WNT5a pellet wasn't resuspended to maintain high concentration of DNA for ligation. The tube containing WNT5a was used to perform the ligation.

2.2.5.3 Ligation of pBABE vector and WNT5a

Ligation of pBABE vector and WNT5a was performed by T4 DNA ligase for 16 h according to the manufacturer's instructions.

2.2.5.4 Bacterial Transformation of pBABE-WNT5a Ligation Product

Bacterial transformation of the ligated pBABE vector was done to obtain positive clones using chemical competent TOP10 bacteria. 2 µl of the ligated product was added to the bacteria, gently mixed by flicking the bottom of the tube. The tube was incubated on ice for 30 mins. 1 µl PUC vector was used as a positive control to check the transformation efficiency in addition to a negative control (bacterial without ligated pBABE vector). After the incubation period, the bacteria were heat shocked for 45 secs at 42°C and then placed back on ice for 2 mins. 300 µl SOC medium was added to each tube and the tubes were incubated for 1 h at 37°C while shaking. After the growth period, the bacteria were plated onto prewarmed 10 cm LB agar plates containing 100 mg/ml ampicillin and the plates were incubated at 37°C overnight.

2.2.5.5 Colony PCR of pBABE-WNT5a colonies

Colony PCR using Taq polymerase was done to check for pBABE-WNT5a positive colonies. Colonies were picked with 10 µl pipette and placed first on LB agar plate for further cultivation. The remaining bacteria from the same tip were placed in a corresponding PCR tubes to check for pBABE-WNT5A positive colonies according to the following protocol (table 2.2.5.5-1,2):

Table 2.2.5.5-1: Colony PCR protocol

Components	Quantity
Picked colonies	1 colony
Fwd primer	1.5 µl
Rev primer	1.5 µl
Taq DNA polymerase master mix	12.5 µl
Nuclease free H_2O (add to 25µl)	9.5 µl

Table 2.2.5.5-2: Colony PCR conditions

Stage		Temperature	Time
Initial Denaturation		95 °C	5 min
Cycling stage (35x)	Denaturation	95 °C	30 sec/cycle
	Annealing	55 °C	
	Elongation	72 °C	
Final Elongation		72 °C	10 min
Storage		8 °C	Forever

2.2.5.6 Minipreparation of pBABE-WNT5A positive colonies

5 ml LB medium containing 100 mg/ml ampicillin was inoculated with the positive bacteria colony and cultivated over night at 37°C while shaking at 180 rpm. The mini-preparation was then performed using the QIAprep Spin Miniprep Kit to obtain pBABE-WNT5A plasmid.

2.2.5.7 Plasmid Preparations for Mammalian Cell Transfections

2.2.5.7.1 Maxi preparation

All plasmids used in the present work were prepared by using EndoFree® Plasmid Maxi Kit to obtain the plasmid DNA for use in cell transfections according to the manufacturer's instructions. DNA concentration was determined by measuring absorption at 260/280 nm using the TECAN Spark microtiter plate reader (TECAN, SPARK 10M, SPARKCONTROL Dashboard System).

2.2.6 WNT5A knock-down and over-expression cell lines establishment

To evaluate the influence of WNT5A on functional and molecular features of RMS tumor cells in vitro and in vivo, generation of RMS cell lines with WNT5A knock down or overexpression was done. To establish the cell lines the following methods was used:

2.2.6.1 Transient transfection

In order to evaluate the relevance of WNT5A, RMS cells were first transiently transfected with pcDNA-WNT5A plasmid using Electroporation (Neon® Transfection system) in 6-wells plate. The cells were seeded out in 10 cm dish to be used the next day while they're in the exponential phase. In next day, the cells were trypsinized and counted. 4 x 10^5 cells (2 x 10^5/well) were transferred to 15 ml falcon tubes (two tubes/cell line for control and WNT5A overexpression) and washed with PBS. The supernatant was discarded and the cells were resuspended in 110 µl resuspension buffer R. The cells were loaded into the Neon pipette tip, which was plugged in the Neon transfection device. The cells were electroporated at a voltage of 1000V and 2 pulses (30 msec/pulse). 50 µl of the transfected cells were pipetted in the corresponding well of 6-wells plate containing 2 ml complete growth medium/well and incubated for 48 h. The cells were checked for the expression of WNT5A by qRT-PCR and western blot and then used for further assays.

Table 2.2.6.1-1: Electroporation protocol

For 2 wells	**Cells number**	**Buffer (µl)**	**DNA (µg)**
Control	4 x 10^5	100	--------
Overexpression	4 x 10^5	100	6

2.2.6.2 Retroviral transduction

Viral transduction was performed to establish stable WNT5A knock-down and over-expression RMS cell lines. All vectors were packaged using 293 T HEK cells in 10 cm dish according to table 2.2.6.2-1. The vectors were transfected using calcium phosphate transfection kit as mentioned before (section *1.2.4.1*) according to table 2.2.6.2-2. RMS cells were transduced with the retroviral supernatant of the 293 T HEK cells containing 5 µg/ml polybrene for 48 h and the cells then were selected for stable transduction using 10 µg/ml puromycin for 7 days. The expression of WNT5A was checked by qRT-PCR and Western Blot.

Table 2.2.6.2-1: Retroviral vector system

	Transfer plasmid	Pakaging plasmid	Envelope Plasmid	Ratio	Selection marker
WNT5A KD	pGIPZ	pCMV	pMDG	3:2:1	Puromycin
WNT5A OE	pBABE	pUMVC	pCMV-VSV-G	1:0.9:0.1	Puromycin

Table 2.2.6.2-2: Transfection protocol

	Tube A			Tube B
Components / 10 cm dish	$CaCl_2$ (µl)	H_2O (µl)	DNA (µg)	HeBS (µl)
	30	245	25	300

2.2.7 Cell viability and proliferation assays

2.2.7.1 MTT

MTT assay was used to check the influence of WNT5A on cell proliferation of RMS cell lines. RMS cells were seeded out in flat bottom 96-wells plates at a density of 5×10^3/well. In the next day, 20 µl of MTT (5 mg/ml, diluted in sterile PBS) was added at different time points (0 h, 24 h, 48 h, 72 h) to each well and incubated for 3 h. Supernatant was discarded and cells were lysed by adding 200 µl DMSO, which is also a solvent for MTT crystals. After plate shaking for 90 sec to dissolve the MTT crystals properly, reduction of MTT by viable RMS cells was colorimetrically determined by a microtiter plate reader from TECAN (TECAN, SPARK 10M, SPARKCONTROL Dashboard system) at 560 nm and a reference wavelength of 620 nm. RMS cells after WNT5A KD and overexpression were used in the assay. Three independent experiments were performed, each in triplicates.

2.2.7.2 Bromodeoxyuridine (BrdU) assay

BrdU is an analog of thymidine, which is incorporated in the newly synthesized DNA by cells entering the S phase of the cell cycle. The incorporated BrdU is then detected by flow cytometry after staining RMS cells with APC conjugated anti-BrdU specific fluorescent antibody. The BrdU staining was coupled with total DNA staining using 7-amino-actinomycin D (7-AAD) dye, which allow the characterization of cells that are actively synthesizing DNA in terms of their cell cycle position.

RMS cells were seeded out in 6-wells plate at a density of 2×10^5/well. When the cells were in the exponential phase, 10 µL of BrdU solution (1 mM BrdU in 1X DPBS) were added directly

to each mL of cell culture medium and cells were incubated for 24 h, the cells then were stained according to the manufacturer's instructions and acquired using FACSLYRIC^{TM} (BD Bioscience).

2.2.8 Migration assay

Migration plays a critical process in rhabdomyosarcoma metastasis. The influence of WNT5A on the migratory ability of rhabdomyosarcoma was examined by Scratch assay as well as Transwell assay.

2.2.8.1 Scratch assay

RMS cells were seeded out in 12-wells plates at a density of 2×10^5/well to achieve a confluent cell layer after 24 h. After incubation a cross scratch was performed with a 200 µl pipette tip in each well. To remove detached cells, each well was washed twice with PBS and fresh growth medium was added into the wells. Pictures were taken at the inverse microscope with phase contrast at 10x (Leica) after 0 h, 6 h and 24 h and were analyzed by using Image J (https://imagej.net).

2.2.8.2 Transwell assay

Cell Biolabs CytoSelect™ Cell Migration Assay Kit (CELL BIOLABS INC.) was used for the quantitative determination of cell migration. It is a fluorometric assay, which was done using polycarbonate membrane inserts (8 µm pore size) in 24-wells plate. After 48 h of starvation, RMS cells were trypsinized and counted. 1 x 10^6 cells were transferred to 15 ml falcon tube, washed with PBS and resuspended in 1 ml growth medium without FCS. The migration plate was let to warm up at room temperature for 10 mins, 500 µl growth medium containing 10% FCS as chemoattractant was added to the lower part of each well of the migration plate. Growth medium without FCS was used as negative control. 300 µl cell suspension were pipetted in each insert and the plate were incubated in the cell culture incubator for 8 h. After the incubation time, the media from the inside of the insert were carefully aspirated and the inserts were transferred to corresponding clean wells containing 200 µl of Cell Detachment Solution, keeping the medium containing the migratory cells to use it later. The plate was incubated for 30 mins at 37ºC, the cells were completely dislodged from the underside of the membrane by tilting the inserts several time in the Cell Detachment solution. The inserts were removed and discarded. 400 µl of medium containing the migratory cells were transferred to the corresponding well that contains 200 µl of Cell Detachment Solution. After mixing well, 180 µl of the mixture were transferred to 96-wells plate. 4X Lysis Buffer/CyQuant® GR dye

solution was prepared for all the samples by diluting the dye 1:75 in 4X Lysis Buffer. 60 µl of 4X Lysis Buffer/CyQuant® GR dye solution was added to each well of the 96-wells plate containing the migratory cells and the plate was incubated for 20 mins at RT. 200 µl of the mixture were transferred to black walled flat 96-wells plate. The plate was fluorometrically quantified by a microtiter plate reader from TECAN (TECAN, SPARK 10M, SPARKCONTROL Dashboard system) at 480 nm/520 nm.

2.2.9 Sphere assay

Sphere forming assay was done as described by Walter et al. to assess the stemness of rhabdomyosarcoma cells and the influence of WNT5A on RMS cells sphere forming ability (Walter et al., 2011). Cancer stem cells have the ability to undergo self-renewal so the number of formed spheres will reflect the stemness of rhabdomyosarcoma cells.
RMS cells were trypsinized, counted and resuspended at a clonal density of 1 cell/µl in sphere media, which was freshly prepared (table 2.2.8.2-1). The cells were seeded out in ultra-low attachment plates (Sigma Aldrich) and maintained at 37°C in a 5% CO_2 incubator for 7 days, fresh sphere medium was added after 4 days. The spheres were counted under the microscope, pictures were taken at the inverse microscope with phase contrast at 10x (Leica). RMS cells with WNT5A knock down and over-expression were used for the assay with and without rWNT3a treatment. Untreated wild type RMS cells were used as a control (Walter et al., 2011).

Table 2.2.8.2-1: Sphere formation assay protocol

	Sphere media					**Treatment**
Component	Neurobasal medium	B27	rhEGF	rhFGF-b	penicillin-streptomycin	rWNT3a
Quantity	200 µl/well	2 x	10 ng/ml	20 ng/mL	100 U/mL	200 ng/ml

2.2.9.1 Sphere passaging

After 7 days the spheres were collected and spun down at a speed corresponding to 300 g for 5 mins. The supernatant was discarded carefully and the spheres were resuspended in trypsin and incubated at 37°C for 5 mins or spheres dissociation. After obtaining single cell suspension, the cells were counted and seeded out at a clonal density of 2 cells/µl.

2.2.10 Flow cytometry assay

For flow cytometric analysis, 2×10^5 cells/well were seeded out in a 6-wells plate. After 24 h and a confluence of a maximum of 70%, cells were harvested and washed twice with ice cold

staining buffer (500 ml HBSS buffer, 2% FCS, 10 mm HEPES, 2 mm EDTA). Cells were incubated with the primary antibody and incubated in ice and dark for 1 h. After the incubation time, cells were washed twice with ice cold staining buffer and incubated with the secondary detection antibody. Before measurement, cells were washed twice with ice cold staining buffer, resuspended in 200 µl staining buffer and acquired by FACSLYRIC^{TM} (BD Bioscience). To detect the expression of fAChR on RMS cells, cells were incubated with 1 µl APC conjugated anti-fAChR antibody, incubated in ice and dark for 20 mins and then acquired after washing the cells and resuspending them in ice cold staining buffer.

For the BrdU assay analysis were done according to the manufacturer's instructions.

CAR T cells' transduction efficiency was detected as mentioned before in section *1.2.3.1*.

2.2.11 Calcium flux assay

Intracellular calcium changes in RMS cells after WNT5A knockdown and over-expression caused by activation of G-protein coupled receptors or calcium channels were detected using Calcium assay kit (BD Bioscience). RMS cells were seeded out in 96-wells black walled plate at a density of 5 x 10^3/well and allowed to grow overnight. In the next day, cells were pretreated with 100 ng/ml pertussis toxin (calcium pathway inhibitor) for 24 h (Grzesk et al., 2014; Nunn et al., 2004). After the pretreatment, 50 µl fresh medium was added to each well in addition to 100 µl 1 X Dye-loading Solution. After 1 h, 50 µl complete growth medium containing 200 ng/ml rWNT5A (final concentration). Growth medium without rWNT5A was used as control and medium containing 10 mm clofibric acid (final concetration) as a positive control (Ikemoto & Endo, 2001). The intracellular calcium changes were quantified by a microtiter plate reader from TECAN (TECAN, SPARK 10M, SPARKCONTROL Dashboard system) at 485 nm/525 nm after 5 mins, 10 mins, 30 mins, 1 h, 2 h, 3 h and 4 h of rWNT5A addition.

2.2.12 RNA isolation, cDNA synthesis and quantitative RT-PCR

2.2.12.1 RNA isolation

Samples were homogenized with 1 ml of TRIzol® Reagent per well of 6 wells and 12 wells plates. Cells were lysed by pipetting the cells up and down several times. TRIzol® samples can be stored at -80°C for at least one month and RNA was isolated according to the manufacturer's instruction. RNA concentration was determined by measuring the absorption at 260 / 280 nm using the TECAN Spark microtiter plate reader (TECAN, SPARK 10M, SPARKCONTROL Dashboard system).

2.2.12.2 cDNA synthesis

500 ng RNA was used to generate cDNA by reverse transcriptase following the manufacture's protocol of PrimeScript™ RT Reagent Kit (TAKARA BIO INC.) (Table 2.2.12.2-1,2).

Table 2.2.12.2-1: cDNA synthesis protocol (per reaction)

Components	Volume
RNA (volume of 500 ng)	v
RNase Free H_2O	7-v
Oligo dT Primer	0.5 µl
PrimeScript Enzyme Mix	0.5 µl
5X PrimeScript Buffer	2.0 µl
Total volume	10.0 µl

Table 2.2.12.2-2: cDNA synthesis conditions

Stage	Temperature	Time
Reverse transcriptase (RT)	37 °C	15 min
Inactivation of RT	85 °C	5 min
Store forever	4 °C	Forever

After cDNA synthesis the samples were stored at -20°C.

2.2.12.3 Quantitative Real-time PCR

For quantitative real time PCR (qRT PCR), cDNA was diluted 1:20 in RNase / DNase free water to a concentration of 2.5 ng/µl. Table 2.2.12.3-1 shows the reaction mix used in the present work.

Table 2.2.12.3-1: reaction mix for qRT-PCR analysis (per well)

Components	Volume
2×SYBR Green Master Mix (TAKARA)	5 µl
Forward primer	1 µl
Reverse primer	1 µl
RNase free H_2O	1 µl
cDNA (2.5 ng/µl)	2 µl
Total volume	8 µl

When preparing the Master Mix, SYBR Green solution should be added as the last component, and the Master Mix should be mixed completely by pipetting up and down several times. 8 µl of the Master Mix and 2 µl of the cDNA sample (5 ng) were loaded into each well. Each cDNA sample was measured in duplicates.

Before running the program, the PCR plate was briefly centrifuged. StepOnePlus Real-Time PCR system, StepOne™ software v2.3 was used for the qRT-PCR, with the conditions showed in table 2.2.12.3-2 and the primers listed in section 2.1.8.2.

Table 2.2.12.3-2: qRT-PCR conditions

Stage	Temperature	Time
Holding stage	95 ºC	10 min
Cycling stage (40 cycles)	95 ºC	15 sec
	60 ºC	1 min
Melting-Curve stage	95 ºC	15 sec
	60 ºC	1 min
	95 ºC	15 sec

2.2.13 Protein isolation and western blot

2.2.13.1 Protein Isolation

2.2.13.1.1 Whole cell lysate

The supernatants of the treated cells were discarded, and cell layers were washed 3 times with ice cold PBS. 150 µl of the protein isolation buffer RIPA completed with the protease inhibitor cOmplete™ Tablets, Mini EDTA-free, EASYpack were added per well of 6-wells plates, or 80 µl for 12-wells plates. Cell suspensions were transferred into microcentrifuge tubes after detaching cells by using a cell scraper and 10 µg/ml of DNase was added into each tube. The protein mixtures were incubated for 30 min at 37°C until genomic DNA is digested. Proteins were stored at -80°C.

2.2.13.1.2 Protein isolation from cultivation supernatant

1ml of Trichloroacetic acid 20% was added to 1 ml of cell culture supernatant. The supernatants were kept on ice for 20 min and were centrifuged at 10,000 g for 5 min. The supernatants were discarded leaving only the protein pellets, which were washed twice with 0.5 ml of cold acetone (100%). The pellets were kept to air dry after discarding the supernatants and were re-suspended with protein loading buffer (4x Laemmli buffer). Proteins can be stored at -80°C for several months.

2.2.13.1.3 Nuclear and cytoplasmic protein

NE-PER™ Nuclear and Cytoplasmic Extraction kit was used to separate nuclear and cytoplasmic protein according to the manufacturer's instructions.

2.2.13.2 Western blot

To separate proteins according to their molecular weight a discontinuous SDS gel electrophoresis was used. Gels were prepared as shown in Table 2.2.13.2-1 and used within 7 days.

Table 2.2.13.2-1: SDS polyacrylamide gel

Separating gel					**Stacking gel**	
	8%	**10%**	**12%**	**15%**		
Acrylamide 30% (ml)	2.7	3.3	4.0	5.0	Acrylamide 30% (ml)	0.65
Tris PH 8.8 (ml)	2.5				Tris PH 6.8 (ml)	1.25
H_2O (ml)	4.6	4.0	3.3	2.3	H_2O (ml)	3
SDS 10%	100 µl				SDS 10%	50 µl
APS 10%	100 µl				APS 10%	50 µl
TEMED	10 µl				TEMED	10 µl

For sample preparation, 6,6 µl of loading buffer (4x Laemmli buffer) was mixed with 20 µl of the protein sample and boiled for 5 min at 95°C. Samples were cool down at room temperature and centrifuged for short time to spin down evaporated sample. The whole protein solution was loaded on the gel with a syringe. Proteins were separated at 100 V for the stacking gel. Voltage was increased as soon as the proteins migrate to the separating gel at 120 V.

After gel electrophoresis, the proteins were transferred to a nitrocellulose membrane by wet transfer. The gel and the nitrocellulose membrane were sandwiched between sponge and paper (as shown in Figure 2.2-1). After removing any air bubbles, they were clamped tightly, submerged in the transfer buffer and connected to the electrodes where the negatively charged proteins traveled towards the positively charged electrode. 0.12 A was used to blot the proteins onto the membrane and the transferring time was 3 h.

To check for success of transfer, Ponceau staining was performed to visualize the protein bands reversible. The membranes were incubated with 5% dry milk resuspended in 1xTBS at room temperature for 1 h to block the nonspecific protein binding sites on the membrane. Primary antibody was diluted with 1% milk in 1xTBS according to the dilution ratio recommended in the standard protocol of the antibody. After the incubation of the blots with the primary antibody at 4°C overnight, 1xTBS was used to wash blots 3 times for 10 min at room temperature.

Secondary antibody (anti-mouse/anti-rabbit) was diluted 1:1000 and was incubated with the blots at room temperature for 1 h. The blots were washed with 1xTBS three times for 10 min.

The blots were kept in PBS and proceeded to images detection. For band detection, enhanced chemiluminiscence (ECL) kit was used as substrate and FUSION imaging system for images capturing and data analysis.

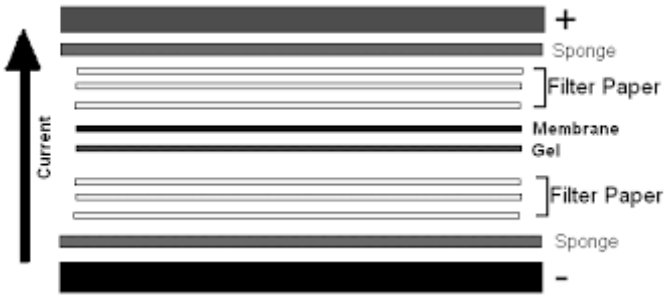

Figure 2.2-2: The gel-membrane sandwich

Table 2.2.13.2-2: Buffers used in the present work

Buffer	Composition
TBS	10mM TRIS (PH 8) 150mM Sodium Chloride

2.2.14 Animal and genetic engineering experiments

2.2.14.1 Animal experiments

The mouse experiments were done in collaboration with Prof. Heidi Hahn (Department of Human Genetics, University Medical Center, Göttingen 37073, Germany); the established knock down and overexpression cell lines were used to transplant these cell lines in nude mice and monitor the tumor growth rate in vivo. After termination of the mice, tumors were removed and the tumor tissue w sent to Mannheim for further examination; inter alia qRT-PCR and protein analysis after in vivo cultivation. Permission has already been obtained from the Regierungspräsidium for the lab in Göttingen (file number 33.9-42502-04-12/0805).

2.2.14.2 Gene engineering experiments

The viral transduction experiments for the knockdown, overexpression of WNT5A and for CAR T cells generation will be done in a biosafety level 2 (S2) laboratory

2.2.15 Statistical Analysis

Three independent experiments were performed, each in triplicates. Data were analyzed with student's t-test using the software tool Graphpad Prism 8.0 mac (GraphPad Software, La Jolla California USA, www.graphpad.com), a p value < 0.05 was considered to be statistically significant. FACS measurments were analyzed with FlowJo™ v10 software (BD Bioscience).

3 RESULTS

3.1 Non-canonical WNT pathway ligand, WNT receptors and co-receptors were highly expressed in embryonal rhabdomyosarcoma

As the activation of different WNT pathways depends on the ligand and the context of receptors/co-receptors expressed in the cells, the expression of the non-canonical WNT ligand WNT5A in addition to different FZD receptors and the non-canonical pathway co-receptors ROR1/ROR2 were tested in PAX3 FOXO1 translocation positive ARMS CRL206, RH30 and RH41 cell lines (RMA+), translocation negative FLOH1 cell line (RMA-) and ERMS RD and TE671 cell lines. Levels of mRNA and protein were measured to get an idea about the expression profile of WNT5A and WNT pathway receptors/co-receptors in RMS cell lines and the differences between ARMS and ERMS. In addition, an independent validation set composed out of RNA samples from 10 RMA (translocation positive) and 10 RME patients were checked.

As shown in Figure 3.1-1, the ERMS cell lines expressed higher levels of the non-canonical WNT ligand WNT5A on mRNA and protein levels compared to ARMS cell lines. A similar expression profile was also observed in the independent dataset. This result was further confirmed by mining two publicly available datasets, which show a higher expression of WNT5A in RME compared to RMA tumors (Davicioni et al., 2009; Williamson et al., 2010).

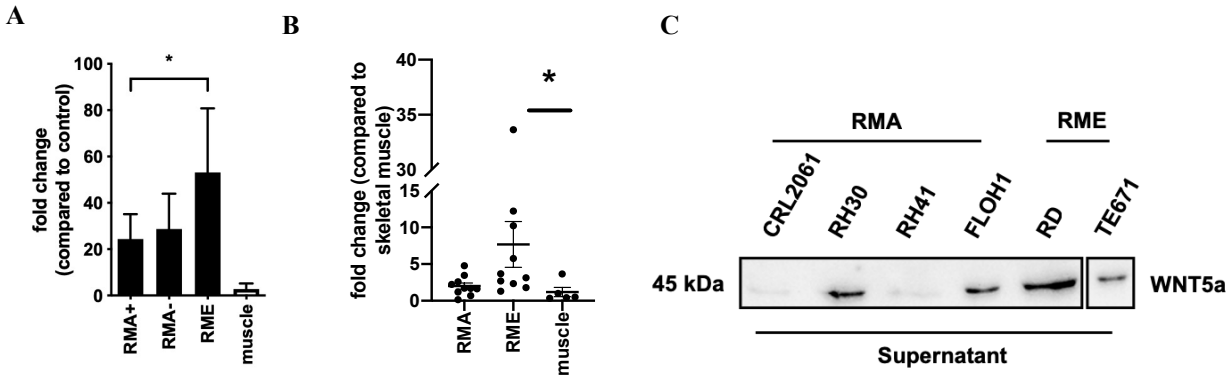

Figure 3.1-1: WNT5A expression in RMS cell lines and patient samples
(A, B) qRT-PCR of WNT5A gene in RMS cell lines **(A)** and alveolar (n=) and embryonal (n=) RMS biopsy specimens **(B)**. Gene expression of the skeletal muscle was set to 1. GAPDH expression was used for normalization. Shown is the mean and SEM of three independent experiments measured in duplicates **(A)**. **(C)** Western Blot of WNT5A expression in the supernatant of RMS cell lines. Data were compared by t-Test using 95% confidence interval (* 0.05 > p > 0.01, ** 0.01 > p > 0.005, ***0.005 > p > 0.001, ****P<0.0001) by GraphPad PRISM® 8.

In addition to WNT5A, the ERMS cell lines showed higher expression of the non-canonical co-receptors ROR1 and ROR2 compared to ARMS cell lines. Moreover, FZD receptors were expressed in all RMS cell lines with higher expression of FZD 2,3,6 and 7 in the ERMS cell

lines. In contrast, the translocation positive ARMS showed higher expression of ß-catenin (Figure 3.1-2). Regarding the receptor composition and the strong WNT5A expression in RME, an activation of the non-canonical WNT signaling pathway is more likely.

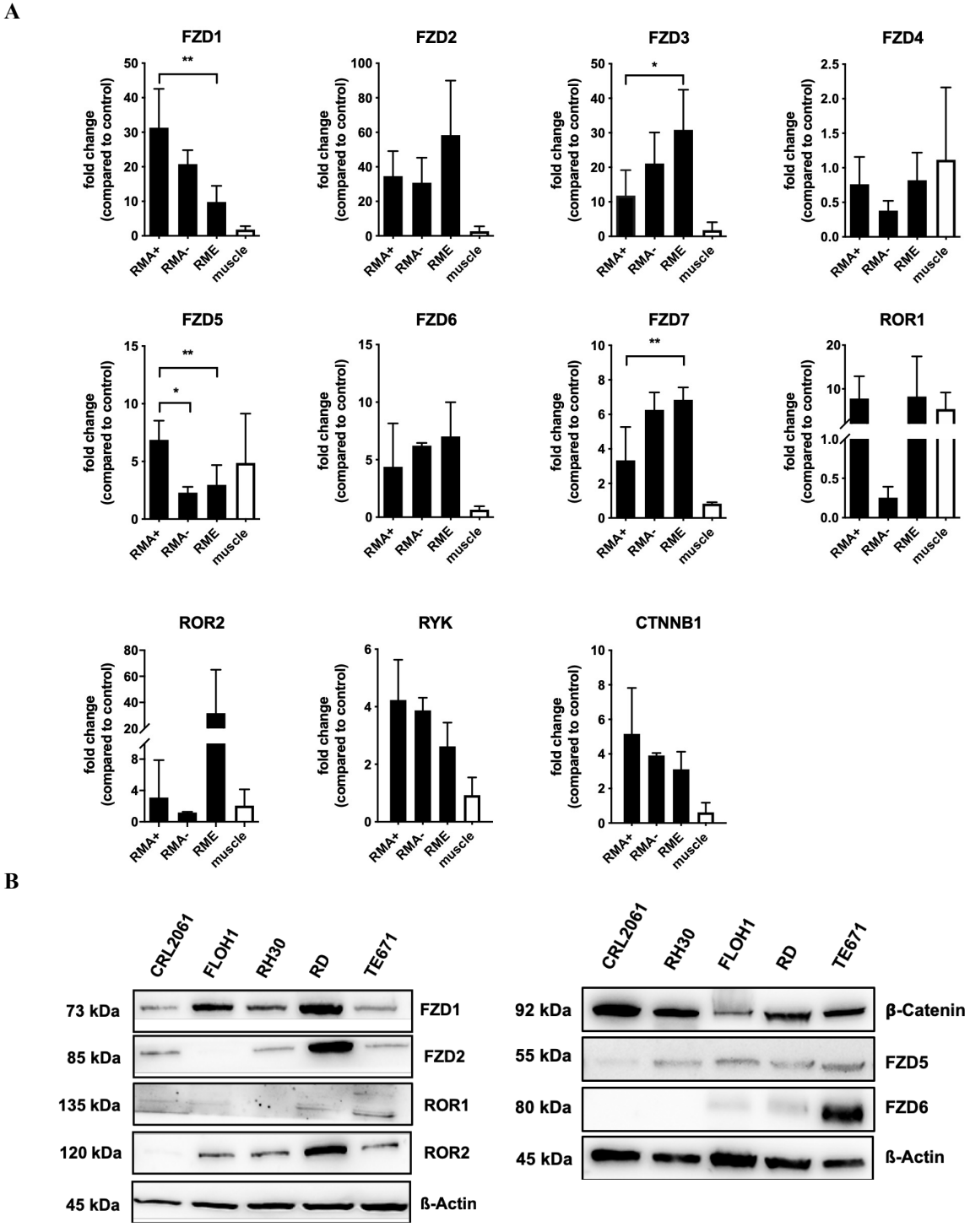

Figure 3.1-2: WNT receptors expression in RMS cell lines
(A) qRT-PCR of FZD 1-7 receptors, ROR 1and 2 co-receptors in addition to ß-catenin genes in RMS cell lines. Gene expression of the skeletal muscle was set to 1. GAPDH expression was used for normalization. Shown is the mean and SEM of two independent experiments measured in duplicates. **(B)** Western Blot of FZD 1, 2, 5 and 6, receptors, ROR 1and 2 co-receptors in addition to ß-catenin expression in RMS cell lines. B-Actin served as loading control. . Data were compared by t-Test using 95% confidence interval (* 0.05 > p > 0.01, ** 0.01 > p > 0.005, ***0.005 > p > 0.001, ****P<0.0001) by GraphPad PRISM® 8.

3.2 Knockdown of WNT5A promoted RMS cell proliferation and cell growth

WNT5A was previously demonstrated to influence proliferation in many tumors either by promoting the cell proliferation as in lung cancer (Y. Huang et al., 2010) or inhibiting the cell proliferation as in hepatocellular carcinoma (T. Wang, Liu, & Wang, 2019) and colon cancer (Cheng et al., 2014).

In contrast, the influence of WNT5A on RMS cell proliferation is still ambiguous. Therefore, we measured the metabolic activity (as a surrogate feature for proliferation and survival) of RMS cell lines by MTT after stable WNT5A knockdown (KD) and overexpression (OE) (Figure 3.2-1A) with or without recombinant WNT5A (rWNT5A) treatment. As shown in Figure 3.2-1B, WNT5A KD promoted the proliferation of RMS cells, which was more significant in the embryonal RMS cell lines. This effect was opposed by the over-expression of WNT5A (Figure 3.2-2A). Furthermore, both WNT5A KD and OE cell lines in addition to the wild type cell lines were treated with 200 ng/ml rWNT5A. As shown in Figure 3.2-2B, rWNT5A inhibited the proliferation of WNT5A KD cell lines but not the controls or the WNT5A OE cell lines.

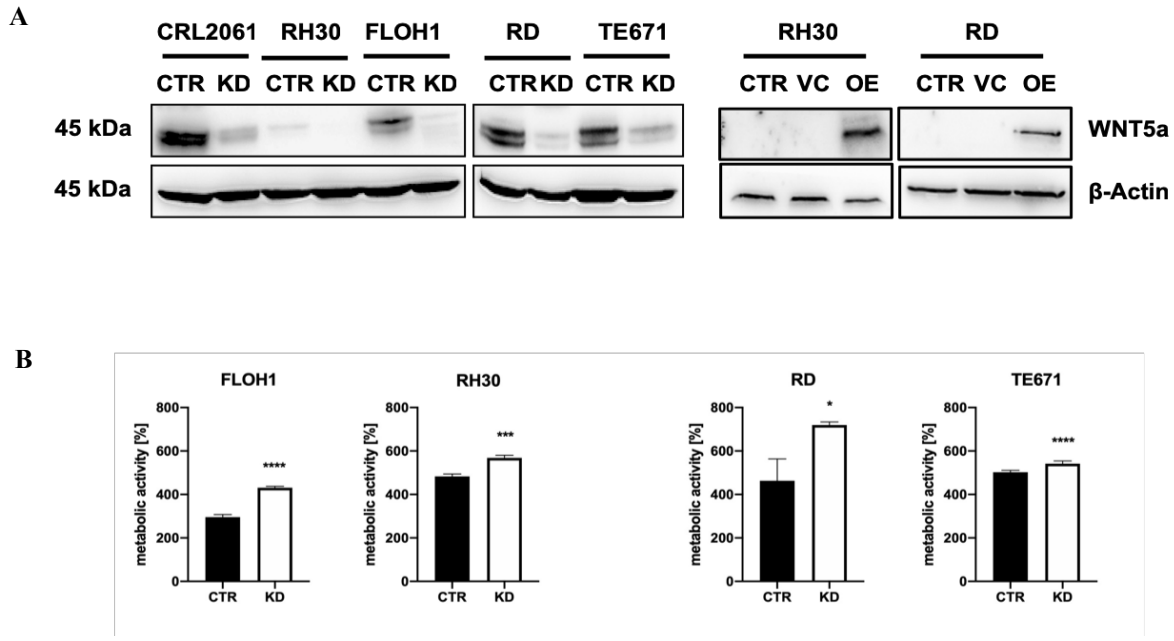

Figure 3.2-1: Influence of WNT5A KD on the proliferation of RMS cell lines in vitro
(A) Western Blot of WNT5A expression in RMS cell lines after stable WNT5A KD and OE. B-Actin served as loading control **(B)** Shows MTT assay to examine the influence of WNT5A KD on the metabolic activity of RMS cell lines over 72 h, wild type cells at time point zero served as 100%. Bars represent the metabolic activity of RMS cell lines after WNT5A KD on time point 72 h. The graphs show mean and SEM of one of three independent experiments measured in triplicates and compared by t-Test using 95% confidence interval (* $0.05 > p > 0.01$ ** $0.01 > p > 0.005$, *** $0.005 > p > 0.001$, **** $P<0.0001$) by GraphPad PRISM® 8.

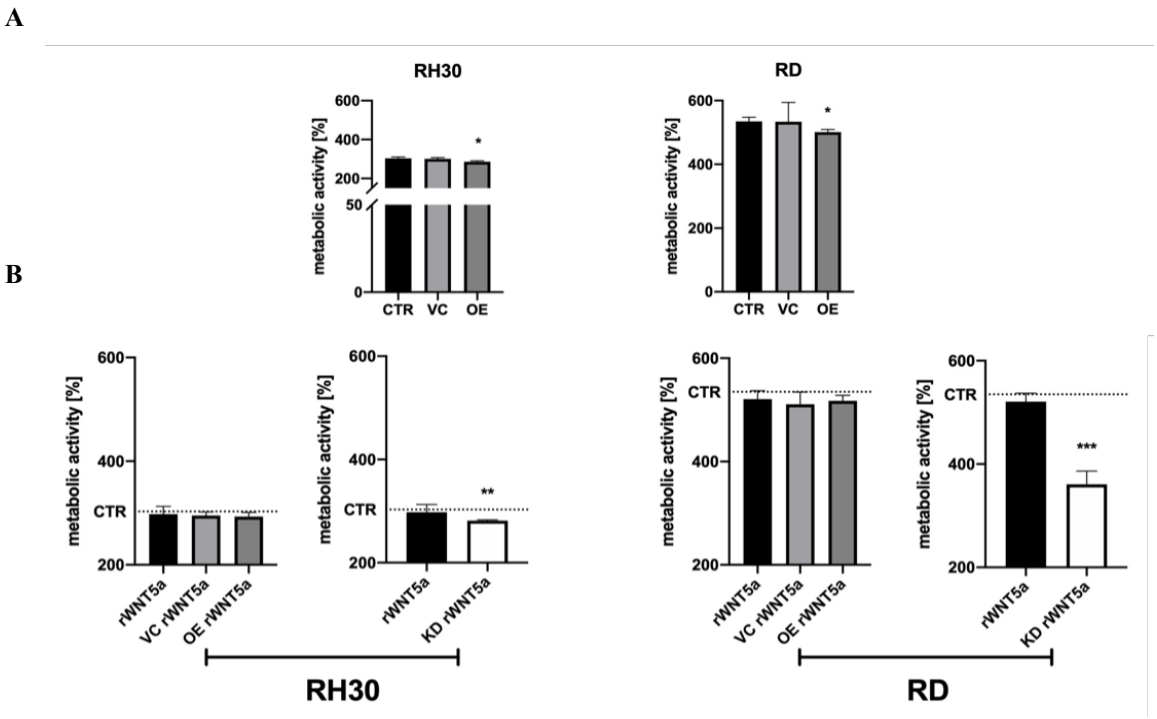

Figure 3.2-2: Influence of WNT5A OE on the proliferation of RMS cell lines in vitro
(A) Bar chart represents the metabolic activity of RMS cell lines after WNT5A OE at time point 72 h. The bars show mean and SEM of one of three independent experiments measured in triplicates. **(B)** Bar chart represents the metabolic activity of the indicated RMS cell lines after 72 h of 200 ng/ml rWNT5A treatment of the KD and OE cell lines. Dashed line represents untreated wild type at time point 72. The bars show mean and SEM of one of three independent experiments measured in triplicates. Data was compared by t-Test using 95% confidence interval (* 0.05 > p > 0.01, ** 0.01 > p > 0.005, ***0.005 > p > 0.001, ****P<0.0001) by GraphPad PRISM® 8.

To further confirm the MTT assay result, BrdU incorporation studies were performed and showed that higher number of cells in the S-phase was detected after WNT5A KD in RMS tumor cells compared to control (Figure 3.2-3).

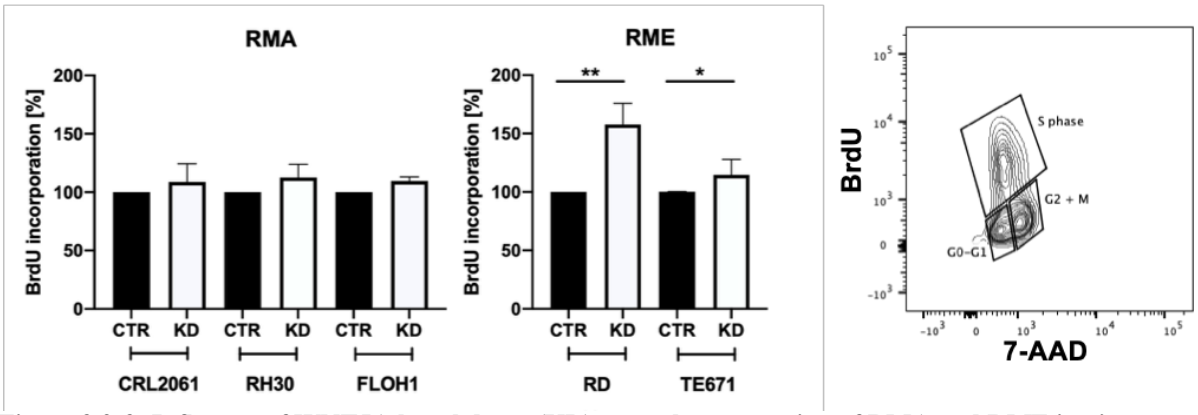

Figure 3.2-3: Influence of WNT5A knockdown (KD) on cycle progression of RMA and RME in vitro
BrdU incorporation in RMS cells after WNT5A KD. BrdU incorporation of the respective wild type cells of each cell line was set as 100%. The graphs show mean and SEM of three (CRL2061, RD, TE671) or two (RH30, FLOH1) independent experiments. Representative BrdU/7-AAD blot showing the gating strategy, depicting $BrdU^{high}$ S-phase cells as basis to calculate BrdU incorporation [%] in the column graphs. Data were compared by t-Test using 95% confidence interval (* 0.05 > p > 0.01, ** 0.01 > p > 0.005, ***0.005 > p > 0.001, ****P<0.0001) by GraphPad PRISM® 8.

As cell cycle progression is controlled by regulatory proteins, the cyclin-dependent kinase inhibitors p16 and p27 are among these proteins and their expression leads to cell cycle arrest (Wong, Chan, Lee, & Hsiao, 2001). The alteration in p16 and p27 expression has been noticed in different tumors like lung and bladder cancer (Ciesielska et al., 2017). Therefore, the expression of p16 and p27 were checked in RMS after WNT5A KD, which showed that the higher proliferation of RMS tumor cells after WNT5A KD was associated with modified expression of p16 and p27, which was cell line specific. As shown in Figure 3.2-4A and B, the analyzed RME cell lines RD and TE671 showed downregulation of p27, while, the analyzed RMA cell lines were more regulated by p16. On mRNA level, the ß-catenin target genes MYC and SURVIVIN were measured, which are pro-proliferative proteins (Bretones, Delgado, & Leon, 2015; Ghoshal & Ghosh, 2016). cMYC was found to be upregulated after WNT5A KD, while the expression of SURVIVIN did not change (Figure 3.2-4C).

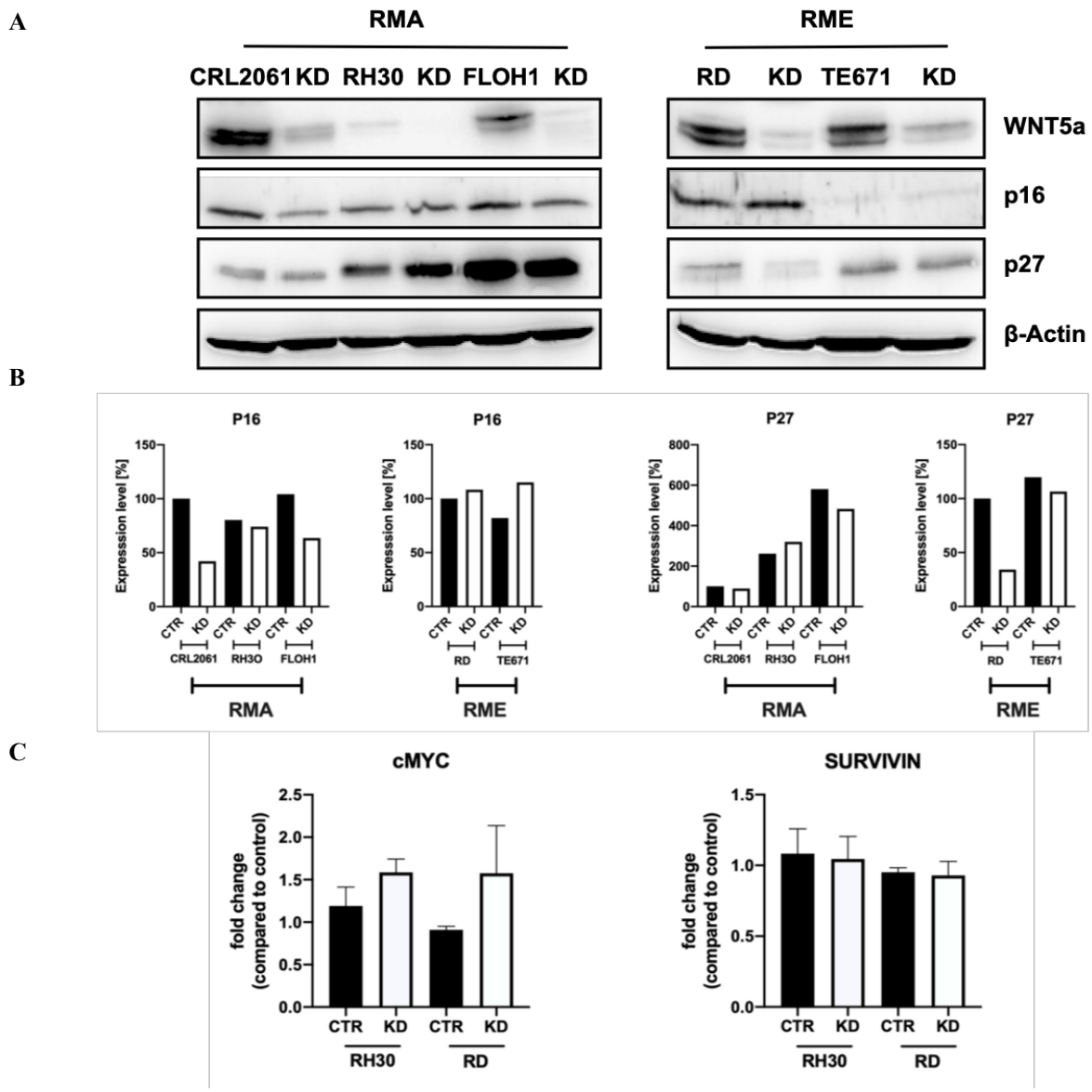

Figure 3.2-4: Influence of WNT5A KD on RMS cell cycle progression in vitro
(A) Western Blot of the cell cycle inhibitors p16 and p27 expression in RMS cell lines after stable WNT5A KD. ß-Actin served as loading control. **(B)** Quantification of the Western Blot showed in **(A)** using ImageJ. Expression of p16 and p27 after WNT5A KD was normalized to ß-actin and compared to untreated cells. The expression in the wild type CRL2061 and RD cells were set to 100% for RMA and RME, respectively. (C) qRT-PCR of MYC and SURVIVIN (BIRC5) genes expression after WNT5A KD. Gene expression of the wild type cells was set to 1. GAPDH expression was used for normalization. Shown is the mean and SEM of three independent experiments measured in duplicates.

3.3 WNT5A KD lead to ß-catenin activation and its subsequent localization to the nucleus

WNT5A has been shown to repress the ß-catenin signaling pathway, but due to pathways cross talk complexity the exact mechanism is not well known (Mikels & Nusse, 2006; Park et al., 2015; Sato, Yamamoto, Sakane, Koyama, & Kikuchi, 2010). In order to investigate whether

the expression of ß-catenin is controlled by the cross talk between the canonical and the non-canonical pathways in RMS tumor cells, the activated form of ß-catenin (the dephosphorylated form) was check in separated cytoplasmic and nuclear protein fractions of RMS after WNT5A KD (and respective controls). Figures 3.3-1A and B show that WNT5A KD lead to stabilization and subsequent nuclear localization of ß-catenin. This effect was significant in the embryonal RMS cell line RD than the alveolar RMS cell line RH30. This cross talk was also confirmed using the TOP/FOP assay, which showed stronger Luciferase activation after WNT5A KD (Figure 3.3-1C). In contrast, WNT5A OE showed reduced ß-catenin protein levels (Figure 3.3-1D).

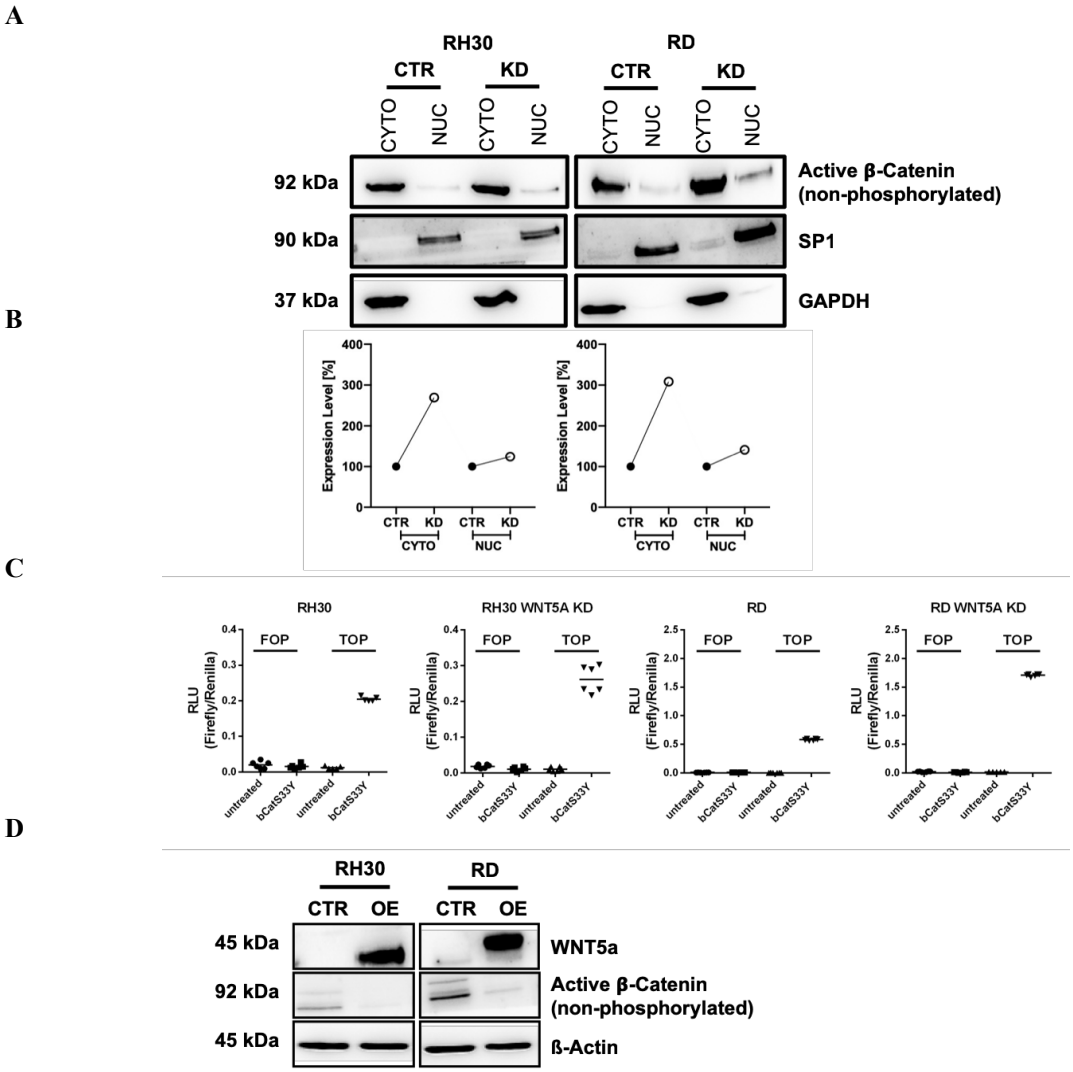

Figure 3.3-1: Influence of WNT5A on ß-catenin expression and subcellular localization in RMS in vitro
(A) Subcellular localization of ß-catenin in RMS cell lines after WNT5A KD. GAPDH served as cytoplasmatic marker and SP1 as nuclear marker to check purity of the protein isolation. CYTO, cytoplasmatic component; NUC, nuclear component. (B) Quantification of the Western Blot shown in (A) using ImageJ. Expression of ß-catenin was normalized to GAPDH and SP1 in case of cytoplasmic and nuclear proteins, respectively and compared to untreated cells. (C) luciferase-based TOP/FOP assay showing the effect of WNT5A KD on RH30 and RD. Values were normalized to a renilla transfection control (D) Western Blot of WNT5A and ß-catenin expression in RMS cell lines after **transient** WNT5A OE. ß-Actin served as loading control.

WNT5A KD also induced a higher expression of the ß-catenin associated transcription factor LEF1, while LEF1 was downregulated in WNT5A OE cell lines (Figure 3.3-2).

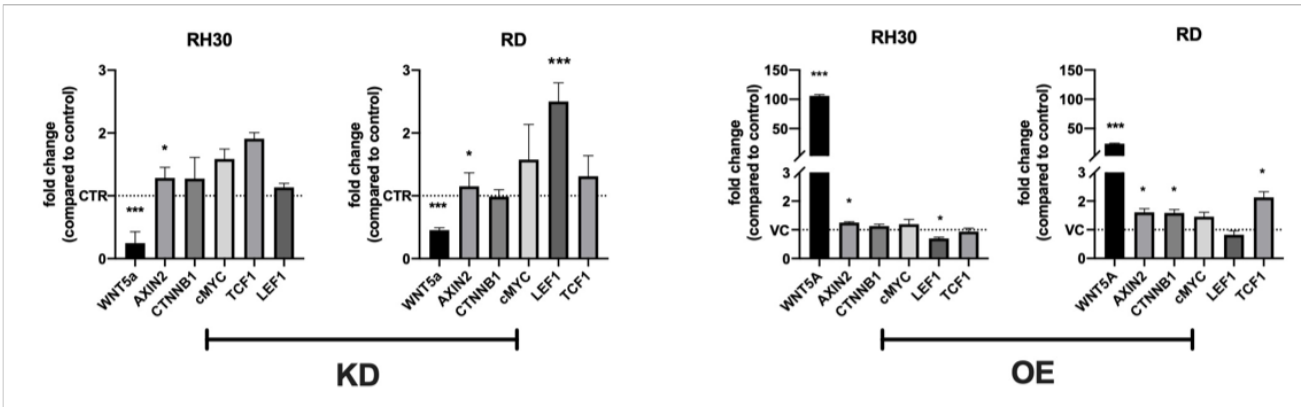

Figure 3.3-2: Influence of WNT5A on ß-catenin target genes expression
qRT-PCR of WNT signaling transcription factors LEF1 and TCF1 genes, in addition to CTTNB1 and its target genes AXIN2, and MYC after WNT5A KD and OE. Gene expression of the CTR and VC was set to 1 for WNT5A KD and OE cell lines, respectively. GAPDH expression was used for normalization. Shown is the mean and SEM of three independent experiments measured in duplicates and compared by t-Test using 95% confidence interval (* 0.05 > p > 0.01 ** 0.01 > p > 0.005, ***0.005 > p > 0.001, ****P<0.0001) by GraphPad PRISM® 8.

3.4 Migration and invasion of RMS tumor cells were induced after WNT5A KD

Since metastasis is the main contributor to cancer mortality, and given the role of the WNT pathway in the progression and metastasis of many cancers (Polakis, 2012), scratch assays in addition to trans-well assays were performed to investigate the influence of WNT5A on RMS tumor cell migration and invasion. Gap closure was faster with RMS cells after WNT5A KD than in control wild type RMS cells (Figure 3.4-1A and B). In order to confirm the results, an experiment using boyden chambers was done. The embryonal RMS cell line RD showed faster migration after WNT5A KD. In contrast, WNT5A OE had virtually no effect on the ERMS cell line RD, while migration of the ARMS cell line RH30 was significantly blocked (Figure 3.4-1C).

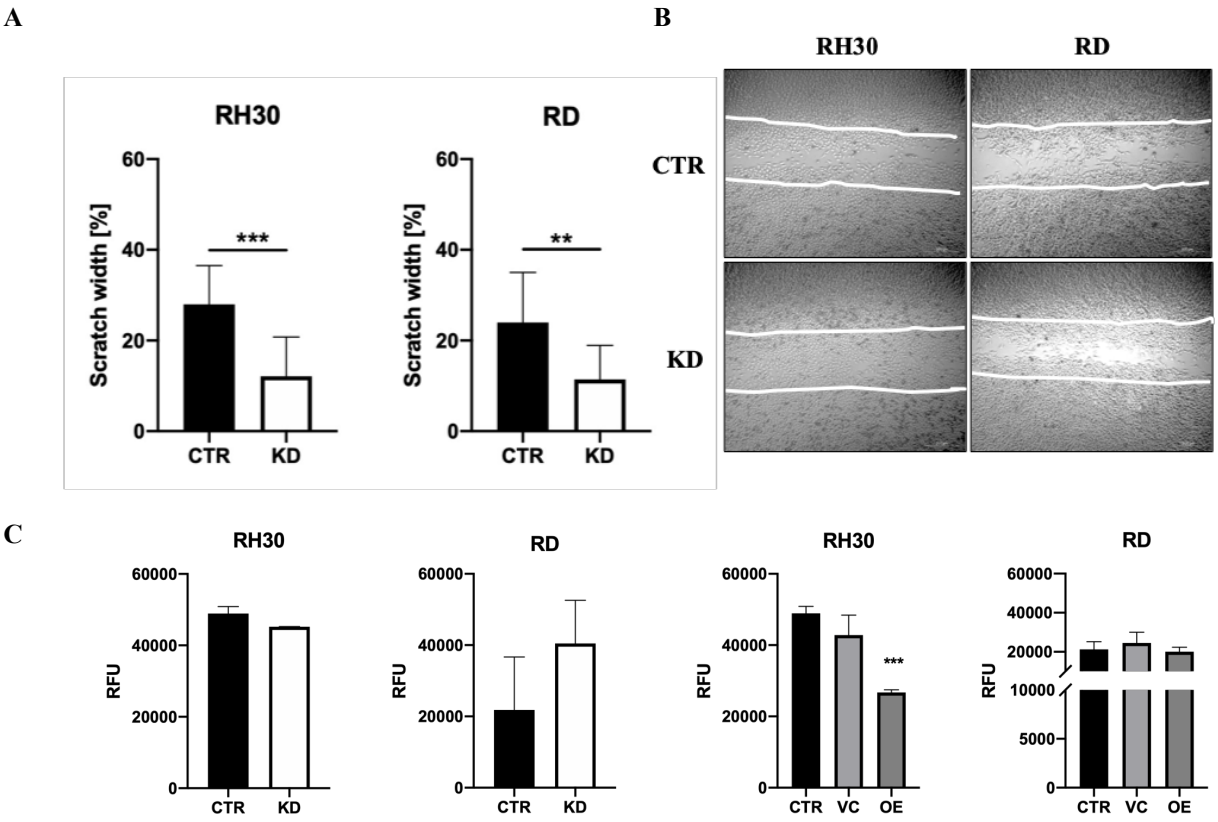

Figure 3.4-1: Influence of WNT5A on the migration and invasion of RMS cells
(A) Scratch assay of RMS cells after WNT5A KD. Scratch width of RMS wild type cells at time zero was set to 100%. After 0, 12 and 24 h, the scratch width at four different positions was measured with ImageJ. The graphs show the mean and SEM of 4 replicates after 24 h. **(B)** Pictures of the scratch in control and WNT5A KD cells after 24 h using phase contrast with a magnification of 100x. **(C)**Transwell assay of RMS cells after WNT5A KD and OE using 10% FCS as chemoattractant, the cells from the underside of the membrane were detached, lyzed, dyed with CyQuant® GR dye solution and fluorometrically quantified by a microtiter plate reader from TECAN at 480 nm/520 nm. The number of invasive cells of the wild type was set to 100%. Bars represent mean and SEM of 3 replicates after 8 h. Data were compared by t-Test using 95% confidence interval (* 0.05 > p > 0.01, ** 0.01 > p > 0.005, ***0.005 > p > 0.001, ****P<0.0001) by GraphPad PRISM® 8.

3.5 WNT5A lead to the induction of stem cell genes in RMS tumor cells and increased sphere formation

Sphere formation assays were performed with cells of the ERMS cell line RD after WNT5A KD and OE. The assays showed an increased number of spheres after WNT5A KD, while the number of spheres was significantly reduced after WNT5A OE, with disintegration of spheres on continued cultivation (Figure 3.5-1A). We speculated that this effect might be attributed to the influence of WNT5A on the expression of the stem cell genes, CD133 and SOX2, as expression of these genes was strongly upregulated after WNT5A KD and down regulated after WNT5A OE (Figure 3.5-1B). Treatment of spheres with rWNT3A to induce the canonical WNT signaling pathway did not change the number of spheres (Figure 3.5-1C). rWNT3A mainly induced expression of SOX2 which was repressed in WNT5A OE cell lines (Figure 3.5-1D).

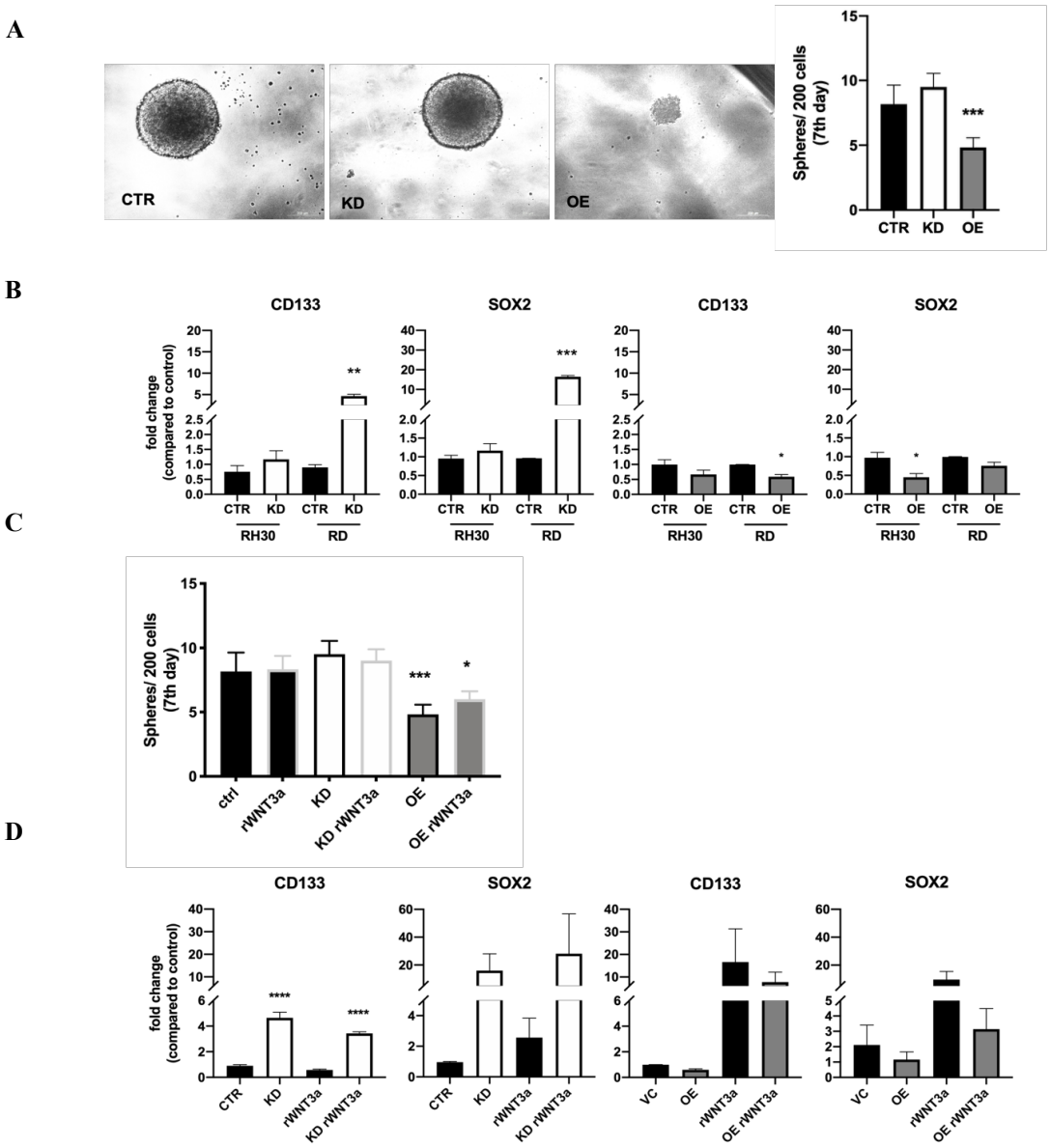

Figure 3.5-1: Influence of WNT5A on RMS cells stem genes expression and sphere formation
(A) Pictures of sphere formation after 7 days in the RME RD cell line. Pictures were taken using phase contrast with a 200-x magnification Bars shows quantification of spheres after WNT5A KD and OE. Shown are the mean values of one experiment performed in 6 replicates. **(B)** qRT-PCR of stem cell genes SOX2 and CD133 after WNT5A KD and OE. GAPDH expression was used for normalization. Shown is the mean and SEM of three independent experiments measured in duplicates. **(C)** Bars show quantification of spheres after long term treatment with WNT3A. Shown are the mean values of one experiment performed in 6 replicates. **(D)** qRT-PCR of stem cell genes SOX2 and CD133 after 48 h of 200ng/ml rWNT3a treatment of WNT5A KD and OE cell lines. Gene expression of the untreated wild type was set to 1. GAPDH expression was used for normalization. Shown is the mean and SEM of three independent experiments measured in duplicates. Data were compared by t-Test using 95% confidence interval (* 0.05 > p > 0.01, ** 0.01 > p > 0.005, ***0.005 > p > 0.001, ****P<0.0001) by GraphPad PRISM® 8.

3.6 WNT5A influenced myogenic markers in RMS tumor cells in vitro

The WNT pathway plays a crucial role in mesenchymal stem cells differentiation (Visweswaran et al., 2015), which is impaired in RMS tumor cells and leads to poorly differentiated tumor with abortive skeletal muscle cell features. However, the reason behind this is poorly

understood (Jothi, Mal, Keller, & Mal, 2013). Therefore, the expression of important myogenic markers was measured in RMS tumor cells after WNT5A KD and OE. The filament protein DESMIN and the fetal AChR gamma-subunit were upregulated after WNT5A KD and downregulated after WNT5A OE, which was more significant on the protein than mRNA level. In both RMA and RME cell lines, the influence of WNT5A on MYOGENIN expression was negligible on the mRNA level, but the myogenin protein was upregulated after WNT5A KD in ERMS RD cells and downregulated after WNT5A OE in RD cells as well as ARMS RH30 cells (Figure 3.6-1A and B).

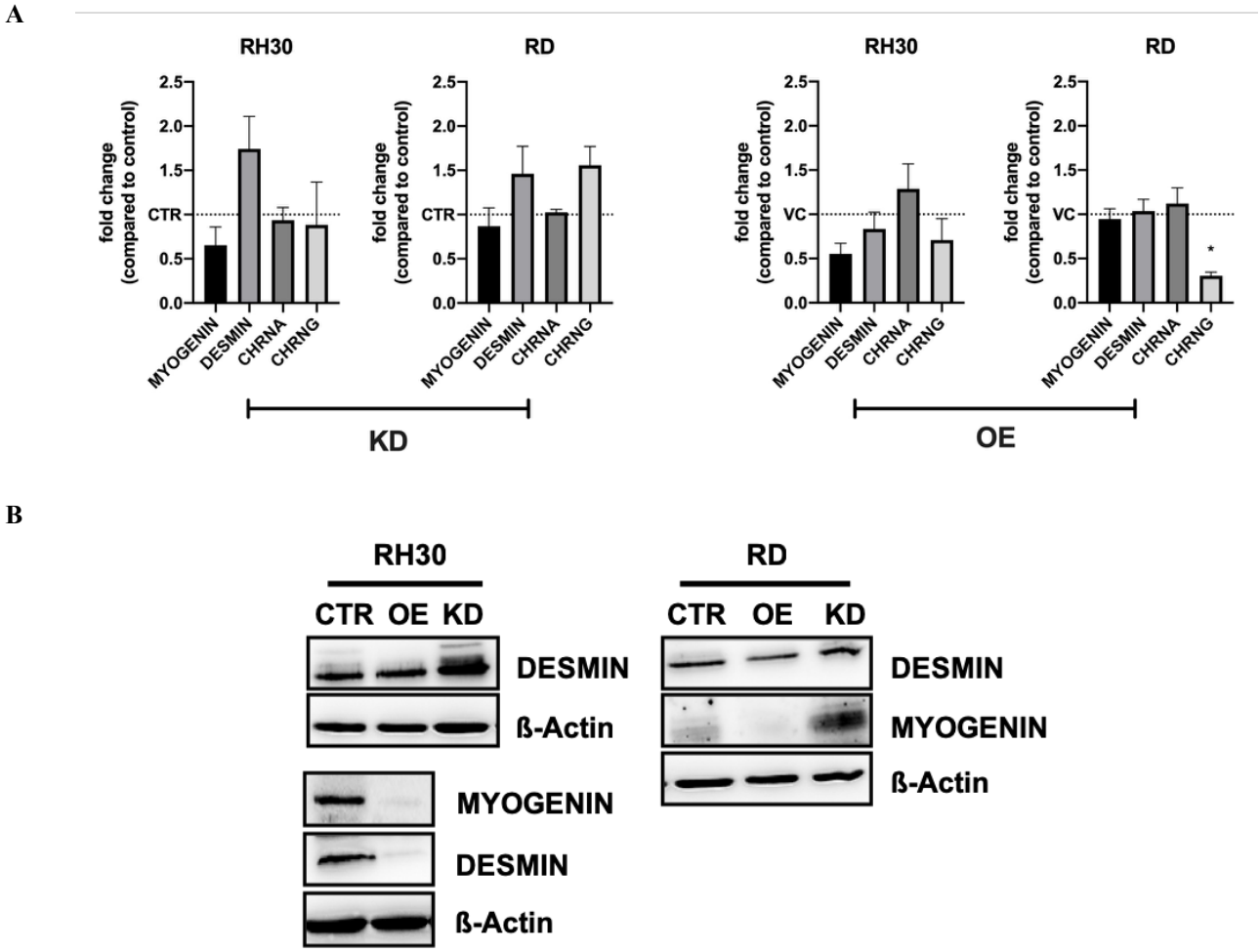

Figure 3.6-1: Influence of WNT5A on myogenic makers in RMS tumor cells.
(A) qRT-PCR of WNT signaling transcription factors LEF1 and TCF1 genes, in addition to CTTNB1 and its target genes AXIN2, and MYC after WNT5A KD and OE. Gene expression of the VC was set to 1. GAPDH expression was used for normalization. Shown is the mean and SEM of three independent experiments measured in duplicates. **(B)** Western Blot of DESMIN and MYOGENIN expression in RMS cell lines after WNT5A KD and OE. ß-Actin served as loading control. Data were compared by t-Test using 95% confidence interval (* 0.05 > p > 0.01, ** 0.01 > p > 0.005, ***0.005 > p > 0.001, ****P<0.0001) by GraphPad PRISM® 8.

3.7 WNT5A influenced the expression of the fetal AChR γ-subunit, which is a target for a CART therapy in RMS tumor cells

As shown in Figure 3.7-1A and B, WNT5A KD in ERMS RD cells but not ARMS RH30 cells significantly induced the expression of the fetal Acetylcholine receptor (fAChR), while WNT5A OE reduced mRNA and protein levels. Therefore, a chimeric antigen receptor (CAR) against fAChR was developed (Figure 3.7-2A) to check whether WNT5A will influence the susceptibility of RMS tumor cells towards fAChR CAR T cells. As shown in Figure 3.7-2B, cytotoxicity assay of alveolar and embryonal RMS cells after coincubation with fAChR CAR-T cells demonstrated that after WNT5A OE the RMS cells became resistant to CAR-T cells. However, WNT5A KD has almost no effect on the killing in vitro compared to the control.

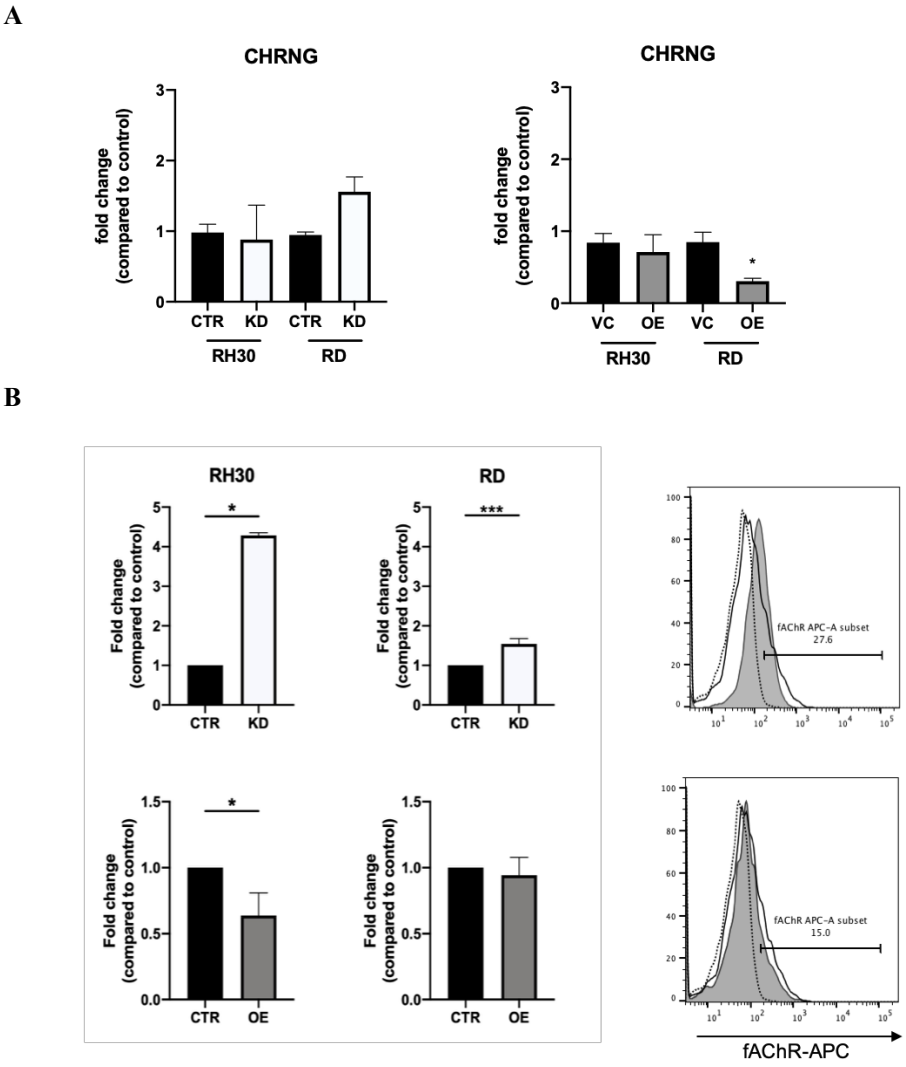

Figure 3.7-1: Influence of WNT5A on CHRNG gene expression
(A) qRT-PCR of CHRNG after WNT5A KD and OE. GAPDH expression was used for normalization. Shown is the mean and SEM of three independent experiments measured in duplicates. **(B)** Flow cytometry analysis of fAChR expression in RMS cell lines after WNT5A KD and OE. The bars show the mean and SEM of three independent experiments. Data were compared by t-Test using 95% confidence interval (* 0.05 > p > 0.01, ** 0.01 > p > 0.005, ***0.005 > p > 0.001, ****P<0.0001) by GraphPad PRISM® 8.

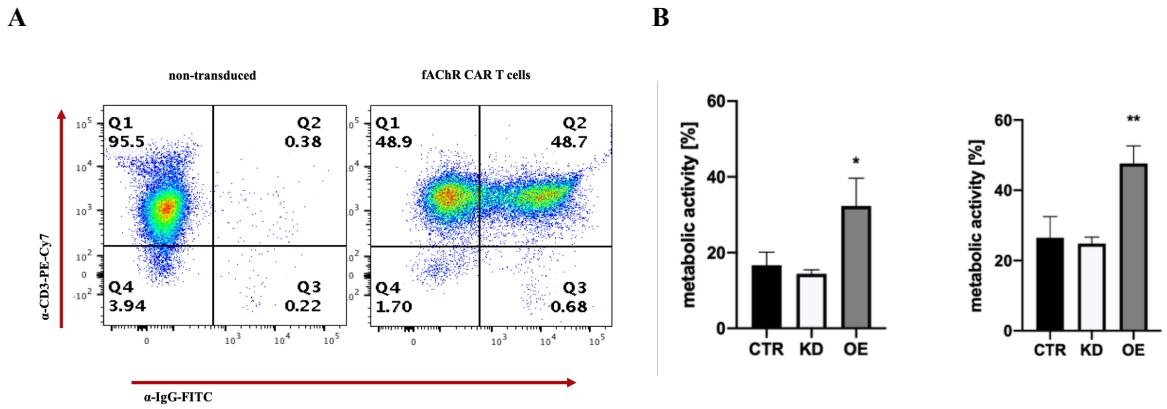

Figure 3.7-2: Impact of WNT5A KD and OE on the survival (metabolic activity) of RMS cells after coculturing with fAChR CAR T cells, i.e. T cells expressing a chimeric antigen receptor (CAR) directed to the fetal acetylcholine receptor (fAChR).
(A) Flow cytometric analysis of CAR expression on the surface of fAChR CAR T cells that were derived from peripheral blood T lymphocytes (non-transduced controls) on transduction with an expression vector encoding a CAR directed to the fAChR (Keller & Guttridge, 2013). **(B)** Survival of manipulated (KD, OE) and control (CTR) ERMS RD cells and ARMS RH30 after 48 h of coincubation with fAChR specific CAR T cells (2.5 x 10^4) measured by MTT. Survival of RMS cells coincubated with PBMCs (6.25 x 10^3) was set to 100% (not shown). Graphs represent the mean value and SEM of triplicates. Data were compared by t-Test using 95% confidence interval (* 0.05 > p > 0.01, ** 0.01 > p > 0.005, ***0.005 > p > 0.001, ****P<0.0001) by GraphPad PRISM® 8.

3.8 WNT5A lead to the activation of the WNT/PCP pathway in RMS tumor cells in vitro

WNT5A is known to activate the non-canonical pathway which is subdivided into two main signaling pathways WNT/Ca^{2+} and WNT/PCP (Asem, Buechler, Wates, Miller, & Stack, 2016; Endo, Nishita, & Minami, 2012; Klaus & Birchmeier, 2008; MacMillan et al., 2014). In order to investigate which non-canonical pathway is involved in RMS tumor cell pathogenesis, different key proteins of the non-canonical pathway were detected by WB after WNT5A KD and OE. Since WNT/Ca^{2+} pathway effector proteins NFAT and NLK are activated through Ca^{2+} release (De, 2011), Ca^{2+} flux after WNT5A KD and OE was firstly detected by Ca^{2+} flux assay. A reduced and an enhanced calcium release in RMA cell line RH30 were detected after WNT5A KD and OE, respectively, which indicated a positive correlation between calcium release and WNT5A signaling in RH30 cells, while this effect was insignificant in the RME cell line RD (Figure 3.8-1A and B). At the protein level, WNT5A did not influence the expression of WNT/Ca^{2+} effector proteins NLK and NFAT in the RME cell line RD (Figure 3.8-2A). In contrast, the activation of c-JUN was influenced by WNT5A: WNT5A KD abolished phospho-c-JUN expression, while its expression was upregulated after WNT5A OE (Figure 3.8-2B).

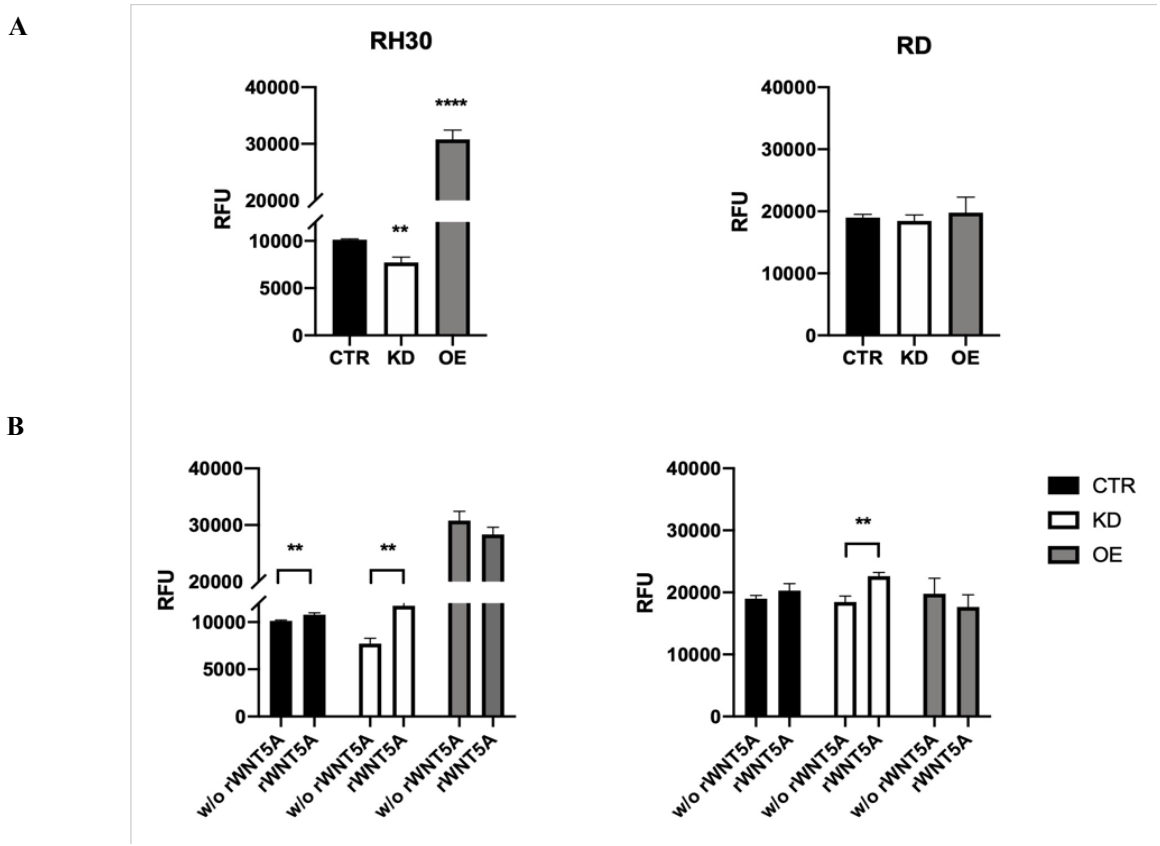

Figure 3.8-1: Influence of WNT5A on intracellular calcium changes and the protein expression of non-canonical pathway components after WNT5A KD and OE
(A) Calcium flux assay of the indicated RMS cell lines after WNT5A KD and OE. **(B)** Calcium flux assay of the indicated RMS cell lines after WNT5A KD and OE with and without 200 ng rWNT5A treatment after 1 h. Graphs represent the mean value and SD of triplicates. Data were compared by t-Test using 95% confidence interval (* $0.05 > p > 0.01$, ** $0.01 > p > 0.005$, ***$0.005 > p > 0.001$, ****$P<0.0001$) by GraphPad PRISM® 8.

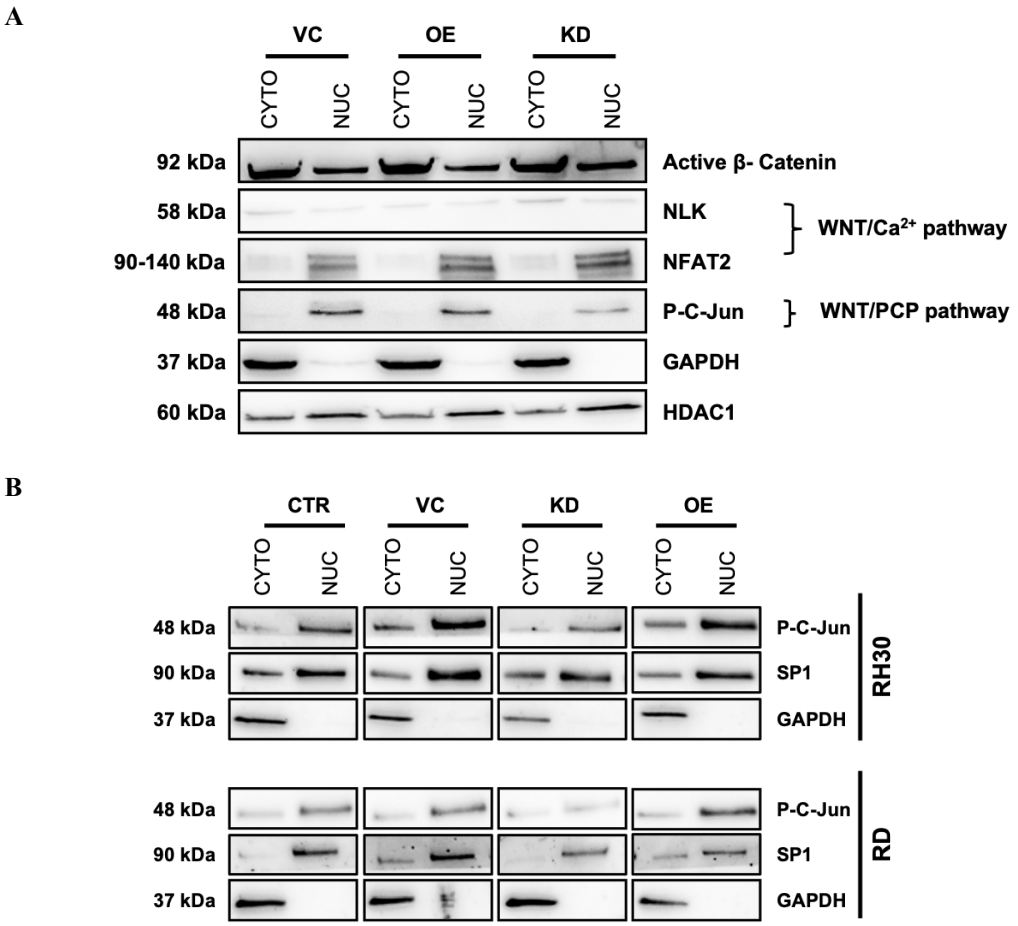

Figure 3.8-2: Influence of WNT5A on the protein expression of non-canonical pathway components after WNT5A KD and OE
(A) Subcellular localization of ß-catenin and non-canonical pathway components in RD cell line after WNT5A KD and OE. GAPDH served as cytoplasmic marker and HDAC1 as nuclear marker to check purity of the protein isolation from different compartments. CYTO, cytoplasmic; NUC, nuclear. **(B)** Subcellular localization of P-c-JUN in RH30 and RD cell lines after WNT5A KD and OE. GAPDH served as cytoplasmic marker and HDAC1 as nuclear marker to check purity of the protein isolation from different compartments. CYTO, cytoplasmic; NUC, nuclear.

3.9 WNT5A KD induced faster tumor growth of RME in vivo

In a xenograft model, a faster tumor cell growth was observed for the WNT5A KD RME cell line TE671 compared to the control, whereas the ARMS cell line RH30 did not show any difference in tumor growth (Figure 3.9-1A and B). As observed above for the RME tumor cell line RD in vitro, CD133 and SOX2 were upregulated in the WNT5A KD ERMS TE671 tumor cells in vivo (Figure 3.9-1C). By contrast, MYOG and CHRNG were downregulated (Figure 3.9-1D). RD cell line didn't grow tumor when injected to the mice, so TE671 as RME cell line was used instead.

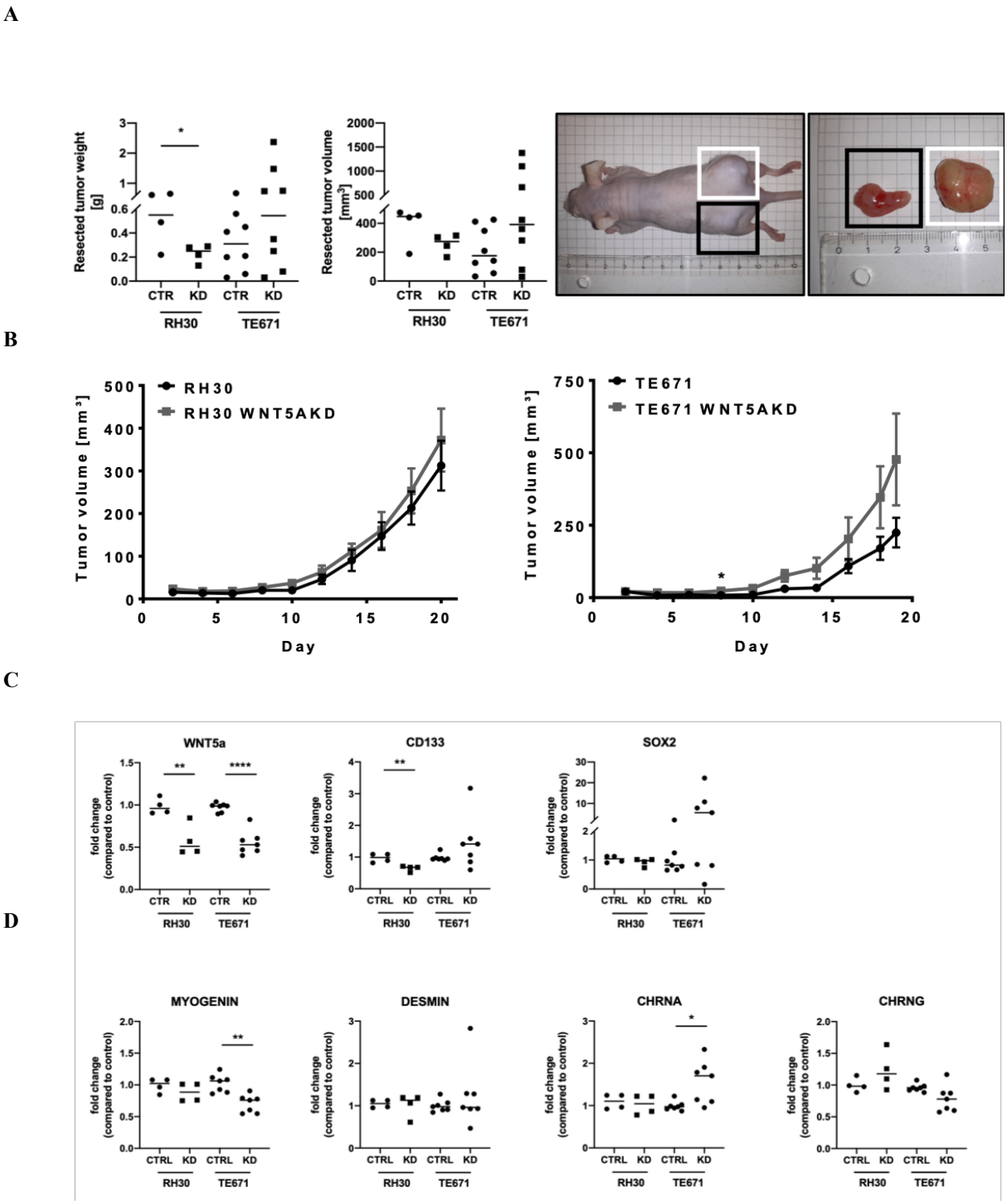

Figure 3.9-1: Influence of WNT5A on RMS tumor weight and volume in a xenograft model
(A) Graphs show weights and volumes of resected RMS tumor 20 days after transplantation in nude mice. Tumor volume was calculated with the modified ellipsoidal formula (Tumor volume= width× width × length × 0.52). The photographs show the mouse in addition to the resected tumor after 20 days of transplantation with the wild type TE671 cell line (black square) and the WNT5A KD TE671 cell line (white square). **(B) (C)** qRT-PCR of stem cell genes SOX2 and CD133 of mRNA extracted from the resected tumor. GAPDH expression was used for normalization. Gene expression of the wild type was set to 1. **(D)** qRT-PCR of muscle differentiation genes MYOGENIN, DESMIN, CHRNA and CHRNG of mRNA extracted from the resected tumor. GAPDH expression was used for normalization. Gene expression of the wild type was set to 1. Data were compared by t-Test using 95% confidence interval (* 0.05 > p > 0.01, ** 0.01 > p > 0.005, ***0.005 > p > 0.001, ****P<0.0001) by GraphPad PRISM® 8.

4 DISCUSSION

WNT signaling pathways control many developmental processes and contribute to a variety of human cancers. In mammals, WNT signaling is divided into the β-catenin-dependent canonical, and the β-catenin-independent non-canonical WNT signaling pathways. To our knowledge, nothing is known about the role of the **non-canonical** pathway in RMS pathogenesis. In contrast, virtually all previous studies focused on canonical WNT/β-catenin signaling, which was found to be activated and associated with the initiation and maintenance of many childhood malignancies like Ewing sarcoma (Pedersen et al., 2016), osteosarcoma (Fang et al., 2018), retinoblastoma (Wu, Wang, Tang, & Sun, 2017), childhood T-ALL (Ng et al., 2014) and Hepatoblastoma (Mavila & Thundimadathil, 2019).

The results of studies done to investigate the role of **canonical** WNT signaling pathway in RMS pathogeneses were controversial. Annavarapu and colleagues described that stimulation of WNT/β-catenin pathway by rWNT3A had tumor suppressive effect, but only in RMA. They reported an induction of MYOG, MYOD, and MYF5 expression in response to the subsequent translocation of β-catenin into the nucleus, as well as inhibiting RMS cell growth in the RMA cell lines RH4 and RH30 but not in the RME cell lines RD and RD18 (Annavarapu et al., 2013). Singh et al. and Chen et al. showed similar results, but used either LiCl or a GSK3 inhibitor to activate the canonical WNT/β-catenin pathway. In contrast, expression of muscle differentiation genes was induced only in RME but not in RMA as shown by Annavarapu (Annavarapu et al., 2013; Singh et al., 2010).

Contrary to these studies, we showed in a previous work that activation or inhibition of canonical WNT/β-catenin signaling in vitro affected proliferation, apoptosis and myodifferentiation of RMS tumor cells only marginally. Moreover, the conditional knockout of β-catenin in Ptchdel/+ RMS mice, which spontaneously developed murine RMS, did not alter RMS incidence or multiplicity (Ragab et al., 2018). The study done by Bharathy et al. is in line with this result, showing that activation of the WNT/β-catenin pathway by using an irreversible GSK3β inhibitor had no effect on growth and myodifferentiation of patient-derived xenografts (Bharathy et al., 2017). Based on these findings, we assumed that either the canonical pathway does not play a role in RMS pathogenesis or there is another pathway, which inhibits the canonical pathway at the transcriptional level.

It was the aim of this study to explore the influence of WNT5A, as a non-canonical pathway ligand, on the 'aggressiveness' of RMS cell lines in vitro and in vivo, and its impact on the WNT/β-catenin pathway. The study showed that WNT5A has a tumor suppressive effect in terms of inhibiting proliferation, stemness and migration of RMS tumor cells by antagonizing WNT/β-catenin pathway. In addition, it was found that WNT5A KD induced RMS myodifferentiation by upregulating fAChR, which made it an attractive target for fAChR-specific CAR T cells.

It is known that there is a crosstalk between the canonical WNT/β-catenin and non-canonical pathways, as the non-canonical pathway inhibits WNT/β-catenin pathway, however, due to pathways complexity, the exact mechanism is not totally understood (Mikels & Nusse, 2006). In this study we found that WNT5A is strongly expressed in the less aggressive RME and PFmin RMA compared to PFplus RMA. WNT5A expression was analyzed on both mRNA and protein level in different RMS cell lines and found to be overexpressed in the RME cell lines RD and TE671 compared to the RMA cell lines CRL2061, RH30 and Rh41. Independent validation dataset was also measured via qRT-PCR to evaluate the expression level of WNT5A in RMS tumors compared to normal skeletal muscle. A significant higher WNT5A expression ($p - 0.015$) was detected in RME tumors, while RMA tumors have an expression level comparable to that of skeletal muscle samples. Our results were supported by data that we retrieved from two publicly available datasets (Davicioni et al., 2009; Williamson et al., 2010).

WNT5A was previously found to play different roles in carcinogenesis depending on the tumor entity, exerting either tumor-suppressive or oncogenic functions. In nasopharyngeal cancer (NPC), WNT5A exerted oncogenic effect, as WNT5A induced NPC cells migration and invasion, in addition, it was correlated with inducing pulmonary metastases in NPC patients (Qin et al., 2015). Similar to NPC, WNT5A induced cancer cells migration and invasion in gastric carcinoma (Liu et al., 2013), oral squamous cell carcinoma (Prgomet, Axelsson, Lindberg, & Andersson, 2015) and melanoma (Sinnberg et al., 2018). WNT5A also induced proliferation of non-small-cell lung cancer (NSCLC) (Y. Huang et al., 2010) and human glioblastoma (Yu et al., 2007). On the other hand, the tumor-suppressive effect of WNT5A was shown in prostate cancer cell as it induced apoptosis and reduced proliferation (Thiele et al., 2015). This was also shown for hematopoietic malignancies (Liang et al., 2003) as well as inhibition of proliferation and migration of colorectal cancer (Castell & Larsson, 2015) and breast cancer (Safholm et al., 2008), respectively.

The ambiguous role of WNT5A in carcinogenesis might be due to the fact that WNT5A can activate different WNT pathways depending on receptors context (FZD receptors) or cofactors (LRP5/6, ROR1/2 coreceptors), that can be differentially expressed by individual tumors (van Amerongen & Nusse, 2009).

For RMS tumor cell lines, we found that typical receptors for the WNT5A mediated non-canonical pathway were expressed higher in the RME cell lines compared to the RMA cell lines. Especially FZD 2,3,6 and 7 in addition to the non-canonical pathway coreceptors ROR1 and ROR2 were found to be active in the RME cell liens compared to the RMA cell lines. The difference in the expression of ROR1 and ROR2 in ARMS and ERMS cell lines was supported by the two publicly available data sets, as ERMS showed higher expression of ROR1 ($p - 0.0177$, $p - 0.0001$ in Davicioni et al. and Williamson et al. data sets, respectively), and ROR2 ($p - 0.0097$ in Davicioni et al. data set) (Davicioni et al., 2009; Williamson et al., 2010).

In order to investigate the impact of WNT5A on RMS aggressiveness and to figure out which other roles WNT5A might play in RMS, stable WNT5A KD and OE RMS cell lines were established. We found that after WNT5A KD, RMS cells showed a higher proliferation rate, associated with an increased amount of S phase cells. This was more significant in the RME cell lines compared to RMA cell lines. On the other hand, proliferation of RMS tumor cells was inhibited after WNT5A OE in RH30 and RD cell lines. We confirmed that this effect was exerted by WNT5A through treating WNT5A KD cell lines with rWNT5A, which lead to proliferation inhibition.

Our results demonstrated that the induction of proliferation after WNT5A KD could partially be explained by the induction of the pro-proliferative gene, like cMYC as well as the expression of cell cycle inhibitors p16 and p27, however, this effect was cell lines specific. P16 and p27 are regulatory proteins that inhibit cell cycle progression, and their expression was found to be altered in different tumors like lung and bladder cancer (Ciesielska et al., 2017; Wong et al., 2001). cMYC, in contrast, was found to be amplified in many tumors and correlates to disease aggressiveness like in neuroblastoma (Beltran, 2014) and colorectal cancer (Castell & Larsson, 2015). In a study made by Wiegering and colleagues, it was described that inhibiting cMYC translation lead to inhibition of MYC-dependent proliferation of colorectal tumor cells in vitro and in vivo (Castell & Larsson, 2015). cMYC itself is also a direct target gene of the canonical WNT signaling pathway (Ghoshal & Ghosh, 2016) suggesting a direct interaction of cMYC overexpression and activation of the canonical WNT signaling pathway (Rennoll & Yochum, 2015).

The influence of WNT5A on RMS cells proliferation is similar to what Cheng et al. observed in colon cancer. They demonstrated that WNT5A OE inhibited cell proliferation, while proliferation was induced after WNT5A KD. This effect was due to the antagonizing effect of WNT5A on the canonical WNT signaling in general and the subsequent induction of cMYC expression (Cheng et al., 2014). A similar tumor suppressor effect of WNT5A was also described by Wang et al. in hepatocellular carcinoma. They performed a cell cycle analysis after WNT5A expression in a hepatocellular carcinoma cell line and found that WNT5A OE lead to a significant reduction of cells in S phase (T. Wang et al., 2019). In addition, these WNT5A overexpressing cells showed diminished migration in association with increased level of phosphorylated β-catenin.

In the present study, comparable effects were detected in RMS tumor cells, in which WNT5A OE contributed to the inhibition of migration and invasion of RMS cells, while WNT5A KD promoted migration. We assume that the ability of WNT5A to inhibit RMS cells migration is analogous to its role in antagonizing the WNT/β-catenin pathway as shown for colon cancer and hepatocellular carcinoma (Cheng et al., 2014; T. Wang et al., 2019).

Expression of WNT5A was shown to correlate with reduced metastatic spread of many tumors. Canesin et al. reported that prostate cancer patients with high WNT5A expression showed better prognosis compared to patients with low expression. By using the WNT5A mimicking peptide, foxy-5 in $WNT5A^{low}$ cancer cells, metastasis to regional and distal lymph nodes could be reduced by 90% and 75%, respectively, in an orthotopic mouse model. They further concluded in that study that WNT5A inhibits cancer cell invasion through different mechanisms, including cMYC downreagulation (Canesin et al., 2017). The same group studied the influence of WNT5A on breast cancer cells, in which they found that intraperitoneal injections of foxy-5 following the inoculation of breast cancer cells reduced their metastatic spread (Safholm et al., 2008).

Based on what was described, we found that β-catenin was activated after WNT5A KD leading to its stabilization and subsequent localization in the nucleus, which was more significant in the RME cell line RD. Stronger Luciferase activation in a performed TOP/FOP assay was observed especially for the RME cell line RD. In contrast, WNT5A OE showed reduced β-catenin protein levels. We also found that WNT5A KD induced a higher expression of the β-catenin associated transcription factor LEF1, which was downregulated after WNT5A OE. Therefore, we hypothesize that the tumor suppressive effect of WNT5A in RMS cells is due to its ability to antagonize the WNT/β-catenin pathway. These observations support our previous studies with

RMS tumors, in which the canonical WNT-β-catenin pathway seems to play only a marginal role (Drager et al., 2017; Ragab et al., 2018).

To further identify the different downstream components of the non-canonical pathways after WNT5A KD and OE, the expression of NLK, NFAT and p-c-JUN were analyzed. Although NLK and NFAT do not correlate with WNT5A expression, p-c-JUN showed a strong positive correlation with WNT5A expression and was downregulated after WNT5A KD.

NFAT and NLK have a dual role in tumorgenesis as they induced cell proliferation in breast cancer (X. Huang et al., 2015; Iampietro, Gravel, & Flamand, 2014), while they have tumorsuppressive effect in hepatocellular carcinoma (Xu et al., 2018) and non-small cell lung cancer (Shi et al., 2019), respectively.

c-JUN is part of the AP1 transcription factor and plays a role in tumorigenesis. A study done by Tseng et al., showed that Caffeic acid phenethyl ester (CAPE) treatment suppressed the migration and invasion of prostate cancer cells through WNT5A mediated activation of WNT/PCP and inhibition of WNT/β-catenin pathway. After CAPE treatment, they observed that the non-canonical signaling proteins ROR2, WNT5A and phospho-JNK, that phosphorylates cJUN, were upregulated, while, the β-catenin dependent signaling target proteins, nuclear β-catenin, c-Myc and cyclin D1 were suppressed (Tseng et al., 2016).

Beside the tumor suppressive effect of WNT5A, WNT5A KD also promoted the myodifferentiation of RMS. We showed that the filament protein DESMIN and the fAChR were upregulated after WNT5A KD and downregulated after WNT5A OE. This effect might be due to LEF1 upregulation induced by WNT5A KD. This is supported by our previous observation, that myodifferentiation of RMS is mainly dependent on LEF1 and TCF transcription factors, rather than β-catenin activation (Drager et al., 2017).

Although some differentiation genes were induced by WNT5A KD, we also found a stronger expression of the stem cell genes, SOX2 and CD133 in RMS cells after WNT5A KD, which affected RMS sphere formation. The sphere formation assay showed increased number of spheres after WNT5A KD, while the number of spheres were significantly reduced and spheres disintegrated during prolonged cultivation after WNT5A OE.

This observation is in line with a study by Osman et al.. These authors demonstrated a reduced number of cancer stem cells in colon cancer after WNT5A treatment. They intraperitoneally injected nude mice with foxy-5 after the presentation of palpable xenograft tumor in the animals and observed a reduced number of cancer stem cells (CSCs) in tumor tissue. However, they

could not elucidate the mechanism by which foxy-5 reduced the number of colonic CSCs (Osman, Bellamkonda, Liu, Andersson, & Sjolander, 2019). In addition, a study by Ying et al. showed that WNT5A is silenced in most CRC cell lines and tumors, and that WNT5A expression could be reactivated by pharmacologic or genetic demethylation. They found that WNT5A ectopic expression lead to an inhibition of tumor cell clonogenicity by antagonizing the WNT/β-catenin pathway (Ying et al., 2008).

We confirmed the tumor suppressive effect of WNT5A also in vivo by inoculating RMS wild type and WNT5A KD cell lines RH30 and TE671 in nude mice, which was kindly performed and provided by the Institute of Human Genetics, AG Hahn, Göttingen. We found that WNT5A KD in RMS xenografts resulted in the formation of tumors with higher volumes and weights compared to controls. However, this effect was observed in the RME cell line TE671, rather than in the RMA cell line RH30. The observed difference between in vitro and in vivo data for the RMA cell line is a matter of speculation. In vitro experiments lead to data corresponding to a pure culture, while the role of the tumor microenvironment in in vivo experiments should not be underestimated. This is all the more relevant since WNT5A is a secretable factor that might influence the cells in the surrounding of the formed tumor.

We also isolated mRNA from the explanted tumor tissue and analyzed it for gene expression, which showed significant upregulation of the transcription factor, LEF1 and of the stem cell genes, CD133 and SOX2 only in the RME cell line TE671. These findings were in line with the results of our vitro experiments (Figure 3.3-2, 3.5-1A).

The same influence of WNT5A on colonic tumor growth was reported by Cheng et al., who showed that after WNT5A OE, tumors of smaller size were formed compared to control which was associated with a decreased expression of cytoplasmic/nuclear β-catenin (Cheng et al., 2014). This was also noticed in hepatocellular carcinoma; with formation of smaller tumors after the subcutaneous transplantation of WNT5A OE cells compared to control (T. Wang et al., 2019).

Considering the fact that the KD of WNT5A enhanced the expression of fAChR expression in RMS cells, such modified RMS tumor cells may become good target for fAChR-specific CAR T cells that have been engineered in our lab (K. Simon-Keller, S. Barth, A. Vincent, & A. Marx, 2013). Despite the upregulated expression of fAChR after WNT5A KD, WNT5A KD has almost no effect on the killing of RMS *in vitro* compared to the control. We hypothesized that improved CAR T cell-mediated killing of RMS cells due to their higher fAChR levels is counteracted by WNT5A KD-driven higher proliferation.

The receptors/coreceptors expression and the strong WNT5A expression in the less aggressive RME make us hypothesize that the WNT5A-driven non-canonical WNT signaling pathway plays an important role in the pathogenesis of RMS.

Risk stratification and treatment outcomes for RMS patients are counted on clinical and histologic features but not prognostic markers. Discovering new prognostic molecular markers is needed to improve patients' outcome by precisely customizing the current therapy to match each patient risk. This might also help in defining new therapeutic targets (Arnold & Barr, 2017). The fact that WNT5A and ROR1/2 coreceptors were found to be upregulated in the less aggressive RME, which makes it warranted to check, whether WNT5A in combination with ROR1/ROR2 may be of prognostic value in RMS tumors. However more studies should be done to investigate whether this combination can be applied as prognostic and predictive biomarkers in the clinic.

The current findings also suggest that WNT5A could potentially be a therapeutic target. In a study done by Prasad et al., they described WNT5A as an attractive anti-metastatic therapeutic approach for WNT5A-negative breast cancer patients by using the WNT5A mimicking peptide foxy-5, which reached phase 1 clinical trial (Prasad, Manchanda, Mohapatra, & Andersson, 2018). This approach would be promising in treating RMS with low WNT5A expression, which can be accompanied with dual CAR T cells targeting both fAChR and ROR1/ROR2 coreceptors.

In any case, this study will open new therapeutic perspectives in medicine which is urgently needed, since the survival rates of RMS patients are very unsatisfactory and have stagnated since the 1990s (C. Chen et al., 2019).

5 SUMMARY

Rhabdomyosarcomas (RMS) are common pediatric soft tissue tumors, show skeletal muscle features, unsatisfying responses to current therapies and a poor outcome that reflects the limited knowledge of RMS' pathogenesis. Since WNT signaling impacts skeletal muscle development, our preliminary expression study addressed this pathway in RMS cell lines, revealing over-expression of WNT5A, i.e. a ligand that activates the non-canonical branch of the WNT pathway in the less aggressive RME. Therefore, the current study had the following **aims:** 1. Assess the impact of WNT5A on functional features of RMS. 2. Investigate the crosstalk between the canonical and the non-canonical pathway in RMS and its impact on RMS features, particularly on skeletal muscle differentiation *in vitro*. 3. Figure out which non-canonical pathway plays a role in RMS tumorigenesis? 4. Assess the impact of overexpressed or downregulated non-canonical WNT signaling in RMS xenografts. 5. Determine whether WNT5A-mediated signaling affect the susceptibility of RMS towards an established, fAChR CAR T cell based immune therapy?

The spectrum of **experimental methods** comprised molecular (genes and proteins expression) and functional studies (proliferation, migration, invasion and sphere formation assays) of various representative RMS cell lines following stable WNT5A knock down and over-expression in vitro and an RMS xenograft model to validate the impact of WNT5A on RMS proliferation in vivo. The **key results** were the following: WNT5A attenuated RMS tumor cells proliferation, migration, invasion and sphere formation ability. These effects were accompanied by decreased ß-catenin nuclear localization and the expression of the associated transcription factor LEF1. In the xenograft model, faster tumor growth was observed for the WNT5A KD embryonal RMS cell line compared to control, whereas the alveolar RMS cell line didn't show any difference in tumor growth.

Additionally, WNT5A inhibited myogenic markers DESMIN and fetal AChR subunit gamma expression in RMS tumor cells, which made the RMS cells resistant to fAChR CAR-T cells. WNT5A effect on RMS tumorigenesis was associated with planar cell polarity (PCP) pathway regulation as WNT5A induced phospho-c-JUN expression.

In conclusion we hypothesized that the non-canonical WNT signaling pathway derived by WNT5A plays a role in RMS tumorgenesis and that it's associated with a more favorable RMS phenotype. These findings indicate that WNT5A could be a promising diagnostic and therapeutic target.

6 REFERNCE

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7 APPENDIX

7.1 Tissue samples

Table 7.1: RMS biopsies used in this work

	Embryonal RMS (n=10)	Alveolar RMS (n=10)
Age (years)	3.6 (±2.9)	12.3 (±3.4)
Female:male ratio	4:6	6:3; NK, *n* = 1
Tumor size (cm)	≤5, *n* = 2; >5, *n* = 8	≤5, *n* = 1; >5, *n* = 8; NK, *n* = 1
Tumor stage	I, *n* = 1; II, *n* = 1; III, *n* = 5; IV, *n* = 3	III, *n* = 3; IV, *n* = 7
Tumor localization	EXT, *n* = 1; OTH, *n* = 6; PM, *n* = 1; NBP, *n* = 1; BP, *n* = 1	EXT, *n* = 4; OTH, *n* = 3; PM, *n* = 3
Overall survival (years)	4.7 (±3.63)	2.1 (±0.92)
Disease free survival (years)	3.8 (±3.9)	1.5 (±1.2)

BP, bladder/prostate; EXT, extremities; NBP, genitourinary tract (not bladder/prostate); NK, not known; OTH, other sites; PM, parameningeal (Dantonello et al., 2008).

8 CURRICULUM VITAE

Personal Information

Name and first name	Nada Ragab
Birth date:	11/07/1992
Birth place:	El Beheira, Egypt
Marital status:	Single
Father:	Ibrahim Ibrahim Ragab
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School education

July 2009	High School Diploma, Nasser Privet School, Damanhour, Egypt (Grade: 96.9%)

University education

(2009) – (2014)	Bachelor degree, Faculty of Pharmacy, Alexandria university, Egypt
(06.2014)	cGPA: 3.8/4
(2016) – (2017)	Master's degree, Medical faculty of Mannheim, Heidelberg university, Germany (Characterization of the non-canonical WNT pathway and its role in antagonizing the canonical WNT/ β-catenin pathway in Rhabdomyosarcoma tumor cell.)
(16.08.2017)	cGPA: 1.5/1

Publications

Ragab, N., Viehweger, F., Bauer, J., Geyer, N., Yang, M., Seils, A., . . . Simon-Keller, K. (2018). Canonical WNT/beta-Catenin Signaling Plays a Subordinate Role in Rhabdomyosarcomas. *Front Pediatr, 6*, 378.

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