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The role of Wnt-Frizzled pathway in neuroblastoma

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## Abstract

Neuroblastoma is a tumor that arises from developing of the peripheral sympathetic nervous system and is the second most common extracranial malignant tumor of childhood. Previous studies have shown that aberrant regulation of wingless/int1 (Wnt)/Frizzled (FZD) signaling pathways may lead to cancer. However, it is not clear whether the Wnt/FZD signaling pathways are involved in neuroblastoma growth.

The aim of this study was to investigate the role of Wnt-FZD2 signaling pathways in neuroblastoma cells and the crosstalk with macrophages. For this study the *MYCN*-single copy SK-N-AS and *MYCN*-amplified SK-N-DZ high-risk neuroblastoma cells and tumor samples from established SK-N-AS and SK-N-DZ xenograft models were used. Neuroblastoma cell lines were treated with recombinant Wnt3a, and Wnt5a protein or FZD2-siRNA to determine the effects on malignancy-defining features of cancer cells such as proliferation and migration. In addition, activation of Wnt signaling pathway components was examined by Western blot analysis. Furthermore, the correlation between FZD2-signaling in neuroblastoma cells and the recruitment and number of tumor associated macrophages (TAMs) was studied in tumor xenograft tissue.

The results demonstrate that stimulation of FZD2 by Wnt3a and Wnt5a regulates  $\beta$ -catenin-dependent and -independent Wnt signaling factors. FZD2 knockdown suppressed canonical Wnt signaling activity but also increased phosphorylation of PKC, AKT, and ERK *in-vitro*, consistent with upregulation of non-canonical Wnt signaling activity. The number of TAMs in tumor tissue was not affected by treatment. In conclusion, the data suggest a role for FZD2 in high-risk neuroblastoma cell growth and provide a potential candidate for therapeutic inhibition in FZD2-expressing neuroblastoma patients.

## Zusammenfassung

Das Neuroblastom ist ein Tumor, der bei der Entwicklung des peripheren sympathischen Nervensystems aus der Neuralleiste entsteht und ist der zweithäufigste extrakranielle bösartige Tumor im Kindesalter.

Frühere Studien haben gezeigt, dass eine permanente Aktivierung von wingless/int1 (Wnt)/Frizzled (FZD) Signalwegen zu Krebs führen kann. Es ist aber unklar, ob die Wnt/FZD Signalwege beim Wachstum des Neuroblastoms beteiligt sind. Das Ziel dieser Studie war die Untersuchung der Rolle von Wnt-FZD2 Signalwegen in Neuroblastomazellen und die mögliche Wechselwirkung mit Makrophagen. Für diese Studie wurden zwei Hochrisiko-Neuroblastomzelllinien verwendet, nämlich die SK-N-AS Zelllinie ohne *MYCN*-Amplifikation und die SK-N-DZ Zelllinie mit *MYCN*-Amplifikation. Überdies wurden auch Tumorproben von SK-N-AS und SK-N-DZ Xenotransplantationsmodellen untersucht.

Neuroblastomazellen wurden mit rekombinanten Wnt3a und Wnt5a Proteinen behandelt, um die Auswirkungen auf maligne Eigenschaften von Krebszellen, wie Proliferation und Migration, zu bestimmen. Zusätzlich wurde die Aktivierung von Komponenten verschiedener Wnt Signalwege durch Western blot-Analysen untersucht. Der Einfluss einer Aktivierung von FZD2 Signalwegen in den Neuroblastomzellen auf die Rekrutierung bzw. Zahl von Tumor-assoziierten Makrophagen (TAMs) im Tumorgewebe wurde auch untersucht.

Die Ergebnisse dieser Studie zeigen, dass die Stimulation von FZD2 durch Wnt3a und Wnt5a  $\beta$ -Catenin-abhängige und -unabhängige Wnt Signalfaktoren reguliert. Die Blockade von FZD2 unterdrückt den  $\beta$ -Catenin-abhängigen Wnt-Signalweg und erhöht die Phosphorylierung von PKC, AKT und ERK *in vitro*, was eine Hochregulation von  $\beta$ -Catenin-unabhängiger Signalaktivität zeigt. Die Anzahl der TAMs im Tumorgewebe wurde durch die Hemmung von FZD2 nicht beeinflusst.

Zusammenfassend deuten die Daten auf eine Rolle von FZD2 bei der Proliferation von Hochrisiko-Neuroblastomazellen hin und FZD2 ist daher ein potentieller Kandidat für eine therapeutische Hemmung bei FZD2-exprimierenden Neuroblastom Patienten.

# Introduction

## 1. Neuroblastoma

Neuroblastoma (NB) is a cancer of the sympathetic nervous system [1]. It is the most common extra cranial solid tumor in childhood and the most common cancer in infancy [2]. Nearly half of neuroblastoma cases occur in children younger than two years [3]. The great majority of cases are sporadic and non-familial [4]. About 1-2% of cases run in families and have been linked to specific gene mutations [5]. Recent advances in understanding the biology and genetics of neuroblastomas allowed classification into low-, intermediate- and high-risk groups [6]. In most children diagnosed with neuroblastoma, the tumors harbored genetic and biological features that highly correlated with a poor treatment outcome [7]. In 1988, a proposal was made to establish an international neuroblastoma staging system (INSS), as well as consistent criteria for confirming the diagnosis and determining the response to therapeutic regimens [8].

Like other cancers, neuroblastoma can metastasize to other parts of the body, such as the lymph nodes, skin, liver, and bones [3]. The invasive, metastatic and hypervascular nature of high-stage neuroblastoma may be one of the key obstacles to the cure of this disease, but the underlying molecular pathways are not fully understood [9].

A subset of neuroblastoma tumors is characterized by amplification of the *MYCN* proto-oncogene, which occurs in approximately 25% of tumors [10, 11]. However, absence of *MYCN* amplification, stage 4 and age of 12 or higher at diagnosis are also categorized as high-risk neuroblastoma suggesting that other factors contribute to high-risk neuroblastoma [12, 13].

Abnormalities in the Wnt signaling pathway are found in a wide range of cancers, and the Wnt signaling pathway also plays a significant role in the pathogenesis of neuroblastoma [14, 15]. The present work focuses on this pathway and its effect in neuroblastoma.

## **1.1.Metastasis**

Metastasis constitutes the most deadly aspect of cancer that results from several interconnected processes including increased cell proliferation, angiogenesis, cell adhesion, migration and invasion into the surrounding healthy tissue. [16]. In over 90% of cases, metastases are ultimately responsible for mortality associated with solid tumors [17]. Metastasis is characterized by the spread of neoplastic cells from a primary site where the tumor originated to distant organs [16]. The comparison of gene expression profiles between metastatic and non-metastatic cells have clearly shown that various genes have a differential expression pattern, which is associated with the metastatic potential of cancer cells [18].

Notably, neuroblastoma is in 70% of patients metastatic [19]. The most common sites of metastasis in neuroblastoma are the bone and bone marrow that are found in the majority of children with newly diagnosed metastatic neuroblastoma [20].

## **1.2. Tumor Microenvironment**

The tumor microenvironment (TME) is the cellular environment in which the tumor exists and includes surrounding blood vessels, immune cells, fibroblasts, bone marrow-derived inflammatory cells, lymphocytes, signaling molecules and the extracellular matrix (ECM) [21]. Each of these components can have either a pro-tumorigenic or anti-tumor effect depending on a number of different factors released by them or the cancer cells [22].

Cancer cells in an aggressive primary mass are able to exploit the particular tissue microenvironment; if once they leave their surroundings, they must possess traits that allow them to survive in new environments [23]. Metastasis occurs when unstable cancer cells adapt to a tissue microenvironment that is distant from the primary tumor [24].

Recent research highlighted that the ECM regulates cell behavior because it is

composed of highly variable and dynamic components [25]. During the progression of cancer, many ECM proteins are significantly deregulated, causing both biochemical and biomechanical changes that together finally can promote the formation of metastasis [26].

## **2. Wnt Signaling Pathways**

Like many other signal transduction pathways, the Wnt pathways are a group of different signaling pathways made of proteins that pass signals from outside of a cell through binding to different cell surface receptors to the inside of the cell [27]. Wnt signaling pathways regulate crucial aspects of cell fate determination, cell migration, cell polarity, neural patterning and organogenesis during embryonic development [14, 28]. Canonical Wnt signaling pathway, noncanonical planar cell polarity (PCP) pathway, and noncanonical Wnt/calcium pathway are three Wnt signaling pathways that have been characterized, which are relevant in cancer development [29, 30]. As their names suggest, these pathways belong to one of two categories: canonical or non-canonical. The difference between the categories is that the canonical pathway involves the protein  $\beta$ -catenin, while noncanonical pathways operate independently of it [31]. All three Wnt signaling pathways are activated when a Wnt-protein ligand binds to a Frizzled family receptor. After binding to Frizzled, the receptor passes the biological signal to the protein Dishevelled, which is located inside the cell [32]. Low-density lipoprotein receptor-related protein 6 (LRP6) is a Wnt co-receptor in the canonical signaling pathway, while there are other co-receptors in non-canonical Wnt signaling pathways, such as receptor tyrosine kinase-like orphan receptors (RORs), receptor tyrosine kinase (Ryc), neurotrophin receptor homolog (NRH1) and tyrosine-protein kinase-like 7 (PTK7) [28, 33]. Activation of the canonical Wnt pathway leads to regulation of gene transcription, while the noncanonical planar cell polarity pathway is primarily involved in regulation of the cytoskeleton that is responsible for the shape of the cell, and the Wnt/calcium pathway, also noncanonical, regulates intracellular calcium levels [30]. Interestingly, aberrant regulation of the Wnt signaling pathway is a prevalent theme in cancer biology [34].

In the canonical Wnt signaling pathway, binding of a Wnt ligand member to one of the Frizzled receptors and the LRP5/6 co-receptor leads to signaling events that

stabilize cytoplasmic  $\beta$ -catenin and subsequently result in transcriptional activation of Wnt target genes that include *MYC*, *CCND1* (cyclin D1) and others [14, 35]. In high-risk neuroblastoma without *MYCN* amplification, increased Wnt ligand levels have been shown together with strongly expressed  $\beta$ -catenin [36].

In cancer research, non-canonical Wnt signaling has been considered a tumor suppressor; however, recent studies have shown that the signaling also enhances cancer progression in the later stages of disease [37]. Moreover Wnt/ $\beta$ -catenin signaling effects in human disease include congenital malformations, cancer and osteoporosis [38].

There is also growing evidence that canonical and non-canonical signaling pathways undergo crosstalk. For example, PCP and  $\beta$ -catenin-dependent Wnt signaling are known to antagonize each other and it has been shown that inhibiting one will typically upregulate the other. Another  $\beta$ -catenin-independent pathway, the Wnt/ $\text{Ca}^{2+}$  pathway can increase the intracellular  $\text{Ca}^{2+}$  concentration and signal transduction in this pathway involves activation of protein kinase C (PKC) [39, 40].

## 2.1 Wnt ligands

The *Wnt* gene was first discovered in *Drosophila* as a mutation causing absence of wings and was named *wingless*. As the name suggests, the *wingless* gene is required to pattern the drosophila wings and other adult body structures [40]. Subsequently, *int* was discovered, a cellular oncogene that is often activated by insertion of proviral DNA of the mouse mammary tumor virus [40-42]. The drosophila and mouse genes were found to be homologous. Therefore the gene was named Wnt by combining the two names [40].

The Wnt gene family consists of structurally related genes, which encode secreted signaling proteins [43]. These proteins have been implicated in oncogenesis and in several developmental processes [44].

Wnt ligands are secreted, hydrophobic, and cysteine-rich glycoproteins, which are 350-400 amino acids in length [38, 42, 45]. Wnts are produced as precursor proteins



that contain a short N-terminal signal sequence, which is required for Wnt secretion [38, 46].

In vertebrates, there are 19 different Wnt protein ligands. The expression of these ligands is tightly controlled during development and spatial as well as temporal regulation of expression takes place and they play a critical role in oncogenesis [38, 47]. Their effects are mediated by binding to a receptor complex that consists one of several Frizzled family receptors and a co-receptor, such as lipoprotein receptor-related protein (LRP)-5, LRP-6, related to tyrosine kinase (Ryk), or receptor tyrosine kinase-like orphan receptor (ROR). Intracellular signaling pathways activated by Wnt regulate cell survival, cell proliferation, cell fate determination, cell polarity, tissue patterning, and tissue homeostasis [33,34,28, 38].

Several Wnt proteins, including Wnt1, have shown to be overexpressed in a number of malignancies, for example colon, lung and melanoma [14, 48, 49]. Furthermore, Wnt1, -2, 5A, -11; and Friz4 and -6 expression is increased in prostate cancer tissues [49].

## **2.2 Wnt Receptor Frizzled**

Frizzled proteins are seven trans membrane G protein-coupled cell-surface receptors for the Wnt family of glycoproteins [50]. Structurally, all members of the Frizzled family are similar [51]. Each contains a divergent N-terminal signal peptide, a highly conserved extracellular cysteine-rich domain (CRD), a variable length linker region, a seven-pass trans membrane domain, and a variable length C-terminal end [52]. Up to now ten different of frizzled receptors have been detected in humans [31]. Wnt ligands bind to Frizzled receptors through the cysteine-rich domain, and may require a co-receptor, such as LRP-5, LRP-6, ROR, or Ryk, to activate either the canonical Wnt/ $\beta$ -catenin signaling pathway or one of the  $\beta$ -catenin-independent, non-canonical signaling pathways [31, 52].

Frizzled genes play essential roles in the development of the embryo, in tissue and cell polarity, the formation of neural synapses, the regulation of proliferation, and in

many other processes involved in both, the developing and the adult organism. [51]. Notably, some Frizzled receptors are expressed in the developing nervous system and neural crest and could play a role in neuroblastoma [53].

## **2.3 Wnt inhibitors**

Wnt signaling can be inhibited by several known antagonists, which bind either to the Wnt ligand itself, or to Wnt receptors. Proteins that act as Wnt antagonists include Dickkopf (Dkk) proteins, Wnt Inhibitory Factor-1 (WIF-1), and secreted Frizzled-Related Proteins (sFRPs) [54]. Dkk proteins function as antagonists of canonical Wnt signaling by preventing the LRP-5/6 co-receptor from interacting with Wnt-Frizzled complexes, or by binding to Kremen-1 or -2 and triggering the internalization of LRP-5/6 [55]. WIF-1 and sFRPs antagonize Wnt signaling by binding directly to Wnt proteins and inhibiting their activity. Different members of the sFRP family, namely sFRP-1-5, are able to bind to Wnt proteins through their N-terminal cysteine-rich domains (CRD), and Wnt proteins prevent the latter from binding the FZD receptors. In addition to this antagonistic effect, it has also been proposed that they may also enhance Wnt signaling by stabilizing or transporting Wnt ligands [30, 38, 54].

## **2.4. Wnt Modulators**

A number of different proteins are known, which can either enhance or inhibit Wnt signal transduction including Glypicans, R-Spondin proteins, Kremen-1, Kremen-2, and Norrin, to name a few. [54]. For example Glypican-3 enhances Wnt signaling by stabilizing the interaction between Wnt proteins and Frizzled receptors [56]. Another group of positive regulators consists of members of the R-Spondin family of proteins (Rspo1-4). Rspo proteins interfere with Dkk-1-mediated internalization of the Wnt co-receptor, LRP-6, thereby functioning as positive regulators of Wnt/ $\beta$ -catenin signaling [57]. In contrast, Kremen-1 and Kremen-2 antagonize Wnt signaling by forming a complex with Dkk proteins and LRP-5/6 that stimulates LRP-5/6 internalization [55].

Some proteins may regulate Wnt signaling by directly binding to Wnt ligands or Wnt receptors. Norrin is a secreted protein associated with the extracellular matrix surrounding cells. Norrin is not related to the Wnt family, but it can bind to Frizzled-4/LRP and activates the  $\beta$ -catenin dependent Wnt signaling pathway [30, 38].

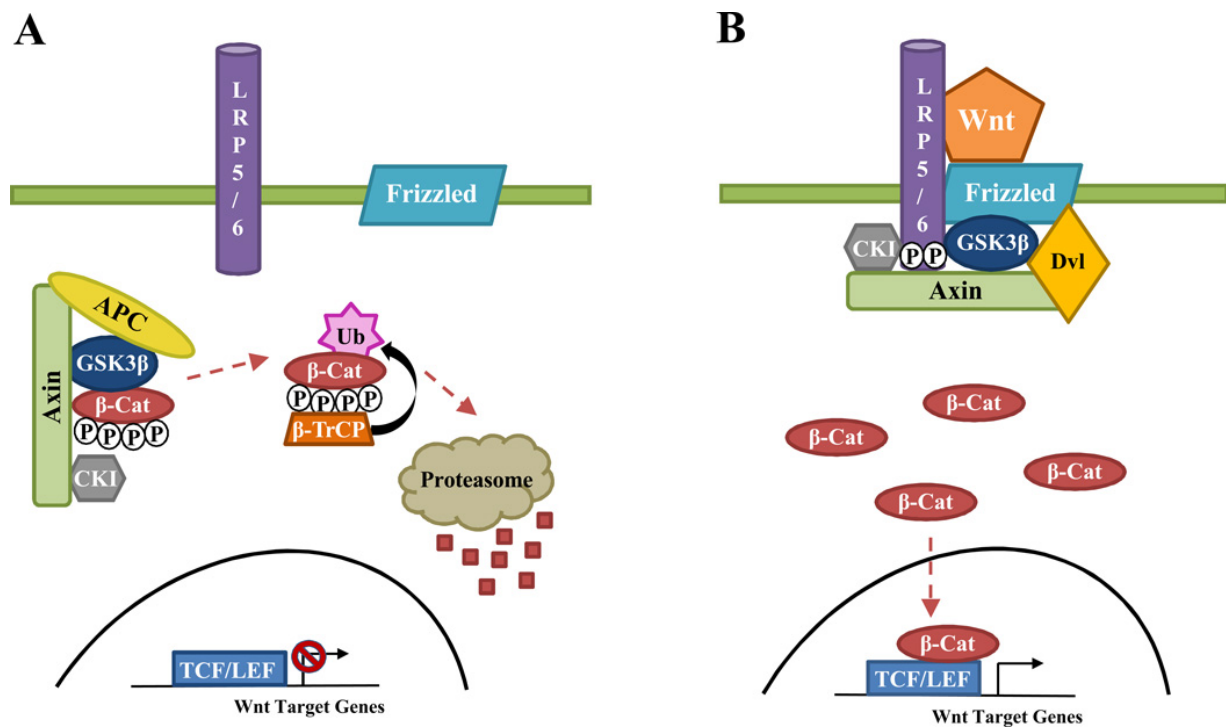
### **3. Wnt Signaling Pathway**

#### **3.1. Canonical Wnt signaling pathway**

The canonical Wnt signaling pathway is the Wnt pathway that causes an accumulation of  $\beta$ -catenin in the cytoplasm and its eventual translocation into the nucleus to act as a transcriptional co-activator of transcription factors that belong to the T-cell factor/lymphoid enhancer factor (TCF/LEF) family [58].  $\beta$ -catenin was first discovered as a part of the adherens junction complex, before being identified as a transcriptional coactivator of Wnt target gene expression [58].

In the absence of Wnt, signaling,  $\beta$ -catenin would not accumulate in the cytoplasm since a destruction complex would normally degrade it. This destruction complex includes Axin, adenomatosis polyposis coli (APC), protein phosphatase 2A (PP2A), glycogen synthase kinase 3 (GSK3), and casein kinase 1 $\alpha$  (CK1 $\alpha$ ) protein which degrade  $\beta$ -catenin by targeting it for ubiquitination, that subsequently sends it to the proteasome to be digested (Figure1A) [38, 59, 58].

When Wnt binds FZD and LRP-5/6, the destruction complex function becomes disrupted. Axin is removed from the "destruction complex" and binds to the cytoplasmic tail of LRP-5/6 then becomes de-phosphorylated and its stability and levels are decreased. In the following step Dsh becomes activated via phosphorylation and its DIX and PDZ domains then inhibit the GSK3 activity of the destruction complex. As a consequence of these signaling events,  $\beta$ -catenin can accumulate and localize to the nucleus. The subsequent induction of a specific cellular response is mediated by induction of gene transduction in conjunction with the TCF/LEF transcription factors (Figure1B) [38, 58, 60].



**Figure 1. Canonical Wnt signaling pathway**

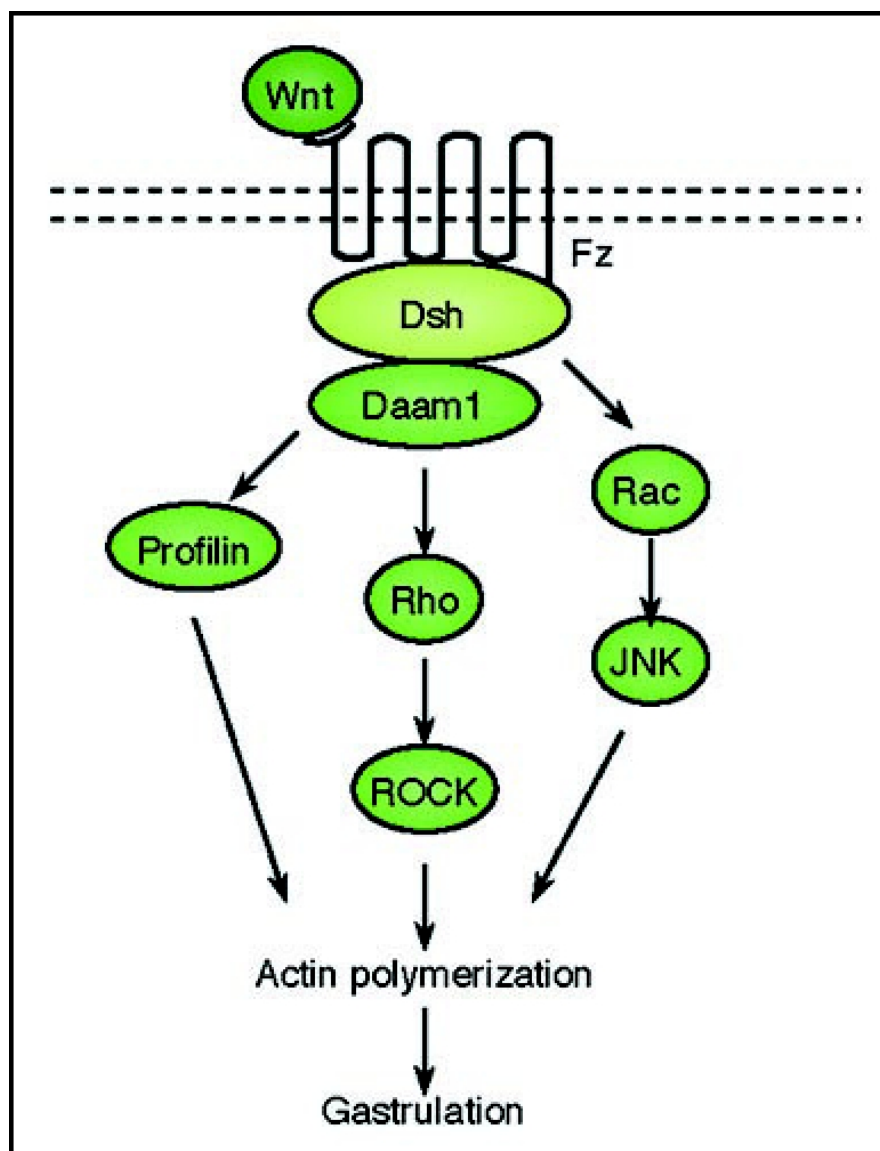
This figure briefly summarizes canonical Wnt signaling. **A**, In the absence of Wnt ligand, cytosolic  $\beta$ -catenin is phosphorylated, ubiquitinated, and degraded by the proteasome to prevent the expression of Wnt target genes. **B**, However, in the presence of Wnt ligand, cytosolic  $\beta$ -catenin is stabilized and translocated to the nucleus, where it acts as a co-activator and facilitates the transcription of Wnt target genes [61].

### 3.2. Non-canonical planar cell polarity pathway

The non-canonical planar cell polarity (PCP) pathway does not involve  $\beta$ -catenin. It uses NRH1, Ryk, PTK7 or ROR2 as co-receptor [28]. The PCP pathway is also activated via the binding of Wnt to FZD and its co-receptor. The activated receptor then recruits Dsh, which uses its PDZ and DEP domains to form a complex with another protein, Dishevelled-associated activator of morphogenesis 1 (DAAM1). Daam1 activates the small G-protein Rho through a guanine exchange factor and

Rho activates Rho-associated kinase (ROCK), which is one of the major regulators of the cytoskeleton. Of interest, Dsh can also form a complex with a family member of small GTPase of the Rho family, Rac1 and mediate profilin binding to actin, which modulates the cytoskeleton. Rac1 in turn can potentially also activate the c-Jun-N-terminal kinase (JNK), which can also lead to actin polymerization [62, 63, 28]. Profilins are actin binding proteins, which regulate actin polymerization (Figure 2) [64] [65].

In addition, the PCP signaling pathway has recently been implicated in tumorigenesis [66]. Notably, activity of the non-canonical Wnt/PCP pathway is important for neuroblastoma [66].

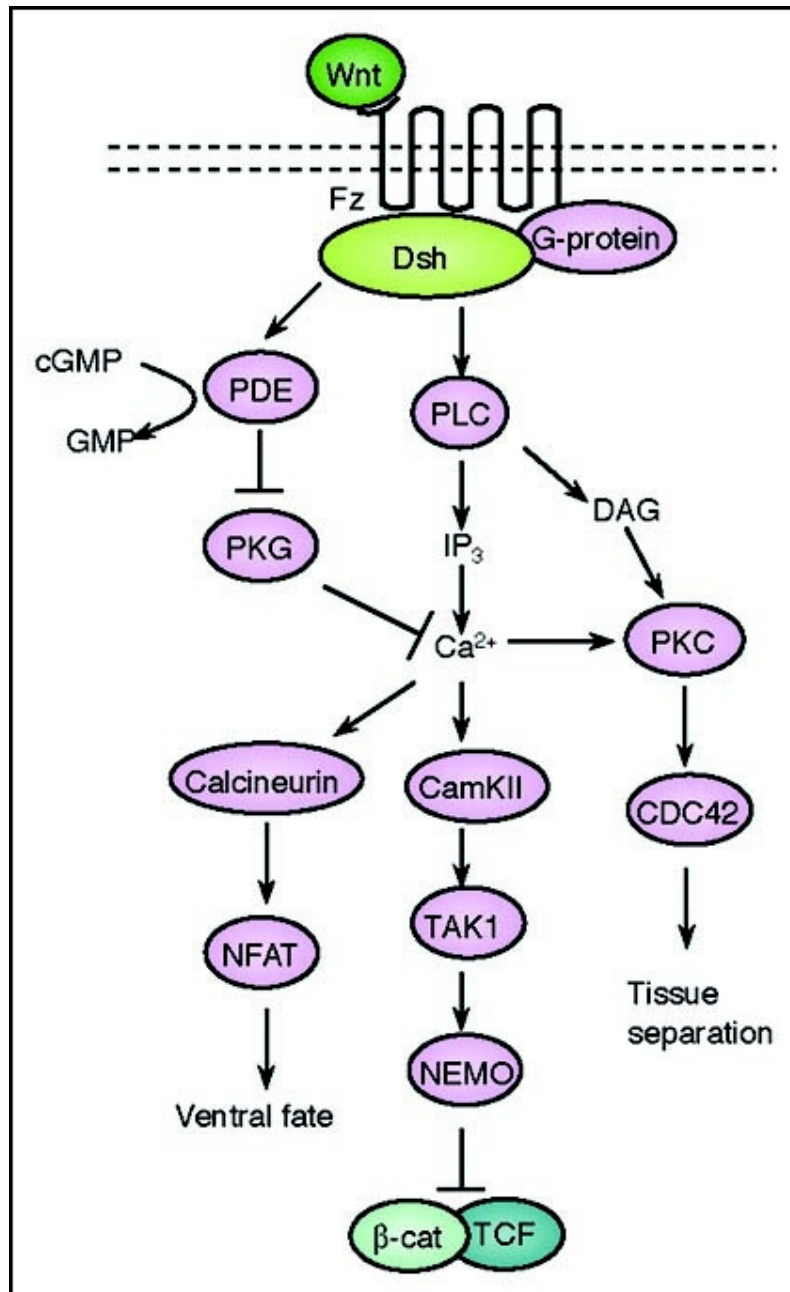


**Figure 2.** Planar cell polarity pathway [28].

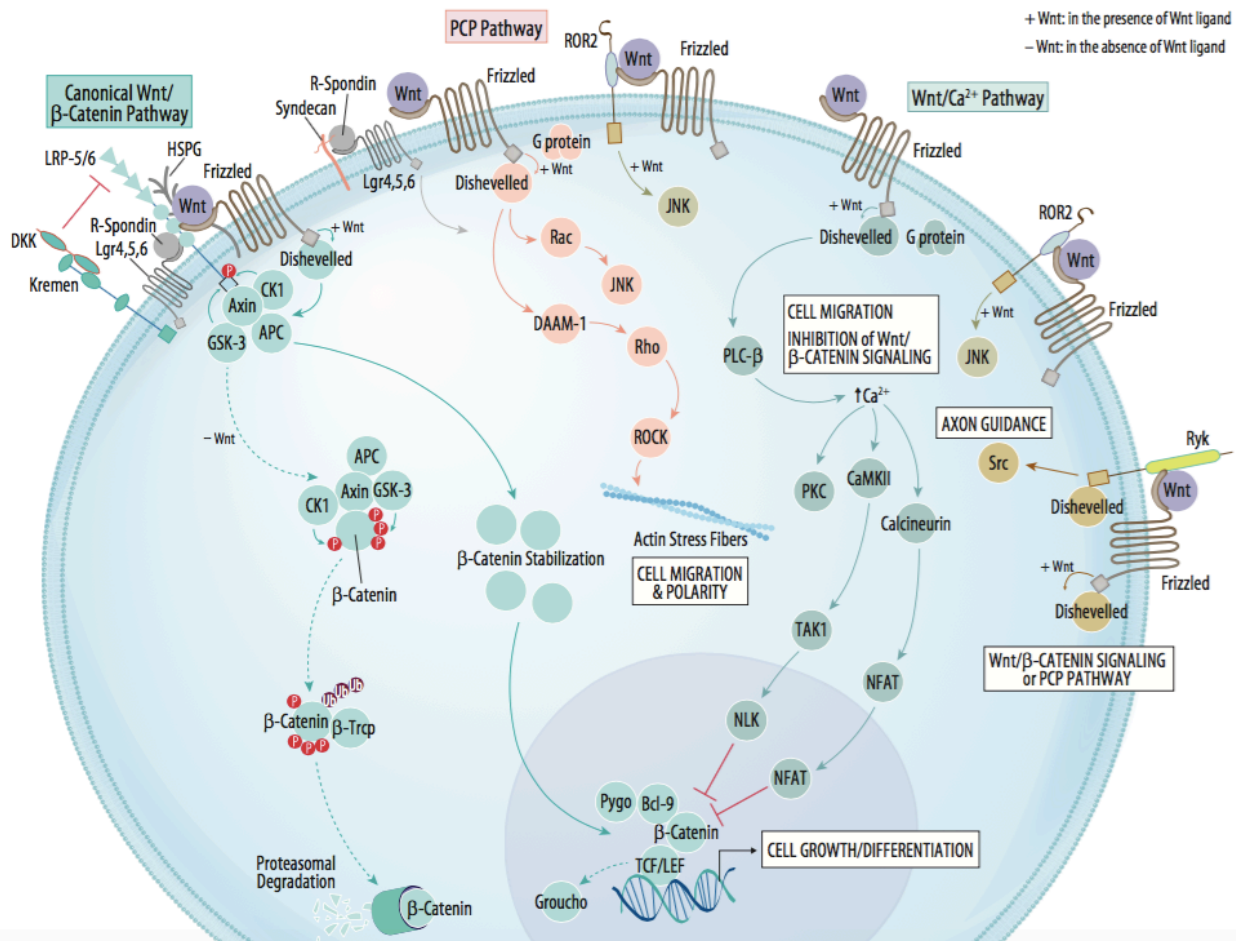
### 3.3. Non-canonical Wnt/calcium pathway

The non-canonical Wnt/calcium pathway does not stimulate accumulation of  $\beta$ -catenin, rather its role is help to regulate calcium release from the endoplasmic reticulum (ER) in order to control intracellular calcium levels. When Wnt binds to the FZD receptor, the activated FZD receptor directly interacts with Dsh and activates specific Dsh-protein domains that are involved in Wnt/calcium signaling, namely the PDZ and DEP domains. Interestingly, unlike other Wnt pathways, the FZD receptor also directly interacts with a trimeric G-protein. This co-stimulation of Dsh and the G-protein can lead to the activation of either Phospholipase C (PLC) or cGMP-specific phosphodiesterase (PDE). If PLC is activated, the plasma membrane component Phosphatidylinositol 4,5-bisphosphate (PIP2) is cleaved into diacylglycerol (DAG) and Inositol triphosphate (IP3). IP3 then binds its receptor on the ER, which causes the release of calcium from the ER. As a consequence the concentrations of calcium increase and DAG can activate Cell division control protein 42 homolog (CDC42), a small GTPase of the Rho family, through Protein kinase C (PKC). CDC42 controls diverse cellular functions, including regulation of cell morphology and ventral patterning. Increased calcium also activates calcineurin and  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II (CaMKII). Calcineurin activates transcription factor NF-AT to regulate ventral cell fates. CamKII activates TAK1 and NLK kinase, which can antagonize TCF/ $\beta$ -catenin signaling in the canonical Wnt pathway. However, activation of the cGMP-specific PDE leads to inhibition of the calcium release from the ER. The mechanism, by which PDE mediates this inhibition of the calcium release involves inhibition of protein kinase G (PKG) (Figure 3) [30, 28, 40].

Wnt5a, through Wnt/ $\text{Ca}^{2+}$  signaling, plays also a role in the pathogenesis of neuroblastoma [67].



**Figure 3.** Non-canonical Wnt/calcium pathway [28].



**Figure 4.** Overview of the Canonical Wnt pathway, the non-canonical planar cell polarity pathway, and the non-canonical Wnt/calcium pathway [68].

#### 4. Wnt signaling and embryonic development

Tight control of cell-cell communication is very essential and important for the generation of a normally patterned embryo. The Wnt family constitutes an important mediator of key cell-cell signaling events during embryogenesis [69]. Additionally, Wnt signaling is crucial for embryonic development in all animal species to date [70]. It is now well established that Wnt signaling controls a plethora of key cellular events thereby regulating cell proliferation, stem cell maintenance and cell fate decisions, as well as organized cell movements and the establishment of tissue polarity. In addition, it is frequently deregulated in human cancers and has been implicated in



degenerative diseases [70].

Figure 4 gives an overview of signal transduction factors regulating the different Wnt signaling pathways involved in cell growth, cell differentiation, cell migration and polarity.

## **5. Wnt signaling and cancer**

When Wnt1 was discovered, it was first identified as a proto-oncogene in a mouse model. Wnt1 is a homolog of Wingless (Wg) and involved in embryonic development, which often calls for rapid cell division and migration, therefore misregulation of this processes can cause unwanted cell growth and movement, which can lead to tumor development [27]. Aberrant regulation of Wnt signaling pathway can be detected in several cancer types such as neuroblastoma, colorectal cancer, melanoma, prostate cancer and lung cancer [71].

One of the reasons that makes Wnt signaling pathways important is association between adenomatous polyposis coli (APC) protein and  $\beta$ -catenin. APC can downregulate  $\beta$ -catenin whereas Wnt-1 can upregulate it and thus provide further support for the Wnt cancer connection [34]. It is well known that APC is mutated in the vast majority of all sporadic colorectal cancers. In order to promote tumorigenesis, a loss of function in both alleles is required. The total loss of functional APC is structurally linked to the ability of APC to regulate the protein stability of  $\beta$ -catenin [34].

Embryonic development is controlled by a small set of signal transduction pathways, including the Wnt signaling pathway, which plays a critical role in axis patterning, cell fate specification, cell proliferation, and cell migration, thus aberrant regulation or mutation of Wnt signaling pathway can lead to cancer [72, 73]. The Wnt/  $\beta$ -catenin signaling displays an extraordinary functional flexibility that can be seen by its ability to act not only in stem cells of the embryo but also in stem cells of the adult as well as in cancer stem cells. During embryogenesis,  $\beta$ -catenin is an essential component in stem cells, which is necessary to mediate the proper Wnt/FZD signaling response for their maintenance and transition from a pluripotent state into the next developmental steps. In adult stem cells, Wnt signaling functions has been shown to

act not always at the same hierarchical level but instead is involved in different hierarchical levels, thus contributing to the specification of different tissues. Based on these facts it has been assumed that aberrant activation or regulation of the tightly regulated self-renewal mediated by Wnt signaling in stem and progenitor cells can be subverted into growth promoting features in cancer cells to allow cell growth and subsequent malignant progression [74].

In hepatocellular carcinoma (HCC) and many other types of cancer, aberrant activation of the canonical Wnt/ $\beta$ -catenin signaling pathway is an important contributor to tumorigenesis. In HCC an increased level of constitutive transcriptional activation by  $\beta$ -catenin/TCF complexes can be observed in many cases. The reason for this increased  $\beta$ -catenin activity is frequently caused through mutations in the N-terminal region of  $\beta$ -catenin, thereby stabilizing the protein and leading to the transcriptional activity ([75].

Taken together, Wnts via activation of different signaling pathways and their corresponding signaling effectors regulate all key processes that contribute to cancer progression. These processes include tumor initiation, tumor growth, cell senescence, cell death, differentiation and finally metastasis. Although Wnt signaling pathways have been difficult to target, improved drug-discovery platforms and new technologies have facilitated the discovery of agents that can alter Wnt signaling in preclinical models, thus setting the stage for clinical trials in humans [76]. Nowadays siRNA as innovative nucleic acid medicines is used for treatment of disease such as cancer because of its sequence-specific posttranscriptional gene silencing effect [77, 78].

## Aim of the study

Most recent genetic discoveries gleaned from tumor genome sequencing indicate that the Wnt pathway continues to evolve as a central mechanism in cancer biology [34]. Wnt signaling pathways play a significant role in different cancer types such as colorectal cancer, melanoma, prostate cancer and lung cancer, but the role of Wnt signaling pathways in neuroblastoma is unclear. No studies have reported an association between FZD2 signaling and high-risk neuroblastoma so far.

This study addresses the question, whether activation of Wnt-Frizzled signaling pathways in neuroblastoma cells is associated with invasive cancer cell behavior and whether these pathways affect infiltration of tumor-associated macrophages (TAMs).

To analyze the role of Wnt/FZD2 signaling pathways in neuroblastoma cells, the effect of Wnt3a and Wnt5a ligands on FZD2 signaling was examined in human high-risk SK-N-AS and SK-N-DZ neuroblastoma cells. By using recombinant Wnt proteins it was analyzed whether the FZD2 ligands Wnt3a and Wnt5a may increase or decrease baseline Wnt-signaling activity in neuroblastoma cells. In addition small interfering RNA (siRNA) specific to FZD2 was used to study the effect of FZD2 blockade.

Furthermore, observations in the Wnt/PCP pathway showed that Wnt binding to FZD receptor was followed by activating Rho/Rac small GTPase and JNK to assist in the subsequent regulation of cytoskeletal organization and gene expression [79]. Because of that, this study also investigated the effects of Wnt on alteration of the cytoskeleton leading to alteration in the migratory behavior of neuroblastoma cells.

Isolated tumor tissue from neuroblastoma xenograft in mice allowed investigation of the expression of downstream Wnt signaling molecules *in vivo*. The results of this study contributed to the study of Zins et al. in the Journal Oncotarget [13].

# MATERIALS AND METHODS

## 1. Cell lines

Two epithelial-like human neuroblastoma cell lines were used. These cell lines, SK-N-AS and SK-N-DZ, form tumors in nude mice and grow in adherent monolayers in culture.

### 1.1 SK-N-AS

SK-N-AS is a *MYCN* single copy human neuroblastoma cell line derived from a 6 year old girl with a poorly differentiated neuroblastoma and was obtained from American Type Culture Collection (ATCC, Manassas, VA).

### 1.2 SK-N-DZ

SK-N-DZ is a *MYCN*-amplified human neuroblastoma cell line derived from a 2 year old girl with poorly differentiated neuroblastoma and was obtained from ATCC.

## 2. Cell culture

Culturing cells in cell culture dishes allows cells to be manipulated and investigated for a number of applications. The growth in an artificial environment by the adjustment of several factors, such as media composition, pH and temperature enables cells a controlled proliferation.

SK-N-AS and SK-N-DZ were cultured in Dulbecco's Modified Eagle's medium (DMEM) growth medium supplemented with 10% fetal bovine serum (FBS), 0.1 M non-essential amino acids (NEAA), 0.1 M sodium pyruvate (S0-Py) and 100 U/ml penicillin-streptomycin (Pen/Strep) The cells were stored in a humidified incubator at 37°C and 5% CO<sub>2</sub>.

For experiments with defined cell numbers, cells were counted with Neubauer cell

counter before seeding.

The cell lines and their specific media are summarized in Table 1.

| Cell line | Medium | supplements             | Growth properties | Cancer type   |
|-----------|--------|-------------------------|-------------------|---------------|
| SK-N-AS   | DMEM   | FBS, NEAA,<br>Pen/Strep | adherent          | neuroblastoma |
| SK-N-DZ   | DMEM   | FBS, NEAA,<br>Pen/Strep | adherent          | neuroblastoma |

**Table 1.** Overview of used neuroblastoma cell lines and their specific media.

## 2.1 Subculturing process

Cell culture media and PBS were pre-warmed at room temperature or by placing them into the incubator or a water bath. After removing the exhausted medium, the cells were washed twice with phosphate buffered saline (PBS) and subsequently 1x Trypsin-EDTA was added. After an incubation of 5-10 minutes at 37°C the trypsinization was stopped with fresh medium and the cells were harvested and transferred from the plate to a tube. The cells were pelleted by centrifugation (1500 rpm) and resuspended in fresh medium corresponding to the respective split ratio. Finally, new cell culture dishes were inoculated and placed in the incubator (37°C, 5% CO<sub>2</sub>). Medium renewal was carried out twice to thrice per week.

## **2.2. Cell counting**

If a defined cell number was necessary, the cells were counted using a Neubauer chamber before seeding. This hemocytometer allows to determine the cell concentration under a microscope through ruled grids etched inside the glass. The calculation of the cell concentration considered the number of counted cells, the number of squares, the volume and the dilution applied.

The cells were trypsinized and collected as for subcultivation and resuspended in 300  $\mu\text{L}$  PBS. 10  $\mu\text{L}$  of this suspension were diluted in 990  $\mu\text{L}$  PBS in a tube, resulting in a dilution factor 1:100. The counting chamber and glass cover slip were cleaned with 70% ethanol and the slip was placed on the chamber. The diluted cell suspension was pipetted onto the border of the glass cover slip and sucked into the counting chamber by capillary action. Cells were counted in the four outer 1  $\text{mm}^2$  squares of both counting areas and the result was averaged. The result of the initial suspension therefore is  $N$  (number of cells)  $\times 10^6$  cells/mL.

## **2.3. Cryopreservation**

For freezing the cells, pellets were resuspended in cryomedium (Appropriate medium containing 10% dimethyl sulfoxide (DMSO)), transferred into a cryotube and frozen in styrofoam container at  $-80^\circ\text{C}$  for at least overnight to achieve a freezing rate of approximately  $1^\circ\text{C}$  per minute. For long-term storage, tubes were transferred to liquid nitrogen.

# **3. Cell culture experiments *in vitro***

## **3.1 Immunofluorescence cell staining**

Immunofluorescence is a powerful technique that utilizes fluorescent-labelled antibodies to detect specific target antigens located onto the cell surface or intracellular. The method used involves a primary, unlabeled antibody, which is antigen-specific and a secondary antibody, which is conjugated to a fluorescence dye.

In this study SK-N-AS cells were used.  $5 \times 10^4$  cells were seeded in each well of camber slide. The cells were incubated overnight. After growing, the cells were washed briefly with PBS and then fixed in acetone at  $-20^{\circ}\text{C}$  for 8 minutes and then washed 3 times for 5 minutes. The cells were incubated with blocking serum (5% goat serum+ 0,3% Triton X in PBS) for 60 minutes. After blocking the cells were incubated overnight with primary antibody at  $4^{\circ}\text{C}$  (Table 2).

The antibody buffer that was used consists of 1% BSA and 0.3% Triton X-100 in PBS. The slides were washed 3 times for 5 minutes then incubated with fluorescence-labeled secondary antibody (Texas Red anti-rabbit IgG) diluted in antibody buffer for 60 minutes at room temperature in the dark. The slide was washed 3 times for 5 minutes. The cells were then incubated with 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI, Invitrogen) diluted 1:1000 in PBS for 10 minutes. At the end, Citifluor AF was added onto the slide and mounted the coverslip with the cells. Cells were observed under a Nikon fluorescent microscope at different magnifications.

| Primary antibody                                                             | Dilution of primary antibody |
|------------------------------------------------------------------------------|------------------------------|
| Anti $\beta$ -catenin-rabbit polyclonal (Abcam)                              | 1:100                        |
| non phospho (active) $\beta$ -catenin rabbit mAb (Cell Signaling Technology) | 1:100                        |
| c-MYC rabbit mAb (Cell Signaling Technology)                                 | 1:200                        |

**Table 2.** Primary antibody and their dilutions used in immunofluorescence cell staining.

### **3.2 Protein isolation and Western blotting (WB)**

Samples were lysed in Frackelton lysis buffer (10mM Tris, 30 mM sodium pyrophosphate, 50 mM sodium chloride, 1% Triton X-100) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF), 100 $\mu$ M Na<sub>3</sub>VO<sub>4</sub> and 1x Complete<sup>TM</sup>-EDTA-free protease Inhibitor Cocktail (Roche) immediately before use. Cell lysates were spun at 15,000 g for 10 minutes in a 4°C pre-cooled centrifuge. Supernatant was transferred to a fresh tube and then were determined the protein concentration for each cell lysates. The samples were prepared for loading into gels. Cell lysates (20  $\mu$ g) were separated by 8-15% SDS-PAGE for approximately 60 minutes at 100 volt. Protein transfer was done in “semi-dry” blotting apparatus for about 45 min at 12 volt. The quality of protein transfer was checked by staining with Ponceau S solution. The nitrocellulose sheets were blocked with blocking buffer (powder milk/TBST+5%BSA) at room temperature for 1-2h. Afterwards, the membrane was incubated overnight with primary antibodies at 4° C (Table 3). To remove excess of primary antibodies, the sheets were washed three times with TBS-T buffer then the membrane was incubated with the secondary antibody, anti-rabbit HRP diluted 1:10000 in 5% BSA in TBST (Table 3). Finally, membranes were washed again three times with TBST before color detection of proteins by chemiluminescence (Ace Glow, Peqlab). Signals were scanned using FUSION-FX7 (Vilber Lourmat) and quantified by Fusion-CAPT-Software 16.07 (Vilber Lourmat).



| Type                                     | Primary antibody                                                                              | Dilution of Primary Antibody | Secondary antibody             | Dilution of Secondary Antibody |
|------------------------------------------|-----------------------------------------------------------------------------------------------|------------------------------|--------------------------------|--------------------------------|
| <b>FZD2</b>                              | Rabbit anti-Frizzled2 polyclonal (ZYMED Laboratories)                                         | 1:1000                       | HRP-conjugated anti-rabbit IgG | 1:10000                        |
| <b>p-LRP6</b>                            | Phospho-LRP6 (Ser1490) polyclonal Antibody (Cell Signaling Technology)                        | 1:1000                       | HRP-conjugated anti-rabbit IgG | 1:10000                        |
| <b>Total <math>\beta</math>-catenin</b>  | Anti- $\beta$ -catenin antibody-rabbit polyclonal (Abcam)                                     | 1:4000                       | HRP-conjugated anti-rabbit IgG | 1:10000                        |
| <b>Active <math>\beta</math>-catenin</b> | Non phospho (active) $\beta$ -catenin (Ser337377Thr41) Rabbit mAb (Cell Signaling Technology) | 1:1000                       | HRP-conjugated anti-rabbit IgG | 1:10000                        |
| <b>MYC</b>                               | c-MYC Rabbit mAb (Cell Signaling Technology)                                                  | 1:1000                       | HRP-conjugated anti-rabbit IgG | 1:10000                        |
| <b>Cyclin D1</b>                         | Cyclin D1 polyclonal Antibody (Cell Signaling Technology)                                     | 1:1000                       | HRP-conjugated anti-rabbit IgG | 1:10000                        |
| <b>p-PKC</b>                             | Phospho-PKC (pan) ( $\beta$ II Ser660) polyclonal Antibody (Cell Signaling Technology)        | 1:1000                       | HRP-conjugated anti-rabbit IgG | 1:0000                         |
| <b>JNK</b>                               | SPAK/JNK polyclonal Antibody (Cell Signaling Technology)                                      | 1:1000                       | HRP-conjugated anti-rabbit IgG | 1:10000                        |
| <b>p-JNK</b>                             | Phospho-SPAK/JNK (Thr183/Tyr185) Rabbit mAb (Cell Signaling Technology)                       | 1:1000                       | HRP-conjugated anti-rabbit IgG | 1:10000                        |
| <b>AKT</b>                               | Akt polyclonal Antibody (Cell Signaling Technology)                                           | 1:1000                       | HRP-conjugated anti-rabbit IgG | 1:10000                        |

|               |                                                                                   |         |                                |         |
|---------------|-----------------------------------------------------------------------------------|---------|--------------------------------|---------|
| <b>p-AKT</b>  | Phospho-Akt (Ser473) polyclonal Antibody (Cell Signaling Technology)              | 1:1000  | HRP-conjugated anti-rabbit IgG | 1:10000 |
| <b>ERK1/2</b> | P44/42 MAPK (Erk1/2) polyclonal antibody (Cell Signaling Technology)              | 1:2000  | HRP-conjugated anti-rabbit IgG | 1:10000 |
| <b>p-ERK</b>  | Phospho-p44 MAPK (Erk1/2) (Thyr202/Tyr204) Rabbit mAB (Cell Signaling Technology) | 1:2000  | HRP-conjugated anti-rabbit IgG | 1:10000 |
| <b>GAPDH</b>  | Anti-GAPDH mAb antibody; Loading Control (HRP) (Abcam)                            | 1:10000 | HRP-conjugated anti-rabbit IgG | 1:10000 |

**Table 3.** Primary and secondary antibodies used in Western blotting. mAb, monoclonal antibody.

### 3.3. Visualization of the actin cytoskeleton and fluorescence microscopy using Phalloidin staining

Human SK-N-AS cells ( $5 \times 10^4$ ) were grown on a chambered cover glass coated with fibronectin/gelatin (0.001% fibronectin/0.2% gelatin in distilled water) in culture medium and were then transfected with human/mouse frizzled siRNA or human frizzled siRNA or stimulated with recombinant Wnt1 and Wnt5a protein and incubated overnight at 37°C. Cells were then fixed with 3.7% formaldehyde solution in PBS for 10 minutes at RT. After washing with PBS, 0,1% Triton in PBS was added to cells for 5 minutes at RT then washed twice with PBS. Next, cells were labelled with Atto-488 phalloidin (Sigma-Aldrich) and counterstained with DAPI (2ml PBS+10µl phalloidin+5µl DAPI) at RT for 20 minutes. After washing twice with PBS, Citifluor AF was added onto slides and mounted the coverslips with the cells. Fluorescence was observed with a Nikon Eclipse 80i (Tokyo, Japan) microscope equipped with DAPI and fluorescein-isothiocyanate (FITC) filters at different magnifications and images were digitally acquired.

### 3.4 Migration assay

Human SK-N-AS ( $1 \times 10^5$  in 2 ml DMEM with 1% FCS) and SK-N-DZ ( $1 \times 10^5$  in 2 ml DMEM with 1% FCS) neuroblastoma cells were added to the top of each Boyden migration chamber (8- $\mu$ m, 12 well plate format; BD Biosciences). After 24 h the cells were transfected with FZD2 siRNA (FZD2si) or nonspecific scrambled control siRNA (scrCo) (Table 4). After another 24 h of transfection, cells were incubated with 100 ng/ml of recombinant Wnt3a or Wnt5a protein (Table 4). After 48 h, medium was removed and membranes were washed twice with PBS. Cells from the upper side of the membrane were removed with cotton swabs. The membranes were excised using a scalpel, inverted and transferred to a PBS filled tissue culture well. Then membranes were fixed in methanol for 10 min at  $-20^{\circ}\text{C}$ . After washing in PBS, membranes were stained with 1  $\mu\text{g/ml}$  DAPI in PBS (1:1000) for 10 min at room temperature and washed again in PBS. Membranes were then embedded in Cityfluor (Cityfluor) on glass slides. Representative sectors of migrated neuroblastoma cancer cells were counted under a fluorescence microscope.

|   | SK-N-AS               | SK-N-DZ               |
|---|-----------------------|-----------------------|
| 1 | Control Lipofectamine | Control Lipofectamine |
| 2 | Lipofectamine+ Wnt3a  | Lipofectamine+ Wnt3a  |
| 3 | Lipofectamine + Wnt5a | Lipofectamine+ Wnt5a  |
| 4 | Scrambled siRNA       | Scrambled siRNA       |
| 5 | FZD2siRNA             | FZD2siRNA             |
| 6 | FZD2siRNA + Wnt3a     | FZD2siRNA + Wnt3a     |
| 7 | FZD2siRNA + Wnt5a     | FZD2siRNA + Wnt5a     |

**Table 4.** Migration assay.

### 3.5 Cell proliferation assay

Cells (SK-N-AS, SK-N-DZ) were starved overnight in the absence of serum and then washed and scraped off the plate. After centrifugation, the pellet was resuspended in appropriate medium containing 10% FBS.  $1 \times 10^4$  cells were seeded in 100  $\mu$ l DMEM supplemented with 10% FBS in 96-well plates. After 24 h cells were transfected with 100 nM FZD2 siRNA or scrambled siRNA using Lipofectamine and Plus reagent (Thermo Fisher Scientific). After another 24 h culture medium was replaced with DMEM with 1% FBS with or without 100 ng/ml recombinant Wnt3a or Wnt5a protein (R&D Systems). Triplicate cultures were maintained for 24h, 48h and 72h for each condition. Cell proliferation was determined by adding 10  $\mu$ l cell proliferation reagent WST-1 (Roche) per well and after addition of WST-1, absorbance was measured after 3h with a plate reader (Multiscan FC, Thermo Scientific). The absorbance value at 620 nm was subtracted from the value at 450 nm (software SkanIt 3.0).

## **4. *In vivo* experiments**

### **4.1 Immunohistochemistry of tissue sections**

Immunohistochemistry (IHC) is a method for demonstrating the presence and location of proteins in tissue sections and IHC-Paraffin refers to the staining of tissues that have been fixed (usually in neutral buffered formalin) and then embedded in paraffin before being sectioned.

Paraffin-embedded tumors from the mouse xenograft experiment were cut into 5 µm thick sections. Tissue sections were initially deparaffinized and rehydrated in graded alcohols (3' Xylene, 3' 100% EtOH, 3' 96% EtOH, 3' 70% EtOH, 3' PBS). Endogenous peroxidase activity was blocked in 5% H<sub>2</sub>O<sub>2</sub> for 20', and antigens were then demasked for 40 min. in DAKO-solution (pH 6.5) in a water bath at 97°C. The sections were washed three times for 5 minutes in PBS and tissue was labeled with a DAKO pen. Subsequently, slides were blocked with 10% horse serum/fetal bovine serum (depending on the primary antibody) diluted in PBS for 30 minutes and then incubated overnight with primary antibody diluted in 5% horse serum/fetal bovine serum at 4°C (Table 5). The slides were washed 3 times for 5 minutes then incubated with secondary antibody in 5% horse serum/fetal bovine serum for 60 minutes at RT (Table 5). Slides were then washed three times for 5 minutes and incubated with DAKO HRP-streptavidin diluted 1:500 in PBS for 30 minutes and washed again. Slides were treated with DAB to reveal specific staining and washed with ddH<sub>2</sub>O. The tissue sections were then stained for 30 seconds with haematoxylin and were subsequently rinsed with tap water for 2 minutes. The tissue sections were dehydrated in graded alcohols (3' 70% EtOH 3' 96% EtOH, 3' 100% EtOH, 3' Xylene) and mounted in DPX (Merck). Digitalized images were generated with a Nikon Eclipse 80i microscope and analyzed using NIS Elements imaging software (Nikon).

| Tissue section     | Primary antibody                                                                              | Dilution of primary antibody | Secondary antibody                  | Dilution of secondary antibody |
|--------------------|-----------------------------------------------------------------------------------------------|------------------------------|-------------------------------------|--------------------------------|
| SK-N-AS<br>SK-N-DZ | Anti- $\beta$ -catenin antibody-rabbit polyclonal (Abcam)                                     | 1:500                        | Anti-mouse IgG/Anti-Rabbit IgG      | 1:200                          |
| SK-N-AS SK-N-DZ    | Non phospho (active) $\beta$ -catenin (Ser337377Thr41) Rabbit mAb (Cell Signaling Technology) | 1:300                        | Anti-mouse IgG/Anti-Rabbit IgG      | 1:200                          |
| SK-N-AS SK-N-DZ    | Anti-Mouse F4/80 Antigen Biotin mAb (Bio-Rad AbD Serotec)                                     | 1:100                        | -----                               | -----                          |
| SK-N-AS SK-N-DZ    | Ki67 (Dako, Glostrup, Denmark)                                                                | 1:75                         | Anti mouse IgG/Anti Rabbit IgG(H+L) | 1:200                          |

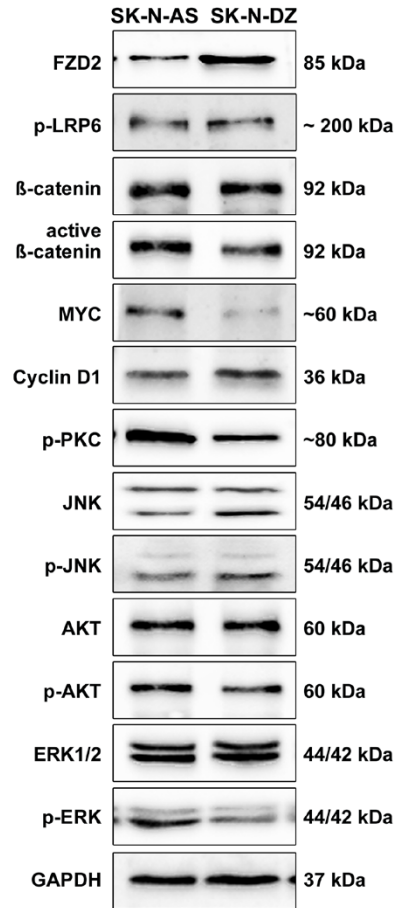
**Table 5.** List of primary and secondary antibodies used for immunohistochemistry. mAb, monoclonal antibody.

## Results

### 1. In vitro experiments

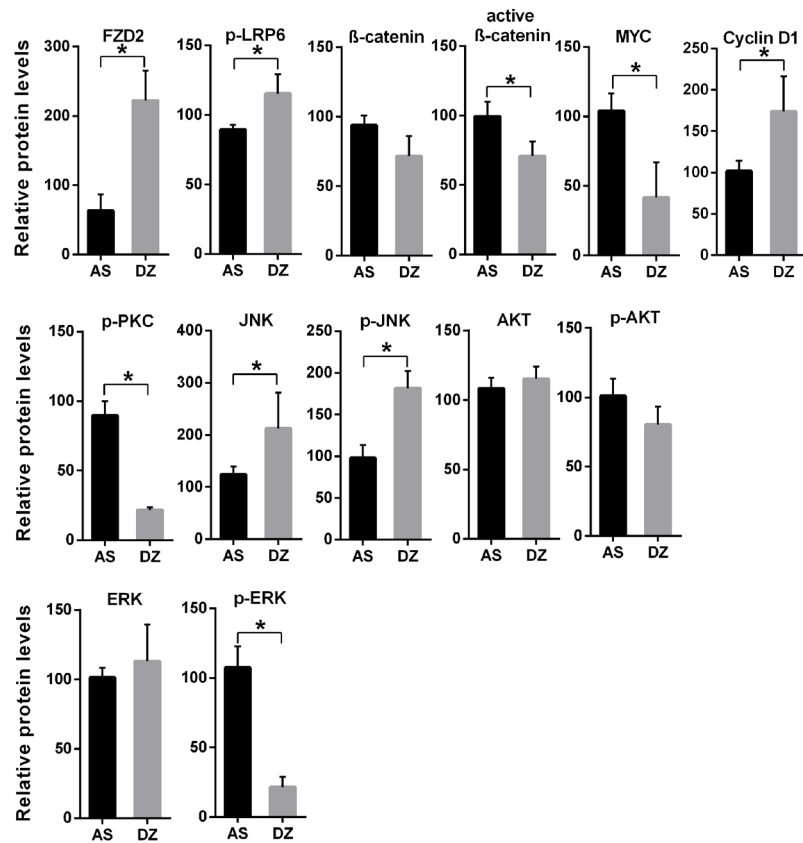
#### 1.1. Basal Wnt signaling activity differs in the neuroblastoma cell lines examined

Untreated SK-N-AS and SK-N-DZ cells were harvested to analyze the levels of FZD2, phospho-LRP6, total  $\beta$ -catenin, active  $\beta$ -catenin, MYC, cyclin D1, pan-phospho-PKC, total JNK, phospho-JNK, total AKT, phospho-AKT, total ERK and phospho-ERK by Western blotting (Figures 5 and 6). First, the basal activity of  $\beta$ -catenin-dependent-canonical Wnt signaling components was examined by analyzing LRP6 phosphorylation, total  $\beta$ -catenin, stabilized active  $\beta$ -catenin, MYC and cyclin D1 protein levels. This experiment showed that in SK-N-AS cells, FZD2 and phosphorylated LRP6 protein levels were lower compared with SK-N-DZ cells. In addition levels of total  $\beta$ -catenin, active  $\beta$ -catenin and MYC were more abundantly expressed in SK-N-AS. SK-N-DZ cells expressed higher levels of cyclin D1. Next the basal activity of  $\beta$ -catenin-independent non-canonical Wnt signaling components was examined by analysis of phosphorylation of PKC, JNK, AKT and ERK. SK-N-AS neuroblastoma cells expressed phosphorylated PKC at higher levels than SK-N-DZ cells. In contrast, SK-N-DZ cells expressed higher levels of JNK and phosphorylated JNK. SK-N-AS cells and SK-N-DZ cells showed similar high expression of AKT and phosphorylated AKT. These data indicated that basal Wnt signaling activity differs in SK-N-AS and SK-N-DZ cell lines.



**Figure 5. Representative Western blot images of protein expression in SK-N-AS and SK-N-DZ neuroblastoma cells.** Cells were harvested to analyze protein levels by Western blotting. GAPDH, loading control. For quantification, see “Figure 6”.





**Figure 6. Quantitative determination of protein expression in SK-N-AS and SK-N-DZ cells.** Active  $\beta$ -catenin (non-phospho  $\beta$ -catenin; Ser33/37/Thr41), MYC, cyclin D1, pan-phospho-PKC, ( $\beta$ II Ser660; p-PKC), total JNK, phospho- JNK (JNK phosphorylated at Thr183/Tyr185; p-JNK), total AKT, phospho-AKT (AKT phosphorylated at Ser473; p-AKT), total ERK and phospho-ERK (ERK1/2 phosphorylated at Thr202/Tyr204; p-ERK) were analyzed by Western blotting. Comparison of protein expression profiles between SK-N-AS and SK-N-DZ cells revealed differences in basal expression and activation levels of investigated signaling proteins. Graphs represent the mean of 3 independent experiments  $\pm$  SD (\*  $P < 0.05$ ).

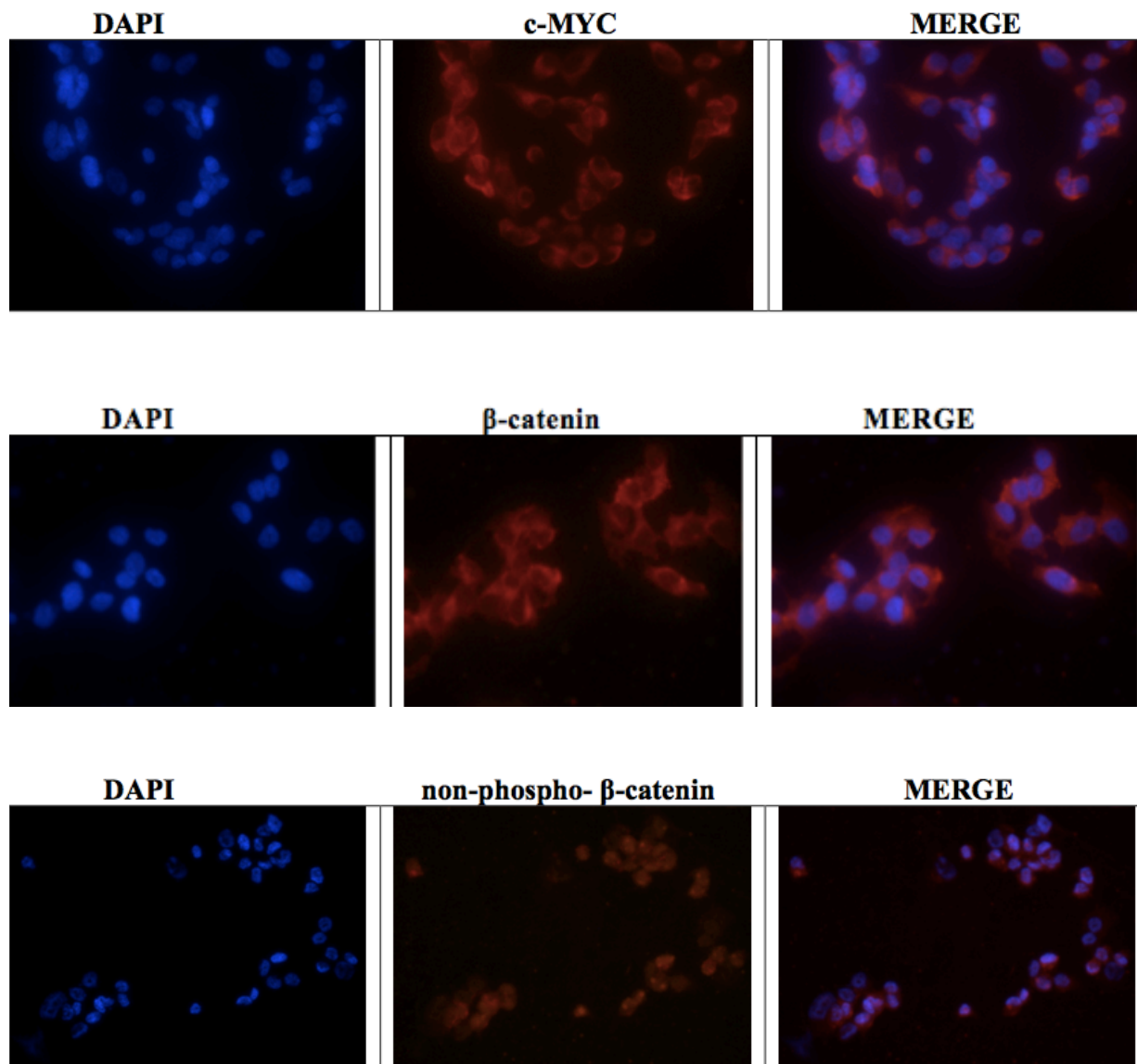
## 1.2. SK-N-AS cells express high level of c-MYC

Dysregulation of the Wnt signaling pathway allows  $\beta$ -catenin to accumulate and translocate to the nucleus, while it can lead to cancer [80]. A recent study showed hyperactive  $\beta$ -catenin/TCF signaling in a significant percentage of almost all types of cancer [81]. Another important protein in this study was c-MYC, which is a proto-oncogene and found to be activated in various tumors as well [82]. Interestingly, transcription of c-MYC occurred in 21 of 26 neuroblastomas [83]. Recently, a study revealed that c-MYC is highly expressed in breast cancer cells, but after silencing  $\beta$ -

catenin, c-MYC expression was reduced [84]. In addition to c-MYC, cyclin D1 contributes to tumor proliferation and malignant progression, which is also target gene of  $\beta$ -catenin [85].

First expression of c-MYC,  $\beta$ -catenin, and non-phospho  $\beta$ -catenin was assessed by immunoblot analysis in SK-N-AS cells as shown above. Then to investigate its role in neuroblastoma *in vivo*, we analyzed the nuclear expression of c-MYC and  $\beta$ -catenin in tumor tissue of neuroblastoma xenografts (Figure 7). This experiment revealed c-MYC,  $\beta$ -catenin, and non-phospho  $\beta$ -catenin protein around the nucleus. As known, increased cellular levels of  $\beta$ -catenin allow  $\beta$ -catenin to translocate to the nucleus and activate transcription factors in the cell. The level of  $\beta$ -catenin and c-MYC was higher around the nuclei compared to non-phospho  $\beta$ -catenin. The finding that high levels of  $\beta$ -catenin are present in SK-N-AS cells is in line with the fact that Wnt binding to FZD prevents phosphorylation of  $\beta$ -catenin and results in its stabilization and cytoplasmic accumulation [86].

This study showed high expression of c-MYC in the SK-N-AS cell line. Additionally, immunofluorescence analysis revealed high  $\beta$ -catenin and active  $\beta$ -catenin levels around the nucleus. Taken together, these data suggest translocation of active  $\beta$ -catenin and subsequent transcription of c-MYC as  $\beta$ -catenin target gene in neuroblastoma cells. It is important to know, that high MYCN/c-MYC target gene expression is a hallmark of malignant neuroblastoma progression [87].



**Figure 7. Expression of total  $\beta$ -catenin, active (non-phospho)  $\beta$ -catenin and c-MYC in SK-N-AS cells.** Immunofluorescence analysis of SK-N-AS cells using anti-human  $\beta$ -catenin, non-phospho-  $\beta$ -catenin, and c-MYC rabbit monoclonal antibodies as primary antibodies and Texas red anti-rabbit IgG as secondary antibody (red). DAPI was used to stain the cell nuclei. (40 x objective).

### **1.3. FZD2 regulates $\beta$ -catenin-dependent and -independent pathways in high-risk neuroblastoma cell lines**

Studying the effect of gene silencing in different cancers is very important and the use of siRNA provides an efficient tool for analyzing signal transduction mechanisms and also constitutes a potential therapeutic modality for cancer and other diseases.

Therefore siRNA specific to FZD2 was used to study the effect of FZD2 blockade in SK-N-AS and SK-N-DZ neuroblastoma cells. Nonspecific scrambled siRNA was used as control.

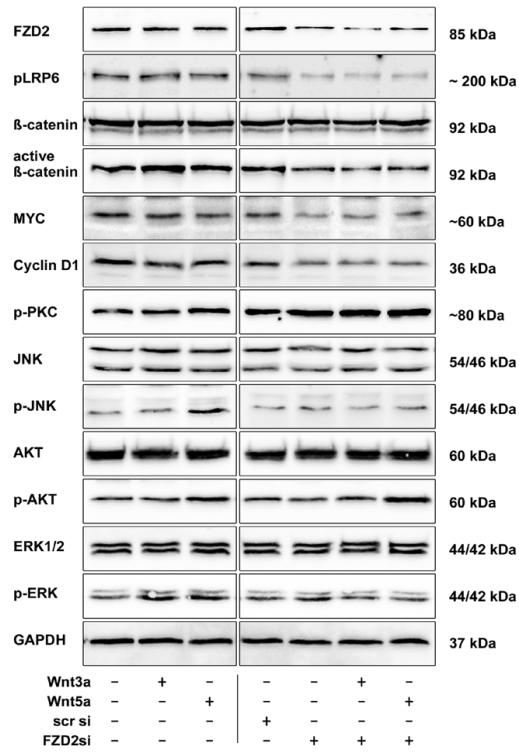
In addition we used recombinant Wnt3a and Wnt5a proteins to analyze whether these FZD2 ligands may increase or decrease baseline Wnt-signaling activity in neuroblastoma cells (Figures 8 and 9).

First, we analyzed activation of factors involved in signaling of the  $\beta$ -catenin – dependent Wnt pathway. This experiment showed that FZD2 siRNA reduced the levels of FZD2 protein in SK-N-AS and SK-N-DZ cells, as compared to cells treated with scrambled siRNA. Knockdown of FZD2 resulted in decreased levels of phosphorylated LRP6 and active  $\beta$ -catenin in SK-N-AS and SK-N-DZ cells stimulated with Wnt3a and Wnt5a and unstimulated controls. In addition, knockdown of FZD2 led to decreased MYC and cyclin D1 levels, while Wnt3a and Wnt5a treatment did not lead to any changes. In this experiment total  $\beta$ -catenin levels were not significantly changed compared to baseline levels.

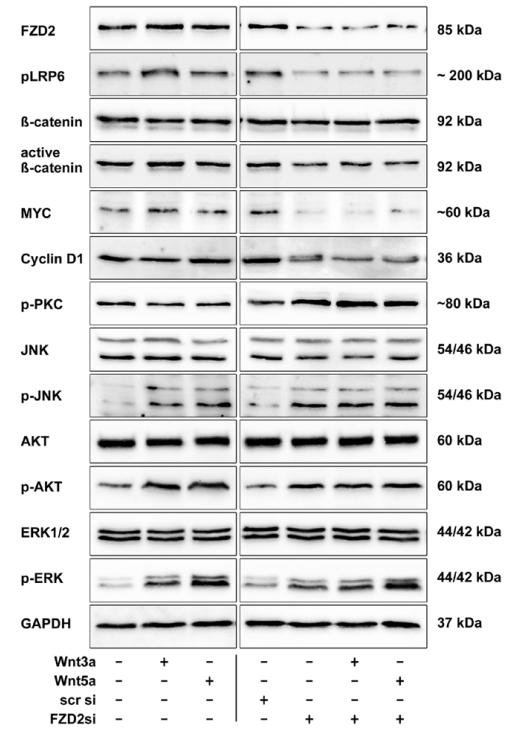
Next, activation of factors involved in signaling of noncanonical,  $\beta$ -catenin-independent Wnt signaling pathways were analyzed. Stimulated cells with recombinant Wnt3a and Wnt5a did not change PKC levels compared to baseline levels, while knockout of FZD2 significantly increased phosphorylation of PKC in SK-N-AS and SK-N-DZ cells. Looking at the total protein levels of AKT, JNK and ERK revealed unchanged levels of all these factors in both cell lines under the observed conditions.

FZD2 knockdown had no suppressing effect on JNK activity in SK-N-AS and SK-N-DZ cell lines, whereas treatment with Wnt5a increased phosphorylation of JNK in both cell lines, but Wnt3a increased phosphorylation of JNK only in SK-N-DZ cells (Figures 8 and 9).

### A SK-N-AS

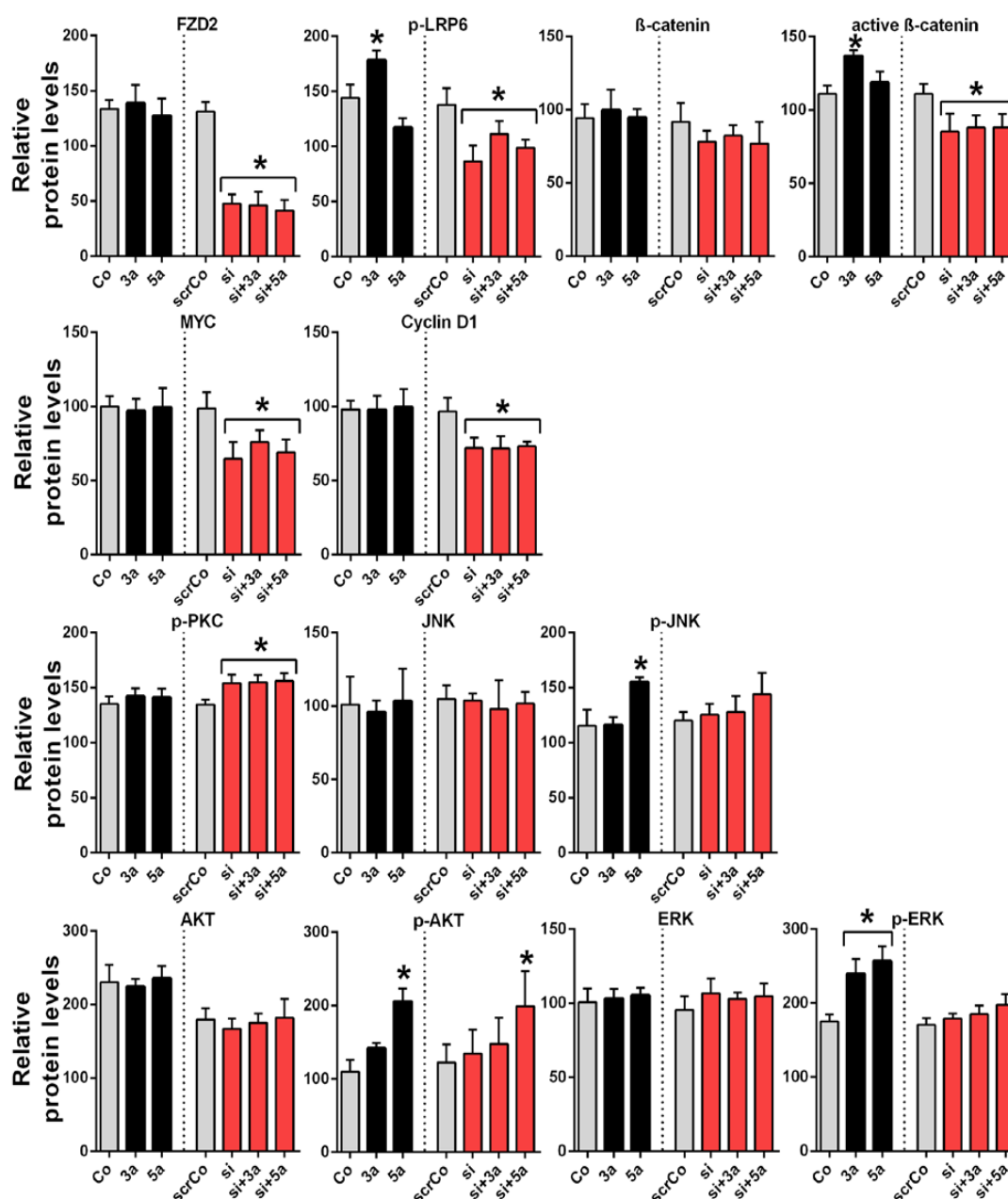


### B SK-N-DZ



**Figure 8. Western blot images of neuroblastoma cell lines. A.**SK-N-AS and **B.** SK-N-DZ.neuroblastoma cells. Untreated neuroblastoma cells (Control; Co) or cells treated with Wnt3a (3a) and Wnt5a (5a) for 15 min, with or without FZD2 siRNA were analyzed. Nonspecific scrambled siRNA (scr si) served as an additional control. Then proteins were isolated and analyzed by Western blotting (FZD2, phospho-LRP6 (LRP6 phosphorylated at Ser1490; p-LRP6), total  $\beta$ -catenin, active  $\beta$ -catenin (non-phospho  $\beta$ -catenin; Ser33/37/Thr41), MYC, cyclin D1, pan-phospho-PKC, ( $\beta$ II Ser660); p-PKC), total JNK, phospho-JNK (JNK phosphorylated at Thr183/Tyr185; p-JNK), total AKT, phospho-AKT (AKT phosphorylated at Ser473serine-473; p-AKT), total ERK and phospho-ERK (ERK1/2 phosphorylated at Thr202/Tyr204; p-ERK)).

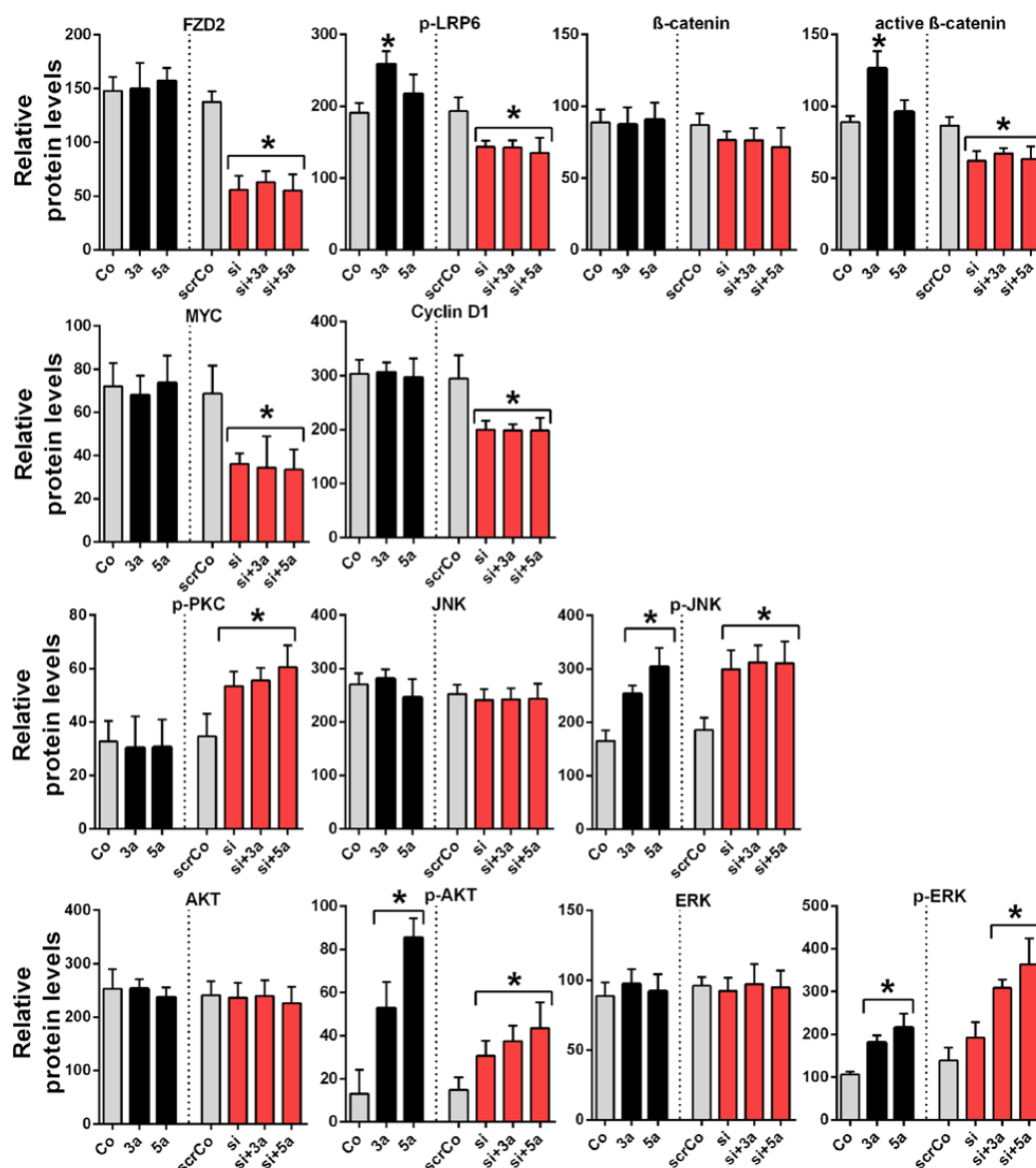
## A SK-N-AS



**Figure 9A. Quantification of Western blots from Wnt3a and Wnt5a stimulated or unstimulated SK-N-AS cells.** SK-N-AS neuroblastoma cells were left untreated (Control; Co) or treated with Wnt3a (3a) and Wnt5a (5a) for 15 min, with or without FZD2 siRNA (si) pretreatment. Nonspecific scrambled siRNA (scrCo) served as an additional control. Activation levels of signaling factors p-LRP6 and active  $\beta$ -catenin, which are associated with the  $\beta$ -catenin-dependent signaling pathway, and of the  $\beta$ -catenin target genes MYC and cyclin D1 were reduced below baseline levels in Wnt stimulated and unstimulated neuroblastoma cells pretreated with FZD2 siRNA in contrast to activation levels of p-PKC, p-

AKT, p-JNK, and p-ERK signaling factors, which are associated with  $\beta$ -catenin-independent signaling. Graphs represent the mean of 3 independent replicates  $\pm$  SD. Asterisks (\*) indicate  $P < 0.05$  vs. untreated controls, GAPDH, loading control.

## B SK-N-DZ



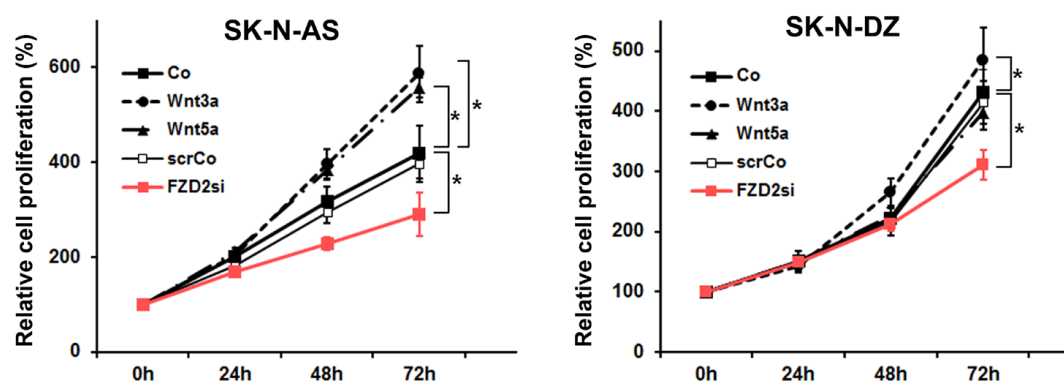
**Figure 9B. Quantification of Western blots from Wnt3a and Wnt5a stimulated or unstimulated SK-N-DZ cells.** SK-N-DZ neuroblastoma cells were left untreated (Control; Co) or treated with Wnt3a (3a) and Wnt5a (5a) for 15 min, with or without FZD2 siRNA (si) pretreatment. Nonspecific scrambled siRNA (scrCo) served as an additional control. Activation levels of signaling factors associated with  $\beta$ -catenin-dependent signaling pathway (p-LRP6, active  $\beta$ -catenin) and  $\beta$ -catenin target genes (MYC, cyclin D1) were reduced below

baseline levels in Wnt stimulated and unstimulated neuroblastoma cells pretreated with FZD2 siRNA in contrast to activation levels of signaling factors associated with  $\beta$ -catenin-independent signaling (p-PKC, p-AKT, p-JNK, p-ERK). Graphs represent the mean of 3 independent replicates  $\pm$  SD. Asterisks (\*) indicate  $P < 0.05$  vs. untreated controls, GAPDH, loading control.

#### 1.4. FZD2 stimulates cell proliferation of human neuroblastoma cells

Relative density of cancer cells was measured using the WST-1 cell proliferation assay up to 72 hrs following stimulation with Wnt3a and Wnt5a or pretreatment with FZD2 siRNA or nonspecific scrambled siRNA. Treatment of neuroblastoma cells with recombinant Wnt3a increased SN-N-AS and SK-N-DZ cell proliferation, whereas Wnt5a stimulation increased cellular proliferation of SK-N-AS but not SK-N-DZ cells as compared with unstimulated cells. Furthermore, treatment of unstimulated neuroblastoma cells with FZD2 siRNA significantly decreased proliferation of SK-N-AS and SK-N-DZ cells compared to untreated and scrambled siRNA transfected control cells (Figure 10).

From these experiments, we conclude that Wnt3a and Wnt5a stimulated FZD2 increasing the proliferation of both, MYCN-unamplified and MYCN-amplified neuroblastoma cell lines.

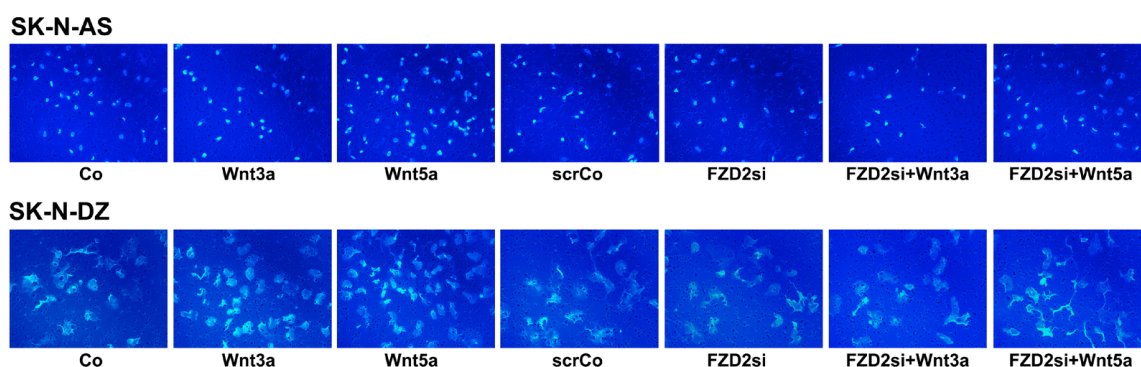


**Figure 10.** Effects of Wnt3a and Wnt5a stimulation and FZD2 blockade on cell proliferation. Graphs represent the mean of 3 independent experiments  $\pm$  SD. Asterisks (\*) indicate  $P < 0.04$  vs. untreated controls (Co, scrCo).

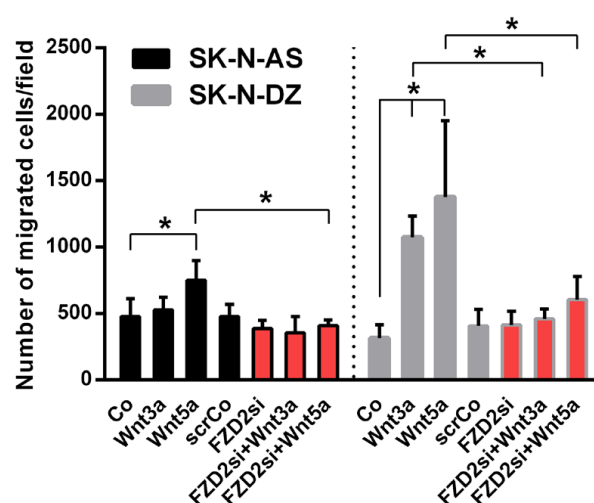


### **1.5. FZD2 promotes cell migration in neuroblastoma cell**

The effect of FZD2 on Wnt3a- and Wnt5a-stimulated neuroblastoma cell migration was evaluated in transwell assays. Migration of Wnt3a-stimulated SK-N-AS cells remained unchanged during 48 hours, whereas Wnt5a stimulation significantly enhanced the ability of SK-N-AS neuroblastoma cells to migrate as compared to control cells. In SK-N-DZ cells, Wnt3a and Wnt5a stimulation promoted transwell migration compared to control. FZD2 knockdown completely suppressed Wnt5a-induced migration of SK-N-AS cells and Wnt3a and/or Wnt5a-induced migration of SK-N-DZ cells. These data suggest that FZD2 promotes neuroblastoma cell migration through a process that involves stimulation by Wnt5a in SK-N-AS cells and by Wnt3a and/or Wnt5a in SK-N-DZ cells (Figures 11 and 12).



**Figure 11. Effect of Wnt3a and Wnt5a stimulation and FZD2 blockade on neuroblastoma cell migration.** Representative images of migrated SK-N-AS and SK-N-DZ cells from an *in vitro* migration assay.



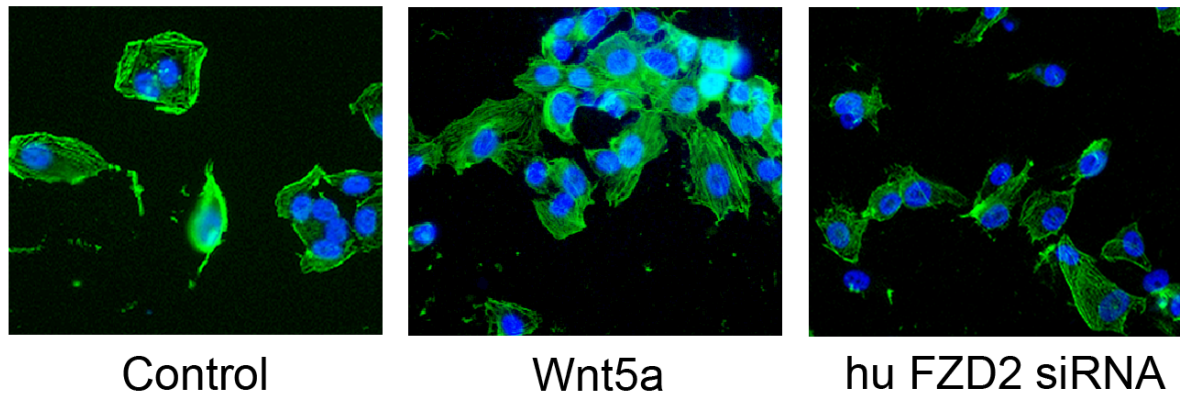
**Figure 12. Quantification of cell migration.** SK-N-AS and SK-N-DZ cells were stimulated with 100 ng/ml recombinant proteins Wnt3a and Wnt5a with or without pretreatment with FZD2 siRNA or scrambled siRNA (scrCo) for 24 h followed by quantification of migrated cancer cells. FZD2 blockade suppressed Wnt-induced migration of neuroblastoma cells. Graphs represent the mean of 3 independent experiments  $\pm$  SD. Asterisks (\*) indicate  $P < 0.05$  vs. untreated controls (Co, scrCo) and vs. Wnt3a and Wnt5a stimulated cells, respectively.

### **1.6. Treatment with FZD2 siRNA changes SK-N-AS cells morphology**

One of the important aspects of cancer cells are defects in cellular morphogenesis [88]. The actin filaments together with intermediate filaments and microtubules are responsible for mechanical stabilization of the cellular structure and migration [89]. The cytoskeleton in normal cells can be subverted in cancer cells and contributes to changes in cell growth, movement and invasiveness [90]. Molecules that link migratory signals to the actin cytoskeleton are upregulated in invasive and metastatic cancer cells [91]. Upon binding the ligand molecules to cell surface receptors, the molecules stimulate intracellular signaling pathways to reorganize the actin cytoskeleton [92].

First the effect of the FZD2 ligand Wnt5a on reorganization of the actin cytoskeleton was investigated. Stimulation of SK-N-AS cells with recombinant Wnt5a protein followed by staining of the actin cytoskeleton with phalloidin showed a rearrangement of the actin cytoskeleton and enriched numbers of filopodia in Wnt5a stimulated cells compared to control cells. These finding suggests an increased migratory capacity of Wnt5a-stimulated SK-N-AS cells mediated by FZD2 (Figure 13).

Then, to directly investigate the effect of the Wnt/FZD2 pathway on cellular migration and morphology, SK-N-AS cells were transfected with human FZD2 siRNA followed by phalloidin staining. The fluorescence microscopic analysis showed reduced spreading of SK-N-AS cells after FZD siRNA transfection as compared to control and Wnt5a stimulated cancer cells. These data are indicative of downregulated activity of the actin cytoskeleton and the corresponding migratory capacity in SK-N-AS cells elicited by blockade of FZD2-signaling.



**Figure 13. Cytoskeletal staining of SK-N-AS cells.** Nuclei were stained with blue-fluorescent DAPI. Actins filaments were stained with Alexa Fluor 448 Phalloidin. SK-N-AS cells were stimulated with recombinant Wnt5a or treated with human FZD2 siRNA or left untreated (control) (60 x objective).

## **2. In vivo experiments**

### **2.1. FZD2 blockade reduces growth of neuroblastoma xenografts**

Histology and immunohistochemistry was performed on tumor tissue obtained from previous experiments in athymic nude mice in which SK-N-AS or SK-N-DZ cells were injected subcutaneously into mice. Mice bearing human neuroblastoma xenografts received five intratumoral injection of FZD2 siRNA or scrambled siRNA. Tumor growth was significantly reduced after treatment with FZD2 siRNA in both SK-N-AS and SK-N-DZ xenografts compared to control groups as described by Zins et al. [13].

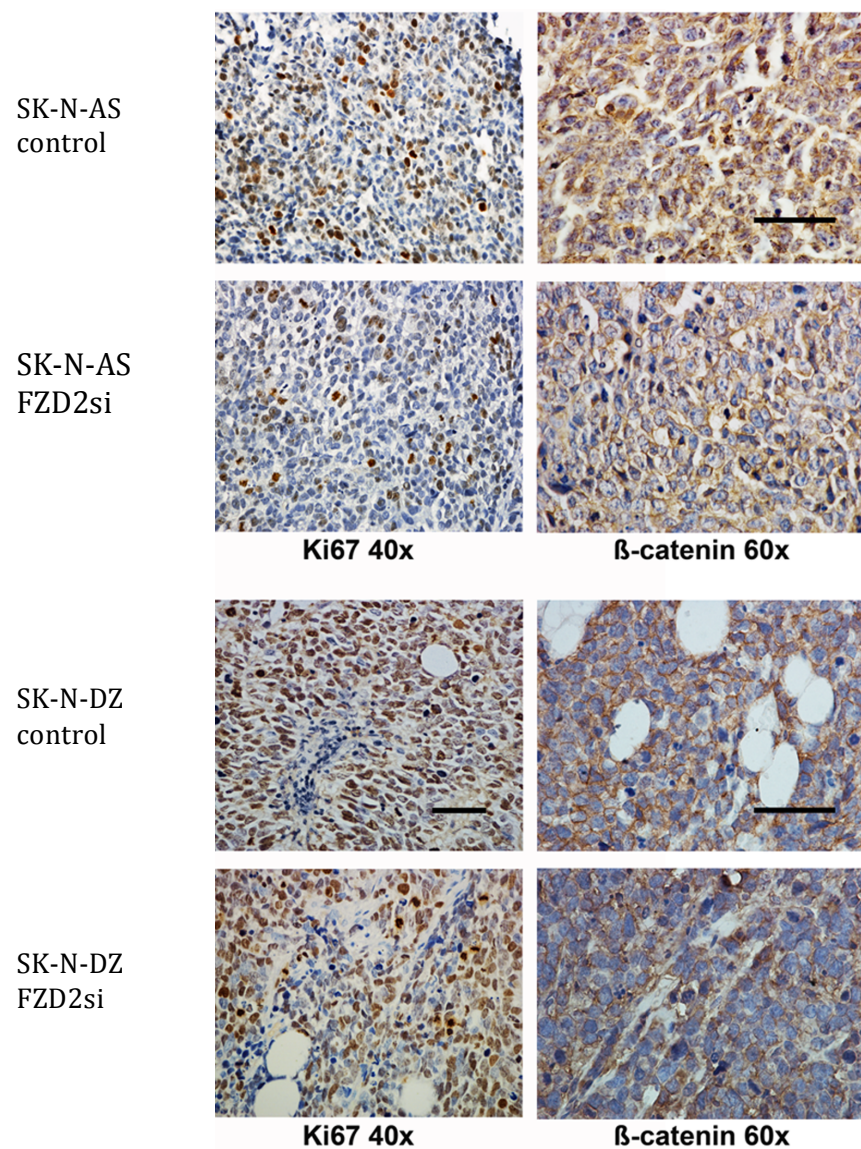
### **2.2. FZD2 blockade decreases $\beta$ -catenin staining intensity in SK-N-AS and SK-N-DZ tumors**

Immunohistochemical staining of tumor sections was performed with anti-human  $\beta$ -catenin and anti-human Ki67 antibodies. Antigen Ki67 is a nuclear protein that is associated with and may be important for cellular proliferation and it is associated with ribosomal RNA transcription [93].

Histological analysis of tumors demonstrated that FZD2 siRNA treated tumors showed morphological changes. Ki67+ proliferating cells were significantly reduced in both FZD2 siRNA groups by 42% in SK-N-AS, and 40% in SK-N-DZ xenografts (Figures 14 and 15).

Immunohistochemistry staining for  $\beta$ -catenin showed higher  $\beta$ -catenin expression in SK-N-AS neuroblastoma tumors as compared to SK-N-DZ neuroblastoma tumors. On the other hand, FZD2 blockade decreased  $\beta$ -catenin staining intensity in SK-N-AS and SK-N-DZ tumors (Figures 14 and 15).

From these data it can be suggested that a blockade of FZD2 reduces tumor growth and tumor cell proliferation and that these changes are associated with decreased  $\beta$ -catenin levels in both, MYCN-unamplified SK-N-AS and MYCN-amplified SK-N-DZ neuroblastomas.

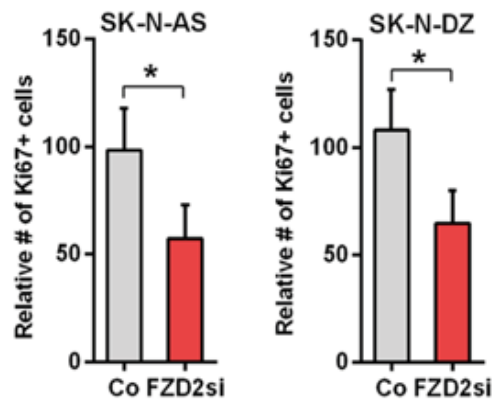


**Figure 14. Immunohistochemistry of neuroblastoma xenograft tumor sections.**

Representative images of tumor tissue sections from mice treated with FZD2 siRNA on day 24 after tumor inoculation.

Left column: Neuroblastoma xenografts stained with the proliferation marker Ki67. The scale bar in this column represents 50  $\mu$ m.

Right column: Neuroblastoma tumors stained for total  $\beta$ -catenin.  $\beta$ -catenin expression was higher in SK-N-AS tumors. In both, SK-N-AS and SK-N-DZ xenografts,  $\beta$ -catenin staining intensity was reduced in the FZD2 siRNA group. The scale bar in the right column represents 50  $\mu$ m.



**Figure 15.** Quantitative histomorphometric analysis of Ki67-positive, proliferating tumor cells. Cellular proliferation was significantly reduced following FZD2 blockade. Data are mean  $\pm$  SD (n = 8 per group). Asterisks (\*) indicate  $P < 0.05$  vs. controls.

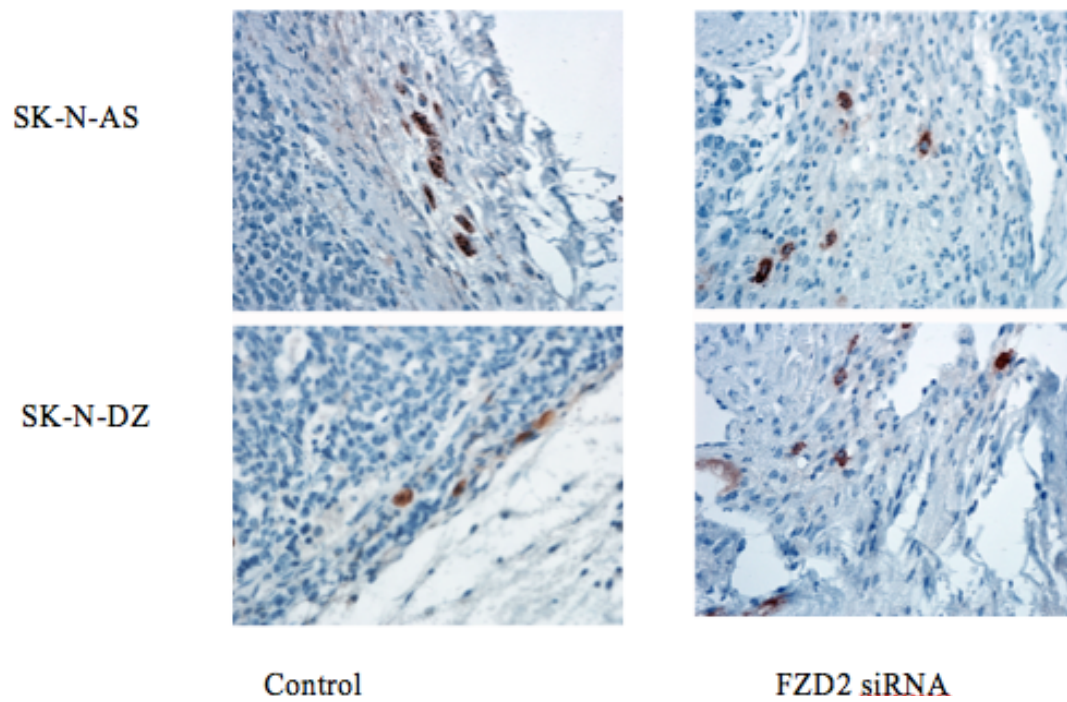
### 2.3. Blocking FZD2 has no effect on tumor-associated macrophages

To address whether FZD2 siRNA affects the number of tumor-associated macrophages (TAMs) in neuroblastomas, we analyzed TAMs in tumor tissue sections from mice treated with FZD2 siRNA by immunohistochemistry. TAMs belong to the macrophage lineage and can be found in tumor tissue. Notably, TAMs can promote tumor initiation, growth, migration, and angiogenesis in different cancers [94, 95].

Immunohistochemical staining was performed on tumor tissue sections of SK-N-AS and SK-N-DZ xenografts using an anti-F4/80 antibody conjugated to biotin. The F4/80 antigen is expressed by the majority of mature murine macrophages and is one of the best markers for this population of cells [96].

Staining showed a similar number and distribution of F4/80 positive cells (Figure 16). These data suggest that TAMs found in the tumor are not affected by treatment with FZD2 siRNA. However, they may still contribute to malignant behavior of cancer cells in neuroblastoma.





**Figure 16. Immunohistochemical staining of neuroblastoma tumor sections.** F4/80 positive TAMs are stained brown. Left column: untreated SN-N-AS and SK-N-DZ tumors (control). Right column: FZD2 siRNA treated SK-N-AS and SK-N-DZ tumors. Nuclei were stained with DAPI.



## Discussion

Neuroblastoma is the most common extracranial solid tumor in children that arises from the developing sympathetic nervous system [12]. Four stages are used for neuroblastoma. In stage 1, the cancer is still in the area where it started. Stage 2 is divided into stages 2A and 2B. In both of them the tumor can be completely removed during surgery, but in stage 2B cancer cells are found in the lymph nodes near the tumor. Although the cancer has not spread to distant parts of body in stage 3, the tumor cannot be removed completely by surgery. Finally in stage 4, the tumor has spread to distant lymph nodes or other parts of the body [97, 98].

In neuroblastoma many factors, such as age at diagnosis, stage of disease at diagnosis, and the molecular, cellular, and genetic features of the tumor determine whether it will spontaneously regress or metastasize and become refractory to therapy [12]. The spontaneous regression usually consists of complete disappearance of the disease and various factors may influence regression, whereas in some neuroblastomas maturation takes place [99]. The standard therapy for patient with high-risk neuroblastoma consists of surgery, intensive chemotherapy, radiotherapy, and stem cell transplantation [100-104]. Unfortunately, more than half of the children with high-risk neuroblastoma relapse regardless of the treatment intensity [104]. Additionally, resistance formation after initial therapy response is common in high-risk neuroblastoma patients [105].

The Wnt signaling transduction cascade controls a myriad of biological phenomena like development, differentiation, proliferation, and cellular homeostasis, and thus it is not surprising that aberrant Wnt signaling underlies a wide range of pathologies in humans [42, 86]. Wnt signaling pathways have been characterized to the canonical or Wnt/ $\beta$ -catenin dependent pathway and non-canonical or  $\beta$ -catenin-independent pathways, which can be further divided into the planar cell polarity and the Wnt/ $\text{Ca}^{2+}$  pathways and these branches are being actively dissected at the molecular and biochemical levels [28].

The extracellular Wnt signal stimulates several intracellular signal transduction cascades in canonical and non-canonical Wnt signaling pathways [28]. Without Wnt signaling, cytoplasmic  $\beta$ -catenin is degraded by a  $\beta$ -catenin destruction complex but

in the presence of Wnt ligand  $\beta$ -catenin remains unphosphorylated [28, 106]. The stabilized  $\beta$ -catenin translocates to the nucleus where it regulates downstream gene expression by binding to TCF/LEF factors [106]. The TCF/LEF family of transcription factors modulates expression of target genes critical for cell proliferation, differentiation, survival and apoptosis, such as cyclin D1 and c-MYC [107]. MYC and cyclin D1 are involved in oncogenesis during cancer formation [28]. Both, c-MYC and cyclin D1 have been shown to induce cell-cycle progression in breast cancer cell lines [108, 109].

Since it is known that Wnt signaling pathways and its components play a critical role in several cancers [34], we examined basal canonical Wnt pathway activity in SK-N-AS and SK-N-DZ cells using several markers. These experiments show higher levels of  $\beta$ -catenin, active  $\beta$ -catenin, and c-MYC in SK-N-AS and higher levels of cyclin D1, FZD2, and phosphorylated LRP6 in SK-N-DZ cells. These data suggest different expression levels of canonical Wnt signaling components in SK-N-AS and SK-N-DZ cells.

The already mentioned non-canonical Wnt pathways branch off into the planar cell polarity pathway and the Wnt/ $\text{Ca}^{2+}$  pathway [28, 42]. Planar cell polarity refers to the polarization of a field of cells within the plane of a cell sheet, and it is required for diverse cellular processes in vertebrates, such as body axes patterning [110, 111]. Dysregulation of the planar cell polarity pathway also occurs in human cancers [112]. The second branch of non-canonical Wnt signaling, the Wnt/ $\text{Ca}^{2+}$  pathway, plays a role in development and the maintenance of neural and blood stem cells and is involved in cancer too [40].

The Wnt/ $\text{Ca}^{2+}$  pathway involves activation of phospholipase C, increasing  $\text{Ca}^{2+}$  levels, and subsequent activation of PKC,  $\text{Ca}^{2+}$ -calmodulin-dependent protein kinase II, and calcineurin. The PCP pathway is activated when Wnt ligands binds to a FZD receptor followed by transduction of signals that lead to activation of small GTPases, such as Rac and RhoA, and activation of JNK, finally affecting cell polarity and movement [113]. Consequently we examined basal non-canonical Wnt signaling components in SK-N-AS and SK-N-DZ cells. Comparison of protein expression profiles between SK-N-AS and SK-N-DZ cells revealed differences in basal expression and activation levels of phospho-PKC, JNK, phospho-JNK, and phospho-ERK.

FZD2 is one of the most important receptors in non-canonical Wnt pathways and data from our group showed high levels of mRNA expression of FZD2 in neuroblastoma cell lines [13]. Wnt3a and Wnt5a can serve as a ligand for the FZD2 receptor. Wnt5a can also bind to membrane-bound receptor tyrosine kinase (RTK), Ror1/2, and mediate non-canonical signal transduction [114]. The functions of Ror2 in mediating the non-canonical Wnt signaling have been well established [115]. The secreted regulatory proteins Wnt3a and Wnt5a may lead to activation of both canonical (Wnt3a) and non-canonical (Wnt5a) Wnt signaling pathways in cancer [115, 116]. Interestingly, high expression of both Wnt3 and Wnt5a has been shown in neuroblastoma tumors and cell lines [2]. Thus, we examined the function of FZD2 and its potential ligands Wnt3a and Wnt5a in MYCN-single copy SK-N-AS and MYCN-amplified SK-N-DZ neuroblastoma cell lines using recombinant Wnt3a, Wnt5a and FZD2 siRNA.

This study has shown Wnt/FZD2 activity in two neuroblastoma cell lines by increased phospho-LRP6 and active  $\beta$ -catenin levels in neuroblastoma cells treated with recombinant Wnt3a, consistent with canonical  $\beta$ -catenin signaling. In supported of this, other studies have shown a potential relationship between increased Wnt/ $\beta$ -catenin signaling and neuroblastoma [36, 117, 118]. In addition, evidence is provided that FZD2 is also involved in regulation of non-canonical Wnt signaling in high-risk neuroblastoma cells. This includes the PCP pathway, in which FZD receptors activate a cascade that involves the small GTPase Rac1 and JNK, but also the Wnt/ $\text{Ca}^{2+}$  pathway, in which Wnts trigger FZD-mediated  $\text{Ca}^{2+}$  release and activate PKC [119].

In the cell lines used in this study, Wnt5a increased phosphorylation of JNK in both cell lines, whereas Wnt3a had no effect. Moreover, Wnts increased ERK phosphorylation in our cell lines. Of interest, there are reports showing the involvement of Wnt5a in metastatic melanoma progression and invasive breast cancer [67]. Moreover, Wnt5a has been suggested to be involved in the pathogenesis of neuroblastoma through the Wnt/ $\text{Ca}^{2+}$  pathway [67].

One way to understand the function of a gene is a biological system that lacks that gene. RNA interference (RNAi) has become an important tool in basic life science research for validating gene function through knock down of the expression of

individual genes [120]. In addition, RNA interference provides a promising therapeutic modality for cancer and other diseases [120, 121]. In this study we have used FZD2 siRNA to understand the role of FZD2 receptor in neuroblastoma growth. FZD2 blockade reduced  $\beta$ -catenin staining intensity in both neuroblastoma xenografts. It is known that MYC expression is dependent upon canonical Wnt/ $\beta$ -catenin signaling [122]. Accordingly, expression of the  $\beta$ -catenin target genes MYC and cyclin D1 were coordinately reduced by FZD2 blockade associated with a lower proliferation rate. Together, analyzing signaling factors of the  $\beta$ -catenin-dependent Wnt pathway demonstrated that FZD2 activates the canonical Wnt signaling pathway.

FZD2 blockade not only reduced canonical signaling factors like active  $\beta$ -catenin. We showed that FZD2 blockade led to increased PKC-phosphorylation associated with downregulation of the  $\beta$ -catenin-dependent pathway, indicating a move from Wnt/ $\beta$ -catenin to Wnt/ $\text{Ca}^{2+}$  signaling in the investigated neuroblastoma cells. Total protein levels of AKT, JNK and ERK remained constant in both cell lines after FZD2 knockdown. Moreover, this study showed that FZD2 blockade suppressed Wnt-dependent Rac activation and concomitant migration in both cell lines. FZD2 blockade affected not only Wnt5a-dependent Rac1 activation in SK-N-AS and SK-N-DZ cells, but also Wnt3a-dependent Rac activation in SK-N-DZ cells, indicating cell type-specific differences in Rac activation. [13].

Taken together, these data suggest the crosstalk between canonical and non-canonical signaling pathway in neuroblastoma. In support of this, another study reported a considerable degree of overlap between Wnt pathways [40].

Besides mutations and aberrant expression of specific components of the canonical Wnt pathway, which can be oncogenic, mitogen-activated protein kinase (MAPK) pathways have unique and provocative roles that impact canonical Wnt signaling [123]. Additionally, relapsed neuroblastomas had frequent RAS-ERK pathway mutations [124]. The MAPK signaling cascades play a critical role in regulation of cell proliferation and contribute to the amplification and specificity of the transmitted signals that eventually activate several regulatory molecules in the cytoplasm and in the nucleus to initiate cellular processes such as proliferation, differentiation, development transformation, and apoptosis [125, 126]. The MAPK-ERK pathway

represents one of the best characterized MAPK signaling pathways. The stimulation of tyrosine kinase receptors (RTKs) stimulates the activation of MAPKs in a multistep process. Involving a cascade of protein kinases defined sequentially as MAPKKK and MAPKK such as MEK1 and MEK2. MEKs ultimately phosphorylate ERK1 and ERK2 respectively, thereby increasing their enzymatic activity. Then the activated ERKs translocate to the nucleus and activate transcription factors, changing gene expression to promote growth and differentiation. On the other hand, it is known that activation of G protein-coupled receptors (GPCRs) leads to stimulation of a large number of complex signaling cascades, which can mediate the activation of MAPKs. In this case, stimulation of GPCRs leads to activation of RTKs by tyrosine phosphorylation, which then results in downstream signaling events ultimately leading to ERK activation [126, 127].

Consistent with this, our experiments showed that perturbation of FZD2 by siRNA led to downregulation of LRP6 phosphorylation with concomitant upregulation of ERK phosphorylation in neuroblastoma indicating a FZD2-dependent crosstalk between Wnt and ERK signaling. Another potential interpretation of these findings may be that the inverse relationship of LRP6 and ERK1/2 phosphorylation is the result of a compensatory signaling response. Furthermore, suppression of the FZD2/LRP6 pathway caused over-activation of RTK pathways, including AKT and ERK pathways that potentially oppose the antiproliferative effects of FZD2 inhibitors. However, further investigation using FZD inhibitors is needed to confirm our FZD2 siRNA data and to fully understand how neuroblastoma cells adapt their signaling circuitry, taking advantage of routes of feedback and crosstalk to maintain their function. A recent study has highlighted that MEK inhibitors are a promising molecular-targeted therapeutic option for neuroblastoma [124]. We hypothesize that simultaneous MEK inhibition could be more effective than FZD inhibition alone to decrease neuroblastoma cell proliferation and cancer progression.

Different RTK elicit different cellular response, however, usually they all activate RAS/ERK, SCR-family, and PI3K/AKT pathways [13]. Apart from that, a study investigating cyclin D knockdown cells revealed that, these cells required higher p-AKT and lower pERK signals to increase cyclin D protein stability [13]. Cell cycle progression and growth factor signaling are linked by cyclin D1, whose gene is induced as secondary response gene following mitogenic stimulation [126].

Interestingly, high expression of cyclin D1 occurs in approximately two thirds of neuroblastoma cell lines and tumors [128]. Thus, based on these findings, one might speculate that the shift of the p-AKT/p-ERK ratio to high p-AKT/high p-ERK in FZD2 siRNA treated neuroblastomas, which was found in this study, translates the p-ERK/p-AKT decision towards decreased proliferation of neuroblastoma cells. In further support of this hypothesis it is worth mentioning that this was associated with reduced cyclin D1 expression levels.

Many non-tumor host cells such as inflammatory cells, fibroblasts and endothelial cells are present in the tumor microenvironment and affect the malignant potential and chemo-resistance of the tumor cells. An example for these inflammatory cells are tumor-associated macrophages (TAMs) [129]. Macrophage functions are broadly classified into two distinct types; the classically activated macrophage (M1) and the alternatively activated macrophage (M2). M1 macrophages are characterized by the production of pro-inflammatory cytokines, which have the capacity for bactericidal and tumor-suppressive activities. M2 macrophages are characterized by the production of anti-inflammatory cytokines. TAMs are close in character to M2 macrophages [130, 131]. There are reports showing that TAMs can stimulate neuroblastoma growth in human cell lines and their xenografts [130, 132]. Another report showed that metastatic neuroblastoma had higher infiltration of TAMs than locoregional tumors [133]. Notably, Macrophage migration inhibitory factor regulated neuroblastoma growth, angiogenesis, and metastasis and was associated with dedifferentiation in *MYCN* amplified tumors [12]. Therefore, in the present study we examined the association of TAMs with neuroblastoma cells. Findings from the present study showed no significant changes in TAM quantity after FZD2 siRNA treatment in SK-N-AS and SK-N-DZ cells. However, this does not mean that TAMs do not affect the malignant behavior of neuroblastomas, and further investigations are needed to determine the effect of FZD2 blockade on TAMs in neuroblastoma. A very recent study also demonstrated that cell-cell interaction between TAMs and cancer-associated fibroblasts induced recruitment and activation of each other, and the combined activities of these cells were involved in neuroblastoma progression [129].

In cancer metastasis, cell adhesion and cell migration mechanisms play an important role. Cell migration involves coordinated and dynamic changes in the actin

cytoskeleton and cell adhesion. When epithelial cells start to migrate, cell-cell junctions are first disrupted. During migration, membrane protrusions like lamellipodia and filopodia, are seen externally at the cell front, and retraction at the cell rear. Additionally, dynamic reorganization of the actin cytoskeleton, such as disassembly and reassembly of stress fibers at cell-cell and cell-matrix junctions and membrane protrusions, is observed internally [134]. The GTP-binding proteins Rho, Rac and Cdc42 are known to regulate actin organization. In fibroblasts, Rho mediates the formation of actin stress fibers, whereas Rac regulates lamellipodium formation and Cdc42 controls filopodium formation [135, 136]. In this study, we observed Wnt5a-dependent Rac1 activation in both, SK-N-AS and SK-N-DZ neuroblastoma cell lines. Moreover, Wnt3a-dependent Rac activation was found in SK-N-DZ but not SK-N-AS cells. These data suggest cell type-specific differences in Rac activation and of the associated rearrangement of the actin cytoskeleton in high-risk neuroblastoma cell lines. Of interest, FZD2 blockade suppressed Wnt-dependent Rac activation and the migration rate in both cell lines.

### **Concluding remarks**

In summary, we conclude that FZD2 is likely a control point for cell growth in high-risk neuroblastoma tumors by regulating  $\beta$ -catenin-dependent and  $\beta$ -catenin-independent signaling pathways. FZD2 blockade by siRNA inhibited neuroblastoma cell proliferation, and reduced cell motility. Thus, FZD2 represents a potential therapeutic target in FZD2-driven high-risk neuroblastoma tumors. Moreover our studies demonstrated that TAMs showed no change by FZD2 siRNA blockade. The latter phenomenon, however, deserves further work.

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## Appendix

### 1. Abbreviations

|                    |                                                          |
|--------------------|----------------------------------------------------------|
| AKT (PBC)          | protein kinase B                                         |
| APC                | protein kinase C                                         |
| BSA                | bovine serum albumin                                     |
| Ca <sup>2+</sup>   | calcium                                                  |
| CamKII             | Ca <sup>2+</sup> /calmodulin-dependent protein kinase II |
| CDC42              | Cell division control protein 42 homolog                 |
| CK1                | casein kinase 1                                          |
| CK1 $\alpha$       | casein kinase 1 $\alpha$                                 |
| CRD                | cytine-rich domain                                       |
| DAAM1              | Dishelved associated activator of morphogenesis 1        |
| DAG                | di-glyceride                                             |
| DAPI               | 4',6-diamidino-2-phenylindole                            |
| ddH <sub>2</sub> O | double-distilled water = DIW                             |
| Dkk                | Dickkopf                                                 |
| DMSO               | Dimethyl sulphoxide                                      |
| DNA                | deoxyribonucleic acid                                    |
| DPX                | Distyrene Plasticizer Xylene                             |
| Dsh                | Disheveled                                               |
| ECL                | enhanced chemoluminescence                               |
| ECM                | extracellular matrix                                     |
| EDTA               | ethylenediaminetetraacetic acid                          |
| ER                 | endoplasmic reticulum                                    |
| ERK                | extracellular signal-regulated kinase                    |
| EtOH               | Ethanol                                                  |

|                                                    |                                                                                     |
|----------------------------------------------------|-------------------------------------------------------------------------------------|
| FBS                                                | fetal bovine serum                                                                  |
| FZD                                                | frizzled                                                                            |
| GPCRs                                              | G-protein coupled receptor                                                          |
| GSK-3                                              | glycogen synthase kinase 3                                                          |
| H                                                  | hour(s)                                                                             |
| HCC                                                | hepatocellular carcinoma                                                            |
| HRP                                                | Horseradish peroxidase                                                              |
| IgG                                                | Immunoglobulin G                                                                    |
| IHC                                                | immunohistochemistry                                                                |
| IP3                                                | Inositol triphosphate                                                               |
| JNK                                                | c-Jun N-terminal kinase                                                             |
| kDa                                                | kilo Dalton                                                                         |
| Ki67 (MKI67)                                       | protein encoded by the MKI67 gene (antigen identified by monoclonal antibody Ki-67) |
| LRP5/6                                             | Low-density lipoprotein receptor-related protein5/6                                 |
| MAP                                                | mitogen-activated protein                                                           |
| MAPK                                               | mitogen-activated protein kinase                                                    |
| Min                                                | minute(s)                                                                           |
| mL                                                 | milliliter                                                                          |
| mM                                                 | milimolar                                                                           |
| MQ                                                 | macrophage                                                                          |
| Na <sub>3</sub> VO <sub>4</sub>                    | sodium orthovanadate                                                                |
| Na <sub>3</sub> VO <sub>4</sub> ·2H <sub>2</sub> O | sodium orthovanadate dehydrate                                                      |
| NaCl                                               | sodium chloride                                                                     |
| NB                                                 | Neuroblastoma                                                                       |
| NEAA                                               | non-essential amino acids                                                           |
| NRH1                                               | neurotrophin receptor homolog                                                       |

|           |                                            |
|-----------|--------------------------------------------|
| PBS       | phosphate buffered saline                  |
| PCP       | Planar cell polarity pathway               |
| PDE       | phosphodiesterase                          |
| Pen/Strep | penicillin-streptomycin                    |
| pH        | power of hydrogen                          |
| PIP2      | Phosphatidylinositol 4,5-bisphosphate      |
| PKC       | protein kinase C                           |
| PKG       | protein kinase G                           |
| PLC       | Phospholipase C                            |
| PMSF      | phenylmethanesulfonyl fluoride             |
| PP2A      | protein phosphatase 2A                     |
| Rac1      | ras-related C3 botulinum toxin substrate 1 |
| RNA       | ribonucleic acid                           |
| ROCK      | Rho-associated kinase                      |
| ROR       | Retinoid-related orphan receptor           |
| Rpm       | rounds per minute                          |
| RT        | room temperature                           |
| RTK7      | tyrosine-protein kinase-like7              |
| Ryc       | receptor-like tyrosine kinase              |
| S         | second(s)                                  |
| scrCo     | nonspecific scrambled siRNA                |
| SD        | standard deviation                         |

|                |                                                            |
|----------------|------------------------------------------------------------|
| SDS-PAGE       | Sodium dodecyl sulphate polyacrylamide gel electrophoresis |
| sFRPs          | secreted Frizzled-Related Proteins                         |
| siRNA          | small interfering RNA                                      |
| SK-N-AS        | human neuroblastoma cell line                              |
| SK-N-DZ        | human neuroblastoma cell line                              |
| So-Py          | sodium pyruvate                                            |
| TAMs           | tumor associated macrophages                               |
| TBST           | tris-buffered saline = TBS                                 |
| TCF/LEF        | T-cell factor/lymphocyte enhance factor                    |
| TME            | tumor microenvironment                                     |
| Tris           | Tris (hydroxymethyl)- aminomethane                         |
| WB             | Western blotting                                           |
| Wg             | Wingless                                                   |
| WIF-1          | Wnt Inhibitory Factor-1                                    |
| Wnt            | Wingless-type MMTV integration site family member          |
| WST-1          | water soluble tetrazolium                                  |
| Xylene (xylol) | dimethylbenzene                                            |
| μL             | microliter                                                 |
| ‘              | minute(s)                                                  |
| “              | second(s)                                                  |
| °              | degree symbol                                              |
| °C             | degree Celsius                                             |

## 2. Figures

**Figure 1. Canonical Wnt signaling pathway.** This figure briefly summarizes canonical Wnt signaling. **A**, In the absence of Wnt ligand, cytosolic  $\beta$ -catenin is phosphorylated, ubiquitinated, and degraded by the proteasome to prevent the expression of Wnt target genes. **B**, However, in the presence of Wnt ligand, cytosolic  $\beta$ -catenin is stabilized and translocated to the nucleus, where it acts as a co-activator and facilitates the transcription of Wnt target genes.

**Figure 2.** Planar cell polarity pathway.

**Figure 3.** Non-canonical Wnt/calcium pathway.

**Figure 4.** Overview of the Canonical Wnt pathway, the non-canonical planar cell polarity pathway, and the non-canonical Wnt/calcium pathway

**Figure 5. Representative Western blot images of protein expression in SK-N-AS and SK-N-DZ neuroblastoma cells.** Cells were harvested to analyze protein levels by Western blotting. GAPDH, loading control. For quantification, see “Figure 6”.

**Figure 6. Quantitative determination of protein expression in SK-N-AS and SK-N-DZ cells.** active  $\beta$ -catenin (non-phospho  $\beta$ -catenin; Ser33/37/Thr41), MYC, cyclin D1, pan-phospho-PKC, ( $\beta$ II Ser660; p-PKC), total JNK, phospho- JNK (JNK phosphorylated at Thr183/Tyr185; p-JNK), total AKT, phospho-AKT (AKT phosphorylated at Ser473; p-AKT), total ERK and phospho-ERK (ERK1/2 phosphorylated at Thr202/Tyr204; p-ERK) were analyzed by Western blotting. Comparison of protein expression profiles between SK-N-AS and SK-N-DZ cells revealed differences in basal expression and activation levels of investigated signaling proteins. Graphs represent the mean of 3 independent experiments  $\pm$  SD (\*  $P < 0.05$ ).

**Figure 7. Expression of total  $\beta$ -catenin, active (non-phospho)  $\beta$ -catenin and c-MYC in SK-N-AS cells.** Immunofluorescence analysis of SK-N-AS cells using anti-human  $\beta$ -catenin, non-phospho-  $\beta$ -catenin, and c-MYC rabbit monoclonal antibodies as primary antibodies and Texas red anti-rabbit IgG as secondary antibody (red).

DAPI was used to stain the cell nuclei. (40 x objective).

**Figure 8. Western blot images of neuroblastoma cell lines. A.SK-N-AS and B. SK-N-DZ.**neuroblastoma cells. Untreated neuroblastoma cells (Control; Co) or cells treated with Wnt3a (3a) and Wnt5a (5a) for 15 min, with or without FZD2 siRNA were analyzed. Nonspecific scrambled siRNA (scr si) served as an additional control. Then proteins were isolated and analyzed by Western blotting (FZD2, phospho-LRP6 (LRP6 phosphorylated at Ser1490; p-LRP6), total  $\beta$ -catenin, active  $\beta$ -catenin (non-phospho  $\beta$ -catenin; Ser33/37/Thr41), MYC, cyclin D1, pan-phospho-PKC, ( $\beta$ II Ser660); p-PKC), total JNK, phospho-JNK (JNK phosphorylated at Thr183/Tyr185; p-JNK), total AKT, phospho-AKT (AKT phosphorylated at Ser473serine-473; p-AKT), total ERK and phospho-ERK (ERK1/2 phosphorylated at Thr202/Tyr204; p-ERK)).

**Figure 9A. Quantification of Western blots from Wnt3a and Wnt5a stimulated or unstimulated SK-N-AS cells.** SK-N-AS neuroblastoma cells were left untreated (Control; Co) or treated with Wnt3a (3a) and Wnt5a (5a) for 15 min, with or without FZD2 siRNA (si) pretreatment. Nonspecific scrambled siRNA (scrCo) served as an additional control. Activation levels of signaling factors p-LRP6 and active  $\beta$ -catenin, which are associated with the  $\beta$ -catenin-dependent signaling pathway, and of the  $\beta$ -catenin target genes MYC and cyclin D1 were reduced below baseline levels in Wnt stimulated and unstimulated neuroblastoma cells pretreated with FZD2 siRNA in contrast to activation levels of p-PKC, p-AKT, p-JNK, and p-ERK signaling factors, which are associated with  $\beta$ -catenin-independent signaling. Graphs represent the mean of 3 independent replicates  $\pm$  SD. Asterisks (\*) indicate  $P < 0.05$  vs. untreated controls, GAPDH, loading control.

**Figure 9B. Quantification of Western blots from Wnt3a and Wnt5a stimulated or unstimulated SK-N-DZ cells.** SK-N-DZ neuroblastoma cells were left untreated (Control; Co) or treated with Wnt3a (3a) and Wnt5a (5a) for 15 min, with or without FZD2 siRNA (si) pretreatment. Nonspecific scrambled siRNA (scrCo) served as an additional control. Activation levels of signaling factors associated with  $\beta$ -catenin-dependent signaling pathway (p-LRP6, active  $\beta$ -catenin) and  $\beta$ -catenin target genes (MYC, cyclin D1) were reduced below baseline levels in Wnt stimulated and unstimulated neuroblastoma cells pretreated with FZD2 siRNA in contrast to

activation levels of signaling factors associated with  $\beta$ -catenin-independent signaling (p-PKC, p-AKT, p-JNK, p-ERK). Graphs represent the mean of 3 independent replicates  $\pm$  SD. Asterisks (\*) indicate  $P < 0.05$  vs. untreated controls, GAPDH, loading control.

**Figure 10.** Effects of Wnt3a and Wnt5a stimulation and FZD2 blockade on cell proliferation. Graphs represent the mean of 3 independent experiments  $\pm$  SD. Asterisks (\*) indicate  $P < 0.04$  vs. untreated controls (Co, scrCo).

**Figure 11. Effect of Wnt3a and Wnt5a stimulation and FZD2 blockade on neuroblastoma cell migration.** Representative images of migrated SK-N-AS and SK-N-DZ cells from an *in vitro* migration assay.

**Figure 12. Quantification of cell migration.** SK-N-AS and SK-N-DZ cells were stimulated with 100 ng/ml recombinant proteins Wnt3a and Wnt5a with or without pretreatment with FZD2 siRNA or scrambled siRNA (scrCo) for 24 h followed by quantification of migrated cancer cells. FZD2 blockade suppressed Wnt-induced migration of neuroblastoma cells. Graphs represent the mean of 3 independent experiments  $\pm$  SD. Asterisks (\*) indicate  $P < 0.05$  vs. untreated controls (Co, scrCo) and vs. Wnt3a and Wnt5a stimulated cells, respectively.

**Figure 13. Cytoskeletal staining of SK-N-AS cells.** Nuclei were stained with blue-fluorescent DAPI. Actins filaments were stained with Alexa Fluor 448 Phalloidin. SK-N-AS cells were stimulated with recombinant Wnt5a or treated with human FZD2 siRNA or left untreated (control) (60 x objective).

**Figure 14. Immunohistochemistry of neuroblastoma xenograft tumor sections.** Representative images of tumor tissue sections from mice treated with FZD2 siRNA on day 24 after tumor inoculation.

Left column: Neuroblastoma xenografts stained with the proliferation marker Ki67. The scale bar in this column represents 50  $\mu$ m.

Right column: Neuroblastoma tumors stained for total  $\beta$ -catenin.  $\beta$ -catenin

expression was higher in SK-N-AS tumors. In both, SK-N-AS and SK-N-DZ xenografts,  $\beta$ -catenin staining intensity was reduced in the FZD2 siRNA group. The scale bar in the right column represents 50  $\mu$ m.

**Figure 15.** Quantitative histomorphometric analysis of Ki67-positive, proliferating tumor cells. Cellular proliferation was significantly reduced following FZD2 blockade. Data are mean  $\pm$  SD (n = 8 per group). Asterisks (\*) indicate  $P < 0.05$  vs. controls.

**Figure 16. Immunohistochemical staining of neuroblastoma tumor sections.** F4/80 positive TAMs are stained brown. Left column: untreated SN-N-AS and SK-N-DZ tumors (control). Right column: FZD2 siRNA treated SK-N-AS and SK-N-DZ tumors. Nuclei were stained with DAPI.



### 3.Tables

**Table 1.** Overview of used neuroblastoma cell lines and their specific media.

**Table 2.** Primary antibody and their dilutions used in immunofluorescence cell staining.

**Table 3.** Primary and secondary antibodies used in Western blotting. mAB, monoclonal antibody.

**Table 4.** Migration assay.

**Table 5.** List of primary and secondary antibodies used for Western blotting.