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NEW INSIGHTS INTO ROR1, ROR2, AND PTK7 MEDIATED WNT SIGNALING

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Tiivistelmä

Tutkimuksen tausta ja tavoitteet: Wnt-signaloinnilla on keskeinen rooli alkionkehityksessä ja aikuisen kudosten homeostasiassa. Tämän tärkeän signaloinnin virheellinen toiminta voi aiheuttaa erilaisia kehityshäiriöitä ja syöpiä. Wnt-signalointi jaetaan kanoniseen ja ei-kanoniseen signalointiin, pitäen sisällään useita osittain päällekkäisiä reseptoreja, ligandeja ja signalointireittejä. Wnt-signalointi on monimutkaisuutensa takia vajavaisesti kuvattu ja lisätutkimuksille on tarvetta. Tämä maisterintyö pyrkii karakterisoimaan ei-kanonisten Wnt-reseptorien Ror1, Ror2, ja Ptk7 ligandeja sekä solunsisäisiä signalointireittejä.

Tutkimusmenetelmät: 15 Wnt-ligandin ilmentyminen ja eritys solun ulkopuolelle tutkittiin systemaattisesti transfektoimalla Wnt plasmidit HEK293T-soluihin ja analysoimalla Wnt-tasot solumediasta ja -lysaateista western blotilla. Wnt-ligandien sitoutumista Ror1, Ror2, ja Ptk7-reseptoreihin selvitettiin immunopresipitaatiolla HEK293T-soluissa. Tässä kokeessa 15 Wnt-ligandia ilmennettiin yhdessä tutkittavan reseptorin (Ror1, Ror2, tai Ptk7) kanssa ja analysoitiin western blotilla mitkä Wnt-ligandit saostuvat yhdessä kohdereseptorin kanssa. HEK293T-soluissa ilmennettiin myös mutatoitua Ror1-reseptoria, josta puuttui joko kysteiini-rikas domeeni tai Kringle domeeni ja selvitettiin immunopresipitaatiolla näiden domeenien puuttumisen vaikutus Wnt-ligandien (Wnt5a ja Wnt16) sitoutumiseen. Tutkimuksen toisessa osassa selvitettiin Ror1, Ror2 ja Ptk7-välitteistä solunsisäistä Wnt-signalointia. Kanonista Wnt-signalointia tutkittiin lusiferaasi-kokeella, jossa Wnt-ligandeja ilmennettiin joko erikseen tai yhdessä Ror1, Ror2, tai Ptk7 reseptorien kanssa. Ei-kanonista Wnt-signalointia tutkittiin stimuloimalla Wnt-ligandeilla BaF3-soluja, jotka ilmentävät stabiilisti näitä reseptoreja ja analysoimalla western blotilla muutokset ERK/AKT-fosforylaatiotasoissa.

Tulokset: Kaikki 15 Wnt-ligandia ilmentyivät transfektoiduissa HEK293T-soluissa ja erittyivät solun ulkopuolelle hyvin vaihtelevissa määrin. Wnt-ligandeilla oli keskenään päällekkäisyyttä Ror1, Ror2, ja Ptk7-reseptoreihin sitoutumisessa, vaikka jonkin verran reseptorispesifisyyttä olikin havaittavissa. Jotkin Wnt-ligandit eivät sitoutuneet yhteenkään kolmesta reseptorista, kun taas toiset sitoutuivat kaikkiin kolmeen. Wnt-ligandin sitoutuminen oli riippuvaista sekä kysteiini-rikkaasta että Kringle domeenista Ror1-reseptorissa. Wnt3a oli ainoa Wnt, joka pystyi aktivoimaan kanonisen Wnt-signaloinnin, ja tämä signalointi hiljentyi 62–82% ilmennettäessä Wnt-ligandia yhdessä Ror1, Ror2 tai Ptk7-reseptorin kanssa. Jotkin Wnt-ligandit pystyivät näiden reseptorien kautta lisäämään ERK/AKT-fosforylaatiota, joka on merkki ei-kanonisen Wnt-signaloinnin aktivaatiosta.

Johtopäätökset: Tämä työ vastaa tarpeeseen laaja-alaisille ja perusteellisille tutkimuksille toistaiseksi vaillinaisesti tunnetusta ei-kanonisesta Wnt-signaloinnista. Tulokset ovat kuitenkin toistaiseksi alustavia ja ne pitäisi vielä toistaa muissa solulinjoissa ja koejärjestelyissä. Tästä huolimatta tulokset osoittavat, kuinka monimutkaisia Wnt-signalointireitit todella ovat, ja tarjoavat tärkeää tietoa Ror1, Ror2, ja Ptk7-välitteisen Wnt-signaloinnin perusbiologiasta.

Avainsanat: Wnt-signalointi, β-kateniini, alkionkehitys, ei-kanoniset Wnt reseptorit

MASTER'S THESIS

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Abstract

Background and aims: Wnt signaling has central role during embryonic development and adult tissue homeostasis. Dysregulation of this important signaling can lead to different developmental disorders and other diseases such as cancers. Wnt signaling is divided into canonical and non-canonical branches, involving multiple partially overlapping receptors, ligands, and pathways. Wnt signaling is not fully understood due to its complexity and more studies are needed. This Master thesis work aims to characterize ligands and downstream signaling of the non-canonical Wnt receptors Ror1, Ror2, and Ptk7.

Methods: The expression and secretion profiles of 15 Wnt ligands were studied by transfecting Wnt-encoding plasmids into HEK293T cells and analysing Wnt levels in media and cell lysates by western blotting. To determine the Wnt ligands for Ror1, Ror2, and Ptk7 receptors, coimmunoprecipitation assay was performed in HEK293T cells. 15 Wnt ligands were individually over-expressed with either Ror1, Ror2, or Ptk7 and analysed which Wnt ligands become co-precipitated with the receptor. To understand which domains of the receptor are required for binding Wnts, deletion constructs of Ror1 that lack either Cysteine-rich domain or Kringle domain were expressed in HEK293T cells together with Wnt5a or Wnt16 ligands and subjected to coimmunoprecipitation assay. In other part of the study, downstream signaling of Ror1, Ror2, and Ptk7 in response to Wnt binding was investigated. Canonical Wnt signaling was studied by luciferase assay with expressing Wnts alone or together with Ror1, Ror2, or Ptk7 in HEK293T cells. Non-canonical Wnt signaling was studied by stimulating BaF3 cells stably expressing Ror1, Ror2, or Ptk7 with Wnt ligands and analysing ERK/AKT phosphorylation levels by western blotting.

Results: Wnts were secreted at very variable levels by the transfected HEK293T cells. Wnt ligands exhibited highly overlapping binding profile to Ror1, Ror2, and Ptk7 – some Wnts interacted with all three receptors, while others had no interactions at all. However, there was some degree of specificity in which Wnts Ror1, Ror2, and Ptk7 bind, even though the binding profiles were rather similar between the receptors. It was shown that both Cysteine-rich and Kringle domains of Ror1 have indispensable role in the Wnt binding. Wnt3a was the only Wnt ligand that could activate the canonical Wnt signaling, and this activation was repressed by 62-82% when co-expressed with Ror1, Ror2, or Ptk7. On the other hand, it was shown that certain Wnt ligands act through these receptors to increase ERK/AKT phosphorylation levels, indicating the activation of non-canonical Wnt signaling.

Conclusions: This work answers the need for large-scale screens focusing into the components of the less known non-canonical Wnt signaling. The results are preliminary and should be repeated in other cell lines and experimental settings. Nevertheless, the findings show how complex and interconnected the Wnt signaling pathways are and provide important fundamental information about the Ror1, Ror2, and Ptk7-mediated Wnt signaling.

Keywords: Wnt signaling, β-catenin, embryonic development, non-canonical Wnt receptors

Preface

This research project was done in the Cancer Signaling research group under the Faculty of Medicine and Health Technology of the Tampere University. I would like to use this opportunity to thank my supervisor, docent Daniela Ungureanu and Cancer Signaling group members Hanna Karvonen (PhD), Wilhelmiina Niininen (B.A.Sc.), and Laura Kaleva (MSc.) who always provided their guidance during the two years that I worked in the team. I am also grateful to have been involved in several interesting cancer research projects outside the scope of this Master thesis.

Considering my whole journey as a student at Tampere University, I want to commend all my past university teachers and professors who are responsible for the high-level education that our faculty offers and teaching unique skills valued by both academia and industry. I also want to thank my family and fellow students and friends for all the memorable time spent together during the past six years. Now, it is time to turn the page and explore what the next life chapter holds.

Tampere, 27th of April 2021

Robert Perttilä

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Abbreviations

ADC	Antibody-drug conjugate
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
APC	Adenomatosis polyposis coli
BCL-2	B-cell lymphoma 2
BSA	Bovine Serum Albumin
CAMKII	Calmodulin-dependent kinase II
CKIα/ε/γ	Casein kinase 1α/ε/γ
CLL	Chronic lymphocytic leukemia
CRD	Cysteine-rich Wnt-binding domain
DAAM1	Dvl-associated activator of morphogenesis 1
DKK1	Dickkopf 1
DSRT	Drug Sensitivity and Resistance Testing
DVL2	Dishevelled 2
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
ERK	Extracellular signal-regulated kinase
FIMM	Institute for Molecular Medicine Finland
FZD	Frizzled
GSK3β	Glycogen synthase kinase-3β
HEK293T	Human Embryonic Kidney 293T cells
IL3	Interleukin-3
IP	Immunoprecipitation
JNK	cJun-N-terminal kinase
LRP5/6	Low-density lipoprotein receptor-related protein 5/6
MCL	Mantle cell lymphoma
MMP	Matrix metalloproteinase
NFAT	Nuclear factor of activated T cells
ONPG	ortho-Nitrophenyl-β-galactoside
PCP	Planar cell polarity
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PLC	Phospholipase C
PRD	Proline-rich domain
PTEN	Phosphatase And Tensin Homolog
PTK7	Protein tyrosine kinase 7
RhoA	Ras homolog family member A
ROR1/2	Receptor tyrosine kinase-like orphan receptor 1/2
RTK	Receptor tyrosine kinase
RYK	Related to receptor tyrosine kinase
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Ser/Thr	Serine/Threonine rich domain
SFRP	Secreted Frizzled-related protein
STAT3	Signal transducer and activator of transcription 3
STF	Super8XTOPflash reporter
TCF/LEF1	T cell factor/Lymphoid enhancer-binding factor 1
TERT	Telomerase reverse transcriptase
TKD	Tyrosine kinase domain
TBS	Tris-buffered saline
WIF	Wnt inhibitory factor
WNT	Wingless/Int1
WNT/STOP	Wnt-dependent stabilization of proteins

1 INTRODUCTION

Cellular behaviours, such as growth, proliferation, migration, and death, are regulated by extracellular molecules that activate signaling pathways inside cells. The signaling pathway that is the focus of this work is Wnt (Wingless/Int1) pathway. This pathway plays a fundamental role in embryonic and fetal development, including development of bones, muscles, and neurons [1]. It is also important in adults, where it regulates for example self-renewal of stem cells and maturation of B-cells [2, 3]. Wnt ligands are secreted growth factors that interact with various receptors to activate either canonical or non-canonical Wnt signaling [2]. Canonical Wnt signaling is characterized by β-catenin mediated transcriptional activation of TCF/LEF1 (T cell factor/Lymphoid enhancer-binding factor 1) target genes. In contrast, non-canonical signaling, which comprises several pathways, does not involve β-catenin in the signaling cascade [2].

Almost 50 years after the discovery of first Wnt [4], we still lack detailed understanding of many of the components of Wnt signaling due to exceptionally overlapping and complex nature of this signaling network [2]. For example, while several Wnt ligands preferentially activate either β-catenin-dependent or β-catenin-independent pathways, the exact pathway depends on the cellular context and on the expression of receptors [2]. This context-specific mechanism of activation is one of the key unsolved questions in the Wnt signaling and is just beginning to be elucidated [2]. A clear need for more detailed studies into Wnt signaling networks has been expressed in the literature [5-7]. Especially non-canonical Wnt signaling remains only partially understood, with lot of small-scale experiments reported in different experimental settings with conflicting results [7, 8]. Hence, this work aims to provide much-needed large-scale screens in a well-controlled experimental setting to clarify signaling and ligands associated with the non-canonical Wnt receptors.

Non-canonical Wnt receptors in the centre of this work are Ror1 (receptor tyrosine kinase-like orphan receptor 1), Ror2, and Ptk7 (protein tyrosine kinase 7, also known as CCK4).

These receptors are upregulated in neurogenerative diseases and several types of cancers, including hepatocellular carcinoma, melanoma, ovarian, prostate, and colorectal cancer as well as several hematological malignancies [3, 9, 10]. Their expression is linked to cell motility, proliferation, and drug-resistance [3, 11]. They also have generally low expression in healthy adult tissues, which makes them especially attractive therapeutic targets [10, 11]. However, the clinical translation is currently hindered by the complexity of Wnt biology and many open questions regarding the roles and activities of different Wnt proteins in humans [12]. This was another motivator for this work – incompletely understood non-canonical Wnt signaling is in need for more fundamental research to the underlying biology in order to fully realize the clinical potential, with only few therapies having advanced yet to clinical trials despite several being in preclinical development [10, 11, 13].

2 LITERATURE REVIEW

2.1 Overview of the Wnt signaling

Wnt signaling is one of the main regulators of development [14]. It regulates cell differentiation, proliferation, polarity, and migration, all important processes during development [15, 16]. Wnt signaling was first discovered in *Drosophila* [4], where it regulates embryonic axis formation, segmentation, and appendage patterning [2]. In mammals, it has a critical role in the organogenesis of the brain, heart, lung, bones, and muscles [1, 17]. In developed adults, Wnt signaling retains its important role in the self-renewal of stem cells, including the production of B cells [11], proliferation of intestinal crypt cells [18], and maintenance of bone and muscle mass [1, 19]. Because of this important role in embryonic development and adult tissue homeostasis, it is no surprise that dysregulation of Wnt signaling can lead to solid and hematological cancers as well as other diseases like osteoporosis and arthritis [5, 20, 21].

Wnt signaling is not a single pathway but is instead a complex group of different pathways [2]. 19 secreted Wnt ligands and more than 15 receptors or co-receptors mediating Wnt signaling have been discovered to date [2, 12]. These different ligand-receptor complexes form higher-order signaling networks that are becoming increasingly complex as more information about them is being generated [2]. On a general level, Wnt signaling is divided into canonical (β-catenin dependent) and non-canonical (β-catenin independent) Wnt pathways, the former being markedly better characterized pathway [9]. The activation of certain pathway depends on many factors like the type of Wnt ligand, cellular context, and expression of Wnt receptors [12]. However, the pathways are closely intertwined with each other, sharing many components at ligand level, receptor level, as well as intracellularly. Wnt pathways, their components, and association in cancer will be discussed in better detail in the following chapters.

2.2 Canonical Wnt signaling

Canonical Wnt pathway is highly conserved Wnt pathway [11]. Central component of the canonical Wnt signaling is cytosolic β-catenin, which is a coactivator of Wnt target gene transcription [2]. When the canonical Wnt signaling is inactive, cytosolic β-catenin is phosphorylated by glycogen synthase kinase 3 (GSK3) that is part of an intact 'destruction complex'. Other components of the destruction complex are Axin, casein kinase 1α (CKIα), and adenomatosis polyposis coli (APC) as illustrated in Figure 1. Phosphorylated β-catenin is polyubiquitinylated by E3 ligase and targeted to proteasomal degradation [22]. Without β-catenin, Wnt target genes cannot be transcribed, since repressors Groucho/TLE bind transcription factors TCF/LEF1 to repress gene transcription as shown in Figure 1.

Wnt signaling is activated when one of the canonical Wnts binds to a member of cell surface receptor family Frizzled (Fzd) and co-receptor LRP5/6 (low-density lipoprotein receptor-related protein 5 or 6) [12]. Established canonical Wnt ligand is Wnt3a but other ligands such as Wnt1 and Wnt8 can also activate canonical Wnt signaling [12]. It is characteristic to Wnt signaling that one Wnt can bind multiple different Fzd receptors, and each Fzd can respond to multiple Wnts [15, 23]. As an example, both Fzd2 and Fzd7 are known to mediate Wnt3a-induced Wnt signaling in 293T cells [16].

Wnt binding initiates downstream signaling cascade, where Serine and Threonine residues on the cytoplasmic tail of LRP5/6 receptor are phosphorylated by CKIγ and GSK3 on [15]. Axin, a key component of the previously described destruction complex, binds to phosphorylated LRP [24]. This leads to inactivation of the destruction complex, and GSK3 cannot anymore phosphorylate β-catenin to send it for degradation (Figure 1). Instead, stabilized β-catenin accumulates in the cytosol and enters the nucleus, where it acts as a coactivator. It displaces Groucho/TLE from TCF/LEF1, which initiates transcription of several target genes, such as c-Myc and Cyclin D that enhance cell proliferation and differentiation [1, 9].

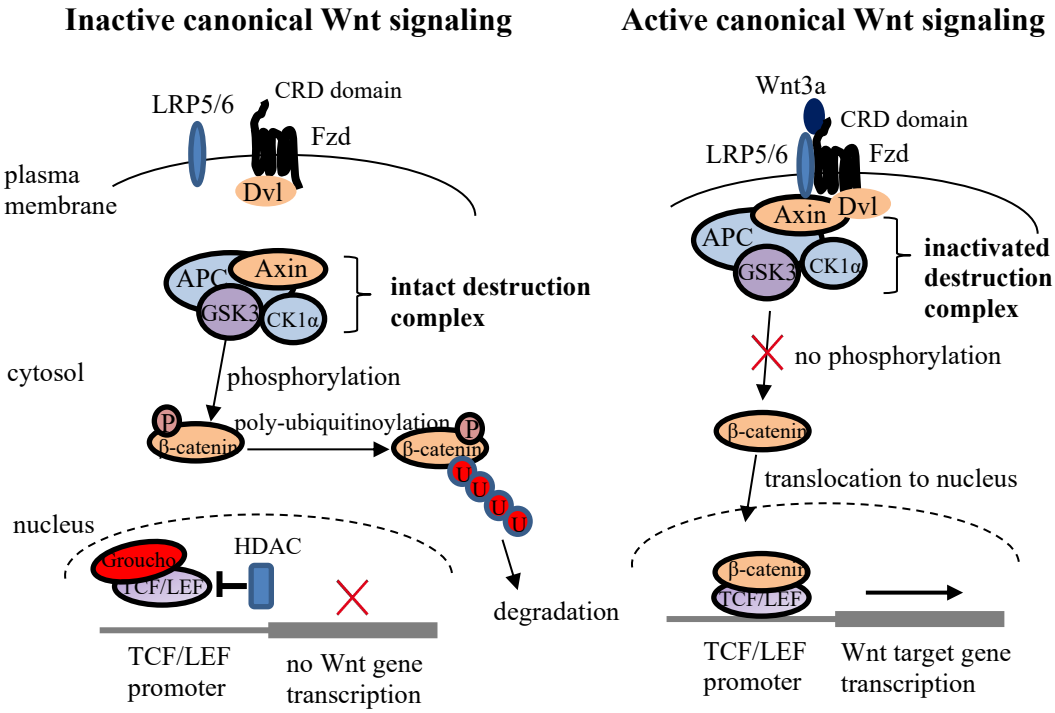

Figure 1. Illustration of the canonical Wnt signaling. In the absence of the Wnt ligand (left panel), destruction complex is functional and targets β-catenin for proteasomal degradation via phosphorylation by GSK3. When β-catenin is degraded, corepressor Groucho binds to TCF/LEF and recruits HDACs to repress Wnt target gene transcription. Wnt3a binding to the CRD domain of the Fzd receptor (right panel) inhibits β-catenin phosphorylation through inactivation of the destruction complex. This results in β-catenin stabilization and translocation to nucleus, where it displaces Groucho and coactivates TCF/LEF1 transcription factors. HDACs are not recruited and Wnt target genes can be transcribed. Abbreviations: P, phosphorylation; U, ubiquitination; DAC, histone deacetylase; Dvl, Dishevelled; CRD, cysteine-rich domain. Adapted from [2, 5].

Dishevelled 2 (Dvl2) is an important phosphoprotein in Wnt signaling, where it interacts with Frizzled on its cytoplasmic side [25]. CKIε-mediated phosphorylation of Dvl2 promotes Dvl2-Fzd interaction, Axin binding to LRP5/6 and subsequent disruption of the destruction complex and stabilization of β-catenin in the cytosol [15, 24]. It has been shown that Dvl2 also binds Src in a Wnt3a-dependent manner activating this kinase, which in turn enhances the activation of canonical Wnt pathway [25]. However, canonical Wnt signaling can also be activated in a Dvl2-independent manner [24]. In this Src/Fyn/STAT3 signaling branch, receptor Fzd2 is phosphorylated by Src, leading to binding and activation of Fyn kinase [24]. Activated Fyn can then phosphorylate STAT3 and β-catenin on Tyr142, releasing β-catenin from α-catenin and E-cadherin [24]. This

activating phosphorylation turns β-catenin from structural protein into transcriptional activator [24]. Besides Fyn and Src, also Fer, c-Met, and EGFR (epidermal growth factor receptor) can downregulate this E-cadherin mediated adhesion by phosphorylating β-catenin and releasing it to act as transcription coactivator [25].

Wnt signaling is tightly regulated to prevent from being excessively active, which would enhance cell differentiation, proliferation and migration and predispose individual to cancer formation [2]. Wnt receptors are regulated by phosphorylation, ubiquitination, proteolytic degradation, and endocytosis [2]. Many of these regulation events are necessary for active Wnt signaling: for example, phosphorylation of intracellular domain of LRP is essential for its ability to activate canonical Wnt signaling, and clathrin-dependent endocytosis of the Wnt-receptor complex is also required for signal transduction [2]. Proteolytic release of the cytoplasmic portion of the receptor and transport from plasma membrane to the nucleus can also function as key step in signal transduction [15].

Wnt signaling is also regulated by various secreted molecules either negatively or positively. Antagonistic (negative) regulators include Cerberus, DKK1 (Dickkopf 1), SFRP (secreted Frizzled-related protein), WIF (Wnt inhibitory factor), and Sclerostin [2]. Wnt agonists (activators) include Norrin and R-spondin family [2]. Norrin functions by binding to the Fzd4 and activating canonical Wnt signaling independently of Wnt ligand or any co-receptor [2]. R-spondins on the other hand protect Fzd receptors from degradation by ubiquitin ligases ZNRF3 and RNF43 by removing these ligases from cell surface and sending them to lysosomal degradation [2, 9]. These two ligases are also transcriptional targets of Wnt signaling, which forms a negative feedback loop – when Wnt signaling is active, these ligases are produced and suppress Wnt signaling, and vice versa [9].

Wnt signaling can also be regulated by YAP/TAZ transcription factors of the Hippo pathway [9]. They can associate with the β-catenin destruction complex and recruit β-catenin ubiquitinylating ligase beta-TrCP, functioning as antagonists of Wnt signaling. However, when the destruction complex is disassembled in response to Wnt binding, YAP/TAZ and beta-TrCP are displaced from Axin and become positive transcriptional regulators of Wnt signaling [26]. This dual role of YAP/TAZ factors in the regulation of Wnt signaling underlines a close connection of the Wnt and Hippo signaling pathways [9].

2.3 Non-canonical Wnt signaling

Non-canonical Wnt signaling involves all Wnt pathways that signal independently of β-catenin. They are less well characterized than canonical Wnt signaling [2, 7, 9]. Similar to canonical Wnt signaling, these pathways involve Frizzled receptors and a co-receptor. However, typically different set of co-receptors are involved, including Ror1, Ror2, Ptk7, and Ryk (related to receptor tyrosine kinase) [2]. It is thought that when these (co)receptors displace the LRP5/6 in the Wnt binding, non-canonical Wnt signaling is preferably activated instead of the canonical pathway [12, 15].

While canonical Wnt signaling promotes cell differentiation and proliferation, non-canonical pathways mainly affect cell polarity and migration. They have an important role in morphogenesis, meaning spatial organization of cells, during embryonic development [15]. Established non-canonical Wnt ligands are Wnt5a and Wnt11, even though Wnt ligands cannot be rigorously classified since their function might be affected depending on the cellular context and Wnt receptor profiles [2, 12]. The Wnt ligands and their receptors will be discussed in more detail in the later chapters. Key intracellular signaling mediators of non-canonical Wnt pathways are small GTPases, including Rac1, cdc42, and RhoA (Ras homolog family member A) [27].

PCP pathway (Reviewed in [28]) is the best characterized non-canonical Wnt pathway [2] (Figure 2). A diverse set of different receptors are involved in this pathway: multi-pass receptors (Fzd3, Fzd6, Vangl1-2, CELSR1), protocadherin family receptors (FAT4, PAPC), and pseudokinase receptors (Rors, Ptk7) [29]. In a typical initiation of PCP signaling, Wnt ligand binds to the Ror-Fzd receptor complex to activate Dvl, a shared mediator of both canonical and non-canonical Wnt signaling [9]. Next to Dvl, Vangl2 is phosphorylated and activated by CKδ [9]. Activation of Dvl also leads to de-inhibition of DAAM1 (Dvl associated activator of morphogenesis 1) and subsequent binding to RhoA. RhoA together with Rac1 then trigger ROCK and JNK (cJun-N-terminal kinase), as shown in Figure 2. JNK activates transcription factor Jun, which leads to transcription of the PCP target genes, while ROCK induces actin polymerization, leading to rearrangements of the cytoskeleton [9, 15]. Main phenotypic outcome is orientation of cells within

tissue, which is key event during gastrulation [15, 30] – the process in embryonic development where a single-cell layer embryo (blastula) becomes multi-layer embryo called gastrula [35].

Wnt/Ca^{2+} pathway is another non-canonical Wnt pathway, being first described in zebrafish and *Xenopus* embryos [31]. As shown in Figure 2, Wnt-binding to Fzd and a co-receptor (e.g., Rors) initiates this pathway in a similar way as with PCP signaling. Subsequently, phospholipase C (PLC) is activated through heterotrimeric G proteins [2]. PLC stimulates diacylglycerol and inositol-1,4,5-trisphosphate (Ins(1,4,5) P3) production [2]. This triggers release of calcium from intracellular stores and activation of effector kinases protein kinase C (PKC) and calmodulin-dependent kinase II (CAMKII) [2]. Signaling results in calcium-dependent cytoskeletal re-arrangements as well as transcription of target genes through transcription factor NFAT (Nuclear factor associated with T cells) [2, 9]. Cell fate and cell migration are main phenotypic consequences of this pathway [2].

Non-canonical PCP and Wnt/Ca^{2+} pathways are known to downregulate β-catenin-dependent Wnt signaling [1, 8, 24]. It has been proposed that since Fzd receptors are the shared components of both canonical and non-canonical Wnt signaling, Wnt5a and Wnt3a compete for binding to it, and Wnt5a binding prevents Wnt3a binding and subsequent activation of the canonical Wnt signaling [15]. This way, the type of Wnt ligand dictates whether β-catenin dependent or independent signaling is activated by determining the type of co-receptor (e.g., LRP5/6 or Ror1/2) [15]. Different adaptor proteins in different cell types might promote the use of one co-receptor over another and hence prefer the activation of either canonical or non-canonical pathway in that cell type [15]. The repression of canonical Wnt signaling can also occur at β-catenin level (by upregulation of Siah-2 that targets β-catenin to proteasomal degradation) or even in the nucleus through inhibition of TCF/LEF1 transcription [7, 15].

Wnt-dependent stabilization of proteins (Wnt/STOP) has been described as a third non-canonical Wnt pathway [32]. It is fundamentally different from the two other non-canonical Wnt pathways since it does not lead to transcriptional activation of target genes but instead functions on the level of post-translational modifications [9]. In addition, it does not compete with, but is rather accentuated, by canonical Wnt signaling [9]. Wnt/STOP

pathway stabilizes proteins, comprising up to 20% of the genome, in a β-catenin independent manner [32]. This protein stabilization occurs through inhibition of GSK3, leading to blockage of poly-phosphorylation and poly-ubiquitinoylation of target proteins that would otherwise be targeted to proteasomal degradation [9]. As a result, Wnt/STOP signaling increases cell size and affects chromosomal stability and endolysosomal biogenesis [32, 33].

Besides the main non-canonical Wnt pathways, Wnt proteins can trigger additional tissue-specific pathways that do not belong into any of these non-canonical pathways discussed previously [2]. For example, non-canonical Wnt signaling can function in a Dvl-independent manner through Ror2-bound Src [24]. In addition, Wnt5a-Ror signaling has been described as a separate pathway but it has significant overlap at least with the PCP pathway in vertebrates, and the extent of this overlap may depend on the cellular context [2, 21].

2.4 Wnt ligands

Wnts are secreted glycoproteins that belong to growth factors [22]. They control critical processes during developmental, such as embryonic patterning, cell growth, differentiation, and migration [22]. Wnts differ from other growth factors by being able to provide shape to growing tissues by instructing new cells to be arranged in a way that organized, rather than amorphous, structures are formed [14].

Wnts are present in all animal species as well as sponges, but none have been found from single-cell organisms, suggesting that Wnts might be as old as multicellularity [22]. Their name originates from the combination of wg and int-1; wg indicating the wingless phenotype of *Drosophila* when this gene is mutated, while int-1 indicates integration of proviral oncogene, relating to early insertional mutagenesis studies in mice leading to the discovery of Wnt genes in mammals [22].

There are 19 different Wnt ligands identified to date in humans [2, 7]. They can be roughly classified as canonical or non-canonical ligands based on which of the pathways they activate (Table 1). However, as mentioned earlier this classification is not rigorous, since the exact pathway that Wnt will activate may depend also on the cell type and receptor

context [2, 12]. At least Wnt5a, Wnt7a, Wnt10b, and Wnt11 have been reported to have potential to activate both canonical and non-canonical Wnt pathways in different situations [1, 7, 34].

Wnts, such as Wnt5a, are secreted outside the cells to signal in an auto- and paracrine manner [35, 36]. In most tissues, Wnts act only locally between neighbouring cells that contact each other [14]. They can be secreted either directly by solubilization, encapsulated in extracellular vesicles called exosomes, or bound to lipid protein carriers [9, 22]. In addition, some Wnts, such as Wnt3, can be tethered to plasma membrane [37]. The different release mechanisms may be related to variety of roles that Wnt have in development processes, since they need to act over wide ranges of gradients at different stages of development [9, 38].

Production and export of Wnt ligands is a complex step which is still not completely understood [14]. After Wnt proteins have been transcribed, they need to undergo several modifications before they are secreted outside the cell [22]. An important modification step is their acylation by Porcupine [38]. This endoplasmic reticulum (ER) resident acyl transferase attaches palmitoleic acid to a conserved serine residue (Ser239) in the Wnt ligand [22]. Next, acylated Wnts can bind to a transmembrane protein (Wntless/Evi) which transports them to the Golgi apparatus where N-linked glycosylations are added, and from there to the cell surface [9, 22]. Also p24 proteins assist in the transportation from Golgi to plasma membrane [9]. After Wnts are secreted outside the cell, Wntless/Evi

Table 1. Classification of Wnts based on their ability to activate canonical (left) or non-canonical (right) Wnt signaling. The classification is only directional, as Wnt-activated pathway type also depends on the cellular context including the receptor profile.

Canonical Wnts	**Ref.**	**Non-canonical Wnts**	**Ref.**
Wnt1	[12, 19]	Wnt2	[39]
Wnt2b	[40]	Wnt4	[1, 27, 41, 42]
Wnt3a	[12, 41]	Wnt5a	[1, 27]
Wnt6	[43]	Wnt5b	[44]
Wnt8a	[12, 41]	Wnt7a	[1, 34, 45]
Wnt10b	[43, 46]	Wnt9a	[1]
		Wnt11	[1, 27, 42]
		Wnt16	[3]

is recycled through endocytosis back inside the cell and into the ER membrane where it resides [9].

The primary function of the Wnt acylation (attachment of palmitoleic acid) is Wnt interaction with its receptor [14, 15]. Some of the identified receptors for different Wnts are shown in Table 2. A breakthrough crystallization structure of Wnt8-Fzd8 complex showed that palmitoleic acid is inserted into a hydrophobic groove of the CRD in Fzd [23]. However, it was recently shown that Wnt3a can also bind and signal without acylation but Wnt1 and Wnt5a cannot, so the dependence of acylation for receptor binding may differ for individual Wnts [38]. Acylation also makes Wnt proteins hydrophobic and increases their binding to cell membranes, which may have a role in restricting Wnt

Table 2. Some of the receptors for different Wnt ligands identified by coimmunoprecipitation assay in different cell lines. The table is not inclusive and there are likely other receptors for Wnts reported in the literature besides those shown in this table.

Wnt ligand	**Reported receptor(s)**	**Cell line(s)**	**Reference**
Wnt1	Ror2	U2OS	[47]
Wnt2	otk/Ptk7	Drosophila S2R+	[39]
Wnt2b	Fzd4	Intestinal cells	[48]
Wnt3a	Ptk7 Ror2 Fzd5	Xenopus U2OS HEK293T	[41] [47] [49]
Wnt4	Vangl2 otk/Ptk7	NIH 3T3 Xenopus embryos	[1] [41]
Wnt5a	Ror1 Ror2, Ptk7, Ryk, CD146, Vangl2, Fzd7	Several 293T	[3, 36] [29, 50, 51]
Wnt5b	Ror1 Ryk	293T Zebrafish embryos	[36] [44]
Wnt7a	Fzd7, Vangl2	Myoblasts	[1]
Wnt8a	Ptk7	Xenopus	[41]
Wnt9a	Ror1, Ror2	HEK293T	[1, 8]
Wnt9b	Ror1, Ror2	HEK293T	[8]
Wnt11	Ror2 Vangl2 Ryk, Fzd7	HEK293T NIH 3T3 HEK293T	[27] [1] [51]
Wnt16b	Ror1 Ror2, CD146, AP2b1	293T 293T	[3] [50, 52]

spreading and range of action [14]. The requirement of Wnt acylation for their secretion is controversial. As mentioned previously, transport protein Wntless/Evi binds acylated Wnts, and mutating the acylation site in Wnts abrogated both secretion and signaling activity of Wnts [22]. However in other studies, mutating acylation site of Wnt8 did not prevent its secretion from mammalian cells [38], so acylation requirement for secretion may also vary for different Wnts or in different contexts.

Several Wnts regulate the development of bones. Vertebrate skeleton develops during early stages of embryogenesis and is remodeled by bone formation and resorption [46]. The formation of new bone mass is facilitated by osteoblasts, which are derived from mesenchymal stem cells, while hematopoietic stem cell-derived osteoclasts degrade bone tissue [46]. The correct balance between bone degradation and formation is important for the maintenance of calcium homeostasis [2]. At least Wnt1 [2], Wnt3a [19], Wnt5a [21], Wnt6 [43], Wnt9a [2], Wnt10a [43], Wnt10b [46], and Wnt16 [6] have important roles in the regulation of bone mass balance. For example, Wnt3a and Wnt5a protect osteoblasts from apoptosis through MAPK/ERK and PI3K/AKT/mTOR pathways (discussed later in the section 2.5) and Wnt5a mutant mice exhibit dwarfism, short limbs, and short nose [19]. Moreover, Wnt9a mutant mice have shortened humerus (arm bone) [2], and Wnt16 prevents bone fragility fractures by suppressing the production of osteoclasts [7].

Another developmental process where various Wnts play important role is the formation of muscles. During embryonic development, skeletal muscles are formed from myogenic progenitor cells [2]. These cells fuse with each other to produce multinucleated myofibers, whose polarized elongation is regulated by Wnt11 [2]. Then, motor neurons localize to the center of the myofibers to form the neuromuscular junctions mediated by Wnt4 and Wnt11 in mouse embryos [2]. After birth, various growth factors including Wnt5a, Wnt10, and Wnt11 secreted by mesenchymal stem cells promote proliferation and differentiation of postnatal myogenic cells called satellite cells [2, 46]. Satellite cells have critical role in the muscle regeneration especially after muscle damage [2]. Also Wnt7a activates expansion of satellite cells by PI3K/AKT/mTOR pathway in muscle fibers and has been shown to enhance stem cell therapy for skeletal muscle [1, 45]. It has also been investigated in preclinical studies as a treatment for Duchenne muscular dystrophy, a muscle disease that is characterized by muscle atrophy because of impaired proliferation and migration of satellite cells [2].

Besides muscle and bone formation, many other developmental processes are also regulated by Wnt ligands. 12 Wnts have been reported to have roles in immune system and hematopoiesis, most notably Wnt2b, Wnt3a, Wnt5a, and Wnt10b [46]. Wnt2b regulates liver formation as well as retinal cell proliferation and differentiation in developing vertebrate eye [53, 54]. Wnt5a is important for generation of neurons during development [2]. Wnt5b regulates cell movements during gastrulation [35]. Wnt6, Wnt10a, and Wnt10b inhibit adipogenesis (formation of fat cells from stem cells) by suppressing expression of adipogenic transcription factors [43]. Lastly, Wnt16 delays maturation of the chondrocytes (cartilage cells) [52].

2.5 Receptor tyrosine kinases

Receptor tyrosine kinases (RTKs) constitute a large family of cell surface receptors that have ligand-binding extracellular portion, single-pass transmembrane portion, and cytosolic portion that contains tyrosine kinase (TK) domain [55]. There are 58 different RTKs in humans that are divided into 20 subfamilies [55], some of which are shown in Figure 3. Their general structure, activation mechanisms, and intracellular pathways are highly conserved in evolution [55]. This conservation from worms to humans is explained by their essential role in the cellular processes, such as proliferation, differentiation, survival, migration, and metabolism. Because of this key regulatory role, mutations in RTKs are linked to many pathologies like cancers, bone malformations, inflammation, arteriosclerosis, and diabetes [55].

RTK signaling is typically activated through receptor homo- or heterodimerization in response to growth factor binding [55], as shown in Figure 4. The receptor dimerization induces activation of cytosolic TK domains and autophosphorylation using ATP, with important exception being pseudokinases that cannot catalyse ATP, so they transduce signal by other mechanisms [55, 56] (discussed later). The phosphate groups added to TK domains act as docking sites for signaling mediators, such as PI3K that is part of PI3K/AKT/mTOR signaling pathway [56] (Figure 4). The activated mediators can then initiate downstream signaling cascades that transduce the signal to transcription factors in the nucleus that initiate transcription of the target genes.

It is important to note that the activation of RTKs does not always rely on receptor dimerization, since some RTKs exist in a dimeric form even without the presence of the activating ligand. For example, insulin receptor family is expressed on cell surface in a disulfide-linked dimeric form in both active and inactive states (Figure 3). Activation of these RTKs happens through ligand-induced conformational changes within the dimeric receptor [55].

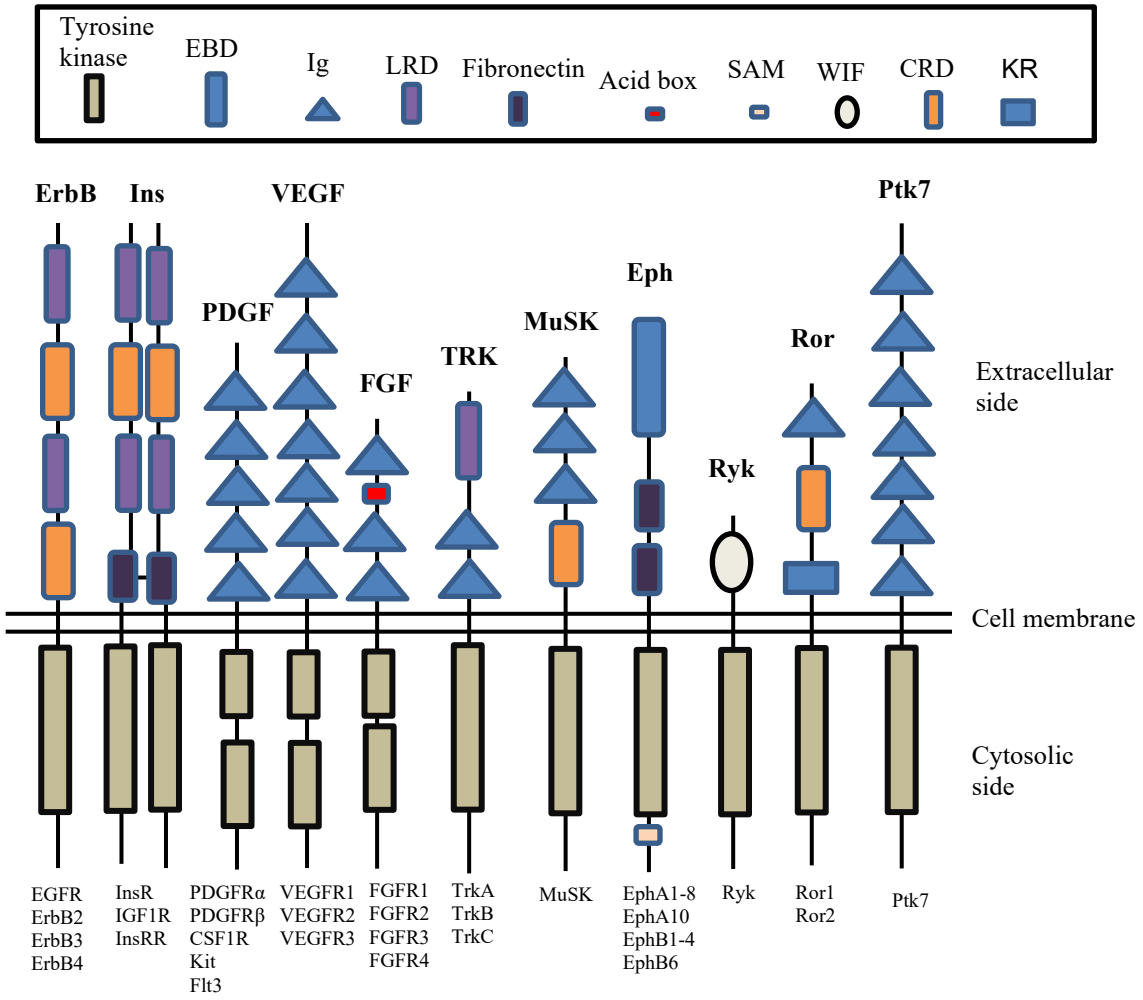

Figure 3. Selected RTK subfamilies and their general domain structures. Members of each subfamily are listed below the structural illustrations. Abbreviations: EBD, Ephrin-binding domain; Ig, immunoglobulin domain; LRD, leucine-rich domain; WIF, Wnt-inhibitory factor; CRD, cysteine-rich domain; KR, Kringle. Adapted from [55].

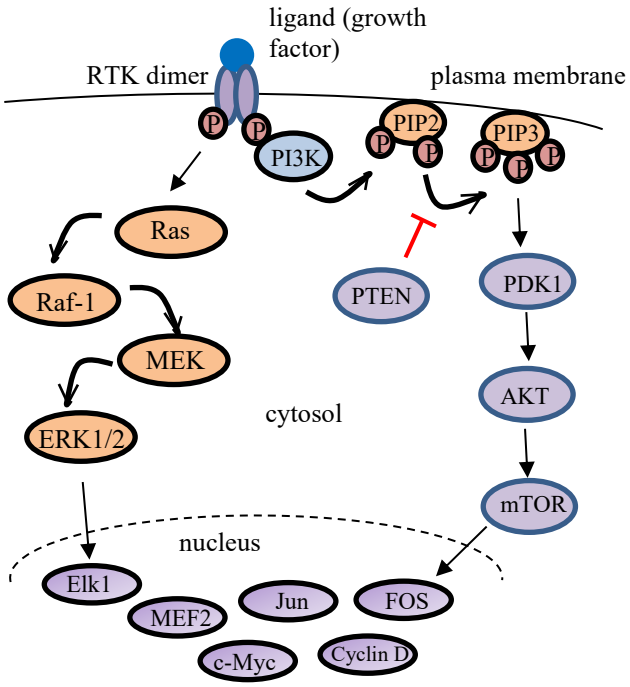

Figure 4. RTK-mediated signaling pathways MAPK/ERK (on the left side) and PI3K/AKT/mTOR (on the right side). Ligand binding induces formation of RTK homo- or heterodimers, which can then autophosphorylate their tyrosine kinase domains. The phosphate groups act as docking sites for downstream signaling mediators like PI3K, which phosphorylates PIP2 to form PIP3, a process that is negatively regulated by PTEN. Activated second-messenger PIP3 then transduces signal to mTOR through PDK1 and AKT, which leads to mTOR phosphorylating and activating key transcription factors like FOS and Cyclin D in the nucleus. Another signaling cascade that is activated by RTK dimerization includes Ras, Raf-1, MEK, and ERK1/2 that phosphorylate each other to transduce signal to transcription factors like Elk1 and c-Myc. The signaling pathways are in reality more complex including more elements and are simplified here for illustration purposes. Abbreviations: PI3K, phosphoinositide 3-kinase; PIP2, phosphatidylinositol 4,5-diphosphate; PIP3, phosphatidylinositol 3,4,5-triphosphate; PTEN, Phosphatase And Tensin Homolog; P, phosphate group. Adapted from [56, 57].

2.5.1 Wnt receptors Ror1, Ror2, and PTK7

Non-canonical Wnt receptors Ror1, Ror2, and Ptk7 that are the focus of this work belong into RTK superfamily. However, these RTKs are catalytically inactive pseudokinases, meaning that they cannot catalyse ATP with their tyrosine kinase domain (TKD) as a result of critical mutations in their catalytic motifs making them catalytically inactive [58]. Pseudokinases are a large class of enzymes, representing around 10% of the whole human kinome [59]. Despite inability to signal by ATP phosphorylation, pseudokinases have evolved alternative mechanisms for signaling, such as recruitment of downstream scaffolding molecules and cytosolic kinases as a response to dimerization and/or conformation change of the receptor [10, 58]. The exact signaling mechanisms are still in many parts poorly understood for all pseudokinases [1].

Ror family consists of only Ror1 and Ror2 [15]. They were first identified in a PCR-based screen for closely resembling Trk family tyrosine kinases [60]. Like RTKs in general, Rors are highly conserved between different organisms across vertebrate and invertebrate species, underlining their evolutionary importance [15, 61]. Ror1 and Ror2 are single-pass transmembrane receptors and have similar domain structures, as shown in Figure 5. Their extracellular portion consists of immunoglobulin (Ig) like domain and two cysteine-rich domains (CRD/FZD and KR), while intracellular portion consists of inactive TKD, two Serine/Threonine rich domains (Ser/Thr) flanking a Proline-rich domain (PRD) and C-terminus domain [61].

Ptk7 is another evolutionarily conserved RTK [30]. It has similar inactive tyrosine kinase homology domain as Rors while its extracellular portion is different, consisting of seven extracellular IgG-like domains [30] (Figure 5). For comparison, also the representative structure of Fzd receptor family, which includes 10 receptors (Fzd1-10) is shown in Figure 5. Fzd receptors are seven-pass transmembrane receptors and have similar CRD domain as Rors that can bind Wnt ligands, but they are not classified as RTKs since they lack the TK domain [2].

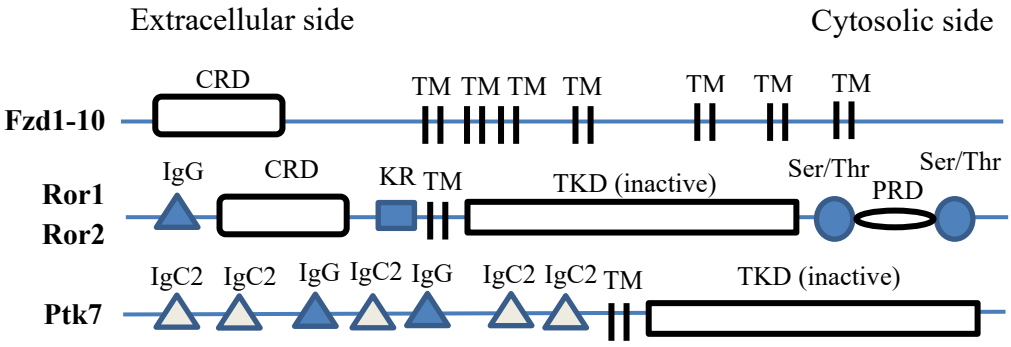

Figure 5. Domain structures of the relevant Wnt (co)receptors Fzd1-10, Ror1-2, and Ptk7. Abbreviations: CRD, cysteine-rich domain; TM, transmembrane domain; IgG, immunoglobulin G; KR, Kringle; TKD, Tyrosine kinase domain; Ser/Thr, Serine/Threonine rich domain; PRD, Proline-rich domain; IgC, immunoglobulin constant. Adapted from [2, 27].

Rors were for a long time classified as orphan receptors since their ligands and signaling pathways were unknown [15, 63]. However, the identification of CRD domain of Rors and its close resemblance to the Wnt-binding domain of Frizzled receptors (hence CRD is also called FZD) suggested that in addition to Frizzled, Wnts could be ligands also for Ror receptors [62]. This has later been confirmed to be the case [63]. While Wnt5a is the most widely reported non-canonical Wnt ligand for Rors and Ptk7, many other Wnts such as Wnt8, Wnt9a, Wnt11 and Wnt16 have later been shown to also interact with these receptors [8, 27, 41, 50].

Rors have a well-defined role in the non-canonical Wnt signaling, especially in PCP pathway, which in turn represses canonical Wnt signaling [7, 64], as described previously. Interestingly, Ror2 can also activate canonical Wnt signaling when it is engaged with a canonical Wnt ligand [1, 16]. Ror1 has role in in MAPK/ERK and PI3K/AKT/mTOR pathways [19, 61, 65] which are shown in Figure 4. These pathways are downregulated when Ror1 is inhibited [65, 66]. For example, knockdown of Ror1 decreases phosphorylation levels of AKT and mTOR [66].

Rors can function as stand-alone receptors as well as co-receptors together with both Frizzled receptors and Vangl2 [2, 15]. Frizzled is not always required for Ror-mediated signaling, since Ror1 and Ror2 can heterodimerize in response to Wnt5a through their KR domains [38, 64]. Ror1 could also be cleaved to form a splice variant that lacks both extracellular and transmembrane domains, which is then directed to nucleus to activate

gene transcription [15]. It has also been shown that Ror1 can directly associate with actin-binding protein Filamin A in response to Wnt5a to induce filopodia formation and subsequent cell migration [67].

The detailed signaling mechanism of Ptk7 remains much less characterized [29, 41] despite Ptk7 being described already in 1995 [68]. Ptk7 belongs to the PCP pathway where it associates with Ror2 to form heterodimeric signaling complex in response to Wnt5a [29]. This results in the activation of JNK and proteolytic cleavage of Ptk7 by ADAMs and MT1-MMP [69]. Nuclear translocation of the cleaved intracellular domain activates *papc* expression, while the remaining soluble Ptk7 fragments might enhance cell invasion by affecting cytoskeletal organization [29]. Another Ptk7-mediated PCP pathway involves Ptk7 dimerization with Fzd7 and subsequent recruitment of Dvl through RACK1 and PKCδ1 [41]. Ptk7 also has a role in the canonical Wnt signaling similar to Rors [70]. Fine-tuning different Wnt signaling networks might be important function of the Ptk7-mediated signaling [30].

Rors have important role in organogenesis with partially overlapping expression patterns [15]. Complete Ror knockdown results in pervasive developmental abnormalities in the lung, heart, urogenital tracts, and other organs [64]. For example, mutations in Ror2 are linked with Robinow syndrome that manifests with brachydactyly, short stature and skeletal dysfunctions [8, 15, 71]. Best known role of Ror2 is in bone morphogenesis – loss-of-function of Ror2 in mice causes craniofacial abnormalities and an embryonic skeletal dysplasia phenotype with severe shortening of the limbs, which has also been documented in humans [8, 15]. Ror1 has less significant role in bone development: homozygous Ror1 mutant mice are viable with normally developing bones [8]. Also Ptk7 has a role in development, where it regulates processes that require coordinated cellular movements. For example, Ptk7 regulates stereociliary bundle orientation and neural tube closure in vertebrates [72].

After embryonic development, Ror1 is strongly downregulated in adult human cells, but a small level of Ror1 expression remains in adipose tissues, lung, and pancreas [73]. Ror1 is also transiently upregulated during intermediate stages of B cell development in coordination with the assembly of pre-BCR complex [74]. Pre-BCR drives activation of PI3K/AKT pathway, which inhibits B-cell differentiation while promoting proliferation

and survival of B cells (Figure 6a). During B-cell maturation, loss of PI3K/AKT activity allows cell differentiation to proceed. However, this results in the loss of pro-survival signal, and transient upregulation of Ror1 at this stage offers important compensatory mechanism for prosurvival signaling through MEK/ERK activation [74], as shown in Figure 6b. Ror2 is expressed on normal CD19+ B cells [11]. Gene expression of Ror1, Ror2, and Ptk7 in different hematological tissues is presented in [11].

2.6 Wnt signaling in cancer

Since Wnt pathways regulate cell proliferation, polarity and motility as previously described, it is not surprising that these pathways are dysregulated in carcinogenesis. Oncogenic activity of Wnt signaling was first reported almost four decades ago, when Wnt1 was found to have a role in the regulation of mammary tumorigenesis in mice [75]. Since then, our understanding of the role of Wnt pathway in cancer has increased dramatically. Some examples of Wnt signaling in cancer are shortly presented in this section, while a more detailed overview on the subject is provided in a recent, excellent review by Menck and colleagues [13].

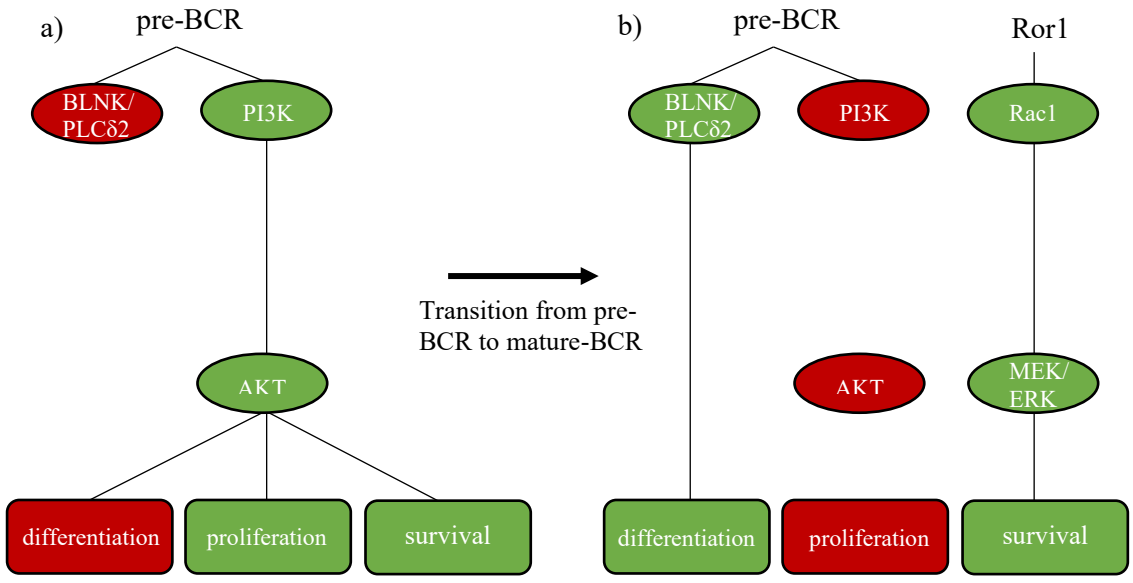

Figure 6. The pro-survival role of Ror1 in B-cell development. a) At B-cell progenitor stage, PI3K/AKT pathway maintains cell proliferation and survival, while inhibiting differentiation. b) During pre-BCR/mature-BCR transition, AKT activity is lost which allows cell differentiation through the BLNK/PLCδ2 pathway, but this also means loss of pro-survival signal. Ror1 is expressed at this stage to provide this pro-survival signal through MEK/ERK during cell differentiation. Abbreviations: BLNK, B-cell linker protein; PLCδ2, phospholipase C δ-type 2; BCR, B-cell receptor. Adapted from [74].

Wnt signaling has important roles in the initiation, progression, metastasis, and therapy resistance in many solid and hematological malignancies [9]. Both canonical and non-canonical Wnt signaling have been associated with enhanced cancer cell invasion and EMT (epithelial-to-mesenchymal transition), both key events in cancer dissemination [9, 76]. However, there are also data showing that non-canonical signaling can act as tumor suppressor in certain cell contexts by repressing oncogenic canonical Wnt signaling [7, 64]. It might be that Wnt signaling either enhances or inhibits formation of cancer depending on the cancer type and possibly other, yet unknown factors.

Wnt signaling is associated with formation of cancer stem cells [9]. Cancer stem cells are a small fraction of cells within the tumor that have infinite proliferative potential and are associated with cancer initiation, metastasis, therapy resistance, and poor prognosis [9]. β-catenin can directly bind to TERT (telomerase reverse transcriptase) promoter in cancer stem cells and induce expression of telomerase that is an enzyme capable of elongating the telomeres (chromosome ends) and this way making cells immortal [9].

Wnt signaling has a well-defined role in colorectal cancer. More than 90% of colorectal cancers involve overactive canonical Wnt signaling [2]. Often, the tumor-suppressor APC that functions as part of β-catenin destruction complex has deactivating mutations, which causes β-catenin stabilization and accumulation independent of the Wnt ligand [9]. The restoration of APC levels causes the regression of adenomas in mouse tumor models [77].

Wnt signaling is also overactive in many leukemias, importantly in CLL (chronic lymphocytic leukemia), ALL (acute lymphoblastic leukemia), and AML (acute myeloid leukemia) [9]. Active canonical Wnt signaling appears to be requirement for the self-renewal of leukemia-initiating cells [9]. From non-canonical Wnt signaling, Ror1 receptor, but not Ror2, has been shown to contribute to CLL [11, 61]. Ror1 has also oncogenic properties in B-ALL (B cell acute lymphoblastic leukemia), where the transient crosstalk between Ror1 and pre-BCR during normal B cell maturation (Figure 6) is exploited by cancer cells for sustained survival [74]. Moreover, Ror1 is upregulated in Burkitt's lymphoma/leukemia as well as MCL (mantle cell lymphoma), suggesting that Ror1 may be expressed in most B cell malignancies [20, 74]. Because Ror1 is not expressed in healthy mature B cells, it can be a useful biomarker in the diagnosis of these leukemias [61]. Also

Ror2 has also been shown to be an oncogene in many cancer types [13]. For example, Ror2 is highly expressed in multiple myeloma, where it mediates adhesion of cancer cells to the microenvironment through PI3K/AKT/mTOR signaling pathway [71].

Besides Rors, also the expression of Ptk7 is often dysregulated in cancers. Ptk7 was originally named CCK4 (colon carcinoma kinase 4) because it was found to be upregulated in colon cancer but was later renamed after it was shown to be involved in many other cancers and normal physiological processes as well [30]. Ptk7 is upregulated in esophageal, breast, colorectal, gastric, and prostate cancers, while downregulated in lung squamous cell carcinoma, and metastatic melanoma [30]. Ptk7 also promotes migration and survival of leukemic cells [41]. Ptk7 is associated with drug resistance: its overexpression increases cancer resistance to anthracycline-based chemotherapeutics such as doxorubicin, while Ptk7 silencing reverses this resistance [78].

2.6.1 Wnt signaling as a therapeutic target

The complex and overlapping nature of Wnt signaling has hampered the entrance of Wnt targeting molecules into clinical trials. In addition, active Wnt signaling has been sometimes associated with better survival, making targeted inhibition of Wnt signaling even more complicated and it is evidently not suitable as a universal strategy to treat all cancers [9, 35]. Hence, targeting Wnt signaling is a viable option for distinct tumor subclasses or with specific mutational backgrounds [9].

There has been substantial increase in the development of strategies for targeting Wnt signaling in the past years, with many Wnt inhibitors having entered clinical trials [35]. Indications of the trials include colorectal, head & neck, breast, ovarian, pancreatic, liver, and prostate cancers, among others [9]. One of these Wnt signaling targeting agents is Porcupine inhibitor LGK974, which is currently in Phase 1/2 trials sponsored by Novartis for several solid cancers (clinical trial identifier NCT01351103). Another agent is Ipafricept, a Fzd8-Fc decoy receptor that aims to scavenge Wnt ligands out from the extracellular Wnt pool capable of activating Wnt signaling [9]. It showed promising results in a first-in-human Phase 1 study in patients with solid tumors [79]. Another interesting example is Foxy-5, which is a Wnt5a mimetic. It is currently in a large Phase 2 trial in 100

patients with Wnt5a-low colon cancer, where the lack of Wnt5 expression correlates with poor prognosis (clinical trial identifier NCT03883802).

Promising results in preclinical and early clinical studies are beginning to unravel the huge potential of targeting Wnt receptor Ror1 [13]. Ror1 is in theory a good candidate for anticancer therapies, since it is strongly detectable only in embryonic tissue and to a big extent absent in adult tissues [15]. However, Rors and Ptk7 being pseudokinases poses significant challenge for drug development. Typically, tyrosine kinases can be inhibited with small-molecule inhibitors targeting the active intracellular TK domain of these kinases (Figure 3), and there are more than 30 FDA-approved small molecule kinase inhibitors already in the clinic [10]. However, targeting pseudokinases Ror1, Ror2, and Ptk7 is much more difficult since their 'druggable' pocket, TKD, is inactive as discussed earlier in the chapter 2.5.1. Despite this, two ATP-competitive small-molecule inhibitors for Ror1 were recently identified in a small-molecule screening assay [58]. However, as of 2019 no therapeutic agent targeting pseudokinases has been clinically approved [10], and only one small-molecule inhibitor of Ror1 (ARI-1) has shown anticancer efficacy at preclinical level [65].

Despite the scarcity of small-molecule inhibitors for Ror1, immunotherapies targeting the extracellular portion of Ror1 have been more successful. Several Ror1 targeting monoclonal antibodies have been developed and one of them, cirmtuzumab (Oncternal Therapeutics), is being investigated in several clinical studies, such as in Phase 1b/2 trial involving 156 patients with B-cell lymphoid malignancies including CLL and MCL (clinical trial identifier NCT03088878). Other antibody-based Ror1 targeting options that have entered clinical evaluations include antibody-drug conjugates (ADC) VLS-101 and NBE-002 as well as chimeric antigen receptor T (CAR-T) cells [11, 13]. These therapies together with some other preclinical Ror1-targeting agents are illustrated in Figure 7.

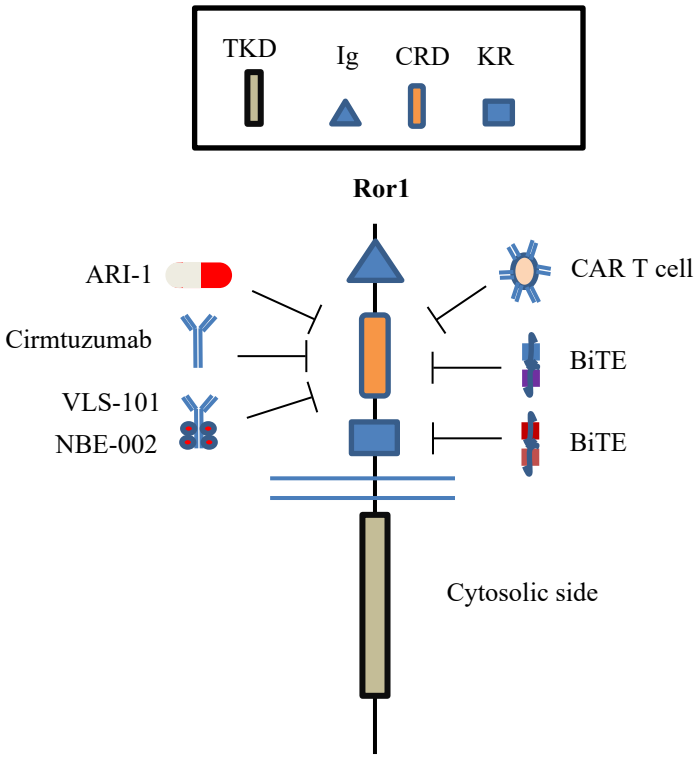

Figure 7. Different Ror1-targeting agents and their targeting sites in Ror1. ARI-1 is a novel small-molecule inhibitor or Ror1, cirmtuzumab is Ror1-targeting antibody, VLS-101 is ADC that links cirmtuzumab to antineoplastic agent monomethyl auristatin E (MMAE), NBE-002 is another ADC that links Ror1-targeting antibody with anthracycline PNU-159682. CAR T cells offer cellular alternative for Ror1-targeted therapy, while BiTEs (bi-specific T cell engagers) link Ror1 with activated T cells through their CD3-specific arm. Most of Ror1-targeting agents bind to the CRD of Ror1, while BiTEs can also bind to the Kringle domain. Abbreviations: TKD, Tyrosine kinase domain; Ig, immunoglobulin domain; CRD, Cysteine-rich domain; KR, Kringle domain; ADC, antibody-drug conjugate; CAR, chimeric antigen receptor; BiTE, bi-specific T cell engager. Adapted from [13].

There is significantly less clinical activity with Ror2 targeting strategies compared to Ror1 [13]. Presently, Ror2-targeting strategies mostly focus on adoptive immunotherapy approaches, including monoclonal antibodies, bispecific antibodies, CAR T cells, and ADCs [13]. Only one of these, a Ror2-targeting ADC, is currently in clinical studies (clinical trial identifier NCT03504488), while Ror2-targeting CAR T cells are about to enter clinical trials [13]. In addition, FDA-approved PI3K inhibitors Idelalisib and Duvelisib have shown efficacy in abrogating Ror2-mediated adhesion in multiple myeloma patient samples [71].

Upregulation of Ptk7 in multiple cancer types makes it also interesting candidate for targeted therapies [30]. ADC targeting Ptk7 consists of Ptk7 antibody linked to auristatin-0101, which is a microtubule inhibitor [80]. This ADC reduced the number of tumor-initiating cells and induced tumor regression in preclinical studies [80]. It is currently in a Phase 1 evaluation involving patients with recurrent non-small cell lung cancer (clinical trial identifier NCT04189614).

2.7 Background of the used methods

Interleukin-3 (IL3) dependent Ba/F3 murine pro-B cell line was used in this work to have stably expressing cells for Ror1, Ror2, and Ptk7. The use of this cell line in drug discovery is reviewed in [81]. IL3-dependent Ba/F3 cells die shortly after IL3-withdrawal. However, when a receptor of interest (in this case Ror1, Ror2, or Ptk7) is expressed on Ba/F3 cells, the receptor can prolong the survival of the cells without IL3 if the receptor can provide a compensatory survival pathway for the cells. Ba/F3-Ror1 cells achieved IL3-independent proliferation in our other study [58] and were hence cultured without IL3 in this work. On the other hand, Ba/F3-Ror2 and Ba/F3-Ptk7 cells were cultured with IL3, since Ror2 and Ptk7, despite having modest effect on cell survival, did not give cells full IL3-independency [58]. Supernatant from myelomonocytic cell line WEHI-3 was used as a source for IL3 since these cells secrete high levels of biologically active IL3 [82].

Immunoprecipitation assay (https://ruo.mbl.co.jp/bio/e/support/method/immunoprecipitation.html) was used in this work to study protein-ligand interactions. This assay aims to isolate the antigen of interest together with all proteins that are bound to it (its binding partners), while the rest unbound antigens are washed away. First, a primary antibody against the antigen of interest (in this case Ror1, Ror2, or Ptk7) is added to the cell lysate (Figure 8, 1st step). The incubation period allows antigen-antibody complexes to form (2nd step). Because even high-quality monoclonal antibodies may bind to nonspecific proteins, it is important to also include irrelevant antibody (non-target antibody) sample to be able to identify relevant target interactions from non-specific binding.

After antibody-antigen complexes have formed, agarose beads crosslinked with secondary antibody (Protein G) are added, which precipitates this antibody-antigen complex together with any proteins bound to the antigen (Fig. 8, 3rd step). The beads are then washed

to remove all unbound proteins from the sample (Fig. 8, 4th step) and eluted into SDS (Fig. 8, 5th step). Ideally, only those Wnts that interact with the receptor (shown as blue and green spheres in Fig. 8) will be isolated with the receptor while all other antigens, including other Wnts that do not bind with the receptor (shown as red and yellow spheres) will be removed. The receptor-interacting, isolated proteins can then be analyzed and identified by SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) and Western blot.

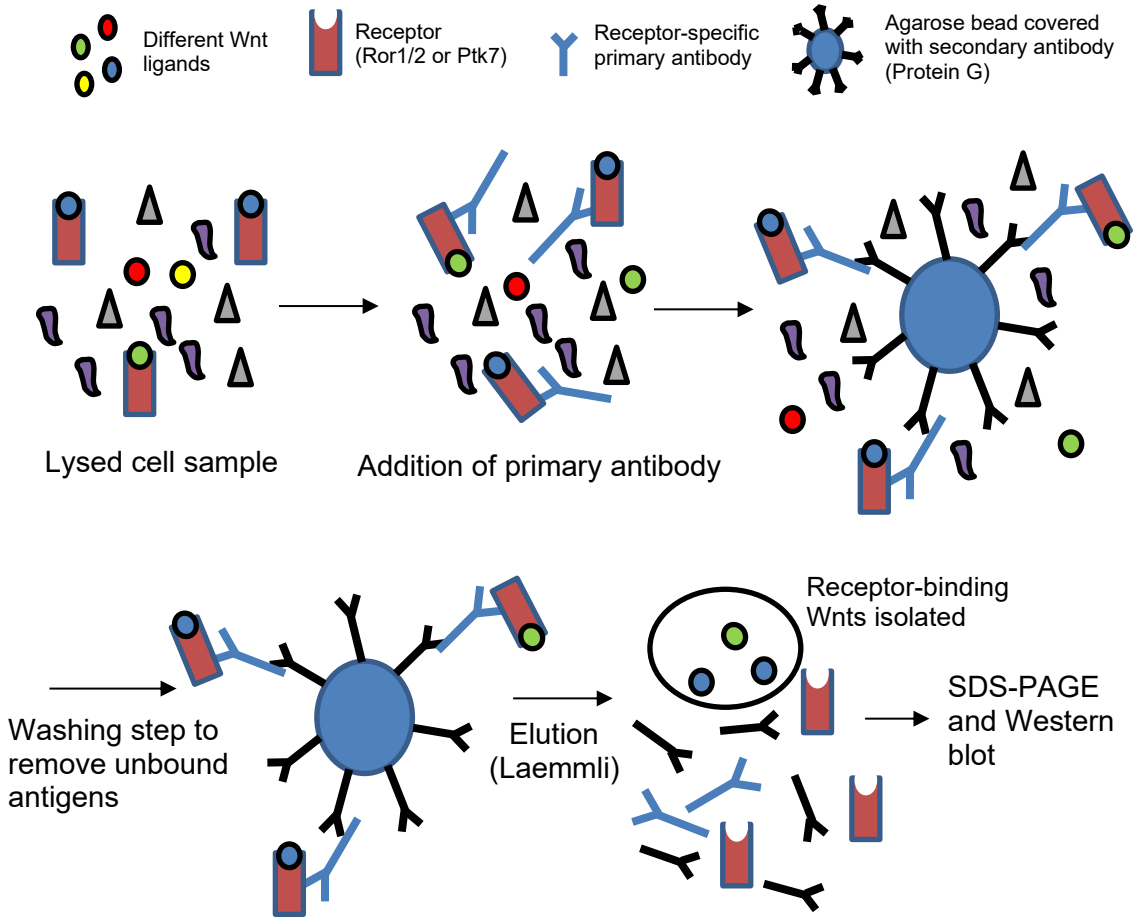

Figure 8. Steps of the immunoprecipitation protocol. The Wnts that interact with the receptor are co-precipitated with the receptor and subjected to identification by SDS-PAGE and Western blot. Adapted from https://ruo.mbl.co.jp/bio/e/support/method/immunoprecipitation.html, accessed 27th March 2020).

Luciferase assay was used in this work to study canonical Wnt signaling transcriptional activity. M50 Super8xTOPFlash (STF) reporter plasmid, described in [83], contains eight TCF/LEF1 binding sites upstream of a luciferase reporter (in the luciferase promoter region). When canonical Wnt signaling is active, β-catenin is stabilized and coactivates the TCF/LEF1 transcription activity, as described in the earlier sections. The activated TCF/LEF1 factors bind to promoter region of M50 plasmid and initiates the transcription of firefly luciferase, as shown in Figure 9. When luciferase substrate, beetle luciferin, is added, oxyluciferin and light (luminescence) are produced from the chemical reaction. The detected luminescence signal correlates with the activity of β-catenin dependent Wnt signaling.

It is also important to include negative control to verify that the luciferase assay is activated only when TCF/LEF1 can bind to the promoter region of the plasmid. The negative control plasmid M51 Super8XFOPflash contains mutant TCF/LEF1 binding sites (M50 TOPFlash mutant). These mutations prevent the binding of the TCF/LEF1 to the promoter region and subsequent transcription of the luciferase gene even when β-catenin dependent Wnt signaling is active. Thus, the detected luminescence when using M51 control plasmid determines the background noise of the assay (ideally there should not be any signal). The mechanism of action is illustrated in Figure 9.

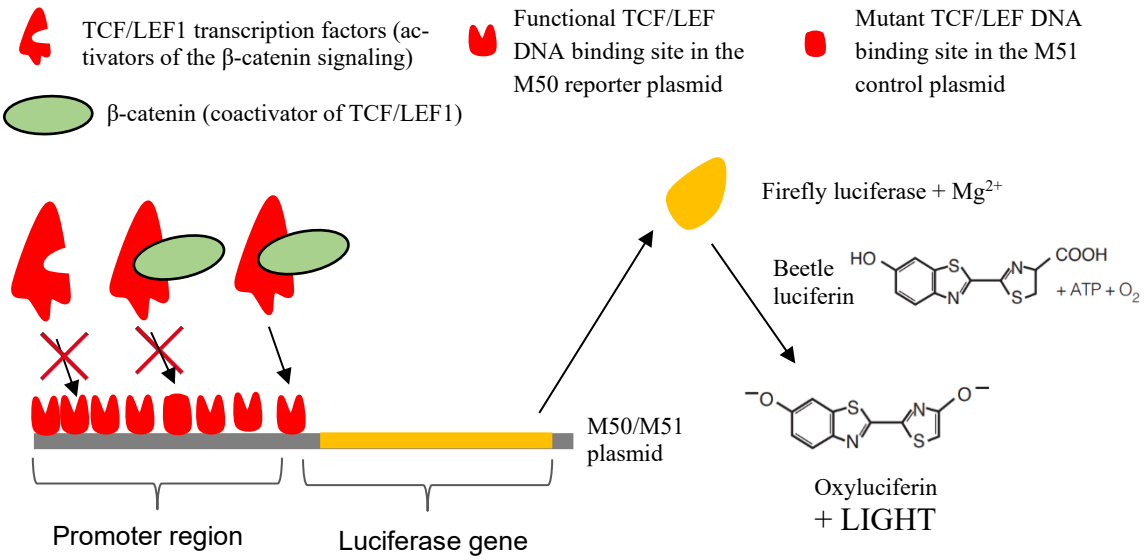

Figure 9. Illustration of the mechanism of luciferase assay with M50 and M51 plasmids. TCF/LEF1 are transcription factors of the canonical Wnt pathway. Transcription is activated when TCF/LEF1 associates with coactivator β-catenin, after which the activated TCF/LEF1 can bind to the M50 plasmid's promoter region that contains eight TCF/LEF1 binding sites (mutated in the M51 Super8XFOPflash negative control plasmid). As a result, firefly luciferase reporter protein is produced. Luciferase produces oxyluciferin and light when the substrate, beetle luciferin, is added in the assay. Note that the sizes and shapes shown here are not relative to each other.

3 AIMS OF THE STUDY

Three main aims of this work were to study:

1) Expression and secretion profiles of Wnt ligands in HEK293T cells
2) Interaction of the Wnt ligands with the non-canonical Wnt receptors Ror1, Ror2, and Ptk7
3) Wnt ligand-induced activation of downstream Wnt signaling pathways through Ror1, Ror2, and Ptk7

4 MATERIALS AND METHODS

4.1 Cell culture

Experiments were performed *in vitro* using the cell lines described below. All used cell lines were cultured in +37 °C temperature and 5% CO_2 and were split about twice a week in sterile conditions.

1) Human embryonic kidney cells (HEK293T) cultured in DMEM media (Dulbecco's Modified Eagle Medium media, Thermo Fisher Scientific), supplemented with 10% FBS (Fetal Bovine Serum), 2 mM L-Glutamine, 1% sodium pyruvate, and 100 U/ml penicillin/streptomycin.
2) *Mus musculus* BaF3 cells (pro B cell line, DSMZ, ACC 300) and Ba/F3 cells with stable expression of Ror1 (Ba/F3-Ror1), Ror2 (Ba/F3-Ror2), or Ptk7 (Ba/F3-Ptk7) created in our lab by cloning and electroporation as described earlier [58]. Ba/F3, Ba/F3-Ror2, and Ba/F3-Ptk7 cells were cultured in RPMI 1640 media (Lonza, Basel, Switzerland) supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin/streptomycin and 10% WEHI supernatant (IL3). Ba/F3-Ror1 cells were cultured in same media but without WEHI supernatant.

4.2 Transfections

15 human-origin Wnt ligands (Addgene) were included in this study, as shown in Table 3. To study expression and secretion profiles of these Wnt ligands, HEK293T cells were plated on 6-well plates (200,000 cells/well). Cell counts were determined by Trypan-blue exclusion using a Countess II automated cell counter (Thermo Fisher). After overnight incubation, the Wnt-encoding plasmids were transfected. For transfections, a separate transfection mix was prepared for every transfection containing one of the Wnt-encoding plasmids (2 µg), Gibco Opti-MEM reduced serum media (Thermo Fisher Scientific) and FuGENE (Promega) transfection reagent (2 µl for 1 µg of DNA). The mixes were pipetted on top of the cells on 6-well plates and incubated for 68 hours (time point where Wnt secretion was determined to be highest in the media in a separate experiment, data not shown). This resulted in total of 15 transfections.

Table 3. V5-tagged Wnt-encoding plasmids (Addgene) included in this study.

Plasmid	Addgene plasmid reference
Wnt1-V5	#35924
Wnt2-V5	#43809
Wnt2b-V5	#43808
Wnt3a-V5	#35927
Wnt4-V5	#35929
Wnt5a-V5	#43813
Wnt5b-V5	#43814
Wnt6-V5	#35932
Wnt7a-V5	#35933
Wnt8a-V5	#43818
Wnt9a-V5	#43820
Wnt9b-V5	#43821
Wnt10-V5	#35939
Wnt11-V5	#35941
Wnt16-V5	#35942

To study the interactions between Wnt ligands and the receptors of interest, each Wnt ligand together with one of the receptors (Ror1, Ror2, and Ptk7) was overexpressed for 24 hours in HEK293T cells in four separate experiments (one for each receptor-Wnt set plus additional set for Ptk7 to be used for irrelevant antibody control, as explained later under immunoprecipitations section). HEK293T cells (10^6 cells counted with Countess II) were plated on Petri dishes (100 mm diameter) in DMEM media and cultured overnight to reach c. 70% confluence for optimal transfection efficiency. 2 µg of the receptor plasmid and 1.5 µg of each Wnt-encoding plasmid were transfected together for 24 hours (FuGENE as transfection reagent). Also, a sample without any Wnt transfection was included in all four sets as a negative control. Empty pSG5 vector (1.5 µg) was used in the negative control to replace the Wnt-encoding plasmid so that the amount of transfected DNA would be balanced between all transfections. This resulted in 16 transfections in each of the four sets.

To get better information on what receptor domains are required for binding to the Wnt ligand, Ror1 wild type plasmid as well as Ror1 plasmids with a deleted domain (ΔCRD and ΔKR) were transfected together with Wnt5a or Wnt16 according to Table 4 for 24 hours. HEK293T cells (200,000/well, counted with Countess II) were transfected for 24 hours as described above, except Turbofect (Thermo Fisher Scientific) was used as transfection reagent (4 µl for 1 µg of DNA) instead of FuGENE.

For the luciferase assay, HEK293T cells were plated on 24-well plates (50,000 cells/well in DMEM media) and transfected either in triplicates (for reporter plasmid, shown in orange in Table 5) or in duplicates (for control plasmid, shown in grey in Table 5) for 24 hours. The transfected reporter plasmid was M50 Super8xTOPFlash (Addgene plasmid #12456; http://n2t.net/addgene:12456; RRID: Addgene_12456) and control plasmid was M51 Super8XFOPflash (Addgene plasmid #12457; http://n2t.net/addgene:12457; RRID: Addgene_12457).

Opti-MEM reduced serum media (Thermo Fisher Scientific) and TurboFect (Thermo Fisher Scientific) were used for all transfections. FuGENE (Promega) was initially used as transfection reagent but for an unknown reason, the transfection efficiency with FuGENE was very variable between samples in luciferase assay even within a single plate (nonexistent in some samples and high in others, shown by β-galactosidase color reaction), so FuGENE was replaced with TurboFect and the problem did not happen again. Empty pSG5 plasmid was used to balance the total amount of transfected DNA (710 ng) between the transfections, as shown in Table 5.

Table 4. Plasmids and amounts used for transfections to study what Ror1 domain(s) is required for the Wnt interaction. Each column showing transfected amount (µg) marks one separate transfection. wt = wild type, CDR = cysteine rich domain, KR = Kringle domain.

Plasmid	**Amount per transfection**					
Ror1 wt	1 µg	-	-	1 µg	-	-
Ror1 ΔCRD	-	1 µg	-	-	1 µg	-
Ror1 ΔKR	-	-	1 µg	-	-	1 µg
Wnt5a	1 µg	1 µg	1 µg	-	-	-
Wnt16	-	-	-	1 µg	1 µg	1 µg

Table 5. Luciferase assay transfections with M50 Super8xTOPFlash and M51 Super8xFOPFlash. For M50, each transfection was done in triplicates to get averages from 3 separate readout values. 4 different transfection sets (master mixes) with different receptor plasmids conducted in 3 independent experiments. For a negative control M51, it was decided that duplicates and 2 independent experiments for each set would be sufficient due to very low expected signal.

Reporter plasmid	Receptor plasmid[1] (ng)	Wnt plasmid[2] (ng)	Reporter plasmid (ng)	β-gal reporter (ng)	Empty pSG5 (ng)	Number of transfections
M50 Super8xTOPFlash	-	-	200	10	500	3
	250	-	200	10	250	3
	250	250	200	10	-	3x15 = 45
M51 Super8xFOPFlash	-	-	200	10	500	2
	250	-	200	10	250	2
	250	250	200	10	-	2x15 = 30

[1] Transfection set 1: no receptor plasmid, set 2: Ror1-HA, set 3: Ror2-Flag, set 4: Ptk7-HA. Each set was repeated 3 times (M50) or 2 times (M51) independent of each other. [2] Wnt1, Wnt2, Wnt2b, Wnt3a, Wnt4, Wnt5a, Wnt5b, Wnt6, Wnt7a, Wnt8a, Wnt9a, Wnt9b, Wnt10, Wnt11, Wnt16 (each separately). All Wnt-encoding plasmids were V5-tagged.

4.3 SDS-PAGE and Western blot analysis

Sample preparation for SDS-PAGE: The cells were lysed in cold NP40 cell lysis buffer (50 mM Tris-HCl pH 7.4, 10% glycerol, 50 mM NaCl, 0.5% sodium deoxycholate, 1% NP-40, 20 mM sodium fluoride) supplemented with 1/100 protease inhibitor cocktail (Bimake, Houston, TX, USA) just prior to use. Cell lysates were incubated for 15 minutes on ice, clarified by centrifugation (4 °C, 20 min, 20,000 x g), resuspended in 4x Laemmli sample buffer (Bio-Rad) and boiled at 95 °C for 5 minutes to denature the proteins for the following SDS-PAGE analysis.

SDS-PAGE: The samples were separated with SDS-PAGE using Mini Trans-Blot Cell electrophoresis system (Bio-Rad) and 7.5% mini-gels (10 x 7.5 cm) that were made of H_2O, 30% Acrylamide/Bis Solution 37.5:1, Tris–HCl (1.5 M, pH 8.8), TEMED (tetramethylethylenediamine) and APS (ammonium persulfate). The samples were transferred to nitrocellulose membrane using Trans-Blot SD Semi-Dry Transfer Cell instrument (Bio-Rad).

Western blot: Nitrocellulose membranes containing the transferred samples were blocked in 4% BSA (bovine serum albumin) containing 0.05% Tween 20 in 1xTBS (tris-buffered

Table 6. Primary and secondary antibodies used in the western blot.

Antibody	Manufacturer
anti-V5	Cell Signaling Technology (Danvers, MA, USA)
6D4-Ror1	Cell Signaling Technology
Ror2	BD Biosciences (San Jose, CA, USA)
Ptk7	Cell Signaling Technology
ERK1/2	Cell Signaling Technology
pERK1/2	Cell Signaling Technology
AKT	Cell Signaling Technology
pAKT	Cell Signaling Technology
IRDye 800CW Donkey anti-Mouse IgG	LI-COR (Lincoln, NE, USA)
IRDye 680RD Donkey anti-Rabbit IgG	LI-COR (Lincoln, NE, USA)

saline) for one hour and incubated in primary antibodies (Table 6) overnight. After washes (TBS/0.1% Tween 20 buffer), the blots were stained with secondary antibodies (IRDye, Table 6) at 1:10.000 dilution for one hour. The blots were scanned with Odyssey CLx imaging system (LI-COR) and expression levels were quantified with Image Studio Lite (LI-COR). GraphPad Prism was used to make figures from the blots.

4.4 Wnt secretion profile

To determine the expression and secretion profile of the Wnts in HEK293T cells, the cells were transfected and lysed for SDS-PAGE as described previously. However, also culture media was separately collected to analyze how much each Wnt is secreted out from the cells. In the case of media, the sample preparation for SDS-PAGE included suspending 30 µl of collected culture media into 10 µl 4x Laemmli sample buffer (Bio-Rad) and boiling at 95 °C for 5 minutes (lysis step was excluded). The expression levels of Wnts in cell lysates and in media were analyzed by Western blot as described earlier using anti-V5 antibody for detecting all Wnts (Table 6). To make the Wnt expression levels in cell lysate and media comparable with each other, the expression levels of Wnts in media were multiplied by 7.5. This normalization compensated the differences in volume from

where Wnt samples were taken for analysis (1.5 ml media and 0.2 ml cell lysate). The relative secretion of each Wnt could be then calculated by dividing the expression level of the Wnt in cell lysate by the normalized expression level of the Wnt in culture media.

4.5 Immunoprecipitations

Immunoprecipitation assay was used to study protein-ligand interactions. From the lysed cell lysates, 50 µl per sample was used for the western blot protein expression level analysis, while the rest was used for immunoprecipitation. First, primary antibody against the receptor of interest was added to the lysates: 3 µl 4A5 (against Ror1-HA), 1 µl 1/3 diluted anti-FLAG (against Ror2-FLAG) or anti-HA (against Ptk7-HA), see Table 7 for details about used antibodies. Additionally, anti-FLAG was also used with another Ptk7-HA set for an irrelevant antibody precipitation. Lysates were incubated with the antibody in 1.5 ml Eppendorf tubes overnight (+4 ºC, 12-14 hours in rotation). The antigen-antibody complexes were precipitated with Protein G PLUS-Agarose beads (Santa Cruz Biotechnology) 20 µl per sample for one hour (+4 °C in rotation).

Immunocomplexes were washed 3 times with cold NP40 lysis buffer and the NP40 buffer was removed carefully not to lose any beads. Samples were eluted into 2x Laemmli buffer (Bio-Rad), boiled in +95 °C for 5 minutes and boiled sample elusion was subjected to SDS-PAGE and western blot analysis as described in the previous section. A heat map was created with Excel to visualize the Wnt-receptor interaction profiles.

Table 7. Antibodies used for immunoprecipitations.

Antibody	**Manufacturer**
4A5-Ror1	BD Biosciences (San Jose, CA, USA)
anti-FLAG	BioLegend (San Diego, CA)
anti-HA	BioLegend (San Diego, CA)

4.6 Luciferase assay

Transfected cells (see section 4.2 for transfections) were lysed in a reporter lysis buffer (RLB, Promega) and luciferase assay (Promega) was performed according to manufacturer's instructions (https://fi.promega.com/products/luciferase-assays/reporter-assays/luciferase-assay-system/?catNum=E1500#protocols, accessed 28th Feb 2021). Luminescence was measured in black 96-well plates (Luminometer with automatic dispenser for luciferase reagent 75 µl/well) and β-galactosidase values in white 96-well plates (Multiskan Ex, Thermo LabSystems) with manual ONPG (ortho-Nitrophenyl-β-galactoside) substrate addition 20 µl/well to start the color formation. β-galactosidase readings were used to normalize the results with respect to transfection efficiency between the wells, and average values were calculated from three separate replicates within a single plate. After normalization of luciferase readings in respect to b-galactosidase, they were further normalized against the luciferase reporter transfections alone without Wnt or receptor-encoding plasmids (first set of transfections in Table 5). All experiments were done in triplicates and for three independent experiments, and figure was created with GraphPad Prism software.

4.7 Wnt stimulations

The non-canonical Wnt pathway signaling was studied by stimulating the Ba/F3 cell lines up to 4 hours with recombinant Wnt ligands Wnt3a, Wnt5a, and Wnt16. These ligands were selected to be representative of both canonical Wnt signaling (Wnt3a) and non-canonical Wnt signaling (Wnt5a and Wnt16). 4 x 10^6 cells for each cell line (Ba/F3, Ba/F3-Ror1, Ba/F3-Ror2, and Ba/F3-Ptk7) were washed once with PBS and resuspended into 4 ml media. Each of the three Wnt ligands (50 ng/ml) was then added to cells in separate tubes for each cell line and incubated in +37 °C and 5% CO_2. Samples (10^6 cells in 1 ml) were collected at different time points (0, 1/2, 2, 4 hours). The 0-hour time point was collected for analysis without Wnt addition but kept in incubator until the last time point (4h) was collected to give the cells to recover from initial manipulations (Wnts were added to cells after the collection of 0-hour time point). Collected samples at each time point were lysed in cold NP-40 lysis buffer with protease inhibitor cocktail and subjected to SDS-PAGE. MAPK/ERK and PI3K/AKT/mTOR pathways were studied with western blot (antibodies in Table 6). Wnt expression levels in western blot were quantified with Image Studio Lite (LI-COR).

5 RESULTS AND DISCUSSION

5.1 Relative secretion of Wnts by the HEK293T cells

The relative secretion profiles of Wnts were studied in HEK293T cells. The cells and culture media were separately collected and analysed by Western blot. The relative secretion levels of Wnts were calculated by normalizing to expression levels of the Wnt ligands (Supplementary Table 1). All Wnts were secreted from the cells into the media but at highly varying levels (Figure 10). Wnt7a had highest relative secretion from the cells (50% secreted), while Wnt2 was secreted at the lowest level (4%).

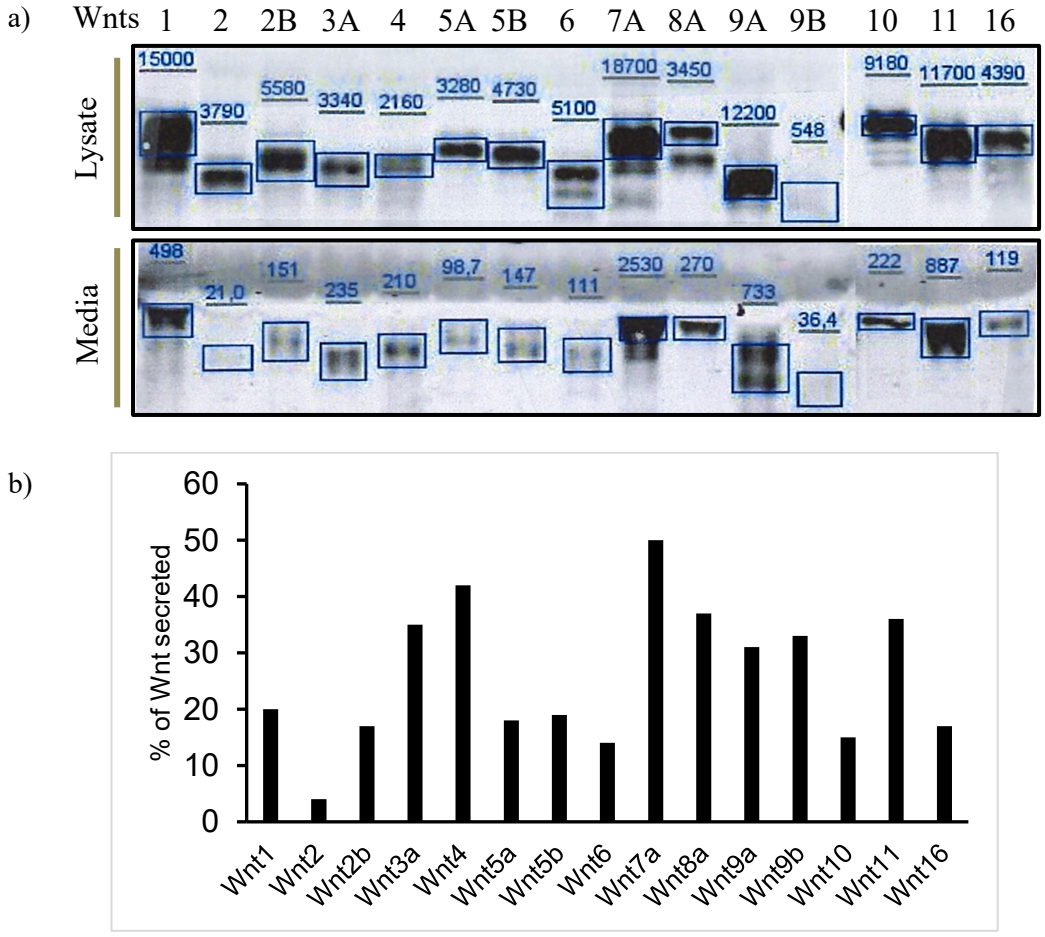

Figure 10. Relative secretion profile of the 15 Wnts studied in HEK293T cells. a) Western blot analysis of expression of each Wnt in cell lysates (upper panel) and in the culture media (lower panel) after transfecting Wnts in HEK293T cells for 68 hours. The expression level of each Wnt in the blots was quantified with Image Studio Lite (LI-COR). b) Relative secretion profiles of each Wnt ligand based on the expression levels of Wnts in the western blot presented in (a). Secretion-% was calculated by dividing Wnt expression levels in cell lysates with normalized Wnt expression levels in the culture media.

This result was confirmatory to previously published directionally similar result [12]. Since Wnts show only small variation at mRNA level [12], the variations at the protein level might be due to variations in the Wnt stability and/or in the mRNA translation. In addition, different Wnt ligands have variable numbers of isoforms (i.e., number of bands in a western blot), for example Wnt8a has two bands while Wnt5a has only one (Figure 10a). Interestingly, some isoform bands visible in the lysates are not visible in the media (e.g., for Wnt1 and Wnt8a), suggesting that there exists at least some degree of specificity in the secretion of different Wnt isoforms outside the cell.

It is important to note that this was an overexpression setting, and the naturally produced Wnt ligands might have different secretion profiles. In addition, acylation levels of secreted Wnts should be studied in future since the acylation might have effect on Wnt secretion and biological activity depending on the type of Wnt ligand [38]. Also, the levels of cell membrane bound Wnts should be determined in future studies, since if Wnt binds to cell membrane as has been previously observed for Wnt3a [37], the bound Wnt is not detected from collected cell media and might result in the underestimation of the total Wnt secretion level. However, these secretion results are important because Wnts signal in autocrine manner [36], and variable secretion levels of different Wnts might function as an additional regulation step in the subsequent activation of Wnt-mediated signaling pathways.

5.2 Wnt binding to Ror1, Ror2, and Ptk7

After confirming that all Wnts are produced and secreted in HEK293T cells, next step was to study their physical binding to pseudokinase receptors Ror1, Ror2, and Ptk7 by an immunoprecipitation assay. The pseudokinase receptor was precipitated together with the proteins bound to it, including any of the Wnts. Western blots of the lysates (Figure 11a-c, upper panels) confirm the sufficient expression of the Wnts (with some minor exceptions, specifically Wnt9b was expressed weakly), while IP blots (Figure 11a-c, lower panels) show that all the receptors Ror1, Ror2, and Ptk7 bind to several Wnt ligands, as marked by red asterisks. The highly overlapping binding pattern is visualized in a heatmap (Figure 11d).

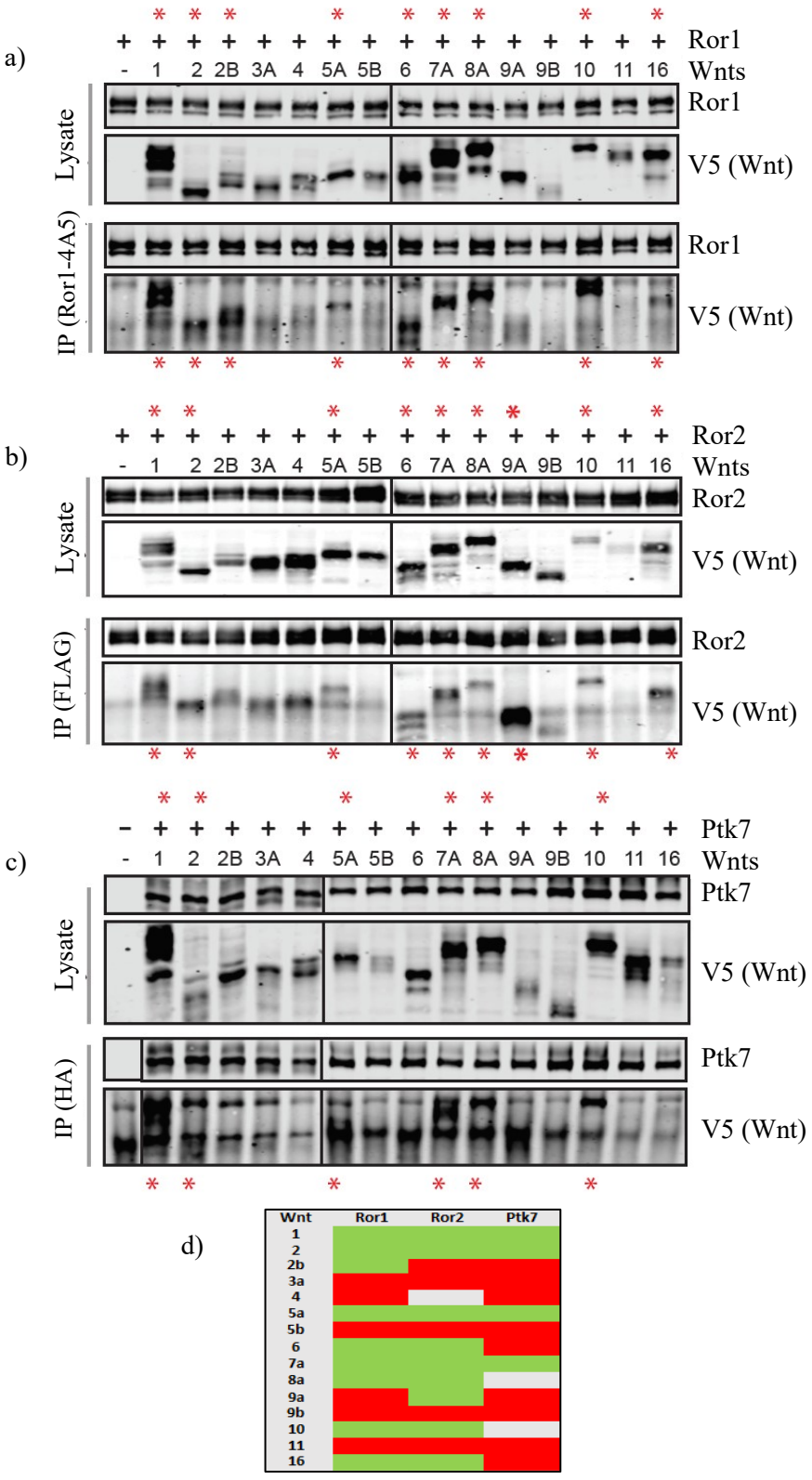

Figure 11. Immunoblots showing the Wnt binding with Ror1 (a), Ror2 (b), or Ptk7 (c), and heatmap (d) summarizing these interactions. Western blots of the lysates are shown in the upper panel and western blots of the immunoprecipitations in the lower panel. Asterisks in a-c denote the observed positive binding between the pseudokinase receptor and Wnt ligand. (d). Heatmap summarizing results of Wnt interactions with receptors. Binding shown in green colour, weak or no observed binding shown in red color while uncertain results (that can be unspecific background and not the actual binding) in grey color.

The irrelevant antibody IP (Figure 12) shows rather strong background noise for unspecific binding, which makes interpretation of the IP results challenging in some cases – weakly binding Wnts can in fact be background noise, or vice versa. These uncertain cases are marked in grey color in the heatmap (Figure 11d). Results shown here are rather qualitative than quantitative, and the purpose was rather to provide starting point for further studies. For example, Ror1-Wnt16 interaction found in this work (Figure 11a) was later validated and reported by our group in a separate publication [3].

The binding profiles of Ror1 and Ror2 were rather resembling, both having some degree of binding with 9 out of 15 studied Wnt ligands. In contrast, the Wnt binding profile of Ptk7 was different with 6 binding Wnts (Figure 11d). This is logical, since Ror1 and Ror2 belong to the same protein family with similar domain architecture [61], while extracellular portion of Ptk7 is markedly different from Rors (Figure 5). However, conservation of Rors is mostly restricted to the intracellular TKD, while extracellular domains have more variation [15]. This opens up the possibility that Ror1 and Ror2 can interact with distinct Wnt ligands despite their close homology, and some differences in binding profiles were in fact also seen in this study (Figure 11d). Studies analysing binding of Ptk7 with Wnt ligands have been largely lacking from the literature [30]. At least Wnt5a has been shown to both physically and functionally interact with Ptk7 in HEK293T cells [29], which is in line with results of this work.

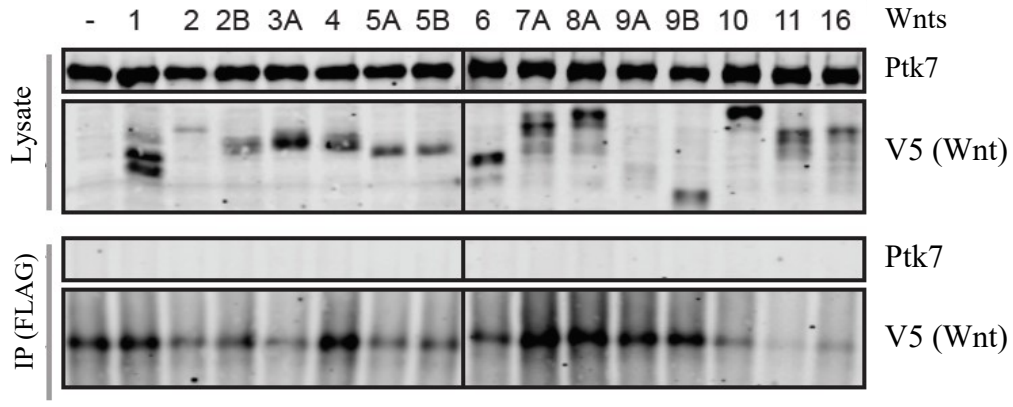

Figure 12. Negative control for co-immunoprecipitations with irrelevant antibody. Ptk7-HA was expressed together with all Wnt-V5 ligands (upper part of the figure: lysate). The immunoprecipitation was done with FLAG antibody that does not co-precipitate Ptk7 or Wnt ligands (lower part of the figure: IP). The background of the assay is visible as dark unspecific bands in the V5 (Wnt) immunoprecipitation blot.

From Wnt ligand perspective, some Wnts (for example, Wnt1, Wnt7a) co-precipitated with all three receptors, some (e.g., Wnt9a, Wnt16) with 1-2 receptors, while others (e.g., Wnt3a, Wnt11) did not bind to any of the three receptors (Figure 11d). This overlapping binding profile of Wnts is in line with the literature [2, 7, 12]. To analyze a few examples, Wnt3a is a well-known canonical Wnt ligand [41, 63], so its lack of precipitation with non-canonical Rors and Ptk7 in this work is expected result. Wnt4 and Wnt11, on the other hand, are classified as non-canonical Wnt ligands [2, 7], so their lack of binding to these receptors was unexpected. Wnt4 has been reported to bind to *Drosophila* Ptk7 ortholog Otk (off-track) [41] and activate noncanonical PCP signaling via an unknown mechanism [27]. Wnt11, on the other hand, regulates cell migration and morphology zebrafish gastrulation through Ror2 and has also been shown to co-precipitate with Ror2 in HEK293T cells [27]. The low expression of Wnt11 in this work (Figure 11b) might have resulted in a false negative and repeating the experiment with better Wnt11 expression level could be informative.

It is especially relevant to consider the experimental setting used when immunoprecipitation results are being compared to earlier findings reported in the literature. Especially the cell line is an important factor, since differently expressed (co)receptors in different cell lines can influence the Wnt-receptor interaction dynamics, further demonstrating the complex nature of Wnt signaling and overlapping binding kinetics of the Wnt ligands [8]. Even if the studied receptor is expressed on the cell surface, the lack or presence of certain dimerizing co-receptor partner(s) in that cell line might affect Wnt binding to the receptor of interest. In addition, several secreted Wnt binding factors (such as WIFs) might vary between different cell types and conditions, and even additional yet unknown Wnt (co)receptors might be existing [15]. This might underlie the mixed results in literature: for example, Wnt3a was shown to co-precipitate with Ptk7 inly in the presence of Fzd7 in Xenopus cells [41]. Moreover, Ror1 strongly co-precipitated with Wnt1 in HEK293T cells (Figure 11a), while no co-precipitation was detected in U2OS cells [47]. Similarly, Wnt3a co-precipitates with Ror2 in U2OS cells [63], while no interaction was observed in HEK293 cells (Figure 11b). It will be important to identify the exact set of Wnt (co)receptors expressed by each cell line in future studies and include cell line information with the immunoprecipitation results, as shown in Table 2.

Wnt5b, a known ligand for mammalian Ryk receptor [27], did not immunoprecipitate with any of the three receptors, even though closely related Wnt5a precipitated with all three receptors (Figure 11d). Wnt5a and 5b share very similar amino acid sequences so it is a surprising result that their binding profiles with the three receptors are so different. Notably, Wnt5b is well expressed in the lysates, so expression level should not be problem in this case. Potential explanation could be the presence of other factors, for example Wnt5a is known to dimerize with Wnt11 [84] which might change the receptor binding profile compared to Wnt5b. Further studies are required to explain the difference. Would be interesting to overexpress Wnt5a/5b together with Wnt11 and study if both overexpressed Wnt ligands co-precipitate together with the receptor or if the binding profile is altered.

After identifying which Wnt ligands interact with Ror1, Ror2, and Ptk7, the next step was to determine which receptor domains are required for this interaction. Full-length Ror1 and truncated forms of Ror1 receptor with either CRD or KR domain missing were used to study binding with Wnt ligands (Wnt5a and Wnt16). Figure 13 shows that when either the CRD or the KR domain of Ror1 is absent, the Wnt binding to Ror1 is completely abolished. This result shows that both of these extracellular domains are required for the Wnt binding to happen.

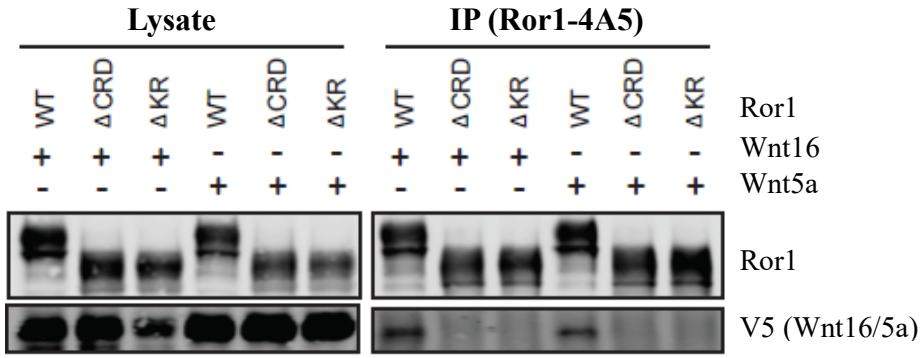

Figure 13. Ror1 domains and Wnt binding in Western blot analysis. Lysates (left side) show comparable expression levels of the transfected plasmids (Wnt5a, Wnt16, Ror1 wt, ΔCRD, ΔKR). IP with Ror1 antibody (right side) show Wnt16/5a binding only with wt Ror1, while no Wnt interaction is observed with truncated forms of Ror1. Co-immunoprecipitations demonstrate that both CRD and KR domains of Ror1 receptor are required for the Wnt binding. The blot is representative of three independent experiments. Abbreviations: IP, immunoprecipitation; WT, wild-type; CRD, cysteine-rich domain; KR, Kringle domain.

CRD is a well-known Wnt-binding domain of Ror and Fzd receptor families [8, 38, 64], so the result for CRD was expected. However, the role of KR in the Wnt binding has not been reported to my knowledge, even though KR is known to be required for Wnt-induced Ror1-Ror2 heterodimerization [11, 64]. Because neither Wnt5a nor Wnt16 precipitated with Ror1 in this work when KR was missing, this is interesting new finding suggesting that not only CRD but also KR is indispensable for Wnt binding at least for these two tested Wnts. The exact mechanism for this remains to be elucidated in future studies. One possibility is that absence of KR domain impairs Ror1 binding to some co-receptor partner and that way indirectly also Wnt binding is disturbed, as illustrated in Figure 14.

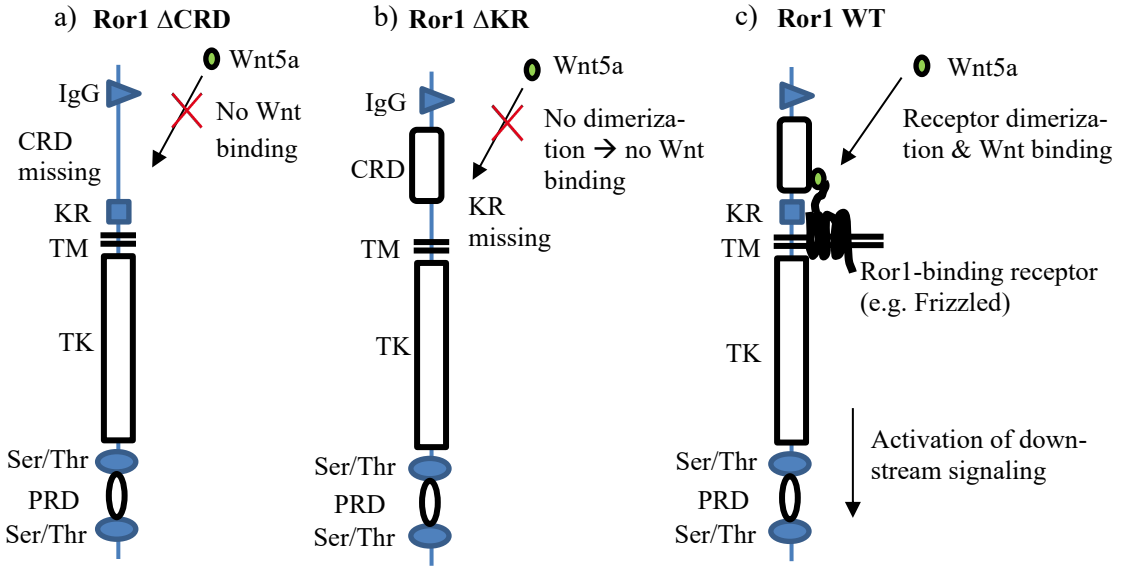

Figure 14. Proposed model for the Wnt-Ror1 binding based on the experimental results. a) When CRD is missing (Ror1 ΔCRD), Wnt binding is abrogated. b) When KR domain is missing (Ror1 ΔKR), Ror1 cannot bind to dimerization partner and Wnt binding is abrogated. c) Full-length Ror1 (Ror1 WT) forms complex with another receptor and Wnt5a binds to this complex. Note that the model presented here is significantly simplified and in reality, the situation is much more complex, involving many additional components such as other co-receptors. Abbreviations: IgG, immunoglobulin G; CRD, cysteine-rich domain; KR, Kringle domain; WT, wild-type; TM, trans-membrane; TK, tyrosine kinase; PRD, proline-rich domain.

5.3 Ror1, Ror2, and Ptk7 mediated signaling

After confirming the binding dynamics of different Wnts to Ror1, Ror2, and Ptk7, next step was to study the Wnt signaling pathways activated by this binding. Both canonical and non-canonical Wnt signaling pathways were studied in response to the Wnt binding. The canonical Wnt signaling was studied with STF luciferase reporter assay in HEK293T cells. Out of 15 studied Wnts, only Wnt3a could induce transcriptional activation of canonical Wnt signaling (Figure 15a). This Wnt3a-dependent signaling activation was significantly attenuated when the receptors Ror1 (79% attenuation), Ror2 (82%), or Ptk7 (62%) were overexpressed together with the Wnts. Figure 15 also shows that neither Ror1, Ror2, nor Ptk7 could activate canonical Wnt signaling. In addition, no significant transcriptional activation was observed with a negative control plasmid M51 that has mutated TCF/LEF binding sites, validating the functionality of the used assay (Figure 15b).

The result of Rors and Ptk7 attenuating Wnt3a-induced canonical Wnt signaling supports the classification of these receptors as part of non-canonical Wnt pathways, which are known to compete with β-catenin-dependent (canonical) Wnt signaling [24]. Also direct evidence has been reported in the literature for these receptors. For example, Ror2 attenuated Wnt3a-activated STF reporter activity (canonical Wnt signaling) in osteoblastic cells [47], and STF reported activity was attenuated by Wnt5a only when Ror2 was co-expressed in HEK293T cells [7]. Wnt3a/8a induced β-catenin activation was inhibited by Ptk7 in HEK293 cells and in *Xenopus* embryos [41]. There are several proposed mechanisms to explain this inhibition. Receptor might bind and sequester canonical Wnt ligands, or the lack of Axin-binding potency leaves Axin in the cytoplasm to block canonical Wnt signaling (in contrast to LRP5/6 that can bind Axin and disrupt the destruction complex) [41]. Ptk7 has also been shown to directly bind β-catenin [70], which provides additional mechanism by scavenging β-catenin from activating canonical Wnt signaling.

Also completely opposite results have been reported for Rors and Ptk7. For example, Ror2 potentiated Wnt3a-induced canonical Wnt signaling in both H441 lung carcinoma cells [85] and 786-0 renal cancer cells [16]. One study reported Ptk7 to potentiate canonical Wnt signaling in *Xenopus* and other cell lines [70]. These conflicting results could also be explained by the presence of different set of receptors in different cellular contexts that determine what signaling pathway is activated. For example, Fzd2 has been reported

to cooperate with Ror2 in enhancing the canonical Wnt signaling [16], and Ptk7-mediated canonical Wnt signaling inhibition requires dimerization with the Fzd7 receptor [41].

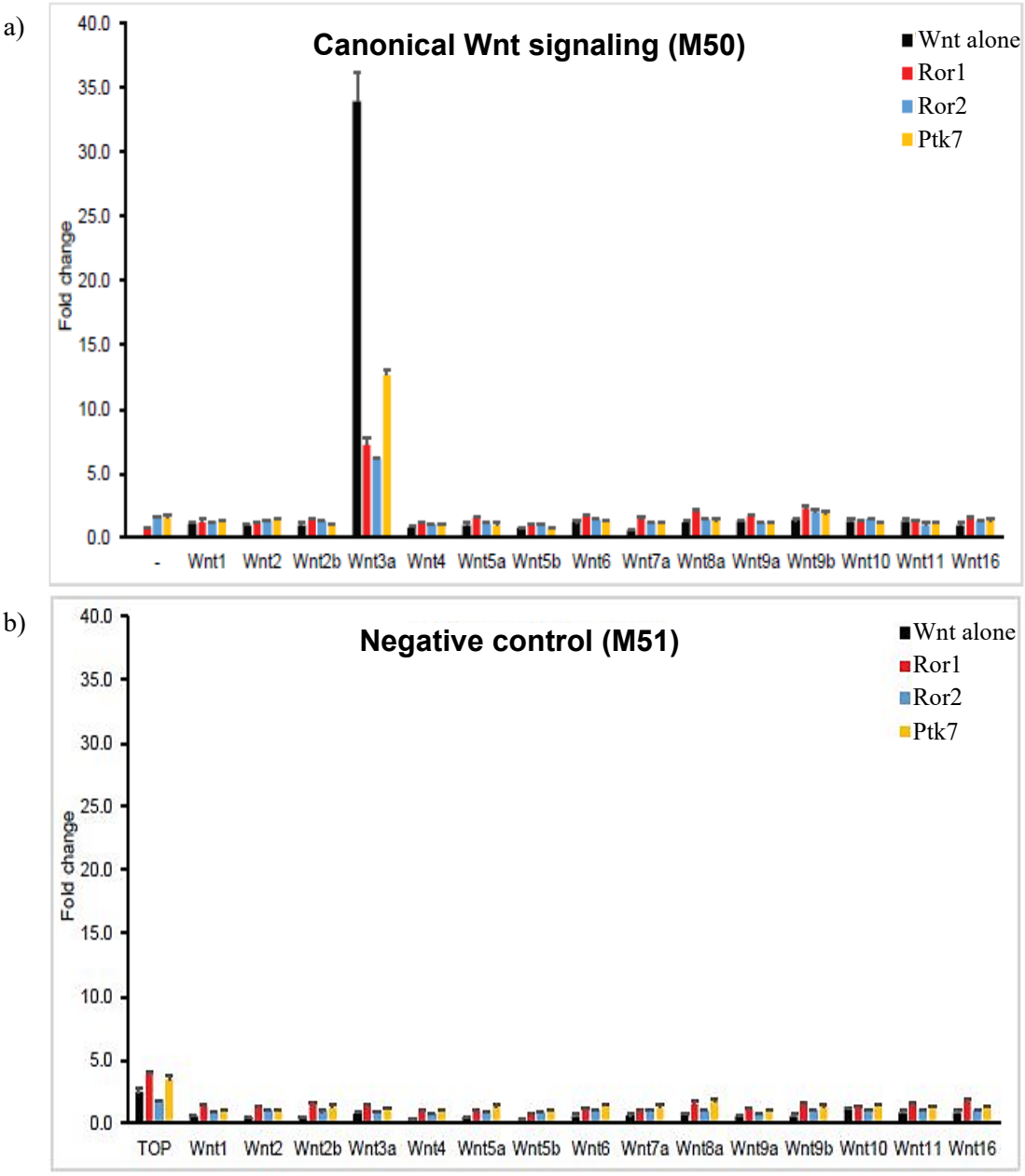

Figure 15. β-catenin dependent Wnt signaling studied by a STF luciferase assay. Each Wnt was expressed in HEK293T cells either alone (black bars) or together with a one of the pseudokinase receptors Ror1, Ror2, or Ptk7 (coloured bars). a) Transcriptional activation of canonical Wnt signaling is measured by M50 reporter plasmid that has functional TCF/LEF1 binding sites. b) M51 plasmid acts as negative control for the assay since it has mutated TCF/LEF1 binding sites while the rest of its DNA sequence is unchanged from M50 reporter plasmid. Error bars represent standard deviation (STD) from three independent experiments.

Wnt3 being the only ligand that activated canonical Wnt signaling in this work is also in line with the classification of Wnt3a as a "canonical Wnt ligand" [41]. Similar result for Wnt3a has been previously reported in both HEK293T (46) and U2OS cells [47]. However, whether the ligand activates canonical or non-canonical Wnt signaling can also be influenced by the set of receptors expressed on the cell surface. For example, non-canonical Wnt ligand Wnt5a can activate canonical Wnt signaling when Fzd4 and LRP5 are expressed together on the cell surface [7]. This expression is absent in HEK293 cells [7], which can explain why Wnt5a could not activate canonical Wnt signaling in this work (Figure 15a). Interestingly, Wnt5a could also activate canonical Wnt signaling by heterodimerizing with another non-canonical ligand, Wnt11, at least in three tested cell lines (*Xenopus* oocytes, mouse L cells, and human embryonic stem cells) [84]. Hence, transfecting Wnt11 together with Wnt5a could decrease the threshold for β-catenin activation in HEK293T that have low endogenous expression of correct set of receptors for Wnt5a-induced β-catenin signaling to occur.

To my knowledge, only one comprehensive Wnt screening is reported in the literature with 19 Wnts included [12]. The study found that 12 Wnt ligands activated canonical Wnt signaling in a luciferase assay, most active Wnts being Wnt1 (450-fold), Wnt3/3a (120-fold), Wnt7b (59-fold), Wnt9b (59-fold), and Wnt2 (49-fold). When Wnt ligands with C-terminal V5-tag were used in luciferase assay, the activation was completely abolished for all Wnts [12]. This indicates that tagging can interfere with downstream signaling of Wnts and could explain why no other ligands than Wnt3a activated canonical Wnt signaling in this study where V5-tagged Wnts were used (Figure 15). Thus, it would be interesting to repeat the luciferase assay with untagged Wnts in the future, especially since it would not add any inconvenience to the workflow of the assay (compared to western blot, where tagging enables detecting all Wnts simultaneously using only one antibody specific for the tag).

The luciferase assay could have also been done using recombinant Wnt proteins to replace transfections with Wnt-encoding plasmids, as was also done in some other studies [7, 41]. Recombinant Wnts could potentially yield stronger signals, since all introduced Wnt protein is present outside the cell available for receptor binding/activation, while in case of transfections, only part of the intracellularly produced Wnt is secreted in the media where

it is functionally active. However, recombinant Wnts are not often commercially available. This is because unlike many other growth factors, they are not freely soluble due to their palmitoylation, making production of stable and biologically active Wnts difficult [22].

Since this pseudokinase-induced attenuation effect suggests the activation of competing non-canonical Wnt signaling, it was studied next with selected Wnts (Wnt3a, Wnt5a, Wnt16). Parental Ba/F3 cells and Ba/F3 cells with expression of Ror1, Ror2, or Ptk7 were stimulated by recombinant Wnts to understand changes in ERK/AKT phosphorylations that serve as indicator of the non-canonical Wnt signaling. None of the tested Wnts were able to activate ERK/AKT phosphorylations in the absence of Ror1, Ror2, or Ptk7 (Figure 16a, Ba/F3). When Ror1 was expressed on the cell surface, canonical Wnt ligand Wnt3a had still no effect, which is well in line with the negative co-immunoprecipitation result between Ror1 and Wnt3a (Figure 11a). In contrast, Wnt5a and Wnt16 induced the phosphorylation of ERK and AKT in all time points (Figure 16a, Ba/F3-Ror1). With Wnt5a, phosphorylation of AKT was strongest at the first (30 min) time point and faded at later time points, while with Wnt16, the activation became stronger at later time points. However, ERK phosphorylations faded at the last time point (4h) with both Wnt5a and Wnt16.

When Ror2 or Ptk7 were expressed in Ba/F3 cells (Figure 16b), all three Wnt ligands could induce ERK phosphorylation which was strongest at first time point (30 min) and faded at later time points. However, the expression of Ror2 on the Ba/F3 cells was rather low as shown in the western blot. Especially the activation of ERK in these cells by Wnt3a was surprising, since Wnt3a is a classical canonical Wnt ligand [12] and it did not bind with these receptors in the co-immunoprecipitation assay. One explanation could be that handling of cells when setting up the experiment can itself induce ERK/AKT phosphorylation, since 0h time point samples without any Wnt additions had elevated ERK/AKT levels when the samples were immediately lysed and analysed (data not shown). After this problem was identified, 0h sample was incubated for 4 hours until the collection of last time point to give the cells time to recover from handling. Possibly, the first time points (0.5h and 2h) have not yet recovered and are ERK/AKT phosphorylated irrespective of the Wnt presence, as ERK phosphorylation is strongest in the 0.5h time point and fades towards the later time points (Figure 16b). This problem could be solved by more gentle handling of the cells and by adding an incubation/recovery period for cells after

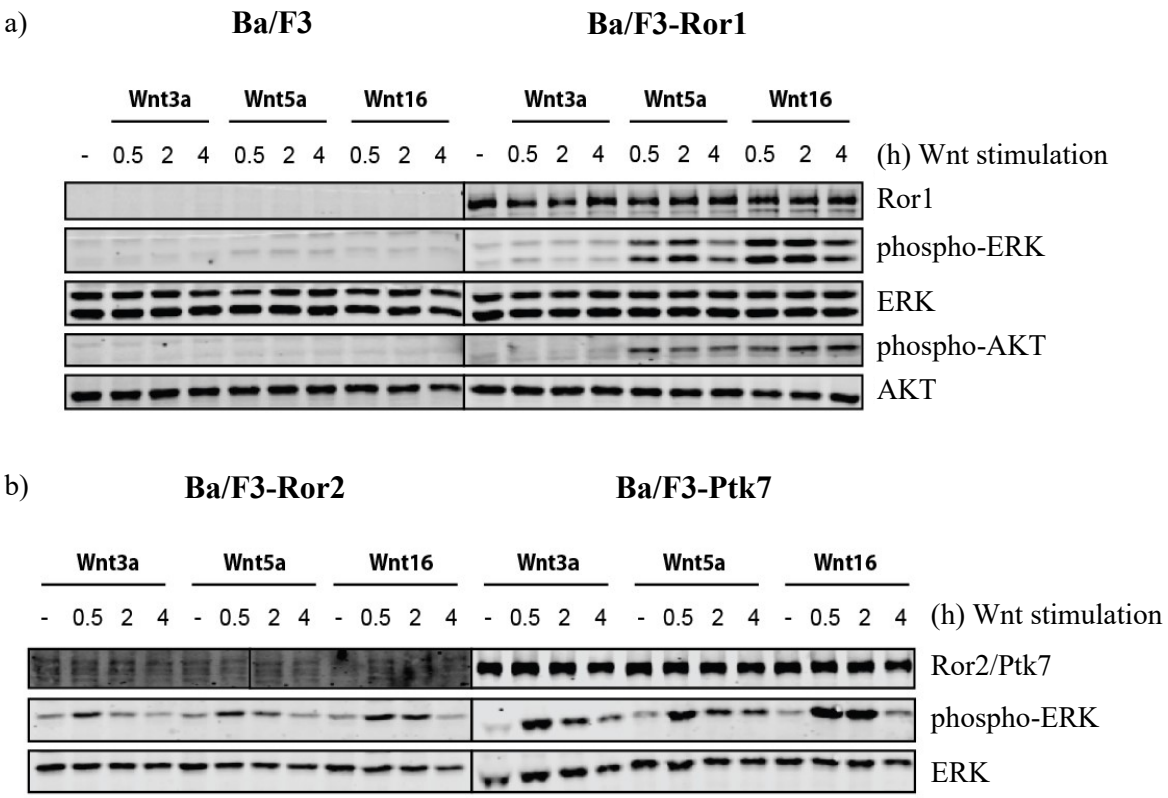

Figure 16. Non-canonical Wnt signaling in response to Wnt stimulations. Induction of ERK and AKT phosphorylations in response to stimulations by Wnt ligands (Wnt3a, Wnt5a, or Wnt16) at different timepoints (0, 0.5, 2, and 4 hours of stimulation). a) Ba/F3 cells (left side) and Ba/F3-Ror1 (right side) Wnt stimulation results. b) Ba/F3-Ror2 (left side) and Ba/F3-Ptk7 (right side) Wnt stimulation results.

setting up the experiment before adding the Wnts to the cells. In addition, it would be good to repeat the experiment with cells expressing better Ror2.

Interestingly, the expression of Ptk7 alone has been shown to activate non-canonical Wnt signaling in *Xenopus* embryos almost to similar extent as by Wnt5a expression [41]. Thus, the expression of non-canonical Wnt receptor (such as Ptk7) could be also alone sufficient to activate ERK/AKT signaling to some extent. If Wnt stimulation does not significantly increase this signaling, it does not directly indicate that the signaling pathway of interest is unresponsive to Wnt, but the signaling pathway might already be active even without Wnt because of stable expression of Ptk7 or some other relevant receptor.

Even though ERK/AKT phosphorylation was studied here as an indicator of non-canonical Wnt signaling, it is important to note that increase in ERK/AKT phosphorylation is not exclusive indicator of non-canonical Wnt signaling. For example, it has been shown

ERK/AKT mediate anti-apoptotic actions of osteoblastic cells as part of Wnt3a-induced canonical Wnt signaling [19]. ERK is required for GSK3 inactivation leading to stabilization and nuclear accumulation of β-catenin [19]. This could be one explanation why canonical Wnt3a induced the ERK/AKT phosphorylation in BaF3-Ror2 and Ba/F3-Ptk7 cells, since both Ror2 and Ptk7 can also activate canonical Wnt signaling depending on the cellular context [16, 70]. Yet another explanation could be that a "signalosome" composed of multiple components and capable of triggering Wnt3a signal is formed at the cell surface only when Ror2 or Ptk7 are expressed but is not formed in case of Ror1.

When considering the Wnt binding (Figure 11) and downstream signaling (Figures 15-16), it is important to note that results are not directly comparable between each other since different cell lines were utilized in these experiments. For example, Wnt16 did not precipitate with Ptk7 in HEK293T cells (Figure 11), but it could still activate downstream signaling in Ba/F3-Ptk7 cells (Figure 16b). Possibly Ba/F3 cells express some co-receptors required for Wnt16 binding and signaling that are not present in HEK293T cells. On the other hand, Wnt binding to receptor does not always translate into downstream signaling. This is also supported in the literature: even though both Wnt3a and Wnt5a can bind to Ror2, only Wnt5a could activate Ror2 homodimerization and non-canonical signaling in HEK293T cells [24]. In fact, even when downstream signaling is not activated, receptor binding to canonical Wnt ligands can sequester them out from the ligand pool and this way inhibit canonical Wnt signaling, mechanism proposed for both Ror2 [15] and Ptk7 [41].

Only three Wnts were included into the Wnt stimulation study due to time constrains. However, it would be interesting to conduct stimulations in future with a comprehensive set of Wnts in a similar way as was done for immunoprecipitations (Figure 11) and luciferase assay (Figure 15). Non-canonical Wnt pathway could be also studied with luciferase assay using reporter plasmids specific for non-canonical Wnt signaling. cJun and NFAT plasmids are commercially available luciferase reporters for PCP pathway and Wnt/Ca^{2+} pathway, respectively. With these reporters, receptors Ror1, Ror2, and Ptk7 would likely enhance the luciferase assay activity, in contrast to inhibition with TCF/LEF reporter plasmid (canonical Wnt pathway) shown in this study.

It is important to note that the results presented in this Master thesis are preliminary. To improve the reliability of immunoprecipitation and Wnt stimulation studies, these experiments should also be repeated at least three times as was done with luciferase assay and preferably also in different cell lines. Nevertheless, the experiments shown in this thesis provide an important starting point for further investigations and validations by other research groups.

6 CONCLUSION

Non-canonical Wnt signaling is complex and currently inadequately characterized despite its role in many malignancies. To answer this need, pseudokinase receptors Ror1, Ror2, and Ptk7 belonging to non-canonical Wnt signaling were characterized for their Wnt ligands and downstream signaling. 15 out of 19 currently known human Wnt ligands [2] were included in the study and showed highly overlapping binding profiles as well as downstream signaling functions. Ror1, Ror2, and Ptk7 precipitated with several Wnt ligands, even though some degree of specificity for certain Wnts was existing. The more detailed domain-level analysis showed that neither CRD nor KR domains of Ror1 can be removed without fully abrogating the Wnt-binding capacity. Out of 15 Wnt ligands overexpressed in HEK293T cells, Wnt3a was the only ligand that could induce canonical Wnt signaling, which was attenuated by Ror1, Ror2, and Ptk7, indicating activation of the competing non-canonical Wnt signaling by these receptors.

Importantly, the results of his work are cell line specific. Despite these results are preliminary, they show well the bigger picture of the highly complex and overlapping signaling network where the linear view of ligand-receptor pair leading to a certain signaling outcome is not applicable. Rather, many different factors, some of which are likely unknown, play a role in what is the final signaling result of ligand-receptor pair. The clinical relevance of the non-canonical Wnt signaling makes it important to better identify and understand these factors and the signaling networks and hopefully utilize this information more efficiently in the future for developing new targeted therapies against cancers and other diseases.

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8 APPENDICES

Supplementary Table 1. Expression levels of each Wnt ligand in both lysate (cells) and in the media after overexpression of each Wnt in HEK293T cells for 68 hours. Media results normalized by multiplying by 7.5 to make Wnt concentrations comparable between lysate (0.2 ml) and media (1.9 ml). Secretion-% of each Wnt calculated by dividing the Wnt expression in the lysate by normalized expression in the media.

Ligand	Lysate	Media	Media normalized	Secretion-%
Wnt1	15000	498	3735	20
Wnt2	3790	21	157.5	4
Wnt2b	5580	151	1132.5	17
Wnt3a	3340	235	1762.5	35
Wnt4	2160	210	1575	42
Wnt5a	3280	98.7	740.25	18
Wnt5b	4730	147	1102.5	19
Wnt6	5100	111	832.5	14
Wnt7a	18700	2530	18975	50
Wnt8a	3450	270	2025	37
Wnt9a	12200	733	5497.5	31
Wnt9b	548	36.4	273	33
Wnt10	9180	222	1665	15
Wnt11	11700	887	6652.5	36
Wnt16	4390	119	892.5	17