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MECHANISMS OF WNT SIGNALING: FROM EMBRYONIC STEM CELLS TO DOPAMINERGIC NEURONS

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ABSTRACT

The ability of a cell to respond in a specific way to certain signals represents key biological phenomena governing development of multicellular organism. Cellular signaling regulates all aspects of cell biology such as proliferation, migration, differentiation, and death. Detailed understanding of mechanisms by which various signals are interpreted into certain cellular responses is crucial in order to efficiently manipulate these processes. Guiding a stem cell via specific cues to a cell type of interest, such as dopaminergic (DA) neurons, is a necessary prerequisite for cell replacement therapy (CRT) of diseases, such as Parkinson's disease (PD), where DA neurons are progressively lost. This thesis examines molecular mechanisms of action of Wnts, a group of factors providing such cues, and their functional role in midbrain development and DA neuron differentiation.

In our first study we manipulated Wnt/β-catenin signaling pathway in mouse embryonic stem cells (mESCs) to analyze its impact on mESC differentiation into DA neurons. We show that pathway impairment at the ligand (Wnt1) or receptor (LRP6) level enhances neuronal and DA differentiation of mESCs. Similarly, application of Dkk1 (Wnt/β-catenin pathway inhibitor) also increased the yield of mESC-derived DA neurons. Combined, our data demonstrate that Wnt1 and LRP6 are dispensable for mESC DA differentiation, that mESC differentiation into DA neurons is facilitated by attenuated Wnt/β-catenin signaling, and that inhibitors of Wnt/β-catenin pathway can be used to increase efficiency of DA differentiation protocols.

Earlier reports from our lab demonstrated enhancement of DA differentiation by Wnt5a, an activator of Wnt/β-catenin-independent pathways in DA cells. Thus, we focused on mechanisms of Wnt/β-catenin-independent signaling and its functional aspects in our following studies, as these were not elucidated before this thesis. We show by analyses of Wnt5a -/- mice embryos the importance of Wnt5a for proper midbrain morphogenesis. Moreover, absence of Wnt5a led to increase in proliferation of DA progenitors, accumulation of Nurr1+ precursors and attenuated differentiation of these precursors into TH+ DA neurons.

To characterize Wnt5a-mediated effect on DA differentiation we analyzed possible activation of putative downstream pathway components. We demonstrate that Wnt5a effects on DA differentiation are mediated via small GTPase Rac1, which is a

downstream effector of Wnt5a/Dvl signaling in DA cells. Subsequently, we examined molecular aspects of the Wnt5a/Dvl/Rac signaling in closer detail. We demonstrate that β-arrestin is a crucial component of Wnt5a/Dvl/Rac signaling route and we show its critical role in regulation of CE movements during *Xenopus* gastrulation. Moreover, we found that specification of Wnt-mediated signaling at the level of Dvl is further controlled by phosphorylation of Dvl by casein kinases CK1 and CK2. Therefore, CK1 and CK2 act as switches between distinct branches of Wnt/β-catenin-independent signaling. Next, to get further insight into Wnt5a/Dvl-mediated activation of Rac1 we analyzed the Dvl-Rac1 interaction and performed a proteomic screen for Dvl-binding regulators of Rac1 activity. We show that Dvl and Rac1 form a complex, and the N-terminal part of Dvl mediates this interaction. Further, we demonstrate that Tiam1, a novel Dvl-binding partner found in our study, is required both for Rac1 activation in the Wnt5a/Dvl/Rac signaling branch and for DA neuron differentiation. Collectively, we identified β-arrestin, CK1, CK2, and Tiam1 as novel regulators of Wnt5a-induced signaling.

In sum, data in the presented thesis describes molecular mechanisms and functional consequences of Wnt-driven signaling pathways and pinpoints the modulation of Wnt signaling as a possible tool to improve PD therapies.

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TABLE OF CONTENTS

1 INTRUDUCTION 1

1.1 Context: Parkinson's disease and regenerative medicine 1

1.2 Embryonic stem cells 3

1.2.1 Regulation of pluripotency 3

1.3 Embryogenesis: from zygote to midbrain and DA neurons 5

1.3.1 Early embryogenesis and gastrulation 5

1.3.1.1 Patterning the embryo by morphogens 6

1.3.1.2 Neural induction 6

1.3.1.3 Neurulation 7

1.3.2 Midbrain and DA neuron development 7

1.3.2.1 Patterning of midbrain by Shh and FGF8 7

1.3.2.2 Wnts in VM development and DA differentiation 9

1.3.2.3 Intrinsic factors regulate DA neuron development and differentiation 11

1.3.2.4 Maturation and function of DA neurons 12

1.3.2.5 How to get DA neurons from ESCs 12

1.4 Wnt signaling: mechanisms and consequences 13

1.4.1 Wnt ligands 14

1.4.2 Specificity in Wnt signaling 15

1.4.3 Wnt/β-catenin signaling pathway 15

1.4.4 PCP and Wnt/PCP pathways 17

1.4.4.1 PCP signaling 18

1.4.4.1.1 Wnt/Dvl/RhoA signaling 19

1.4.4.1.2 Wnt/Dvl/Rac signaling 19

1.4.4.1.3 Wnt/Cdc42 signaling 21

1.4.5 Wnt/Ca^{2+}signaling pathway 21

1.4.6 Wnt receptors 22

1.4.6.1 Frizzleds 22

1.4.6.2 LRPs 24

1.4.6.3 Ror receptors 25

1.4.6.4 Ryk receptor 26

1.4.7 Soluble modulators of Wnt signaling 27

1.4.8 Dishevelled 28

1.4.9 Casein kinases 30

1.4.9.1 CK1 30

1.4.9.2 CK2 31

1.4.10 β-arrestin 31

1.4.11 Downstream effectors: Rho family of small GTPases 32

1.4.11.1 Regulation and function 32

1.4.11.2 Rac small GTPases 34

1.4.11.2.1 Tiam1-a Rac1 GEF 35

1.4.12 Wnt pathways crosstalk 36

2 AIMS 38

3 RESULTS AND DISCUSSION 39

3.1 Paper I 39

3.1.1 Absence of Wnt1 or LRP6 increases neuronal differentiation and number of DA neurons in mESC cultures 39

3.1.2 Increased generation of DA neurons is not due to factors produced by feeder cells 40

3.1.3 Attenuation of Wnt/β-catenin pathway increases the yield of mESC-derived DA neurons 41

3.1.4 How can decreased Wnt/β-catenin signaling contribute to neuronal and DA differentiation? 41

3.2 Prelude to Paper II, III, and IV 43

3.3 Paper II 44

3.3.1 Analyses of Wnt5a -/- mice 44

3.3.2 Wnt5a activates small GTPase Rac1, activity of which is required for Wnt5a-induced DA differentiation in primary culture 45

3.3.3 Possible mechanisms of Wnt5a signaling in the developing VM 45

3.3.4 Is Rac1 Wnt5a effector *in vivo*? 47

3.4 Paper III 47

3.4.1 β-arrestin is a novel component of the Wnt/Dvl/Rac signaling route 48

3.4.2 Multiple roles of β-arrestin-which of them matters for the Wnt/Dvl/Rac signaling? 49

3.4.3 Roles of CK1 and CK2 in Wnt-driven signaling pathways 50

3.5 Paper IV 52

3.5.1 Search for Rac1 activators 52

3.5.2 Tiam1, a Rac1 GEF, is expressed in developing VM and interacts with Dvl 53

3.5.3 Functional aspects of Tiam1 in the Wnt/Dvl/Rac signaling and DA differentiation 54

4 CONCLUSIONS 57

5 ACKNOWLEDGEMENTS 58

6 REFERENCES 59

LIST OF ABBREVIATIONS

APC	Adenomatous polyposis coli
BDNF	Brain-derived neurothrophic factor
BIO	6-bromo-indirubin-39-oxime
BMP	Bone morphogenetic protein
β-TrCP	Beta-transducin repeat containing protein
CamKII	Calcium/calmodulin-dependent protein kinase II
Cdc42	Cell division cycle 42
CE	Convergence and extension
Celsr1	Cadherin, EGF LAG sevel pass G-type receptor 1
cGMP	Cyclic guanosine monophosphate
CK	Casein kinase
CM	Conditioned medium
CNS	Central nervous system
CRD	Cysteine rich domain
CRT	Cell replacement therapy
DA	Dopaminergic
Daam1	Dishevelled associated activator of mophogenesis 1
DAG	Diacylglycerol
DAT	Dopamine transporter
DEP	Dishevelled., EGL-10. pleckstrin
DH	Dbl homology
DIX	Dishevelled, axin
Dkk	Dickkopf
Dvl or Dsh	Dishevelled
E	Embryonic day
EGF	Epidermal growth factor
ESC (m/h)	Embryonic stem cell (mouse/human)
FGF	Fibroblast growth factor
Foxa2	Forkhead box A2
FRET	Fluorescence resonance energy transfer
GAP	GTPase-activating protein
Gbx2	Gastrulation brain homeobox 2
GDI	Guanine nucleotide exchange inhibitor
GDNF	Glial cell derived neurothrophic factor
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GOF	Gain of function
GPCR	G-protein coupled receptor
GSK3	Glycogen synthase kinase 3
GTP	Guanosine triphosphate
ICM	Inner cell mass
Id	Inhibitor of differentiation
Ig	Immunoglobulin
IP3	Inositol triphosphate

iPSC	Induced pluripotent stem cells
ISH	In situ hybridization
JAK	Janus kinase
JNK	cJUN N-terminal kinase
L-DOPA	Levodopa (L-dihydroxyphenylalanine)
LIF	Leukemia inhibitory factor
Lmx1a/b	LIM homeobox transcription factor 1a/b
LOF	Loss of function
LRP	Low density lipoprotein receptor
Mash1	Mammalian achaete-scute complex homolog 1
MEF	Mouse embryonic fibroblast
MMTV	Mouse mammary tumor virus
Msx1	Msh homeobox homolog 1
mTOR	Mammalian target of rapamycin
NFAT	Nuclear factor of activated T-cells
NGF	Nerve growth factor
Ngn	Neurogenin
Nurr1	Nuclear receptor-related protein 1
Oct3/4	Octamer binding transcription factor 3/4
Otx2	Orthodenticle homeobox 2
Pax	Paired box
PCP	Plannar cell polarity
PD	Parkinson’s disease
PDZ	PSD95, DlgA, zo-1
PDGF	Platelet-derived growth factor
PH	Pleckstrin homology
Pitx3	Paired-like homeodomain 3
PKC	Protein kinase C
Q-PCR	Quantitative polymerase chain reaction
RA	Retinoid acid
Rac	Ras-related C3 botulinum toxin substrate
Rap	Ras-related protein
RhoA	Ras homolog gene family, member A
ROCK	Rho-associated, coiled coil containing protein kinase
Ror	Receptor tyrosine kine-like orphan receptor protein
Ryk	Related to protein kinase
RTK	Receptor tyrosine kinase
SFRP	Secreted Frizzled-related protein
Shh	Sonic hedgehog
SMAD	Similar to mothers decapentaplegic homolog
SN	Substantia nigra
Sox2	Sry (sex determining region) box 2
STAT3	Signal transducer and activator of transcription 3
TCF/LEF	T-cell specific transcription factor/ Lymphoid enhancer factor
TGF	Transforming growth factor
Tiam1	T-cell lymphoma invasion and metastasis 1
TH	Tyrosine hydroxylase
Tuj	Tubulin beta 3

Vangl	Vang-like protein (van gogh, Drosophila)
VM	Ventral midbrain
VTA	Ventral tegmental area
WIF	Wnt inhibitory factor
Wnt	Wingless-type MMTV integration site family

1 INTRUDUCTION

1.1 CONTEXT: PARKINSON'S DISEASE AND REGENERATIVE MEDICINE

Parkinson's disease (PD) is the second most common neurodegenerative disease; current demographic trends predict a doubling in the number of cases by 2050 (Schapira, 2009). PD is diagnosed by the onset of motor manifestations such as tremor, rigidity, hypokinesia, and gait and balance impairment. Recent evidence suggests that PD may have a pre-motor phase, associated with depression and/or sleep abnormalities as most common signs (Langston, 2006). As the disease progresses, other non-motor symptoms may appear, including various cognitive impairments and dementia.

At a pathological level, PD is characterized by a progressive loss of substantia nigra (SN) dopaminergic (DA) neurons innervating the striatum. Loss of these striatal connections is causative of the motor symptoms of the disease. However, as the appearance of further symptoms suggests, other neuronal populations in the peripheral and central nervous system are also affected in later phases of the PD.

The cause of PD is largely unknown, with 95% of the cases being sporadic. In addition to the mitochondrial dysfunctions, which were proposed as contributing factor leading to death of DA neurons (Schapira et al., 1989), there is evidence of free-radical-mediated damage to proteins and lipids in the SN (Owen et al., 1996), impairment of ubiquitin-proteasome system (Olanow and McNaught, 2006), and inflammatory changes contributing to the pathology of PD. Recent studies also proposed involvement of prion-like mechanisms in progression and/or spreading of the disease (Angot et al., 2010). Together, all this evidence suggests that PD is rather a syndrome with different underlining causative factors leading to common endpoint, death of DA neurons (Langston, 2006; Obeso et al., 2010). Furthermore, it is also not known to which extent the degeneration of SN DA neurons precedes the onset of PD symptoms. It is estimated that about 70-80% dopamine depletion is necessary for the classical PD signs to manifest (Schapira, 2009).

Currently, treatment of PD patients is entirely symptomatic, mainly utilizing application of L-DOPA (precursor of dopamine) to replenish the dopamine levels. Alternatively, surgical treatment of PD is based on the use of deep brain electrical stimulation. While these treatments provide symptomatic relief, they do not affect the

course of the disease as such. Therefore, strategies aiming at either preventing the progressive cell loss or restoring the pool of dopamine producing cells are of a great interest in context of regenerative medicine (Arenas, 2010).

Cell replacement therapy (CRT) represents one of the most attractive avenues for restoration of striatal levels of dopamine with one-time only treatment (Arenas, 2010; Correia et al., 2005; Hedlund and Perlmann, 2009; Parish and Arenas, 2007). Transplantation of fetal midbrain tissue has been demonstrated as viable therapeutic alternative for the treatment of PD (Lindvall et al., 1989; Piccini et al., 1999). Nonetheless, apart from the obvious ethical issue of using aborted human fetuses (6-8 per one patient), this therapeutic approach is limited by the poor survival and integration of transplanted progenitors and/or neurons, and the causing diskinesia. These complications have been mainly attributed to poor standardization of the transplanted material and the presence of undesired cell types (Hagell et al., 2002; Olanow et al., 2003).

Stem cells, either embryonic stem cells (ESC) or induced pluripotent stem cells (iPSC) obtained by reprogramming of more differentiated cell types (Takahashi et al., 2007) are an attractive alternative source of cells for the purpose of CRT. Compared to primary fetal cells, less starting material would be required, as these cells can be expanded in culture and directed to differentiate into the cell type of interest (DA neurons).

In order for ESC-derived DA neurons to induce a functional recovery following the stem cell-based CRT, it is necessary to obtain sufficient number of correctly specified DA neurons using highly efficient protocols for DA differentiation. Moreover, these neurons have to survive and create functional connections with host cells upon transplantation. Efforts from many researchers have thus been devoted to study the development, differentiation, and survival of midbrain DA neurons. Understanding the mechanisms controlling those processes is critical for establishing such protocols and thereby a necessary prerequisite for successful implementation of the CRT in the future. In this regard, mouse models and mouse primary cells have been widely used in order to elucidate the role of both intrinsic and extrinsic factors in DA neuron development and differentiation. For this purpose, mouse ESCs (mESCs) represent a valuable tool since they allow to study the development and differentiation of DA neurons, as well as to screen for novel regulators and/or optimal differentiation protocols that will ultimately be applied in hESCs/iPSCs (Niwa, 2010).

Among the factors important for the development of midbrain DA neurons, but still not fully understood in terms of their mechanisms of action, are Wnts. Work in this thesis focuses on characterization of molecular mechanisms and functional consequences of signaling events mediated by Wnts that are relevant to midbrain DA neurons.

1.2 EMBRYONIC STEM CELLS

Successful derivation of mESC lines from the inner cell mass (ICM) of mouse pre-implantation blastocyst was reported already 30 years ago (Evans and Kaufman, 1981; Martin, 1981). ESCs are undifferentiated cells capable of self-renewing (copying themselves). Since their discovery, mESCs have been immediately recognized as tools to address mechanisms underlying the proces of differentiation (giving rise to more specialized progeny). Analysis of chimeric mouse embryos produced by the injection of ICM cells and mESCs into blastocysts, has shown that ICM cells/mESCs are able to give rise to cell progeny of any of the three germ layers: ectoderm, endoderm, and mesoderm but do not contribute to the trophectoderm lineage (Niwa, 2007; Solter, 2006). mESCs are therefore pluripotent. Only totipotent cells give rise to all cell types including trophectoderm. Moreover, ESCs can give rise to germ cells in chimaeras and these germ cells should in turn be able to give rise to normal, fertile adults (Solter, 2006). This, for obvious ethical reasons, has been demonstrated only for mESCs (Bradley et al., 1984; Nagy et al., 1993), and not for hESCs. Importantly, the ability of mESCs to give germ line transmission enabled transgenesis and, in combination with another powerful approach - gene targeting by homologous recombination - turned mESCs into tools to study gene function, a technology which was recognized by a Nobel Prize in 2007.

Apart from ESCs, tissue-specific stem cells have a more restricted potential and give rise to a more limited progeny. Such cells are thus often referred to as multi/oligo/bi-potent and have been described during embryogenesis and postnataly *in vivo* and/or expanded *in vitro* (Falk and Frisen, 2005; Li and Xie, 2005; Slack, 2008; Wagers et al., 2002).

1.2.1 Regulation of pluripotency

Self-renewal, the ability of both ESCs and tissue-specific stem cells to go through numerous cycles of cell division while maintaining their undifferentiated status, the so

called “stemness” (Boiani and Scholer, 2005; Niwa, 2007; Solter, 2006), is a property that has attracted well-deserved attention of many researchers. Stemness of mESCs is maintained during self-renewal by the inhibition of differentiation promoting signals and by promotion of proliferation (Lanner and Rossant, 2010; Niwa, 2007; Silva et al., 2008; Wray et al., 2010; Ying et al., 2008). Leukemia inhibitory factor (LIF) has been identified as a critical factor for the long-term self-renewal of mESCs via activation of JAK/STAT3 signaling (Nichols et al., 2001; Smith et al., 1988; Williams et al., 1988). Moreover, BMP4 (bone morphogenetic protein 4) has been shown to contribute to the LIF signaling cascade, enhancing the self-renewal of mESCs by activating the SMAD4/Id pathway (Ying et al., 2003a). However, BMP4-mediated signaling is not sufficient to maintain mESCs undifferentiated in the absence of LIF. Further, compound 6-bromo-indirubin-39-oxime (BIO), an inhibitor of GSK3 (glycogen synthase kinase 3) has been reported to sustain the undifferentiated state of both mESCs and hESCs (Sato et al., 2004). GSK3 is negative regulator of Wnt/β-catenin pathway (see chapters 1.4.2 and 1.4.3) and is also involved in many other processes, including the regulation of cell metabolism (Doble and Woodgett, 2003). Therefore, it is not clear which of the functions of GSK3, if any, were responsible for observed effects, as the use of other GSK3 inhibitors failed to prevent mESCs differentiation in the absence of LIF (Wray et al., 2010; Ying et al., 2008). However, the Wnt/β-catenin pathway has been shown to promote the stemness of mESCs in a manner similar to that of BMP4, by synergizing with/depending on LIF-driven signaling (Anton et al., 2007; Lee et al., 2009; Miyabayashi et al., 2007; Ogawa et al., 2006).

Activation of the above mentioned signaling pathways results in the transcriptional induction or repression of genes that are responsible for implementing stem-cell pluripotency. The core circuitry of pluripotency-associated transcription factors consists of Oct3/4 (Nichols et al., 1998; Niwa et al., 2000; Scholer et al., 1989), Sox2 (Yuan et al., 1995), and Nanog (Chambers et al., 2003; Mitsui et al., 2003). Published evidence suggests that none of those transcription factors acts as “the master gene of pluripotency”. It is their coordinated action and mutual regulation that is responsible for maintaining of the undifferentiated state of mESCs (Boiani and Scholer, 2005). Moreover, it is becoming obvious that many other genes, apart from those already mentioned (STAT3, Nanog, Oct3/4, and Sox2), are connected to the circuitry of pluripotency-regulating genes and are therefore important for the maintenance of the undifferentiated state of mESCs (Cole et al., 2008; Guo and Smith, 2010; Niwa et al., 2009).

1.3 EMBRYOGENESIS: FROM ZYGOTE TO MIDBRAIN AND DA NEURONS

1.3.1 Early embryogenesis and gastrulation

Vertebrate embryogenesis represents a remarkable process where proliferation, cell fate specification, and cell migration are exquisitely orchestrated in order to give rise to a highly organized embryo from a single cell zygote. This process involves an ordered series of lineage specifications and cell movements that first result in the development of a morula, then a blastocyst, and, later, in the formation of the embryo itself, with determined anterior-posterior, dorsal-ventral, and medial-lateral axes. At the blastocyst stage, the cells of the ICM segregate into two layers - epiblast and hypoblast (also called primitive endoderm). The former gives rise to all embryonic tissues, the latter, together with trophoblast cells, gives rise to extraembryonic tissues (Rossant and Tam, 2009; Zernicka-Goetz, 2002). During induction of the germ layers (ectoderm, mesoderm, and endoderm), the rather unstructured early embryo (blastula) is transformed by gastrulation movements into a multilayered embryo (gastrula). Several types of morphogenetic movements are involved in vertebrate gastrulation: internalization, epiboly, convergence and extension. Gastrulation starts by internalization of cells of future mesoderm and endoderm beneath the prospective ectoderm via opening in the blastula, blastopore (termed primitive streak in mammals), thus allowing a new layers of cells to form. Epiboly movements lead to spreading and thinning, while simultaneous convergence and extension (CE) movements narrow the newly formed germ layers medio-laterally, therefore cause elongation of the embryo/tissue along the rostral-caudal axis (Solnica-Krezel, 2005)(Figure 1). Crucial regulators of the CE movements are the Wnt/β-catenin-independent pathways (Roszko et al., 2009; Veeman et al., 2003a) (see chapter 1.4.2).

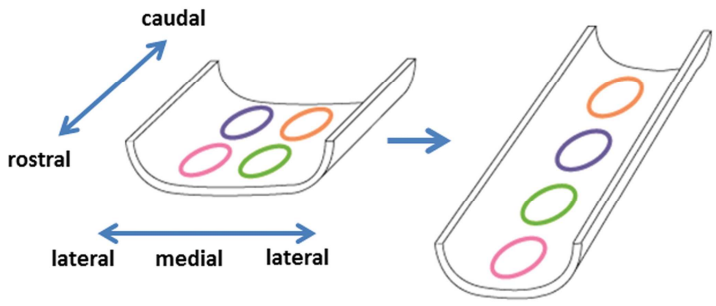

Figure 1: CE movements illustrated on an example of forming neural tube. CE movements within the neural tube result in narrower and longer neural tube.

1.3.1.1 Patterning the embryo by morphogens

Induction and specification of the germ layers to produce specific tissues and organs take place before and during gastrulation, as the mesodermal and endodermal precursors migrate through the blastopore/primitive streak to their destinations. Specification of cell fate is accomplished by signaling molecules (morphogens) forming gradients along anterior-posterior, dorsal-ventral, and/or medial-lateral axes. Gradients of morphogens are interpreted by activation of transcription of specific sets of genes, which then determine the spatial and temporal position of cell(s) within the embryo or organ/tissue and the acquisition of specific cell fate. These inductive processes are controlled by signaling centers (organizers) producing such morfogens (e.g. Shh, TGF-β, FGF, RA, and Wnt) during development of the embryo (De Robertis et al., 2000).

ESCs also offer a system to study these processes *in vitro* in cell culture. When grown in suspension, in the absence of LIF, mESCs are able to spontaneously differentiate and form spheroid aggregates. As those mimicked postimplantation embryonic tissues, they were termed embryoid bodies (Doetschman et al., 1985). More importantly, embryoid bodies have been shown to recapitulate key events of gastrulation, such as primitive streak formation, establishment of gradients of morphogens, and formation of cell lineages of all three germ layers (Desbaillets et al., 2000; ten Berge et al., 2008).

1.3.1.2 Neural induction

The ectodermal region of the embryo gives rise to neuroectoderm and epidermis. It is well accepted that inhibition of BMP pathway is required for acquisition of neural fate (neural induction) in frog, while BMP activity promotes formation of epidermis (Hemmati-Brivanlou and Melton, 1997; Stern, 2005). Further, activation of Wnt/β-catenin pathway is required at very early stages for dorsalisation of the *Xenopus* embryo and formation of the Spemann organizer (McMahon and Moon, 1989; Niehrs, 2001; Sokol et al., 1991; Tao et al., 2005). The Spemann organizer is critical for the initiation of gastrulation, formation of body axis and consequently for the formation of neural tissue (Baker et al., 1999; De Robertis et al., 2000; Stern, 2006). The Spemann organizer produces inhibitors of BMP and Wnt signaling pathways (e.g. Noggin, Chordin, and Dkk), which are required for specification of head neuroectoderm (del

Barco Barrantes et al., 2003; Niehrs, 2001). Therefore, it seems that the Wnt/β-catenin signaling pathway has to be later inhibited for the efficient neural induction (Heeg-Truesdell and LaBonne, 2006). Further evidence suggests that a similar model applies also to neural induction in mammals (Gaulden and Reiter, 2008; Levine and Brivanlou, 2007). Moreover, results obtained from mESC differentiation experiments have further supported the negative role of BMP and Wnt in neural induction/specification in culture. In this case, efficiency of acquisition of neural fate is enhanced by serum-free conditions (serum used in culture media contains BMP), low cell density (dilution of soluble signaling factors produced by cells), and application of BMP and/or Wnt inhibitors (Aubert et al., 2002; Haegele et al., 2003; Chambers et al., 2009; Smukler et al., 2006; ten Berge et al., 2008; Tropepe et al., 2001; Verani et al., 2007).

1.3.1.3 Neurulation

As gastrulation proceeds, the neuroectoderm forms a neural plate, which subsequently starts to develop folds at the junctions with the non-neural ectoderm (prospective epidermis). These folds elevate, come into contact and subsequently fuse, thereby giving rise to the neural tube. It is not surprising that this rather complex process is underpinned by changes in cell polarity and by morphogenetic CE movements. Importantly, both the cell polarity and the CE movements are under control of Wnt/β-catenin-independent pathways (Copp et al., 2003; Montcouquiol et al., 2006).
Following neurulation, the forming CNS is specified by gradients of morphogens along its rostral-caudal as well as dorsal-ventral axis. This allows differentiation of the neural tube into forebrain, midbrain, hindbrain, and spinal cord, together with acquisition of many specialized neuronal fates based on both the instructive and positional information provided (Edlund and Jessell, 1999; Jessell et al., 1989).

1.3.2 Midbrain and DA neuron development

1.3.2.1 Patterning of midbrain by Shh and FGF8

Organization of the midbrain, specification of progenitors and their subsequent differentiation into specialized neuronal subtypes, such as ventral midbrain (VM) dopaminergic (DA) neurons, are under orchestrated action of extrinsic signaling clues and intrinsic fate determinants. Two main organizing centers, the isthmic organizer at

the midbrain-hindbrain boundary, and the floor plate at the midline of the neural tube, instruct together the fate of neural progenitors at the intersection of their “spheres of influence”(Hynes and Rosenthal, 1999). The floor plate provides signals that specify midbrain progenitors along dorsal-ventral axis and it is also the place where VM DA neurons are born (Bonilla et al., 2008; Kittappa et al., 2007; Ono et al., 2007). It has been experimentally demonstrated that the floor plate tissue is capable of inducing ectopic DA neurons in dorsal midbrain (Hynes et al., 1995b), and that secreted Sonic hedgehog (Shh) mediates this inductive activity (Hynes et al., 1995a; Wang et al., 1995). Mice lacking Shh fail to develop any VM DA neurons, which underscores the important role of Shh signaling in dorsal-ventral patterning of the neural tube and regulation of progenitor proliferation (Agarwala et al., 2001; Ishibashi and McMahon, 2002).

Formation of the isthmic organizer at the boundary between midbrain and hindbrain is controlled by expression of two transcriptional factors, orthodenticle homologue 2 (Otx2) in the presumptive forebrain and midbrain, and gastrulation brain homeobox 2 (Gbx2) in the region giving rise to hindbrain and spinal cord (Acampora et al., 1997; Simeone, 2000; Wassarman et al., 1997). Nonetheless, further studies have demonstrated that proper establishment of the isthmic organizer is defined not just by these two transcription factors but also by a complex genetic interaction between morphogens (Wnt1, FGF8) and additional transcriptional factors Engrailed and Pax (Bally-Cuif et al., 1995; Danielian and McMahon, 1996; Joyner, 1996; Prakash et al., 2006; Prakash and Wurst, 2006; Reifers et al., 1998; Wurst and Bally-Cuif, 2001).

Similar to the floor plate, transplantation of tissue from the midbrain-hindbrain boundary can induce ectopic formation of both the midbrain as such and VM DA neurons (Martinez et al., 1991; Wurst and Bally-Cuif, 2001; Ye et al., 1998). This activity has been attributed to the secretion of FGF8, which can mimic the effect of transplanted isthmic organizer (Martinez et al., 1999). Importantly, both Shh and FGF8 are necessary for specification of the VM and development of DA neurons. Interestingly, neither of the two factors is sufficient in absence of the other (Ye et al., 1998). Thus, integration of both anterior-posterior and dorsal-ventral signals is required (Figure 2).

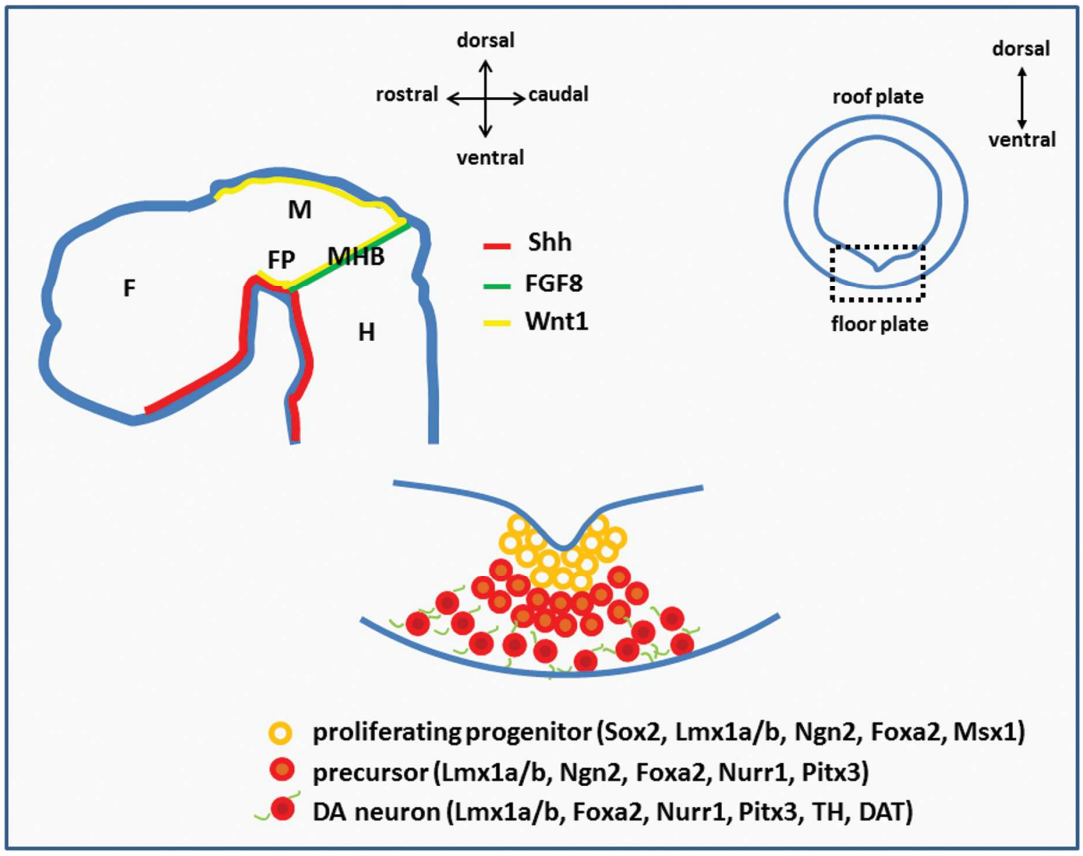

Figure 2: Development of midbrain and DA neurons. On the left: Differentiation of neural tube into forebrain (F), midbrain (M), and hindbrain (H) and specification of VM by secreted factors produced by floor plate (FP) and isthmic organizer at the midbrain-hindbrain boundary (MHB). Coronal section of neural tube in the midbrain region (to the right). The area within the dashed rectangle is magnified in the middle. Differentiation of progenitors and precursors is controlled by sets of transcription factors. Terminally differentiated DA neurons express TH and DAT. Not all DA markers are depicted.

1.3.2.2 Wnts in VM development and DA differentiation

Wnt1, a ligand from the Wnt protein family (see chapter 1.4.1) is expressed at the prospective midbrain-hindbrain boundary already at E8, and also later in the developing VM (McMahon et al., 1992; Parr et al., 1993) (see Figure 2). Wnt1 deficiency in mice leads to a deletion of most of the midbrain and a great reduction in the number of DA neurons (McMahon and Bradley, 1990; Prakash et al., 2006; Thomas and Capecchi, 1990), which has been attributed to loss of expression of Engrailed, its target gene (Danielian and McMahon, 1996). Interestingly, Wnt1 is required for the induction of ectopic DA neurons by Shh and FGF8, as Shh and FGF8 failed to do that in Wnt1 -/- background (Prakash et al., 2006). Moreover, its ectopic expression leads to both increase in proliferation and induction of ectopic DA neurons (Panhuysen et al., 2004; Prakash et al., 2006). It is thus becoming clear that Wnt1 and Wnt/β-catenin pathway,

respectively, regulates different aspects of VM development and DA differentiation (Bally-Cuif et al., 1995; Castelo-Branco et al., 2003; Joksimovic et al., 2009; Tang et al., 2009) (Figure 3). The use of partially purified Wnt1 conditioned media (CM) has increased number of VM DA neurons obtained in mouse primary culture (Castelo-Branco et al., 2003). Furthermore, analyses of mutant mice either with a point mutation in the Wnt1 gene or lacking LRP6, a receptor important for the Wnt/β-catenin pathway (see chapters 1.4.3 and 1.4.6.2), have suggested an involvement of this pathway in maintenance of the isthmic organizer (Bally-Cuif et al., 1995; Pinson et al., 2000). Conversely, expression of Wnt1 under the Engrailed1 promoter resulted in a caudal expansion of the VM DA domain and an increase in the number of DA neurons, (Prakash et al., 2006). Moreover, recent work has proposed an involvement of Wnt1 in the regulation of neurogenic potential of the VM floor plate through a genetic interaction with Shh pathway (Joksimovic et al., 2009).

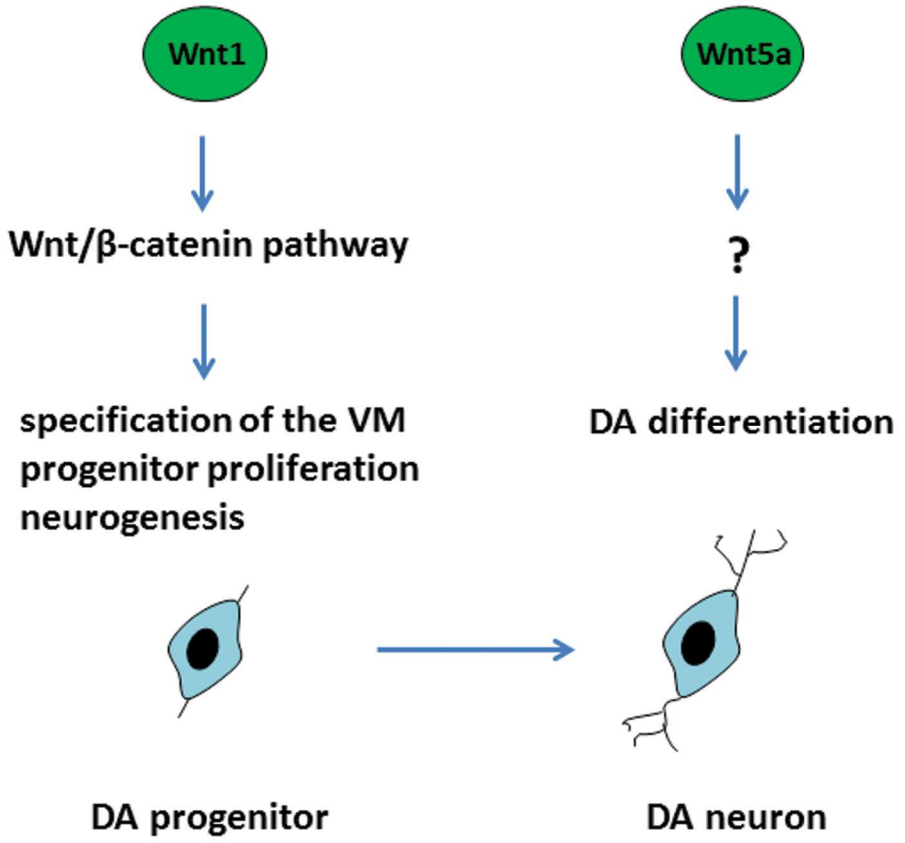

Figure 3: Brief summary of the roles of Wnt1 and Wnt5a in the development of DA neurons.

Other Wnt ligands are expressed in the developing VM at the time when DA neurons are born (Castelo-Branco et al., 2003; Rawal et al., 2006). Some of those are possibly functionally redundant to Wnt1, based on their ability to activate Wnt/β-catenin pathway (Ikeya et al., 1997; Rawal et al., 2006; Sousa et al., 2010), while others may

have distinct signaling properties. In regard of the latter, Wnt5a, which fails to activate Wnt/β-catenin signaling in DA cells (Bryja et al., 2007c; Schulte et al., 2005), promotes differentiation of precursors into DA neurons in various culture systems, including primary VM precursors, VM-derived neurospheres, and ESCs (Castelo-Branco et al., 2003; Hayashi et al., 2008; Parish et al., 2008; Sanchez-Pernaute et al., 2008; Schulte et al., 2005) (Figure 3).

1.3.2.3 Intrinsic factors regulate DA neuron development and differentiation

Genetic pathways of cell intrinsic factors are regulated, both temporally and spatially, by secreted morphogens. Moreover, complex genetic interactions including regulatory loops, activation and/or repression of transcription, as well as crosstalk at different levels between these genetic pathways takes place to ensure correct fate specification of progenitors and precursors, and their proper differentiation into mature VM DA neurons (Abeliovich and Hammond, 2007; Ang, 2006; Arenas, 2008; Smidt and Burbach, 2007; Wallen and Perlmann, 2003).

Several transcription factors have been demonstrated to be important for proliferation and/or subsequent specification of progenitor/precursor differentiation towards the DA lineage: Sox2 (Graham et al., 2003), Ngn2 (Andersson et al., 2006a; Kele et al., 2006; Thompson et al., 2006), Mash1 (Kele et al., 2006; Park et al., 2006), Msx1 (Andersson et al., 2006b), Foxa2 (Ferri et al., 2007; Kittappa et al., 2007; Lin et al., 2009), and Lmx1a/1b (Andersson et al., 2006b; Guo et al., 2007; Guo et al., 2008; Chung et al., 2009; Lin et al., 2009; Smidt et al., 2000) (Figure 2). Further, nuclear receptor Nurr1 has been demonstrated to be essential for both the development and differentiation of postmitotic DA precursors into mature VM DA neurons. As a consequence, markers of terminally differentiated DA neurons such as the enzyme critical for dopamine synthesis, tyrosine hydroxylase (TH), or the dopamine transporter (DAT) fail to be expressed in Nurr1 -/- mice (Le et al., 1999; Saucedo-Cardenas et al., 1998; Zetterstrom et al., 1997). Nurr1 directly regulates expression of many DA genes by binding to their promoter regions, in cooperation with other nuclear receptors (Sacchetti et al., 2002; Sacchetti et al., 2001; Sakurada et al., 1999) and/or other transcription factors, such as Pitx3 (Jacobs et al., 2009).

1.3.2.4 Maturation and function of DA neurons

Newly born DA neurons have to send axonal projections into their target areas and maintain them in order to function appropriately in adulthood. The majority of differentiated DA neurons born in the VM subsequently contribute to formation of the SN and the ventral tegmental area (VTA). As already mentioned, DA neurons arising from the SN project to the striatum, to regulate motor control, and their degeneration is associated with the motor symptoms of PD. Several neurotrophic factors such as BDNF and GDNF are involved in controlling the survival of both DA neurons and their projections (Hyman et al., 1991; Krieglstein, 2004; Moore et al., 1996; Sauer et al., 1995). Interestingly, homeobox transcription factor Pitx3 has been identified as a pro-survival factor critical for the SN DA neurons, but not for DA neurons located in the VTA (Hwang et al., 2003; Nunes et al., 2003; Smidt et al., 2004).

Many recent reports have proposed that some of the genes employed in specification of DA progenitors and/or their differentiation during embryogenesis are also functionally relevant for fully matured DA neurons during the adulthood, and thus their impaired function may represent another piece in the puzzle in terms of the mechanisms contributing to pathogenesis of PD (Fuchs et al., 2009; Kadkhodaei et al., 2009; Kittappa et al., 2007; Le et al., 2009).

1.3.2.5 How to get DA neurons from ESCs

Strategies to differentiate mESCs or hESCs into DA neurons aim at mimicking the conditions found during VM DA neuron development *in vivo*. Differentiation protocols usually combine efficient acquisition of neuroectodermal fate with appropriate specification of neural progenitors by morphogens, followed by differentiation of progenitors and/or precursors induced by mitogen withdrawal (Arenas, 2010; Kim et al., 2007; Perrier and Studer, 2003). The former is often achieved by attenuation of BMP and/or Wnt pathways (Aubert et al., 2002; Haegele et al., 2003; Chambers et al., 2009; Smukler et al., 2006; ten Berge et al., 2008; Tropepe et al., 2001; Verani et al., 2007). Moreover, efficiency of neural differentiation can be monitored by verification of expression of several neural markers (e.g. Sox1/2, Nestin, and Tuj). Specification of progenitors is done by the administration of morphogens, which are involved in patterning of the VM *in vivo* (e.g. Shh, FGF8), and/or the overexpression of cell intrinsic factors regulating DA neuron development (e.g. Lmx1a/b, Foxa2, Nurr1, and

Pitx3). Expression of those intrinsic regulators of VM DA fate also suggests whether or not the obtained neurons are properly specified. Further, expression of the genes involved in dopamine synthesis or transport (e.g. TH, DAT) reflects terminal differentiation into VM DA neurons.

Interestingly, co-culture systems of mESC with various feeder cells, e.g. stromal cells (Barberi et al., 2003; Kawasaki et al., 2000) or meningeal cells (Hayashi et al., 2008) have proven to be quite efficient. In fact, factors secreted by the feeder cells are, to some extent, sufficient for both neural and DA differentiation of mESCs. However, factors produced by these feeder cell lines and mediating the pro-neural and pro-DA differentiation effects are not entirely characterized. This obviously represents a drawback of this system for certain applications. Therefore, significant effort has been invested into developing feeder-free differentiation protocols with comparable or better DA neuron yield. These protocols take advantage of the use of growth factors and/or morphogenes, small molecules, or transgene expression, either in mESCs grown in monolayer or as embryoid bodies (Andersson et al., 2006b; Han et al., 2009; Kim et al., 2007; Kim et al., 2002; Lee et al., 2000; Ying et al., 2003b). DA neurons obtained in these cultures showed properties of *bona fide* VM DA neurons (expression of DA neuron markers and electrochemical properties). Moreover, mESC have been successfully used to screen for novel regulators of DA development and differentiation, and to elucidate mechanisms of their action in more accessible system of a cell culture.

1.4 WNT SIGNALING: MECHANISMS AND CONSEQUENCES

Wnt signaling governs numerous aspects of embryogenesis and adult tissue homeostasis throughout the animal kingdom (from sponges, through worms, flies, and frogs, to human) by controlling cell proliferation, polarity and migration, cell fate specification, and differentiation via several downstream pathways (Angers and Moon, 2009; Ciani and Salinas, 2005; Clevers, 2006; Inestrosa and Arenas, 2009; Lai et al., 2009; MacDonald et al., 2009; Montcouquiol et al., 2006; Strutt, 2003; van Amerongen and Nusse, 2009; Veeman et al., 2003a).

1.4.1 Wnt ligands

Wnt stands for wingless-related MMTV integration site. The wingless mutation was first described in *Drosophila* (Sharma, 1973; Sharma and Chopra, 1976), and after that the int1 oncogene was identified in mammals (Nusse et al., 1984; Nusse and Varmus, 1982). It became apparent later that they both encoded for homologs of the same gene (Rijsewijk et al., 1987). Thus, the term "Wnt" was created to designate a novel family of signaling molecules (Nusse et al., 1991).

Wnt ligands represent a family of 19 different genes in mammals. Originally they were subdivided into two groups: "canonical/Wnt1 class " Wnts (e.g. Wnt1, Wnt3a, Wnt8a/b) and "non-canonical/Wnt5a class" (e.g. Wnt4, Wnt5a, Wnt11) based on the ability of Wnts from the "canonical" class to induce morphological transformation of C57MG cells (Wong et al., 1994) and a secondary body axis in *Xenopu*s embryos (Du et al., 1995; McMahon and Moon, 1989; Sokol et al., 1991; Torres et al., 1996) by activation of the Wnt/β-catenin signaling pathway (Guger and Gumbiner, 1995; McCrea et al., 1993). On the other hand, Wnts from the "non-canonical" class, such as Wnt5a, triggered "non-canonical" signaling independently of β-catenin, thereby regulating CE movements during gastrulation (Moon et al., 1993). Further studies have demonstrated that different Wnt ligands have preferences for triggering distinct type of downstream signaling. As mentioned already, Wnt5a has been shown to activate Wnt/β-catenin-independent signaling in DA cells (Bryja et al., 2007c; Schulte et al., 2005), which is in agreement with its potential to regulate CE movements and failure to induce the axis duplication in *Xenopus* embryos. However, as also discussed in the following chapters, the capability of Wnt ligand to activate particular downstream signaling pathway seems to be very context dependent. In this regard, various reports have shown activation of the "canonical" Wnt/β-catenin signaling pathway by Wnts from "non-canonical" class (He et al., 1997; Cha et al., 2008; Cha et al., 2009; Mikels and Nusse, 2006; Tao et al., 2005) and vice versa (Habas et al., 2003; Spinsanti et al., 2008) in specific context.

Wnts are secreted proteins, their efficient secretion requires membrane protein Wntless/Evi/Sprinter (Banziger et al., 2006; Bartscherer et al., 2006; Goodman et al., 2006). Furthermore, Wnt ligands carry several posttranslational modifications (glycosylation, palmitoylation, palmitoleoylation) which make them rather hydrophobic and possibly membrane associated. These modifications are important for secretion and/or signaling capabilities of Wnts (Ching et al., 2008; Kurayoshi et al., 2007;

Takada et al., 2006; Willert et al., 2003) and account for their very poor solubility. Consequently, only a few Wnts have been purified and demonstrated to retain their ability to trigger signaling events (Mikels and Nusse, 2006; Schulte et al., 2005; Sousa et al., 2010; Willert et al., 2003). Due to this fact, most studies regarding Wnt signaling utilize either Wnt3a or Wnt5a, the first two Wnt ligands purified.

Wnts are highly hydrophobic and poorly soluble, yet they are supposed to act as morphogens (Charron and Tessier-Lavigne, 2005; Slack, 1993). Therefore, several experimental models explaining long distance transport of Wnt proteins have been proposed (Bartscherer and Boutros, 2008; Lorenowicz and Korswagen, 2009; Port and Basler, 2010). In light of recent evidence, it seems that formation of multimers, interaction with proteoglycans and/or lipoprotein carriers facilitates their long-range transport and the formation of morphogenetic gradients within a tissue (Bartscherer and Boutros, 2008; Neumann et al., 2009; Panakova et al., 2005; Yan and Lin, 2009).

1.4.2 Specificity in Wnt signaling

Wnt ligands activate several downstream signaling pathways. The character of the signaling (activation of a specific pathway) seems to be determined by: 1. specificity of Wnt ligands' interactions with various receptors in certain cellular contexts (defined by presence of specific set of receptor and by their subcellular distribution). 2. cytosolic pathway components specifically interacting with particular receptors and/or downstream pathway effectors. Wnts are known to activate three main pathways: Wnt/β-catenin pathway, Wnt/PCP pathway, and Wnt/Ca^{2+} pathway. As the latter two do not signal via β-catenin, they are together referred as Wnt/β-catenin-independent pathways.

In the next sections, key steps of each individual Wnt-driven signaling branch will be presented, and then selected pathway components will be described in greater detail.

1.4.3 Wnt/β-catenin signaling pathway

In the absence of Wnt ligand, a destruction complex is formed, consisting of glycogen synthase kinase 3 (GSK3), casein kinase 1α (CK1α), Axin and adenomatous polyposis coli (APC). CK1α and GSK3 phosphorylate β-catenin (CK1α at Ser45 and GSK3 at Ser33/37/Thr41), thus priming it for ubiquitination by β-TrCP E3 ligase and subsequent degradation by proteasome (Clevers, 2006; MacDonald et al., 2009). Interaction of Wnt

ligands with Frizzled receptors and the receptor LRP5/6 activates cytosolic protein Dishevelled (Dsh in *Drosophila*, Dvl in mammals, Dvl used as abbreviation for Dishevelled(s) in this thesis), which is recruited to the Frizzled receptor and facilitates Axin and GSK3 co-recruitment. GSK3 together with casein kinase 1γ (CK1γ) phosphorylate LRP5/6, thus promoting Axin binding to LRP5/6. Recruitment and subsequent inhibition of GSK3 together with recruitment of Axin leads to the disassembly of the destruction complex (Davidson et al., 2005; Tamai et al., 2004; Zeng et al., 2007; Zeng et al., 2005). Therefore, β-catenin level starts to increase and β-catenin is subsequently translocated into the nucleus. Increasing evidence also indicate that Wnt-induced endocytosis of the receptor complexes acts upstream of β-catenin stabilization (Cruciat et al., 2010; Yamamoto et al., 2006; Yamamoto et al., 2008).

Upon entering the nucleus, β-catenin interacts with transcription factors TCF/LEF; either by replacing co-repressors bound to TCF/LEF at promoter regions or by directly binding to TCF/LEF. Together with the TCF/LEF, β-catenin regulates the expression of target genes of the Wnt/β-catenin pathway (Behrens et al., 1996; Mosimann et al., 2009) (Figure 4).

More than 100 direct target genes of Wnt/β-catenin pathway have been identified, many of those acting as critical regulators of cell cycle progression/proliferation, cell fate specification, and differentiation (Nusse, 2011).

Interestingly, the existence of a mechanism which allows the cell cycle to tune the level of Wnt/β-catenin signaling at the level of LRP5/6 phosphorylation has been recently proposed (Davidson et al., 2009). This not only strengthens the view of Wnt/β-catenin signaling pathway as regulator of proliferation, but also suggests that activation of the pathway may have different outcomes in different stages of the cell cycle.

This recent finding underscores the complexity of Wnt/β-catenin pathway regulation. Several high throughput screens have been recently performed to identify critical components of the pathway. Based on these studies it seems that many regulators of Wnt/β-catenin signaling act in cell specific context. In line with this, the number of "core components", which have been identified by multiple screens as essential for Wnt/β-catenin signal transduction, is rather small (James et al., 2009; Major et al., 2007; Major et al., 2008; Miller et al., 2009; Tang et al., 2008). Moreover, many pathways regulators are functionally redundant and fulfill their regulatory role at multiple levels within the pathway.

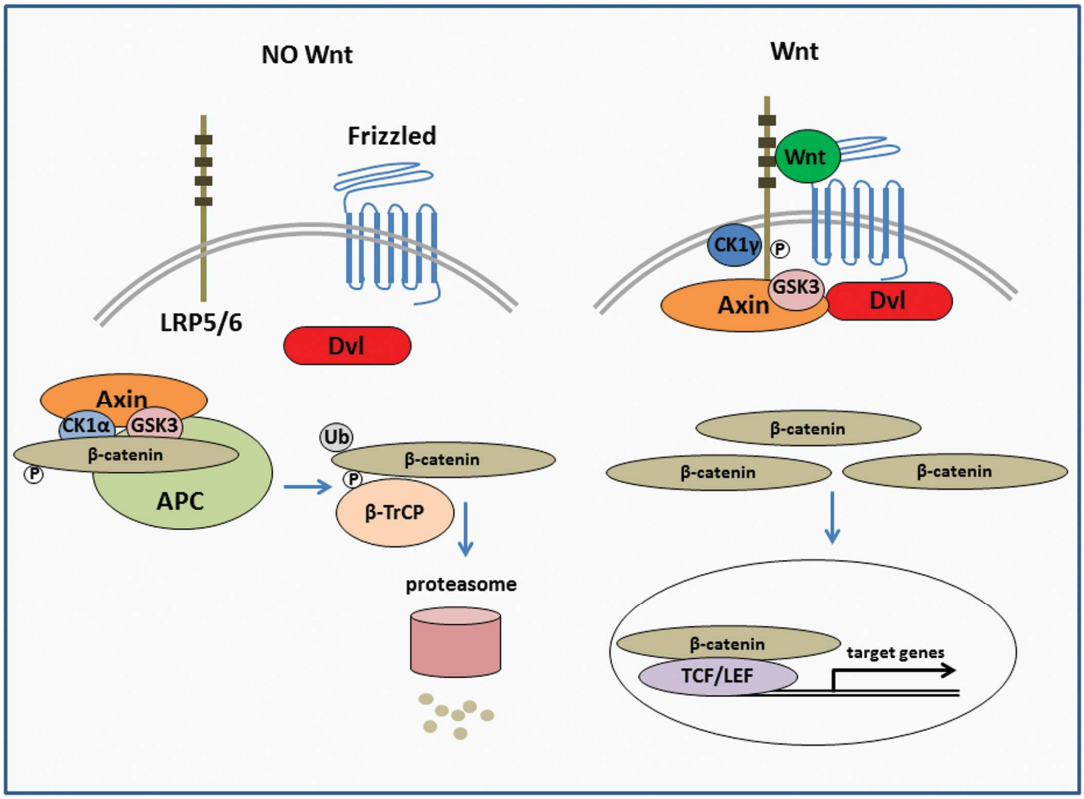

Figure 4: Wnt/β-catenin signaling pathway. In the absence of Wnt ligand, β-catenin is phosphorylated by kinases of the destruction complex, CK1α and GSK3, and subsequently degraded in the proteasome. Upon ligand binding to Frizzled and LRP5/6, Dishevelled is recruited and interacts with Frizzled. This allows phosphorylation of LRP5/6 as well as inhibition of GSK3 and disassemble of the destruction complex. β-catenin is no longer degraded and enters the nucleus where together with TCF/LEF regulates gene expression.

1.4.4 PCP and Wnt/PCP pathways

PCP (Planar cell polarity) refers to the generation of uniform orientation of individual cells within a plane of a single layered sheet of cells. In *Drosophila*, PCP signaling is implicated in regulation of the orientation of hairs on the wing and ommatidia in the eye (Seifert and Mlodzik, 2007). In vertebrates, Wnt/PCP signaling has been implicated in the regulation of CE movements, neural tube closure, and orientation of stereocilia in the inner ear (Montcouquiol et al., 2006). Importantly, while the vertebrate Wnt/PCP pathway is regulated by Wnt ligands (Habas et al., 2003; Heisenberg et al., 2000), genetic evidence suggests that the *Drosophila* PCP pathway acts in a Wnt-independent manner (James et al., 2008). Interestingly, downstream pathway components are highly conserved between *Drosophila* and vertebrates, including Frizzled, Dishevelled (Dvl), Flamingo (Celsr1 in vertebrates), Strabismus (Vangl in vertebrates), Diego (Diversin/Inversin), and Prickle. These represent the so called “core PCP components” (Seifert and Mlodzik, 2007).

1.4.4.1 PCP signaling

Evidence mainly from *Drosophila* suggests that the PCP is determined by asymmetrical localization of the core PCP components within a cell. Strabismus and Prickle forms a complex at proximal side of the cell, while Frizzled and Dvl forms a complex in the distal portion of the cell (Axelrod, 2001). Specific subcellular localization of these complexes seems to be promoted by the activities of the Strabismus and Prickle complex, which antagonize the interaction between Frizzled and Dvl, preventing the formation of Frizzled-Dvl complexes at the proximal side (Bastock et al., 2003; Strutt, 2001). Further, Diego acts as a positive regulator of the Frizzled-Dvl complex at the distal side, where it prevents the inhibitory action of the Strabismus-Prickle complex (Jenny et al., 2005). Moreover, this asymmetry in distribution of these protein complexes is further tuned by interactions with Flamingo (Seifert and Mlodzik, 2007; Shimada et al., 2001; Usui et al., 1999), as well as by Frizzled-Strabismus interactions between neighboring cells (Wu and Mlodzik, 2008). As already mentioned, this model is strongly supported by epistatic experiments from *Drosophila*, and by some functional evidence from vertebrates (Montcouquiol et al., 2003; Park and Moon, 2002; Wallingford et al., 2000). Nonetheless, the molecular basis underlying this process is largely unknown.

RhoA, Rac1, and Cdc42, small GTPases from the RhoA family, have been shown to act as effector proteins downstream of Frizzled/Dvl and/or Wnt mainly to regulate rearrangement of the cytoskeleton (Fanto et al., 2000; Habas et al., 2003; Munoz-Descalzo et al., 2007; Nishita et al., 2010; Sato et al., 2010; Schambony and Wedlich, 2007; Schlessinger et al., 2009; Schlessinger et al., 2007; Strutt et al., 1997; Witze et al., 2008). Both Rac1 and RhoA have been demonstrated to be able to compensate for PCP phenotype caused by loss of Dvl in *Drosophila* (Fanto et al., 2000; Strutt et al., 1997). Interestingly, further experiments have showed that roles of RhoA and Rac1 are not fully functionally redundant, as loss of one cannot be completely rescued by overexpression of the other (Tahinci and Symes, 2003). They seem to act in two parallel pathways, because depletion of Rac1 has no effect on Wnt or Dvl-mediated RhoA activation (Habas et al., 2003). Furthermore, stimulation of cells by various Wnt ligands has been demonstrated to trigger activation of either RhoA, or Rac1 or their combination, depending on the specific cellular context (Endo et al., 2005; Habas et al., 2003; Sato et al., 2010). Moreover, additional small GTPases (Rap, RhoB) have been proposed as downstream effectors of Wnt signaling (Tsai et al., 2007; Witze et al.,

2008). Therefore, taking into account all of the above, it seems that activation of various GTPases by Wnts is utilized via separate but interconnected signaling routes, branching at the level of Dvl or the receptor(s).

1.4.4.1.1 Wnt/Dvl/RhoA signaling

As already mentioned, RhoA has been identified as component of the Wnt/β-catenin-independent pathway downstream of Dvl. Moreover, overexpression of Dvl has been demonstrated to be sufficient to induce RhoA activation (Habas et al., 2001). In vertebrates, upon binding of Wnt ligand to Frizzled receptor, the signal is transduced via Dvl to activate RhoA (Habas et al., 2001; Schlessinger et al., 2009). Whether any additional receptor is employed in activation of this pathway is not known (Figure 5).
The Formin homology adaptor protein Daam1 has been identified as critical regulator of this pathway at the level of Dvl. Daam1 mediates the interaction between Dvl and RhoA, which is necessary for the activation of RhoA induced by Wnt or Dvl (Habas et al., 2001). Binding of Dvl to Daam1 induces a conformational change in Daam1, enabling its interaction with and subsequent activation of RhoA. Moreover, Daam1 is able, to some extent, to rescue the CE movement defects caused by Dvl loss of function (LOF) during *Xenopus* embryogenesis (Liu et al., 2008a). Further, ROCK has been proposed to mediate the effects of RhoA downstream of Wnt/Dvl (Kishida et al., 2004; Marlow et al., 2002). However, at this point it is not clear whether ROCK is the only effector of Wnt/Dvl/RhoA signaling route, because RhoA has been reported to activate numerous additional downstream effectors (Aspenstrom, 1999; Bishop and Hall, 2000; Chardin, 2003).

1.4.4.1.2 Wnt/Dvl/Rac signaling

Similar to RhoA, Rac1 also acts downstream of Dvl which is sufficient to induce its activation (Habas et al., 2003). However, mechanism by which Wnt signals via Dvl to activate Rac1 has not been characterized.
Ror receptors have been recently described to participate together with Frizzled receptors in Wnt-mediated Rac1 activation in mouse cells (Nishita et al., 2010; Sato et al., 2010). However, Ror cannot be considered as a Wnt/Dvl/Rac-specific receptor, as in *Xenopus* it is employed in transduction of Wnt5a-mediated Cdc42 activation (see the following section about Wnt/Cdc42 signaling). Moreover, it is not clear whether or not

is Ror involved in Dvl/Rac1 activation also in the context of PCP signaling in *Drosophila* (Green et al., 2008; Hendrickx and Leyns, 2008). Thus, its role needs to be considered in the context of ligand, receptors, and other pathway components available in a particular system.

Dvl overexpression or Wnt stimulation have been shown to trigger JNK activation in various systems, this effect has been abrogated by a dominant negative version of Rac1, thus positioning JNK downstream of Wnt/Dvl/Rac (Boutros et al., 1998; Habas et al., 2003; Nishita et al., 2010; Rosso et al., 2005) (Figure 5). However, not only Rac1, but also RhoA as well as Cdc42 have been proposed to regulate JNK in the context of the Wnt/PCP pathway (Kim and Han, 2005; Schambony and Wedlich, 2007).

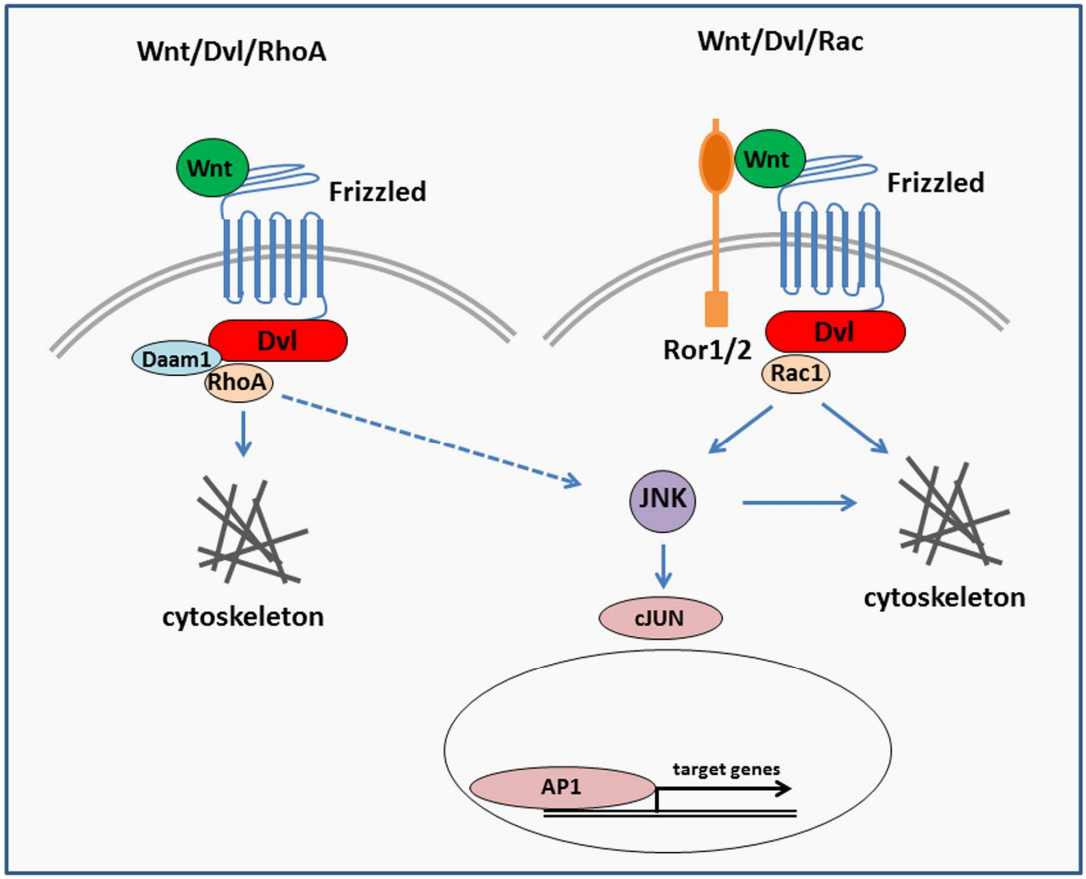

Figure 5: Wnt/Dvl/RhoA and Wnt/Dvl/Rac signaling. On the left: Wnt binds to Frizzled and via the Dvl/Daam1 complex induces activation of RhoA which regulate cytoskeleton via its effectors (not depicted). Dashed arrow indicates a possible activation of JNK (see main text). On right: Wnt binds to Frizzled and Ror1/2. Subsequently, the signal from the receptor complex is transduced via Dvl to activate Rac1 (and possibly other Rac proteins, not depicted). Rac1 induces changes in cytoskeleton via set of effectors (not depicted) and/or via activation of JNK. JNK can also phosphorylate transcription factor cJUN, which enters the cell nucleus and together with transcription factor cFos forms the AP1 complex, which regulates gene expression.

It is generally accepted that activation of JNK represents a means by which Wnt/β-catenin-independent signaling may regulate (via downstream effector of JNK-transcription factor cJUN) gene transcription (Fanto et al., 2000; Habas et al., 2003).

Nonetheless, the evidence for direct target gene regulation via this signaling branch is rather sparse (Schambony and Wedlich, 2007; Yamamoto et al., 2009; Yamamoto et al., 2010), if compared to numerous genes regulated by the Wnt/β-catenin pathway (Nusse, 2011). As JNK is also well characterized to directly regulate cytoskeleton rearrangements (Bishop and Hall, 2000; Huang et al., 2004; Xia and Karin, 2004), the exact contribution of nuclear JNK signaling to the Wnt/PCP pathway remains unclear.

1.4.4.1.3 Wnt/Cdc42 signaling

Functional studies from *Drosophila* proposed a Dvl-independent role for Cdc42 in the regulation of PCP (Boutros et al., 1998). This finding has been confirmed in *Xenopus*, as dominant negative Cdc42 (LOF) fails to rescue effects of Dvl gain of function (GOF) on CE movements (Choi and Han, 2002). Interestingly, Cdc42 activation in *Xenopus* has been recently shown to be under the control of Wnt5a and Ror2, respectively, thus regulating the CE movements via activation of JNK (Schambony and Wedlich, 2007) (Figure 6).

1.4.5 Wnt/Ca^{2+}signaling pathway

Calcium (Ca^{2+}) has been identified as a second messenger of Wnt/β-catenin-independent signaling based on experiments where the injection of mRNA of Dvl, Wnt5a, or Wnt11 has elevated levels of intracellular Ca^{2+} in zebrafish (Sheldahl et al., 2003; Slusarski et al., 1997b; Westfall et al., 2003). Moreover, stimulation with Wnt ligand (Wnt5a) has been reported to trigger Wnt/Ca^{2+} signaling in mammalian cells (Dejmek et al., 2006; Ma and Wang, 2006).

As shown in Figure 6, the Wnt signal is transduced by Frizzled receptors, possibly with cooperation of other receptor Ryk (Kohn and Moon, 2005; Kuhl et al., 2000; Li et al., 2009; Li et al., 2010; Slusarski et al., 1997a; Slusarski and Pelegri, 2007). The signal is subsequently transduced via phospholipase C (PLC) and/or phosphodiesterase (PDE) (Ahumada et al., 2002; James et al., 2008; Ma and Wang, 2006; Slusarski et al., 1997a). Interestingly, despite the fact that Dvl has been shown to be sufficient to activate Wnt/Ca^{2+} signaling (Sheldahl et al., 2003), it seems to be dispensable for some aspect of this signaling, as elevation of intracellular Ca^{2+} can be triggered by Wnt ligand in cells depleted of Dvl proteins (Ma and Wang, 2007).

Downstream of Ca^{2+} elevation, kinases PKC and CamKII, and transcription factor NFAT have been identified as mediators of Wnt/Ca^{2+} signaling, having a broad range of effects (Cook et al., 1996; Kuhl et al., 2000; Saneyoshi et al., 2002; Sheldahl et al., 1999).

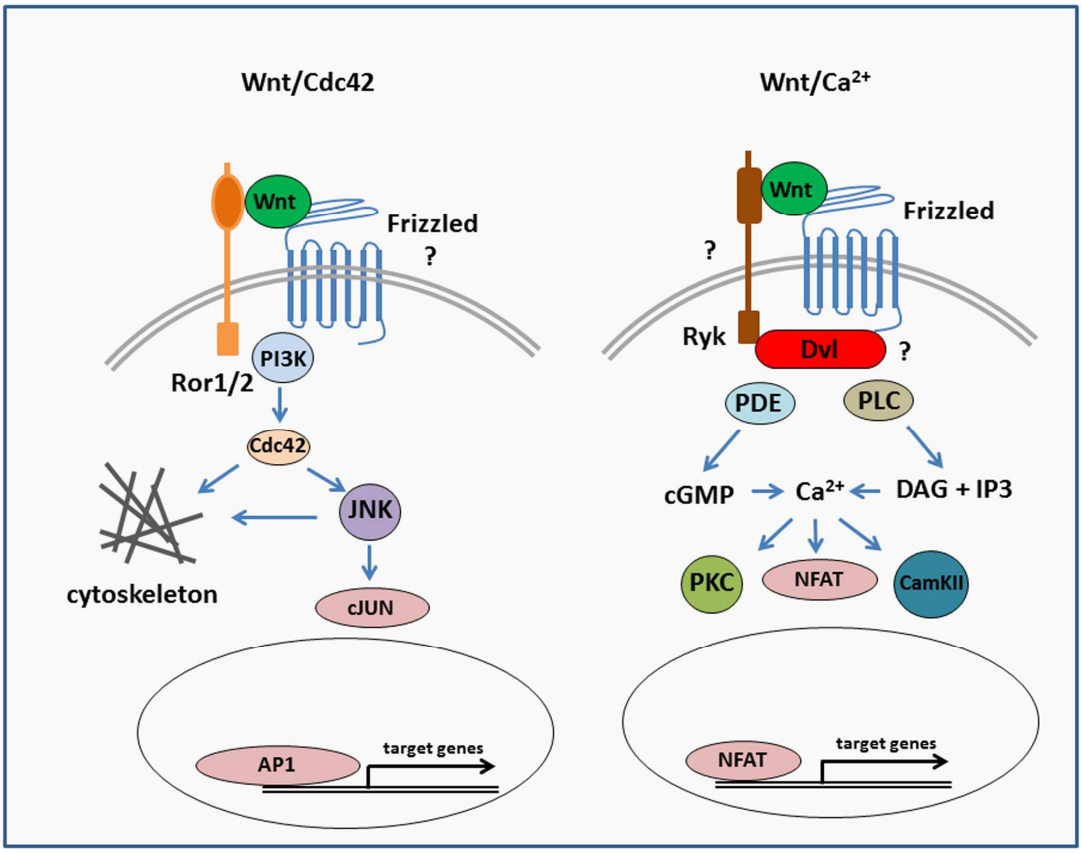

Figure 6: Wnt/Cdc42 and Wnt/Ca^{2+} signaling. On the left: Wnt binds to Ror2 and possibly also to Ror1. The involvement of Frizzled has not been demonstrated in this pathway. Cdc42 is activated in a PI3K-dependent manner and can regulate either cytoskeleton or gene expression via activation of the JNK/cJUN cascade (AP1 – complex of cJUN and cFos). On the right: Wnt interacts with Frizzled and possibly also with the receptor Ryk. Dvl seems to be dispensable for some aspects of this pathway (see main text). Further, PDE (via production of cGMP) and/or PLC (via production of DAG and IP3) regulate Ca^{2+} channels (not depicted) and thereby levels of intracellular Ca^{2+}. Increased levels of Ca^{2+} lead to activation of kinases PCK and CamKII, and of transcription factor NFAT.

1.4.6 Wnt receptors

1.4.6.1 Frizzleds

The name "Frizzled" came from a study in *Drosophila*, where the "Frizzled" mutant showed irregularly arranged and tightly curled hairs and bristles on the thorax, wings, and feet (Bridges and Brehme, 1944). Later, Frizzled1 as well as other Frizzled genes were characterized. Subsequently, Frizzled2 was identified as a Wnt receptor, while

Frizzled1 was shown to act in *Drosophila* in a Wnt-independent manner (Bhanot et al., 1996; Seifert and Mlodzik, 2007; Vinson and Adler, 1987; Vinson et al., 1989; Wang et al., 1996). In mammals, 10 Frizzled receptors have been described. All Frizzleds have an extracellular N-terminal CRD domain involved in ligand binding, a 7-transmembrane domain, and an intracellular C-terminal domain containing highly conserved PDZ binding KTxxxW motif critical for signal transduction (Schulte, 2010; Schulte and Bryja, 2007; Wang et al., 2006a; Wang and Malbon, 2004). Interestingly, while the CRD domain is required for ligand binding and possibly for dimerization/oligomerization of the receptors, it is dispensable for signal transduction as such (Carron et al., 2003; Dann et al., 2001; Chen et al., 2004; Povelones and Nusse, 2005). Moreover, although the extracellular part is relatively well conserved between different Frizzleds, the intracellular part differs rather substantially (Schulte and Bryja, 2007; Wang et al., 2006a). This opens a possibility for protein-protein interactions specific for the particular Frizzled receptor. Due to the presence of the 7-transmembrane domain and the ability of Frizzleds to form homo- or heterodimers, these receptors are considered as a class of G-protein coupled receptors (GPCRs). Despite the fact that evidence demonstrating the involvement of G-proteins in most if not all Wnt-driven signaling pathways is rather strong (Ahumada et al., 2002; Katanaev et al., 2005; Liu et al., 2001; Liu et al., 2005; Slusarski et al., 1997a), the question whether the action of G-proteins indeed takes place at the level of Frizzled receptor has not been fully answered (Egger-Adam and Katanaev, 2008; Schulte, 2010).

In terms of function, Frizzled receptors show certain degree of redundancy, which is expected provided the similarities in their structure and patterns of expression (Fischer et al., 2007; van Amerongen and Berns, 2006). On the other hand, individual Frizzled receptors have been shown to be sufficient to activate distinct downstream signaling pathways (Ahumada et al., 2002; Gazit et al., 1999; Holmen et al., 2002; Wang and Malbon, 2004). However, as these studies were done in the presence of other receptors, the requirement of individual Frizzled receptors for a specific downstream signaling is still not well understood. Moreover, due to the nature of Wnt ligands already mentioned (poorly soluble and difficult to purify), the specificity and binding affinities between individual Wnt-receptor pairs (Hsieh et al., 1999; Wu and Nusse, 2002) remain some of the least characterized areas in the field.

1.4.6.2 LRPs

Low density lipoprotein receptor-related proteins (LRPs) are a subfamily of single transmembrane receptors from the low density lipoprotein (LDL) receptor family, proteins with diverse functions in endocytosis, cell metabolism, and embryogenesis (May et al., 2007). *Drosophila* LRP Arrow and its vertebrate homologues LRP5/6 have been demonstrated to be critical pathway components required for Wnt/β-catenin signaling (He et al., 2004; Kelly et al., 2004; Tamai et al., 2000; Wehrli et al., 2000). *Drosophila* Arrow mutant phenocopies the LOF phenotype of Wnt mutant (Wingless) (Wehrli et al., 2000), and injection of LRP6 mRNA is sufficient to induce secondary body axis in *Xenopus* embryo (Tamai et al., 2000). LRP5 and LRP6 are highly functionally redundant, as mice lacking both LRP5 and LRP6 (Kelly et al., 2004) show much more severe phenotype (failure to finish gastrulation) compared to the phenotype of mice lacking only one of these receptors. Further, LRP6 seems to be more functionally important for embryogenesis than LRP5. Mice lacking LRP6 are perinatal lethal, compared to LRP5-deficient mice, which grow to adulthood and are fertile, but show signs of osteoporosis (Kato et al., 2002; Pinson et al., 2000).

LRP5/6 receptor has been shown to function in the proximity of Frizzled receptor (Schweizer and Varmus, 2003; Tolwinski et al., 2003), to form clusters/signalosomes (Bilic et al., 2007; Cong et al., 2004b), and ternary complexes with Frizzled and Wnt (Bourhis et al., 2010; Semenov et al., 2001). EGF-like domains and β-propeller motifs, located at the extracellular part of LRP5/6, are important for the binding of Wnt ligand (Kato et al., 2002; Liu et al., 2009; Tamai et al., 2000). Interestingly, LRP6 mutant lacking its extracellular domain can activate Wnt/β-catenin signaling in a ligand-independent manner (Brennan et al., 2004; Liu et al., 2003). Therefore, the extracellular part the receptor is dispensable for the actual signal transduction in the Wnt/β-catenin pathway. Intracellular part of LRP5/6 contains 5 PPPSP motifs flanked by Ser/Thr clusters, which are phosphorylated by GSK3 and CK1γ, respectively (Davidson et al., 2005; Tamai et al., 2004; Zeng et al., 2007; Zeng et al., 2005). Interestingly, several other kinases have been recently proposed to participate in LRP5/6 phosphorylation (Cervenka et al., 2010; Davidson et al., 2009; Chen et al., 2009). Importantly, phosphorylated PPPSP motifs are both necessary and sufficient for the activation of Wnt/β-catenin pathway (Tamai et al., 2004). Moreover, they seem to directly inhibit GSK3 activity (Wu et al., 2009).

LRP5/6 is required for Wnt/β-catenin pathway activation (Angers and Moon, 2009; He et al., 2004; Macdonald et al., 2007; MacDonald et al., 2009). However, recent evidence has also suggested functional implication for LRP5/6 in the regulation of CE movements via modulation of Wnt/β-catenin-independent pathway(s) (Bryja et al., 2009; Tahinci et al., 2007).

Similar to what was described for Wnt-Frizzled binding, the specificity of LRP5/6 towards any particular Wnt ligand is largely unknown. Interestingly, recent mapping studies have identified unique binding sites for different Wnts in the extracellular part of LRP5/6, suggesting the possibility of simultaneous binding of different ligands to the LRP5/6 receptor (Bourhis et al., 2010; Ettenberg et al., 2010).

1.4.6.3 Ror receptors

A role of receptor tyrosine kinase-like orphan receptor (Ror) proteins, members of the receptor tyrosine kinases (RTKs) family, in Wnt signaling has only recently emerged (Green et al., 2008; Minami et al., 2010). Despite Ror homologs are well conserved among different species, it is mostly human Ror1 and Ror2, which have been relatively well characterized. This is mainly due to their implication in diseases such as brachydactyly, Robinow syndrome, and chronic lymphocytic leukemia) (Afzal et al., 2000; Fukuda et al., 2008; Oldridge et al., 2000; van Bokhoven et al., 2000). In mice, Ror1 and Ror2 are already expressed during gastrulation as well as later during embryonal development in a rather overlapping pattern, suggesting functional redundancy (Al-Shawi et al., 2001; Matsuda et al., 2001; Oishi et al., 2003; Oishi et al., 1999). Ror2-deficient as well as Ror1/2-deficient mice largely phenocopy mice lacking Wnt5a in terms of outgrowth defects of multiple structures during embryogenesis (Nomi et al., 2001; Oishi et al., 2003; Yamaguchi et al., 1999). Furthermore, Ror2 GOF leads to CE movement defects in *Xenopus* embryos (Hikasa et al., 2002; Oishi et al., 2003) in a similar manner as Wnt5a GOF does (Moon et al., 1993). As was already pointed out, the Wnt5a/Ror2-mediated control of CE movements in *Xenopus* seems to be underlined by direct signaling into the nucleus, and subsequent upregulation of expression of protocadherin PAPC (Schambony and Wedlich, 2007). Nonetheless, whether or not this distinct signaling branch operates also in other systems is not clear.

The extracellular region of vertebrate Ror1/2 contains an Ig-like domain, a CRD domain (similar to CRD domain of Frizzled receptors), and a Kringle domain. The CRD domain of vertebrate Ror1/2 has been shown to bind to Wnt5a as well as several

other Wnt ligands (Billiard et al., 2005; Fukuda et al., 2008; Mikels and Nusse, 2006; Oishi et al., 2003). Although the CRD domain seems to be sufficient to bind Wnt5a as well as Frizzled 2&5 (Oishi et al., 2003), deletion of the Ig-like domain also has a negative impact on the ligand-receptor interaction (Mikels et al., 2009).

The cytoplasmic region of the Ror1/2 contains conserved tyrosine kinase domain, which has been shown to mediate receptor homodimerization and autophosphorylation upon ligand binding to the extracellular part (Liu et al., 2008b; Mikels et al., 2009). On the other hand, phosphorylations of Ser/Thr residues of Ror2 by several other kinases has also been reported (Grumolato et al., 2010; Kani et al., 2004; Yamamoto et al., 2007). In this regard, it is possible that the phosphorylation status of different residues of the intracellular part of Ror1/2 plays a key role in Ror1/2-mediated signal transduction by regulating association with various adaptor proteins, in a manner similar to that seen with other RTKs.

Precise mechanisms controlling signaling downstream of Ror1/2 to one or more signaling routes are currently not well characterized. So far, many different signaling events have been attributed to Ror1/2 in different cellular systems and context (Billiard et al, 2005; Enomoto et al., 2009; Fukuda et al., 2008; Liu et al., 2007; Mikels and Nusse, 2006; Witte et al., 2010). For instance, Ror2 has been either shown to promote or inhibit Wnt/β-catenin signaling pathway in response to different Wnt ligands in different cell types (Billiard et al, 2005; Li et al., 2008; Mikels and Nusse, 2006). Nonetheless, despite the incompletely defined aspects of signaling, the role of Ror1/2 as Wnt receptor is very well established, especially for Wnt5a.

1.4.6.4 Ryk receptor

Ryk (Related to tyrosine kinase) is another RTK involved in Wnt-mediated signal transduction. In terms of structure, Ryk is a transmembrane protein with an extracellular WIF (Wnt inhibitory factor) domain (implicated in ligand binding), and an intracellular domain bearing the kinase domain (not fully conserved, therefore considered kinase-inactive) and the PDZ-binding domain (Fradkin et al., 2010; Hendrickx and Leyns, 2008). The intracellular part of Ryk is required for Ryk-mediated signal transduction, possibly due to its role in recruitment of adaptor proteins and/or PDZ domain scaffolding proteins (Bonkowsky et al., 1999; Lu et al., 2004).

Its function as a receptor for Wnts has been demonstrated both in *Drosophila* (with Wnt5) (Yoshikawa et al., 2003) and in mice (Wnt1 and Wnt3a) (Lu et al., 2004).

Moreover, Ryk forms ternary complexes with Wnt1 and Frizzled-CRD, and enhances Wnt-mediated activation of the Wnt/β-catenin pathway (Lu et al., 2004). Further studies have also identified both biochemical and functional interactions between Ryk and Wnt5a during the axon guidance, possibly via Wnt/β-catenin-independent signaling (Wnt/Ca^{2+} pathway) (Keeble et al., 2006; Li et al., 2009; Li et al., 2010). The view of Ryk as a receptor capable of also signaling via Wnt/β-catenin-independent pathways has been further strengthen by recent findings identifying Ryk as critical regulator of CE movements in both *Xenopus* (Kim et al., 2008) and zebrafish (Lin et al., 2010). Similarly to the other receptors already discussed, mechanisms underlying its signaling specificity in different cellular contexts are currently unknown.

1.4.7 Soluble modulators of Wnt signaling

Wnts or their receptors interact both functionally and biochemically with several secreted factors distinct from Wnts, which modulate Wnt signaling in either positive or negative manner.

First class of the Wnt modulators is formed by Secreted Frizzled-related proteins (SFRPs). As the name suggest, SFRPs are similar to Frizzled receptors in that they contain the CRD domain. However, they lack the transmembrane and intracellular parts (Kawano and Kypta, 2003). It is therefore not surprising that SFRPs are able to interfere with Wnt/β-catenin signaling. They have been shown to block Wnt-induced axis duplication in *Xenopus* embryos and Wnt-induced accumulation of nuclear β-catenin in mammalian cells (Finch et al., 1997; Leyns et al., 1997; Uren et al., 2000). Nonetheless, the view on the function of SFRPs has evolved from being simple extracellular scavengers of Wnt ligands to multifunctional modulators of Wnt signaling. It has been proposed that SFRPs have biphasic effects. It seems that low concentrations have promoting and high concentrations inhibiting effects on the Wnt/β-catenin signaling pathway (Uren et al., 2000). Further, ability of SFRPs to interfere with Wnt-driven signaling seems to vary, depending on the particular Wnt ligand, from highly potent block to no effect (Wang et al., 1997). Moreover, SFRPs have been demonstrated to directly bind to the CRD of Frizzled receptors, and to trigger downstream signaling events (Rodriguez et al., 2005).

Another group of Wnt signaling modulators are Dickkopfs (Dkk1-4 in vertebrates, no Dkk in *Drosophila*). Dkks contain two CRD domains separated by a linker region

(Kawano and Kypta, 2003). Dkk1 is the best characterized member of the Dkk protein family. During early embryogenesis in *Xenopus*, Dkk1 is produced by the Spemann organizer and mediates some of the organizer's activity necessary for the formation of the body axis and head structures by inhibiting the Wnt/β-catenin signaling pathway (Glinka et al., 1998; Niehrs, 2001). This role of Dkk1 is conserved in mammals as Dkk1-deficient mice fail to develop anterior neuroectoderm (lack of forebrain and major part of midbrain) (Mukhopadhyay et al., 2001). Dkk1 has been demonstrated to block Wnt-induced axis duplication in *Xenopus* and Wnt/β-catenin pathway activation in mammalian cells (Fedi et al., 1999; Glinka et al., 1998).

Dkk1 binds to the extracellular part of LRP5/6 and prevents the formation of Wnt-Frizzled-LRP5/6 trimeric complexes (Bourhis et al., 2010; Mao et al., 2001; Semenov et al., 2001; Semenov et al., 2008). Moreover, Dkk1 has been proposed to interfere with Wnt/β-catenin signaling by also promoting endocytosis of LRP5/6, thus regulating the level of LRP5/6 receptor available for Wnt signaling at the membrane (Mao et al., 2002; Yamamoto et al., 2008). Interestingly, as LRP5/6 has been recently implicated in the regulation of CE movements, similar role in modulation of the Wnt/PCP pathway has also been attributed to Dkk1 (Caneparo et al., 2007). Nonetheless, the inhibitory role of Dkk1 in the Wnt/β-catenin pathway has not been challenged.

1.4.8 Dishevelled

"Dishevelled" mutation was first described in *Drosophila*, causing failure to orient hairs on the wings and legs (Fahmy and Fahmy, 1959). Connection to the Wnt pathway was demonstrated much later, when Dvl (Dsh in *Drosophila*) was identified as a critical component of both Wnt/β-catenin and Wnt/β-catenin-independent pathways in various animal models (Klingensmith et al., 1994; Krasnow et al., 1995; Li et al., 1999; Noordermeer et al., 1994; Sheldahl et al., 2003; Sokol et al., 1995). Three homologs have been identified in mice, functional analyses of single Dvl- and double Dvl-deficient mice (lacking two Dvls) has revealed both functional redundancy between Dvls, and differences in ability to compensate for lack of other Dvls. In this regard, Dvl1 seems to be the least functionally important for mouse development. In fact, Dvl1-deficient mice are viable and mice lacking both Dvl2 and Dvl3 show the most severe phenotype compared to other double-deficient mouse strains (Etheridge et al., 2008; Gao and Chen, 2010; Wang et al., 2006b).

Three main highly conserved domains have been characterized in the Dishevelled proteins: an N-terminal DIX (Dishevelled, Axin) domain of 80 amino acids, a central PDZ (Postsynaptic density 95, Discs Large, Zonula occludens-1) domain of about 90 amino acids, and a C-terminal DEP (Dvl, Egl-10, Pleckstrin) domain (Gao and Chen, 2010; Wallingford and Habas, 2005) (Figure 7).

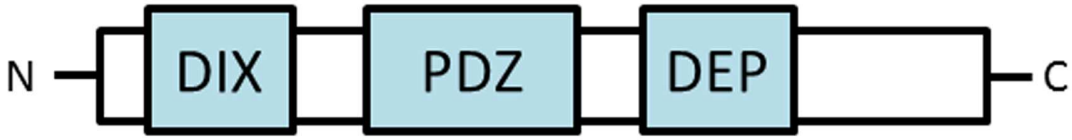

Figure 7: Scheme of the conserved motifs of Dvl

Mechanistically, there is substantial evidence that Dvl and its distinct domains contribute to the branching of Wnt signaling into separate pathways (Axelrod et al., 1998; Capelluto et al., 2002; Pan et al., 2004; Sheldahl et al., 2003). As already mentioned, Dvl is involved in the inhibition of the destruction complex, likely via recruitment of GSK3 and Axin to the plasma membrane. In this regard, membrane recruitment of Dvl has been attributed to the activation of Wnt signaling (Axelrod et al., 1998; Cliffe et al., 2003; Cong et al., 2004b; Park et al., 2005; Zeng et al., 2007). Further, Dvl interacts with the PDZ binding motifs of Frizzled and Ryk via its PDZ domain (Lu et al., 2004; Punchihewa et al., 2009; Wong et al., 2003), and with Ror via its C-terminal part (Witte et al., 2010). Dvl undergoes dynamic polymerization, a feature which requires the DIX domain (Schwarz-Romond et al., 2007a; Schwarz-Romond et al., 2007b). This ability to polymerize is critical for activation of the Wnt/β-catenin pathway, as it is required for clustering and phoshorylation of LRP5/6 (Bilic et al., 2007; Metcalfe et al., 2010). Interestingly, Dvl polymerization seems to also be relevant for the Wnt/Dvl/Rac signaling, as has been recently demonstrated (Nishita et al., 2010).

Further, Dvl is a scaffolding protein, providing platform for many protein-protein interactions. More than 50 different proteins have been identified as Dvl-binding partners in different cellular systems (Gao and Chen, 2010). As it is unlikely that these interactions occur simultaneously, simply due to steric limitations, it is clear that the presence or absence of specific interactors (due to competition for docking sites) represents a way to further specify the transduced signal (Habas et al., 2001; Kishida et

al., 1999). Moreover, recruitment of Dvl into different subcellular compartments has also been attributed to differential activation of distinct downstream signaling pathways (Capelluto et al., 2002; Park et al., 2005).

Yet another level of complexity at the level of Dvl is secured by its phosphorylation by several kinases, including CK1δ/ε, CK2, PAR1, PKC, and MAK (Cong et al., 2004a; Hino et al., 2003; Chen et al., 2003; Kibardin et al., 2006; Ossipova et al., 2005; Willert et al., 1997). It has been proposed that phosphorylation of Dvl by different "Dvl kinases" is required mainly for Wnt/β-catenin pathway activation and thus represents a switch between the Wnt/β-catenin-dependent and -independent signaling pathways (Cong et al., 2004a; Dominguez et al., 2004; Hino et al., 2003; Peters et al., 1999). However, recent data have suggested that this model may be a bit simplified, because phosphorylation of Dvl seems also to be involved in regulation of Wnt/β-catenin-independent signaling (Bryja et al., 2007c; Klein et al., 2006).

Several Wnt ligands (demonstrated for Wingless, Wnt3a, and Wnt5a) have been shown to trigger phosphorylation of Dvl at multiple sites (Gonzalez-Sancho et al., 2004; Schulte et al., 2005; Yanagawa et al., 1995). This leads to a phosphorylation-dependent shift in the mobility of Dvl in polyacrylamide gel, referred to as PS-Dvl (phosphorylated and shifted Dvl). Apearence of PS-Dvl is considered as one of the hallmarks of Wnt signaling activation (Gonzales-Sancho et al., 2004; Schulte and Bryja, 2007). However, a causative link between PS-Dvl and its employment in activation/inhibition of downstream signaling has not been made.

1.4.9 Casein kinases

1.4.9.1 CK1

CK1 represents a group within the superfamily of serine/threonine kinases. The CK1 family is highly evolutionary conserved (homologs even in yeasts), and its members are expressed ubiquitously. In mammals, 7 isoforms have been identified in the CK1 family (α, β, γ1-3, δ, ε), which mostly differ in length of their N-terminal and C-terminal noncatalytic regions (Knippschild et al., 2005). They act as monomers and are considered to be constutively active kinases. In this regard, their subcellular localization and autoinhibitory phosphorylation of their C-terminus play important roles in the regulation of kinase-substrate interactions (Knippschild et al., 2005; Price, 2006).

Interestingly, Wnt has been described to induce dephosphorylation and subsequent activation of CK1 δ/ε (Swiatek et al., 2004).

Members of the CK1 family fulfill many roles in the regulation of Wnt signaling, acting both as positive and negative regulators. Apart from their role in phosphorylation of Dvl, different CK1 isoforms have been shown to phosphorylate many other components of Wnt pathway, such as Ror2 (Kani et al., 2004), LRP5/6 (Swiatek et al., 2006), β-catenin (Liu et al., 2002; Sakanaka, 2002), APC (Ha et al., 2004) and TCF (Lee et al., 2001). Moreover, overexpression of CK1α, CK1δ or CK1ε has been described to mimic Wnt-induced formation of a secondary body axis in *Xenopus* embryos (Cheong and Virshup, 2010; Peters et al., 1999; Sakanaka et al., 1999).

However, list of substrates of CK1 extends beyond the Wnt pathway. Members of the CK1 protein family are also implicated in the regulation of Shh signaling, circadian rhythms, DNA repair, and apoptosis (Cheong and Virshup, 2010; Knippschild et al., 2005; Price, 2006). CK1 may thus provide a link between Wnt signaling and other pathways.

1.4.9.2 CK2

CK2 is a serine/threonine protein kinase as well, but evolutionary unrelated to CK1 family. CK2 is also conserved through evolution and ubiquitously expressed, but compared to CK1 is mostly present as a tetramer of two catalytic (α and α′) and two regulatory subunits (β) (Litchfield, 2003). Interestingly, CK2β subunits have been proposed to also function independently of the CK2 holoenzyme (Olsten and Litchfield, 2004). Similarly to the CK1, CK2 is rather "promiscuous" kinase with over 100 substrates identified (Litchfield, 2003), including Dvl (Song et al., 2003; Willert et al., 1997), β-catenin (Song et al., 2003), and LEF1 (Wang and Jones, 2006). Prior Paper III, CK2 has been exclusively considered as a positive regulator of Wnt/β-catenin pathway (Dominguez et al., 2004) and its possible function in other Wnt pathways had not been known.

1.4.10 β-arrestin

There are four members of the β-arrestin family in mammals, all of which are very closely related (70% sequence homology). Interestingly, while β-arrestins 1/2 are ubiquitously expressed, the remaining two members are specifically expressed in the

visual system where they participate in the desensitization of rhodopsin (Lefkowitz and Shenoy, 2005).

β-arrestins were originally described as adaptor proteins involved in desensitization, internalization, and degradation of GPCRs (Lefkowitz and Shenoy, 2005). Further studies pinpointed their role as multifunctional scaffolding proteins, employed in various signaling pathways including the Wnt pathway (Kovacs et al., 2009; Lefkowitz et al., 2006; Schulte et al., 2010).

First, β-arrestin1 has been shown to positively regulate the effect of Dvl on Wnt/β-catenin pathway activation (Chen et al., 2001). Furthermore, β-arrestin1/2 has been demonstrated to bind Dvl and Axin, and therefore represents an important component of the Wnt/β-catenin pathway, possibly acting at the level of β-catenin destruction complex (Bryja et al., 2007b; Rosano et al., 2009).

Moreover, a role for β-arrestins in the Wnt/β-catenin-independent pathways has been proposed, because of the involvement of β-arrestin2 in Wnt5a and PKC mediated endocytosis of Frizzled4 (Chen et al., 2003). Interestingly, β-arrestin2 did not mediate Frizzled internalization via directly binding to it, as it is the case for the classical GPCRs, but rather through interaction with Dvl and an adaptor protein AP-2 (Chen et al., 2003; Yu et al., 2007).

1.4.11 Downstream effectors: Rho family of small GTPases

1.4.11.1 Regulation and function

The Rho GTPase family represents a highly conserved group of proteins regulating numerous cellular processes from yeasts to mammals. Twenty-two mammalian genes encoding Rho GTPases have been described, with the Rac1, RhoA, and Cdc42 being the most well studied members. They were identified as regulators of the actin cytoskeleton and as such they regulate cell migration, adhesion, morphogenesis, and axon guidance (Etienne-Manneville and Hall, 2002; Govek et al., 2005; Ng et al., 2002). In addition to this, Rho GTPases are involved in the regulation of microtubular dynamics, cell polarity, gene expression, cell cycle, vesicle trafficking/endocytosis, and reactive oxygen species metabolism (Bosco et al., 2009; Govek et al., 2005; Jaffe and Hall, 2005; Schlessinger et al., 2009). The very broad spectrum of cellular functions controlled by Rho GTPases is further underscored by the fact that about 1% of all

known genes encode for proteins which either regulate or are regulated by interaction with Rho GTPases (Jaffe and Hall, 2005).

Such a broad spectrum of functions obviously requires involvement of rather complex regulatory mechanisms which control both spatial (specific subcellular compartment) and temporal activation of Rho GTPases. Naturally, not only Wnt signaling, but also signaling by other morphogens and growth factors is involved in the control of Rho GTPases activity (e.g. TGF, FGF, PDGF, and NGF) (Chardin, 2003; Zhang, 2009). Interestingly, while the number of regulators is high, the mechanism enabling precise control of the activation/inactivation of Rho GTPases is relatively simple and well conserved (Figure 8).

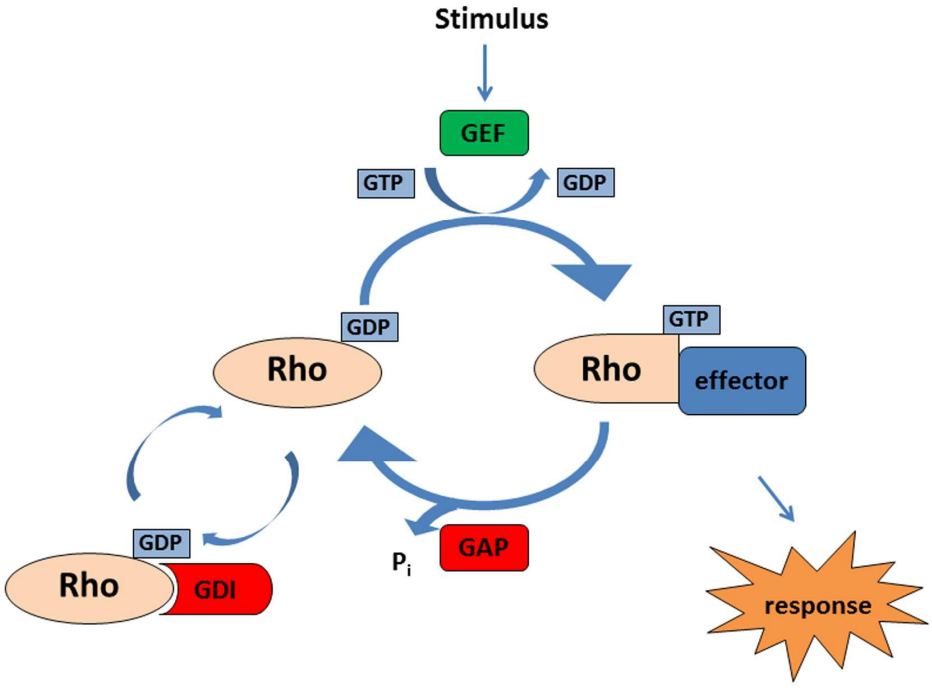

Figure 8: Regulation of the activity of small GTPases. Upon stimulation, GEF interacts with small GTPase from Rho family and induces GDP to GTP exchange. GTP-bound Rho changes its conformation, leaves the complex with GEF and activates its effector by protein-protein interaction. See main text for details on functional aspects. Binding of GAP increases the activity of Rho to hydrolyze GTP to GDP and leads to the inactivation of the small GTPase. GDP-bound Rho protein can be sequestered from its subcellular compartment by interactions with GDI.

Similar to GTPases from other families, Rho GTPases act as molecular switches cycling between two conformational states: an active GTP-bound state and an inactive GDP-bound state. In the active GTP-bound state the Rho GTPases fulfill their regulatory functions through a conformation-specific interaction with their effector proteins. Together, over 50 different effectors have been identified for RhoA, Rac1, and Cdc42 in different cellular contexts (Aspenstrom, 1999; Bishop and Hall, 2000; Jaffe and Hall, 2005). The transition from the inactive to active state is controlled by guanine

nucleotide exchange factors (GEFs), which catalyze the exchange of GDP for GTP. This is followed by a conformational change of the Rho GTPase (Cherfils and Chardin, 1999). There are over 70 GEFs in mammals. The intrinsic activity of Rho GTPases to hydrolyze GTP is low, and is enhanced by interaction with GTPase-activating proteins (GAPs), which leads to the inactivation of the GTPase (Etienne-Manneville and Hall, 2002). More than 80 GAPs have been described. Further, guanine nucleotide dissociation inhibitors (GDIs) bind to the GDP-bound GTPase and sequester it from its native subcellular compartment, thus preventing its possible "re-activation" by interaction with GEFs (Etienne-Manneville and Hall, 2002).

Clearly, such multiplicity in molecules that converge to regulate Rho GTPases enables multiple levels of specificity control. Both GEFs and GAPs often show specificity towards certain members of Rho GTPase family (Schmidt and Hall, 2002). Further, many of these regulators are specifically expressed in particular cell type(s). Moreover, recent evidence has suggested that protein-protein interactions between GEFs and scaffolding proteins are employed in targeting GEFs into specific cell compartments/microdomains (Garcia-Mata and Burridge, 2007; Marinissen and Gutkind, 2005). It has been also demonstrated that these interactions with scaffolding proteins can affect the route of the signal from the "activated" GTPase towards particular effector, simply by linking them together at the surface of the scaffolding protein (Jaffe et al., 2005).

1.4.11.2 Rac small GTPases

Three Rac genes have been identified in vertebrates (Rac1, Rac2, and Rac3). They share high sequence homology (90%), and diverge in the last 15 amino acids at the C-terminus. This C-terminal region is important for specifying the subcellular localization of Rac proteins and interaction with their effectors (Ando et al., 1992; Kinsella et al., 1991).

In mice, Rac1 is expressed rather ubiquitously. Rac2 is expressed mainly in the hematopoietic system and Rac3 in the developing brain (de Curtis, 2008). Thanks to their structural similarity, they seem to be highly redundant in terms of their function (Corbetta et al., 2005; Corbetta et al., 2009).

1.4.11.2.1 Tiam1-a Rac1 GEF

Tiam1 (T-cell lymphoma invasion and metastasis 1) is one of the many GEFs described as regulating Rac1 activity. Tiam1 was originally identified as a gene inducing the invasive phenotype of otherwise non-malignant T-lymphoma cells (Habets et al., 1994), and its overexpression caused membrane ruffling and cytoskeletal changes in a Rac1-dependent manner (Michiels et al., 1995). It seems that Tiam1 acts as a Rac specific GEF *in vivo* (cells and animal models), although it is capable, to some extent, of also inducing GDP-to-GTP exchange for other Rho GTPases in true *in vitro* situation (purified recombinant proteins in a tube) (Michiels et al., 1995; Minard et al., 2004).
In terms of structure, its N-terminal part carries a lipid modification important for association with the plasma membrane. Further, Tiam1 contains several distinct protein domains. Its DH domain is crucial for interaction with Rac1 and mediates actual GDP-GTP exchange (Worthylake et al., 2000). There are two PH domains that are located at the N-terminus and in the C-terminus, next to the DH domain, thus flanking the PDZ domain located in the central part of Tiam1 (Figure 9).

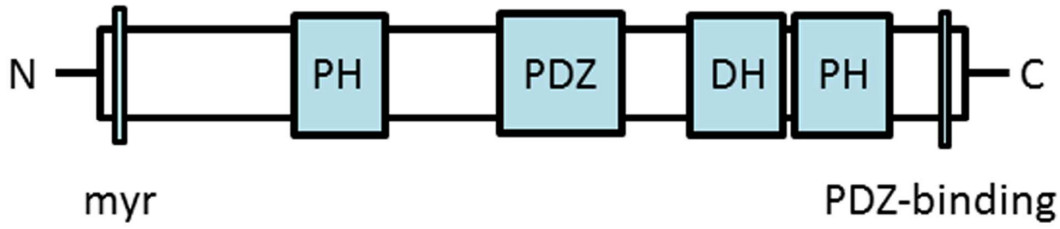

Figure 9: The scheme of the conserved motifs of Tiam1. A signal for myristoylation is located at the N-terminus, while the C-terminus contains a putative PDZ binding motif. Not all structural motifs are depicted.

The activity of Tiam1 is modulated at several levels. Phosphoinositol lipids have been proposed to bind to the PH domain and thereby enhance ability of Tiam1 to activate Rac1 (Mertens et al., 2003). Moreover, Ca^{2+}-regulated kinases PKC and CamKII have been shown to phosphorylate Tiam1, thus affecting its membrane versus cytosolic localization (Buchanan et al., 2000). Finally, Tiam1 has been described to interact with several scaffolding proteins, and this interaction seems to have a great impact on specification of signaling downstream of Rac1 (Buchsbaum et al., 2003; Marinissen and Gutkind, 2005).
However, the possible involvement of Tiam1 in Wnt/PCP signaling has not been addressed prior Paper IV in this thesis.

1.4.12 Wnt pathways crosstalk

The number of signaling routes described as Wnt-driven goes beyond the number of pathways described here (Macdonald et al., 2007; Semenov et al., 2007; Schulte, 2010). As the "toolkit" (pathway components) utilized by many of the Wnt signaling cascades is often shared, it is becoming obvious that individual branches of Wnt signaling are highly interconnected, thus forming a network with many nodes/options for crosstalk.

Although unavailability of purified Wnt ligands has hampered rigorous pharmacological analyses of ligand-receptor binding, there is good reason to believe that different Wnt ligands bind to different receptors with different affinities. The ability of Wnt/β-catenin-independent pathway(s) to interfere with the activation of the Wnt/β-catenin pathway is well documented (Bryja et al., 2007c; James et al., 2008; Mikels et al., 2009; Mikels and Nusse, 2006; Torres et al., 1996; Westfall et al., 2003). In this regard, competition between different Wnt ligands for binding to the receptor may to some extent account for this. Interestingly, recent reports have demonstrated that Wnt5a-mediated inhibition of Wnt/β-catenin pathway activation by Wnt3a is underpinned by competition for LRP6 (Bryja et al., 2009) or Frizzled2 (Sato et al., 2010).

Another important node in the network is Dvl thanks to its numerous binding partners, utilization of its distinct domains by distinct downstream pathways, and further modulability by several kinases. It is therefore very possible that competition between different modules of Wnt-driven signaling also occurs at the level of Dvl. Moreover, Dvl also represents a platform for crosstalk with other signaling pathways (e.g. Notch, Hippo, and mTOR) (Axelrod et al., 1996; Mak et al., 2005; Varelas et al., 2010).

Apart from the competitions already mentioned, the Wnt/β-catenin pathway is antagonized by Wnt/β-catenin-independent signaling downstream of receptors and/or Dvl. In this regard, Wnt5a has been demonstrated to promote β-catenin degradation via Siah2 E3 ubiquitin ligase (Topol et al., 2003). Moreover, Wnt/Ca^{2+} signaling has been shown to inhibit Wnt/β-catenin pathway either by targeting interaction between TCF/LEF and β-catenin (Ishitani et al., 2003; Ishitani et al., 1999) or by promoting β-catenin degradation in a PKC-dependent manner (Gwak et al., 2006).

The Wnt/Ca^{2+} pathway also seems to very closely interact with Wnt/PCP signaling in a reciprocal manner (James et al., 2008; Kohn and Moon, 2005). Wnt5a-mediated activation of Cdc42 has been demonstrated to simultaneously suppress activation of the Wnt/Ca^{2+} pathway at the level of NFAT (Dejmek et al., 2006). On the other hand,

Cdc42 has also been suggested to act downstream of Wnt/Ca^{2+} pathway, as its dominant negative form rescues the phenotype caused by overexpression of PKC (Choi and Han, 2002). Interestingly, the picture is getting more complicated, as many small GTPases can be modulated by Ca^{2+} and moreover, they can affect levels of intracellular Ca^{2+} by binding to Ca^{2+} channels and/or transporters (Aspenstrom, 2004). The close interconnection between Wnt/PCP and Wnt/Ca^{2+} signaling is further supported by interactions on a functional level, as both regulate CE movements during embryogenesis (Slusarski and Pelegri, 2007; Veeman et al., 2003a).

2 AIMS

This thesis examined molecular mechanisms of Wnt signal transduction and the functional relevance of Wnt-mediated signaling for the development of the VM and the differentiation of VM DA neurons. Specifically, the following questions were addressed:

- What is the role of the Wnt/β-catenin signaling pathway in the differentiation of mESCs into VM DA neurons?
- Does Wnt5a play a role in DA neuron development *in vivo*?
- What are the mechanisms and consequences of Wnt5a signaling during DA differentiation?
- Which possible novel regulators of Wnt/β-catenin-independent pathways are employed in Wnt5a-mediated signaling and how do they contribute to specification and/or transduction of the signal?

3 RESULTS AND DISCUSSION

3.1 PAPER I

3.1.1 Absence of Wnt1 or LRP6 increases neuronal differentiation and number of DA neurons in mESC cultures

Prior this study, results from our lab showed that partially purified Wnt1 CM increased the number of TH+ DA neurons obtained in mouse primary culture (Castelo-Branco et al., 2003), and that Wnt1 overexpression in mouse progenitor neurosphere cultures increased the number of Nurr1+ DA precursors (Parish et al., 2008). Due to strong evidence for the involvement of Wnt1 and the Wnt/β-catenin pathway in DA progenitor pool expansion and specification, we decided to examine the role of the Wnt/β-catenin signaling pathway in DA differentiation of mESC by genetically targeting the pathway at the ligand (Wnt1) and receptor (LRP6) level.

First, we developed a highly efficient protocol for derivation of mESC lines from C57/BL6 genetic background, which was considered a rather non-permissive strain for mESC line derivation (Bryja et al., 2006a; Bryja et al., 2006b; Nagy and Vintersten, 2006). Using this protocol we derived mESCs lacking Wnt1 (Wnt1-/-) or LRP6 (LRP6 -/-) as well as their corresponding wild type counterparts.

Next, we differentiated these mESCs using a protocol that combines the use of recombinant growth factors and PA6 stromal cell line co-culture (Barberi et al., 2003; Kawasaki et al., 2000). Unexpectedly, we obtained more TH+ neurons (TH - a rate limiting enzyme in synthesis of dopamine) as well as Tuj+ neurons (Tuj - a neuronal specific form of tubulin expressed in immature neurons) in cultures of Wnt1 -/- mESC compared to Wnt1 +/+. This finding was truly surprising, given the great reduction in number of DA neurons in Wnt1 -/- mice (Danielian and McMahon, 1996; McMahon and Bradley, 1990; Prakash et al., 2006; Thomas and Capecchi, 1990). To exclude possibility that the obtained results were a simple artifact of one specific cell line, we confirmed the enhanced generation of TH+ neurons with another pair of Wnt1 -/- and Wnt1 +/+ mESC lines (unpublished observation). Further, comparable increases in number of TH+ colonies, TH+ cells (unpublished observation), and TH protein expression level were observed in differentiated LRP6 -/- mESCs compared to their respective controls. Moreover, if the mESCs were differentiated for shorter period of

time (5-7 days instead of 14 days), increases in proportion of TH+ and Tuj+ colonies were detected both in Wnt1-deficient as well as in the LRP6-deficient cells.

Further, we did not observe any morphological differences between TH+ DA neurons obtained from mESCs lacking Wnt1 or LRP6 and control mESCs. Expression analyses of mRNA revealed that these cultures expressed typical DA neuron markers including Nurr1, Pitx3, Map2, DAT, Lmx1a, and Foxa2 both in Wnt1 -/- and LRP6 -/- cells as well as in their respective controls, indicating that they contained VM DA neurons. It would be of interest to elucidate how these neurons behave in terms of electrophysiological properties and whether they are capable of forming functional networks upon transplantation in a Parkinsonian mouse model.

Our findings were in contradiction to phenotypes described in mice lacking Wnt1 (Danielian and McMahon, 1996; McMahon and Bradley, 1990; Prakash et al., 2006; Thomas and Capecchi, 1990) or LRP6 (Castelo-Branco et al., 2010), but in agreement with reports linking impairment of Wnt/β-catenin pathway to expansion of neuroectoderm *in vivo* (Glinka et al., 1998; Kelly et al., 2004; Mukhopadhyay et al., 2001; Yoshikawa et al., 1997) and to enhanced mESC neuronal differentiation in culture (Aubert et al., 2002; Engberg et al., 2010; Haegele et al., 2003; Verani et al., 2007). Nonetheless, as such enhanced formation of neuroectoderm had not been demonstrated in Wnt1 or LRP6 deficient mice, respectively, we went for a more detailed validation of our findings.

3.1.2 Increased generation of DA neurons is not due to factors produced by feeder cells

Experiments with mESCs lacking LRP6, a critical receptor of the Wnt/β-catenin pathway, suggested that the enhanced generation of TH+/Tuj+ neurons we observed was based on a cell autonomous mechanism. However, the differentiation protocol we used was based on co-culture with PA6 cell line producing uncharacterized set of factors. This raised a possibility that a deficiency of the secreted ligand, Wnt1, was simply “rescued” by Wnt ligand(s) supplied by the PA6. To test this possibility, we differentiated the cells using a feeder-free protocol (Ying et al., 2003b). Quite remarkably, we obtained similar results showing enhanced yield of DA neurons in the Wnt1-deficient mESC cultures, which was further potentiated by addition of Shh/FGF8/bFGF. These results indicated that the differences observed between the +/+ and -/- mESCs resided in the cell autonomous ability of ESCs to differentiate into

TH+/Tuj+ neurons, independently of any recombinant growth factor added or produced by the feeder cells.

3.1.3 Attenuation of Wnt/β-catenin pathway increases the yield of mESC-derived DA neurons

As both Wnt1 and LRP6 are components of the Wnt/β-catenin pathway, we tested whether or not this pathway is affected in Wnt1 -/- and LRP6 -/- mESCs. We found an attenuated response to Wnt3a ligand stimulation in the case of LRP6 -/- mESCs, and a decreased expression of Wnt/β-catenin pathway target genes, Axin2 and Brachyury, in the case of mESC lacking Wnt1. Moreover, we were able to mimic the genetic ablation of Wnt1 or LRP6 to some extent by administrating Dkk1 during mESCs differentiation. Further, adding Wnt3a into the culture media during feeder-free differentiation of Wnt1 -/- mESCs led to a decrease in Tuj, TH, and Nurr1 mRNA levels (unpublished observation), thus "rescuing" the phenotype. Together, these experiments suggest that observed phenotypes are indeed caused by deficiency in Wnt/β-catenin signaling pathway during the mESC DA differentiation. Moreover, experiments with Dkk1 further demonstrate that an increased yield of DA neurons can also be achieved by the attenuation of Wnt/β-catenin signaling by soluble inhibitors.

3.1.4 How can decreased Wnt/β-catenin signaling contribute to neuronal and DA differentiation?

The enhanced generation of Tuj+/TH+ DA neurons in Wnt1 -/- and LRP6 -/- mESCs, respectively, is in agreement with the negative role of Wnt/β-catenin signaling on differentiation into neuroectodermal lineages both in ESC cultures (Aubert et al., 2002; Engberg et al., 2010; Haegele et al., 2003; Verani et al., 2007) and *in vivo* (Glinka et al., 1998; Kelly et al., 2004; Mukhopadhyay et al., 2001; Yoshikawa et al., 1997). However, these results from Wnt1 -/- and LRP6 -/- mESCs differentiation experiments did not fully reflect DA neuron development in Wnt1 -/- and LRP6 -/- mice *in vivo*.

The evidence obtained from mutant mice analyses suggests that the Wnt/β-catenin pathway is implicated in patterning the VM (Bally-Cuif et al., 1995; Pinson et al., 2000; Prakash et al., 2006) and in DA neurogenesis (Joksimovic et al., 2009; Tang et al., 2009). However, there is a difference in the extent of patterning and neurogenic defects

between Wnt1-deficient and LRP6-deficient mice. The absence of Wnt1 causes much more pronounced phenotypes in the VM than the absence of LRP6. Interestingly, there seem to be no other Wnt ligands capable to compensate for the Wnt1 loss. Therefore, one can assume that the lack of Wnt1, during critical period of midbrain development, also causes more severe impairment of Wnt signaling. On the other hand, the VM phenotypes in LRP6 -/- mice suggest that decreased Wnt/β-catenin signaling causes only minor patterning defects and/or a developmental delay in DA differentiation (Castelo-Branco et al., 2010; Pinson et al., 2000). In fact, selective attenuation of Wnt/β-catenin pathway in VM floor plate leads to decreases in the number of DA neuron being generated, but those that are born continue to differentiate and express markers of mature DA neurons such as DAT (Tang et al., 2009). Therefore, it seems that decreased Wnt/β-catenin signaling, to certain extent, still allows the generation and maturation of VM DA neurons.

In light of this data and our results we conclude that level of Wnt/β-catenin signaling present during differentiation of Wnt1 or LRP6-deficient mESCs is not a limiting factor for the efficient generation of DA neurons. This could be related to the fact that mESC cultures differentiate asynchronously, meaning that even at later stages of differentiation the cultures still contain some undifferentiated mESCs and proliferating neural progenitors. Further, cultures of differentiated mESCs tend to be heterogeneous, containing additional types of differentiated cells beside the DA neurons. Thus, cells in such cultures might be exposed to factors/conditions which their corresponding *in vivo* counterparts do not experience during development. We believe that this asynchrony and heterogeneity may have an impact on the level of Wnt/β-catenin signaling in differentiating mESC cultures.

Further, Wnt/β-catenin signaling is linked to cell cycle progression in various tissues (He et al., 1998; Panhuysen et al., 2004; Tetsu and McCormick, 1999). Therefore, the accelerated appearance of Tuj+ and TH+ neurons we observed in our cultures could have been a the consequence of a cell cycle defect, leading to premature cell cycle exit and differentiation. We did not observe any changes in proliferation of Wnt1 -/- mESCs, possibly due to low expression level of Wnt1 in undifferentiated mESC. However, mESCs lacking LRP6 showed slower proliferation rate (unpublished observation). Moreover, the attenuation of the Wnt/β-catenin pathway could have led to augmented Wnt/β-catenin-independent signaling (Bryja et al., 2009; Tahinci et al., 2007) and thereby to enhancement on DA differentiation (Bryja et al., 2007c; Castelo-

Branco et al., 2005; Parish et al., 2008). Which of these mechanisms accounted for the observed phenotypes is currently unclear.

In sum, this study demonstrated that neither Wnt1 nor LRP6 are *per se* required for the generation of DA neuron with characteristics of VM DA neurons in mESC cultures. Moreover, diminished Wnt/β-catenin signaling turned out to be beneficial for the yield of DA neurons derived from mESCs. All of these represent novel findings which have the potential to facilitate the development of CRT. In light of this study and current literature, it is clear that Wnt/β-catenin pathway regulates multiple aspects of mESC differentiation towards mature DA neurons. Dissecting out the precise temporal and spatial regulatory mechanisms and their orchestrated action in heterogeneous mESC cultures seems thus rather challenging. In this regard, different cell sorting strategies, isolation of more specified neural progeny and/or the use of strategies allowing conditional genetic ablation of Wnt pathway components will undoubtedly prove useful. As mentioned earlier, it is uncertain at this point whether DA neurons generated under conditions of attenuated Wnt/β-catenin signaling were fully functional or not. They showed characteristics of VM DA neurons; however whether or not all these neurons were indeed indistinguishable from *bona fine* VM DA neurons is at this point not entirely clear. Therefore, this issue should be addressed in the future, as interfering with Wnt/β-catenin signaling represents a promising tool for reducing the risk of excessive proliferation/tumor formation upon grafting of ESC-derived VM DA neurons in CRT for PD.

3.2 PRELUDE TO PAPER II, III, AND IV

The interest of our lab in the mechanisms and consequences of Wnt5a action on DA differentiation goes back to year 2003, when Goncalo-Castelo Branco et al. showed a positive effect of Wnt5a conditioned media (CM) on generation of TH+ neurons from Nurr1+ precursors in mouse primary neuron culture. Subsequent follow-up report identified Wnt5a as a DA differentiation-promoting factor secreted by VM glia (Castelo-Branco et al., 2005). Further insights into the role of Wnt5a brought its purification (Schulte et al., 2005), which allowed us to further analyze mechanisms of Wnt5a signaling. Our lab showed that purified Wnt5a did not activate Wnt/β-catenin

pathway in DA cells, but promoted DA differentiation, possibly via CK1δ/ε-mediated phosphorylation of Dvl (Bryja et al., 2007c).
Importantly, neither the role of Wnt5a in VM development nor the mechanisms underlying its functions in DA differentiation have been fully addressed before Papers II-IV.

3.3 PAPER II

3.3.1 Analyses of Wnt5a -/- mice

To address functional relevance of Wnt5a for the development of DA neurons, we analyzed Wnt5a -/- mice (Yamaguchi et al., 1999) for morphogenetic, proliferation, and/or differentiation defects in the VM. The number of TH+ neurons in the Wnt5a -/- mice seemed unchanged, except of transient increase at E14.5. Further, we checked for possible changes in proliferation and detected an increased proliferation at E11.5 in the VM (increase in number of BrdU+ and Ki67+ cells). Moreover, Wnt5a -/- mice showed an increased number of Nurr1+ DA precursors at E12.5. This implied that loss of Wnt5a led to an accumulation of Nurr1+ precursors and that their further differentiation into TH+ DA neuron was to some extent impaired. Indeed, we detected decreased proportion of TH+/Nurr1+ cells in the VM of Wnt5a -/- mice at E12.5. Nonetheless, Wnt5a *per se* was clearly not as crucial for DA differentiation *in vivo* as originally hypothesized based on experiments with Wnt5a CM in primary neuronal culture.
Interestingly though, while the total number of TH+ neurons was barely altered, the actual distribution of TH+ neurons in the midbrain was affected in Wnt5a -/- mice. The domain occupied by TH+ DA neurons expanded medio-laterally and shortened rostro-caudaly, thus mimicking defects in CE movements during neural tube formation. Moreover, additional neuronal populations in the VM were similarly affected. We conclude from these experiments that Wnt5a contributes to proper VM morphogenesis via regulation of CE movements. This conclusion is in agreement with the view of Wnt5a as a regulator of the Wnt/PCP pathway, since Wnt5a has been shown to genetically interact with core PCP components Vangl and Prickle during gastrulation and to regulate CE movements during formation of neural tube (Montcouquiol et al., 2006; Qian et al., 2007; Veeman et al., 2003b).

3.3.2 Wnt5a activates small GTPase Rac1, activity of which is required for Wnt5a-induced DA differentiation in primary culture

As already mentioned, previous studies in our lab pinpointed Wnt5a as a regulator of Wnt/β-catenin-independent signaling in SN4741 DA cells (Bryja et al., 2007c; Schulte et al., 2005). In light of these findings and our data from the analysis of Wnt5a -/- mice we checked the ability of Wnt5a to activate effectors of Wnt/PCP and other Wnt-driven β-catenin-independent signaling pathways in the SN4741 cells. Interestingly, we found that treatment with recombinant mouse Wnt5a activated small GTPases Rac1 and Rap1 (unpublished observation). Moreover, using the small molecule NCS23766, which acts as a competitive inhibitor of Rac1 activation (Gao et al., 2004), we abolished the effect of Wnt5a on DA neuron differentiation in E11.5 primary neuron culture. This data suggests that Rac1 also acts downstream of Wnt5a in primary cells and that Rac1 activation is a necessary component of the Wnt5a-mediated pro-differentiation effects on VM DA precursors.

3.3.3 Possible mechanisms of Wnt5a signaling in the developing VM

An earlier report from our lab suggested that additional Wnt ligands could be present in the developing VM, since mRNA transcripts of 13 different Wnts have been detected by Q-PCR (Rawal et al., 2006). It is therefore possible to speculate whether other Wnt ligands with comparable signaling capabilities and receptor affinities might have compensated for the lack of Wnt5a in the midbrain to some extent. In this regard, a strategy targeting pathway receptors may be more promising in elucidating the functional role of Wnt/β-catenin-independent signaling in DA neuron differentiation and midbrain morphogenesis. Nonetheless, there are 7 Frizzled receptors expressed in the VM at E9.5 (detected by ISH) (Fischer et al., 2007), and as many as 10 Frizzleds detected in the VM by Q-PCR (Rawal et al., 2006). As already mentioned, no study has systematically addressed binding affinities of Wnt5a to various Frizzled receptors. Frizzled2, 4, 5, 7, and 8 have been proposed as receptors of Wnt5a, as they mediate some aspects of Wnt5a-triggered signaling events and/or co-immunoprecipitate with Wnt5a (He et al., 1997; Chen et al., 2003; Ishitani et al., 2003; Mikels and Nusse, 2006; Nishita et al., 2010; Safholm et al., 2006; Sato et al., 2010; Wallingford et al., 2001). However, due to functional redundancy between different Frizzleds, the importance of individual Frizzled receptors for Wnt5a-mediated signaling is likely very context

dependent. Moreover, a recent study, aiming at elucidating binding affinities of Wnt3a, Wnt5a, and Wnt7a to Frizzled5 and Frizzled10, failed to detect an interaction between Wnt5a and Frizzled5-CRD (Carmon and Loose, 2010), despite of the fact that Wnt5a has been shown to functionally interact with Frizzled5 in other studies (He et al., 1997; Ishitani et al., 2003; Safholm et al., 2006).

On the other hand, Ror1/2 has been described to bind Wnt5a and mediate its effect, including activation of Rac1, in various mammalian cell types (Fukuda et al., 2008; Liu et al., 2008b; Mikels and Nusse, 2006; Nishita et al., 2006; Oishi et al., 2003; Sato et al., 2010). Therefore, current effort of our lab aims at analyzing the possible implication of Ror1/2 in DA differentiation *in vivo* (JC Villaescusa, in preparation). Another receptor proposed to mediate some aspects of Wnt5a-mediated signaling is Ryk. However, morphogenetic defects described in Ryk-deficient mice seem to be much less pronounced than in case of Wnt5a -/- mice (Halford et al., 2000; Lu et al., 2004; Yamaguchi et al., 1999).

Currently, questions remaining to answer are whether the impaired differentiation of Nurr1+ precursor into Nurr1+/TH+ neuron is in some way a consequence of the morphogenetic defect in the midbrain, and to what extent Rac1 is involved in DA differentiation and/or midbrain morphogenesis *in vivo*. In regards of the former, recent work from the W. Wurst lab has described gross morphogenetic defects (including collapse of the brain ventricles) in the midbrain of mice lacking Frizzled3/6. Interestingly, the patterning of the midbrain and DA differentiation were largely unaffected by the lack of both receptors (Stuebner et al., 2010). This suggests that the severity of the defect in CE movements is not linearly translated into a similar level of DA differentiation impairment, and, in light of our data, implies that Wnt5a signaling may separatedly contribute to both midbrain morphogenesis and DA differentiation *in vivo*.

Wnt5a stimulation of SN4741 cells and/or mouse embryonic fibroblast (MEFs) activated additional signaling components, such as CK1δ/ε and Rap1. CK1δ/ε was already functionally related to DA differentiation (Bryja et al., 2007c) (see Paper III for additional information). Whether the Wnt5a-mediated activation of Rap1 has any functional implication for DA neuron differentiation is currently being elucidated.

As our experiments have suggested, Wnt5a can activate more than one signaling route in our *in vitro* cellular systems, which in terms of expression of receptors and cytosolic components are relatively uniform. Therefore, it seems reasonable to expect that Wnt5a

activates multiple signaling routes also during development of the midbrain, since the expression of many pathway components changes during VM development (Rawal et al., 2006). Thus, although we do not have any experimental proof for our claim, in the light of our *in vitro* data, and published *in vivo* evidence from other systems, we think that Wnt5a activates multiple signaling routes in developing VM *in vivo*.

3.3.4 Is Rac1 Wnt5a effector *in vivo*?

Rac1 has a profound role in the regulation of cytoskeleton rearrangements, cell polarization, cell migration, and neuronal maturation. Moreover, the functional role of Dvl/Rac signaling in the regulation of PCP and/or CE movements has been well demonstrated (Fanto et al., 2000; Habas et al., 2003; Tahinci and Symes, 2003; Paper III). However, it is currently not clear to what extent Rac1 LOF is responsible for phenotypes observed in Wnt5a -/- mice. Direct experimental evidence addressing functional relevance of Rac1 for the development of VM is missing, as Rac1 -/- mice fail to finish gastrulation and die before E9.5, thereby hampering possible analyses of midbrain development (Sugihara et al., 1998). Thus, future strategies should aim at conditional ablation of Rac function in the developing VM to address the functional requirement of Rac for midbrain morphogenesis and/or DA neuron differentiation. However, Rac3, another member of the Rac protein family highly redundant to Rac1, is expressed together with Rac1 in almost all areas of the developing brain and compensates for loss of Rac1 (de Curtis, 2008; Tahirovic et al., 2010). Alternatively, tissue explants culture combined with live imaging techniques using biosensors of Rac1 activation may represent a potent strategy to uncover novel aspects of both spatial and temporal regulation of Rac1 activity in the VM.

3.4 PAPER III

At the time when this study began, results from our lab demonstrated that Wnt5a activated the Wnt/Dvl/Rac signaling route of the Wnt/PCP pathway and inhibited Wnt/β-catenin signaling in DA cells. Furthermore, various studies have suggested that Wnt-signaling pathways are mutually interconnected to form a signaling network. However, despite the fact that many reports had characterized new Wnt-driven signaling cascades and identified novel pathway components, the relations between different Wnt/β-catenin-independent signaling pathways and individual pathway

components with the network had not been fully understood. In this regard, we realized that some of our questions regarding mechanisms of Wnt5a action were difficult to address by relying on DA cells only. We thus sought to investigate the mechanisms and consequences of Wnt5a signaling in a broader context of other systems.

Prior to the beginning of this study, a link connecting β-arrestin to Wnt/PCP signaling had been demonstrated by showing the requirement for β-arrestin2 in Wnt5a and PKA induced Frizzled4 internalization (Chen et al., 2003). However, the importance of β-arrestin for Wnt/PCP signaling as such had not been addressed.

3.4.1 β-arrestin is a novel component of the Wnt/Dvl/Rac signaling route

We tested the functional requirement for β-arrestin in Wnt5a-induced Rac1 activation and phoshorylation of Dvl in MEFs lacking β-arrestin1/2. While phoshorylation and the mobility shift of Dvl (PS-Dvl) after Wnt5a treatment showed slower kinetics compared to control, activation of Rac1 was completely abrogated in cells lacking β-arrestin1/2. This observation fitted well with previous studies identifying β-arrestin as a Dvl-binding partner (Bryja et al., 2007b; Chen et al., 2003) and positioned β-arrestin upstream/at the level of Dvl in the Wnt/Dvl/Rac signaling branch (note that β-arrestin is required for the activation of Rac1 by Wnt5a but not by Dvl). To get further insight into the functional aspects of β-arrestin in the context of the Wnt/PCP pathway we took advantage of the *Xenopus* embryo Keller explants assay (Figure 10).

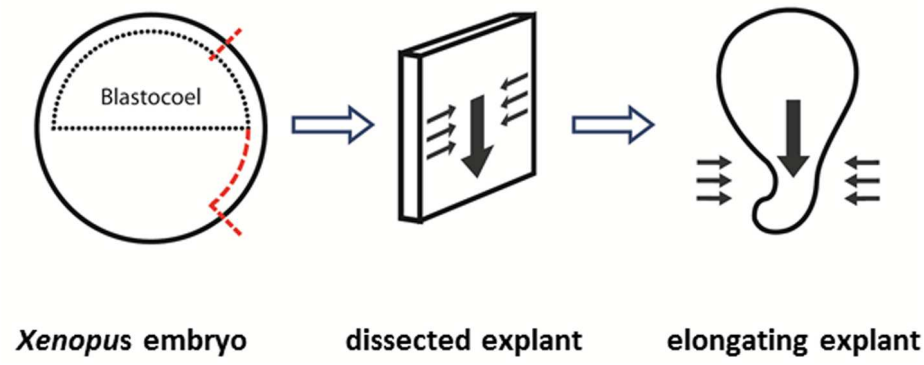

Figure 10: Keller explants of pre-gastrulation *Xenopus* embryos represent useful tools to study CE movements in a situation very close to *in vivo*. Upon dissection (indicated by dashed line) and subsequent cultivation, the prospective mesoderm tissue from the dissected explant narrows and elongates (Keller, 1991).

We confirmed the requirement of β-arrestin for CE movements, as knockdown of β-arrestin expression impaired elongation of explants. Moreover, we showed that this phenotype could be rescued by constitutive active Rac1 and RhoA, thus confirming the status of β-arrestin as a regulator of both Wnt/Dvl/Rac and Wnt/Dvl/RhoA signaling routes during CE movements. The findings were in agreement with a report published during the preparation of our study, which identified β-arrestin2 as an upstream regulator of RhoA during *Xenopus* gastrulation (Kim and Han, 2007).

Next we aimed at identifying the Wnt ligand acting upstream of β-arrestin in elongating *Xenopus* embryo explants. Our results had clearly demonstrated that β-arrestin is required for CE movements/Wnt/PCP signaling in *Xenopus*. However, Wnt5a, the lingand which we used in our experiments with mammalian cells, was not required for elongation of explants (Schambony and Wedlich, 2007; Unterseher et al., 2004). In light of this data we hypothesized that signaling of Wnt5a in *Xenopus* Keller explants is β-arrestin independent. Indeed, we did not find any evidence of genetic interactions between Wnt5a and β-arrestin in this system. On the other hand, our rescue experiments demonstrated genetic interactions between β-arrestin and another Wnt ligand, Wnt11, which supported the view of Wnt11 as the main regulator of Wnt/PCP pathway during CE in gastrulating *Xenopus* embryo (Habas et al., 2003; Habas et al., 2001).

Finally, it is worth noting that the mechanisms accounting for different downstream signaling of Wnt5a in mammalian cells (Wnt5a/Dvl/Rac signaling route) and in *Xenopus* explants (Wnt5a/PI3K/Cdc42) have not been characterized. Since both signaling routes seem to utilize Ror2, it is the employment of different Frizzled receptors and/or cytosolic adaptor proteins which may account for the divergence of signaling downstream of Wnt5a/Ror2.

3.4.2 Multiple roles of β-arrestin-which of them matters for the Wnt/Dvl/Rac signaling?

Interestingly, recent evidence has proposed that β-arrestin-dependent clathrin-mediated endocytosis of Frizzled plays an important role in the regulation of the Wnt/PCP pathway during CE movements (Chen et al., 2003; Kim et al., 2008). However, evidence from the GPCR field also pinpoints β-arrestin as a scaffolding protein important for activation of downstream signaling. Interestingly, such a role has also recently been proposed for β-arrestin in the context of Wnt/β-catenin-independent

signaling (Kim and Han, 2007). In this regard, it is interesting to speculate whether is the requirement of β-arrestin for efficient Wnt/Dvl/Rac signaling underpinned by its involvement in receptor endocytosis or its role as scaffolding protein. We attempted to address this issue by using hyperosmotic sucrose or K+ depletion to block endocytosis and thereby assess its impact on Wnt/Dvl/Rac signaling. Surprisingly, we found that block of clathrin-mediated endocytosis by the means just mentioned led to efficient depletion of Dvl at protein level (Bryja et al., 2007a). Despite this is an interesting finding, these experiments are rather inconclusive regarding the precise mechanistical role of β-arrestin in Wnt/Dvl/Rac signaling.

3.4.3 Roles of CK1 and CK2 in Wnt-driven signaling pathways

Our finding that β-arrestin was critical for Wnt5a-induced Rac1 activation in MEFs but not essential as such for the appearance of PS-Dvl raised an interesting question. Is the formation of PS-Dvl dispensable for Wnt/Dvl/Rac signaling activation? To address this possibility we looked at whether manipulation of the phosphorylation status of Dvl somehow affected Rac1 activity. CK1 and CK2 seemed to be good candidates for testing our hypothesis, both were shown as Wnt-dependent kinases able to phosphorylate Dvl and capable of interacting with β-arrestin (Swiatek et al., 2004; Willert et al., 1997; Xiao et al., 2007). Interestingly, when we performed the experiment in MEFs, using CK1 and CK2 inhibitors, we found that a block of Dvl phosphorylation was accompanied by strong activation of Rac1. This was also observed in lysates from *Xenopus* explants, proposing that PS-Dvl acts as a negative regulator of Wnt/Dvl/Rac signaling. However, as CK2 as well as the members of CK1 family are rather “promiscuous” kinases in terms of number of targets they phosphorylate, and the inhibitors we used could have side effects, we went for further confirmation experiments. First, overexpression of CK1ε decreased the level of activated Rac1 induced by Dvl overexpression in MEFs. Next, inhibition CK1 rescued the LOF defect in *Xenopus* explants, thus mimicking the effect of constitutive active Rac1. Importantly, both the rescue effect on elongation of explants and the positive effect on Rac1 activation were dependent on the presence of Dvl. These experiments therefore confirmed the status of CK1 as a negative regulator of the Wnt/Dvl/Rac signaling route acting at the level of Dvl.

We showed that Wnt/Dvl/Rac signaling is also involved in Wnt5a-mediated effect on DA neuron differentiation (Paper II). Therefore, one would assume that inhibition of CK1, which leads to activation of Rac1, would have an effect opposite to Rac1 inhibition on DA differentiation. However, earlier work from our lab has suggested that the Wnt5a-mediated DA neuron differentiation is also blocked by CK1 inhibitor (Bryja et al., 2007c). Therefore, as was already emphasized, it is very possible that Wnt5a triggers multiple signaling cascades during DA differentiation, and it is either their orchestrated or sequential action which is actually needed for the DA differentiation-promoting effect of Wnt5a. In this regard, there is a clear parallel to experiments with *Xenopus* embryo explants, where both LOF and GOF also give rise to similar phenotypes. While in the frog this is attributed to loss of cell polarity, it is unclear whether similar mechanisms underlie the effects of CK1 and Rac1 inhibition and how they translate to the block of DA differentiation.

Compared to CK1, CK2 showed a slightly different behavior during our validation experiments. Biochemical experiments with depletion of Dvl protein suggested that the effect of CK2 inhibitor on Rac1 activation did not required Dvl *per se*. Assuming a relative specificity of the CK2 inhibitor used in this study, this finding argued for employment of CK2 in both Dvl/β-arrestin-dependent and independent events, which is not that surprising in light of the numerous cellular processes controlled by CK2. A series of experiments in frog suggested that CK2 acted as a negative regulator of β-arrestin dependent signaling (CK2 failed to rescue the β-arrestin LOF phenotype), but at the same time might have had a positive function in other signaling routes, regulated by Wnt11 during explants elongation, but not requiring β-arrestin. Interestingly, β-arrestin2 knockdown does not affect Wnt/Ca^{2+} signaling via PKC in *Xenopus* (Kim and Han, 2007) and some aspects of Wnt/Ca^{2+} signaling seem to be independent of β-arrestin binding partner Dvl (Ma and Wang, 2007). Moreover, CK2 has been shown to phosphorylate Ca^{2+}-binding protein Calmodulin and thereby affects signaling events induced by Ca^{2+} transients (Arrigoni et al., 2004). Thus, the Wnt/Ca^{2+} pathway is a plausible candidate for a Wnt11-driven β-arrestin-independent pathway regulating gastrulation movements and utilizing CK2 activity (Garriock and Krieg, 2007; Panakova et al., 2010). However, direct experimental evidence of CK2 employment downstream of Wnt/Ca^{2+} signaling has not been demonstrated and should be addressed by future experiments. Moreover, due to increasing evidence of interactions between Wnt/PCP signaling routes and Wnt/Ca^{2+} signaling, it would be interesting to try to

dissect out nodes of interaction within this network. In this regard, application of small GTPase and Ca^{2+}biosensors combined with live cell imaging techniques may prove useful.

In summary, our study demonstrated a function of β-arrestin as a novel regulator of Wnt/Dvl/Rac signaling and pointed out CK1 and CK2 as negative regulators of this signaling route and switches between different cascades of Wnt-induced β-catenin-independent signaling, more specifically between Wnt/Dvl/Rac and Wnt/PS-Dvl.

3.5 PAPER IV

3.5.1 Search for Rac1 activators

In this study we aimed to elucidate the mechanism of Wnt5a-mediated activation of Rac1 in DA cells, since our data linked the pro-differentiation effect of Wnt5a with the activation of Rac1 (Paper II). Specifically, our effort mainly aimed at the identification of GEF(s) responsible for Rac1 activation in the context of the Wnt/Dvl/Rac signaling route. Interestingly, while the role of GEFs in the activation of Rho GTPases had been studied for decades, and Rho GTPases were identified as downstream components of Wnt signaling more than 10 years ago, there was very sparse evidence regarding the use of particular GEF in Wnt-driven signaling cascades at the beginning of this study.
Our initial strategy was to immunoprecipitate Rac1, and to subsequently identify its binding partners by mass spectrometry. Unfortunately, this approach in its original design did not lead to the identification of any candidate GEF of Rac1. In this regard, interactions between GEFs and small GTPases seem to be transient (Worthylake et al., 2000). This transient character of the interactions could account for the failure of our first strategy, by preventing us to obtain sufficient amounts of GEF-Rac1 protein complexes for identification by mass spectrometry. Therefore we searched for alternative bait for our proteomic experiment. Dvl seemed as a good candidate, thanks to its function as scaffolding protein and regulator of Rac1 activation.

First, we decided to characterize the Dvl-Rac1 interaction, which had been proposed in earlier studies (Habas et al., 2003; Rosso et al., 2005). Indeed, we detected recruitment of endogenous Rac1 into Dvl2/3 polymers, which appeared in a form of cytosolic puncta upon Dvl2/3 overexpression. Moreover, we confirmed by FRET analyses that

Rac1 and Dvl were located in close proximity within these protein assemblies. We mapped the region of Dvl mediating the formation of the Dvl-Rac1 complex to its N-terminal part. As the ability of Dvl to form polymers had been attributed to its N-terminal DIX domain, and this polymerization ability has been proposed as a prerequisite for efficient Dvl-Rac1 interaction (Nishita et al., 2010), we tested if the Dvl-Rac1 interaction was somehow affected by the inability of Dvl to polymerize. Interestingly, we found that Dvl-point mutants, lacking the polymerization ability, interacted with Rac1 in a manner very similar to the wild type form of Dvl. Thus, we concluded that the DIX-mediated polymerization of Dvl was not required *per se* for Dvl-Rac1 interaction. Future experiments should address whether the Dvl-Rac1 interaction is direct or possibly mediated by another binding partner.

3.5.2 Tiam1, a Rac1 GEF, is expressed in developing VM and interacts with Dvl

The results we obtained upon characterization of the Dvl-Rac1 interaction prompted us to carry on the proteomic experiment using Dvl as bait, which subsequently led to identification of Tiam1 as a candidate Dvl-interacting protein. As the initial observation of Dvl-Tiam1 interaction came from a non-neuronal cell line, we analyzed the expression of Tiam1 in the SN4741 DA cell line and in the midbrain, and found that Tiam1 is expressed in the developing VM as early as at E10.5, at the onset of DA neurogenesis.

Next, to confirm the Dvl-Tiam1 interaction, we overexpressed Tiam1 and Dvl, and detected their colocalization in puncta and their interaction by mutual co-immunoprecipitation. Moreover, we found that the N-terminal region of Dvl, which was critically required for Dvl-Rac1 co-immunoprecipitation, was not required for the Dvl-Tiam1 interaction. Thus, we concluded that Rac1 was not mediating the Dvl-Tiam1 interaction. However, which parts of Dvl and Tiam1 are involved in their mutual interaction needs to be addressed in future experiments. In this regard, the PDZ domain of Dvl looks like a plausible candidate, as Tiam protein contains a PDZ-binding motif at its C-terminus (Garcia-Mata and Burridge, 2007). Preliminary results suggest that the Dvl-PDZ domain is indeed involved in the interaction with Tiam1.

3.5.3 Functional aspects of Tiam1 in the Wnt/Dvl/Rac signaling and DA differentiation

Tiam1 is well known as a GEF for Rac1 (Collard et al., 1996; Mertens et al., 2003; Michiels et al., 1995; Sasaki et al., 2010). Since our data suggested that Tiam1 forms a complex with Dvl, we decided to test if Tiam1 was involved in the transduction of signal between Dvl and Rac1. Interestingly, we did not detect almost any Dvl-mediated activation of Rac1 after knockdown of Tiam1 expression by siRNA, while Dvl overexpression induced Rac1 activation in control condition. Moreover, our preliminary results suggest that the ability of Wnt5a to trigger Rac1 activation is also impaired by Tiam1 siRNA. Thus, based on these experiments we conclude that Tiam1 is required for Rac1 activation in the context of the Wnt/Dvl/Rac signaling.

Agonist-induced membrane recruitment of both Tiam1 and Dvl has been previously documented (Buchanan et al., 2000; Cliffe et al., 2003; Park et al., 2005). In our experiment, the membrane associated pool of Tiam1 did not colocalize with Dvl. This data suggested that the recruitment of Tiam1 to the membrane is not sufficient to induce translocation of Dvl to the membrane in the absence of exogenous Wnt ligand. Furthermore, it is interesting to speculate how the Dvl-Tiam1 complex described here is affected by acute Wnt ligand stimulation, presence/absence of another Dvl-interacting protein(s), and/or the status of Dvl phosphorylation. In fact, data not included in this manuscript suggests that overexpression of CK1ε negatively regulates Dvl-Tiam1 interaction. This is in agreement with the role of CK1ε as a negative regulator of the Wnt/Dvl/Rac signaling route (Paper III) and suggests that changes in protein-protein interactions between Dvl and Tiam1 may mediate activation of Rac1 via Tiam1. Further studies should address this possibility in detail.

We described expression of Tiam1 in the developing mibrain and demonstrated the requirement of Tiam1 for efficient Wnt/Dvl/Rac signaling. As the Wnt/Dvl/Rac signaling route is involved in DA neuron differentiation (Paper II), our obvious next step in this study was to test the role of Tiam1 in DA differentiation. Due to difficulties with transfection of primary cells, we used expanded DA progenitor neurosphere culture and their subsequent differentiation as a model to address this hypothesis. Our experiments showed a decrease in mRNA levels of the neuronal marker Tuj, and DA neuron marker TH, as well as a decrease in the number of TH+ DA neurons after

Tiam1 siRNA. This suggested a role for Tiam1 in the differentiation of postmitotic precursors into TH+ DA neurons, as expression levels of early DA markers Nurr1 and Pitx3 remained unchanged. The knockdown of Tiam1 also led to a decrease in Tuj mRNA. This finding suggests that Tiam1 may also regulate other aspects of neuronal development in non-DA neurons. Together, our experiments demonstrate a functional role of Tiam1 in the generation of DA neurons, but their interpretation in the context of Wnt/Dvl/Rac signaling is less straightforward, since no exogenous Wnt ligand was applied during neurosphere differentiation. Therefore, to fully link Tiam1 deficiency to attenuated Wnt/Dvl/Rac signaling, an experiment combining Wnt5a treatment/overexpression with Tiam1 si/sh RNA should be performed in the near future.

On a functional level, Tiam1-mediated Rac1 activation has been mostly studied for its role in cancer (Habets et al., 1994; Malliri et al., 2002; Mertens et al., 2003; Strumane et al., 2009). Moreover, Tiam1 was identified as direct target gene of Wnt/β-catenin signaling pathway in colon cancer cells (Malliri et al., 2006). However, Tiam1 is also expressed in the developing mouse brain, and has been linked to the regulation of axon and neurite outgrowth, and neuronal migration (Ehler et al., 1997; Kawauchi et al., 2003; Leeuwen et al., 1997; Tanaka et al., 2004). Moreover, our experiments also show expression of Tiam1 in the developing midbrain. However, whether Tiam1 expression in the midbrain is also controlled by Wnt/β-catenin signaling is not clear. Analyses of Wnt/β-catenin pathway activation in the developing midbrain of the Top-gal reporter mice revealed a peak of activity at E10.5 (Castelo-Branco et al., 2003). Therefore, in light of this finding it seems plausible that the gradual increase in Tiam1 mRNA expression we observed between E10.5 and E15.5 was maintained independently of the Wnt/β-catenin pathway. Further, as no gross developmental defects in the CNS, resembling Wnt/PCP pathway defects, have been reported in Tiam1-deficient mice (Malliri et al., 2002), it is possible that other Rac1 GEFs compensate for lack of Tiam1 in the developing brain. However, a detailed analyses of VM development in Tiam1 -/- mice has not been reported.

Attenuation of Rac1 activity is linked to impaired cytoskeleton remodeling and loss of cell polarity, which in the context of neuronal differentiation can be reflected by impaired neurogenic cell division and/or neuritogenesis (Govek et al., 2005; Heasman and Ridley, 2008; Mertens et al., 2006; Minobe et al., 2009; Zhang and Macara, 2006).

Wnt5a/Dvl-mediated signaling events are also implicated in the regulation of polarity in differentiating neurons (Endo and Rubin, 2007; Li et al., 2010; Schlessinger et al., 2009; Zhang et al., 2007). Thus, the cell polarity may underlie the requirement of Wnt5a/Dvl/Rac signaling for efficient DA differentiation. A recent report has described a novel function of Wnt5a in the regulation of the distribution of chemokine receptors at the cell membrane, thus enabling efficient cell response to a chemokine gradient (Witze et al., 2008). Interestingly, the role of chemokines as factors regulating DA differentiation has recently emerged (Edman et al., 2008). Whether or not the Wnt5a signaling indeed regulates responsiveness of DA cells to chemokine gradient remains to be experimentally tested. Moreover, the regulation of gene expression via Tiam/Rac/AP1 represents another possible mechanism controlling DA differentiation. Interestingly, AP1 binding sites have been identified in the promoters of several DA genes (Nagamoto-Combs et al., 1997; Seo et al., 1996). However, to which extent AP1-mediated transcriptional regulation is implicated in Wnt5a signaling is not entirely clear, because very few target genes of AP1 have been shown to be regulated in a Wnt5a-dependent manner. Moreover, we attempted to address the possible contribution of Wnt5a signaling to direct changes in gene expression in mESCs using Illumina technology, and found hardly any genes differentially expressed after 2h or 6h stimulation with Wnt5a (unpublished observation). Therefore, the contribution of Wnt5a/Rac1-mediated regulation of transcription to DA differentiation remains an interesting, but at the moment a rather theoretical possibility. We currently have no evidence supporting that.

Thus, at this point is not clear which function of Tiam1/Rac makes these proteins important for DA differentiation. Further studies will be needed to elucidate this matter.

4 CONCLUSIONS

Based on the work compiled in this thesis, the following conclusions have been made:

- Attenuation of the Wnt/β-catenin pathway increases the yield of midbrain DA neurons obtained upon DA differentiation of mESCs
- Wnt5a controls both proper morphogenesis of the midbrain and differentiation of precusors into DA neurons
- Small GTPase Rac1 is a mediator of Wnt5a-induced DA neuron differentiation
- β-arrestin is a necessary component of the Wnt/Dvl/Rac signaling route
- CK1 and CK2 regulate distinct routes of Wnt/β-catenin-independent signaling at the level of Dvl
- Tiam1 is responsible for Wnt5a/Dvl-induced Rac1 activation and is involved in DA differentiation

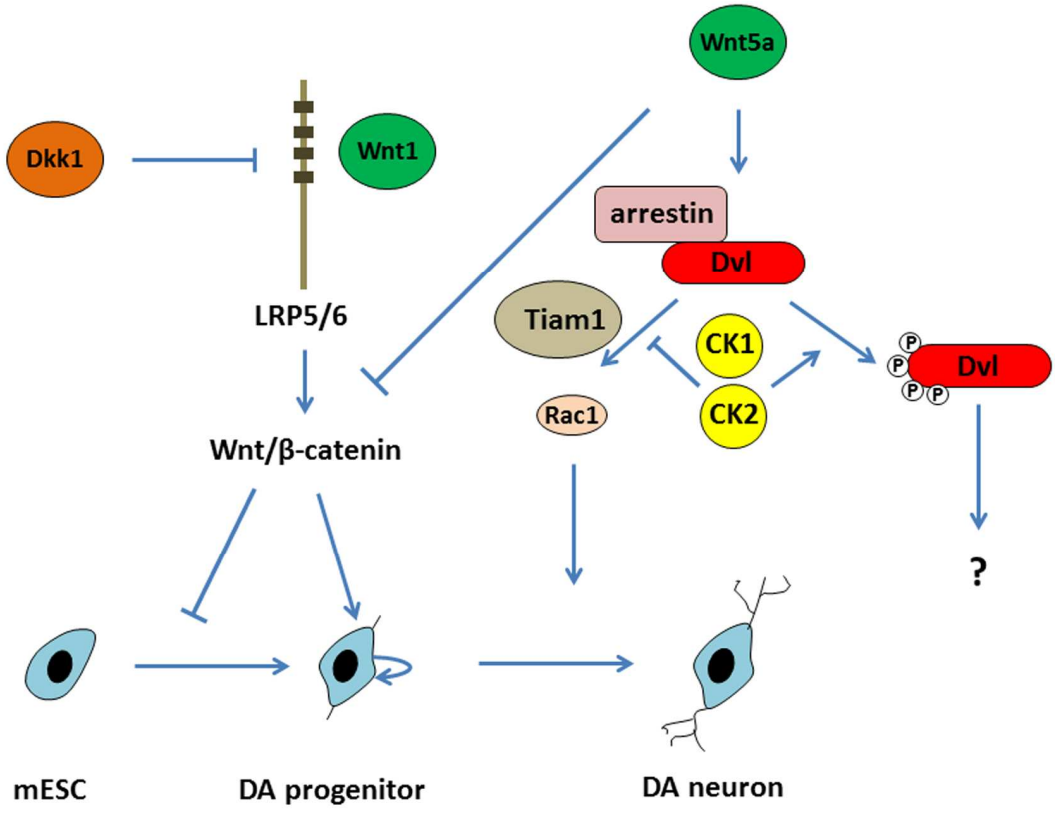

Figure 11: Model of Wnt1/LRP6 and Wnt5a functions in DA differentiation based on published evidence and findings presented in this thesis.

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