Division of Molecular Neurobiology
Department of Medical Biochemistry and Biophysics
Karolinska Institutet, Stockholm, Sweden

THE WNT PATHWAY IN VENTRAL MIDBRAIN DOPAMINERGIC DIFFERENTIATION

Diogo Ribeiro

Karolinska Institutet

Stockholm 2011

Published by Karolinska Institutet. Printed by LarsErics Digital AB

ISBN 978-91-7457-194-3

Cover:

On the cover is a picture of dopaminergic neurons derived from human fetal ventral midbrain cells in the presence of Wnt5a. Dopaminergic neurons express the pan-neuronal marker Tuj1 (in green) and tyrosine hydroxylase (in red). Hoechst staining (in blue) labels the cells nuclei. This thesis has focused, amongst other topics, on strategies to improve the dopaminergic yield from human fetal mesencephalon tissue for cell-replacement therapies.

"The doubter is a true man of science; he doubts only himself and his interpretations, but he believes in science."

Claude Bernard

ABSTRACT

Parkinson's disease (PD) is a debilitating neurodegenerative disease in which dopaminergic (DA) neurons in the substantia nigra are lost. Current treatments such as administration of levodopa (L-DOPA) are initially effective but the disease eventually progresses, highlighting the need for a treatment that restores and maintains the otherwise lost functions. Cell-replacement therapy (CRT), where DA neurons and/or precursors are grafted into the striatum in order to restore the lost striatal DA transmission, are considered a promising treatment. In order to implement CRT, large numbers of correctly specified ventral midbrain (VM) dopaminergic (DA) neurons should be obtained. Therefore, a lot of effort has been put into identifying cell sources from which high numbers of DA neurons can be generated and also into the study of the intrinsic and extrinsic factors that regulate DA neuron development. In our lab, we have focused on the Wnt pathway and its extracellular modulators which we believe to be of value for CRT as it does not imply genetic modification of the cells. In this thesis, we have investigated the role of Wnt pathway components such as Wnt1, Wnt5a, Lrp6 and Dkk1 in VM DA neuron development and in stem cell DA differentiation.

The Wnt pathway regulates several important processes such as cell proliferation, fate determination, differentiation and patterning. The ligand Wnt1 plays a crucial role in DA VM midbrain patterning and specification: in its absence most of the midbrain fails to be formed; Wnt1 promotes the specification of the DA progenitor domain and differentiation *in vivo*, and the proliferation of DA precursors *in vitro*. Prior to this thesis, the role of the Wnt receptor Lrp6 in VM DA development and specification had not been elucidated. To determine the role of the Wnt/β-catenin pathway in DA specification, mouse embryonic stem cells (mESC) lacking the Wnt1 ligand or the Lrp6 receptor were induced to differentiate towards a VM DA phenotype using established protocols. Our results revealed that an impaired Wnt/β-catenin pathway, at the ligand or receptor level, improved neurogenesis and DA differentiation from mESC, Moreover, addition of Dickkopf 1 (Dkk1), a Wnt inhibitor, mimicked this effect in a mESC line, confirming that mESC-derived DA differentiation is improved by impairing the Wnt/β-catenin pathway and that current protocols to induce DA differentiation can be enhanced by addition of soluble factors such as Dkk1.

We next sought to investigate the role of Lrp6 in VM DA neuron development *in vivo*. Analysis of *Lrp6*$^{-/-}$ embryos revealed a normal midbrain patterning and a decreased

differentiation of DA neurons, which later recovered. Thus, in the absence of *Lrp6* there is a delay in DA neuron differentiation.

Given the results obtained with mESC, we decided to further assess the role of Dkk1 in DA development. Our results revealed that *Dkk1* is expressed in the VM just prior to the onset of DA neurogenesis. In *Dkk1*$^{+/-}$ mice we could not detect any changes in the number of DA progenitors and neurons at E11.5. However, at E13.5 there was a significant reduction in the number of DA neurons. DA progenitors and precursors were not affected, there were no differences in cell death and other ventral midbrain populations were unchanged, suggesting a DA specific differentiation impairment. At later stages, we could still detect a 30% reduction in the number of DA neurons and an abnormal distribution in the VM. Analysis of the few surviving *Dkk1*$^{-/-}$ embryos at E17.5 revealed very few or almost absent DA neurons which, when present, were abnormally distributed and had a very immature morphology. Together, these results suggest a role of Dkk1 in VM DA differentiation and morphogenesis.

Finally, in order to address some of the limitations of using human fetal VM cells for CRT, we have evaluated whether a protocol that allows the generation of large and functional numbers of DA neurons from mouse VM cells could be applied to human cells. Cells were expanded in the presence of DA-appropriate factors for 2 weeks and induced to differentiate to assess their DA potential. Our results show that human fetal VM cells can be successfully expanded 2 to 3-fold, retain their VM DA identity during expansion and give rise to large and increasing numbers of DA neurons. Moreover, addition of Wnt5a, which has been shown to promote DA differentiation in several systems, also promoted a significant increase in the numbers of DA neurons after 2 weeks of expansion.

In sum, the results presented in this thesis describe new functions of the Wnt/ β-catenin pathway in DA differentiation from embryonic stem cells and *in vivo*, a novel regulator of DA differentiation and an efficient protocol for expansion and differentiation of human fetal VM cells. We believe this knowledge can be successfully applied and improve current and future stem cell therapies in PD.

LIST OF PUBLICATIONS

I. Čajánek, L., Ribeiro, D., Liste, I., Parish, C. L., Bryja, V. and Arenas, E. (2009), **Wnt/β-Catenin Signaling Blockade Promotes Neuronal Induction and Dopaminergic Differentiation in Embryonic Stem Cells.** Stem Cells, 27: 2917–2927

II. Castelo-Branco, G., Andersson, E. R., Minina, E., Sousa, K. M., Ribeiro, D., Kokubu, C., Imai, K., Prakash, N., Wurst, W. and Arenas, E. (2010), **Delayed dopaminergic neuron differentiation in *Lrp6* mutant mice**. Developmental Dynamics, 239: 211–221

III. Diogo Ribeiro, Kristina Ellwanger*, Désirée Glagow*, Spyridon Theofilopoulos, Nina S. Corsini, Ana Martin-Villalba, Christof Niehrs and Ernest Arenas, **Dkk1 regulates ventral midbrain dopaminergic differentiation and morphogenesis.** PLoS ONE, *in press*

IV. Diogo Ribeiro, Rocio Laguna Goya, Tobias Piroth, Clare L. Parish, Malin Parmar, Olle Lindvall, Guido Nikkhah, Roger Barker and Ernest Arenas, **Efficient expansion and differentiation of human ventral midbrain neural stem cells.** *Manuscript*

*denotes equal contribution

CONTENTS

LIST OF ABBREVIATIONS

APC	Adenomatous Polyposis Coli
AADC	Aromatic L-amino acid decarboxylase
AVE	Anterior visceral endoderm
β-TrCP	Beta-transducin repeat containing protein
bFGF/FGF	Basic fibroblast growth factor
BDNF	Brain derived neurotrophic factor
BMP	Bone morphogenetic protein
Ca^{2+}	Calcium
CAMIIK	Ca^{2+}/calmodulin-dependent protein kinase
Cdc42	Cell division control protein 42 homolog
Cdx2	Caudal-type homeobox protein 2
CE	Convergent-extension
CK1	Casein kinase
CNS	Central nervous system
CRD	Cysteine rich domain
CRT	Cell replacement therapy
DA	Dopaminergic
Dat	Dopamine transporter
dcAMP	Dibutyryl adenosine monophosphate
Dkk1	Dickkopf1
DVE	Distal visceral endoderm
Dvl	Dishevelled
E	Embryonic day
EGF	Epidermal growth factor
EMT	Epithelial-to-mesenchyme transition
En	Engrailed
ESC	Embryonic stem cells
FGF	Fibroblast growth factor
Fgf8	Fibroblast growth factor 8
Foxa2	Forkhead box a2
Fzd	Frizzled
Gbx2	Gastrulation brain homeobox 2
GDNF	Glial derived neurotrophic factor
GFP	Green fluorescent protein
GSK-3β	Glycogen synthase kinase
HDAC	Histone deacetylases
hESC	Human embryonic stem, cells
Hnf3 β	Hepatocyte nuclear factor 3 β
ICM	Inner cell mass
ICD	Intracellular domain
iPS	Induced pluripotent stem cells
IZ	Intermediate zone
JNK	c-Jun NH2-terminal kinase
L-DOPA	Levodopa (L-dihydroxyphenylalanine)

Lmx1a/b	LIM homeodomain transcription factor
LXR	Liver X receptor
LDLR	Low density lipoprotein receptor
LRP	LDL receptor-related proteins
MHB	Midbrain-hindbrain boundary
mESC	Mouse embryonic stem cells
MMTV	Mouse mammary tumor virus
mRNA	Messenger RNA
Msx1	Muscle segment homeobox 1
MZ	Marginal zone
Ncor2	Nuclear receptor co-repressor 2
Ngn2	Neurogenin 2
Nurr1	Nuclear receptor related 1 protein
Oct4/Pou5f1	POU domain, class 5, transcription factor 1
Otx2	Orthodenticle homologue 2
PCP	Planar cell polarity
PD	Parkinson's disease
Pitx3	Paired-like homeodomain 3
PP1	Protein phosphatase 1
PP2A	Protein phosphatase 2a
Ror2	Receptor tyrosine kinase-like orphan receptor 2
Ryk	Receptor-like tyrosine kinase
TGF β	Transforming growth factor β
TCF/LEF	T-cell specific transcription factor/ lymphoid enhancer factor
TLE	Transducin-like enhancer of split
Shh	Sonic hedgehog
sFRP	Soluble Frizzled-related protein
SN	Substantia nigra
SNpc	Substantia nigra pars compacta
VTA	Ventral tegmental area
VZ	Ventricular zone
Wg	Wingless
Wnt	Wingless-related MMTV integration site

1 INTRODUCTION

Parkinson's disease (PD) is a neurodegenerative disorder characterized by resting tremor, rigidity and slowness of movement (hypokinesia). The motor symptoms of PD are a result of a progressive loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc), which project to the striatum and are involved in motor control. The gold standard for PD treatment is the administration of levodopa (L-DOPA), which is converted into dopamine by the remaining DA neurons. Although the initial use of L-DOPA is effective in symptomatic treatment of PD, its efficacy often declines after long-term therapy with additional disabling side-effects such as dyskinesias (Chase et al., 1993). Moreover, current treatments such as L-DOPA and deep brain stimulation do not halt the progression of the disease, demonstrating the need for treatments which are not only able to restore lost functions but also maintain function and provide symptomatic relieve. A promising approach is cell-replacement therapy (CRT), where DA neurons and/or progenitors are transplanted into the striatum to restore DA transmission. In order to be used as a valid therapeutic approach, it is necessary to obtain large numbers of correctly specified DA neurons that can elicit a functional recovery. Several cell types have been considered as potential sources of DA neurons, such as embryonic stem cells (ES) and induced pluripotent stem cell (iPS) due to their capacity to generate large numbers of DA neurons and induce behavioral improvement in animal models of PD (Kawasaki et al., 2000, Lee et al., 2000, Kim et al., 2002, Wernig et al., 2008, Hargus et al., 2010). However, there are a few limitations when using these cells as cell-sources for CRT, mainly because the molecular mechanisms regulating DA neuron differentiation are still not fully understood and/or have not been completely implemented. Thus, a lot of research has been focused on understanding the sequence of events and intrinsic and extrinsic factors that regulate DA neuron development *in vivo*. The use of soluble factors has the advantage of no genetic manipulation being involved, which is of interest in CRT. One of the most studied factors in DA development are the Wnt ligands, which have been shown to be involved in several critical steps of DA neuron development (McMahon and Bradley, 1990, Thomas and Capecchi, 1990, Castelo-Branco et al., 2003, Andersson et al., 2008, Joksimovic et al., 2009, Prakash et al., 2006) and *in vitro* differentiation (Castelo-Branco et al., 2003, Parish et al., 2008).

Clinical trials with human fetal ventral midbrain derived from aborted fetuses have been performed and support CRT as a valid therapy by providing proof-of-principle evidence that these DA neurons can survive and provide significant benefits in some patients (Kordower et al., 1995, Piccini et al., 1999, Mendez et al., 2005). However, there are a few technical limitations, such as the need for a high number of fetuses per patient, which must be addressed before the widespread implementation of CRT for PD.

This thesis focuses on the function of modulators of Wnt signaling in DA neuron differentiation *in vivo* and *in vitro*, and how this knowledge can be used to improve CRT strategies for PD using fetal tissue as source of stem/progenitor cell-derived DA neurons.

1.1 MIDBRAIN DEVELOPMENT

The development of an adult organism from a single cell is a fascinating event comprising complex mechanisms such as patterning, cell division, specification, migration and differentiation. The development of the different organs and body structures occurs due to an intricate network of conserved transcription factors, secreted factors and their signaling pathways. In this chapter, I will describe the ontogeny of midbrain DA neurons in the mouse and some of the secreted factors and transcription factors involved at each step.

1.1.1 E0-E7.5: From inception to “the most important time of your life”

Upon fusion of the egg and sperm (embryonic day 0, E0), the fertilized murine egg initiates a series of divisions. By E2.5, at the 8-cell stage, the first differentiation event takes place as in the next round of division cells divide asymmetrically and generate cells which are different in size, polarity and in the expression of the transcription factors Oct4 (also known as Pou5f1, POU domain class 5 transcription factor 1) and Cdx2 (caudal-type homeobox protein 2). These factors are involved in the first cell fate decision: cells expressing Oct4 will give rise to inner cell mass (ICM) and cells expressing Cdx2 will become trophectoderm (Niwa et al., 2005). By E3.5 the fertilized egg is now a blastocyst, a spherical structure with the ICM cells on the inside surrounded by a layer of trophectoderm cells. One day later, the ICM further

specializes into 2 distinct populations: the epiblast and the primitive endoderm or hypoblast (the outermost layer of cells of the ICM). The trophectoderm, epiblast and primitive endoderm will give rise to the placenta, embryo proper and the yolk sac, respectively.

At E5.5, after implantation, the embryonic axes start to be formed. At the most distal part of the embryo, Nodal, a member of the transforming growth factor (TGFβ) family, induces hypoblast-derived cells to specialize forming the distal visceral endoderm (DVE, (Mesnard et al., 2006). These cells start to express Wnt and bone morphogenetic protein (BMP)/Nodal antagonists such as Cerberus-like 1 (Cer1), Dickkopf1, (Dkk1) and Lefty1 as part of a negative feedback loop, thus creating a gradient of high Nodal/BMP/Wnt signaling in the proximal pole of the embryo and low in the most distal part (Yamamoto et al., 2004). At E6.0, the DVE cells start to migrate to the most anterior part of the embryo forming the very first organizer, the anterior visceral endoderm (AVE, a head organizer). Migration of DVE and formation of the AVE is dependent on Nodal signaling on the posterior side of the embryo and repression of Wnt signaling at the anterior side of the DVE brought about by Dkk1 (Kimura-Yoshida et al., 2005). The position of the AVE dictates the anterior-posterior axis. The secretion of the Wnt/Nodal antagonists from the AVE in the most anterior part of the embryo will induce neuroectoderm formation; conversely, in the most posterior side of the embryo there is activation of these pathways and cells will be instructed to become mesoderm and endoderm.

E6.5 is the onset of a very important process (the most important event of our lives, as elegantly phrased by Lewis Wolpert): gastrulation. By this point, the already molecularly regionalized embryo breaks radial symmetry and epiblast cells on the opposite side of the AVE (proximal epiblast) ingress and form the primitive streak. The establishment and correct placement of the primitive streak is dependent on a balance between Nodal and its antagonists and Wnt signaling (Brennan et al., 2001, Perea-Gomez et al., 2002, Liu et al., 1999b, Kelly et al., 2004). By E7.5, at the anterior end of the primitive streak, another signaling centre is formed, the node. Cell migration through the primitive streak involves epithelial-to-mesenchyme transition (EMT) and convergent-extension (CE) movements, and is dependent on FGF (fibroblast growth factor) signaling (Ciruna and Rossant, 2001, Sun et al., 1999). The cells passing through the node will form the prechordal plate and notochord (important signaling

centers for the formation of the nervous system), whilst the cells passing through other portions of the streak will give rise to mesoderm and endoderm. Cells which do not migrate end up anterior to the primitive streak and form the ectoderm. Thus, by the end of gastrulation all three germ layers of the embryo are formed: an outside layer of ectodermal cells separated from an internal endodermal cell layer by the mesoderm (for a more detailed and comprehensive description of early embryogenesis, please see reviews from Tam and Loebel, 2007, Rossant and Tam, 2009, Arnold and Robertson, 2009).

1.1.2 E7.5: Neurulation

During late gastrulation, signals from the underlying notochord and head organizer (Wnt and BMP antagonists such as Dkk1 and Noggin, respectively, (Barrantes et al., 2003) signal dorsal ectodermal cells opposite to the primitive streak to thicken and elongate forming the neural plate. Shortly after, the neural plate and the notochord, through CE movements, lengthen and become narrower; in addition, the neural plate also bends and folds in upon itself. The CE movements and bending induce the neural plate to form the neural tube, which closes at the dorsal midline and separates from and is covered by the surface ectoderm. As the neural tube closes, a number of vesicles form in the most anterior part which will give rise to the forebrain, followed by the midbrain, hindbrain and spinal cord thus cementing an anterior-posterior axis. Signals produced by the non-neural ectoderm and notochord such as BMP (Lee and Jessell, 1999) and Sonic Hedgehog (Echelard et al., 1993) are responsible for creating the dorsoventral axis. As a result, the future central nervous system (CNS) is patterned as a Cartesian coordinate system of positional identities.

1.1.3 E7.5-E9.5: Midbrain induction

DA neurons are born in the ventral part of the midbrain. A crucial event in midbrain DA development is the induction of two important signaling centers shortly after neurulation: the isthmus organizer and the floor plate. The isthmus forms at the junction of the presumptive mid- and hindbrain, and it is both necessary (Nieuwkoop, 1991) and sufficient (Martinez et al., 1991, Gardner and Barald, 1991, Martinez et al., 1995, Marin and Puelles, 1994) to induce midbrain and hindbrain structures. It is characterized by the expression of specific transcription and secreted factors which are

able to regulate patterning of the adjacent tissues in a set of complex genetic interactions that include positive and negative feedback loops. The floor plate is a specialized glial structure which forms in the ventral midline of the developing neural tube induced by the notochord (Placzek, 1995, Jessell, 2000). Floor plate-derived signals, such as Shh, are responsible for ventralizing neural progenitors of the neural tube (Yamada et al., 1993, Marti et al., 1995, Ericson et al., 1996, Lupo et al., 2006). Due to its importance in midbrain DA development (Figure1), I will discuss several of the isthmus and floor plate- associated signals and factors in more detail.

1.1.3.1 Isthmus-associated factors

1.1.3.1.1 Otx2/Gbx2

The homeobox-domain-containing transcription factors orthodenticle homologue 2 (Otx2) and gastrulation brain homeobox 2 (Gbx2) are responsible for the proper positioning of the isthmus. *Otx2* is first detected in the AVE where it plays a role in inducing rostral neural plate (Acampora et al., 1995) and becomes restricted to the anterior region of the mouse embryo in all three germ layers during gastrulation (Simeone et al., 1992). *Gbx2* is expressed in the posterior part of the embryo across all germ layers (Wassarman et al., 1997). After neurulation (E7.5), *Otx2* and *Gbx2* are expressed in the anterior and posterior part of the neuroectoderm, respectively. The expression domain of *Otx2* defines the forebrain and midbrain and the *Gbx2* expression domain defines the hindbrain and spinal cord, as genetic depletion of these two genes leads to a concomitant failure of induction of anterior or posterior head structures (Acampora et al., 1995, Ang et al., 1996, Rhinn et al., 1998, Matsuo et al., 1995, Wassarman et al., 1997). There is a reciprocal antagonism between *Otx2* and *Gbx2*, and the meeting point of these domains gives rise to midbrain-hindbrain border (MHB), where the isthmus forms (Simeone, 2000, Wurst and Bally-Cuif, 2001). Elegant experiments have shown that *Otx*2 and *Gbx2* are important for the proper positioning and maintenance of the isthmus: in mice with only one functional *Otx* allele (Acampora et al., 1997) or in mice where *Gbx2* is ectopically expressed in the caudal midbrain the isthmus is shifted rostrally and there is replacement of midbrain structures by hindbrain. Conversely, upon ectopic expression of *Otx2* in rostral hindbrain or deletion of *Gbx2* the isthmus is shifted caudally and there is a transformation of rostral hindbrain to midbrain (Broccoli et al., 1999, Katahira et al., 2000, Wassarman et al., 1997). The

position of the isthmus is also responsible for specifying the location and size of the DA and serotoninergic neurons in the ventral midbrain and hindbrain, respectively: a caudal shift of the isthmus leads to ectopic expression of DA neurons in the hindbrain at the expense of serotoninergic neurons and a rostral shift leads to reposition of DA neurons in the forebrain and an increase in the size of the serotoninergic population (Brodski et al., 2003). *Otx2* also has a specific role in neurogenesis and the specification of ventral midbrain DA progenitors and neurons (Vernay et al., 2005, Puelles et al., 2004, Omodei et al., 2008, Prakash et al., 2006).

In spite of their importance, *Otx2* and *Gbx2* are not required for the isthmus' initial induction and activity as in their absence there is still expression of isthmus-derived mid-hindbrain organizer factors (Liu and Joyner, 2001), and a residual MHB is still formed in *Gbx2*-deficient mice (Wassarman et al., 1997).

1.1.3.1.2 Wnt1

At E8.0 the secreted molecule Wnt1 starts being expressed initially in a broad domain of the presumptive midbrain and at around E9.0-E9.5 becomes restricted to a ring in the caudal midbrain (rostral to the isthmus), a narrow stripe along the dorsal midline and in two stripes at both sides of the ventral midbrain (Parr et al., 1993, McMahon et al., 1992, Wilkinson et al., 1987). Deletion of *Wnt1* leads to an early deletion of the midbrain and later of the rostral hindbrain as a result of multiple defects (McMahon and Bradley, 1990, Thomas and Capecchi, 1990). However, ectopic expression of *Wnt1* does not alter either the positioning or the activity of the isthmus (Panhuysen et al., 2004, Matsunaga et al., 2002, Lee et al., 1997), indicating that *Wnt1* is not required for the initial induction and activity of the isthmus but is necessary to maintain the region-specific pattern of gene expression at the midbrain–hindbrain boundary. *Wnt1* overexpression induces enhanced cell proliferation and an increase in the size of the inferior colliculi, a caudal-dorsal midbrain derivative (Panhuysen et al., 2004). *Wnt1* is expressed at least until E14.5 (Wilkinson et al., 1987) and in addition to its its isthmic activity, *Wnt1* is also important for DA neuron development: in *Wnt1*$^{-/-}$ mutants few DA neurons are born, and the few that are born are largely lost at later stages (Prakash et al., 2006) due to a dual role of Wnt1 in establishing the DA progenitor domain and a later activity required for the proper differentiation of DA precursors into mature DA neurons (Prakash et al., 2006).

1.1.3.1.3 En1/En2

Shortly after *Wnt1*, *Engrailed 1* (*En1*) starts to be expressed in the prospective midbrain and rostral hindbrain. *En1* shows a similar pattern of expression as *Wnt1* (McMahon et al., 1992, Davis and Joyner, 1988, Davidson et al., 1988) and in *Wnt1*$^{-/-}$ embryos *En1* is initially expressed but not maintained (McMahon et al., 1992), suggesting that *Wnt1* maintains *En1* expression. In agreement with this, *En1* is able to rescue the *Wnt1*$^{-/-}$ phenotype when expressed under the *Wnt1* regulatory element in *Wnt1*$^{-/-}$ embryos (Danielian and McMahon, 1996). *En2* expression starts later, but overlaps with the expression of *Wnt1* and *En1* (Davis and Joyner, 1988, Davis et al., 1988). *En1*$^{-/-}$ mice have a deletion of the dorsal and ventral midbrain and rostral hindbrain (Wurst et al., 1994), *En2*$^{-/-}$ exhibit minor cerebellar defects (Millen et al., 1994), but in compound *En1*$^{-/-}$*;En2*$^{-/-}$ mutants there is a complete deletion of the mid/hindbrain region, similar to the *Wnt1*$^{-/-}$ phenotype (Simon et al., 2001, Liu and Joyner, 2001). The expression of the *En* is also maintained by *Otx2* (Rhinn et al., 1998). The *En* genes are expressed into later fetal and post-natal stages (Simon et al., 2001) and also play a role in DA differentiation and survival given that in *En1*$^{+/-}$ and *En1*$^{-/-}$*;En2*$^{-/-}$ mutants DA neurons are born but are lost soon after (Simon et al., 2001, Sonnier et al., 2007, Sgado et al., 2006).

1.1.3.1.4 Fgf8

Fibroblast growth factor 8 (Fgf8) is first expressed in the embryo during gastrulation (Sun et al., 1999). Between E8.0 and E8.5 it starts to be expressed in the MHB, but also at the rostral end of the developing neural plate (Crossley and Martin, 1995). By E9.0 to E9.5, the *Fgf8* expression domain in the MHB is restricted to a sharp, narrow ring on the rostral hindbrain (caudal to the isthmus), much like a mirror image of the expression of *Wnt1* (Crossley and Martin, 1995, Wurst and Bally-Cuif, 2001). Fgf8 is considered one of the key isthmic organizer molecules as it can mimic the properties of the isthmus and it is required for the development of the prospective midbrain and cerebellum. Indeed, *Fgf8* hypomorphs exhibit, amongst other defects, a deletion of posterior midbrain and cerebellar tissue (Meyers et al., 1998) and in *Fgf8* conditional knockout animals where *Fgf8* is specifically removed from the MHB the midbrain, isthmus and cerebellum are deleted due to extensive cell death (Chi et al., 2003). Deletion of *Fgf8* induces loss of expression of isthmus-specific genes such as *Wnt1*,

Gbx2, *Fgf17* and *Fgf18* prior to the onset of cell death (Chi et al., 2003), indicating that *Fgf8* is important for the maintenance of the isthmus. Furthermore, gain-of-function studies have revealed that Fgf8-soaked beads are able to induce ectopic isthmus, midbrain, cerebellum and isthmus-associated genes such as *En1/2* in forebrain (Martinez et al., 1999, Crossley et al., 1996, Ye et al., 1998. Liu et.al, 1999a). *Fgf8* acts by repressing *Otx2* in the hindbrain and is capable of inducing and is regulated by *Gbx2* (Martinez et al., 1999, Irving and Mason, 1999, Liu et al., 1999a); thus, the increase in *Otx2* expression detected upon deletion of *Gbx2* is at least in part due to decreased *Fgf8* expression.

There are several *Fgf8* isoforms but the two most abundant in the isthmus are *Fgf8a* and *Fgf8b* (Blunt et al., 1997, Sato et al., 2001) which have distinct properties: *Fgf8a* does not possess any patterning activity since in mice lacking *Fgf8a* the midbrain and hindbrain develop normally, although there is a general growth retardation and postnatal lethality (Guo et al., 2010). In transgenic mice where *Fgf8a* is driven by a *Wnt1* promoter (*Wnt1-Fgf8a*) there is an enlargement of the midbrain and caudal diencephalon due to overproliferation, but no changes at the level of the MHB are detected (Lee et al., 1997). Depletion of *Fgf8b* induces a near complete deletion of the dorsal midbrain and the cerebellum, similar to the phenotype resulted from specific deletion of *Fgf8* in the isthmus (Guo et al., 2010). Moreover, in *Wnt1-Fgf8b* mice there is a transformation of the midbrain and caudal forebrain into an anterior hindbrain fate through expansion of the *Gbx2* domain and repression of *Otx2* (Liu et al., 1999a). These results indicate that *Fgf8b* is the patterning molecule of the MHB. There seems to be an interesting connection between *Fgf8* and *Wnt1*: deletion of *Fgf8* induces loss of *Wnt1* expression (Chi et al., 2003), and explant experiments have shown that Fgf8-soaked beads can induce *Wnt1* expression non cell-autonomously (Liu et al., 1999a, Liu and Joyner, 2001). Interestingly, in *Wnt1*$^{-/-}$ mutants *Fgf8* expression in the hindbrain is initiated but soon after disappears, indicating that *Wnt1* is capable of maintaining *Fgf8* expression (Lee et al., 1997, Danielian and McMahon, 1996). This suggests the presence of an integrated regulatory network that controls the maintenance of the MHB. *Fgf8* expression is detected in the isthmus until at least E12.5 (Crossley and Martin, 1995) and persists in the CNS until adult stage (Tanaka et al., 2001). In addition to its isthmic organizer activity, Fgf8 activity is necessary for the development of midbrain DA neurons: *Fgf8* hypomorphs generate a population of midbrain DA neurons, but their numbers are dramatically reduced (Ye et al., 1998). Moreover, E9.0 rat ventral mid/hindbrain explants treated with a high-affinity blocking receptor for

Fgf8 fail to generate DA neurons, while other ventral midbrain populations are not affected (Ye et al., 1998), indicating that DA neurons (which are born adjacent to the isthmus) are differentially affected by reduced levels of Fgf8. Together with Shh, Fgf8 is capable of ectopically inducing DA neurons in caudal forebrain (Ye et al., 1998). However, Fgf8-soaked beads are unable to induce ectopic DA neurons in the forebrain of *Wnt1*$^{-/-}$ mutants (Prakash et al., 2006) indicating that Fgf8's role in DA development might be mediated through *Wnt1*.

1.1.3.1.5 Lmx1b

The LIM homeodomain transcription factor Lmx1b is first detected in the MHB at E7.5 (Guo et al., 2007, Adams et al., 2000), becoming progressively restricted to the isthmus between E9.0 and E10.5 (Guo et al., 2007). *Lmx1b* has an important role in the activity of the isthmic organizer during mid/hindbrain development: it is necessary to initiate the expression of *Fgf8* (Matsunaga et al., 2002, Guo et al., 2007) and to maintain the expression of *Wnt1*and *En1/2* (Guo et al., 2007, Adams et al., 2000), which are responsible for the mid/hindbrain-inducing activity of the isthmus. Deletion of *Lmx1b* leads to a loss of *Fgf8* expression at the isthmus (Guo et al., 2007, O'Hara et al., 2005) and a reduction and subsequent loss of *Wnt1* and *En1/2* and expression (Guo et al., 2007). Conversely, misexpression of *Lmx1b* induces ectopic expression of *Wnt1* and *Fgf8* (Matsunaga et al., 2002, Adams et al., 2000). *Lmx1b* might act on *Fgf8* expression by repressing it cell-autonomously but inducing it through Wnt1 in neighboring cells (Matsunaga et al., 2002), and Fgf8 is able to maintain the expression of *Lmx1b* in a self-regulation process (Matsunaga et al., 2002, Adams et al., 2000). *Lmx1b* is expressed into later fetal and post-natal stages and has also a later role in proper specification of the ventral midbrain DA neurons as DA neurons are formed in *Lmx1b*$^{-/-}$ mice but are lost by birth (Smidt et al., 2000).

1.1.3.2 Floor plate-associated factors

1.1.3.2.1 Shh

Sonic hedgehog (Shh) expression is first detected during late gastrulation in the node and prechordal plate (Echelard et al., 1993). By E8.5 expression is detected at the ventral midline of the prospective midbrain, subsequently expanding rostrally into the forebrain and caudally into the hindbrain and spinal cord (Echelard et al., 1993). Shh

secretion from the notochord induces cells at the ventral midline to become floor plate cells (Jessell et al., 1989, Marti et al., 1995, Jessell, 2000, Chiang et al., 1996, Yamada et al., 1993), a group of specialized cells that influence the development of other neural cells. The floor plate cells secret Shh which is sufficient and necessary to ventralize neural progenitor (Yamada et al., 1993, Hynes et al., 1995a). In *Shh*-null mice the floor plate is absent, the notochord degenerates, ventral markers such as *Islet1* are lost, and the midbrain is smaller and has an abnormal morphology (Chiang et al., 1996). In these animals, the expression of *Otx2* in the midbrain is also reduced while the expression of *En2* remains unaltered. Ectopic dorsal expression of *Shh* leads to activation of floor plate genes (Echelard et al., 1993). *Shh* expression in the notochord and floor plate lasts until E13.5 (Echelard et al., 1993) but it is downregulated in ventral midbrain midline cells at E11.5 (Andersson et al., 2006b). Both the floor plate and Shh play a crucial role in DA neuron development: when forebrain and midbrain-derived explants in which the floor plate was removed are cultured in the presence of exogenous floor plate explants (derived from the spinal cord), there is generation of DA neurons (Hynes et al., 1995b). Moreover, floor plate explants are able to induce an ectopic supernumerary dorsal floor plate *in vivo* and ectopic dorsal DA neurons (Hynes et al., 1995b), and recent studies have demonstrated that the floor plate can not only induce but also directly generate DA neurons (Ono et al., 2007, Bonilla et al., 2008, Joksimovic et al., 2009). Regarding the role of *Shh* in DA neuron development, timing is crucial: fate mapping studies have revealed that DA neurons only respond to Shh from E7.75 to E9.0 (Zervas et al., 2004). In agreement with this, in conditional mutants where *Shh* is inactivated by Cre recombinase expression under the *En1* promoter (≈E9.0) there is a strong reduction in midbrain DA neurons due to a proliferation defect (Perez-Balaguer et al., 2009) whereas when *Shh* is removed by Cre under the *Nestin* promoter (E10.5) DA neurons develop normally (Ferri et al., 2007). Recombinant Shh is sufficient to induce DA neurons in midbrain and rostral forebrain explants (Ye et al., 1998) and in floor plate-depleted midbrain explants (Hynes et al., 1995a). Moreover, blocking endogenous Shh with a neutralizing antibody impairs the DA neuron development in midbrain explants, and Shh and Fgf8 are able to cooperate to specify DA neurons in ectopic locations (Ye et al., 1998).

Thus, in agreement with the model of cell fate patterning in a Cartesian grid, the intersection of the secretion of Fgf8 (along the anterior-posterior axis) and Shh (along the dorsal-ventral axis) specifies the domain where DA neurons are born. Interestingly,

a recent study has demonstrated that Shh inhibits the neurogenic potential of the midbrain floor plate and that *Wnt1* is able to suppress *Shh* (Joksimovic et al., 2009), suggesting that initially Shh is necessary for the establishment and proliferation of the DA neuron precursors but later on it must be inhibited (by Wnt1) to promote DA neurogenesis. Moreover, another recent study has also demonstrated a cell-context dependent antagonistic interaction between Wnt/β-catenin and Shh during DA neurogenesis (Tang et al., 2010).

1.1.3.2.2 Hnf3β/Foxa2

The hepatocyte nuclear factor 3β *Hnf3*β (or forkhead box a2, *Foxa2*) is a member of the *fork head/Hnf-3* family of DNA-binding transcription factors (Lai et al., 1990) and it is first detected during gastrulation in the node, notochord and overlying floor plate (Sasaki and Hogan, 1993, Echelard et al., 1993). It precedes and resembles the expression of *Shh* in the notochord, indicating that initial activation of *Shh* expression is regulated by *Hnf3*β*/Foxa2* (Echelard et al., 1993, Jeong and Epstein, 2003, Epstein et al., 1999); *Shh* in turn activates the expression of *Hnf3*β*/Foxa2* in the floor plate (Sasaki et al., 1997). The importance of *Foxa2* during development is demonstrated by the analysis of *Foxa2*$^{-/-}$ mutants, which die at E9.5 due to gastrulation defects (Ang et al., 1993) and lack a node and notochord with consequent defects in floor plate development and patterning (Ang and Rossant, 1994, Weinstein et al., 1994). When ectopically expressed in the dorsal hindbrain, *Foxa2* is able to induce an ectopic floor plate (Sasaki and Hogan, 1994) indicating a role in the development of the floor plate. *Foxa2* is expressed into post-natal stages (Kittappa et al., 2007) and is important for midbrain DA neuron development: *Foxa1* (also expressed in ventral midbrain progenitors) and *Foxa2* single mutants exhibit an impaired DA differentiation, whereas in *Foxa1*$^{-/-;}$*Foxa2*$^{-/-}$ double mutants there is a reduction in VM neurogenesis and incomplete differentiation of DA neurons (Ferri et al., 2007). Analysis of the conditional *Foxa1/2*-null mutants have shown that they are implicated in maintaining Shh expression in the midbrain floor plate, specifying DA progenitors markers such as *Lmx1a/b* and inhibit expression of other ventral midbrain cell fates (Lin et al., 2009). Moreover, E8.5 midbrain explants from *Foxa2*$^{-/-}$ mutants are not able to generate DA neurons and overexpression of *Foxa2* in midbrain cells and embryonic stem cells-derived cell line induces an increase in the number of DA neurons (Kittappa et al.,

2007). In addition, *Foxa2*$^{+/-}$ mice develop a late-onset condition associated with a significant loss of midbrain DA neurons (Kittappa et al., 2007).

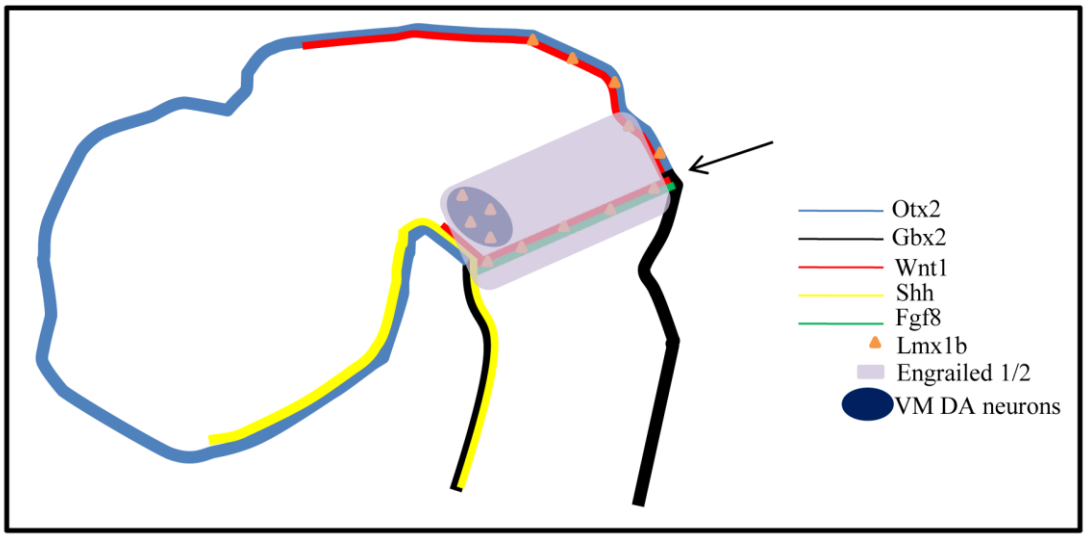

Figure 1: Expression of isthmus and floor plate-associated genes at E9.5. The arrow indicates the isthmus and the blue circle corresponds to the place where DA neurons will be born.

1.1.4 E9.5-E11.5: Ventral midbrain dopaminergic specification

By E9.5 the isthmus becomes a fully morphologically discernible structure, the isthmic constriction that separates the midbrain from the hindbrain. The neural tube is divided along the dorsal-ventral axis into the roof plate, alar plate, basal plate and floor plate. The ventral midbrain is further subdivided into three layers, the ventricular (VZ), intermediate (IZ) and marginal zones (MZ). Cells in the VZ acquire an epithelial-like structure forming the neuroepithelium; these cells are neural stem cells, capable of dividing symmetrically to allow self-renewal and asymmetrically to originate neural progenitors with limited self-renewal potential. Some of these cells move out of the ventricular zone and start migrating ventrally and laterally, exiting the cell cycle and differentiating as they migrate. Migration progresses along radial glia, a type of cells characterized by long processes extending from the VZ to the pial surface (MZ) that serve not only as scaffold but also as neural precursors (Malatesta et al., 2000, Bonilla et al., 2008). Cells in the IZ are post-mitotic DA precursors that become fully differentiated DA neurons once they reach the MZ (Figure 2). E9.5 to E11.5 is the period where the DA domain is specified in the ventral midbrain. In the next paragraphs, I will discuss the intrinsic factors involved in more detail.

1.1.4.1 Lmx1a/Msx1

The expression of the LIM homeobox transcription factor 1 *Lmx1a* is first detected in the midbrain at E9 in ventral midline cells (Andersson et al., 2006b) and throughout adult life (Zou et al., 2009). The expression of the muscle segment homeobox 1 *Msx1* starts at E9.5 following the same expression pattern as *Lmx1a*, indicating that *Msx1* is downstream of *Lmx1a* (Andersson et al., 2006b, Nakatani et al., 2010). Besides the midbrain, these two factors are also detected in the most caudal part of the diencephalon (Andersson et al., 2006b). From E9.5 onwards the expression of *Lmx1a* and *Msx1* becomes restricted to the DA lineage: *Lmx1a* is expressed in the DA progenitor cells in the ventricular zone and persists later in the post-mitotic precursors and the differentiated DA neurons whereas *Msx1* expression remains confined to the proliferating DA progenitors in the ventricular zone. Thus, *Lmx1a* labels the whole DA lineage (Andersson et al., 2006b, Ono et al., 2007). The expression pattern of these genes is similar to *Shh* expression, as they are expressed early in floor plate-ventral midline cells, suggesting that they might be induced by *Shh*. However, the role of Shh in *Lmx1a* induction is controversial: in basal midbrain explants exposed to Shh there is induction of *Lmx1a* and *Msx1* accompanied by a ventralization of progenitor cells (Andersson et al., 2006b), but analysis of the $En1^{KI/Cre/+}$;$Shh^{flox/flox}$ mutants, where *Shh* expression is lost in basal and floor plate progenitors already at E8.75, revealed that Shh is not necessary to maintain *Lmx1a* expression in the floor plate as there are no significant changes in $Lmx1a^{+}$ cells (Lin et al., 2009). Moreover, in embryonic stem cell-derived neural progenitors treated with Shh or cyclopamine (a Shh inhibitor) there are no significant changes in the *Lmx1a* mRNA levels, suggesting that Lmx1a is not a direct target of Shh (Chung et al., 2009). Interestingly, in an effort to identify downstream targets of Wnt1, Chung and colleagues demonstrated that the expression of *Lmx1a* is directly regulated by *Wnt1* in VM DA development forming an autoregulatory loop (Chung et al., 2009). Furthermore, in *Shh/Cre*-mediated conditional removal of *β-catenin* (a Wnt1 downstream effector) and in *Shh–Cre*;$Ctnnb1^{Lox(Ex3)}$ where β-catenin is constitutively activated upon *Shh* expression, there is a reduction and an increase in the levels of Lmx1a, respectively (Joksimovic et al., 2009), (Tang et al., 2010). Gain and loss-of-function experiments have revealed that *Lmx1a* is necessary and required for the generation of DA neurons, as forced expression (Andersson et al., 2006b, Nakatani et al., 2010, Lin et al., 2009) or knock-down (Andersson et al., 2006b) of *Lmx1a* induces ectopic generation or a substantial

reduction of VM DA neurons, respectively. *Msx1*, on the other hand, can cooperate with *Lmx1a* in inducing DA neurons but is not sufficient *per se*; instead, *Msx1* promotes suppression of alternative cell fates and cell-cycle exit by induction of Neurogenin 2 (*Ngn2/Neurog2/Atoh4)*, a pro-neural marker (Andersson et al., 2006b, Nakatani et al., 2010, Lin et al., 2009). *Msx1*$^{-/-}$ embryos exhibit a 40% reduction in the normal number of DA neurons, probably as a result of the downregulation of *Ngn2* expression (Andersson et al., 2006b). *Lmx1a*-mediated induction of *Msx1* induces VM DA neuronal differentiation by suppressing the floor plate characteristics of the ventral midline cells (Andersson et al., 2006b), further confirming that *Lmx1a*$^{+}$ floor plate cells give rise to the DA progenitors in the ventral midbrain (Andersson et al., 2006b, Ono et al., 2007). However, other factor(s) must be involved given that *Lmx1a* alone cannot induce neurogenesis in caudal floor plate cells (Ono et al., 2007): possible candidates include *Otx2* (Ono et al., 2007) and *Foxa1/2* since a recent report has shown that *Foxa1/2* can cooperate with *Lmx1a/b* in floor plate cell differentiation (Nakatani et al., 2010). Interestingly, it has been shown that *Foxa1/2* are able to cooperate with *Lmx1a* in DA neuron fate determination: *Lmx1a/b* expression initiates but is subsequently lost when *Foxa1/2* are removed at an early stage suggesting that *Foxa1/2* are able to maintain the *Lmx1a/b* expression (Lin et al., 2009). Furthermore, they can induce ectopic DA neurons when co-expressed (Lin et al., 2009), but this cooperation seems to be dependent on the context of ventral midbrain progenitors (Nakatani et al., 2010). In *dreher* mice, which carry a mutation in the *Lmx1a* locus, VM DA development is reduced due to a downregulation in proneural gene expression in Lmx1a^{+} progenitor cells and a mild reduction in the number of DA neurons (Ono et al., 2007). However, progenitor identity is not affected, suggesting more of a proneural gene induction role for *Lmx1a* (Ono et al., 2007). The somewhat discrepant results from Andersson et al (done mostly in chick) and Ono et al might be due to a hypomorphic mutation rather than a null mutation in *dreher* mice, species differences, or redundant roles of *Lmx1a* and *Lmx1b* in mouse development; analysis of the *Lmx1a*- null mutants or conditional *Lmx1a/b* knockout mice could provide a more clear understanding of the role of *Lmx1a* in VM DA neuron specification. Interestingly, *Lmx1a* and *Lmx1b* do seem to be redundant *in vivo* (Nakatani et al., 2010) and *in vitro* DA differentiation (Chung et al., 2009). Downstream targets of Lmx1a including *Pitx3* and *Nurr1* (DA neuron differentiation markers) have been shown to be regulated by *Wnt1* through the Wnt1-Lmx1a autoregulatory loop (Chung et al., 2009); these results are in agreement with other studies where *Pitx3* expression was shown to not be initiated in the absence of

Wnt1 (Prakash et al., 2006) and further suggest a *Shh/Foxa2*-independent role of *Lmx1a* in DA phenotype specification and survival.

1.1.4.2 Nurr1

The nuclear receptor related 1 protein (Nurr1 or NR4A2) is a member of the nuclear receptor family of transcription factors and it is able to recognize and bind DNA sequences in the absence of a ligand (Wang et al., 2003). *Nurr1* is expressed in many areas of the CNS (Law et al., 1992) and in the VM it is first detected in post-mitotic cells in the IZ and MZ at E10.5 (Zetterstrom et al., 1997) and its expression persists into adulthood (Backman et al., 1999). The importance of *Nurr1* in DA neuron development is demonstrated by the failure of generation or maintenance of midbrain specific-DA neurons in *Nurr1*$^{-/-}$ mice (Zetterstrom et al., 1997, Saucedo-Cardenas et al., 1998, Le et al., 1999) assessed by the lack of expression of *Th* (Tyrosine hydoxylase, a marker of differentiated DA neurons). In these animals most DA neuron markers are absent at birth but some DA markers such as *En*, *Lmx1b* and *Pitx3* are present at earlier stages, although later downregulated (Thuret et al., 2004, Saucedo-Cardenas et al., 1998, Wallen et al., 1999, Smidt et al., 2000), indicating that *Nurr1* is important for the differentiation and survival of early developing DA cells and that it is not necessary for the induction of all DA genes. In agreement with a role for *Nurr1* in the maintenance and maturation of the DA neurons, deletion of *Nurr1* at late stages of VM DA neuron development (E15.5), or in adult mice, leads to decreased levels of *Th* expression (Kadkhodaei et al., 2009). Moreover, in *Nurr1*-null mice expression of *Ret*, a co-receptor for trophic factors such as the glial derived neurotrophic factor (GDNF), is also absent (Wallen et al., 2001); GDNF is important for postnatal survival of DA neurons (Backman et al., 1999). Thus, this suggests that Nurr1 is able to directly activate genes such as *Th* (Sakurada et al., 1999) and *Ret* (Galleguillos et al., 2010). *Nurr1* is important for the acquisition of the neurotransmitter identity as it is required for the expression of *Vmat2* (vesicular monoamine transporter 2) and *Dat* (dopamine transporter, Smits et al., 2003, Sacchetti et al., 1999, Sacchetti et al., 2001, Kadkhodaei et al., 2009). *Nurr1* is able to induce cell cycle arrest *in vitro* (Castro et al., 2001), but no changes in cell cycle are observed in *Nurr1*-null animals (Wallen et al., 1999). Disturbances in cell migration, target innervation and cell death are also detected in *Nurr1*-null mice (Wallen et al., 1999, Saucedo-Cardenas et al., 1998). A neuroprotective role for *Nurr1* by limiting the production of neurotoxic mediators by

microglia and astrocytes in the midbrain has also been reported (Saijo et al., 2009). *Nurr1* expression has been shown to be regulated by *Foxa2* (Ferri et al., 2007) and *Lmx1a* (Chung et al., 2009).

1.1.4.3 Ngn2

Neurogenin-2 (Ngn2/Neurog2/Atoh4), a proneural gene, is a member of the basic helix-loop-helix family of transcription factors. It is first expressed in the ventral midline of midbrain at E10.75 (Andersson et al., 2006b). At E11.5, the onset of DA neurogenesis, *Ngn2* expression is confined to the cells within the proliferative VZ and also in Nurr1^{+} cells in the IZ (Kele et al., 2006, Andersson et al., 2006a). In the absence of *Ngn2* there is a near complete loss of DA neurons in the midbrain at E11.5, as assessed by the reduction in the expression of *Th*, *Pitx3* and *Nurr1*, with the cells in the ventral midbrain midline being the most affected (Kele et al., 2006, Andersson et al., 2006a); the phenotype is due to a failure of VZ progenitors to differentiate into post-mitotic DA precursors. At later stages of development there is a partial rescue, as some DA neurons are born (but still in low numbers when compared to wild type embryos) due to a compensation from *Mash1*, another proneural gene expressed in the VM (Kele et al., 2006). Thus, *Ngn2* is required, but not sufficient, for DA differentiation, as it does not promote DA neuron differentiation when ectopically expressed in dorsal and ventral midbrain E11.5 primary cultures (Andersson et al., 2006a). *Ngn2* seems to be a downstream target of *Foxa1/2*, as its expression is substantially reduced in *Foxa1*$^{-/-}$;*Foxa2*$^{-/-}$ mutants (Ferri et al., 2007), and also is a target of *Lmx1a* (most likely through *Msx1*) (Ono et al., 2007).

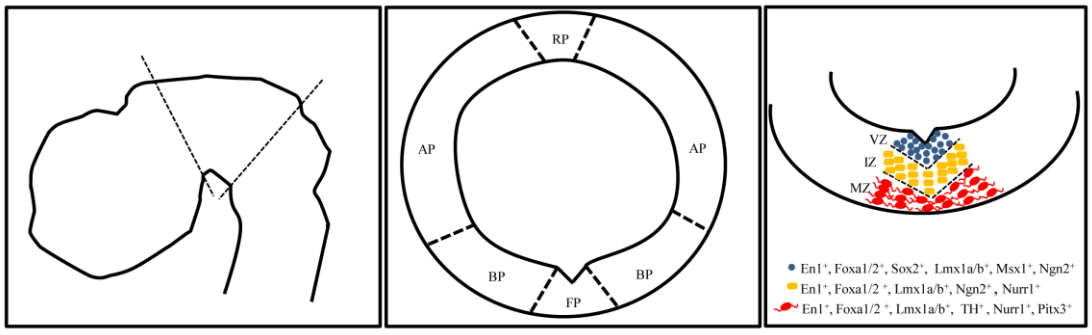

Figure 2: Ventral midbrain DA specification. A coronal cut at the level of the midbrain at E11.5 (left) reveals that the neural tube is divided, from dorsal to ventral, into roof plate, alar plate, basal plate and floor plate (middle panel). As DA progenitors move out of the ventricular zone (VZ) they exit the cell cycle giving rise to post-mitotic DA precursors in the intermediate zone (IZ) which become fully differentiated DA neurons when they reach the marginal zone (MZ, right panel). Throughout this process, cells will express a specific set of transcription factors.

1.1.5 E11.5-E15.5: Ventral Midbrain Dopaminergic Differentiation

By E11.5 the first DA neurons in the VM are born in the MZ, as assessed by the expression of tyrosine hydroxylase (TH), the rate limiting enzyme in dopamine synthesis, catalyzing the conversion of tyrosine to L-dihydroxyphenylalanine (L-DOPA), which is then converted to dopamine by the aromatic amino acid decarboxylase (AADC, Nagatsu et al., 1964). TH is first detected in the midbrain at E11.5 and persists throughout adulthood and *Th*-null mutants die peri- or postnatally (Kobayashi et al., 1995).

During the subsequent days of embryonic development (and first postnatal weeks), neurogenesis and final differentiation proceed as cells start to express markers related to neurotransmitter identity and survival and to extend neurites towards their projection areas in the forebrain (Kawano et al., 1995). I will address some of the markers associated with DA differentiation.

1.1.5.1 Pitx3

The paired-like homeodomain 3 Pitx3 is a transcription factor of the bicoid class of homeodomain proteins. It is detected in the midbrain from E11-E11.5 until adulthood (Smidt et al., 1997) but it is also expressed transiently in the eye lens and skeletal muscle (Smits et al., 2006). In the brain *Pitx3* is exclusively associated with VM DA neurons with an almost 100% overlap in TH and Pitx3 expression (Maxwell et al., 2005, Zhao et al., 2004, Smidt et al., 1997). At early stages of development (E12) some $Pitx3^+$ cells are not TH^+, but by E14 the vast majority of $Pitx3^+$ cells become TH^+, thus suggesting that the expression of *Pitx3* precedes and regulates *Th* expression (Zhao et al., 2004, Maxwell et al., 2005). In agreement with this, it has been shown that *in vitro* Pitx3 can activate the *Th* promoter (Lebel et al., 2001, Messmer et al., 2007). Analysis of the *aphakia* mice, where there is a double deletion within the *Pitx3* gene (Semina et al., 2000), revealed that *Pitx3* is important for the differentiation and survival of TH^+ DA neurons: in these animals DA neurons are initially born but from E12.5 onwards the most lateral population is absent (Smidt et al., 2004, Nunes et al., 2003, van den Munckhof et al., 2003). In the adult, this phenotype is reflected in an almost complete absence of DA neurons in the substantia nigra (SN), and a partial decrease in the

ventral tegmental area (VTA) due to neuronal loss (Nunes et al., 2003, Maxwell et al., 2005). As a result, target innervations and motor behavior are also severely affected (Smidt et al., 2004, Nunes et al., 2003, van den Munckhof et al., 2003) The remaining DA neurons express most of the DA developmental and neurotransmitter markers, indicating they are fully matured DA neurons (Smidt et al., 2004), and the reason why SN neurons are more severely affected than VTA neurons is still unclear. Analysis of *Pitx3*$^{-/-}$*-GFP* mice has revealed that at E12.5 cells that are lost in *Pitx3*-null mutants are GFP^{+}/TH^{+}, while GFP^{+}/TH^{-} cells are not affected indicating that DA neurons, but not the progenitors and precursors, are affected by the loss of Pitx3(Maxwell et al., 2005). In spite of evidence that Pitx3 is able to activate the *Th* promoter, the fact that some TH^{+} neurons are born in the absence of Pitx3 (Smidt et al., 2000, Maxwell et al., 2005) indicates that *Pitx3* is important, but not required, for *Th* expression in DA midbrain neurons and/or that its requirement is region-specific. In agreement with this, in *Pitx3*$^{-/-}$*-GFP* mice there is a reduction in the percentage of GFP^{+}/TH^{+} cells and an increase in the number of GFP^{+}/TH^{-} cells in the SN, indicating that *Pitx3* is required in the GFP^{+} cells to induce *Th* expression specifically in the SN (Maxwell et al., 2005). *Pitx3* has also been shown to regulate the expression of *Vmat2* and *Dat* (Hwang et al., 2009), suggesting a possible cooperation between *Pitx3* and *Nurr1* in the DA neuron specification; indeed, a recent report has demonstrated that Pitx3 and Nurr1 are able to bind to the same regions on promoters of Nurr1 target genes and that Nurr1-regulated genes such as *Dat* and *Vmat2* are also affected in *Pitx3*-null mutants, thus indicating that combined actions of Pitx3 and Nurr1 are necessary to induce a DA phenotype *in vivo* (Jacobs et al., 2009). Moreover, the authors showed that Pitx3 decreases the interaction of Nurr1 with the nuclear receptor co-repressor 2 (Ncor2), which normally represses the Nurr1 activity through histone deacetylases (HDACs), while interference with HDAC-mediated repression in *Pitx3*$^{-/-}$ embryos restores Nurr1 target gene expression. Cooperation between Nurr1 and Pitx3 in the midbrain DA phenotype has also been demonstrated in human embryonic stem cells (Martinat et al., 2006).

Besides Pitx3, several other factors expressed early on in midbrain development have a role in the survival and maturation of DA neurons. **Otx2** is one of these factors: when it is removed after the isthmus has been formed, there is a reduction in the DA neurons as assessed by reduced levels of TH, Nurr1, Pitx3 and Lmx1a/b (Vernay et al., 2005, Omodei et al., 2008, Puelles et al., 2004). The DA neuron deficit is due to a decrease in neurogenesis, as determined by reduced levels of Ngn2 and Mash1 (*Mash1/Ascl1*,

another pro-neural gene), and a decrease in DA progenitor proliferation. Otx2's role in DA differentiation is thus due to its ability to control the proliferation of DA progenitors (Omodei et al., 2008), the expression of proneural genes that mediate their transition into post-mitotic cells (Vernay et al., 2005) and also to suppress alternative VM fates (Vernay et al., 2005, Prakash et al., 2006). Conversely, in mice where *Otx2* is overexpressed there is an increase in DA neurons due to an increase in the DA progenitor domain (Omodei et al., 2008).

Wnt1 also has a role in DA neuron survival by maintaining *Otx2* expression in the ventral midbrain (Prakash et al., 2006). Moreover, in the absence of *Wnt1* very few TH^+ neurons are born and those that are born are not maintained (Prakash et al., 2006). These few neurons fail to express *Pitx3*, indicating that Wnt1 might, directly or indirectly, regulate *Pitx3* expression.

En1/2 are important for DA neuron survival given that TH^+ neurons in $En1^{-/-}/En2^{-/-}$ mice are initially born in the VM but are lost by E14 and are undetectable at birth (Simon et al., 2001). En1 and En2 are able to compensate for each other: $En2^{-/-}$ mice do not have a VM DA phenotype and in $En1^{-/-}$ mice, despite major developmental defects (Wurst et al., 1994), there are TH^+ neurons in the VM at levels comparable to wild type (Simon et al., 2001). Interestingly, $En1^{+/-}/En2^{-/-}$ and $En1^{+/-}/En2^{+-}$, which are viable and fertile, show a progressive loss of VM DA neurons during their first 3 months, which resembles the progressive loss of DA neurons in PD (Sonnier et al., 2007, Sgado et al., 2006).

Lmx1b has been shown to regulate DA neuron maintenance: after its initial induction in the isthmus, *Lxm1b* expression in the VM is similar to *Lmx1a*, albeit in a slightly broader domain at early stages that includes basal plate populations (Lin et al., 2009, Andersson et al., 2006b). In $Lmx1b^{-/-}$ embryos, $Nurr1^+$ and TH^+ neurons are born but fail to express *Pitx3*, and by E16 they are lost (Smidt et al., 2000).

1.1.5.2 LXR's and oxysterols: new players

The liver X receptors α/β (Lxrα and β) are nuclear receptors which, upon binding to oxysterols (cholesterol derivatives), are able to bind to DNA and regulate transcription. Recently it has been shown that *Lxr*'s are able to regulate cell division, neurogenesis

and DA neuron development in the VM (Sacchetti et al., 2009). *Lxrα* and *Lxrβ* transcripts are first detected in the midbrain at E9.5 and are upregulated at E10.5 and E11.5, and in *Lxrαβ* $^{-/-}$ mice there is an impairment in DA neurogenesis as assessed by the reduction in the expression of *Lmx1b*, *Wnt1*, *Ngn2*, *Nurr1*, *Pitx3* and a decrease in the numbers of Tuj1^{+} (an early neuronal marker) and TH^{+} cells; this phenotype is still present at later stages, despite a partial recovery. The DA neurogenesis impairment is due to a decrease in cell cycle exit of the floor plate progenitors, leading to their accumulation. Moreover, oxysterols are able to specifically increase DA neurogenesis during DA differentiation of VM and human embryonic stem cell cultures, further confirming the importance of Lxrs in DA development.

1.2 WNT SIGNALING

The Wnt signalling pathway, highly conserved during evolution, has a crucial role during embryonic development and adult tissue homeostasis affecting processes such as cell proliferation, migration, polarity and fate determination. In 1982, the mouse *Int-1* gene was identified: activation of this gene by a proviral insertion of the mouse mammary tumor virus (MMTV) was able to induce breast tumors (Nusse and Varmus, 1982). *Int-1* encodes a secreted protein and it was subsequently shown that *Int-1* and the *Drosophila wingless (wg)* gene, which controls segment polarity during larval development (Sharma and Chopra, 1976, Nussleinvolhard and Wieschaus, 1980), were homologues (Rijsewijk et al., 1987). Thus, the term *Wnt* was coined to designate this new signaling pathway.

The diversity present already at the cell-membrane level indicates that Wnt signaling is far from being simple: in mammals there are 19 Wnt ligands, at least 15 receptors (10 Frizzleds, 2 Lrps, 1 Ryk and 2 Rors), 2 classes of inhibitors (Wnt-binding such as soluble Frizzled-related proteins (sFRPs), or receptor-binding such as Dickkopfs) and 2 classes of agonists (Norrin and R-spondin). Moreover, considering that the intracellular signal transduction involves several proteins, phosphorylation events and feedback loops, it is easy to see the intricate nature of this pathway.

Initially, functional assays were used to classify Wnt signaling activity: *Wnt* mRNAs which were able to induce axis duplication when injected into *Xenopus* embryos and that were able to transform mouse C57MG mammary epithelial cells were classified as

canonical (McMahon and Moon, 1989, Du et al., 1995, Wong et al., 1994). The canonical pathway involves the stabilization and subsequent transcriptional activity of β-catenin. Wnt ligands which did not induce secondary axis, were able to disturb morphogenetic movements and failed to induce transformation in C57MG mammary epithelial cells were classified as non-canonical. In the non-canonical pathway other downstream effectors are involved, such as RhoA, Rac, protein kinase C (PKC), calcium/calmodulin-dependent kinase (CAMKII) and the c-Jun N-terminal kinase (JNK, Smith et al., 2000, Tada and Smith, 2000). However, increasing evidence has demonstrated that a particular Wnt may activate β-catenin and/or non-canonical pathways depending on the receptor complement (He et al., 1997, Mikels and Nusse, 2006, van Amerongen et al., 2008). Moreover, many of the components involved during signaling are common to the two branches (Wallingford and Habas, 2005, Bryja et al., 2009, Wu et al., 2008, Bryja et al., 2007, Grumolato et al., 2010) suggesting extensive cross-talk between them. A more appropriate designation of these pathways has emerged: the canonical pathway is now referred to as Wnt/β-catenin pathway; the non-canonical is now subdivided into Wnt/PCP and Wnt/Calcium (Ca^{2+}) pathways.

1.2.1 Wnt/β-catenin signaling

A crucial event in the Wnt/β-catenin signaling is the stabilization and accumulation of β-catenin in the cytosol, which is then able to translocate into the nucleus and regulate gene expression.

In the absence of Wnt ligand, free cytosolic β-catenin is constantly target to degradation by the actions of the serine/threonine kinases casein kinase 1 (CK1) and glycogen synthase kinase 3β (GSK-3β). Together with the scaffolding proteins Axin and Adenomatous Polyposis Coli (APC) they form what it is called the destruction complex. Axin is the key scaffolding protein, as it interacts with all other components and seems to be rate limiting in the complex assembly (Lee et al., 2003). Axin binds to and coordinates the sequential phosphorylation of β-catenin at serine 45 by CK1 and at threonine 41, serine 37 and serine 33 by GSK-3β (Kimelman and Xu, 2006, MacDonald et al., 2009). CK1 and GSK-3β also phosphorylate Axin and APC, increasing their association with β-catenin and enhancing its phosphorylation. Phosphorylated β-catenin is then recognized by β-TrCP (beta-transducin repeat

containing protein), a component of the E3 ubiquitin ligase and targeted for proteosomal degradation (Aberle et al., 1997).

When Wnt ligands are present, they bind to Frizzled (Fz in Drosphila/Fzd in mammals), a seven transmembrane receptor, and to the LDL receptor-related proteins 5 and 6 (Lrp5/6), forming a Wnt-induced Fzd-Lrp complex. Accordingly to the most accepted model of Wnt activation, the Fzd-Lrp6 complex recruits Disheveled (Dvl), a cytoplasmic scaffolding protein, to bind to Fzd (Wong et al., 2003, Wallingford and Habas, 2005). Dvl is able to interact and recruit GSK-3β-bound Axin to the membrane (Cliffe et al., 2003, Wallingford and Habas, 2005) and GSK-3β induces phosphorylation in PPPPSPxS motifs in the Lrp5/6 receptor (P-proline; S-serine or threonine; x- variable residue) (Tamai et al., 2004, Zeng et al., 2005, Zeng et al., 2008). GSK-3β is responsible for the PPPSP phosphorylation whereas the xS phosphorylation is mediated by another kinase, CK1 (Davidson et al., 2005, Zeng et al., 2005). The dually phosphorylated PPPSPxS motifs recruit the Axin complex to the membrane (Zeng et al., 2005, Mao et al., 2001b, Davidson et al., 2005) in a positive feedback loop to ensure that all PPPSPxS motifs are phosphorylated. It has been suggested that this initiation event is further amplified by the Dvl-mediated clustering of individual Wnt-Lrp-Fzd complexes in the membrane, forming the "signalosomes"(Bilic et al., 2007, Figure 3).

The mechanisms by which Wnt activation leads to β-catenin stabilization remain uncertain. Removal of Axin from the destruction complex due to its recruitment to Lrp6 or Fzd resulting in β-catenin stabilization has been suggested (Cliffe et al., 2003). Axin degradation might also be one possible mechanism, as overexpression of activated Wnt receptors or recombinant Dvl can lead to Axin degradation (Lee et al., 2003, Mao et al., 2001b, Tolwinski et al., 2003). In addition, there are two serine/threonine phosphatases, PP1 and PP2A, which associate with Axin (Hsu et al., 1999, Luo et al., 2007) and promote the dephosphorylation of Axin and β-catenin, respectively (Luo et al., 2007, Su et al., 2008). However, how the activity of these phosphatases is regulated by Wnt signals is not known. Alternatively (or in parallel), it has been proposed that phosphorylated Lrp6 is able to directly inhibit GSK-3β phosphorylation of β-catenin (Cselenyi et al., 2008, Piao et al., 2008, Wu et al., 2009).

Upon stabilization, β-catenin is able to translocate to the nucleus where it binds to the T cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors. In the absence of Wnt ligands, TCF represses gene expression by interacting with the repressor Groucho/TLE1. Upon nuclear accumulation β-catenin interacts with TCF/LEF, thus displacing Groucho/TLE1 (Daniels and Weis, 2005, Figure 3) and recruiting other co-activators for gene activation. TCF/LEF target genes are involved in cell proliferation, fate specification, and differentiation (for a more comprehensive overview of TCF target genes please see the Wnt homepage: http://www.stanford.edu/group/nusselab/cgi-bin/wnt/). Interestingly, Wnt signaling components are positively or negatively regulated by TCF/β-catenin (Chamorro et al., 2005, Kazanskaya et al., 2004, Khan et al., 2007), thus indicating that feedback control is a key feature of Wnt signaling regulation.

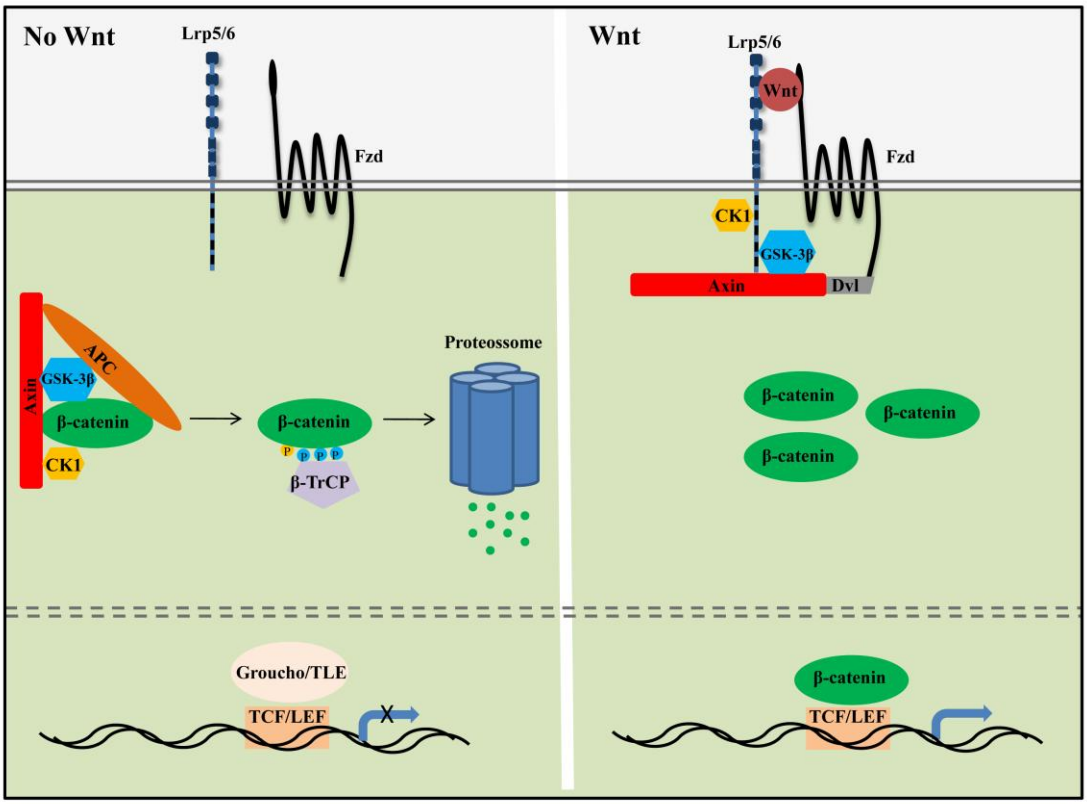

Figure 3: Wnt/β-catenin signaling. In the absence of a Wnt ligand, free cytosolic β-catenin is targeted for degradation by the destruction complex. Upon binding of a Wnt ligand to Lrp5/6 and Fzd, β-catenin is stabilized in the cytoplasm and can translocate into the nucleus where it displaces repressors such as Groucho/TLE1, binds to TCF and promotes gene transcription.

Due to the work described in this thesis, I will focus on some of the membrane-associated members of the Wnt/β-catenin pathway.

1.2.1.1 Membrane-associated members of the Wnt/β-catenin pathway

1.2.1.1.1 The ligand: Wnt1

The *Wnt1* mRNA encodes a 370 amino acids/41 kDA protein characterized by a strongly hydrophobic amino terminus, a cysteine-rich carboxy terminus, and four potential glycosylation sites (Fung et al., 1985). Like other Wnts, Wnt1 is quite hydrophobic and poorly soluble due to lipid modifications (Zhai et al., 2004) which are essential for its activity, and transform Wnt1 into a membrane-anchored protein localized in specialized lipid raft microdomains before secretion (Zhai et al., 2004). As a morphogen, Wnt1 is capable of short and long distance signaling, circumventing its insolubility in the aqueous space possibly by the formation of Wnt1 multimers that bury lipid modifications (Katanaev et al., 2008, MacDonald et al., 2009) or Wnt1 binding to lipoproteins (Panakova et al., 2005, MacDonald et al., 2009). Wnt1 is able to bind to the cysteine-rich domain (CRD) at the amino terminus of Fzd (Bhanot et al., 1996, Dann et al., 2001) and EGF-like domains of Lrp5/6 (Mao et al., 2001a).

As mentioned above, *Wnt1* overactivation has been linked with breast tumors (Nusse and Varmus, 1982, Li et al., 2000) and enhanced cell proliferation (Panhuysen et al., 2004). During development Wnt1 has a crucial role in midbrain and cerebellar formation (McMahon and Bradley, 1990, Thomas and Capecchi, 1990). Consistent with its proliferation-inducing activity, Wnt1 is able to increase the proliferation of $Nurr1^+$ DA precursors *in vitro* (Castelo-Branco et al., 2003).

1.2.1.1.2 The receptor: Lrp5/6

Lrp5/6 are homologues of the *Drosophila arrow* gene, which has been shown to be necessary for Wingless signaling events in *Drosophila* (Wehrli et al., 2000); similarly to *arrow*, they are able to act as co-receptors and activate Wnt/β-catenin signaling, as demonstrated by gain and loss-of-function studies (Tamai et al., 2000, Kelly et al., 2004, He et al., 2004). Lrp5/6 are part of the LRP subfamily of the LDLR family (Brown et al., 1998, Hey et al., 1998). They are type I single-span transmembrane proteins with 1615 and 1613 amino acid residues, respectively. Lrp5 and Lrp6 each contain an extracellular domain with 4 YWTD (tyrosine, tryptophan, threonine and aspartic acid) β-propeller domains, 4 EGF (epidermal growth factor) repeats (E1-E4) and LDLR repeats (Brown et al., 1998, Hey et al., 1998, He et al., 2004), followed by a

transmembrane region and a cytoplasmic domain with 5 PPP(S/T)P motifs. The prevalent view is that EGF repeats E1-E2 are necessary for Wnt binding and the E3-E4 are necessary for Dickkopf1 binding, a Wnt/β-catenin antagonist (Mao et al., 2001a, Liu et al., 2009). However, recent studies have challenged this view suggesting that Wnts can bind to the E3-E4 domain and that Dickkopf1 can bind both E1-E2 and E3-E4 (Binnerts et al., 2009, Bourhis et al., 2010). The intracellular domain is necessary for Wnt/β-catenin activation, given that a Lrp6 mutant lacking the intracellular domain is defective in Wnt signaling (Tamai et al., 2000); conversely, Lrp5/6 mutants lacking the extracellular domain (but anchored on the membrane) are constitutively active (Mao et al., 2001a, Mao et al., 2001b). The PPP(S/T)P motifs are phosphorylated by GSK-3β and CK1 upon Wnt binding and are required for Axin binding and the subsequent activation of the pathway (Tamai et al., 2004). Exogenous expression of the free (i.e., not anchored to the membrane) Lrp6 intracellular domain (Lrp6-ICD) is also able to induce a constitutively active Wnt/β-catenin pathway (Mi and Johnson, 2005) even in the absence of PPP(S/T)P motif phosphorylation (Beagle et al., 2009), suggesting that free Lrp6-ICD may act distinctly (and perhaps complementarily) from the membrane-anchored form in Wnt/β-catenin activation. In addition to their role in the Wnt/β-catenin pathway, Lrp5/6 have been shown to modulate the Wnt/PCP pathway, as gain and loss of function of Lrp6 disrupts CE-extension in *Xenopus* embryos (CE movements are normally mediated by the Wnt/PCP pathway) (Tahinci et al., 2007); moreover, Lrp5/6 have been shown to interact with Wnt5a, a Wnt/PCP ligand (Mikels and Nusse, 2006, Bryja et al., 2009, Andersson et al., 2010).

Lrp5/6 are expressed in several tissues during embryogenesis and in adult tissues (Kim et al., 1998, Pinson et al., 2000, Kelly et al., 2004). *Lrp5*$^{-/-}$ mice have normal embryogenesis and are fertile, but show osteoporosis (Kato et al., 2002). *Lrp6*$^{-/-}$ mice die perinatally and exhibit mid/hindbrain defects (dorsocaudal midbrain deletion, less defined MHB and a disorganized cerebellum), posterior embryonic truncation and abnormal limb patterning, which resemble the defects of mice mutant for *Wnt1, Wnt3a* and *Wnt7a* (Pinson et al., 2000). The milder phenotype in *Lrp5*$^{-/-}$ indicates that Lrp6 has a more influential role than Lrp5. They do seem to be fairly redundant as the *Lrp6*$^{-/-}$ mutants have a less severe phenotype than those observed in individual Wnt mutants (Pinson et al., 2000), suggesting that Lrp5 is able to substitute, at least partially, for Lrp6. Indeed, the *Lrp5*$^{-/-}$*; Lrp6*$^{-/-}$ double mutant mice die during gastrulation, as they fail to form the primitive streak, similarly to the phenotype observed in the *Wnt3*

mutant (Liu et al., 1999b). Thus, at least during embryogenesis, Lrp6 seems to have a more crucial role than Lrp5. In the *Lrp6*$^{-/-}$ mutants there are also PCP-associated defects such as exencephaly and spina bifida due to neural tube closure failure (Pinson et al., 2000, Bryja et al., 2009, Andersson et al., 2010), which can be rescued by removal of one or both *Wnt5*a alleles (Bryja et al., 2009), suggesting that the neural tube closure defects observed in *Lrp6*$^{-/-}$ mutants are a result of a gain-of-function of the Wnt/PCP pathway and that Lrp6 is able to block this pathway.

1.2.1.1.3 The antagonist: Dickkopf 1

Dickkopf 1 (Dkk1) is a secreted 35-45 kDA glycoprotein belonging to the Dkk family, which consists of four members (Dkk-1, -2, -3, -4). Dkk1 has an N-terminal signal peptide and contains two conserved cysteine-rich domains (Cys-1 and Cys-2) separated by a linker region (Glinka et al., 1998, Krupnik et al., 1999). Both human and murine Dkk-1 have one conserved potential *N*-glycosylation site located close to the C-terminus of the protein (Krupnik et al., 1999, Fedi et al., 1999), indicating that Dkk1 is post-translationally modified. As with other Dkks, Dkk1 contains several potential sites of proteolytic cleavage by furin-type proteases (Nakayama, 1997), suggesting that the proteins may be subject to post-translational proteolytic processing (Krupnik et al., 1999). Dkk1 is a potent Wnt/β-catenin pathway inhibitor: it binds to Lrp5/6 with high affinity (Mao et al., 2001a, Bafico et al., 2001), blocking the Wnt pathway (Figure 4). The exact mechanism regarding the Dkk1-mediated inhibition of Wnt/Lrp6 signaling remains to be elucidated: according to the most common model, Dkk1 competes with the Wnt protein for Lrp6 binding, disrupting the Wnt-induced Fzd- Lrp6 complex formation which is necessary for signal transduction (Semenov et al., 2001). In addition, it has been suggested that Dkk1 can also block the Wnt/β-catenin pathway by inducing Lrp6 endocytosis in the presence of kringle containing transmembrane proteins (Kremens, Mao et al., 2002), single-pass transmembrane, high-affinity receptors for Dkk1 (Figure 4). In support of the former model, it has been shown that Dkk1 inhibits binding of Lrp6 to Wnt3a but does not disrupt the Wnt3a-Fzd8 CRD interaction (Bourhis et al., 2010) and that Dkk1 can antagonize the Wnt/β-catenin pathway without Lrp6 degradation/internalization (Semenov et al., 2008). However, Dkk1-mediated Lrp6 endocytosis has also been reported (Yamamoto et al., 2008, Sakane et al., 2010). Interestingly, in a recent report, Dkk1 has been shown to decrease

Lrp6 expression and Wnt/Lrp6 signaling in the presence of Kremen but to stabilize Lrp6 in the membrane by blocking Wnt3a-induced Lrp6 turnover (Li et al., 2010).

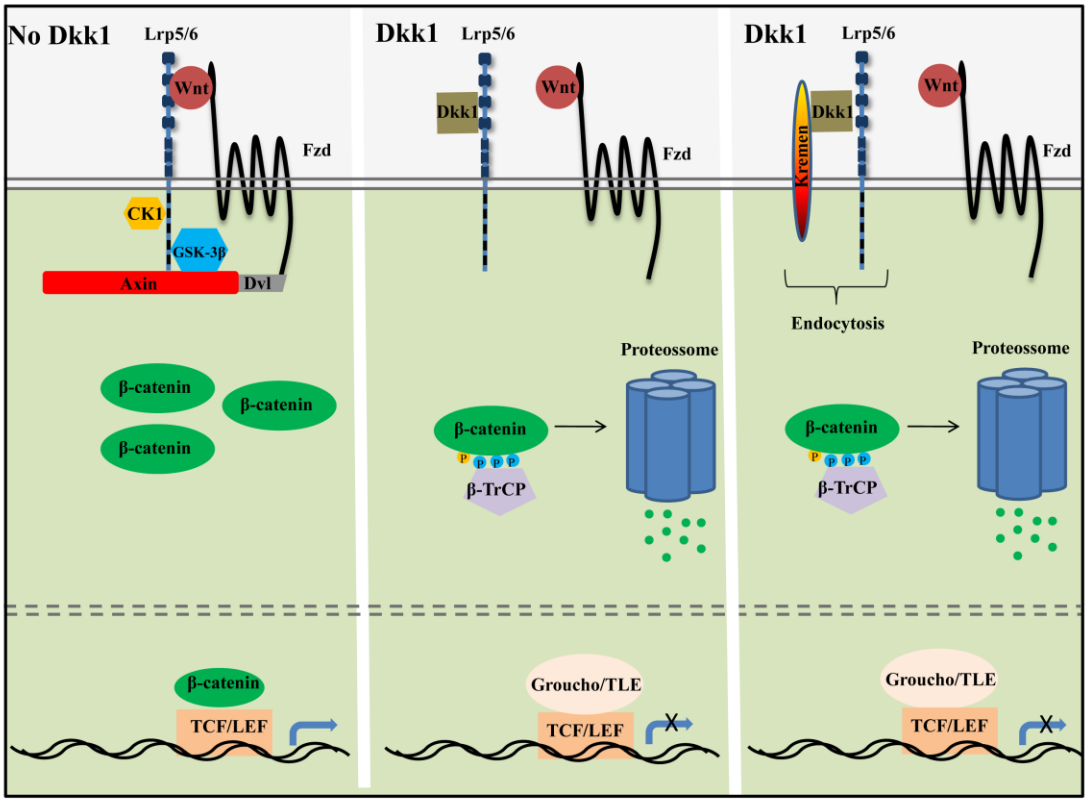

Figure 4: Dkk1 inhibits the Wnt/β-catenin pathway. Dkk1 binds to Lrp5/6 and prevents the formation of the with the Lrp-Fzd-Wnt complex. Dkk1 is also able to mediate Lrp6 endocytosis in the presence of Kremen proteins (right panel).

During embryonic development *dkk1* is first expressed in *Xenopus* in the Spemann organizer of the early gastrula and in mouse in the AVE (Glinka et al., 1998), and then later in the anterior mesendoderm (which comprises the prechordal plate and notochord) and foregut endoderm. The Spemman organizer (equivalent to the node in mouse) and the AVE (in combination with the node) are crucial for head induction during development through a dual BMP and Wnt inhibition (Barrantes et al., 2003). Indeed, injection of *dkk1* mRNA into *Xenopus* embryos leads to anteriorized embryos with big heads and enlarged cement glands. Together with a dominant-negative mutant of the BMP2/4 receptor, *dkk1* mRNA is also able to induce secondary axes with complete heads (Glinka et al., 1998). The importance of Dkk1 in development is demonstrated by loss-of-function studies: *Xenopus* embryos injected with an anti-Dkk1 antibody (Glinka et al., 1998) and *Dkk1* knockout mice (Mukhopadhyay et al., 2001) lack anterior head structures. The head-inducing activity of Dkk1 is mediated through the inhibition of the Wnt/β-catenin posteriorizing activity in early gastrula embryos

(Kazanskaya et al., 2000). In addition, Dkk1 is involved in limb formation (Grotewold et al., 1999, Adamska et al., 2004), vertebral development (MacDonald et al., 2004), bone formation (Li et al., 2006, Morvan et al., 2006) and bilateral eye induction (Kazanskaya et al., 2000). Dkk1 has also been described to regulate cell proliferation and programmed cell death (Mukhopadhyay et al., 2001, Gregory et al., 2003, Wang et al., 2000), and to have a role in diseases such as cancer (Tian et al., 2003) and Alzheimer's disease (Caricasole et al., 2004). Consistent with its role *in vivo*, Dkk1 is able to induce neural differentiation from embryonic stem cells (Watanabe et al., 2005, Verani et al., 2007, Kong and Zhang, 2009).

A possible role of *Dkk1* in the establishment of the floor plate has been suggested. A recent study has demonstrated that in human embryonic stem cells (hESC) induced to differentiate into floor plate cells treatment with Shh induces a decrease in Dkk1 in both mRNA and protein levels. Furthermore, Dkk1 expression inhibits Foxa2 expression (Fasano et al., 2010), and *Foxa2* knockout embryos show increased expression of Dkk1 in the ectoderm at E7.5 (Kimura-Yoshida et al., 2007).

The majority of the Dkk1 effects are a result of a direct inhibition of the Wnt/β-catenin pathway. However, other roles for Dkk1 have been suggested. *In vitro*, Dkk1 has been shown to antagonize Wnt signaling independent of β-catenin and possibly through the Wnt/JNK pathway (Lee et al., 2004). Moreover, *in vivo* evidence has also implicated Dkk1 both positively and negatively in the Wnt/PCP pathway: Caneparo et al. have shown that gain and loss of Dkk1 function can modulate CE-associated gastrulation movements (Caneparo et al., 2007); this effect was independent of Wnt/β-catenin pathway since up-regulation of the β-catenin pathway did not induce the gastrulation movement defects observed in Dkk1 morphants. Moreover, it was also shown that Dkk1 is able to cooperate with glypican4/6 member Knypek, a receptor which is required for Wnt/PCP activity (Topczewski et al., 2001). On the other hand, another study has revealed that Dkk1 antagonizes both canonical and non-canonical signaling to regulate the dorsal/ventral patterning of the early pre-gastrulation *Xenopus* (Cha et al., 2008). These results suggest that the differential regulation of the Wnt signaling by Dkk1 might be dependent on the developmental context.

A β-catenin-independent action of the Dkk1 amino terminal has also been demonstrated (Korol et al., 2008). Previous studies have suggested that the N terminal of Dkk1 is

responsible for its inhibitory activity (Brott and Sokol, 2002), and it would be interesting to further characterize the influence of the full length protein versus N terminal peptide in the disruption of Wnt signaling.

Dkk1 and Lrp5/6 have also been shown to interact *in vivo*: the extra digit number and anterior head truncation phenotypes resulting from hypomorphic expression of *Dkk1* can be corrected by genetically reducing the amount of *Lrp6* (MacDonald et al., 2004), and limb abnormalities in these mutants can be corrected by reduced *Lrp5* levels. Conversely, removal of *Dkk1* improves survival, limb and axial development in *Lrp6*$^{-/-}$ animals (MacDonald et al., 2004), demonstrating the importance of correctly balancing positive and negative regulation of Wnt signaling during mammalian development.

1.2.2 "Non-canonical" signaling: the Wnt/PCP and Wnt/Ca^{2+} pathways

In the "non-canonical" pathway, Wnts are still able to bind to Fzd and activate Dvl (Gonzalez-Sancho et al., 2004) but the downstream targets do not promote β-catenin stabilization. These pathways are involved in CE movements (medio-lateral cell movements that promote narrowing along one axis and elongation along a perpendicular axis) and planar cell polarity (PCP, refers to the uniform orientation of a population of cells within a single epithelial plane). The Wnt ligands associated with the "non-canonical" pathway do not induce axis duplication and lead to defective gastrulation when injected in *Xenopus* embryos (Du et al., 1995, Moon et al., 1993). In the Wnt/Ca^{2+} pathway, binding of Wnt to Fzd leads to the activation of Dvl and to an increase in intracellular Ca^{2+} levels and activation of PKC and CamKII, which will then mediate several intracellular responses. In the Wnt/PCP pathway, activated Dvl signals through small GTPases such as Rho, Rac, Cdc42 (Habas et al., 2001, Habas et al., 2003, Choi and Han, 2002) and also through JNK (Boutros et al., 1998, Yamanaka et al., 2002), leading to cytoskeletal modifications. Even though they are grouped collectively as a Wnt/PCP pathway, signaling through small GTPases have distinct intracellular targets and are considered by some as separate. Moreover, it is possible that the Wnt/PCP and Wnt/Ca^{2+} are not mechanistically distinct as overexpression of "non-canonical" Wnt ligands leads to CE defects as well as increases in intracellular Ca^{2+} levels (Du et al., 1995, Moon et al., 1993). Another interesting observation is that gain and loss-of-function on the Wnt/PCP pathway can produce similar phenotypes (Schambony and Wedlich, 2007).

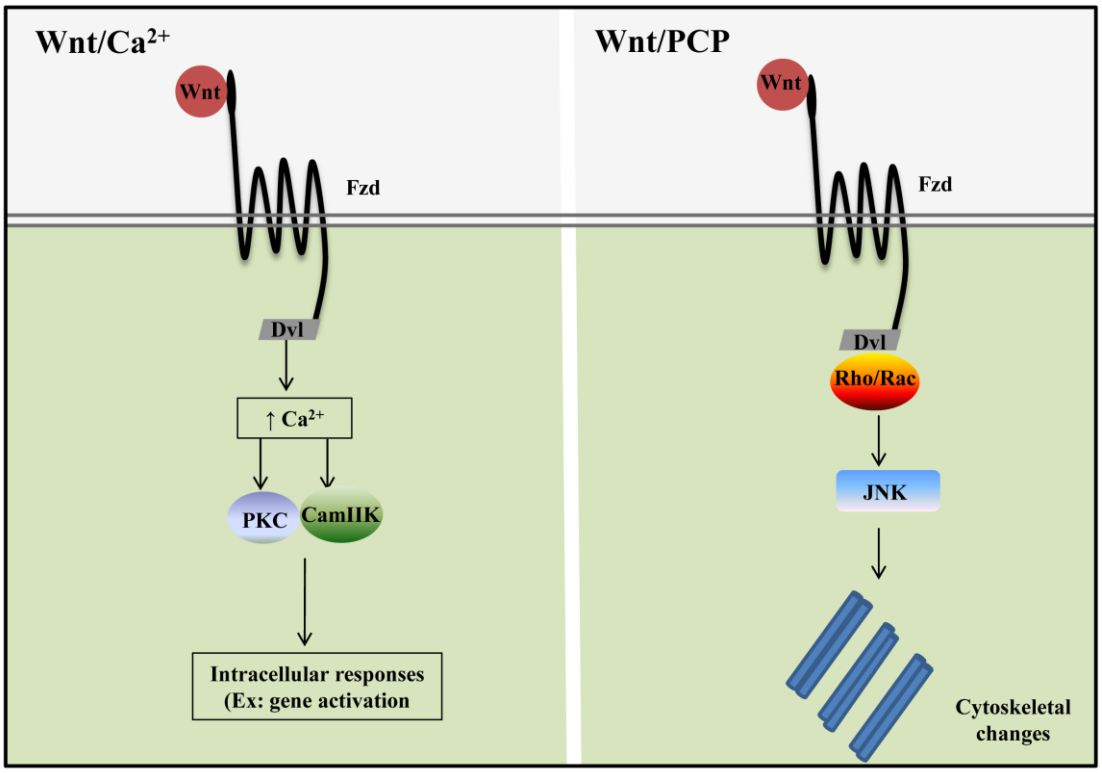

Figure 5: The Wnt/Ca^{2+} and Wnt/PCP and pathways. In Wnt/Ca^{2+} pathway, Wnt binding to Fzd promotes an increase in intracellular Ca^{2+} levels and activation of PKC and CamIIK. In the Wnt/PCP pathway, activated Dvl signals through small GTPases such as Rho and Rac and JNK.

1.2.2.1 Wnt5a

Wnt5a is a 46 kDa protein and one of the prototypical ligands of the "non-canonical" pathway. Injection of *Wnt5a* mRNA in *Xenopus* induces defective morphogenetic movements (Moon et al., 1993, Du et al., 1995) and no axis duplication, as well as stimulating intracellular Ca^{2+} (Slusarski et al., 1997). Like other Wnts, Wnt5a can signal through Fzd receptors but roles for Ror1/2 and Ryk, an orphan tyrosine kinase and a tyrosine-related receptor, respectively, have been described in Wnt5a signaling (Angers and Moon, 2009, Green et al., 2008, Hikasa et al., 2002, Schambony and Wedlich, 2007, Oishi et al., 2003, Grumolato et al., 2010).

Wnt5a has been shown to inhibit the Wnt/β-catenin pathway: coexpression of *X*Wnt5a with *X*Wnt8 (a Wnt/β-catenin ligand) in *Xenopus* embryos impairs the ability of *X*Wnt8 to induce a secondary axis (Torres et al., 1996) and *Wnt5a*$^{-/-}$ knockout mice show increased β-catenin signaling in the distal limb, indicating that Wnt5a may inhibit β-catenin stabilization (Yamaguchi et al., 1999). However, the exact mechanism by which Wnt5a is able to inhibit the Wnt/β-catenin pathway remains elusive: a GSK- 3β -independent β-catenin degradation has been suggested (Topol et al., 2003), but Wnt5a-

mediated inhibition of canonical Wnt signaling at the level of TCF transcription (with no changes in the protein levels of β-catenin, Mikels and Nusse, 2006) as well as a reciprocal pathway inhibition of "canonical" and "non canonical" Wnts at the cell surface by competition for Fzd binding (Grumolato et al., 2010) have been reported. To add even further complexity, Wnt5a has been shown to activate TCF when co-expressed with Fzd4 and Lrp5 (Mikels and Nusse, 2006), to activate β-catenin when fused to the Lrp5/6-binding domain of Dkk2 (Liu et al., 2005) and to induce axis duplication in the presence of Fzd5 (He et al., 1997) indicating that Wnt5a-mediated inhibition or activation of the β-catenin pathway might be dependent on the receptor context.

Wnt5a is firstly expressed during gastrulation in several tissues and *Wnt5a*$^{-/-}$ mice display anterior-posterior extension defects (Yamaguchi et al., 1999). *Wnt5a* is expressed in the midbrain as early as E8.75 (Yamaguchi et al., 1999) and its expression continues throughout DA neuron development (Castelo-Branco et al., 2003, Andersson et al., 2008). Wnt5a promotes DA differentiation of Nurr1^{+} precursors in a DA cell line (Schulte et al., 2005, Bryja et al., 2007), VM-derived neurospheres (Parish et al., 2008) and primary mesencephalic cultures (Castelo-Branco et al., 2003, Andersson et al., 2008) through activation of Dvl (Schulte et al., 2005, Bryja et al., 2007) and Rac (Andersson et al., 2008). In *Wnt5a*$^{-/-}$ mice there is a transient increase in the number of proliferating progenitors and Nurr1^{+} precursors and an impairment in the DA differentiation at E12.5, as assessed by the TH^{+}/Nurr1^{+} ratio (Andersson et al., 2008), which is later recovered. In these mice VM morphology and DA population distribution are altered in a typical CE phenotype, indicating that Wnt5a mediates several functions in the VM and DA neuron development.

1.3 CELL REPLACEMENT THERAPY IN PARKINSON'S DISEASE

One of the most promising therapeutic approaches in PD is cell-replacement therapy (CRT), in which DA neurons and/or precursors are grafted into the striatum in order to restore the lost striatal DA transmission. Several cell types have been considered as potential sources of DA neurons; the work presented in this thesis focuses on embryonic stem cells and human fetal ventral midbrain cells.

1.3.1 Embryonic Stem Cells (ESC)

Embryonic stem cells are derived from the ICM of a blastocyst. The first mouse ESC lines were generated in 1981 (Evans and Kaufman, 1981, Martin, 1981) and in 1998 the first human ESC were derived (Thomson et al., 1998). ESCs are able to self-renew and are pluripotent (they can give rise to all cells from all three germ layers). These properties mean that ESCs can be expanded indefinitely *in vitro* and that they can generate large numbers of DA neurons. The majority of the protocols used to induce DA differentiation from ESC make use of basic concepts of Developmental Biology: induction of a neuroectoderm-like population by modulation of BMP and/or Wnt signaling (Gerrard et al., 2005, Watanabe et al., 2005, Kong and Zhang, 2009, Sonntag et al., 2007), followed by patterning events to promote DA neuron specification. The most common approaches to induce DA differentiation are based on (a) the generation of embryoid bodies, from which neural progenitors are derived (Lee et al., 2000) (b) co-culturing of the ESCs on feeder layers (Kawasaki et al., 2000, Barberi et al., 2003, Yue et al., 2006, Roy et al., 2006) or (c) adherent monolayer cultures (Ying et al., 2003). The patterning events are achieved through addition (or secretion by the feeder cells) of soluble factors involved in midbrain patterning and DA specification such as Shh and Fgf8 (Lee et al., 2000, Barberi et al., 2003) or by genetic modification of ESCs by overexpression of midbrain-specific transcription factors. Indeed, in ESC engineered to express Nurr1 (Kim et al., 2002, Martinat et al., 2006), Pitx3 (Chung et al., 2005, Hedlund et al., 2008), Lmx1a (Andersson et al., 2006b, Friling et al., 2009) and Foxa2 (Kittappa et al., 2007), DA differentiation is robustly increased. It seems, though, that the most efficient DA induction is through a combination of soluble factors and gene overexpression (Kim et al., 2006, Andersson et al., 2006b). Moreover, mESC-derived DA neurons have been able to induce behavioral improvement in animal models of PD (Kawasaki et al., 2000, Kim et al., 2002, Lee et al., 2000). In spite of its promising potential, the clinical application of ESC-derived DA neurons has been hindered by some ESC specific characteristics: the purity of the cultures (ESC cultures develop asynchronously and heterogeneously) and the risk of excessive proliferation and teratoma formation due to incomplete differentiation and their genetic instability (Morizane et al., 2008). Furthermore, important differences between mouse and human ES cells have been detected in terms of the factors their require for their maintenance and differentiation (Sato et al., 2003, Xu et al., 2005, Levenstein et al., 2006, Ying et.al, Sonntag et al., 2007) and human ES cell–derived DA cells have been found to survive

poorly after transplantation in animal models (Roy et al., 2006, Sonntag et al., 2007). Thus, further work is required in order to develop hESC as tools for CRT.

1.3.2 Human Fetal Ventral Midbrain Cells

Human ventral mesencephalons from aborted fetuses have been considered to be an excellent cell source for CRT: due to their origin, these cells are correctly specified towards a midbrain DA fate. Moreover, even though mesencephalic tissue contains proliferating progenitors they are less prone to form tumors after transplantation and appear to be genomically more stable than ESC. More importantly, human fetal ventral midbrain cells have been used in clinical trials and provided proof-of-principle evidence that DA neurons obtained from fetal human ventral midbrain cells can survive and provide significant benefits in some patients (Kordower et al., 1995, Piccini et al., 1999, Mendez et al., 2005). The first open label clinical trials with human fetal mesencephalic cells were performed in the late 80's (Lindvall et al., 1988, Lindvall et al., 1989) and since then several similar trials have followed, with reported clinical benefits associated with graft survival (Lindvall et al., 1990, Hagell et al., 1999, Brundin et al., 2000, Kordower et al., 1998) and motor function improvement (Piccini et al., 1999). However, the follow up double-blind, sham surgery-controlled studies were not as successful with modest or poor clinical outcomes (Freed et al., 2001, Olanow et al., 2003). These different results are probably a reflection of the high experimental variability between these studies regarding patient selection, tissue preparation/composition and immune suppression (Lindvall et al., 2004, Winkler et al., 2005). Moreover, one of the major limiting factors in the widespread use of human fetal mesencephalic tissue is the need for high numbers of fetuses per patient (6 to 8) in order to obtain the necessary numbers of DA neurons that can elicit a positive outcome (100,000 TH^+ cells, (Kordower et al., 1995)).

Isolation and propagation of human ventral midbrain-derived cells has been reported by several groups (Storch et al., 2001, Sanchez-Pernaute et al., 2001, Wang et al., 2004, Jin et al., 2005, Milosevic et al., 2006, Maciaczyk et al., 2008, Hovakimyan et al., 2006). In these studies, cells have been expanded for as long as 8 months (Storch et al., 2001) but in general there is a decrease in the numbers of neurons after multiple passages and limited numbers of TH^+ neurons upon differentiation. So far, the best differentiation protocol yielded 21% of TH^+ neurons after 7 days of proliferation when cells were differentiated in the presence of dibutyryl adenosine monophosphate

(dcAMP, Sanchez-Pernaute et al., 2001) In a recent report, in long-term expanded midbrain-derived cultures (≈ 1 year) it was possible to detect 50% of neuronal cells and of those approximately 15% were immunopositive for TH (Maciaczyk et al., 2008) when cells were differentiated for longer periods and in the presence of brain derived neurotrophic factor (BDNF). Cells are typically cultured in the presence of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF/FGF2) during proliferation (Storch et al., 2001, Sanchez-Pernaute et al., 2001, Hovakimyan et al., 2006) and differentiation is achieved by removal of these mitogens and enhanced by the addition of neurotrophins such as BDNF (Maciaczyk et al., 2008) and GDNF (Storch et al., 2001, Jin et al., 2005), ascorbic acid, cyclic adenosine monophosphate (cAMP, Sanchez-Pernaute et al., 2001) or cytokines (Storch et al., 2001, Jin et al., 2005). Immortalized human mesencephalic cell lines have also been established (Donato et al., 2007, Villa et al., 2009, Lotharius et al., 2002). Notably, overexpression of Bcl-X_L, an anti-apoptotic marker, enhances the maintenance of the neuronal and DA competence in long term expanded cultures and protects the cells from apoptotic cell death during differentiation (Courtois et al., 2010). These Bcl-X_L-overexpressing cell lines are also able to survive transplantation and generate mature DA neurons in a rat model of PD, thus enhancing functional recovery of Parkinsonian rats.

2 AIMS OF THE STUDY

Understanding how DA neurons develop in the midbrain is crucial for the successful establishment of CRT. The Wnt pathway is involved in several important processes in development and adult tissue homeostasis, and has been implicated in ventral midbrain DA neuron development. The main goal of this thesis was to further investigate the role of different components of the Wnt signaling pathway in DA neuron development and to determine their potential use in CRT:

- To evaluate the impact of impaired Wnt/β-catenin signaling on mouse ESC-derived DA differentiation (Paper I)

- To determine the role of two Wnt signaling components, Lrp6 (Paper II) and Dkk1 (Paper III), on midbrain DA development *in vivo*

- To improve protocols to obtain significant amounts of DA neurons from human fetal ventral midbrain tissue (Paper IV)

3 RESULTS & DISCUSSION

3.1 PAPER I

3.1.1 Absence of *Wnt1* promotes neuronal differentiation and an increase in the number of DA neurons in mESC cultures

In the *Wnt1*$^{-/-}$ mutants there is a substantial loss of midbrain structures and only 10% of the normal numbers of DA neurons are born (McMahon and Bradley, 1990, Thomas and Capecchi, 1990, Prakash et al., 2006). Furthermore, we have previously shown that Wnt1 promotes proliferation of DA neurons precursors in mouse VM primary cultures (Castelo-Branco et al., 2003). In order to further investigate the role of Wnt1 in VM DA neuron development we induced DA differentiation in *Wnt1*$^{-/-}$ mESC (Bryja et al., 2006) using an established protocol based on co-culture of ESCs on PA6 cells and addition of soluble factors such as Shh, Fgf8 and Fgf2 (Barberi et al., 2003). After 14 days of differentiation we could detect an increase in the proportion of Tuj1 positive colonies (Tuj1/βIII tubulin, an early neuronal marker) in *Wnt1*$^{-/-}$ cells. Surprisingly, we detected an increase in the number of TH^{+} colonies and cells in the *Wnt1*$^{-/-}$ mESC when compared wild type cells. Furthermore, analysis of other DA markers such as *Nurr1* confirmed this increase in DA yield. To test that the absence of Wnt1 was impairing the Wnt/β-catenin pathway, we differentiated *Wnt1*$^{-/-}$ mESC until day 4 and 5 of the protocol, given that Wnt1 expressed at very low levels in undifferentiated ES cells and it is up-regulated during day 4-5 of mESC differentiation. We then examined the expression of *Axin* and *Brachyury*, two well known downstream targets of the Wnt/β-catenin pathway and found lower levels in *Wnt1*$^{-/-}$ mESC compared to wild type cells, thus confirming that the absence of Wnt1 impairs the Wnt/β-catenin pathway.

3.1.2 Impairment of the Wnt/β-catenin at the receptor level increases DA differentiation

To further confirm that disturbing Wnt/β-catenin signaling promotes DA differentiation in mESC, we differentiated *Lrp6*$^{-/-}$ mESC (Bryja et al., 2006) towards a DA phenotype using the same protocol as for Wnt1 cells. After 14 days of differentiation, we could detect an increase in TH^{+} colonies, but not Tuj1, in the *Lrp6*$^{-/-}$ cultures when compared to wild type. This specific increase in DA differentiation was further verified by the

increased protein levels of TH (but not Tuj1) in our cultures and by the increase of other DA markers such as *Foxa2, Lmx1a, Nurr1, Pitx3, Dat* (dopamine transporter). The inability of *Lrp6*$^{-/-}$ to respond to Wnt ligands, as assessed by Western Blot analysis and luciferase assays, confirmed that indeed in these cells the Wnt/β-catenin signaling was impaired.

3.1.3 Increases in neuronal and DA differentiation in *Wnt1*$^{-/-}$ and *Lrp6*$^{-/-}$ mESC are not due to factors secreted from the feeder cells

PA6 cells are known to secrete factors which are involved in DA specification (Swistowska et al., 2010, Catherine Schwartz, manuscript). To investigate the contribution of PA6-derived factor(s) to the observed phenotype, we used a feeder-free protocol to differentiate the *Wnt1*$^{-/-}$ mESC. When cells were differentiated for 8 days in these conditions, we could still detect an increase in the number of Tuj1^{+} and TH^{+} colonies and in the number of TH^{+} cells within the colonies in *Wnt1*$^{-/-}$ mESC comparative to wild type mESC. Moreover, mRNA levels of DA markers such as *Foxa2, Lmx1a, Nurr1,* and *Pitx3* were increased in *Wnt1*$^{-/-}$ cells, indicating that the increases in neuronal and DA differentiation were a direct effect of *Wnt1* deletion. Interestingly, the DA differentiation was further increased when *Wnt1*$^{-/-}$ cells were cultured in the presence of the patterning factors Shh, Fgf8 and Fgf2 in the feeder-free protocol and expression of *Dat* was only detected after exposure to patterning factors and increased by deletion of *Wnt1*. Together, these data suggest that the observed increase in neuronal and DA differentiation in *Wnt1*$^{-/-}$ mESC is cell-intrinsic (not due to factors secreted by the feeder cells) and can be enhanced by soluble factors involved in DA neuron specification.

3.1.4 Neuronal and DA differentiation are accelerated in the absence of *Wnt1* and *Lrp6*

To determine at which time the deletion of Wnt1 and Lrp6 were exerting their effect in neuronal and DA differentiation, we examined *Wnt1*$^{-/-}$ and *Lrp6*$^{-/-}$ mESC at earlier times during differentiation on PA6 cells. Already at day 7, when very few Tuj1^{+} and TH^{+} cells were present in wild type ESC, we could detect mature Tuj1-expressing cells and some TH^{+} neurons in *Wnt1*$^{-/-}$ cells and an increase in the proportion of Tuj1^{+} and TH^{+} colonies. In the *Lrp6*$^{-/-}$ cells analyzed at day 5 of differentiation, we could

detect neural rosettes, seldom seen in differentiating mESC, and by day 6 mature $Tuj1^+$ and (occasionally) TH^+ cells were found. Moreover, at day 6 of differentiation almost all the colonies were $Tuj1^+$ and TH^+ colonies where present in *Lrp6*$^{-/-}$ mESC, whereas in control wild type mESC only half of the colonies were positive for Tuj1 and very few TH^+ colonies were present. These results indicate that neuronal and DA specification occurs earlier in differentiating mESC cultures in the absence of *Wnt1* or of *Lrp6*.

3.1.5 Dkk1 treatment promotes DA neurogenesis in mESC

Given the observed phenotype in *Wnt1* and *Lrp6* mutant mESC, we asked ourselves whether the effect of the deletion could be mimicked in wild type cells by treatment with Dkk1, a known Wnt/β-catenin inhibitor. R1 mESC were induced to differentiate in a feeder-free protocol and treated with Dkk1 from day1 to 5 or during the whole differentiation protocol (12 days). Although treatment from day1 to 5 promoted only a small increase in *Th* and *Tuj* expression, addition of Dkk1 during the whole protocol increased the proportion and numbers of TH^+ colonies and cells (even in the presence of patterning factors), albeit to a lesser extent than the absence of *Wnt1* or *Lrp6*. Western Blot analyses revealed that Dkk1 treatment in mESC was able to decrease the Wnt3a-induced activation of the Wnt/β-catenin, and Dkk1's effects were competed against by Wnt3a in a dose-dependent manner.

3.1.6 What is the contribution of the Wnt/β-catenin pathway in neurogenesis and DA differentiation in mESC?

From our work we can conclude that impairment of the Wnt/β-catenin pathway in mESC promotes neuroectodermal and DA differentiation. While the former can be explained by basic Developmental Biology concepts and both alterations can be attributed to the differences between *in vitro* and *in vivo* data, the latter observation was a surprising one. Indeed, the protocols used to induce mESC differentiation try to recapitulate the events that occur during development; a crucial point is the induction of a neuroectoderm which is mediated by Wnt and BMP antagonists. Thus, in order to induce head formation there must be an inhibition of the Wnt/β-catenin pathway shortly after gastrulation. This is further confirmed by the analysis of the *Dkk1* mutants which fail to generate most of the anterior head structures (Mukhopadhyay et al., 2001) and *in*

vitro studies where overexpression of *sFrp2* (which can act as a Wnt inhibitor) was shown to stimulate the production of $Sox1^+$ neural progenitors, while forced expression of *Wnt1* and treatment with lithium chloride (which activates the Wnt/β-catenin pathway by inhibiting the activity of GSK-3β) inhibit neural differentiation (Aubert et al., 2002). Thus, increased and accelerated neuralization in *Wnt1*$^{-/-}$ and *Lrp6*$^{-/-}$ mESC is most likely due to an early inhibition of the Wnt/β-catenin pathway. However, both positive (Otero et al., 2004) and negative (Aubert et al., 2002, Haegele et al., 2003) roles of the Wnt/β-catenin pathway in neural differentiation from mESC have been described, and activation of the Wnt/β-catenin pathway has been implicated in promoting the neuronal differentiation from neural precursors (Hirabayashi et al., 2004, Muroyama et al., 2004), suggesting a time-dependent dual role of this pathway in neurogenesis. Given the importance of Wnt1 for midbrain and DA neuron development, the observation that midbrain DA differentiation was enhanced by the absence of Wnt1 or Lrp6 was unexpected. While at earlier stages the increase in TH^+ neurons and colonies might be due to an overall increase in neurogenesis, the fact that in *Lrp6*$^{-/-}$ mESC there is a recovery in Tuj1 expression but not TH at later stages suggests a more specific effect. Moreover, Dkk1's effect was more pronounced when added during the whole protocol in comparison with its presence only during the first 5 days of differentiation, and in *Dkk1* deficient mice there is a DA differentiation impairment (Paper III). Together, these data suggest that inhibition of the Wnt/β-catenin pathway is involved in the differentiation of mESC into correctly specified DA neurons. Given the asynchrony and heterogeneity of mESC cultures, inappropriate secretion from different cell types and/or at different time points might provide an explanation for the *Wnt1*$^{-/-}$ cultures. Moreover, disturbance of the Wnt/β-catenin might also affect other signaling pathways which might also be responsible for the observed phenotype. One could speculate that, as "canonical" and "non-canonical" Wnt signaling are known to negatively regulate each other, increased DA differentiation in our cultures might be due to an increase in Wnt5a-induced or non-canonical signaling. Another important conclusion form our work is that current protocols to induce DA differentiation can be enhanced by modulation of the Wnt/β-catenin pathway with soluble factors such as Dkk1, which is of importance for CRT.

3.2 PAPER II

3.2.1 Deletion of *Lrp6* does not alter VM patterning, cell survival or proliferation

In light of previous studies, we decided to investigate whether Lrp6 is required for midbrain or DA neuron development by analyzing *Lrp6*$^{-/-}$ mice. *In situ* hybridization and quantitative PCR (qPCR) confirmed that *Lrp5* and *Lrp6* are ubiquitously expressed in the brain at E11.5. As previously described (Pinson et al., 2000) we could detect a less well-defined isthmus at a dorsal level at E9.5 and E10.5 in *Lrp6*$^{-/-}$ mice, and a deletion of dorsal mid/hindbrain tissue at E12.5, but analysis of the ventral expression of several patterning factors such as *Otx2*, *En1*, *Lmx1b*, *Shh* and *Wnt5a* revealed no differences in *Lrp6*$^{-/-}$ embryos in comparison to wild type embryos; in fact, a normal patterning of the VM was also present even in *Lrp6*$^{-/-}$ exencephalic embryos. Moreover, we did not detect any changes in proliferation, as assessed by analysis of phospho-histone-3^{+} cells and the 5-ethynyl-2´-deoxyuridine (EdU) and 5-bromo-2-deoxyuridine (BrdU) incorporation. Cell survival was not affected as determined by the similar numbers of Caspase-3^{+} cells in wild type and *Lrp6* mutants and progenitor markers such as Nestin and *Aldehyde dehydrogenase 2* (*Ahd2*) were also unaffected in *Lrp6*$^{-/-}$ embryos.

3.2.2 *Lrp6*$^{-/-}$ mice display a delayed DA differentiation and an altered VM morphology

To investigate if DA development was affected by the absence of *Lrp6*, we examined the number of TH^{+} cells at E11.5 and found a 50% reduction, as assessed by immunohistochemistry, qPCR and *in situ* hybridization, with no apparent change in the number of total neurons (as determined by Tuj1 expression). Moreover, we could also detect a 40% reduction in the number of Nurr1^{+} cells, a 60% reduction in *Nurr1* mRNA levels at E11.5, as well as a decrease in the levels of *Pitx3* mRNA at E12.5 in *Lrp6*$^{-/-}$ embryos, indicating that there was a differentiation impairment. However, by E13.5 there was a recovery in these defects, as we found no differences in Nurr1 expression between wild type and *Lrp6*$^{-/-}$ embryos, and only a 25% reduction in the number of TH^{+} cells in *Lrp6* mutants which was restored to wild type levels at later stages. *Lrp6*$^{-/-}$ mice also displayed a narrower V-shaped VM VZ.

3.2.3 What is the contribution of Lrp6 to VM DA development?

Despite the pronounced effects of Lrp6 in mESC-derived DA differentiation, our *in vivo* studies revealed a transient decrease in DA neurogenesis and differentiation, which was recovered at later stages. An imbalance between "canonical" and "non-canonical" signaling in the VM might account for the phenotype (given the established role of Wnt5a in promoting DA differentiation, more Wnt5a-mediated "non-canonical" signaling could rescue differentiation); an imbalance between these 2 branches of Wnt signaling is further suggested by an alteration in VM morphology. On the other hand, the compensation of the phenotype of the *Lrp6*$^{-/-}$ at later stages and the lack of and early phenotype could be explained by a compensation by Lrp5, the other member of the LRP family that signals through canonical Wnt signalling. Indeed, *Lrp5* is expressed in the developing VM, and between E11.5 and E15.5, *Lrp5* expression was up-regulated in *Lrp6* homozygote mutant embryos (Gonçalo Castelo-Branco, unpublished data).

3.3 PAPER III

3.3.1 Dkk1 is expressed in the VM

The results obtained with Dkk1 in mESC-derived DA differentiation led us to investigate whether Dkk1 had a role in midbrain DA development. *In situ* hybridization revealed that *Dkk1* was expressed in the ventral midbrain at E9.5 and E10.5, after which its expression was no longer detected. At E9.5, *Dkk1* was expressed in a salt-and-pepper pattern in the medial and lateral floor plate, overlapping with and in between the two Wnt1 stripes. *Dkk1* was more highly expressed rostrally and less caudally. At E10.5, expression of *Dkk1* was detected in all the three layers of the developing VM but restricted to the medial part of the floor plate in Lmx1a^{+} cells adjacent to the midline.

3.3.2 Initial neurogenesis is not affected but differentiation is impaired in *Dkk1* mutants

To assess the contribution of Dkk1 to midbrain development we analyzed *Dkk1*$^{+/-}$ animals, given the severity of the phenotype of *Dkk1*-null mice. At E11.5, the onset of DA neurogenesis, we did not detect any changes in the number of TH^{+} neurons or in the expression of Tuj1. Moreover, analysis of the number of Lmx1a^{+} cells revealed no

differences between wild type and *Dkk1*$^{+/-}$ embryos. However, at E13.5 we detected a 40% reduction in the number of TH^{+} neurons in *Dkk1*$^{+/-}$ mice. While the number of precursors was not affected, as determined by the expression of Nurr1, there was a reduction in the number of Pitx3^{+} cells, suggesting a differentiation impairment. Proliferation, neurogenesis and cell death were not affected in *Dkk1*$^{+/-}$ embryos as assessed by the expression of Ki67, Tuj1 and Caspase-3, respectively. Moreover, other ventral midbrain populations such as the Islet1^{+} oculomotor neurons and the Brn3a^{+} red nucleus cells were not affected indicating that the observed phenotype was specific to DA neurons. In E17.5 *Dkk1*$^{+/-}$ animals we could still detect a 30% reduction in the numbers of TH^{+} neurons, indicating that the differentiation impairment persists at later stages.

3.3.3 In addition to a differentiation impairment, VM cell distribution and morphogenesis is also affected in *Dkk1* mutants

Besides the DA differentiation deficit, in E17.5 *Dkk1*$^{+/-}$ animals we detected an abnormal distribution of TH^{+} DA neurons at different anterior-posterior levels in the VM. To further characterize the role of Dkk1 in VM DA neuron development, we decided to analyze the few *Dkk1*$^{-/-}$ embryos obtained at E17.5. As expected, these embryos had a deletion of most anterior head structures, with different levels of midbrain deletion. In these animals, we could detect a severe reduction or an almost complete absence of DA neurons which, when present, were very abnormally distributed forming clusters and exhibited few or no processes. Some of these TH^{+} neurons were also positive for Pitx3, indicating that despite the severe VM and DA defects, some midbrain tissue was still formed in the absence of *Dkk1*. However, given the lack of forebrain structures and the early role of Dkk1 in development, we cannot exclude the possibility of an indirect mechanism. Thus, these data suggest that *Dkk1* is required for DA differentiation and midbrain morphogenesis.

3.3.4 Dkk1: a novel regulator of VM DA development and morphogenesis?

Our finding that Dkk1 is expressed in the VM at early stages suggests a role for Dkk1 in midbrain DA development. In *Dkk1* mutants, we could detect a DA differentiation deficit most likely due to modifications in the Wnt/β-catenin pathway. Indeed, a

balanced Wnt/ β-catenin pathway is of utmost importance during development, and Dkk1 and Wnt ligands are quite often expressed in adjacent domains and are known to genetically interact (Lewis et al., 2008). Defects in *Dkk1*-null mice (where one can expect an increase in Wnt/ β-catenin signaling) can be ameliorated by removing one allele of *Lrp6* (MacDonald et al., 2004, Lewis et al., 2008) or of *Wnt3a* (Lewis et al., 2008). Several lines of evidence implicate a balance between positive and negative regulators of Wnt/β-catenin signaling in DA neuron development: (a) Wnt1 and *Dkk1* show a similar temporal expression pattern in the VM as both are highly expressed at E9.5 in a complementary anterior-posterior and ventro-lateral gradient (b) *Dkk1* expression peaks at E10.5 and β-catenin expression and transcriptional activity, as assessed in the TOPGAL reporter mice, has been found to follow a similar spatial-temporal expression pattern (Castelo-Branco et al., 2003) (c) We have reported that, as in other systems, *Dkk1* is most likely a direct transcriptional target of β-catenin in the VM as part of a negative feedback loop. Indeed, we found that treatment of primary E10.5 VM cultures with Wnt3a, a known activator of Wnt/β-catenin signaling in this culture (Castelo-Branco et al., 2003), acutely upregulates the expression of *Dkk1* (Paper III).

Given the *Dkk1* expression in the floor plate and ventral midline, it is tempting to speculate that *Dkk1* might be co-expressed in *Shh*-expressing cells. Recent evidence for a Shh and Wnt1 antagonism has been demonstrated (Joksimovic et al., 2009), and in early VM development *Shh* and *Wnt1* expression are complementary to each other (*Shh* is more highly expressed in the medial floor plate and lower in the lateral floor plate, whereas Wnt1 is more abundant in the lateral floor plate). As demonstrated by Joksimovic and colleagues, after an initial role in establishing the DA progenitor domain, a Wnt1-mediated antagonism of Shh is necessary to induce DA neurogenesis (Joksimovic et al., 2009). One possible explanation would be that a Dkk1-mediated Wnt/β-catenin inhibition in the medial floor plate prevents a premature downregulation of Shh in the floor plate, allowing thus the initial DA induction. Between E10.5-E11.5 *Shh* expression is downregulated in the ventral midline (Joksimovic et al., 2009), coinciding with a decrease in *Dkk1* in the same region; the latter event would possibly explain the former by creating a permissive region for Wnt1. However, in the *Dkk1* mutants we did not detect any defect at the level of DA progenitors nor in neurogenesis, indicating that early events in DA development are not affected. One possible

explanation for the differentiation deficit would be an excessive and/or premature activation Wnt/β-catenin signaling in these animals: indeed, in animals where β-catenin is constitutively expressed early in VM development, there is an accumulation of DA progenitors, impaired DA differentiation and reduced number of midbrain DA neurons (Tang et al., 2010). It is thus possible that the loss of Dkk1 results in an excess of Wnt/β-catenin signaling in the VM niche and affect the process of differentiation. The role of the Wnt/β-catenin pathway in midbrain DA neuron development is thus quite complex and has not been fully elucidated given the multiple roles of the Wnt/β-catenin pathway in regulating proliferation, cell-cycle exit and differentiation (Tang et al., 2009, Tang et al., 2010). Moreover, a recent study has indicated that the role of the Wnt/β-catenin pathway in the midbrain seems to be cell context-dependent (Tang et al., 2010): while constitutive activation of Wnt/β-catenin using *Shh–Cre* perturbs cell cycle progression and antagonizes the expression of Shh, contributing to a reduction of the number of DA neurons, a cell-type-specific activation of Wnt/β-catenin in the midline progenitors using *Th–IRES–Cre* leads to a increase in DA neuron numbers. The importance of timing and dosage of Shh adds a further level of complexity to an already intricate mechanism of Wnt1-Shh antagonism.

Another observation from our studies was the altered cell distribution in *Dkk1* mutants. An alteration of the DA population distribution in the midbrain has also been reported in *Wnt5a*$^{-/-}$ mice (Andersson et al., 2008). Interestingly, 20% of the *Dkk1*$^{+/-}$ embryos at E13.5 had exencephaly (failure of neural tube closure at the dorsal midbrain line), similarly to *Lrp6*-null embryos and to some *Wnt5a*$^{-/-}$ embryos (Qian et al., 2007). Both processes have been linked with the Wnt/PCP pathway and since Dkk1 has been shown to modulate gastrulation movements through the activation of this pathway, it is possible that the observed morphological defects in *Dkk1* mutants are due to a dysregulation in Wnt/PCP signaling. If so, these results suggest, and are in agreement with, the notion that a decrease in the Wnt/PCP pathway might be due to a more active Wnt/β-catenin signaling and with the concept that gain and loss-of-function of the Wnt/PCP pathway produce similar phenotypes.

3.4 PAPER IV

3.4.1 Human fetal VM cells can be expanded *in vitro* while retaining their VM DA identity

We have previously shown that mouse VM neural stem cells can be propagated as spheres in the presence of key factors involved in VM DA neuron development, and give rise to a large number of functional DA neurons that were able to survive, integrate and induce functional recovery after transplantation in animal models (Parish et al., 2008). In order to address some of the limitations of using fetal human tissue in CRT, we examined whether this protocol could be applied to human VM neural stem cells. VM tissue obtained from 6-11 weeks aborted fetuses was dissociated and cells were grown as neurospheres for 14 days with passaging every 7 days in the presence of the DA-appropriate factors Shh, Fgf8, Fgf2 and BDNF. One of our first observations was that initial cell numbers may be important for optimal expansion, given that preparations with initial cell counts lower than 500,000 cells could be expanded 2 to 3-fold after two weeks in proliferation whereas initial preparations with high cell numbers were not expanded successfully. As embryos from approximately the same age gave rise to both low and high initial cell numbers, this observation might reflect accuracy and/or stringency of dissection. After one week in proliferation we could detect a decrease in cell numbers, more obvious in cultures with medium and higher initial counts, whereas preparations with low initial cell numbers were able to recover; this event might reflect cell death in our cultures due to damage after tissue dissociation. Further characterization of the spheres in proliferation revealed the expression of neural stem cells markers such as *Sox2 and Nestin,* the latter being upregulated after 14 days of expansion when compared with VM primary tissue. Moreover, we could also detect *Lmx1b*, a marker of DA progenitors, and *Nurr1* which were present in higher levels when compared with primary tissue. Interestingly, we could also detect expression of high levels of *Tuj1* and *Th*, indicating that some differentiation is ongoing in proliferating spheres. Thus, we conclude that cells from human fetal VM expanded 2 to 3-fold retain their VM DA identity.

3.4.2 Human fetal VM cells retain their differentiation potential with passaging

To determine their DA potential, cells were induced to differentiate after tissue dissociation, and after 7 and 14 days of expansion. Cells were induced to differentiate

in the presence of factors known to regulate maturation and/or survival of DA neurons and the effects of Wnt5a were examined. Primary VM tissue-derived cells in differentiation were primarily neurons (65%) and 6% of total numbers of cells were TH^+ with a mature morphology. Addition of Wnt5a increased the numbers of TH^+ cells to 8%, as well as the number of TH^+ cells counted per field. After 7 days in proliferation, we could still detect high expression of Tuj1 and TH, albeit a less clear effect of Wnt5a. In cells expanded for 14 days we could still detect a high number of TH^+ cells with long processes, and addition of Wnt5 promoted a 40% increase in the number of TH^+ cells per field when compared with control. Upon differentiation, cells in proliferation conditions for 21 days still expressed appropriate VM markers and were able to differentiate efficiently, with Wnt5a promoting again an increase in *Th* levels. Thus, expanded cells retain their DA differentiation potential even after 21 days. However, at this stage the number of TH^+ cells in the culture declined. This result indicated that the optimal expansion time is 14 days in order to achieve abundant numbers of midbrain DA neurons

3.4.3 Increase in human DA neurons by combined passaging and differentiation: a new light in CRT?

In contrast with our observation with human VM cells, rodent VM neuorsphere cultures showed a reduction in the number of TH^+ cells with passaging, and after 2 weeks of expansion, very few TH^+ cells were present (Parish et al., 2008). In our human preparations, however, when comparing the direct differentiation of the VM tissue with cells cultivated for 14 days that were passaged 2 times and differentiated in the presence of Wnt5a there was significant four-fold increase in the number of TH^+ cells/field. Thus, our results show that the expansion and subsequent differentiation of primary human VM progenitors gives rise to an increase in human midbrain DA neurons. These results suggest that it may be possible to reduce the number of embryos necessary for CRT in PD patients. The next logical step will be to determine if these cells are able to survive and induce functional recovery in an animal model of PD.

4 CONCLUSIONS

From the work presented in this thesis, we can conclude:

1. Impairing the Wnt/β-catenin promotes neurogenesis and DA differentiation in mESC
2. Addition of Wnt/β-catenin inhibitors such as Dkk1 can be used to promote DA differentiation in mESC
3. The Lrp6 receptor is important for the timely onset of DA differentiation *in vivo*
4. The Wnt/β-catenin inhibitor Dkk1 regulates VM DA differentiation and morphogenesis
5. Human fetal VM cells can be expanded 2-3 fold in vitro and retain their DA differentiation potential; the latter is increased by addition of Wnt5a

In sum, the work herein presented reveals important functions of the Wnt signaling in DA differentiation and provides useful and important knowledge to improve cell-replacement therapy in Parkinson's disease.

5 ACKNOWLEDGEMENTS

Professor **Ernest Arenas**, thank you for welcoming me into your lab and for allowing me to conduct my PhD in such a vibrant environment! Above all, thank you for your enthusiastic and positive approach to science and life in general, for your ability to see the bigger picture and to extract the information from every single experiment! For giving me the freedom to work but always keeping your office door open and always being up for a "quick" meeting (I still remember our 6,5 h meeting!), for your openness to our comments and suggestions, and for enduring my fast talking and my sometimes "peculiar" sense of humor!

I would like to thank Dr. **Meng Li** for acting as my opponent and to Profs. **Erik Sundström**, **Jonas Frisén** and **Zaal Kokaia** for accepting my invitation to be part of the Examination Board.

I would like to thank **Emma Andersson** for the feedback on the thesis.

To our collaborators:

In Heidelberg- A big thank you to Prof. **Cristof Niehrs**, Dr. **Kristina Ellwanger**, **Désirée Glagow**, Dr. **Nina S. Corsini**, and Dr. **Ana Martin-Villalba** for all the help with the Dkk1 mice, comments and suggestions!

In Lund- Prof**. Olle Lindvall** and Dr. **Malin Parmar** for all the help with the human fetal cells project and for the quick response with the all the documents I needed for my PhD application!

In Freiburg- All of the members of Prof. **Guido Nikkhah**'s, in particular to Dr. **Tobias Piroth**, for your warm welcome when I went there!

In Cambridge- Dr **Roger Barker**'s group, in particular **Rocio Laguna Goya**: thank you for all those "planning-experiments-over-the-phone" moments, your enthusiasm and commitment!

To the ones with me during my scientific "baby-steps":

So many to thank! In Lund University: Profs. **Jia-Yi Li** and **Patrik Brundin** for welcoming me and introducing me to science. **Laurent Roybon**, for your patience to teach me everything from immunos to molecular biology, your advices on life and science and for always trying to answer my infinite questions! The Portuguese crowd in the lab: **Joana Gil** and **Sofia Correia** thank you for all the laughs and for creating such a fun environment! A huge thank you to **Jelena Pesic**, **Emma Lane**, **Sergey Anisimov**, **Jorien van der Burg**, **Tomas Deierborgh**, **Ruben Smith**, **Hinfan Chung, Manolo Carta, Birgit Haraldsson, Britt Lindberg.**

To EA group, past:

My buddies **Kyle** and **Paul** (honorary member), thanks for all the good times, crazy lunches and the "not-so-politically-correct" fun moments during my first years at Mol Neuro. **Julianna a.k.a Jules,** for your friendship and support during the good and not-so-good times, for the after-work social moments and expensive bottles of wine, the pep-talks, ruling the dancefloor, for the passionate way you live life! **Emma a.k.a Lil'E** for your true kindness, for getting my jokes, our nice fikas, your inquisitive mind and for all the dinner/video game nights at your place! **Paola S.**, for always caring and willingness to help, your warm welcome the first day I arrived to lab and during my visit to the US, your sharp mind, all of our interesting discussions and your sound advices! **Clare**, for introducing me to the world of cell transplantation (and your patience while doing it), your professionalism and your positive, "down-to-earth" attitude! **Solange**, for your "joie de vivre" and your bright personality, our nice lunches and your help with everything from housing to site-directed mutagenesis! **Isabel,** one of the hardest workers I've ever met, for being my "partner in crime" in ESC work and for embracing our own version of "portunhol". **Linda E.**, for the always entertaining lunch-time conversations and for your expressiveness, it's always fun to be around you! **Sonia**, for your friendliness and for your endurance with my "expansion" when we where bench-neighbors! **Nina R.**, for your sense of humor, knowledge and determination. **Alejandro**, another hard worker, for your endurance, the company during those late nights in the lab and for being a great roommate during our lab meeting in London. **Vita**, for your enthusiasm and sharing your knowledge on Western

Blots and Slivovitz! **Lenka**, for always bringing a smile to lab and for being a perfect hostess when a few of us dropped by unannounced at late hours!

To EA group, present:

Lukas, for your witty observations, for always managing to capture in photos our most embarrassing moments and for being a great colleague to work with. **Spyros**, for your genuine nature, what you see is what you get! Thanks for all the good moments and stories you've shared with us. **Carlos,** for all the fun while attending conferences, (specially the "I-think-we-missed-the-boat/plane…"moments), your drive and commitment. **Carmen S.**, the "lab mamma", for always caring for everyone, for offering me dinner after that 6.5h meeting and for always being in a good mood. **Lottie**, don't know how our lab would be without your organizing skills, thank you for bringing some order! **Linda A.**, for your thoroughness and authenticity and for also being a fan of adjectives! **Enrique**, for always keeping our spirits up with your jokes. **Shanzeng**, for your dedication, hard work and knowledge. **Magda**, for your impressive multi-tasking. The visiting students, **Kristina**, **Daniel**, **Chao**, hope you are having a great time!

To PE, present and past:

Ruani, for your good-hearted nature, great cooking and for always bringing your upbeat attitude into lab! **Blanchi** (or shall I say Blonnn-chi) for keeping the positive energy levels high, you're a master at putting everyone at ease, all of our office discussions and lunches, and for not freaking out every time you found some of my stuff on your desk! **Moritz**, the group leader in the "Lunch Movement", for your patience ("5 min! Really, just need 5 min!!") and for being a fellow "pop culture" connoisseur. **François**, for your ability to balance successfully science and family life, for always seeing the bright side of things and for keeping it real! **Igor**, your passion for science and eagerness to learn more, thanks for always having an answer to my questions (or trying) and for explaining us what to do if we ever get lost in a forest during winter! **Isa,** for the microscope conversations, help with *in situs*, the fun trip to Italy and airplane moments! The good thing about missing that flight was that I got to know you better. **Boris**, for being a fellow "loud-laughter", bringing music into the lab

and for your making sure that I'm eating right! **Natalia**, member of the "Portuguese-speaking corner" in our office, for all the fun and "dogma-challenging" discussions (nunca aborrecidas!) **Alessandro**, discussing Italian politics with you is always entertaining! **Mitja**, the official photographer, for never shying away from a complicated experiment and your resourcefulness! **Satish**, for managing to get things done and the always interesting conversations about "living" in the lab. **Olga**, for introducing us to some peculiar office pets! **Helena S.**, for being proactive and keeping our unit rolling! **Marina**, for your funny remarks. **Sergi**, for being so methodical! **Hind**, for your on point comments and common love of olive oil on bread! **Anna S**, for the nice discussions about the cultural differences between Portugal and Sweden and PhD life! **Jorge**, for your attention to every detail and good mood. **Patrik Enfors**, for being so practical.

To PU, present and past:

Nicolas, for your balanced approach to life, all the fun moments in lab, parties (the legacy of the "Nico dancing face"!), cheesy boat karaokes and soccer games! **Seth**, for showing me the Swedes' "modus operandis", your "tech savvyness" and for those "lost-in-translation" moments. **Paola R.**, for the socializing at "coffee time" and after work, and the fun time during our stay in Toronto. **Marie**, for sharing with me the adventures of the final stage of a PhD! **Songbai¸** for your entertaining presentations! **Per Uhlén**, for all the fun moments changing lamps on microscopes, and for teaching me how to use the confocal microscope. **Hiromi**, for enduring our teasing **Cristian**, another member of the increasing group of the (at least) "tri-lingual" people! **Ivar**, **Simone, Erik**

To MA group:

Shermaine, for our teasing but funny moments and inappropriate jokes! **Michael Andäng**, for thinking outside the box and your enthusiasm. **Petra**, for our nice trip to Riga. **Anna**, for your indisputably recognizable and contagious laughter. **Mia** and **Shaimaa¸** for always contributing with the Swedish point of view in our sometimes weird lunch conversations. **Helena J**

To SL group**:**

Una, for your strong opinions and for never dodging an interesting debate. **Pawel**, your advices on qPCR, for introducing our "junk-food Fridays" and the nice chatty after-work moments. **Saiful**, for out fun trip to Toronto. **Sten Linnarsson**, for all the qPCR troubleshooting and that interview you gave me for one of my courses, it was really helpful!

Sandro, chilled and always in a good mood! Thank you for being a sports and your help when I had to move (couldn't have done it without you!)

Ben, for all the good moments when you were here and for the very nice (and very Swedish) Midsommer celebration!

Göran Månsson, for your expertise on microscopes, you have made our live so much easier!

Misha, for being a fellow martial arts practitioner.

Tibor Harkany, for your advices and suggestions, and for the enlightening conversations over a pizza every time you pay us a visit.

Alessandra Nani, for always wanting to know how everyone is doing and for going that extra mile to help us! Thank you for taking such good care of us (from housing to Skatteverket and everything in between), the Swedish lessons and for your uplifting and encouraging words!

Johnny Söderlund, everytime you go on vacations, chaos takes over the lab! Thank you for you for assuring we can do our experiments, for motivating us to speak Swedish and for making sure we all get your pieces of fruit!

Annika Käller, for your help when I first arrived.

To my friends:

Catarina Oliveira, from Coimbra, to Lund to Stockholm…you've been a constant presence in my life for the past 10 years, so much to thank you for! For your ability to appreciate the small things and live life to the fullest, your love for art and travelling, for always striving for perfection when it comes to science and for always trying to make me put things in perspective!

João Paredes, even though you're no stranger to Stockholm…welcome! Who would have thought 10 years ago we would be together in Sweden? Thank you for the great times together, for your good character and integrity, for being easy-going, for all the stories and for making us laugh with your funny punch lines!

The Portuguese crowd in Stockholm:

Ricardo, thank you for all the fun times going out in Stockholm, trips to Kiruna and Gotland and for being the engine behind most of the social events, if not for you I would have spent even more nights and weekends in the lab! **Vasco**, there's always something fun happening when we are with you, thanks also for your knowledge on the ins and outs of Sweden! **Gonçalo S**, for reaching out even when I get stuck in my own little world **Gonçalo Castelo-Branco**, for your dedication to science and for making sure that everyone knows about Portugal! **Orlando**, for the crazy stories! **Paulo S.**, you have truly embraced Sweden! **João A.,** always ready with a sharp remark! **Pedro M**, all the nice discussions about PhD life. **Cláudia**, always friendly! **João Noutel** and **Sofia Azevedo**, for the good times when you where here.

The multi-cultural crowd in Stockholm:

Mustafah, **Haythem**, **Clarisa**, **Joana**, **Pedro R.**, **Nigel**, **Rami**, **Jamie, Roberto, Santi** thanks for all the great moments in the past 2 years, it has been a pleasure to meet you and it's always fun to hang out with you guys!

Mats, for also believing in the power of exercise-induced endorphins! Thanks mate for the trainings, movie sessions, after-work drinks and that 5 hours-long trip from Uppsala to Stockholm!

My flat mates:

Stef, for your lively personality, thanks for putting up with me and for always asking me if everything was well! **Valeria**, always so friendly and for your passion for art!

The Coimbra crowd:

Rita, **Ana V.**, **Angie**, **Bruno C**, **Inês M.**, **Pit**, **Fil**, **Jota**, **Carla**, **Vera**, **Tavinho**, **Carina**, for the great years spent in Coimbra, all the shenanigans everytime we're together and for making me feel like we've just seen each other yesterday everytime we meet!

The Lisboa crowd:

Catarina C., Dúnia, Catarina R., Emanuel, Abílio, Cláudia, Pedro, Raquel, Hélder, Inês, most of you know me since high school, thanks for the friendship even though I wasn't there as often as I would have wanted, your warm welcome and support everytime I'm there and our nice vacations, New Years' celebrations and trips!

Last, but definitely not least..Family!:

Nádia, apesar de não o dizer vezes suficientes, obrigado por todo o teu apoio, a tua coragem e determinação, pela maneira como enfrentas e vences as adversidades que vão surgindo. Ser irmã mais velha não é fácil, mas tu estiveste sempre à altura!

Mãe, se hoje estou aqui muito devo-o a ti! Obrigado por tentares sempre dar o melhor possível aos teus filhos, por todos os sacrifícios que passaste, a tua força de espírito, por me deixares seguir o meu caminho mesmo quando implica estar longe!

I would also like to thank the **Portuguese Foundation for Science and Technology (FCT)** which has provided financial support during my PhD.

6 REFERENCES

Aberle, H., Bauer, A., Stappert, J., Kispert, A. and Kemler, R. (1997) 'beta-catenin is a target for the ubiquitin-proteasome pathway', *Faseb Journal* 11(9): 3232.

Acampora, D., Avantaggiato, V., Tuorto, F. and Simeone, A. (1997) Genetic control of brain morphogenesis through Otx gene dosage requirement, *Development* 124(18): 3639-3650.

Acampora, D., Mazan, S., Lallemand, Y., Avantaggiato, V., Maury, M., Simeone, A. and Brulet, P. (1995) Forebrain and midbrain regions are deleted in otx2(-/-) mutants due to a defective anterior neuroectoderm specification during gastrulation, *Development* 121(10): 3279-3290.

Adams, K. A., Maida, J. M., Golden, J. A. and Riddle, R. D. (2000) The transcription factor Lmx1b maintains Wnt1 expression within the isthmic organizer, *Development* 127(9): 1857-1867.

Adamska, M., MacDonald, B. T., Sarmast, Z. H., Oliver, E. R. and Meisler, M. H. (2004) En1 and Wnt7a interact with Dkk1 during limb development in the mouse, *Developmental Biology* 272(1): 134-144.

Andersson, E., Bryjova, L., Biris, K., Yamaguchi, T. P., Arenas, E. and Bryja, V. (2010) Genetic Interaction Between Lrp6 and Wnt5a During Mouse Development, *Developmental Dynamics* 239(1): 237-245.

Andersson, E., Jensen, J. B., Parmar, M., Guillemot, F. and Bjorklund, A. (2006a) Development of the mesencephalic dopaminergic neuron system is compromised in the absence of neurogenin 2, *Development* 133(3): 507-516.

Andersson, E., Tryggvason, U., Deng, Q. L., Friling, S., Alekseenko, Z., Robert, B., Perlmann, T. and Ericson, J. (2006b) Identification of intrinsic determinants of midbrain dopamine neurons, *Cell* 124(2): 393-405.

Andersson, E. R., Prakash, N., Cajanek, L., Minina, E., Bryja, V., Bryjova, L., Yamaguchi, T. P., Hall, A. C., Wurst, W. and Arenas, E. (2008) Wnt5a Regulates Ventral Midbrain Morphogenesis and the Development of A9-A10 Dopaminergic Cells In Vivo, *Plos One* 3(10).

Ang, S. L., Jin, O., Rhinn, M., Daigle, N., Stevenson, L. and Rossant, J. (1996) A targeted mouse Otx2 mutation leads to severe defects in gastrulation and formation of axial mesoderm and to deletion of rostral brain, *Development* 122(1): 243-252.

Ang, S. L. and Rossant, J. (1994) Hnf-3-beta is essential for node and notochord formation in mouse development, *Cell* 78(4): 561-574.

Ang, S. L., Wierda, A., Wong, D., Stevens, K. A., Cascio, S., Rossant, J. and Zaret, K. S. (1993) The formation and maintenance of the definitive endoderm lineage in the mouse - involvement of hnf3/forkhead proteins, *Development* 119(4): 1301-1315.

Angers, S. and Moon, R. T. (2009) Proximal events in Wnt signal transduction, *Nature Reviews Molecular Cell Biology* 10(7): 468-477.

Arnold, S. J. and Robertson, E. J. (2009) Making a commitment: cell lineage allocation and axis patterning in the early mouse embryo, *Nature Reviews Molecular Cell Biology* 10(2): 91-103.

Aubert, J., Dunstan, H., Chambers, I. and Smith, A. (2002) Functional gene screening in embryonic stem cells implicates Wnt antagonism in neural differentiation, *Nature Biotechnology* 20(12): 1240-1245.

Backman, C., Perlmann, T., Wallen, A., Hoffer, B. J. and Morales, M. (1999) A selective group of dopaminergic neurons express Nurr1 in the adult mouse brain, *Brain Research* 851(1-2): 125-132.

Bafico, A., Liu, G. Z., Yaniv, A., Gazit, A. and Aaronson, S. A. (2001) Novel mechanism of Wnt signalling inhibition mediated by Dickkopf-1 interaction with LRP6/Arrow, *Nature Cell Biology* 3(7): 683-686.

Barberi, T., Klivenyi, P., Calingasan, N. Y., Lee, H., Kawamata, H., Loonam, K., Perrier, A. L., Bruses, J., Rubio, M. E., Topf, N. et al. (2003) Neural subtype specification of fertilization and nuclear transfer embryonic stem cells and application in parkinsonian mice, *Nature Biotechnology* 21(10): 1200-1207.

Barrantes, I. D., Davidson, G., Grone, H. J., Westphal, H. and Niehrs, C. (2003) Dkk1 and noggin cooperate in mammalian head induction, *Genes & Development* 17(18): 2239-2244.

Beagle, B., Mi, K. H. and Johnson, G. V. W. (2009) Phosphorylation of PPP(S/T)P Motif of the Free LRP6 Intracellular Domain Is Not Required to Activate the Wnt/beta-Catenin Pathway and Attenuate GSK3 beta Activity, *Journal of Cellular Biochemistry* 108(4): 886-895.

Bhanot, P., Brink, M., Samos, C. H., Hsieh, J. C., Wang, Y. S., Macke, J. P., Andrew, D., Nathans, J. and Nusse, R. (1996) A new member of the frizzled family from Drosophila functions as a Wingless receptor, *Nature* 382(6588): 225-230.

Bilic, J., Huang, Y. L., Davidson, G., Zimmermann, T., Cruciat, C. M., Bienz, M. and Niehrs, C. (2007) Wnt induces LRP6 signalosomes and promotes dishevelled-dependent LRP6 phosphorylation, *Science* 316(5831): 1619-1622.

Binnerts, M. E., Tomasevic, N., Bright, J. M., Leung, J., Ahn, V. E., Kim, K. A., Zhan, X. M., Liu, S. C., Yonkovich, S., Williams, J. et al. (2009) The First Propeller Domain of LRP6 Regulates Sensitivity to DKK1, *Molecular Biology of the Cell* 20(15): 3552-3560.

Blunt, A. G., Lawshe, A., Cunningham, M. L., Seto, M. L., Ornitz, D. M. and MacArthur, C. A. (1997) Overlapping expression and redundant activation of mesenchymal fibroblast growth factor (FGF) receptors by alternatively spliced FGF-8 ligands, *Journal of Biological Chemistry* 272(6): 3733-3738.

Bonilla, S., Hall, A. C., Pinto, L., Attardo, A., Gotz, M., Huttner, W. B. and Arenas, E. (2008) Identification of midbrain floor plate radial glia-like cells as dopaminergic progenitors, *Glia* 56(8): 809-820.

Bourhis, E., Tam, C., Franke, Y., Bazan, J. F., Ernst, J., Hwang, J., Costa, M., Cochran, A. G. and Hannoush, R. N. (2010) Reconstitution of a Frizzled8.Wnt3a.LRP6 Signaling Complex Reveals Multiple Wnt and Dkk1 Binding Sites on LRP6, *Journal of Biological Chemistry* 285(12): 9172-9179.

Boutros, M., Paricio, N., Strutt, D. I. and Mlodzik, M. (1998) Dishevelled activates JNK and discriminates between JNK pathways in planar polarity and wingless signaling, *Cell* 94(1): 109-118.

Brennan, J., Lu, C. C., Norris, D. P., Rodriguez, T. A., Beddington, R. S. P. and Robertson, E. J. (2001) Nodal signalling in the epiblast patterns the early mouse embryo, *Nature* 411(6840): 965-969.

Broccoli, V., Boncinelli, E. and Wurst, W. (1999) The caudal limit of Otx2 expression positions the isthmic organizer, *Nature* 401(6749): 164-168.

Brodski, C., Weisenhorn, D. M. V., Signore, M., Sillaber, I., Oesterheld, M., Broccoli, V., Acampora, D., Simeone, A. and Wurst, W. (2003) Location and size of dopaminergic and serotonergic cell populations are controlled by the position of the midbrain hindbrain organizer, *Journal of Neuroscience* 23(10): 4199-4207.

Brott, B. K. and Sokol, S. Y. (2002) Regulation of Wnt/LRP signaling by distinct domains of Dickkopf proteins, *Molecular and Cellular Biology* 22(17): 6100-6110.

Brown, S. D., Twells, R. C. J., Hey, P. J., Cox, R. D., Levy, E. R., Soderman, A. R., Metzker, M. L., Caskey, C. T., Todd, J. A. and Hess, J. F. (1998) Isolation and characterization of LRP6, a novel member of the low density lipoprotein receptor gene family, *Biochemical and Biophysical Research Communications* 248(3): 879-888.

Brundin, P., Pogarell, O., Hagell, P., Piccini, P., Widner, H., Schrag, A., Kupsch, A., Crabb, L., Odin, P., Gustavii, B. et al. (2000) Bilateral caudate and putamen grafts of embryonic mesencephalic tissue treated with lazaroids in Parkinson's disease, *Brain* 123: 1380-1390.

Bryja, V., Andersson, E. R., Schambony, A., Esner, M., Bryjova, L., Biris, K. K., Hall, A. C., Kraft, B., Cajanek, L., Yamaguchi, T. P. et al. (2009) The Extracellular Domain of Lrp5/6 Inhibits Noncanonical Wnt Signaling In Vivo, *Molecular Biology of the Cell* 20(3): 924-936.

Bryja, V., Bonilla, S., Cajanek, L., Parish, C. L., Schwartz, C. M., Luo, Y. Q., Rao, M. S. and Arenas, E. (2006) An efficient method for the derivation of mouse embryonic stem cells, *Stem Cells* 24(4): 844-849.

Bryja, V., Schulte, G., Rawal, N., Grahn, A. and Arenas, E. (2007) Wnt-5a induces Dishevelled phosphorylation and dopaminergic differentiation via a CK1-dependent mechanism, *Journal of Cell Science* 120: 586-595.

Caneparo, L., Huang, Y. L., Staudt, N., Tada, M., Ahrendt, R., Kazanskaya, O., Niehrs, C. and Houart, C. (2007) Dickkopf-1 regulates gastrulation movements by coordinated modulation of Wnt/beta catenin and Wnt/PCP activities, through interaction with the Dally-like homolog Knypek, *Genes & Development* 21(4): 465-480.

Caricasole, A., Copani, A., Caraci, F., Aronica, E., Rozemuller, A. J., Caruso, A., Storto, M., Gaviraghi, G., Terstappen, G. C. and Nicoletti, F. (2004) Induction of Dickkopf-1, a negative modulator of the Wnt pathway, is associated with neuronal degeneration in Alzheimer's brain, *Journal of Neuroscience* 24(26): 6021-6027.

Castelo-Branco, G. A., Wagner, J., Rodriguez, F. J., Kele, J., Sousa, K., Rawal, N., Pasolli, H. A., Fuchs, E., Kitajewski, J. and Arenas, E. (2003) Differential regulation of midbrain dopaminergic neuron development by Wnt-1, Wnt-3a, and Wnt-5a, *Proceedings of the National Academy of Sciences of the United States of America* 100(22): 12747-12752.

Castro, D. S., Hermanson, E., Joseph, B., Wallen, A., Aarnisalo, P., Heller, A. and Perlmann, T. (2001) Induction of cell cycle arrest and morphological differentiation by Nurr1 and retinoids in dopamine MN9D cells, *Journal of Biological Chemistry* 276(46): 43277-43284.

Cha, S. W., Tadjuidje, E., Tao, Q., Wylie, C. and Heasman, J. (2008) Wnt5a and Wnt11 interact in a maternal Dkk1-regulated fashion to activate both canonical and non-canonical signaling in Xenopus axis formation, *Development* 135(22): 3719-3729.

Chamorro, M. N., Schwartz, D. R., Vonica, A., Brivanlou, A. H., Cho, K. R. and Varmus, H. E. (2005) FGF-20 and DKK1 are transcriptional targets of beta-catenin and FGF-20 is implicated in cancer and development, *Embo Journal* 24(1): 73-84.

Chi, C. L., Martinez, S., Wurst, W. and Martin, G. R. (2003) The isthmic organizer signal FGF8 is required for cell survival in the prospective midbrain and cerebellum, *Development* 130(12): 2633-2644.

Chiang, C., Ying, L. T. T., Lee, E., Young, K. E., Corden, J. L., Westphal, H. and Beachy, P. A. (1996) Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function', *Nature* 383(6599): 407-413.

Choi, S. C. and Han, J. K. (2002) Xenopus Cdc42 regulates convergent extension movements during gastrulation through Wnt/Ca2+ signaling pathway, *Developmental Biology* 244(2): 342-357.
Chung, S., Hedlund, E., Hwang, M., Kim, D. W., Shin, B. S., Hwang, D. Y., Kang, U. J., Isacson, O. and Kim, K. S. (2005) The homeodomain transcription factor Pitx3 facilitates differentiation of mouse embryonic stem cells into AHD2-expressing dopaminergic neurons, *Molecular and Cellular Neuroscience* 28(2): 241-252.
Chung, S., Leung, A., Han, B. S., Chang, M. Y., Moon, J. I., Kim, C. H., Hong, S., Pruszak, J., Isacson, O. and Kim, K. S. (2009) Wnt1-Imx1a Forms a Novel Autoregulatory Loop and Controls Midbrain Dopaminergic Differentiation Synergistically with the SHH-FoxA2 Pathway, *Cell Stem Cell* 5(6): 646-658.
Ciruna, B. and Rossant, J. (2001) FGF signaling regulates mesoderm cell fate specification and morphogenetic movement at the primitive streak, *Developmental Cell* 1(1): 37-49.
Cliffe, A., Hamada, F. and Bienz, M. (2003) A role of dishevelled in relocating axin to the plasma membrane during wingless signaling, *Current Biology* 13(11): 960-966.
Courtois, E. T., Castillo, C. G., Seiz, E. G., Ramos, M., Bueno, C., Liste, I. and Martinez-Serrano, A. (2010) In Vitro and in Vivo Enhanced Generation of Human A9 Dopamine Neurons from Neural Stem Cells by Bcl-X-L, *Journal of Biological Chemistry* 285(13): 9881-9897.
Crossley, P. H. and Martin, G. R. (1995) The mouse Fgf8 gene encodes a family of polypeptides and is expressed in regions that direct outgrowth and patterning in the developing embryo, *Development* 121(2): 439-451.
Crossley, P. H., Martinez, S. and Martin, G. R. (1996) Midbrain development induced by FGF8 in the chick embryo, *Nature* 380(6569): 66-68.
Cselenyi, C. S., Jernigan, K. K., Tahinci, E., Thorne, C. A., Lee, L. A. and Lee, E. (2008) LRP6 transduces a canonical Wnt signal independently of Axin degradation by inhibiting GSK3's phosphorylation of beta-catenin, *Proceedings of the National Academy of Sciences of the United States of America* 105(23): 8032-8037.
Danielian, P. S. and McMahon, A. P. (1996) Engrailed-1 as a target of the Wnt-1 signalling pathway in vertebrate midbrain development, *Nature* 383(6598): 332-334.
Daniels, D. L. and Weis, W. I. (2005) beta-catenin directly displaces Groucho/TLE repressors from Tcf/Lef in Wnt-mediated transcription activation, *Nature Structural & Molecular Biology* 12(4): 364-371.
Dann, C. E., Hsieh, J. C., Rattner, A., Sharma, D., Nathans, J. and Leahy, D. J. (2001) Insights into Wnt binding and signalling from the structures of two Frizzled cysteine-rich domains, *Nature* 412(6842): 86-90.
Davidson, D., Graham, E., Sime, C. and Hill, R. (1988) A gene with sequence similarity to drosophila-Engrailed is expressed during the development of the neural-tube and vertebrae in the mouse, *Development* 104(2): 305-316.
Davidson, G., Wu, W., Shen, J. L., Bilic, J., Fenger, U., Stannek, P., Glinka, A. and Niehrs, C. (2005) Casein kinase 1 gamma couples Wnt receptor activation to cytoplasmic signal transduction, *Nature* 438(7069): 867-872.
Davis, C. A. and Joyner, A. L. (1988) Expression patterns of the homeo box-containing genes En-1 and En-2 and the proto-oncogene Int-1 diverge during mouse development, *Genes & Development* 2(12B): 1736-1744.
Davis, C. A., Nobletopham, S. E., Rossant, J. and Joyner, A. L. (1988) Expression of the homeo box containing gene En-2 delineates a specific region of the developing mouse-brain, *Genes & Development* 2(3): 361-371.

Donato, R., Miljan, E. A., Hines, S. J., Aouabdi, S., Pollock, K., Patel, S., Edwards, F. A. and Sinden, J. D. (2007) Differential development of neuronal physiological responsiveness in two human neural stem cell lines, *Bmc Neuroscience* 8.

Du, S. J., Purcell, S. M., Christian, J. L., McGrew, L. L. and Moon, R. T. (1995) Identification of distinct classes and functional domains of Wnts through expression of wild-type and chimeric proteins in Xenopus embryos, *Molecular and Cellular Biology* 15(5): 2625-2634.

Echelard, Y., Epstein, D. J., Stjacques, B., Shen, L., Mohler, J., McMahon, J. A. and McMahon, A. P. (1993) Sonic-hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity, *Cell* 75(7): 1417-1430.

Epstein, D. J., McMahon, A. P. and Joyner, A. L. (1999) Regionalization of Sonic hedgehog transcription along the anteroposterior axis of the mouse central nervous system is regulated by Hnf3-dependent and -independent mechanisms, *Development* 126(2): 281-292.

Ericson, J., Morton, S., Kawakami, A., Roelink, H. and Jessell, T. M. (1996) Two critical periods of Sonic Hedgehog signaling required for the specification of motor neuron identify, *Cell* 87(4): 661-673.

Evans, M. J. and Kaufman, M. H. (1981) Establishment in culture of pluripotential cells from mouse embryos, *Nature* 292(5819): 154-156.

Fasano, C. A., Chambers, S. M., Lee, G., Tomishima, M. J. and Studer, L. (2010) Efficient Derivation of Functional Floor Plate Tissue from Human Embryonic Stem Cells, *Cell Stem Cell* 6(4): 336-347.

Fedi, P., Bafico, A., Soria, A. N., Burgess, W. H., Miki, T., Bottaro, D. P., Kraus, M. H. and Aaronson, S. A. (1999) Isolation and biochemical characterization of the human Dkk-1 homologue, a novel inhibitor of mammalian Wnt signaling, *Journal of Biological Chemistry* 274(27): 19465-19472.

Ferri, A. L. M., Lin, W., Mavromatakis, Y. E., Wang, J. C., Sasaki, H., Whitsett, J. A. and Ang, S. L. (2007) Foxa1 and Foxa2 regulate multiple phases of midbrain dopaminergic neuron development in a dosage-dependent manner, *Development* 134(15): 2761-2769.

Freed, C. R., Greene, P. E., Breeze, R. E., Tsai, W. Y., DuMouchel, W., Kao, R., Dillon, S., Winfield, H., Culver, S., Trojanowski, J. Q. et al. (2001) Transplantation of embryonic dopamine neurons for severe Parkinson's disease, *New England Journal of Medicine* 344(10): 710-719.

Friling, S., Andersson, E., Thompson, L. H., Jonssonc, M. E., Hebsgaard, J. B., Nanou, E., Alekseenko, Z., Marklund, U., Kjellander, S., Volakakis, N. et al. (2009) Efficient production of mesencephalic dopamine neurons by Lmx1a expression in embryonic stem cells, *Proceedings of the National Academy of Sciences of the United States of America* 106(18): 7613-7618.

Fung, Y. K. T., Shackleford, G. M., Brown, A. M. C., Sanders, G. S. and Varmus, H. E. (1985) Nucleotide-sequence and expression invitro of cDNA derived from messenger-rna of Int-1, a provirally activated mouse mammary oncogene, *Molecular and Cellular Biology* 5(12): 3337-3344.

Galleguillos, D., Fuentealba, J. A., Gomez, L. M., Saver, M., Gomez, A., Nash, K., Burger, C., Gysling, K. and Andres, M. E. (2010) Nurr1 regulates RET expression in dopamine neurons of adult rat midbrain, *Journal of Neurochemistry* 114(4): 1158-1167.

Gardner, C. A. and Barald, K. F. (1991) The cellular environment controls the expression of Engrailed-like protein in the cranial neuroepithelium of quail chick chimeric embryos, *Development* 113(3): 1037-1048.
Gerrard, L., Rodgers, L. and Cui, W. (2005) Differentiation of human embryonic stem cells to neural lineages in adherent culture by blocking bone morphogenetic protein signaling, *Stem Cells* 23(9): 1234-1241.
Glinka, A., Wu, W., Delius, H., Monaghan, A. P., Blumenstock, C. and Niehrs, C. (1998) Dickkopf-1 is a member of a new family of secreted proteins and functions in head induction, *Nature* 391(6665): 357-362.
Gonzalez-Sancho, J. M., Brennan, K. R., Castelo-Soccio, L. A. and Brown, A. M. C. (2004) Wnt proteins induce dishevelled phosphorylation via an LRP5/6-Independent mechanism, irrespective of their ability to stabilize beta-catenin, *Molecular and Cellular Biology* 24(11): 4757-4768.
Green, J. L., Kuntz, S. G. and Sternberg, P. W. (2008) Ror receptor tyrosine kinases: orphans no more, *Trends in Cell Biology* 18(11): 536-544.
Gregory, C. A., Singh, H., Perry, A. S. and Prockop, D. J. (2003) The Wnt signaling inhibitor dickkopf-1 is required for reentry into the cell cycle of human adult stem cells from bone marrow, *Journal of Biological Chemistry* 278(30): 28067-28078.
Grotewold, L., Theil, T. and Ruther, U. (1999) Expression pattern of Dkk-1 during mouse limb development, *Mechanisms of Development* 89(1-2): 151-153.
Grumolato, L., Liu, G. Z., Mong, P., Mudbhary, R., Biswas, R., Arroyave, R., Vijayakumar, S., Economides, A. N. and Aaronson, S. A. (2010) Canonical and noncanonical Wnts use a common mechanism to activate completely unrelated coreceptors, *Genes & Development* 24(22): 2517-2530.
Guo, C., Qiu, H. Y., Huang, Y., Chen, H., Yang, R. Q., Chen, S. D., Johnson, R. L., Chen, Z. F. and Ding, Y. Q. (2007) Lmx1b is essential for Fgf8 and Wnt1 expression in the isthmic organizer during tectum and cerebellum development in mice, *Development* 134(2): 317-325.
Guo, Q. X., Li, K. R., Sunmonu, N. A. and Li, J. Y. H. (2010) Fgf8b-containing spliceforms, but not Fgf8a, are essential for Fgf8 function during development of the midbrain and cerebellum, *Developmental Biology* 338(2): 183-192.
Habas, R., Dawid, I. B. and He, X. (2003) Coactivation of Rac and Rho by Wnt/Frizzled signaling is required for vertebrate gastrulation, *Genes & Development* 17(2): 295-309.
Habas, R., Kato, Y. and He, X. (2001) Wnt/Frizzled activation of Rho regulates vertebrate gastrulation and requires a novel formin homology protein Daam1, *Cell* 107(7): 843-854.
Haegele, L., Ingold, B., Naumann, H., Tabatabai, G., Ledermann, B. and Brandner, S. (2003) Wnt signalling inhibits neural differentiation of embryonic stem cells by controlling bone morphogenetic protein expression, *Molecular and Cellular Neuroscience* 24(3): 696-708.
Hagell, P., Schrag, A., Piccini, P., Jahanshahi, M., Brown, R., Rehncrona, S., Widner, H., Brundin, P., Rothwell, J. C., Odin, P. et al. (1999) Sequential bilateral transplantation in Parkinson's disease - Effects of the second graft, *Brain* 122: 1121-1132.
Hargus, G., Cooper, O., Deleidi, M., Levy, A., Lee, K., Marlow, E., Yow, A., Soldner, F., Hockemeyer, D., Hallett, P. J. et al. (2010) Differentiated Parkinson patient-derived induced pluripotent stem cells grow in the adult rodent brain and reduce motor asymmetry in Parkinsonian rats, *Proceedings of the National Academy of Sciences of the United States of America* 107(36): 15921-15926.

He, X., SaintJeannet, J. P., Wang, Y. S., Nathans, J., Dawid, I. and Varmus, H. (1997) A member of the frizzled protein family mediating axis induction by Wnt-5A, *Science* 275(5306): 1652-1654.

He, X., Semenov, M., Tamai, K. and Zeng, X. (2004) LDL receptor-related proteins 5 and 6 in Wnt/beta-catenin signaling: Arrows point the way, *Development* 131(8): 1663-1677.

Hedlund, E., Pruszak, J., Lardaro, T., Ludwig, W., Vinuela, A., Kim, K. S. and Isacson, O. (2008) Embryonic stem cell-derived Pitx3-enhanced green fluorescent protein midbrain dopamine neurons survive enrichment by fluorescence-activated cell sorting and function in an animal model of Parkinson's disease, *Stem Cells* 26(6): 1526-1536.

Hey, P. J., Twells, R. C. J., Phillips, M. S., Nakagawa, Y., Brown, S. D., Kawaguchi, Y., Cox, R., Xie, G. C., Dugan, V., Hammond, H. et al. (1998) Cloning of a novel member of the low-density lipoprotein receptor family, *Gene* 216(1): 103-111.

Hikasa, H., Shibata, M., Hiratani, I. and Taira, M. (2002) The Xenopus receptor tyrosine kinase Xror2 modulates morphogenetic movements of the axial mesoderm and neuroectoderm via Wnt signaling, *Development* 129(22): 5227-5239.

Hirabayashi, Y., Itoh, Y., Tabata, H., Nakajima, K., Akiyama, T., Masuyama, N. and Gotoh, Y. (2004) The Wnt/beta-catenin pathway directs neuronal differentiation of cortical neural precursor cells, *Development* 131(12): 2791-2801.

Hovakimyan, M., Haas, S. J. P., Schmitt, O., Gerber, B., Wree, A. and Andressen, C. (2006) Mesencephalic human neural progenitor cells transplanted into the neonatal hemiparkinsonian rat striatum differentiate into neurons and improve motor behaviour, *Journal of Anatomy* 209(6): 721-732.

Hsu, W., Zeng, L. and Costantini, F. (1999) Identification of a domain of axin that binds to the serine/threonine protein phosphatase 2A and a self-binding domain, *Journal of Biological Chemistry* 274(6): 3439-3445.

Hwang, D. Y., Hong, S., Jeong, J. W., Choi, S., Kim, H., Kim, J. and Kim, K. S. (2009) Vesicular monoamine transporter 2 and dopamine transporter are molecular targets of Pitx3 in the ventral midbrain dopamine neurons, *Journal of Neurochemistry* 111(5): 1202-1212.

Hynes, M., Porter, J. A., Chiang, C., Chang, D., Tessierlavigne, M., Beachy, P. A. and Rosenthal, A. (1995a) Induction of midbrain dopaminergic-neurons by sonic hedgehog, *Neuron* 15(1): 35-44.

Hynes, M., Poulsen, K., Tessierlavigne, M. and Rosenthal, A. (1995b) Control of neuronal diversity by the floor plate - contact-mediated induction of midbrain dopaminergic-neurons, *Cell* 80(1): 95-101.

Irving, C. and Mason, I. (1999) Regeneration of isthmic tissue is the result of a specific and direct interaction between rhombomere 1 and midbrain, *Development* 126(18): 3981-3989.

Jacobs, F. M. J., van Erp, S., van der Linden, A. J. A., von Oerthel, L., Burbach, J. P. H. and Smidt, M. P. (2009) Pitx3 potentiates Nurr1 in dopamine neuron terminal differentiation through release of SMRT-mediated repression, *Development* 136(4): 531-540.

Jeong, Y. and Epstein, D. J. (2003) Distinct regulators of Shh transcription in the floor plate and notochord indicate separate origins for these tissues in the mouse node, *Development* 130(16): 3891-3902.

Jessell, T. M. (2000) Neuronal specification in the spinal cord: Inductive signals and transcriptional codes, *Nature Reviews Genetics* 1(1): 20-29.

Jessell, T. M., Bovolenta, P., Placzek, M., Tessierlavigne, M. and Dodd, J. (1989) Polarity and patterning in the neural-tube - the origin and function of the floor plate, *Ciba Foundation Symposia* 144: 255-280.

Jin, G. H., Tan, X. F., Tian, M. L., Qin, J. B., Zhu, H. X., Huang, Z. and Xu, H. J. (2005) The controlled differentiation of human neural stem cells into TH-immunoreactive (ir) neurons in vitro, *Neuroscience Letters* 386(2): 105-110.

Joksimovic, M., Yun, B. A., Kittappa, R., Anderegg, A. M., Wchang, W., Taketo, M. M., McKay, R. D. G. and Awatramani, R. B. (2009) Wnt antagonism of Shh facilitates midbrain floor plate neurogenesis, *Nature Neuroscience* 12(2): 125-131.

Kadkhodaei, B., Ito, T., Joodmardi, E., Mattsson, B., Rouillard, C., Carta, M., Muramatsu, S. I., Sumi-Ichinose, C., Nomura, T., Metzger, D. et al. (2009) Nurr1 Is Required for Maintenance of Maturing and Adult Midbrain Dopamine Neurons, *Journal of Neuroscience* 29(50): 15923-15932.

Katahira, T., Sato, T., Sugiyama, S., Okafuji, T., Araki, I., Funahashi, J. and Nakamura, H. (2000) Interaction between Otx2 and Gbx2 defines the organizing center for the optic tectum, *Mechanisms of Development* 91(1-2): 43-52.

Katanaev, V. L., Solis, G. P., Hausmann, G., Buestorf, S., Katanayeva, N., Schrock, Y., Stuermer, C. A. O. and Basler, K. (2008) Reggie-1/flotillin-2 promotes secretion of the long-range signalling forms of Wingless and Hedgehog in Drosophila, *Embo Journal* 27(3): 509-521.

Kato, M., Patel, M. S., Levasseur, R., Lobov, I., Chang, B. H. J., Glass, D. A., Hartmann, C., Li, L., Hwang, T. H., Brayton, C. F. et al. (2002) Cbfa1-independent decrease in osteoblast proliferation, osteopenia, and persistent embryonic eye vascularization in mice deficient in Lrp5, a Wnt coreceptor, *Journal of Cell Biology* 157(2): 303-314.

Kawano, H., Ohyama, K., Kawamura, K. and Nagatsu, I. (1995) Migration of dopaminergic-neurons in the embryonic mesencephalon of mice, *Developmental Brain Research* 86(1-2): 101-113.

Kawasaki, H., Mizuseki, K., Nishikawa, S., Kaneko, S., Kuwana, Y., Nakanishi, S. and Sasai, Y. (2000) Induction of midbrain dopaminergic neurons from ES cells by stromal cell-derived inducing activity, *Neuron* 28(1): 31-40.

Kazanskaya, O., Glinka, A., Barrantes, I. D., Stannek, P., Niehrs, C. and Wu, W. (2004) R-spondin2 is a secreted activator of Wnt/beta-catenin signaling and is required for Xenopus myogenesis, *Developmental Cell* 7(4): 525-534.

Kazanskaya, O., Glinka, A. and Niehrs, C. (2000) The role of Xenopus dickkopf1 in prechordal plate specification and neural patterning, *Development* 127(22): 4981-4992.

Kele, J., Simplicio, N., Ferri, A. L. M., Mira, H., Guillemot, F., Arenas, E. and Ang, S. L. (2006) Neurogenin 2 is required for the development of ventral midbrain dopaminergic neurons, *Development* 133(3): 495-505.

Kelly, O. G., Pinson, K. I. and Skarnes, W. C. (2004) The Wnt co-receptors Lrp5 and Lrp6 are essential for gastrulation in mice, *Development* 131(12): 2803-2815.

Khan, Z., Vijayakumar, S., de la Torre, T. V., Rotolo, S. and Bafico, A. (2007) Analysis of endogenous LRP6 function reveals a novel feedback mechanism by which Wnt negatively regulates its receptor, *Molecular and Cellular Biology* 27(20): 7291-7301.

Kim, D. H., Inagaki, Y., Suzuki, T., Ioka, R. X., Yoshioka, S. Z., Magoori, K., Kang, M. J., Cho, Y., Nakano, A. Z., Liu, Q. et al. (1998) A new low density lipoprotein receptor belated protein, LRP5, is expressed in hepatocytes and adrenal cortex, and recognizes apolipoprotein E, *Journal of Biochemistry* 124(6): 1072-1076.

Kim, D. W., Chung, S., Hwang, M., Ferree, A., Tsai, H. C., Park, J. J., Nam, T. S., Kang, U. J., Isacson, O. and Kim, K. S. (2006) Stromal cell-derived inducing activity, Nurr1, and signaling molecules synergistically induce dopaminergic neurons from mouse embryonic stem cells, *Stem Cells* 24(3): 557-567.

Kim, J. H., Auerbach, J. M., Rodriguez-Gomez, J. A., Velasco, I., Gavin, D., Lumelsky, N., Lee, S. H., Nguyen, J., Sanchez-Pernaute, R., Bankiewicz, K. et al. (2002) Dopamine neurons derived from embryonic stem cells function in an animal model of Parkinson's disease, *Nature* 418(6893): 50-56.

Kimelman, D. and Xu, W. (2006) beta-Catenin destruction complex: insights and questions from a structural perspective, *Oncogene* 25(57): 7482-7491.

Kimura-Yoshida, C., Nakano, H., Okamura, D., Nakao, K., Yonemura, S., Belo, J. A., Aizawa, S., Matsui, Y. and Matsuo, I. (2005) Canonical Wnt signaling and its antagonist regulate anterior-posterior axis polarization by guiding cell migration in mouse visceral endoderm, *Developmental Cell* 9(5): 639-650.

Kimura-Yoshida, C., Tian, E., Nakano, H., Amazaki, S., Shimokawa, K., Rossant, J., Aizawa, S. and Matsuo, I. (2007) Crucial roles of Foxa2 in mouse anterior-posterior axis polarization via regulation of anterior visceral endoderm-specific genes, *Proceedings of the National Academy of Sciences of the United States of America* 104(14): 5919-5924.

Kittappa, R., Chang, W. W., Awatramani, R. B. and McKay, R. D. G. (2007) The foxa2 gene controls the birth and spontaneous degeneration of dopamine neurons in old age, *Plos Biology* 5(12): 2875-2884.

Kobayashi, K., Morita, S., Sawada, H., Mizuguchi, T., Yamada, K., Nagatsu, I., Hata, T., Watanabe, Y., Fujita, K. and Nagatsu, T. (1995) Targeted disruption of the tyrosine-hydroxylase locus results in severe catecholamine depletion and perinatal lethality in mice, *Journal of Biological Chemistry* 270(45): 27235-27243.

Kong, X. B. and Zhang, C. (2009) Dickkopf (Dkk) 1 promotes the differentiation of mouse embryonic stem cells toward neuroectoderm, *In Vitro Cellular & Developmental Biology-Animal* 45(3-4): 185-193.

Kordower, J. H., Freeman, T. B., Chen, E. Y., Mufson, E. J., Sanberg, P. R., Hauser, R. A., Snow, B. and Olanow, C. W. (1998) Fetal nigral grafts survive and mediate clinical benefit in a patient with Parkinson's disease, *Movement Disorders* 13(3): 383-393.

Kordower, J. H., Freeman, T. B., Snow, B. J., Vingerhoets, F. J. G., Mufson, E. J., Sanberg, P. R., Hauser, R. A., Smith, D. A., Nauert, G. M., Perl, D. P. et al. (1995) Neuropathological evidence of graft-survival and striatal reinnervation after the transplantation of fetal mesencephalic tissue in a patient with parkinsons-disease, *New England Journal of Medicine* 332(17): 1118-1124.

Korol, O., Gupta, R. W. and Mercola, M. (2008) A novel activity of the Dickkopf-1 amino terminal domain promotes axial and heart development independently of canonical Wnt inhibition, *Developmental Biology* 324(1): 131-138.

Krupnik, V. E., Sharp, J. D., Jiang, C., Robison, K., Chickering, T. W., Amaravadi, L., Brown, D. E., Guyot, D., Mays, G., Leiby, K. et al. (1999) Functional and structural diversity of the human Dickkopf gene family, *Gene* 238(2): 301-313.

Lai, E., Prezioso, V. R., Smith, E., Litvin, O., Costa, R. H. and Darnell, J. E. (1990) Hnf-3a, a hepatocyte-enriched transcription factor of novel structure is regulated transcriptionally, *Genes & Development* 4(8): 1427-1436.

Law, S. W., Conneely, O. M., Demayo, F. J. and Omalley, B. W. (1992) Identification of a new brain-specific transcription factor, Nurr1, *Molecular Endocrinology* 6(12): 2129-2135.

Le, W. D., Conneely, O. M., Zou, L. L., He, Y., Saucedo-Cardenas, O., Jankovic, J., Mosier, D. R. and Appel, S. H. (1999) Selective agenesis of mesencephalic dopaminergic neurons in Nurr1-deficient mice, *Experimental Neurology* 159(2): 451-458.

Lebel, M., Gauthier, Y., Moreau, A. and Drouin, J. (2001) Pitx3 activates mouse tyrosine hydroxylase promoter via a high-affinity binding site, *Journal of Neurochemistry* 77(2): 558-567.

Lee, A. Y., He, B., You, L., Xu, Z. D., Mazieres, J., Reguart, N., Mikami, I., Batra, S. and Jablons, D. M. (2004) Dickkopf-1 antagonizes Wnt signaling independent of beta-catenin in human mesothelioma, *Biochemical and Biophysical Research Communications* 323(4): 1246-1250.

Lee, E., Salic, A., Kruger, R., Heinrich, R. and Kirschner, M. W. (2003) The roles of APC and axin derived from experimental and theoretical analysis of the Wnt pathway, *Plos Biology* 1(1): 116-132.

Lee, K. J. and Jessell, T. M. (1999) The specification of dorsal cell fates in the vertebrate central nervous system, *Annual Review of Neuroscience* 22: 261-294.

Lee, S. H., Lumelsky, N., Studer, L., Auerbach, J. M. and McKay, R. D. (2000) Efficient generation of midbrain and hindbrain neurons from mouse embryonic stem cells, *Nature Biotechnology* 18(6): 675-679.

Lee, S. M. K., Danielian, P. S., Fritzsch, B. and McMahon, A. P. (1997) Evidence that FGF8 signalling from the midbrain-hindbrain junction regulates growth and polarity in the developing midbrain, *Development* 124(5): 959-969.

Levenstein, M. E., Ludwig, T. E., Xu, R. H., Llanas, R. A., VanDenHeuvel-Kramer, K., Manning, D. and Thomson, J. A. (2006) Basic fibroblast growth factor support of human embryonic stem cell self-renewal, *Stem Cells* 24(3): 568-574.

Lewis, S. L., Khoo, P. L., De Young, R. A., Steiner, K., Wilcock, C., Mukhopadhyay, M., Westphal, H., Jamieson, R. V., Robb, L. and Tam, P. P. L. (2008) Dkk1 and Wnt3 interact to control head morphogenesis in the mouse, *Development* 135(10): 1791-1801.

Li, J., Sarosi, I., Cattley, R. C., Pretorius, J., Asuncion, F., Grisanti, M., Morony, S., Adamu, S., Geng, Z. P., Qiu, W. R. et al. (2006) Dkk1-mediated inhibition of Wnt signaling in bone results in osteopenia, *Bone* 39(4): 754-766.

Li, Y., Hively, W. P. and Varmus, H. E. (2000) Use of MMTV-Wnt-1 transgenic mice for studying the genetic basis of breast cancer, *Oncogene* 19(8): 1002-1009.

Li, Y. H., Lu, W. Y., King, T. D., Liu, C. C., Bijur, G. N. and Bu, G. J. (2010) Dkk1 Stabilizes Wnt Co-Receptor LRP6: Implication for Wnt Ligand-Induced LRP6 Down-Regulation, *Plos One* 5(6).

Lin, W., Metzakopian, E., Mavromatakis, Y. E., Gao, N., Balaskas, N., Sasaki, H., Briscoe, J., Whitsett, J. A., Goulding, M., Kaestner, K. H. et al. (2009) Foxa1 and Foxa2 function both upstream of and cooperatively with Lmx1a and Lmx1b in a feedforward loop promoting mesodiencephalic dopaminergic neuron development, *Developmental Biology* 333(2): 386-396.

Lindvall, O and Bjorklund, A. (2004) Cell therapy in Parkinson's disease. *NeuroRx.* 1(4): 382–393

Lindvall, O., Brundin, P., Widner, H., Rehncrona, S., Gustavii, B., Frackowiak, R., Leenders, K. L., Sawle, G., Rothwell, J. C., Marsden, C. D. et al. (1990) Grafts of fetal dopamine neurons survive and improve motor function in Parkinsons-disease, *Science* 247(4942): 574-577.

Lindvall, O., Gustavii, B., Astedt, B., Lindholm, T., Leenders, K. L., Frackowiak, R., Johnels, B., Freedman, R., Rehncrona, S., Brundin, P. et al. (1988) Fetal dopamine-rich mesencephalic grafts in Parkinsons-disease, *Lancet* 2(8626-7): 1483-1484.

Lindvall, O., Rehncrona, S., Brundin, P., Gustavii, B., Astedt, B., Widner, H., Lindholm, T., Bjorklund, A., Leenders, K. L., Rothwell, J. C. et al. (1989) Human-fetal dopamine neurons grafted into the striatum in 2 patients with severe Parkinsons-disease - a detailed account of methodology and a 6-month follow-up, *Archives of Neurology* 46(6): 615-631.

Liu, A. M. and Joyner, A. L. (2001) EN and GBX2 play essential roles downstream of FGF8 in patterning the mouse mid/hindbrain region, *Development* 128(2): 181-191.

Liu, A. M., Losos, K. and Joyner, A. L. (1999a) FGF8 can activate Gbx2 and transform regions of the rostral mouse brain into a hindbrain, *Development* 126(21): 4827-4838.

Liu, C. C., Pearson, C. and Bu, G. J. (2009) Cooperative Folding and Ligand-binding Properties of LRP6 beta-Propeller Domains, *Journal of Biological Chemistry* 284(22): 15299-15307.

Liu, G. Z., Bafico, A. and Aaronson, S. A. (2005) The mechanism of endogenous receptor activation functionally distinguishes prototype canonical and noncanonical Wnts, *Molecular and Cellular Biology* 25(9): 3475-3482.

Liu, P. T., Wakamiya, M., Shea, M. J., Albrecht, U., Behringer, R. R. and Bradley, A. (1999b) Requirement for Wnt3 in vertebrate axis formation, *Nature Genetics* 22(4): 361-365.

Lotharius, J., Barg, S., Wiekop, P., Lundberg, C., Raymon, H. K. and Brundin, P. (2002) Effect of mutant alpha-synuclein on dopamine homeostasis in a new human mesencephalic cell line, *Journal of Biological Chemistry* 277(41): 38884-38894.

Luo, W., Peterson, A., Garcia, B. A., Coombs, G., Kofahl, B., Heinrich, R., Shabanowitz, J., Hunt, D. F., Yost, H. J. and Virshup, D. M. (2007) Protein phosphatase 1 regulates assembly and function of the beta-catenin degradation complex, *Embo Journal* 26(6): 1511-1521.

Lupo, G., Harris, W. A. and Lewis, K. E. (2006) Mechanisms of ventral patterning in the vertebrate nervous system, *Nature Reviews Neuroscience* 7(2): 103-114.

MacDonald, B. T., Adamska, M. and Meisler, M. H. (2004) Hypomorphic expression of Dkk1 in the doubleridge mouse: dose dependence and compensatory interactions with Lrp6, *Development* 131(11): 2543-2552.

MacDonald, B. T., Tamai, K. and He, X. (2009) Wnt/beta-Catenin Signaling: Components, Mechanisms, and Diseases, *Developmental Cell* 17(1): 9-26.

Maciaczyk, J., Singec, Y., Maciaczyk, D. and Nikkhah, G. (2008) Combined use of BDNF, ascorbic acid, low oxygen, and prolonged differentiation time generates tyrosine hydroxylase-expressing neurons after long-term in vitro expansion of human fetal midbrain precursor cells, *Experimental Neurology* 213(2): 354-362.

Malatesta, P., Hartfuss, E. and Gotz, M. (2000) Isolation of radial glial cells by fluorescent-activated cell sorting reveals a neuronal lineage, *Development* 127(24): 5253-5263.

Mao, B. Y., Wu, W., Davidson, G., Marhold, J., Li, M. F., Mechler, B. M., Delius, H., Hoppe, D., Stannek, P., Walter, C. et al. (2002) Kremen proteins are Dickkopf receptors that regulate Wnt/beta-catenin signalling, *Nature* 417(6889): 664-667.

Mao, B. Y., Wu, W., Li, Y., Hoppe, D., Stannek, P., Glinka, A. and Niehrs, C. (2001a) LDL-receptor-related protein 6 is a receptor for Dickkopf proteins, *Nature* 411(6835): 321-325.

Mao, J. H., Wang, J. Y., Liu, B., Pan, W. J., Farr, G. H., Flynn, C., Yuan, H. D., Takada, S., Kimelman, D., Li, L. et al. (2001b) Low-density lipoprotein receptor-related protein-5 binds to Axin and regulates the canonical Wnt signaling pathway, *Molecular Cell* 7(4): 801-809.

Marin, F. and Puelles, L. (1994) Patterning of the embryonic avian midbrain after experimental inversions - a polarizing activity from the isthmus, *Developmental Biology* 163(1): 19-37.

Marti, E., Bumcrot, D. A., Takada, R. and McMahon, A. P. (1995) Requirement of 19k form of Sonic hedgehog for induction of distinct ventral cell-types in CNS explants, *Nature* 375(6529): 322-325.

Martin, G. R. (1981) Isolation of a pluripotent cell-line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem-cells, *Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences* 78(12): 7634-7638.

Martinat, C., Bacci, J. J., Leete, T., Kim, J., Vanti, W. B., Newman, A. H., Cha, J. H., Gether, U., Wang, H. G. and Abeliovich, A. (2006) Cooperative transcription activation by Nurr1 and Pitx3 induces embryonic stem cell maturation to the midbrain dopamine neuron phenotype, *Proceedings of the National Academy of Sciences of the United States of America* 103(8): 2874-2879.

Martinez, S., Crossley, P. H., Cobos, I., Rubenstein, J. L. R. and Martin, G. R. (1999) FGF8 induces formation of an ectopic isthmic organizer and isthmocerebellar development via a repressive effect on Otx2 expression, *Development* 126(6): 1189-1200.

Martinez, S., Marin, F., Nieto, M. A. and Puelles, L. (1995) Induction of ectopic engrailed expression and fate change in avian rhombomeres - intersegmental boundaries as barriers, *Mechanisms of Development* 51(2-3): 289-303.

Martinez, S., Wassef, M. and Alvaradomallart, R. M. (1991) Induction of a mesencephalic phenotype in the 2-day-old chick prosencephalon is preceded by the early expression of the homeobox gene En, *Neuron* 6(6): 971-981.

Matsunaga, E., Katahira, T. and Nakamura, H. (2002) Role of Lmx1b and Wnt1 in mesencephalon and metencephalon development, *Development* 129(22): 5269-5277.

Matsuo, I., Kuratani, S., Kimura, C., Takeda, N. and Aizawa, S. (1995) Mouse Otx2 functions in the formation and patterning of rostral head, *Genes & Development* 9(21): 2646-2658.

Maxwell, S. L., Ho, H. Y., Kuehner, E., Zhao, S. L. and Li, M. (2005) Pitx3 regulates tyrosine hydroxylase expression in the substantia nigra and identifies a subgroup of mesencephalic dopaminergic progenitor neurons during mouse development, *Developmental Biology* 282(2): 467-479.

McMahon, A. P. and Bradley, A. (1990) The Wnt-1 (Int-1) protooncogene is required for development of a large region of the mouse-brain, *Cell* 62(6): 1073-1085.

McMahon, A. P., Joyner, A. L., Bradley, A. and McMahon, J. A. (1992) The midbrain hindbrain phenotype of Wnt-1--Wnt-1- mice results from stepwise deletion of engrailed-expressing cells by 9.5 days postcoitum, *Cell* 69(4): 581-595.

McMahon, A. P. and Moon, R. T. (1989) Ectopic expression of the proto-oncogene Int-1 in xenopus embryos leads to duplication of the embryonic axis, *Cell* 58(6): 1075-1084.

Mendez, I., Sanchez-Pernaute, R., Cooper, O., Vinuela, A., Ferrari, D., Bjorklund, L., Dagher, A. and Isacson, O. (2005) Cell type analysis of functional fetal dopamine cell

suspension transplants in the striatum and substantia nigra of patients with Parkinson's disease, *Brain* 128: 1498-1510.

Mesnard, D., Guzman-Ayala, M. and Constam, D. B. (2006) Nodal species embryonic visceral endoderm and sustains pluripotent cells in the epiblast before overt axial patterning, *Development* 133(13): 2497-2505.

Messmer, K., Remington, M. P., Skidmore, F. and Fishman, P. S. (2007) Induction of tyrosine hydroxylase expression by the transcription factor Pitx3, *International Journal of Developmental Neuroscience* 25(1): 29-37.

Meyers, E. N., Lewandoski, M. and Martin, G. R. (1998) An Fgf8 mutant allelic series generated by Cre- and Flp-mediated recombination, *Nature Genetics* 18(2): 136-141.

Mi, K. H. and Johnson, G. V. W. (2005) Role of the intracellular domains of LRP5 and LRP6 in activating the Wnt canonical pathway, *Journal of Cellular Biochemistry* 95(2): 328-338.

Mikels, A. J. and Nusse, R. (2006) Purified Wnt5a protein activates or inhibits beta-catenin-TCF signaling depending on receptor context, *Plos Biology* 4: 570-582.

Millen, K. J., Wurst, W., Herrup, K. and Joyner, A. L. (1994) Abnormal embryonic cerebellar development and patterning of postnatal foliation in 2 mouse Engrailed-2 mutants, *Development* 120(3): 695-706.

Milosevic, J., Brandt, A., Roemuss, U., Arnold, A., Wegner, F., Schwarz, S. C., Storch, A., Zimmermann, H. and Schwarz, J. (2006) Uracil nucleotides stimulate human neural precursor cell proliferation and dopaminergic differentiation: involvement of MEK/ERK signalling, *Journal of Neurochemistry* 99(3): 913-923.

Moon, R. T., Campbell, R. M., Christian, J. L., McGrew, L. L., Shih, J. and Fraser, S. (1993) Xwnt-5a - a maternal wnt that affects morphogenetic movements after overexpression in embryos of Xenopus-laevis, *Development* 119(1): 97-111.

Morizane, A., Li, J. Y. and Brundin, P. (2008) From bench to bed: the potential of stem cells for the treatment of Parkinson's disease, *Cell and Tissue Research* 331(1): 323-336.

Morvan, F., Boulukos, K., Clement-Lacroix, P., Roman, S. R., Suc-Royer, I., Vayssiere, B., Ammann, P., Martin, P., Pinho, S., Pognonec, P. et al. (2006) Deletion of a single allele of the Dkk1 gene leads to an increase in bone formation and bone mass, *Journal of Bone and Mineral Research* 21(6): 934-945.

Mukhopadhyay, M., Shtrom, S., Rodriguez-Esteban, C., Chen, L., Tsukui, T., Gomer, L., Dorward, D. W., Glinka, A., Grinberg, A., Huang, S. P. et al. (2001) Dickkopf1 is required for embryonic head induction and limb morphogenesis in the mouse, *Developmental Cell* 1(3): 423-434.

Muroyama, Y., Kondoh, H. and Takada, S. (2004) Wnt proteins promote neuronal differentiation in neural stem cell culture, *Biochemical and Biophysical Research Communications* 313(4): 915-921.

Nagatsu, T., Levitt, M. and Udenfriend, S. (1964) Tyrosine hydroxylase - initial step in norepinephrine biosynthesis, *Journal of Biological Chemistry* 239(9): 2910-&.

Nakatani, T., Kumai, M., Mizuhara, E., Minaki, Y. and Ono, Y. (2010) Lmx1a and Lmx1b cooperate with Foxa2 to coordinate the specification of dopaminergic neurons and control of floor plate cell differentiation in the developing mesencephalon, *Developmental Biology* 339(1): 101-113.

Nakayama, K. (1997) Furin: a mammalian subtilisin/Kex2p-like endoprotease involved in processing of a wide variety of precursor proteins, *Biochemical Journal* 327: 625-635.

Nieuwkoop, P. D. (1991) The successive steps in the pattern-formation of the amphibian central-nervous-system, *Development Growth & Differentiation* 33(2): 149-154.
Niwa, H., Toyooka, T., Shimosato, D., Strumpf, D., Takahashi, K., Yagi, R. and Rossant, J. (2005) Interaction between Oct3/4 and Cdx2 determines trophectoderm differentiation, *Cell* 123(5): 917-929.
Nunes, I., Tovmasian, L. T., Silva, R. M., Burke, R. E. and Goff, S. P. (2003) Pitx3 is required for development of substantia nigra dopaminergic neurons, *Proceedings of the National Academy of Sciences of the United States of America* 100(7): 4245-4250.
Nusse, R. and Varmus, H. E. (1982) Many tumors induced by the mouse mammary-tumor virus contain a provirus integrated in the same region of the host genome, *Cell* 31(1): 99-109.
Nussleinvolhard, C. and Wieschaus, E. (1980) Mutations affecting segment number and polarity in Drosophila, *Nature* 287(5785): 795-801.
O'Hara, F. P., Beck, E., Barr, L. K., Wong, L. L., Kessler, D. S. and Riddle, R. D. (2005) Zebrafish Lmx1b.1 and Lmx1b.2 are required for maintenance of the isthmic organizer, *Development* 132(14): 3163-3173.
Oishi, I., Suzuki, H., Onishi, N., Takada, R., Kani, S., Ohkawara, B., Koshida, I., Suzuki, K., Yamada, G., Schwabe, G. C. et al. (2003) The receptor tyrosine kinase Ror2 is involved in non-canonical Wnt5a/JNK signalling pathway, *Genes to Cells* 8(7): 645-654.
Olanow, C. W., Goetz, C. G., Kordower, J. H., Stoessl, A. J., Sossi, V., Brin, M. F., Shannon, K. M., Nauert, G. M., Perl, D. P., Godbold, J. et al. (2003) A double-blind controlled trial of bilateral fetal nigral transplantation in Parkinson's disease, *Annals of Neurology* 54(3): 403-414.
Omodei, D., Acampora, D., Mancuso, P., Prakash, N., Di Giovannantonio, L. G., Wurst, W. and Simeone, A. (2008) Anterior-posterior graded response to Otx2 controls proliferation and differentiation of dopaminergic progenitors in the ventral mesencephalon, *Development* 135(20): 3459-3470.
Ono, Y., Nakatani, T., Sakamoto, Y., Mizuhara, E., Minaki, Y., Kumai, M., Hamaguchi, A., Nishimura, M., Inoue, Y., HayashiO, H. et al. (2007) Differences in neurogenic potential in floor plate cells along an anteroposterior location: midbrain dopaminergic neurons originate from mesencephalic floor plate cells, *Development* 134: 3213-3225.
Otero, J. J., Fu, W. M., Kan, L. X., Cuadra, A. E. and Kessler, J. A. (2004) beta-catenin signaling is required for neural differentiation of embryonic stem cells, *Development* 131(15): 3545-3557.
Panakova, D., Sprong, H., Marois, E., Thiele, C. and Eaton, S. (2005) Lipoprotein particles are required for Hedgehog and Wingless signalling, *Nature* 435(7038): 58-65.
Panhuysen, M., Weisenhorn, D. M. V., Blanquet, V., Brodski, C., Heinzmann, U., Beisker, W. and Wurst, W. (2004) Effects of Wnt1 signaling on proliferation in the developing mid-/hindbrain region, *Molecular and Cellular Neuroscience* 26(1): 101-111.
Parish, C. L., Castelo-Branco, G., Rawal, N., Tonnesen, J., Sorensen, A. T., Salto, C., Kokaia, M., Lindvall, O. and Arenas, E. (2008) Wnt5a-treated midbrain neural stem cells improve dopamine cell replacement therapy in parkinsonian mice, *Journal of Clinical Investigation* 118(1): 149-160.
Parr, B. A., Shea, M. J., Vassileva, G. and McMahon, A. P. (1993) Mouse Wnt genes exhibit discrete domains of expression in the early embryonic CNS and limb buds, *Development* 119(1): 247-261.

Perea-Gomez, A., Vella, F. D. J., Shawlot, W., Oulad-Abdelghani, M., Chazaud, C., Meno, C., Pfister, V., Chen, L., Robertson, E., Hamada, H. et al. (2002) Nodal antagonists in the anterior visceral endoderm prevent the formation of multiple primitive streaks, *Developmental Cell* 3(5): 745-756.

Perez-Balaguer, A., Puelles, E., Wurst, W. and Martinez, S. (2009) Shh dependent and independent maintenance of basal midbrain, *Mechanisms of Development* 126(5-6): 301-313.

Piao, S., Lee, S. H., Kim, H., Yum, S., Stamos, J. L., Xu, Y. B., Lee, S. J., Lee, J., Oh, S., Han, J. K. et al. (2008) Direct Inhibition of GSK3 beta by the Phosphorylated Cytoplasmic Domain of LRP6 in Wnt/beta-Catenin Signaling, *Plos One* 3(12): 10.

Piccini, P., Brooks, D. J., Bjorklund, A., Gunn, R. N., Grasby, P. M., Rimoldi, O., Brundin, P., Hagell, P., Rehncrona, S., Widner, H. et al. (1999) Dopamine release from nigral transplants visualized in vivo in a Parkinson's patient, *Nature Neuroscience* 2(12): 1137-1140.

Pinson, K. I., Brennan, J., Monkley, S., Avery, B. J. and Skarnes, W. C. (2000) An LDL-receptor-related protein mediates Wnt signalling in mice, *Nature* 407(6803): 535-538.

Placzek, M. (1995) THe role of the notochord and floor plate in inductive interactions, *Current Opinion in Genetics & Development* 5(4): 499-506.

Prakash, N., Brodski, C., Naserke, T., Puelles, E., Gogoi, R., Hall, A., Panhuysen, M., Echevarria, D., Sussel, L., Weisenhorn, D. M. V. et al. (2006) A Wnt1-regulated genetic network controls the identity and fate of midbrain-dopaminergic progenitors in vivo, *Development* 133(1): 89-98.

Puelles, E., Annino, A., Tuorto, F., Usiello, A., Acampora, D., Czerny, T., Brodski, C., Ang, S. L., Wurst, W. and Simeone, A. (2004) Otx2 regulates the extent, identity and fate of neuronal progenitor domains in the ventral midbrain, *Development* 131(9): 2037-2048.

Qian, D., Jones, C., Rzadzinska, A., Mark, S., Zhang, X. H., Steel, K. P., Dai, X. and Chen, P. (2007) Wnt5a functions in planar cell polarity regulation in mice, *Developmental Biology* 306: 121-133.

Rhinn, M., Dierich, A., Shawlot, W., Behringer, R. R., La Meur, M. and Ang, S. L. (1998) Sequential roles for Otx2 in visceral endoderm and neuroectoderm for forebrain and midbrain induction and specification, *Development* 125(5): 845-856.

Rijsewijk, F., Schuermann, M., Wagenaar, E., Parren, P., Weigel, D. and Nusse, R. (1987) The drosophila homolog of the mouse mammary oncogene int-1 is identical to the segment polarity gene wingless, *Cell* 50(4): 649-657.

Rossant, J. and Tam, P. P. L. (2009) Blastocyst lineage formation, early embryonic asymmetries and axis patterning in the mouse, *Development* 136(5): 701-713.

Roy, N. S., Cleren, C., Singh, S. K., Yang, L., Beal, M. F. and Goldman, S. A. (2006) Functional engraftment of human ES cell-derived dopaminergic neurons enriched by coculture with telomerase-immortalized midbrain astrocytes, *Nature Medicine* 12(11): 1259-1268.

Sacchetti, P., Brownschidle, L. A., Granneman, J. G. and Bannon, M. J. (1999) Characterization of the 5 '-flanking region of the human dopamine transporter gene, *Molecular Brain Research* 74(1-2): 167-174.

Sacchetti, P., Mitchell, T. R., Granneman, J. G. and Bannon, M. J. (2001) Nurr1 enhances transcription of the human dopamine transporter gene through a novel mechanism, *Journal of Neurochemistry* 76(5): 1565-1572.

Sacchetti, P., Sousa, K. M., Hall, A. C., Liste, I., Steffensen, K. R., Theofilopoulos, S., Parish, C. L., Hazenberg, C., Richter, L. A., Hovatta, O. et al. (2009) Liver X Receptors

and Oxysterols Promote Ventral Midbrain Neurogenesis In Vivo and in Human Embryonic Stem Cells, *Cell Stem Cell* 5(4): 409-419.
Saijo, K., Winner, B., Carson, C. T., Collier, J. G., Boyer, L., Rosenfeld, M. G., Gage, F. H. and Glass, C. K. (2009) A Nurr1/CoREST Pathway in Microglia and Astrocytes Protects Dopaminergic Neurons from Inflammation-Induced Death, *Cell* 137(1): 47-59.
Sakane, H., Yamamoto, H. and Kikuchi, A. (2010) LRP6 is internalized by Dkk1 to suppress its phosphorylation in the lipid raft and is recycled for reuse, *Journal of Cell Science* 123(3): 360-368.
Sakurada, K., Ohshima-Sakurada, M., Palmer, T. D. and Gage, F. H. (1999) Nurr1, an orphan nuclear receptor, is a transcriptional activator of endogenous tyrosine hydroxylase in neural progenitor cells derived from the adult brain, *Development* 126(18): 4017-4026.
Sanchez-Pernaute, R., Studer, L., Bankiewicz, K. S., Major, E. O. and McKay, R. D. G. (2001) In vitro generation and transplantation of precursor-derived human dopamine neurons, *Journal of Neuroscience Research* 65(4): 284-288.
Sasaki, H. and Hogan, B. L. M. (1993) Differential expression of multiple fork head-related genes during gastrulation and axial pattern-formation in the mouse embryo, *Development* 118(1): 47-59.
Sasaki, H. and Hogan, B. L. M. (1994) Hnf-3-beta as a regulator of floor plate development, *Cell* 76(1): 103-115.
Sasaki, H., Hui, C. C., Nakafuku, M. and Kondoh, H. (1997) A binding site for Gli proteins is essential for HNF-3 beta floor plate enhancer activity in transgenics and can respond to Shh in vitro, *Development* 124(7): 1313-1322.
Sato, T., Araki, I. and Nakamura, H. (2001) Inductive signal and tissue responsiveness defining the tectum and the cerebellum, *Development* 128(13): 2461-2469.
Sato, N., Sanjuan, I. M., Heke, M., Uchida, M., Naef, F. and Brivanlou, A. H. (2003) Molecular signature of human embryonic stem cells and its comparison with the mouse, *Developmental Biology* 260(2): 404-413.
Saucedo-Cardenas, O., Quintana-Hau, J. D., Le, W. D., Smidt, M. P., Cox, J. J., DeMayo, F., Burbach, J. P. H. and Conneely, O. M. (1998) Nurr1 is essential for the induction of the dopaminergic phenotype and the survival of ventral mesencephalic late dopaminergic precursor neurons, *Proceedings of the National Academy of Sciences of the United States of America* 95(7): 4013-4018.
Schambony, A. and Wedlich, D. (2007) Wnt-5A/Ror2 regulate expression of XPAPC through an alternative noncanonical signaling pathway, *Developmental Cell* 12(5): 779-792.
Schulte, G., Bryja, V., Rawal, N., Castelo-Branco, G., Sousa, K. M. and Arenas, E. (2005) Purified Wnt-5a increases differentiation of midbrain dopaminergic cells and dishevelled phosphorylation, *Journal of Neurochemistry* 92(6): 1550-1553.
Semenov, M. V., Tamai, K., Brott, B. K., Kuhl, M., Sokol, S. and He, X. (2001) Head inducer Dickkopf-1 is a ligand for Wnt coreceptor LRP6, *Current Biology* 11(12): 951-961.
Semenov, M. V., Zhang, X. and He, X. (2008) DKK1 antagonizes Wnt signaling without promotion of LRP6 internalization and degradation, *Journal of Biological Chemistry* 283(31): 21427-21432.
Semina, E. V., Murray, J. C., Reiter, R., Hrstka, R. F. and Graw, J. (2000) Deletion in the promoter region and altered expression of Pitx3 homeobox gene in aphakia mice, *Human Molecular Genetics* 9(11): 1575-1585.

Sgado, P., Alberi, L., Gherbassi, D., Galasso, S. L., Ramakers, G. M. J., Alavian, K. N., Smidt, M. P., Dyck, R. H. and Simon, H. H. (2006) Slow progressive degeneration of nigral dopaminergic neurons in postnatal Engrailed mutant mice, *Proceedings of the National Academy of Sciences of the United States of America* 103(41): 15242-15247.

Sharma, R. P. and Chopra, V. L. (1976) Effect of Wingless (Wg1) mutation on wing and haltere development in Drosophila-Melanogaster, *Developmental Biology* 48(2): 461-465.

Simeone, A. (2000) Positioning the isthmic organizer - where Otx2 and Gbx2 meet, *Trends in Genetics* 16(6): 237-240.

Simeone, A., Acampora, D., Gulisano, M., Stornaiuolo, A. and Boncinelli, E. (1992) Nested expression domains of 4 homeobox genes in developing rostral brain, *Nature* 358(6388): 687-690.

Simon, H. H., Saueressig, H., Wurst, W., Goulding, M. D. and O'Leary, D. D. M. (2001) Fate of midbrain dopaminergic neurons controlled by the engrailed genes, *Journal of Neuroscience* 21(9): 3126-3134.

Slusarski, D. C., YangSnyder, J., Busa, W. B. and Moon, R. T. (1997) Modulation of embryonic intracellular Ca^{2+} signaling by Wnt-5A, *Developmental Biology* 182(1): 114-120.

Smidt, M. P., Asbreuk, C. H. J., Cox, J. J., Chen, H., Johnson, R. L. and Burbach, J. P. H. (2000) A second independent pathway for development of mesencephalic dopaminergic neurons requires Lmx1b, *Nature Neuroscience* 3(4): 337-341.

Smidt, M. P., Smits, S. M., Bouwmeester, H., Hamers, F. P. T., van der Linden, A. J. A., Hellemons, A., Graw, J. and Burbach, J. P. H. (2004) Early developmental failure of substantia nigra dopamine neurons in mice lacking the homeodomain gene Pitx3, *Development* 131(5): 1145-1155.

Smidt, M. P., vanSchaick, H. S. A., Lanctot, C., Tremblay, J. J., Cox, J. J., vanderKleij, A. A. M., Wolterink, G., Drouin, J. and Burbach, J. P. H. (1997) A homeodomain gene Ptx3 has highly restricted brain expression in mesencephalic dopaminergic neurons, *Proceedings of the National Academy of Sciences of the United States of America* 94(24): 13305-13310.

Smith, J. C., Conlon, F. L., Saka, Y. and Tada, M. (2000) Xwnt11 and the regulation of gastrulation in Xenopus, *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences* 355(1399): 923-930.

Smits, S. M., Burbach, J. P. H. and Smidt, M. P. (2006) Developmental origin and fate of meso-diencephalic dopamine neurons, *Progress in Neurobiology* 78(1): 1-16.

Smits, S. M., Ponnio, T., Conneely, O. M., Burbach, J. P. H. and Smidt, M. P. (2003) Involvement of Nurr1 in specifying the neurotransmitter identity of ventral midbrain dopaminergic neurons, *European Journal of Neuroscience* 18(7): 1731-1738.

Sonnier, L., Le Pen, G., Hartmann, A., Bizot, J. C., Trovero, F., Krebs, M. O. and Prochiantz, A. (2007) Progressive loss of dopaminergic neurons in the ventral midbrain of adult mice heterozygote for Engrailed1, *Journal of Neuroscience* 27(5): 1063-1071.

Sonntag, K. C., Pruszak, J., Yoshizaki, T., van Arensbergen, J., Sanchez-Pernaute, R. and Isacson, O. (2007) Enhanced yield of neuroepithelial precursors and midbrain-like dopaminergic neurons from human embryonic stem cells using the bone morphogenic protein antagonist Noggin, *Stem Cells* 25(2): 411-418.

Storch, A., Paul, G., Csete, M., Boehm, B. O., Carvey, P. M., Kupsch, A. and Schwarz, J. (2001) Long-term proliferation and dopaminergic differentiation of human mesencephalic neural precursor cells, *Experimental Neurology* 170(2): 317-325.

Su, Y. Y., Fu, C. J., Ishikawa, S., Stella, A., Kojima, M., Shitoh, K., Schreiber, E. M., Day, B. W. and Liu, B. (2008) APC Is Essential for Targeting Phosphorylated beta-Catenin to the SCF beta-TrCP Ubiquitin Ligase, *Molecular Cell* 32(5): 652-661.
Sun, X., Meyers, E. N., Lewandoski, M. and Martin, G. R. (1999) Targeted disruption of Fgf8 causes failure of cell migration in the gastrulating mouse embryo, *Genes & Development* 13(14): 1834-1846.
Swistowska, A. M., da Cruz, A. B., Han, Y., Swistowski, A., Liu, Y., Shin, S., Zhan, M., Rao, M. S. and Zeng, X. M. (2010) Stage-Specific Role for Shh in Dopaminergic Differentiation of Human Embryonic Stem Cells Induced by Stromal Cells, *Stem Cells and Development* 19(1): 71-82.
Tada, M. and Smith, J. C. (2000) Xwnt11 is a target of Xenopus Brachyury: regulation of gastrulation movements via Dishevelled, but not through the canonical Wnt pathway, *Development* 127(10): 2227-2238.
Tahinci, E., Thorne, C. A., Franklin, J. L., Salic, A., Christian, K. M., Lee, L. A., Coffey, R. J. and Lee, E. (2007) Lrp6 is required for convergent extension during Xenopus gastrulation, *Development* 134: 4095-4106.
Tam, P. P. L. and Loebel, D. A. F. (2007) Gene function in mouse embryogenesis: get set for gastrulation, *Nature Reviews Genetics* 8(5): 368-381.
Tamai, K., Semenov, M., Kato, Y., Spokony, R., Liu, C. M., Katsuyama, Y., Hess, F., Saint-Jeannet, J. P. and He, X. (2000) LDL-receptor-related proteins in Wnt signal transduction, *Nature* 407(6803): 530-535.
Tamai, K., Zeng, X., Liu, C. M., Zhang, X. J., Harada, Y., Chang, Z. J. and He, X. (2004) A mechanism for Wnt coreceptor activation, *Molecular Cell* 13(1): 149-156.
Tanaka, A., Kamiakito, T., Hakamata, Y., Fujii, A., Kuriki, K. and Fukayama, M. (2001) Extensive neuronal localization and neurotrophic function of fibroblast growth factor 8 in the nervous system, *Brain Research* 912(2): 105-115.
Tang, M., Villaescusa, J., Luo, S., Guitarte, C., Lei, S., Miyamoto, Y., Taketo, M., Arenas, E. and Huang, E. (2010) Interactions of Wnt/beta-catenin signaling and sonic hedgehog regulate the neurogenesis of ventral midbrain dopamine neurons, *J Neurosci* 30(27): 9280-91.
Tang, M. Z., Miyamoto, Y. and Huang, E. J. (2009) Multiple roles of beta-catenin in controlling the neurogenic niche for midbrain dopamine neurons, *Development* 136(12): 2027-2038.
Thomas, K. R. and Capecchi, M. R. (1990) Targeted disruption of the murine Int-1 protooncogene resulting in severe abnormalities in midbrain and cerebellar development, *Nature* 346(6287): 847-850.
Thomson, J. A., Itskovitz-Eldor, J., Shapiro, S. S., Waknitz, M. A., Swiergiel, J. J., Marshall, V. S. and Jones, J. M. (1998) Embryonic stem cell lines derived from human blastocysts, *Science* 282(5391): 1145-1147.
Thuret, S., Bhatt, L., O'Leary, D. D. M. and Simon, H. H. (2004) Identification and developmental analysis of genes expressed by dopaminergic neurons of the substantia nigra pars compacta, *Molecular and Cellular Neuroscience* 25(3): 394-405.
Tian, E., Zhan, F. H., Walker, R., Rasmussen, E., Ma, Y. P., Barlogie, B. and Shaughnessy, J. D. (2003) The role of the Wnt-signaling antagonist DKK1 in the development of osteolytic lesions in multiple myeloma, *New England Journal of Medicine* 349(26): 2483-2494.
Tolwinski, N. S., Wehrli, M., Rives, A., Erdeniz, N., DiNardo, S. and Wieschaus, E. (2003) Wg/Wnt signal can be transmitted through Arrow/LRP5,6 and Axin independently of Zw3/Gsk3 beta activity, *Developmental Cell* 4(3): 407-418.

Topczewski, J., Sepich, D. S., Myers, D. C., Walker, C., Amores, A., Lele, Z., Hammerschmidt, M., Postlethwait, J. and Solnica-Krezel, L. (2001) The zebrafish glypican knypek controls cell polarity during gastrulation movements of convergent extension, *Developmental Cell* 1(2): 251-264.

Topol, L., Jiang, X. Y., Choi, H., Garrett-Beal, L., Carolan, P. J. and Yang, Y. Z. (2003) Wnt-5a inhibits the canonical Wnt pathway by promoting GSK-3-independent beta-catenin degradation, *Journal of Cell Biology* 162(5): 899-908.

Torres, M. A., YangSnyder, J. A., Purcell, S. M., DeMarais, A. A., McGrew, L. L. and Moon, R. T. (1996) Activities of the Wnt-1 class of secreted signaling factors are antagonized by the Wnt-5A class and by a dominant negative cadherin in early Xenopus development, *Journal of Cell Biology* 133(5): 1123-1137.

van Amerongen, R., Mikels, A. and Nusse, R. (2008) Alternative Wnt Signaling Is Initiated by Distinct Receptors, *Science Signaling* 1(35).

van den Munckhof, P., Luk, K. C., Ste-Marie, L., Montgomery, J., Blanchet, P. J., Sadikot, A. F. and Drouin, J. (2003) Pitx3 is required for motor activity and for survival of a subset of midbrain dopaminergic neurons, *Development* 130(11): 2535-2542.

Verani, R., Cappuccio, I., Spinsanti, P., Gradini, R., Caruso, A., Magnotti, M. C., Motolese, M., Nicoletti, F. and Melchiorri, D. (2007) Expression of the Wnt inhibitor Dickkopf-1 is required for the induction of neural markers in mouse embryonic stem cells differentiating in response to retinoic acid, *Journal of Neurochemistry* 100(1): 242-250.

Vernay, B., Koch, M., Vaccarino, F., Briscoe, J., Simeone, A., Kageyama, R. and Ang, S. L. (2005) Otx2 regulates subtype specification and neurogenesis in the midbrain, *Journal of Neuroscience* 25(19): 4856-4867.

Villa, A., Liste, I., Courtois, E. T., Seiz, E. G., Ramos, M., Meyer, M., Juliusson, B., Kusk, P. and Martinez-Serrano, A. (2009) Generation and properties of a new human ventral mesencephalic neural stem cell line, *Experimental Cell Research* 315(11): 1860-1874.

Wallen, A., Castro, D. S., Zetterstrom, R. H., Karlen, M., Olson, L., Ericson, J. and Perlmann, T. (2001) Orphan nuclear receptor Nurr1 is essential for Ret expression in midbrain dopamine neurons and in the brain stem, *Molecular and Cellular Neuroscience* 18(6): 649-663.

Wallen, A., Zetterstrom, R. H., Solomin, L., Arvidsson, M., Olson, L. and Perlmann, T. (1999) Fate of mesencephalic AHD2-expressing dopamine progenitor cells in Nurr1 mutant mice, *Experimental Cell Research* 253(2): 737-746.

Wallingford, J. B. and Habas, R. (2005) The developmental biology of Dishevelled: an enilgmatic protein governing cell fate and cell polarity, *Development* 132(20): 4421-4436.

Wang, J., Shou, J. and Chen, X. B. (2000) Dickkopf-1, an inhibitor of the Wnt signaling pathway, is induced by p53, *Oncogene* 19(14): 1843-1848.

Wang, X., Li, X. X., Wang, K., Zhou, H. F., Xue, B., Li, L. S. and Wang, X. M. (2004) Forskolin cooperating with growth factor on generation of dopaminergic neurons from human fetal mesencephalic neural progenitor cells, *Neuroscience Letters* 362(2): 117-121.

Wang, Z. L., Benoit, G., Liu, J. S., Prasad, S., Aarnisalo, P., Liu, X. H., Xu, H. D., Walker, N. P. C. and Perlmann, T. (2003) Structure and function of Nurr1 identifies a class of ligand-independent nuclear receptors, *Nature* 423(6939): 555-560.

Wassarman, K. M., Lewandoski, M., Campbell, K., Joyner, A. L., Rubenstein, J. L. R., Martinez, S. and Martin, G. R. (1997) Specification of the anterior hindbrain and

establishment of a normal mid/hindbrain organizer is dependent on Gbx2 gene function, *Development* 124(15): 2923-2934.
Watanabe, K., Kamiya, D., Nishiyama, A., Katayama, T., Nozaki, S., Kawasaki, H., Watanabe, Y., Mizuseki, K. and Sasai, Y. (2005) Directed differentiation of telencephalic precursors from embryonic stem cells, *Nature Neuroscience* 8(3): 288-296.
Wehrli, M., Dougan, S. T., Caldwell, K., O'Keefe, L., Schwartz, S., Vaizel-Ohayon, D., Schejter, E., Tomlinson, A. and DiNardo, S. (2000) Arrow encodes an LDL-receptor-related protein essential for Wingless signalling, *Nature* 407(6803): 527-530.
Weinstein, D. C., Altaba, A. R. I., Chen, W. S., Hoodless, P., Prezioso, V. R., Jessell, T. M. and Darnell, J. E. (1994) The winged-helix transcription factor Hnf-3-beta is required for notochord development in the mouse embryo, *Cell* 78(4): 575-588.
Wernig, M., Zhao, J. P., Pruszak, J., Hedlund, E., Fu, D. D., Soldner, F., Broccoli, V., Constantine-Paton, M., Isacson, O. and Jaenisch, R. (2008) Neurons derived from reprogrammed fibroblasts functionally integrate into the fetal brain and improve symptoms of rats with Parkinson's disease, *Proceedings of the National Academy of Sciences of the United States of America* 105(15): 5856-5861.
Wilkinson, D. G., Bailes, J. A. and McMahon, A. P. (1987) Expression of the protooncogene Int-1 is restricted to specific neural cells in the developing mouse embryo, *Cell* 50(1): 79-88.
Winkler, C., Kirik, D. and Bjorklund, A. (2005) Cell transplantation in Parkinson's disease: how can we make it work?, *Trends in Neurosciences* 28(2): 86-92.
Wong, G. T., Gavin, B. J. and McMahon, A. P. (1994) Differential transformation of mammary epithelial-cells by Wnt genes, *Molecular and Cellular Biology* 14(9): 6278-6286.
Wong, H. C., Bourdelas, A., Krauss, A., Lee, H. J., Shao, Y. M., Wu, D. Q., Mlodzik, M., Shi, D. L. and Zheng, J. (2003) Direct binding of the PDZ domain of Dishevelled to a conserved internal sequence in the C-terminal region of frizzled, *Molecular Cell* 12(5): 1251-1260.
Wu, G., Huang, H., Abreu, J. G. and He, X. (2009) Inhibition of GSK3 Phosphorylation of beta-Catenin via Phosphorylated PPPSPXS Motifs of Wnt Coreceptor LRP6, *Plos One* 4(3).
Wu, X. M., Tu, X. L., Joeng, K. S., Hilton, M. J., Williams, D. A. and Long, F. X. (2008) Rac1 activation controls nuclear localization of beta-catenin during canonical Wnt signaling, *Cell* 133(2): 340-353.
Wurst, W., Auerbach, A. B. and Joyner, A. L. (1994) Multiple developmental defects in Engrailed-1 mutant mice - an early mid-hindbrain deletion and patterning defects in forelimbs and sternum, *Development* 120(7): 2065-2075.
Wurst, W. and Bally-Cuif, L. (2001) Neural plate patterning: Upstream and downstream of the isthmic organizer, *Nature Reviews Neuroscience* 2(2): 99-108.
Xu, R. H., Peck, R. M., Li, D. S., Feng, X. Z., Ludwig, T. and Thomson, J. A. (2005) Basic FGF and suppression of BMP signaling sustain undifferentiated proliferation of human ES cells, *Nature Methods* 2(3): 185-190
Yamada, T., Pfaff, S. L., Edlund, T. and Jessell, T. M. (1993) Control of cell pattern in the neural-tube - motor-neuron induction by diffusible factors from notochord and floor plate, *Cell* 73(4): 673-686.
Yamaguchi, T. P., Bradley, A., McMahon, A. P. and Jones, S. (1999) A Wnt5a pathway underlies outgrowth of multiple structures in the vertebrate embryo, *Development* 126(6): 1211-1223.

Yamamoto, H., Sakane, H., Michiue, T. and Kikuchi, A. (2008) Wnt3a and Dkk1 regulate distinct internalization pathways of LRP6 to tune the activation of beta-catenin signaling, *Developmental Cell* 15(1): 37-48.

Yamamoto, M., Saijoh, Y., Perea-Gomez, A., Shawlot, W., Behringer, R. R., Ang, S. L., Hamada, H. and Meno, C. (2004) Nodal antagonists regulate formation of the anteroposterior axis of the mouse embryo, *Nature* 428(6981): 387-392.

Yamanaka, H., Moriguchi, T., Masuyama, N., Kusakabe, M., Hanafusa, H., Takada, R., Takada, S. and Nishida, E. (2002) JNK functions in the non-canonical Wnt pathway to regulate convergent extension movements in vertebrates, *Embo Reports* 3(1): 69-75.

Ye, W. L., Shimamura, K., Rubenstein, J. L. R., Hynes, M. A. and Rosenthal, A. (1998) FGF and Shh signals control dopaminergic and serotonergic cell fate in the anterior neural plate, *Cell* 93(5): 755-766.

Ying, Q. L., Stavridis, M., Griffiths, D., Li, M. and Smith, A. (2003) Conversion of embryonic stem cells into neuroectodermal precursors in adherent monoculture, *Nature Biotechnology* 21(2): 183-186.

Yue, F. M., Cui, L., Johkura, K., Ogiwara, N. and Sasaki, K. (2006) Induction of midbrain dopaminergic neurons from primate embryonic stem cells by coculture with Sertoli cells, *Stem Cells* 24(7): 1695-1706.

Zeng, X., Huang, H., Tamai, K., Zhang, X. J., Harada, Y., Yokota, C., Almeida, K., Wang, J., Doble, B., Woodgett, J. et al. (2008) Initiation of Wnt signaling: control of Wnt coreceptor Lrp6 phosphorylation/activation via frizzled, dishevelled and axin functions, *Development* 135(2): 367-375.

Zeng, X., Tamai, K., Doble, B., Li, S. T., Huang, H., Habas, R., Okamura, H., Woodgett, J. and He, X. (2005) A dual-kinase mechanism for Wnt co-receptor phosphorylation and activation, *Nature* 438(7069): 873-877.

Zervas, M., Millet, S., Ahn, S. and Joyner, A. L. (2004) Cell behaviors and genetic lineages of the mesencephalon and rhombomere 1, *Neuron* 43(3): 345-357.

Zetterstrom, R. H., Solomin, L., Jansson, L., Hoffer, B. J., Olson, L. and Perlmann, T. (1997) Dopamine neuron agenesis in Nurr1-deficient mice', *Science* 276(5310): 248-250.

Zhai, L., Chaturvedi, D. and Cumberledge, S. (2004) Drosophila Wnt-1 undergoes a hydrophobic modification and is targeted to lipid rafts, a process that requires porcupine, *Journal of Biological Chemistry* 279(32): 33220-33227.

Zhao, S. L., Maxwell, S., Jimenez-Beristain, A., Vives, J., Kuehner, E., Zhao, J. X., O'Brien, C., de Felipe, C., Semina, E. and Li, M. (2004) Generation of embryonic stem cells and transgenic mice expressing green fluorescence protein in midbrain dopaminergic neurons, *European Journal of Neuroscience* 19(5): 1133-1140.

Zou, H. L., Su, C. J., Shi, M., Zhao, G. Y., Li, Z. Y., Guo, C. and Ding, Y. Q. (2009) Expression of the LIM-homeodomain gene Lmx1a in the postnatal mouse central nervous system, *Brain Research Bulletin* 78(6): 306-312.