Generation and analysis of mouse models of aberrant β-catenin function

A thesis submitted in partial fulfilment for the degree of Doctor of Philosophy to the University of London

by

Pedro Manuel Ródenas Cuadrado BSc (Hons)

University College London

Declaration

I, Pedro Manuel Ródenas Cuadrado, confirm that the work presented in this thesis is my original research work. Where contributions of others are involved, this has been clearly indicated in the thesis.

Acknowledgements

I would like to thank my supervisor, Professor Sebastian Brandner, for his guidance and advice. Similarly, I would like to thank my secondary supervisor, Dr. Thomas Jacques. In addition, big thanks go out to all members of the Department of Neurodegenerative disease, UCL Institute of Neurology for their help and advice during my PhD. Also thanks to Wakefield Street staff, especially Steven Morrell for all his hard work.

My immense gratitude goes to Heike Naumann for her teaching, dedication and endless patience. I am a better scientist because of you. I would also like to thank Dr. Nico Henriquez for his advice with experiments and helping me start writing this thesis. My thanks go to my PhD buddy Alex Swales -the terror of chickens worldwide- for your positive attitude and great sense of humour. You helped me have a good time even when the going was tough. I would also like to thank Ilaria Mirabile and Samira Rabbanian (also Drs-to-be) for being great friends and being there for a chat when I needed it.

Above all, I would like to thank all my family, especially my mother and my grandfather for their love and for making me the person I am today; there are no words to express my love for them.

Finally, I would like to give my eternal gratitude to Pela for her endless patience when dealing with me, for her emotional support during the bleakest moments, for helping me so much with this thesis when it wasn't required of her and for her unconditional affection. I wouldn't be me without you. I love you.

For my grandfather

Abstract

The key component of the Wnt/β-catenin pathway is β-catenin, a 780 amino acid protein which was originally identified as a component of E-cadherin junctions, where it links E-cadherin with α-catenin and consequently to the actin cytoskeleton. In the absence of Wnt signalling, cytoplasmic β-catenin is phosphorylated and subsequently degraded by ubiquitinylation. Activation of Wnt signalling stabilises cytoplasmic β-catenin, which translocates into the nucleus where it binds to nuclear transcription factors TCF/LEF and regulates the expression of over thirty genes, including cell cycle activating genes.

The Wnt/β-catenin pathway is essential for nervous system development and it is aberrantly active in common malignant tumours of the central nervous system, such as glioblastoma in adults and medulloblastoma in children.

We have generated a mouse model expressing a Cre-recombinase-inducible form of degradation-resistant (oncogenic) β-catenin. We generated compound mutant mice to activate the Wnt/β-catenin pathway in specific brain regions and during several developmental time points. Compound mutant transgenic mice expressed dominant active β-catenin *in vivo*, but did not show developmental abnormalities or brain tumours even in combination with inactivated p53 alleles. Similarly, neural stem cells expressing dominant active β-catenin did not show a growth advantage or altered self-renewal. We attributed these results to low level expression of dominant active β-catenin.

In addition, we later acquired mice that carry an inducible deletion of the exon 3 of β-catenin (containing the phosphorylation site). Expression of this form of β-catenin did not increase neural stem cell growth and self-renewal. However, *in vivo* expression results in brain malformation and lethality depending on the cells in which β-catenin was activated, however, recombination in adult stem cells *in vivo* did not result in brain tumours.

Table of contents

Index of figures and tables

List of abbreviations and acronyms

aa	amino acid
ACC	Acinal cell carcinoma
ADP	Adenosine diphosphate ribose
AIM1	Absent in melanoma 1
APAF-1	Apoptotic protease-activating factor 1
APC	Adenomatous poliposis coli
APS	Ammonium Persulfate
ARF	ADP rybosylation factor
AVL	Avian leukosis virus
Bax	Bcl-2 associated X protein
BCA	Bicinchoninic acid
Bcl-2	B-cell lymphoma 2
BMP	Bone morphogenic protein
bp	base pair
BrdU	5-Bromo-2-deoxyuridine
BSA	Bovine serum albumin
β-TrCP	β-transducin repeats-containing protein
Ca^{2+}	Calcium
CAK	Cyclin activating kinase
CaMK	Calmodulin-dependent kinase
cAMP	cyclin Adenosine monophosphate
CBP	cAMP-binding protein
CCNC	Cyclin C
Cdc42	Cell division cycle 42
CDK	Cyclin-dependent kinase
CDKN1A	Cyclin-dependent kinase inhibitor 1A
cDNA	complementary DNA
Ck1α	Casein kinase 1α
cm	centimetre
CNS	Central nervous system
CRC	Colorectal cancer
CRD	Cysteine rich domain
Cre	Causes recombination
Cre-ER	Cre-Oestrogen receptor binding-domain
CSC	Cancer stem cells
CtBP	C-terminal binding protein
Daam1	Dishevelled associated activator of morphogenesis 1
DAG	Diacylglycerol
Dally	Division abnormally delayed
DNA	Deoxyribonucleic acid
DEP	Dvl-egl10-Pleckstrin
Dgo	Diego

DIX	Dishevelled-Axin
Dkk	Dickkopf
DLG	Discs-large protein
DlgA	*Drosophila* disc large tumour suppressor
Dly	Dally-like
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
dNTP	deoxyribonucleotide triphosphate
Drl	Derailed
Dsh/Dvl	Dishevelled
DTT	Dithriothreitol
E2F	E2 transcription factor
EB1	End-binding protein 1
ECM	Extracellular cell matrix
EDTA	Ethylenediaminetetraacetic acid
EF3	Elongation factor 3
EGF	Epidermal growth factor
EGL	External granule layer
En1/2	Engrailed 1/2
ER	Endoplasmic reticulum
ER	Oestrogen receptor
ErB4	Oestrogen receptor β4
ES	Embryonic stem
FAP	Familial Adenomatous polyposis
FCS	Foetal calf serum
FGFβ	Fibroblast growth factor β
Fmi	Flamingo
Fz	Frizzled
g	gram
g	gravitational force
GAG	Glycosaminoglycan
GBM	Glioblastoma
GBP	Gsk3β binding protein
Gbx2	Gastrulation brain homeobox 2
GCN5	General control nonderepressible 5
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GPI	Glycosylphosphatidylinisotol
GPCR	G-protein coupled receptor
Gsk3β	Glycogen synthase kinase 3β
H&E	Haematoxylin and Eosin
H3/4	Histone 3/4
HAT	Histone acetyltransferase
HCC	Hepatocellular carcinoma

HDAC	Histone deacetylase
HEAT	Huntingtin-EF3-PP2A-TOR1
HEK	Human embryonal kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HLH-LZ	Helix-loop-helix leucine zipper
HMG	High mobility group
HMT	Histone methyltransferase
HNPCC	Hereditary non-polyposis colorectal carcinoma
HRP	Horseradish peroxidase
HSPG	Heparin sulfate proteoglycans
IGL	Internal granule layer
IO	Isthmic organizer
IP3	Inositol 1,4,5-triphosphate
IPTG	isopropyl β-D-1-thiogalactopyranoside
IQ	Intelligence quotient
IRES	Internal ribosome entry site
JNK	Jun kinase
kb	Kilo base
kDa	Kilo Dalton
Krm	Kremen
L	Litre
LATS1	Large tumour suppresor 1
LB	Luria-Bertani
LDL	Low-density lipoprotein
LDLR	LDL receptor
LEF	Lymphoid enhancer factor
loxP	Locus of crossover (x) in P1
LPP	Lipoprotein
LRP	LDLR-related protein
M	Molar
MAP2	Microtubule associated protein-2
MAPK	Mitogen activated protein kinase
MAPKKK	Mitogen activated protein kinase kinase kinase
Mcl-1	Myeloid cell leukaemia-1
MCR	Mutational cluster region
MDCK	Madin Darby canine kidney
µg	microgram
mg	milligram
min	minute
µL	microlitre
mL	millilitre
MLH1/2	MutL homolog 1/2
mM	millimolar
mm	millimetre

µm	micrometer
µM	micromolar
MMP-7	Matrix metalloproteinase-7
MOI	Multiplicity of infection
MOMP	Mitochondrial outer membrane permeabilisation
mRNA	messenger RNA
Nck2	NCK adaptor protein 2
NeuN	Neuronal nuclei
NF200	Neurofilament 200
NFAT	Nuclear factor of activated T cells
NFκB	kappa-light-chain-enhancer of activated B cells
ng	nanogram
NLK	Nemo-like kinase
NLS	Nuclear localisation signal
nm	nanometre
NOL7A	Nucleolar protein 7
NOS	not otherwise specified
NS	Neurospehere
NSC	Neuronal stem cell
OD	Optical density
Otx2	Orthodenticle homeobox 2
P/S	Penicillin/Streptamicin
pA	polyAdenosine
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PCP	Planar cell polarity
PCR	Polymerase chain reaction
PDZ	PSD95-DlgA-Zo1
PI	Phosphatidylinositol
PI3K	Phosphatidylinositol-3-kinase
PIP2	Phosphatidylinositol (4,5) biphosphate
Pk	Prickle
PKC	Protein kinase C
PLAGL1	Pleiomorphic Adenoma gene-like 1
PLC	Phospholipase C
pM	picomolar
PMS2	Postmeiotic segregation increased 2
PNET	Primary neuroectodermal tumour
porc	Porcupine
PP2A	Protein phosphatase 2A
PrP	Prion protein
PSD95	Post synaptic density protein 95
PTCH	Patched
PTEN	Phosphatase and tensin homolog

PTPRK	Protein tyrosine phosphatase receptor K
Rac	Ras-related C3 botulinum toxin substrate
Rb	Retinoblastoma
RGS	Regulator of G protein signalling
Rho	Rhodopsin
RhoA	Ras homolog gene A
RIPA	Radio-immunoprecipitation assay
RMS	Rostral migratory stream
RNA	Ribonucleic acid
Rok	Rho kinase
rpm	revolution per minute
rRNA	ribosomal RNA
RT	Room temperature
RTK	Receptor tyrosine kinase
S37A	Serine37Alanine
SAMP	Serine-Alanine-Methionine-Proline
SASH1	SAM and SH3 domain containing 1
SCF	Skp1-Cullin1-F box protein
SCID	Severe combined immuno-defficiency
SCZ	Sub-callosal zone
SDS	Sodium dodecyl sulphate
sFRP	secreted frizzled related protein
SGZ	Sub-granular zone
shh	sonic hedgehog
SL-IC	SCID leukaemia-initiating cell
sPNET	supratentorial PNET
SPT	Solid-pseudopapillary tumour
Stbm	Strabismus
SVZ	Sub-ventricular zone
Syp	Synaptophysin
TAE	Tris base-acetic acid-EDTA
TBP	TATA-binding protein
TBST	Tris-buffered saline Tween-20
TCF	T-cell factor
TE	Tris-EDTA
TEAD1	TEA domain 1
TEMED	Tetramethylethylenediamine
TGFβ	Tumour necrosis factor β
TIP	TATA-interactive protein
TLE-1	Transducin-like enhancer of split-1
tRNA	transfer RNA
TRRAP	Transformation/transcription domain-associated protein
U	units
V	volt

Vps	Vacuolar sorting protein
Wg	Wingless
WHO	World health organisation
WIF	Wnt inhibitory factor
Wls	Wntless
Wnt	Wg/int
Wts	Warts
WTX	Wilms tumour gene on the chromosome X
YAP1	Yes-associated protein 1
Yki	Yorkie
Zo-1	Zonula occludens-1 protein

1 Introduction

Brain tumours are associated with poor prognosis and a high mortality rate. The current approach to treating these tumours is a combination of surgical resection, radiotherapy and chemotherapy depending on the specific type of tumour. Evidence suggests that a population of cells with stem-like properties exist within tumours, called 'cancer stem cells' (CSCs) (Al Hajj *et al*., 2003; Bonnet and Dick, 1997; Singh *et al*., 2004). As these cells are rare, a small residual population may survive treatment; continue dividing and regenerate the tumour mass. Studies have demonstrated that these cells are resistant to chemotherapy and often have aberrant DNA repair mechanisms making them also resistant to radiotherapy (Bao *et al*., 2006; Woodward *et al*., 2007). Therefore, it is imperative to carefully analyse the molecular and genetic mechanisms underlying the transformation of these cells in order to develop more specific and targeted treatments. Stem cells and 'cancer stem cells' share many common pathways, one of which is the Wnt/β-catenin pathway, important in differentiation, development and tumourigenesis (Haegele *et al*., 2003; Koch *et al*., 1999; Nusse and Varmus, 1982; Thomas and Capecchi, 1990). In this pathway, β-catenin, a nuclear protein, has a key role (Siegfried *et al*., 1994).

To study the role of the Wnt/β-catenin pathway in tumourigenesis of the central nervous system (CNS) we developed a mouse model that inducibly expresses an oncogenic form of β-catenin. More specifically, the well-established Cre-loxP system was used to induce expression in the CNS or in adult neural stem cells to determine the oncogenic potential of activating this pathway.

1.1 **Brain tumours**

A tumour is an abnormal mass of tissue, which proliferates due to increased cell division and/or resistance to apoptosis. These properties, especially resistance to apoptosis, significantly increase the rate by which tumour cells accumulate random mutations. Some of these mutations confer improved growth/survival characteristics to tumour cells, which can result in a more aggressive phenotype (Sidransky *et al*., 1992). Brain tumours can be either primary (arising from brain tissue) or secondary (arising from other organs and spreading to the brain by metastasis or direct invasion). Brain tumours are classified according to the patterns or features they express. The World Health Organisation (WHO) classification of tumours of the CNS (2007) provides morphological and immunohistochemical criteria by which to classify brain tumours and predict their biological behaviour. This WHO classification also grades tumours according to their malignancy in a scale from I to IV.

WHO grade I brain tumours are generally tumours with low proliferation, can be cured by surgical resection and have a low risk of recurrence. Grade II tumours also proliferate slowly, but they often recur after surgical resection, sometimes transforming to higher-grade tumours. Patients with WHO grade II tumours typically survive for more than five years following clinical diagnosis. In contrast, patients with WHO grade III tumours tend to survive for two to three years post-diagnosis. WHO grade IV tumours are the most malignant type and are rapidly fatal if left untreated. The prognosis of patients with WHO grade IV tumours is greatly determined by the availability of effective treatment regimens.

1.1.1 **Medulloblastomas**

Medulloblastomas (WHO grade IV) are the most common malignant brain tumours in childhood, affecting 1 in 200,000 children younger than fifteen years old (Central Brain Tumor Registry of the United States, 1995). Medulloblastomas are largely made up of poorly differentiated cells (Ellison, 2002). There have been significant advances in the treatment of childhood medulloblastomas; in the last five years 60-70 % of patients, compared to 30 % previously, survive for five years (Central Brain Tumor Registry of the United States, 2005). However, most patients suffer from severe long-term cognitive problems (Copeland *et al*., 1999; Mulhern *et al*., 1998; Mulhern *et al*., 1999; Mulhern *et al*., 2001; Palmer *et al*., 2001; Palmer *et al*., 2003; Ris *et al*., 2001). The most common method for assessing these neuro-cognitive problems has been the Intelligence Quotient (IQ) test. Longitudinal and cross-sectional studies on patients receiving treatment for medulloblastomas have consistently shown a significant correlation of IQ deficiency with tumour resection, radiation dose and age of treatment (Mulhern *et al*., 1999; Palmer *et al*., 2001; Palmer *et al*., 2003; Ris *et al*., 2001). Deficits in memory and attention have also been observed (Copeland *et al*., 1999; Mulhern *et al*., 1998; Mulhern *et al*., 2001). Histopathologically, medulloblastomas can be sub-divided into classic medulloblastoma, desmoplastic/nodular medulloblastoma, medulloblastoma with extensive nodularity, anaplastic medulloblastoma and large cell medulloblastoma. These sub-divisions correlate with prognosis and are therefore clinically relevant. Large cell and anaplastic medulloblastomas have significantly poorer prognosis, while medulloblastomas with nodular features tend to have a favourable outcome (Brown *et al*., 2000; Eberhart *et al*., 2002a; Giangaspero *et al*., 1992; Giangaspero *et al*., 2006; Lamont *et al*., 2004; McManamy *et al*., 2003; Rutkowski *et al*., 2005; Sure *et al*., 1995).

The most common genetic event in medulloblastoma is isochromosomy of 17q. This entails loss of 17p and gain of a second copy of 17q, and occurs in up to 40 % of medulloblastoma cases (Bigner *et al*., 1988; Griffin *et al*., 1988). Other common chromosomal losses include 10q (21 %), 8p (15-22 %), 16q (16-17 %) and 11q (11-16 %) (Eberhart *et al*., 2002b; Rickert and Paulus, 2004).

1.1.1.1 **De-regulation of developmental signalling pathways in medulloblastomas**

The over-activation of developmentally important pathways such as Sonic Hedgehog, Notch and Wnt is common in medulloblastoma (Fan *et al*., 2004; Lasser *et al*., 1994; Pomeroy *et al*., 2002). The Sonic Hedgehog (Shh) pathway is activated in a subset of medulloblastomas and the most common event is inactivation of Patched (PTCH) (8 % of medulloblastomas), which results in constitutive activation of the pathway (Pietsch *et al*., 1997; Raffel *et al*., 1997; Vorechovsky *et al*., 1997; Wolter *et al*., 1997; Zurawel *et al*., 2000). Shh is an important mitogen for cerebellar granule cell progenitors, which are thought to be the cells of origin for the majority of medulloblastomas (Wechsler-Reya and Scott, 1999). Activation of the Shh pathway is associated with desmoplastic/nodular medulloblastomas (Pomeroy *et al*., 2002; Schofield *et al*., 1995; Thompson *et al*., 2006).

Adenomatous polyposis coli (APC) protein is a key negative regulator of the Wnt pathway originally identified as a mutant form in familial Adenomatous polyposis (FAP), an inherited colon cancer syndrome (Groden *et al*., 1991; Joslyn *et al*., 1991; Kinzler *et al*., 1991). FAP patients have an increased susceptibility for other types of cancer, one of which is medulloblastoma. Subsequently, mutations of components of the Wnt pathway have been found in approximately 20 % of sporadic medulloblastomas (**Table 1.5**). However, pathway activation has been identified in as many as 25 % of medulloblastomas (Eberhart *et al*., 2000; Ellison *et al*., 2005; Yokota *et al*., 2002). Interestingly, although Wnt/β-catenin pathway activation is normally associated with enhanced proliferation and tumour aggressiveness, pathway activation in medulloblastomas appears to be a marker of favourable prognosis (Bondi *et al*., 2004; Clifford *et al*., 2006; Ellison *et al*., 2005; Kotsinas *et al*., 2002; Nhieu *et al*., 1999; Zechner *et al*., 2003). Studies have shown that 92 % of patients with nucleo-positive β-catenin medulloblastomas have an overall survival of five years in contrast to 65 % percent of patients with non-nuclear (i.e. cytoplasm or membrane-bound) β-catenin medulloblastomas (Clifford *et al*., 2006; Ellison *et al*., 2005). This prolonged survival may be due to the presence of fewer genetic mutations in medulloblastomas with an aberrant Wnt/β-catenin pathway (when compared to other medulloblastomas) (Clifford *et al*., 2006). Genomic alteration analysis of three β-catenin nucleo-positive medulloblastomas using array-comparative genomic hybridization techniques found that these tumours possessed few chromosomal mutations other than the loss of a copy of chromosome 6 (chromosome 6 monosomy) (Clifford *et al*., 2006). In contrast, sixteen β-catenin nucleo-negative medulloblastomas harboured widespread chromosomal abnormalities (Clifford *et al*., 2006).

1.1.2 **CNS Primary neuroectodermal tumours (PNETs)**

Another type of CNS tumours, in which aberrant activation of the Wnt/β-catenin pathway has been identified, is the primitive neuroectodermal tumours of the CNS (CNS PNETs). These are a group of heterogeneous embryonal tumours, which predominantly arise in children (Central Brain Tumor Registry of the United States, 2006). 'CNS PNET, not otherwise specified' (CNS PNET, NOS), also termed supratentorial PNET (sPNET), is a WHO grade IV tumour composed of undifferentiated or poorly differentiated neuroepithelial cells arising from within the brain. The incidence of these tumours is difficult to determine due to their rarity and unclear classification. The percentage of cells undergoing proliferation in sPNET is generally high, reaching up to 85 % within a high-power field of view. Although medulloblastomas are also called infratentorial PNETs, they do not share genetic similarity with sPNETs (Pomeroy *et al*., 2002). SPNETs are scarce, making it hard to determine the way in which these tumours arise. Wnt/β-catenin pathway activation has been observed in these tumours, but its role remains unclear (Koch *et al*., 2001; Rogers *et al*., 2009).

1.1.3 **Turcot's syndrome**

Turcot's syndrome is a rare hereditary disease and presents with CNS tumours and colorectal polyps or colorectal cancer (Hamilton *et al*., 1995; Lasser *et al*., 1994; Mori *et al*., 1994; TURCOT *et al*., 1959). According to the underlying genetic cause, Turcot's syndrome can be divided into two types (Hamilton *et al*., 1995). Type I involves glioblastomas or astrocytomas along with hereditary non-polyposis colorectal carcinoma (HNPCC) and germline mutations in the DNA mismatch repair genes, postmeiotic segregation increased 2 (PMS2), mutL homolog 1 (MLH1) and/or MSH2 (Hamilton *et al*., 1995). Type II is characterised by medulloblastomas and FAP, as a result of APC truncation mutations in the germline (Hamilton *et al*., 1995; Lasser *et al*., 1994; Mori *et al*., 1994).

1.2 **Stem cells & cancer stem cells**

There are two hypotheses that explain how tumours arise and develop: the stochastic model and the hierarchical model [reviewed in (Dick, 2009)] (**Figure 1.1**). The stochastic model predicts that all the cells in a tumour are heterogeneous, but can promote tumour progression due to intrinsic or extrinsic variables [reviewed in (Dick, 2009)]. The hierarchical model predicts that only a small subset of cells in a tumour is capable of re-generating the bulk of the tumour, with the rest of the cells being more differentiated [reviewed in (Dick, 2009)]. The hierarchical model corroborates with the cancer stem cell (CSC) hypothesis, which postulates that tumours are initiated and maintained by a population of cells with similar properties to adult stem cells; termed CSCs (Al Hajj *et al*., 2003; Bonnet and Dick, 1997). The first of these cells to be described was the severe combined immunodeficiency (SCID) leukaemia-initiating cell (SL-IC) in 1997 (Bonnet and Dick, 1997). Similarly, CSCs have been identified in breast and colon cancer and it is widely thought that the same may be true for most, if not all, cancers (Al Hajj *et al*., 2003; O'Brien *et al*., 2007; Ricci-Vitiani *et al*., 2007). Recently, a third model containing elements of both the hierarchical and stochastic models has been suggested [reviewed in (Greaves, 2010)]. This model proposes that CSCs in a tumour may be heterogeneous, with sub-clones becoming more or less dominant than others due to stochastic and seemingly random variations.

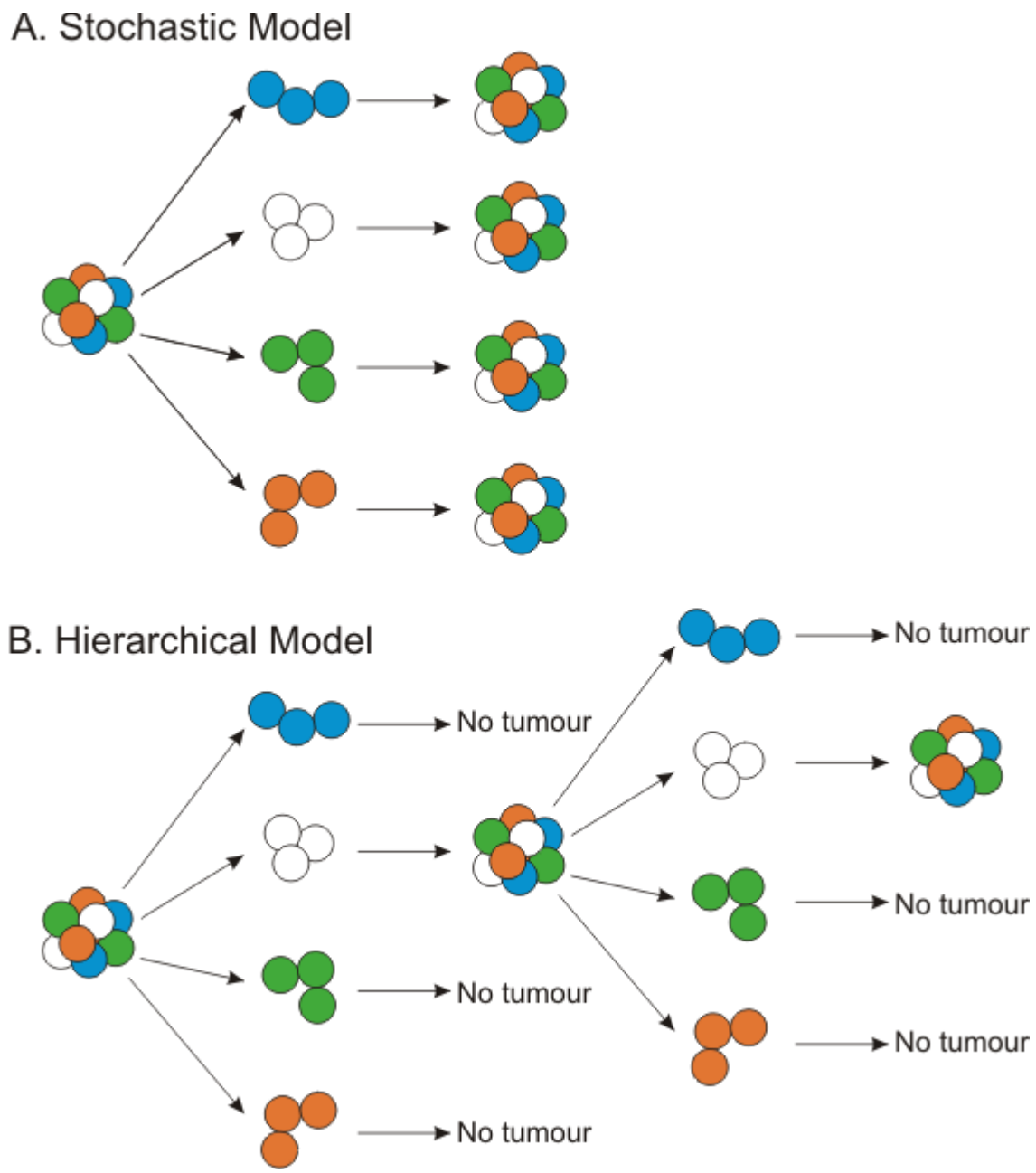


Figure 1.1 Models for tumour development[1]

(**A**) Stochastic model for tumour development. This model predicts that all cells types in a tumour have the potential to be tumourigenic. (**B**) The hierarchical model predicts that only specific cells in a tumour have the capacity to drive tumour progression and re-generate the bulk of the tumour after treatment. The other cell populations in the tumour are made up of more differentiated cells.

[1] Adapted by permission from Macmillan Publishers Ltd: Nature Biotechnology, John E. Dick, Looking ahead in cancer stem cell research, **27**, pp. 44-46, copyright (2009).

Stem cells are defined by two key properties: their ability to differentiate into multiple cell types of the organ they are derived from (multipotency), and their ability to divide symmetrically or asymmetrically to maintain the stem cell pool (self-renewal).

For many years, the adult brain was thought to be capable of proliferation (Altman and Das, 1965; Dacey and Wallace, 1974; MESSIER *et al*., 1958; Reynolds and Weiss, 1992). Since then, this has been convincingly demonstrated in rats, mice, humans and many other species (Eriksson *et al*., 1998; Gould *et al*., 1998; Kirschenbaum *et al*., 1994; Kukekov *et al*., 1999; Lois and Alvarez-Buylla, 1993; Luskin, 1993).

To date, four distinct stem cell niches have been identified in the murine brain. These include (i) the sub-ventricular zone (SVZ) of the lateral ventricle, (ii) the sub-granular zone (SGZ) in the dentate gyrus of the hippocampus, (iii) the sub-callosal zone (SCZ) located between the hippocampus and the corpus callosum, and (iv) a reservoir between the internal granule layer (IGL) and the white matter of the cerebellum (Doetsch *et al*., 1999; Lee *et al*., 2005; Seri *et al*., 2001; Seri *et al*., 2006). The largest of these areas is the SVZ (**Figure 1.2**). Alvarez-Buylla and colleagues identified a subset of cells within this area, which possess characteristics of both stem cells and astrocytes (Doetsch *et al*., 1999). These were termed B-type cells and were shown to regenerate the whole SVZ cell population following *in vitro* ablation (Doetsch *et al*., 1999). B-type cells are capable of slow division and differentiation into transiently amplifying C-type cells, which can rapidly proliferate and differentiate to give rise to large numbers of young neuroblasts (type A cells) (Doetsch *et al*., 1999). Type A cells are restricted to a neuronal fate and migrate along the rostral migratory stream (RMS) until they reach the olfactory bulb where they differentiate into interneurones (Doetsch and Alvarez-Buylla, 1996; Lois *et al*., 1996; Lois and Alvarez-Buylla, 1994).

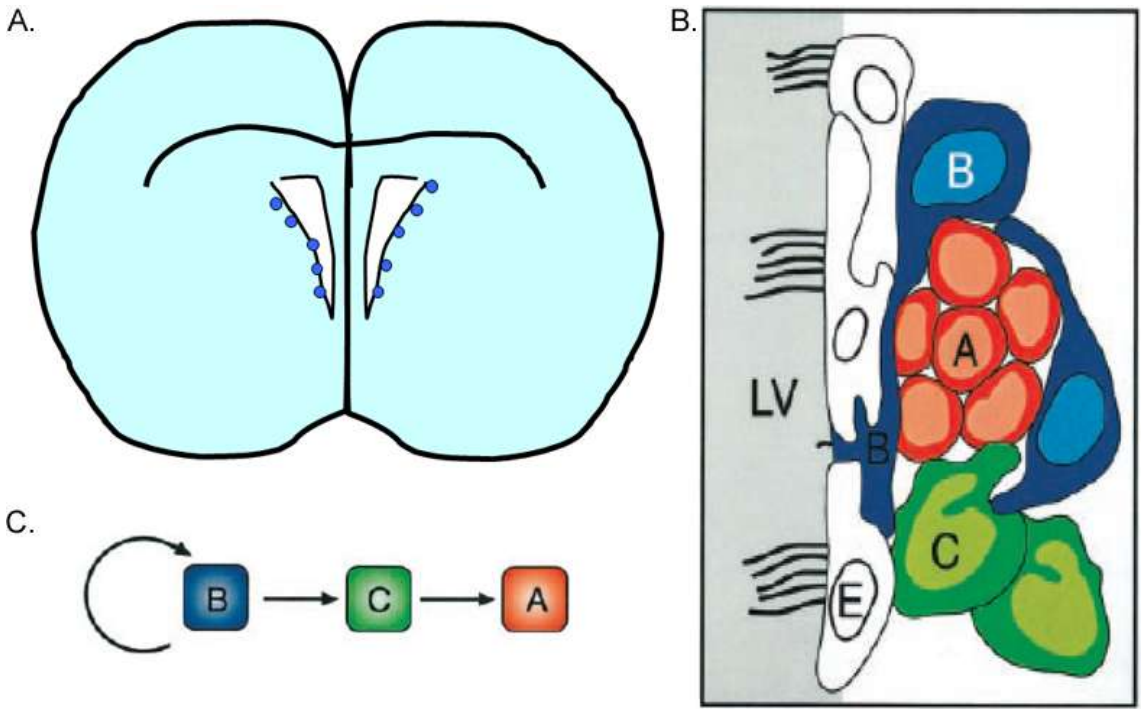


Figure 1.2 Neurogenesis at the sub-ventricular zone of adult mice[2]

(**A**) Diagram of a coronal section of a mouse brain. Neural stem cells (dark blue dots) are located in the sub-ventricular zone (SVZ) in the lateral wall of the lateral ventricle. These cells migrate out of the SVZ as they undergo differentiation. (**B**) Schematic diagram of cellular organisation of the SVZ at the lateral ventricle (LV), which comprises ependymal cells (E), type A neuroblasts (A), astrocytic putative stem cells (B), and transiently amplifying stem cells (C). (**C**) Type B cells undergo asymmetric division to generate a sister Type B cell and a Type C cell. Type C cells divide rapidly and differentiate to give rise to Type A neuroblasts, which migrate to the olfactory bulb to integrate as interneurones.

[2] Adapted by permission of Expert Reviews Ltd: Samantha J. Richardson *et al.*, Hormones and adult neurogenesis in mammals, Expert reviews of Endocrinology & Metabolism, **2**, pp. 261-276, copyright (2007).

Neuronal stem cell (NSC) properties can be investigated *in vitro* by growing them under non-adherent conditions in medium rich in mitogens such as epidermal growth factor (EGF) and fibroblast growth factor β (FGFβ) (Doetsch *et al*., 1999; Doetsch *et al*., 2002; Gritti *et al*., 1996; Gritti *et al*., 1999). Under these conditions most differentiated cells undergo apoptosis, while NSCs grow in response to the growth factors. An interesting property of *in vitro* cultured stem cells is their ability to grow as spherical conglomerates, termed 'neurospheres' (NS). NSCs can be serially passaged and cultured under these conditions confirming their ability to self-renew (Gritti *et al*., 1996). Upon removal of growth factors NSCs differentiate into neurones, astrocytes or oligodendrocytes (Gritti *et al*., 1996; Gritti *et al*., 1999). Cells with stem-like characteristics were discovered in glioblastoma multiforme and medulloblastoma after use of such mitogen-enriched medium (Hemmati *et al*., 2003; Ignatova *et al*., 2002). The study by Ignatova and colleagues showed that these cells form spheres and that they could be serially passaged and differentiated along neuronal and glial lineages (astrocytes, oligodendrocytes) (Ignatova *et al*., 2002). Furthermore, Hemmati *et al*, 2003 were able to show that transplantation of glioblastoma cells, grown *in vitro*, into nude mice reproduced the original tumour (Hemmati *et al*., 2003). Similarly, neurosphere growing cells from these tumours can be isolated and re-grafted to recapitulate glioblastoma, thus showing the *in vivo* self-renewal potential of these CSCs (Galli *et al*., 2004) .

The growth similarities between intrinsic NSCs and CSCs are a good indication that parallel growth, survival and proliferation pathways are active in both types of cells. An important pathway in stem cells proliferation and differentiation is the Wnt signalling pathway, which is the focus of this thesis.

1.3 **Wnt Signalling**

Correct development requires precise coordination between evolutionary conserved signalling pathways [reviewed in (Chopra and Levine, 2009; Raible, 2006)]. One of these pathways is the Wnt signalling pathway, which has been shown to play a critical role in embryonic patterning/cell polarity, differentiation (Funayama *et al*., 1995; Huelsken *et al*., 2000; Lie *et al*., 2005) and is de-regulated in tumours (**Table 1.3** and **Table 1.5**). There are currently 19 Wnt proteins described in mammals (www.stanford.edu/~rnusse/Wntwindow.html). Loss of function of Wnt genes has been shown to cause severe developmental abnormalities (**Table 1.6**). There are at least three pathways attributed to Wnt proteins; the Wnt/planar cell polarity (PCP) pathway (also known as the Wnt/JNK pathway in vertebrates), the Wnt/Calcium (Ca^{2+}) pathway and the canonical Wnt/β-catenin pathway. The focus of this thesis is on the canonical pathway, mainly because of its de-regulation in human cancers (**Table 1.3** and **Table 1.5**). The Wnt/PCP and the Wnt/Ca^{2+} pathway will also be briefly described.

Table 1.1 Gene nomenclature of key Wnt pathway proteins, antagonists and receptors

Values in parentheses are the number of known homologues

Species	Function in Wnt pathway	Nomenclature (gene)		
		Human	Mouse	*Drosophila*
Wnt	Ligand	WNT (19)	Int/Wnt (19)	Wg, DWnt (7)
Porc	Palmitoylation	PORC (4)	Porc (4)	porc
Wls	Wnt secretion	WLS	Wls	wls
Dkk	Wnt antagonist	DKK (4)	Dkk	-
sFRP	Wnt antagonist	SFRP1, 2, 4, 5, FRZB	Sfrp1, 2, 4, 5, FrzB	-
Fz	Receptor	FZD (10), SMO	Fzd (10), smo	Fz, dfz (5)
LRP	Co-receptor	LRP (2)	Lrp (11)	arrow
Ryk	Co-receptor	RYK	Ryk	Derailed
Ror	Co-receptor	ROR (2)	Ror (2)	Ror
Knypek	Co-receptor	GPC4, GPC6	Gpc4, Gpc6	dlp
Krm	Co-receptor	KREMEN (2)	Kremen (2)	-

1.3.1 **Wnt synthesis and secretion**

1.3.1.1 **Wnt Synthesis**

Wnt proteins are excreted as lipid-modified glycoproteins, which contain 23 cysteine residues, an N-terminal signal sequence and some possible N-glycosylation sites (Kurayoshi *et al*., 2007; van Ooyen *et al*., 1985). The highly conserved spacing of the cysteine residues suggests that intra-molecular disulphide bridges may be important for correct Wnt protein folding. Although not evident from the primary sequence, Wnt proteins are lipid modified by palmitoylation at two residues (Takada *et al*., 2006; Willert *et al*., 2003). The first modification discovered was in Cysteine77 on Wnt3A (Willert *et al*., 2003). This residue, and by extrapolation the palmitate group, is conserved in all Wnt proteins. Mutation at this residue greatly impairs the signalling activity of Wnt proteins (Willert *et al*., 2003). Recently, a second lipid modification was identified at Serine209 on Wnt3A (Takada *et al*., 2006). This palmitoylation event is essential for proper intracellular trafficking of Wnt proteins as mutations have been demonstrated to lead to the accumulation of Wnt in the endoplasmic reticulum (ER) (Takada *et al*., 2006). The acyltransferase porcupine (porc) has been suggested to catalyse palmitoylation at this residue, as loss of function of this protein mimics the Serine209 phenotype (Takada *et al*., 2006). Porc encodes an ER multi-pass transmembrane protein from the O-acyltransferase superfamily (Hofmann, 2000; Kadowaki *et al*., 1996). However, it is unclear if it also catalyses Cysteine77 palmitoylation, since any possible phenotype is probably masked by loss of the lipid modification at Serine209.

1.3.1.2 **Wnt Secretion**

Recent studies have identified factors involved in Wnt protein secretion (Bartscherer *et al*., 2006; Coudreuse *et al*., 2006). These factors consist of the retromer complex and the multi-pass transmembrane protein Wntless (WLS). Three independent laboratories have identified a protein called Wntless (Wls), which is required for secretion of Wnt proteins, *in vivo* and *in vitro* (Banziger *et al*., 2006; Bartscherer *et al*., 2006; Goodman *et al*., 2006). Wls is a multi-pass transmembrane protein conserved in *C. elegans* and mammals, which directly interacts with Wnt proteins (Banziger *et al*., 2006; Bartscherer *et al*., 2006; Goodman *et al*., 2006). It has been localised at the plasma membrane, the Golgi and endosomes (Bartscherer *et al*., 2006; Belenkaya *et al*., 2008; Port *et al*., 2008). Whereas the molecular function of Wls remains to be elucidated, it has been proposed to help localisation and secretion of Wnt proteins (Yang *et al*., 2008). Studies using a Wls-green fluorescent protein (GFP) fusion protein have shown that Wls is enriched in the apical membrane of polarised *Drosophila* follicle cells and largely absent at the basolateral membrane (Bartscherer *et al*., 2006). Wingless (Wg), the *Drosophila* Wnt protein, accumulates at the apical side of polarised cells, but is retained in the ER in the absence of Wls (Marois *et al*., 2006; Port *et al*., 2008). This supports the idea that Wls might work as a cargo receptor assisting Wg/Wnt to reach the apical membrane, from where it is released (**Figure 1.3**).

In 2006, the retromer was shown to be able to regulate Wnt proteins (Coudreuse *et al*., 2006; Prasad and Clark, 2006). The retromer is a complex of evolutionary conserved proteins from the Vps family. In yeast, the retromer complex is made up of Vacuolar sorting protein (Vps) 5, Vps17, Vps26, Vps29 and Vps35 and orthologs exist in *C. Elegans*, *Drosophila* and mammals [reviewed in (Seaman, 2005)]. These proteins function to selectively retrieve cargo receptors to the Golgi to rescue them from endosome-mediated degradation (Arighi *et al*., 2004; Seaman, 2004). A key example of this is the recovery of mannose-6-phosphate in mammals (Arighi *et al*., 2004). In the absence of retromer, Wnt protein secretion is abrogated (Coudreuse *et al*., 2006). This phenotype closely resembles loss of Wls, suggesting that the retromer might act by recycling Wls back to the Golgi (Belenkaya *et al*., 2008; Franch-Marro *et al*., 2008; Port *et al*., 2008). This hypothesis is strengthened by assays showing co-immunoprecipitation of Vps35 and Wls and antibody-uptake assays showing recycling of Wls (Belenkaya *et al*., 2008; Franch-Marro *et al*., 2008).

1.3.1.3 **Extra-cellular transport**

Extra-cellular detection of Wg in wing imaginal discs reveals two types of signalling: a steep and narrow gradient at the apical side termed short-range signalling and a broad Wg gradient across the basolateral surface, termed long-range signalling (Panakova *et al*., 2005; Strigini and Cohen, 2000). The latter type is capable of up-regulating genes up to twenty to thirty cells away from the secreting cells (Zecca *et al*., 1996). The way this is achieved is currently unclear as Wnt proteins are hydrophobic and possibly membrane-bound through lipid modifications (Takada *et al*., 2006; Willert *et al*., 2003). Currently, there are two hypotheses to explain how long-range signalling may occur. First, studies have indicated that palmitoylation may allow Wnt proteins to interact with each other to form multimeric complexes (Takada *et al*., 2006; Willert *et al*., 2003). Palmitoylation has been implicated in the extracellular movement of Hedgehog, another lipid-modified secreted morphogen (Chen *et al*., 2004). In this manner Wnt proteins could overcome their inherent hydrophobicity and potentiate their signalling capacity. Second, recent studies in *Drosophila* have suggested that Wnt proteins may be secreted bound to lipoproteins (LPPs), which could act as vehicles facilitating long-range signalling (Panakova *et al*., 2005). LPPs are spherical bodies consisting of a lipid monolayer, apolipoproteins and cholesterol, which contain triacylglycerides in their core [reviewed in (Brown and Goldstein, 1986)]. Once they bind to low density lipoprotein (LDL) receptors on receptor cells, they are endocytosed and their cargo is used by the cell [reviewed in (Brown and Goldstein, 1986)]. In *Drosophila*, Wg has been found to be associated with LPPs in the extracellular space and loss of lipophorin (the *Drosophila* LPP) reduces the range of Wg signalling (Panakova *et al*., 2005). Both Wg and lipophorin localise to endosomes (Chen *et al*., 2004). As such, it is possible that they become loaded into lipophorin and subsequently secreted at the basolateral membrane (Chen *et al*., 2004).

Accumulating evidence suggests that glycosaminoglycan (GAG)-modified proteins might facilitate the extracellular movement of Wnt proteins (Baeg *et al*., 2001; Lin and Perrimon, 1999; Nakato *et al*., 1995; Perrimon *et al*., 1996; Tsuda *et al*., 1999). In *Drosophila*, mutations in two enzymes involved in the synthesis of heparin sulphate proteoglycans (HSPGs), namely sugarless (encodes a UDP-glucose dehydrogenase) and sulfateless (encodes a heparin sulphate N-deacetylase/N-sulfotransferase), result in aberrant Wg signalling (Binari *et al*., 1997; Hacker *et al*., 1997; Haerry and Gehring, 1997; Lin and Perrimon, 1999). HSPGs are extracellular cell membrane and cell surface proteins with attached heparin sulphate GAGs [reviewed in (Bernfield *et al*., 1999)]. Various genetic screens have shown that two HSPGs, Dally (division abnormally delayed) and Dally-like (Dly) appear to be required for Wnt signalling (Baeg *et al*., 2001; Tsuda *et al*., 1999). This became

apparent when a study using dsRNA to knock-down the expression of these proteins recapitulated the phenotype seen when Wg is lost (Baeg *et al*., 2001; Tsuda *et al*., 1999). Dally and Dly belong to the glypican family of cell surface HSPGs, and are linked to the plasma membrane *via* a glycosylphosphatidylinisotol (GPI) anchor (Tsuda *et al*., 1999). The exact method by which these receptors mediate Wnt protein signalling is not understood. It has been hypothesised that they may interact with secreted Wnt proteins to sequester them at the membrane and promote binding to Wnt receptors (Cadigan *et al*., 1998). Alternatively, interaction between Wnt proteins and Dally and Dly may be required for endocytosis and recycling of Wnt proteins. Finally, it may be that GPI-linked Dally and Dly stabilise Wnt proteins as they are transported in LPPs. Experimental evidence for this later hypothesis came from GFP studies in LPPs of *Drosophila* wing imaginal disc cells, which were found to express GPI-linked GFP (Greco *et al*., 2001).

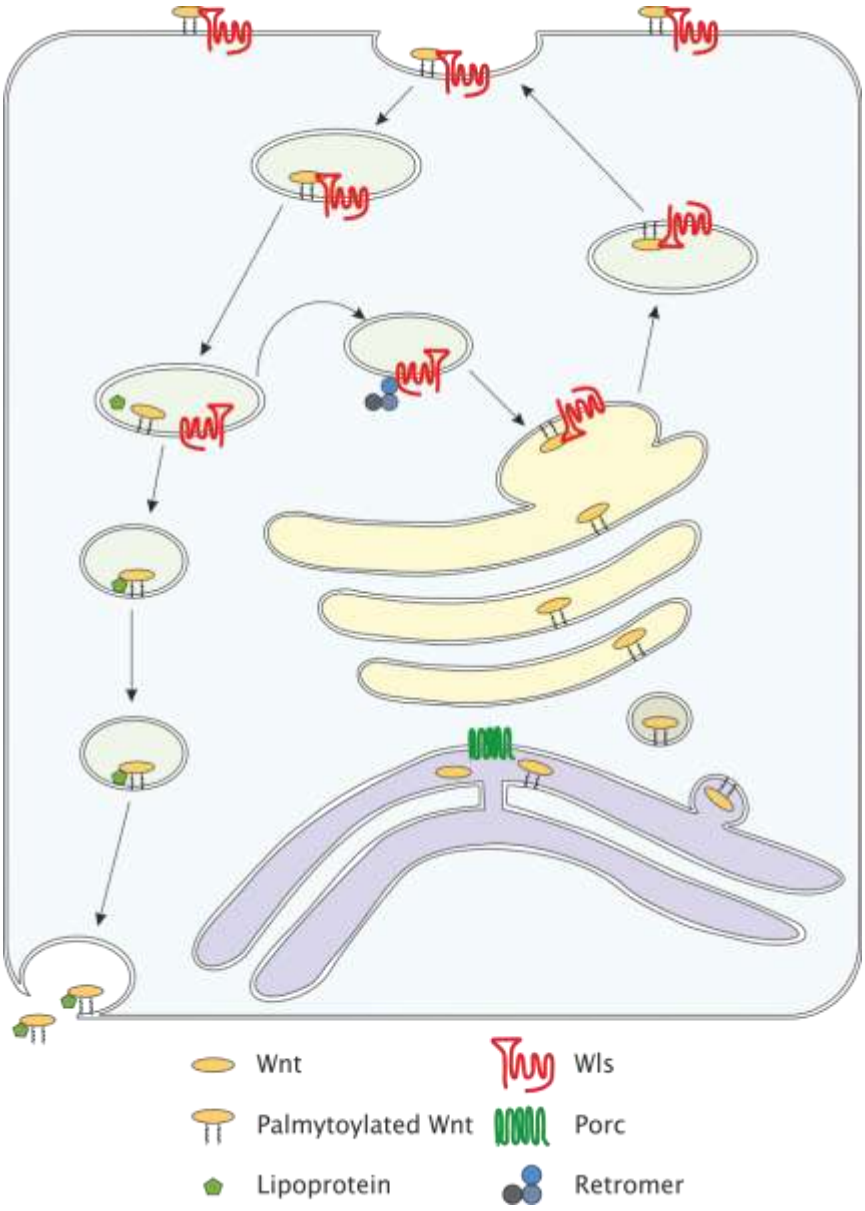


Figure 1.3 A possible model for Wg synthesis and secretion[3]

Wingless (Wg) is palmytoylated by Porcupine (Porc) in the endoplasmic reticulum. Subsequently, Wg is transported through the Golgi apparatus and secreted to the apical side in a complex with Wntless (Wls). At the apical side, Wg can mediate short range signalling. For basolateral secretion, Wg is endocytosed alongside Wls. In the endosome, Wls dissociates from Wg and is recycled to the Golgi apparatus by the retromer complex. Wg complexes with lipoproteins and is secreted at the basolateral side, where it can mediate long-range signalling.

[3] Adapted by permission of Macmillan Publishers Ltd: EMBO Reports, Kerstin Bartscherer & Michael Boutros, Regulation of Wnt protein secretion and its role in gradient formation, **9**, pp. 977-982, copyright (2008).

1.3.2 **Receptors**

There are two main types of receptors required for Wnt protein signal transduction: Frizzled receptors (Fz) and LDL receptor (LDLR)-related receptor proteins (LRPs) (Wehrli *et al*., 2000; Yang-Snyder *et al*., 1996). Fz are seven-pass transmembrane receptors, which are a common component of all three Wnt pathways. All Fz proteins share a highly glycosylated N-terminus containing ten invariantly spaced cysteines which is called the cysteine-rich domain (CRD) (Wang *et al*., 1996). Additionally, Fz proteins contain a conserved seven-pass transmembrane domain and an intracellular domain with little homology between family members (Wang *et al*., 1996) (**Figure 1.4**). Wnt proteins interact with Fz receptors *via* their CRD domain (He *et al*., 1997; Yang-Snyder *et al*., 1996). This interaction has been successfully characterised in *Drosophila* where Wg binds to *Drosophila* Fz1 (DFz1) and DFz2 with an affinity coefficient of 10^{-8}M and 10^{-9}M respectively (Bhanot *et al*., 1996; Rulifson *et al*., 2000). The precise mechanism by which Wnt protein signals are transduced is not known, but evidence suggests that heterotrimeric G proteins are involved (Liu *et al*., 2001; Slusarski *et al*., 1997a). This leads to the activation of either of the known Wnt signalling pathways (Wnt/PCP, Wnt/Ca^{2+}, Wnt/β-catenin). This is an inherently complicated process, not only because of the large diversity of Wnts (19 in mammals) and Fzs (10 in humans and mice), but also because of difficulties encountered in attempts to purify Wnt proteins (Willert *et al*., 2003). Furthermore, some Wnt proteins can induce distinct Wnt pathways by interacting with different Wnt receptors. For example, Wnt5a can induce the Wnt/β-catenin pathway when it interacts with Fz5 or Fz4 (He *et al*., 1997; Mikels and Nusse, 2006). On the contrary, when the same protein interacts with Ror2, an alternative Wnt receptor, it is capable of inducing the Wnt/PCP pathway (Mikels and Nusse, 2006). Therefore, the action of Wnt proteins depends on the various Wnt protein receptors expressed by cells or tissues.

Whilst Wnt/Fz interaction is sufficient for inducing non-canonical Wnt pathways, activation of the Wnt/β-catenin pathway also requires the involvement or LRPs (Semenov *et al*., 2001; Wehrli *et al*., 2000). In *Drosophila*, the co-receptor is named Arrow (Wehrli *et al*., 2000) and in vertebrates two LRP proteins have been identified; LRP5 and LRP6 (Pinson *et al*., 2000; Tamai *et al*., 2000). *Drosophila* mutants lacking Arrow reproduced Wg loss whereas LRP6 knockout mice resembled Wnt protein knockout defects (e.g. mid and hindbrain defects, ventralization of limbs etc.) (Pinson *et al*., 2000). Furthermore, truncated LRP5/6 lacking the extracellular domain, but still anchored to membrane, where shown to constitutively activate the canonical pathway (Brennan *et al*., 2004; Mao *et al*., 2001a; Mao *et al*., 2001b). Whilst the precise method of signal transduction is not known, it is thought that trimerisation of Wnt, Fz and LRP may be required (Schweizer and Varmus, 2003).

In addition to Fz and LRP two more types of potential transducers of Wnt signalling have been discovered. The first of these is Ror2, a single-pass transmembrane protein of the receptor tyrosine kinase (RTK) (Oishi *et al*., 2003). This receptor interacts with Wnt5a to activate the Wnt/JNK pathway (Mikels and Nusse, 2006; Oishi *et al*., 2003). Ror2 deficient mice exhibit skeletal, genital and cardiovascular abnormalities that have been attributed to aberrant convergent extension during gastrulation, a process mediated by the Wnt/JNK pathway in vertebrates (Oishi *et al*., 2003). The second type of receptor is Ryk and its *Drosophila* (Derailed, Drl) and *C. Elegans* (Lin-18) homologues (Hovens *et al*., 1992; Inoue *et al*., 2004; Yoshikawa *et al*., 2003). Studies in human embryonic kidney cells (HEK293) have shown that Ryk induces the Wnt/β-catenin pathway as a co-receptor with Fz and Wnt proteins (Lu *et al*., 2004). Functionally, Ryk appears to be required for neurite outgrowth, induced by Wnt3a and Wnt1, and for corticospinal tract axon repulsion, induced by Wnt1 and Wnt5a (Liu *et al*., 2005; Lu *et al*., 2004). Interestingly, Ryk contains an extracellular domain similar to the secreted Wnt inhibitor factor-1 (WIF), which is thought to mediate Ryk/Wnt interaction (Patthy, 2000).

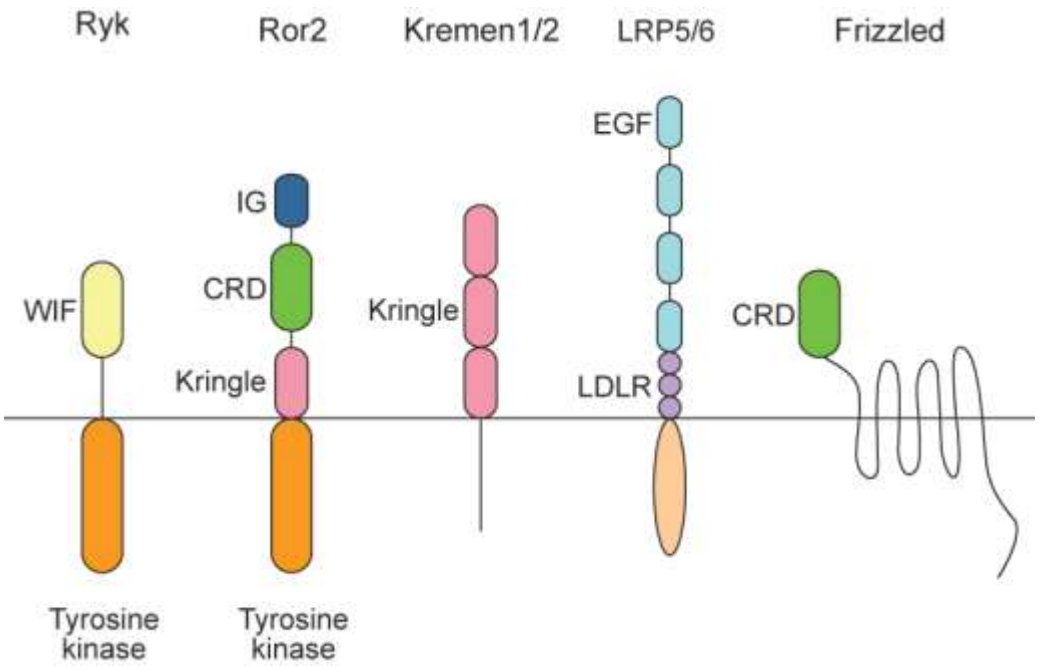


Figure 1.4 Domains of the receptors of the Wnt signalling pathway (not to scale)

Ryk contains an intracellular tyrosine kinase and an extracellular Wnt inhibitory factor (WIF) domain. Ror2 contains an intracellular tyrosine kinase and an extracellular Kringle domain, cysteine-rich domain (CRD) and an immunoglobulin (IG) domain. Kremen1/2 contains three extracellular kringle domains. LRP5/6 contains three low-density lipopropretin receptor (LDLR) domains and four epidermal growth factor-like (EGF) domains. Frizzled contains an extracellular CRD domain.

1.3.3 **Extracellular Antagonists of the Wnt pathway**

Binding of Wnt proteins to receptors is modulated by endogenously-secreted antagonists (Bafico *et al*., 1999; Glinka *et al*., 1998) (**Figure 1.5**). These antagonists can be divided into two functional classes: secreted frizzled related proteins (sFRPs) and Dickkopf (DKK) proteins (Bafico *et al*., 1999; Semenov *et al*., 2001). Members of the sFRP group include the sFRP family, WIF-1 and Cerberus. There are currently eight known sFRP family members, namely sFRP1 to 5, Sizzled, Sizzled2 and Crescent. These can be further sub-divided based on sequence homology: sFRP1, sFRP2 and sFRP5 form one group, and sFRP3 and sFRP4 form the second group. SFRP1 to 5 have not been identified in invertebrates, and Sizzled, Sizzled2 and Crescent, which make up the final subgroup, have not been identified in mammals. Structurally, sFRPs show homology with the Fz CRD domain at the N-terminus and with Netrin, an axonal guidance protein, at the C-terminus (Banyai and Patthy, 1999; Melkonyan *et al*., 1997). The antagonistic effect of sFRPs is thought to arise from the interaction of their CRD-like domain with Wnt proteins (Lin *et al*., 1997). However, sFRP1 has also been found to interact with Fz (Bafico *et al*., 1999). Thus, sFRPs might modulate Wnt signalling by physically interacting with Wnt proteins or by preventing Wnt proteins from binding to Fz.

However, sFRPs do not always act as antagonists (Melkonyan *et al*., 1997; Uren *et al*., 2000). *In vitro* experiments with Madin Darby canine kidney (MDCK) cells demonstrated that sFRP1 could potentiate Wg signalling at low concentrations (Uren *et al*., 2000). This potentiation was observed with truncated sFRP1 lacking the CRD domain, suggesting that the Netrin-like domain (NTR) may serve as a high affinity binding site for Wg (Uren *et al*., 2000). An alternative hypothesis is that at high concentrations sFRP1 could interact with Frizzled, preventing Frizzled/Wg interaction. Indeed, sFRP1 was found to interact with human Fz6 (Bafico *et al*., 1999). The obvious caveat with these assays is that they involve *Drosophila* Wg rather than mammalian Wnt. Although there is a high degree of conservation between Wg and Wnt proteins, it is possible that they interact differently with sFRPs. Similarly to sFRP1, sFRP2 can also function both as an antagonist and as an agonist (Ladher *et al*., 2000; Melkonyan *et al*., 1997). Injection of sFRP2 into *Xenopus* embryos blocks the dorsalising activity of Wnt-8 (Ladher *et al*., 2000). However, sFRP2 has been shown to block the antagonistic activity of sFRP1 when co-expressed in the metanephric kidney (Yoshino *et al*., 2001).

Although sFRPs have been postulated to be tumour suppressor genes, no genetic mutations have been discovered to date. Nonetheless, sFRP1 is found to be downregulated in cervical, kidney, breast and ovary carcinoma (Ko *et al*., 2002; Ugolini *et al*., 2001; Zhou *et al*.,

1998). Furthermore, chromosome 8p21, where sFRP1 is located, is frequently lost in human cancers (Wright *et al*., 1998). Lastly, sFRP promoters have been shown to be commonly hypermethylated in human cancers (**Table 1.4** and **Table 1.5**).

The other group of antagonists, the Dickkopf (DKK) family, comprises five members: DKK1, DKK2, DKK3, DKK4, and a DKK3 related protein called Soggy. The most extensively studied member of this family is DKK1. Studies with Dkk1 knockout mice and experiments using injection of Dkk1 messenger RNA (mRNA) into *Xenopus* embryos have demonstrated a lack of anterior head structures, highlighting the importance of Dkk1 in head formation (Glinka *et al*., 1998). In a similar fashion to sFRP2 and sFRP3, DKK1 can also block *Xenopus* Wnt-8 effects (XWnt8) (Glinka *et al*., 1998). Unlike sFRPs, DKK1 does not inhibit activation of the Wnt signalling pathway by binding to Wnt proteins; it binds to LRP5/6 instead (Bafico *et al*., 2001; Semenov *et al*., 2001). DKK1 mediates the internalisation of LRP5/6 by forming a ternary complex with the single-pass transmembrane protein Kremen1 (Krm1) and Krm2 (Mao *et al*., 2002). As mentioned in **section 1.3.2**, LRP5 and LRP6 are solely required for the activation of the canonical Wnt/β-catenin pathway. This is exemplified by the inability of DKK1 and degradation-resistant LRP6 to inhibit the Wnt/PCP pathway (Semenov *et al*., 2001). Moreover, there is also evidence to suggest that DKK1 may be able to activate the Wnt/PCP pathway (Caneparo *et al*., 2007).

Whereas DKK4 functions in the same manner as DKK1, DKK3 and Soggy have no effect on the Wnt signaling pathway (Krupnik *et al*., 1999; Mao and Niehrs, 2003). DKK2 appears to have both antagonistic and agonistic effects on Wnt signaling. DKK2 can bind to LRP6 and Krm2 to antagonise Wnt1 and XWnt8 binding (Brott and Sokol, 2002; Mao *et al*., 2002; Wu *et al*., 2000). However, ectopic expression of DKK2 in *Xenopus* embryos has shown that it synergises with LRP6 to potentiate axis duplication, an effect of Wnt/β-catenin pathway activation (Brott and Sokol, 2002). This synergism was shown to be prevented by co-expression of Krm2, and therefore suggests that the relative levels of LRP5/6 and Krm2 dictate whether DKK2 functions as an agonist or antagonist (Mao and Niehrs, 2003).

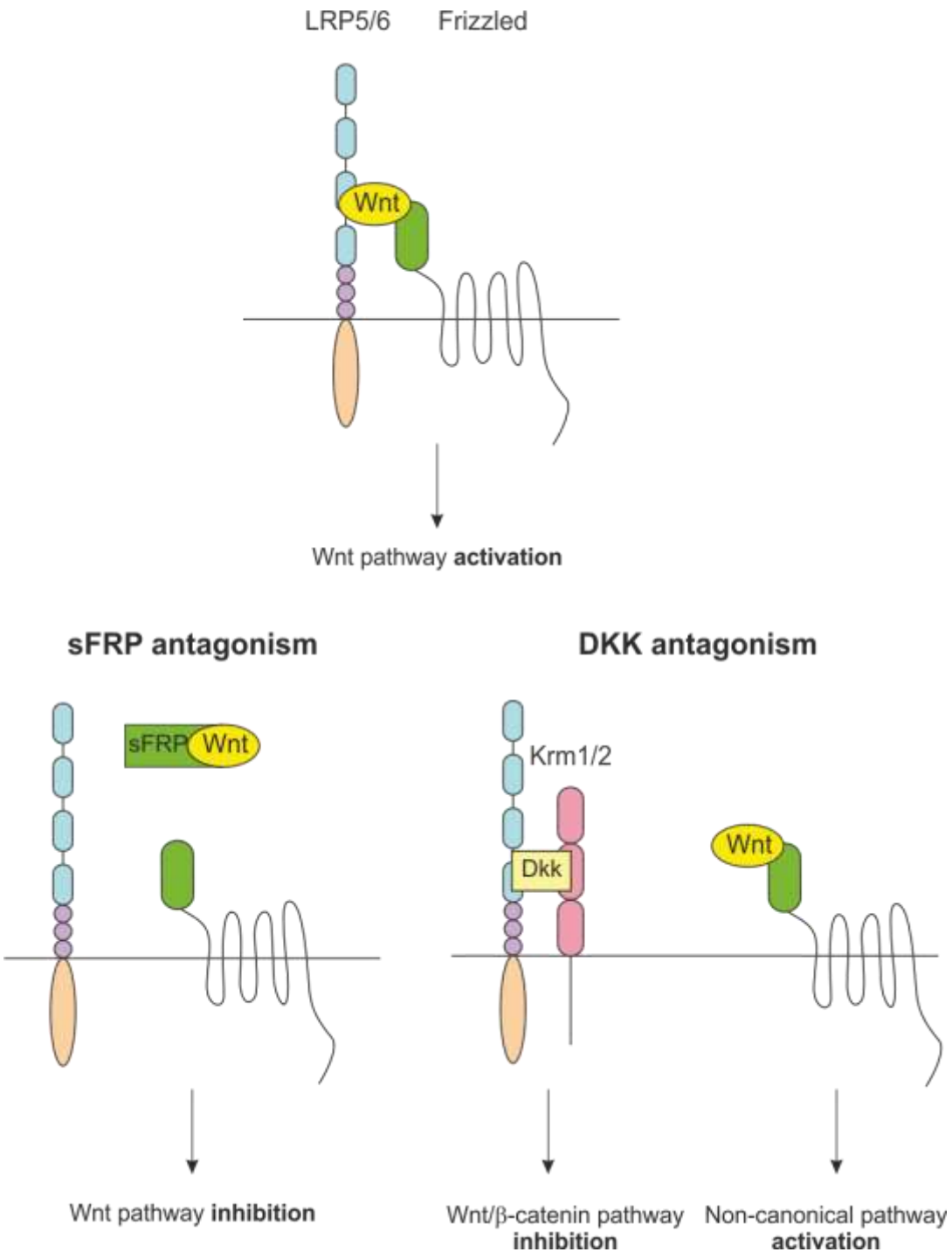


Figure 1.5 Mechanism for Wnt protein antagonism

Wnt proteins interact with Frizzled and Frizzled/LRP to activate the Wnt pathway. Secreted frizzled related proteins (sFRPs) inhibit the Wnt pathway by sequestering Wnt proteins. On the other hand Dickkopf (DKK) proteins specifically inhibit the Wnt/β-catenin pathway by promoting the internalisation of LRP5/6.

1.3.4 **The Wnt/PCP or Wnt/JNK pathway**

Cells in tissues are highly coordinated both in terms of their arrangement and function. A key feature of this coordination is polarisation. The most common form of polarisation is the apical/basal polarisation of epithelial cells [reviewed in (Simons and Fuller, 1985)]. Nevertheless, polarisation has also been found to be essential for wing hair and eye photoreceptor orientation in *Drosophila*, mitotic spindle orientation in invertebrates, hair orientation, neural tube closure, convergent extension in mammals and ciliogenesis (De Calisto *et al*., 2005; Gubb and Garcia-Bellido, 1982; Park *et al*., 2006; Veeman *et al*., 2003; Zheng *et al*., 1995). This polarisation is controlled by the Wnt/Planar cell polarity (PCP) pathway in *Drosophila*, which in vertebrates is called the Wnt/JNK pathway (**Figure 1.6**).

PCP was first recognised in *Oncopeltus fasciatus* and studies in *Drosophila* helped to elucidate many of the components of the pathway (Lawrence, 1966). The two most studied PCP events in *Drosophila* are wing hair orientation and the polarity of photoreceptors in ommatidia (the units that make up the compound eyes). In the wing, planar cell polarity is dependent on the arrangement of six proteins, Fz, Dishevelled (Dsh), Flamingo (Fmi), Diego (Dgo), Strabismus (Stbm) and Prickle (Pk) (Chae *et al*., 1999; Klingensmith *et al*., 1994; Taylor *et al*., 1998; Vinson *et al*., 1989). These localise proximally or distally in the cells to coordinate actin microfilaments (Chae *et al*., 1999; Klingensmith *et al*., 1994; Taylor *et al*., 1998; Vinson *et al*., 1989). In vertebrates, activation is achieved by Wnt11 orWnt5A binding to Fz receptor (Heisenberg *et al*., 2000; Moon *et al*., 1993). The intracellular domain of Fz interacts with Dsh to activate the Rhodopsin (Rho) GTPases ras homolog gene A (RhoA), ras-related C3 botulinum toxin substrate (Rac) and cell division cycle 42 (Cdc42) (Fukukawa *et al*., 2009; Tanegashima *et al*., 2008). These GTPases regulate the activity of Rho kinase (Rok), which activates the mitogen activated protein kinase (MAPK) cascade culminating in the activation of Jun kinase (JNK) (Fukukawa *et al*., 2009; Winter *et al*., 2001). JNK in turn phophorylates c-Jun, which activates gene transcription (Pulverer *et al*., 1991; Smeal *et al*., 1991). The process by which c-Jun promotes cell polarity is not yet fully understood.

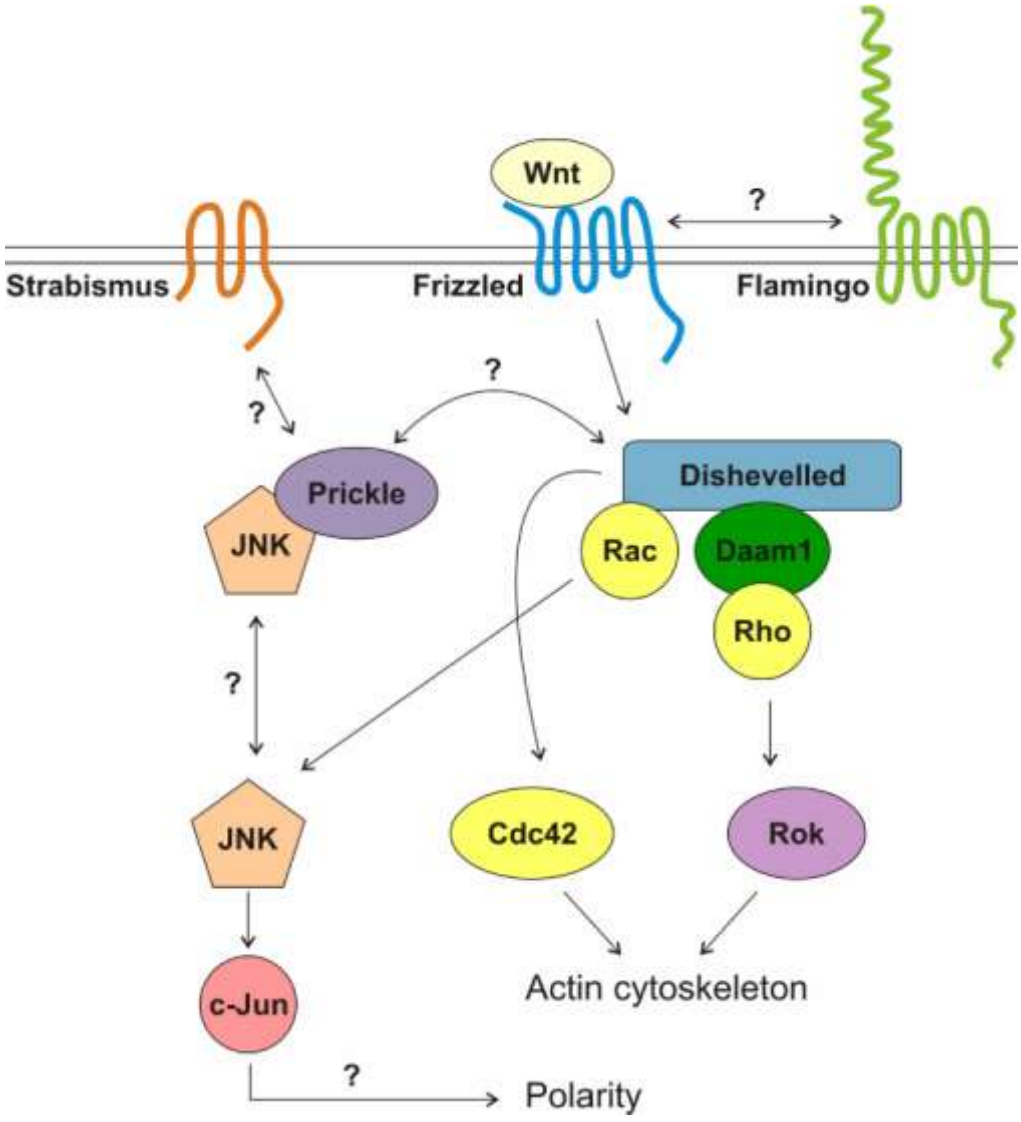


Figure 1.6 Schematic representation of the Wnt/PCP pathway in *Drosophila*[4]

Induction of the Wnt/Planar cell polarity pathway (Wnt/PCP) activates dishevelled (Dsh in *Drosophila*). Dsh in turn mediates the activation of ras-related C3 botulinum toxin substrate (Rac), cell division cycle 42 (Cdc42) and Rhodopsin (Rho). Cdc42 and Rho kinase mediate actin cytoskeleton remodelling. On the other hand, Rac promotes the activation of Jun Kinase (JNK). JNK phosphorylates c-jun and activates it. Activated c-jun promotes the expression of genes required for cell polarity. Prickle, Strabismus and Flamingo localise to the proximal or distal side of the cell, where they help organise PCP. However, the precise mechanism remains to be elucidated.

[4] Adapted from Seminars in Cell and Developmental Biology, **20**, Roszko *et al*., Regulation of convergence and extension movements during vertebrate gastrulation by the Wnt/PCP pathway, pp. 986, 997, copyright (2009), with permission from Elsevier.

1.3.5 **The Wnt/Ca^{2+} pathway**

The latest pathway to be linked to Wnt proteins is the Wnt/Ca^{2+} pathway (**Figure 1.7**). Wnt proteins were first linked to calcium signalling ten years ago, following the injection of *Xenopus* Wnt5A and/or rat Fz2 mRNA into early Zebrafish embryos, which resulted in intracellular Ca^{2+} release in a manner dependent on G-protein coupled receptors (GPCRs) and the phosphatydilinositol (PI) cycle (Ma and Wang, 2006; Slusarski *et al*., 1997a; Slusarski *et al*., 1997b). Furthermore, the expression of Pk, Wnt4, Wnt5 and Wnt11 has also been shown to stimulate Ca^{2+} release in Zebrafish embryos (Veeman *et al*., 2003; Westfall *et al*., 2003). One of the main classes of receptors that activate the PI cycle are G-protein coupled receptors (GPCRs) (Ma and Wang, 2006; Slusarski *et al*., 1997a; Slusarski *et al*., 1997b). These receptors activate PI-specific phospholipase C (PLC) that cleaves membrane-bound phosphatidylinositol (4,5) biphosphate (PIP2) into membrane-bound diacylglycerol (DAG) and cytoplasmic inositol 1,4,5-triphosphate (IP3) [reviewed in (Berridge *et al*., 2000)]. IP3 subsequently binds to Ca^{2+} channels in the ER, thereby triggering the rapid release of Ca^{2+} into the cytoplasm [reviewed in (Berridge *et al*., 2000)]. Intracellular Ca^{2+} ions are rapidly taken up by proteins, which either act as Ca^{2+} buffers or require Ca^{2+} for activation. Two notable enzymes activated by Ca^{2+} ions are calmodulin and protein kinase C (PKC) (Haiech *et al*., 1981; Oancea and Meyer, 1998; Sakai *et al*., 1997). Calmodulin activates protein kinases like Ca^{2+}/calmodulin-dependent kinases (CaMK), phosphatases, ion transporters and cytoskeletal proteins (Hoeflich and Ikura, 2002). A vital phosphatase activated in this manner is calcineurin, which activates the transcription factor nuclear factor of activated T cells (NFAT) (Ruff and Leach, 1995). Wnt-induced release of intracellular Ca^{2+} has since been shown to activate PKC, Calmodulin/CaMKII, and Calcineurin/NFAT, suggesting that Wnt proteins can activate the PI cycle (Kuhl *et al*., 2000; Saneyoshi *et al*., 2002; Sheldahl *et al*., 1999). Interestingly, the Wnt/Ca^{2+} pathway has been linked with Nemo-like kinase (NLK) activation, which can antagonise the Wnt/β-catenin pathway (Ishitani *et al*., 2003). Finally, the Wnt/Ca^{2+} pathway has also been shown to be important for the patterning of embryos, regulation of the Wnt/β-catenin pathway and tumourigenesis (Weeraratna *et al*., 2002).

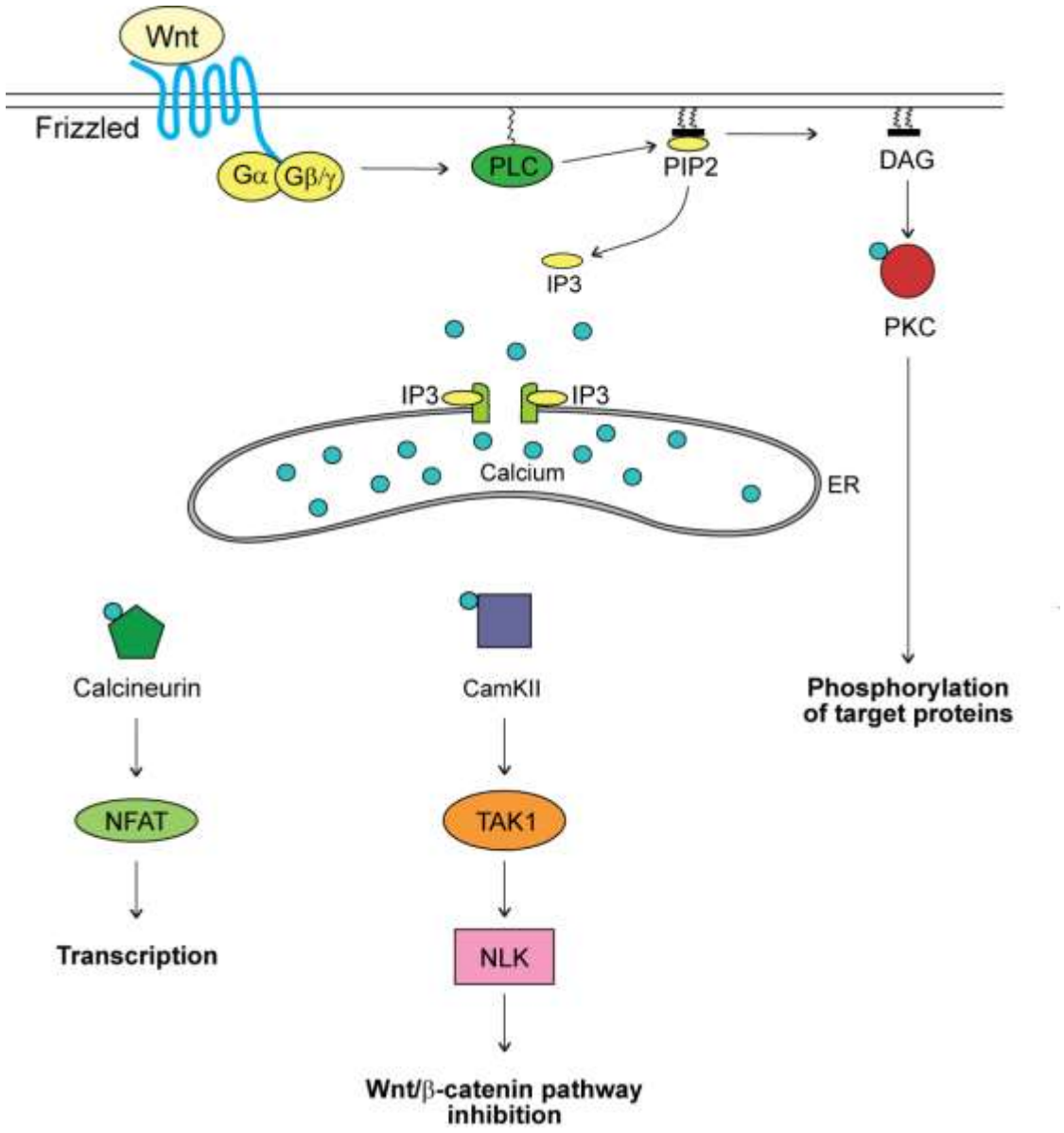
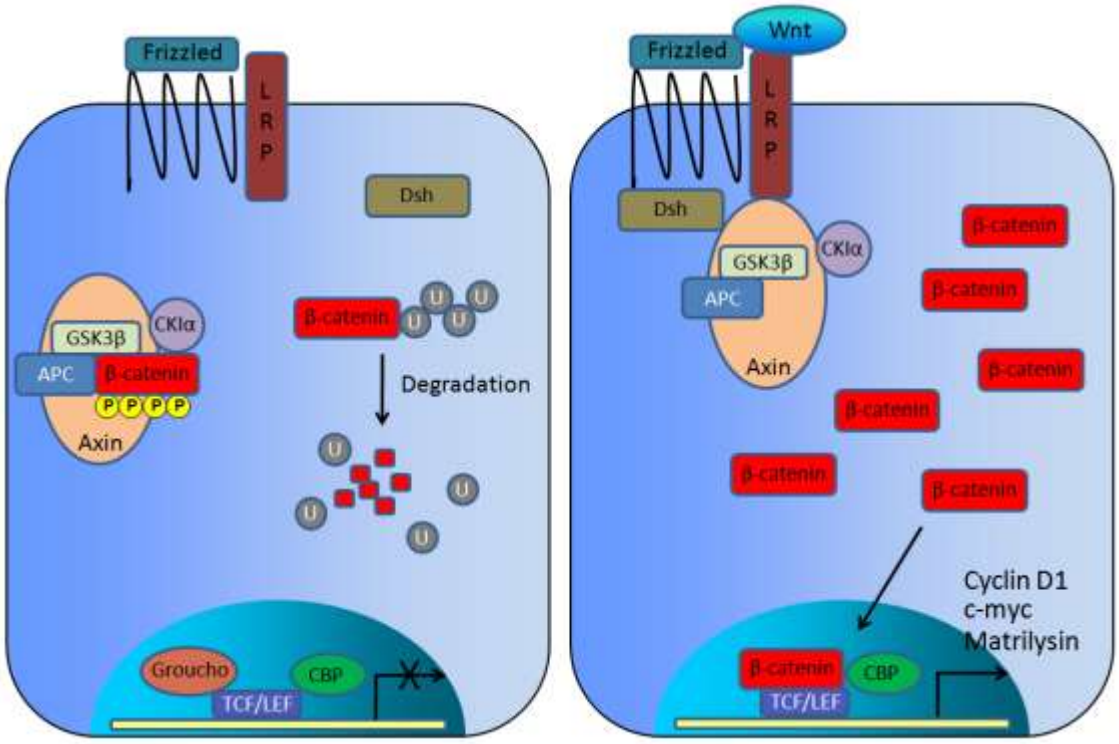


Figure 1.7 Wnt/Calcium pathway

Activation of the Wnt/Calcium pathway promotes the activation of phospholipase C, which cleaves membrane-bound phosphatidylinositol (4,5) biphosphate (PIP2) into membrane-bound diacylglycerol (DAG) and cytoplasmic inositol 1,4,5-triphosphate (IP3). IP3 binds to Calcium channels in the endoplasmic reticulum (ER), which promotes the release of Calcium ions into the cytoplasm. Three proteins activated by calcium ions are Calcineurin, Ca^{2+}/calmodulin-dependent kinases (CamKII) and Protein kinase C (PCK). PCK additionally can be activated by DAG. Calcineurin promotes transcription, whilst PCK promotes the phosphorylation of target genes. CamKII phosphorylates and activates TGFβ activated kinase 1 (TAK1). TAK1 in turn phosphorylates nemo-like kinase (NLK), which translocates into the nucleus and inhibits β-catenin-dependent gene transcription.

1.3.6 **The canonical Wnt/β-catenin pathway**

As the name implies, the key component of the Wnt/β-catenin pathway is β-catenin. β-catenin is a 780 amino acid (aa) protein, which was originally identified as a component of E-cadherin junctions, where it links E-cadherin with α-catenin and consequently to the actin cytoskeleton (Aberle *et al*., 1996; Redies and Takeichi, 1996; Takeichi, 1991). However, β-catenin was subsequently shown to be a component of the Wnt signalling pathway (Funayama *et al*., 1995). As shown in **Figure 1.8**, in the absence of Wnt proteins (OFF state), cytoplasmic β-catenin is efficiently degraded *via* phosphorylation-dependent ubiquitinylation (Hart *et al*., 1999; Marikawa and Elinson, 1998). However, in the presence of Wnt proteins (ON state) this phosphorylation, and thus degradation, is abolished (Liu *et al*., 2002). Stable cytoplasmic β-catenin translocates into the nucleus and interacts with T-cell factor/Lymphoid enhancer factor (TCF/LEF) to promote the expression of over thirty genes; including oncogenes such as the cell cycle regulator cyclin D1, the transcriptional factor c-myc and the extracellular cell matrix (ECM)-degrading enzyme matrilysin (Crawford *et al*., 1999; He *et al*., 1998; Shtutman *et al*., 1999).



OFF State ON State

Figure 1.8 Schematic representation of the Wnt/β-catenin pathway signalling cascade

In the absence of Wnt proteins, cytoplasmic β-catenin is phosphorylated by CKIα and GSK3β and targeted for degradation by ubiquitination. The interaction of Wnt proteins with Frizzled/LRP stabilises β-catenin by disrupting the 'destruction complex' (Dvl, Axin, APC, CKIα and GSK3β). Stable cytoplasmic β-catenin translocates into the nucleus and activates gene expression by interacting with TCF/LEF.

Table 1.2 Nomenclature for genes involved in the Wnt/β-catenin pathway

Numbers in parentheses represent the number of known homologues

Species	Function in Wnt pathway	Nomenclature (gene)		
		Human	Mouse	*Drosophila*
Ck1ε	Dvl phosphorylation	CKIε	Ck1ε	dco
Dvl	Disrupts the 'destruction complex'	DVL (3)	Dvl (3)	dsh
Axin	Scaffold	AXIN (2)	Axin (2)	daxin
Gsk3β	Phosphorylation	GSK3B	Gsk3β	sgg
Ck1α	Phosphorylation	CKIα	Ck1α	ck1α
Apc	Scaffold	APC, APC2/ APCL	Apc (2)	dapc, E-apc
β-catenin	Transcriptional factor	CTNNB1	Ctnnb1	armadillo
β-TrCP	Ubiquitination	β-TrcP (2)	β-TrcP (2)	slimb
GBP	GSK3β antagonist	FRAT (2)	Frat (2)	-
TCF/LEF	Transcription factor	TCF, LEF (4)	Tcf/Lef (4)	pangolin
Groucho	Transcription repressor	TLE1, 2, 3, 4, 6	Tle1, 2, 3, 4, 6	groucho
CtBP	Transcription repressor	CTBP (2)	Ctbp (2)	CtBP
CBP	Transcription repressor/activator	CREBBP	Crebbp	CBP
Legless	Transcription factor	BCL9	Bcl9	legless
Pygopus	Transcription factor	PYGO (2)	Pygo (2)	pygopus
TBP	Transcription factor	TBP	Tbp	tbp
TIP48	Transcription factor	RUVBL2/TIP48	Ruvbl2/Tip48	rept
TIP49	Transcription factor	RUVBL1/TIP49	Ruvbl1/Tip49	pont

1.3.6.1 **OFF state: Absence of Wnt signals**

In the absence of Wnt proteins, β-catenin is phosphorylated and targeted for degradation by a complex of proteins designated the 'destruction complex'; APC, Axin, Glycogen synthase kinase 3β (Gsk3β), Casein kinase I α (Ck1α) and Protein phosphatase 2A (PP2A) (**Figure 1.9**).

1.3.6.1.1 ***APC***

APC is a 2843 amino-acid, 312 kDa multifunctional protein that was originally discovered as a truncated mutant in FAP (**section 1.1**). The role of APC in the Wnt/β-catenin pathway is to stabilise the 'destruction complex' to promote β-catenin phosphorylation (Behrens *et al*., 1998; Rubinfeld *et al*., 1993; Su *et al*., 1993b). APC has also been implicated in cell adhesion, cytoskeletal integrity, DNA repair/apoptosis and cell migration in a Wnt-independent manner (Balusu *et al*., 2007; Barth *et al*., 1997; Mahmoud *et al*., 1997; Munemitsu *et al*., 1994). Additionally, APC has been shown to interact with End-binding protein 1 (EB1), which regulates microtubule assembly and Discs-large protein (DLG), a tumour suppressor protein that regulates cell cycle progression from G0/G1 to S phase (Ishidate *et al*., 2000; Nakamura *et al*., 2001b). Structurally, APC contains several heptad domains that mediate homodimerisation, followed by seven armadillo repeats (Kinzler *et al*., 1991; Su *et al*., 1993a). The central part of the protein contains three 15 aa and seven 20 aa repeats shown to mediate β-catenin binding (Rubinfeld *et al*., 1997a; Su *et al*., 1993b). Interspersed within the 20 aa repeats are three Serine-Alanine-Methionine-Proline (SAMP) repeats, which have been demonstrated to be vital in Axin binding (Behrens *et al*., 1998). At the C-terminus, APC contains a basic domain for microtubule interaction, an EB1 binding domain and a DLG binding domain (Matsumine *et al*., 1996; Munemitsu *et al*., 1994; Su *et al*., 1995). APC expression is high during development; it is expressed in adult epithelial cells, especially in the brain (Midgley *et al*., 1997; van Es *et al*., 1999). Its homologue APC2 was identified in 1998 and it was found to be highly expressed in the brain (Nakagawa *et al*., 1998; van Es *et al*., 1999). APC2 is smaller than APC and does not contain the 15 aa repeats, but retains the 20 aa and two SAMP repeat motifs (van Es *et al*., 1999). This suggests that it may down-regulate β-catenin (van Es *et al*., 1999). However, this redundant role may not fully compensate for APC loss during tumourigenesis since C-terminal APC truncation mutations are sufficient to induce polyposis in the intestine (Andreu *et al*., 2005; Fodde *et al*., 1994; Oshima *et al*., 1995). Recent studies have shown that APC can regulate β-catenin-mediated gene transcription at the nucleus (Hamada and Bienz, 2004; Sierra *et al*., 2006). APC bound to β-transducin repeats-containing protein (β-TrCP) and to transcription repressors [C-terminal binding protein (CtBP), transducin-like enhancer of split-1(TLE-

1) and histone deacetylase 1 (HDAC1)] could interact with β-catenin at the MYC gene and facilitate its repression (Hamada and Bienz, 2004; Sierra *et al*., 2006). Intriguingly, C-terminal truncated APC mutants commonly seen in human cancers are missing this CtBP binding domain and may account for some of the tumourigenic potential of these mutants (Hamada and Bienz, 2004; Sierra *et al*., 2006).

1.3.6.1.2 ***Axin***

Axin is the product of the *Fused* gene, which was originally identified as an inhibitor of the Wnt/β-catenin pathway due to its ability to inhibit axis duplication in *Xenopus* (Zeng *et al*., 1997). The Axin gene is conserved in mammals, *Droshophila* and *Xenopus* and although the protein it lacks enzymatic activity, it has been found to play a vital role in organising the 'destruction complex' (Hart *et al*., 1998; Hedgepeth *et al*., 1999; Ikeda *et al*., 1998; Zeng *et al*., 1997). It binds GSK3β and β-catenin in its centre, physically facilitating their interaction (Behrens *et al*., 1998; Ikeda *et al*., 1998). Additionally, Axin contains an N-terminal regulator of G protein signalling (RGS) domain (that binds to APC) and a C-terminal DIX (acronym of Dishevelled-Axin) domain important for binding to Dishevelled and for Axin oligomerisation (Hsu *et al*., 1999; Kishida *et al*., 1998; Kishida *et al*., 1999). These domains are critical, and loss of the DIX domain has been shown to severely disrupt β-catenin down-regulation (Kawahara *et al*., 2000; Sakanaka and Williams, 1999). Regardless, Axin mutations are relatively rare in human cancers and have been mainly identified in hepatocellular carcinomas and medulloblastomas (**Table 1.5**). Similar to APC, Axin has also been found to interact with many Wnt-independent partners (Cowan and Henkemeyer, 2001; Zhang *et al*., 1999). These include MAPK kinase kinase 1 (MAPKKK1) and MAPKKK4 and Smad3 [a member of the tumour growth factor β (TGF-β) pathway] (Furuhashi *et al*., 2001; Luo *et al*., 2003; Zhang *et al*., 1999). Additionally, it can interact with NCK adaptor protein 2 (Nck2), an enzyme which modulates Ephrin B1 involved in axon growth (Cowan and Henkemeyer, 2001). These pathways have been implicated in cell proliferation, differentiation and apoptosis, suggesting that Axin may be a vital scaffold protein for regulating these processes (Furuhashi *et al*., 2001; Luo *et al*., 2003; Zhang *et al*., 1999). A homologue of Axin, named Axin2, was identified by its interaction with GSK3β using a yeast two-hybrid screen (Yamamoto *et al*., 1998). Axin2 shares 44 % identity with Axin and can also promote β-catenin degradation (Behrens *et al*., 1998; Yamamoto *et al*., 1998). Interestingly, Axin2 is up-regulated by β-catenin, suggesting that it may serve as a feedback loop to modulate the duration and intensity of the active Wnt/β-catenin pathway (Jho *et al*., 2002; Lustig *et al*., 2002; Yan *et al*., 2001).

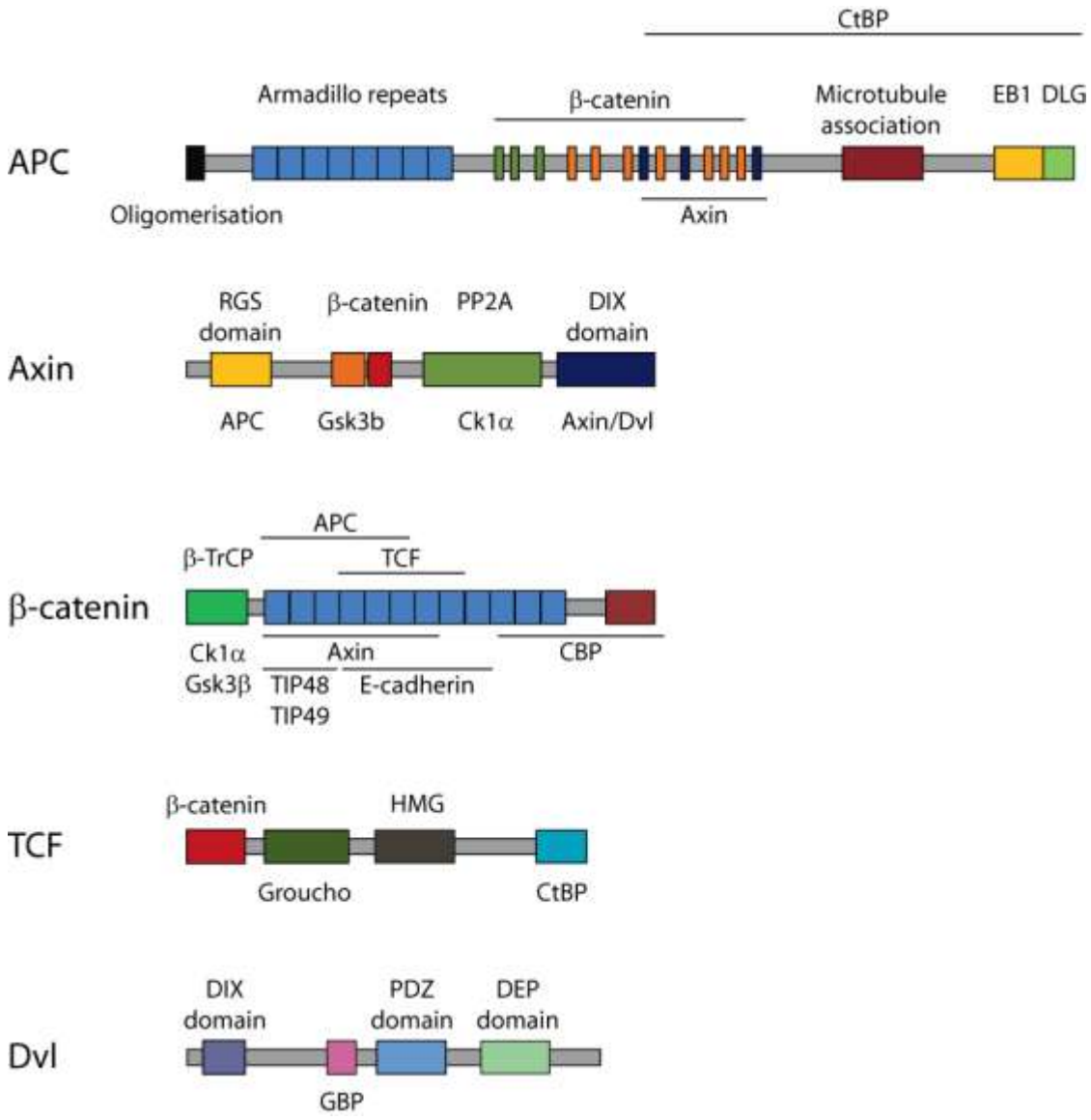


Figure 1.9 Schematic representation of Wnt/β-catenin pathway intracellular members (not to scale)
APC contains an oligomerisation domain, eight armadillo repeats, three 15 amino acid (aa) and seven 20 aa repeats that mediate β-catenin binding, three SAMP repeats important for binding to Axin, a microtubule association domain, an End-binding protein 1 (EB1) binding domain and a Discs-large protein (DLG) binding domain. Axin contains an RGS domain for binding to APC and a C-terminal DIX domain important for binding to Dishevelled and for Axin oligomerisation. Axin binds to β-catenin and Gsk3β at the centre. The N-terminus of β-catenin is phosphorylated by Gsk3β and Ck1α. Phosphorylation allows β-TrCP to bind to β-catenin and promote its degradation. Additionally, β-catenin contains twelve armadillo repeats. These repeats mediate the interaction of β-catenin with APC, TCF, E-cadherin, TIP48/49 and CBP among other binding partners. TCF interacts with β-catenin and Groucho at the N-terminus, a central HMG domain for binding to DNA and a CtBP binding domain at the C-terminus. Finally, Dvl contains a DIX domain, which mediates binding with Axin, a GBP binding domain and a PDZ and DEP domain, which are important for binding to proteins involved in the Wnt/PCP and Wnt/Ca^{2+} pathway.

1.3.6.1.3 *The destruction complex kinases: Gsk3β and Ck1α*

β-catenin degradation is dependent on the phosphorylation of four key serine/threonine residues at its C-terminus domain (Aberle *et al*., 1997; Morin *et al*., 1997; Yost *et al*., 1996) (**Figure 1.10**). The two kinases responsible for this are Ck1α and Gsk3β (Amit *et al*., 2002; Yost *et al*., 1996). Both of these enzymes are unable to bind to β-catenin directly and require Axin and APC for interaction (Behrens *et al*., 1998; Ikeda *et al*., 1998; Rubinfeld *et al*., 1996). The Ck1 family of protein kinases is highly conserved across species and regulates many critical processes such as Wnt signalling, nuclear import (Fish *et al*., 1995; Vielhaber *et al*., 2000). Ck1α interacts with Axin and enhances β-catenin degradation by phosphorylating it at Serine45, priming it for subsequent phosphorylation by Gsk3β (Amit *et al*., 2002; Zhang *et al*., 2002). Gsk3β is an essential protein kinase that is involved in many cellular processes [reviewed in(Forde and Dale, 2007)]. It was originally identified by its ability to regulate glycogen synthase (Embi *et al*., 1980). Subsequently, it has been implicated in microtubule dynamics, transcription factor regulation and many other cellular events (Lovestone *et al*., 1996; Sears *et al*., 2000)[reviewed in (Forde and Dale, 2007)]. In the Wnt/β-catenin pathway, its main role is to sequentially phosphorylate β-catenin at Threonine41, Serine37 and Serine33, following priming (Liu *et al*., 2002). Gsk3β also phosphorylates Axin and APC, thereby increasing their stability and affinity for β-catenin (Hart *et al*., 1998; Yamamoto *et al*., 1999b). Phosphorylated Serine33/37 forms a docking site for β-TrCP1/2, a component of the SCF (acronym of Skp1-Cullin1-F-box protein) E3 ubiquitin ligase, which recognises Aspartic acid-Serine (phosphorylated)-Glycine-X-X-Serine (phosphorylated) [DS(p)GXXS(p)] motifs (Alkalay *et al*., 1995; Fuchs *et al*., 1999; Winston *et al*., 1999). Phosphorylation at both residues in absolutely required and disruption of this motif inhibits the degradation of β-catenin (Miyoshi *et al*., 1998a; Morin *et al*., 1997). The SCF E3 ubiquitin ligase interacts with an E2 enzyme and sequentially transfers ubiquitin from E2 to the bound protein, in this case β-catenin [reviewed in (Krek, 1998)]. Polyubiquitinated β-catenin is then degraded by the 26S proteasome (Fuchs *et al*., 1999; Hart *et al*., 1999; Winston *et al*., 1999).

Paradoxically, CKIα and GSK3β appear to perform opposite functions at the cell membrane. There, they have been shown to phosphorylate Proline-Proline-Proline-Serine-Proline (PPPSP) motifs in the intracellular domain of LRP6 (Brennan *et al*., 2004). LRP5 contains three of these motifs and interaction with GSK3β promotes its binding to Axin (Mao *et al*., 2001b). The outcome of this interaction remains to be fully determined. However, a study in *Drosophila* using Arrow (homolog of LRP5/6), suggests that it may result in Axin degradation (Tolwinski *et al*., 2003). As mentioned previously, LRP5/6 truncated mutants lacking the extracellular domain can constitutively activate the Wnt/β-catenin pathway (Brennan *et al*., 2004; Mao *et al*., 2001a; Mao *et al*., 2001b). Interestingly, LRP5 mutants-containing the intracellular and transmembrane domain were shown to activate the pathway more than mutants containing only the intracellular domain, indicating that membrane localisation of Axin may be important for activation (Mao *et al*., 2001b). Recently, a role for caveolin in β-catenin stabilisation was also reported (Yamamoto *et al*., 2006). In this study, Wnt3a protein promoted the internalisation of LRP6 by its interaction with caveolin in the human embryonic kidney cell line HEK293 (Yamamoto *et al*., 2006). Furthermore, immunoprecipitation analysis of LRP6/GSK3β/Axin/β-catenin showed that caveolin inhibits the binding of β-catenin to Axin (Yamamoto *et al*., 2006). Even though the exact method by which caveolin acts remains to be elucidated, it is possible that it accounts for the importance of membrane localisation of Axin in activation of the Wnt/β-catenin pathway. In conclusion, it appears that CKIα and GSK3β promote the degradation of β-catenin in the absence of Wnt, but promote its stability during Wnt/β-catenin signalling by promoting the interaction between LRP5/6 and Axin.

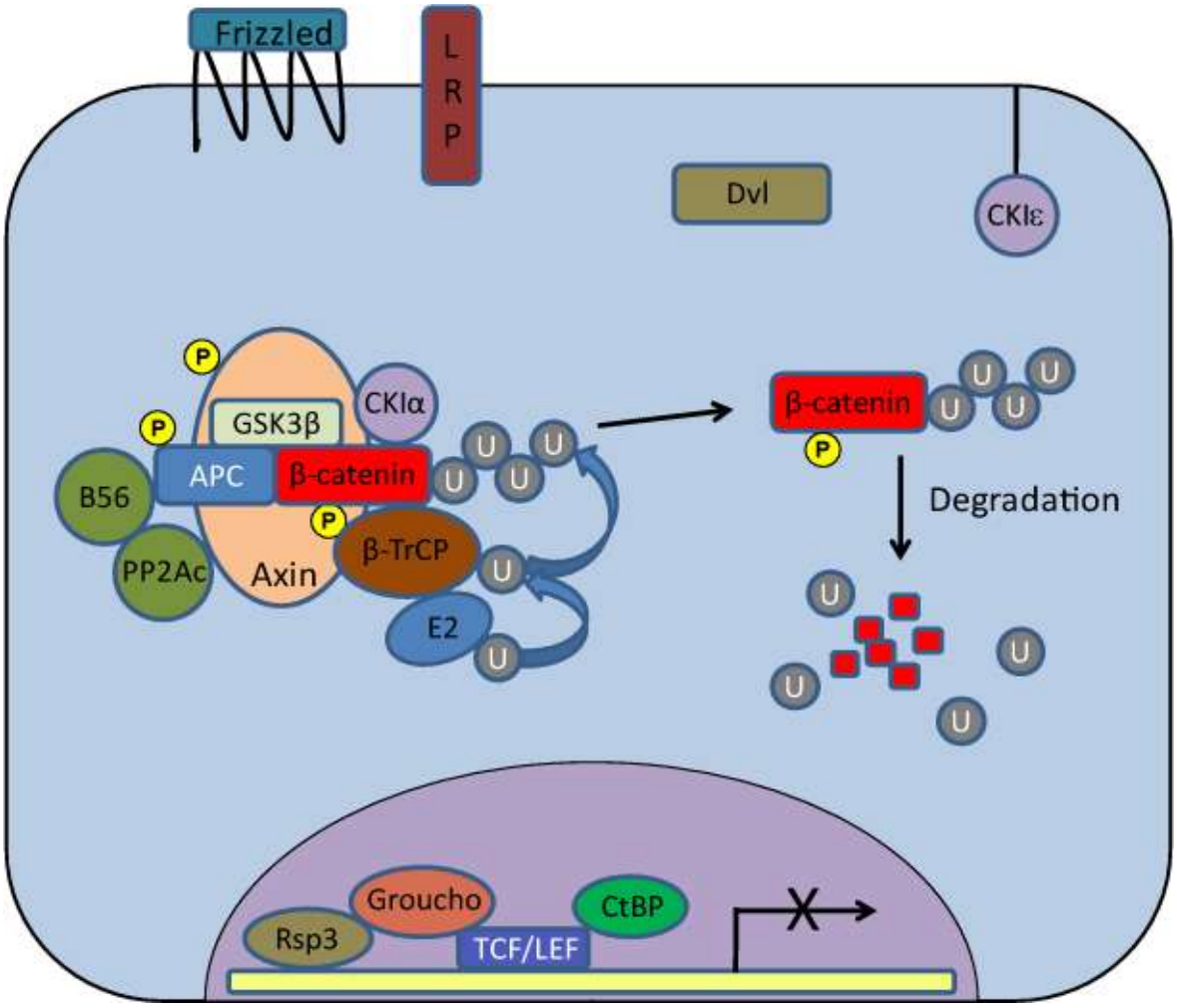


Figure 1.10 Wnt/β-catenin pathway OFF state

In the absence of Wnt proteins, an efficient complex made up of Axin, Gsk3β, APC, Ck1α and PP2A promotes the hyperphosphorylation of β-catenin. Phosphorylated β-catenin is recognised by β-TrCP, wich targets it for ubiquitination and degradation by the 26S proteasome. In the absence on nuclear β-catenin, Wnt responsive genes are inhibited by Groucho, histone deacetylases (HDAC) and C-terminal binding protein (CBP).

1.3.6.2 **ON state: Presence of Wnt signals**

Activation of the Wnt/β-catenin pathway recruits Dishevelled (Dvl in vertebrates) to the cell membrane, where it is hyperphosphorylated by membrane-bound Ck1ε and thereby activated (Hino *et al*., 2003; Kishida *et al*., 2001; Peters *et al*., 1999) (**Figure 1.11**). Dvl contains an N-terminal DIX domain, a central PDZ domain (acronym of post synaptic density protein [PSD95], *Drosophila* disc large tumor suppressor [DlgA], zonula occludens-1 protein [zo-1]) and a C-terminal DEP domain (acronym of Dishevelled, egl-10 and Pleckstrin). Hyperphosphorylated Dvl interacts with Axin *via* its DIX and PDZ domain (Kishida *et al*., 1999; Li *et al*., 1999). This interaction inhibits Gsk3β-mediated phosphorylation of β-catenin (Kishida *et al*., 1999). The mechanism of inhibition is not fully understood, but it may involve conformation changes in axin leading to a decreased affinity between Gsk3β and β-catenin. Alternatively, Dvl has been shown to bind Gsk3β binding protein (GBP) *via* its PDZ domain and recruit it to the 'destruction complex' (Li *et al*., 1999). GBP can therefore interact with the C- terminus of GSK3β and inactivate it (Li *et al*., 1999).

Once Gsk3β is inactivated, β-catenin is no longer phosphorylated and cannot be recognised by β-TrCP (Yost *et al*., 1996). Nevertheless, β-catenin still needs to dissociate from Axin and APC before it can translocate into the nucleus. As abovementioned, GSK3β phosphorylates Axin and APC to enhance their affinity to β-catenin (Hart *et al*., 1998; Yamamoto *et al*., 1999b). Therefore, they need to be de-phosphorylated. This is mediated by PP2A, one of the four major serine/threonine phosphatases (Cohen, 1989). PP2A is a heterotrimeric protein comprised of a catalytic subunit (C), a regulatory subunit (A), and one of variable regulatory subunits (B), which modulate its activity (Cohen, 1989). Axin directly interacts with PP2A C, and its inhibition (by okadaic acid) has been shown to prevent the de-phosphorylation of Axin and APC (Hsu *et al*., 1999; Ikeda *et al*., 2000a; Willert *et al*., 1999). Interestingly B56, a regulatory subunit of PP2A, binds to APC and its over-expression promotes β-catenin down-regulation, probably by inhibiting PP2A C (Seeling *et al*., 1999). On the other hand, the process by which Wnt proteins mediate a change from inactive to active PP2A remains to be elucidated.

1.3.6.3 **Nuclear translocation of β-catenin and gene transactivation**

β-catenin lacks any obvious nuclear localisation signal (NLS) and two different mechanisms for its translocation into the nucleus have been proposed. Microinjection or expression of TCF in *Xenopus* embryos promotes translocation of β-catenin indicating that they may translocate as a complex (Behrens *et al*., 1996; Huber *et al*., 1996; Molenaar *et al*., 1996). However, deletion of the β-catenin TCF binding site did not prevent β-catenin translocation, suggesting a TCF independent mechanism (Orsulic and Peifer, 1996; Prieve and Waterman, 1999). This was further substantiated *in vitro*, where β-catenin was able to enter the nucleus independent of importins, a family of proteins involved in recognising proteins with NLS (Fagotto *et al*., 1998; Yokoya *et al*., 1999). Interestingly, the central armadillo repeats appear to be required for translocation (Funayama *et al*., 1995). These repeats can also be found in importin-α and are structurally related to the importin-β HEAT repeats (acronym for Huntingtin, elongation factor 3 [EF3], PP2A, and TOR1) which bind to the nuclear pore complex (Gorlich *et al*., 1995; Kutay *et al*., 1997; Malik *et al*., 1997). Once in the nucleus, β-catenin is either anchored to DNA by interacting with TCF/LEF or exported from the nucleus by interacting with nuclear Axin or APC (Cong and Varmus, 2004; Rosin-Arbesfeld *et al*., 2000).

There are at least four mammalian TCF/LEF members; LEF1, TCF1, TCF2 and TCF4 (Korinek *et al*., 1997; Travis *et al*., 1991; van de Wetering *et al*., 1991; Waterman *et al*., 1991). They bind to the minor groove of DNA *via* a high motility group (HMG) box and induce a sharp bend (Giese *et al*., 1992). However, they do not possess any transactivation activity (Giese *et al*., 1992; Molenaar *et al*., 1996). This activity is provided by the N- and C-terminus transactivation domains of β-catenin (Hecht *et al*., 1999; Hsu *et al*., 1998; van de Wetering *et al*., 1997). In the absence of Wnt proteins, Groucho and CtBP interacts with TCF/LEF and represses gene expression by in/directly modifying chromatin structure (Brannon *et al*., 1999; Cavallo *et al*., 1998; Roose *et al*., 1998). Groucho inhibits expression by recruiting HDAC2, which condenses chromatin by deacetylating histones (Chen *et al*., 1999). On the other hand, the CtBP complex contains both HDAC and histone methyltransferase (HMT) activities which deacetylate and methylate histones to condense the structure of chromatin (Shi *et al*., 2003). A further level of repression is observed in *Drosophila*, where cyclic Adenosine monophosphate (cAMP) response element binding protein (CBP) interacts with TCF/LEF and acetylates it at the β-catenin-binding domain weakening its interaction for the armadillo repeats (Waltzer and Bienz, 1998). Interaction of β-catenin with TCF/LEF displaces Groucho and CtBP from TCF/LEF and recruits transcriptional coactivators (Chen *et al*., 1999).

Interestingly, the vertebrate CBP is recruited in this way and acetylates β-catenin to promote its transactivation activity (Hecht *et al*., 2000; Takemaru and Moon, 2000). Furthermore, it binds to the TATA-binding protein (TBP), TATA-interactive protein 48 (TIP48) and TIP49, which are proposed to form a bridge between β-catenin and RNA polymerase II (Bauer *et al*., 1998; Hecht *et al*., 1999). In summary, binding of β-catenin to TCF/LEF promotes the organisation of an efficient complex that activates the expression of canonical Wnt pathway target genes.

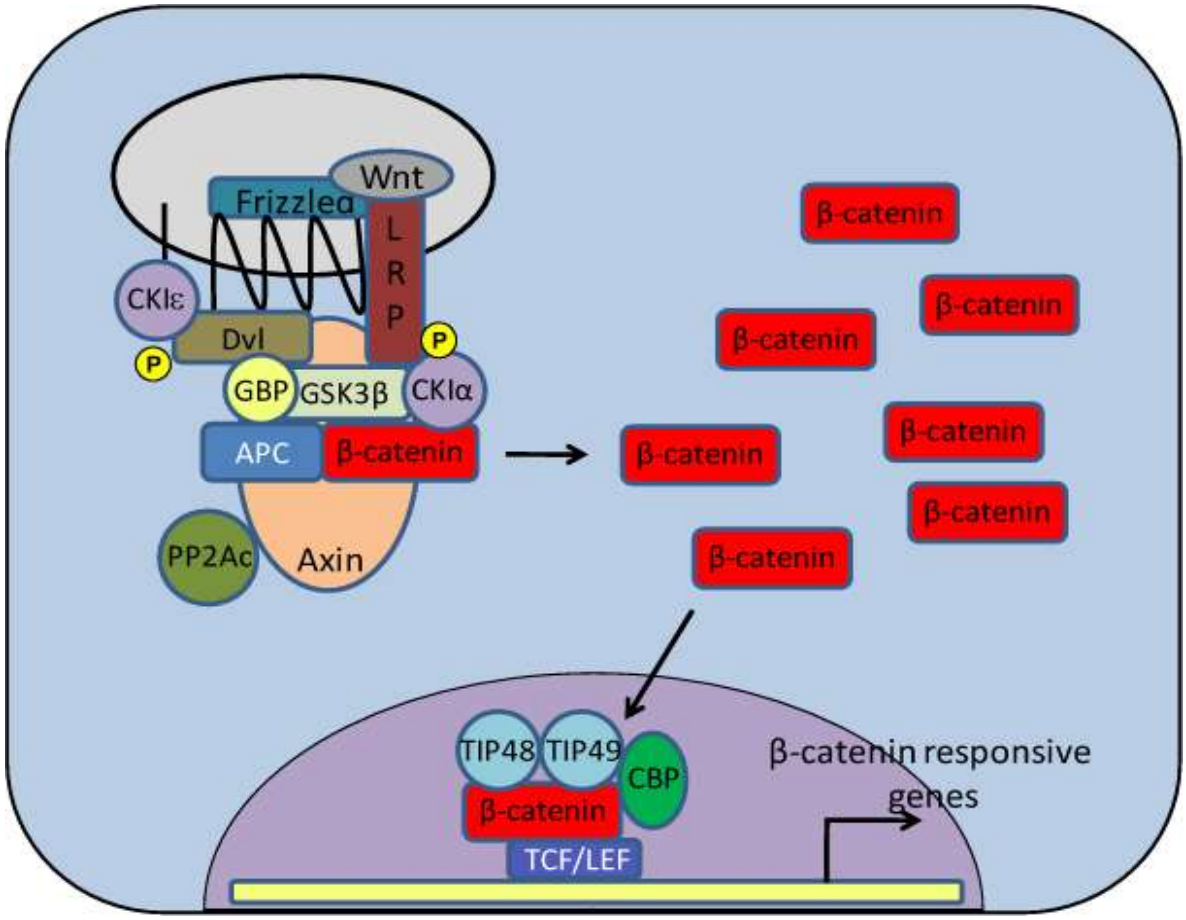


Figure 1.11 Wnt/β-catenin pathway ON state

In the presence of Wnt proteins, Gsk3β and Ck1α phosphorylate the intacellular domain of LRP5/6 at PPPSP motifs. Phosphorylated LRP5/6 binds to Axin and disrupts the 'destruction complex'. Additionally, dishevelled (Dvl) becomes hyperphosphorylated by casein kinases. Phosphorylated Dvl promotes Gsk3β inactivation by binding to Gsk3β binding protein (GBP). PP2A dephosphorylates Axin and APC to decrease their affinity for β-catenin. These three events de-stabilise the 'destruction compplex', thereby inhibiting β-catenin degradation. Stable β-catenin translocates into the nucleus where is promotes transcription by displacing the transcriptional repressors Groucho and C-terminal binding protein (CtBP) and binding to the transcriptional activator cAMP response element binding protein (CBP). Additionally, β-catenin binds to TIP48 and TIP49, which are thought to form a bridge β-catenin and RNA polymerase II.

1.3.6.4 **Wnt/β-catenin target genes**

More than 30 genes are regulated by β-catenin. Among these, cyclin D1, c-myc and matrilysin stand out for their oncogenic potential (Crawford *et al*., 1999; He *et al*., 1998; Mann *et al*., 1999; Shtutman *et al*., 1999; Tetsu and McCormick, 1999). Cyclin D1 is a critical regulator of the cell cycle and is frequently activated in human cancers [reviewed in (Baldin *et al*., 1993; Donnellan and Chetty, 1998)]. This activation can occur by chromosomal translocation of the cyclin D1 gene, gene amplification and deregulation of the Ras or Wnt/β-catenin pathways [reviewed in (Donnellan and Chetty, 1998)]. Cyclin D1 is expressed during early to mid G1 phase and is involved in promoting cell cycle progression (Baldin *et al*., 1993) (**Figure 1.12**). It functions as a regulatory subunit for cyclin-dependent-kinase 4 (CDK4) and CDK6 holoenzyme complex (Matsushime *et al*., 1992; Meyerson and Harlow, 1994). One of the main targets of cyclin D1/CDK4 is the retinoblastoma (Rb) protein (Ewen *et al*., 1993; Kato *et al*., 1993; Matsushime *et al*., 1992). Hypophosphorylated Rb suppresses cell cycle progression by sequestering E2 transciption factor proteins (E2F), an important family of transcription factors implicated in cell cycle progression (Ross *et al*., 2001; Wu *et al*., 2001a). Cyclin D1/CDK4 partially phosphorylates Rb, but it does not disrupt its interaction with E2F (Lundberg and Weinberg, 1998). Additionally, cyclin D1 and CDK4 have been shown to promote the expression of the transcriptional factor Sp1 (Tapias *et al*., 2008). Mutation of Sp1 binding sequences in the cyclin E promoter prevents full expression of the gene (Kim *et al*., 2006). These results suggest that Cyclin D1/CDK4-mediated expression of Sp1 may promote cyclin E expression. Cyclin E/CDK2 complexes fully phosphorylate Rb, disrupting its interaction with E2F (Lundberg and Weinberg, 1998). This prompts the cell to progress to S phase (Muller *et al*., 2001). Overexpression of cyclin D1 *in vitro* shortens the G1 phase but requires co-operation with other proto-oncogenes to promote cell transformation (Jiang *et al*., 1993; Musgrove *et al*., 1994; Zhou *et al*., 1995). Cyclin D1 can transform rat kidney cells and rat embryo fibroblasts in conjunction with Ras and can co-operate with c-myc to induce B cell lymphomas in transgenic mice (Lovec *et al*., 1994a; Lovec *et al*., 1994b). Interestingly, studies in human cancers have shown that cyclin D1 overexpression can promote carcinogenesis independently of CDKs (Lamb *et al*., 2003; Oyama *et al*., 1998; Zukerberg *et al*., 1996). To ascertain the significance of this activity, a comprehensive analysis of deregulated genes in breast cancer was undertaken (Lamb *et al*., 2003). This study showed that in breast cancer, overexpression of cyclin D1 did not correlate with Rb disruption and cell cycle deregulation. In oestrogen sensitive tissues, cyclin D1 has been shown to regulate growth by activating the oestrogen receptor (ER) (Neuman *et al*., 1997; Zwijsen *et al*., 1997). Cyclin D1 can bind to ER and promote ER-mediated transcription in the absence of

oestrogen ligand (Neuman *et al*., 1997; Zwijsen *et al*., 1997). This may underlie the oncogenic role of cyclin D1 in ER-positive breast cancer. Cyclin D1 can therefore promote tumourigenesis by shortening cell cycle progression or by CDK-independent gene transcription.

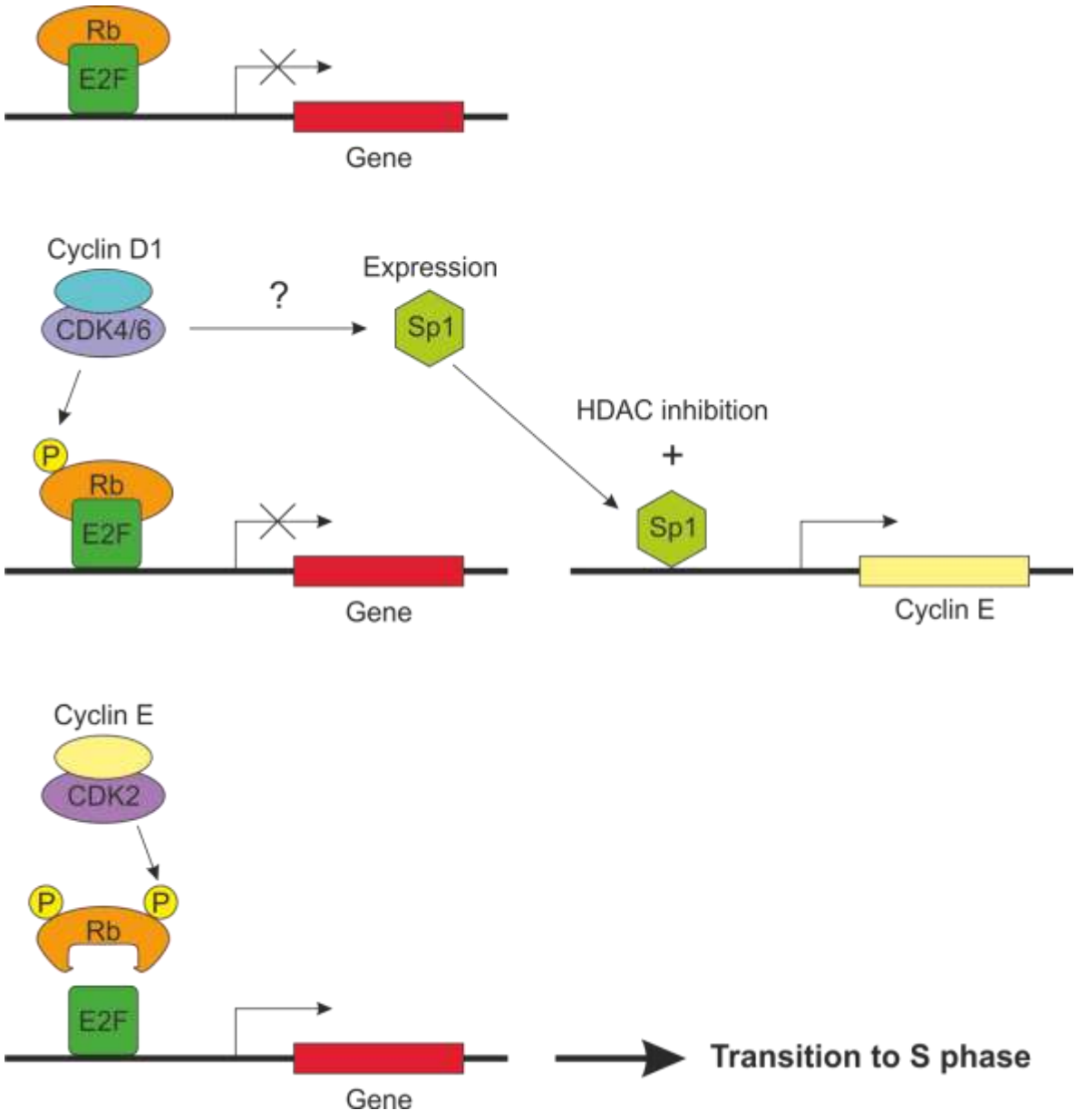


Figure 1.12 Activation of the cell cycle by cyclin D1

At G0, the Retinoblastoma protein (Rb) binds to E2 trasncription factors (E2F) and prevents their transcription activity. At mid-G1 phase cyclin D1 binds to cyclin dependent kinases 4 or 6 (CDK4/6) and phosphorylates Rb. This phosphorylation event induces a conformation change in Rb, but it does not disrupt Rb-E2F interaction. Cyclin D1-CDK4/6 promote the expression of the transcription factor Sp1 by an unkown mechanism. Inhibition of histone deacetylases promotes the expression of cyclin E. Expression of cyclin E depends on Sp1. Cyclin E interacts with CDK2 and phosphorylates Rb in a site exposed by the conformation change induced by cyclin D1-CDK4/6 phosphorylation. Phosphorylation at this site disrupts the interaction between Rb and E2F. Subsequently, E2F mediates the expression of genes required for G1 to S phase transition.

C-myc is a member of the MYC family of helix-loop-helix leucine zipper (HLH-LZ) transcriptional factors (Kato *et al*., 1990; Murre *et al*., 1989). It dimerizes with Max to bind specific DNA sequences, such as the E-box sequences (CACGTC) to promote target gene expression (Blackwood and Eisenman, 1991; Prendergast and Ziff, 1991). C-myc/Max dimers promote gene transcription by modulating chromatin condensation (McMahon *et al*., 2000). This process is dependent on recruited factors like TIP48/49/60, General control nonderepressible 5 (GCN5) and transformation/transcription domain-associated protein (TRRAP) (Bellosta *et al*., 2005; Bouchard *et al*., 2001; McMahon *et al*., 1998; McMahon *et al*., 2000). TRRAP is a protein that interacts with c-myc during chromatin binding and is believed to bridge c-myc to histone acetyltransferases (HATs) (McMahon *et al*., 1998). TIP60 and GCN5 are HATs, which interact with c-myc to acetylase histone 3 (H3) and H4 to relax chromatin condensation and promote gene transcription (Allard *et al*., 1999; McMahon *et al*., 2000; Xu *et al*., 1998). C-myc promotes proliferation and aberrant expression is associated with aggressive, poorly differentiated tumours (Fults *et al*., 2002; Lamont *et al*., 2004). C-myc induces G1→S phase transition by several mechanisms. C-myc promotes the activity of cyclin E/CDK2 (Berns *et al*., 1997; Steiner *et al*., 1995). It also directly promotes the expression of cyclin D2 and CDK4 (Bouchard *et al*., 1999; Hermeking *et al*., 2000). Furthermore, c-myc promotes the degradation of p27, and inhibitor of cyclin E/CDK4 (O'Hagan *et al*., 2000). Inhibitor-free cyclin/CDK complexes are subsequently activated by cyclin activating kinase (CAK) and promote cell cycle progression by hyperphosphorylating Rb (Kato *et al*., 1993; Russo *et al*., 1996). C-myc also induces proliferation by up-regulating RNA polymerase III, an enzyme involved in generating transfer RNA (tRNA) and 5S ribosomal RNA (rRNA), which is required for protein synthesis during cell growth (Gomez-Roman *et al*., 2003). Whilst being a potent inducer of cell proliferation, c-myc can also induce apoptosis. This can be achieved in several ways. In certain cell lines, c-myc has been shown to increase the expression of B-cell lymphoma 2 (Bcl-2) associated X protein (Bax), an inducer of mitochondrial outer membrane permeabilisation (MOMP) (Juin *et al*., 2002; Mitchell *et al*., 2000). This releases cytochrome c from mitochondria, which associates with apoptotic protease-activating factor 1 (APAF-1) to induce activation of Caspase-9 and promote apoptosis (Acehan *et al*., 2002). C-myc can also down-regulate members of the anti-apoptotic Bcl-2 protein family (Eischen *et al*., 2001; MacLean *et al*., 2003). Finally, c-myc can promote apoptosis by inhibiting nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and indirect activation of p53 *via* Adenosine diphosphate ribose (ADP) ribosylation factors (ARFs) (Klefstrom *et al*., 1997; You *et al*., 2002; Zindy *et al*., 1998). The decision between proliferation and apoptosis is

likely to be dependent on the cell type, the presence or absence of additional mutations and the presence of mitogenic signal and nutrients.

Matrix metalloproteinase-7 (MMP-7, also known as matrilysin) is an enzyme that belongs to a family of zinc dependent endopeptidases which cleave ECM components [reviewed in (Roy *et al*., 2009)]. Matrilysin recognises many components of the ECM including elastin, type IV collagen, fibronectin, vitronectin, aggrecan and proteoglycans (Miyazaki *et al*., 1990; Wilson and Matrisian, 1996; Woessner, Jr. and Taplin, 1988). Tumour metastasis is dependent on ECM degradation so MMPs are commonly up-regulated in malignant tumours [reviewed in (Roy *et al*., 2009)]. Matrilysin is no exception and its over-expression has been identified in oesophageal, colon, pancreatic, liver and stomach invasive tumours among others (Aihara *et al*., 2005; Jones *et al*., 2004; Yamamoto *et al*., 1997; Yamamoto *et al*., 1999a; Yamamoto *et al*., 2001; Yamamoto *et al*., 2003). Apart from degrading ECM components, matrilysin has also been shown to promote tumourigenesis by indirectly inhibiting E-cadherin junctions, activating the Oestrogen receptor β4 (ErB4) receptor and inhibiting apoptosis (Noe *et al*., 2001; Yu *et al*., 2002).

1.3.6.5 **Wnt/β-catenin pathway mutations in human cancers**

The Wnt/β-catenin pathway is de-regulated in many types of cancer (**Table 1.3** and **Table 1.5**). The most commonly mutated proteins in the Wnt/β-catenin pathway in tumours are β-catenin, APC and Axin1 (**Table 1.3** and **Table 1.5**). Activating β-catenin mutations occur mostly at exon 3, which corresponds to the CKIα/GSK3β phosphorylation site (Wright *et al*., 1999). Mutations in this region tend to be missense mutations either adjacent to (Asparagine32, Glycine34) or on codons (Serine33, Serine37, Threonine41, Serine45) which are phosphorylation sites of β-catenin (Garcia-Rostan *et al*., 2001). These mutations interfere with β-catenin phosphorylation resulting in protein stabilisation (Miyoshi *et al*., 1998a). In contrast, most mutations in APC are either nonsense mutations (C-terminal truncation) or frameshift mutations [reviewed in (Beroud and Soussi, 1996)]. Around 68-77 % of somatic mutations fall on a region called the mutation cluster region (MCR) which corresponds to 8 % of the gene (codons 1286-1513) (Nishisho *et al*., 1991). Whilst APC truncation products generally retain the β-catenin binding domain, they lose the Axin-binding domain, highlighting the importance of Axin-APC interaction in β-catenin down-regulation [reviewed in (Beroud and Soussi, 1996)]. Interestingly, complete deletion of both APC alleles has not been observed in human cancers. The N-terminal part of the APC protein contains proline-free blocks with heptad repeats of hydrophobic residues (Joslyn *et al*., 1993; Su *et al*., 1993b). This pattern is characteristic of coiled coils and has been shown to mediate APC homodimerisation *in vitro* (Joslyn *et al*., 1993; Su *et al*., 1993a). Dimerisation suggests that truncated APC may negatively affect full length APC. Indeed, expression of truncated APC in enterocytes increases β-catenin activity and extends proliferation (Mahmoud *et al*., 1997). Furthermore, the ability of wild-type APC in controlling β-catenin transcription is strongly attenuated when a mutant APC truncated at codon 1309 is present (Dihlmann *et al*., 1999). Colorectal cancers (CRCs) with truncated APC retaining one or two β-catenin binding sites seem to have an increased selective advantage (Albuquerque *et al*., 2002; Smits *et al*., 2000). These APC species can partially regulate β-catenin mediated transcription (Dihlmann *et al*., 1999; Munemitsu *et al*., 1995; Rubinfeld *et al*., 1997a). Therefore N-terminal APC seems to promote tumourigenesis by retaining optimal control over β-catenin. This has been named the 'just-right' hypothesis.

AXIN1 plays a central role in the 'destruction complex' by interacting with all of its members (Amit *et al*., 2002; Behrens *et al*., 1998; Ikeda *et al*., 1998). However, AXIN1 mutations are unexpectedly rare in cancer and are mostly restricted to CRC, medulloblastoma and hepatocellular carcinoma (HCC) (**Table 1.3** and **Table 1.5**). By far the most common types of mutations in AXIN1 are missense mutations (Satoh *et al*., 2000; Taniguchi *et al*., 2002; Wu *et al*.,

2001b). These are predominantly found in the APC-GSK3β binding domains, which are hypothesised to disrupt the 'destruction complex' (Satoh *et al*., 2000; Taniguchi *et al*., 2002; Wu *et al*., 2001b). Indeed, Leucine396Methionine mutation in the GSK3β binding domain has been shown to disrupt Axin-GSK3β interaction (Webster *et al*., 2000). Interestingly, in medulloblastoma, AXIN1 deletions are much more frequent than missense mutations (Dahmen *et al*., 2001; Yokota *et al*., 2002). These can occur at the C-terminus, ablating the APC-GSK3β-β-catenin binding domains or at the N-terminus, deleting the DIX domain essential for AXIN1 oligomerisation and down-regulation of β-catenin (Dahmen *et al*., 2001; Yokota *et al*., 2002). Mutations in β-catenin, APC and AXIN1 promote β-catenin stabilisation and are mostly mutually exclusive; e.g. a tumour with an APC mutation will be likely to be wild-type for β-catenin or AXIN1 (Sparks *et al*., 1998; Yokota *et al*., 2002).

By contrast, GSK3β mutations have not been found in human cancers to date. GSK3β is involved in glycogen metabolism, transcription, translation, cytoskeletal regulation, differentiation, proliferation and apoptosis so inactivating mutations are likely to be detrimental for survival [reviewed in(Forde and Dale, 2007)]. Instead, tumours seem to control GSK3β through phosphorylation. Phosphorylation at Ser9 inhibits GSK3β whereas phosphorylation at Tyr216 fully activates the enzyme (Hughes *et al*., 1993; Sutherland *et al*., 1993). GSK3β negatively regulates many oncogenic proteins and cell cycle regulators so it was predicted to act as a tumour suppressor (de Groot *et al*., 1993; Nikolakaki *et al*., 1993). Indeed, in a mouse model for skin carcinogenesis phosphorylation and Ser9 is increased whilst phosphorylation at Tyr216 is decreased (Leis *et al*., 2002). Furthermore, a recent study found that pGSK3β(Ser9) was strongly expressed in squamous cell carcinomas and basal cell carcinomas (Ding *et al*., 2007b; Ma *et al*., 2007). This also appears to be the case for mammary tumours (Farago *et al*., 2005). Expression of inactive degradation-resistant GSK3β in mammary glands promotes tumourigenesis (Farago *et al*., 2005). Interestingly, this transformation was accompanied by cytoplasmic/nuclear accumulation of β-catenin and cyclin D1, suggesting that tumourigenesis was mediated by the activation of the Wnt/β-catenin pathway (Farago *et al*., 2005). Mutagenesis studies with primary breast tumours have shown that β-catenin and APC mutations are rare events (**Table 1.3** and **Table 1.5**). However, nuclear accumulation of β-catenin and cyclin D1 are frequent and are associated with poorer prognosis (**Table 1.3**). Studies in breast cancer cell lines show that expression GSK3β or constitutively active GSK3β (Serine9Alanine mutant) can induce cell cycle arrest or apoptosis (Ding *et al*., 2007b; Wang *et al*., 2006). This implicates GSK3β as a tumour suppressor in breast tumourigenesis. In contrast, GSK3β protein appears to play an oncogenic role in human ovarian,

colon and pancreatic carcinomas, suggesting an oncogenic role (Cao *et al*., 2006; Ougolkov *et al*., 2006). Pharmacological inhibition of GSK3β suppresses proliferation of ovarian cancer cells *in vitro* and prevents tumour formation after injection of these cells into nude mice (Cao *et al*., 2006). Similarly, inhibition of GSK3β decreases survival and proliferation of colon and pancreatic cancer cells (Ougolkov *et al*., 2005; Ougolkov *et al*., 2006; Shakoori *et al*., 2005; Shakoori *et al*., 2007). An oncogenic role of GSK3β in hepatocellular, prostate, lymphocytic leukaemia and medullary thyroid cancer cells have also been suggested (Erdal *et al*., 2005; Kunnimalaiyaan *et al*., 2007; Liao *et al*., 2003; Mazor *et al*., 2004; Ougolkov *et al*., 2007). However, these effects may not be related to activation of the Wnt/β-catenin pathway. For example, GSK3β-induced suppression of mammary tumours has been suggested to be mediated by myeloid cell leukaemia-1 (Mcl-1) (Ding *et al*., 2007a; Ding *et al*., 2007b). Mcl-1 is an anti-apoptotic member of the Bcl-2 family, which is related to high grade breast cancer and poor survival (Ding *et al*., 2007b). Inactivation of GSK3β promotes accumulation of Mcl-1 which has been linked with increased mammary tumour chemoresistance (Ding *et al*., 2007a; Ding *et al*., 2007b).

Table 1.3 Mutations of the Wnt/β-catenin pathway in selected human cancers

Cancer type	Gene	Mutation frequency	Comments	References
Primary Breast Cancer	β-catenin	0/177	Mutations in components of the Wnt/β-catenin pathway are rare but nuclear β-catenin and nuclear cyclin D1 are associated with poor prognosis	(Jonsson *et al*., 2000; Lin *et al*., 2000)
	APC	4/271		(Jonsson *et al*., 2000; Sorlie *et al*., 1998)
	Nuclear β-catenin	74/123		(Lin *et al*., 2000)
	Nuclear cyclin D1	49/123		
Breast Fibromatosis	β-catenin	15/33	Breast fibromatosis are rare non-metastasing tumours	(Abraham *et al*., 2002b)
	APC	11/33		
	Nuclear β-catenin	27/33		
Desmoid tumours	β-catenin	39/85	Desmoid tumours are a locally invasive soft tissue tumour composed of spindle cells	(Miyoshi *et al*., 1998b; Saito *et al*., 2001; Saito *et al*., 2002; Tejpar *et al*., 1999)
	APC	12/48		(Alman *et al*., 1997; Tejpar *et al*., 1999)
	Nuclear β-catenin	19/38		(Saito *et al*., 2001)
Colorecetal carcinoma	APC	128/164	The 27 samples tested for β-catenin mutations corresponded to APC wild-type tumours.	(Jen *et al*., 1994; Powell *et al*., 1993; Samowitz *et al*., 2007)
	β-catenin	13/27		(Sparks *et al*., 1998)
Gastric carcinoma	β-catenin	26/161	Reduced expression of β-catenin is associated with metastasis and larger tumours. Of the 90 tumours with nuclear β-catenin only 19 possessed mutations in β-catenin	(Clements *et al*., 2002; Kim *et al*., 2009; Woo *et al*., 2001; Yoon *et al*., 2008)
	Nuclear β-catenin	90/311		(Clements *et al*., 2002)
Gastric Adenoma without associated Adenocarcinoma	β-catenin	0/96	Gastric Adenomas are rare gastric polyps that often progress to Gastric Carcinoma	(Abraham *et al*., 2003; Lee *et al*., 2002)
Gastric intestinal-type Adenocarcinoma	β-catenin	7/26	40 of the 41 tumours with nuclear β-catenin were early gastric cancer suggesting that de-regulation of the Wnt/β-catenin pathway may play a role in tumourigenesis	(Park *et al*., 1999)
	Nuclear β-catenin	41/154		(Miyazawa *et al*., 2000; Song *et al*., 2004)
	APC	67/96		(Park *et al*., 1999) (Miyazawa *et al*., 2000; Song *et al*., 2004)

Cancer type	Gene	Mutation frequency	Comments	References
Hepatocellular carcinoma	β-catenin	91/223	Hepatocellular carcinoma is one of the most common types of cancers worldwide and has poor prognosis	(De La *et al*., 1998; Legoix *et al*., 1999; Miyoshi *et al*., 1998a; Nhieu *et al*., 1999; Satoh *et al*., 2000; Taniguchi *et al*., 2002; Wong *et al*., 2001)
	AXIN1	15/179		(Satoh *et al*., 2000; Taniguchi *et al*., 2002)
	AXIN2	2/73		(Taniguchi *et al*., 2002)
Hepatoblastoma	β-catenin	125/223	Hepatoblastoma is a malignant childhood neoplasm and represents the most common malignant liver tumour in childhood	(Blaker *et al*., 1999; Jeng *et al*., 2000; Koch *et al*., 1999; Park *et al*., 2001; Takayasu *et al*., 2001; Taniguchi *et al*., 2002; Udatsu *et al*., 2001; Wei *et al*., 2000)
	APC	0/68		(Takayasu *et al*., 2001)
	APC	9/13		(Oda *et al*., 1996)
	AXIN1	2/27		(Taniguchi *et al*., 2002)
Lung Adenocarcinoma	β-catenin	3/137	Patients with low β-catenin expression have significantly poorer prognosis	(Nozawa *et al*., 2006; Shigemitsu *et al*., 2001; Sunaga *et al*., 2001)
Solid-pseudopapillary tumours (SPTs)	β-catenin	18/20	SPTs account for around 1 % of pancreatic cancers have a low malignant potential. These tumours mostly arise in young women	(Abraham *et al*., 2002a)
	Nuclear β-catenin	19/20		(Abraham *et al*., 2002a)
Wilms Tumour	β-catenin	39/286	Wilms tumour is a paediatric kidney cancer that affects 1 in 100,000 children	(Koesters *et al*., 1999; Kusafuka *et al*., 2002; Maiti *et al*., 2000; Zirn *et al*., 2006)
	WTX	15/51	Wilms tumour gene on the chromosome X (WTX) is a protein that has been recently linked with promoting β-catenin degradation	(Rivera *et al*., 2007)Rivera at al 2007 Science, Major *et al*, 2007 Science

Cancer type	Gene	Mutation frequency	Comments	References
Prostate cancer	β-catenin	14/264	-	(Chesire *et al*., 2000; Gerstein *et al*., 2002; Voeller *et al*., 1998)
	APC	2/22		(Gerstein *et al*., 2002)
	β-TrCP	2/22		(Gerstein *et al*., 2002)
Uterine endometrial	β-catenin	33/182	Nuclear β-catenin generally corresponds to type I endometrial tumours, which have a improved prognosis	(Ashihara *et al*., 2002; Fukuchi *et al*., 1998; Ikeda *et al*., 2000b; Mirabelli-Primdahl *et al*., 1999; Moreno-Bueno *et al*., 2001b; Nei *et al*., 1999)
	Nuclear β-catenin	74/319		(Machin *et al*., 2002; Ng *et al*., 2005; Scholten *et al*., 2003)
Ovarian Carcinoma (emdometrial type)	β-catenin	47/153	Common ovarian cancer	(Moreno-Bueno *et al*., 2001b; Palacios and Gamallo, 1998; Wright *et al*., 1999; Wu *et al*., 2001b)
	APC	1/47		(Wu *et al*., 2001b)
	AXIN1	2/47		(Wu *et al*., 2001b)
	AXIN2	1/47		(Wu *et al*., 2001b)
Malignant melanoma	β-catenin	11/221	The Wnt/β-catenin pathway plays an important role in melanocyte development. However nuclear β-catenin is associated with improved prognosis. De-regulation of the Wnt/β-catenin pathway is hypothesised to be important for the early stages of melanoma development, but not for later stages	(Omholt *et al*., 2001; Pollock and Hayward, 2002; Reifenberger *et al*., 2002; Rimm *et al*., 1999; Rubinfeld *et al*., 1997b)
	APC	3/63		(Reifenberger *et al*., 2002; Rubinfeld *et al*., 1997b)
	Nuclear β-catenin	28/111		(Chien *et al*., 2009; Omholt *et al*., 2001; Rimm *et al*., 1999)
Pilomatrixoma	β-catenin	36/57	Pilomatrixoma is a common skin tumour arising from the hair follicles	(Chan *et al*., 1999; Kajino *et al*., 2001; Moreno-Bueno *et al*., 2001a; Xia *et al*., 2006)
Thyroid carcinoma	β-catenin	26/57	Nuclear β-catenin or mutations are associated with poor prognosis in these tumours	(Garcia-Rostan *et al*., 2001)

Cytoplasmic and nuclear β-catenin accumulation is associated with activation of the Wnt/β-catenin pathway and is commonly used as a diagnostic marker in cancer (Eberhart *et al*., 2000; Terris *et al*., 1999). Interestingly, nuclear β-catenin is observed more frequently than mutations, suggesting the presence of undiscovered mutations and alternative mechanisms for Wnt/β-catenin pathway activation. For example, the Wilms tumour gene on the chromosome X (WTX) is a recently discovered gene that is mutated in Wilms tumour, a paediatric cancer of the kidney (Rivera *et al*., 2007). WTX protein has been suggested to form a complex with β-catenin, AXIN1, β-TrCP, and APC and promote β-catenin ubiquitination and degradation (Major *et al*., 2007). Alternative tumour mechanisms of activating the Wnt/β-catenin pathway include promoter hypermethylation of Wnt protein antagonists, Wnt protein up-regulation and down-regulation of E-cadherin. Promoter hypermethylation of SFRP and DKK silences their expression and is a common event in tumourigenesis (**Table 1.4**). Up-regulation of Wnt5a mRNA occurs in gastric, colorectal and uterine cancer and the Wnt2 proto-oncogene is up-regulated in breast, uterine, colorectal and kidney cancer (Katoh, 2001; Saitoh *et al*., 2002; Taki *et al*., 2003). β-catenin associates with E-cadherin to mediate epithelial cell-cell adhesion (Rimm *et al*., 1995). Down-regulation of E-cadherin in the absence of Wnt/β-catenin pathway mutations does not promote tumourigenesis (Caca *et al*., 1999; van de Wetering *et al*., 2001), but down-regulation in the presence of APC mutations has been reported in colorectal tumours and exacerbates pathway activation (Gottardi *et al*., 2001; Kuphal and Behrens, 2006).

Table 1.4 Hypermethylation of Wnt antagonists in selected human cancers

Cancer type	Gene involved	Mutation Frequency	Comments	References
Hepatocellular carcinoma	SFRP1	9/19	Hypermethylation is also detected in liver cirrhosis. This suggests that hypermethylation could be an early event in liver carcinogenesis	(Takagi *et al*., 2008)
	SFRP2	12/19		
	SFRP5	8/19		
Acute myeloid leukaemia	SFRP1	29/100	No activating mutations in the Wnt/β-catenin identified have been indentified in acute myeloid leukaemia to date	(Jost *et al*., 2008)
	SFRP2	19/100		
	SFRP4	0/100		
	SFRP5	9/100		
Pancreatic cancer	SFRP1	42/60	Hypermethylation and subsequent loss of expression of sFRPs appears to be an early event in the pathogenesis of pancreatic cancer	(Bu *et al*., 2008)
	SFRP2	29/60		
	SFRP4	36/60		
	SFRP5	46/60		
Oral squamous Cell carcinoma	SFRP1	7/17	No mutations have been identified in β-catenin or APC in these tumours to date. These hypermethylation events were found in cell lines grown from tumours	(Sogabe *et al*., 2008)
	SFRP2	16/17		
	SFRP5	14/17		
Breast cancer	SFRP1	31/78	Methylation of SFRP5 is associated with reduced survival	(Suzuki *et al*., 2008; Veeck *et al*., 2008)
	SFRP2	60/78		
	DKK1	15/78		
	SFRP5	123/168		
Gastric cancer	SFRP1	42/46	-	(Nojima *et al*., 2007)
	SFRP2	44/46		
	SFRP5	30/46		

As mentioned in **section 1.1** aberrant activation of the Wnt/β-catenin pathway has been implicated in CNS tumours, including medulloblastomas, CNS PNET, NOS and glioblastomas (**Table 1.5**). However not much is known about the role the Wnt/β-catenin pathway plays in these tumours. In order to address these issues transgenic mouse models are necessary to study the role of β-catenin in CNS tumourigenesis.

Table 1.5 De-regulation of the Wnt/β-catenin pathway in CNS tumours

Tumour	Abnormality	Mutation frequency	Description	References
Diffuse astrocytoma	β-catenin mutations	0/16	-	(Gotze *et al*., 2009)
WHO grade II	APC allelic imbalance	1/5	-	(Nikuseva-Martic *et al*., 2007)
	Increased levels of Dvl3 mRNA	-	Dvl3 de-stabilises of the 'destruction complex'	(Sareddy *et al*., 2009b)
	Increased levels of β-catenin mRNA and protein	-	Higher expression of these targets correlates with increased malignancy	
	Increased expression of TCF4, LEF1, c-myc, cyclin D1	-		
	NKD2 promoter hypermethylation	1/16	Promoter hypermethylation correlates with decreased expression of the target genes (6/16 tumours possessed Wnt antagonist promoter hypermethylation)	(Gotze *et al*., 2009)
	SFRP1 promoter hypermethylation	1/16		
	SFRP2 promoter hypermethylation	2/16		
	SFRP4 promoter hypermethylation	1/16		
	SFRP5 promoter hypermethylation	1/16		
Anaplastic astrocytoma	β-catenin mutations	1/19	-	(Gotze *et al*., 2009; Nikuseva-Martic *et al*., 2007)
WHO grade III	Increased levels of Dvl3 mRNA	-	Dvl3 de-stabilises of the 'destruction complex'	(Sareddy *et al*., 2009b)
	Increased levels of β-catenin mRNA and protein	-	Higher expression of these targets correlates with increased malignancy	
	Increased expression of TCF4, LEF1, c-myc and cyclin D1	-		
	Patients with Turcot's syndrome type 2	4/24	-	(Attard *et al*., 2007)
	DKK1 promoter hypermethylation	1/14	Promoter hypermethylation correlates with decreased expression of the target gene	(Gotze *et al*., 2009)
	SFRP1 promoter hypermethylation	2/14		

Tumour	Abnormality	Mutation frequency	Description	References
	SFRP4 promoter hypermethylation	1/14	(4/16 tumours possessed Wnt antagonist promoter hypermethylation)	
	SFRP5 promoter hypermethylation	1/14		
Glioblastoma (GBM) **WHO grade IV**	APC loss of heterozygosity or allelic imbalances	6/18	-	(Nikuseva-Martic *et al*., 2007)
	β-catenin mutations	1/58	-	(Gotze *et al*., 2009; Nikuseva-Martic *et al*., 2007)
	Glioblastoma cell lines (T98G, GO-G-CCM, U373 and C6) overexpress β-catenin protein	-	Inhibition of Wnt signalling in these cell lines decreases proliferation	(Sareddy *et al*., 2009b)
	Increased levels of β-catenin mRNA and protein	-	Higher expression of these targets correlates with increased malignancy	
	Increased expression of TCF4, LEF1, c-myc, cyclin D1	-		
Primary GBM	NKD2 promoter hypermethylation	13/20	Promoter hypermethylation correlates with decreased expression of the target gene (23/30 tumours possessed Wnt antagonist promoter hypermethylation)	(Gotze *et al*., 2009)
	SFRP1 promoter hypermethylation	16/30		
	SFRP2 promoter hypermethylation	13/30		
	SFRP4 promoter hypermethylation	2/30		
	SFRP5 promoter hypermethylation	1/30		
Secondary GBM	NKD2 promoter hypermethylation	2/10	Promoter hypermethylation correlates with decreased expression of the target gene (7/10 tumours possessed Wnt antagonist promoter hypermethylation)	(Gotze *et al*., 2009)
	DKK1 promoter hypermethylation	5/10		
	DKK3 promoter hypermethylation	2/10		
	SFRP1 promoter hypermethylation	1/10		
	SFRP2 promoter hypermethylation	2/10		
	SFRP5 promoter hypermethylation	2/10		

Tumour	Abnormality	Mutation frequency	Description	References
CNS PNET, NOS **WHO grade IV**	β-catenin mutations	2/30	-	(Koch *et al*., 2001; Rogers *et al*., 2009)
	APC mutations	0/24	-	
	Nuclear β-catenin	10/28	-	(Rogers *et al*., 2009)
	Nuclear Cyclin D1	12/28	8 of the 12 tumours positive for cyclin D1 were also positive for nuclear β-catenin. However, no correlation was found between proliferation and cyclin D1 overexpression	
Medulloblastoma **WHO grade IV**	β-catenin mutations	16/253	-	(Huang *et al*., 2000; Koch *et al*., 2001; Rogers *et al*., 2009; Yokota *et al*., 2002; Zurawel *et al*., 1998)
	APC mutations	4/162	-	(Dahmen *et al*., 2001; Huang *et al*., 2000; Rogers *et al*., 2009)
	Axin1 mutations	9/109	-	(Dahmen *et al*., 2001; Yokota *et al*., 2002)
	Nuclear β-catenin	51/220	-	(Eberhart *et al*., 2000; Ellison *et al*., 2005; Rogers *et al*., 2009; Yokota *et al*., 2002)
	Nuclear Cyclin D1	12/28	There was no significant correlation between nuclear β-catenin and cyclin D1 overexpression. Additionally, no correlation was found between proliferation and cyclin D1 overexpression	(Rogers *et al*., 2009)
	Patients with Turcot's syndrome type 2	17/26	Turcot's syndrome arises from APC germline mutations	(Attard *et al*., 2007)
Ependymoma	Patients with Turcot's syndrome type 2	3/28	Turcot's syndrome arises from APC germline mutations	(Attard *et al*., 2007)
	β-catenin mutations	0/77	-	(Onilude *et al*., 2006)
	APC mutations	0/77	-	
Germinoma	β-catenin mutations	1/1	-	(Nikuseva-Martic *et al*., 2007)

Tumour	Abnormality	Mutation frequency	Description	References
Meningioma	APC loss of heterozygosity	4/16	-	(Nikuseva-Martic *et al*., 2007)
WHO grade II	β-catenin mutations	2/16	-	
	miRNA-200a downregulation	10/14	miRNA-200a downregulation results in downregulation of E-cadherin and upregulation of β-catenin	(Saydam *et al*., 2009)
Neurinoma	APC loss of heterozygosity	1/6	-	(Nikuseva-Martic *et al*., 2007)
Oligoastrocytoma	APC loss of heterozygosity	1/1	-	(Nikuseva-Martic *et al*., 2007)

1.4 **Mouse models**

1.4.1 **Conventional loss-of-function/knockout Models**

Mice are widely used to model human diseases due to their genetic similarity and genetic techniques available. One of the greatest breakthroughs in disease modelling was the cellular targeting of genes to introduce mutations (Folger *et al*., 1985). This was achieved by 'homologous recombination', which involves the recombination between two homologous DNA sequences (Folger *et al*., 1982).

Another breakthrough in disease modelling was the development of techniques that allowed embryonic stem (ES) cells to be cultured *in vitro* (Evans and Kaufman, 1981; Martin, 1981). These cells remain undifferentiated *in vitro*, but retain the ability to differentiate into all cell types, including germ cells, when returned to the environment of the early embryo (Bradley *et al*., 1984). ES cells carrying genetic mutations can be generated using homologous recombination (Thomas and Capecchi, 1987). Subsequently, these cells can be injected into the inner cell mass of blastocysts, where they can differentiate and contribute to the development of the embryo (**Figure 1.13**) (Bradley *et al*., 1984). To generate a stable transgenic mouse line, the mutant ES cells need to incorporate into the germline so the null mutations can be inherited (Bradley *et al*., 1984). In this manner, mice carrying null mutations for specific genes could be generated (Koller and Smithies, 1989; Thomas and Capecchi, 1990). These mice were named knockout mice.

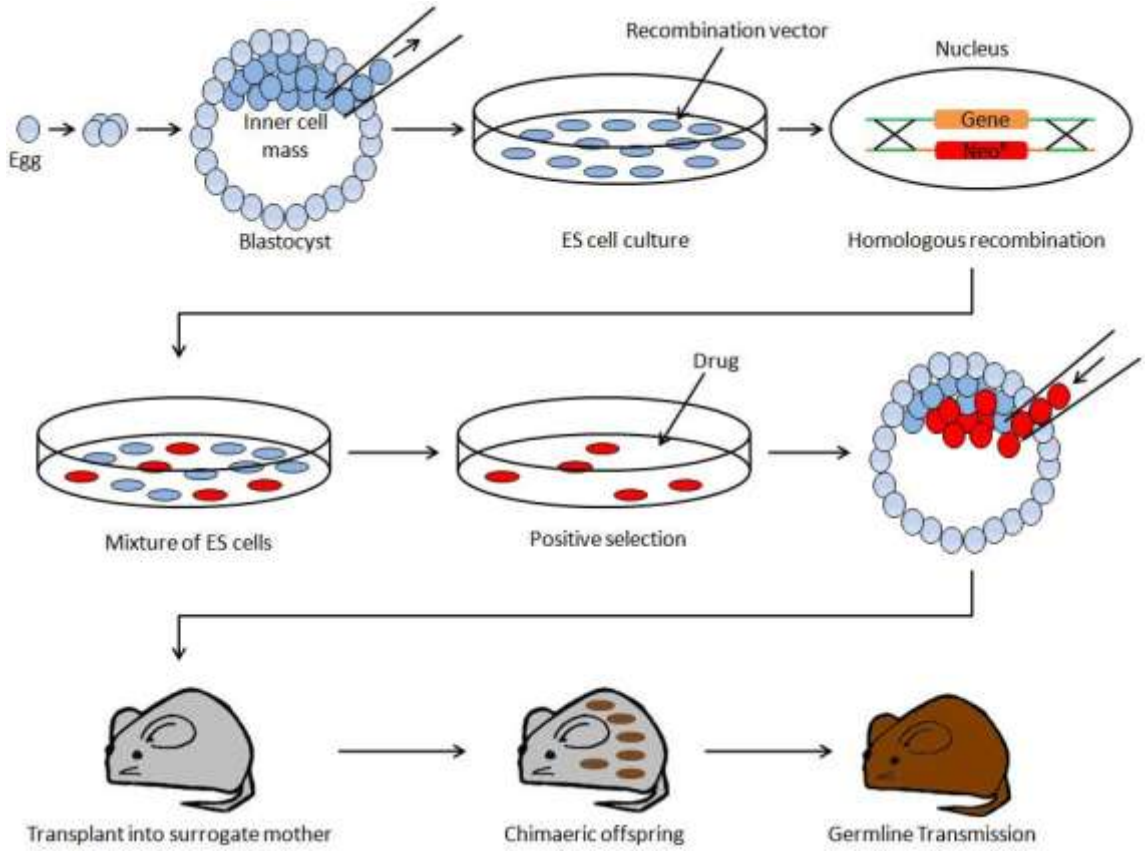


Figure 1.13 Generation of knockout mice

Embryonic stem cell cultures are established from inner cell mass cells. Once in culture, these cells are electroporated with vectors containing the null allele and a drug resistant gene. In the example above, the Neo^R gene confers resistance to neomycin. Electroporation results in a mixed culture containing recombined and non-recombined cells. The recombined cells are positively selected by administering neomycin. Only the recombined cells will survive the treatment. Recombined ES cells are subsequently injected into blastocyts, which are transplanted into a surrogate mother. The ES cells are derived from mice with agouti (brown) fur whilst the surrogate mother is non agouti. In the image above, the agouti patches are derived from the ES knockout cells. This allows for chimaeric mice to be identified. If the ES cells were incorporated into the germline, the null allele can be transmitted to the offspring of the chimaeric mice.

There are two major draw-backs to knockout mice. First, many genes are essential for embryonic development and ablation can result in embryonic lethality (Thomas and Capecchi, 1990). Hence, only the first vital function of a developmentally important gene can be studied. Second, if a gene knockout is not embryonic lethal, null expression occurs in all tissues of the body. This often results in complex phenotypes, which can complicate the interpretation. The Wnt pathway plays a very important role during development and 'conventional' knockout models of many its components result in embryonic lethality (**Table 1.6, Table 1.7** and **Table 1.8**).

Table 1.6 Phenotypes of Wnt protein 'conventional' knockout mice

Gene	Phenotype	References
Wnt1	Severe mid-hindbrain abnormalities	(Mcmahon and Bradley, 1990; Thomas and Capecchi, 1990)
Wnt2	Placental defects	(Monkley *et al*., 1996)
Wnt3	Lack of Anterior-Posterior patterning, no primitive streak formed	(Liu *et al*., 1999)
Wn3a	Disruption of notochord, no tailbud or caudal somites	(Takada *et al*., 1994)
	Paraxial mesoderm defects	(Yoshikawa *et al*., 1997)
	Underproliferation of hippocampal progenitors	(Lee *et al*., 2000)
	Abnormalities in left-right body symmetry	(Nakaya *et al*., 2005)
	Truncation of rostral hindlibms and absence of tail bud	(Greco *et al*., 1996)
Wnt4	Failure to form Kidney tubules	(Stark *et al*., 1994)
	Ectopic testosterone synthesis in female mice and absence of Mullerian duct	(Vainio *et al*., 1999)
	Role in male gonad development	(Jeays-Ward *et al*., 2004)
Wnt5a	Truncation of Anterior-Posterior axis	(Yamaguchi *et al*., 1999)
	Abnormalities in distal lung morphogenesis	(Li *et al*., 2002)
	Abnormal pituitary gland development	(Cha *et al*., 2004)
	Required for pancreatic insulin-cell migration	(Kim *et al*., 2005)
Wnt7a	Dorsal-Ventral defects, lack of posterior digits	(Parr and Mcmahon, 1995)
	Role in Anterior-Posterior patterning, defects in uterus and vagina	(Miller and Sassoon, 1998)
	Mullerian duct regression failure in males	(Parr and Mcmahon, 1998; Stevens *et al*., 1991)
	Delayed synaptic maturation in cerebellum	(Hall *et al*., 2000)
Wnt7b	Placental abnormalities	(Parr *et al*., 2001)
	Defects in pulmonary vessels	(Shu *et al*., 2002)
Wnt9a	Defects in skelotogenesis, loss of synovial chondroid metaplasia	(Spater *et al*., 2006)
Wnt9b	Essential for caudal extension of Mullerian duct in females	(Carroll *et al*., 2005)
Wnt10A	Unknown	-
Wnt10b	Excess lipid accumulation in regenerating muscle	(Vertino *et al*., 2005)
Wnt11	Kidney hypoplasia from ureteric branching defects	(Majumdar *et al*., 2003)

Table 1.7 Phenotypes of Fzd and Lrp 'conventional' knockout mice

Gene	Phenotype	References
Fzd3	Defects in axon tracts in the forebrain	(Wang *et al*., 2002)
Fzd4	Progressive cerebellar, auditory and oesophageal dysfunction	(Wang *et al*., 2001)
	Infertility due to impaired formation of corpora lutea	(Hsieh *et al*., 2005)
Fzd5	Yolk sac and placental angiogenesis defects	(Ishikawa *et al*., 2001)
Fzd6	Hair patterning defects	(Guo *et al*., 2004)
Fzd9	Defects in B-cell development	(Ranheim *et al*., 2005)
	Hippocampal and visuospatial learning defects	(Zhao *et al*., 2005)
Fzd10	Unknown	-
Lrp5	Low bone mass as a result of decreased osteoblast proliferation, impaired vascular regression	(Kato *et al*., 2002)
	Increased plasma cholesterol and decreased glucose-induced insulin secretion	(Fujino *et al*., 2003)
Lrp6	Truncated Anterior-Posterior axis, neural tube closure, mid-hindbrain and dorsal-ventral patterning defects	(Pinson *et al*., 2000)
	Small eyes and aberrant lenses	(Stump *et al*., 2003)
	Reduced dentate granule neurone production, abnormalities of the radial glia scaffolding	(Zhou *et al*., 2004b)
	Smaller and severely disorganized dorsal thalamus	(Zhou *et al*., 2004a)

Table 1.8 Phenotypes of Wnt/β-catenin downstream component 'conventional' knockout mice

Gene	Phenotype	References
Dvl1	Abnormal social interaction	(Lijam *et al*., 1997; Long *et al*., 2004)
Dvl2	Cardiovascular outflow tract defect, vertebral and rib malformations, neural tube closure defects	(Hamblet *et al*., 2002)
Dvl3	Cardiac outflow tract abnormalities	(Etheridge *et al*., 2008)
GBP1-2-3	No obvious defects in Wnt signal transduction	(van Amerongen *et al*., 2005)
Axin1	No knockout generated. Fused mutant allele results in Anterior-Posterior duplication, neuroectodermal and cardiac abnormalities	(Zeng *et al*., 1997)
Axin2	Malformation of skull due to increased osteoblast proliferation and differentiation	(Yu *et al*., 2005)
Gsk3β	Liver degeneration due to defects in NFκB signalling	(Hoeflich *et al*., 2000)
	Hypertrophic cardiomyopathy and cardiomyoblast hyperproliferation	(Kerkela *et al*., 2008)
Apc1	Development of primitive ectoderm fails before gastrulation	(Moser *et al*., 1995)
Apc2	Unknown	-
β-catenin	Failure in Anterior-Posterior formation	(Huelsken *et al*., 2000)
	Defects in the embryonic ectoderm cell layer	(Haegel *et al*., 1995)
Lef1	Defects in pro-B-cell proliferation and survival	(Reya *et al*., 2000)
	Defects in the formation of organs that require inductive tissue interaction	(van Genderen *et al*., 1994)
	Varying degrees of hippocampal abnormalities	(Galceran *et al*., 2000)
Tcf1	Defects in thymocyte proliferation and differentiation	(Schilham and Clevers, 1998; Verbeek *et al*., 1995)
Tcf3	Failure in Anterior-Posterior axis formation	(Merrill *et al*., 2004)
Tcf4	Depletion of epithelial stem cell compartments in the intestine	(Korinek *et al*., 1998)

1.4.2 **Mouse models of conditional gene inactivation (Cre-loxP system)**

Conditional gene inactivation allows for inactivation of genes in a time- and/or tissue-specific manner (Gu *et al*., 1993). This relies on enzymes that can recombine specific DNA sequences with high fidelity. The most common system for conditional gene inactivation is the Cre-loxP system. Cre-recombinase is a bacteriophage P1 enzyme that belongs to a large family of DNA recombinases (Sternberg and Hamilton, 1981). It recognises sequences called locus of crossover (x) in P1 (loxP), which consist of two 13bp inverted repeats separated by an 8bp asymmetric spacer (Hoess and Abremski, 1984; Sternberg and Hamilton, 1981). Cre-recombinase accomplishes recombination by binding to two loxP sites and mediating strand cleavage, exchange and ligation at the asymmetric spacer (Sadowski, 1995, Hoess 1985). In this manner, Cre-recombinase can mediate the excision or inversion of DNA flanked by loxP sites (**Figure 1.14**). Furthermore, it can also mediate the insertion of circular DNA molecules into linear molecules or the exchange of DNA between two linear molecules as long as each one contains a loxP site (**Figure 1.14**).

By flanking a gene with loxP sites its excision can be triggered in the presence of Cre-recombinase (Gu *et al*., 1994). Therefore, by controlling Cre-recombinase expression it is possible to conditionally inactivate gene function (Gu *et al*., 1994). The most common method for controlling Cre-recombinase is by generating mice that express the enzyme under the control of a specific promoter (Orban *et al*., 1992). As a result, only the cells in which the promoter is active will express Cre-recombinase and be recombined (Orban *et al*., 1992). The limitation of this approach is the need of multiple Cre-recombinase-expressing mice to study the effect of a gene in different cell populations.

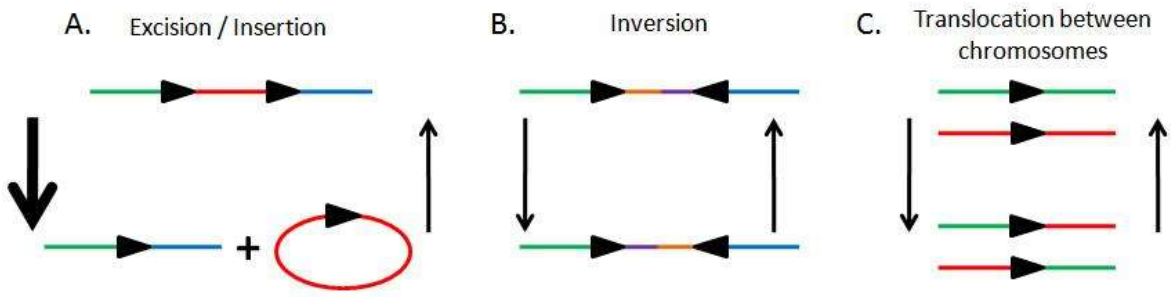


Figure 1.14 Cre-recombinase recombination events

Cre-recombinase can mediate excision/insertion (**A**) inversion (**B**) and translocation between chromosomes (**C**) depending of the location and the direction of the loxP sites. The arrows represent the direction of recombination and the thickness represents the likelihood of the recombination event taking place.

Cre-recombinase expression can also be controlled by inducible systems. An example of this is the Cre-Tamoxifen system (Metzger *et al*., 1995). Tamoxifen is a synthetic drug which competes with oestrogen for binding to the oestrogen receptor (ER) (Wakeling *et al*., 1984). In the Cre-Tamoxifen inducible system, transgenic mice, which express a Cre-Oestrogen receptor binding-domain fusion protein (Cre-ER), are used (Feil *et al*., 1996). When tamoxifen binds to the oestrogen receptor it translocates from the cytosol into the nucleus (Dix and Jordan, 1980). Therefore, in the presence of Tamoxifen, the Cre-ER fusion protein is translocated into the nucleus where it can mediate loxP site recombination (Metzger *et al*., 1995). This method is highly versatile as Tamoxifen can be administered to localised areas within tissues *via* postnatal injection (Andersson *et al*., 2010). Alternatively, it can be delivered orally to pregnant mice for recombination during embryonic development (Andersson *et al*., 2010).

Finally, Adenoviral vectors expressing Cre-recombinase can also be used to mediate loxP site recombination (Anton and Graham, 1995; Sakai *et al*., 1995). In this system, all infected cells transiently express Cre-recombinase and are irreversibly recombined.

The nature of the scientific investigation determines which of the methods for Cre-mediated recombination is most appropriate. Cre-recombinase-expressing mouse lines are useful for studying gene inactivation in specific cell populations. On the other hand, inducible and Adenoviral systems are useful for studying the effect of null mutations in targeted areas.

1.4.3 Conditional Models for studying the Wnt/β-catenin pathway in CNS tumours

De-regulation of the Wnt/β-catenin pathway has been observed in CNS tumours (**Table 1.5**). However, the role of the Wnt/β-catenin pathway in the development and maintenance of these tumours is poorly understood. To study this role, mouse models in which the pathway is constitutively active are necessary. The most common method used to achieve this is mutating the four phosphorylation residues of β-catenin. As discussed in **section 1.3.6**, phosphorylation at those residues is necessary for β-catenin degradation. Hence, abolishing their phosphorylation prevents β-catenin from being degraded, resulting in constitutive activation of the pathway.

Several β-catenin gain-of-function conditional mouse models have been generated. The first of these models expressed Serine37Alanine (S37A) β-catenin under the control of the prion protein (PrP) promoter (E11.5) (Kratz *et al*., 2002). PrP is a GPI-anchored membrane protein predominantly expressed in neurones (Bailly *et al*., 2004). Although a doubling in nuclear S37A β-catenin was observed in the cerebella of transgenic mice, no brain tumours were detected. In this model S37A β-catenin was expressed in neuronally committed cells. However, evidence suggests that stem/progenitor cells are the cell of origin of CNS tumours (Jacques *et al*., 2010; Sanai *et al*., 2005). Therefore, degradation-resistant β-catenin may need to be expressed in neural stem cells for tumourigenesis to take place. This possibility was explored by Holland's group (Fults *et al*., 2002; Momota *et al*., 2008). In the first study, transgenic mice were infected with an avian leukosis virus (ALV) expressing S37A β-catenin (ALV-S37A-β-catenin) (Fults *et al*., 2002). Murine cells are not susceptible to AVL. However, the transgenic mice used in this study expressed TVA, the avian receptor for ALV, in Nestin-expressing cells (Nestin-TVA mice). This allowed for Nestin-expressing cells to be specifically infected with ALV-S37A-β-catenin. These mice did not develop tumours within eight weeks, the endpoint of the experiment. However, this is unsurprising since inactivation of two tumour suppressor genes in the SVZ results in the development of tumours after up to thirty weeks (Jacques *et al*., 2010). Additionally, infection of Nestin-TVA mice with an avian leukosis virus (AVL) expressing the potent oncogene c-myc (ALV-c-myc) did not yield any tumours (Fults *et al*., 2002). Hence, the lack of tumours may be explained by the lack of additional mutations and/or short follow-up of the experiment. In their second study, Holland's group addressed the issue of additional mutations by using mice expressing TVA under the control of the GFAP promoter (GFAP-TVA) on a $p53^{-/-}$ genetic background (Momota *et al*., 2008). GFAP-TVA; $p53^{-/-}$ mice infected with ALV-c-myc, but not with ALV-S37A-β-catenin, formed tumours (Momota *et al*., 2008). However, the endpoint of the experiment was at three months due to the development of lymphomas and sarcomas, a known phenotype of p53 knockout mice (Harvey

et al., 1993). Interestingly, combined infection with ALV-c-myc and ALV-S37A-β-catenin increased the phenotypic diversity and halved the tumour latency in GFAP-TVA; $p53^{-/-}$ mice without increasing tumour incidence in comparison to ALV-c-myc infected mice (Momota *et al*., 2008). These results suggest that constitutive activation of the Wnt/β-catenin pathway does not promote tumourigenesis. Instead, it appears to influence tumour phenotype and behaviour. However, the possibility that degradation-resistant β-catenin may promote tumourigenesis in the long-term can not be ruled out.

In order to address these unresolved issues and investigate further, a conditional mouse model, which expresses a degradation-resistant form of β-catenin (ΔGSK-β-catenin) was generated (**Figure 3.1**). ΔGSK-β-catenin contains point mutations at the four phosphorylation sites required for β-catenin down-regulation (S33A, S37A, T41A and S45A). These mutations abolish GSK3β and CKIα mediated phosphorylation and therefore prevent ΔGSK-β-catenin from being degraded (**section 1.3.6**). Expression of ΔGSK-β-catenin in embryonic stem (ES) cells up-regulated the expression of the Wnt/β-catenin downstream targets (Haegele *et al*., 2003). This phenotype was reproduced by the expression of a truncated form of APC (Haegele *et al*., 2003). These results confirmed that this ΔGSK-β-catenin construct can effectively activate the Wnt/β-catenin pathway. Therefore, transgenic mice expressing this construct **(Figure 3.1)** were generated to study the Wnt/β-catenin pathway in the CNS.

2 Materials and methods

2.1 Mice

All mice were housed and maintained under specific pathogen-free conditions in accordance with Institutional and UK Home Office guidelines (Project license 70/6603). They were kept on a 12 hour light-dark cycle and given food and water *ad libitum*. All procedures, including culling of the mice, were carried out in accordance with Home Office regulations.

2.1.1 Genotyping

Mice were assessed for the presence of transgenes by ear biopsies, which were used for DNA extraction and subsequent analysis by polymerase chain reaction (PCR) (**section 2.7**).

2.1.2 Embryonic LacZ expression

After timed matings, embryos were harvested at embryonic day 10.5, 12.5, or 16.5 and β-galactosidase-stained (**section 2.2.2**). They were then photographed using a Nikon Coolpix 995 camera mounted on a Zeiss Stemi SV11 microscope and paraffin-embedded.

2.1.3 BrdU injection of mice

5-Bromo-2-deoxyuridine (BrdU) (Sigma; 5 mg/ml or 10 mg/ml) was made in distilled water and dissolved using a sonicator (Fisherbrand FP11002). To label proliferating cells, mice were given intraperitoneal injections of BrdU at a concentration of 50 µg per gram of body weight. As a base analogue of thymidine, BrdU substitutes for thymidine during DNA synthesis and incorporates into the newly synthesized DNA. Therefore, by BrdU administration, dividing (stem) cells are labelled. BrdU-positive cells can be detected on brain sections by immunohistochemical techniques.

2.1.4 Adenovirus-Cre intraventricular injection

Viral infection of SVZ cells was achieved by unilateral stereotaxic injections of 10^9 plaque-forming units of Adenovirus-Cre in phosphate buffered saline (PBS) into anaesthetized mice placed in a Narishige SR 6N stereotaxic frame. The injection in relation to bregma was anterior 0 mm; lateral 0.5 mm and ventral 2.5 mm. Injections (usually of 2-4 µL volume) were administered with a Hamilton syringe 1701RN and a 26 gauge needle (Hamilton).

2.2 Tissue harvesting and preparation

2.2.1 Brain and peripheral organ harvesting

For histology (**section 2.5**) and β-galactosidase staining (**section 2.2.2**), the mice were killed by increasing concentration of CO_2 in order to retain the structural integrity of the entire brain. For primary cell cultures (**section 2.4**), mice were killed by cervical dislocation and their brains removed under sterile conditions, to minimise cell death.

For Western blotting (**section 2.6**) brains were sagittally cut with one hemisphere used for β-galactosidase staining (**section 2.2.2**). The other hemisphere was sub-divided into forebrain and cerebellum, which were further sub-divided into halves. The pieces were then submerged in isopentane (Sigma) and snap-frozen in liquid nitrogen. Excess isopentane was blotted out and the samples were stored at -80°C until use. Peripheral organs such as heart, kidney, lung, liver, spleen, and testis of mice were removed for β-galactosidase staining (**section 2.2.2**).

2.2.2 β-galactosidase staining assay

Brains were incubated in formalin (Pioneer Research) for 30 min at room temperature (RT), cut sagittally and then incubated for a further 30 min in formalin. They were then permeabilised (by shaking) in permeabilisation buffer, which consisted of β-gal staining solution (10 mM Phosphate buffer [pH: 7-7.2][Fisher Chemicals], 150 mM NaCl [Fisher Chemicals], 1 mM $MgCl_2$ [Sigma], 3.3 mM $K_4Fe(CN)_6$ x $3H_2O$ [Sigma], 3.3 mM $K_3Fe(CN)_6$ [Sigma]), 1 % $MgCl_2$ [w/v] [Sigma], 0.02 % Igepal (v/v) (Fluka BioChemika) and 0.01 % Sodium Deoxycholate (w/v) (Sigma). The brains were then incubated overnight at 37°C (shaking) in permeabilisation buffer and 1 % X-Gal (w/v) (Merck; dissolved in N,N-Dimethylformamide [Sigma]). X-Gal is cleaved by β-galactosidase, when the latter is expressed by the LacZ gene, yielding galactose and 5-bromo-4-chloro-3-hydroxyindole. The resulting compound is then oxidised into 5,5'-dibromo-4,4'-dichloro-indigo, an insoluble blue product. The brains were subsequently photographed using a Nikon Coolpix 995 camera mounted on a Zeiss Stemi SV11 microscope to record the staining pattern of β-galactosidase. Somatic organs were prepared in exactly the same way, but they were not sectioned. When staining for embryos, permeabilisation was not required.

2.3 Generation of the pcall2-ΔGSK-β-catenin-IRES-GFP vector

2.3.1 Restriction enzyme digestion and ligation

The pUHD10-3/ΔGSK-β-catenin vector containing ΔGSK-β-catenin was a kind gift from Dr Nelson, Department of Molecular and Cellular Physiology, Stanford University School of Medicine, Stanford, California, United States of America (Barth *et al*., 1999). A BamH I restriction enzyme site (GGATCC) was inserted upstream of ΔGSK-β-catenin cDNA sequence by doing a PCR with the pUHD10-3/ΔGSK-β-catenin vector and primers TJ1 and TJ3 (**section 2.7.2**). The PCR product and pUHD10-3/ΔGSK-β-catenin vector were digested with 0.5 U/µl Sac II and 0.5 U/µl Sph I (both from New England Biolabs), NE buffer 4 (New England Biolabs) for three hours at 37°C. The digests were run on a 2 % agarose gel (w/v) (Sigma) containing 75 ng/ml ethidium bromide (Sigma) for 30 min. The bands were briefly visualised under UV light, cut out of the gel and purified using the QIAquick gel purification kit (QIAGEN). The digested PCR product and the digested pUHD10-3/ΔGSK-β-catenin vector were subsequently ligated with T4 DNA ligase (20 U/ml, New England Biolabs) in T4 DNA ligase buffer (New England Biolabs) overnight at 16°C.

The modified pUHD10-3/ΔGSK-β-catenin vector was restriction enzyme digested with 0.5 U/µl BamH I (New England Biolabs), NE Buffer 2 (New England Biolabs) supplemented with 100 µg/ml bovine serum albumin (BSA) (Sigma) and incubated at 37°C for three hours. The digest was run on gel and purified as mentioned above to obtain the ΔGSK-β-catenin cDNA. Subsequently, the pcall2-updated-IRES-GFP vector was digested with 0.5 U/µl Bgl II (New England Biolabs), NE Buffer 3 (New England Biolabs) at 37°C for three hours. The pcall2-updated-IRES-GFP digest was gel purified as mentioned above. Both BamH I and Bgl II leave the same overhang so the digested ΔGSK-β-catenin cDNA and pcall2-updated-IRES-GFP can be ligated together. For ligation, the DNA digests were incubated with 20 U/ml T4 DNA ligase (New England Biolabs) in T4 DNA ligase buffer (New England Biolabs) overnight at 16°C.

2.3.2 Bacterial transformation and purification

Prior to bacterial transformation, Luria Bertani (LB) agar plates containing neomycin were prepared. For this purpose, 37 g/L LB agar (Sigma) was made and autoclaved. Subsequently, Isopropyl β-D-1-thiogalactopyranoside (IPTG) (100 µM final concentration) (Promega), Neomycin (100 µg/ml final concentration) (Sigma) and X-gal (20 mg/ml final concentration) (Merck; dissolved in N,N-Dimethylformamide [Sigma]) were added. This mixture was poured into 10 cm tissue culture plates and allowed to cool and solidify.

For transformation, 2 µl of ligation product was added to 90 µl of thawed JM109 competent cells (Promega). This mixture was incubated on ice for 20 min. Following

incubation, the cells were heat-shocked by heating for 45 seconds at 42°C followed by incubation on ice for 5 min. 1 mL of LB broth (25 g/L, sterile) (Sigma) was added and the cells were incubated at 37°C for one hour (shaking). After incubation, cells were pelleted for 10 min at 3000 x *g* and re-suspended in 100 µl of LB broth. The cells were plated on an LB agar plate and incubated at 37°C overnight. The following day, single colonies were picked with a pipette tip and transferred to a 15 mL Falcon tube containing 2 mL of LB broth supplemented with neomycin 100 µg/ml (Sigma) and incubated at 37°C overnight (shaking). The cells were pelleted for 10 min at 3000 x *g.* The DNA from the pellet was harvested using the QIAprep spin miniprep kit (QIAGEN). Finally, the plasmid DNA was linearised by restriction enzyme digestion with 0.5 U/µl Sca I (New England Biolabs), NE Buffer 3 (New England Biolabs) incubated at 37°C for three hours and purified using the QIAquick PCR purification kit (QIAGEN).

2.3.3 ES cells electroporation

The ES cell medium (Dulbecco's modified Eagle's medium [DMEM] [Sigma], 20 % foetal calf serum (v/v) [FCS] [Invitrogen], 1 mM Na pyruvate [Sigma], 2 mM L-glutamine [Sigma], 0.1 mM non-essential amino acids [Sigma], 0.1 mM 2-mercaptoethanol [Sigma], 103 units/ml ESGRO murine LIF [Millipore], and 1.000U/ml Penicillin/Streptomycin [P/S] [Lonza]) of C57BL/6J ES cell cultures was changed two to three hours prior to electroporation. The C57BL/6J ES cells were harvested following a 5 min incubation with trypsin (Sigma). The trypsin was inactivated with surplus ES cell medium. The cells were subsequently centrifuged for 10 min at 300 x *g* and re-suspended in ES cell medium to a concentration of 1 x 10^7 cells/ml. 30 µg of the linearised plasmid (**section 2.3.2**) was added per 0.8 mL of re-suspended cells and aliquoted into electroporation cuvettes (Biorad). The cells were electroporated using a Biorad GenePulser at 230V, 500 µF. The time constant was set to 7.0. Following electroporation, the cuvettes were incubated at room temperature for 5 min and the cells were transferred to 10 cm plates with fresh ES cell medium.

2.3.4 ES cell selection for LacZ

Electroporated ES cells were treated with trypsin (Sigma) for 5 min. The trypsin was inactivated with surplus ES cell medium. The cells were counted and diluted to a concentration of 200 cells/ml. 200 µl/well of this mixture was plated on wells of a 96-well plate (around 1 cell/well final concentration). The plate was incubated at 37°C in an atmosphere of 5 % CO_2 and 95 % rH (relative humidity) for three days. Subsequently, the cells from each well were harvested individually. The cells were plated in duplicate into 96-well plates. One of the 96-well plates was used for β-galactosidase staining (**section 2.2.2**). The A7, B4, C11, E3, E6, E11, F9, G1 and H4 clones (the names correspond to the well position) were positive for β-galactosidase. The corresponding ES cell clones from the other 96-well plate were harvested

and used for southern blotting (**section 2.3.5**) and microinjection into blastocysts. The blastocysts were injected into pseudo-pregnant 129sv female mice.

2.3.5 Southern blot analysis

2.3.5.1 Gel electrophoresis and transfer

DNA was extracted from the B4, C11, E3, E6, E11, F9, G1 and H4 ES cell clones using the Dynabeads DNA DIRECT Kit (Invitrogen). Subsequently the DNA was digested with 0.5 U/µl EcoR I (New England Biolabs), EcoR I Buffer (New England Biolabs) incubated at 37°C for 19 hours. The digested DNA was separated on a 1 % agarose gel (w/v) (Sigma). The DNA on the gel was then depurinated by immersing it in 0.25 M HCl and gently rocking it for 10 min. Afterwards the gel was neutralised and alkaline denatured with three washes in 0.4M NaOH for 15 min each wash. Subsequently, the gel was submerged in 20x SSC buffer (3 M NaCl [Fisher Chemicals], 300 mM Na_3Citrate [Trisodium citrate], pH 7) for 5 min (shaking). The blotting set-up was as follows (from bottom to top):

1. 20x SCC buffer reservoir
2. Glass plate
3. 2x pieces of wick-blotting paper wetted with 20x SCC buffer cut to the width of the gel and lenth such that the wick is in contact with the bottom of the dish
4. Agarose gel
5. Plastic wrap to cover the entire gel
6. Hybond N+ nylon membrane (GE healthcare) wetted with distilled water cut to the size of the gel
7. 4x pieces of blotting paper cut to size of the gel (the first piece wetted with 20x SSC)
8. Glass plate
9. A weight to keep blot in place

The blot was transferred overnight. Subsequently the membrane was cross-linked by UV using the auto-crosslink program of a Stratalinker (Stratagene).

2.3.5.2 Probe synthesis, hybridisation and visualisation

The membrane was prehybridised in CG solution (0.5 M NaP [Sigma] pH7.2, 7 % sodium dodecyl sulphate [v/v] [SDS] [BioChemika], 1 % BSA [w/v] [Sigma] and 1 mM ethylenediaminetetraacetic acid [EDTA] [Fluka BioChemika] pH8.0) for four to six hours. Whilst the membrane was prehybridising, the ΔGSK-β-catenin probe was synthesised using the NEBlot kit (New England Biolabs). The probe was then added to the membrane and incubated at 65°C overnight. The following day, the membrane was washed three times with CG wash

solution (40 mM NaP [Sigma] pH7.2, 1 % SDS [v/v] [BioChemika] and 1 mM EDTA [Fluka BioChemika] pH8.0) at 65°C for 10 min/wash. The membrane was dried, placed in Saran Wrap and exposed overnight in a PhosphorImaging cassette.

2.4 Cell culture

2.4.1 Transfection of N2a cells

N2a cells were grown in DMEM supplemented with L-glutamine (BioWhittaker), 10 % FCS (v/v) (Invitrogen) and 1.000 U/ml P/S (Lonza). 3 µL of Fugene 6 reagent (Roche) was diluted in 95 µL of serum free media per transfection reaction. A total of 1 µg of plasmid DNA was added to each Fugene/Medium mixture. N2a cells were grown at a density of $4x10^5$ cells/well in seven wells of two 6-well plates overnight. The following day the cells were transfected as follows (each combination corresponds to one well):

1. 0.5 µg pEGFP + 0.5 µg pBlue II Ks+ (vehicle)
2. 0.5 µg pCre + 0.5 µg pBlue II Ks+
3. 0.5 µg pcall2-ΔGSK-β-catenin-IRES-GFP #3 + 0.5 µg pBlue II Ks+
4. 0.5 µg pcall2-ΔGSK-β-catenin-IRES-GFP #18 + 0.5 µg pBlue II Ks+
5. 0.5 µg pCre + 0.5 µg pcall2-updated-IRES-GFP
6. 0.5 µg pcall2-updated-IRES-GFP + 0.5 µg pBlue II Ks+
7. 0.5 µg pCre + 0.5 µg pcall2-ΔGSK-β-catenin-IRES-GFP #3
8. 0.5 µg pCre + 0.5 µg pcall2-ΔGSK-β-catenin-IRES-GFP #18

48 hours post-transfection, the cells were washed with PBS and harvested. The cells were then centrifuged for five minutes at 300 x *g* (4°C). Finally, the cell pellets were re-suspended in 100 µL of Radio-immunoprecipitation assay (RIPA) buffer (50 mM Tris pH7.4 [Sigma], 1 % Igepal [v/v] [Fluka BioChemika], 0.25 % Sodium Deoxycholate [w/v] [Sigma], 150 mM NaCl [Fisher Chemicals], 1 mM EGTA [Sigma]) containing protease inhibitor (1 tablet in 7 mL RIPA buffer)(Roche) and kept for Western blotting (**section 2.6**).

2.4.2 SVZ dissection and growth of neural stem cells

All surgical instruments were sterilised by heat or by immersing in 70 % ethanol. Mice were killed as previously mentioned (**section 2.2.1**). Brains were removed for NSC isolation; they were placed in a 10 cm dish containing ice-cold HIB buffer to preserve the tissue. Each brain was processed individually by transferring it into a sterile Petri dish and dissected under a Zeiss Stemi SV11 microscope as illustrated in **Figure 2.1**.

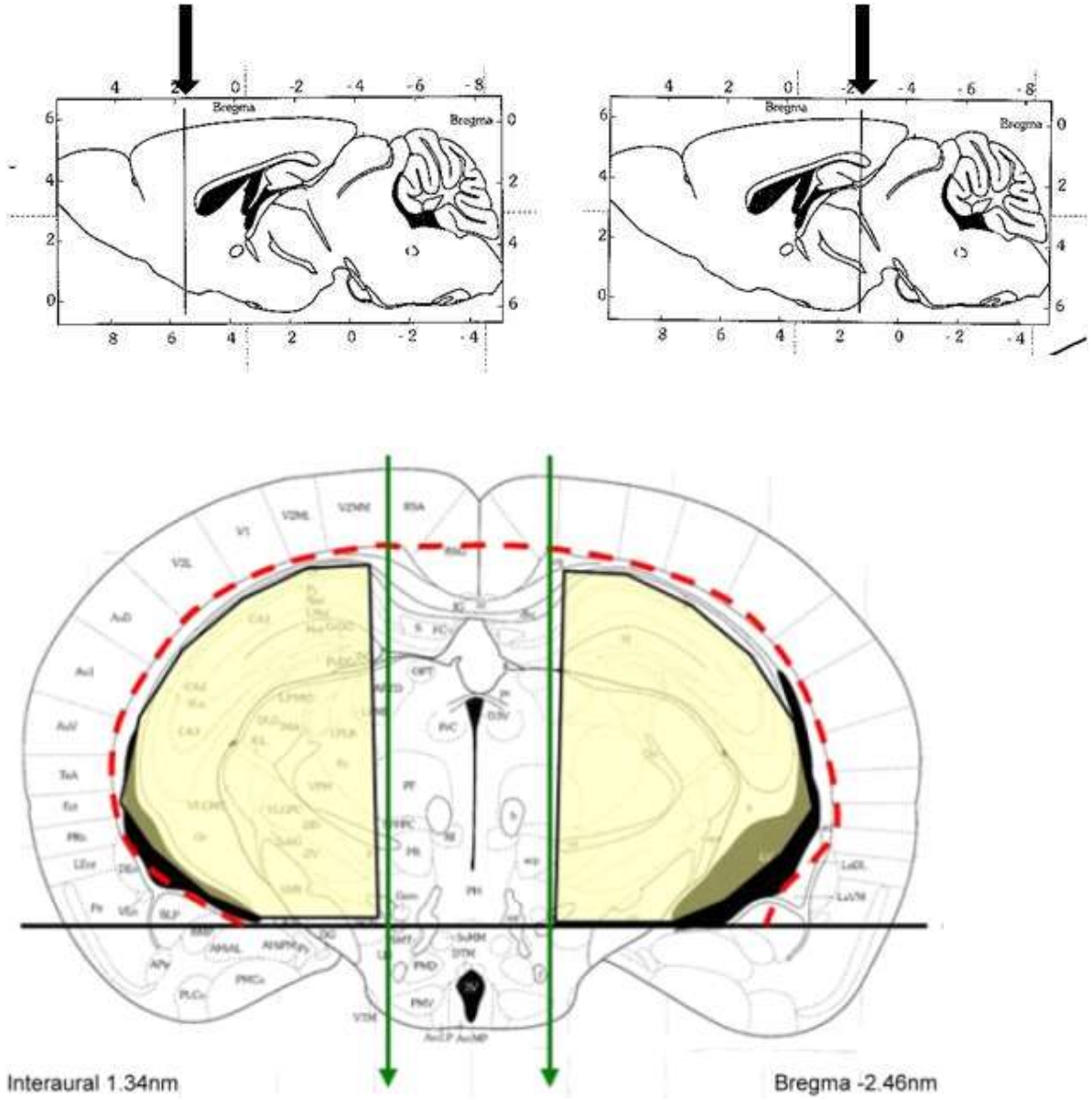


Figure 2.1 Dissection of the SVZ part 1

Top panel: The brain is cut along the arrows shown. The brain slice is placed with the rostral side facing down. **Bottom panel:** The brain slice is cut along the green, black and red lines and the tissue represented in yellow is used for subsequent steps in the protocol.

The dissected tissue was transferred into ice-cold HIB buffer whilst the other brains were dissected. Following dissection, the tissue was transferred onto a sterile Teflon disk and minced five times (in different positions) using a Tissue Chopper (McIlwain) set to cut at 400 µm intervals. The tissue was then treated with Papain (Worthington, USA) and mechanically dissociated into a single cell suspension. The cells were pelleted by centrifugation at 300 x *g* for 5min at RT. The pellet was reconstituted in a DNAse-albumin-inhibitor mixture and spun in a discontinuous density dependent gradient over an albumin-inhibitor mixture at 70 x *g* for 6 min at RT. The purified cell pellet was re-suspended in NSC medium (DMEM/Ham's F12 [Sigma] supplemented with 1x B27 [Gibco], 1.000 U/ml P/S [Lonza], 20 ng/ml recombinant murine EGF [Peprotech] and 20 ng/ml recombinant human FGFβ [Peprotech]). The cells were plated in wells of a 6-well plate for five days before replacing the medium. The cells then grew as free-floating aggregates called neurospheres (NS). For the first passage NS were incubated with Accumax (Chemicon) for 5 min at 37°C and mechanically dissociated. Following this, media was replaced every two to three days. Further passages were performed approximately every seven days depending on NS size. These passages were performed by incubation in Accumax (Chemicon) for 20 min at 37°C and by mechanical dissociation. All cultures were maintained at 37°C in an atmosphere of 5 % CO_2 and 95 % relative humidity.

2.4.3 Infection of cultures with Adenovirus

NS were collected in a 15 or a 50 mL tube and allowed to settle. All but 1 mL media was aspirated and a 50 µL aliquot was taken and incubated in 350 µL AccuMax (Chemicon) at 37°C for 20 min. Following incubation, the cells were mechanically dissociated to a single cell suspension and counted using a Haemocytometer. The number of cells in the remaining 1 ml of medium was calculated. The cells were divided equally among three 10 cm plates with 10 mL NS medium for each. One plate was for Adeno-Cre infection, one plate was for Adeno-GFP infection and the remaining plate was left untreated. For infection with Adeno-Cre and Adeno-GFP, five times more virus was added than cells in a 10 cm plate (both viral titres were ~$2.5x10^9$ pfu/ml). The NS medium was changed 2 days after infection and the NS were passaged the following day. Infection efficiency was determined by visualising GFP immunofluoresence at 488 nm excitation wavelength using a Zeiss Axiovert 200 fluorescence microscope. Photographs were taken with a Nikon Coolpix 995 3 Megapixel digital camera.

2.4.4 **WST-1 assay**

The WST-1 assay was used to measure the relative proliferation rates of cells *in vitro*. The assay principle is based on the conversion of the tetrazolium salt WST-1 into an orange coloured dye (formazan salt) by mitochondrial dehydrogenase enzymes. Over time, this reaction produces a colour change of the medium, which is directly proportional to the amount of mitochondrial dehydrogenase, and therefore cell number, in a given culture. This reaction product can be detected on a spectrophotometer (Tecan Sunrise microplate reader with XFluor 4 software) at detection wavelength of 440 nm, with a reference of 620 nm.

This analysis was performed in a 96-well plate. NS were dissociated with Accumax (**section 2.4.2**) and seeded at 6 x 10^4 cells/well in 10 wells containing 100 µL of neural stem cell media. The cells were left for 3 days before the addition of 10 µL WST-1 reagent per well and the optical density (OD) was recorded at 4, 6 and 8 hours. Alternatively, cells were plated in triplicate at either 6 x 10^4 cells/well or 3.5 x 10^3 cells/well (specified in text in the relevant results chapters). WST-1 reagent was added to one plate at day one post-passage and OD was measured at 4 hours. The other plates were treated at day two and three post-passage, respectively, and OD was measured as previously.

All samples were run in groups of 10 or 5 repeats (specified in text in the relevant results chapters) and corrected against control wells containing NSC media. Statistical analysis was performed using two-tailed t-test.

2.4.5 **Hoechst proliferation assay**

NS were dissociated with AccuMax (**section 2.4.2**) to a single cell suspension. Subsequently, cells were plated in quintuplate for each treatment in wells of a 96-well plate at a cell density of 3.5 x 10^3 cells/well in 100 µL of NSC medium. 96-well plates were prepared in triplicates. At day one post-passage, 50 µL of 5 µg/µl of Hoechst 33342 (Sigma) dissolved in dimethylsulfoxide (DMSO) was added to each well of one of the plates and incubated for 30 min at 37°C. The plate was centrifuged for 1 min at 1000 x *g*. Subsequently, the medium was removed from the wells to remove as much unbound Hoechst as possible and replaced with 1X PBS. Fluorescence was detected using a spectrophotometer (Tecan Sunrise microplate reader with XFluor 4 software) with an excitation wavelength of 360 nm and an emission wavelength of 465 nm. This was repeated at day 3 and 5 post-plating or day 2 and 3 post-plating using the remaining 96-well plates (specified in text in the relevant results chapters). Statistical analysis was performed using a two-tailed t-test.

2.4.6 **NS size**

NS were dissociated with AccuMax (**section 2.4.2**) and plated on a 6 cm plate at a cell density of 0.5 x 10^6 cells/well. On the fourth day post-passage, the cells were media changed. The cultures were media changed every two days thereafter. On day ten post-passage, the diameter of the cells was measured using an Axiovert 135 microscope (Zeiss) and Openlab 5 software. An average diameter was calculated from ≥100 neurospheres (each diameter was measured twice). Statistical analysis was performed using a two-tailed t-test.

2.4.7 **Limited dilution assay**

100 µL of NSC media was pipetted into each well of a 96-well plate. NS were dissociated with AccuMax (**section 2.4.2**) and 100 µL of single cell suspension (5 x 10^3 cells/ml) was pipetted into each well of the row of wells of the plate. Using a 12-channel pipette, 100 µL from the top row of wells was serially diluted with each row of wells. This results in a gradient of cell concentrations ranging from around 250 cells/well for the top row and 1 cells/well for the bottom row.

2.4.8 **Differentiation of neural stem cells**

NS differentiation was induced by plating NS on laminin (1 mg/ml) coated glass coverslips (13 mm) in DMEM/Ham's F12 (Sigma) with 1 % FCS (v/v) (Invitrogen), for two days. Cells were fixed in 4 % paraformaldehyde (w/v) for 30 min and processed for immunofluorescence (**section 2.4.9**).

2.4.9 **Immunostaining of differentiated neurospheres**

Coverslips were washed with PBS and permeabilised with 0.1 % TritonX-100 (v/v) (Sigma) diluted in PBS for 10 min. The cells were washed three times with PBS for 5 min each time and blocked with 0.1 % BSA (w/v) for 15 min. For anti-O4 immunostaining, coverslips were blocked in 5 % FCS (v/v) for 30 min. Following blocking, the cells were washed three times with PBS for 5 min/wash and incubated with primary antibody (1:500 rabbit polyclonal anti-GFAP [Dako], 1:500 mouse monoclonal anti- microtubule associated protein 2 [MAP2] [Chemicon], 1:200 mouse monoclonal anti-Nestin [BD Pharmingen], 1:500 mouse monoclonal anti-β-catenin [BD Pharmingen] and 1:400 mouse monoclonal anti-O4 [Chemicon]) for 1 hour at room temperature. The cells were washed three times with PBS for 5 min/wash and incubated with 1:1000 secondary antibody (goat anti-mouse IgG Alexa 546 [Molecular probes], goat anti-mouse IgM Alexa 546 [Molecular probes] for anti-O4 immunostaining and goat anti-rabbit Alexa 488 [Molecular probes] for anti-GFAP immunostaining) and 1:1000 Hoechst 33342 diluted in DMSO (250 ng/ml) to label the nuclei. The coverslips were washed once in PBS and once in distilled water. Excess water was removed and the coverslips were placed on glass

slides face down with DAKO fluorescent mounting medium (DAKO). The slides were kept in the dark at 4°C.

2.5 **Immunohistochemistry (Paraffin Histology)**

Brains were fixed in formalin, embedded in paraffin, cut into sections of 3 µm nominal thickness and stained with haematoxylin and eosin (H&E). On selected sections, immunostains were carried out according to the manufacturer's instructions: anti-GFAP (rabbit polyclonal, 1:1000 [DAKO]), anti-neuronal nuclei (NeuN; mouse monoclonal, 1:2000 [Chemicon]), anti-Nestin (mouse monoclonal IgG1, 1:100, [BD Pharmingen]), anti-synaptophysin (rabbit polyclonal, prediluted; [Zymed]), anti-BrdU (rat monoclonal, 1:100, [Abcam]); anti-GFP (rabbit polyclonal; 1:300, [Abcam]); anti-MAP2 (mouse monoclonal; 1:200, [Chemicon]).

Biotinylated secondary antibodies were used in conjunction with the primary antibodies, and visualisation was carried out using the horseradish peroxidase-conjugated streptavidin system with diaminobenzidine as a chromogen.

All immunostaining was carried out using the automated NEXES (Ventana Medical Systems; www.ventanamed.com), or Vision Biosystems (www.vision-bio.com) automated staining apparatus in accordance with the manufacturer's guidelines. Photographs were obtained on a ColorView II digital camera (www.soft-imaging.de) mounted on a ZEISS Axioplan microscope.

2.6 **Protein extraction and Western blotting**

2.6.1.1 **Brain and NS homogenisation**

The entire procedure is performed on ice. Brain pieces were homogenised using a glass mortar and pestle in RIPA buffer (50 mM Tris pH7.4 [Sigma], 1 % Igepal [v/v] [Fluka BioChemika], 0.25 % Sodium Deoxycholate [w/v] [Sigma], 150 mM NaCl [Fisher Chemicals], 1 mM EGTA [Sigma]) containing protease inhibitor (1 tablet in 7ml RIPA buffer) (Roche). The protein extracts were stored at -80°C.

NS from a 10 cm plate were allowed to settle, washed with PBS and re-suspended in 100 µl of RIPA buffer containing protease inhibitor. The protein extracts were stored at -80°C.

2.6.1.2 **Nuclear fractionation**

The procedure is performed on ice or at 4°C (where stated). NS were NS from a 10 cm plate were allowed to settle and re-suspended in 500 µl of buffer A (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES] [Sigma], 1.5 mM MgCl2 [Fisher Chemicals], 10 mM KCl [Fisher Chemicals], 0.5 mM dithriothreitol (DTT) [Sigma], 0.05 % Igepal [v/v] [Fluka BioChemika] pH7.9) containing protease inhibitor (1 tablet in 7ml RIPA buffer [Roche]). Subsequently, the cells were centrifuged at 3000 rpm for 10 min (4°C). The supernatant was removed and the pellet, containing the nuclei was re-suspended in 374 µl of buffer B (5 mM HEPES [Sigma], 1.5 mM MgCl2 [Fisher Chemicals], 0.2 mM EDTA [Fluka BioChemika], 0.5 mM DTT [Sigma], 26 % glycerol (v/v) [Sigma] pH 7.9) and 26 µl of 4.6 M NaCl (final conc. 300mM) (Fisher Chemicals) and incubated for 30 min. Finally, the solution was centrifuged at 24000 x *g* for 20 min (4°C). The supernatant contained the proteins and was stored at -80°C.

2.6.1.3 **Determination of protein concentration.**

BSA standards of 0.2 mg/ml to 1.2 mg/ml in 0.2mg/ml increments were prepared by diluting BSA (stock 2mg/ml) (Fisher chemicals) in RIPA buffer. The forebrain and cerebellum homogenates were diluted 20-fold and 10-fold respectively with RIPA/protease inhibitor. Bicinchoninic acid (BCA) protein assay reagent A and B (Fisher Chemicals) were mixed in a 50:1 ratio and 200 µL were pipetted into the wells of a 96-well plate. 10 µL of each sample were added in duplicate and the plate was incubated at 37°C for 30 min before analysis by a Tecan Sunrise microplate reader (with XFluor 4 software). Detection wavelength was 570 nm and reference 690 nm. Using the BSA standard, the protein concentration of the brain homogenates was calculated.

2.6.1.4 **Polyacrylamide gel**

0.75 mm polyacrylamide gels were made with 10 % separating gel (10 % Bis-acrylamide [v/v] [Promega], separating gel buffer [0.38 M Tris-base [Sigma], 1.4 mM SDS [BioChemika] pH8.8], 0.08 % ammonium persulfate [w/v] [APS] [Sigma], 0.2 % tetramethylethylenediamine [TEMED] [w/v] [Sigma]) and 5 % stacking gel (5 % Bis-acrylamide [v/v] [Promega], stacking gel buffer [0.13 M Tris-base {Sigma}, 3.5 mM SDS {BioChemika}, traces of Bromphenolblue [Sigma] pH6.8, 0.08 % APS [w/v] [Sigma], 0.2 % TEMED [v/v] [Sigma]) for the wells. Prior to loading, the protein samples were made up to 1mg/ml with 1x XT sample buffer (BioRad), 0.35 M DTT (Sigma) and distilled water. The samples were then boiled for 5' at 95°C. 10 µL corresponding to 10 µg of protein were loaded into each well. Two different protein markers were used, biotinylated protein ladder (Cell Signalling) and Precision Plus protein ladder (BioRad). Empty wells were loaded with 10 µL 1x XT sample buffer (BioRad) to ensure even running of the bands. The gel was then run at 200V for 30 min in 'running buffer' (250 mM Tris-base [Sigma], 1.92 M Glycine [Sigma], 1 % SDS [v/v] [BioChemika]). The stacking gel and any unused wells were then removed.

2.6.1.5 **Immunoblotting**

Immobilon-P membrane (Millipore) and gel bloating paper (Schleicher & Schuell) were cut to the size of the gel. The membrane was dipped in 100 % methanol for 15 seconds, washed in distilled water and equilibrated in cold transfer buffer (20 % methanol [v/v] [Fisher Chemicals], 25 mM Tris [Sigma], 0.2 M Glycine [Sigma]) for 5 min. The gel, four filter paper sheets and two foam pads were also equilibrated in cold transfer buffer for 15 min. The blot was assembled as follows; 1x foam pad, 2x filter paper, equilibrated gel, Immobilon-P membrane, 2x filter paper, 1x foam pad. A glass tube was used to gently roll air bubbles trapped in between the layers. The blot was placed in a cassette into the mini-trans blot cell (BioRad) filled with cold 'transfer buffer' for 1 hour at 100V. The membrane was air-dried to improve protein binding, dipped in 100 % methanol [Fisher Chemicals] for 10 seconds and air dried again. After drying, the blot was washed in tris-buffered saline tween-20 (TBST) (1 mM Tris-base pH7.8 [Sigma], 0.1 M NaCl (Fisher Chemicals), 0.05 % Tween-20 [v/v] [Fisher Scientific]) and blocked overnight in 5 % milk powder (w/v) (Marvel, Premier International Foods)/TBST at 4°C. After washing, the blot was incubated overnight with primary antibody (see below) in 1 % milk powder (w/v)/TBST at 4°C. Excess antibody was washed off with TBST and the blot was incubated for 1 hour with horseradish peroxidase (HRP)-conjugated secondary antibody (see below) in 1 % milk powder (w/v)/TBST. Excess antibody was washed off with TBST and the blot was incubated with SuperSignal WestPico Chemiluminescent

substrate (Fisher Chemicals) to enhance the HRP signal. The bands were visualised with an Image Station 440 (Kodak) with Kodak 1D v3.6 software.

Antibodies: anti-β-catenin (mouse monoclonal, 1:500, [BD transduction Laboratories]), anti-GFP (mouse monoclonal, 1:500, [Chemicon]), anti-KT-3 (rabbit polyclonal, 1:5000, [Abcam]), anti-β-actin (mouse monoclonal, 1:1000, [Sigma]), anti-mouse IgG-HRP (Sigma) and anti-rabbit IgG-HRP (Sigma).

2.7 Techniques involving nucleic acids

2.7.1 Extraction of DNA from tails or ear biopsies

The tails of mice were anaesthetised with ethyl chloride and up to 0.5 cm were biopsied into separate sterile Eppendorf tubes. Each tail was submerged in 0.5 mL of tail lysis buffer (50 mM Tris pH8 [Sigma], 100 mM EDTA pH8 [Fluka BioChemika], 100 mM NaCl [Fisher Chemicals], 1 % SDS [v/v] [BioChemika]) containing 3 µL of Proteinase K (Roche) and digested for at least 2 hours at 55°C (shaking).

After complete digestion, the samples were spun for 10 min at ≥16000 rpm using an Eppendorf centrifuge 5415D to settle debris at the bottom. The supernatant was transferred to Eppendorf tubes and 0.5 mL of Isopropanol (Fisher Chemicals) was added to precipitate the DNA. The samples were centrifuged for 1 min at ≥16000 rpm. The supernatant was discarded and the DNA was air-dried for 5-10 min. Finally 200 µL of tris-EDTA (TE) buffer (10 mM Tris pH7.5 [Sigma], 1 mM EDTA [Fluka BioChemika]) pH7 was added and the samples were incubated for ≥2 hours (shaking) at 37°C to dissolve the DNA. Samples were kept at 4°C thereafter until used for polymerase chain reaction (PCR) analysis.

DNA was also extracted from mouse embryos tails. In this case smaller amount of reagents were used and the 10' centrifugation step was omitted as no debris needed to be separated.

2.7.2 Polymerase Chain Reaction

GoTaq Flexi DNA polymerase kit (Promega) was used and kept on ice at all times. All PCR primers were obtained from MGW Biotech, dissolved in sterile water to a concentration of 100 pmol/µl.

A mastermix containing 5x Green GoTaq Flexi Buffer (1x final concentration), $MgCl_2$ (1.5 mM), GoTaq DNA polymerase (1U), deoxyribonucleotide triphosphates (dNTPs) (0.25 mM) (PCR nucleotide mix [Promega]), primers (0.5 pmol/µl) (MGW Biotech) was made. Per sample, 1 µL of DNA was dissolved in 24 µL of mastermix. The PCR was perfomed using an AB Applied Biosystems GeneAmp PCR System 9700. All PCRs had a common initial heating phase of 94°C for 2 min to activate the Taq polymerase and a final phase of 72°C for 7 min. Annealing temperatures varied with the primers (see below). The PCR products were stored at 4°C for 2 days or at -20°C until used.

LacZ/ JunD

Primers :	JunD 1:	5′ TCG CTC TTG GCA ACA GCG GCC GCC ACC AGG 3′
	JunD 2:	5′ GGC CGC TCA GCG CCT CCT CGC CAT AGA AGG 3′
	LacZ -f:	5′ CGT CAC ACT ACG TCT GAA CG 3′
	LacZ -r:	5′ CGA CCA GAT GAT CAC ACT CG 3′
Band size:	LacZ:	500bp
	JunD:	300bp (internal control)
PCR program:	Denaturing	94°C 30′′
	Annealing	58 °C 40′′ 35 cycles
	Extension	72 °C 1′30′′

eGFP/β-actin

Primers:	eGFP-F:	5′ CCT ACG GCG TGC AGT GCT TCA GC 3′
	eGFP-R:	5′ CGG CGA GCT GCA CGC TGC GTC CTC 3′
	Actin-F:	5′ GAT GAC GAT ATC GCT GCG CTG GTCG 3′
	Actin-R:	5′ GCC TGT GGT ACG ACC AGA GGC ATA CAG 3′
Band size:	β-Actin:	1Kb (internal control)
	eGFP:	300bp
PCR program:	Denaturing	94°C 30′′
	Annealing	60 °C 45′′ 35 cycles
	Extension	72 °C 45′′

Cre/p53

Primers	Cre 1:	5′ ACC AGC CAG CTA TCA ACT C 3′
	Cre 2:	5′ TAT ACG CGT GCT AGC GAA GAT CTC CAT CTT CCA GCA G 3′
	p53 Int10 fwd:	5′ AAG GGG TAT GAG GGA CAA GG 3′
	p53 Int10 rev:	5′ GAA GAC AGA AAA GGG GAG GG 3′
Band size:	Cre:	300bp
	p53 wt:	400bp (internal control)
	p53 floxed:	600bp
PCR program:	Denaturing	94°C 30′′
	Annealing	56 °C 30′′ 35 cycles
	Extension	72 °C 50′′

Rb

Primers	Rb18:	5′ GGC GTG TGC CAT CAA TG 3′	
	Rb19:	5′ AAC TCA AGG GAG ACC TG 3′	
Band size:	Rb wt:	690bp	
	Rb floxed:	740bp	
PCR program:	Denaturing	94°C 30′′	
	Annealing	58°C 40′′	35 cycles
	Extension	72°C 1′	

β-catenin exon 3

Primers	β-cat ex3-F:	5′ GCT GCG TGG ACA ATG GCT AC 3′	
	β-cat ex3-R:	5′ GCT TTT CTG TCC GGC TCC AT 3′	
Band size:	Rb wt:	690bp	
	Rb floxed:	740bp	
PCR program:	Denaturing	94°C 30′′	
	Annealing	58°C 40′′	35 cycles
	Extension	72°C 1′	

2.7.3 **Agarose Gel Electrophoresis**

The PCR products were separated by agarose gel electrophoresis. 1.5-2 % Agarose (w/v) (Sigma) was dissolved in 1x Tris base-acetic acid-EDTA (TAE) buffer (Sigma). 1.5 µl of 10 mg/ml ethidium bromide (Sigma) was added per 200 mL of Agarose gel. The gel was poured into a mould and allowed to solidify. It was then transferred to a running chamber and filled with enough 1x TAE buffer to completely submerge the gel. 10 µL of PCR samples were pipetted into the wells. 6 µL of 100 bp ladder (Promega) was loaded as a marker and the gel was run at 140V for 1 hour. The bands were visualised with a BioRad Gel Doc 1000 and QuantityOne software.

3 Generation and expression analysis of ΔGSK-β-catenin transgenic mice

3.1 Background

The Wnt/β-catenin pathway plays an important role in development, stem cell maintenance and tumourigenesis (chapter 1). The central member of this pathway is β-catenin, a 780 aa protein, which was originally identified as a component of E-cadherin junctions (Takeichi, 1991). In the absence of Wnt proteins, cytoplasmic β-catenin is phosphorylated at key residues and is thereby targeted for degradation (Aberle *et al*., 1997; Morin *et al*., 1997; Yost *et al*., 1996). However, in the presence of Wnt proteins this phosphorylation, and thus degradation, is abolished (Liu *et al*., 2002). Under these circumstances, stable β-catenin can translocate into the nucleus where it interacts with transcription factors to promote the expression of genes involved in proliferation and differentiation (Orsulic and Peifer, 1996; Prieve and Waterman, 1999). Some of the genes that are up-regulated by β-catenin include the potent oncogenes c-myc, cyclin D1 and MMP-7 (Crawford *et al*., 1999; He *et al*., 1998; Shtutman *et al*., 1999).

Mutations in components of the Wnt/β-catenin pathway are common in human cancers (**Table 1.3** and **Table 1.5**). This pathway is aberrantly activated in central nervous system (CNS) tumours such as medulloblastomas, 'CNS PNET, NOS', Turcot's syndrome and glioblastomas (**Table 1.5**). Of these, the most extensively studied are medulloblastomas, the most common malignancy in children (Central Brain Tumor Registry of the United States, 1995; Stevens *et al*., 1991). In medulloblastomas, nuclear β-catenin, a marker of pathway activation, has been observed in around 23 % of cases (Eberhart *et al*., 2000; Ellison *et al*., 2005; Rogers *et al*., 2009; Yokota *et al*., 2002). Interestingly, even though activation of the Wnt/β-catenin pathway is generally associated with enhanced proliferation/aggressiveness, activation in medulloblastomas appears to be a marker of a favourable outcome (Bondi *et al*., 2004; Clifford *et al*., 2006; Ellison *et al*., 2005; Kotsinas *et al*., 2002; Nhieu *et al*., 1999; Zechner *et al*., 2003). However, patients suffer severe neuro-cognitive defects as a result of current treatment regimes (Copeland *et al*., 1999; Mulhern *et al*., 1998; Mulhern *et al*., 1999; Mulhern *et al*., 2001; Palmer *et al*., 2001; Palmer *et al*., 2003; Ris *et al*., 2001). Therefore, it is important to develop more targeted therapies in order to improve the quality of life of these individuals. Nevertheless, not much is known about the role of the Wnt/β-catenin pathway in medulloblastomas. A recent report suggests that the Wnt/β-catenin pathway may promote

the progression of anaplastic astrocytomas (WHO grade II) to glioblastomas (WHO grade IV) (Sareddy *et al*., 2009b).

To understand the role of the Wnt/β-catenin pathway in tumours of the CNS, a transgenic mouse model was generated in which the Wnt/β-catenin pathway is constitutively activated. This model relies on the conditional expression of ΔGSK-β-catenin (Barth *et al*., 1999). ΔGSK-β-catenin is a form of β-catenin, which contains the point mutations Serine33Alanine, Serine37Alanine, Threonine41Alanine and Serine45Alanine. These mutations abolish Gsk3β and Ck1α mediated-phosphorylation of β-catenin and prevent this molecule from being degraded (**section 1.3.6.1.3**). As such, ΔGSK-β-catenin can up-regulate the expression of genes downstream of β-catenin in the absence of Wnt proteins (Barth *et al*., 1999; Haegele *et al*., 2003).

3.1.1 Aims

The aim of this study was to generate a transgenic mouse model to investigate the role of the Wnt/β-catenin pathway in CNS tumours.

3.1.2 Methods

Restriction digestion and ligation were used to generate the pcall2-ΔGSK-β-catenin-IRES-GFP vector (**section 2.3.1**). The vector was transfected into neuroblastoma (N2a) cells (**section 2.4.1**) and Western blotting was used to confirm recombination and expression of ΔGSK-β-catenin (**section 2.6**). ES cells were electroporated with the vector (**section 2.3.3**) and β-galactosidase staining was used to confirm integration and expression of the vector (**section 2.2.2**). Southern blot analysis was used to select ES cell clones with few copies of the pcall2-ΔGSK-β-catenin-IRES-GFP vector (**section 2.3.5**). Vector expression and recombination was assessed in mouse brains by β-galactosidase staining (**section 2.2.2**). Western blotting was performed to detect ΔGSK-β-catenin expression (**section 2.6**). Finally, mouse brains were immunohistochemically stained to visualise brain morphology (**section 2.5**).

3.2 Results

3.2.1 Generation of the pcall2-ΔGSK-β-catenin-IRES-GFP vector

The pUHD10-3/ΔGSK-β-catenin vector containing ΔGSK-β-catenin was a kind gift from Dr Nelson (Barth *et al.*, 1999). In order to excise the intact ΔGSK-β-catenin cDNA from the pUHD10-3/ΔGSK-β-catenin vector, a BamH I restriction site was inserted 5' to the start codon. Subsequent BamH I restriction enzyme digestion yielded a 2.49 kb fragment containing the full ΔGSK-β-catenin cDNA. This fragment was inserted into the Bgl II site of the pcall2-updated-IRES-GFP vector. Integration of the 2.49 kb fragment into the correct site was confirmed by sequencing (data not shown). This new vector was named pcall2-ΔGSK-β-catenin-IRES-GFP (**Figure 3.1**).

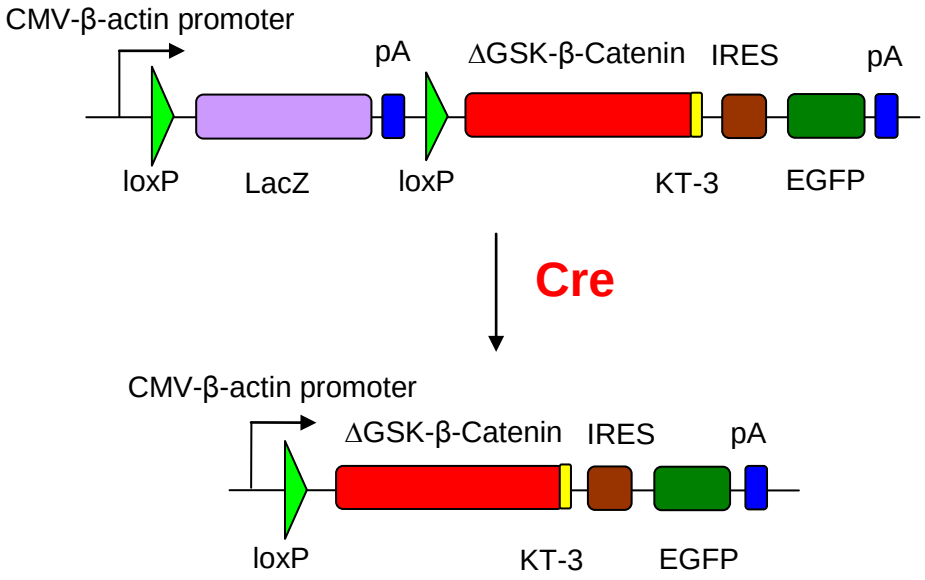


Figure 3.1 pcall2-ΔGSK-β-catenin-IRES-GFP vector

Transgenic mice, which express this construct, were generated. The construct uses the CMV-β-actin promoter which confers ubiquitous expression. In non recombined cells, β-galactosidase (LacZ) is expressed but not ΔGSK-β-catenin. Following Cre-recombinase administration, the loxP sites are recombined resulting in the excision of the LacZ stop cassette. Recombination induces the expression of ΔGSK-β-catenin.

3.2.2 N2a cells transfected with pcall2-ΔGSK-β-catenin-IRES-GFP vector express ΔGSK-β-catenin following Cre-mediated recombination

To confirm that expression of ΔGSK-β-catenin and EGFP occurs only after Cre-mediated recombination, the pcall2-ΔGSK-β-catenin-IRES-GFP vector was transiently transfected into neuroblastoma (N2a) cells in the presence, or absence, of a vector expressing Cre-recombinase. Immunoblotting of transfected cells with anti-GFP and anti-β-catenin antibodies showed expression of both ΔGSK-β-catenin and EGFP only when Cre-recombinase was present (**Figure 3.2**).

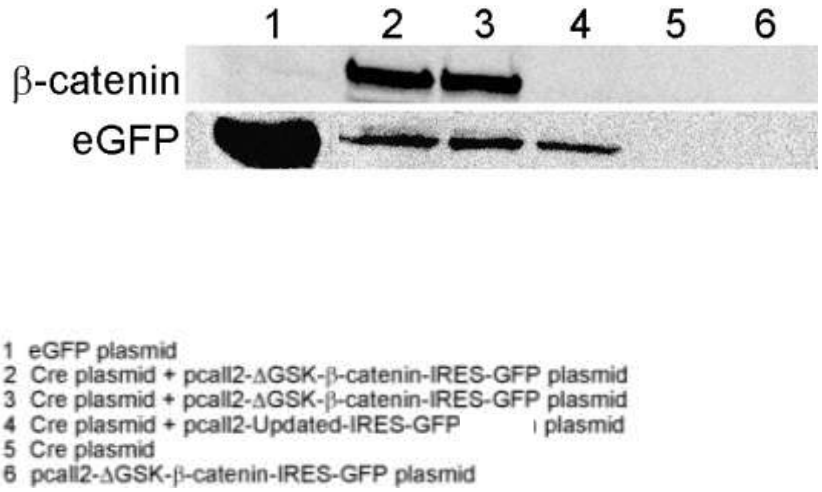


Figure 3.2 Cre-mediated recombination of pcall2-ΔGSK-β-catenin-IRES-GFP results in expression of ΔGSK-β-catenin and EGFP in N2a cells

Cell lysates from transfected neuroblastoma (N2a) cells were immunoblotted using anti-β-catenin and anti-GFP antibodies. 20 µg of total protein were loaded per lane. Co-transfection of a Cre-expressing plasmid with the pcall2-ΔGSK-β-catenin-IRES-GFP plasmid resulted in the expression of ΔGSK-β-catenin and EGFP (lanes 2 and 3). *This experiment was performed by H. Naumann, UCL Institute of Neurology, and Thomas Jacques, UCL Institute of Child Health.*

3.2.3 Selection of embryonic stem cells transfected with the pcall2-ΔGSK-β-catenin-IRES-GFP vector for blastocyst injection

The pcall2-ΔGSK-β-catenin-IRES-GFP vector expresses LacZ in the absence of Cre-recombinase (**Figure 3.2**). LacZ encodes the bacterial enzyme β-galactosidase, which hydrolyzes β-galactosidase sugars. β-galactoside expressing cells can be detected by the addition of X-gal, which is a synthetic compound. In the presence of β-galactosidase, X-gal can be hydrolysed into galactose and 5-bromo-4-chloro-3-hydroxyindole (X), a blue insoluble compound.

To insert the pcall2-ΔGSK-β-catenin-IRES-GFP vector into the genome of embryonic stem (ES) cells, ES cells were electroporated with the vector and clones were plated into duplicate 96-well plates. ES cells from one plate were stained using X-gal to confirm the expression of the pcall2-ΔGSK-β-catenin-IRES-GFP vector. Nine clones (A7, B4, C11, E3, E6, E11, F9, G1 and H4) were positive for β-galactosidase expression (data not shown; *from Dr. Behrens, Cancer Reasearch UK*). Transfected cells may contain more than one copy of the transfected vector. However, β-catenin mutations in tumours occur only in one allele, with only one copy of the mutant gene present (Morin *et al*., 1997; Zurawel *et al*., 1998). To model this scenario, the ES cell clones, which were positive for LacZ expression, were analysed for the insertion of pcall2-ΔGSK-β-catenin-IRES-GFP vector. DNA from each clone was harvested and then digested with the EcoR I restriction enzyme. Subsequently, the DNA fragments were separated by gel electrophoresis and analysed by southern blot hybridisation. A probe, which recognises the C-terminal domain of β-catenin cDNA was used to detect DNA fragments derived from the mutant transgene. Clones A7, C11, E11 and H4 were found to contain the smallest amounts of the transgene and were selected for injection into blastocysts (**Figure 3.3**). To this end, pseudopregnant foster mice were injected with A7, C11 or H4 ES cells, and produced chimaeric offspring. To test whether LacZ was expressed in the offspring of the chimaeric mice, a PCR was performed, which confirmed that the injected pcall2-ΔGSK-β-catenin-IRES-GFP vector had been inserted into the germline and could therefore be inherited. Three transgenic mouse lines were generated in this manner, which were named ΔGSK-β-catenin^{A}, ΔGSK-β-catenin^{C} and ΔGSK-β-catenin^{H}, corresponding with the ES cell clone from which they originate (A7, C11 and H4).

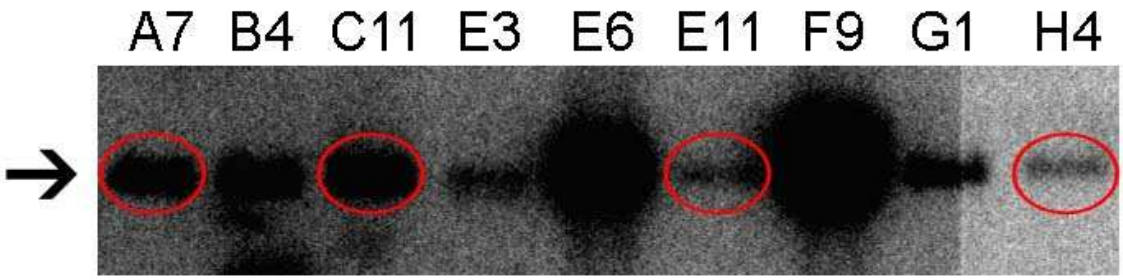


Figure 3.3 Transfected ES cell clones were selected according to their EcoR I band pattern

DNA from embryonic stem (ES) cell clones A7, B4, C11, E3, E6, E11, F9, G1 and H4 was digested with EcoR I restriction enzyme and analysed by Southern blot. The probe was generated to target the C-terminal domain of β-catenin cDNA. A7, C11, E11 and H4 were selected for injection into blastocysts. *This experiment was performed by Dr Thomas Jacques, in the laboratory of Dr. Behrens, Cancer Research UK.*

3.2.4 ΔGSK-β-catenin mice express LacZ in the brain and other organs

During electroporation, vectors are inserted randomly into the genome. Hence, the location of insertion influences the expression level and the expression pattern. To determine the expression pattern of the pcall2-ΔGSK-β-catenin-IRES-GFP insert in ΔGSK-β-catenin mice, brains and somatic organs were stained for β-galactosidase expression. ΔGSK-β-catenin$^{A/-}$ and ΔGSK-β-catenin$^{C/-}$ mice showed strong β-galactosidase expression in the brain, heart and testis (**Figure 3.4** and **Figure 3.5**). Additionally, ΔGSK-β-catenin$^{C/-}$ mice showed weak β-galactosidase expression in the kidney and liver (**Figure 3.4** and **Figure 3.5**). In contrast, ΔGSK-β-catenin$^{H/-}$ mice weakly expressed β-galactosidase in the brain (**Figure 3.4**). ΔGSK-β-catenin$^{-/-}$ littermate control mice did not show any β-galactosidase staining in the organs tested (**Figure 3.4** and **Figure 3.5**).

During development, β-galactosidase expression was strong in ΔGSK-β-catenin$^{A/-}$ and ΔGSK-β-catenin$^{C/-}$ embryos and weak in ΔGSK-β-catenin$^{H/-}$ embryos (**Figure 3.6**).

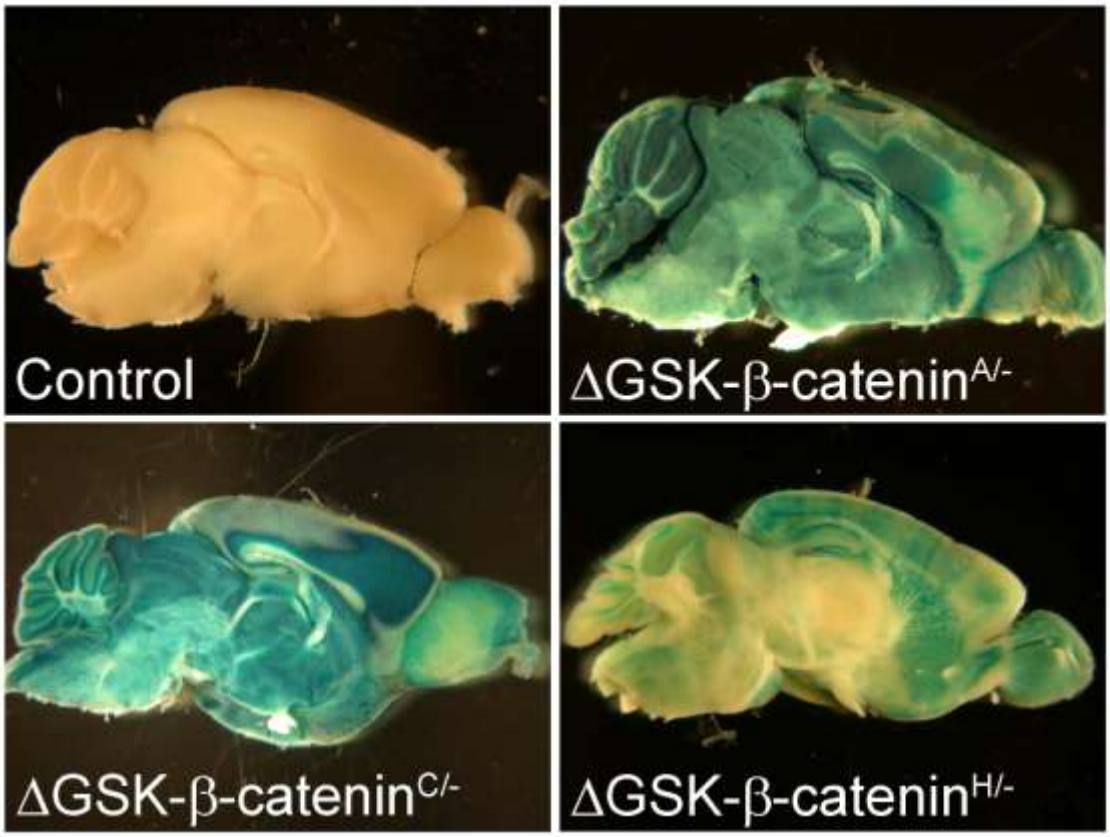


Figure 3.4 ΔGSK-β-catenin transgenic mice express LacZ in the brain

Brains from ΔGSK-β-catenin$^{A/-}$, ΔGSK-β-catenin$^{C/-}$, ΔGSK-β-catenin$^{H/-}$ transgenic mice and ΔGSK-β-catenin$^{-/-}$ littermate control mice were formalin-fixed and stained using the β-galactosidase staining assay. β-galactosidase metabolises X-gal into galactose and an insoluble blue indole compound (5-bromo-4-chloro-3-hydroxyindole). ΔGSK-β-catenin$^{A/-}$ (top right panel) and ΔGSK-β-catenin$^{C/-}$ (bottom left panel) transgenic mice expressed LacZ in all visible areas of the brain (blue staining) except for the corpus callosum and the white matter of the cerebellum. Furthermore, in these mice weak LacZ expression could be detected in the brain stem and olfactory bulb. By comparison, ΔGSK-β-catenin$^{H/-}$ (bottom right panel) expressed LacZ (blue staining) weakly in the cortex, cerebellum, caudate putamen, hippocampus and olfactory bulb. There was no LacZ expression seen in ΔGSK-β-catenin$^{-/-}$ littermate control mice (top left panel).

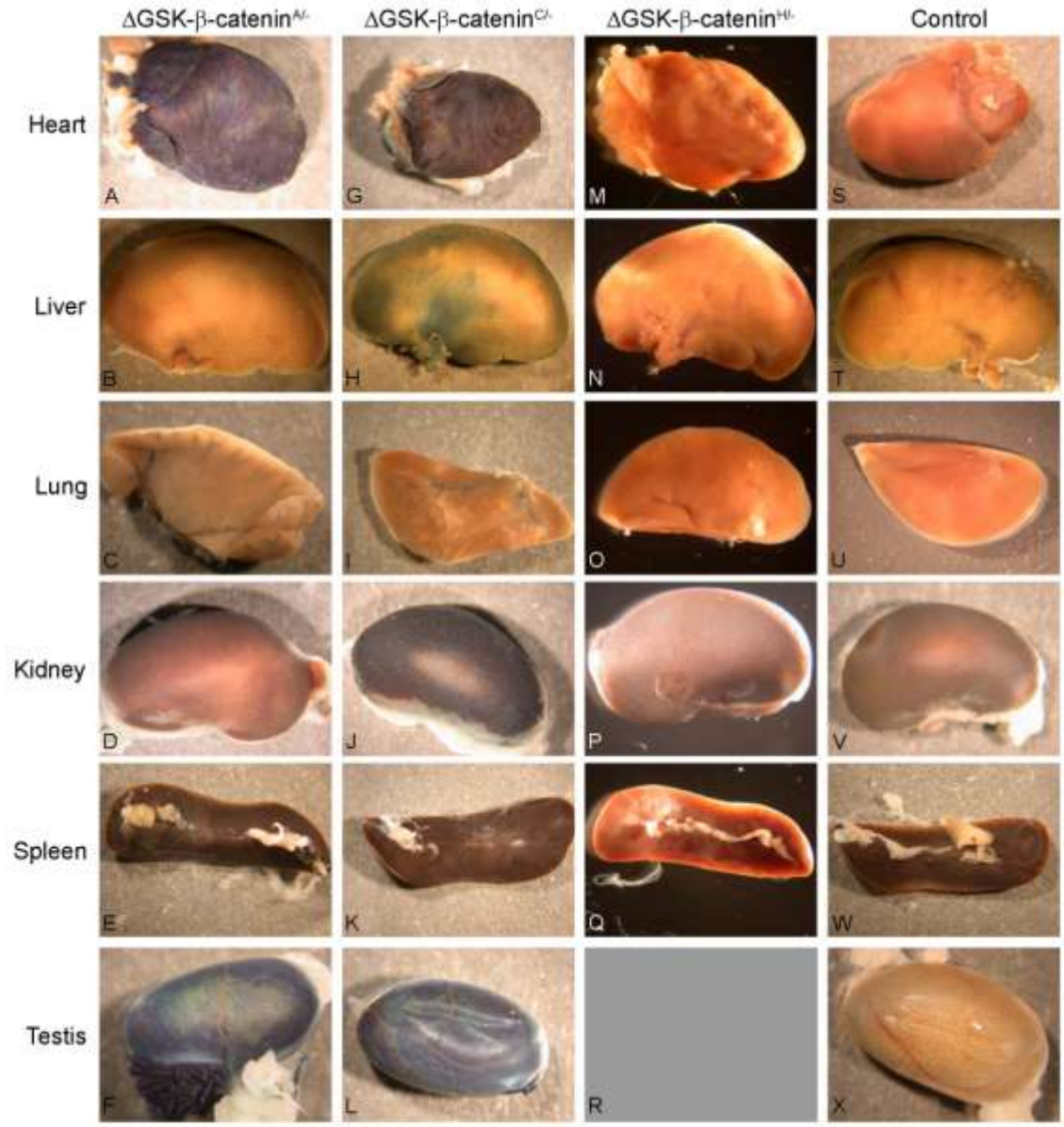


Figure 3.5 LacZ expression in somatic organs of ΔGSK-β-catenin transgenic mice

The heart, liver, lung, kidney, spleen and testis were dissected out from ΔGSK-β-catenin$^{A/-}$ **(A-F)**, ΔGSK-β-catenin$^{C/-}$ **(G-L)**, ΔGSK-β-catenin$^{H/-}$ **(M-R)** and ΔGSK-β-catenin$^{-/-}$ littermate control mice **(S-X)**, were formalin-fixed and stained using the β-galactosidase staining assay. LacZ expression (blue staining) was seen in the heart, lung and testis of ΔGSK-β-catenin$^{A/-}$ and ΔGSK-β-catenin$^{C/-}$ transgenic mice. ΔGSK-β-catenin$^{C/-}$ transgenic mice also showed LacZ expression in the liver and kidney. In contrast, ΔGSK-β-catenin$^{H/-}$ transgenic mice showed no LacZ expression in any of the organs tested. There was no LacZ expression seen in ΔGSK-β-catenin$^{-/-}$ littermate control control mice. The ΔGSK-β-catenin$^{H/-}$ transgenic mouse tested was female, hence no testes were tested.

3.2.5 **Nestin-Cre; ΔGSK-β-catenin mice do not show embryonic lethality**

Previous work by Chenn and Walsh has shown that expression of oncogenic β-catenin (Δ90N-β-catenin) in neuronal progenitor cells results in a gross increase in brain size, which culminates in embryonic lethality (Chenn and Walsh, 2002). To confirm these results in our mouse model, the ΔGSK-β-catenin transgenic mice were crossed with Nestin-Cre transgenic mice. Nestin-Cre transgenic mice express Cre-recombinase under the control of the Nestin promoter, which is active in neural stem cells during development and in adulthood (Lendahl *et al*., 1990; Reynolds and Weiss, 1992). However, Nestin is also expressed in the developing skeletal muscle (Sejersen and Lendahl, 1993). To overcome this, the Nestin-Cre transgenic mice used contained the Nestin second intron downstream of the promoter. These mice express Cre-recombinase specifically in neural stem cells and neural crest cells (Lothian and Lendahl, 1997). In contrast to Chenn and Walsh's findings, Nestin-Cre; ΔGSK-β-catenin transgenic mice were not embryonic lethal, despite strong β-galactosidase expression during development (**Figure 3.6**).

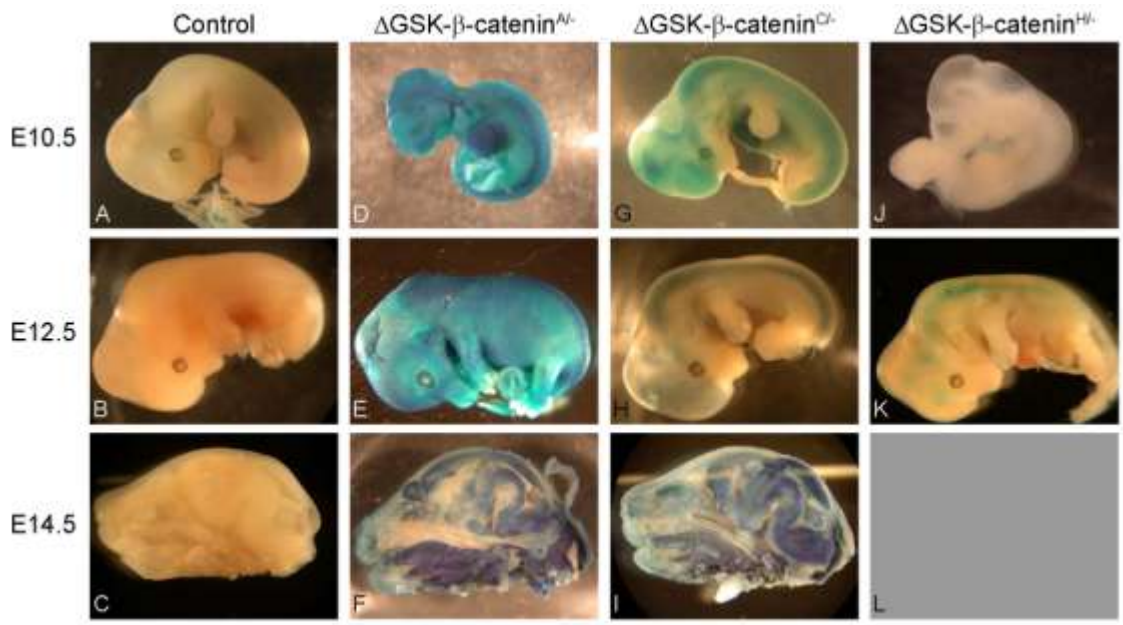


Figure 3.6 The ΔGSK-β-catenin construct is expressed in the CNS during development

ΔGSK-β-catenin$^{-/-}$ littermate control **(A-C)**, ΔGSK-β-catenin$^{A/-}$ **(D-F)**, ΔGSK-β-catenin$^{C/-}$ **(G-I)** and ΔGSK-β-catenin$^{H/-}$ **(J and K)** embryos from embryonic (E) days E10.5, E12.5 and E14.5 were formalin-fixed and stained using the β-galactosidase assay. ΔGSK-β-catenin$^{A/-}$ embryos showed strong LacZ expression in the skin and the CNS during development. Similarly, ΔGSK-β-catenin$^{C/-}$ embryos expressed LacZ in the CNS. In contrast, ΔGSK-β-catenin$^{H/-}$ embryos only expressed LacZ weakly in the CNS. E14.5 embryos from the ΔGSK-β-catenin$^{H/-}$ mouse line were not tested as a result of the weak expression observed at E12.5. ΔGSK-β-catenin$^{-/-}$ littermate control mice showed no LacZ expression at all time points tested.

3.2.6 **Recombination in Nestin-Cre; ΔGSK-β-catenin mouse brains**

To analyse which CNS regions in Nestin-Cre; ΔGSK-β-catenin mice were recombined, brains from Nestin-Cre; ΔGSK-β-catenin$^{A/-}$, Nestin-Cre; ΔGSK-β-catenin$^{C/-}$ and Nestin-Cre; ΔGSK-β-catenin$^{H/-}$ were stained for β-galactosidase expression. In the mouse model studied here, the LacZ gene, which encodes β-galactosidase, is flanked by loxP sites and is excised upon Cre-mediated recombination. Hence, cells that are recombined do not express β-galactosidase and therefore remain unstained. β-galactosidase staining of Nestin-Cre; ΔGSK-β-catenin$^{A/-}$ and Nestin-Cre; ΔGSK-β-catenin$^{C/-}$ brains showed widespread recombination in comparison to controls (**Figure 3.7** and **Figure 3.8**). Recombination was marked in the thalamus, cerebellum, caudate putamen and SVZ (**Figure 3.7** and **Figure 3.8**). Additionally, the hippocampus, hypothalamus, cortex and brainstem were partially recombined (**Figure 3.7** and **Figure 3.8**). Nestin-Cre; ΔGSK-β-catenin$^{H/-}$ brains showed extensive recombination (**Figure 3.7**).

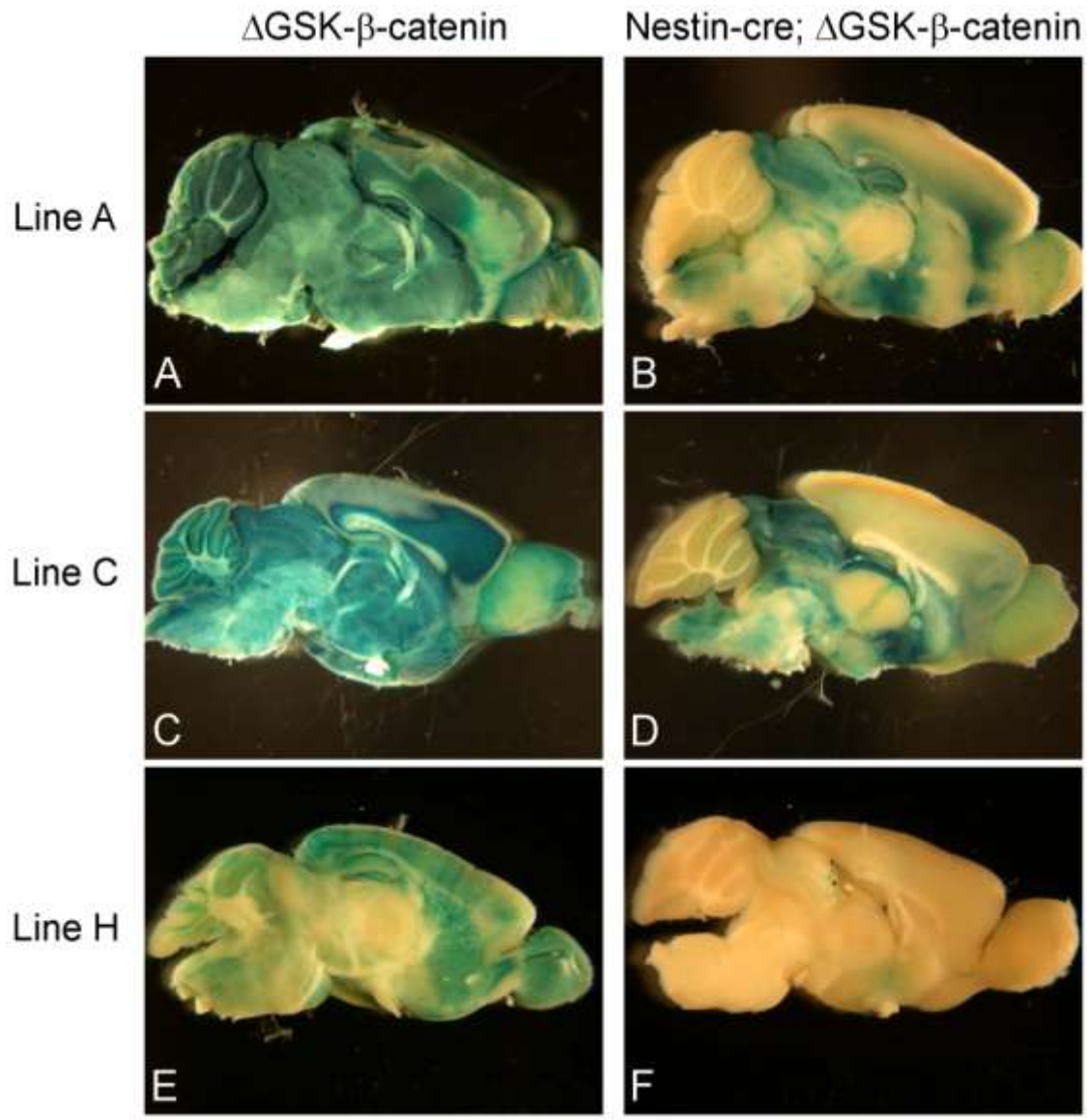


Figure 3.7 Nestin-Cre recombination of ΔGSK-β-catenin mouse brains (sagittal sections)

Following dissection, brains from ΔGSK-β-catenin and Nestin-Cre; ΔGSK-β-catenin transgenic mice were formalin-fixed, sectioned in the sagittal plane and stained using the β-galactosidase assay. Nestin-Cre; ΔGSK-β-catenin$^{A/-}$ (**B**) showed extensive recombination in the cerebellum and thalamus, and partial recombination in the brainstem, cortex and olfactory bulb when compared to a ΔGSK-β-catenin$^{A/-}$ control brains (**A**). Nestin-Cre; ΔGSK-β-catenin$^{C/-}$ brains showed a very similar recombination pattern (**D**). Finally, Nestin-Cre; ΔGSK-β-catenin$^{H/-}$ brains (**F**) were also extensively recombined, compared to control brains **(E)**.

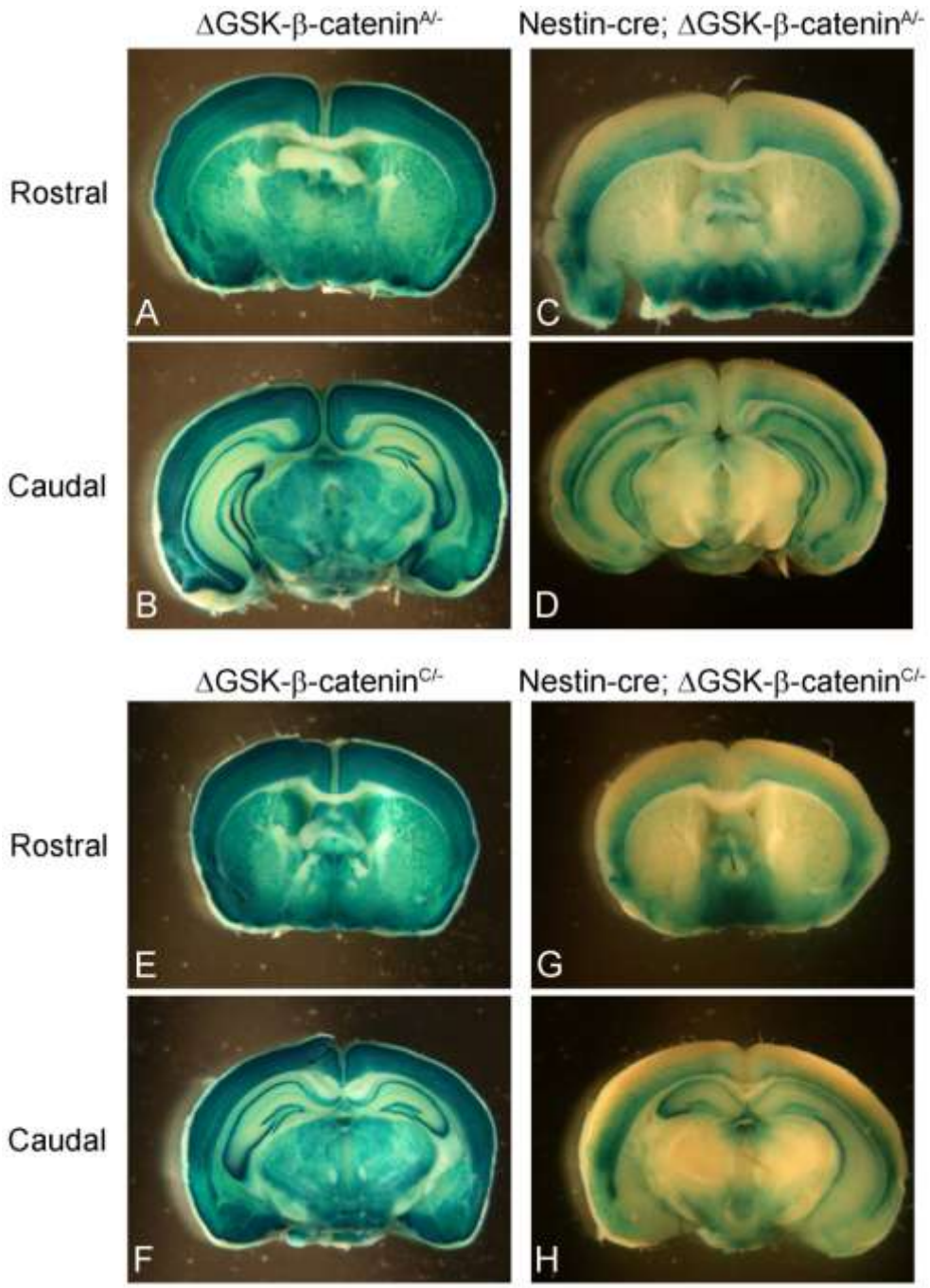
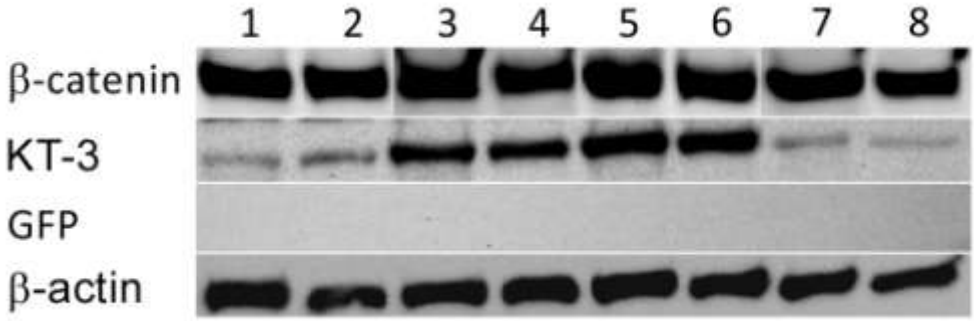


Figure 3.8 Nestin-Cre recombination of ΔGSK-β-catenin mouse brains (coronal sections)

Following dissection, brains from ΔGSK-β-catenin and Nestin-Cre; ΔGSK-β-catenin mice were formalin-fixed, sectioned coronally and stained using the β-galactosidase staining assay. Nestin-Cre; ΔGSK-β-catenin$^{A/-}$ (**C and D**) showed complete recombination in the caudate putamen and the tissue surrounding the lateral ventricle, with partial recombination in the cortex and hippocampus in comparison to ΔGSK-β-catenin$^{A/-}$ control brains (**A and B**). Nestin-Cre; ΔGSK-β-catenin$^{C/-}$ brains (**G and H**) showed a similar recombination pattern compared to ΔGSK-β-catenin$^{C/-}$ control brains (**E and F**).

3.2.7 **Nestin-Cre; ΔGSK-β-catenin mice express ΔGSK-β-catenin in the brain**

Nestin-Cre; ΔGSK-β-catenin brains were recombined but were not found to be enlarged. This could be attributed to a lack of ΔGSK-β-catenin protein expression post-recombination. To investigate this, forebrain and cerebella fractions of brains from Nestin-Cre; ΔGSK-β-catenin$^{A/-}$, Nestin-Cre; ΔGSK-β-catenin$^{C/-}$ and Nestin-Cre; ΔGSK-β-catenin$^{H/-}$ mice were homogenised and tested for β-catenin expression by Western blot analysis. Immunoblotting with an anti-β-catenin antibody showed that Nestin-Cre; ΔGSK-β-catenin$^{A/-}$ and Nestin-Cre; ΔGSK-β-catenin$^{C/-}$ mouse brains expressed marginally higher levels of total β-catenin compared to control brains (**Figure 3.9**). However, in order to selectively visualise ΔGSK-β-catenin protein, brain homogenates were immunoblotted with an anti-KT-3 antibody. KT-3 is a thirteen amino acid tag at the C-terminus of ΔGSK-β-catenin. This demonstrated that ΔGSK-β-catenin protein could be detected in brain homogenates of Nestin-Cre; ΔGSK-β-catenin$^{A/-}$ and Nestin-Cre; ΔGSK-β-catenin$^{C/-}$ mice (**Figure 3.9**). In contrast, very low levels of ΔGSK-β-catenin protein were detected in Nestin-Cre; ΔGSK-β-catenin$^{H/-}$ brain homogenates in comparison to controls (**Figure 3.9**). Control brain homogenates from C57Bl/6J mice also showed a weak band of the same size as ΔGSK-β-catenin (**Figure 3.9**). This was one of several non-specific bands seen as a result of the cross-reaction of the anti-KT-3 antibody with other targets present in the homogenates. GFP protein was not detected after recombination (**Figure 3.9**).



1 Nestin-cre; ΔGSK-β-catenin$^{H/-}$ forebrain
2 Nestin-cre; ΔGSK-β-catenin$^{H/-}$ cerebellum
3 Nestin-cre; ΔGSK-β-catenin$^{C/-}$ forebrain
4 Nestin-cre; ΔGSK-β-catenin$^{C/-}$ cerebellum
5 Nestin-cre; ΔGSK-β-catenin$^{A/-}$ forebrain
6 Nestin-cre; ΔGSK-β-catenin$^{A/-}$ cerebellum
7 C57Bl/6 forebrain
8 C57Bl/6 cerebellum

Figure 3.9 Nestin-Cre recombination activates the expression of ΔGSK-β-catenin

Forebrain and cerebellar homogenates from Nestin-Cre; ΔGSK-β-catenin$^{A/-}$, Nestin-Cre; ΔGSK-β-catenin$^{C/-}$ and Nestin-Cre; ΔGSK-β-catenin$^{H/-}$ transgenic mice as well as C57Bl/6J control mice were immunoblotted for β-catenin (1:500), KT3 (1:5000), GFP (1:500) and β-actin (1:1000). 10 µg of total protein was loaded per lane. Expression levels of total β-catenin were not significantly increased after recombination. ΔGSK-β-catenin is tagged with KT-3 at the C-terminus. Immunoblotting with anti-KT-3 antibody showed that ΔGSK-β-catenin protein could be detected in the forebrain and cerebellum of Nestin-Cre; ΔGSK-β-catenin$^{A/-}$ (**lanes 5 and 6**), Nestin-Cre; ΔGSK-β-catenin$^{C/-}$ (**lanes 3 and 4**) mice. Nestin-Cre; ΔGSK-β-catenin$^{H/-}$ mice (**lanes 1 and 2**) expressed little or no ΔGSK-β-catenin. C57Bl/6J brains (**lanes 7 and 8**) showed a faint band with an equivalent size to ΔGSK-β-catenin protein. This was one of several unspecific bands seen as a result of the cross-reaction of the anti-KT-3 antibody with other targets. β-actin was used as an internal loading control .

3.2.8 ΔGSK-β-catenin expression does not alter brain architecture or cellular morphology

Expression of ΔGSK-β-catenin in Nestin-positive cells was not embryonic lethal as previously reported (Chenn and Walsh, 2002). However, it is possible that ΔGSK-β-catenin expression could exert a more a subtle effect on brain morphogenesis. To examine this possibility, brains from Nestin-Cre; ΔGSK-β-catenin$^{A/-}$ mice were immunohistochemically stained for the neuronal markers NeuN, MAP2 and neurofilament 200 (NF200), the glial marker GFAP, and for β-catenin protein. Stronger β-catenin staining was detected in the hippocampus, cortex and thalamus, but not in the cerebellum of recombined mice in comparison to ΔGSK-β-catenin$^{A/-}$ control brains (**Figure 3.10, Figure 3.11** and **Figure 3.12**). However, no morphological defects or tumours were detected in any region analysed (**Figure 3.10, Figure 3.11** and **Figure 3.12**). Of note, some morphological differences were observed in the cerebellum and hippocampus (**Figure 3.10** and **Figure 3.11**). However, these were attributed to variations in the depth of the brain sections rather than being caused by expression of ΔGSK-β-catenin.

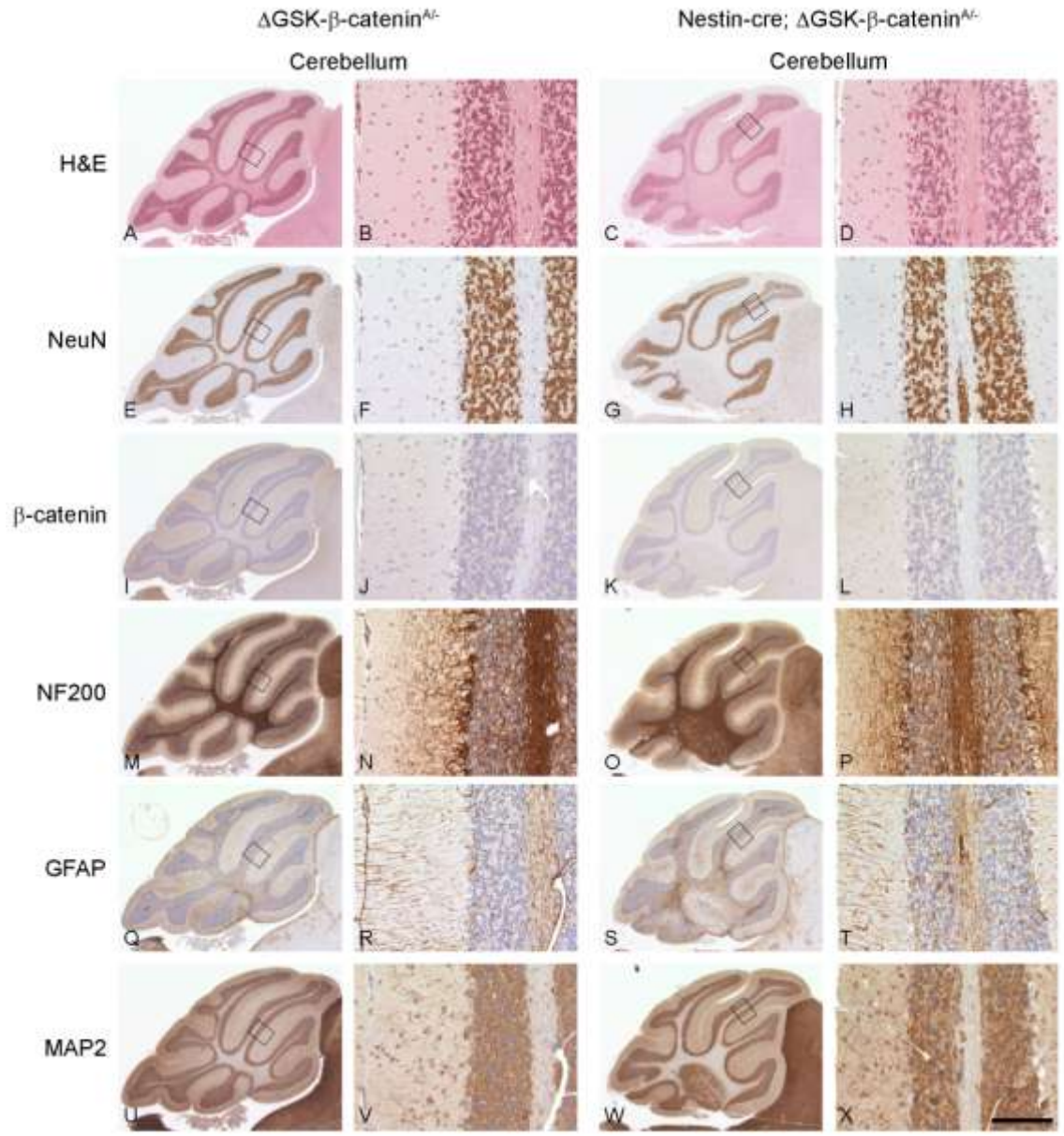


Figure 3.10 No abnormalities were detected in the cerebellum of Nestin-Cre; ΔGSK-β-catenin mice

Immunohistochemical studies of control ΔGSK-β-catenin$^{A/-}$ and Nestin-Cre; ΔGSK-β-catenin$^{A/-}$ mice. Cerebella were stained with H&E (**A-D**), NeuN (**E-H**), β-catenin (**I-L**), NF200 (**M-P**), GFAP (**Q-T**) and MAP2 (**U-X**). The first and second columns correspond to low and high power magnification of a control ΔGSK-β-catenin$^{A/-}$ cerebellum. The third and fourth columns correspond to low and high power magnification of a Nestin-Cre; ΔGSK-β-catenin$^{A/-}$ cerebellum (note the slightly deeper (i.e. lateral) section of this brain compared to the control). High power magnification images correspond to the fifth and sixth lobule of the cerebellum (black box). β-catenin staining in Nestin-Cre; ΔGSK-β-catenin$^{A/-}$ was not altered after recombination (**K and L**) compared to control (**I and J**). Furthermore, recombined brains showed no abnormalities in comparison to controls. The structural differences observed between ΔGSK-β-catenin$^{A/-}$ and Nestin-Cre; ΔGSK-β-catenin$^{A/-}$ are explained by the lateral depth of the histological sections. The scale bar in **X** corresponds to 1300 μm and 140 μm for low power and high power magnification respectively.

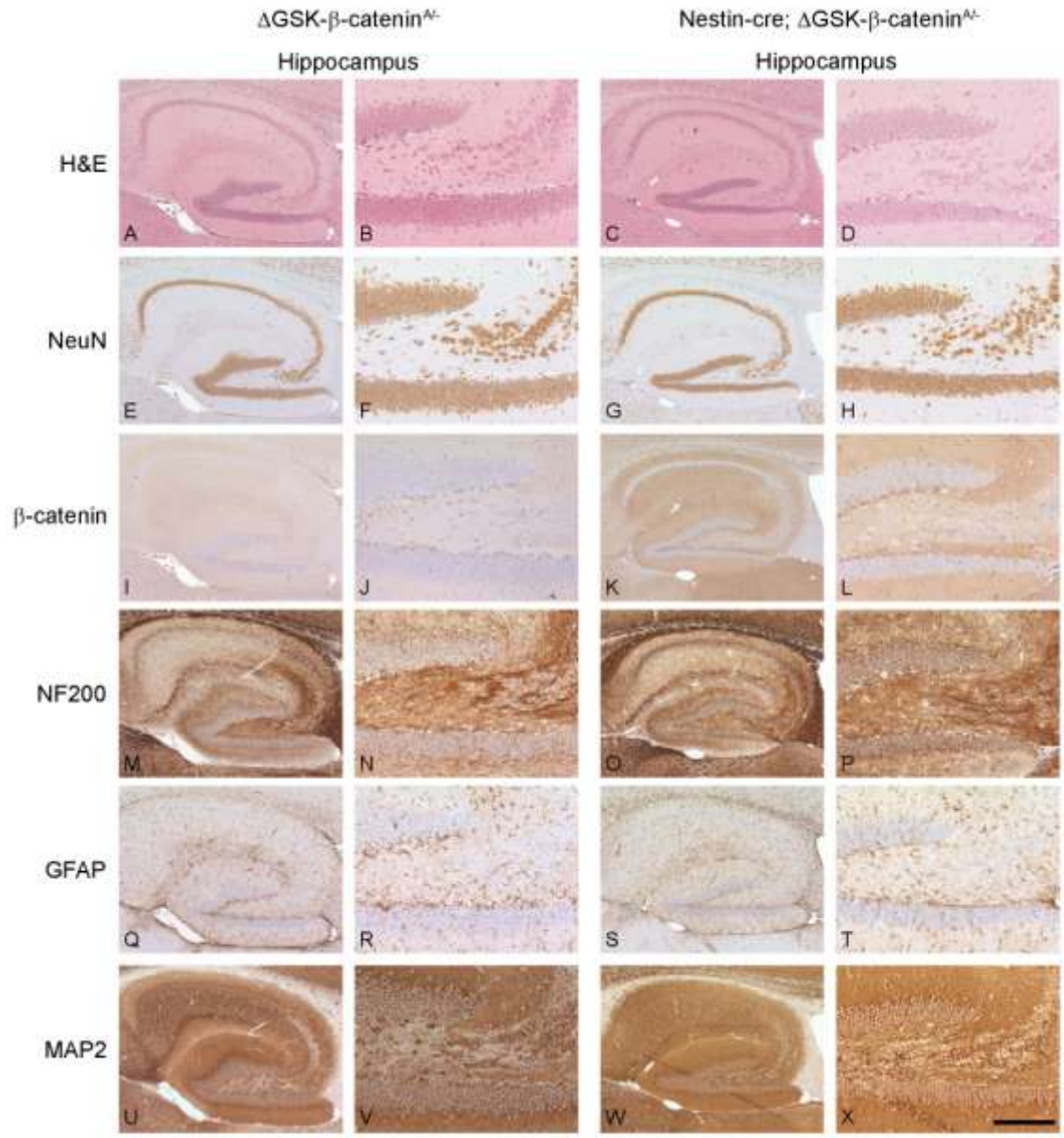


Figure 3.11 No abnormalities in the hippocampus of Nestin-Cre; ΔGSK-β-catenin brains

Immunohistochemical studies of control ΔGSK-β-catenin$^{A/-}$ and Nestin-Cre; ΔGSK-β-catenin$^{A/-}$ mice. Hippocampi were stained with H&E (**A-D**), NeuN (**E-H**), β-catenin (**I-L**), NF200 (**M-P**), GFAP (**Q-T**) and MAP2 (**U-X**). The first and second columns correspond to low and high power magnification of a control ΔGSK-β-catenin$^{A/-}$ hippocampus. The third and fourth columns correspond to low and high power magnification of a Nestin-Cre; ΔGSK-β-catenin$^{A/-}$ hippocampus. β-catenin staining was stronger after recombination (**K and L**) compared to control (**I and J**), but no abnormalities were detected. The structural differences between ΔGSK-β-catenin$^{A/-}$ and Nestin-Cre; ΔGSK-β-catenin$^{A/-}$ are explained by the lateral depth of the histological sections. The scale bar in **X** corresponds to 700 μm and 220 μm for low power and high power magnification respectively.

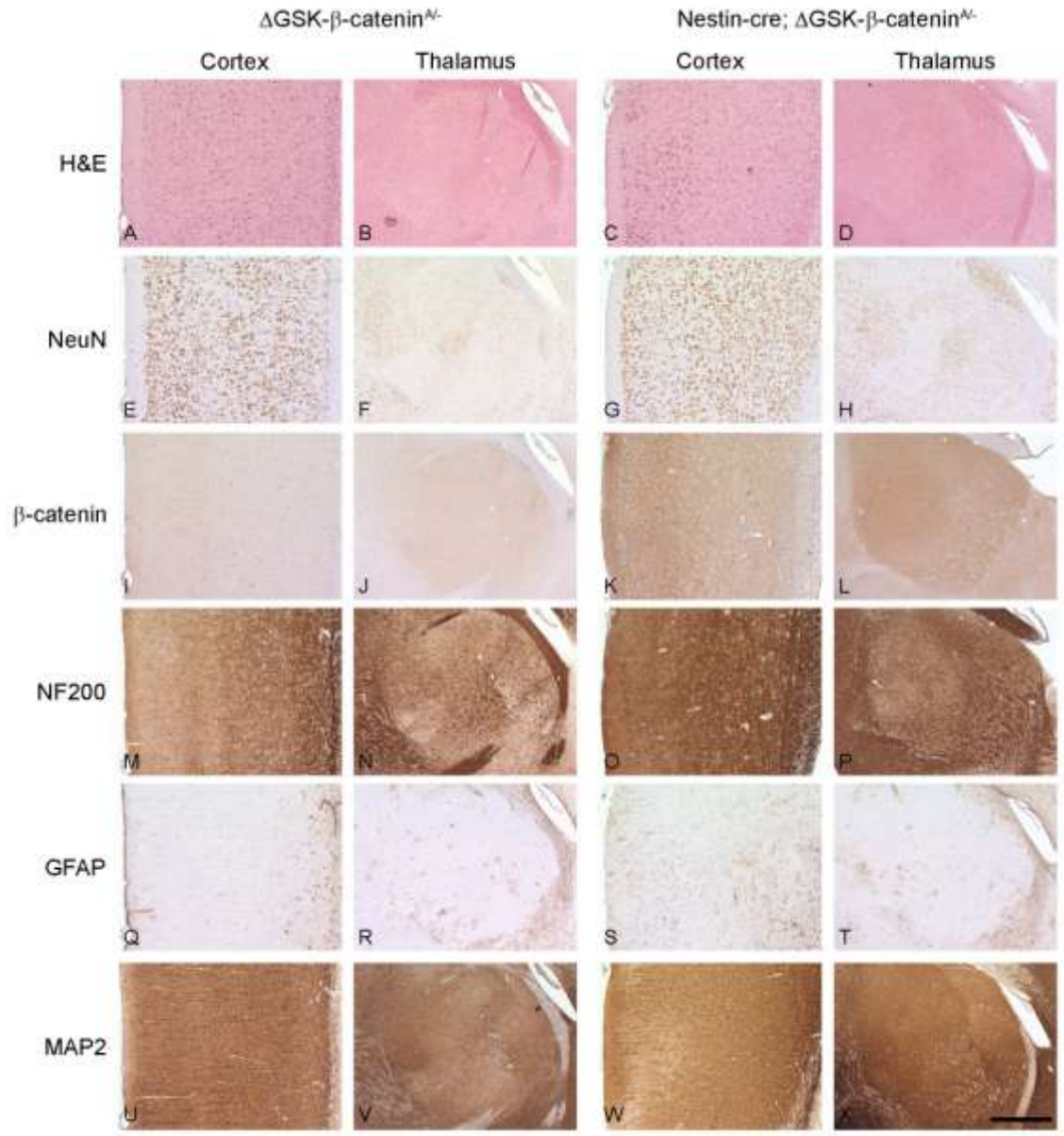


Figure 3.12 No abnormalities in the cortex and thalamus of Nestin-Cre; ΔGSK-β-catenin brains

Immunohistochemical studies of control ΔGSK-β-catenin$^{A/-}$ and Nestin-Cre; ΔGSK-β-catenin$^{A/-}$ mice. Cortex and thalamus was stained with H&E (**A-D**), NeuN (**E-H**), β-catenin (**I-L**), NF200 (**M-P**), GFAP (**Q-T**) and MAP2 (**U-X**). The first and second columns correspond to control ΔGSK-β-catenin$^{A/-}$ cortex and thalamus respectively. The third and fourth columns correspond to Nestin-Cre; ΔGSK-β-catenin$^{A/-}$ cortex and thalamus respectively. β-catenin staining in Nestin-Cre; ΔGSK-β-catenin$^{A/-}$ (**K and L**) was stronger after recombination compared to control (**I and J**), but no abnormalities were detected. The structural differences between ΔGSK-β-catenin$^{A/-}$ and Nestin-Cre; ΔGSK-β-catenin$^{A/-}$ are explained by the lateral depth of the histological sections. The scale bar in **X** corresponds to 850 μm and 450 μm for low power and high power magnification respectively.

3.3 **Discussion**

The Wnt/β-catenin pathway plays an important role in the development of the CNS and maintenance of neural stem cells (Thomas and Capecchi, 1990; Wexler *et al*., 2009). Certain CNS tumours show aberrant activation of the Wnt/β-catenin pathway (**Table 1.5**). For example, patients with APC germline mutations have an increased risk of developing medulloblastomas, astrocytomas and ependymomas (Attard *et al*., 2007). Similarly, mutations of β-catenin, APC and Axin have been found in approximately 17 % of medulloblastomas (Dahmen *et al*., 2001; Huang *et al*., 2000; Koch *et al*., 2001; Rogers *et al*., 2009; Yokota *et al*., 2002; Zurawel *et al*., 1998). Furthermore, mutations have also been detected in CNS PNET, NOS and glioblastomas, albeit rarely (Gotze *et al*., 2009; Koch *et al*., 2001; Nikuseva-Martic *et al*., 2007; Rogers *et al*., 2009). On the other hand, the Wnt/β-catenin pathway is commonly de-regulated by other mechanisms, as shown by nuclear β-catenin immunoreactivity, a marker of pathway activity (Eberhart *et al*., 2000; Terris *et al*., 1999). Indeed, around 23 % of medulloblastomas and 35 % of CNS PNET, NOS cases have been shown to be positive for nuclear β-catenin, a much higher rate than detected pathway mutations (Eberhart *et al*., 2000; Ellison *et al*., 2005; Rogers *et al*., 2009; Yokota *et al*., 2002). Only about 1 % of glioblastomas harbour β-catenin mutations (Gotze *et al*., 2009; Nikuseva-Martic *et al*., 2007). However, a recent study has reported that promoter hypermethylation of Wnt inhibitors is common in primary and secondary glioblastomas (Gotze *et al*., 2009). Furthermore, expression levels of β-catenin, TCF4, LEF1 and β-catenin downstream targets are increased in glioblastomas and lower grade astrocytic tumours (Sareddy *et al*., 2009b). Interestingly, the expression levels of these targets correlate with malignancy, which suggests that the Wnt/β-catenin pathway may play a role in glioma progression (Sareddy *et al*., 2009a; Sareddy *et al*., 2009b).

The role of the Wnt/β-catenin pathway in tumours of the CNS is not completely understood. To study putative roles of this pathway in CNS tumours, transgenic mice, which conditionally express ΔGSK-β-catenin, were generated. ΔGSK-β-catenin has four point mutations at key phosphorylation residues, which renders the protein resistant to degradation (Barth *et al*., 1999). As a result, ΔGSK-β-catenin can constitutively activate the Wnt/β-catenin pathway (Barth *et al*., 1999). This was also demonstrated in ES cells, where ΔGSK-β-catenin expression up-regulates the expression of Wnt/β-catenin downstream target genes and inhibits neuronal differentiation (Haegele *et al*., 2003). This phenotype has been reproduced by expressing truncated APC (Haegele *et al*., 2003). Collectively, these studies provide strong evidence for the ability of ΔGSK-β-catenin to activate the Wnt/β-catenin pathway.

The transgenic mice described in this chapter express the LacZ gene prior to recombination. This allowed for identification of cells expressing the pcall2-ΔGSK-β-catenin-IRES-GFP insert using the β-galactosidase staining assay. LacZ expression was widespread in the CNS during development and in adulthood (**Figure 3.4** and **Figure 3.6**). Expression could also be detected in somatic organs, especially in the testis and heart (**Figure 3.5**). In 2002, Chenn and Walsh reported that the expression of dominant active β-catenin (Δ90N-β-catenin) in Nestin-expressing cells resulted in severe CNS abnormalities (Chenn and Walsh, 2002). This was attributed to an increase in the proportion of ventricular zone (VZ) neural precursors, which continued to divide instead of differentiating (Chenn and Walsh, 2002). To compare this result to our experimental models, ΔGSK-β-catenin mice were crossed with Nestin-Cre transgenic mice. In the ΔGSK-β-catenin mice, recombination can be detected by loss of LacZ expression (**Figure 3.1**). However, despite widespread recombination, Nestin-Cre; ΔGSK-β-catenin transgenic mice did not show any developmental abnormalities (**Figure 3.7** and **Figure 3.8**). Of relevance, recombination was observed in the tissue surrounding the lateral ventricle, where the SVZ is located, which is anatomically related to the developmental VZ (**Figure 3.8**). However, the SVZ was not enlarged in these mice. These results suggest that the ΔGSK-β-catenin protein may not be expressed after recombination. No difference in total β-catenin protein levels was detected between ΔGSK-β-catenin and Nestin-Cre; ΔGSK-β-catenin mouse brains (**Figure 3.9**). Of note, ΔGSK-β-catenin protein contains a C-terminal KT3 tag. Using an antibody raised against KT-3, ΔGSK-β-catenin protein was detected in brain homogenates from Nestin-Cre; ΔGSK-β-catenin$^{A/-}$ and Nestin-Cre; ΔGSK-β-catenin$^{C/-}$ mice, but not in brain homogenates from control mice. A faint band could be detected in ΔGSK-β-catenin$^{H/-}$ brain homogenates. However a similar band was also detected in C57Bl/6 homogenates. Therefore this faint band is likely to be non-specific binding of the antibody to other targets. In cancers with β-catenin mutations, 50 % of the expressed β-catenin is degradation-resistant. This raises the possibility that low-level expression of ΔGSK-β-catenin may not be sufficient to promote CNS abnormalities. However, in theory, degradation-resistant β-catenin should be able to constitutively activate the Wnt/β-catenin pathway irrespective of expression levels. This suggests that Nestin-Cre; ΔGSK-β-catenin mice may have a more subtle phenotype than that observed in the Nestin-Δ90N-β-catenin mice.

To assess this phenotype, brains from Nestin-Cre; ΔGSK-β-catenin mice were processed for immunohistological analysis. Brains were immunostained for the neuronal markers, NeuN, MAP2 and NF200, the glial marker GFAP, and β-catenin. No morphological differences were found between Nestin-Cre; ΔGSK-β-catenin and control ΔGSK-β-catenin mice (**Figure 3.10, Figure 3.11** and **Figure 3.12**). β-catenin staining was stronger in the hippocampus,

cortex and thalamus of Nestin-Cre; ΔGSK-β-catenin brains. These findings correlated with the loss of LacZ staining (**Figure 3.7** and **Figure 3.8**). However, no difference in β-catenin staining was detected in the cerebellum, despite extensive recombination (**Figure 3.7** and **Figure 3.10**). The β-catenin antibody detects both wild-type β-catenin and ΔGSK-β-catenin. Therefore, the small increase in total β-catenin levels observed by Western blotting may not be sufficient to be visualised histologically in the cerebellum (**Figure 3.9**).

3.4 **Summary**

Transgenic mice, which conditionally expressed degradation-resistant β-catenin were generated. However, despite extensive recombination in the CNS and expression of the mutant β-catenin no developmental abnormalities were detected. To investigate the lack of a phenotype, we then extended our studies to *in vitro* systems to detect potential subtle functional effects of ΔGSK-β-catenin in neural stem cells.

4 *In vitro* analysis of ΔGSK-β-catenin expression

4.1 Background

The hierarchical model for tumour development (**section 1.2**) postulates that only a subset of cells in a tumour is capable of re-generating the bulk of the tumour. This model is in keeping with the CSC hypothesis, which suggests that tumours are initiated and maintained by a population of cells with stem-like properties (Al Hajj *et al*., 2003; Bonnet and Dick, 1997). These cells have been identified in many types of cancer (Al Hajj *et al*., 2003; Bonnet and Dick, 1997; O'Brien *et al*., 2007; Ricci-Vitiani *et al*., 2007). Evidence suggests that stem cells may be the cell of origin of tumours and CSCs (**section 1.2**).

Developmental pathways are vital for stem cell maintenance and are often dysregulated in CSCs. Such pathways include the Shh, Notch, Bone morphogenic protein (BMP), TGF-β and the Wnt pathway. The Wnt/β-catenin pathway plays a complex role in stem cells, regulating proliferation and differentiation (Chenn and Walsh, 2002; Hirabayashi *et al*., 2004; Lie *et al*., 2005; Wexler *et al*., 2008; Wrobel *et al*., 2007). For example, activation of the Wnt/β-catenin pathway in crypt progenitor cells of the gut has been shown to promote their proliferation (Sansom *et al*., 2004). Interestingly, constitutive activation of the Wnt/β-catenin pathway in these progenitors promotes the development of Adenomas (Barker *et al*., 2009).

The Wnt/β-catenin pathway plays an important role in NSC maintenance during development (Lee *et al*., 2000; Thomas and Capecchi, 1990). For example, Wnt1 knockout mice show a marked decrease in the expansion of neural precursors during mid/hindbrain development (Mcmahon and Bradley, 1990; Thomas and Capecchi, 1990). Similarly, Wnt3a knockout mice show a reduced proliferation of precursor cells in the caudomedial cortical epithelium (Lee *et al*., 2000). Conversely, increasing Wnt1 expression in turn increases proliferation and leads to the expansion of the developing mid/hindbrain (Castelo-Branco *et al*., 2003; Panhuysen *et al*., 2004).

The Wnt/β-catenin pathway is also vital for adult NSCs. Several Wnt family members are expressed in the dentate gyrus, the stem cell niche of the hippocampus (Lie *et al*., 2005; Shimogori *et al*., 2004). Injection of a lentivirus expressing Wnt3 into the dentate gyrus of adult rats doubled the number of newly generated neurones (Lie *et al*., 2005). In contrast, injection with a lentivirus expressing DKK1, a Wnt antagonist, into the same area, decreased the amount of new neurones by 13 % (Lie *et al*., 2005). These results suggest that the Wnt/β-catenin pathway is an important regulator of hippocampal progenitor proliferation and differentiation. However, the role of the Wnt/β-catenin pathway in the sub-ventricular zone (SVZ) stem cell

niche is not completely understood. Interestingly, a report by Adachi *et al*. suggests that activation of this pathway in SVZ precursors promotes proliferation and inhibits differentiation (Adachi *et al*., 2007).

4.1.1 **Aims**

The aims of this study were to determine the effect of ΔGSK-β-catenin expression in NSCs and to study the synergism between Wnt/β-catenin pathway activation and p53 or Rb knockout in NSCs and brain tumourigenesis.

4.1.2 **Methods**

NSC cultures were derived from the sub-ventricular zone (SVZ) and hippocampus of adult ΔGSK-β-catenin$^{A/-}$, ΔGSK-β-catenin$^{C/-}$, ΔGSK-β-catenin$^{A/A}$; p53$^{loxP/loxP}$ and ΔGSK-β-catenin$^{A/A}$; Rb$^{loxP/loxP}$ mice (**section 2.4.2**). NSCs were infected with Adeno-Cre to mediate recombination (**section 2.4.3**). Western blotting was used to detect levels of ΔGSK-β-catenin and cyclin D1 (**section 2.6**). NSC growth was assayed with the WST-1 assay (**section 2.4.4**), Hoechst assay (**section 2.4.5**) and neurosphere size assay (**section 2.4.6**). Self-renewal was determined with the limited dilution assay (**section 2.4.7**). Neurospheres were differentiated (**section 2.4.8**), and the multi-lineage potential of NSCs was assayed using immunocytochemistry (**section 2.4.9**). Nestin-Cre; ΔGSK-β-catenin$^{A/-}$; p53$^{loxP/loxP}$ were analysed histologically (**section 2.5**). ΔGSK-β-catenin$^{A/-}$; p53$^{loxP/loxP}$ mice were injected intra-ventricularly with Adeno-Cre to assess the interaction between p53 and the Wnt/β-catenin pathway, and ultimately to test a potentially tumourigenic effect (**section 2.1.4**).

4.2 Results

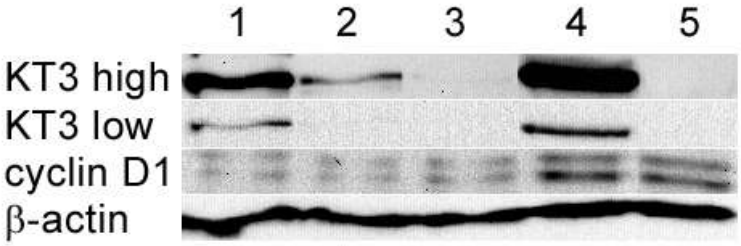
4.2.1 *In vitro* expression of ΔGSK-β-catenin does not influence neuronal stem cell biology

Nestin-Cre; ΔGSK-β-catenin mice did not develop tumours or gross abnormalities (**chapter 3**). Transgenic mice expressing Δ90Nβ-catenin under the control of the Nestin promoter showed enlarged ventricles as a result of an increase in the pool of sub-ventricular zone (SVZ) progenitors (Chenn and Walsh, 2002). Chapter 3 shows that although the SVZ appears to be recombined in Nestin-Cre; ΔGSK-β-catenin mice, it is not enlarged (**Figure 3.8**). As previously discussed, absence of abnormalities may be explained by low expression of ΔGSK-β-catenin, but this could still result in a more subtle effect on NSCs. To test this hypothesis, NSCs from adult ΔGSK-β-catenin mice were cultured *in vitro*. Expression of ΔGSK-β-catenin was achieved by infecting the cells with an Adenovirus expressing Cre-recombinase (Adeno-Cre) (**Figure 4.1**). To control for the effect of the virus, a percentage of the cells was infected with Adenovirus expressing GFP (Adeno-GFP).

The WST-1 proliferation assay and neurosphere size assay were used to measure the growth of ΔGSK-β-catenin$^{A/-}$ and ΔGSK-β-catenin$^{C/-}$ expressing NSCs. Despite ΔGSK-β-catenin expression after recombination, there was no significant growth difference in Adeno-Cre recombined NSCs compared to controls (**Figure 4.2** and **Figure 4.3**).

The Ccnd1 gene encodes the protein Cyclin D1, which is up-regulated during Wnt/β-catenin signalling (Shtutman *et al*., 1999; Tetsu and McCormick, 1999). Immunoblotting with anti-Cyclin D1 antibody did not show increased levels of Cyclin D1 in Adeno-Cre treated ΔGSK-β-catenin (**Figure 4.1**).

Similarly, no difference in self-renewal was observed using the limited dilution assay (**Figure 4.4**).



1 Nestin-cre; ΔGSK-β-catenin$^{A/-}$ forebrain
2 Adeno-cre ΔGSK-β-catenin$^{C/-}$ NSCs
3 Adeno-GFP ΔGSK-β-catenin$^{C/-}$ NSCs
4 Adeno-cre ΔGSK-β-catenin$^{A/-}$ NSCs
5 Adeno-GFP ΔGSK-β-catenin$^{A/-}$ NSCs

Figure 4.1 Adeno-Cre infected NSCs express ΔGSK-β-catenin, but do not show increased cyclin D1 levels

Nestin-Cre; ΔGSK-β-catenin$^{A/-}$ brain homogenates (**lane 1**), ΔGSK-β-catenin$^{A/-}$ and ΔGSK-β-catenin$^{C/-}$ NSCs (**lanes 2-5**) were immunoblotted using anti-KT-3 (1:5000), cyclin D1 (1:2000) and β-actin (1:1000) antibodies. 10 μg of total protein was loaded per lane. 'KT-3 high' refers to long blot exposure and 'KT-3 low' refers to short blot exposure. ΔGSK-β-catenin protein is only expressed in ΔGSK-β-catenin$^{A/-}$ and ΔGSK-β-catenin$^{C/-}$ NSCs that have been infected with Adeno-Cre (**lanes 2 and 4 respectively**). Adeno-Cre-treated ΔGSK-β-catenin$^{A/-}$ NSCs express markedly more ΔGSK-β-catenin than Adeno-Cre-treated ΔGSK-β-catenin$^{C/-}$ NSCs. Cyclin D1 protein levels were unchanged (**lane 2 versus lane 3 and lane 4 versus lane 5**). Nestin-Cre; ΔGSK-β-catenin$^{A/-}$ brain homogenates were used as positive controls. β-actin was used as an internal loading control.

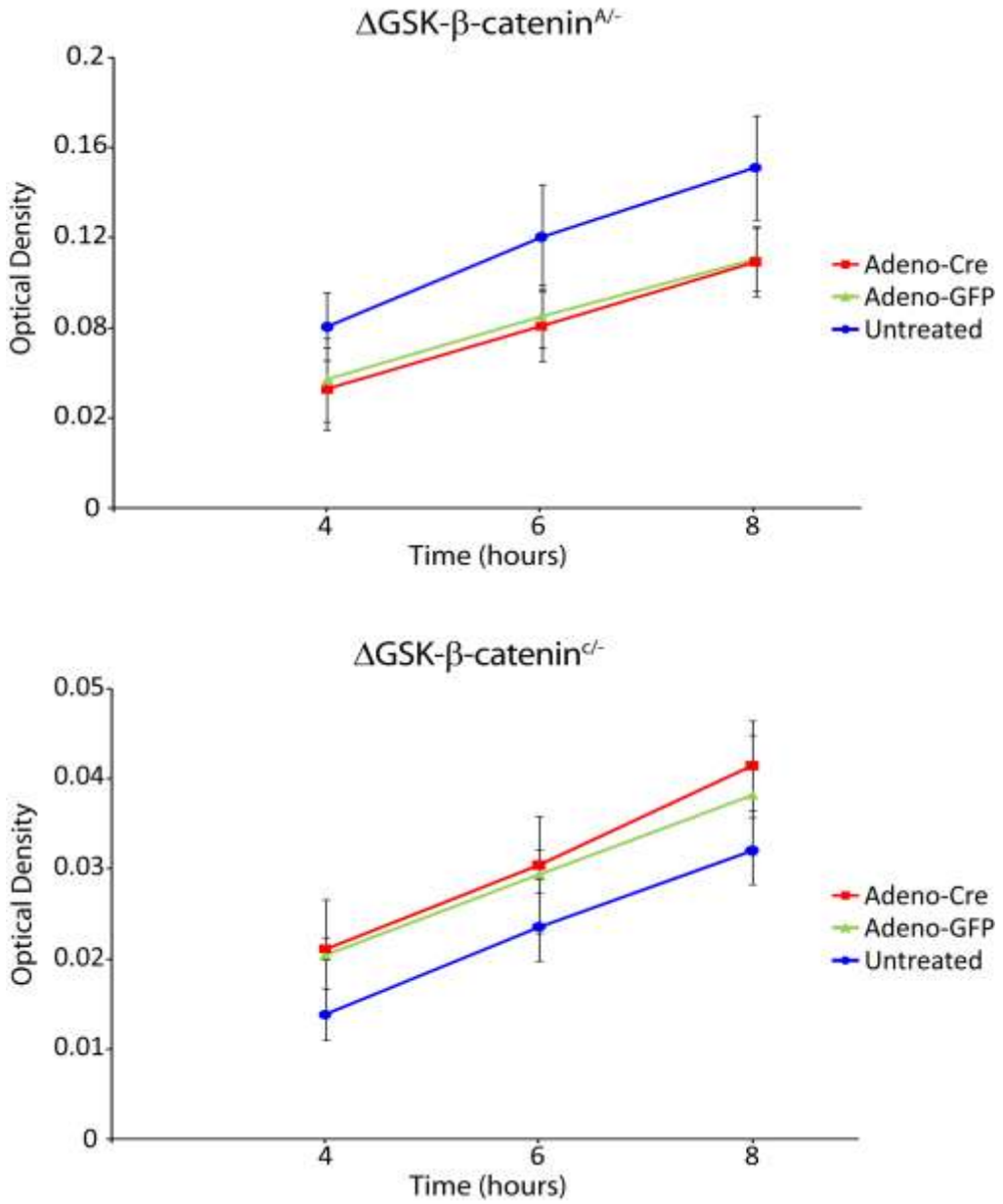


Figure 4.2 Adeno-Cre infected NSCs do not show increased proliferation potential

Neurospheres were passaged to a single cell suspension and plated at a concentration of $6x10^5$ cells/ml. Three days following passage, the WST-1 reagent was added. The reagent is metabolised by mitochondria into a coloured formazan salt and the optical density (OD) measured at 440nm, which correlates with cell number. The OD was measured 4, 6 and 8 hours after WST-1 addition. The results are from three independent cultures for each line. Each experiment represents an average of ten wells. No significant difference was detected between any two treatment groups at any time point for ΔGSK-β-catenin$^{A/-}$ and ΔGSK-β-catenin $^{C/-}$ NSCs (two-tailed t-test). OD values where tested for normality and homoscedasticity (i.e. all random variables have the same variance).

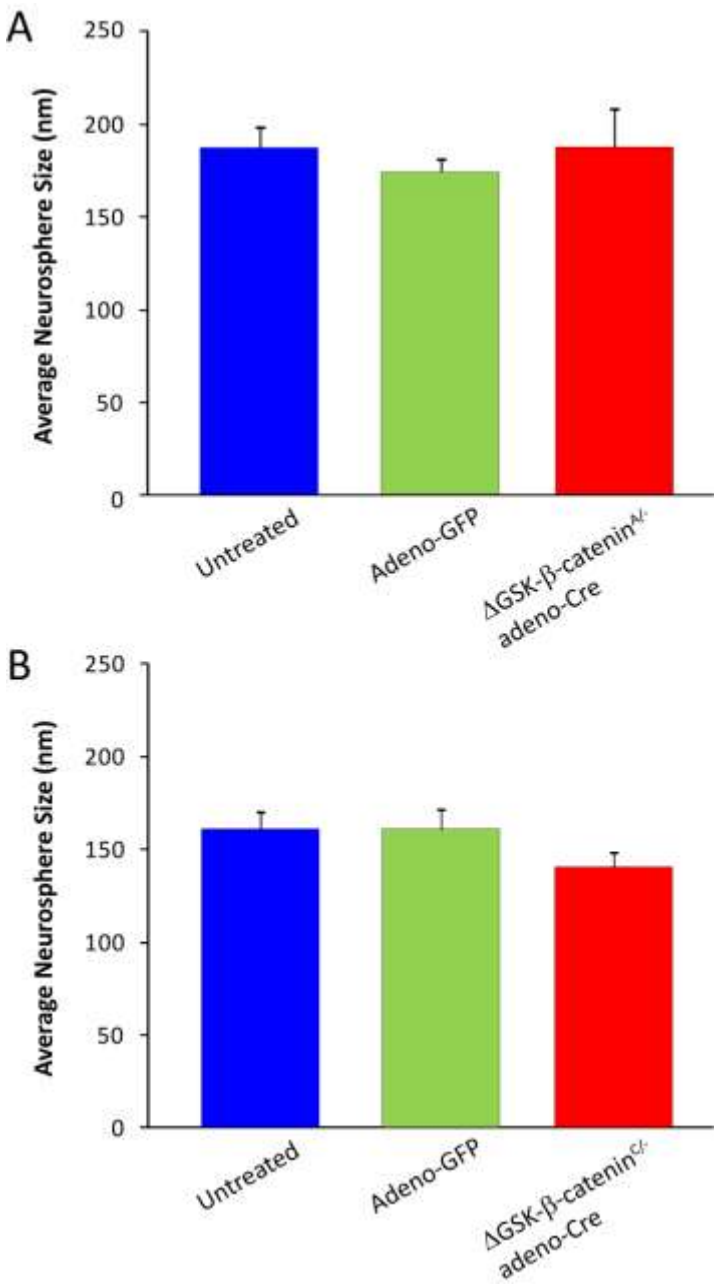


Figure 4.3 Adeno-Cre infected NSCs are not bigger than controls

Neurospheres were passaged to a single cell suspension and $0.5x10^6$cells were plated on a 6 cm plate. Ten days following passage, the diameter of the neurospheres was measured using an Axiovert 135 microscope (Zeiss) and the Openlab 5 software. An n=3 was calculated from three separate cultures for each cell line. Each experiment represents the average of 100 neurospheres. There was no significant difference in neurosphere diameter for ΔGSK-β-catenin$^{A/-}$ (**A**) or ΔGSK-β-catenin$^{C/-}$ (**B**) NSCs. Neurosphere diameter measurements where checked for normality and homoscedasticity.

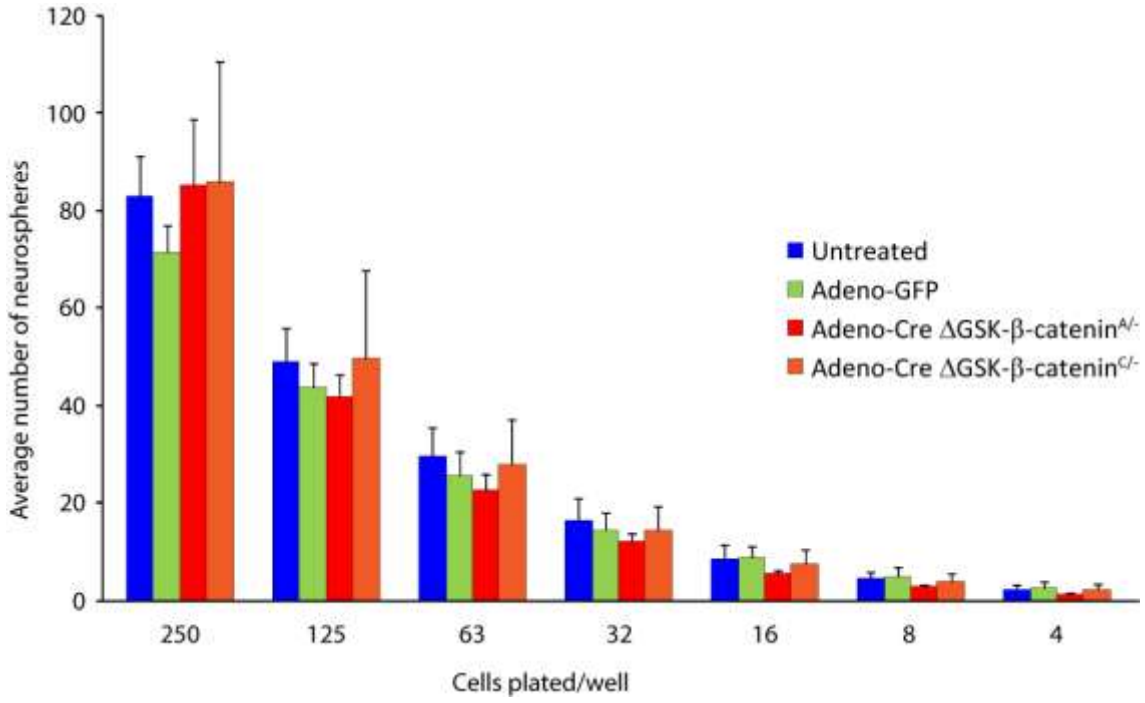


Figure 4.4 Adeno-Cre infected NSCs do not show increased self-renewal potential

Neurospheres were passaged to a single cell suspension. Around 500 cells were plated into each well of the top row of a 96-well plate. The cells were serially diluted for each remaining row of wells. Ten days following passage, the number of neurospheres was counted. An n=3 was calculated from three separate cultures for each line. Each single experiment represents the average of twelve wells. The untreated and Adeno-GFP-treated results from ΔGSK-β-catenin$^{A/-}$ and ΔGSK-β-catenin$^{C/-}$ were pooled for an n=6. There was no significant difference in the number of neurospheres for both ΔGSK-β-catenin$^{A/-}$ and ΔGSK-β-catenin$^{C/-}$ at any concentration. Neurosphere numbers where checked for normality and homoscedasticity.

One of the hallmarks of NSCs is their ability to differentiate into neurones, astrocytes and oligodendrocytes. To investigate the effect of ΔGSK-β-catenin in differentiation ΔGSK-β-catenin$^{A/-}$ NSCs were differentiated *in vitro* and analysed by immunofluorescence. Neurones were detected using an antibody against MAP2, astrocytes with an antibody against GFAP and oligodendrocytes with an antibody against O4. Type B NSCs were detected by double staining with an anti-GFAP and anti-Nestin antibodies (Doetsch, *et al* 1999). No obvious difference was observed for any cell population (**Figure 4.5**). To detect β-catenin, differentiated cells were immunostated with an anti-β-catenin antibody. However, no increase in nuclear β-catenin was observed (data not shown). As a result, an anti-KT3 antibody was used to specifically detect ΔGSK-β-catenin, but the staining pattern of this antibody was unspecific (data not shown). As a result of this and of the negative results with ΔGSK-β-catenin mice thus far, the cells were not counted.

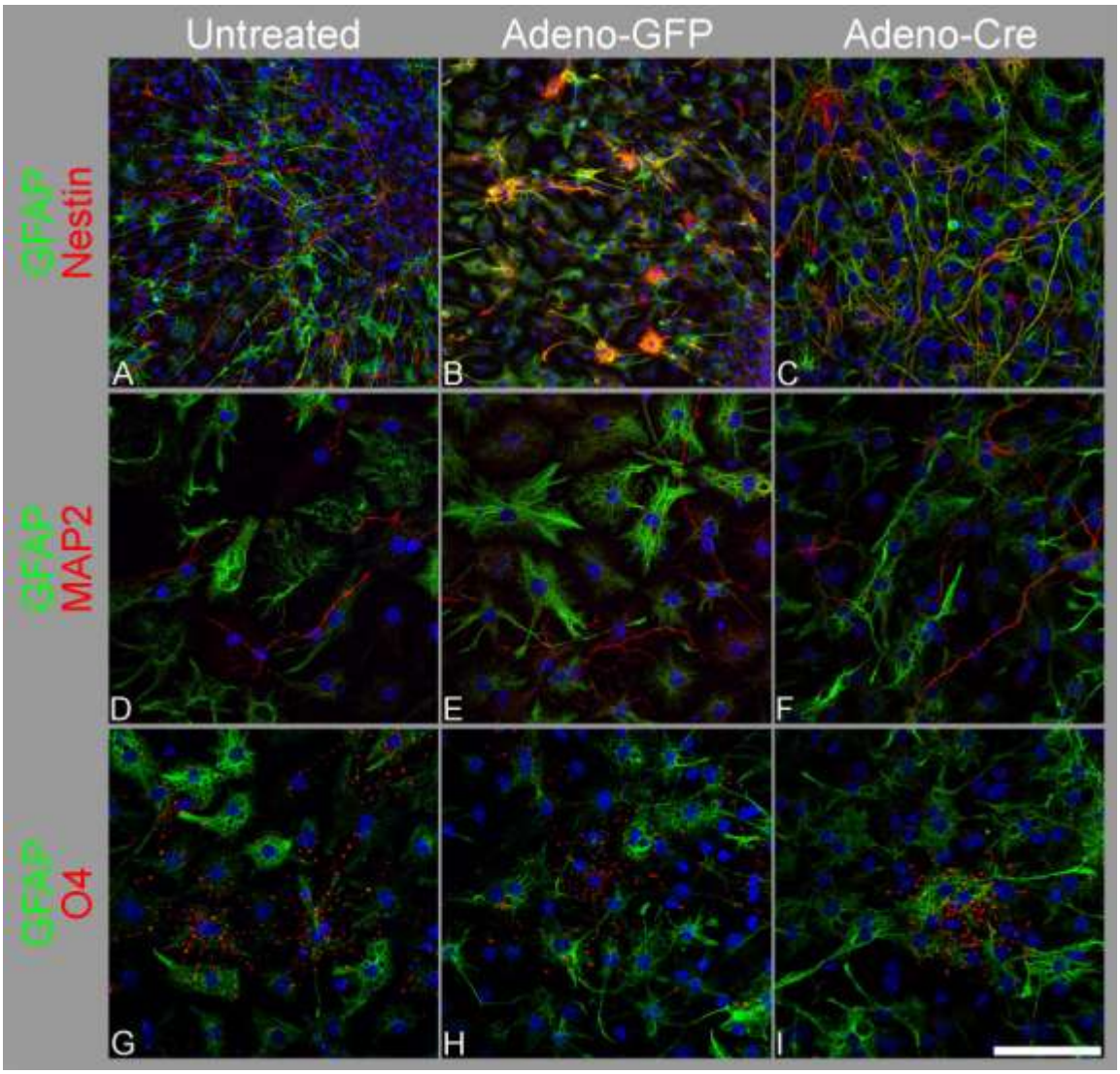


Figure 4.5 No obvious difference in the differentiation of ΔGSK-β-catenin$^{A/-}$ NSCs

Adeno-Cre, Adeno-GFP treated and untreated ΔGSK-β-catenin$^{A/-}$ neurospheres were plated on laminin-coated glass slides in DMEM/Ham-F12 media supplemented with 10 % FCS. Forty eight hours post-culture, cells were fixed with 4 % formaldehyde and immunostained with rabbit polyclonal anti-GFAP (1:500), mouse monoclonal anti-MAP2 (1:500) and mouse monoclonal anti-O4 (1:300). Hoechst 33342 (1:1000) was added to label the nuclei. The scale bar in **H** corresponds to 76 µm (**A and B**) and 153 µm (**C-I**). No obvious difference in the differentiation pattern was detected

4.2.2 Additional deletion of p53 does not affect brain morphology and does not promote neoplastic transformation

TP53, the gene encoding p53, is one of the most commonly mutated genes in human cancers (Hainaut and Hollstein, 2000). For example, TP53 is mutated in up to 25 % of primary and 65 % of secondary glioblastomas (Ohgaki *et al*., 2004; Watanabe *et al*., 1996; Watanabe *et al*., 1997). However, p53 knockout in mice does not result in brain tumour development (Donehower *et al*., 1992; Harvey *et al*., 1993). In contrast, combination of p53 knockout with phosphatase and tensin homolog (PTEN) or Rb knockout in mice, promotes the development of PNETs and glial tumours corresponding to human oligoastrocytomas (Jacques *et al*., 2010). Therefore, Nestin-Cre; ΔGSK-β-catenin mice were crossed with $p53^{loxP/loxP}$ conditional mice to promote neoplastic transformation.

Brains from ΔGSK-β-$catenin^{A/-}$; $p53^{loxP/loxP}$ and Nestin-Cre; ΔGSK-β-$catenin^{A/-}$; $p53^{loxP/loxP}$ mice were immunohistochemically stained for NeuN, MAP2 and NF200, GFAP and β-catenin but no malformations or brain tumours were detected (**Figure 4.6, Figure 4.7** and **Figure 4.8**). Increased GFAP staining was detected in Nestin-Cre; ΔGSK-β-$catenin^{A/-}$; $p53^{loxP/loxP}$ mice, but not in ΔGSK-β-$catenin^{A/-}$; $p53^{loxP/loxP}$ mice or Nestin-Cre; ΔGSK-β-catenin mice. This suggests that p53 loss of function is associated with this phenotype. GFAP marks astrocytes and type B NSCs (Doetsch, *et al* 1999). However, type B NSCs are unlikely to be present in an adult cerebellum. This suggests that the increased GFAP staining corresconds with an increase in gliosis. Additionally, there was no difference in brain weight between Nestin-Cre; ΔGSK-β-$catenin^{A/-}$; $p53^{loxP/loxP}$ and ΔGSK-β-$catenin^{A/-}$; $p53^{loxP/loxP}$ mice (**Figure 4.9**).

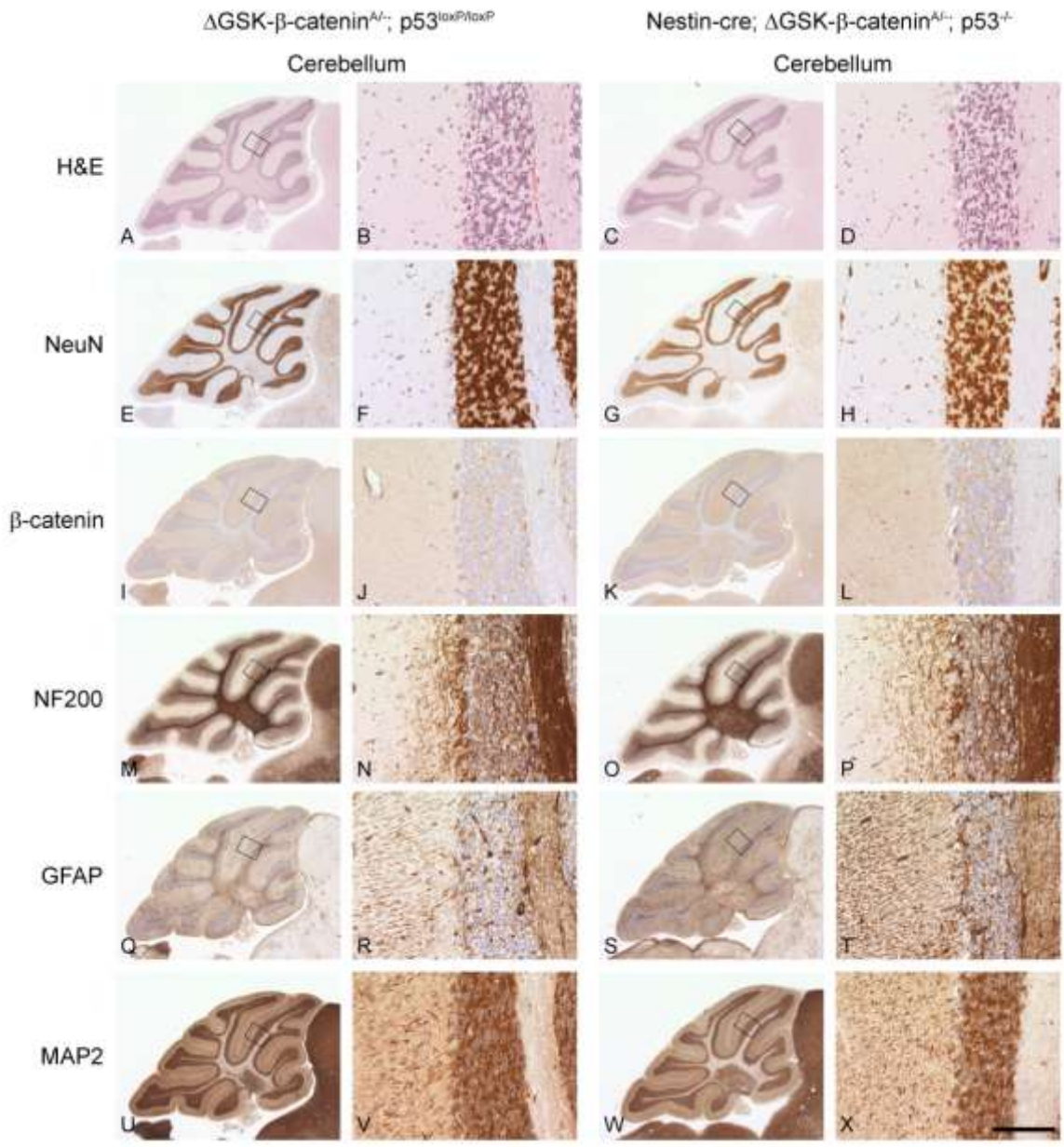


Figure 4.6 There are no abnormalities in the cerebellum of Nestin-Cre; ΔGSK-β-catenin; p53$^{-/-}$ brains

Cerebella from ΔGSK-β-catenin$^{A/-}$; p53$^{loxP/loxP}$ and Nestin-Cre; ΔGSK-β-catenin$^{A/-}$; p53$^{loxP/loxP}$ mice were stained for H&E (**A-D**), NeuN (**E-H**), β-catenin (**I-L**), NF200 (**M-P**), GFAP (**Q-T**) and MAP2 (**U-X**). The first and second columns correspond to low and high power magnification of a ΔGSK-β-catenin$^{A/-}$; p53$^{loxP/loxP}$ cerebellum. The third and fourth columns correspond to low and high power magnification of a Nestin-Cre; ΔGSK-β-catenin$^{A/-}$; p53$^{-/-}$ cerebellum. High power magnification images correspond to the fifth and sixth lobule of the cerebellum (black box). β-catenin staining was unchanged after recombination. Furthermore, recombined brains showed no abnormalities when compared to controls. Stronger gliosis was observed in Nestin-Cre; ΔGSK-β-catenin$^{A/-}$; p53$^{loxP/loxP}$ cerebella. The scale bar in **X** corresponds to 1300 µm and 140 µm for low power and high power magnifications respectively.

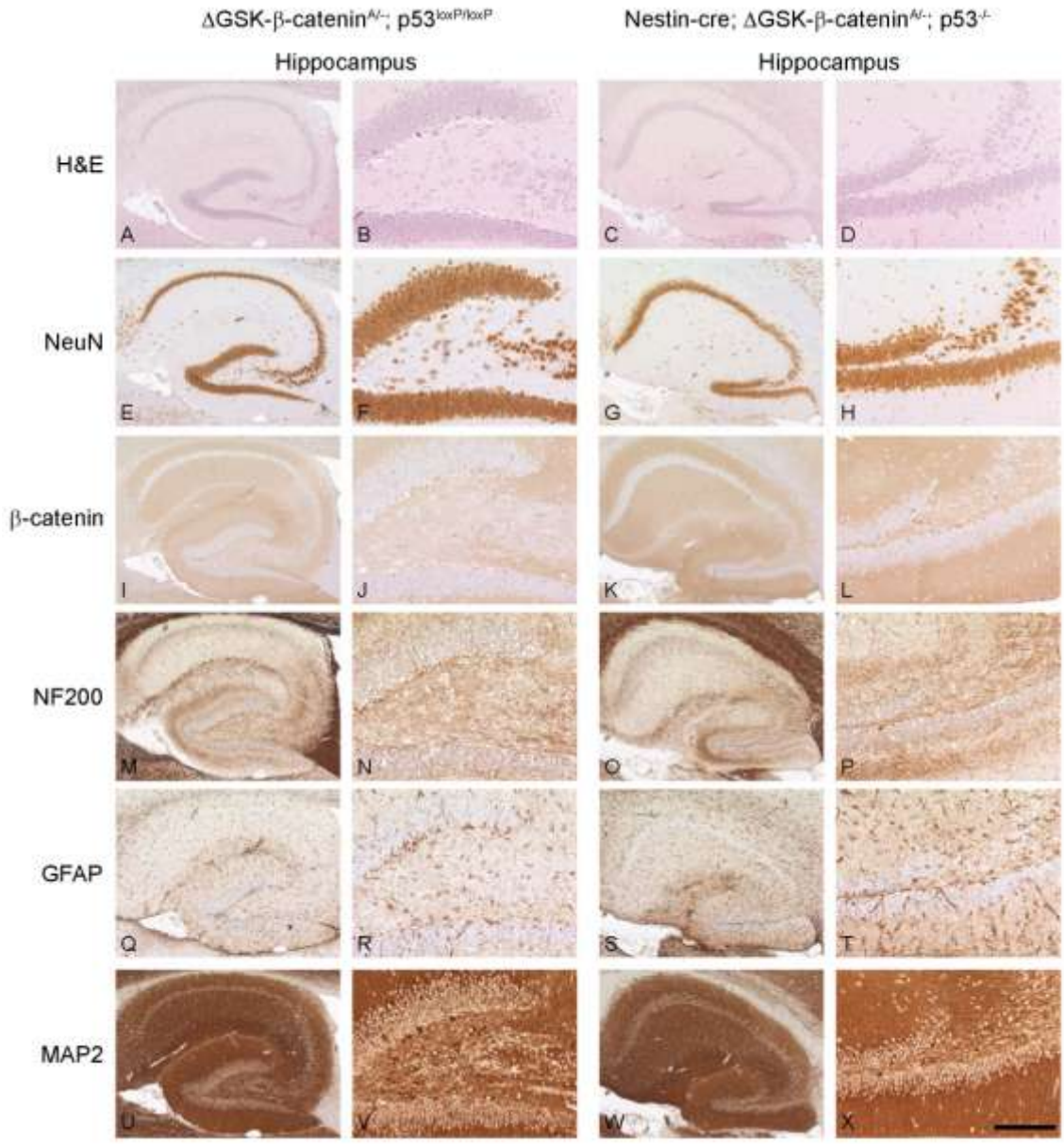


Figure 4.7 There are no abnormalities in the hippocampus of Nestin-Cre; ΔGSK-β-catenin; p53$^{loxP/loxP}$ brains

Hippocampi from ΔGSK-β-catenin$^{A/-}$; p53$^{loxP/loxP}$ and Nestin-Cre; ΔGSK-β-catenin$^{A/-}$; p53$^{loxP/loxP}$ mice were stained for H&E (**A-D**), NeuN (**E-H**), β-catenin (**I-L**), NF200 (**M-P**), GFAP (**Q-T**) and MAP2 (**U-X**) The first and second columns correspond to low and high power magnification of a ΔGSK-β-catenin$^{A/-}$; p53$^{loxP/loxP}$ hippocampus. The third and fourth columns correspond to low and high power magnification of a Nestin-Cre; ΔGSK-β-catenin$^{A/-}$; p53$^{-/-}$ hippocampus. β-catenin staining was unchanged after recombination. Furthermore, recombined brains showed no abnormalities when compared to controls. Stronger gliosis was observed in Nestin-Cre; ΔGSK-β-catenin$^{A/-}$; p53$^{loxP/loxP}$ brains. The structural differences between the hippocampi of ΔGSK-β-catenin$^{A/-}$ and Nestin-Cre; ΔGSK-β-catenin$^{A/-}$ are explained by the lateral depth of the histological sections. The scale bar in **X** corresponds to 700 µm and 220 µm for low power and high power magnifications respectively.

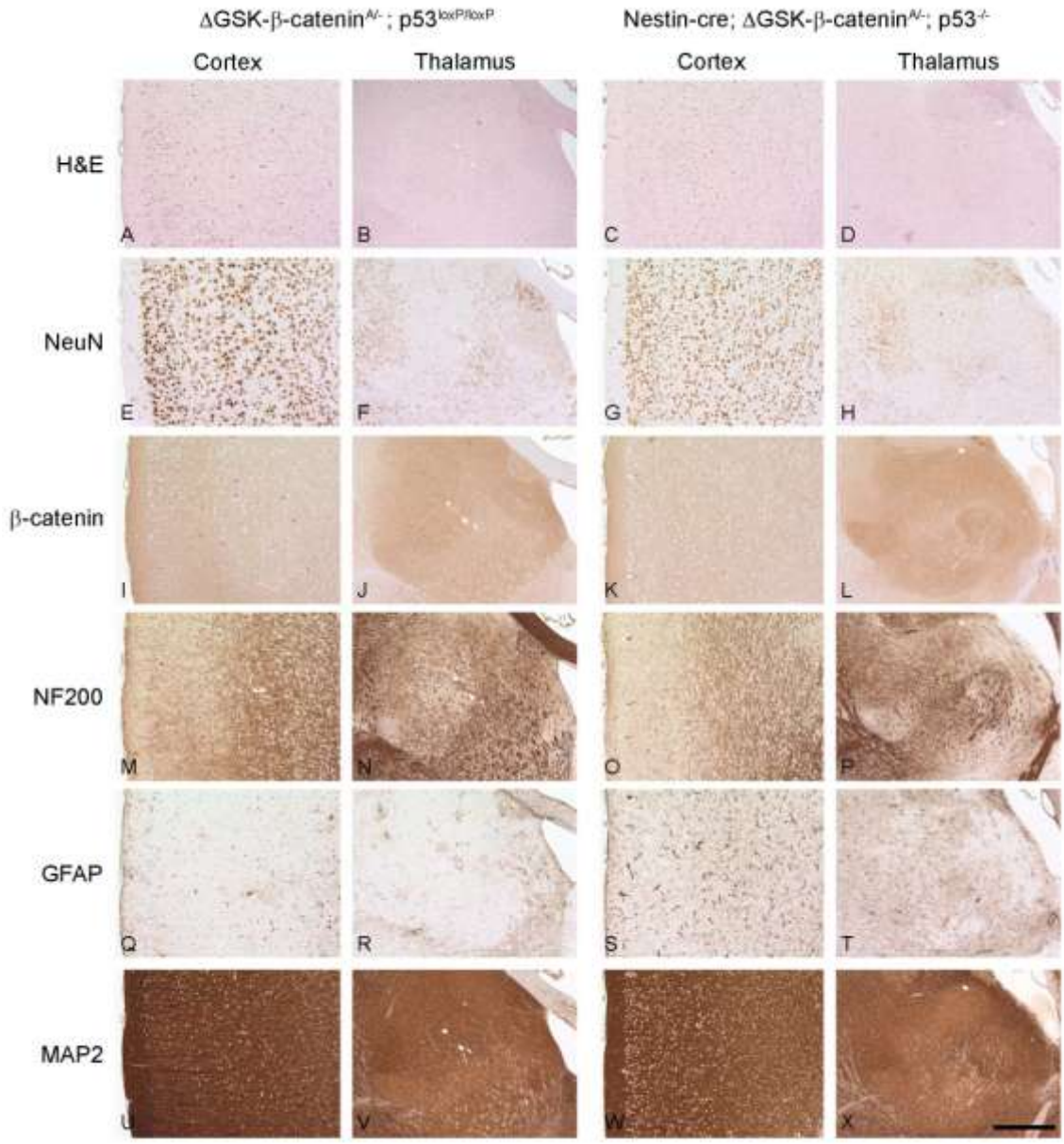


Figure 4.8 There are no abnormalities in the cortex and thalamus of Nestin-Cre; ΔGSK-β-catenin; p53$^{loxP/loxP}$ brains

Cortices and thalami from ΔGSK-β-catenin$^{A/-}$; p53$^{loxP/loxP}$ and Nestin-Cre; ΔGSK-β-catenin$^{A/-}$; p53$^{loxP/loxP}$ mice was stained for H&E (**A-D**), NeuN (**E-H**), β-catenin (**I-L**), NF200 (**M-P**), GFAP (**Q-T**) and MAP2 (**U-X**) The first and second columns correspond to the cortex and thalamus of a ΔGSK-β-catenin$^{A/-}$; p53$^{loxP/loxP}$ mouse. The third and fourth columns correspond to the cortex and thalamus of a Nestin-Cre; ΔGSK-β-catenin$^{A/-}$; p53$^{-/-}$ mouse. β-catenin staining was unchanged after recombination. Furthermore, recombined brains showed no abnormalities when compared to controls. Stronger gliosis was observed in Nestin-Cre; ΔGSK-β-catenin$^{A/-}$; p53$^{loxP/loxP}$ brains. The scale bar in **X** corresponds to 850 µm and 450 µm for low power and high power magnifications respectively.

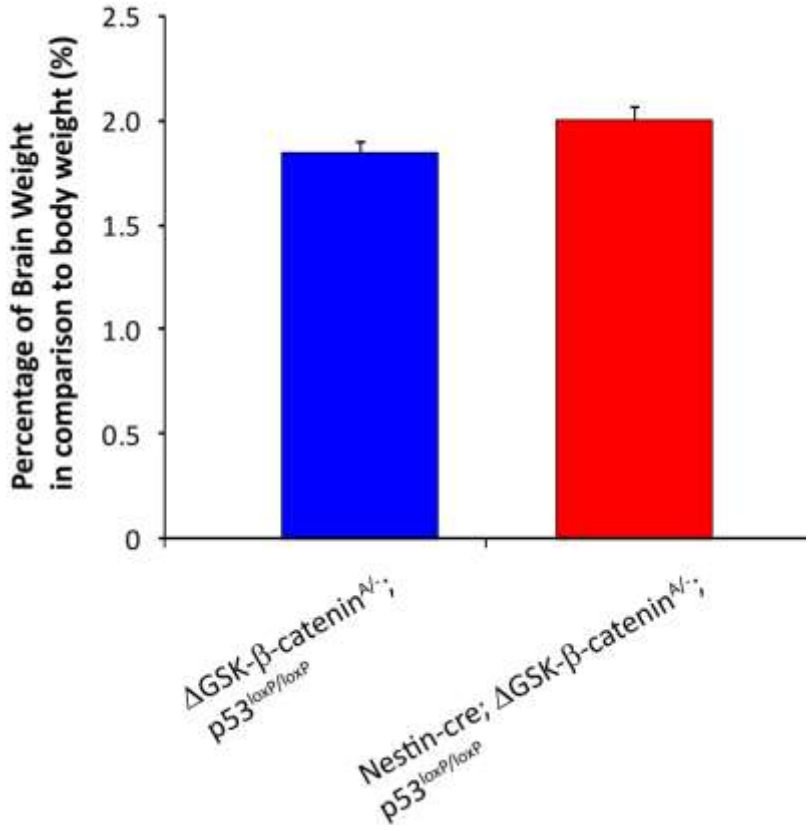


Figure 4.9 No significant difference in brain weight between ΔGSK-β-catenin$^{A/-}$; p53$^{loxP/loxP}$ and Nestin-Cre; ΔGSK-β-catenin$^{A/-}$; p53$^{loxP/loxP}$ mice

ΔGSK-β-catenin$^{A/-}$; p53$^{loxP/loxP}$ (n=14) and Nestin-Cre; ΔGSK-β-catenin$^{A/-}$; p53$^{loxP/loxP}$ (n=17) brains were weighed and the value converted to a percentage of body weight. There was no significant difference between ΔGSK-β-catenin$^{A/-}$; p53$^{loxP/loxP}$ and Nestin-Cre; ΔGSK-β-catenin$^{A/-}$; p53$^{loxP/loxP}$ mice (p=0.06, two-tailed t-test).

4.2.3 Additional deletion of p53 or Rb in NSCs does not have a consistent effect on proliferation

As ΔGSK-β-catenin expression was predicted to be low, ΔGSK-β-catenin$^{A/A}$ homozygous mice were generated to increase expression. These mice were then crossed with either p53$^{loxP/loxP}$ or Rb$^{loxP/loxP}$ conditional knockout mice to assess the synergistic effect, if any, of these mutations in NSCs. Subsequently, NSC cultures were derived and grown as previously discussed.

NSC growth was measured using the Hoechst proliferation assay. Hoechst 33342 is a cell-permeant nuclear counterstain that emits blue fluorescence when bound to dsDNA; fluorescence is proportional to the amount DNA, which is an indication of the number of cells in culture. Using this assay, the amount of fluorescence was measured at days 1, 3 and 6 post-plating. A modest increase in the proliferation of Adeno-Cre infected ΔGSK-β-catenin$^{A/A}$; p53$^{loxP/loxP}$ and ΔGSK-β-catenin$^{A/A}$; Rb$^{loxP/loxP}$ NSCs (n=1) was detected (**Figure 4.10**).

Proliferation was also assessed using the neurosphere size assay. Adeno-Cre recombined ΔGSK-β-catenin$^{A/A}$; p53$^{loxP/loxP}$ and ΔGSK-β-catenin$^{A/A}$; Rb$^{loxP/loxP}$ NSCs grew smaller in size than controls (n=1) (**Figure 4.11**).

The limited dilution assay was used to determine self-renewal and indicated that Adeno-Cre treated ΔGSK-β-catenin$^{A/A}$; Rb$^{loxP/loxP}$ cultures grew fewer neurospheres than Adeno-GFP treated controls (n=1) (**Figure 4.12**). Conversely, Adeno-Cre-infected ΔGSK-β-catenin$^{A/A}$; p53$^{loxP/loxP}$ cultures produced more neurospheres than Adeno-GFP infected controls (n=1) (**Figure 4.12**). However, the number of neurospheres was similar between untreated cultures and Adeno-Cre treated cultures for both cell lines.

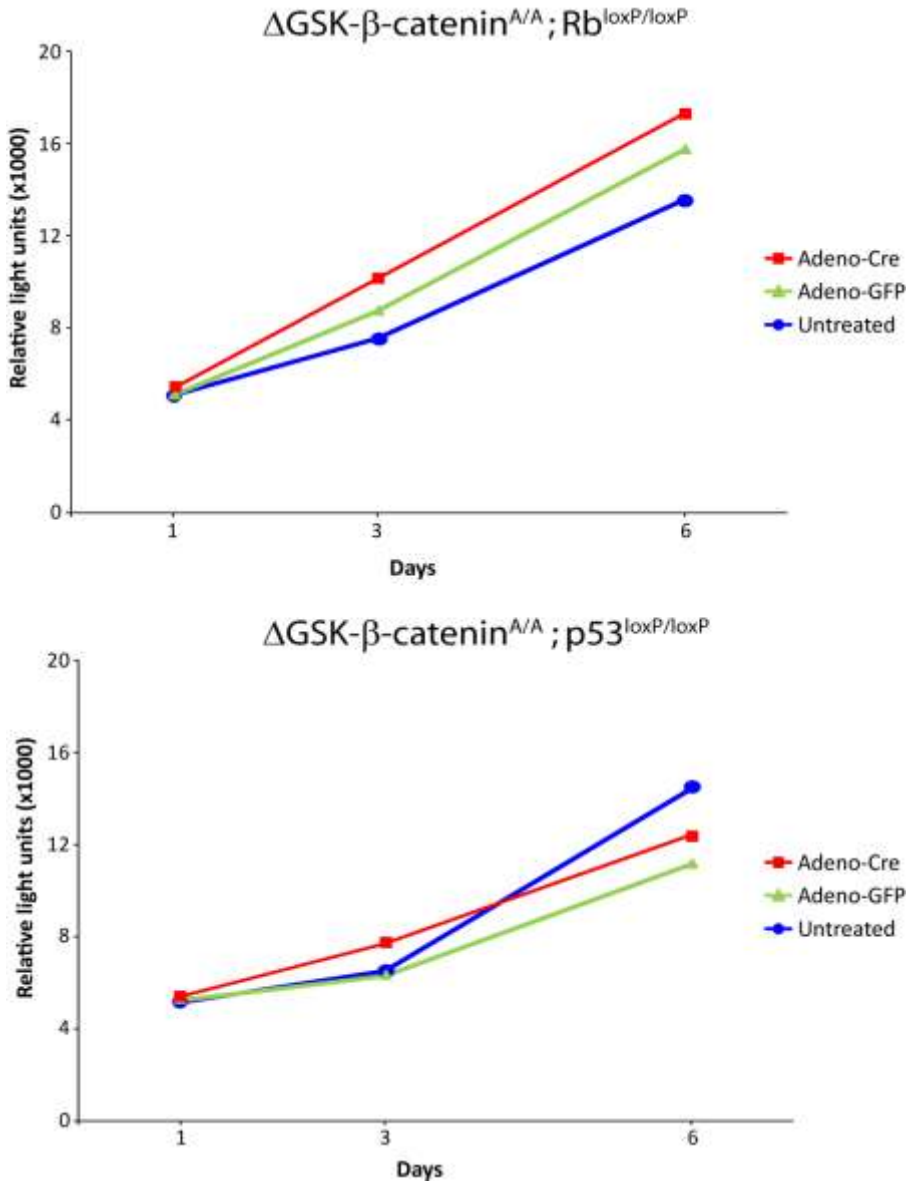


Figure 4.10 Adeno-Cre-infected ΔGSK-β-catenin$^{A/A}$; Rb$^{loxP/loxP}$ and ΔGSK-β-catenin$^{A/A}$; p53$^{loxP/loxP}$ NSCs show a slightly enhanced proliferation potential

Neurospheres from ΔGSK-β-catenin$^{A/A}$; Rb$^{loxP/loxP}$ (**A**) or ΔGSK-β-catenin$^{A/A}$; p53$^{loxP/loxP}$ (**B**) cultures were passaged to a single cell suspension. Cells were plated in quintuplet for each treatment at a density of 3.5x10^{3} cells/well. Three identical 96-well plates were prepared. On day one post-plating, Hoechst 33342/DMSO was added to each well of one of the plates and incubated for 30 min at 37 °C. As much medium as possible was removed and replaced with PBS to lower unbound background Hoechst. Fluorescence was measured at 360 nm excitation and 465 nm emission. The same procedure was repeated on days 3 and day 6 post-plating with the remaining 96-well plates. Adeno-Cre-infected NSCs grew slightly faster than controls. This was a single experiment, therefore the statistical significance cannot be determined.

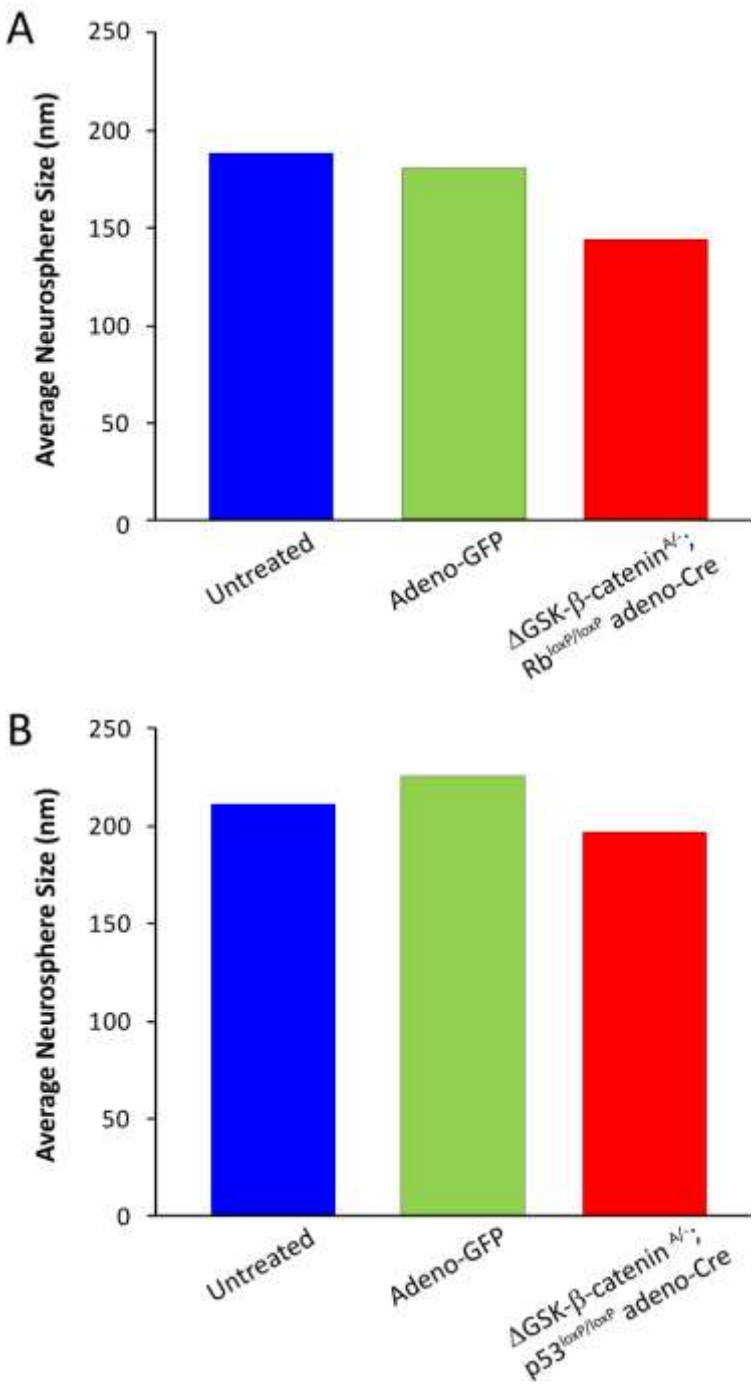


Figure 4.11 Adeno-Cre-infected ΔGSK-β-catenin$^{A/A}$; Rb$^{loxP/loxP}$ and ΔGSK-β-catenin$^{A/A}$; p53$^{loxP/loxP}$ neurospheres are smaller than controls

Neurospheres were passaged to a single cell suspension and 0.5x10^{6}cells were plated on a 6 cm plate. Ten days after passage, the diameter of the neurospheres was measured using an Axiovert 135 microscope (Zeiss) and the Openlab 5 software. Each experiment was the average of 100 neurospheres. Adeno-Cre-infected ΔGSK-β-catenin$^{A/A}$; Rb$^{loxP/loxP}$ (**A**) or ΔGSK-β-catenin$^{A/A}$; p53$^{loxP/loxP}$ (**B**) neurospheres grew smaller than controls. This was a single experiment, therefore the statistical significance cannot be determined.

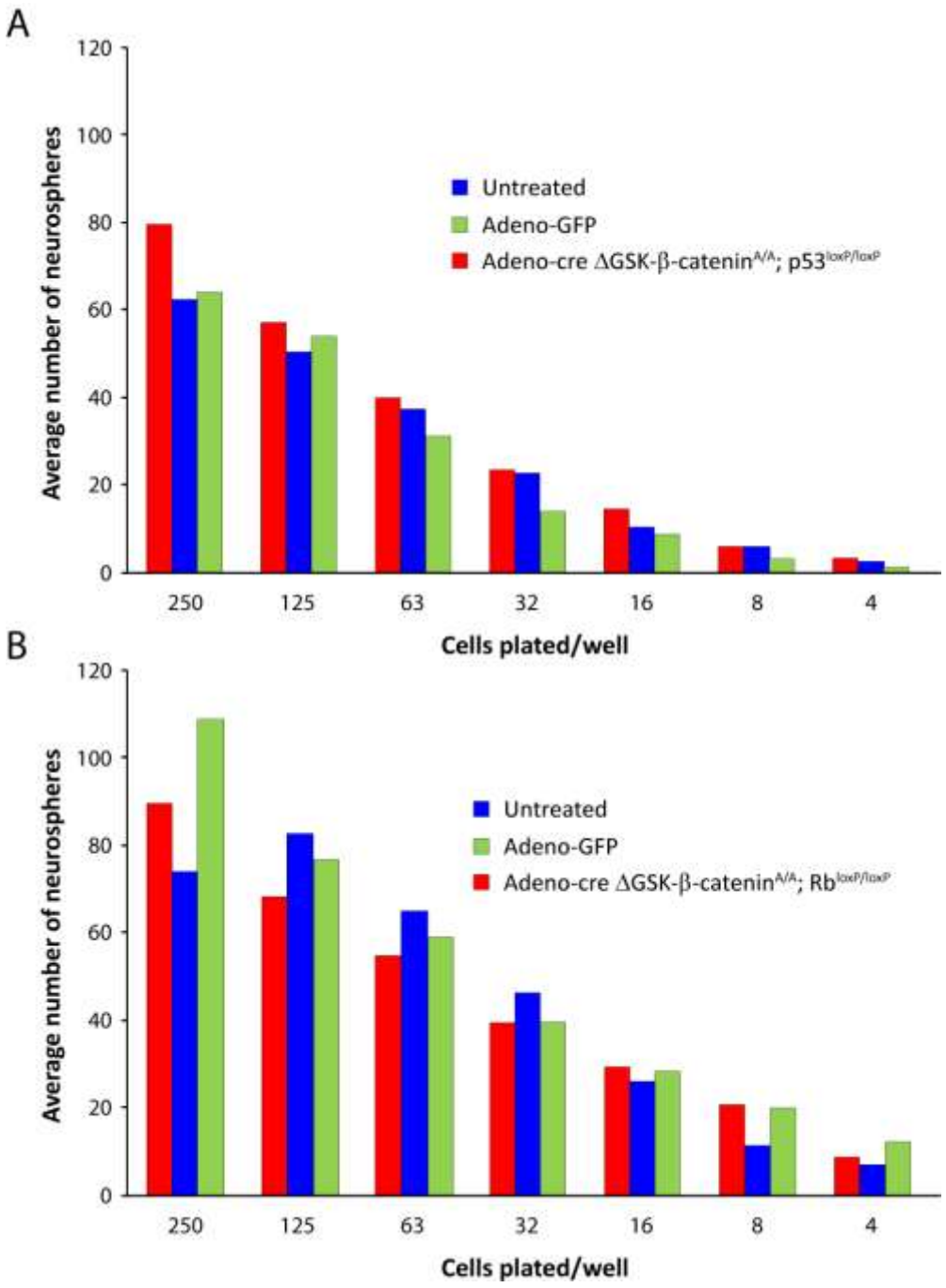


Figure 4.12 Adeno-Cre-infected ΔGSK-β-catenin$^{A/A}$; p53$^{loxP/loxP}$ NSCs show higher self-renewal, but ΔGSK-β-catenin$^{A/A}$; Rb$^{loxP/loxP}$ NSCs do not

Neurospheres were passaged to a single cell suspension. About 500 cells were plated into each well of the top row of a 96-well plate. The cells were serially diluted for each row of wells. Ten days after passage, the number of neurospheres was counted. Each result was an average of twelve wells. Adeno-Cre treated ΔGSK-β-catenin$^{A/A}$; p53$^{loxP/loxP}$ (**A**) grew more spheres than Adeno-GFP controls. Conversely, Adeno-Cre infected ΔGSK-β-catenin$^{A/A}$; Rb$^{loxP/loxP}$ grew fewer spheres than Adeno-GFP treated controls (**B**). This was a single experiment, therefore the statistical significance cannot be determined.

4.2.4 *In vivo* recombination in the SVZ niche of ΔGSK-β-catenin$^{A/-}$; p53$^{flox/flox}$ mice does not cause CNS neoplasia

An alternative method for inducing recombination *in vivo* is injecting Adeno-Cre virus directly into lateral ventricles, resulting in an infection of cells in the SVZ (Jacques *et al*., 2010). Nine ΔGSK-β-catenin$^{A/-}$; p53$^{loxP/loxP}$ mice and nine p53$^{loxP/loxP}$ control mice were injected with Adeno-Cre and culled three hundred days post-infection. No hyperplasias or tumours were found in the SVZ (**Figure 4.13**).

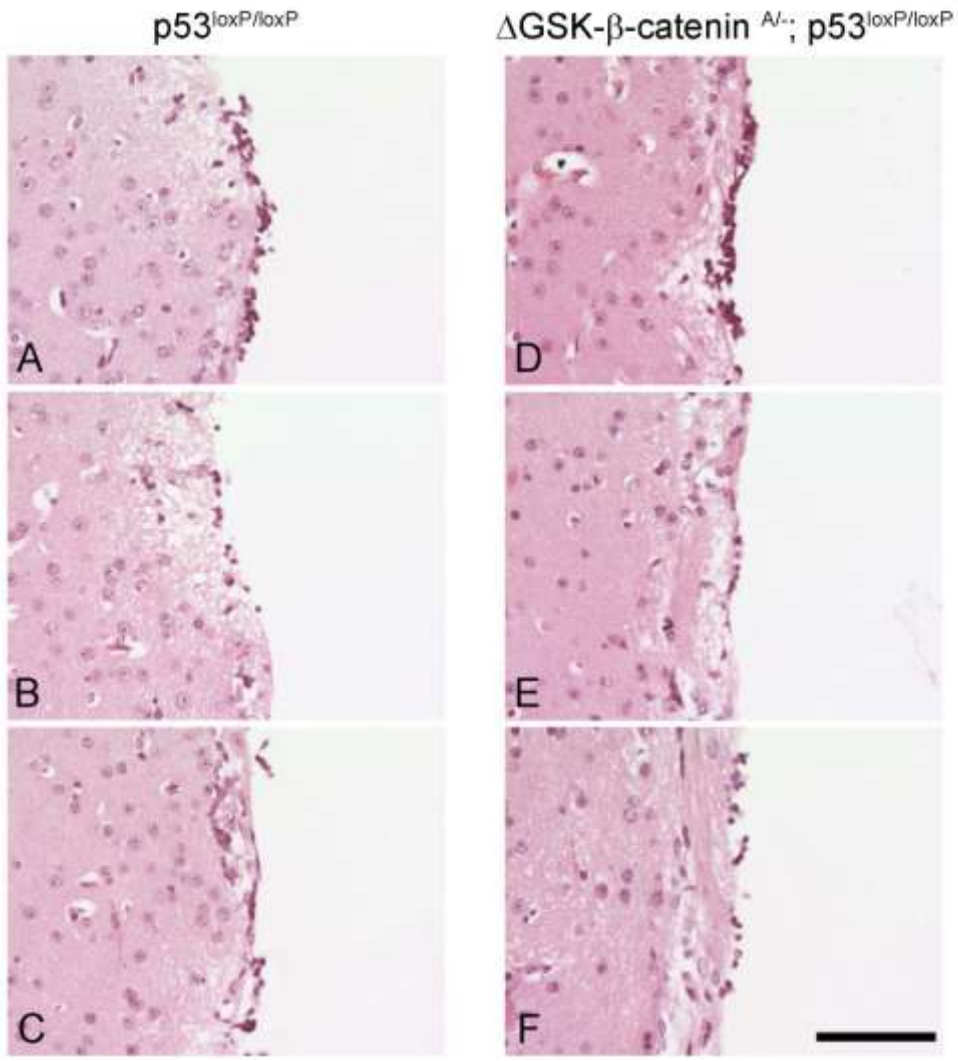


Figure 4.13 SVZ recombined ΔGSK-β-catenin$^{A/-}$; p53$^{flox/flox}$ mice do not develop abnormal cell proliferations

The lateral ventricles of ΔGSK-β-catenin$^{A/-}$; p53$^{flox/flox}$ (n=9) (**D-F**) and control p53$^{flox/flox}$ (n=9) (**A-C**) mice were injected with Adeno-Cre. Three hundred days post-injection the mice were culled and the brains were processed for histology. Hyperplasia or tumours were not found. The scale bar in **F** corresponds to 130 μm.

4.3 **Discussion**

Nestin-Δ90N-β-catenin transgenic mice show severe CNS developmental abnormalities and die prior to birth (Chenn and Walsh, 2002). In this mouse model, expression of Δ90N-β-catenin in NSCs increases the proportion of neural stem cells, which continue to proliferate instead of differentiating (Chenn and Walsh, 2002). As previously discussed, Nestin-Cre; ΔGSK-β-catenin mice do no show CNS developmental abnormalities (**chapter 3**). Although the protein is expressed in the brain, the SVZ is not enlarged as would be expected. This may be attributed to the weak expression of ΔGSK-β-catenin. However, the existence of a more subtle phenotype, which may not be detected, by histological examination, cannot be excluded. To investigate this possibility, the effect of ΔGSK-β-catenin expression in NSCs was investigated. For this purpose, NSC cultures were grown from the SVZ of adult ΔGSK-β-catenin mice. To induce ΔGSK-β-catenin expression, NSCs were infected with an Adenovirus expressing Cre-recombinase. An Adenovirus expressing GFP was used as a control. Western blots of NSCs showed that ΔGSK-β-catenin was expressed after Adeno-Cre mediated recombination (**Figure 4.1**). Of the two ΔGSK-β-catenin transgenic mouse lines tested, ΔGSK-β-catenin$^{A/-}$ NSCs expressed significantly more mutant protein than ΔGSK-β-catenin$^{C/-}$ NSCs.

The WST-1 proliferation assay was used to determine NSC growth. In this assay, the WST-1 reagent is metabolised by mitochondria into formazan, a dark red compound. The amount of formazan can be measured by optical density and is proportional to the number of cells in culture. Adeno-Cre recombined ΔGSK-β-catenin$^{A/-}$ and ΔGSK-β-catenin$^{C/-}$ NSCs did not show an increased growth potential in comparison to controls. However, the caveat of this method is that growth is not measured directly, but as a by-product of mitochondrial activity. Hence, if expression of ΔGSK-β-catenin alters mitochondrial metabolism, this would have affected the interpretation of the data. Indeed, expression of degradation-resistant β-catenin has been shown to increase the mitochondrial activity of intestinal epithelial and colon cancer cells (Mezhybovska *et al*., 2009). This suggests that Adeno-Cre recombined ΔGSK-β-catenin NSCs may have an increased rate of formazan production, which could be misunderstood for an increase in proliferation. However, in our studies, Adeno-Cre recombination did not significantly alter formazan formation. This can be explained if ΔGSK-β-catenin expression is not having an effect in these cells. This is in agreement with the results obtained in this chapter.

Another method used to determine NSC growth was to measure NS size. NSCs grown under non-adherent conditions form spherical bodies, termed neurospheres. If a treatment has the potential to affect the growth of NSCs it can be hypothesised that it would also have an impact on the size of the neurospheres. To test this theory, NSCs were grown for ten days

before measuring the diameter of one hundred spheres for each treatment. Adeno-Cre treated ΔGSK-β-catenin$^{A/-}$ and ΔGSK-β-catenin$^{C/-}$ neurospheres were not significantly larger or smaller than controls. However, it is possible that neurosphere growth may have reached a plateau by day 10. This would indicate that growth differences may have been missed. Indeed, a report by Mori *et al.* suggests that neuropshere growth starts decreasing at around 250 μm in diameter (Mori *et al*., 2006). Moreover, in our study neurospheres rarely reached a size ≥ 250 μm.

One of the key characteristics of stem cells is their ability to self-renew. To assess self-renewal, cells were plated in decreasing concentrations from 250 cells/well to 4 cells/well and grown for 10 days. Only stem cells have the ability to form spheres. Hence, the number of secondary neurospheres is an indication of self-renewal potential. A caveat to this assay is that neurospheres freely fuse with each other (Singec *et al*., 2006). Coles-Takabe *et al.* (2008), closely studied the formation of non-clonal neurospheres and found that when cells were plated at a density of 131 cells/cm^2, the percentage of non-clonal spheres was negligible if the cultures were left undisturbed (Coles-Takabe *et al*., 2008). In the limited dilution assay, cells are grown at 168 cells/cm^2, for the highest concentration and 1 cell/cm^2, for the lowest. They are disturbed only twice during the experiment for the addition of fresh medium and growth factors. Therefore, non-clonal sphere formation is likely to be negligible, especially for the lower concentrations. The results presented in this chapter show that there is no significant difference in self-renewal after Adeno-Cre recombination for either ΔGSK-β-catenin$^{A/-}$ and ΔGSK-β-catenin$^{C/-}$ NSCs.

Collectively, the results here largely disagree with published data (Hirabayashi *et al*., 2004; Qu *et al*., 2010; Yu *et al*., 2006). A study investigating the expression of S33Y β-catenin in cortical neural precursors demonstrated that S33Y β-catenin does not affect their growth and self-renewal (Hirabayashi *et al*., 2004). Although this report provides evidence in favour of the results presented in this chapter, the precursors in the Hirabayashi *et al*. study were from E11.5 embryos, which would have had a different expression pattern and chromatin structure than what occurs in adult NSCs. In disagreement with the data shown here, a report by Yu and colleagues suggests that activation of the Wnt/β-catenin pathway increases the proliferation and self-renewal of SVZ neural stem cells (Yu *et al*., 2006). In this report, activation of the pathway was induced by transfection with Wnt3a or Wnt5a. Proliferation was measured by BrdU incorporation, which labelled dividing cells and daughter cells. However, there are a few counter arguments for their results. For example, the medium used to culture the cells contained 10 % FCS, which has been shown to cause the irreversible differentiation of NSCs (Gage *et al*., 1995; Lee *et al*., 2006; McKay, 1997; Reynolds and Weiss, 1992). Hence, this may have influenced their results. Additionally, for self-renewal measurement, cells were plated at 2x10^3 cell/cm^2, which was shown by Coles-Takabe *et al.,* to result in 15-100 % of non-clonal

spheres, depending on how often the plates were disturbed(Coles-Takabe *et al*., 2008; Yu *et al*., 2006). However, a recent report by Qu and colleagues agrees with Yu and colleagues results. In this report, expression of Δ90N-β-catenin is shown to increase the percentage of BrdU-positive cells from 34 to 49 % in SVZ NSC cultures (Qu *et al*., 2010). Similarly, expression of Wnt7a also increased proliferation and self-renewal (Qu *et al*., 2010). In this study, self-renewal was measured by plating single cells under adherent conditions and counting the progeny, thereby removing the risk of non-clonal sphere formation.

Despite expression of ΔGSK-β-catenin protein in NSCs, their growth was not significantly increased. Therefore, mice homozygous for ΔGSK-β-catenin$^{A/A}$ were generated to increase expression levels. Subsequently, these mice were crossed with p53 or Rb conditional knockout mice to promote growth and tumour development. p53 is involved in the cellular response to DNA damage and is often mutated in cancers [reviewed in (Hainaut and Hollstein, 2000)]. With respect to CNS tumours, p53 is mutated in up to 25 % of primary and 65 % of secondary glioblastomas (Ohgaki *et al*., 2004; Watanabe *et al*., 1996; Watanabe *et al*., 1997). Similarly, TP53 is mutated in medulloblastomas, albeit rarely (up to 11 % of cases) (Adesina *et al*., 1994; Alderson *et al*., 1996; Ohgaki *et al*., 1993). However, the p53 pathway is altered by alternative mechanisms in up to 21 % of medulloblastoma cases (Frank *et al*., 2004). Additionally, several medulloblastoma and PNET mouse models have been generated, which involve p53 inactivation (Frappart *et al*., 2009; Momota *et al*., 2008). The RB gene encodes the retinoblastoma protein (Rb), which plays a crucial role in G1→S phase transition (Weinberg, 1995). Loss of heterozygosity at 13q, which affects the RB gene, was found in 12 % of primary and 38 % of secondary glioblastomas (Nakamura *et al*., 2000). Similarly, promoter hypermethylation of the RB gene, which has been correlated with loss of expression, was found in 43 % of primary and 14 % of secondary glioblastomas (Nakamura *et al*., 2001a).

In vitro, Adeno-Cre treated ΔGSK-β-catenin$^{A/A}$; p53$^{loxP/loxP}$ and ΔGSK-β-catenin$^{A/A}$; Rb$^{loxP/loxP}$ NSCs grew marginally faster than controls. To measure growth, the Hoechst proliferation assay was used. In contrast to the WST-1 proliferation assay which measures mitochondrial metabolism, this assay measures DNA content. The amount of DNA was measured on days 1, 3 and 6 post-plating to more accurately follow the growth of the cells. Neurospheres from Adeno-Cre treated ΔGSK-β-catenin$^{A/A}$; p53$^{loxP/loxP}$ and ΔGSK-β-catenin$^{A/A}$; Rb$^{loxP/loxP}$ NSCs grew smaller than controls. Additionally, some differences in self-renewal were observed. However, in general, these effects were very mild.

Nestin-Cre; ΔGSK-β-catenin$^{A/-}$; p53$^{loxP/loxP}$ mouse brains were not larger than control and did not show CNS abnormalities. Nestin-Cre; ΔGSK-β-catenin$^{A/-}$; p53$^{loxP/loxP}$ brains showed increased level of gliosis, although this was not the case for Nestin-Cre; ΔGSK-β-catenin$^{A/-}$

brains. These results imply that gliosis was a result of p53 inactivation and were independent of ΔGSK-β-catenin expression.

Expression of degradation-resistant β-catenin may not be sufficient for neoplastic transformation. For example, Patched$^{+/-}$ mice expressing S33Y β-catenin in Nestin-expressing cells do not develop any tumours after eight weeks (Fults *et al*., 2002). Similarly, p53$^{-/-}$ mice expressing S33Y β-catenin in GFAP-expressing cells do not develop tumours within three months (Momota *et al*., 2008). The latter study was limited to three months due to the development of lymphomas and sarcomas, a known phenotype of p53 knockout mice (Harvey *et al*., 1993). Collectively, both studies suggest that β-catenin does not promote tumourigenesis. However, degradation-resistant β-catenin may promote tumour formation in the long-term. By using p53 conditional knockout mice the risk of sarcoma and lymphoma development is minimised, since p53 is not inactivated in connective tissue or the lymphatic system in this study. Hence, to address this possibility, the lateral ventricles of a cohort of ΔGSK-β-catenin$^{A/-}$; p53$^{loxP/loxP}$ mice were injected with Adeno-Cre. However, 300 days post-infection no brain tumours or hyperplasia were detected in the SVZ (**Figure 4.13**). Taken together these results suggest that degradation-resistant β-catenin does not have a neoplastic potential. Nonetheless, degradation-resistant β-catenin may be able to affect a tumour phenotype. For example, p53$^{-/-}$ mice expressing c-myc in GFAP-expressing cells developed PNET-like tumours with a latency of fourteen weeks (Momota *et al*., 2008). However, when S33Y β-catenin was co-expressed, the tumour latency shortened to eight weeks (Momota *et al*., 2008). Furthermore, these tumours showed a larger range of histological features (Momota *et al*., 2008). This was observed without an increase in the number of tumours detected, suggesting that S33Y β-catenin was not involved in the genesis of these tumours.

4.4 **Summary**

Expression of ΔGSK-β-catenin did not alter neural stem cell proliferation or self-renewal. In addition, NSC proliferation was not increased, despite ΔGSK-β-catenin expression in conjunction with p53 or Rb inactivation. *In vivo*, Nestin-Cre; ΔGSK-β-catenin$^{A/-}$; p53$^{loxP/loxP}$ mice did not develop tumours or CNS abnormalities. Similarly, ΔGSK-β-catenin$^{A/-}$; p53$^{loxP/loxP}$ mice injected with Adeno-Cre into the lateral ventricles did not develop hyperplastic or neoplastic foci. As discussed above, most of these findings are in variance with existing literature. We assumed that the expression level of ΔGSK-β-catenin was responsible for the lack of a phenotype. Deletion of Rb or p53 alone does not show a significant change in stem cell proliferation *in vitro*. Additional activation of β-catenin does not further alter the proliferation of self-renewal of the stem cells. It was therefore concluded that expression of ΔGSK-β-catenin in this model system is not able to elicit a significant biological effect alone or in combination with inactivated Rb or p53 function. It was therefore decided not to further investigate our model system and obtain an alternative transgenic mouse model for expressing degradation-resistant β-catenin.

5 Analysis of β-catenin$^{\Delta ex3}$ expression in the CNS

5.1 Background

As discussed in chapters 3 and 4, the results obtained from the ΔGSK-β-catenin transgenic mouse model do not agree with previous published reports (Chenn and Walsh, 2002; Wrobel *et al*., 2007). Therefore, an alternative mouse model, in which degradation-resistant β-catenin is conditionally expressed, was utilised. In this model, exon 3 of the β-catenin gene, which encodes the Gsk3β/Ck1α phosphorylation site among other domains, is flanked by loxP sites. Cre-mediated recombination excises this exon and degradation-resistant β-catenin$^{\Delta ex3}$ is expressed. Deletion of exon 3 has been identified in some human cancer cell lines (Iwao *et al* 1998, Sparks *et al* 1998). Since the endogenous β-catenin gene is mutated, this model accurately depicts spontaneous mutations in human cancers. Furthermore, the target gene will be expressed at endogenous levels.

The β-catenin$^{lox(ex3)}$ mice were originally generated by the group of Taketo, who demonstrated that expression of β-catenin$^{\Delta ex3}$ promoted intestinal polyposis (Harada *et al*., 1999). Subsequent reports have indicated that expression of β-catenin$^{\Delta ex3}$ can promote prostate and liver tumourigenesis (**Table 5.1**). Currently, there is limited research into CNS tumourigenesis using the β-catenin$^{lox(ex3)}$ mouse model. Of particular interest, expression of β-catenin$^{\Delta ex3}$ in Nestin-expressing cells has been shown to delay the maturation of radial glia (Wrobel *et al*., 2007). As a result, there is a gross expansion of the ventricular zone, the precursor to the SVZ, which is equivalent to the phenotype observed in Nestin-Δ90N-β-catenin mice (Chenn and Walsh, 2002; Wrobel *et al*., 2007).

5.1.1 **Aims**

The aim of this study was to assess the role of the Wnt/β-catenin pathway in the CNS using β-catenin$^{lox(ex3)}$ transgenic mice and to test whether expression of β-catenin$^{\Delta ex3}$ at endogenous levels results in a different biological phenotype.

5.1.2 **Methods**

NSC cultures were derived from the SVZ and the hippocampus of adult mice (**section 2.4.2**). NSCs were infected with Adeno-Cre to mediate recombination (**section 2.4.3**). Western blot analysis was used to detect β-catenin$^{\Delta ex3}$ and cyclin D1 protein levels (**section 2.6**). NSC growth was assayed using the WST-1 proliferation assay (**section 2.4.4**), Hoechst proliferation assay (**section 2.4.5**) and neurosphere size assay (**section 2.4.6**). Self-renewal was assessed by the limited dilution assay (**section 2.4.7**). Nuclear fractions were obtained from Adeno-Cre, Adeno-GFP and untreated β-catenin$^{lox(ex3)/wt}$ NSC homogenates in order to assess levels of nuclear β-catenin (**section 2.6.1.2**). Neurospheres were differentiated (**section 2.4.8**) and multi-lineage potential of NSCs was analysed by immunocytochemical methods (**section 2.4.9**). En2-Cre; β-catenin$^{\Delta ex3}$ mouse brains were analysed histologically (**section 2.5**). β-catenin$^{lox(ex3)}$ mice were injected intra-cranially with Adeno-Cre to recombine the SVZ (**section 2.1.4**).

Table 5.1 Selected literature regarding β-catenin$^{\Delta ex3}$ transgenic mice

Tissue	Cre expression	Cre expression	Effect	References
Bone	Col2a1-Cre	Chondrocytes	Death ~E18, very severe chondrodysplasia	(Akiyama *et al*., 2004)
Breast	WAP-Cre	Mammary alveolar epithelium	Hyperplastic structures and squamous metaplasia	(Miyoshi *et al*., 2002)
	Pdgfb-iCreER	Capillary and small vessel endothelial cells	Transcriptional activation of β-catenin accelerates blood-brain-barrier (BBB) maturation	(Liebner *et al*., 2008)
CNS	Wnt1-Cre (cell culture)	Neural Crest cells	Sensory neural cell differentiation	(Lee *et al*., 2004)
	Nestin-Cre	Neural precursors	Increase in radial glia population resulting in gross enlargement of SVZ,	(Wrobel *et al*., 2007)
Embryo	Nes8-Cre	Telencephalon	Up-regulation of dorsal markers and down-regulation of ventral markers	(Backman *et al*., 2005)
	Brn4-Cre, Wnt1-Cre	Neural tube	Expansion of the progenitor pool	(Ille *et al*., 2007)
Eye	αMHC-CrePR1	Cardiomyocytes	Impaired cardiac growth or cardiac muscular atrophy	(Baurand *et al*., 2007)
Heart	Islet1-Cre (E7.0)	Second heart field (SHF) progenitors	Stabilisation resulted in a marked enhancement of SHF progenitors	(Kwon *et al*., 2007)
Intestine	Fabpl-Cre, Ck19-Cre	Intestinal epithelium	Cells escape mitotic arrest and apoptosis which results in chromosomal instability	(Aoki *et al*., 2007)
Liver	Adeno-Cre (adult)	Injected into tail vein	Stable β-catenin and H-ras promoted hepatocarcinogenesis	(Harada *et al*., 2002; Harada *et al*., 2004; Mucenski *et al*., 2005)
Lung	CCSP-Cre (E14.5)	Respiratory epithelial cells	Goblet cell hyperplasia, and pulmonary tumours	(Mucenski *et al*., 2005)
Prostate	PB-Cre	Postrate secretory epithelium	Squamous metaplasia and prostate Adenocarcinoma	(Cheon *et al*., 2006)
Thymus	Foxn1-Cre (E10.5)	Thymic epithelial cells	Impaired thymus microenvironment, unable to support T cell development	(Maatouk *et al*., 2008)
XY gonads	Sf1-Cre (E11.5)	Gonadal somatic cells	Male to female sex reversal	(Maatouk *et al*., 2008)

5.2 **Results**

5.2.1 **Adeno-Cre treated β-catenin$^{lox(ex3)/wt}$ NSCs do not show an enhanced growth potential**

NSC cultures were derived from adult β-catenin$^{lox(ex3)/wt}$ mouse brains in order to assess the effect of β-catenin$^{\Delta ex3}$ on these cells. Expression of β-catenin$^{\Delta ex3}$ was induced by infecting the NSCs with Adenovirus-Cre. As expected, Adeno-Cre infected β-catenin$^{lox(ex3)/wt}$ NSCs expressed β-catenin$^{\Delta ex3}$ (**Figure 5.1**). Expression levels were equivalent to those of wild-type β-catenin, confirming efficient infection and recombination. However, there was no increase in cyclin D1 detected (**Figure 5.1**), suggesting that proliferation may not be increased.

Subsequently, growth was measured using the WST-1 and Hoechst proliferation assays. Growth was assessed over the course of three days. Using the standard WST-1 protocol NSC proliferation was found to decrease over this time period (**Figure 5.2**). To this end the starting cell number was optimised to 3.5 x 10^3 cells/well (**Figure 5.2**). No significant growth difference was detected between Adeno-Cre treated, Adeno-GFP treated or untreated NSCs (n=3) (**Figure 5.3** and **Figure 5.4**). Similarly, no significant difference was found in the diameter of neurospheres (n=3) (**Figure 5.5**).

NSC self-renewal was assessed using the limited dilution assay. Figure 5.6 shows no significant difference between Adeno-Cre and Adeno-GFP infected β-catenin$^{lox(ex3)/wt}$ NSCs (n=3) (**Figure 5.6**).

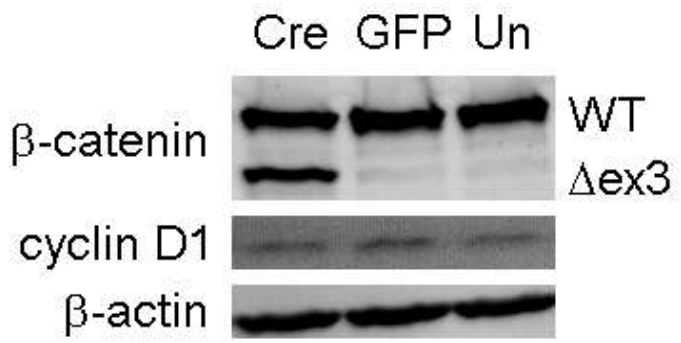


Figure 5.1 Adeno-Cre infected β-catenin$^{lox(ex3)/wt}$ NSCs express β-catenin$^{\Delta ex3}$, but do not have increased cyclin D1 levels

Untreated (Un), Adeno-GFP (GFP) and Adeno-Cre (Cre)-treated β-catenin$^{lox(ex3)/wt}$ NSC protein extracts were immunoblotted with antibodies against β-catenin (1:500), cyclin D1 (1:2000) and β-actin (1:1000). 10 µg of total protein was loaded per lane. This is a representative western blot. Adeno-Cre infected β-catenin$^{lox(ex3)/wt}$ NSCs expressed β-catenin$^{\Delta ex3}$ at an equivalent level to that seen with wild-type β-catenin. However, no increase in cyclin D1 was detected. β-actin was used as an internal loading control.

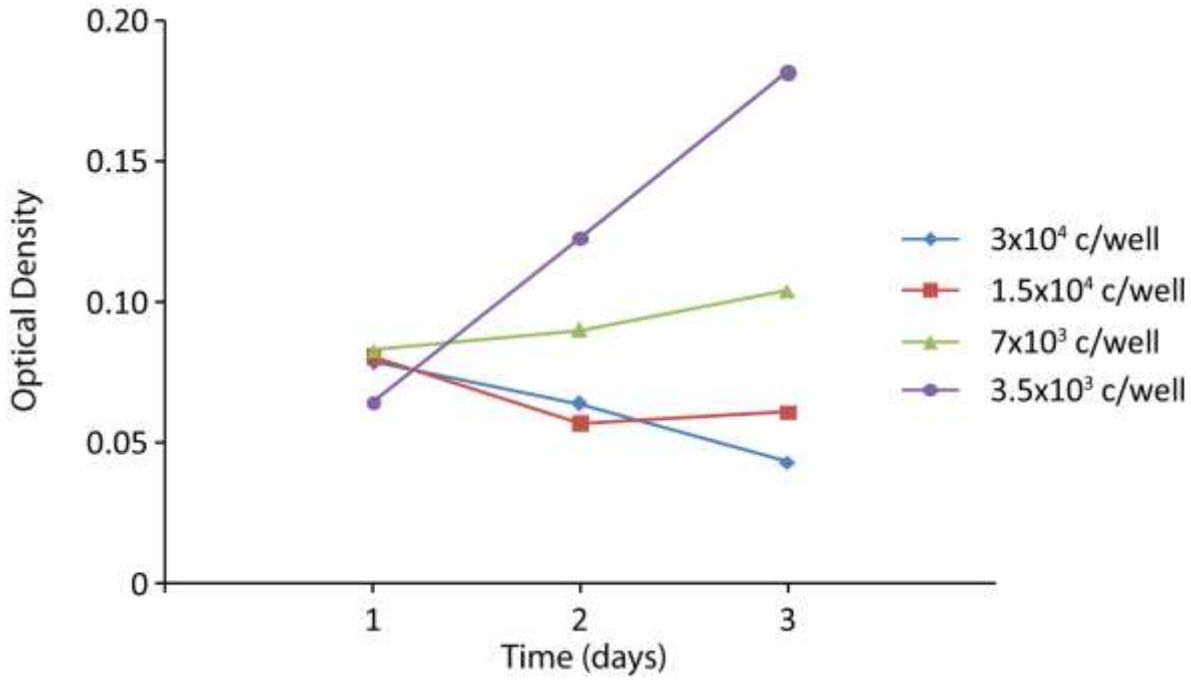


Figure 5.2 The optimal starting cell number for the WST-1 proliferation assay is 3.5 x 10^3 cells/ml

Neurospheres were passaged to a single cell suspension and untreated β-catenin$^{lox(ex3)/wt}$ cells plated in quintuplet at densities of 3 x 10^4, 1.5 x 10^4, 7 x 10^3, 3.5 x 10^3 cells/ml in wells of a 96-well plate. Three identical plates were prepared. At day one, two and three after passage, WST-1 reagent was added to one 96-well plate. WST-1 is metabolised by mitochondria into a coloured formazan salt. The OD of the formazan salt can be measured at 440nm and correlates with cell number. Over the course of three days, the proliferation of cells plated at 3 x 10^4 and 1.5 x 10^4 cells/ml decreased. The proliferation of cells plated at 7 x 10^3 cells/ml was found to be marginally increased. In contrast, the proliferation of cells plated at 3.5 x 10^3 cells/ml was markedly higher.

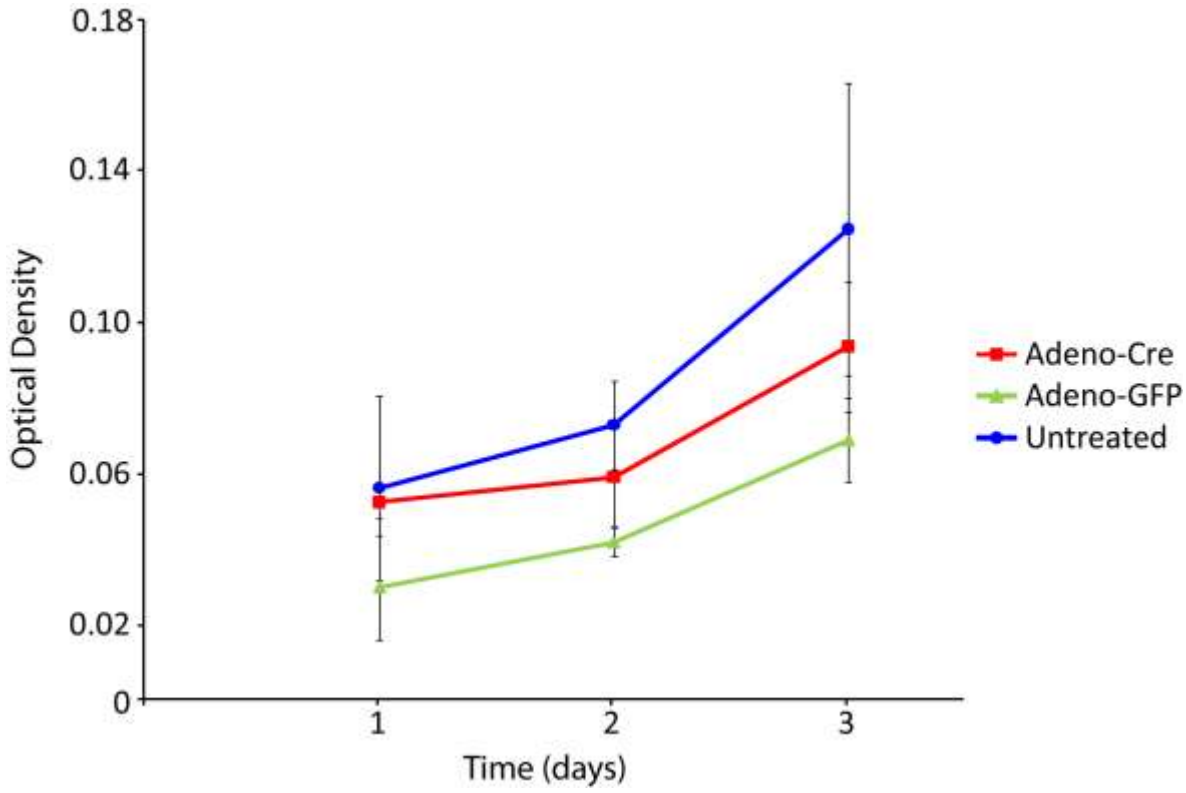


Figure 5.3 No difference in the proliferation of β-catenin$^{\Delta ex3}$ expressing NSCs (WST-1 proliferation assay)

Neurospheres from untreated, Adeno-GFP-treated and Adeno-Cre-treated β-catenin$^{lox(ex3)/wt}$ cultures were passaged to a single cell suspension. Cells were plated in quintuplet for each treatment at 3.5 x 10^3 cells/well. Three identical 96-well plates were prepared. At day one, two and three after passage, WST-1 reagent was added to one 96-well plate. WST-1 is metabolised by mitochondria into a coloured formazan salt. The OD of the formazan salt can be measured at 440nm and correlates with cell number. The results shown represent the average of three independent cultures. No significant difference in proliferation was detected between any two treatment groups at any time point (two-tailed t-test). OD values were tested for normality and homoscedasticity.

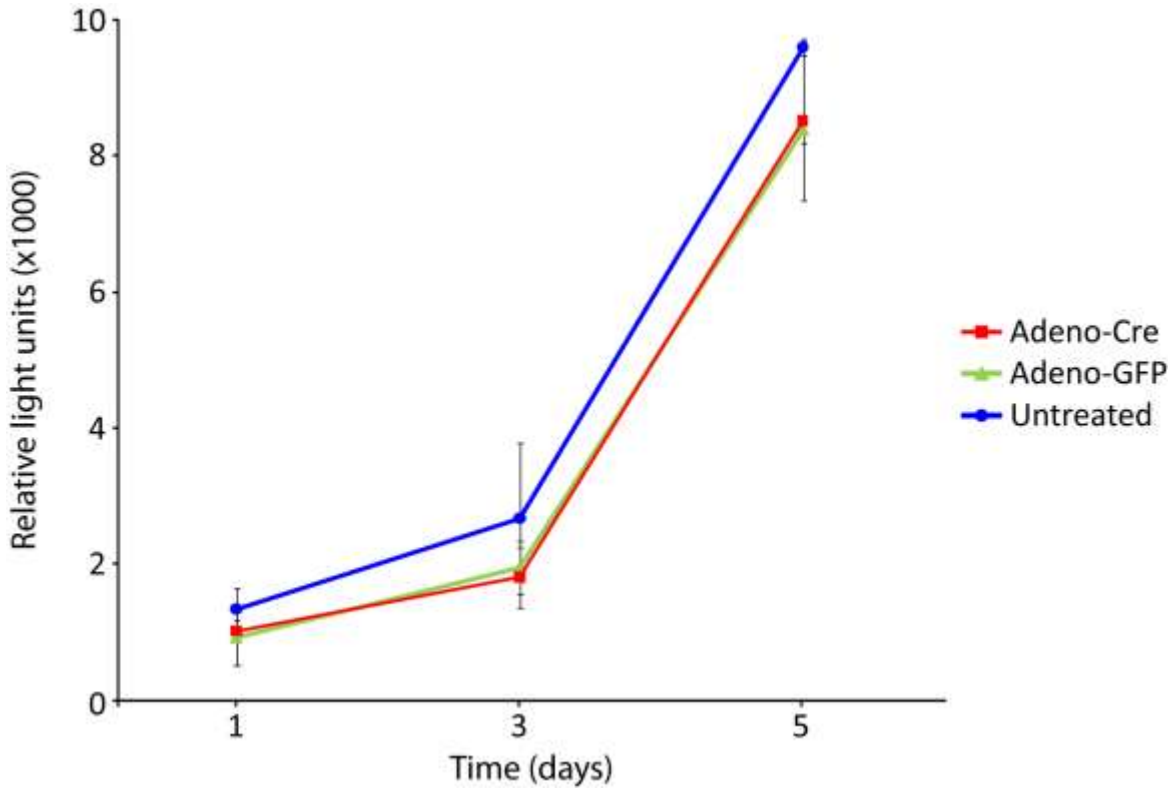


Figure 5.4 No difference in the proliferation of β-catenin$^{\Delta ex3}$ expressing NSCs (Hoechst proliferation assay)

Neurospheres from untreated, Adeno-GFP-treated and Adeno-Cre-treated β-catenin$^{lox(ex3)/wt}$ cultures were passaged to a single cell suspension. Cells were plated in quintuplet for each treatment at a cell density of $3.5x10^3$ cells/well. Three identical 96-well plates were prepared. At day one post-plating, 50 ng of Hoechst 33342/DMSO was added to each well of one of the plates and incubated for 30 min at 37°C. The plate was subsequently centrifuged for 1 min at 1000 x *g* and as much medium as possible was removed and replaced with 1X PBS, which allowed the amount of unbound Hoechst to be lowered. Hoechst fluorescence was detected at 360 nm excitation and 465 nm emission. The same procedure was repeated at days two and three post plating with the remaining 96-well plates. No significant growth difference was detected between any two treatment groups (two-tailed T-test). OD values where tested for normality and homoscedasticity.

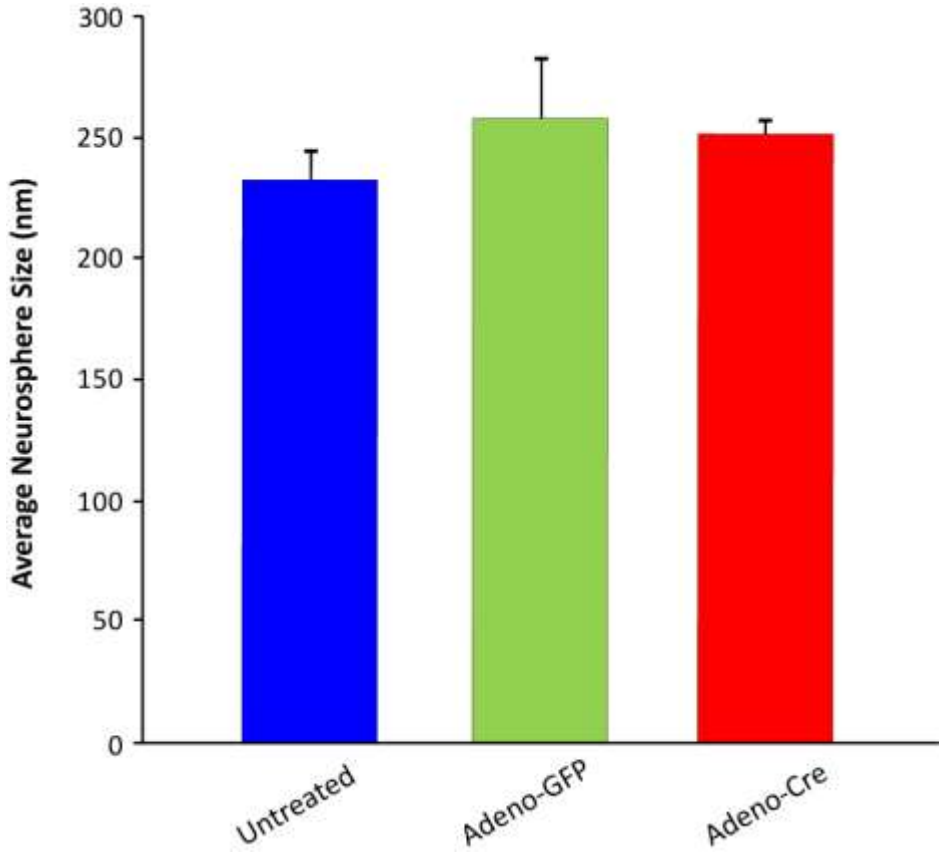


Figure 5.5 No difference in the size of neurospheres from β-catenin$^{\Delta ex3}$ expressing NSCs

Neurospheres from untreated, Adeno-GFP-treated and Adeno-Cre-treated β-catenin$^{lox(ex3)/wt}$ cultures were passaged to a single cell suspension and plated at 0.5 x 10^6 cells in individual 6 cm diameter plates. Ten days after passaging, the diameter of the neurospheres was measured using an Axiovert 135 microscope (Zeiss) and the Openlab 5 software. The diameter of a 100 neurospheres was measured for each treatment group. An n=3 was calculated from three separate cultures for each treatment group. No significant difference in neurosphere size was found (two-tailed T-test). Neurosphere diameter measurements where checked for normality and homoscedasticity.

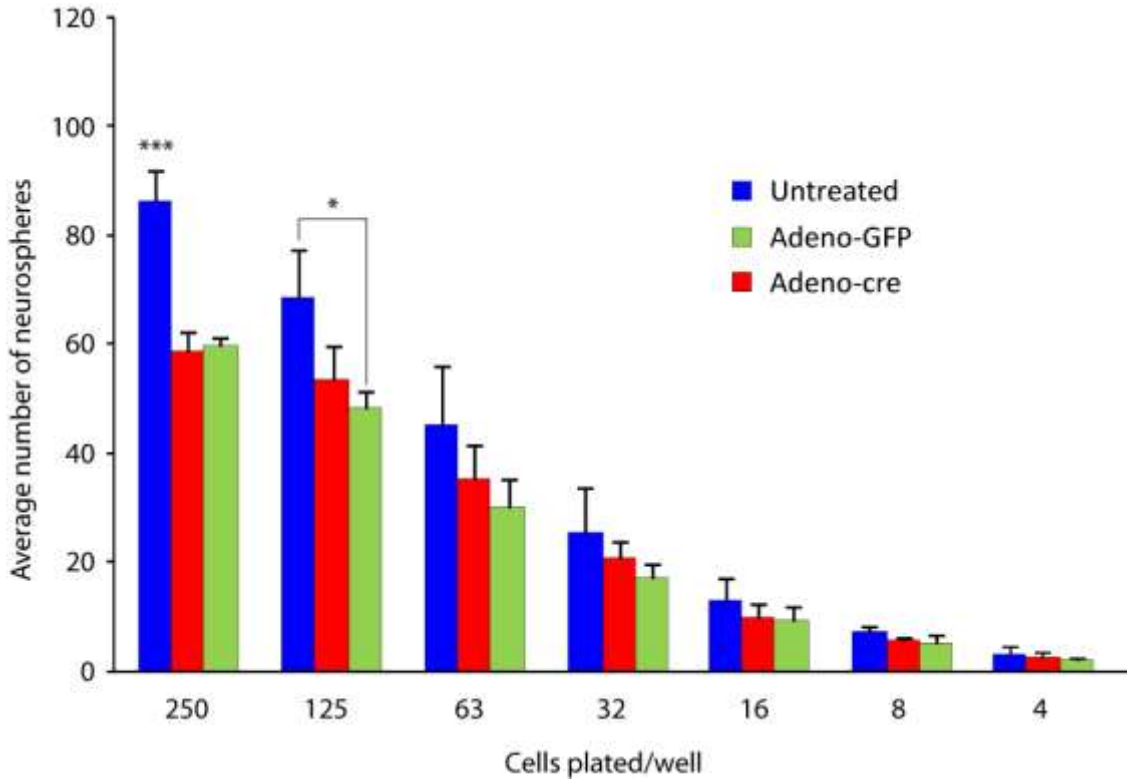


Figure 5.6 There is no difference in self-renewal between Adeno-Cre and Adeno-GFP treated NSCs

Neurospheres from untreated, Adeno-GFP-treated and Adeno-Cre-treated β-catenin$^{lox(ex3)/wt}$ cultures were passaged to a single cell suspension. Around 500 cells were plated into each well of the top row of a 96-well plate. The cells were serially diluted for each row of wells. Ten days after passage the number of neurospheres was counted. The results from each cell concentration were the average of twelve wells. An n=3 was calculated from three individual cultures for each treatment group. No significant difference in self-renewal was detected between Adeno-Cre and Adeno-GFP treated NSCs. The self-renewal of untreated β-catenin$^{lox(ex3)/wt}$ NSCs was significantly higher than Adeno-Cre and Adeno-GFP treated NSCs at 250 cells/well ($p \leq 0.001$). Similarly, the self-renewal of untreated β-catenin$^{lox(ex3)/wt}$ NSCs was significantly higher than Adeno-GFP treated NSCs at 125 cells/well ($p \leq 0.05$).

5.2.2 Expression of β-catenin$^{lox(ex3)/wt}$ does not increase differentiation

The role of the Wnt/β-catenin pathway in NSC differentiation is not completely understood. To assess the effect of β-catenin$^{\Delta ex3}$ expression in NSC differentiation, Adeno-Cre and Adeno-GFP treated β-catenin$^{lox(ex3)/wt}$ neurospheres were cultured on laminin-coated chamber slides. To promote differentiation, the neurospheres were cultured in media complemented with 10 % foetal calf serum, in the absence of growth factors. Forty eight hours post-culture, cells were immunostained for GFAP (marker for astrocytes), MAP2 (marker for neurones), O4 (marker for oligodendrocytes), Nestin (marker for undifferentiated cells) and β-catenin (**Figure 5.7** and **Figure 5.8**). In view of the fact that MAP2 immunostaining failed for two of the three experiments, quantification was not possible. O4-positive cells were rare in both treatment groups (≤ 1 % of cells per field of view). Interestingly, O4-positive cells were predominantly found near neurospheres. However, the number of neurospheres plated per well was variable. To this end, quantification of O4-positive cells would be biased by the number of neurospheres plated. As expected, nuclear β-catenin was significantly increased in Adeno-Cre treated cells (**Figure 5.9**). No significant difference was detected in the percentage of GFAP- or Nestin-expressing cells (**Figure 5.9**).

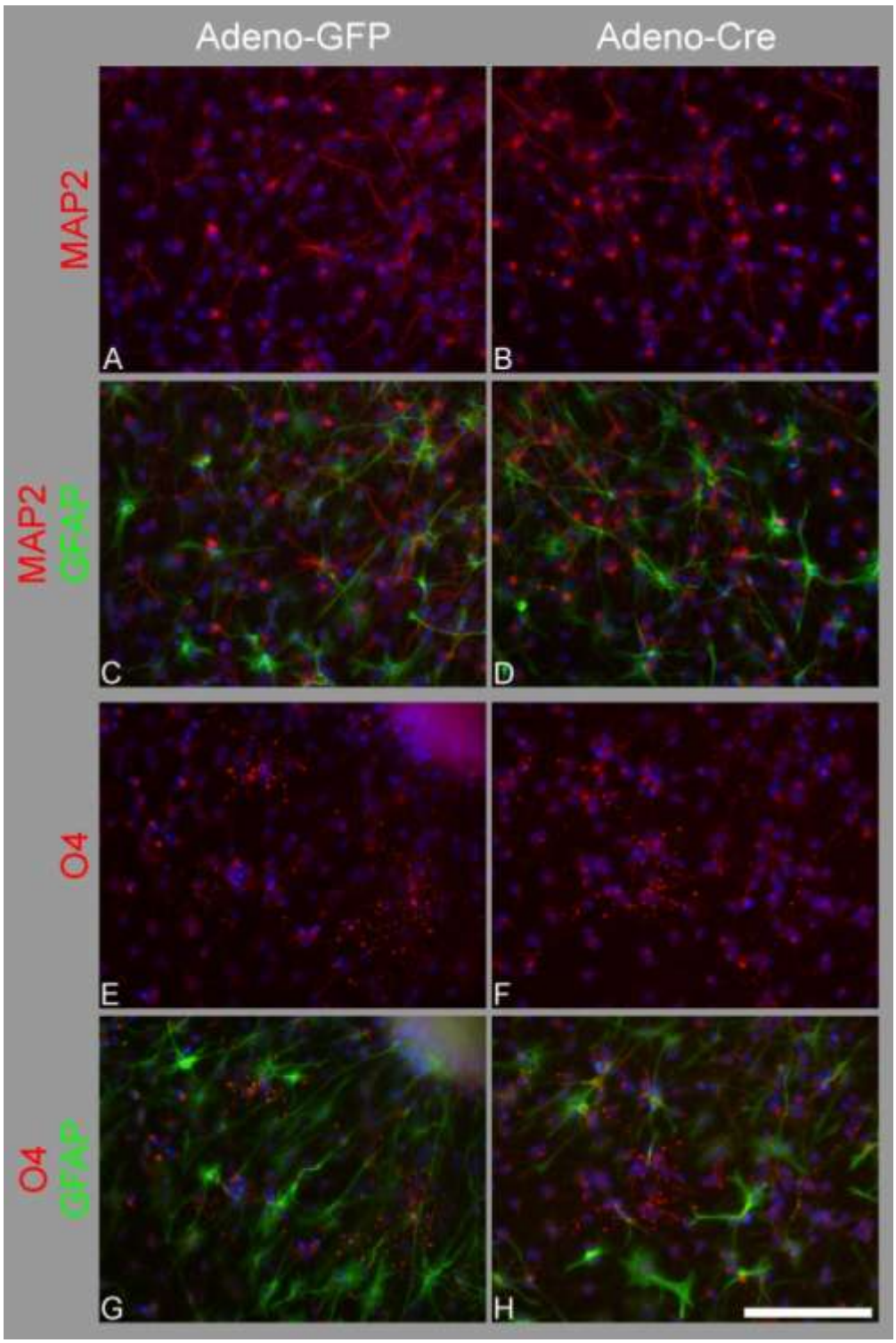


Figure 5.7 β-catenin$^{lox(ex3)/wt}$ NSCs express MAP2, O4 and GFAP

Adeno-Cre and Adeno-GFP treated β-catenin$^{lox(ex3)/wt}$ neurospheres were plated on laminin-coated chamber slides in DMEM/Ham-F12 media supplemented with 10 % foetal calf serum (FCS). Forty eight hours post-culture, cells were fixed with 4 % formaldehyde and immunostained with rabbit polyclonal anti-GFAP (1:500) (**C, D, G and H**), mouse monoclonal anti-MAP2 (1:500) (**A-D**) and mouse monoclonal anti-O4 (1:300) (**E-H**). Hoechst 33342 (1:1000) was added to label the nuclei. The scale bar in **H** corresponds to 150 µm

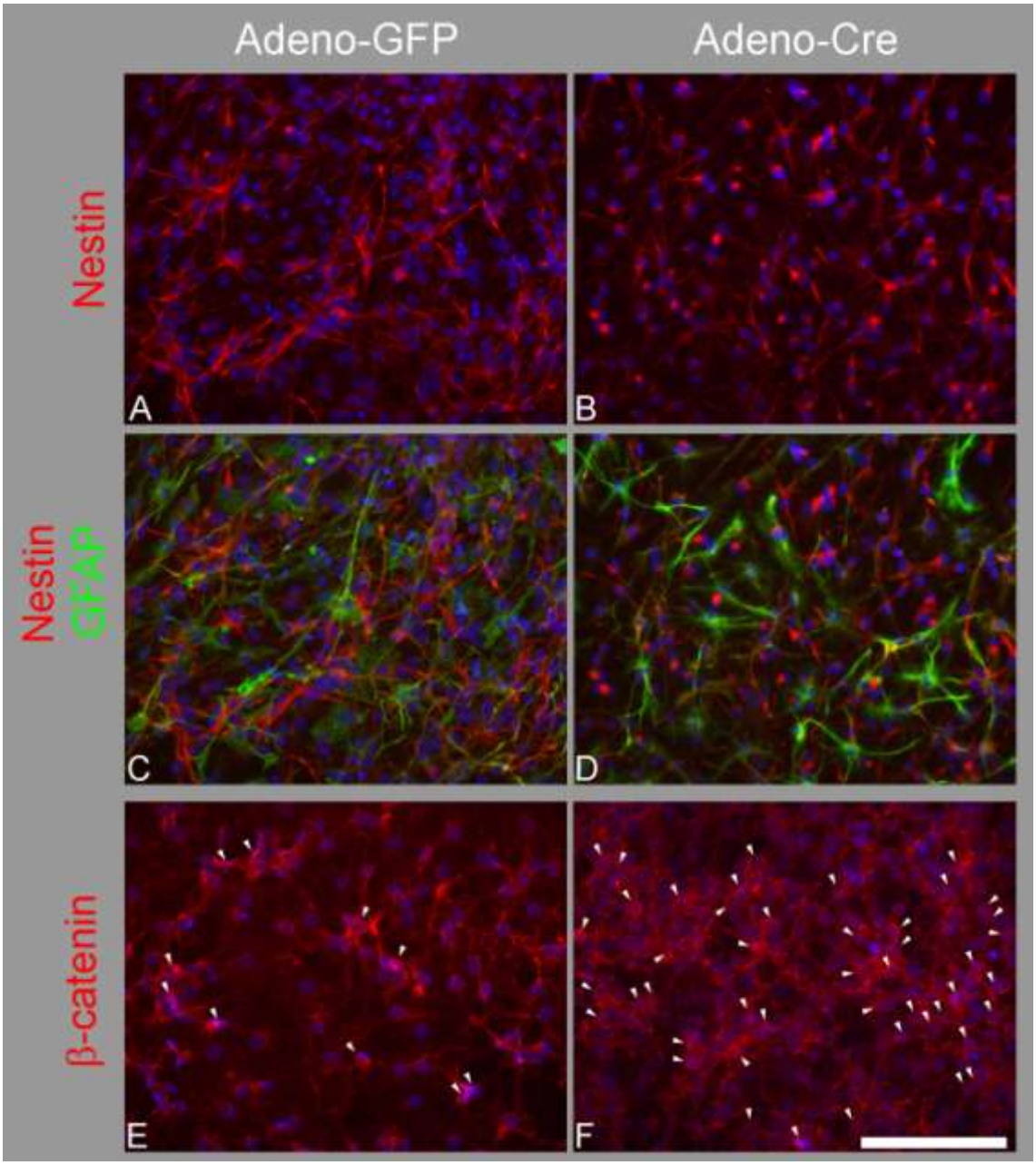


Figure 5.8 β-catenin$^{lox(ex3)/wt}$ NSCs express Nestin, β-catenin and GFAP

Adeno-Cre and Adeno-GFP treated β-catenin$^{lox(ex3)/wt}$ neurospheres were plated on laminin-coated chamber slides in DMEM/Ham-F12 media supplemented with 10 % foetal calf serum (FCS). Forty eight hours post-culture, cells were fixed with 4 % formaldehyde and immunostained with rabbit polyclonal anti-GFAP (1:500) (**C and D**), mouse monoclonal anti-Nestin (1:200) (**A-D**) and mouse monoclonal anti-β-catenin (1:500) (**E and F**). Hoechst 33342 (1:1000) was added to label the nuclei. The white arrows in (**E and F**) represent β-catenin nucleo-positive cells. The scale bar in **F** corresponds to 150 µm

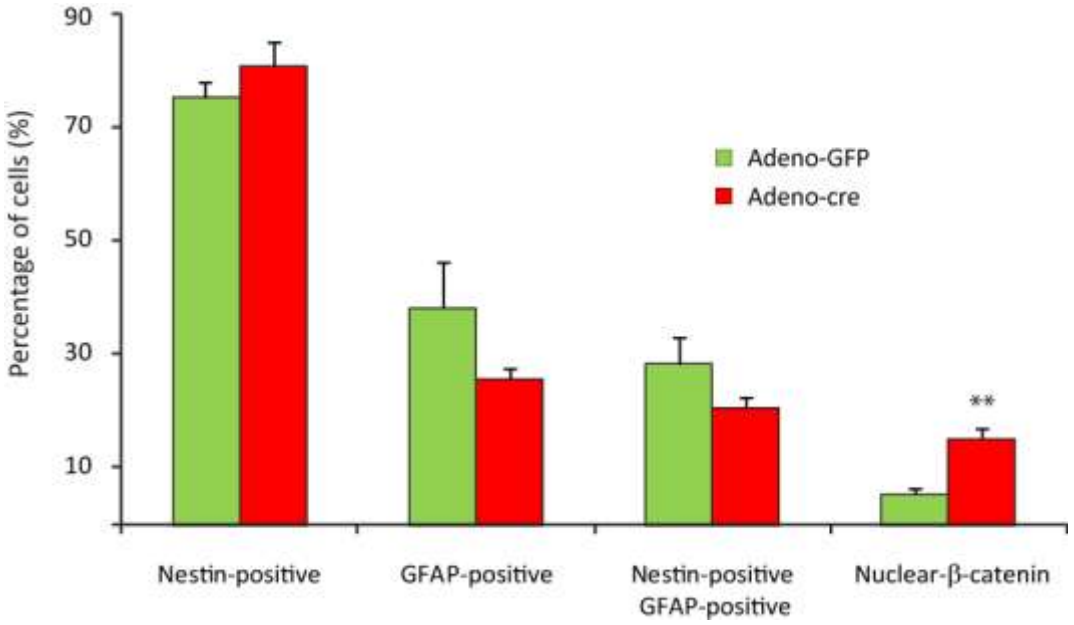
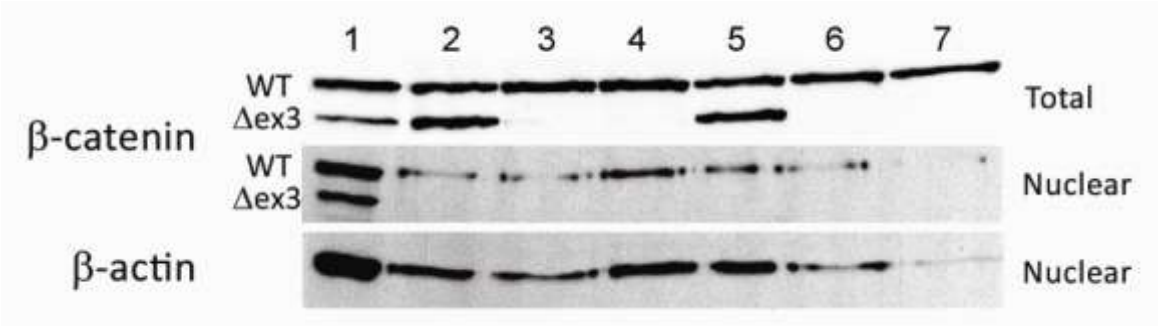


Figure 5.9 No difference in differentiation between Adeno-Cre and Adeno-GFP treated β-catenin$^{lox(ex3)/wt}$ NSCs

Adeno-Cre and Adeno-GFP treated β-catenin$^{lox(ex3)/wt}$ neurospheres were plated on laminin-coated chamber slides in DMEM/Ham-F12 media supplemented with 10 % foetal calf serum (FCS). Forty eight hours post-culture, cells were fixed with 4 % formaldehyde and immunostained for GFAP, Nestin and β-catenin. Cells were counted from three random fields of view for each immunostaining. The results presented are an average from three independent cultures for each treatment. There was no significant difference in the percentage of GFAP and Nestin positive cells between Adeno-Cre and Adeno-GFP treated β-catenin$^{lox(ex3)/wt}$ NSCs. However, the percentage of cells with nuclear β-catenin was significantly higher in Adeno-Cre treated cells (p= 0.002) (two-tailed T-test).

5.2.3 Nuclear wild type β-catenin is more abundant than nuclear β-catenin$^{\Delta ex3}$ in Adeno-Cre treated cells

As mentioned previously, Adeno-Cre treated β-catenin$^{lox(ex3)/wt}$ NSCs do not show enhanced growth or self-renewal. These results are different from published data. One possible explanation may be that β-catenin$^{\Delta ex3}$ did not translocate into the nucleus. Although β-catenin$^{\Delta ex3}$ has been shown to translocate into the nucleus of ES cells (Harada *et al*., 1999), activating mutations in β-catenin are not always associated with nuclear translocation in tumours (Abraham *et al*., 2001; Anna *et al*., 2000; Kobayashi *et al*., 2000). To assess nuclear β-catenin protein levels, Adeno-Cre, Adeno-GFP and untreated β-catenin$^{lox(ex3)/wt}$ NSC nuclear fractions were isolated. Subsequently, protein extracts from these fractions were immunoblotted for β-catenin. Interestingly, all three NSC treatment groups showed high levels of nuclear wild-type β-catenin (**Figure 5.10**). In contrast, no β-catenin$^{\Delta ex3}$ was detected in Adeno-Cre treated cultures (n=2) (**Figure 5.10**). This may explain why no increase in growth and self-renewal was detected in these cells.



1 Adeno-Cre β-catenin$^{\Delta ex3/wt}$ total protein control
2 Adeno-Cre β-catenin$^{\Delta ex3/wt}$ (I)
3 Adeno-GFP β-catenin$^{lox(ex3)/wt}$ (I)
4 Untreated β-catenin$^{lox(ex3)/wt}$ (I)
5 Adeno-Cre β-catenin$^{\Delta ex3/wt}$ (II)
6 Adeno-GFP β-catenin$^{lox(ex3)/wt}$ (II)
7 Untreated β-catenin$^{lox(ex3)/wt}$ (II)

Figure 5.10 β-catenin$^{\Delta ex3}$ protein is not detected in the nucleus of Adeno-Cre recombined NSCs

Nuclear fractions from untreated, Adeno-GFP and Adeno-Cre treated β-catenin$^{lox(ex3)/wt}$ NSC were immunoblotted with antibodies against β-catenin (1:500) and β-actin (1:1000). 10 µg of total protein was loaded per lane, except for **lane 7** where nuclear protein yield was low. Control Adeno-Cre treated β-catenin$^{lox(ex3)/wt}$ NSC total protein was loaded into **lane 1**. Nuclear fractions in **lanes 2-4 and 5-7** are from two independent cultures respectively. All treatment groups contained nuclear wild-type β-catenin. However, there was no nuclear β-catenin$^{\Delta ex3/wt}$ detected, despite its strong expression in total protein fractions. β-actin was used as an internal loading control.

5.2.4 **En2-Cre; β-catenin$^{lox(ex3)/wt}$ mice show developmental abnormalities**

To assess the role of degradation-resistant β-catenin in the CNS, β-catenin$^{lox(ex3)/wt}$ mice were crossed with Nestin-Cre and En2-Cre mice. No Nestin-Cre; β-catenin$^{\Delta ex3/wt}$ double mutant mice were born from this crossing. These results are in agreement with published reports; a recent study demonstrated that Nestin-Cre; β-catenin$^{\Delta ex3/wt}$ mice are embryonic lethal (Wrobel *et al*., 2007). This phenotype is similar to that observed in Nestin-Δ90N-β-catenin mice (Chenn and Walsh, 2002).

Instead, En2-Cre; β-catenin$^{\Delta ex3/wt}$ double mutant mice were viable. Engrailed 2 (En2) belongs to a family of evolutionary conserved transcription factors. During early development, En2 is expressed in spatially-restricted domains across the mid/hind-brain junction, the region from which the cerebellum develops (Davis and Joyner, 1988; Davis *et al*., 1988; Davis *et al*., 1991; Davidson *et al*., 1988). En2-Cre; β-catenin$^{\Delta ex3/wt}$ mouse brains showed marked reduction of the cerebellar vermis, resulting in a tissue defect (**Figure 5.11** and **Figure 5.12**). The phenotype was so pronounced that the vermis was not fused at the midline (**Figure 5.11**). Furthermore, the cerebella showed severe lobular abnormalities. In **Figure 5.12 A**, all lobules but the tenth were missing. Similarly, in **Figure 5.12 B**, only lobules III, IX and X are visible. However, in this example lobule III and IX are clearly abnormal when compared to controls (**Figure 5.12 C**). Of note, lobule II fails to fold back resulting in the inner granule layer meeting with the inferior colliculus (**Figure 5.12**).

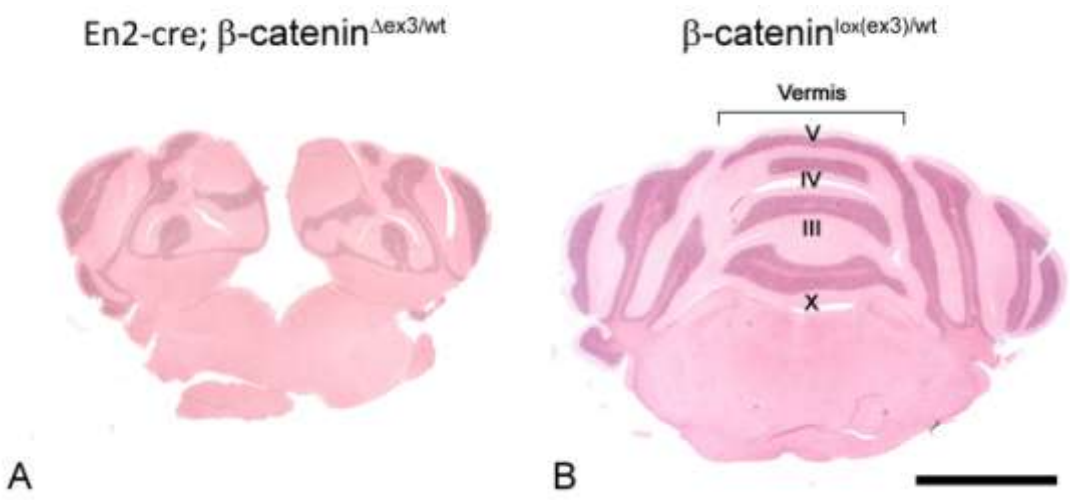


Figure 5.11 The cerebella of En2-Cre; β-catenin$^{\Delta ex3/wt}$ mice show signs of severe hypoplasia (coronal)
Coronal sections of cerebella from En2-Cre; β-catenin$^{lox(ex3)/wt}$ (**A**) and control β-catenin$^{lox(ex3)/wt}$ (**B**) mice were stained for H&E. Compared to the control, the En2-Cre; β-catenin$^{\Delta ex3/wt}$ cerebellum is showing severe hypoplasia in the vermis. The roman numerals represent the different cerebellar lobules. The scale bar in **B** corresponds to 1500 µm.

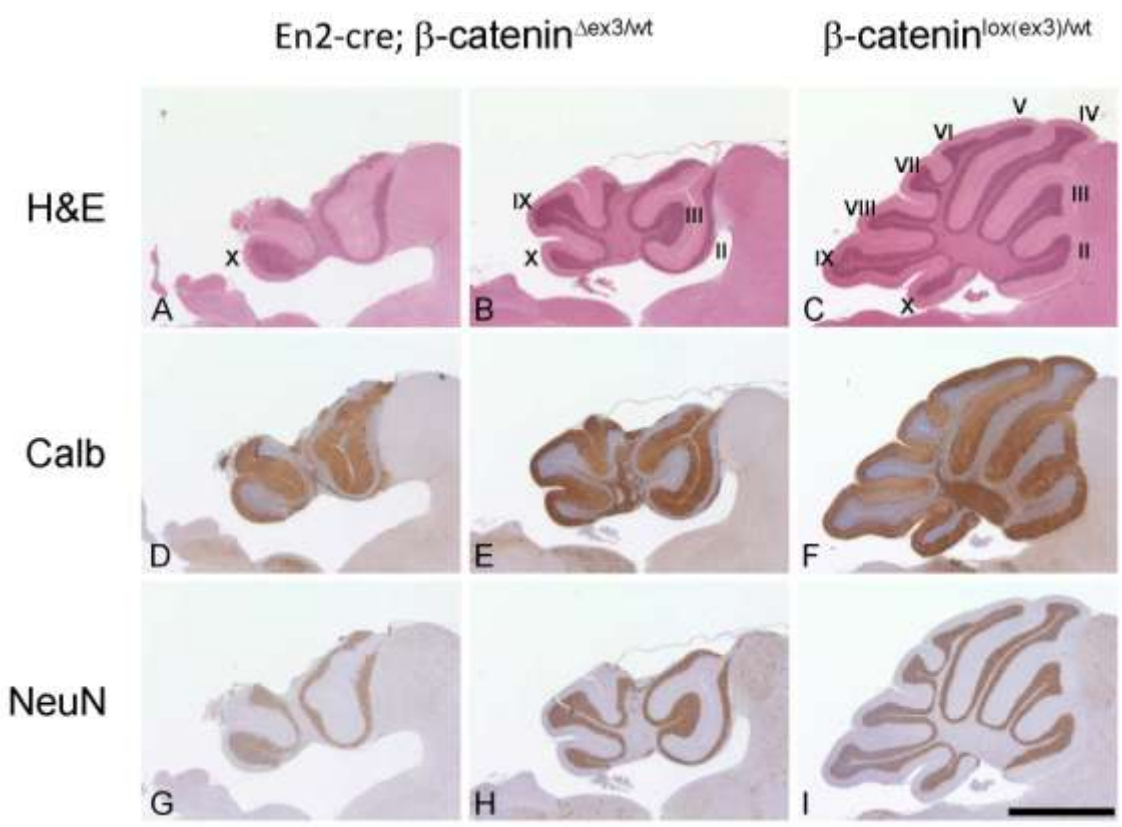


Figure 5.12 The cerebella of En2-Cre; β-catenin$^{lox(ex3)/wt}$ mice show signs of severe hypoplasia (sagittal) Sagittal sections of cerebella from En2-Cre; β-catenin$^{lox(ex3)/wt}$ (A-B; D-E; G-H) and β-catenin$^{lox(ex3)/wt}$ (C, F, I) mice were stained for H&E (**A-C**), calbindin (Calb) (**D-F**) and NeuN (**G-I**). The roman numerals represent the different cerebellar lobules. En2-Cre; β-catenin$^{\Delta ex3/wt}$ cerebella (**A**) show severe lobular abnormalities compared to the control. Although lobules III and IX are present in (**B**), they are abnormal. Lobule X appears unaffected in En2-Cre; β-catenin$^{\Delta ex3/wt}$. Interestingly, lobule II fails to fold back in En2-Cre; β-catenin$^{\Delta ex3/wt}$ mice, resulting in the internal granule layer fusing with the inferior colliculus. The scale bar in **I** corresponds to 1300 μm.

5.2.5 β-catenin$^{lox(ex3)/wt}$ mice develop SVZ hyperpasia, but no neoplasia following Adeno-Cre injection

To express β-catenin$^{\Delta ex3}$ in the SVZ, the ventricles of adult β-catenin$^{lox(ex3)/wt}$ mice were injected with Adeno-Cre virus. 100 days post-infection, mice were injected with BrdU and then sacrificed (n=5). The brains were then sectioned coronally and stained for GFAP (astrocytes), Synatophysin (SYP; neurones) and BrdU (dividing cells and daughter cells). Small growths were detected in the SVZ of Adeno-Cre injected β-catenin$^{lox(ex3)/wt}$ mice. These growths consisted of an underlying layer of GFAP+/SYP-/BrdU+ cells and a mass of GFAP-/SYP-/BrdU- cells, which protruded into the ventricle (**Figure 5.13**). Although GFAP+/SYP-/BrdU+ cells share the expression profile of SVZ neural stem cells, more work is needed for confirmation (Doetsch, *et al* 1999). In contrast, the identity of the GFAP-/SYP-/BrdU- cells was not clear. It is unlikely that they were transit-amplifying daughter cells, as such cells have a high proliferative potential and would have stained strongly for BrdU.

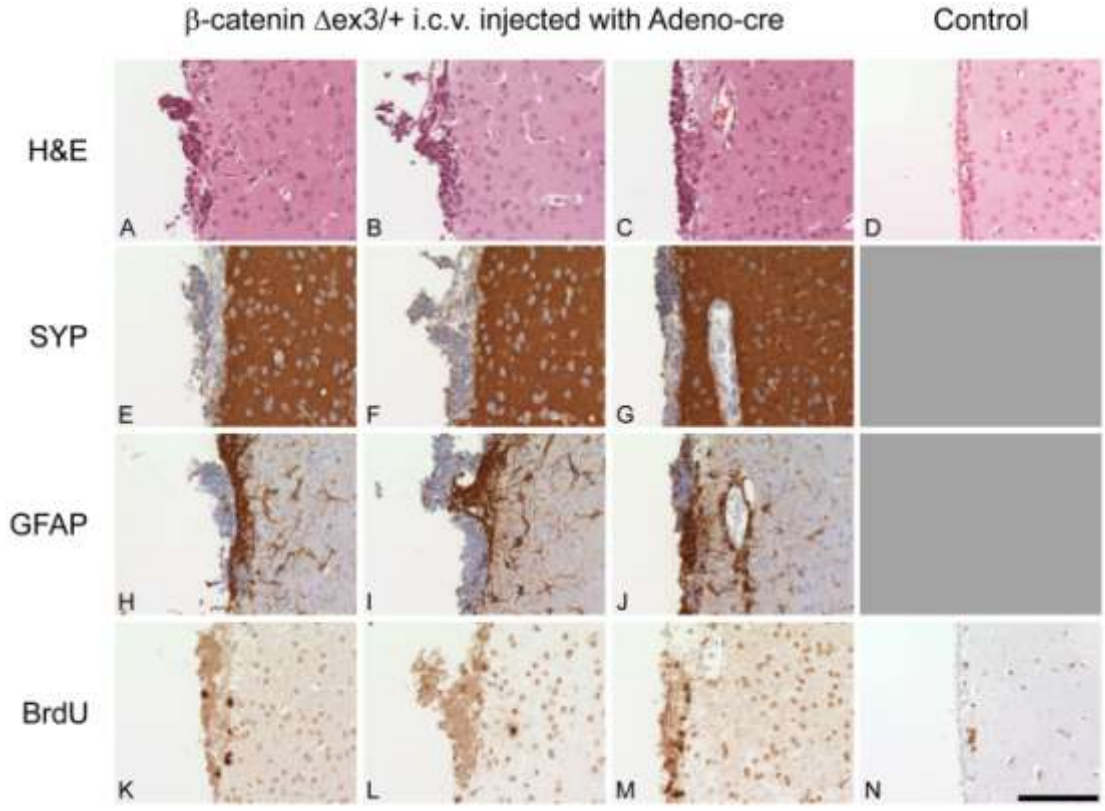


Figure 5.13 β-catenin$^{lox(ex3)/wt}$ mice develop SVZ enlargements after Adeno-Cre intra cranial (i.c.) injections

β-catenin$^{lox(ex3)/wt}$ mice were injected with Adeno-Cre into the ventricles. After 100 days, mice were injected with BrdU and culled. SVZ staining for H&E (**A-C**), synaptophysin (SYP) (**E-G**), GFAP (**H-J**) and BrdU (**K-M**) shows abnormal growths. These growths are composed of a layer of GFAP+/SYP-/BrdU+ cells and GFAP-/SYP-/BrdU- cells. The GFAP+/SYP-/BrdU+cells may be stem cells. The identity of the GFAP-/SYP-/BrdU- cells is unclear. The control panels (**D and N**) correspond with a Rosa26 LacZ mouse injected with Adeno-Cre (90 days incubation). The scale bar in **N** corresponds to 130 µm.

5.3 **Discussion**

Results from the ΔGSK-β-catenin mouse model and NSC cultures, presented in chapters 3 and 4, did not show a significant phenotype and after careful testing it was decided to use an alternative mouse model. The β-catenin$^{lox(ex3)/wt}$ mouse model is the most commonly used model for studying the effect of degradation-resistant β-catenin (Harada *et al*., 1999). In this model, exon 3 of the endogenous β-catenin gene, which encodes the phosphorylation motif of β-catenin is flanked by loxP sites. Following Cre-mediated recombination, exon 3 is deleted and degradation-resistant β-catenin$^{\Delta x3}$ is expressed. This model confers two key advantages. First, as β-catenin$^{\Delta ex3}$ expression is controlled by the endogenous β-catenin promoter and regulatory elements, this model depicts β-catenin mutations in cancer more accurately than the ΔGSK-β-catenin mouse model. Second, as β-catenin$^{\Delta ex3}$ is 76 aa smaller than wild-type β-catenin, they can be distinguished from one another by simple immunoblotting based on size (using the same antibody).

To assess the role of β-catenin$^{\Delta ex3}$ expression on NSCs *in vitro*, neurospheres were derived, expanded and recombined as described in chapter 2. Immunoblotting analysis of Adeno-Cre-treated NSCs showed expression of β-catenin$^{\Delta ex3}$, the protein levels of which were similar to those of wild-type β-catenin (expressed by the other allele), and confirmed efficient recombination. However, expression of β-catenin$^{\Delta ex3}$ did not elicit an increase of cyclin D1 protein levels. Similarly, Adeno-Cre-treated cells did not grow significantly faster or larger than controls and no differences were detected in NSC self-renewal. One possible explanation for these results is that β-catenin$^{\Delta ex3}$ may not translocate into the nucleus of our cultured cells. β-catenin$^{\Delta ex3}$ protein has been shown to translocate into the nucleus of ES cells (Harada *et al*., 1999). However, activating mutations in β-catenin are not always associated with nuclear translocation in tumours (Abraham *et al*., 2001; Anna *et al*., 2000; Kobayashi *et al*., 2000). Therefore, to test this hypothesis, nuclear extracts were isolated from Adeno-Cre, Adeno-GFP and untreated β-catenin$^{lox(ex3)/wt}$ NSCs. Western blotting analysis (using an anti-β-catenin antibody) demonstrated high levels of wild-type β-catenin, but not β-catenin$^{\Delta ex3}$. This is in contrast to the results obtained with total protein fractions. One reason for this could be loss of protein during nuclear fractionation. However, no β-catenin$^{\Delta ex3}$ was detected in either of the Adeno-Cre treated NSC nuclear protein fraction samples. These results suggest that in undifferentiated NSC cultures, β-catenin$^{\Delta ex3}$ does not appear to translocate into the nucleus. This would explain the lack of any significant differences in growth and self-renewal, but more evidence is needed to substantiate these results.

Results presented in this chapter contradict with published data. Expression of degradation-resistant β-catenin has been shown to increase the proliferation and self-renewal

of NSCs *in vitro* (Lie *et al*., 2005; Qu *et al*., 2010; Yu *et al*., 2006). A possible explanation for this discrepancy could be the NSC culture conditions in our system. For example, in the study by Yu *et al.*, NSCs were cultured in medium containing DMEM/HAMS F12, EGF, FGF, B27 supplement and 10 % FCS. Of note, serum has been shown to cause irreversible differentiation of NSCs and therefore was not considered as a suitable condition (Gage *et al*., 1995; Lee *et al*., 2006; McKay, 1997; Reynolds and Weiss, 1992). Similarly, during isolation of adult hippocampal NSCs by Lie and colleagues (2005), cultures were treated with DMEM/HAMS F12 and 10 % FCS for 24 hours before treatment with media supplemented with N2 and FGF (Lie *et al*., 2005)**.** Apart from irreversible differentiation, culturing NSCs in FCS allows for the permanent decrease of NSC proliferation (Lee *et al*., 2006). Another study however, disagrees with these findings and their interpretation. In a study by Qu and colleagues, NSCs were cultured under similar conditions as in our study (Qu *et al*., 2010). However, the NSCs grew in monolayers and not as free-floating spheres. It is possible that the cells in the study of Qu and colleagues were grown in laminin-coated culture plates. It remains to be shown whether growth as a monolayer influences the translocation of degradation-resistant β-catenin into the nucleus.

The Wnt/β-catenin pathway plays a complex role in differentiation and maintenance of NSCs. To assess the role of β-catenin$^{\Delta ex3}$ expression in differentiation, NSCs were grown in laminin-coated chamber slides and cultured in differentiation medium (DMEM/HAMS F12 supplemented with 10 % FCS). 48h post-culture, cells where immunostained for GFAP (astrocytes), MAP2 (neurones), O4 (oligodendrocytes), β-catenin and Nestin (undifferentiated cells). Expression of β-catenin$^{\Delta ex3}$ increased the fraction of cells with nuclear β-catenin. However, there was no significant difference in the fraction of GFAP-positive or Nestin-positive cells. Results from MAP2 immunostaining were inconclusive. O4-positive cells were rare, ranging from 0-5 cells per field of view. They were more prominently found in close proximity to neurospheres. However, the number of neurospheres plated per well was variable. Therefore, it was not possible to accurately compare the O4 immunostaining between the different treatment groups. Taking into account the lack of a significant difference in Nestin staining, these results suggest that expression of β-catenin$^{\Delta ex3}$ is not increasing differentiation. This is in disagreement with known roles of the Wnt/β-catenin in NSC differentiation. The Wnt/β-catenin pathway appears to have a dual role in differentiation depending on the developmental stage of cells. Constitutive activation of the Wnt/β-catenin pathway during early development appears to inhibit NSC differentiation without an increase in proliferation (Chenn and Walsh, 2002; Wrobel *et al*., 2007). Similarly, during adulthood, low-level Wnt/β-catenin pathway activity has been proposed to maintain NSCs in an undifferentiated state (Wexler *et al*., 2009). However, constitutive activation of the pathway during late development

and in adult NSCs increases neuronal differentiation (Hirabayashi *et al*., 2004; Lie *et al*., 2005; Wexler *et al*., 2008). These results could be explained by an increase in the proliferation of cell committed to neuronal differentiation rather than by a putative increase in differentiation. Lie *et al*. reported that Wnt3 can increase the amount of new neurones by five- to ten-fold under non-proliferating conditions. However, even under such conditions, five percent of cells were found to still be proliferating (Lie *et al*., 2005). These authors do not mention the percentage of cells proliferating without Wnt3 treatment. Therefore, the possibility remains that five percent of proliferating cells could give rise to this increase in neurogenesis. In this case the prospect that the Wnt/β-catenin pathway may promote the proliferation of neuronally committed precursor cells rather than increasing proliferation is still possible. If so, it may explain why the percentage of cells expressing Nestin were not decreased in NSC cultures expressing β-catenin$^{\Delta ex3}$.

To assess the effect of β-catenin$^{\Delta ex3}$ in the CNS, β-catenin$^{lox(ex3)/wt}$ mice were crossed with two CNS Cre-recombinase-expressing mouse lines: Nestin-Cre (expression in neural precursors and adult neural stem cells) and En2-Cre, which drives expression in spatially-restricted domains across the mid/hind-brain junction, which develop into the cerebellum (Davis and Joyner, 1988; Davis *et al*., 1988; Davis *et al*., 1991; Davidson *et al*., 1988). As expected, no Nestin-Cre; β-catenin$^{\Delta ex3/wt}$ double mutant mice were born. These mice have been reported to be embryonic lethal due to abnormalities in the maturation of radial glia (Wrobel *et al*., 2007). Instead, En2-Cre; β-catenin$^{\Delta ex3/wt}$ double mutant mice were viable and showed ataxia after 3 weeks, caused by a defect in the cerebellar vermis, where the Cre transgene is expressed (Zinyk *et al*., 1998). Furthermore, most of the cerebellar lobules were either missing or were abnormal, except for the tenth lobule. Interestingly, this phenotype was similar to that observed in mice that express orthodenticle homeobox 2 (Otx2), a gene involved in cerebellar patterning, under the control of Engrailed 1 (En1$^{wt/Otx2LacZ}$ mice) (Broccoli *et al*., 1999). Like in En2-Cre; β-catenin$^{\Delta ex3/wt}$ cerebella, the vermis of these mice was reduced and not fused at the midline (Broccoli *et al*., 1999). Furthermore, only the posterior lobules VIII, IX and X are present (Broccoli *et al*., 1999). In order to compare these two transgenic mouse models it is necessary to discuss certain aspects of cerebellar development.

From anterior to posterior, the neural tube is composed of the prosencephalon, the mesencephalon, the metencephalon and the myelencephalon. Studies in quail-chick chimaeras have indicated that both the mesencephalon and metencephalon contribute to cerebellum development (Hallonet *et al*., 1990; Hallonet and Le Douarin, 1993; Hatten and Heintz, 1995). The isthmic organizer (IO), located just caudally to the junction of these two regions, functions in releasing signalling proteins required for the correct patterning of the mid-hindbrain (Bally-

Cuif *et al*., 1992; Bally-Cuif and Wassef, 1994; Martinez *et al*., 1991) (**Figure 5.14**). The IO is in turn organised by a complex gene expression pattern. Specifically, the negative interaction of two genes, namely Otx2 and gastrulation brain homeobox 2 (Gbx2), defines IO positioning in the brain (Broccoli *et al*., 1999; Katahira *et al*., 2000; Millet *et al*., 1999). One of the main signalling proteins originating from the IO is Fgf8, which induces Wnt1 expression (Adams *et al*., 2000; Reifers *et al*., 1998). Subsequently, Wnt1 regulates the expression of En1 and En2 (Bally-Cuif *et al*., 1992; Mcmahon *et al*., 1992).

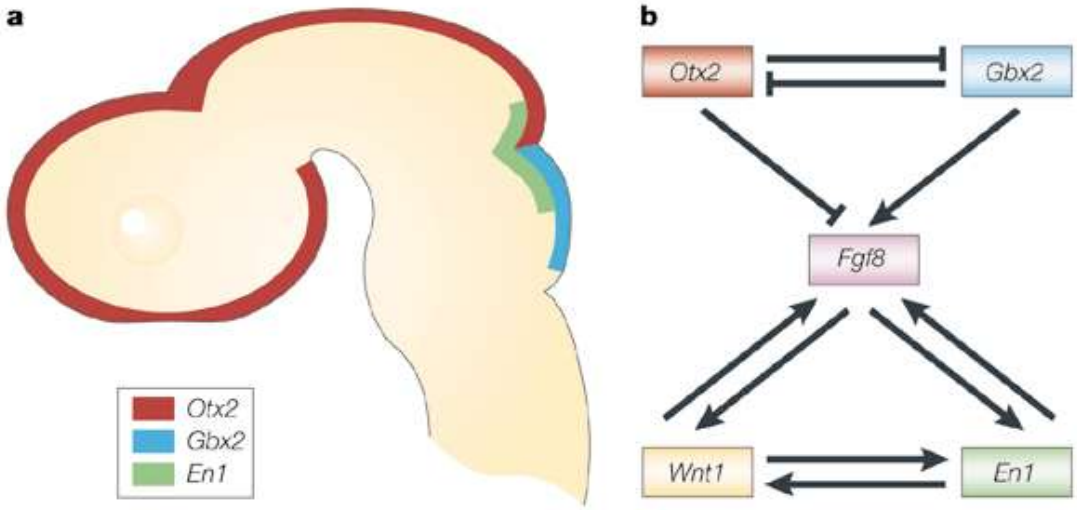


Figure 5.14 Patterning of the midbrain/hindbrain region[5]

(**a**) Formation of the mid-/hindbrain region is dependent on proper functioning of the isthmus organizer, which is formed by the interaction, or reciprocal repression, of Otx2 and Gbx2. En1 is expressed in both the mid- and hindbrain regions. (**b**) The genetic interactions among Otx2, Gbx2, Fgf8, Wnt1 and En1. En1, engrailed 1; Fgf8, fibroblast growth factor 8; Gbx2, gastrulation brain homeobox 2; Otx2, orthodenticle homologue 2.

[5] Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Neuroscience, Vincent Y. Wang and Huda Y. Zoghbi, Genetic regulation of cerebellar development, **2**: 484-491, copyright (2001).

In $En1^{wt/Otx2LacZ}$ transgenic mice, Otx2 expression is pushed caudally (Broccoli *et al*., 1999). As a result, Wnt1 expression is shifted into the rhombic lip, the germinal area from which granule neurones, glutamergic deep nuclear neurones and unipolar brush cells originate (Alder *et al*., 1996; Englund *et al*., 2006; Fink *et al*., 2006; Machold and Fishell, 2005; Wang *et al*., 2005) (**Figure 5.15**). In the $En1^{wt/Otx2LacZ}$ mouse model, Wnt1 is expressed in the anterior region of the rhombic lip. Interestingly, mapping experiments have shown that precursors from this area give rise to the cerebellar vermis (Sgaier *et al*., 2005). Furthermore, Wnt1 acts through the canonical pathway in cerebellar development (Panhuysen *et al*., 2004). Collectively, these studies suggest that activation of the Wnt/β-catenin pathway in the rhombic lip is detrimental to cerebellar development. This is in agreement with the data obtained from the En2-Cre; $\beta\text{-catenin}^{\Delta ex3/wt}$ mouse model presented here. Of note, conditional APC loss-of-function in neural crest-derived cells results in a massive apoptosis of neural crest cells (Hasegawa *et al*., 2002). Hence, activation of the Wnt/β-catenin pathway may promote the apoptosis of cerebellar rhombic lip precursors.

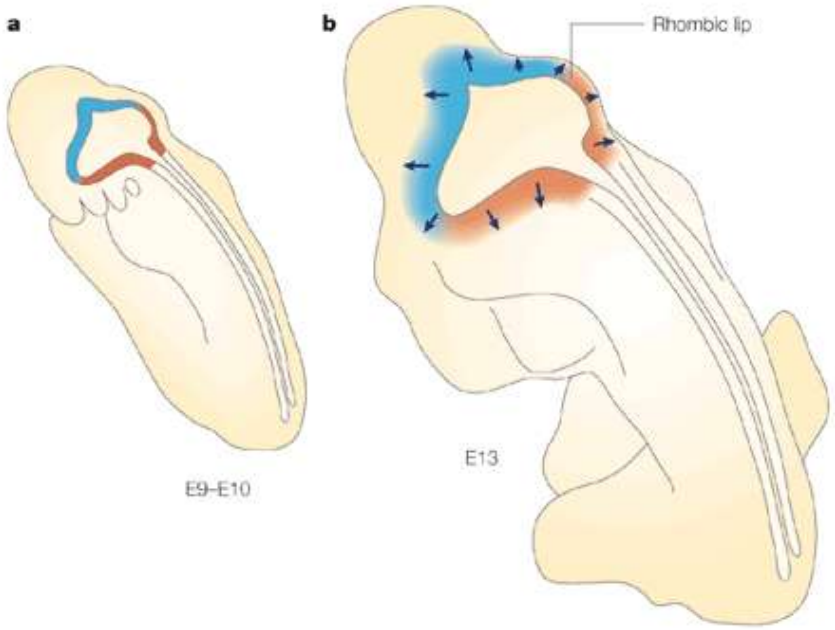


Figure 5.15 Granule neurone development in mice[6]

(**a**) At embryonic day (E) 9, the rhombic lip (blue and orange) is a zone of proliferation between the fourth ventricle and the neural tube. The cells in the blue region give rise to the cerebellar granule neurons and pontine nucleus, whereas cells in the red region yield other rhombic lip derivatives such as the inferior olivary nucleus. (**b**) At about E13, cells from the rhombic lip migrate outwards to populate the cerebellar anlage.

[6] Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Neuroscience, Vincent Y. Wang and Huda Y. Zoghbi, Genetic regulation of cerebellar development, **2**: 484-491, copyright (2001).

De-regulation of the Wnt/β-catenin pathway has been documented for tumours of the CNS (**Table 1.5**). Previous reports have documented that constitutive activation of the Wnt/β-catenin pathway in SVZ neural stem cells was not sufficient to elicit tumours (Fults *et al*., 2002; Momota *et al*., 2008). However, the latest time point in these studies was three months. Jacques *et al*. showed that conditional inactivation of two or even three tumour suppressor genes in the SVZ results in brain tumours after up to eight months (Jacques *et al*., 2010). Therefore, constitutive activation of the Wnt/β-catenin pathway may still promote tumourigenesis in the long-term. To assess this, β-catenin$^{lox(ex3)/wt}$ mice were injected with Adenovirus-Cre into the lateral ventricles, where the SVZ is located. This experiment is currently ongoing. However, a cohort of mice was culled for analysis 100 days post-infection. Encouragingly, these mice showed abnormal enlargements in the SVZ. Immunohistochemical staining of these growths for GFAP, BrdU and synaptophysin (SYP) indentified two cell populations. The first population consisted of GFAP+/SYP-/BrdU+ cells, which correspond with markers expressed by type B neuronal stem cells. The second population consisted of GFAP-/SYP-/BrdU- cells, which do not match with markers for either type C transit-amplifying cells or type A neuroblasts. Hence, these preliminary results presented here suggest that activation of the Wnt/β-catenin pathway in the SVZ promotes neural stem cell expansion *in vivo*. However, one year post injection the mice did not develop any tumours (Brandner, S, private communication).

5.4 **Summary**

Expression of β-catenin$^{\Delta ex3}$ in NSCs does not significantly increase growth or self-renewal potential *in vitro*. This may be attributed to β-catenin$^{\Delta ex3}$ not translocating into the nucleus. However, more data is needed to confirm this result. However, mice, which expressed β-catenin$^{\Delta ex3}$ under the control of the En2 promoter, had a developmental defect in the area corresponding to embryonal Cre expression, which later becomes the cerebellar vermis. Finally, activation of dominant active β-catenin$^{lox(ex3)/wt}$ mice in the stem/progenitor cell compartment of the SVZ in the lateral ventricles resulted in small hyperplasias, but no neoplastic lesions in the SVZ. Experiments are ongoing to assess the collaboration with other oncogenic signals, such as p53 or RB pathway inactivation.

6 Conclusion and future work

6.1 Summary

To study the role of the Wnt/β-catenin pathway in the CNS, we generated transgenic mice that conditionally express degradation-resistant β-catenin (ΔGSK-β-catenin). However, as described in chapter 3 and 4, ΔGSK-β-catenin expression in developing and the adult CNS did not result in detectable developmental abnormalities (with or without p53 expression). *In vitro* expression of ΔGSK-β-catenin did not alter the proliferation or self-renewal of adult NSCs. Similarly, additional deletion of the tumour suppressor genes Tp53 or Rb did not convincingly alter the proliferation or self-renewal of these cells. To directly recombine the SVZ neural stem cells, ΔGSK-β-catenin; $p53^{loxP/loxP}$ mice were injected with Adeno-Cre into the lateral ventricles. Nonetheless, no mice were found to have developed hyperplasias or tumours as late as one year after intraventricular injection. The most plausible explanation for the absence of a phenotype is that the expression levels of ΔGSK-β-catenin might have been insufficient to elicit an effect. However, this hypothesis has not been confirmed experimentally. Instead, we obtained an alternative transgenic mouse model for expressing degradation-resistant β-catenin; these were β-$catenin^{lox(ex3)/wt}$ transgenic mice. Expression of β-$catenin^{\Delta ex3}$ in NSCs did not significantly alter growth or self-renewal. This may be attributed to β-$catenin^{\Delta ex3}$ not translocating into the nucleus. However, more data is needed to confirm this result. *In vivo*, no Nestin-cre; β-$catenin^{\Delta ex3}$ were born as expected, whilst mice expressing β-$catenin^{\Delta ex3}$ under the control of the En2 promoter survived to term. These mice showed severe cerebellar vermis abnormalities. The cause for this phenotype was not determined in this study as time did not permit. However, existing literature suggests that constitutive activation of the Wnt/β-catenin pathway during early development can cause severe apoptosis of neuronal crest derived cells. Finally, β-$catenin^{lox(ex3)/wt}$ mice injected with Adeno-Cre into the lateral ventricles developed growths in the SVZ one hundred days post-injection. These growths did not appear to be highly proliferative, but there are mice available for analysis at later time-points.

6.2 Future work

6.3 Synergism between Wnt/β-catenin, p53, PTEN and Rb pathways in CNS tumours

One of the goals of this study was to investigate the synergism between the Wnt/β-catenin pathway and the p53, Rb and PTEN pathway in brain tumourigenesis. However, due to complications with the ΔGSK-β-catenin mice this was not achieved. Therefore, the alternative mouse model, β-catenin$^{lox(ex3)/wt}$,should be used to ascertain the role of the abovementioned pathways in tumourigenesis.

6.3.1 TP53, PTEN and RB in gliomas

Astrocytic gliomas progress from low-grade diffuse astrocytomas (WHO grade II) to anaplastic astrocytomas (WHO grade III) and subsequently to (secondary) glioblastomas (WHO grade IV). TP53 is mutated in more than 60 % of diffuse astrocytomas (Okamoto *et al*., 2004; Reifenberger *et al*., 1996; Watanabe *et al*., 1996). The frequency of these mutations does not change during tumour progression, which suggests that TP53 mutations are important for tumourigenesis (Sidransky *et al*., 1992; von *et al*., 1992; Watanabe *et al*., 1996; Watanabe *et al*., 1997).

The RB1 gene encodes the retinoblastoma protein, which plays a crucial role in G1 to S phase transition during proliferation (Weinberg, 1995). The RB1 gene is found at chromosome 13q, which is lost in 12 % of primary and 38 % of secondary glioblastomas (Nakamura *et al*., 2000). Additionally, promoter hypermethylation occurs in 12 % of primary and 43 % of secondary glioblastomas (Nakamura *et al*., 2001a). Interestingly, de-regulation of the Rb pathway also occurs in anaplastic astrocytomas (Reifenberger *et al*., 1994; Schmidt *et al*., 1994). These findings suggest that the pathway is important for progression from diffuse astrocytomas to anaplastic astrocytomas.

PTEN is a tumour suppressor that antagonises the phosphatidylinositol-3-kinase (PI3K) pathway (Maehama and Dixon, 1998). Loss of chromosome 10q, where PTEN is located, occurs in 70 % of glioblastomas (Ohgaki *et al*., 2004). This incidence is similar between primary and secondary glioblastomas (Tohma *et al*., 1998). Loss of chromosome 10q is infrequent in low grade gliomas, but has been reported in 35 % to 60 % of anaplastic astrocytomas, suggesting a role in progression (Balesaria *et al*., 1999; Bigner and Vogelstein, 1990; Ichimura *et al*., 1998; James *et al*., 1988).

β-catenin is rarely mutated in gliomas (Gotze *et al*., 2009). However, β-catenin, TCF4, LEF1, c-myc and cyclin D1 expression increases with glioma malignancy grade (Sareddy *et al*., 2009b). This effect may be partially attributed to an increase in promoter hypermethylation of Wnt antagonists (Gotze *et al*., 2009). Similarly, Wnt antagonist promoter hypermethylation is common in secondary glioblastomas (Gotze *et al*., 2009). These results suggest that activation of the Wnt/β-catenin pathway may be involved in both glioma progression and spontaneous development of primary glioblastomas.

In summary, aberrations in the p53 pathway are vital for glioma tumourigenesis. On the other hand mutations in the PTEN, Rb and Wnt/β-catenin pathways also seem to play a role in tumour progression. Taking these findings into account, it is unlikely that β-catenin$^{lox(ex3)/wt}$; Rb$^{loxP/loxP}$ or β-catenin$^{lox(ex3)/wt}$; PTEN$^{loxP/loxP}$ mice will develop gliomas. However, β-catenin$^{lox(ex3)/wt}$; p53$^{loxP/loxP}$ mice might develop gliomas, which would shed some light into the role of the Wnt/β-catenin pathway in these tumours.

Finally, p53$^{loxP/loxP}$; Rb$^{loxP/loxP}$ and p53$^{loxP/loxP}$; PNET$^{loxP/loxP}$ mice develop PTEN-like and glioma-like tumours (Jacques *et al*., 2010). Therefore it would be interesting to determine how the expression of β-catenin$^{\Delta ex3}$ may influence the tumour phenotype of these mice.

6.3.2 **TP53, PTEN and RB in medulloblastomas**

TP53 mutations are rare in medulloblastomas (5-10 %), but if present are associated with poor prognosis (Adesina *et al*., 1994; Alderson *et al*., 1996; Ohgaki *et al*., 1993) (Tabori *et al*., 2010). Additionally, the p53 pathway is altered by alternative mechanisms in up to 21 % of medulloblastomas (Frank *et al*., 2004). Interestingly, mice null for double-strand break repair genes and p53 develop medulloblastomas (Frappart *et al*., 2009; Yan *et al*., 2006). These tumours often acquire mutations in components of the Shh pathway (Frappart *et al*., 2009; Yan *et al*., 2006). This is relevant since mutations in the Wnt and Shh pathways are mutually exclusive events in medulloblastoma (Thompson *et al*., 2006). This suggests that mutations in the p53 pathway may not correlate with medulloblastomas with Wnt/β-catenin pathway de-regulation. Hence, β-catenin$^{lox(ex3)/wt}$; p53$^{loxP/loxP}$ mice may not be biologically relevant for modelling medulloblastomas.

PTEN mutations are also rare in medulloblastomas (Rasheed *et al*., 1997). However, loss of 10q is found in 18 % to 42 % of medulloblastoma cases (Avet-Loiseau *et al*., 1999; Gilhuis *et al*., 2000; Mendrzyk *et al*., 2005; Reardon *et al*., 1997; Shlomit *et al*., 2000). Furthermore, PTEN promoter hypermethylation can be detected in 50 % of medulloblastomas (5/10 cases investigated) (Hartmann *et al*., 2006).

No mutations in the RB gene have been detected in medulloblastomas (Lee *et al*., 1987). However, mice with conditional deletions of Rb and p53 in GFAP-expressing cells develop tumours with medulloblastoma features and combined inactivation of Rb and p53 in SVZ stem/progenitor cells give rise to PNETs, which are histologically similar to medulloblastomas (Jacques *et al*., 2010; Marino *et al*., 2000). Hence, de-regulation of the Rb pathway may play a role in medulloblastoma development.

As discussed in chapter 1, activation of the Wnt/β-catenin pathway has been reported in around 23 % of medulloblastomas (**Table 1.5**). Interestingly, medulloblastomas with nuclear β-catenin immunoreactivity tend to:

1. Have β-catenin mutations (92 %, 23/25)
2. Have lost of a copy of chromosome 6 (70 %, 14/20)
3. Show few other chromosomal abnormalities
4. Show histological features of classical medulloblastomas (85 %, 40/47)

(Clifford *et al*., 2006; Ellison *et al*., 2005; Thompson *et al*., 2006). This suggests that loss of chromosome 10 and 13, which are associated with PTEN and Rb loss, does not correlate with the Wnt/β-catenin pathway in medulloblastomas. However, genes lost on chromosome 6 may be involved in the p53, PTEN or Rb pathway. Hence, β-catenin$^{lox(ex3)/wt}$; Rb$^{loxP/loxP}$ or β-catenin$^{lox(ex3)/wt}$; PTEN$^{loxP/loxP}$ or β-catenin$^{lox(ex3)/wt}$; p53$^{loxP/loxP}$ mice might develop medulloblastomas.

6.3.3 Tumour suppressor genes on chromosome 6

Full or partial loss of chromosome 6 has been reported for other types of cancer, including breast cancer, lymphomas and melanomas (Noviello *et al*., 1996; Offit *et al*., 1993; Trent *et al*., 1983). This suggests that important tumour suppressor genes are present on this chromosome. In support of this argument, microcell-mediated transfer of chromosome 6 into the breast cancer cell line MDA-MB-231, resulted in reduced *in vitro* invasion and tumourigenic potential (Theile *et al*., 1996). Some tumour suppressor genes located on chromosome 6 are summarised in (**Table 6.1**).

Table 6.1 Selection of tumour suppressor genes located on the human chromosome 6

Human gene	Abbreviation	Chromosomal position
Cyclin-dependent kinase inhibitor 1A	CDKN1A	6p21.2
Nucleolar protein 7	NOL7	6p23
Absent in melanoma 1	AIM1	6q21
Cyclin C	CCNC	6q21
Protein tyrosine phosphatase, receptor type K	PTPRK	6q22.33
Pleiomorphic Adenoma gene-like 1	PLAGL1	6q24.2
SAM and SH3 domaint containing 1	SASH1	6q24.3
Large tumour suppresor, *Drosophila* homolog 1	LATS1	6q25.1

Of the genes listed in Table 6.1, LATS1 and PLAGL1 are of particular interest. LATS1 is the human homolog of *Drosophila* warts (wts), a member of the Hippo pathway (Justice *et al*., 1995). In *Drosophila*, the Hippo pathway inhibits cell proliferation and promotes proliferation by inhibiting Yorkie (Yki) (Huang *et al*., 2005). The mammalian homolog of Yki is Yes-associated protein 1 (YAP1), which is phosphorylated and inhibited by LATS1 (Dong *et al*., 2007; Oh and Irvine, 2009). Interestingly, YAP1 is up-regulated in medulloblastomas with an active Wnt/β-catenin pathway (Fernandez *et al*., 2009). This suggests that de-regulation of the Hippo pathway may be an important event in medulloblastomas with Wnt/β-catenin pathway activation. The Hippo pathway partly regulates proliferation by negatively regulating the Wnt/β-catenin pathway. Small interfering RNA knockdown of LATS1 in a human embryonal kidney cell line (HEK293T) increased the Wnt3a-dependent expression of AXIN2 and enhanced the luciferase expression of the TOPflash Wnt/β-catenin pathway reporter plasmid (Varelas *et al*., 2010). Interestingly, TEA domain 1 (TEAD1), the major transcriptional partner of YAP1, is also up-regulated in medulloblastomas (Fernandez *et al*., 2009; Zhao *et al*., 2008). TEAD1 is negatively regulated by p38α MAPK, which is located on chromosome 6 (Ambrosino *et al*.,

2006). Therefore, deregulating the Hippo pathway seems an attractive target for studying Wnt/β-catenin medulloblastomas.

PLAGL1 is a member of the novel subfamily of zinc-finger transcription factors, consisting of PLAG1, PLAGL1 and PLAGL2. PLAGL1 has been shown to induce apoptosis and G1 arrest, in a manner similar to p53 (Spengler *et al*., 1997). Subsequently, it was found that PLAGL1 could directly bind to p53 and significantly enhance the expression of p53-responsive genes (Huang *et al*., 2001). This effect was not observed in the absence of p53, suggesting that PLAGL1 is a transcriptional co-factor of p53 (Huang *et al*., 2001). In the cerebellum, PLAGL1 expression was detected in the external granule layer from postnatal (P) day 2 to 7 (Ciani *et al*., 2003). Nuclear PLAGL1 was also detected in Purkinje cells at P7 (Ciani *et al*., 2003). In the adult cerebellum, PLAGL1 expression is low and primarily found in Purkinje cells (Ciani *et al*., 2003). Interestingly, PLAGL1 expression co-localises with neuronal stem cell markers (Nestin, GFAP, proliferating cell nuclear antigen [PCNA] and BrdU) during CNS development (Valente *et al*., 2005). Furthermore, promoting PLAGL1 expression by raising cyclic Adenosine monophosphate (cAMP) decreased BrdU incorporation in cerebellar granule neurons (Contestabile *et al*., 2005). These results suggest that PLAGL1 has a key role in controlling neuronal stem cell proliferation. The PLAGL1 gene is paternally imprinted in mice and humans (Kamiya *et al*., 2000; Smith *et al*., 2002). This means that PLAGL1 is only expressed from the paternal chromosome 6. Medulloblastomas with Wnt/β-catenin pathway activation tend to lose one copy of chromosome 6. It would be interesting to assess if this is the copy of chromosome 6 that is lost in these tumours.

No conditional knockout mouse models have been published for either of these genes. Once they have been generated, it will be interesting to cross them with β-catenin$^{lox(ex3)/wt}$ mice to determine if they can synergistically promote medulloblastoma development.

7 References

Aberle,H., Bauer,A., Stappert,J., Kispert,A., and Kemler,R. (1997). beta-catenin is a target for the ubiquitin-proteasome pathway. EMBO J *16*, 3797-3804.

Aberle,H., Schwartz,H., Hoschuetzky,H., and Kemler,R. (1996). Single amino acid substitutions in proteins of the armadillo gene family abolish their binding to alpha-catenin. Journal of Biological Chemistry *271*, 1520-1526.

Abraham,S.C., Klimstra,D.S., Wilentz,R.E., Yeo,C.J., Conlon,K., Brennan,M., Cameron,J.L., Wu,T.T., and Hruban,R.H. (2002a). Solid-pseudopapillary tumors of the pancreas are genetically distinct from pancreatic ductal Adenocarcinomas and almost always harbor beta-catenin mutations. Am J Pathol *160*, 1361-1369.

Abraham,S.C., Nobukawa,B., Giardiello,F.M., Hamilton,S.R., and Wu,T.T. (2001). Sporadic fundic gland polyps: common gastric polyps arising through activating mutations in the beta-catenin gene. Am. J. Pathol. *158*, 1005-1010.

Abraham,S.C., Park,S.J., Lee,J.H., Mugartegui,L., and Wu,T.T. (2003). Genetic alterations in gastric Adenomas of intestinal and foveolar phenotypes. Mod. Pathol *16*, 786-795.

Abraham,S.C., Reynolds,C., Lee,J.H., Montgomery,E.A., Baisden,B.L., Krasinskas,A.M., and Wu,T.T. (2002b). Fibromatosis of the breast and mutations involving the APC/beta-catenin pathway. Hum Pathol *33*, 39-46.

Acehan,D., Jiang,X., Morgan,D.G., Heuser,J.E., Wang,X., and Akey,C.W. (2002). Three-dimensional structure of the apoptosome: implications for assembly, procaspase-9 binding, and activation. Mol Cell *9*, 423-432.

Adachi,K., Mirzadeh,Z., Sakaguchi,M., Yamashita,T., Nikolcheva,T., Gotoh,Y., Peltz,G., Gong,L., Kawase,T., Alvarez-Buylla,A., Okano,H., and Sawamoto,K. (2007). Beta-catenin signaling promotes proliferation of progenitor cells in the adult mouse subventricular zone. Stem Cells *25*, 2827-2836.

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*, 1857-1867.

Adesina,A.M., Nalbantoglu,J., and Cavenee,W.K. (1994). p53 gene mutation and mdm2 gene amplification are uncommon in medulloblastoma. Cancer Res. *54*, 5649-5651.

Aihara,R., Mochiki,E., Nakabayashi,T., Akazawa,K., Asao,T., and Kuwano,H. (2005). Clinical significance of mucin phenotype, beta-catenin and matrix metalloproteinase 7 in early undifferentiated gastric carcinoma. Br J Surg *92*, 454-462.

Akiyama,H., Lyons,J.P., Mori-Akiyama,Y., Yang,X., Zhang,R., Zhang,Z., Deng,J.M., Taketo,M.M., Nakamura,T., Behringer,R.R., McCrea,P.D., and de Crombrugghe,B. (2004). Interactions between Sox9 and beta-catenin control chondrocyte differentiation. Genes Dev *18*, 1072-1087.

Al Hajj,M., Wicha,M.S., Benito-Hernandez,A., Morrison,S.J., and Clarke,M.F. (2003). Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci U S A *100*, 3983-3988.

Albuquerque,C., Breukel,C., van der,L.R., Fidalgo,P., Lage,P., Slors,F.J., Leitao,C.N., Fodde,R., and Smits,R. (2002). The 'just-right' signaling model: APC somatic mutations are selected based

on a specific level of activation of the beta-catenin signaling cascade. Hum Mol Genet *11*, 1549-1560.

Alder,J., Cho,N.K., and Hatten,M.E. (1996). Embryonic precursor cells from the rhombic lip are specified to a cerebellar granule neuron identity. Neuron *17*, 389-399.

Alderson,L., Fetell,M.R., Sisti,M., Hochberg,F., Cohen,M., and Louis,D.N. (1996). Sentinel lesions of primary CNS lymphoma. J. Neurol. Neurosurg. Psychiatry *60*, 102-105.

Alkalay,I., Yaron,A., Hatzubai,A., Orian,A., Ciechanover,A., and Ben Neriah,Y. (1995). Stimulation-dependent I kappa B alpha phosphorylation marks the NF-kappa B inhibitor for degradation via the ubiquitin-proteasome pathway. Proc Natl Acad Sci U S A *92*, 10599-10603.

Allard,S., Utley,R.T., Savard,J., Clarke,A., Grant,P., Brandl,C.J., Pillus,L., Workman,J.L., and Cote,J. (1999). NuA4, an essential transcription adaptor/histone H4 acetyltransferase complex containing Esa1p and the ATM-related cofactor Tra1p. EMBO J. *18*, 5108-5119.

Alman,B.A., Li,C., Pajerski,M.E., Diaz-Cano,S., and Wolfe,H.J. (1997). Increased beta-catenin protein and somatic APC mutations in sporadic aggressive fibromatoses (desmoid tumors). Am J Pathol *151*, 329-334.

Altman,J., and Das,G.D. (1965). Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. J Comp Neurol *124*, 319-335.

Ambrosino,C., Iwata,T., Scafoglio,C., Mallardo,M., Klein,R., and Nebreda,A.R. (2006). TEF-1 and C/EBPbeta are major p38alpha MAPK-regulated transcription factors in proliferating cardiomyocytes. Biochem. J. *396*, 163-172.

Amit,S., Hatzubai,A., Birman,Y., Andersen,J.S., Ben Shushan,E., Mann,M., Ben Neriah,Y., and Alkalay,I. (2002). Axin-mediated CKI phosphorylation of beta-catenin at Ser 45: a molecular switch for the Wnt pathway. Genes Dev *16*, 1066-1076.

Andersson,K.B., Winer,L.H., Mork,H.K., Molkentin,J.D., and Jaisser,F. (2010). Tamoxifen administration routes and dosage for inducible Cre-mediated gene disruption in mouse hearts. Transgenic Res. *19*, 715-725.

Andreu,P., Colnot,S., Godard,C., Gad,S., Chafey,P., Niwa-Kawakita,M., Laurent-Puig,P., Kahn,A., Robine,S., Perret,C., and Romagnolo,B. (2005). Crypt-restricted proliferation and commitment to the Paneth cell lineage following Apc loss in the mouse intestine. Development *132*, 1443-1451.

Anna,C.H., Sills,R.C., Foley,J.F., Stockton,P.S., Ton,T.V., and Devereux,T.R. (2000). Beta-catenin mutations and protein accumulation in all hepatoblastomas examined from B6C3F1 mice treated with anthraquinone or oxazepam. Cancer Res. *60*, 2864-2868.

Anton,M., and Graham,F.L. (1995). Site-specific recombination mediated by an Adenovirus vector expressing the Cre-recombinase protein: a molecular switch for control of gene expression. J. Virol. *69*, 4600-4606.

Aoki,K., Aoki,M., Sugai,M., Harada,N., Miyoshi,H., Tsukamoto,T., Mizoshita,T., Tatematsu,M., Seno,H., Chiba,T., Oshima,M., Hsieh,C.L., and Taketo,M.M. (2007). Chromosomal instability by beta-catenin/TCF transcription in APC or beta-catenin mutant cells. Oncogene *26*, 3511-3520.

Arighi,C.N., Hartnell,L.M., Aguilar,R.C., Haft,C.R., and Bonifacino,J.S. (2004). Role of the mammalian retromer in sorting of the cation-independent mannose 6-phosphate receptor. J Cell Biol *165*, 123-133.

Ashihara,K., Saito,T., Mizumoto,H., Nishimura,M., Tanaka,R., and Kudo,R. (2002). Mutation of beta-catenin gene in endometrial cancer but not in associated hyperplasia. Med Electron Microsc. *35*, 9-15.

Attard,T.M., Giglio,P., Koppula,S., Snyder,C., and Lynch,H.T. (2007). Brain tumors in individuals with familial Adenomatous polyposis: a cancer registry experience and pooled case report analysis. Cancer *109*, 761-766.

Avet-Loiseau,H., Venuat,A.M., Terrier-Lacombe,M.J., Lellouch-Tubiana,A., Zerah,M., and Vassal,G. (1999). Comparative genomic hybridization detects many recurrent imbalances in central nervous system primitive neuroectodermal tumours in children. Br. J. Cancer *79*, 1843-1847.

Backman,M., Machon,O., Mygland,L., van den Bout,C.J., Zhong,W., Taketo,M.M., and Krauss,S. (2005). Effects of canonical Wnt signaling on dorso-ventral specification of the mouse telencephalon. Dev Biol *279*, 155-168.

Baeg,G.H., Lin,X., Khare,N., Baumgartner,S., and Perrimon,N. (2001). Heparan sulfate proteoglycans are critical for the organization of the extracellular distribution of Wingless. Development *128*, 87-94.

Bafico,A., Gazit,A., Pramila,T., Finch,P.W., Yaniv,A., and Aaronson,S.A. (1999). Interaction of frizzled related protein (FRP) with Wnt ligands and the frizzled receptor suggests alternative mechanisms for FRP inhibition of Wnt signaling. J. Biol. Chem. *274*, 16180-16187.

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

Bailly,Y., Haeberle,A.M., Blanquet-Grossard,F., Chasserot-Golaz,S., Grant,N., Schulze,T., Bombarde,G., Grassi,J., Cesbron,J.Y., and Lemaire-Vieille,C. (2004). Prion protein (PrPc) immunocytochemistry and expression of the green fluorescent protein reporter gene under control of the bovine PrP gene promoter in the mouse brain. J Comp Neurol *473*, 244-269.

Baldin,V., Lukas,J., Marcote,M.J., Pagano,M., and Draetta,G. (1993). Cyclin D1 is a nuclear protein required for cell cycle progression in G1. Genes Dev. *7*, 812-821.

Balesaria,S., Brock,C., Bower,M., Clark,J., Nicholson,S.K., Lewis,P., de,S.S., Evans,H., Peterson,D., Mendoza,N., Glaser,M.G., Newlands,E.S., and Fisher,R.A. (1999). Loss of chromosome 10 is an independent prognostic factor in high-grade gliomas. Br. J. Cancer *81*, 1371-1377.

Bally-Cuif,L., Alvarado-Mallart,R.M., Darnell,D.K., and Wassef,M. (1992). Relationship between Wnt-1 and En-2 expression domains during early development of normal and ectopic met-mesencephalon. Development *115*, 999-1009.

Bally-Cuif,L., and Wassef,M. (1994). Ectopic induction and reorganization of Wnt-1 expression in quail/chick chimeras. Development *120*, 3379-3394.

Balusu,R., Jaiswal,A.S., Armas,M.L., Kundu,C.N., Bloom,L.B., and Narayan,S. (2007). Structure/function analysis of the interaction of Adenomatous polyposis coli with DNA polymerase beta and its implications for base excision repair. Biochemistry *46*, 13961-13974.

Banyai,L., and Patthy,L. (1999). The NTR module: domains of netrins, secreted frizzled related proteins, and type I procollagen C-proteinase enhancer protein are homologous with tissue inhibitors of metalloproteases. Protein Sci. *8*, 1636-1642.

Banziger,C., Soldini,D., Schutt,C., Zipperlen,P., Hausmann,G., and Basler,K. (2006). Wntless, a conserved membrane protein dedicated to the secretion of Wnt proteins from signaling cells. Cell *125*, 509-522.

Bao,S., Wu,Q., McLendon,R.E., Hao,Y., Shi,Q., Hjelmeland,A.B., Dewhirst,M.W., Bigner,D.D., and Rich,J.N. (2006). Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. Nature *444*, 756-760.

Barker,N., Ridgway,R.A., van Es,J.H., van de,W.M., Begthel,H., van den,B.M., Danenberg,E., Clarke,A.R., Sansom,O.J., and Clevers,H. (2009). Crypt stem cells as the cells-of-origin of intestinal cancer. Nature *457*, 608-611.

Barth,A.I., Pollack,A.L., Altschuler,Y., Mostov,K.E., and Nelson,W.J. (1997). NH2-terminal deletion of beta-catenin results in stable colocalization of mutant beta-catenin with Adenomatous polyposis coli protein and altered MDCK cell adhesion. J. Cell Biol. *136*, 693-706.

Barth,A.I., Stewart,D.B., and Nelson,W.J. (1999). T cell factor-activated transcription is not sufficient to induce anchorage-independent growth of epithelial cells expressing mutant beta-catenin. Proc. Natl. Acad. Sci. U. S. A *96*, 4947-4952.

Bartscherer,K., Pelte,N., Ingelfinger,D., and Boutros,M. (2006). Secretion of Wnt ligands requires Evi, a conserved transmembrane protein. Cell *125*, 523-533.

Bauer,A., Huber,O., and Kemler,R. (1998). Pontin52, an interaction partner of beta-catenin, binds to the TATA box binding protein. Proc Natl Acad Sci U S A *95*, 14787-14792.

Baurand,A., Zelarayan,L., Betney,R., Gehrke,C., Dunger,S., Noack,C., Busjahn,A., Huelsken,J., Taketo,M.M., Birchmeier,W., Dietz,R., and Bergmann,M.W. (2007). Beta-catenin downregulation is required for adaptive cardiac remodeling. Circ Res *100*, 1353-1362.

Behrens,J., Jerchow,B.A., Wurtele,M., Grimm,J., Asbrand,C., Wirtz,R., Kuhl,M., Wedlich,D., and Birchmeier,W. (1998). Functional interaction of an axin homolog, conductin, with beta-catenin, APC, and GSK3beta. Science *280*, 596-599.

Behrens,J., von Kries,J.P., Kuhl,M., Bruhn,L., Wedlich,D., Grosschedl,R., and Birchmeier,W. (1996). Functional interaction of beta-catenin with the transcription factor LEF-1. Nature *382*, 638-642.

Belenkaya,T.Y., Wu,Y., Tang,X., Zhou,B., Cheng,L., Sharma,Y.V., Yan,D., Selva,E.M., and Lin,X. (2008). The retromer complex influences Wnt secretion by recycling Wntless from endosomes to the trans-Golgi network. Dev Cell *14*, 120-131.

Bellosta,P., Hulf,T., Balla,D.S., Usseglio,F., Pradel,J., Aragnol,D., and Gallant,P. (2005). Myc interacts genetically with Tip48/Reptin and Tip49/Pontin to control growth and proliferation during *Drosophila* development. Proc Natl Acad Sci U S A *102*, 11799-11804.

Bernfield,M., Gotte,M., Park,P.W., Reizes,O., Fitzgerald,M.L., Lincecum,J., and Zako,M. (1999). Functions of cell surface heparan sulfate proteoglycans. Annu. Rev. Biochem. *68*, 729-777.

Berns,K., Hijmans,E.M., and Bernards,R. (1997). Repression of c-Myc responsive genes in cycling cells causes G1 arrest through reduction of cyclin E/CDK2 kinase activity. Oncogene *15*, 1347-1356.

Beroud,C., and Soussi,T. (1996). APC gene: database of germline and somatic mutations in human tumors and cell lines. Nucleic Acids Res. *24*, 121-124.

Berridge,M.J., Lipp,P., and Bootman,M.D. (2000). The versatility and universality of calcium signalling. Nat. Rev. Mol. Cell Biol. *1*, 11-21.

Bhanot,P., Brink,M., Samos,C.H., Hsieh,J.C., Wang,Y., 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*, 225-230.

Bigner,S.H., Mark,J., Friedman,H.S., Biegel,J.A., and Bigner,D.D. (1988). Structural chromosomal abnormalities in human medulloblastoma. Cancer Genet. Cytogenet. *30*, 91-101.

Bigner,S.H., and Vogelstein,B. (1990). Cytogenetics and molecular genetics of malignant gliomas and medulloblastoma. Brain Pathol. *1*, 12-18.

Binari,R.C., Staveley,B.E., Johnson,W.A., Godavarti,R., Sasisekharan,R., and Manoukian,A.S. (1997). Genetic evidence that heparin-like glycosaminoglycans are involved in wingless signaling. Development *124*, 2623-2632.

Blackwood,E.M., and Eisenman,R.N. (1991). Max: a helix-loop-helix zipper protein that forms a sequence-specific DNA-binding complex with Myc. Science *251*, 1211-1217.

Blaker,H., Hofmann,W.J., Rieker,R.J., Penzel,R., Graf,M., and Otto,H.F. (1999). Beta-catenin accumulation and mutation of the CTNNB1 gene in hepatoblastoma. Genes Chromosomes Cancer *25*, 399-402.

Bondi,J., Bukholm,G., Nesland,J.M., and Bukholm,I.R. (2004). Expression of non-membranous beta-catenin and gamma-catenin, c-Myc and cyclin D1 in relation to patient outcome in human colon Adenocarcinomas. APMIS *112*, 49-56.

Bonnet,D., and Dick,J.E. (1997). Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med *3*, 730-737.

Bouchard,C., Dittrich,O., Kiermaier,A., Dohmann,K., Menkel,A., Eilers,M., and Luscher,B. (2001). Regulation of cyclin D2 gene expression by the Myc/Max/Mad network: Myc-dependent TRRAP recruitment and histone acetylation at the cyclin D2 promoter. Genes Dev *15*, 2042-2047.

Bouchard,C., Thieke,K., Maier,A., Saffrich,R., Hanley-Hyde,J., Ansorge,W., Reed,S., Sicinski,P., Bartek,J., and Eilers,M. (1999). Direct induction of cyclin D2 by Myc contributes to cell cycle progression and sequestration of p27. EMBO J *18*, 5321-5333.

Bradley,A., Evans,M., Kaufman,M.H., and Robertson,E. (1984). Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines. Nature *309*, 255-256.

Brannon,M., Brown,J.D., Bates,R., Kimelman,D., and Moon,R.T. (1999). XCtBP is a XTcf-3 co-repressor with roles throughout *Xenopus* development. Development *126*, 3159-3170.

Brennan,K., Gonzalez-Sancho,J.M., Castelo-Soccio,L.A., Howe,L.R., and Brown,A.M. (2004). Truncated mutants of the putative Wnt receptor LRP6/Arrow can stabilize beta-catenin independently of Frizzled proteins. Oncogene *23*, 4873-4884.

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

Brott,B.K., and Sokol,S.Y. (2002). Regulation of Wnt/LRP signaling by distinct domains of Dickkopf proteins. Mol. Cell Biol. *22*, 6100-6110.

Brown,H.G., Kepner,J.L., Perlman,E.J., Friedman,H.S., Strother,D.R., Duffner,P.K., Kun,L.E., Goldthwaite,P.T., and Burger,P.C. (2000). "Large cell/anaplastic" medulloblastomas: a Pediatric Oncology Group Study. J. Neuropathol. Exp. Neurol. *59*, 857-865.

Brown,M.S., and Goldstein,J.L. (1986). A receptor-mediated pathway for cholesterol homeostasis. Science *232*, 34-47.

Bu,X.M., Zhao,C.H., Zhang,N., Gao,F., Lin,S., and Dai,X.W. (2008). Hypermethylation and aberrant expression of secreted frizzled-related protein genes in pancreatic cancer. World J Gastroenterol. *14*, 3421-3424.

Caca,K., Kolligs,F.T., Ji,X., Hayes,M., Qian,J., Yahanda,A., Rimm,D.L., Costa,J., and Fearon,E.R. (1999). Beta- and gamma-catenin mutations, but not E-cadherin inactivation, underlie T-cell factor/lymphoid enhancer factor transcriptional deregulation in gastric and pancreatic cancer. Cell Growth Differ *10*, 369-376.

Cadigan,K.M., Fish,M.P., Rulifson,E.J., and Nusse,R. (1998). Wingless repression of *Drosophila* frizzled 2 expression shapes the Wingless morphogen gradient in the wing. Cell *93*, 767-777.

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 Dev. *21*, 465-480.

Cao,Q., Lu,X., and Feng,Y.J. (2006). Glycogen synthase kinase-3beta positively regulates the proliferation of human ovarian cancer cells. Cell Res *16*, 671-677.

Carroll,T.J., Park,J.S., Hayashi,S., Majumdar,A., and Mcmahon,A.P. (2005). Wnt9b plays a central role in the regulation of mesenchymal to epithelial transitions underlying organogenesis of the mammalian urogenital system. Dev Cell *9*, 283-292.

Castelo-Branco,G., 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. Proc. Natl. Acad. Sci. U. S. A *100*, 12747-12752.

Cavallo,R.A., Cox,R.T., Moline,M.M., Roose,J., Polevoy,G.A., Clevers,H., Peifer,M., and Bejsovec,A. (1998). *Drosophila* Tcf and Groucho interact to repress Wingless signalling activity. Nature *395*, 604-608.

Central Brain Tumor Registry of the United States. First Annual Report. 1995.
Ref Type: Report

Central Brain Tumor Registry of the United States. Statistical Report, 1998-2002. 2005.
Ref Type: Report

Central Brain Tumor Registry of the United States. http://www.cbtrus.org . 2006.
Ref Type: Electronic Citation

Cha,K.B., Douglas,K.R., Potok,M.A., Liang,H., Jones,S.N., and Camper,S.A. (2004). WNT5A signaling affects pituitary gland shape. Mech Dev *121*, 183-194.

Chae,J., Kim,M.J., Goo,J.H., Collier,S., Gubb,D., Charlton,J., Adler,P.N., and Park,W.J. (1999). The *Drosophila* tissue polarity gene starry night encodes a member of the protocadherin family. Development *126*, 5421-5429.

Chan,E.F., Gat,U., McNiff,J.M., and Fuchs,E. (1999). A common human skin tumour is caused by activating mutations in beta-catenin. Nat Genet *21*, 410-413.

Chen,G., Fernandez,J., Mische,S., and Courey,A.J. (1999). A functional interaction between the histone deacetylase Rpd3 and the corepressor groucho in *Drosophila* development. Genes Dev *13*, 2218-2230.

Chen,M.H., Li,Y.J., Kawakami,T., Xu,S.M., and Chuang,P.T. (2004). Palmitoylation is required for the production of a soluble multimeric Hedgehog protein complex and long-range signaling in vertebrates. Genes Dev. *18*, 641-659.

Chenn,A., and Walsh,C.A. (2002). Regulation of cerebral cortical size by control of cell cycle exit in neural precursors. Science *297*, 365-369.

Cheon,S.S., Wei,Q., Gurung,A., Youn,A., Bright,T., Poon,R., Whetstone,H., Guha,A., and Alman,B.A. (2006). Beta-catenin regulates wound size and mediates the effect of TGF-beta in cutaneous healing. FASEB J *20*, 692-701.

Chesire,D.R., Ewing,C.M., Sauvageot,J., Bova,G.S., and Isaacs,W.B. (2000). Detection and analysis of beta-catenin mutations in prostate cancer. Prostate *45*, 323-334.

Chien,A.J., Moore,E.C., Lonsdorf,A.S., Kulikauskas,R.M., Rothberg,B.G., Berger,A.J., Major,M.B., Hwang,S.T., Rimm,D.L., and Moon,R.T. (2009). Activated Wnt/beta-catenin signaling in melanoma is associated with decreased proliferation in patient tumors and a murine melanoma model. Proc Natl Acad Sci U S A *106*, 1193-1198.

Chopra,V.S., and Levine,M. (2009). Combinatorial patterning mechanisms in the *Drosophila* embryo. Brief. Funct. Genomic. Proteomic. *8*, 243-249.

Ciani,E., Frenquelli,M., and Contestabile,A. (2003). Developmental expression of the cell cycle and apoptosis controlling gene, Lot1, in the rat cerebellum and in cultures of cerebellar granule cells. Brain Res. Dev. Brain Res. *142*, 193-202.

Clements,W.M., Wang,J., Sarnaik,A., Kim,O.J., MacDonald,J., Fenoglio-Preiser,C., Groden,J., and Lowy,A.M. (2002). beta-Catenin mutation is a frequent cause of Wnt pathway activation in gastric cancer. Cancer Res *62*, 3503-3506.

Clifford,S.C., Lusher,M.E., Lindsey,J.C., Langdon,J.A., Gilbertson,R.J., Straughton,D., and Ellison,D.W. (2006). Wnt/Wingless pathway activation and chromosome 6 loss characterize a distinct molecular sub-group of medulloblastomas associated with a favorable prognosis. Cell Cycle *5*, 2666-2670.

Cohen,P. (1989). The structure and regulation of protein phosphatases. Annu Rev Biochem *58*, 453-508.

Coles-Takabe,B.L., Brain,I., Purpura,K.A., Karpowicz,P., Zandstra,P.W., Morshead,C.M., and van der,K.D. (2008). Don't look: growing clonal versus nonclonal neural stem cell colonies. Stem Cells *26*, 2938-2944.

Cong,F., and Varmus,H. (2004). Nuclear-cytoplasmic shuttling of Axin regulates subcellular localization of beta-catenin. Proc Natl Acad Sci U S A *101*, 2882-2887.

Contestabile,A., Fila,T., Bartesaghi,R., and Ciani,E. (2005). Cyclic AMP-mediated regulation of transcription factor Lot1 expression in cerebellar granule cells. J. Biol. Chem. *280*, 33541-33551.

Copeland,D.R., deMoor,C., Moore,B.D., III, and Ater,J.L. (1999). Neurocognitive development of children after a cerebellar tumor in infancy: A longitudinal study. J Clin Oncol *17*, 3476-3486.

Coudreuse,D.Y., Roel,G., Betist,M.C., Destree,O., and Korswagen,H.C. (2006). Wnt gradient formation requires retromer function in Wnt-producing cells. Science *312*, 921-924.

Cowan,C.A., and Henkemeyer,M. (2001). The SH2/SH3 adaptor Grb4 transduces B-ephrin reverse signals. Nature *413*, 174-179.

Crawford,H.C., Fingleton,B.M., Rudolph-Owen,L.A., Goss,K.J., Rubinfeld,B., Polakis,P., and Matrisian,L.M. (1999). The metalloproteinase matrilysin is a target of beta-catenin transactivation in intestinal tumors. Oncogene *18*, 2883-2891.

Dacey,M.L., and Wallace,R.B. (1974). Postnatal neurogenesis in the feline cerebellum: a structural-functional investigation. Acta Neurobiol Exp. (Wars.) *34*, 253-263.

Dahmen,R.P., Koch,A., Denkhaus,D., Tonn,J.C., Sorensen,N., Berthold,F., Behrens,J., Birchmeier,W., Wiestler,O.D., and Pietsch,T. (2001). Deletions of AXIN1, a component of the WNT/wingless pathway, in sporadic medulloblastomas. Cancer Res *61*, 7039-7043.

De Calisto,J., Araya,C., Marchant,L., Riaz,C.F., and Mayor,R. (2005). Essential role of non-canonical Wnt signalling in neural crest migration. Development *132*, 2587-2597.

de Groot,R.P., Auwerx,J., Bourouis,M., and Sassone-Corsi,P. (1993). Negative regulation of Jun/AP-1: conserved function of glycogen synthase kinase 3 and the *Drosophila* kinase shaggy. Oncogene *8*, 841-847.

De La,C.A., Romagnolo,B., Billuart,P., Renard,C.A., Buendia,M.A., Soubrane,O., Fabre,M., Chelly,J., Beldjord,C., Kahn,A., and Perret,C. (1998). Somatic mutations of the beta-catenin gene are frequent in mouse and human hepatocellular carcinomas. Proc Natl Acad Sci U S A *95*, 8847-8851.

Dick,J.E. (2009). Looking ahead in cancer stem cell research. Nat. Biotechnol. *27*, 44-46.

Dihlmann,S., Gebert,J., Siermann,A., Herfarth,C., and von Knebel,D.M. (1999). Dominant negative effect of the APC1309 mutation: a possible explanation for genotype-phenotype correlations in familial Adenomatous polyposis. Cancer Res *59*, 1857-1860.

Ding,Q., He,X., Hsu,J.M., Xia,W., Chen,C.T., Li,L.Y., Lee,D.F., Liu,J.C., Zhong,Q., Wang,X., and Hung,M.C. (2007a). Degradation of Mcl-1 by beta-TrCP mediates glycogen synthase kinase 3-induced tumor suppression and chemosensitization. Mol Cell Biol *27*, 4006-4017.

Ding,Q., He,X., Xia,W., Hsu,J.M., Chen,C.T., Li,L.Y., Lee,D.F., Yang,J.Y., Xie,X., Liu,J.C., and Hung,M.C. (2007b). Myeloid cell leukemia-1 inversely correlates with glycogen synthase

kinase-3beta activity and associates with poor prognosis in human breast cancer. Cancer Res *67*, 4564-4571.

Dix,C.J., and Jordan,V.C. (1980). Modulation of nuclear oestrogen receptor levels by oestrogen and antioestrogen [proceedings]. Br. J. Pharmacol. *68*, 170P-171P.

Doetsch,F., and Alvarez-Buylla,A. (1996). Network of tangential pathways for neuronal migration in adult mammalian brain. Proc. Natl. Acad. Sci. U. S. A *93*, 14895-14900.

Doetsch,F., Caille,I., Lim,D.A., Garcia-Verdugo,J.M., and Alvarez-Buylla,A. (1999). Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. Cell *97*, 703-716.

Doetsch,F., Petreanu,L., Caille,I., Garcia-Verdugo,J.M., and Alvarez-Buylla,A. (2002). EGF converts transit-amplifying neurogenic precursors in the adult brain into multipotent stem cells. Neuron *36*, 1021-1034.

Donehower,L.A., Harvey,M., Slagle,B.L., McArthur,M.J., Montgomery,C.A., Jr., Butel,J.S., and Bradley,A. (1992). Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. Nature *356*, 215-221.

Dong,J., Feldmann,G., Huang,J., Wu,S., Zhang,N., Comerford,S.A., Gayyed,M.F., Anders,R.A., Maitra,A., and Pan,D. (2007). Elucidation of a universal size-control mechanism in *Drosophila* and mammals. Cell *130*, 1120-1133.

Donnellan,R., and Chetty,R. (1998). Cyclin D1 and human neoplasia. Mol. Pathol. *51*, 1-7.

Eberhart,C.G., Kepner,J.L., Goldthwaite,P.T., Kun,L.E., Duffner,P.K., Friedman,H.S., Strother,D.R., and Burger,P.C. (2002a). Histopathologic grading of medulloblastomas: a Pediatric Oncology Group study. Cancer *94*, 552-560.

Eberhart,C.G., Kratz,J.E., Schuster,A., Goldthwaite,P., Cohen,K.J., Perlman,E.J., and Burger,P.C. (2002b). Comparative genomic hybridization detects an increased number of chromosomal alterations in large cell/anaplastic medulloblastomas. Brain Pathol. *12*, 36-44.

Eberhart,C.G., Tihan,T., and Burger,P.C. (2000). Nuclear localization and mutation of beta-catenin in medulloblastomas. J Neuropathol Exp. Neurol *59*, 333-337.

Eischen,C.M., Packham,G., Nip,J., Fee,B.E., Hiebert,S.W., Zambetti,G.P., and Cleveland,J.L. (2001). Bcl-2 is an apoptotic target suppressed by both c-Myc and E2F-1. Oncogene *20*, 6983-6993.

Ellison,D. (2002). Classifying the medulloblastoma: insights from morphology and molecular genetics. Neuropathology and Applied Neurobiology *28*, 257-282.

Ellison,D.W., Onilude,O.E., Lindsey,J.C., Lusher,M.E., Weston,C.L., Taylor,R.E., Pearson,A.D., and Clifford,S.C. (2005). beta-Catenin status predicts a favorable outcome in childhood medulloblastoma: the United Kingdom Children's Cancer Study Group Brain Tumour Committee. J Clin Oncol *23*, 7951-7957.

Embi,N., Rylatt,D.B., and Cohen,P. (1980). Glycogen synthase kinase-3 from rabbit skeletal muscle. Separation from cyclic-AMP-dependent protein kinase and phosphorylase kinase. Eur. J. Biochem. *107*, 519-527.

Englund,C., Kowalczyk,T., Daza,R.A., Dagan,A., Lau,C., Rose,M.F., and Hevner,R.F. (2006). Unipolar brush cells of the cerebellum are produced in the rhombic lip and migrate through developing white matter. J. Neurosci. *26*, 9184-9195.

Erdal,E., Ozturk,N., Cagatay,T., Eksioglu-Demiralp,E., and Ozturk,M. (2005). Lithium-mediated downregulation of PKB/Akt and cyclin E with growth inhibition in hepatocellular carcinoma cells. Int J Cancer *115*, 903-910.

Eriksson,P.S., Perfilieva,E., Bjork-Eriksson,T., Alborn,A.M., Nordborg,C., Peterson,D.A., and Gage,F.H. (1998). Neurogenesis in the adult human hippocampus. Nat Med *4*, 1313-1317.

Etheridge,S.L., Ray,S., Li,S., Hamblet,N.S., Lijam,N., Tsang,M., Greer,J., Kardos,N., Wang,J., Sussman,D.J., Chen,P., and Wynshaw-Boris,A. (2008). Murine dishevelled 3 functions in redundant pathways with dishevelled 1 and 2 in normal cardiac outflow tract, cochlea, and neural tube development. PLoS. Genet. *4*, e1000259.

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

Ewen,M.E., Sluss,H.K., Sherr,C.J., Matsushime,H., Kato,J., and Livingston,D.M. (1993). Functional interactions of the retinoblastoma protein with mammalian D-type cyclins. Cell *73*, 487-497.

Fagotto,F., Gluck,U., and Gumbiner,B.M. (1998). Nuclear localization signal-independent and importin/karyopherin-independent nuclear import of beta-catenin. Curr Biol *8*, 181-190.

Fan,X., Mikolaenko,I., Elhassan,I., Ni,X., Wang,Y., Ball,D., Brat,D.J., Perry,A., and Eberhart,C.G. (2004). Notch1 and notch2 have opposite effects on embryonal brain tumor growth. Cancer Res. *64*, 7787-7793.

Farago,M., Dominguez,I., Landesman-Bollag,E., Xu,X., Rosner,A., Cardiff,R.D., and Seldin,D.C. (2005). Kinase-inactive glycogen synthase kinase 3beta promotes Wnt signaling and mammary tumorigenesis. Cancer Res *65*, 5792-5801.

Feil,R., Brocard,J., Mascrez,B., LeMeur,M., Metzger,D., and Chambon,P. (1996). Ligand-activated site-specific recombination in mice. Proc. Natl. Acad. Sci. U. S. A *93*, 10887-10890.

Fernandez,L., Northcott,P.A., Dalton,J., Fraga,C., Ellison,D., Angers,S., Taylor,M.D., and Kenney,A.M. (2009). YAP1 is amplified and up-regulated in hedgehog-associated medulloblastomas and mediates Sonic hedgehog-driven neural precursor proliferation. Genes Dev. *23*, 2729-2741.

Fink,A.J., Englund,C., Daza,R.A., Pham,D., Lau,C., Nivison,M., Kowalczyk,T., and Hevner,R.F. (2006). Development of the deep cerebellar nuclei: transcription factors and cell migration from the rhombic lip. J. Neurosci. *26*, 3066-3076.

Fish,K.J., Cegielska,A., Getman,M.E., Landes,G.M., and Virshup,D.M. (1995). Isolation and characterization of human casein kinase I epsilon (CKI), a novel member of the CKI gene family. J Biol Chem *270*, 14875-14883.

Fodde,R., Edelmann,W., Yang,K., van Leeuwen,C., Carlson,C., Renault,B., Breukel,C., Alt,E., Lipkin,M., Khan,P.M., and . (1994). A targeted chain-termination mutation in the mouse Apc gene results in multiple intestinal tumors. Proc. Natl. Acad. Sci. U. S. A *91*, 8969-8973.

Folger,K.R., Thomas,K., and Capecchi,M.R. (1985). Nonreciprocal exchanges of information between DNA duplexes coinjected into mammalian cell nuclei. Mol. Cell Biol. *5*, 59-69.

Folger,K.R., Wong,E.A., Wahl,G., and Capecchi,M.R. (1982). Patterns of integration of DNA microinjected into cultured mammalian cells: evidence for homologous recombination between injected plasmid DNA molecules. Mol. Cell Biol. *2*, 1372-1387.

Forde,J.E., and Dale,T.C. (2007). Glycogen synthase kinase 3: a key regulator of cellular fate. Cell Mol. Life Sci. *64*, 1930-1944.

Franch-Marro,X., Wendler,F., Guidato,S., Griffith,J., Baena-Lopez,A., Itasaki,N., Maurice,M.M., and Vincent,J.P. (2008). Wingless secretion requires endosome-to-Golgi retrieval of Wntless/Evi/Sprinter by the retromer complex. Nat Cell Biol *10*, 170-177.

Frank,A.J., Hernan,R., Hollander,A., Lindsey,J.C., Lusher,M.E., Fuller,C.E., Clifford,S.C., and Gilbertson,R.J. (2004). The TP53-ARF tumor suppressor pathway is frequently disrupted in large/cell anaplastic medulloblastoma. Brain Res. Mol. Brain Res. *121*, 137-140.

Frappart,P.O., Lee,Y., Russell,H.R., Chalhoub,N., Wang,Y.D., Orii,K.E., Zhao,J., Kondo,N., Baker,S.J., and McKinnon,P.J. (2009). Recurrent genomic alterations characterize medulloblastoma arising from DNA double-strand break repair deficiency. Proc. Natl. Acad. Sci. U. S. A *106*, 1880-1885.

Fuchs,S.Y., Chen,A., Xiong,Y., Pan,Z.Q., and Ronai,Z. (1999). HOS, a human homolog of Slimb, forms an SCF complex with Skp1 and Cullin1 and targets the phosphorylation-dependent degradation of IkappaB and beta-catenin. Oncogene *18*, 2039-2046.

Fujino,T., Asaba,H., Kang,M.J., Ikeda,Y., Sone,H., Takada,S., Kim,D.H., Ioka,R.X., Ono,M., Tomoyori,H., Okubo,M., Murase,T., Kamataki,A., Yamamoto,J., Magoori,K., Takahashi,S., Miyamoto,Y., Oishi,H., Nose,M., Okazaki,M., Usui,S., Imaizumi,K., Yanagisawa,M., Sakai,J., and Yamamoto,T.T. (2003). Low-density lipoprotein receptor-related protein 5 (LRP5) is essential for normal cholesterol metabolism and glucose-induced insulin secretion. Proc. Natl. Acad. Sci. U. S. A *100*, 229-234.

Fukuchi,T., Sakamoto,M., Tsuda,H., Maruyama,K., Nozawa,S., and Hirohashi,S. (1998). Beta-catenin mutation in carcinoma of the uterine endometrium. Cancer Res *58*, 3526-3528.

Fukukawa,C., Nagayama,S., Tsunoda,T., Toguchida,J., Nakamura,Y., and Katagiri,T. (2009). Activation of the non-canonical Dvl-Rac1-JNK pathway by Frizzled homologue 10 in human synovial sarcoma. Oncogene *28*, 1110-1120.

Fults,D., Pedone,C., Dai,C., and Holland,E.C. (2002). MYC expression promotes the proliferation of neural progenitor cells in culture and *in vivo*. Neoplasia *4*, 32-39.

Funayama,N., Fagotto,F., McCrea,P., and Gumbiner,B.M. (1995). Embryonic axis induction by the armadillo repeat domain of beta-catenin: evidence for intracellular signaling. J Cell Biol *128*, 959-968.

Furuhashi,M., Yagi,K., Yamamoto,H., Furukawa,Y., Shimada,S., Nakamura,Y., Kikuchi,A., Miyazono,K., and Kato,M. (2001). Axin facilitates Smad3 activation in the transforming growth factor beta signaling pathway. Mol Cell Biol *21*, 5132-5141.

Gage,F.H., Ray,J., and Fisher,L.J. (1995). Isolation, characterization, and use of stem cells from the CNS. Annu. Rev. Neurosci. *18*, 159-192.

Galceran,J., Miyashita-Lin,E.M., Devaney,E., Rubenstein,J.L., and Grosschedl,R. (2000). Hippocampus development and generation of dentate gyrus granule cells is regulated by LEF1. Development *127*, 469-482.

Galli,R., Binda,E., Orfanelli,U., Cipelletti,B., Gritti,A., De Vitis,S., Fiocco,R., Foroni,C., Dimeco,F., and Vescovi,A. (2004). Isolation and characterization of tumorigenic, stem-like neural precursors from human glioblastoma. Cancer Res *64*, 7011-7021.

Garcia-Rostan,G., Camp,R.L., Herrero,A., Carcangiu,M.L., Rimm,D.L., and Tallini,G. (2001). Beta-catenin dysregulation in thyroid neoplasms: down-regulation, aberrant nuclear expression, and CTNNB1 exon 3 mutations are markers for aggressive tumor phenotypes and poor prognosis. Am J Pathol *158*, 987-996.

Gerstein,A.V., Almeida,T.A., Zhao,G., Chess,E., Shih,I., Buhler,K., Pienta,K., Rubin,M.A., Vessella,R., and Papadopoulos,N. (2002). APC/CTNNB1 (beta-catenin) pathway alterations in human prostate cancers. Genes Chromosomes Cancer *34*, 9-16.

Giangaspero,F., Rigobello,L., Badiali,M., Loda,M., Andreini,L., Basso,G., Zorzi,F., and Montaldi,A. (1992). Large-cell medulloblastomas. A distinct variant with highly aggressive behavior. Am. J. Surg. Pathol. *16*, 687-693.

Giangaspero,F., Wellek,S., Masuoka,J., Gessi,M., Kleihues,P., and Ohgaki,H. (2006). Stratification of medulloblastoma on the basis of histopathological grading. Acta Neuropathol. *112*, 5-12.

Giese,K., Cox,J., and Grosschedl,R. (1992). The HMG domain of lymphoid enhancer factor 1 bends DNA and facilitates assembly of functional nucleoprotein structures. Cell *69*, 185-195.

Gilhuis,H.J., Anderl,K.L., Boerman,R.H., Jeuken,J.M., James,C.D., Raffel,C., Scheithauer,B.W., and Jenkins,R.B. (2000). Comparative genomic hybridization of medulloblastomas and clinical relevance: eleven new cases and a review of the literature. Clin. Neurol. Neurosurg. *102*, 203-209.

Glavic,A., Gomez-Skarmeta,J.L., and Mayor,R. (2002). The homeoprotein Xiro1 is required for midbrain-hindbrain boundary formation. Development *129*, 1609-1621.

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*, 357-362.

Gomez-Roman,N., Grandori,C., Eisenman,R.N., and White,R.J. (2003). Direct activation of RNA polymerase III transcription by c-Myc. Nature *421*, 290-294.

Goodman,R.M., Thombre,S., Firtina,Z., Gray,D., Betts,D., Roebuck,J., Spana,E.P., and Selva,E.M. (2006). Sprinter: a novel transmembrane protein required for Wg secretion and signaling. Development *133*, 4901-4911.

Gorlich,D., Kostka,S., Kraft,R., Dingwall,C., Laskey,R.A., Hartmann,E., and Prehn,S. (1995). Two different subunits of importin cooperate to recognize nuclear localization signals and bind them to the nuclear envelope. Curr Biol *5*, 383-392.

Gottardi,C.J., Wong,E., and Gumbiner,B.M. (2001). E-cadherin suppresses cellular transformation by inhibiting beta-catenin signaling in an adhesion-independent manner. J Cell Biol *153*, 1049-1060.

Gotze,S., Wolter,M., Reifenberger,G., Muller,O., and Sievers,S. (2009). Frequent promoter hypermethylation of Wnt pathway inhibitor genes in malignant astrocytic gliomas. Int. J. Cancer.

Gould,E., Tanapat,P., McEwen,B.S., Flugge,G., and Fuchs,E. (1998). Proliferation of granule cell precursors in the dentate gyrus of adult monkeys is diminished by stress. Proc Natl Acad Sci U S A *95*, 3168-3171.

Greco,T.L., Takada,S., Newhouse,M.M., McMahon,J.A., Mcmahon,A.P., and Camper,S.A. (1996). Analysis of the vestigial tail mutation demonstrates that Wnt-3a gene dosage regulates mouse axial development. Genes Dev *10*, 313-324.

Greco,V., Hannus,M., and Eaton,S. (2001). Argosomes: a potential vehicle for the spread of morphogens through epithelia. Cell *106*, 633-645.

Griffin,C.A., Hawkins,A.L., Packer,R.J., Rorke,L.B., and Emanuel,B.S. (1988). Chromosome abnormalities in pediatric brain tumors. Cancer Res. *48*, 175-180.

Gritti,A., Frolichsthal-Schoeller,P., Galli,R., Parati,E.A., Cova,L., Pagano,S.F., Bjornson,C.R., and Vescovi,A.L. (1999). Epidermal and fibroblast growth factors behave as mitogenic regulators for a single multipotent stem cell-like population from the subventricular region of the adult mouse forebrain. J Neurosci *19*, 3287-3297.

Gritti,A., Parati,E.A., Cova,L., Frolichsthal,P., Galli,R., Wanke,E., Faravelli,L., Morassutti,D.J., Roisen,F., Nickel,D.D., and Vescovi,A.L. (1996). Multipotential stem cells from the adult mouse brain proliferate and self-renew in response to basic fibroblast growth factor. J Neurosci *16*, 1091-1100.

Groden,J., Thliveris,A., Samowitz,W., Carlson,M., Gelbert,L., Albertsen,H., Joslyn,G., Stevens,J., Spirio,L., Robertson,M., and . (1991). Identification and characterization of the familial Adenomatous polyposis coli gene. Cell *66*, 589-600.

Gu,H., Marth,J.D., Orban,P.C., Mossmann,H., and Rajewsky,K. (1994). Deletion of a DNA polymerase beta gene segment in T cells using cell type-specific gene targeting. Science *265*, 103-106.

Gu,H., Zou,Y.R., and Rajewsky,K. (1993). Independent control of immunoglobulin switch recombination at individual switch regions evidenced through Cre-loxP-mediated gene targeting. Cell *73*, 1155-1164.

Gubb,D., and Garcia-Bellido,A. (1982). A genetic analysis of the determination of cuticular polarity during development in *Drosophila* melanogaster. J. Embryol. Exp. Morphol. *68*, 37-57.

Guo,N., Hawkins,C., and Nathans,J. (2004). Frizzled6 controls hair patterning in mice. Proc. Natl. Acad. Sci. U. S. A *101*, 9277-9281.

Hacker,U., Lin,X., and Perrimon,N. (1997). The *Drosophila* sugarless gene modulates Wingless signaling and encodes an enzyme involved in polysaccharide biosynthesis. Development *124*, 3565-3573.

Haegel,H., Larue,L., Ohsugi,M., Fedorov,L., Herrenknecht,K., and Kemler,R. (1995). Lack of beta-catenin affects mouse development at gastrulation. Development *121*, 3529-3537.

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*, 696-708.

Haerry,T.E., and Gehring,W.J. (1997). A conserved cluster of homeodomain binding sites in the mouse Hoxa-4 intron functions in *Drosophila* embryos as an enhancer that is directly regulated by Ultrabithorax. Dev. Biol. *186*, 1-15.

Haiech,J., Klee,C.B., and Demaille,J.G. (1981). Effects of cations on affinity of calmodulin for calcium: ordered binding of calcium ions allows the specific activation of calmodulin-stimulated enzymes. Biochemistry *20*, 3890-3897.

Hainaut,P., and Hollstein,M. (2000). p53 and human cancer: the first ten thousand mutations. Adv. Cancer Res. *77*, 81-137.

Hall,A.C., Lucas,F.R., and Salinas,P.C. (2000). Axonal remodeling and synaptic differentiation in the cerebellum is regulated by WNT-7a signaling. Cell *100*, 525-535.

Hallonet,M.E., and Le Douarin,N.M. (1993). Tracing neuroepithelial cells of the mesencephalic and metencephalic alar plates during cerebellar ontogeny in quail-chick chimaeras. Eur. J. Neurosci. *5*, 1145-1155.

Hallonet,M.E., Teillet,M.A., and Le Douarin,N.M. (1990). A new approach to the development of the cerebellum provided by the quail-chick marker system. Development *108*, 19-31.

Hamada,F., and Bienz,M. (2004). The APC tumor suppressor binds to C-terminal binding protein to divert nuclear beta-catenin from TCF. Dev Cell *7*, 677-685.

Hamblet,N.S., Lijam,N., Ruiz-Lozano,P., Wang,J., Yang,Y., Luo,Z., Mei,L., Chien,K.R., Sussman,D.J., and Wynshaw-Boris,A. (2002). Dishevelled 2 is essential for cardiac outflow tract development, somite segmentation and neural tube closure. Development *129*, 5827-5838.

Hamilton,S.R., Liu,B., Parsons,R.E., Papadopoulos,N., Jen,J., Powell,S.M., Krush,A.J., Berk,T., Cohen,Z., Tetu,B., and . (1995). The molecular basis of Turcot's syndrome. N Engl. J Med *332*, 839-847.

Harada,N., Miyoshi,H., Murai,N., Oshima,H., Tamai,Y., Oshima,M., and Taketo,M.M. (2002). Lack of tumorigenesis in the mouse liver after Adenovirus-mediated expression of a dominant stable mutant of beta-catenin. Cancer Res *62*, 1971-1977.

Harada,N., Oshima,H., Katoh,M., Tamai,Y., Oshima,M., and Taketo,M.M. (2004). Hepatocarcinogenesis in mice with beta-catenin and Ha-ras gene mutations. Cancer Res *64*, 48-54.

Harada,N., Tamai,Y., Ishikawa,T., Sauer,B., Takaku,K., Oshima,M., and Taketo,M.M. (1999). Intestinal polyposis in mice with a dominant stable mutation of the beta-catenin gene. EMBO J *18*, 5931-5942.

Hart,M., Concordet,J.P., Lassot,I., Albert,I., del los,S.R., Durand,H., Perret,C., Rubinfeld,B., Margottin,F., Benarous,R., and Polakis,P. (1999). The F-box protein beta-TrCP associates with phosphorylated beta-catenin and regulates its activity in the cell. Curr Biol *9*, 207-210.

Hart,M.J., de los,S.R., Albert,I.N., Rubinfeld,B., and Polakis,P. (1998). Downregulation of beta-catenin by human Axin and its association with the APC tumor suppressor, beta-catenin and GSK3 beta. Curr Biol *8*, 573-581.

Hartmann,W., Digon-Sontgerath,B., Koch,A., Waha,A., Endl,E., Dani,I., Denkhaus,D., Goodyer,C.G., Sorensen,N., Wiestler,O.D., and Pietsch,T. (2006). Phosphatidylinositol 3'-kinase/AKT signaling is activated in medulloblastoma cell proliferation and is associated with reduced expression of PTEN. Clin. Cancer Res. *12*, 3019-3027.

Harvey,M., McArthur,M.J., Montgomery,C.A., Jr., Butel,J.S., Bradley,A., and Donehower,L.A. (1993). Spontaneous and carcinogen-induced tumorigenesis in p53-deficient mice. Nat. Genet. *5*, 225-229.

Hasegawa,S., Sato,T., Akazawa,H., Okada,H., Maeno,A., Ito,M., Sugitani,Y., Shibata,H., Miyazaki,J.J., Katsuki,M., Yamauchi,Y., Yamamura,K.K., Katamine,S., and Noda,T. (2002). Apoptosis in neural crest cells by functional loss of APC tumor suppressor gene. Proc. Natl. Acad. Sci. U. S. A *99*, 297-302.

Hatten,M.E., and Heintz,N. (1995). Mechanisms of neural patterning and specification in the developing cerebellum. Annu. Rev. Neurosci. *18*, 385-408.

He,T.C., Sparks,A.B., Rago,C., Hermeking,H., Zawel,L., da Costa,L.T., Morin,P.J., Vogelstein,B., and Kinzler,K.W. (1998). Identification of c-MYC as a target of the APC pathway. Science *281*, 1509-1512.

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

Hecht,A., Litterst,C.M., Huber,O., and Kemler,R. (1999). Functional characterization of multiple transactivating elements in beta-catenin, some of which interact with the TATA-binding protein *in vitro*. J Biol Chem *274*, 18017-18025.

Hecht,A., Vleminckx,K., Stemmler,M.P., van Roy,F., and Kemler,R. (2000). The p300/CBP acetyltransferases function as transcriptional coactivators of beta-catenin in vertebrates. EMBO J *19*, 1839-1850.

Hedgepeth,C.M., Deardorff,M.A., and Klein,P.S. (1999). *Xenopus* axin interacts with glycogen synthase kinase-3 beta and is expressed in the anterior midbrain. Mech. Dev *80*, 147-151.

Heisenberg,C.P., Tada,M., Rauch,G.J., Saude,L., Concha,M.L., Geisler,R., Stemple,D.L., Smith,J.C., and Wilson,S.W. (2000). Silberblick/Wnt11 mediates convergent extension movements during zebrafish gastrulation. Nature *405*, 76-81.

Hemmati,H.D., Nakano,I., Lazareff,J.A., Masterman-Smith,M., Geschwind,D.H., Bronner-Fraser,M., and Kornblum,H.I. (2003). Cancerous stem cells can arise from pediatric brain tumors. Proc Natl Acad Sci U S A *100*, 15178-15183.

Hermeking,H., Rago,C., Schuhmacher,M., Li,Q., Barrett,J.F., Obaya,A.J., O'Connell,B.C., Mateyak,M.K., Tam,W., Kohlhuber,F., Dang,C.V., Sedivy,J.M., Eick,D., Vogelstein,B., and Kinzler,K.W. (2000). Identification of CDK4 as a target of c-MYC. Proc Natl Acad Sci U S A *97*, 2229-2234.

Hino,S., Michiue,T., Asashima,M., and Kikuchi,A. (2003). Casein kinase I epsilon enhances the binding of Dvl-1 to Frat-1 and is essential for Wnt-3a-induced accumulation of beta-catenin. J Biol Chem *278*, 14066-14073.

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*, 2791-2801.

Hoeflich,K.P., and Ikura,M. (2002). Calmodulin in action: diversity in target recognition and activation mechanisms. Cell *108*, 739-742.

Hoeflich,K.P., Luo,J., Rubie,E.A., Tsao,M.S., Jin,O., and Woodgett,J.R. (2000). Requirement for glycogen synthase kinase-3beta in cell survival and NF-kappaB activation. Nature *406*, 86-90.

Hoess,R.H., and Abremski,K. (1984). Interaction of the bacteriophage P1 recombinase Cre with the recombining site loxP. Proc. Natl. Acad. Sci. U. S. A *81*, 1026-1029.

Hofmann,K. (2000). A superfamily of membrane-bound O-acyltransferases with implications for Wnt signaling. Trends Biochem Sci *25*, 111-112.

Hovens,C.M., Stacker,S.A., Andres,A.C., Harpur,A.G., Ziemiecki,A., and Wilks,A.F. (1992). RYK, a receptor tyrosine kinase-related molecule with unusual kinase domain motifs. Proc. Natl. Acad. Sci. U. S. A *89*, 11818-11822.

Hsieh,M., Boerboom,D., Shimada,M., Lo,Y., Parlow,A.F., Luhmann,U.F., Berger,W., and Richards,J.S. (2005). Mice null for Frizzled4 (Fzd4-/-) are infertile and exhibit impaired corpora lutea formation and function. Biol. Reprod. *73*, 1135-1146.

Hsu,S.C., Galceran,J., and Grosschedl,R. (1998). Modulation of transcriptional regulation by LEF-1 in response to Wnt-1 signaling and association with beta-catenin. Mol Cell Biol *18*, 4807-4818.

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. J Biol Chem *274*, 3439-3445.

Huang,H., Mahler-Araujo,B.M., Sankila,A., Chimelli,L., Yonekawa,Y., Kleihues,P., and Ohgaki,H. (2000). APC mutations in sporadic medulloblastomas. Am J Pathol *156*, 433-437.

Huang,J., Wu,S., Barrera,J., Matthews,K., and Pan,D. (2005). The Hippo signaling pathway coordinately regulates cell proliferation and apoptosis by inactivating Yorkie, the *Drosophila* Homolog of YAP. Cell *122*, 421-434.

Huang,S.M., Schonthal,A.H., and Stallcup,M.R. (2001). Enhancement of p53-dependent gene activation by the transcriptional coactivator Zac1. Oncogene *20*, 2134-2143.

Huber,O., Korn,R., McLaughlin,J., Ohsugi,M., Herrmann,B.G., and Kemler,R. (1996). Nuclear localization of beta-catenin by interaction with transcription factor LEF-1. Mech. Dev *59*, 3-10.

Huelsken,J., Vogel,R., Brinkmann,V., Erdmann,B., Birchmeier,C., and Birchmeier,W. (2000). Requirement for beta-catenin in anterior-posterior axis formation in mice. J. Cell Biol. *148*, 567-578.

Hughes,K., Nikolakaki,E., Plyte,S.E., Totty,N.F., and Woodgett,J.R. (1993). Modulation of the glycogen synthase kinase-3 family by tyrosine phosphorylation. EMBO J. *12*, 803-808.

Ichimura,K., Schmidt,E.E., Miyakawa,A., Goike,H.M., and Collins,V.P. (1998). Distinct patterns of deletion on 10p and 10q suggest involvement of multiple tumor suppressor genes in the development of astrocytic gliomas of different malignancy grades. Genes Chromosomes. Cancer *22*, 9-15.

Ignatova,T.N., Kukekov,V.G., Laywell,E.D., Suslov,O.N., Vrionis,F.D., and Steindler,D.A. (2002). Human cortical glial tumors contain neural stem-like cells expressing astroglial and neuronal markers *in vitro*. Glia *39*, 193-206.

Ikeda,S., Kishida,M., Matsuura,Y., Usui,H., and Kikuchi,A. (2000a). GSK-3beta-dependent phosphorylation of Adenomatous polyposis coli gene product can be modulated by beta-catenin and protein phosphatase 2A complexed with Axin. Oncogene *19*, 537-545.

Ikeda,S., Kishida,S., Yamamoto,H., Murai,H., Koyama,S., and Kikuchi,A. (1998). Axin, a negative regulator of the Wnt signaling pathway, forms a complex with GSK-3beta and beta-catenin and promotes GSK-3beta-dependent phosphorylation of beta-catenin. EMBO J *17*, 1371-1384.

Ikeda,T., Yoshinaga,K., Semba,S., Kondo,E., Ohmori,H., and Horii,A. (2000b). Mutational analysis of the CTNNB1 (beta-catenin) gene in human endometrial cancer: frequent mutations at codon 34 that cause nuclear accumulation. Oncol Rep *7*, 323-326.

Ille,F., Atanasoski,S., Falk,S., Ittner,L.M., Marki,D., Buchmann-Moller,S., Wurdak,H., Suter,U., Taketo,M.M., and Sommer,L. (2007). Wnt/BMP signal integration regulates the balance between proliferation and differentiation of neuroepithelial cells in the dorsal spinal cord. Dev Biol *304*, 394-408.

Inoue,T., Oz,H.S., Wiland,D., Gharib,S., Deshpande,R., Hill,R.J., Katz,W.S., and Sternberg,P.W. (2004). C. elegans LIN-18 is a Ryk ortholog and functions in parallel to LIN-17/Frizzled in Wnt signaling. Cell *118*, 795-806.

Ishidate,T., Matsumine,A., Toyoshima,K., and Akiyama,T. (2000). The APC-hDLG complex negatively regulates cell cycle progression from the G0/G1 to S phase. Oncogene *19*, 365-372.

Ishikawa,T., Tamai,Y., Zorn,A.M., Yoshida,H., Seldin,M.F., Nishikawa,S., and Taketo,M.M. (2001). Mouse Wnt receptor gene Fzd5 is essential for yolk sac and placental angiogenesis. Development *128*, 25-33.

Ishitani,T., Kishida,S., Hyodo-Miura,J., Ueno,N., Yasuda,J., Waterman,M., Shibuya,H., Moon,R.T., Ninomiya-Tsuji,J., and Matsumoto,K. (2003). The TAK1-NLK mitogen-activated protein kinase cascade functions in the Wnt-5a/Ca(2+) pathway to antagonize Wnt/beta-catenin signaling. Mol. Cell Biol. *23*, 131-139.

Jacques,T.S., Swales,A., Brzozowski,M.J., Henriquez,N.V., Linehan,J.M., Mirzadeh,Z., O' Malley,C., Naumann,H., Alvarez-Buylla,A., and Brandner,S. (2010). Combinations of genetic mutations in the adult neural stem cell compartment determine brain tumour phenotypes. EMBO J. *29*, 222-235.

James,C.D., Carlbom,E., Dumanski,J.P., Hansen,M., Nordenskjold,M., Collins,V.P., and Cavenee,W.K. (1988). Clonal genomic alterations in glioma malignancy stages. Cancer Res. *48*, 5546-5551.

Jeays-Ward,K., Dandonneau,M., and Swain,A. (2004). Wnt4 is required for proper male as well as female sexual development. Dev Biol *276*, 431-440.

Jen,J., Powell,S.M., Papadopoulos,N., Smith,K.J., Hamilton,S.R., Vogelstein,B., and Kinzler,K.W. (1994). Molecular determinants of dysplasia in colorectal lesions. Cancer Res *54*, 5523-5526.

Jeng,Y.M., Wu,M.Z., Mao,T.L., Chang,M.H., and Hsu,H.C. (2000). Somatic mutations of beta-catenin play a crucial role in the tumorigenesis of sporadic hepatoblastoma. Cancer Lett *152*, 45-51.

Jho,E.H., Zhang,T., Domon,C., Joo,C.K., Freund,J.N., and Costantini,F. (2002). Wnt/beta-catenin/Tcf signaling induces the transcription of Axin2, a negative regulator of the signaling pathway. Mol Cell Biol *22*, 1172-1183.

Jiang,W., Kahn,S.M., Zhou,P., Zhang,Y.J., Cacace,A.M., Infante,A.S., Doi,S., Santella,R.M., and Weinstein,I.B. (1993). Overexpression of cyclin D1 in rat fibroblasts causes abnormalities in growth control, cell cycle progression and gene expression. Oncogene *8*, 3447-3457.

Jones,L.E., Humphreys,M.J., Campbell,F., Neoptolemos,J.P., and Boyd,M.T. (2004). Comprehensive analysis of matrix metalloproteinase and tissue inhibitor expression in pancreatic cancer: increased expression of matrix metalloproteinase-7 predicts poor survival. Clin Cancer Res *10*, 2832-2845.

Jonsson,M., Borg,A., Nilbert,M., and Andersson,T. (2000). Involvement of Adenomatous polyposis coli (APC)/beta-catenin signalling in human breast cancer. Eur J Cancer *36*, 242-248.

Joslyn,G., Carlson,M., Thliveris,A., Albertsen,H., Gelbert,L., Samowitz,W., Groden,J., Stevens,J., Spirio,L., Robertson,M., and . (1991). Identification of deletion mutations and three new genes at the familial polyposis locus. Cell *66*, 601-613.

Joslyn,G., Richardson,D.S., White,R., and Alber,T. (1993). Dimer formation by an N-terminal coiled coil in the APC protein. Proc Natl Acad Sci U S A *90*, 11109-11113.

Jost,E., Schmid,J., Wilop,S., Schubert,C., Suzuki,H., Herman,J.G., Osieka,R., and Galm,O. (2008). Epigenetic inactivation of secreted Frizzled-related proteins in acute myeloid leukaemia. Br J Haematol. *142*, 745-753.

Juin,P., Hunt,A., Littlewood,T., Griffiths,B., Swigart,L.B., Korsmeyer,S., and Evan,G. (2002). c-Myc functionally cooperates with Bax to induce apoptosis. Mol Cell Biol *22*, 6158-6169.

Justice,R.W., Zilian,O., Woods,D.F., Noll,M., and Bryant,P.J. (1995). The *Drosophila* tumor suppressor gene warts encodes a homolog of human myotonic dystrophy kinase and is required for the control of cell shape and proliferation. Genes Dev. *9*, 534-546.

Kadowaki,T., Wilder,E., Klingensmith,J., Zachary,K., and Perrimon,N. (1996). The segment polarity gene porcupine encodes a putative multitransmembrane protein involved in Wingless processing. Genes Dev *10*, 3116-3128.

Kajino,Y., Yamaguchi,A., Hashimoto,N., Matsuura,A., Sato,N., and Kikuchi,K. (2001). beta-Catenin gene mutation in human hair follicle-related tumors. Pathol Int *51*, 543-548.

Kamiya,M., Judson,H., Okazaki,Y., Kusakabe,M., Muramatsu,M., Takada,S., Takagi,N., Arima,T., Wake,N., Kamimura,K., Satomura,K., Hermann,R., Bonthron,D.T., and Hayashizaki,Y. (2000). The cell cycle control gene ZAC/PLAGL1 is imprinted--a strong candidate gene for transient neonatal diabetes. Hum. Mol. Genet. *9*, 453-460.

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. Mech. Dev. *91*, 43-52.

Kato,G.J., Barrett,J., Villa-Garcia,M., and Dang,C.V. (1990). An amino-terminal c-myc domain required for neoplastic transformation activates transcription. Mol Cell Biol *10*, 5914-5920.

Kato,J., Matsushime,H., Hiebert,S.W., Ewen,M.E., and Sherr,C.J. (1993). Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin D-dependent kinase CDK4. Genes Dev *7*, 331-342.

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

Katoh,M. (2001). Frequent up-regulation of WNT2 in primary gastric cancer and colorectal cancer. Int J Oncol *19*, 1003-1007.

Kawahara,K., Morishita,T., Nakamura,T., Hamada,F., Toyoshima,K., and Akiyama,T. (2000). Down-regulation of beta-catenin by the colorectal tumor suppressor APC requires association with Axin and beta-catenin. J Biol Chem *275*, 8369-8374.

Kerkela,R., Kockeritz,L., Macaulay,K., Zhou,J., Doble,B.W., Beahm,C., Greytak,S., Woulfe,K., Trivedi,C.M., Woodgett,J.R., Epstein,J.A., Force,T., and Huggins,G.S. (2008). Deletion of GSK-3beta in mice leads to hypertrophic cardiomyopathy secondary to cardiomyoblast hyperproliferation. J. Clin. Invest *118*, 3609-3618.

Kim,H.J., Schleiffarth,J.R., Jessurun,J., Sumanas,S., Petryk,A., Lin,S., and Ekker,S.C. (2005). Wnt5 signaling in vertebrate pancreas development. BMC Biol *3*, 23.

Kim,J.H., Kim,M.A., Lee,H.S., and Kim,W.H. (2009). Comparative analysis of protein expressions in primary and metastatic gastric carcinomas. Hum Pathol *40*, 314-322.

Kim,S., Kang,J.K., Kim,Y.K., Seo,D.W., Ahn,S.H., Lee,J.C., Lee,C.H., You,J.S., Cho,E.J., Lee,H.W., and Han,J.W. (2006). Histone deacetylase inhibitor apicidin induces cyclin E expression through Sp1 sites. Biochem. Biophys. Res. Commun. *342*, 1168-1173.

Kinzler,K.W., Nilbert,M.C., Vogelstein,B., Bryan,T.M., Levy,D.B., Smith,K.J., Preisinger,A.C., Hamilton,S.R., Hedge,P., Markham,A., and . (1991). Identification of a gene located at chromosome 5q21 that is mutated in colorectal cancers. Science *251*, 1366-1370.

Kirschenbaum,B., Nedergaard,M., Preuss,A., Barami,K., Fraser,R.A., and Goldman,S.A. (1994). *In vitro* neuronal production and differentiation by precursor cells derived from the adult human forebrain. Cereb. Cortex *4*, 576-589.

Kishida,M., Hino,S., Michiue,T., Yamamoto,H., Kishida,S., Fukui,A., Asashima,M., and Kikuchi,A. (2001). Synergistic activation of the Wnt signaling pathway by Dvl and casein kinase I epsilon. Journal of Biological Chemistry *276*, 33147-33155.

Kishida,S., Yamamoto,H., Hino,S., Ikeda,S., Kishida,M., and Kikuchi,A. (1999). DIX domains of Dvl and axin are necessary for protein interactions and their ability to regulate beta-catenin stability. Mol Cell Biol *19*, 4414-4422.

Kishida,S., Yamamoto,H., Ikeda,S., Kishida,M., Sakamoto,I., Koyama,S., and Kikuchi,A. (1998). Axin, a negative regulator of the Wnt signaling pathway, directly interacts with Adenomatous polyposis coli and regulates the stabilization of beta-catenin. J Biol Chem *273*, 10823-10826.

Klefstrom,J., Arighi,E., Littlewood,T., Jaattela,M., Saksela,E., Evan,G.I., and Alitalo,K. (1997). Induction of TNF-sensitive cellular phenotype by c-Myc involves p53 and impaired NF-kappaB activation. EMBO J *16*, 7382-7392.

Klingensmith,J., Nusse,R., and Perrimon,N. (1994). The *Drosophila* segment polarity gene dishevelled encodes a novel protein required for response to the wingless signal. Genes Dev. *8*, 118-130.

Ko,J., Ryu,K.S., Lee,Y.H., Na,D.S., Kim,Y.S., Oh,Y.M., Kim,I.S., and Kim,J.W. (2002). Human secreted frizzled-related protein is down-regulated and induces apoptosis in human cervical cancer. Exp. Cell Res. *280*, 280-287.

Kobayashi,M., Honma,T., Matsuda,Y., Suzuki,Y., Narisawa,R., Ajioka,Y., and Asakura,H. (2000). Nuclear translocation of beta-catenin in colorectal cancer. Br. J. Cancer *82*, 1689-1693.

Koch,A., Denkhaus,D., Albrecht,S., Leuschner,I., von Schweinitz,D., and Pietsch,T. (1999). Childhood hepatoblastomas frequently carry a mutated degradation targeting box of the beta-catenin gene. Cancer Res *59*, 269-273.

Koch,A., Waha,A., Tonn,J.C., Sorensen,N., Berthold,F., Wolter,M., Reifenberger,J., Hartmann,W., Friedl,W., Reifenberger,G., Wiestler,O.D., and Pietsch,T. (2001). Somatic mutations of WNT/wingless signaling pathway components in primitive neuroectodermal tumors. Int J Cancer *93*, 445-449.

Koesters,R., Ridder,R., Kopp-Schneider,A., Betts,D., Adams,V., Niggli,F., Briner,J., and von Knebel,D.M. (1999). Mutational activation of the beta-catenin proto-oncogene is a common event in the development of Wilms' tumors. Cancer Res *59*, 3880-3882.

Koller,B.H., and Smithies,O. (1989). Inactivating the beta 2-microglobulin locus in mouse embryonic stem cells by homologous recombination. Proc. Natl. Acad. Sci. U. S. A *86*, 8932-8935.

Korinek,V., Barker,N., Moerer,P., van,D.E., Huls,G., Peters,P.J., and Clevers,H. (1998). Depletion of epithelial stem-cell compartments in the small intestine of mice lacking Tcf-4. Nat. Genet. *19*, 379-383.

Korinek,V., Barker,N., Morin,P.J., van Wichen,D., de Weger,R., Kinzler,K.W., Vogelstein,B., and Clevers,H. (1997). Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC-/- colon carcinoma. Science *275*, 1784-1787.

Kotsinas,A., Evangelou,K., Zacharatos,P., Kittas,C., and Gorgoulis,V.G. (2002). Proliferation, but not apoptosis, is associated with distinct beta-catenin expression patterns in non-small-cell lung carcinomas: relationship with Adenomatous polyposis coli and G(1)-to S-phase cell-cycle regulators. Am J Pathol *161*, 1619-1634.

Kratz,J.E., Stearns,D., Huso,D.L., Slunt,H.H., Price,D.L., Borchelt,D.R., and Eberhart,C.G. (2002). Expression of stabilized beta-catenin in differentiated neurons of transgenic mice does not result in tumor formation. BMC Cancer *2*, 33.

Krek,W. (1998). Proteolysis and the G1-S transition: the SCF connection. Curr. Opin. Genet. Dev. *8*, 36-42.

Krupnik,V.E., Sharp,J.D., Jiang,C., Robison,K., Chickering,T.W., Amaravadi,L., Brown,D.E., Guyot,D., Mays,G., Leiby,K., Chang,B., Duong,T., Goodearl,A.D., Gearing,D.P., Sokol,S.Y., and McCarthy,S.A. (1999). Functional and structural diversity of the human Dickkopf gene family. Gene *238*, 301-313.

Kuhl,M., Sheldahl,L.C., Malbon,C.C., and Moon,R.T. (2000). Ca(2+)/calmodulin-dependent protein kinase II is stimulated by Wnt and Frizzled homologs and promotes ventral cell fates in *Xenopus*. J. Biol. Chem. *275*, 12701-12711.

Kukekov,V.G., Laywell,E.D., Suslov,O., Davies,K., Scheffler,B., Thomas,L.B., O'Brien,T.F., Kusakabe,M., and Steindler,D.A. (1999). Multipotent stem/progenitor cells with similar properties arise from two neurogenic regions of adult human brain. Exp. Neurol. *156*, 333-344.

Kunnimalaiyaan,M., Vaccaro,A.M., Ndiaye,M.A., and Chen,H. (2007). Inactivation of glycogen synthase kinase-3beta, a downstream target of the raf-1 pathway, is associated with growth suppression in medullary thyroid cancer cells. Mol Cancer Ther *6*, 1151-1158.

Kuphal,F., and Behrens,J. (2006). E-cadherin modulates Wnt-dependent transcription in colorectal cancer cells but does not alter Wnt-independent gene expression in fibroblasts. Exp Cell Res *312*, 457-467.

Kurayoshi,M., Yamamoto,H., Izumi,S., and Kikuchi,A. (2007). Post-translational palmitoylation and glycosylation of Wnt-5a are necessary for its signalling. Biochem. J. *402*, 515-523.

Kusafuka,T., Miao,J., Kuroda,S., Udatsu,Y., and Yoneda,A. (2002). Codon 45 of the beta-catenin gene, a specific mutational target site of Wilms' tumor. Int J Mol Med *10*, 395-399.

Kutay,U., Bischoff,F.R., Kostka,S., Kraft,R., and Gorlich,D. (1997). Export of importin alpha from the nucleus is mediated by a specific nuclear transport factor. Cell *90*, 1061-1071.

Kwon,C., Arnold,J., Hsiao,E.C., Taketo,M.M., Conklin,B.R., and Srivastava,D. (2007). Canonical Wnt signaling is a positive regulator of mammalian cardiac progenitors. Proc Natl Acad Sci U S A *104*, 10894-10899.

Ladher,R.K., Church,V.L., Allen,S., Robson,L., Abdelfattah,A., Brown,N.A., Hattersley,G., Rosen,V., Luyten,F.P., Dale,L., and Francis-West,P.H. (2000). Cloning and expression of the Wnt antagonists Sfrp-2 and Frzb during chick development. Dev. Biol. *218*, 183-198.

Lamb,J., Ramaswamy,S., Ford,H.L., Contreras,B., Martinez,R.V., Kittrell,F.S., Zahnow,C.A., Patterson,N., Golub,T.R., and Ewen,M.E. (2003). A mechanism of cyclin D1 action encoded in the patterns of gene expression in human cancer. Cell *114*, 323-334.

Lamont,J.M., McManamy,C.S., Pearson,A.D., Clifford,S.C., and Ellison,D.W. (2004). Combined histopathological and molecular cytogenetic stratification of medulloblastoma patients. Clin. Cancer Res. *10*, 5482-5493.

Lasser,D.M., DeVivo,D.C., Garvin,J., and Wilhelmsen,K.C. (1994). Turcot's syndrome: evidence for linkage to the Adenomatous polyposis coli (APC) locus. Neurology *44*, 1083-1086.

Lawrence,P.A. (1966). Development and determination of hairs and bristles in the milkweed bug, Oncopeltus fasciatus (Lygaeidae, Hemiptera). J. Cell Sci. *1*, 475-498.

Lee,A., Kessler,J.D., Read,T.A., Kaiser,C., Corbeil,D., Huttner,W.B., Johnson,J.E., and Wechsler-Reya,R.J. (2005). Isolation of neural stem cells from the postnatal cerebellum. Nat Neurosci *8*, 723-729.

Lee,H.Y., Kleber,M., Hari,L., Brault,V., Suter,U., Taketo,M.M., Kemler,R., and Sommer,L. (2004). Instructive role of Wnt/beta-catenin in sensory fate specification in neural crest stem cells. Science *303*, 1020-1023.

Lee,J., Kotliarova,S., Kotliarov,Y., Li,A., Su,Q., Donin,N.M., Pastorino,S., Purow,B.W., Christopher,N., Zhang,W., Park,J.K., and Fine,H.A. (2006). Tumor stem cells derived from glioblastomas cultured in bFGF and EGF more closely mirror the phenotype and genotype of primary tumors than do serum-cultured cell lines. Cancer Cell *9*, 391-403.

Lee,J.H., Abraham,S.C., Kim,H.S., Nam,J.H., Choi,C., Lee,M.C., Park,C.S., Juhng,S.W., Rashid,A., Hamilton,S.R., and Wu,T.T. (2002). Inverse relationship between APC gene mutation in gastric Adenomas and development of Adenocarcinoma. Am J Pathol *161*, 611-618.

Lee,S.M., Tole,S., Grove,E., and Mcmahon,A.P. (2000). A local Wnt-3a signal is required for development of the mammalian hippocampus. Development *127*, 457-467.

Lee,W.H., Bookstein,R., Hong,F., Young,L.J., Shew,J.Y., and Lee,E.Y. (1987). Human retinoblastoma susceptibility gene: cloning, identification, and sequence. Science *235*, 1394-1399.

Legoix,P., Bluteau,O., Bayer,J., Perret,C., Balabaud,C., Belghiti,J., Franco,D., Thomas,G., Laurent-Puig,P., and Zucman-Rossi,J. (1999). Beta-catenin mutations in hepatocellular carcinoma correlate with a low rate of loss of heterozygosity. Oncogene *18*, 4044-4046.

Leis,H., Segrelles,C., Ruiz,S., Santos,M., and Paramio,J.M. (2002). Expression, localization, and activity of glycogen synthase kinase 3beta during mouse skin tumorigenesis. Mol Carcinog. *35*, 180-185.

Lendahl,U., Zimmerman,L.B., and McKay,R.D. (1990). CNS stem cells express a new class of intermediate filament protein. Cell *60*, 585-595.

Li,C., Xiao,J., Hormi,K., Borok,Z., and Minoo,P. (2002). Wnt5a participates in distal lung morphogenesis. Dev Biol *248*, 68-81.

Li,L., Yuan,H., Weaver,C.D., Mao,J., Farr,G.H., III, Sussman,D.J., Jonkers,J., Kimelman,D., and Wu,D. (1999). Axin and Frat1 interact with dvl and GSK, bridging Dvl to GSK in Wnt-mediated regulation of LEF-1. EMBO J *18*, 4233-4240.

Liao,X., Zhang,L., Thrasher,J.B., Du,J., and Li,B. (2003). Glycogen synthase kinase-3beta suppression eliminates tumor necrosis factor-related apoptosis-inducing ligand resistance in prostate cancer. Mol Cancer Ther *2*, 1215-1222.

Lie,D.C., Colamarino,S.A., Song,H.J., Desire,L., Mira,H., Consiglio,A., Lein,E.S., Jessberger,S., Lansford,H., Dearie,A.R., and Gage,F.H. (2005). Wnt signalling regulates adult hippocampal neurogenesis. Nature *437*, 1370-1375.

Liebner,S., Corada,M., Bangsow,T., Babbage,J., Taddei,A., Czupalla,C.J., Reis,M., Felici,A., Wolburg,H., Fruttiger,M., Taketo,M.M., von Melchner,H., Plate,K.H., Gerhardt,H., and Dejana,E. (2008). Wnt/beta-catenin signaling controls development of the blood-brain barrier. J Cell Biol *183*, 409-417.

Lijam,N., Paylor,R., McDonald,M.P., Crawley,J.N., Deng,C.X., Herrup,K., Stevens,K.E., Maccaferri,G., McBain,C.J., Sussman,D.J., and Wynshaw-Boris,A. (1997). Social interaction and sensorimotor gating abnormalities in mice lacking Dvl1. Cell *90*, 895-905.

Lin,K., Wang,S., Julius,M.A., Kitajewski,J., Moos,M., Jr., and Luyten,F.P. (1997). The cysteine-rich frizzled domain of Frzb-1 is required and sufficient for modulation of Wnt signaling. Proc. Natl. Acad. Sci. U. S. A *94*, 11196-11200.

Lin,S.Y., Xia,W., Wang,J.C., Kwong,K.Y., Spohn,B., Wen,Y., Pestell,R.G., and Hung,M.C. (2000). Beta-catenin, a novel prognostic marker for breast cancer: its roles in cyclin D1 expression and cancer progression. Proc Natl Acad Sci U S A *97*, 4262-4266.

Lin,X., and Perrimon,N. (1999). Dally cooperates with *Drosophila* Frizzled 2 to transduce Wingless signalling. Nature *400*, 281-284.

Liu,C., Li,Y., Semenov,M., Han,C., Baeg,G.H., Tan,Y., Zhang,Z., Lin,X., and He,X. (2002). Control of beta-catenin phosphorylation/degradation by a dual-kinase mechanism. Cell *108*, 837-847.

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

Liu,T., DeCostanzo,A.J., Liu,X., Wang,H., Hallagan,S., Moon,R.T., and Malbon,C.C. (2001). G protein signaling from activated rat frizzled-1 to the beta-catenin-Lef-Tcf pathway. Science *292*, 1718-1722.

Liu,Y., Shi,J., Lu,C.C., Wang,Z.B., Lyuksyutova,A.I., Song,X.J., and Zou,Y. (2005). Ryk-mediated Wnt repulsion regulates posterior-directed growth of corticospinal tract. Nat. Neurosci. *8*, 1151-1159.

Lois,C., and Alvarez-Buylla,A. (1993). Proliferating subventricular zone cells in the adult mammalian forebrain can differentiate into neurons and glia. Proc Natl Acad Sci U S A *90*, 2074-2077.

Lois,C., and Alvarez-Buylla,A. (1994). Long-distance neuronal migration in the adult mammalian brain. Science *264*, 1145-1148.

Lois,C., Garcia-Verdugo,J.M., and Alvarez-Buylla,A. (1996). Chain migration of neuronal precursors. Science *271*, 978-981.

Long,J.M., LaPorte,P., Paylor,R., and Wynshaw-Boris,A. (2004). Expanded characterization of the social interaction abnormalities in mice lacking Dvl1. Genes Brain Behav. *3*, 51-62.

Lothian,C., and Lendahl,U. (1997). An evolutionarily conserved region in the second intron of the human nestin gene directs gene expression to CNS progenitor cells and to early neural crest cells. Eur. J. Neurosci. *9*, 452-462.

Lovec,H., Grzeschiczek,A., Kowalski,M.B., and Moroy,T. (1994a). Cyclin D1/bcl-1 cooperates with myc genes in the generation of B-cell lymphoma in transgenic mice. EMBO J. *13*, 3487-3495.

Lovec,H., Sewing,A., Lucibello,F.C., Muller,R., and Moroy,T. (1994b). Oncogenic activity of cyclin D1 revealed through cooperation with Ha-ras: link between cell cycle control and malignant transformation. Oncogene *9*, 323-326.

Lovestone,S., Hartley,C.L., Pearce,J., and Anderton,B.H. (1996). Phosphorylation of tau by glycogen synthase kinase-3 beta in intact mammalian cells: the effects on the organization and stability of microtubules. Neuroscience *73*, 1145-1157.

Lu,W., Yamamoto,V., Ortega,B., and Baltimore,D. (2004). Mammalian Ryk is a Wnt coreceptor required for stimulation of neurite outgrowth. Cell *119*, 97-108.

Lundberg,A.S., and Weinberg,R.A. (1998). Functional inactivation of the retinoblastoma protein requires sequential modification by at least two distinct cyclin-cdk complexes. Mol. Cell Biol. *18*, 753-761.

Luo,W., Ng,W.W., Jin,L.H., Ye,Z., Han,J., and Lin,S.C. (2003). Axin utilizes distinct regions for competitive MEKK1 and MEKK4 binding and JNK activation. J Biol Chem *278*, 37451-37458.

Luskin,M.B. (1993). Restricted proliferation and migration of postnatally generated neurons derived from the forebrain subventricular zone. Neuron *11*, 173-189.

Lustig,B., Jerchow,B., Sachs,M., Weiler,S., Pietsch,T., Karsten,U., van de,W.M., Clevers,H., Schlag,P.M., Birchmeier,W., and Behrens,J. (2002). Negative feedback loop of Wnt signaling through upregulation of conductin/axin2 in colorectal and liver tumors. Mol Cell Biol *22*, 1184-1193.

Ma,C., Wang,J., Gao,Y., Gao,T.W., Chen,G., Bower,K.A., Odetallah,M., Ding,M., Ke,Z., and Luo,J. (2007). The role of glycogen synthase kinase 3beta in the transformation of epidermal cells. Cancer Res. *67*, 7756-7764.

Ma,L., and Wang,H.Y. (2006). Suppression of cyclic GMP-dependent protein kinase is essential to the Wnt/cGMP/Ca2+ pathway. J. Biol. Chem. *281*, 30990-31001.

Maatouk,D.M., DiNapoli,L., Alvers,A., Parker,K.L., Taketo,M.M., and Capel,B. (2008). Stabilization of beta-catenin in XY gonads causes male-to-female sex-reversal. Hum Mol Genet *17*, 2949-2955.

Machin,P., Catasus,L., Pons,C., Munoz,J., Matias-Guiu,X., and Prat,J. (2002). CTNNB1 mutations and beta-catenin expression in endometrial carcinomas. Hum Pathol *33*, 206-212.

Machold,R., and Fishell,G. (2005). Math1 is expressed in temporally discrete pools of cerebellar rhombic-lip neural progenitors. Neuron *48*, 17-24.

MacLean,K.H., Keller,U.B., Rodriguez-Galindo,C., Nilsson,J.A., and Cleveland,J.L. (2003). c-Myc augments gamma irradiation-induced apoptosis by suppressing Bcl-XL. Mol Cell Biol *23*, 7256-7270.

Maehama,T., and Dixon,J.E. (1998). The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. J. Biol. Chem. *273*, 13375-13378.

Mahmoud,N.N., Boolbol,S.K., Bilinski,R.T., Martucci,C., Chadburn,A., and Bertagnolli,M.M. (1997). Apc gene mutation is associated with a dominant-negative effect upon intestinal cell migration. Cancer Res. *57*, 5045-5050.

Maiti,S., Alam,R., Amos,C.I., and Huff,V. (2000). Frequent association of beta-catenin and WT1 mutations in Wilms tumors. Cancer Res *60*, 6288-6292.

Major,M.B., Camp,N.D., Berndt,J.D., Yi,X., Goldenberg,S.J., Hubbert,C., Biechele,T.L., Gingras,A.C., Zheng,N., Maccoss,M.J., Angers,S., and Moon,R.T. (2007). Wilms tumor suppressor WTX negatively regulates WNT/beta-catenin signaling. Science *316*, 1043-1046.

Majumdar,A., Vainio,S., Kispert,A., McMahon,J., and Mcmahon,A.P. (2003). Wnt11 and Ret/Gdnf pathways cooperate in regulating ureteric branching during metanephric kidney development. Development *130*, 3175-3185.

Malik,H.S., Eickbush,T.H., and Goldfarb,D.S. (1997). Evolutionary specialization of the nuclear targeting apparatus. Proc Natl Acad Sci U S A *94*, 13738-13742.

Mann,B., Gelos,M., Siedow,A., Hanski,M.L., Gratchev,A., Ilyas,M., Bodmer,W.F., Moyer,M.P., Riecken,E.O., Buhr,H.J., and Hanski,C. (1999). Target genes of beta-catenin-T cell-factor/lymphoid-enhancer-factor signaling in human colorectal carcinomas. Proc Natl Acad Sci U S A *96*, 1603-1608.

Mao,B., and Niehrs,C. (2003). Kremen2 modulates Dickkopf2 activity during Wnt/LRP6 signaling. Gene *302*, 179-183.

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

Mao,B., 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*, 321-325.

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

Marikawa,Y., and Elinson,R.P. (1998). beta-TrCP is a negative regulator of Wnt/beta-catenin signaling pathway and dorsal axis formation in *Xenopus* embryos. Mech. Dev. *77*, 75-80.

Marino,S., Vooijs,M., van Der,G.H., Jonkers,J., and Berns,A. (2000). Induction of medulloblastomas in p53-null mutant mice by somatic inactivation of Rb in the external granular layer cells of the cerebellum. Genes Dev. *14*, 994-1004.

Marois,E., Mahmoud,A., and Eaton,S. (2006). The endocytic pathway and formation of the Wingless morphogen gradient. Development *133*, 307-317.

Martin,G.R. (1981). Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc. Natl. Acad. Sci. U. S. A *78*, 7634-7638.

Martinez,S., Wassef,M., and Alvarado-Mallart,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*, 971-981.

Matsumine,A., Ogai,A., Senda,T., Okumura,N., Satoh,K., Baeg,G.H., Kawahara,T., Kobayashi,S., Okada,M., Toyoshima,K., and Akiyama,T. (1996). Binding of APC to the human homolog of the *Drosophila* discs large tumor suppressor protein. Science *272*, 1020-1023.

Matsushime,H., Ewen,M.E., Strom,D.K., Kato,J.Y., Hanks,S.K., Roussel,M.F., and Sherr,C.J. (1992). Identification and properties of an atypical catalytic subunit (p34PSK-J3/cdk4) for mammalian D type G1 cyclins. Cell *71*, 323-334.

Mazor,M., Kawano,Y., Zhu,H., Waxman,J., and Kypta,R.M. (2004). Inhibition of glycogen synthase kinase-3 represses androgen receptor activity and prostate cancer cell growth. Oncogene *23*, 7882-7892.

McKay,R. (1997). Stem cells in the central nervous system. Science *276*, 66-71.

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*, 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*, 581-595.

McMahon,S.B., Van Buskirk,H.A., Dugan,K.A., Copeland,T.D., and Cole,M.D. (1998). The novel ATM-related protein TRRAP is an essential cofactor for the c-Myc and E2F oncoproteins. Cell *94*, 363-374.

McMahon,S.B., Wood,M.A., and Cole,M.D. (2000). The essential cofactor TRRAP recruits the histone acetyltransferase hGCN5 to c-Myc. Mol Cell Biol *20*, 556-562.

McManamy,C.S., Lamont,J.M., Taylor,R.E., Cole,M., Pearson,A.D., Clifford,S.C., and Ellison,D.W. (2003). Morphophenotypic variation predicts clinical behavior in childhood non-desmoplastic medulloblastomas. J. Neuropathol. Exp. Neurol. *62*, 627-632.

Melkonyan,H.S., Chang,W.C., Shapiro,J.P., Mahadevappa,M., Fitzpatrick,P.A., Kiefer,M.C., Tomei,L.D., and Umansky,S.R. (1997). SARPs: a family of secreted apoptosis-related proteins. Proc. Natl. Acad. Sci. U. S. A *94*, 13636-13641.

Mendrzyk,F., Radlwimmer,B., Joos,S., Kokocinski,F., Benner,A., Stange,D.E., Neben,K., Fiegler,H., Carter,N.P., Reifenberger,G., Korshunov,A., and Lichter,P. (2005). Genomic and protein expression profiling identifies CDK6 as novel independent prognostic marker in medulloblastoma. J. Clin. Oncol. *23*, 8853-8862.

Merrill,B.J., Pasolli,H.A., Polak,L., Rendl,M., Garcia-Garcia,M.J., Anderson,K.V., and Fuchs,E. (2004). Tcf3: a transcriptional regulator of axis induction in the early embryo. Development *131*, 263-274.

MESSIER,B., LEBLOND,C.P., and SMART,I. (1958). Presence of DNA synthesis and mitosis in the brain of young adult mice. Exp. Cell Res *14*, 224-226.

Metzger,D., Clifford,J., Chiba,H., and Chambon,P. (1995). Conditional site-specific recombination in mammalian cells using a ligand-dependent chimeric Cre-recombinase. Proc. Natl. Acad. Sci. U. S. A *92*, 6991-6995.

Meyerson,M., and Harlow,E. (1994). Identification of G1 kinase activity for cdk6, a novel cyclin D partner. Mol Cell Biol *14*, 2077-2086.

Mezhybovska,M., Yudina,Y., Abhyankar,A., and Sjolander,A. (2009). Beta-catenin is involved in alterations in mitochondrial activity in non-transformed intestinal epithelial and colon cancer cells. Br. J. Cancer *101*, 1596-1605.

Midgley,C.A., White,S., Howitt,R., Save,V., Dunlop,M.G., Hall,P.A., Lane,D.P., Wyllie,A.H., and Bubb,V.J. (1997). APC expression in normal human tissues. J. Pathol. *181*, 426-433.

Mikels,A.J., and Nusse,R. (2006). Purified Wnt5a protein activates or inhibits beta-catenin-TCF signaling depending on receptor context. PLoS. Biol. *4*, e115.

Miller,C., and Sassoon,D.A. (1998). Wnt-7a maintains appropriate uterine patterning during the development of the mouse female reproductive tract. Development *125*, 3201-3211.

Millet,S., Campbell,K., Epstein,D.J., Losos,K., Harris,E., and Joyner,A.L. (1999). A role for Gbx2 in repression of Otx2 and positioning the mid/hindbrain organizer. Nature *401*, 161-164.

Mirabelli-Primdahl,L., Gryfe,R., Kim,H., Millar,A., Luceri,C., Dale,D., Holowaty,E., Bapat,B., Gallinger,S., and Redston,M. (1999). Beta-catenin mutations are specific for colorectal carcinomas with microsatellite instability but occur in endometrial carcinomas irrespective of mutator pathway. Cancer Res *59*, 3346-3351.

Mitchell,K.O., Ricci,M.S., Miyashita,T., Dicker,D.T., Jin,Z., Reed,J.C., and El Deiry,W.S. (2000). Bax is a transcriptional target and mediator of c-myc-induced apoptosis. Cancer Res *60*, 6318-6325.

Miyazaki,K., Hattori,Y., Umenishi,F., Yasumitsu,H., and Umeda,M. (1990). Purification and characterization of extracellular matrix-degrading metalloproteinase, matrin (pump-1), secreted from human rectal carcinoma cell line. Cancer Res. *50*, 7758-7764.

Miyazawa,K., Iwaya,K., Kuroda,M., Harada,M., Serizawa,H., Koyanagi,Y., Sato,Y., Mizokami,Y., Matsuoka,T., and Mukai,K. (2000). Nuclear accumulation of beta-catenin in intestinal-type gastric carcinoma: correlation with early tumor invasion. Virchows Arch *437*, 508-513.

Miyoshi,K., Shillingford,J.M., Le Provost,F., Gounari,F., Bronson,R., von Boehmer,H., Taketo,M.M., Cardiff,R.D., Hennighausen,L., and Khazaie,K. (2002). Activation of beta -catenin signaling in differentiated mammary secretory cells induces transdifferentiation into epidermis and squamous metaplasias. Proc Natl Acad Sci U S A *99*, 219-224.

Miyoshi,Y., Iwao,K., Nagasawa,Y., Aihara,T., Sasaki,Y., Imaoka,S., Murata,M., Shimano,T., and Nakamura,Y. (1998a). Activation of the beta-catenin gene in primary hepatocellular carcinomas by somatic alterations involving exon 3. Cancer Res *58*, 2524-2527.

Miyoshi,Y., Iwao,K., Nawa,G., Yoshikawa,H., Ochi,T., and Nakamura,Y. (1998b). Frequent mutations in the beta-catenin gene in desmoid tumors from patients without familial Adenomatous polyposis. Oncol Res *10*, 591-594.

Molenaar,M., van de,W.M., Oosterwegel,M., Peterson-Maduro,J., Godsave,S., Korinek,V., Roose,J., Destree,O., and Clevers,H. (1996). XTcf-3 transcription factor mediates beta-catenin-induced axis formation in *Xenopus* embryos. Cell *86*, 391-399.

Momota,H., Shih,A.H., Edgar,M.A., and Holland,E.C. (2008). c-Myc and beta-catenin cooperate with loss of p53 to generate multiple members of the primitive neuroectodermal tumor family in mice. Oncogene.

Monkley,S.J., Delaney,S.J., Pennisi,D.J., Christiansen,J.H., and Wainwright,B.J. (1996). Targeted disruption of the Wnt2 gene results in placentation defects. Development *122*, 3343-3353.

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*, 97-111.

Moreno-Bueno,G., Gamallo,C., Perez-Gallego,L., Contreras,F., and Palacios,J. (2001a). beta-catenin expression in pilomatrixomas. Relationship with beta-catenin gene mutations and comparison with beta-catenin expression in normal hair follicles. Br J Dermatol. *145*, 576-581.

Moreno-Bueno,G., Gamallo,C., Perez-Gallego,L., de Mora,J.C., Suarez,A., and Palacios,J. (2001b). beta-Catenin expression pattern, beta-catenin gene mutations, and microsatellite

instability in endometrioid ovarian carcinomas and synchronous endometrial carcinomas. Diagn Mol Pathol *10*, 116-122.

Mori,H., Ninomiya,K., Kino-oka,M., Shofuda,T., Islam,M.O., Yamasaki,M., Okano,H., Taya,M., and Kanemura,Y. (2006). Effect of neurosphere size on the growth rate of human neural stem/progenitor cells. J. Neurosci. Res. *84*, 1682-1691.

Mori,T., Nagase,H., Horii,A., Miyoshi,Y., Shimano,T., Nakatsuru,S., Aoki,T., Arakawa,H., Yanagisawa,A., Ushio,Y., and . (1994). Germ-line and somatic mutations of the APC gene in patients with Turcot syndrome and analysis of APC mutations in brain tumors. Genes Chromosomes. Cancer *9*, 168-172.

Morin,P.J., Sparks,A.B., Korinek,V., Barker,N., Clevers,H., Vogelstein,B., and Kinzler,K.W. (1997). Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. Science *275*, 1787-1790.

Moser,A.R., Shoemaker,A.R., Connelly,C.S., Clipson,L., Gould,K.A., Luongo,C., Dove,W.F., Siggers,P.H., and Gardner,R.L. (1995). Homozygosity for the Min allele of Apc results in disruption of mouse development prior to gastrulation. Dev. Dyn. *203*, 422-433.

Mucenski,M.L., Nation,J.M., Thitoff,A.R., Besnard,V., Xu,Y., Wert,S.E., Harada,N., Taketo,M.M., Stahlman,M.T., and Whitsett,J.A. (2005). Beta-catenin regulates differentiation of respiratory epithelial cells *in vivo*. Am J Physiol Lung Cell Mol Physiol *289*, L971-L979.

Mulhern,R.K., Kepner,J.L., Thomas,P.R., Armstrong,F.D., Friedman,H.S., and Kun,L.E. (1998). Neuropsychologic functioning of survivors of childhood medulloblastoma randomized to receive conventional or reduced-dose craniospinal irradiation: a Pediatric Oncology Group study. J Clin Oncol *16*, 1723-1728.

Mulhern,R.K., Palmer,S.L., Reddick,W.E., Glass,J.O., Kun,L.E., Taylor,J., Langston,J., and Gajjar,A. (2001). Risks of young age for selected neurocognitive deficits in medulloblastoma are associated with white matter loss. J Clin Oncol *19*, 472-479.

Mulhern,R.K., Reddick,W.E., Palmer,S.L., Glass,J.O., Elkin,T.D., Kun,L.E., Taylor,J., Langston,J., and Gajjar,A. (1999). Neurocognitive deficits in medulloblastoma survivors and white matter loss. Ann Neurol *46*, 834-841.

Muller,H., Bracken,A.P., Vernell,R., Moroni,M.C., Christians,F., Grassilli,E., Prosperini,E., Vigo,E., Oliner,J.D., and Helin,K. (2001). E2Fs regulate the expression of genes involved in differentiation, development, proliferation, and apoptosis. Genes Dev *15*, 267-285.

Munemitsu,S., Albert,I., Souza,B., Rubinfeld,B., and Polakis,P. (1995). Regulation of intracellular beta-catenin levels by the Adenomatous polyposis coli (APC) tumor-suppressor protein. Proc Natl Acad Sci U S A *92*, 3046-3050.

Munemitsu,S., Souza,B., Muller,O., Albert,I., Rubinfeld,B., and Polakis,P. (1994). The APC gene product associates with microtubules *in vivo* and promotes their assembly *in vitro*. Cancer Res. *54*, 3676-3681.

Murre,C., McCaw,P.S., Vaessin,H., Caudy,M., Jan,L.Y., Jan,Y.N., Cabrera,C.V., Buskin,J.N., Hauschka,S.D., Lassar,A.B., and . (1989). Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. Cell *58*, 537-544.

Musgrove,E.A., Lee,C.S., Buckley,M.F., and Sutherland,R.L. (1994). Cyclin D1 induction in breast cancer cells shortens G1 and is sufficient for cells arrested in G1 to complete the cell cycle. Proc Natl Acad Sci U S A *91*, 8022-8026.

Nakagawa,H., Murata,Y., Koyama,K., Fujiyama,A., Miyoshi,Y., Monden,M., Akiyama,T., and Nakamura,Y. (1998). Identification of a brain-specific APC homologue, APCL, and its interaction with beta-catenin. Cancer Res. *58*, 5176-5181.

Nakamura,M., Yang,F., Fujisawa,H., Yonekawa,Y., Kleihues,P., and Ohgaki,H. (2000). Loss of heterozygosity on chromosome 19 in secondary glioblastomas. J. Neuropathol. Exp. Neurol. *59*, 539-543.

Nakamura,M., Yonekawa,Y., Kleihues,P., and Ohgaki,H. (2001a). Promoter hypermethylation of the RB1 gene in glioblastomas. Lab Invest *81*, 77-82.

Nakamura,M., Zhou,X.Z., and Lu,K.P. (2001b). Critical role for the EB1 and APC interaction in the regulation of microtubule polymerization. Curr. Biol. *11*, 1062-1067.

Nakato,H., Futch,T.A., and Selleck,S.B. (1995). The division abnormally delayed (dally) gene: a putative integral membrane proteoglycan required for cell division patterning during postembryonic development of the nervous system in *Drosophila*. Development *121*, 3687-3702.

Nakaya,M.A., Biris,K., Tsukiyama,T., Jaime,S., Rawls,J.A., and Yamaguchi,T.P. (2005). Wnt3a links left-right determination with segmentation and anteroposterior axis elongation. Development *132*, 5425-5436.

Nei,H., Saito,T., Yamasaki,H., Mizumoto,H., Ito,E., and Kudo,R. (1999). Nuclear localization of beta-catenin in normal and carcinogenic endometrium. Mol Carcinog. *25*, 207-218.

Neuman,E., Ladha,M.H., Lin,N., Upton,T.M., Miller,S.J., DiRenzo,J., Pestell,R.G., Hinds,P.W., Dowdy,S.F., Brown,M., and Ewen,M.E. (1997). Cyclin D1 stimulation of estrogen receptor transcriptional activity independent of cdk4. Mol Cell Biol *17*, 5338-5347.

Ng,T.L., Gown,A.M., Barry,T.S., Cheang,M.C., Chan,A.K., Turbin,D.A., Hsu,F.D., West,R.B., and Nielsen,T.O. (2005). Nuclear beta-catenin in mesenchymal tumors. Mod. Pathol *18*, 68-74.

Nhieu,J.T., Renard,C.A., Wei,Y., Cherqui,D., Zafrani,E.S., and Buendia,M.A. (1999). Nuclear accumulation of mutated beta-catenin in hepatocellular carcinoma is associated with increased cell proliferation. Am J Pathol *155*, 703-710.

Nikolakaki,E., Coffer,P.J., Hemelsoet,R., Woodgett,J.R., and Defize,L.H. (1993). Glycogen synthase kinase 3 phosphorylates Jun family members *in vitro* and negatively regulates their transactivating potential in intact cells. Oncogene *8*, 833-840.

Nikuseva-Martic,T., Beros,V., Pecina-Slaus,N., Pecina,H.I., and Bulic-Jakus,F. (2007). Genetic changes of CDH1, APC, and CTNNB1 found in human brain tumors. Pathol. Res. Pract. *203*, 779-787.

Nishisho,I., Nakamura,Y., Miyoshi,Y., Miki,Y., Ando,H., Horii,A., Koyama,K., Utsunomiya,J., Baba,S., and Hedge,P. (1991). Mutations of chromosome 5q21 genes in FAP and colorectal cancer patients. Science *253*, 665-669.

Noe,V., Fingleton,B., Jacobs,K., Crawford,H.C., Vermeulen,S., Steelant,W., Bruyneel,E., Matrisian,L.M., and Mareel,M. (2001). Release of an invasion promoter E-cadherin fragment by matrilysin and stromelysin-1. J Cell Sci *114*, 111-118.

Nojima,M., Suzuki,H., Toyota,M., Watanabe,Y., Maruyama,R., Sasaki,S., Sasaki,Y., Mita,H., Nishikawa,N., Yamaguchi,K., Hirata,K., Itoh,F., Tokino,T., Mori,M., Imai,K., and Shinomura,Y. (2007). Frequent epigenetic inactivation of SFRP genes and constitutive activation of Wnt signaling in gastric cancer. Oncogene *26*, 4699-4713.

Noviello,C., Courjal,F., and Theillet,C. (1996). Loss of heterozygosity on the long arm of chromosome 6 in breast cancer: possibly four regions of deletion. Clin. Cancer Res. *2*, 1601-1606.

Nozawa,N., Hashimoto,S., Nakashima,Y., Matsuo,Y., Koga,T., Sugio,K., Niho,Y., Harada,M., and Sueishi,K. (2006). Immunohistochemical alpha- and beta-catenin and E-cadherin expression and their clinicopathological significance in human lung Adenocarcinoma. Pathol Res Pract *202*, 639-650.

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*, 99-109.

O'Brien,C.A., Pollett,A., Gallinger,S., and Dick,J.E. (2007). A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. Nature *445*, 106-110.

O'Hagan,R.C., Ohh,M., David,G., de Alboran,I.M., Alt,F.W., Kaelin,W.G., Jr., and DePinho,R.A. (2000). Myc-enhanced expression of Cul1 promotes ubiquitin-dependent proteolysis and cell cycle progression. Genes Dev *14*, 2185-2191.

Oancea,E., and Meyer,T. (1998). Protein kinase C as a molecular machine for decoding calcium and diacylglycerol signals. Cell *95*, 307-318.

Oda,H., Imai,Y., Nakatsuru,Y., Hata,J., and Ishikawa,T. (1996). Somatic mutations of the APC gene in sporadic hepatoblastomas. Cancer Res *56*, 3320-3323.

Offit,K., Parsa,N.Z., Gaidano,G., Filippa,D.A., Louie,D., Pan,D., Jhanwar,S.C., Dalla-Favera,R., and Chaganti,R.S. (1993). 6q deletions define distinct clinico-pathologic subsets of non-Hodgkin's lymphoma. Blood *82*, 2157-2162.

Oh,H., and Irvine,K.D. (2009). *In vivo* analysis of Yorkie phosphorylation sites. Oncogene *28*, 1916-1927.

Ohgaki,H., Dessen,P., Jourde,B., Horstmann,S., Nishikawa,T., Di Patre,P.L., Burkhard,C., Schuler,D., Probst-Hensch,N.M., Maiorka,P.C., Baeza,N., Pisani,P., Yonekawa,Y., Yasargil,M.G., Lutolf,U.M., and Kleihues,P. (2004). Genetic pathways to glioblastoma: a population-based study. Cancer Res. *64*, 6892-6899.

Ohgaki,H., Eibl,R.H., Schwab,M., Reichel,M.B., Mariani,L., Gehring,M., Petersen,I., Holl,T., Wiestler,O.D., and Kleihues,P. (1993). Mutations of the p53 tumor suppressor gene in neoplasms of the human nervous system. Mol. Carcinog. *8*, 74-80.

Oishi,I., Suzuki,H., Onishi,N., Takada,R., Kani,S., Ohkawara,B., Koshida,I., Suzuki,K., Yamada,G., Schwabe,G.C., Mundlos,S., Shibuya,H., Takada,S., and Minami,Y. (2003). The receptor tyrosine kinase Ror2 is involved in non-canonical Wnt5a/JNK signalling pathway. Genes Cells *8*, 645-654.

Okamoto,Y., Di Patre,P.L., Burkhard,C., Horstmann,S., Jourde,B., Fahey,M., Schuler,D., Probst-Hensch,N.M., Yasargil,M.G., Yonekawa,Y., Lutolf,U.M., Kleihues,P., and Ohgaki,H. (2004). Population-based study on incidence, survival rates, and genetic alterations of low-grade diffuse astrocytomas and oligodendrogliomas. Acta Neuropathol. *108*, 49-56.

Omholt,K., Platz,A., Ringborg,U., and Hansson,J. (2001). Cytoplasmic and nuclear accumulation of beta-catenin is rarely caused by CTNNB1 exon 3 mutations in cutaneous malignant melanoma. Int J Cancer *92*, 839-842.

Onilude,O.E., Lusher,M.E., Lindsey,J.C., Pearson,A.D., Ellison,D.W., and Clifford,S.C. (2006). APC and CTNNB1 mutations are rare in sporadic ependymomas. Cancer Genet. Cytogenet. *168*, 158-161.

Orban,P.C., Chui,D., and Marth,J.D. (1992). Tissue- and site-specific DNA recombination in transgenic mice. Proc. Natl. Acad. Sci. U. S. A *89*, 6861-6865.

Orsulic,S., and Peifer,M. (1996). An *in vivo* structure-function study of armadillo, the beta-catenin homologue, reveals both separate and overlapping regions of the protein required for cell adhesion and for wingless signaling. J Cell Biol *134*, 1283-1300.

Oshima,M., Oshima,H., Kitagawa,K., Kobayashi,M., Itakura,C., and Taketo,M. (1995). Loss of Apc heterozygosity and abnormal tissue building in nascent intestinal polyps in mice carrying a truncated Apc gene. Proc. Natl. Acad. Sci. U. S. A *92*, 4482-4486.

Ougolkov,A.V., Bone,N.D., Fernandez-Zapico,M.E., Kay,N.E., and Billadeau,D.D. (2007). Inhibition of glycogen synthase kinase-3 activity leads to epigenetic silencing of nuclear factor kappaB target genes and induction of apoptosis in chronic lymphocytic leukemia B cells. Blood *110*, 735-742.

Ougolkov,A.V., Fernandez-Zapico,M.E., Bilim,V.N., Smyrk,T.C., Chari,S.T., and Billadeau,D.D. (2006). Aberrant nuclear accumulation of glycogen synthase kinase-3beta in human pancreatic cancer: association with kinase activity and tumor dedifferentiation. Clin Cancer Res *12*, 5074-5081.

Ougolkov,A.V., Fernandez-Zapico,M.E., Savoy,D.N., Urrutia,R.A., and Billadeau,D.D. (2005). Glycogen synthase kinase-3beta participates in nuclear factor kappaB-mediated gene transcription and cell survival in pancreatic cancer cells. Cancer Res *65*, 2076-2081.

Oyama,T., Kashiwabara,K., Yoshimoto,K., Arnold,A., and Koerner,F. (1998). Frequent overexpression of the cyclin D1 oncogene in invasive lobular carcinoma of the breast. Cancer Res *58*, 2876-2880.

Palacios,J., and Gamallo,C. (1998). Mutations in the beta-catenin gene (CTNNB1) in endometrioid ovarian carcinomas. Cancer Res *58*, 1344-1347.

Palmer,S.L., Gajjar,A., Reddick,W.E., Glass,J.O., Kun,L.E., Wu,S., Xiong,X., and Mulhern,R.K. (2003). Predicting intellectual outcome among children treated with 35-40 Gy craniospinal irradiation for medulloblastoma. Neuropsychology *17*, 548-555.

Palmer,S.L., Goloubeva,O., Reddick,W.E., Glass,J.O., Gajjar,A., Kun,L., Merchant,T.E., and Mulhern,R.K. (2001). Patterns of intellectual development among survivors of pediatric medulloblastoma: a longitudinal analysis. J Clin Oncol *19*, 2302-2308.

Panakova,D., Sprong,H., Marois,E., Thiele,C., and Eaton,S. (2005). Lipoprotein particles are required for Hedgehog and Wingless signalling. Nature *435*, 58-65.

Panhuysen,M., Vogt Weisenhorn,D.M., Blanquet,V., Brodski,C., Heinzmann,U., Beisker,W., and Wurst,W. (2004). Effects of Wnt1 signaling on proliferation in the developing mid-/hindbrain region. Mol. Cell Neurosci. *26*, 101-111.

Park,T.J., Haigo,S.L., and Wallingford,J.B. (2006). Ciliogenesis defects in embryos lacking inturned or fuzzy function are associated with failure of planar cell polarity and Hedgehog signaling. Nat. Genet. *38*, 303-311.

Park,W.S., Oh,R.R., Park,J.Y., Kim,P.J., Shin,M.S., Lee,J.H., Kim,H.S., Lee,S.H., Kim,S.Y., Park,Y.G., An,W.G., Kim,H.S., Jang,J.J., Yoo,N.J., and Lee,J.Y. (2001). Nuclear localization of beta-catenin is an important prognostic factor in hepatoblastoma. J Pathol *193*, 483-490.

Park,W.S., Oh,R.R., Park,J.Y., Lee,S.H., Shin,M.S., Kim,Y.S., Kim,S.Y., Lee,H.K., Kim,P.J., Oh,S.T., Yoo,N.J., and Lee,J.Y. (1999). Frequent somatic mutations of the beta-catenin gene in intestinal-type gastric cancer. Cancer Res *59*, 4257-4260.

Parr,B.A., Cornish,V.A., Cybulsky,M.I., and Mcmahon,A.P. (2001). Wnt7b regulates placental development in mice. Dev Biol *237*, 324-332.

Parr,B.A., and Mcmahon,A.P. (1995). Dorsalizing Signal Wnt-7A Required for Normal Polarity of D-V and A-P Axes of Mouse Limb. Nature *374*, 350-353.

Parr,B.A., and Mcmahon,A.P. (1998). Sexually dimorphic development of the mammalian reproductive tract requires Wnt-7a. Nature *395*, 707-710.

Patthy,L. (2000). The WIF module. Trends Biochem. Sci. *25*, 12-13.

Perrimon,N., Lanjuin,A., Arnold,C., and Noll,E. (1996). Zygotic lethal mutations with maternal effect phenotypes in *Drosophila* melanogaster. II. Loci on the second and third chromosomes identified by P-element-induced mutations. Genetics *144*, 1681-1692.

Peters,J.M., McKay,R.M., McKay,J.P., and Graff,J.M. (1999). Casein kinase I transduces Wnt signals. Nature *401*, 345-350.

Pietsch,T., Waha,A., Koch,A., Kraus,J., Albrecht,S., Tonn,J., Sorensen,N., Berthold,F., Henk,B., Schmandt,N., Wolf,H.K., von,D.A., Wainwright,B., Chenevix-Trench,G., Wiestler,O.D., and Wicking,C. (1997). Medulloblastomas of the desmoplastic variant carry mutations of the human homologue of *Drosophila* patched. Cancer Res. *57*, 2085-2088.

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*, 535-538.

Pollock,P.M., and Hayward,N. (2002). Mutations in exon 3 of the beta-catenin gene are rare in melanoma cell lines. Melanoma Res *12*, 183-186.

Pomeroy,S.L., Tamayo,P., Gaasenbeek,M., Sturla,L.M., Angelo,M., McLaughlin,M.E., Kim,J.Y., Goumnerova,L.C., Black,P.M., Lau,C., Allen,J.C., Zagzag,D., Olson,J.M., Curran,T., Wetmore,C., Biegel,J.A., Poggio,T., Mukherjee,S., Rifkin,R., Califano,A., Stolovitzky,G., Louis,D.N., Mesirov,J.P., Lander,E.S., and Golub,T.R. (2002). Prediction of central nervous system embryonal tumour outcome based on gene expression. Nature *415*, 436-442.

Port,F., Kuster,M., Herr,P., Furger,E., Banziger,C., Hausmann,G., and Basler,K. (2008). Wingless secretion promotes and requires retromer-dependent cycling of Wntless. Nat Cell Biol *10*, 178-185.

Powell,S.M., Petersen,G.M., Krush,A.J., Booker,S., Jen,J., Giardiello,F.M., Hamilton,S.R., Vogelstein,B., and Kinzler,K.W. (1993). Molecular diagnosis of familial Adenomatous polyposis. N Engl J Med *329*, 1982-1987.

Prasad,B.C., and Clark,S.G. (2006). Wnt signaling establishes anteroposterior neuronal polarity and requires retromer in C. elegans. Development *133*, 1757-1766.

Prendergast,G.C., and Ziff,E.B. (1991). Methylation-sensitive sequence-specific DNA binding by the c-Myc basic region. Science *251*, 186-189.

Prieve,M.G., and Waterman,M.L. (1999). Nuclear localization and formation of beta-catenin-lymphoid enhancer factor 1 complexes are not sufficient for activation of gene expression. Mol Cell Biol *19*, 4503-4515.

Pulverer,B.J., Kyriakis,J.M., Avruch,J., Nikolakaki,E., and Woodgett,J.R. (1991). Phosphorylation of c-jun mediated by MAP kinases. Nature *353*, 670-674.

Qu,Q., Sun,G., Li,W., Yang,S., Ye,P., Zhao,C., Yu,R.T., Gage,F.H., Evans,R.M., and Shi,Y. (2010). Orphan nuclear receptor TLX activates Wnt/beta-catenin signalling to stimulate neural stem cell proliferation and self-renewal. Nat. Cell Biol. *12*, 31-40.

Raffel,C., Jenkins,R.B., Frederick,L., Hebrink,D., Alderete,B., Fults,D.W., and James,C.D. (1997). Sporadic medulloblastomas contain PTCH mutations. Cancer Res. *57*, 842-845.

Raible,D.W. (2006). Development of the neural crest: achieving specificity in regulatory pathways. Curr. Opin. Cell Biol. *18*, 698-703.

Ranheim,E.A., Kwan,H.C., Reya,T., Wang,Y.K., Weissman,I.L., and Francke,U. (2005). Frizzled 9 knock-out mice have abnormal B-cell development. Blood *105*, 2487-2494.

Rasheed,B.K., Stenzel,T.T., McLendon,R.E., Parsons,R., Friedman,A.H., Friedman,H.S., Bigner,D.D., and Bigner,S.H. (1997). PTEN gene mutations are seen in high-grade but not in low-grade gliomas. Cancer Res. *57*, 4187-4190.

Reardon,D.A., Michalkiewicz,E., Boyett,J.M., Sublett,J.E., Entrekin,R.E., Ragsdale,S.T., Valentine,M.B., Behm,F.G., Li,H., Heideman,R.L., Kun,L.E., Shapiro,D.N., and Look,A.T. (1997). Extensive genomic abnormalities in childhood medulloblastoma by comparative genomic hybridization. Cancer Res. *57*, 4042-4047.

Redies,C., and Takeichi,M. (1996). Cadherins in the developing central nervous system: an adhesive code for segmental and functional subdivisions. Dev Biol *180*, 413-423.

Reifenberger,G., Reifenberger,J., Ichimura,K., Meltzer,P.S., and Collins,V.P. (1994). Amplification of multiple genes from chromosomal region 12q13-14 in human malignant gliomas: preliminary mapping of the amplicons shows preferential involvement of CDK4, SAS, and MDM2. Cancer Res. *54*, 4299-4303.

Reifenberger,J., Knobbe,C.B., Wolter,M., Blaschke,B., Schulte,K.W., Pietsch,T., Ruzicka,T., and Reifenberger,G. (2002). Molecular genetic analysis of malignant melanomas for aberrations of the WNT signaling pathway genes CTNNB1, APC, ICAT and BTRC. Int J Cancer *100*, 549-556.

Reifenberger,J., Ring,G.U., Gies,U., Cobbers,L., Oberstrass,J., An,H.X., Niederacher,D., Wechsler,W., and Reifenberger,G. (1996). Analysis of p53 mutation and epidermal growth factor receptor amplification in recurrent gliomas with malignant progression. J. Neuropathol. Exp. Neurol. *55*, 822-831.

Reifers,F., Bohli,H., Walsh,E.C., Crossley,P.H., Stainier,D.Y., and Brand,M. (1998). Fgf8 is mutated in zebrafish acerebellar (ace) mutants and is required for maintenance of midbrain-hindbrain boundary development and somitogenesis. Development *125*, 2381-2395.

Reya,T., O'Riordan,M., Okamura,R., Devaney,E., Willert,K., Nusse,R., and Grosschedl,R. (2000). Wnt signaling regulates B lymphocyte proliferation through a LEF-1 dependent mechanism. Immunity. *13*, 15-24.

Reynolds,B.A., and Weiss,S. (1992). Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science *255*, 1707-1710.

Ricci-Vitiani,L., Lombardi,D.G., Pilozzi,E., Biffoni,M., Todaro,M., Peschle,C., and De Maria,R. (2007). Identification and expansion of human colon-cancer-initiating cells. Nature *445*, 111-115.

Rickert,C.H., and Paulus,W. (2004). Comparative genomic hybridization in central and peripheral nervous system tumors of childhood and adolescence. J. Neuropathol. Exp. Neurol. *63*, 399-417.

Rimm,D.L., Caca,K., Hu,G., Harrison,F.B., and Fearon,E.R. (1999). Frequent nuclear/cytoplasmic localization of beta-catenin without exon 3 mutations in malignant melanoma. Am J Pathol *154*, 325-329.

Rimm,D.L., Koslov,E.R., Kebriaei,P., Cianci,C.D., and Morrow,J.S. (1995). Alpha 1(E)-catenin is an actin-binding and -bundling protein mediating the attachment of F-actin to the membrane adhesion complex. Proc. Natl. Acad. Sci. U. S. A *92*, 8813-8817.

Ris,M.D., Packer,R., Goldwein,J., Jones-Wallace,D., and Boyett,J.M. (2001). Intellectual outcome after reduced-dose radiation therapy plus adjuvant chemotherapy for medulloblastoma: a Children's Cancer Group study. J Clin Oncol *19*, 3470-3476.

Rivera,M.N., Kim,W.J., Wells,J., Driscoll,D.R., Brannigan,B.W., Han,M., Kim,J.C., Feinberg,A.P., Gerald,W.L., Vargas,S.O., Chin,L., Iafrate,A.J., Bell,D.W., and Haber,D.A. (2007). An X chromosome gene, WTX, is commonly inactivated in Wilms tumor. Science *315*, 642-645.

Rogers,H.A., Miller,S., Lowe,J., Brundler,M.A., Coyle,B., and Grundy,R.G. (2009). An investigation of WNT pathway activation and association with survival in central nervous system primitive neuroectodermal tumours (CNS PNET). Br. J. Cancer *100*, 1292-1302.

Roose,J., Molenaar,M., Peterson,J., Hurenkamp,J., Brantjes,H., Moerer,P., van de,W.M., Destree,O., and Clevers,H. (1998). The *Xenopus* Wnt effector XTcf-3 interacts with Groucho-related transcriptional repressors. Nature *395*, 608-612.

Rosin-Arbesfeld,R., Townsley,F., and Bienz,M. (2000). The APC tumour suppressor has a nuclear export function. Nature *406*, 1009-1012.

Ross,J.F., Naar,A., Cam,H., Gregory,R., and Dynlacht,B.D. (2001). Active repression and E2F inhibition by pRB are biochemically distinguishable. Genes Dev. *15*, 392-397.

Roy,R., Yang,J., and Moses,M.A. (2009). Matrix metalloproteinases as novel biomarkers and potential therapeutic targets in human cancer. J. Clin. Oncol. *27*, 5287-5297.

Rubinfeld,B., Albert,I., Porfiri,E., Fiol,C., Munemitsu,S., and Polakis,P. (1996). Binding of GSK3beta to the APC-beta-catenin complex and regulation of complex assembly. Science *272*, 1023-1026.

Rubinfeld,B., Albert,I., Porfiri,E., Munemitsu,S., and Polakis,P. (1997a). Loss of beta-catenin regulation by the APC tumor suppressor protein correlates with loss of structure due to common somatic mutations of the gene. Cancer Res *57*, 4624-4630.

Rubinfeld,B., Robbins,P., El Gamil,M., Albert,I., Porfiri,E., and Polakis,P. (1997b). Stabilization of beta-catenin by genetic defects in melanoma cell lines. Science *275*, 1790-1792.

Rubinfeld,B., Souza,B., Albert,I., Muller,O., Chamberlain,S.H., Masiarz,F.R., Munemitsu,S., and Polakis,P. (1993). Association of the APC gene product with beta-catenin. Science *262*, 1731-1734.

Ruff,V.A., and Leach,K.L. (1995). Direct demonstration of NFATp dephosphorylation and nuclear localization in activated HT-2 cells using a specific NFATp polyclonal antibody. J. Biol. Chem. *270*, 22602-22607.

Rulifson,E.J., Wu,C.H., and Nusse,R. (2000). Pathway specificity by the bifunctional receptor frizzled is determined by affinity for wingless. Mol. Cell *6*, 117-126.

Russo,A.A., Jeffrey,P.D., and Pavletich,N.P. (1996). Structural basis of cyclin-dependent kinase activation by phosphorylation. Nat. Struct. Biol. *3*, 696-700.

Rutkowski,S., Bode,U., Deinlein,F., Ottensmeier,H., Warmuth-Metz,M., Soerensen,N., Graf,N., Emser,A., Pietsch,T., Wolff,J.E., Kortmann,R.D., and Kuehl,J. (2005). Treatment of early childhood medulloblastoma by postoperative chemotherapy alone. N. Engl. J. Med. *352*, 978-986.

Saito,T., Oda,Y., Kawaguchi,K., Tanaka,K., Matsuda,S., Tamiya,S., Iwamoto,Y., and Tsuneyoshi,M. (2002). Possible association between higher beta-catenin mRNA expression and mutated beta-catenin in sporadic desmoid tumors: real-time semiquantitative assay by TaqMan polymerase chain reaction. Lab Invest *82*, 97-103.

Saito,T., Oda,Y., Tanaka,K., Matsuda,S., Tamiya,S., Iwamoto,Y., and Tsuneyoshi,M. (2001). beta-catenin nuclear expression correlates with cyclin D1 overexpression in sporadic desmoid tumours. J Pathol *195*, 222-228.

Saitoh,T., Mine,T., and Katoh,M. (2002). Frequent up-regulation of WNT5A mRNA in primary gastric cancer. Int J Mol Med *9*, 515-519.

Sakai,K., Mitani,K., and Miyazaki,J. (1995). Efficient regulation of gene expression by Adenovirus vector-mediated delivery of the Cre-recombinase. Biochem. Biophys. Res. Commun. *217*, 393-401.

Sakai,N., Sasaki,K., Nakashita,M., Honda,S., Ikegaki,N., and Saito,N. (1997). Modulation of serotonin transporter activity by a protein kinase C activator and an inhibitor of type 1 and 2A serine/threonine phosphatases. J. Neurochem. *68*, 2618-2624.

Sakanaka,C., and Williams,L.T. (1999). Functional domains of axin. Importance of the C terminus as an oligomerization domain. J Biol Chem *274*, 14090-14093.

Samowitz,W.S., Slattery,M.L., Sweeney,C., Herrick,J., Wolff,R.K., and Albertsen,H. (2007). APC mutations and other genetic and epigenetic changes in colon cancer. Mol Cancer Res *5*, 165-170.

Sanai,N., Alvarez-Buylla,A., and Berger,M.S. (2005). Neural stem cells and the origin of gliomas. N. Engl. J. Med. *353*, 811-822.

Saneyoshi,T., Kume,S., Amasaki,Y., and Mikoshiba,K. (2002). The Wnt/calcium pathway activates NF-AT and promotes ventral cell fate in *Xenopus* embryos. Nature *417*, 295-299.

Sansom,O.J., Reed,K.R., Hayes,A.J., Ireland,H., Brinkmann,H., Newton,I.P., Batlle,E., Simon-Assmann,P., Clevers,H., Nathke,I.S., Clarke,A.R., and Winton,D.J. (2004). Loss of Apc *in vivo* immediately perturbs Wnt signaling, differentiation, and migration. Genes Dev. *18*, 1385-1390.

Sareddy,G.R., Challa,S., Panigrahi,M., and Babu,P.P. (2009a). Wnt/beta-catenin/Tcf signaling pathway activation in malignant progression of rat gliomas induced by transplacental N-ethyl-N-nitrosourea exposure. Neurochem. Res. *34*, 1278-1288.

Sareddy,G.R., Panigrahi,M., Challa,S., Mahadevan,A., and Babu,P.P. (2009b). Activation of Wnt/beta-catenin/Tcf signaling pathway in human astrocytomas. Neurochem. Int. *55*, 307-317.

Satoh,S., Daigo,Y., Furukawa,Y., Kato,T., Miwa,N., Nishiwaki,T., Kawasoe,T., Ishiguro,H., Fujita,M., Tokino,T., Sasaki,Y., Imaoka,S., Murata,M., Shimano,T., Yamaoka,Y., and Nakamura,Y. (2000). AXIN1 mutations in hepatocellular carcinomas, and growth suppression in cancer cells by virus-mediated transfer of AXIN1. Nat Genet *24*, 245-250.

Saydam,O., Shen,Y., Wurdinger,T., Senol,O., Boke,E., James,M.F., Tannous,B.A., Stemmer-Rachamimov,A.O., Yi,M., Stephens,R.M., Fraefel,C., Gusella,J.F., Krichevsky,A.M., and Breakefield,X.O. (2009). Downregulated microRNA-200a in meningiomas promotes tumor growth by reducing E-cadherin and activating the Wnt/beta-catenin signaling pathway. Mol. Cell Biol. *29*, 5923-5940.

Schilham,M.W., and Clevers,H. (1998). HMG box containing transcription factors in lymphocyte differentiation. Semin. Immunol. *10*, 127-132.

Schmidt,E.E., Ichimura,K., Reifenberger,G., and Collins,V.P. (1994). CDKN2 (p16/MTS1) gene deletion or CDK4 amplification occurs in the majority of glioblastomas. Cancer Res. *54*, 6321-6324.

Schofield,D., West,D.C., Anthony,D.C., Marshal,R., and Sklar,J. (1995). Correlation of loss of heterozygosity at chromosome 9q with histological subtype in medulloblastomas. Am. J. Pathol. *146*, 472-480.

Scholten,A.N., Creutzberg,C.L., van den Broek,L.J., Noordijk,E.M., and Smit,V.T. (2003). Nuclear beta-catenin is a molecular feature of type I endometrial carcinoma. J Pathol *201*, 460-465.

Schweizer,L., and Varmus,H. (2003). Wnt/Wingless signaling through beta-catenin requires the function of both LRP/Arrow and frizzled classes of receptors. BMC Cell Biol *4*, 4.

Seaman,M.N. (2004). Cargo-selective endosomal sorting for retrieval to the Golgi requires retromer. J Cell Biol *165*, 111-122.

Seaman,M.N. (2005). Recycle your receptors with retromer. Trends Cell Biol. *15*, 68-75.

Sears,R., Nuckolls,F., Haura,E., Taya,Y., Tamai,K., and Nevins,J.R. (2000). Multiple Ras-dependent phosphorylation pathways regulate Myc protein stability. Genes Dev. *14*, 2501-2514.

Seeling,J.M., Miller,J.R., Gil,R., Moon,R.T., White,R., and Virshup,D.M. (1999). Regulation of beta-catenin signaling by the B56 subunit of protein phosphatase 2A. Science *283*, 2089-2091.

Sejersen,T., and Lendahl,U. (1993). Transient expression of the intermediate filament nestin during skeletal muscle development. J. Cell Sci. *106 (Pt 4)*, 1291-1300.

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. Curr Biol *11*, 951-961.

Seri,B., Garcia-Verdugo,J.M., McEwen,B.S., and Alvarez-Buylla,A. (2001). Astrocytes give rise to new neurons in the adult mammalian hippocampus. J Neurosci *21*, 7153-7160.

Seri,B., Herrera,D.G., Gritti,A., Ferron,S., Collado,L., Vescovi,A., Garcia-Verdugo,J.M., and Alvarez-Buylla,A. (2006). Composition and organization of the SCZ: a large germinal layer containing neural stem cells in the adult mammalian brain. Cereb. Cortex *16 Suppl 1*, i103-i111.

Sgaier,S.K., Millet,S., Villanueva,M.P., Berenshteyn,F., Song,C., and Joyner,A.L. (2005). Morphogenetic and cellular movements that shape the mouse cerebellum; insights from genetic fate mapping. Neuron *45*, 27-40.

Shakoori,A., Mai,W., Miyashita,K., Yasumoto,K., Takahashi,Y., Ooi,A., Kawakami,K., and Minamoto,T. (2007). Inhibition of GSK-3 beta activity attenuates proliferation of human colon cancer cells in rodents. Cancer Sci *98*, 1388-1393.

Shakoori,A., Ougolkov,A., Yu,Z.W., Zhang,B., Modarressi,M.H., Billadeau,D.D., Mai,M., Takahashi,Y., and Minamoto,T. (2005). Deregulated GSK3beta activity in colorectal cancer: its association with tumor cell survival and proliferation. Biochem Biophys Res Commun *334*, 1365-1373.

Sheldahl,L.C., Park,M., Malbon,C.C., and Moon,R.T. (1999). Protein kinase C is differentially stimulated by Wnt and Frizzled homologs in a G-protein-dependent manner. Curr. Biol. *9*, 695-698.

Shi,Y., Sawada,J., Sui,G., Affar,e.B., Whetstine,J.R., Lan,F., Ogawa,H., Luke,M.P., Nakatani,Y., and Shi,Y. (2003). Coordinated histone modifications mediated by a CtBP co-repressor complex. Nature *422*, 735-738.

Shigemitsu,K., Sekido,Y., Usami,N., Mori,S., Sato,M., Horio,Y., Hasegawa,Y., Bader,S.A., Gazdar,A.F., Minna,J.D., Hida,T., Yoshioka,H., Imaizumi,M., Ueda,Y., Takahashi,M., and Shimokata,K. (2001). Genetic alteration of the beta-catenin gene (CTNNB1) in human lung cancer and malignant mesothelioma and identification of a new 3p21.3 homozygous deletion. Oncogene *20*, 4249-4257.

Shimogori,T., VanSant,J., Paik,E., and Grove,E.A. (2004). Members of the Wnt, Fz, and Frp gene families expressed in postnatal mouse cerebral cortex. J. Comp Neurol. *473*, 496-510.

Shlomit,R., Ayala,A.G., Michal,D., Ninett,A., Frida,S., Boleslaw,G., Gad,B., Gideon,R., and Shlomi,C. (2000). Gains and losses of DNA sequences in childhood brain tumors analyzed by comparative genomic hybridization. Cancer Genet. Cytogenet. *121*, 67-72.

Shtutman,M., Zhurinsky,J., Simcha,I., Albanese,C., D'Amico,M., Pestell,R., and Ben Ze'ev,A. (1999). The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway. Proc Natl Acad Sci U S A *96*, 5522-5527.

Shu,W., Jiang,Y.Q., Lu,M.M., and Morrisey,E.E. (2002). Wnt7b regulates mesenchymal proliferation and vascular development in the lung. Development *129*, 4831-4842.

Sidransky,D., Mikkelsen,T., Schwechheimer,K., Rosenblum,M.L., Cavanee,W., and Vogelstein,B. (1992). Clonal expansion of p53 mutant cells is associated with brain tumour progression. Nature *355*, 846-847.

Siegfried,E., Wilder,E.L., and Perrimon,N. (1994). Components of wingless signalling in *Drosophila*. Nature *367*, 76-80.

Sierra,J., Yoshida,T., Joazeiro,C.A., and Jones,K.A. (2006). The APC tumor suppressor counteracts beta-catenin activation and H3K4 methylation at Wnt target genes. Genes Dev *20*, 586-600.

Simons,K., and Fuller,S.D. (1985). Cell surface polarity in epithelia. Annu. Rev. Cell Biol. *1*, 243-288.

Singec,I., Knoth,R., Meyer,R.P., Maciaczyk,J., Volk,B., Nikkhah,G., Frotscher,M., and Snyder,E.Y. (2006). Defining the actual sensitivity and specificity of the neurosphere assay in stem cell biology. Nat. Methods *3*, 801-806.

Singh,S.K., Hawkins,C., Clarke,I.D., Squire,J.A., Bayani,J., Hide,T., Henkelman,R.M., Cusimano,M.D., and Dirks,P.B. (2004). Identification of human brain tumour initiating cells. Nature *432*, 396-401.

Slusarski,D.C., Corces,V.G., and Moon,R.T. (1997a). Interaction of Wnt and a Frizzled homologue triggers G-protein-linked phosphatidylinositol signalling. Nature *390*, 410-413.

Slusarski,D.C., Yang-Snyder,J., Busa,W.B., and Moon,R.T. (1997b). Modulation of embryonic intracellular Ca2+ signaling by Wnt-5A. Dev. Biol. *182*, 114-120.

Smeal,T., Binetruy,B., Mercola,D.A., Birrer,M., and Karin,M. (1991). Oncogenic and transcriptional cooperation with Ha-Ras requires phosphorylation of c-Jun on serines 63 and 73. Nature *354*, 494-496.

Smith,R.J., Arnaud,P., Konfortova,G., Dean,W.L., Beechey,C.V., and Kelsey,G. (2002). The mouse Zac1 locus: basis for imprinting and comparison with human ZAC. Gene *292*, 101-112.

Smits,R., Ruiz,P., Diaz-Cano,S., Luz,A., Jagmohan-Changur,S., Breukel,C., Birchmeier,C., Birchmeier,W., and Fodde,R. (2000). E-cadherin and Adenomatous polyposis coli mutations are synergistic in intestinal tumor initiation in mice. Gastroenterology *119*, 1045-1053.

Sogabe,Y., Suzuki,H., Toyota,M., Ogi,K., Imai,T., Nojima,M., Sasaki,Y., Hiratsuka,H., and Tokino,T. (2008). Epigenetic inactivation of SFRP genes in oral squamous cell carcinoma. Int J Oncol *32*, 1253-1261.

Song,B.J., Park,Y.J., Kim,H.S., Kim,C.N., and Chang,S.H. (2004). [Expression of beta-catenin and E-cadherin in early gastric cancer: correlation with clinicopathologic parameters]. Korean J Gastroenterol. *43*, 82-89.

Sorlie,T., Bukholm,I., and Borresen-Dale,A.L. (1998). Truncating somatic mutation in exon 15 of the APC gene is a rare event in human breast carcinomas. Mutations in brief no. 179. Online. Hum Mutat *12*, 215.

Sparks,A.B., Morin,P.J., Vogelstein,B., and Kinzler,K.W. (1998). Mutational analysis of the APC/beta-catenin/Tcf pathway in colorectal cancer. Cancer Res *58*, 1130-1134.

Spater,D., Hill,T.P., O'sullivan,R.J., Gruber,M., Conner,D.A., and Hartmann,C. (2006). Wnt9a signaling is required for joint integrity and regulation of Ihh during chondrogenesis. Development *133*, 3039-3049.

Spengler,D., Villalba,M., Hoffmann,A., Pantaloni,C., Houssami,S., Bockaert,J., and Journot,L. (1997). Regulation of apoptosis and cell cycle arrest by Zac1, a novel zinc finger protein expressed in the pituitary gland and the brain. EMBO J. *16*, 2814-2825.

Stark,K., Vainio,S., Vassileva,G., and Mcmahon,A.P. (1994). Epithelial Transformation of Metanephric Mesenchyme in the Developing Kidney Regulated by Wnt-4. Nature *372*, 679-683.

Steiner,P., Philipp,A., Lukas,J., Godden-Kent,D., Pagano,M., Mittnacht,S., Bartek,J., and Eilers,M. (1995). Identification of a Myc-dependent step during the formation of active G1 cyclin-cdk complexes. EMBO J *14*, 4814-4826.

Sternberg,N., and Hamilton,D. (1981). Bacteriophage P1 site-specific recombination. I. Recombination between loxP sites. J. Mol. Biol. *150*, 467-486.

Stevens,M.C., Cameron,A.H., Muir,K.R., Parkes,S.E., Reid,H., and Whitwell,H. (1991). Descriptive epidemiology of primary central nervous system tumours in children: a population-based study. Clin Oncol (R Coll. Radiol.) *3*, 323-329.

Strigini,M., and Cohen,S.M. (2000). Wingless gradient formation in the *Drosophila* wing. Curr. Biol. *10*, 293-300.

Stump,R.J., Ang,S., Chen,Y., von,B.T., Lovicu,F.J., Pinson,K., de Iongh,R.U., Yamaguchi,T.P., Sassoon,D.A., and McAvoy,J.W. (2003). A role for Wnt/beta-catenin signaling in lens epithelial differentiation. Dev. Biol. *259*, 48-61.

Su,L.K., Burrell,M., Hill,D.E., Gyuris,J., Brent,R., Wiltshire,R., Trent,J., Vogelstein,B., and Kinzler,K.W. (1995). APC binds to the novel protein EB1. Cancer Res. *55*, 2972-2977.

Su,L.K., Johnson,K.A., Smith,K.J., Hill,D.E., Vogelstein,B., and Kinzler,K.W. (1993a). Association between wild type and mutant APC gene products. Cancer Res *53*, 2728-2731.

Su,L.K., Vogelstein,B., and Kinzler,K.W. (1993b). Association of the APC tumor suppressor protein with catenins. Science *262*, 1734-1737.

Sunaga,N., Kohno,T., Kolligs,F.T., Fearon,E.R., Saito,R., and Yokota,J. (2001). Constitutive activation of the Wnt signaling pathway by CTNNB1 (beta-catenin) mutations in a subset of human lung Adenocarcinoma. Genes Chromosomes Cancer *30*, 316-321.

Sure,U., Berghorn,W.J., Bertalanffy,H., Wakabayashi,T., Yoshida,J., Sugita,K., and Seeger,W. (1995). Staging, scoring and grading of medulloblastoma. A postoperative prognosis predicting system based on the cases of a single institute. Acta Neurochir. (Wien.) *132*, 59-65.

Sutherland,C., Leighton,I.A., and Cohen,P. (1993). Inactivation of glycogen synthase kinase-3 beta by phosphorylation: new kinase connections in insulin and growth-factor signalling. Biochem. J. *296 (Pt 1)*, 15-19.

Suzuki,H., Toyota,M., Carraway,H., Gabrielson,E., Ohmura,T., Fujikane,T., Nishikawa,N., Sogabe,Y., Nojima,M., Sonoda,T., Mori,M., Hirata,K., Imai,K., Shinomura,Y., Baylin,S.B., and Tokino,T. (2008). Frequent epigenetic inactivation of Wnt antagonist genes in breast cancer. Br J Cancer *98*, 1147-1156.

Tabori,U., Baskin,B., Shago,M., Alon,N., Taylor,M.D., Ray,P.N., Bouffet,E., Malkin,D., and Hawkins,C. (2010). Universal poor survival in children with medulloblastoma harboring somatic TP53 mutations. J. Clin. Oncol. *28*, 1345-1350.

Takada,R., Satomi,Y., Kurata,T., Ueno,N., Norioka,S., Kondoh,H., Takao,T., and Takada,S. (2006). Monounsaturated fatty acid modification of Wnt protein: its role in Wnt secretion. Dev Cell *11*, 791-801.

Takada,S., Stark,K.L., Shea,M.J., Vassileva,G., McMahon,J.A., and Mcmahon,A.P. (1994). Wnt-3a regulates somite and tailbud formation in the mouse embryo. Genes Dev *8*, 174-189.

Takagi,H., Sasaki,S., Suzuki,H., Toyota,M., Maruyama,R., Nojima,M., Yamamoto,H., Omata,M., Tokino,T., Imai,K., and Shinomura,Y. (2008). Frequent epigenetic inactivation of SFRP genes in hepatocellular carcinoma. J Gastroenterol. *43*, 378-389.

Takayasu,H., Horie,H., Hiyama,E., Matsunaga,T., Hayashi,Y., Watanabe,Y., Suita,S., Kaneko,M., Sasaki,F., Hashizume,K., Ozaki,T., Furuuchi,K., Tada,M., Ohnuma,N., and Nakagawara,A. (2001). Frequent deletions and mutations of the beta-catenin gene are associated with overexpression of cyclin D1 and fibronectin and poorly differentiated histology in childhood hepatoblastoma. Clin Cancer Res *7*, 901-908.

Takeichi,M. (1991). Cadherin Cell-Adhesion Receptors As A Morphogenetic Regulator. Science *251*, 1451-1455.

Takemaru,K.I., and Moon,R.T. (2000). The transcriptional coactivator CBP interacts with beta-catenin to activate gene expression. J Cell Biol *149*, 249-254.

Taki,M., Kamata,N., Yokoyama,K., Fujimoto,R., Tsutsumi,S., and Nagayama,M. (2003). Down-regulation of Wnt-4 and up-regulation of Wnt-5a expression by epithelial-mesenchymal transition in human squamous carcinoma cells. Cancer Sci *94*, 593-597.

Tamai,K., Semenov,M., Kato,Y., Spokony,R., Liu,C., Katsuyama,Y., Hess,F., Saint-Jeannet,J.P., and He,X. (2000). LDL-receptor-related proteins in Wnt signal transduction. Nature *407*, 530-535.

Tanegashima,K., Zhao,H., and Dawid,I.B. (2008). WGEF activates Rho in the Wnt-PCP pathway and controls convergent extension in *Xenopus* gastrulation. EMBO J. *27*, 606-617.

Taniguchi,K., Roberts,L.R., Aderca,I.N., Dong,X., Qian,C., Murphy,L.M., Nagorney,D.M., Burgart,L.J., Roche,P.C., Smith,D.I., Ross,J.A., and Liu,W. (2002). Mutational spectrum of beta-catenin, AXIN1, and AXIN2 in hepatocellular carcinomas and hepatoblastomas. Oncogene *21*, 4863-4871.

Tapias,A., Ciudad,C.J., Roninson,I.B., and Noe,V. (2008). Regulation of Sp1 by cell cycle related proteins. Cell Cycle *7*, 2856-2867.

Taylor,J., Abramova,N., Charlton,J., and Adler,P.N. (1998). Van Gogh: a new *Drosophila* tissue polarity gene. Genetics *150*, 199-210.

Tejpar,S., Nollet,F., Li,C., Wunder,J.S., Michils,G., dal Cin,P., Van Cutsem,E., Bapat,B., van Roy,F., Cassiman,J.J., and Alman,B.A. (1999). Predominance of beta-catenin mutations and beta-catenin dysregulation in sporadic aggressive fibromatosis (desmoid tumor). Oncogene *18*, 6615-6620.

Terris,B., Pineau,P., Bregeaud,L., Valla,D., Belghiti,J., Tiollais,P., Degott,C., and Dejean,A. (1999). Close correlation between beta-catenin gene alterations and nuclear accumulation of the protein in human hepatocellular carcinomas. Oncogene *18*, 6583-6588.

Tetsu,O., and McCormick,F. (1999). Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. Nature *398*, 422-426.

Theile,M., Seitz,S., Arnold,W., Jandrig,B., Frege,R., Schlag,P.M., Haensch,W., Guski,H., Winzer,K.J., Barrett,J.C., and Scherneck,S. (1996). A defined chromosome 6q fragment (at D6S310) harbors a putative tumor suppressor gene for breast cancer. Oncogene *13*, 677-685.

Thomas,K.R., and Capecchi,M.R. (1987). Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. Cell *51*, 503-512.

Thomas,K.R., and Capecchi,M.R. (1990). Targeted disruption of the murine int-1 proto-oncogene resulting in severe abnormalities in midbrain and cerebellar development. Nature *346*, 847-850.

Thompson,M.C., Fuller,C., Hogg,T.L., Dalton,J., Finkelstein,D., Lau,C.C., Chintagumpala,M., Adesina,A., Ashley,D.M., Kellie,S.J., Taylor,M.D., Curran,T., Gajjar,A., and Gilbertson,R.J. (2006). Genomics identifies medulloblastoma subgroups that are enriched for specific genetic alterations. J. Clin. Oncol. *24*, 1924-1931.

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/Gsk3beta activity. Dev Cell *4*, 407-418.

Travis,A., Amsterdam,A., Belanger,C., and Grosschedl,R. (1991). LEF-1, a gene encoding a lymphoid-specific protein with an HMG domain, regulates T-cell receptor alpha enhancer function [corrected]. Genes Dev *5*, 880-894.

Trent,J.M., Rosenfeld,S.B., and Meyskens,F.L. (1983). Chromosome 6q involvement in human malignant melanoma. Cancer Genet. Cytogenet. *9*, 177-180.

Tsuda,M., Kamimura,K., Nakato,H., Archer,M., Staatz,W., Fox,B., Humphrey,M., Olson,S., Futch,T., Kaluza,V., Siegfried,E., Stam,L., and Selleck,S.B. (1999). The cell-surface proteoglycan Dally regulates Wingless signalling in *Drosophila*. Nature *400*, 276-280.

TURCOT,J., DESPRES,J.P., and ST PIERRE,F. (1959). Malignant tumors of the central nervous system associated with familial polyposis of the colon: report of two cases. Dis Colon Rectum *2*, 465-468.

Udatsu,Y., Kusafuka,T., Kuroda,S., Miao,J., and Okada,A. (2001). High frequency of beta-catenin mutations in hepatoblastoma. Pediatr Surg Int *17*, 508-512.

Ugolini,F., Charafe-Jauffret,E., Bardou,V.J., Geneix,J., Adelaide,J., Labat-Moleur,F., Penault-Llorca,F., Longy,M., Jacquemier,J., Birnbaum,D., and Pebusque,M.J. (2001). WNT pathway and mammary carcinogenesis: loss of expression of candidate tumor suppressor gene SFRP1 in most invasive carcinomas except of the medullary type. Oncogene *20*, 5810-5817.

Uren,A., Reichsman,F., Anest,V., Taylor,W.G., Muraiso,K., Bottaro,D.P., Cumberledge,S., and Rubin,J.S. (2000). Secreted frizzled-related protein-1 binds directly to Wingless and is a biphasic modulator of Wnt signaling. J. Biol. Chem. *275*, 4374-4382.

Vainio,S., Heikkila,M., Kispert,A., Chin,N., and Mcmahon,A.P. (1999). Female development in mammals is regulated by Wnt-4 signalling. Nature *397*, 405-409.

Valente,T., Junyent,F., and Auladell,C. (2005). Zac1 is expressed in progenitor/stem cells of the neuroectoderm and mesoderm during embryogenesis: differential phenotype of the Zac1-expressing cells during development. Dev. Dyn. *233*, 667-679.

van Amerongen,R., Nawijn,M., Franca-Koh,J., Zevenhoven,J., van der Gulden,H., Jonkers,J., and Berns,A. (2005). Frat is dispensable for canonical Wnt signaling in mammals. Genes Dev. *19*, 425-430.

van de Wetering,M., Barker,N., Harkes,I.C., van der,H.M., Dijk,N.J., Hollestelle,A., Klijn,J.G., Clevers,H., and Schutte,M. (2001). Mutant E-cadherin breast cancer cells do not display constitutive Wnt signaling. Cancer Res *61*, 278-284.

van de Wetering,M., Cavallo,R., Dooijes,D., van Beest,M., van Es,J., Loureiro,J., Ypma,A., Hursh,D., Jones,T., Bejsovec,A., Peifer,M., Mortin,M., and Clevers,H. (1997). Armadillo coactivates transcription driven by the product of the *Drosophila* segment polarity gene dTCF. Cell *88*, 789-799.

van de Wetering,M., Oosterwegel,M., Dooijes,D., and Clevers,H. (1991). Identification and cloning of TCF-1, a T lymphocyte-specific transcription factor containing a sequence-specific HMG box. EMBO J *10*, 123-132.

van Es,J.H., Kirkpatrick,C., van de,W.M., Molenaar,M., Miles,A., Kuipers,J., Destree,O., Peifer,M., and Clevers,H. (1999). Identification of APC2, a homologue of the Adenomatous polyposis coli tumour suppressor. Curr. Biol. *9*, 105-108.

van Genderen,C., Okamura,R.M., Farinas,I., Quo,R.G., Parslow,T.G., Bruhn,L., and Grosschedl,R. (1994). Development of several organs that require inductive epithelial-mesenchymal interactions is impaired in LEF-1-deficient mice. Genes Dev. *8*, 2691-2703.

van Ooyen,A., Kwee,V., and Nusse,R. (1985). The nucleotide sequence of the human int-1 mammary oncogene; evolutionary conservation of coding and non-coding sequences. EMBO J. *4*, 2905-2909.

Varelas,X., Miller,B.W., Sopko,R., Song,S., Gregorieff,A., Fellouse,F.A., Sakuma,R., Pawson,T., Hunziker,W., McNeill,H., Wrana,J.L., and Attisano,L. (2010). The Hippo pathway regulates Wnt/beta-catenin signaling. Dev. Cell *18*, 579-591.

Veeck,J., Geisler,C., Noetzel,E., Alkaya,S., Hartmann,A., Knuchel,R., and Dahl,E. (2008). Epigenetic inactivation of the secreted frizzled-related protein-5 (SFRP5) gene in human breast cancer is associated with unfavorable prognosis. Carcinogenesis *29*, 991-998.

Veeman,M.T., Slusarski,D.C., Kaykas,A., Louie,S.H., and Moon,R.T. (2003). Zebrafish prickle, a modulator of noncanonical Wnt/Fz signaling, regulates gastrulation movements. Curr. Biol. *13*, 680-685.

Verbeek,S., Izon,D., Hofhuis,F., Robanus-Maandag,E., te,R.H., van de Wetering,M., Oosterwegel,M., Wilson,A., MacDonald,H.R., and Clevers,H. (1995). An HMG-box-containing T-cell factor required for thymocyte differentiation. Nature *374*, 70-74.

Vertino,A.M., Taylor-Jones,J.M., Longo,K.A., Bearden,E.D., Lane,T.F., McGehee,R.E., Jr., MacDougald,O.A., and Peterson,C.A. (2005). Wnt10b deficiency promotes coexpression of myogenic and adipogenic programs in myoblasts. Mol Biol Cell *16*, 2039-2048.

Vielhaber,E., Eide,E., Rivers,A., Gao,Z.H., and Virshup,D.M. (2000). Nuclear entry of the circadian regulator mPER1 is controlled by mammalian casein kinase I epsilon. Mol Cell Biol *20*, 4888-4899.

Vinson,C.R., Conover,S., and Adler,P.N. (1989). A *Drosophila* tissue polarity locus encodes a protein containing seven potential transmembrane domains. Nature *338*, 263-264.

Voeller,H.J., Truica,C.I., and Gelmann,E.P. (1998). Beta-catenin mutations in human prostate cancer. Cancer Res *58*, 2520-2523.

von,D.A., Eibl,R.H., Ohgaki,H., Louis,D.N., von,A.K., Petersen,I., Kleihues,P., Chung,R.Y., Wiestler,O.D., and Seizinger,B.R. (1992). p53 mutations are associated with 17p allelic loss in grade II and grade III astrocytoma. Cancer Res. *52*, 2987-2990.

Vorechovsky,I., Tingby,O., Hartman,M., Stromberg,B., Nister,M., Collins,V.P., and Toftgard,R. (1997). Somatic mutations in the human homologue of *Drosophila* patched in primitive neuroectodermal tumours. Oncogene *15*, 361-366.

Wakeling,A.E., Valcaccia,B., Newboult,E., and Green,L.R. (1984). Non-steroidal antioestrogens--receptor binding and biological response in rat uterus, rat mammary carcinoma and human breast cancer cells. J. Steroid Biochem. *20*, 111-120.

Waltzer,L., and Bienz,M. (1998). *Drosophila* CBP represses the transcription factor TCF to antagonize Wingless signalling. Nature *395*, 521-525.

Wang,V.Y., Rose,M.F., and Zoghbi,H.Y. (2005). Math1 expression redefines the rhombic lip derivatives and reveals novel lineages within the brainstem and cerebellum. Neuron *48*, 31-43.

Wang,Y., Huso,D., Cahill,H., Ryugo,D., and Nathans,J. (2001). Progressive cerebellar, auditory, and esophageal dysfunction caused by targeted disruption of the frizzled-4 gene. J. Neurosci. *21*, 4761-4771.

Wang,Y., Lam,J.B., Lam,K.S., Liu,J., Lam,M.C., Hoo,R.L., Wu,D., Cooper,G.J., and Xu,A. (2006). Adiponectin modulates the glycogen synthase kinase-3beta/beta-catenin signaling pathway and attenuates mammary tumorigenesis of MDA-MB-231 cells in nude mice. Cancer Res *66*, 11462-11470.

Wang,Y., Macke,J.P., Abella,B.S., Andreasson,K., Worley,P., Gilbert,D.J., Copeland,N.G., Jenkins,N.A., and Nathans,J. (1996). A large family of putative transmembrane receptors homologous to the product of the *Drosophila* tissue polarity gene frizzled. J. Biol. Chem. *271*, 4468-4476.

Wang,Y., Thekdi,N., Smallwood,P.M., Macke,J.P., and Nathans,J. (2002). Frizzled-3 is required for the development of major fiber tracts in the rostral CNS. J. Neurosci. *22*, 8563-8573.

Watanabe,K., Sato,K., Biernat,W., Tachibana,O., von Ammon,K., Ogata,N., Yonekawa,Y., Kleihues,P., and Ohgaki,H. (1997). Incidence and timing of p53 mutations during astrocytoma progression in patients with multiple biopsies. Clin. Cancer Res. *3*, 523-530.

Watanabe,K., Tachibana,O., Sata,K., Yonekawa,Y., Kleihues,P., and Ohgaki,H. (1996). Overexpression of the EGF receptor and p53 mutations are mutually exclusive in the evolution of primary and secondary glioblastomas. Brain Pathol. *6*, 217-223.

Waterman,M.L., Fischer,W.H., and Jones,K.A. (1991). A thymus-specific member of the HMG protein family regulates the human T cell receptor C alpha enhancer. Genes Dev *5*, 656-669.

Webster,M.T., Rozycka,M., Sara,E., Davis,E., Smalley,M., Young,N., Dale,T.C., and Wooster,R. (2000). Sequence variants of the axin gene in breast, colon, and other cancers: an analysis of mutations that interfere with GSK3 binding. Genes Chromosomes Cancer *28*, 443-453.

Wechsler-Reya,R.J., and Scott,M.P. (1999). Control of neuronal precursor proliferation in the cerebellum by Sonic Hedgehog. Neuron *22*, 103-114.

Weeraratna,A.T., Jiang,Y., Hostetter,G., Rosenblatt,K., Duray,P., Bittner,M., and Trent,J.M. (2002). Wnt5a signaling directly affects cell motility and invasion of metastatic melanoma. Cancer Cell *1*, 279-288.

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*, 527-530.

Wei,Y., Fabre,M., Branchereau,S., Gauthier,F., Perilongo,G., and Buendia,M.A. (2000). Activation of beta-catenin in epithelial and mesenchymal hepatoblastomas. Oncogene *19*, 498-504.

Weinberg,R.A. (1995). The retinoblastoma protein and cell cycle control. Cell *81*, 323-330.

Westfall,T.A., Brimeyer,R., Twedt,J., Gladon,J., Olberding,A., Furutani-Seiki,M., and Slusarski,D.C. (2003). Wnt-5/pipetail functions in vertebrate axis formation as a negative regulator of Wnt/beta-catenin activity. J. Cell Biol. *162*, 889-898.

Wexler,E.M., Geschwind,D.H., and Palmer,T.D. (2008). Lithium regulates adult hippocampal progenitor development through canonical Wnt pathway activation. Mol. Psychiatry *13*, 285-292.

Wexler,E.M., Paucer,A., Kornblum,H.I., Palmer,T.D., and Geschwind,D.H. (2009). Endogenous Wnt signaling maintains neural progenitor cell potency. Stem Cells *27*, 1130-1141.

Willert,K., Brown,J.D., Danenberg,E., Duncan,A.W., Weissman,I.L., Reya,T., Yates,J.R., III, and Nusse,R. (2003). Wnt proteins are lipid-modified and can act as stem cell growth factors. Nature *423*, 448-452.

Willert,K., Shibamoto,S., and Nusse,R. (1999). Wnt-induced dephosphorylation of axin releases beta-catenin from the axin complex. Genes Dev *13*, 1768-1773.

Wilson,C.L., and Matrisian,L.M. (1996). Matrilysin: an epithelial matrix metalloproteinase with potentially novel functions. Int. J. Biochem. Cell Biol. *28*, 123-136.

Winston,J.T., Strack,P., Beer-Romero,P., Chu,C.Y., Elledge,S.J., and Harper,J.W. (1999). The SCFbeta-TRCP-ubiquitin ligase complex associates specifically with phosphorylated destruction motifs in IkappaBalpha and beta-catenin and stimulates IkappaBalpha ubiquitination *in vitro*. Genes Dev *13*, 270-283.

Winter,C.G., Wang,B., Ballew,A., Royou,A., Karess,R., Axelrod,J.D., and Luo,L. (2001). *Drosophila* Rho-associated kinase (Drok) links Frizzled-mediated planar cell polarity signaling to the actin cytoskeleton. Cell *105*, 81-91.

Woessner,J.F., Jr., and Taplin,C.J. (1988). Purification and properties of a small latent matrix metalloproteinase of the rat uterus. J. Biol. Chem. *263*, 16918-16925.

Wolter,M., Reifenberger,J., Sommer,C., Ruzicka,T., and Reifenberger,G. (1997). Mutations in the human homologue of the *Drosophila* segment polarity gene patched (PTCH) in sporadic basal cell carcinomas of the skin and primitive neuroectodermal tumors of the central nervous system. Cancer Res. *57*, 2581-2585.

Wong,C.M., Fan,S.T., and Ng,I.O. (2001). beta-Catenin mutation and overexpression in hepatocellular carcinoma: clinicopathologic and prognostic significance. Cancer *92*, 136-145.

Woo,D.K., Kim,H.S., Lee,H.S., Kang,Y.H., Yang,H.K., and Kim,W.H. (2001). Altered expression and mutation of beta-catenin gene in gastric carcinomas and cell lines. Int J Cancer *95*, 108-113.

Woodward,W.A., Chen,M.S., Behbod,F., Alfaro,M.P., Buchholz,T.A., and Rosen,J.M. (2007). WNT/beta-catenin mediates radiation resistance of mouse mammary progenitor cells. Proc Natl Acad Sci U S A *104*, 618-623.

Wright,K., Wilson,P., Morland,S., Campbell,I., Walsh,M., Hurst,T., Ward,B., Cummings,M., and Chenevix-Trench,G. (1999). beta-catenin mutation and expression analysis in ovarian cancer: exon 3 mutations and nuclear translocation in 16% of endometrioid tumours. Int J Cancer *82*, 625-629.

Wright,K., Wilson,P.J., Kerr,J., Do,K., Hurst,T., Khoo,S.K., Ward,B., and Chenevix-Trench,G. (1998). Frequent loss of heterozygosity and three critical regions on the short arm of chromosome 8 in ovarian Adenocarcinomas. Oncogene *17*, 1185-1188.

Wrobel,C.N., Mutch,C.A., Swaminathan,S., Taketo,M.M., and Chenn,A. (2007). Persistent expression of stabilized beta-catenin delays maturation of radial glial cells into intermediate progenitors. Dev Biol *309*, 285-297.

Wu,L., Timmers,C., Maiti,B., Saavedra,H.I., Sang,L., Chong,G.T., Nuckolls,F., Giangrande,P., Wright,F.A., Field,S.J., Greenberg,M.E., Orkin,S., Nevins,J.R., Robinson,M.L., and Leone,G. (2001a). The E2F1-3 transcription factors are essential for cellular proliferation. Nature *414*, 457-462.

Wu,R., Zhai,Y., Fearon,E.R., and Cho,K.R. (2001b). Diverse mechanisms of beta-catenin deregulation in ovarian endometrioid Adenocarcinomas. Cancer Res *61*, 8247-8255.

Wu,W., Glinka,A., Delius,H., and Niehrs,C. (2000). Mutual antagonism between dickkopf1 and dickkopf2 regulates Wnt/beta-catenin signalling. Curr. Biol. *10*, 1611-1614.

Xia,J., Urabe,K., Moroi,Y., Koga,T., Duan,H., Li,Y., and Furue,M. (2006). beta-Catenin mutation and its nuclear localization are confirmed to be frequent causes of Wnt signaling pathway activation in pilomatricomas. J Dermatol. Sci *41*, 67-75.

Xu,W., Edmondson,D.G., and Roth,S.Y. (1998). Mammalian GCN5 and P/CAF acetyltransferases have homologous amino-terminal domains important for recognition of nucleosomal substrates. Mol. Cell Biol. *18*, 5659-5669.

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*, 1211-1223.

Yamamoto,H., Adachi,Y., Itoh,F., Iku,S., Matsuno,K., Kusano,M., Arimura,Y., Endo,T., Hinoda,Y., Hosokawa,M., and Imai,K. (1999a). Association of matrilysin expression with recurrence and poor prognosis in human esophageal squamous cell carcinoma. Cancer Res *59*, 3313-3316.

Yamamoto,H., Iku,S., Adachi,Y., Imsumran,A., Taniguchi,H., Nosho,K., Min,Y., Horiuchi,S., Yoshida,M., Itoh,F., and Imai,K. (2003). Association of trypsin expression with tumour progression and matrilysin expression in human colorectal cancer. J Pathol *199*, 176-184.

Yamamoto,H., Iku,S., Itoh,F., Tang,X., Hosokawa,M., and Imai,K. (2001). Association of trypsin expression with recurrence and poor prognosis in human esophageal squamous cell carcinoma. Cancer *91*, 1324-1331.

Yamamoto,H., Itoh,F., Adachi,Y., Sakamoto,H., Adachi,M., Hinoda,Y., and Imai,K. (1997). Relation of enhanced secretion of active matrix metalloproteinases with tumor spread in human hepatocellular carcinoma. Gastroenterology *112*, 1290-1296.

Yamamoto,H., Kishida,S., Kishida,M., Ikeda,S., Takada,S., and Kikuchi,A. (1999b). Phosphorylation of axin, a Wnt signal negative regulator, by glycogen synthase kinase-3beta regulates its stability. J Biol Chem *274*, 10681-10684.

Yamamoto,H., Kishida,S., Uochi,T., Ikeda,S., Koyama,S., Asashima,M., and Kikuchi,A. (1998). Axil, a member of the Axin family, interacts with both glycogen synthase kinase 3beta and beta-catenin and inhibits axis formation of *Xenopus* embryos. Mol Cell Biol *18*, 2867-2875.

Yamamoto,H., Komekado,H., and Kikuchi,A. (2006). Caveolin is necessary for Wnt-3a-dependent internalization of LRP6 and accumulation of beta-catenin. Dev Cell *11*, 213-223.

Yan,C.T., Kaushal,D., Murphy,M., Zhang,Y., Datta,A., Chen,C., Monroe,B., Mostoslavsky,G., Coakley,K., Gao,Y., Mills,K.D., Fazeli,A.P., Tepsuporn,S., Hall,G., Mulligan,R., Fox,E., Bronson,R., De,G.U., Lee,C., and Alt,F.W. (2006). XRCC4 suppresses medulloblastomas with recurrent translocations in p53-deficient mice. Proc. Natl. Acad. Sci. U. S. A *103*, 7378-7383.

Yan,D., Wiesmann,M., Rohan,M., Chan,V., Jefferson,A.B., Guo,L., Sakamoto,D., Caothien,R.H., Fuller,J.H., Reinhard,C., Garcia,P.D., Randazzo,F.M., Escobedo,J., Fantl,W.J., and Williams,L.T. (2001). Elevated expression of axin2 and hnkd mRNA provides evidence that Wnt/beta -catenin signaling is activated in human colon tumors. Proc Natl Acad Sci U S A *98*, 14973-14978.

Yang,P.T., Lorenowicz,M.J., Silhankova,M., Coudreuse,D.Y., Betist,M.C., and Korswagen,H.C. (2008). Wnt signaling requires retromer-dependent recycling of MIG-14/Wntless in Wnt-producing cells. Dev Cell *14*, 140-147.

Yang-Snyder,J., Miller,J.R., Brown,J.D., Lai,C.J., and Moon,R.T. (1996). A frizzled homolog functions in a vertebrate Wnt signaling pathway. Curr Biol *6*, 1302-1306.

Yokota,N., Nishizawa,S., Ohta,S., Date,H., Sugimura,H., Namba,H., and Maekawa,M. (2002). Role of Wnt pathway in medulloblastoma oncogenesis. Int J Cancer *101*, 198-201.

Yokoya,F., Imamoto,N., Tachibana,T., and Yoneda,Y. (1999). beta-catenin can be transported into the nucleus in a Ran-unassisted manner. Mol Biol Cell *10*, 1119-1131.

Yoon,C.S., Hyung,W.J., Lee,J.H., Chae,Y.S., Won,N.H., Yeom,B.W., and Choi,J.S. (2008). Expression of S100A4, E-cadherin, alpha- and beta-catenin in gastric Adenocarcinoma. Hepatogastroenterology *55*, 1916-1920.

Yoshikawa,S., McKinnon,R.D., Kokel,M., and Thomas,J.B. (2003). Wnt-mediated axon guidance via the *Drosophila* Derailed receptor. Nature *422*, 583-588.

Yoshikawa,Y., Fujimori,T., Mcmahon,A.P., and Takada,S. (1997). Evidence that absence of Wnt-3a signaling promotes neuralization instead of paraxial mesoderm development in the mouse. Dev Biol *183*, 234-242.

Yoshino,K., Rubin,J.S., Higinbotham,K.G., Uren,A., Anest,V., Plisov,S.Y., and Perantoni,A.O. (2001). Secreted Frizzled-related proteins can regulate metanephric development. Mech. Dev. *102*, 45-55.

Yost,C., Torres,M., Miller,J.R., Huang,E., Kimelman,D., and Moon,R.T. (1996). The axis-inducing activity, stability, and subcellular distribution of beta-catenin is regulated in *Xenopus* embryos by glycogen synthase kinase 3. Genes Dev *10*, 1443-1454.

You,Z., Madrid,L.V., Saims,D., Sedivy,J., and Wang,C.Y. (2002). c-Myc sensitizes cells to tumor necrosis factor-mediated apoptosis by inhibiting nuclear factor kappa B transactivation. J Biol Chem *277*, 36671-36677.

Yu,H.M., Jerchow,B., Sheu,T.J., Liu,B., Costantini,F., Puzas,J.E., Birchmeier,W., and Hsu,W. (2005). The role of Axin2 in calvarial morphogenesis and craniosynostosis. Development *132*, 1995-2005.

Yu,J.M., Kim,J.H., Song,G.S., and Jung,J.S. (2006). Increase in proliferation and differentiation of neural progenitor cells isolated from postnatal and adult mice brain by Wnt-3a and Wnt-5a. Mol. Cell Biochem. *288*, 17-28.

Yu,W.H., Woessner,J.F., Jr., McNeish,J.D., and Stamenkovic,I. (2002). CD44 anchors the assembly of matrilysin/MMP-7 with heparin-binding epidermal growth factor precursor and ErbB4 and regulates female reproductive organ remodeling. Genes Dev *16*, 307-323.

Zecca,M., Basler,K., and Struhl,G. (1996). Direct and long-range action of a wingless morphogen gradient. Cell *87*, 833-844.

Zechner,D., Fujita,Y., Hulsken,J., Muller,T., Walther,I., Taketo,M.M., Crenshaw,E.B., III, Birchmeier,W., and Birchmeier,C. (2003). beta-Catenin signals regulate cell growth and the balance between progenitor cell expansion and differentiation in the nervous system. Dev Biol *258*, 406-418.

Zeng,L., Fagotto,F., Zhang,T., Hsu,W., Vasicek,T.J., Perry,W.L., III, Lee,J.J., Tilghman,S.M., Gumbiner,B.M., and Costantini,F. (1997). The mouse Fused locus encodes Axin, an inhibitor of the Wnt signaling pathway that regulates embryonic axis formation. Cell *90*, 181-192.

Zhang,Y., Neo,S.Y., Wang,X., Han,J., and Lin,S.C. (1999). Axin forms a complex with MEKK1 and activates c-Jun NH(2)-terminal kinase/stress-activated protein kinase through domains distinct from Wnt signaling. J Biol Chem *274*, 35247-35254.

Zhang,Y., Qiu,W.J., Chan,S.C., Han,J., He,X., and Lin,S.C. (2002). Casein kinase I and casein kinase II differentially regulate axin function in Wnt and JNK pathways. J Biol Chem *277*, 17706-17712.

Zhao,B., Ye,X., Yu,J., Li,L., Li,W., Li,S., Yu,J., Lin,J.D., Wang,C.Y., Chinnaiyan,A.M., Lai,Z.C., and Guan,K.L. (2008). TEAD mediates YAP-dependent gene induction and growth control. Genes Dev. *22*, 1962-1971.

Zhao,C., Aviles,C., Abel,R.A., Almli,C.R., McQuillen,P., and Pleasure,S.J. (2005). Hippocampal and visuospatial learning defects in mice with a deletion of frizzled 9, a gene in the Williams syndrome deletion interval. Development *132*, 2917-2927.

Zheng,L., Zhang,J., and Carthew,R.W. (1995). frizzled regulates mirror-symmetric pattern formation in the *Drosophila* eye. Development *121*, 3045-3055.

Zhou,C.J., Pinson,K.I., and Pleasure,S.J. (2004a). Severe defects in dorsal thalamic development in low-density lipoprotein receptor-related protein-6 mutants. J. Neurosci. *24*, 7632-7639.

Zhou,C.J., Zhao,C., and Pleasure,S.J. (2004b). Wnt signaling mutants have decreased dentate granule cell production and radial glial scaffolding abnormalities. J. Neurosci. *24*, 121-126.

Zhou,P., Jiang,W., Zhang,Y.J., Kahn,S.M., Schieren,I., Santella,R.M., and Weinstein,I.B. (1995). Antisense to cyclin D1 inhibits growth and reverses the transformed phenotype of human esophageal cancer cells. Oncogene *11*, 571-580.

Zhou,Z., Wang,J., Han,X., Zhou,J., and Linder,S. (1998). Up-regulation of human secreted frizzled homolog in apoptosis and its down-regulation in breast tumors. Int. J. Cancer *78*, 95-99.

Zindy,F., Eischen,C.M., Randle,D.H., Kamijo,T., Cleveland,J.L., Sherr,C.J., and Roussel,M.F. (1998). Myc signaling via the ARF tumor suppressor regulates p53-dependent apoptosis and immortalization. Genes Dev *12*, 2424-2433.

Zinyk,D.L., Mercer,E.H., Harris,E., Anderson,D.J., and Joyner,A.L. (1998). Fate mapping of the mouse midbrain-hindbrain constriction using a site-specific recombination system. Curr. Biol. *8*, 665-668.

Zirn,B., Samans,B., Wittmann,S., Pietsch,T., Leuschner,I., Graf,N., and Gessler,M. (2006). Target genes of the WNT/beta-catenin pathway in Wilms tumors. Genes Chromosomes Cancer *45*, 565-574.

Zukerberg,L.R., Benedict,W.F., Arnold,A., Dyson,N., Harlow,E., and Harris,N.L. (1996). Expression of the retinoblastoma protein in low-grade B-cell lymphoma: relationship to cyclin D1. Blood *88*, 268-276.

Zurawel,R.H., Allen,C., Chiappa,S., Cato,W., Biegel,J., Cogen,P., de,S.F., and Raffel,C. (2000). Analysis of PTCH/SMO/SHH pathway genes in medulloblastoma. Genes Chromosomes. Cancer *27*, 44-51.

Zurawel,R.H., Chiappa,S.A., Allen,C., and Raffel,C. (1998). Sporadic medulloblastomas contain oncogenic beta-catenin mutations. Cancer Res *58*, 896-899.

Zwijsen,R.M., Wientjens,E., Klompmaker,R., van der,S.J., Bernards,R., and Michalides,R.J. (1997). CDK-independent activation of estrogen receptor by cyclin D1. Cell *88*, 405-415.