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Bachelor Thesis:

Wnt Signaling in Stem Cells and Cancer

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CONTENT

1. ABSTRACT

Wnt signaling is a common theme in animal development and the aberrant activation of the Wnt pathway is also implicated in the cellular transformation and cancer. The signaling cascade initiated by the secreted Wnt ligands is one of a relatively small number of the cell signaling pathways that relay information from the extracellular milieu via the cytosolic components up to the nucleus. The stabilization of cytosolic β-catenin is a central molecular mechanism of Wnt signaling. Stable β-catenin enters the cell nucleus where it associates with the TCF/LEF transcription factors to regulate expression of the specific Wnt-responsive genes. The pathway itself is tightly regulated at various levels. The activity of the Wnt ligands is stimulated or attenuated in the extacellular space by a diverse group of antagonists, cofactors and coreceptors. In the cytoplasm, the amounts of β-catenin are constitutively reduced by a large destruction protein complex. . In the nucleus, the transcriptional stimulation of the target genes is modulated by a complicated interplay between various activators, repressors and other auxiliary factors. Recently, a new nuclear attenuator of Wnt signaling, HIC1 (hypermethylated in cancer 1), has been discovered. A detailed characterization of molecular functions of this newly described Wnt signaling modulator is a main subject of my diploma work.

In this Bachelor thesis I present the basic description of the Wnt pathway and its molecular mechanisms and components. I describe the role of this signaling cascade in survival of the stem cells in gut and bone marrow and, furthermore, the involvement of non-physiological Wnt signaling in development of various forms of cancer. Finally, in the last part, I would like to offer a closer look at HIC1, the tumor suppressor with a novel and specific function in the signaling process. I present here also some experimental results obtained during starting months of my laboratory practice.

2. LIST OF ABBREVIATIONS

aa amino acid

ALL acute lymphocytic leukemia

AML acute myeloid leukemia

APC adematous polyposis coli

Arm Armadillo

BMP bone morphogenic protein

C.e. *Caenorhabditis elegans*

CKI casein kinase I

CLL acute lymphocytic leukemia

CML chronic myeloid leukemia

CNS central nervous system

CRD cysteine-rich domain

CRT β-Catenin/Tcf-regulated transcription

CtBP C-terminal binding protein

Dkk Dickkopf

Dsh/ Dvl Dishevelled

DTT dithiothreitol

ECM extracellular matrix

ER endoplasmic reticulum

FAP familial adenomatous polyposis

FRAT frequently rearranged in advanced T-cell lymphomas

Fz Frizzled

GBP GSK3β-binding protein

GEF guanine nucleotide exchange factor

GFP green fluorescent protein

GSK3 glycogen synthase kinase 3

h, m, x human, mouse, *Xenopus* gene /protein

HDAC human histone deacetylase

HIC-1 hypermethylated in cancer-1

HSC haematopoietic stem cell

HSPG heparan sulfate proteoglycan

ISC intestinal stem cell

LEF lymphocyte-enhancer factor

LOF/GOF loss/gain of function

LOH loss of heterozygosity

MCR mutation cluster region

NES nuclear export signal

NLS nuclear localization signal

OTC oligosaccharyl transferase complex

PCR Polymerase Chain Reaction

por porcupine

S, Ser serine

SDS-PAGE sodium dodecylsulphate polyacrylamide gel electrophoresis

SFRP secreted Frizzled related protein

TCF T-cell factor

Wg wingless

WIF Wnt inhibitory factor

wt, fl wild type, full-length gene/protein

Y, Tyr tyrosine

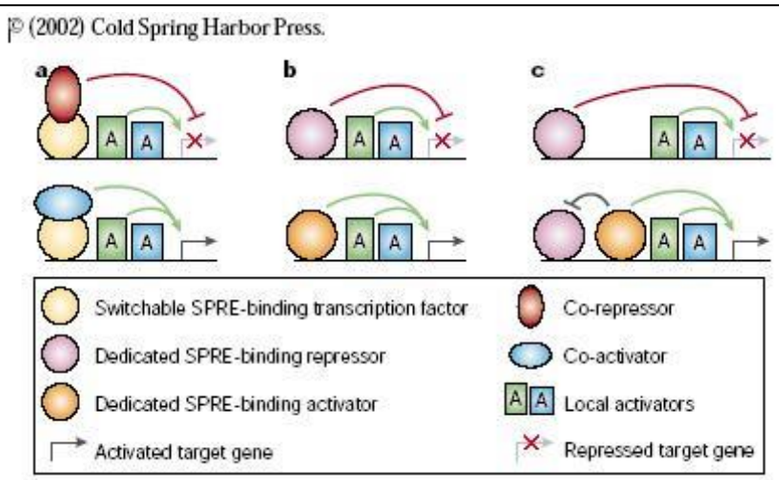
3. INTRODUCTION

An amazing number of cell types and patterns can be found in the animal kingdom, but there is only a handful of signaling pathways directly involved in the cell determination processes. Most of the cell–cell interactions during embryonic development include at least one of the following types of signaling: (1) Hedgehog (Hh); (2) wingless/int-1 (Wnt); (3) transforming growth factor-β (TGF-β); (4) receptor tyrosine kinase (RTK); (5) Notch; (6) Janus kinase/signal transducer and activator of transcription (JAK/STAT); (7) nuclear hormone [17].

The common molecular mechanism found in all these major cell to cell signaling pathways is the transcriptional activation of the pathway-specific target genes. These genes are mostly regulated by signal-dependent transcription factors which are distinct for each type of a signaling process. Basic modes of a transcriptional control can be divided into these three categories (see Figure 1.) [17]:

1. type I (also called "default repression"): the signal-dependent transcription factors that regulate the signaling pathway specific response element (SPRE) function as repressors in the absence of the signal. These factors are converted to the transcriptional activators upon stimulation of the cell with a particular ligand. This is the case of the Wnt, Notch, Hh and nuclear receptor signaling pathways (shown in Fig. 1, panel a).
2. type II : an activator replaces the repressor, both bind to the same enhancer element. The TGF-β and RTK signaling pathways utilize this switching mechanism (panel b). In addition, type I regulation might be also used.
3. type III : the repressor and the activator recognize different binding sites, in the presence of signaling the activator releases the repressor activity (panel c).

Figure 1. Types of transcriptional control [ref. 17; da Silva, A.P. & Sommer,



4. THE WNT SIGNALING PATHWAY

Wnt signaling pathway is an evolutionarily conserved signal tranduction cascade, which has been found virtually in all metazoan organisms including primitive cnidarians and mammals (Figure 2). The pathway plays a crucial role during embryonic development and also in adult mainly self-renewing tissues. Wnt proteins function as concentration-dependent long-range morphogenetic signals regulating various cellular phenotypes as proliferation, differentiation, survival, cell polarity, and movement. Non-physiological activation of Wnt signaling is one of the key features of many cancers. It is generally accepted that the tightly regulated self-renewal and differentiation of stem and progenitor cells is controlled by Wnt signaling. A failure of such control leads to the cell transformation, malignant proliferation and cancer.

Wnt proteins and their downstream cellular effectors were originally described mainly in *Drosophila*, nevertheless many of the signaling pathway mechanisms were also characterized in other animal models as mouse and *Xenopus* [36]. Genetic and biochemical data gained from these models have, to date, identified more than 50 proteins that are directly involved in transducing of the Wnt signals.

Figure 2. Evolutionary conservation of the Wnt pathway [21; Frame, S. & Cohen, P.]

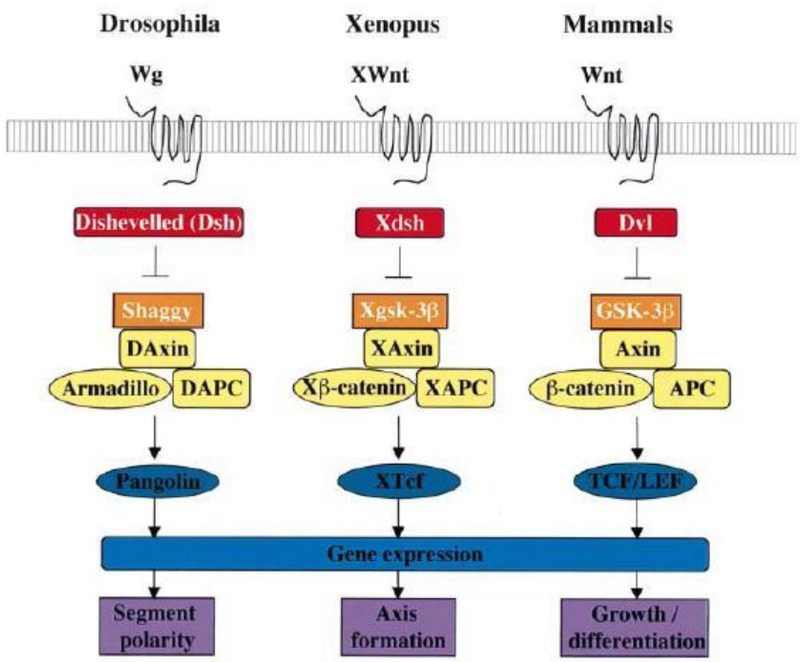


Figure 3 Wnt signalling components are highly conserved between diverse organisms

The Wnt signals can be transmitted via at least three independent cellular pathways, one of which, so called canonical Wnt signaling, is the key topic of my thesis. It includes binding of the Wnt proteins to the Frizzled receptors, activation of

several intracellular proteins that leads to the inhibition of the β-catenin-degradation complex and the accumulation of β-catenin. Stabilized β-catenin translocates to the nucleus where it is engaged with the factors of the Tcf/Lef family. The Tcf(Lef)/β-catenin heterocomplexes drive transcription of a specific set of the Wnt signaling target genes. Like all other signaling pathways, the Wnt cascade is a complex system with various regulatory levels. One of the numerous modulators is the HIC1 tumor suppressor, that participates in control of the final, i.e. nuclear steps, of the pathway. The description of the HIC1 function is included in the last part of this thesis.

4. 1. The Wnts

The Wnt proteins form a family of highly conserved secreted signaling molecules. The term Wnt is an amalgam of *wingless* (*wg*) and *int* (see below) [40].

4. 1. 1. *Wnt* genes

The first Wnt gene, *Wnt1*, was cloned from the mouse genome as a proto-oncogene in 1982 by Nusse and Varmus. *Wnt-1* was isolated during a search for genes that are insertionally activated by proviral DNA in mammary carcinomas induced by the mouse mammary tumor virus (MMTV) [40]. Until 1991 *Wnt1* was called *Int-1* (for MMTV integration site). *Wingless* (*wg*) is the *Wnt1* homolog in *Drosophila*.

Several other Wnt genes were initially cloned as candidate protooncogenes (mouse *Wnt7* and *Wnt3*), or as genes located near the cystic fibrosis locus (human *Wnt2*) [40]. Following the identification of these founding Wnt family members conserved sequences for primers were designed and used in various PCR-based approaches to clone the rest of the Wnt genes. Today, when the primary genomes of many organisms were completed, we can assume that there are 19 Wnt genes in human (see Table 1) and mouse, 16 in *Xenopus*, 11 in chicken, 12 in zebrafish, 7 in *Drosophila*, 5 in *C. elegans* and at least one in *Hydra*. Interestingly, β-catenin is absent in genomes of plants, prokaryotes and unicellular eukaryotic organisms [35].

Between *Drosophila* and mammals, there is a strong conservation of the *Wnt* genes, thus, the real orthologs can be assigned. For example human WNT1 and mouse Wnt1 proteins are 98% identical ; human WNT5a and *Xenopus* Wnt5a share 84% of their amino-acid sequence. The overall sequence identity between orthologous proteins in humans and

flies ranges from 21% (human WNT8a/d vs. Drosophila DWnt8) to 42% (WNT1 and Wingless) [36].

The majority of human WNT genes contain 4 coding exons. The exceptions are WNT9A, with 3 exons, WNT2, WNT5b, and WNT11, with 5 exons, and WNT8b which has 6 exons. WNT2b/13, WNT8a/d, and WNT16 - have alternative amino or carboxyl termini, which result from the use of alternative 5' or 3' exons [36].

Table 1. Human Wnt genes and proteins (based on data from [1][2][27][34])

Gene	Locus	Size (bp)	Protein size (aa)/(Da)	Expression in human tissues	Disease Malignant potential
WNT 1	12q13.12	4,243	370/ 40982	Developing CNS	Schizophrenia Highly transforming protooncogene
WNT 2	7q31.2	46,061	360/ 40418	Developing allantois, pericardium, ventral lateral mesoderm and in adult lung,brain, heart.	Lowly transforming protooncogene
WNT2B/13	1p13.2	54,742	391/ 43770	Breast and gastric cancer	
WNT3	17q21.31	54,221	355/ 39645	Developing nervous system and in adult thalamus, cerebellum, pons. Required for the primary axis formation.	Tetra-Amelia
WNT3A	1q42.13	54,209	352/ 39365	Lung	Highly transforming protooncogene
WNT4	1p32.16	24,020	351/ 39052	Guidance of commisural axons in the spinal chord	Mullerian-duct regression and virilization, Overexpression - abnormal proliferation in human breast tissue, kidney damage, Polycystic kidney disease Non-transforming
WNT5A	3p14.3	21,587	365/ 40887	Developing face, ventral area of midbrain, limbs an in adult brain and lung.	Leukemia Metastasis
WNT5B	12p13.33	30,157	359/ 40323		Associated with Susceptibility to type 2 diabetes Lowly transforming protooncogene
WNT6	2q35	15,496	365/ 39721	Ectoderm, somites, limb and mammary gland in mice. Involved in short-range signaling during embryonic patterning.	Non-transforming
WNT7A	3p25.1	61,536	349/ 38897	Controlling limb development at the dorsoventral axis, upregulating LMX1A expression in limbs mesenchyme and dorsal differentiation. Allows sexually dimorphic development of the mullerian ducts.	Highly transforming protooncogene
WNT7B	22q13.31	28,379	349/ 39327	Specific region of the forebrain, the kidney and the limb ectoderm	Highly transforming protooncogene
WNT8A	5q31.2	8,375	355/ 39527	Development and differentiation of certain forebrain structures, notably the hippocampus.	
WNT8B	10q24.31	20,703	351/ 38721	Developing forebrain, notably the hippocampus	
WNT9A (previously WNT14)			1q42.13	26,902	365/ 40320
WNT9B (previously WNT15)			17q21.31	25,469	357/ 38971
WNT10A	2q35	13,396	417/ 46444	Developing CNS	
WNT10B	12q13.12	6,419	389/ 43000	Breast carcinoma, heart, skeletal muscle	
WNT11	11q13.5	20,204	354/ 39179	The perichondrium of the developing skeleton	
WNT16	7q31.31	15,737	365/ 40690		

The quantity of the Wnt gene family members suggests functional redundancy, but in fact, they exhibit unique patterns of expression and distinct functions, although some overlap

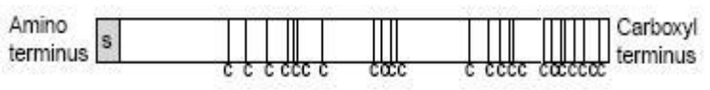
is observed (see below). However, the detection of Wnt proteins in many tissues has been problematic due to lack of suitable antibody reagents.

4. 1. 2. The Wnt proteins

Wnts are cysteine-rich secreted glycoproteins that are defined by their sequence rather than functional properties. In humans WNT proteins share 27% to 83% amino-acid sequence identity and have a conserved pattern of 23 or 24 cysteine residues. They are all very similar in size, their molecular weight ranges from 39 kDa (WNT8a) to 46 kDa (WNT10a) (see Table 1) [36]. In other species these proteins share overall sequence identity with over 100 conserved residues fairly evenly distributed across the entire sequence [40]. Of course they are slightly less conserved between vertebrates and invertebrates.

Wnt proteins display many of the characteristics of secreted growth factors: a hydrophobic signal peptide, a recognition site for signal peptidase, the absence of additional transmembrane domains, abundant and strongly conserved cysteine residues and prospective sites for N-linked glycosylation.(Figure 3.)

Figure 3. Wnt protein [36; Miller, J.R.]



Analysis of chimeric Wnt proteins has shown that the C-terminal region of Wnt proteins may play a role in determining the specificity of responses to different Wnts. In addition, deletion mutants lacking the C-terminal third of the Wnt protein can act as dominant-negative regulators in a cell-non-autonomous manner. This suggests that the N-terminal region may mediate interactions with the Wnt receptors but requires the carboxyl terminus to activate these receptors [36].

4.1.2.1. Post-translational modifications of the Wnt proteins

Little is known about the structure of the Wnt proteins, as they are notoriously insoluble (see below), but the highly conserved spacing of cysteine residues suggests that their folding may depend on the formation of multiple intramolecular disulfide bonds.

Early difficulties in isolating active Wnts are now explained by the finding that the Wnt molecules are palmitoylated and therefore much more hydrophobic than was predicted from the primary amino acid sequence. The palmitoylation appears on a conserved cysteine

(Cys 95 in mouse Wnt1), suggesting that all Wnts have this modification. Treating Wnts with the enzyme acyl protein thioesterase results in a form of protein that is neither hydrophobic, nor active. This underscores the importance of the palmitate for signaling [35].

The enzymes which add the palmitate to Wnts are likely encoded (a direct proof for this has not been provided yet) by the *porcupine* (*por*) gene in *Drosophila*, and *mom-1(more mesoderm-1)* in *Caenorhabditis elegans*. Por and mom-1 seems to be the enzymes dedicated to Wnt signaling. There is a significant sequence similarity between Por and membrane-bound acyltransferases (enzymes in the ER membrane that acylate a variety of substrates). So, it is possible that *por* encodes an enzyme which catalyzes the transfer of palmitate onto Wnt [35].

Wingless is hydrophobic and can associate with membranes, but both characteristics are lost when *O*-acyltransferase activity is inhibited either biochemically or by mutation of the *por* gene. Notably, overexpression of Wg in *Drosophila* can partially circumvent the need for *por*, and moreover, the *Wnt* mutant gene constructs lacking the palmitoylation motif can produce an attenuated signal when overexpressed. It suggests that the lipid modification targets Wnts to membranes but its absence can be overcome by high protein concentrations [35].

Glycosylation is the second crucial modification of Wnt proteins. For instance mouse Wnt1 has four potential *N*-glycosylation sites (Asn^{29}, Asn^{316}, Asn^{346} and Asn^{359}), three of these (Asn^{29}, Asn^{316} and Asn^{359}) are in fact glycosylated. Por and its homologs are possibly involved in this action. In the absence of *por* the *N*-glycosylation of Wg is impaired. In contrast, the ectopic production of Por stimulates the *N*-glycosylation of both endogenous and exogenous Wg. The *N*-glycosylation of Wg in the ER normally occurs posttranslationally, while in the presence of dithiothreitol (a reversible inhibitor of disulfide bond formation of newly synthesized proteins in the ER) it occurs cotranslationally. The cotranslational disulfide bond formation competes with the *N*-glycosylation by an oligosaccharyl transferase complex [51].

Por binds the N-terminal 24-aa domain (residues 83–106) of Wg, which is highly conserved in the Wnt family and stimulates the *N*-glycosylation at Asn^{103}. Under conditions when Cys^{104} forms a disulfide bond, it is likely that the Asn^{103} cannot be *N*-glycosylated. If this is true, por may delay disulfide bond formation at Cys^{104}, thereby enhancing the *N*-glycosylation at Asn^{103}. Por might also anchor the protein at the ER membrane possibly through acylation and as a result, the access of Asn-X-(Ser/Thr) sites to the OST complex would be accelerated. It was proposed that the acylation of Wg by Por is essential for the

posttranslational *N*-glycosylation of Wg and therefore, for its secretion. As the loss of *porcupine* leads to the hypoglycosylation of Wg, calnexin and calreticulin might fail to associate with Wg efficiently. This possibly generates unfolded variants of the Wg polypeptide which can exit ER and thus are retained in the cell [51].

4.1.2.2. Transport of the Wnt proteins between cells and extracellular inhibitors of Wnt proteins

It is not known how are the palmitoylated Wnt molecules actively transported to the cell surface, how are Wnts released from the cell, and how they move as morphogens over long distances. Vesicular transport outside of cells (vesicles called argosomes with Wg as a cargo) has been proposed in the *Drosophila* wing imaginal discs. There is also an evidence of cytonemes, long thin filopodial extensions of cell cytoplasm that might carry Wnts and other growth factors. Nevertheless, no specific exporters of the Wnt molecules have been discovered yet, although *mom-3 (more mesoderm-3*) in *C. elegans* is required in Wnt-producing cells [35].

Once released from the cell, Wnts can interact with a number of binding proteins. Heparan sulfate proteoglycans (HSPGs) are thought to be positive regulators of Wnt activity. The absence on *Drosophila* HSPG *Dally* and mutations in the genes encoding enzymes modifying HSPG result in phenotypes similar to those found in the *wingless* mutants. Nevertheless, Dally 's precise biochemical function is unknown so far [35].

The Wnt proteins can also bind members of the SFRP (secreted Frizzled related protein) family. These proteins resemble the ligand-binding domain of the transmembrane Frizzled Wnt receptors (see below). Another binding partners are the WIF (Wnt inhibitory factor) proteins homologous to the extracellular part of the Derailed/RYK class of the Wnt receptors (see below). In general, it is presumed that these factors function as extracellular inhibitors by sequestering Wnts and by preventing their interaction with the membrane-bound receptors [35]..

The Dickkopf (Dkk) proteins are the best characterized secreted Wnt inhibitors. Dkks have not been found in invertebrates, but mice and humans have multiple Dkk genes. Dkk binds with a high affinity to LRPs (Low-density lipoprotein receptor related proteins) (see below) and to another class of transmembrane molecules, the Kremens. After forming a trimeric complex Dkks promote the internalization of LRPs or disrupt the Fz-LRP complex formation. Dkk1 is a vertebrate head inducer that synergizes with Kremen 1 to inhibit LRP6

function [25]. Dkk2 requires Kremen2 and cannot function with Kremen1 to down-regulate the Wnt signal. Similarly, Kremen2 promotes the inhibitory activity of Dkk4 [35].

Paradoxically, Dkk2 can activate or inhibit LRP5/6 function in different cell types or assays. Another secreted molecule which exhibits such dual activities is called Wise. It can activate the β-catenin target genes *Xnr3* and *siamois* in *Xenopus* embryos, but when combined with the BMP (bone morphogenetic protein) inhibitor, Wise acts like an Wnt inhibitor molecule in the head induction. Thus, it cannot be excluded that these proteins may, depending on expression levels or cellular context, promote Wnt signaling [25].

Figure 4. The extracellular environment of a Wnt responding cell [5; Bejsovec, A.]

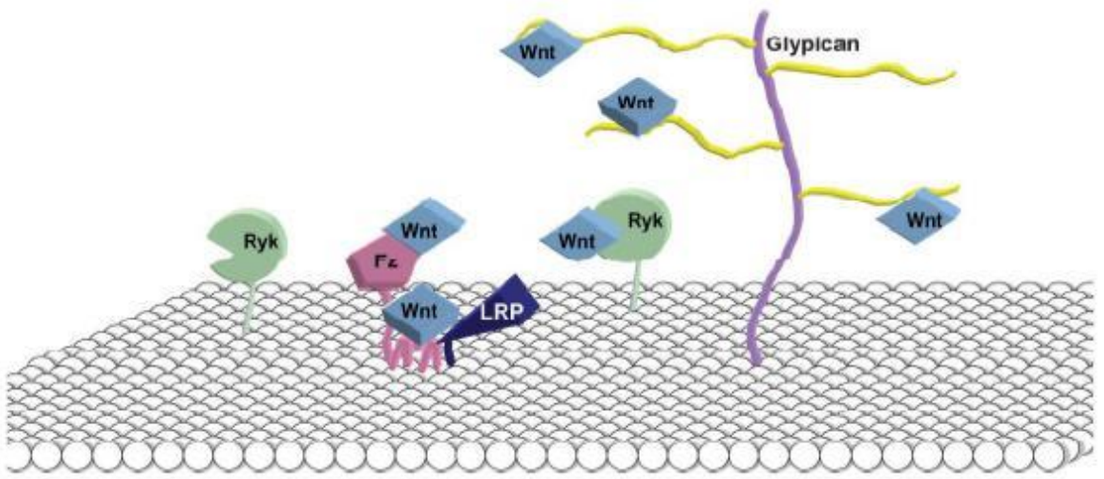


Figure 2. Schematic Diagram of the Extracellular Environment of a Cell Responding to Wnt Signal
The CRD domain that comprises the extracellular amino-terminus of Fz is known to bind Wnt, but may not be required for transducing the signal. This implies that the true ligand binding pocket may be formed by the extracellular loops of the transmembrane-spanning region. The extracellular WIF domain of the Ryk receptor also binds Wnt and this may either transduce signal directly through some novel pathway or may potentiate signaling through the Fz receptor. Sulfated glycosaminoglycans, such as those attached to the glypican proteoglycan core protein, are also known to bind Wnt but are not essential for signaling when ligand is abundant. Thus, they act to concentrate Wnt at the cell surface, perhaps enhancing its interaction with the Fz-LRP receptor complex.

4. 1. 3. The Wnt receptors

Members of the Frizzled family of seven-pass transmembrane proteins are the primary receptors for Wnts. Very recently, another Wnt receptor, Derailed, was discovered. Derailed is entirely different from Frizzleds as it is a transmembrane tyrosine kinase that belongs to the RYK (recertor Y=tyrosine kinase) subfamily. Derailed contains the Wnt-interacting WIF domain in its extracellular region which can bind to the DWnt-5 protein, a regulator of the axon guidance in the *Drosophila* CNS. The Wnt5a receptor has not been identified so far, thus it is possible that in fact it is RYK. Not much is known about the signal which is

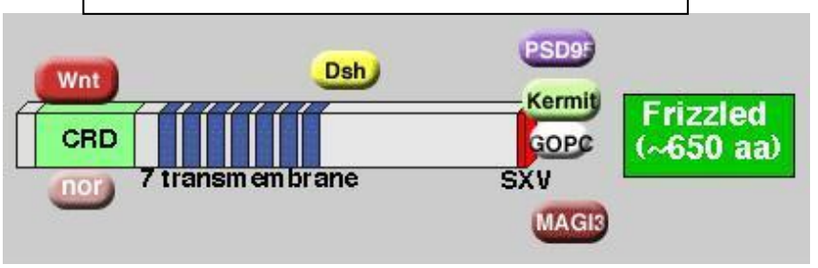
transduced by Derailed. Derailed-related signaling probably involves yet undiscovered co-receptor (likewise Frizzled and its co-receptor LRP), since the Derailed kinase domain appears dispensable for its function [35].

Wnt signaling requires not only a functional Fz, but also a presence of LRP (LDL receptor related protein), a long single-pass transmembrane molecule. The genome of vertebrates encodes two highly homologous genes, *LRP5* and *LRP6,* a single homolog in *Drosophila* is called *arrow*. It has been proposed that the Wnt molecules can bind to LRP and form a trimeric complex with Frizzleds.

4. 1. 3. 1. Frizzled receptors

Frizzled (Fz) is a protein family of serpentine receptors. Ten Fzs genes are found in the human genome [25]. These receptors are so-called seven-pass transmembrane molecules (they encompass the cytoplasmic membrane seven times) with a long amino-terminal extension (120 to 125 aa) called CRD (cysteine-rich domain) - for instance, mouse Frizzled 8 (mFz8) contains 10 conserved cysteines within this domain [18]. They further contain a short cytoplasmic tail with the PDZ domain binding motif Ser/Thr-X-Val located at the C-terminus of the protein (Figure 5).

Figure 5. Frizzled receptor [1; Nusse R.]



The Wnt proteins bind directly to the CRD of Fz (Kd 1.6 nM [25]). One specific Wnt possibly binds several distinct Fzs, and different Fzs may also bind several Wnts, thus there is a large number of various Wnt-Fz combinations.

Notably, Fz can also interact with another ligand called Norrin. This protein possesses no discernible sequence similarity to Wnts, nevertheless, it binds with a high affinity to the Frizzled-4 CRD. Together with LRP, Fz4 and Norrin activate the canonical signaling pathway. This suggests that there could be additional ligands for this receptor family [35].

According to the multiple transmembrane domain structure, Wnt binding might reconfigure the Fz transmembrane domains, and results in the appropriate intracellular response. The heptahelical topology of the Frizzled molecules also implies that these receptors could signal through the heterotrimeric G proteins. Actually, only little is known

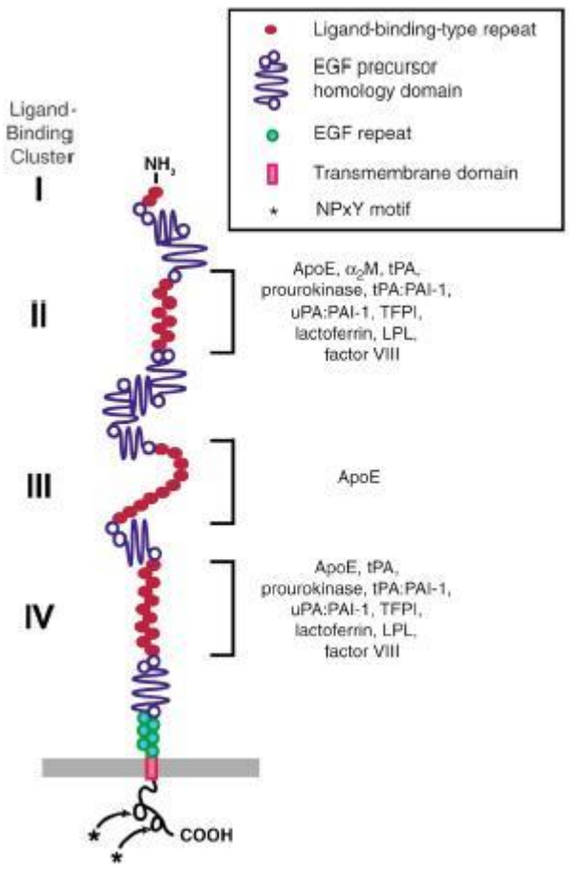
about the G protein-related signaling downstream of Fz. It was only suggested that it is probably not involved in so called canonical pathway.

The member of the Wnt pathway mediating the relay of the signal immediately downstream of Fz is Dishevelled (Dsh). It interacts directly with a short amino acid motif (Lys-Thr-X-X-X-Trp) present in the Fz C-terminal cytoplasmic tail. This motif is strictly required for Fz signaling. As shown recently by NMR Dsh possesses the PDZ domain. Binding of the Wnt ligands to their receptors also leads to differential phosphorylation of Dsh. This process is mediated by several protein kinases, of which Par1 is most likely the most prospective candidate to be regulated by the signal [35].

4. 1. 3. 2. The LRP family

In contrast to the LDL receptor that acts solely in the lipoprotein metabolism, the LRPs have other distinct functions. Besides the lipid metabolism, LRPs also play a role in a homeostasis of proteinases and proteinase inhibitors, cellular entry of viruses and toxins, activation of lysosomal enzymes, cellular signal transduction, and neurotransmission. LRPs can recognize at least 30 different ligands, that represent several families of proteins (Figure 6.) [26].

Figure 6. LRP [26; Herz, J. & Strickland, D.E.]



Both LRP5 and LRP6 are involved in Wnt signaling. LRPs, like all members of the LDL receptor gene family, consist of five structural units - (1) ligand-binding (complement) type cysteine rich repeats (clusters containing from two to eleven individual repeats); (2) epidermal growth factor (EGF) receptor–like cysteine-rich repeats (usually two); (3) six YWTD domains (arranged in a propeller-like structure); (4) additional EGF repeat; (5) a single membrane-spanning segment; (6) the cytoplasmic tail containing two NPxY

motifs that serve as docking sites for the endocytosis machinery and for cytoplasmic adaptor and scaffolding proteins involved in signaling events [26].

Boca in *Drosophila* and Mesd in mice are specific accessory molecules that enable the transport of LRPs from the ER to the cell surface. Mutations in these genes result in phenotypes similar to the loss of LRP (or Arrow in *Drosophila*) [35].

LRPs may interact with the cytosolic protein Axin. LRPs contain several Pro-Pro-Pro-(Ser/Thr)-Pro motifs that can be phosphorylated upon the Wnt stimulation. These phosphorylated motifs mediate docking of Axin to the LRP cytoplasmic tail [35]. This suggests that Wnt signaling can lead to the formation of a complex including the two receptors, Axin and Dsh. Direct interactions between Axin and Dsh, possibly through the DIX domain (present in both proteins), would be the intrinsic connection between the receptor and downstream effectors.

When LRP is not expressed or it is down-modulated through the factors such as Dkks, Wnts may still form a complex with Fz executing signaling independent from Tcf/β-catenin. This "noncanonical" pathway results in cellular responses such as increased calcium flux, cytoskeletal rearrangements and repression of the Tcf-mediated transcription (see below).

4. 2. The canonical Wnt/β-catenin pathway

The canonical Wnt signaling pathway is a term for the main mode of signaling via Frizzled receptors including an intracellular mediator β-catenin. There exist also β-catenin-independent, non-canonical pathways induced by Wnts, briefly described in chapter 4. 3.

4. 2. 1. Overview

In unstimulated cells newly synthesized cytoplasmatic β-catenin is destabilized by a multiprotein complex containing two scaffolding proteins - Adematous polyposis coli (APC) and Axin (or its homolog Conductin). Casein kinase Iα (CKIα) and Glycogen synthase kinase 3β (GSK3β) are kinases residing in a multiprotein complex that phosphorylate conserved Ser/Thr residues in the N-terminal part of β-catenin. Such phosphorylation earmarks β-catenin for ubiquitinilation by the ubiquitin E3 ligase and further degradation in 26S proteasome (Figure 7). When cells are stimulated, the kinase activity of degradation complex is inhibited, β-catenin is stabilized and its level increases. This leads to its nuclear accumulation and complex formation with the Lef/Tcf transcription factors. There β-catenin serves as a coactivator and stimulates transcription of Wnt target genes (see Figure 7). Not all mechanisms involved in this way of signaling are completely understood, but it is clear that the stability of free cytoplasmatic β-catenin is the heart of the canonical Wnt pathway.

Figure 7. The Wnt canonical pathway [45; Reya, T. & Clevers, H.]

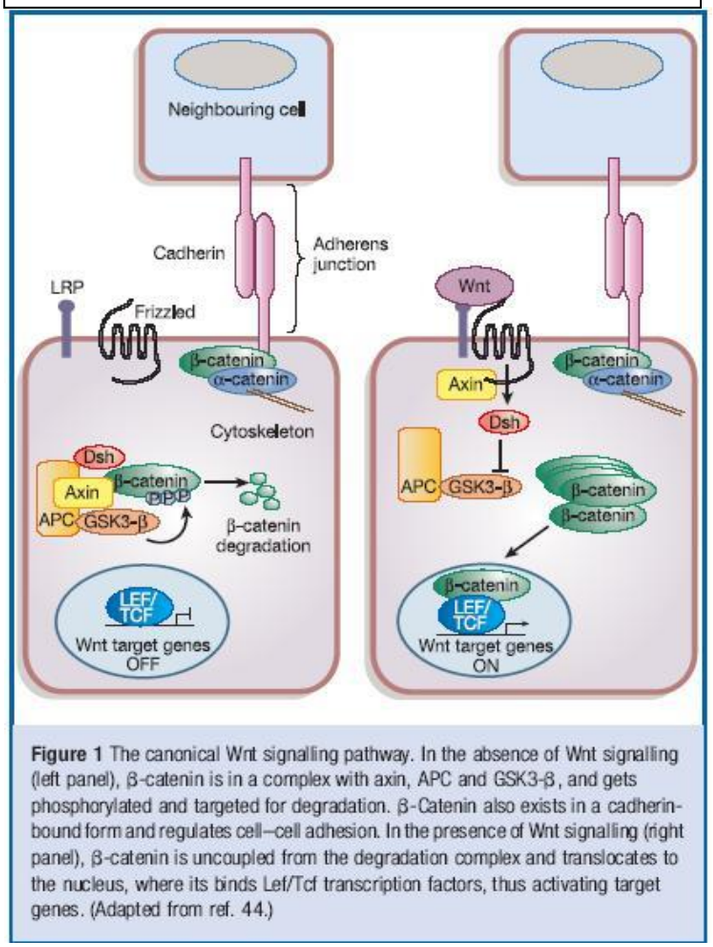


Figure 1 The canonical Wnt signalling pathway. In the absence of Wnt signalling (left panel), β-catenin is in a complex with axin, APC and GSK3-β, and gets phosphorylated and targeted for degradation. β-Catenin also exists in a cadherin-bound form and regulates cell–cell adhesion. In the presence of Wnt signalling (right panel), β-catenin is uncoupled from the degradation complex and translocates to the nucleus, where its binds Lef/Tcf transcription factors, thus activating target genes. (Adapted from ref. 44.)

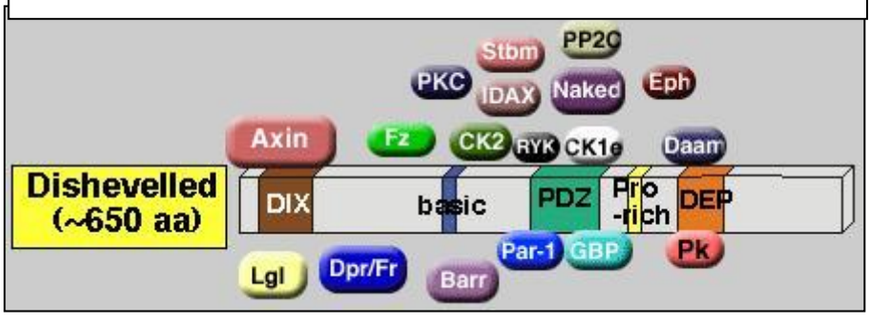
4. 2. 2. Signaling within the cytoplasm

How the destruction complex senses Wnts at the cell surface is so far not fully understood. Thus, I will present the most recent findings describing the individual steps of Wnt signaling in the cytoplasm.

4. 2. 2. 1. Dishevelled (Dsh)

After binding of Wnts to Fz/LRP, the adapter protein Dishevelled (Dsh in *Drosophila*; Dvl1,2 and 3 in mice) is recruited to the membrane via interaction with Fz intracellular domain (see above) or/and phospholipids. There it becomes phophorylated and activated. It was proposed that this may happen through stimulation of casein kinase Iε (CKIε) and/or casein kinase II (CKII) [36]. Although biochemical analysis suggests that it binds directly to Dishevelled's PDZ domain, CKIε has been placed downstream of Dsh and upstream of GSK3β [41]. Note that CKIε was also described as a member of the β-catenin destruction complex (see below), so its precise function is not known at this time.

Figure 10. The APC domains [1; Nusse R.]



Dsh and its homologs contain three highly conserved domains: N-terminal DIX (Dishevelled and axin) domain; central PDZ (PSD-95/SAP90, Discs-large, ZO-1) domain (see above); and DEP (Dishevelled, EGL-10, pleckstrin) domain, critical for the activation of the Jun-N-terminal kinase (Figure 8) [41].

GSK3β-binding protein (GBP), whose mammalian homologues are the FRATs (frequently rearranged in advanced T-cell lymphomas), associates with Dsh and may participate in signal transducing process by promoting the dissociation of GSK3β from the degradation complex [7]. However, recent genetic evidence shows that FRAT is not essential for Wnt signaling, since mice with all three Frat family members deleted develop entirely normally. In parallel, Wnt-induced phosphorylation of the cytoplasmic tail of LRP allows docking of Axin to LRP. Recruitment of Axin to the membrane is thought to disrupt the destruction complex, which subsequently releases β-catenin.

A GFP tag was used as a tool to monitor Axin behavior during Wnt signalization. Axin-GFP is present in the cytoplasm as "dots" but becomes concentrated near the plasma membrane when Wg signaling is active, and in addition, overexpression of Wg increases Axin-GFP localization to the plasma membrane. Dsh (but neither GSK-3 nor APC) is required for Axin-GFP plasma membrane localization after Wg signaling. Dsh-GFP itself shows plasma membrane localization (which does not appear to change during Wg signaling) and also appears in intracellular dots. Dsh might shuttle the Axin complex to the plasma membrane via a vesicular transport mechanism. The DIX domain of Dvl2 binds phospholipids and this activity is required for Dvl2 vesicle-like localization and its ability to activate β-catenin signaling. Consistent with this, Rab GTPases (proteins involved in the vesicular trafficking), are required for Wg response during fly wing development. But so far no direct evidence exists for these hypothetical vesicles [25].

It has been also published that protein phosphatase 2A (PP2A) may promote stabilization of β-catenin. PP2A can bind to Axin and possibly dephosphorylate GSK3β substrates, including β-catenin. Thus, overexpression of the B56 subunit of PP2A results in decreased levels of β-catenin [20].

4. 2. 2. 1. β-catenin destruction complex

The main function of this complex is to promote phosphorylation of β-catenin by CKI and GSK3β at a set of specific serine and threonine residues in its N-terminus.

The functional interactions within this complex (including Axin/Conductin, APC, GSK3β and CKIα) will be described in detail in the following chapter.

4. 2. 2. 1. 1. Axin/ Conductin

Axin has been originally identified as a product of the mouse gene called *Fused*. Mutations in *Fused* cause recessive embryonic lethality at E8–E10. Such embryos show a duplication of the embryonic axis suggesting that Axin normally serves as negative regulator in the response to an axis-inducing signal. Overexpression of Axin stimulates enhanced degradation of β-catenin. There are alternative Axin splicing products, termed forms 1 and 2. Conductin (Axin homolog) has been identified as a β-catenin-binding protein by yeast two-hybrid screening of a mouse cDNA library [29].

Axin has Ser/Thr residues (S^{330},T^{341} and S^{343}) that can be phosphorylated by GSK3β. The phosphorylation of Axin does not affect its binding to GSK3β or β-catenin *in vitro,* but treatment of the cells with LiCl (a GSK3β inhibitor) decreases the levels of Axin, whereas treatment with okadaic acid (a phosphatase inhibitor) increases the Axin levels . These results suggest that GSK3β regulates the stability of Axin by phosphorylation and that the phosphorylated form of Axin is more stable than the unphosphorylated one [29]. Therefore, GSK3β phosphorylates both Axin and β-catenin in the complex.

In addition to Wnt, several growth factors including EGF, insulin, and IGF1, inactivate GSK3 (via phosphorylation at Ser^{21} in GSK3α and Ser^{9} in GSK3β by protein kinase B, PKB; also called Akt). However, there is no evidence that these growth factors cause accumulation of β-catenin. Therefore, Axin may act as a scaffold protein that selectively channels the signal from Wnt ligands to β-catenin [21]. A composition of the destruction complex and the stoichiometry of its components have not been fully solved. Recent data show that the number of Axin molecules in an individual cell is much lower (5000-fold) than other proteins in the complex. Therefore, Axin could be the limiting component of the Wnt signaling cascade that may promote the rapid assembly and disassembly of Wnt pathway components in order to regulate the β-catenin stability in the cell. Since Dsh, APC, GSK3β, and β-catenin participate in other signaling events, low Axin levels may also isolate the Wnt pathway from changes in the abundance of the other Wnt signaling components while they participate in different signaling processes [35].

As a typical scaffold protein Axin possess a lot of domains suitable for interaction with different binding partners - APC, GSK3 β, β-catenin, Dvl, and PP2A (Figure 9).

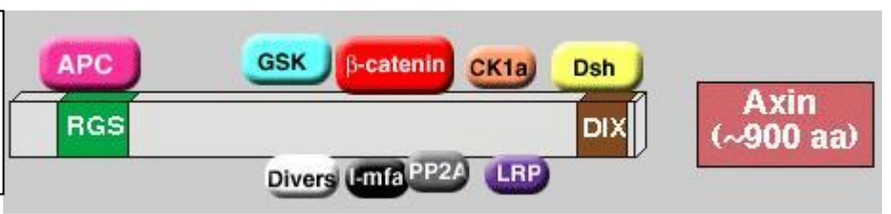


Figure 9. Axin and its binding partners [1; Nusse R.]

Although Axin (Axin1) and Conductin (Axin2) share an overall identity in their amino acids sequence (45%) and they seem to have similar biochemical functions, they display quite different expression patterns. Axin is ubiquitously expressed during embryonic development and in the adults, whereas Conductin appears to be specifically expressed in tissues with active Wnt signaling. Moreover, Conductin (but not Axin) is strongly up-regulated in colon, liver, and ovarian tumors. The promoter of the *conductin/axin2* gene contains functional TCF

binding sites and therefore it is a direct target of the Wnt pathway. Interestingly, it provides a negative feedback loop for the signaling. Taken together, Axin appears to represent the constitutive component of the β-catenin destruction complex, while Conductin is its inducible part. Conductin could be important to prevent an "overshoot" of the signaling, or might act as a rapid switch-off when the Wnt ligands are no longer present. In the absence of wt APC or when β-catenin is mutated, both Axin and Conductin fail to block the accumulation of *β*-catenin. Nevertheless, it cannot be ruled out that these proteins might be able to retain β-catenin in the cytoplasm to some extent and thus attenuate the signal even in the absence of APC [6].

4. 2. 2. 1. 2. APC

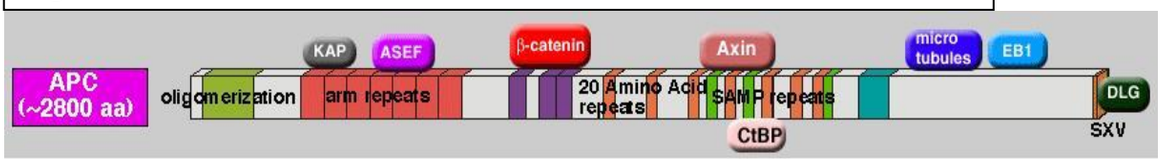
APC stands for adenomatous polyposis coli. The *APC* gene is mutated in familial adenomatous polyposis (FAP), a hereditary form of colon cancer. Carriers of such mutations develop thousands of colon tumors (called polyps), some of which inevitably progress to malignancy. Inactivating mutations in *APC* are also found in the large majority of sporadic colon cancers. APC is therefore an important tumor suppressor in the colon. (See chapter 5.1.3.1.)

APC is a large protein with multiple functions and interactions within the cell, including Wnt signaling transduction, intercellular adhesion, cytoskeleton stabilization and presumably cell cycle regulation and apoptosis.

The *APC* gene is 8535 bp large and has 21 exons. The most common isoform contains 2843-aa. Alternative splicing considers exon 10A that adds further 18 aa. Interestingly, exon 15 includes more than 75% of the coding sequence [20].

APC is highly conserved in vertebrates. *Drosophila* has two redundant *APC* genes which are functionally equivalent to mammalian APC. Human APC and *Drosophila* E-APC are ubiquitous proteins, while human APC2 and *Drosophila* dAPC are predominantly expressed in the nervous system [7]. *C. elegans* encodes a gene for a distantly related APC protein that functions similarly to vertebrate and *Drosophila* APC.

Figure 10. The APC domains [1; Nusse R.]



The APC protein contains an oligomerization domain at its very N-terminus which is followed by the armadillo repeat region. A set of 15- and 20-aa repeats is located in the central part of the protein. The C-terimal part contains the basic domain and the EB1- and HDLG (human discs large) binding sites (Figure 10). The mutation cluster region (MCR) lies between codons 1286 an 1513. The name of this region corresponds to the fact that over 60% of all somatic mutations in *APC* "hit" this region [20].

The oligodimerization domain with its heptad repeats allows APC to generate homodimers - amino acids 6 to 57 are necessary for this interaction. The fact that wt APC can form dimers with both wt and truncated (mutant) APC may explain the dominant negative effect of APC mutants on its tumor suppressor function [20]. The armadillo region possesses seven repeats and is markedly homologic to a similar area in β-catenin. It can bind the B56 regulatory subunit of PP2A and Asef (APC-stimulated guanine nucleotide exchange factor), that is a GEF for the Rac and Rho GTP binding proteins. It was suggested that APC may be involved in the dynamics of the actin cytoskeleton network [20]. The 15-aa repeats provide binding sites for β-catenin. There are three repeats between aa 1020 - 1169. These sites are unique, they do not feature the β-catenin binding sites in the cadherins. Interaction of β-catenin with this portion of APC (in contrast to 20-aa repeats) does not earmark it for proteasome degradation [20].

Cluster of 20-aa is repeated seven times, but only a single repeat is essential for β-catenin binding. It occurs after GSK3β-mediated prosphorylation on each site (as well as 15-aa repeats). At least 3 sites must be present in mutant APC to preserve its ability to downregulate β-catenin. Most of truncated mutant protein found in tumors lack all or most of these repeats, so it can be concluded that this domain is a target to be eliminated during tumorigenesis [20].

Axin binding sites on APC are present within the region of 20-aa repeats where β-catenin binds as well. Axin binds to these sites via its RGS (regulator of the G protein signaling) domain. Conductin possesses a similar domain showing ability to associate with APC. Each Axin binding site has SAMP aa motif (one-letter aa code) that is essential for the interaction. The binding is crucially important for the formation of the degradation complex, which facilitates phosphorylation of both APC and β-catenin by GSK3β. Phosphorylation of APC results in enhanced β-catenin binding and more efficient phosphorylation of the latter molecule by GSK3β [20].

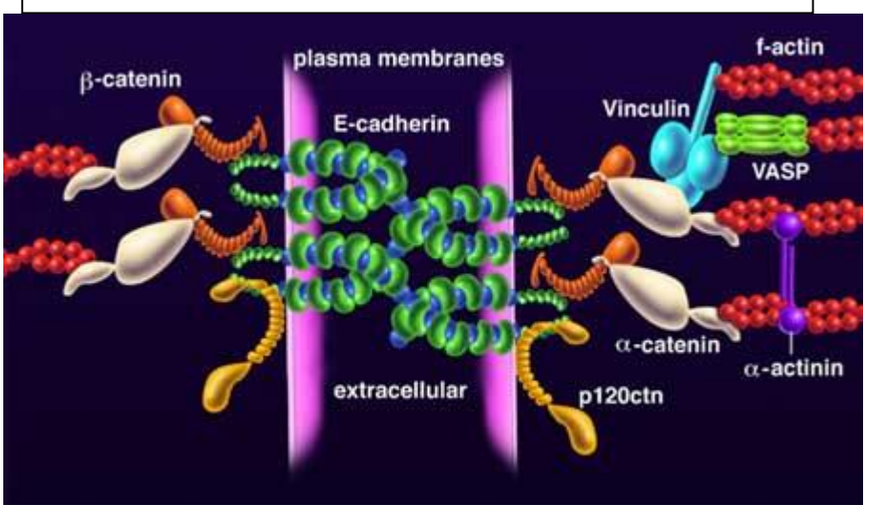
The basic domain of APC is found at its C-terminus between aa 2200 and 2400. It was named according to a remarkable frequency of Arg and Lys residues. Besides basic aa,

it contains an unusually high percentage of prolins; this suggests that it can bind microtubules. Indeed, there are several observations that the C-terminus of APC binds to microtubules and stimulates polymerization of tubulin *in vitro* [20].

EB1 (end-binding protein 1) is closely associated with the centromere, mitotic spindle and (+)tips of microtubules in all stages of the cell cycle. According to studies in yeast it is thought to be involved in checkpoint mechanisms in the cell cycle. The ability of APC to bind EB1 may direct APC to the microtubule (+) tips and therefore facilitate its interaction with specific binding sites at the plasma membrane [20].

All catenins (α-catenin, β-catenin and γ-catenin, also called plakoglobin) are associated with catherins that mediate intercellular adhesion in the epithelia (Figure 11). Thus, because APC can bind β-catenin, it suggests another possible role of APC in the cell. E-catherin is a transmembrane protein in which extracellular domains can homodimerize, keeping neighbouring cell together in zonula adherens junctions. Its cytoplasmatic domain binds to β-catenin (the domain has SLSSL sequence that is also shared by four 20-aa repats in APC). β-catenin additionally binds α–catenin; this provides the link to the actin cytoskeleton.

Figure 11. Adherens junctions [59; humpath.com]



Cytoplasmatic APC accumulates at the leading edges of the cells. This appearance depends on the intact microtubule (but not actin) network. APC is involved in orderly migration of intestinal cells within an intestinal crypt and when it is overexpressed, a migration of these cells in mice gets disordered [20].

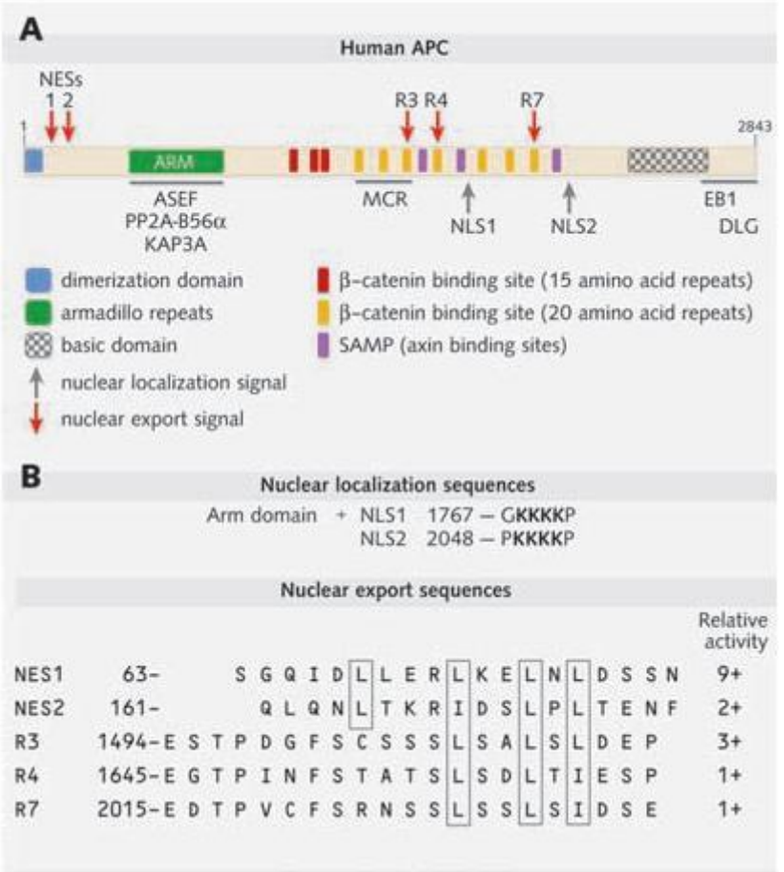
APC's ability to bind HDLG, the human homolog of the *Drosophila* discs large tumor suppressor protein, depends on the final 72 aa. Overexpression of APC suppresses the cell cycle in the transition from G_0/G_1 to S-phase. It was proposed that APC/HDLG may be responsible for this effect [20].

The VSTV motif at the extreme C-terminus of APC binds protein tyrosine phosphatase PTP-BL via its PDZ domain and thus may regulate phosphorylation of other proteins such as β-catenin and GSK3β [20].

APC is also found in the nucleus, where it possibly shuttles β-catenin between the nucleus and the cytoplasm. There are several putative NESs (nuclear export signal) and NLSs (nuclear localization signal) in APC, but various authors differ in their precise location and even in their numbers. According to Henderson and Fagotto [60], there are at least three NLSs, two of these function through the importin-α/β receptor pathway, but they do not seem to be essential for the nuclear localization of APC. Its nuclear export is executed by the CRM1/Exportin receptor pathway and there are at least five NESs in APC (Figure 12).

It is worth mentioning that the 3' end of MCR in APC coincides with one of the NESs suggesting a strong selective pressure to eliminate these NESs during tumorigenesis [20].

Figure 12. NESs and NLSs of APC [60; Henderson, B.R. & Fagotto, F.]



APC can bind directly to DNA as it harbors three potential DNA-binding domains. Possible interaction of APC with both microtubules and DNA indicates its direct role in cell division.

APC in normal human colon is expressed only in the cells in the luminal part of the crypts. These cells are shed after undergoing apoptosis. It is feasible to predict that APC is indirectly involved in regulation of apoptosis. Increased cell death through apopoptosis occurs when expression of wt APC is induced in cancer cell lines containing originally only mutant APC. Apoptosis goes in hand with cell releasing from ECM or from cellular contacts, that's why APC may indirectly provide the stimulus for apoptosis.

The role of APC as a "gatekeeper" in colorectal tumorigenesis is discussed in a chapter about cancer.

4. 2. 2. 2. 3. GSK3β (glycogen synthase kinase 3β) and CKI (casein kinase I)

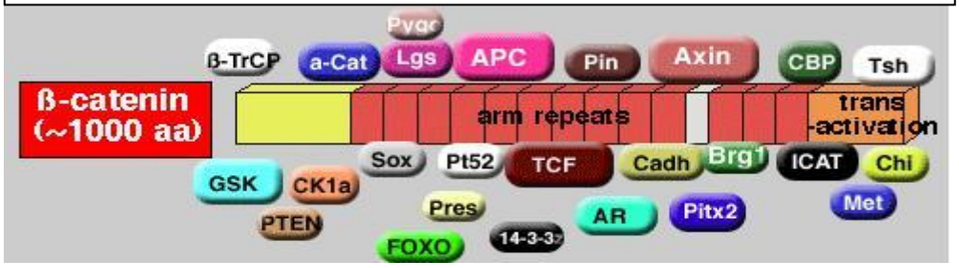
Glycogen synthase kinase-3 was identified originally as a regulator of glycogen metabolism. Today it is known that it may also play important roles in protein synthesis, cell proliferation, cell differentiation, microtubule dynamics and cell motility. There are two closely related isoforms, GSK3α and GSK3β, which are expressed ubiquitously in mammalian tissues. GSK3 homologue in *Drosophila* is called shaggy or zeste-white3 [21].

The substrate specificity of GSK3 is quite unusual as its function requires the presence of another phosphorylated residue optimally located four amino acids downstream to the site of GSK3 phosphorylation. CKI serves as such priming kinase in the β-catenin degradation complex. Despite the observation that GSK3 β phosphorylation of catenin is stimulated 20,000-fold in the presence of Axin, CKI is nonetheless required as a priming kinase in vivo [25]. There are two forms of CKI, CKIα and CKIε, present in the Axin complex. It is believed that CKIα primes β-catenin phosphorylation at S^{45}. Whether CKIε also functions here is not clear as phosphorylation of β-catenin at S^{45} is cell-type specific . Both of these kinases were found to stimulate Wnt/β-catenin signaling [25].

4. 2. 2. 3. β-catenin - not only a key molecule of the Wnt canonical pathway

In the absence of Wnt signaling, β-catenin levels in the cell are kept low owing to its constitutive degradation by the proteasome pathway. Phospho-aa (S^{33}, S^{37}, $T^{41}$and S^{45}) on the N-terminus of this key player act as a tag, promoting interaction of β-catenin with the F-box protein β-TrCP (or Slimb in *Drosophila)*. β-TrCP is the recognition subunit for the E3 ubiquitin ligase complex. In yeast E3 ubiquitin ligases only recognize their substrates when phosphorylated [41]. Ubiquitination of β-catenin serves as a second tag marking it for destruction by the proteasome.

Figure 13. β-catenin and its interaction partners [1; Nusse R.]



β-catenin is encoded by a single gene in most animals;

in humans the gene is termed *CTNNB1*. The *Drosophila* homolog is called *Armadillo*. *C. elegans* has three β-catenin-like genes. HMP-2 is involved in cellular adhesion, BAR-1 functions exclusively in the canonical Wnt pathway and WRM-1 signals during the polarization of early embryonic cells [7].

As mentioned above, β-catenin is a dual-function protein. It is involved in intercellular adhesion (it was initially discovered for this role) as well as in transducing of the Wnt signals from the cytoplasm to the nucleus.

The adhesion function is based on a subcellular pool of β -catenin which is membrane-associated and stable. On the other hand, the signaling function of β-catenin is triggered by a soluble cytoplasmic pool that is highly unstable in the absence of the Wnt signal. Such sharing of a critical component between two fundamental processes may reflect a need for coordinate control between them. Cell signaling is joined with a loosening of adhesion between epithelial cells during epithelial–mesenchymal transitions and also other developmental processes. In addition, cancer progression and malignancy typically depends on inappropriate cell signaling and loss of cadherin-mediated adhesion.

Although the two β-catenin pools are normally functionally separated from each other, experimental manipulations of the levels of one pool can affect the function of the other under some circumstances. The switch between these pools and subsequently functions has been discovered recently [9].

Figure 14. β-catenin and its binding partners [9; Bienz, M.]

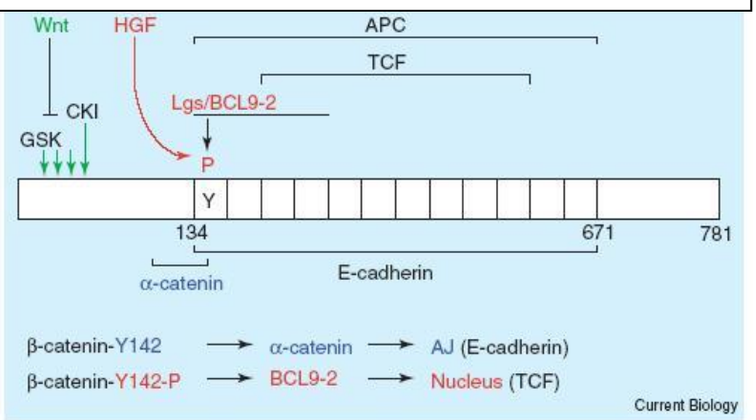


Figure 2. β-catenin and its choice of binding partners in Wnt signaling or cell adhesion.

Schematic drawing of β-catenin (not to scale), showing its amino-terminal phosphorylation sites under the control of Wnt signaling. The core region of β-catenin, consisting of 12 armadillo repeats, is involved in binding to the protein's partners in Wnt signaling (indicated above) or cell adhesion (indicated below); the extents of interactions, indicated by brackets, are based on structural determinations [12,15,17–19], except for Lgs/BCL9-2 (approximate extent of binding [9,10] indicated by dotted line). Phosphorylation of the pivotal Y142 residue by hepatocyte growth factor (HGF) signalling switches the function of β-catenin from cell adhesion to Wnt signaling.

Y^{142}, a tyrosine residue within the armadillo repeat region, needs to be phosphorylated for efficient interaction between β-catenin and BCL9-2. A substitution of this residue with any other amino acid abolishes binding of β-catenin to α-catenin. BCL9-2 is a mammalian relative of the Wnt signaling component Legless in *Drosophila*. Legless and BCL9-2 are crucially required for the function of activated β-catenin/Armadillo in the transcription of the Wnt target genes

(see below). They bind to the N-terminal part of the armadillo repeat domain of β-catenin/Armadillo. Thus, Y^{142} is a pivotal residue within β-catenin that determines whether this protein will bind α-catenin or BCL9-2. Phosphorylation of this important residue by Met receptor tyrosine kinase (also binds hepatocyte growth factor) releases β-catenin from α-catenin to BCL9-2, and thereby from cell adhesion to Wnt signaling (Figure 14) [9].

The question whether Wnt ligands influence the choice of β-catenin for its binding partners remain unclear. According to current evidence they seem not to, or not in a significant way. It would be potentially harmful if the Wnt stimulated cells changes its adhesion properties as a consequence of a diversion of β-catenin from E-cadherin [9].

4. 2. 3. A look inside the nucleus

The increased stability of β-catenin following Wnt signaling leads to its accumulation in the both cytoplasm and nucleus. Once in the nucleus, β-catenin binds members of the Tcf/Lef family of transcription factors and this complex stimulates expression of Wnt/β-catenin target genes. This is the final step of the Wnt canonical cascade.

4. 2. 3. 1. β-catenin

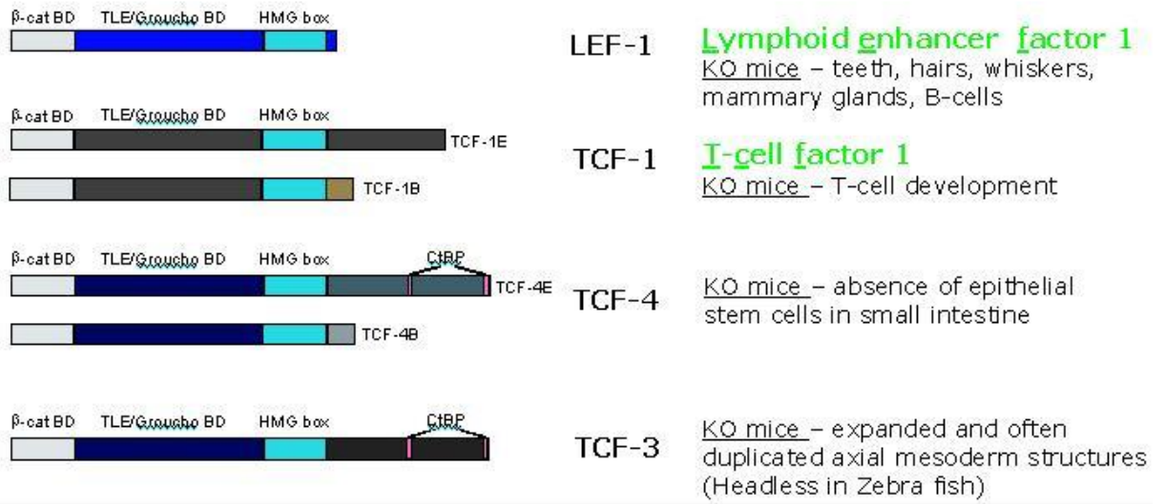
The mechanism of β-catenin transport to the nucleus and regulation of this process remains unclear. For a long time it was believed that APC shuttles β-catenin in and out of the nucleus, as β-catenin contains no recognizable NLS. Recently, it has been proposed that in non-stimulated cells APC actively exports β-catenin from the nucleus, whereas in cancer and Wnt-stimulated cells the accumulated β-catenin can move between the nucleus and cytoplasm independently of APC. It has been also demonstrated that β-catenin can enter the nucleus by binding directly to the nuclear pore machinery. The central armadillo repeats are required for such transport and they are structurally related to the importin β-HEAT repeats which have been shown to associate with the nuclear pore complex [60].

After translocation into the nucleus and binding to TCF/LEF factors, β-catenin activates transcription. Mutational analyses have identified two regions in β-catenin, one near the N-terminus and one at the C-terminus, that are important for transactivation.

4. 2. 3. 2. TCF/LEF transcription factors

There are four TCF/LEF proteins in mammals: TCF-1 (T-cell factor 1), LEF-1 (lymphocyte-enhancer factor 1), TCF-3 and TCF-4 (Figure 15).

Figure 15. TCF/LEF proteins and consequences of their knock outs (KO) in mice [Kořínek V.]



TCFs include an 80 aa HMG (high mobility group) box, that binds to the minor grove of DNA. Its binding dramatically bends DNA and thus TCFs are thought to have an architectural function when organizing the spatial structure of enhancers [55].

In the absence of the Wnt signal, TCFs act as repressors by forming a complex with TLE/GROUCHO corepressors (TLE stands for transducin-like enhancer of split). These corepressors bind both the hypoacetylated N-terminal tail of histone H3 and the human histone deacetylase 1 (HDAC1). Their action is to close DNA to a conformation preventing transcription. The Groucho family members Grg-1, Grg-2, Grg-3 and Grg-4 (in mice; TLE-1-4 in humans) contain five domains and can repress transcriptional activation of any TCF-dependent promoter in a reporter assay. Grg-5 that contains only two domais fails to bind HDAC and acts as a de-repressor [10].

Chibby and ICAT are also negative regulators of β-catenin-mediated transcription. Chibby binds to the C terminus of β-catenin. ICAT (inhibitor of β-catenin and TCF), not only blocks the binding of β-catenin to TCF but also causes dissociation of complexes between β-catenin, LEF, and CBP/p300 [35].

In contrast, β-catenin recruits a number of nuclear factors responsible for the transactivation of the TCF target genes. Two of these factors are the histone acetylase

Figure 16. Nuclear components of the Wnt canonical pathway [35; Logan, C.Y., Nusse, R]

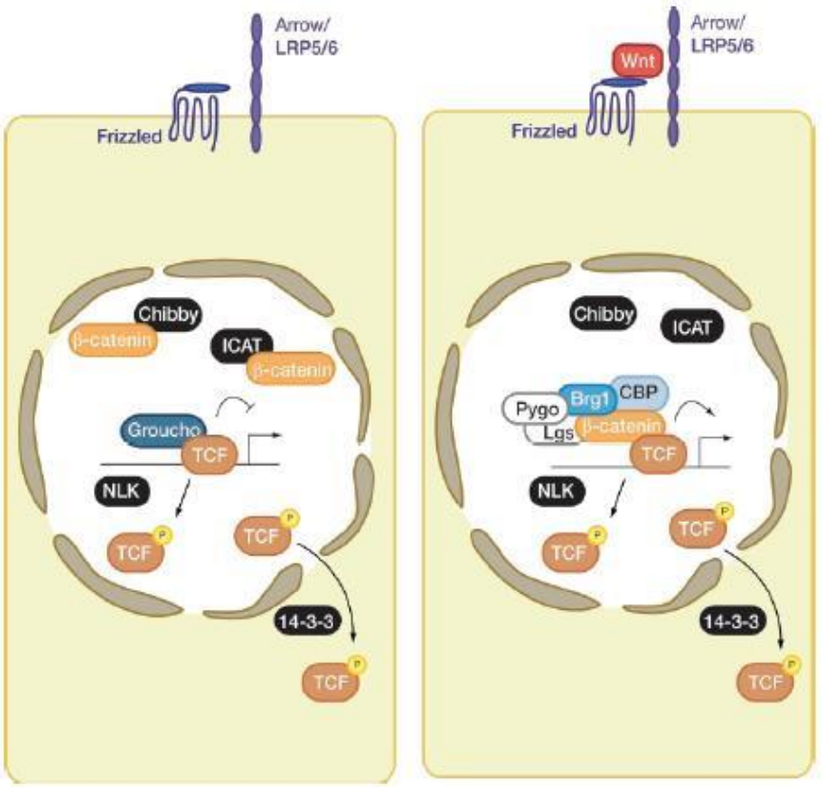


Figure 4 Nuclear factors in Wnt signaling. The interaction between Groucho and TCF is thought to down-regulate transcriptional activation (*left panel*). β-catenin is also negatively regulated by binding to Chibby and Inhibitor of β-catenin and TCF (ICAT). TCF activity in the nucleus can be modulated by phosphorylation by Nemo-like kinase (NLK), and in *C. elegans*, a 14-3-3-like protein has been shown to facilitate nuclear export of TCF (*thin arrow*). β-catenin interferes with the interaction between TCF and Groucho,, and together with TCF, activates gene expression. β-catenin also binds to other components such as Legless (Lgs), Pygopus (Pygo), CREB-binding protein (CBP), and Brg1. Negative regulators are shown in black. Positively acting components are outlined in color.

CBP/p300 (cyclic AMP response element-binding protein) and the SWI/SNF (switching-defective and sucrose nonfermenting) component BRG1 [35]. dCBP/p300 seems to be a negative regulator of Wnt signaling in *Drosophila* cells that are weakly stimulated; there it acetylates TCF and interferes with its binding to Armadillo. In mammalian cells, CBP behaves as a transcriptional coactivator [7]. Activation of the target genes also depends on the nuclear proteins Legless/BCL9-2 and Pygopus. The nuclear location of Legless depends entirely on its direct binding to Pygopus, which is a constitutive nuclear protein of the Wnt cascade. Legless and Pygopus are involved directly in activation of transcription possibly by recruiting chromatin remodeling factors. Humans have two Pygo proteins, both of which are required for TCF-mediated transcription in colorectal cancer cells [52]. Legless is known to transport β-catenin to the nucleus (see above) and thus may function as an adaptor between Pygopus and β-catenin. Mutations in either of these genes result in *wingless*-like phenotypes in *Drosophila*. Both genes also promote Wnt signaling in mammalian cell cultures (Figure 16).

The mitogen-activated protein (MAP) kinase–related protein kinase NLK/Nemo, activated by the mitogen-activated protein (MAP) kinase kinase TAK1, can phophorylate TCF. Such phosphorylation is thought to decrease the DNA-binding affinity of the β-

catenin/TCF complex and in *C. elegans* to export TCF from the nucleus by a 14-3-3 protein (Par5). Recently, another binding partner of β-catenin in the nucleus has been found. It is termed Pitx2 and its binding to β-catenin can convert it from transcriptional repressor into activator [35]. It is likely that more β-catenin interaction partners will be discovered in future. Other nuclear proteins that affect β-catenin-driven transcription, like HIC1 or CtBP will be discussed later.

4. 2. 3. 3. The Wnt target genes

Today there are more than 50 target genes of Wnt signaling. Some of them are components of the pathway itself (Table 2.) providing a feedback control loop.

Table 2. Wnt pathway components as Wnt signaling targets [35; Logan, C.Y., Nusse, R]

TABLE 2 Wnt signaling components as Wnt pathway targets

Target gene	Effect of Wnt signal on target gene expression	Effect of changes in target gene expression on Wnt pathway	Target gene interacts with	Reference
Fz	Down	Inactivate	Wnt	(Muller et al. 1999)
Dfz2	Down	Inactivate	Wnt	(Cadigan et al. 1998)
Dfz3	Up	Activate	Wnt	(Sato et al. 1999)
Fz7	Up		Wnt	(Willert et al. 2002)
Arrow/LRP	Down	Inactivate	Wnt	(Wehrli et al. 2000)
Dally (HSPG)	Down		Wnt	(Baeg et al. 2001)
Wingful/notum	Up	Inactivate	HSPG?	(Giraldez et al. 2002)
naked	Up	Inactivate	Dsh	(Rousset et al. 2001)
Axin2	Up	Inactivate	β-catenin	(Jho et al. 2002)
β-TCRP	Up	Inactivate	β-catenin	(Spiegelman et al. 2000)
TCF1 (dn)	Up	Inactivate	TCF	(Roose et al. 1999)
LEF1	Down	Activate	β-catenin	(Hovanes et al. 2001)
Nemo	Up	Inactivate (*Drosophila*) Activate (*Zebrafish*)	β-catenin/ LEF/TCF	(Zeng & Verheyen 2004, Thorpe & Moon 2004)

A large majority of the Wnt target genes is cell type-specific. Such specificity is common in developmental signaling pathways, and reflects the basic mechanism of gene control by extracellular signals: the cell rather than the signal determines the nature of the response. These genes include developmental regulatory genes such as *siamois*, *twin*, and *Xnr-3* in Xenopus, *ultrabiothorax* in Drosophila. In addition, Wnt signaling also controls genes that are more widely induced (Table 3.). For instance, regulators of the cell growth and proliferation, *c-myc*, *cyclin D1*, c-met tyrosine kinase; a regulator of cell-cell communication, *connexin-43*; the metalloproteinase *matrilysin*/MMP7 and MMP-26, capable of degrading ECM; cell adhesion molecules CD44 and NrCAM; a caspase inhibitor survivin; and VEGH

(vascular endothelial growth factor) that stimulates angiogenesis [37][6]. These target genes provide a clear link to invasion and metastasis of tumor cells.

Table 3. The other Wnt target genes [6; Behrens, J.]

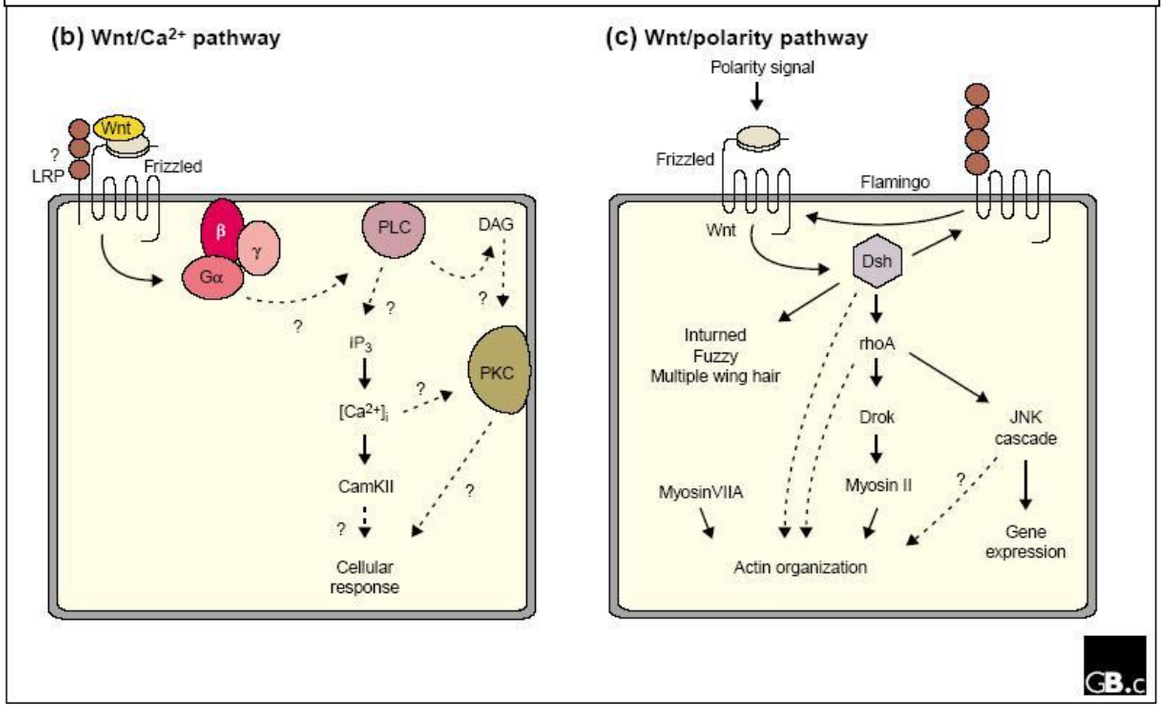
Table 1. *List of β-catenin/Tcf target genes tested functionally* in vitro *or* in vivo

Gene	Type	LOF/GOF	References
c-Myc	-bHLH transcription factor	-knockdown blocks proliferation	He et al. 1998; van de Wetering et al. 2002
Cyclin D1	-cell cycle regulator	-cyclinD1$^{-/-}$/APC$^{min/+}$ show reduced polyp burden	Shtutman et al. 1999; Tetsu and McCormick 1999; Hulit et al. 2004
Id2	-inhibitor of bHLH transcription factors	-Id2$^{-/-}$ develop tumors and show impaired differentiation	Rockman et al. 2001; Russell et al. 2004
ITF-2	-bHLH transcription factor	-overexpression promotes neoplastic transformation	Kolligs et al. 2002
Tcfl	-Wnt signaling	-Tcfl$^{-/-}$/APC$^{min/+}$ show increased polyp burden	Roose et al. 1999
PPARδ	-ligand-activated transcription factors	-PPARδ$^{-/-}$/APC$^{min/+}$ show increased polyp burden -treatment with PPARδ agonist, GW501516, increases number and size of polyps in APC$^{min/+}$	He et al. 1999; Gupta et al. 2004; Harman et al. 2004; Reed et al. 2004
COX-2	-prostaglandin pathway	-COX-2$^{-/-}$/APC$^{\Delta 716/+}$ show reduced polyp burden	Oshima et al. 1996; Hsi et al. 1999; Araki et al. 2003
HDAC2	-histone deactylase	-treatment with HDAC2 inhibitor, valproic acid, reduces polyp number in APC$^{min/+}$ mice	Zhu et al. 2004
FGF18	-growth factor	-knockdown suppresses growth of CRC cells	Shimokawa et al. 2003
FGF20	-growth factor	-knockdown suppresses anchorage-independent growth	Chamorro et al. 2005
Endothelin	-growth factor	-rescues growth arrest and apoptosis resulting from blocking β-catenin	Kim et al. 2005
Gastrin	-gastrointestinal growth factor and hormone	-Gastrin$^{-/-}$/APC$^{min/+}$ show reduced polyp burden	Koh et al. 2000
BAMBI	-BMP and activin membrane-bound inhibitor	-overexpression blocks TGFβ-mediated growth inhibition	Sekiya et al. 2004
MMP7/Matrilysin	-ECM protease	-MMP7$^{-/-}$/APC$^{min/+}$ show reduced polyp burden	Wilson et al. 1997
Nr-CAM	-adhesion	-overexpression increases cellular motility	Conacci-Sorrell et al. 2002
Mdr1	-ABC transporter	-Mdr$^{-/-}$/APC$^{min/+}$ show reduced polyp burden	Yamada et al. 2000, 2003
ENC1	-BTB/Kelch protein family member	-overexpression increases growth rate in CRC cells	Fujita et al. 2001
APCDD1	-unknown	-knockdown inhibits cell/tumor growth	Takahashi et al. 2002

4. 3. The noncanonical Wnt pathways

There are at least two additional intracellular pathways that can transduce Wnt signals. These are the 'Wnt/Ca^{2+}' pathway and the 'Wnt/polarity' pathway (also called the 'planar polarity'pathway). Distinct sets of Wnts and Fz receptors pairs can activate these pathways and lead to the unique cellular responses, different from of the Wnt/β-catenin pathway [36]. (Figure17.) The major function of the Wnt/polarity pathway is regulation of the cytoskeletal organization. The biological function of the Wnt/Ca2+ pathway is unclear, but it is known to be involved in Wnt5a signaling in haematopoetic cells, where it may antagonize the Wnt/β-catenin signaling [34]. Also XWnt4, XWnt5a, and XWnt11 were reported to stimulate this pathway [36].

Figure 17. Wnt/Ca^{2+} pathway and Wnt/polarity pathway [36; Miller, J.R.]
(b) Signaling through the Wnt/Ca2+ pathway appears to involve activation of the two pertussis-toxin-sensitive G proteins, Gαo and Gαt, in combination with G_2. G-protein activation then leads to an increase in intracellular Ca2+ and the subsequent stimulation of Ca2+/calmodulin-dependent kinase II (CamKII). Activation of the Wnt/Ca2+ pathway also results in stimulation of PKC activity in the form of the translocation of PKC to the plasma membrane. Downstream targets of the Wnt/Ca2+ pathway have not been identified. **(c)** The Wnt/polarity pathway, which regulates cytoskeletal organization; the *Drosophila* Wnt/polarity pathway that regulates the polarity of trichomes in the wing is shown as an example. In this case, the nature of the polarity signal is not known.



4. 4. The evolution of the Wnt signaling pathway

As mentioned several times before, components of the Wnt pathway, particularly the Wnt protein, are extensively conserved between *Drosophila* and mammals. The evolutionary relationship between the five Wnt genes of *C. elegans* and the human Wnt genes is less apparent, so it is difficult to determine real orthologs [36]. Cnidaria contain a complete set of the Wnt pathway genes and even primitive organisms, like *Dictyostelium,* have several proteins involved in the pathway, but they are not necessarily regulated by the Wnt signal [35]. The evolutionary relationships between human and also selected invertebrate and vertebrate Wnt genes are shown in Figure 18.

Based on the Drosophila genome, where the paralogous genes wingless, DWnt6 and DWnt10 are located in a close proximity on the chromosome II and all are transcribed in the same orientation, it was proposed that there was an ancient cluster of the Wnt genes consisting of Wnt1, Wnt6 and Wnt10 in a common ancestor of vertebrates and arthropods. In vertebrates, this cluster may have been duplicated with a subsequent loss of some genes [36].

Figure 18. The evolutionary relationships of Wnts [36; Miller, J.R.]

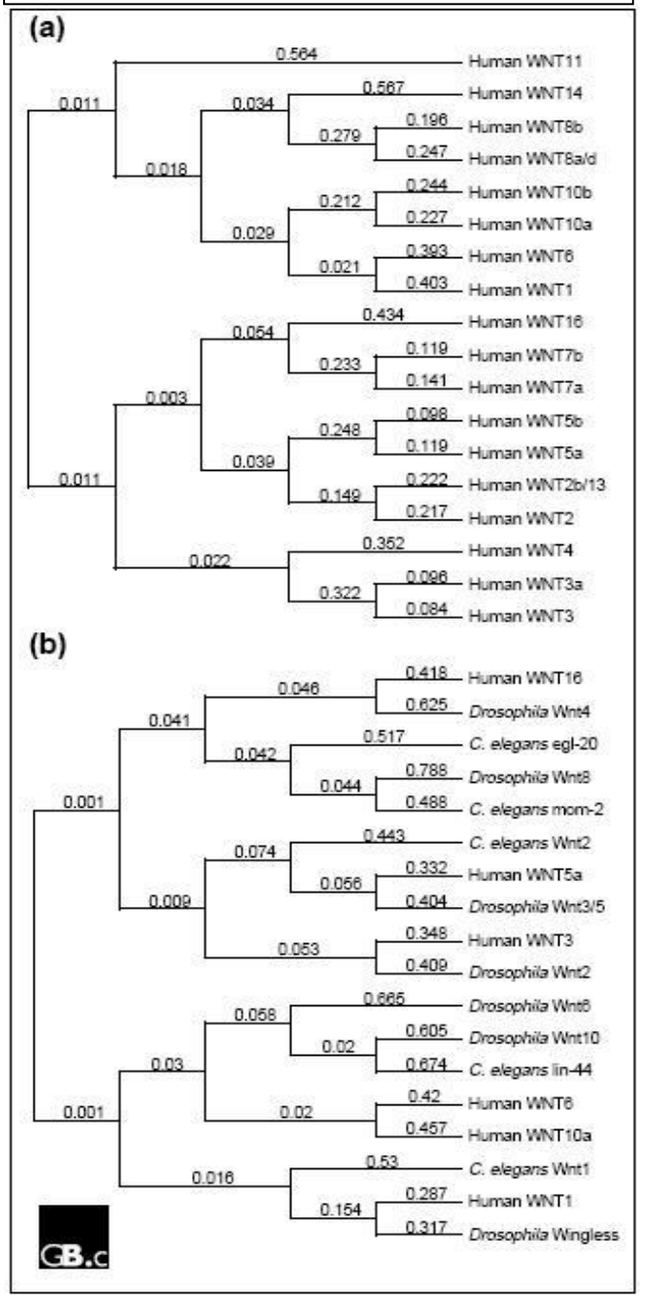


Figure 2
Predicted evolutionary relationships between members of the *Wnt* gene family. **(a)** Predicted relationships between 18 of the 19 known human *WNT* protein sequences; *WNT15* was omitted because only a partial sequence is available. **(b)** Predicted evolutionary relationships between selected human *WNT* proteins (representing each large grouping shown in (a)) and *Wnt* proteins from mouse, *Xenopus*, *Drosophila*, and *Caenorhabditis elegans*. Sequences were aligned using the ClustalW program; trees were constructed from the alignments using the neighbor-joining method and are diagrammed using midpoint rooting. Numbers indicate branch lengths.

5. WNT SIGNALING IN TISSUE DEVELOPMENT, ADULT TISSUE MAINTENANCE, DISEASE AND CANCER

It is not surprising that defects of this fundamental signaling pathway regulating different processes in early development and in the adult tissues can initiate cellular transformation and might lead to cancer.

In this part of my thesis I am going to discuss both signaling and features of normal tissue development as well as deregulated Wnt signaling in cancer.

5. 1. Intestinal epithelium and colorectal cancer

The relevance of Wnt signaling to the biology of the intestine was established around the year 1991, when the tumor suppressor gene *APC* was found mutated in a large number of hereditary and sporadic colorectal cancers [23]. Later it was also shown that the inactivation of APC in colorectal cancer cells results in constitutively active Wnt signaling.

5. 1. 1. Development of the intestinal epithelium

The laboratory mouse is the most common model used in the experimental biology. Thus, at first, I will describe the basic physiology of the mouse gastrointestinal tract (Figure 19.).

Figure 19. Mouse intestinal development [23; Gregorieff, A. & Clevers, H]

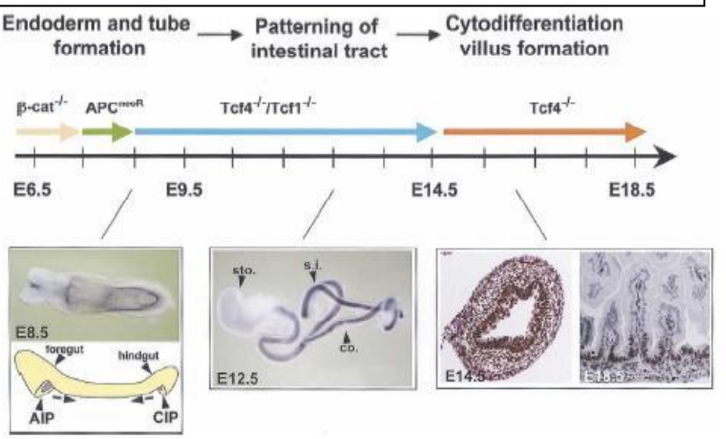


Figure 2. Time line of intestinal development in the mouse. Definitive endodermal cells are specified at E6.0 during gastrulation. The *left* panel shows the bottom view of an E8.5 embryo, along with a schematic representation of the primitive gut. At E8.5 endodermal tube formation is initiated by folding (depicted by arrows) of the endodermal lining at the anterior and posterior ends creating anterior and caudal intestinal portals (AIP and CIP). The endodermal lining is stained with a probe recognizing *Foxa1*. At later stages (E9.5–E14.5), the primitive gut tube is patterned along the anterior–posterior axis. The expression of specific intestinal markers first appears in the hindgut at E9.0 (Maunoury et al. 1992; Braunstein et al. 2002). The *middle* panel shows a whole mount preparation of the entire gastro-intestinal tract (E12.5) stained for the intestinal marker *Villin*. Villus formation and cyto differentiation (formation of enterocytes, goblet cells, enteroendocrine cells and Paneth cells) is initiated at E14.5. In the *right* panel, sections from the small intestine were stained for the proliferation marker Ki67. Mutations associated with the Wnt pathway affect gut development at various stages (see text). Figure adapted and reprinted, with permission, from Wells and Melton (© 1999 by Annual Reviews http://www.annualreviews.org).

5. 1. 2. The intestinal stem cells

The intestinal tract has a rapid epithelial cell turnover, the life cycle of an individual epithelial cell lasts less than a week. In the small intestine of mouse 60 % of crypt cells pass through the cell cycle every 12 hr, around 200 cells are generated per crypt every day and enterocytes pass from base to the top of a villus in 2–3 days. More than 10^8 cells are shed each day in the mouse, and about 10^{11} cells in humans [7]. This process is maintained by a population of stem cells, which give rise to all intestinal epithelial cell lineages - enterocytes, enteroendocrine cells, goblet cells and Paneth cells (Figure 20) [32].

Figure 20. The small intestine [45; Reya, T. & Clevers, H.]

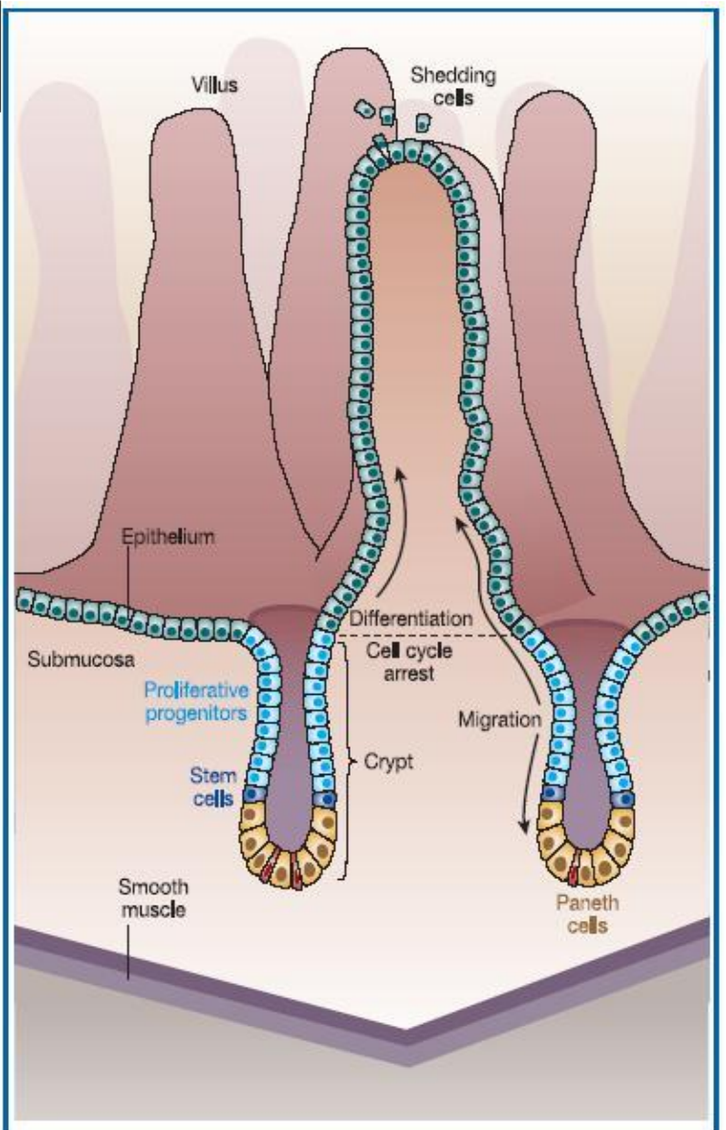


Figure 2 Tissue anatomy of the adult small intestine. Putative stem cells (dark blue) reside immediately above the Paneth cells (yellow) near the crypt bottom. Proliferating progenitor cells occupy the remainder of the crypt. Differentiated cells (green) populate the villus, and include goblet cells, enterocytes and entero-endocrine cells. (Adapted from ref. 89.)

Stem cells are hard to identify as no unique markers are known for ISCs, but they can be labeled *in vivo* with ^{3}H-thymidine or 5-bromodeoxyuridine (BrdU) during early postnatal life or after irradiation. However, it is thought that they reside in a 'niche' towards the base of the crypt at 'position +4' above the Paneth cells and their activity is regulated by the paracrine secretion of growth factors and cytokines from surrounding mesenchymal cells. These cells are possibly intestinal subepithelial myofibroblasts (ISEMFs), that are immediately adjacent to crypt epithelial cells. The number of stem cells vary between one and six per crypt [23] [45] [32].

Division of stem cells is usually asymmetric with the result of an identical daughter stem cell and a committed progenitor cell. Progenitor cells keep the ability to divide until they terminally differentiate. Incidental symmetric division produces either two daughter cells with stem cell loss, or two stem cells [32]. The stem cells cycle slowly, but they produce 'transit-amplifying' cells that proliferate rapidly and are capable to differentiate towards all epithelial lineages. Committed progenitors undergo cell cycle arrest and when they reach the crypt–villus junction, they start expressing differentiation markers. This coincides with the upward migration. The only exception are the Paneth cells, which are generated from progenitors migrating downward to the crypt base. The crypts are monoclonal, but each villus receives cells from multiple crypts, as the differentiated cells stretch along the crypt–villus axis [45]. Polyclonality plays important role in tumor progression as the polyp formation initiates at the crypt-villus boundary [7]. The establishment of the crypt–villus boundaries as well as the positioning of the Paneth cells at the crypt bottom depends on Wnt signaling via control over expression of the EphB/ephrinB sorting receptors and ligands [45].

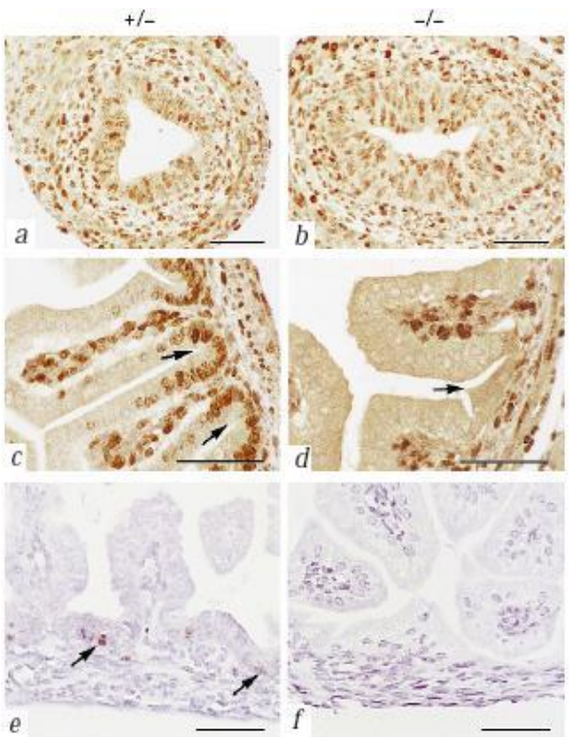


Fig. 6 Absence of cycling cells in *Tcf7l2*$^{-/-}$ small intestinal epithelium. Paraffin sections of the proximal small intestine from E14.5 heterozygous (*a*) or homozygous (*b*), and E16.5 heterozygous (*c*) or homozygous mutant (*d*) embryos stained with an antibody against proliferating cell antigen KI-67, and E17 heterozygous (*e*) or homozygous (*f*) mutant embryos labelled with BrdU and stained with an anti-BrdU monoclonal antibody. At E14.5, numerous proliferating cells are present throughout the epithelium of both heterozygous and homozygous mutant embryos (*a*,*b*). At E16.5, proliferating cells are restricted to the intervillus regions in the epithelium of the *Tcf7l2*$^{+/-}$ embryos (arrows), but are absent from the *Tcf7l2*$^{-/-}$ embryos (arrows in *c*,*d*). There is no apparent difference between the staining of underlying mesenchymal tissue in the heterozygous and the homozygous mutant intestine. BrdU-labelled cells are present in the intervillus regions of the *Tcf7l2*$^{+/-}$ embryos (arrows), but are absent from the *Tcf7l2*$^{-/-}$ embryos (*e*,*f*). Horizontal bars indicate 0.1mm.

Figure 21. Small intestine of mice lacking Tcf-4 [31; Korinek, V. *et al*]

Wnt signaling is required for the gut tube formation. The proliferation of the transit-amplifying cells is also entirely dependent on the continual stimulation of the Wnt pathway [23]. It was well documented that the β-catenin/Tcf-4 complexes drive transcription of the target genes in colorectal cancer cells as well as in the normal stem-cell compartments in the small intestine of mice. *Tcf7l2* $^{-/-}$ (gene *Tcf7l2* encodes Tcf-4) mice die shortly after birth and show a single histopathological abnormality located in the intestinal epithelium. The epithelium of the knockouts is composed entirely of differentiated, non-dividing villus cells (enterocytes) (Figure 21). *Tcf7l2* $^{-/+}$ animals are viable and fertile [31].

5. 1. 3. The colorectal cancer

While in mice the active role of Wnt signaling (physiological and aberrant) considers mainly the small intestine, in humans, it is mostly the large intestine (colon) which suffers from the non-physiological activation of the Wnt signaling cascade.

The nature of colon is quite similar to the small intestine, but it has a flat surface epithelium instead of villi (Figure 22). Stem cells occupy the first cell position at the crypt bottom.

Figure 22. The colon [45; Reya, T. & Clevers, H.]

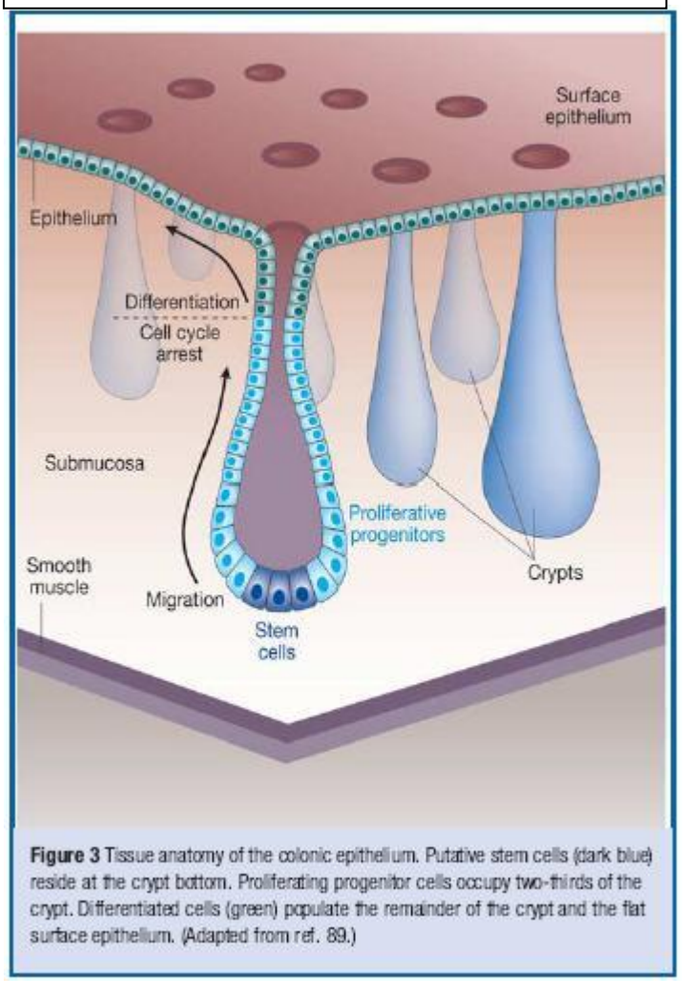


Figure 3 Tissue anatomy of the colonic epithelium. Putative stem cells (dark blue) reside at the crypt bottom. Proliferating progenitor cells occupy two-thirds of the crypt. Differentiated cells (green) populate the remainder of the crypt and the flat surface epithelium. (Adapted from ref. 89.)

According to Hahn and Weinberg, a normal cell has to acquire six phenotypes in order to behave as a fully transformed malignant cell. These phenotypes are resistance to growth inhibition, immortalization, independence from mitogenic stimulation, the ability to acquire their own blood supply, the ability to invade and metastasize and the ability to suppress or evade apoptosis [57].

On a global scale, colorectal cancer is the fourth most common malignant neoplasm after lung, breast and prostate cancer. More than 50% of the Western population develop colorectal adenomas by the age of 70 and at least 85% of these tumors contain mutations in *APC* [30]. The tumors are derived from benign adenomatous lesions that are estimated to take 5–15 years to evolve into invasive cancer. Epidemiological and clinical data suggest that if these premalignant lesions are identified and removed, the subsequent development of colorectal cancer is aborted. Since adenomas are largely asymptomatic, not many cases are caught up in time [57].

Mutations in *APC* are thought to be the primary event in overwhelming majority of sporadic colorectal tumors. These mutations are also the molecular base for inherited FAP syndrome (see below). One of many studies proving the importance of the deletion of the

APC gene for development of the intestinal malignancies described inducible deletion of the single *APC* allele in mice. Within 1–2 days, villi of the experimental animals were entirely populated by crypt-like cells. DNA array analysis revealed the induction of many of the crypt markers previously identified as the Tcf-4 targets in human colorectal cancer cell lines [45]. This and other similar observations resulted in APC's nickname "gatekeeper" of colorectal tumorigenesis.

5. 1. 3. 1. APC - a "gatekeeper" in initiation of colorectal neoplasia

Germline mutation (often at codons 1061 and 1309) of the *APC* gene cause the autosomal dominant inherited disease called FAP (familial adenomatous polyposis). FAP patients, inheriting one defective *APC* allele, develop large numbers of colon polyps, or adenomas. This mostly appears in the third decade of their lives. The polyps are clonal outgrowths of the epithelial cells which lost the second wt *APC* allele (Figure 23).

Figure 23. Adenomas [7 Bienz, M. & Clevers, H]

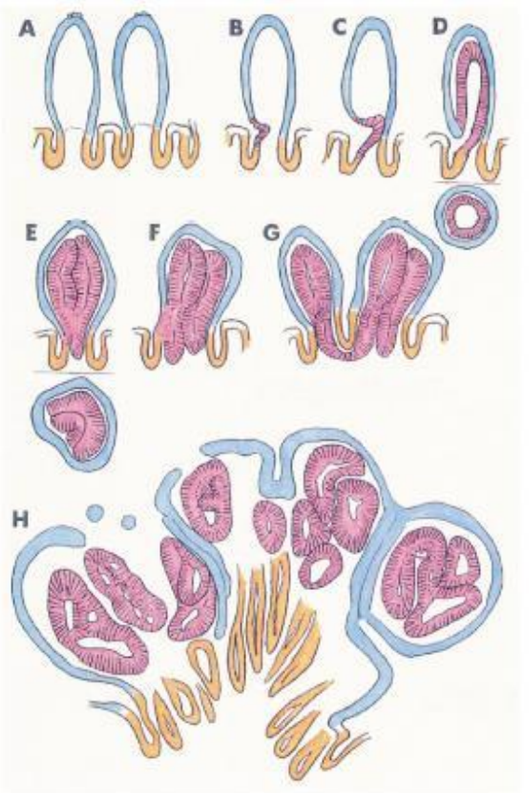


Figure 2. Adenomatous Polyp Formation in *Apc* Mutant Mice
(A) Normal intestinal epithelium (yellow, crypt cells; blue, differentiated cells in the villi). (B and C) Outpocketing pouches form at the proliferative zone of the crypt and protrude into the lacteal side of neighboring villi. (D–F) Microadenoma formation within single villi. (G) Expansion of a microadenoma into a neighboring villus. (H) Multivillus polyp formed by fusion of multiple adenomas, in the process of rupturing through the villus epithelium to protrude into the gut lumen. Adenomatous tissue in pink. This figure is reproduced from Oshima et al. (1997).

When untreated, polyps develop cancer inevitably by the early forties at latest. The risk of cancer seems to be related to the polyp number. It is possibly due to the clonal "evolution" of the individual polyps as they accumulate mutations in additional oncogenes or tumour suppressor genes, such as p53, K-Ras and Smad4. The incidence of FAP is 1 in 8000 and is maintained by mutation *de novo* (1/4 of all cases) [20].

Somatic mutations in *APC* are implicated in majority of sporadic colorectal cancers and occur early in tumorigenesis. Mutations within the MCR (see above; codons 1309 and 1450) are so-called hotspots. Such mutations result in the

truncated APC protein which lacks all the Axin binding sites, most of the β-catenin-intracting repeats and several NESs. In accordance with the Knudson's two-hit hypothesis, the position of the first mutation determines the nature of the second hit. The MCR mutations are associated with LOH (loss of heterozygosity) and the non-MCR mutations with the truncations. It indicates a strong selective pressure for a more advantageous mutant rather than the prime *APC* mutant change that emerges at first [20]. It is not clear why cancer cells retain the truncated APC versions, as these APC variants are not sufficient in downregulating the transcriptional activity of the TCF/β-catenin complexes. Interestingly, various truncated APC proteins differ in their ability to reduce β-catenin/Tcf-regulated transcription (CRT). Figure 24. shows schematic structures of four common APC mutants found in hereditary and sporadic colorectal tumours along with the corresponding CRT luciferase activities.

Figure 24. Truncated APC proteins and their inhibitory effect on CRT [39; Morin, P.J. *et al*]

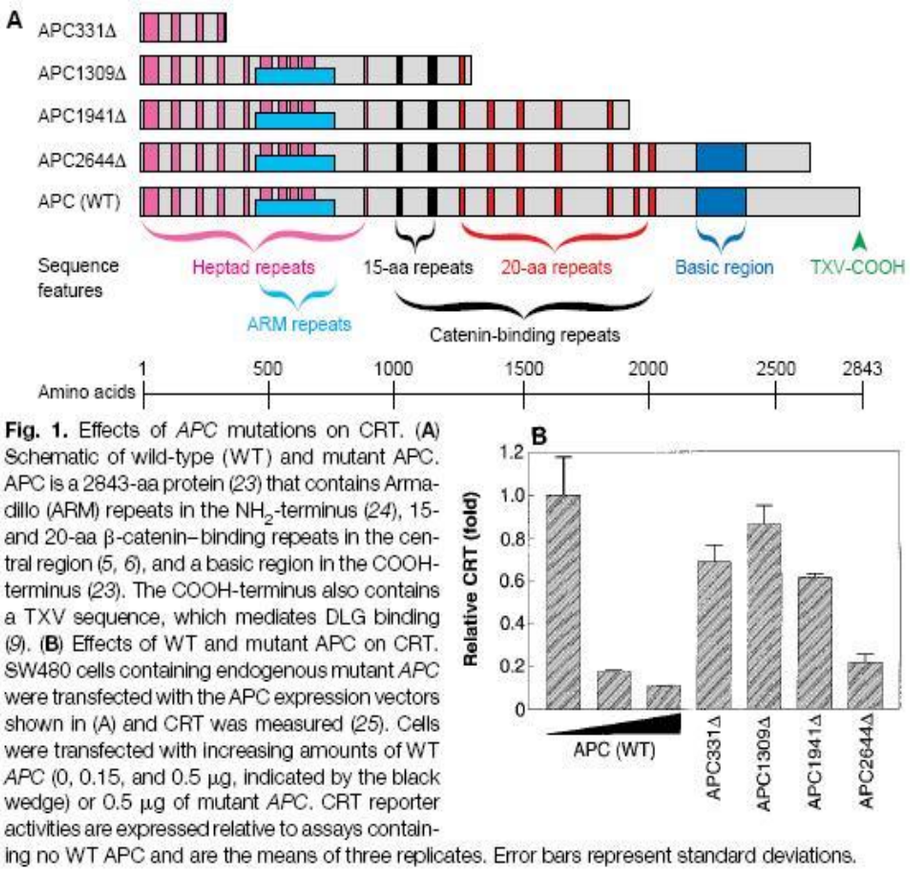


Fig. 1. Effects of *APC* mutations on CRT. (**A**) Schematic of wild-type (WT) and mutant APC. APC is a 2843-aa protein (*23*) that contains Armadillo (ARM) repeats in the NH_2-terminus (*24*), 15- and 20-aa β-catenin–binding repeats in the central region (*5, 6*), and a basic region in the COOH-terminus (*23*). The COOH-terminus also contains a TXV sequence, which mediates DLG binding (*9*). (**B**) Effects of WT and mutant APC on CRT. SW480 cells containing endogenous mutant *APC* were transfected with the APC expression vectors shown in (A) and CRT was measured (*25*). Cells were transfected with increasing amounts of WT *APC* (0, 0.15, and 0.5 μg, indicated by the black wedge) or 0.5 μg of mutant *APC*. CRT reporter activities are expressed relative to assays containing no WT APC and are the means of three replicates. Error bars represent standard deviations.

Tcf-4 seems to be the only member of the Tcf/Lef family expressed in both normal and neoplastic colonic epithelium. The interaction of β -catenin with Tcf-4 is regulated by APC and transcriptional activation of target genes occurs only when Tcf-4 is associated with β-catenin. Colon carcinoma cells with homozygous disruption of *APC* contain large amounts of monomeric, cytoplasmic β-catenin as well as stable β-catenin/Tcf-4 complexes. Treatment with wt APC prevents Tcf-4 binding, reduces the overall amount of β-catenin and lowers the transcription of the Tcf/β-catenin target genes [30]. A key Tcf/β-catenin target gene, *c-myc*, prevents cell-

cycle arrest in the crypt cells via p21 repression. In concordance with results mentioned above, loss of APC in the transit-amplifying cells (located in the upper part of the crypt) seems to cause an expansion of the crypt compartment. This is believed to be the key event that initiates tumor formation [8].

Considering the APC's role in tumorigenesis, its contribution in intercellular adhesion and dynamics of cytoskeleton, both features also involved in cancer progression, cannot be left out. Furthermore, truncated but not wt APC activates the ASEF (see above), which in turn activates the small G-protein Rac and thereby stimulates cell migration. APC also plays a role in the correct establishment of the mitotic spindle and disturbance of the APC function by mutations could lead to chromosomal instability (CIN), which is observed in the majority of colorectal tumors [6].

APC mutations are far more rarely found in other than colorectal tumor types. However, they have been reported in hepatoblastomas, gastric, pancreatic, thyroid, breast, and ovarian cancers. In melanoma cells both genetic and epigenetic alterations of *APC* were found. In 13% of tested cell lines and 17% of melanoma biopsies hypermethylation of the *APC* promoter 1A was present. By reason that these cells do not show increased Wnt-induced transcription - perhaps due to residual APC expressed from promoter 1B- it was proposed that epigenetic silencing of the promoter 1A may contribute to the development of malignant melanoma [28]. Epigenetic silencing of gene transcription by CpG island promoter hypermethylation is a common event in tumor biology. For instance, at least four genes (*SFRPs, WIF-1, DKK-1* and *HIC1*) that inhibit Wnt signaling on different levels of hierarchy have been reported in colon cancer cell lines [4].

5. 1. 3. 2. Mutations in other components of the Wnt cascade

Not all colorectal tumors possess *APC* mutations, so there must be more possibilities how to provoke the hallmark of Wnt signaling, the stabilized β-catenin. Thus, β-catenin itself is the most likely candidate. Indeed, it is mutated in up to 10% of all sporadic colon carcinomas and moreover, *CTNNB1* mutations are observed in other tumor types, such as liver tumors [6].

Experiments done on two colorectal tumor cell lines that express wild-type APC (HCT116 and SW48) revealed mutations in the β-catenin gene (*CTNNB1*) affecting Ser^{45} and Ser^{33}, i.e. the GSK3β phosphorylation sites. These mutations are somatic with a dominant effect, their CRT is insensitive to the effects of wtAPC, but their activity can be inhibited by

dominant-negative Tcf-4 [39]. These and other experiments support the former assumption that GSK3β phosphorylation sites are crucial for regulation of β-catenin amounts available for transducing Wnt signals. A removal of these destruction promoting motifs has immense consequences. Intriguingly, β-catenin and APC mutations are mutually exclusive, which possibly reflects the fact that both components act in the same signaling pathway.

Figure 25. β-catenin mutations in human tumors [37; Miller, J.R. *et al.*]

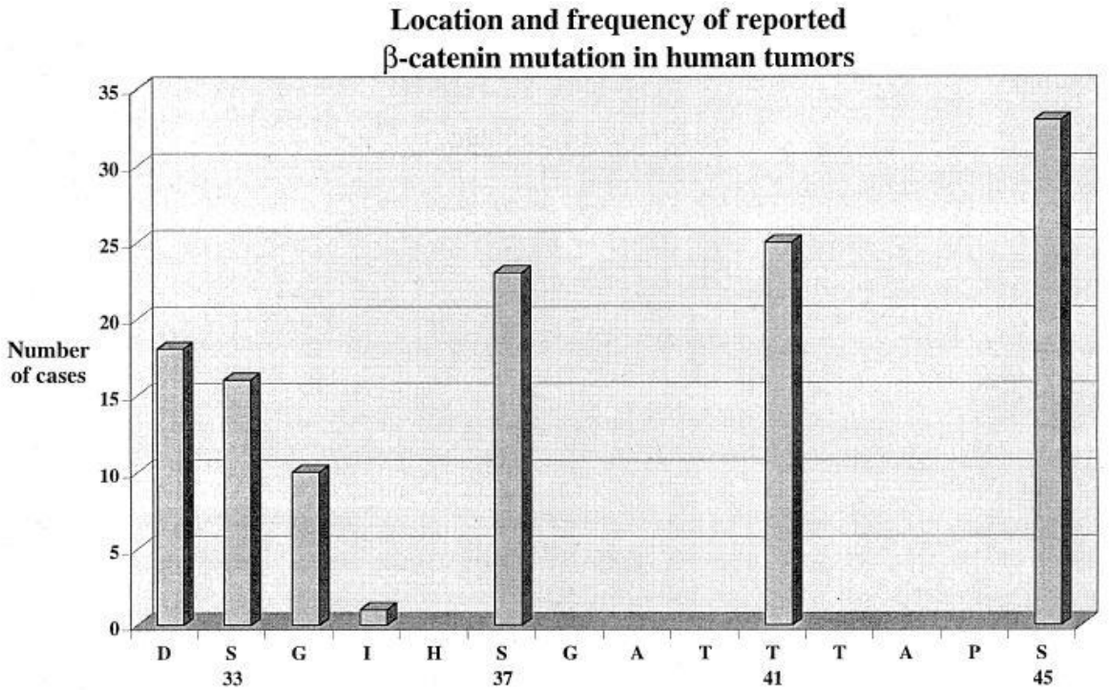


Figure 3 Mutations of *β*-catenin in human cancers. (a) Analysis of the location and frequency of reported *β*-catenin mutation in human tumors. Amino acid sequence of the destruction box of human, mouse and *Xenopus β*-catenin (aa32–45). Numerical superscripts indicate the number of times a particular mutation has been identified. Letter superscripts indicate cited reference. Δe3 indicates a deletion in exon 3. (b) Histogram depicting location and frequency of reported *β*-catenin mutations. [A](Iwao *et al.*, 1998; Morin *et al.*, 1997; Samowitz *et al.*, 1999; Sparks *et al.*, 1998); [B](Kobayashi *et al.*, 1999); [C](Blaker *et al.*, 1999); [D](de La Coste *et al.*, 1998; Legoix *et al.*, 1999; Miyoshi *et al.*, 1998); [E](Iwao *et al.*, 1999); [F](Zurawel *et al.*, 1998); [G](Gamallo *et al.*, 1999; Palacios and Gamallo, 1998; Sagae *et al.*, 1999; Wright *et al.*, 1999); [H](Chan *et al.*, 1999); [I](Voeller *et al.*, 1998); [J](Iwao *et al.*, 1999); [K](Fukuchi *et al.*, 1998); [L](Koesters *et al.*, 1999); *multiple mutations were found in two samples

Mutations of *conductin/axin2* have also been described. They occur in 25% of the microsatellite- unstable colorectal tumors and germline mutations show prevalence for the formation of oligodontia and colorectal neoplasias. Mutations of the *axin* and *conductin/axin2* genes have also been identified in various other tumor entities, like medulloblastomas, endometrioid ovarian carcinomas, hepatomas and hepatocellular carcinomas [6].

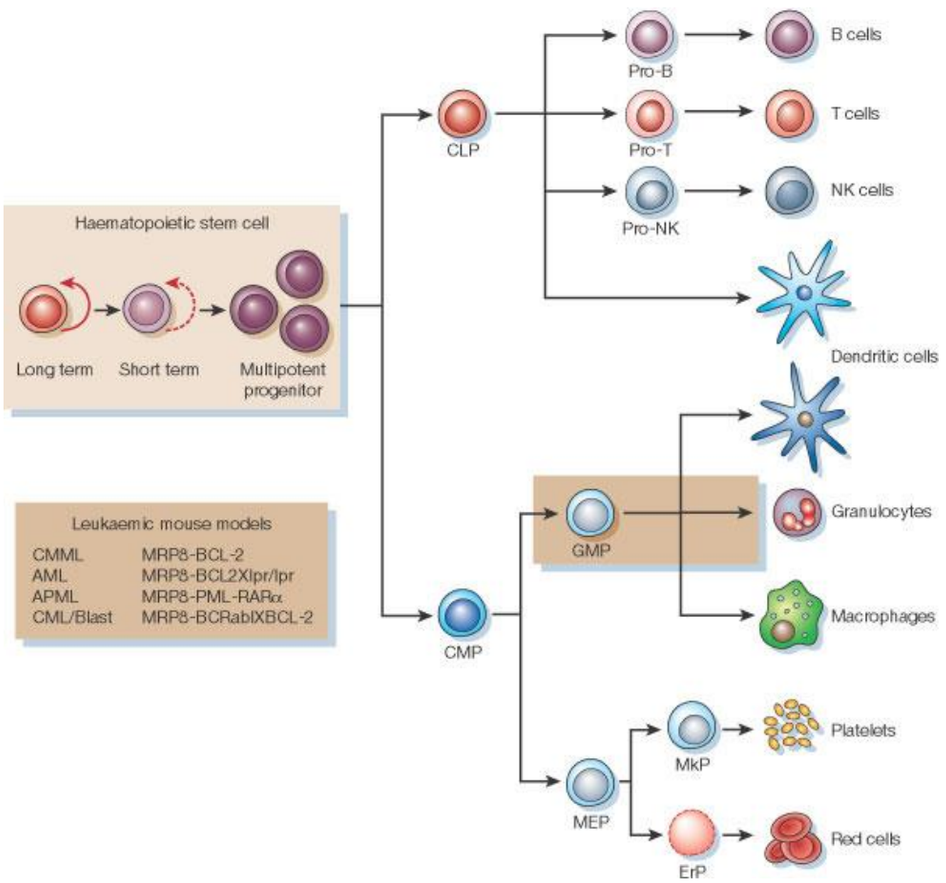
Wnt signaling is not only the initiator of many cancers but also an important player in tumor progression. It seems that Wnt signaling can act differently at different stages of cancerogenesis. This is based on the knowledge of the early dysplastic lesions resulting from

mutations of *APC.* They are non-malignant and do not show any of the properties of invasive cells. It was proposed that the level of Wnt signaling increases during the tumor progression involving stimulation by autocrine mechanisms. β-catenin staining in colorectal carcinomas often shows a heterogeneous pattern with strong nuclear enrichment at the invasion front and mainly cytoplasmic and membrane distribution in the central tumour area [6]. In progression to malignancy further genetic changes are needed. These can be loss of the response to TGF-β (tumour growth factor β) or oncogenic activation of K-ras and/or mutations in the *p53 tumor suppressor* [7].

5. 2. Wnts in haematopoietic stem cells and in leukemia

5. 2. 1. HSCs

Figure 26. HSCs and their development into all types of blood cells [nature.com]



Haemat opoietic stem cells (HSCs) are perhaps the best understood stem cells in human body (Figure 26). HSCs renew themselves and give rise to all lineages of the blood throughout our lifetime.

HSCs make up a very rare population of cells, approximately 0.007% of bone marrow in the mouse (Figure 27). They are characterized by their expression of lineage specific

markers (such as B220, CD3,Mac-1), high levels of c-Kit and Sca-1 and low levels of Thy-1 [46]. The first HSCs in development are derived from mesoderm. Consistent with this, their proliferation and differentiation is regulated by mammalian homologues of hedgehog, BMPs, Notch ligands and Wnts [50]. HSCs directly respond to Wnt signaling *in vivo* – the Wnt proteins synergize with stem cell factors and promote the growth and inhibit the differentiation of the murine haematopoietic progenitors [45].

Overexpression of activated β-catenin, S^{33} mutant, and use of Wnt3a-conditioned medium expand the pool of HSCs in long-term cultures *in vitro*. In contrast, the ectopic expression of axin or a frizzled ligand-binding domain lead to inhibition of HSCs growth *in vitro* and also reduced reconstitution of the haematopoietic system of lethally irradiated mice *in vivo* [46].

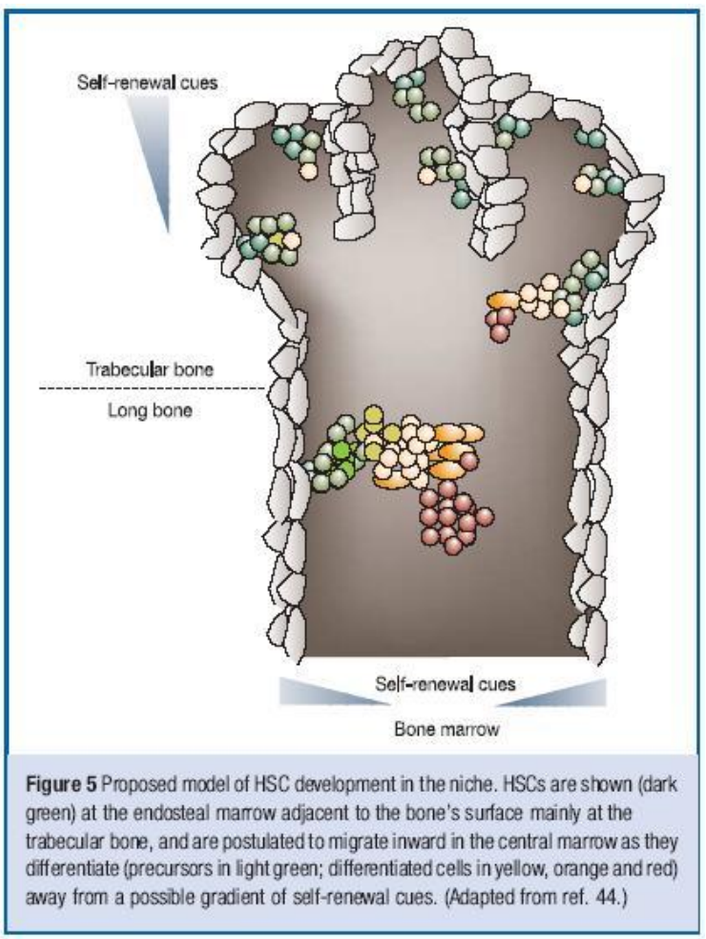


Figure 5 Proposed model of HSC development in the niche. HSCs are shown (dark green) at the endosteal marrow adjacent to the bone's surface mainly at the trabecular bone, and are postulated to migrate inward in the central marrow as they differentiate (precursors in light green; differentiated cells in yellow, orange and red) away from a possible gradient of self-renewal cues. (Adapted from ref. 44.)

Figure 27. Haematopoietic stem cells [45; Reya, T. & Clevers, H.]

Both canonical pathway (e.g., Wnt3a) and non-canonical pathways (e.g., Wnt5a) seem to influence HSCs equivalently. Wnt5a, similarly to other Wnts, can upregulate cell proliferation, when it signals via the canonical pathway. For instance, in the presence of the stromal cells it promotes the expansion of the undifferentiated progenitors. Thus, in fact, it fuctions as a protooncogene. On the other hand, it was reported that when Wnt5a signals through the noncanonical Wnt/Ca^{++} pathway, it suppresses cyclin D1 expression and negatively regulates B-cell proliferation. In additon, $Wnt5a^{-/+}$ mice develop myeloid leukemias and B-cell lymphomas that are of a clonal origin and also display loss of the Wnt5a function in tumors. Furthermore, in human primary leukemias deletion of the *WNT5A* gene and/or loss of WNT5A expression is observed in a majority of the patients, thus aiming Wnt5a to be a

tumor suppressor [34]. It was also published that in some context, Wnt5a may signal through heterotrimeric G proteins to activate PLC-dependent phospholipid turnover in the membranes of ER and release of intracellular Ca^{++}. Such release activates protein kinase C (PKC) and calmodulin-dependent protein kinase II (CamKII). This leads subsequently to a block of the β-catenin signaling cascade and activation of calcium-sensitive transcription factor NFAT [34].

As foreshadowed on the previous page, WNT signaling occurs not only in HSCs but also in more committed lymphoid progenitor cells (Figure 28).

Figure 28. Wnts in haematopoiesis and thymopoiesis. [50; Staal, F.J.T. & Clevers, H]

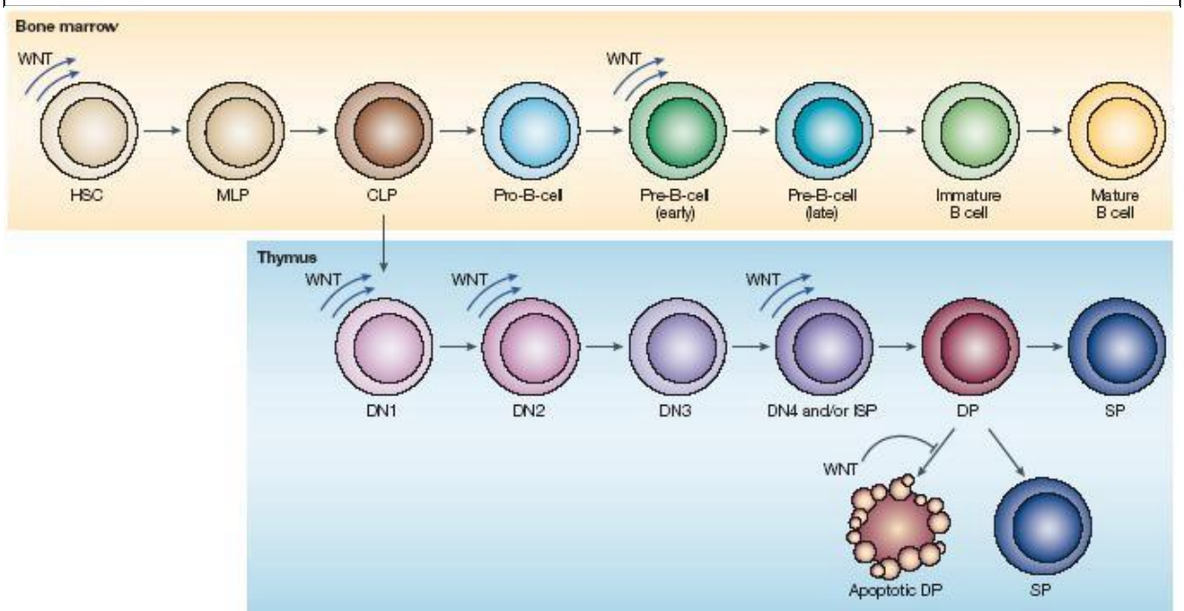


Figure 3 | **WNT signalling in haematopoiesis and thymopoiesis.** A schematic depiction of the various cellular stages of B- and T-cell development in the bone marrow and thymus is shown. Myeloid development is not shown, because the role of WNT signalling in myelopoiesis is unknown. WNT signalling provides proliferative (as indicated by blue arrows) and anti-apoptotic signals at the stages of haematopoiesis shown. Pro-B cells are defined as $CD19^+$cytoplasmic IgM^- or, sometimes (using the Hardy classification scheme), as $B220^+CD43^+$. Precursor (pre)-B cells are defined as $CD19^+$cytoplasmic IgM^+ or, sometimes, as $B220^+CD43^-$cell-surface IgM^- (Hardy classification). CLP, common lymphoid precursor; DN, double-negative thymocyte; DP, double-positive thymocyte; HSC, haematopoietic stem cell; ISP, immature single-positive thymocyte; MLP, multi-lineage precursor; SP, single-positive thymocyte.

TCF/LEF proteins were originally discovered in models for early lymphocyte development - TCF-1 owing to its ability to bind the CD3ε enhancer. In fact TCF-1 is the first definitive T-cell marker that directly precedes CD3ε expression. LEF-1 can bind TCRα enhancer and also plays a key role in B-cell development. In adult tissues TCF-1 expression is exclusive to the T lymphoid lineages, whereas LEF-1 is expressed in pro-B lymphocytes as well. TCF-3 and -4 does not seem to be involved in the immune system. Among TCF/LEF proteins LEF-1 is unique as it contains a context-dependent activation domain (CAD) that activates transcription when ALY coactivator is present. Mice KO approach revealed that TCF-1 is essential for the maintenance of the early thymocyte progenitor compartments but it

is redundant for mature T cells. During embryogenesis the expression of LEF-1 overlaps with TCF-1 and there is a large functional redundancy between these two Tcf/Lef family members at the protein level. The observations made for T cell lineage are similar to those seen for the B-cell lineage. Mature B-cells express neither TCF-1, nor LEF-1 and there is some current evidence that the Wnt cascade does not operate in mature lymphocytes [55].

5. 2. 2. Leukemia

Leukemia is the most common malignancy found in the child's patients. The two main types of leukemia are acute and chronic. Acute leukemia tends to affect younger people. The symptoms develop rapidly and it can be quite life-threatening disease if not treated properly. The most common form is acute lymphocytic leukemia (ALL). Chronic leukemia tends to affect older people. The disease gets worse slowly and has a more prolonged progression. With chronic leukemia, the leucocytes are almost fully grown and normal when they enter the blood stream. They can exert some functions but not at the same levels as the normal cells. Chronic myeloid leukemia (CML) has two phases, a chronic phase that may last several years - during this stage symptoms develop quite slowly, followed by a more aggressive phase (accelerated phase), where symptoms become rapidly worsening.

Only recently it has been proved that oncogenic growth in leukemias of both myeloid and lymphoid lineages is dependent on Wnt signaling (Table 4.). Consistently with this, progenitor cells must adopt a stem-cell-like ability to be transformed [45].

Table 4. Wnts and haematopoiesis malignancies [50; Staal, F.J.T. & Clevers, H]

Table 1 | **Involvement of dysregulated WNT signalling in haematological malignancies**

Disease	Cells studied	WNT-signalling involvement	References
AML	Human cell lines	Increased plakoglobin expression induced by transfection of translocation fusion genes	61
CLL	Human cell lines	High-level expression of WNT and frizzled mRNA	62
Pre-B-ALL	Human cell lines	E2A–PBX1⁺ pre-B-ALL cells express WNT16	63
CML	Human primary cells	Activation of WNT-mediated signalling, as measured by reporter genes and activated β-catenin during blast crises	64
Multiple myeloma	Human primary cells	Expression of the soluble WNT inhibitor DKK1, and high levels of both the total pool of β-catenin and the nuclear, activated form	65,66
B-cell lymphoma	Mouse primary cells	*Wnt5a* functions as a tumour-suppressor gene in B cells	67

AML, acute myeloid leukaemia; CLL, chronic lymphocytic leukaemia; CML, chronic myeloid leukaemia; DKK1, dickkopf 1; E2A, transcription factor E2A; PBX, precursor-B-cell leukaemia transcription factor 1; pre-B-ALL, precursor-B acute lymphoblastic leukaemia.

CML results mainly from a BCR-Abl translocation. It originates in HSCs but exhibits its markers in more committed myeloid precursors. Interestingly, Granulocyte–macrophage progenitors (GMPs) from CML patients and blast crisis cells from patients resistant to therapy display activated Wnt signaling. This was determined by the Wnt reporter activity, accumulation of nuclear β-catenin and by decrease of the replating capacity of these leukaemic cells *in vitro* by the ectopic expression of axin [45]. One of the defining features of AML is impaired differentiation of the malignant cells to mature granulocytes and monocytes. It was found that β -catenin is expressed in primary human CD34+ progenitor cells and is downregulated during myeloid differentiation. CD33+CD34+ cells at the myeloblast stage have very low/undetectable levels of this Wnt effector. Primary AML blasts have a range of β-catenin expression, and this does not appear to correlate with CD34 expression. It indicates that the link between β-catenin downregulation and myeloid differentiation may be uncoupled in leukemia [49].

Multiple myeloma is a cancer of terminally differentiated B cells and in many patients it is characterized by lesions in the bones that are caused by the presence of myeloma cells [50]. Although it is clear that WNT signalling provides proliferative signals for the most immature progenitor cells in both the B- and T-cell lineages as well as self-renewal signals for HSCs, many questions remain to be answered. One of the most interesting ones concerns the interaction of WNT signals with other signaling pathways in immature thymocytes (such as pathways initiated by ligation of Notch, the IL-7 receptor or the pre-TCR) and in immature B cells and HSCs.

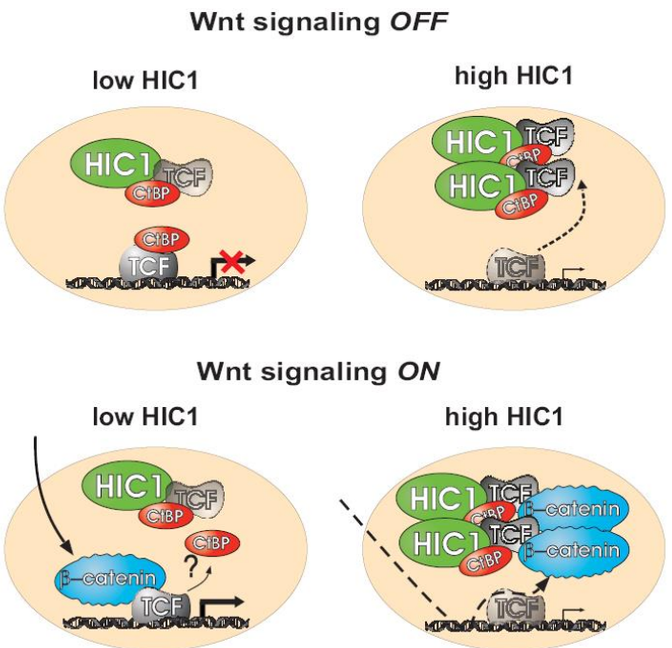
5. 3. Concluding remarks

It has been shown in several studies that the molecular intervention within the frame of the Wnt signaling pathway has a promising therapeutical potential. For example, as mentioned above, Wnts help to retain the stem cells in undifferentiated state during prolonged cultivation *in vitro.* This phenomenon clearly implies a possible use of the Wnt ligands during the bone marrow transplantation procedures. Additionally, inhibitors of Wnt signaling such as 6-bromoindirubin-3'-oxime (BIO) which acts on GSK3 and exisulind, the inhibitor of β-catenin signaling, interfere with growth of cancer cell lines. Currently, various biotechnological and chemical genetics approaches are used to discover compounds which could influence the levels of Wnt signaling in the normal or cancer cell. Such compounds are

of a great hope for people suffering from tumors induced by non-physiological activation of the Wnt signaling cascade.

6. HIC1 TUMOR SUPPRESSOR

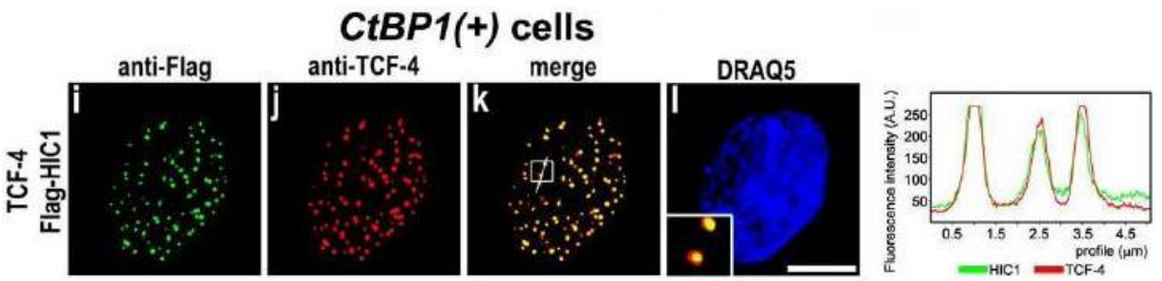
Figure 29. HIC1 and Wnt signaling © NPG press



HIC1 (hypermethylated in cancer 1) was isolated as a candidate tumor suppressor gene during the screening of chromosome 17p, that is frequently altered in human cancers.

HIC1 interacts with at least two proteins involved in Wnt signaling, CtBP and TCF-4. Furthermore, HIC1 can oligomerize via the N-terminal BTB/POZ domain and form discrete nuclear structures called HIC1 bodies. We described recently that HIC1 recruits TCF-4/β-catenin complexes to the nuclear bodies and prevents transcription of the Wnt-responsive genes (Figure 29, 30.) [54]. The characterization of the additional HIC1 functions related to Wnt signaling is a subject of my future diploma thesis (see the final part of the bachelor thesis).

Figure 30. Simultaneous interaction between CtBP, TCF-4 and HIC1 is essential for the efficient nuclear sequestration of TCF-4 into the HIC1 bodies. [54; Valenta *et al.*] Confocal microscopy images of *CtBP1(+)* cells transfected with the indicated constructs (left) and stained with anti-Flag and anti-TCF-4 antibody. The right panel shows the overlap of fluorescence intensity peaks along profiles as indicated in the merged micrographs.



6. 1. *HIC1* gene

HIC-1 gene is located at 17p13.3, locus SMG6. This region is hypermethylated or subject to allelic loss in many human cancers. It spreads also within the 350-kbp critical region deleted in most patients with Miller-Dieker syndrome (MDS) [24].

The gene spans 4.6 kb, it contains three exons, and codes for two alternative transcriptional products. They are transcribed from two promoter elements: the first containing a conserved GC box; the second containing a TATA box [47]. Recently a third promoter has been described but without further functional significance so far [13]. Both transcripts are present in many human tissues, but exon 1a transcripts are predominant [44]. That is a "classical" 714-amino acid BTB/POZ protein, the less common transcript is a protein with 19 additional residues located upstream of the BTB/POZ domain[24].

HIC-1 is extremely GC-rich; exon II is a classic CpG island itself and both promoters are encompassed within CpG islands [47]. In addition, there are six NotI restriction sites (each with 2 CpG dinucleotides) in the 11 kb region around the HIC-1 gene (the average frequency in the genome is one NotI site every 150 kb). The density of CpG dinucleotides in the *HIC-1* gene makes it an ideal target for inactivation through methylation [22].

6. 2. HIC1 protein

Human HIC1 is a 714 aa polypeptide that belongs to the BTB/POZ family (Broad-Complex, Tramtrack, Bric à brac/ Pox virus and Zinc finger) of transcription factors. Besides the N-terminal BTB/POZ domain, it possesses five Krüppel-like C_2H_2 (Cys_2-His_2) zinc finger motifs located towards C-terminus and a CtBP (C-terminal binding protein) interacting motif in the central part (Figure 31).

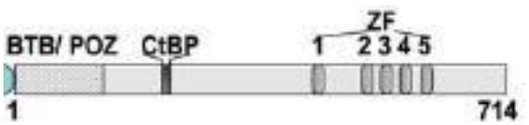


Figure 31. HIC1 protein [54; Valenta, T. *et al.*]

The similar organization of zinc fingers is found in several transcription factors, for example BCL-6 and PLZF that are involved in human neoplasia [24].

During embryonic development, mouse Hic1 is expressed in mesenchymes of the sclerotomes, lateral body wall, limb, and craniofacial regions that embed the outgrowing peripheral nerves during their differentiation. Hic1 is also expressed in mesenchymes opposed to the precartilaginous condensations, at many interfaces to budding epithelia of

inner organs, and weakly in muscles [3]. In adulthood HIC1 is found in all tissues [58]. Constitutive expression of *HIC-1* by stable transfection in various cancer cell lines results in a significant decrease in their clonogenic survival, which suggests that *HIC1* might suppress cell growth [24].

Mice KO approach revealed HIC1 essentiality for development. *Hic1* knockout results in embryonic and perinatal lethality and these mice exhibit a range of gross developmental defects such as acrania, exencephaly, cleft palate, limb abnormalities and omphalocele and are smaller than wt mice [58].

Disruption of only one *Hic1* allele predisposes mice to a gender-dependent spectrum of malignant tumors with an onset after 70 weeks of life. 75% of the malignancies were epithelial cancers in males, whereas among the females 85% of the malignancies were lymphoid and mesenchymal cancers. $Hic1^{+/-}$ showed another distinct phenotype - over 25% of these mice develop severely pruritic ulcerative dermatitis around the facial area.

Knudson's hypothesis says that both alleles of a gene must be inactivated (either by methylation, mutation, or deletion) for a tumor suppressor gene to be inactivated. The complete loss of *Hic1* function in the $Hic1^{+/-}$ mice seems to involve dense methylation of the promoter of the remaining wild-type allele. Interestingly, in malignancies isolated from males, hypermethylation was observed mainly in the downstream promoter (1b) while in females it was mainly in the upstream (1a) promoter.

6. 3. Epigenetical silencing of HIC1

Genes can be inactivated by genetic of epigenetic routes. DNA methylation is one of the mechanisms to silence transcription. The addition of a methyl group at the 5-position of cytosine occurs exclusively at CpG dinucleotides. In the vertebrate genome CpG dinucleotides represent only 1% of the genome. In some regions, known as CpG islands, CpG residues can make 6% or more of all dinucleotides. CpG islands are usually associated with the promoter regions of housekeeping genes and are unmethylated. Methylation of CpG islands occurs only at silenced genes or on the inactive X chromosome and in parentally imprinted genes. In addition, CpG islands may become methylated upon oncogenic transformation. Such silencing is present in tumor suppressor loci such as *p15, p16, Rb, VHL, e-cadherin, ER,* and *HIC1* or *APC.* The silencing involves the packing of methylated DNA into a closed chromatin configuration. It is mediated by MeCP2 (methyl-CpG binding

protein 2), which has a methylation-specific DNA binding site and a transcriptional repression site, that recruits histone deacetylases. HDACs make histones bind DNA more tightly, by deacetylating their lysine-rich tails and potentially blocking the access of the transcription factors [47].

HIC1 has been found methylated in a large number of adult solid tumors, including those of the colon, lung, breast, brain, kidney, liver, cervix, ovary and prostate [44]. It has been also observed in various pediatric tumors, like medulloblastomas (the most common malignant brain tumor in children), ependymomas, retinoblastomas, rhabdomyosarcomas, germ cell and neurouroblastic tumors, neuroblastomas, ganglioneuromas and ganglioneuroblastomas [44][47][56]. 100% incidence of this methylation is in recurrent acute lymphocytic leukemias [44]. Intriguingly, the *HIC1* promoter is methylated also in some healthy human tissues. Normal breast ductal tissues and normal prostate epithelium includes approximately equal amounts of densely methylated and completely unmethylated HIC1 [22]. A baseline level of methylation was also found in normal brain tissues [47]. However, the correlation between hypermethylation of *HIC1* and reduced or lost expression did not reach statistical significance in ependymomas [56]. There was also no statistically significant correlation between methylation status and LOH. Methylation was neither correlated with clinical risk category and is therefore an independent prognostic factor. Furthermore, LOH status was not correlated with overall survival [47].

6. 3. 1. HIC1 and p53

TP53 is a very potent tumor suppressor gene. Its product, p53, is a transcription factor that integrates a number of pathways that mediate apoptosis in response to a wide range of cellular stresses, including DNA damage, hypoxia and nutrient deprivation, cell survival and proliferation. Thus, it couples stimuli promoting cell division to those promoting cell death.

TP53 resides at 17p13.1. So two tumour suppressor genes are located closely on the same chromosome and additionally, *HIC1* expression can be up-regulated by p53 as a functional p53 binding site was found in its promoter. Deletion of these regions on at least one chromosome is frequently observed in cancers. The intact copy for *p53* is then often mutated, and that for *Hic1* is frequently hypermethylated .

In mice, these genes are localized closely on chromosome 11. A double KO approach was used to study the relationship between these tumor suppressors. $Hic1^{+/-}$, $p53^{+/-}$ mice were either *trans* (alleles deleted on separate chromosomes) or *cis* (on the same chromosome).

Each of these cases yields distinct tumor phenotypes. *Trans* mice develop breast and ovarian carcinomas and metastatic osteosarcomas with epigenetic inactivation of the wild-type *Hic1* allele and interstitial deletion of the wt copy of *p53*, whereas *cis* germline deletion results in earlier appearance and increased prevalence and aggressiveness of osteosarcomas with genetic deletion of both wt alleles. It underscores the selection pressure for rapid tumorigenesis as the deletion can simultaneously include the wild-type *p53* on the same chromosome, whereas promoter hypermethylation inactivates only *Hic1* [14].

6. 4. Miller-Dieker syndrome (MDS)

MDS is a contiguous gene deletion syndrome, all cases of which are found to be associated with haploinsufficiency on chromosome 17p13.3. The frequency in population is 1:50,000. Another syndrome connected with deletion in this region is Isolated lissencephaly sequence (ILS). Patients with MDS have a severe grade of lissencephaly, a heterogeneous human developmental brain disorder caused by defects in neuronal migration events that normally occur at 9–13 week of embryonic development. Lissencephaly encompasses varying degrees of agyria or pachygyria (the absence or thickening, respectively, of the convolutions of the cerebral cortex). MDS patients display also a characteristic dysmorphic facial appearance, including prominent forehead with bitemporal hollowing, short nose with upturned nostrils, thickened upper lip, widely spaced eyes, low ears, and small jaw. MDS has been associated with other abnormalities, including central hypotonia, heart malformations, or omphalocele. Taken together, these patients exhibit mental and motor impairment, epilepsy, and a reduced life span [58].

The lisssencephaly and mental retardation have been clearly attributed to haploinsufficiency in the *LIS1* gene. Other anomalies, like craniofacial dysmorphology, defects of the limbs and digits, and omphalocele, are also observed in *Hic-1*$^{-/-}$ mouse embryos and additionaly, parts of the *HIC-1* expression territories as defined by *in situ* hybridization studies of mouse embryos overlap with regions that exhibit abnormalities in MDS patients [24].

7. THE RESULTS OF THE STARTING PROJECT

In this chapter I am going to briefly sketch out, what I have done on my project in the last few months, and also the line I am going to follow in the near future.

The aims of the project:

(1) HIC1 expression profiles in mouse and human tissues (real time qRT-PCR, immunohistochemistry, immunocytochemistry)

(2) Searching for HIC1 interaction partners (GST pull-down assays, MALDI-TOF)

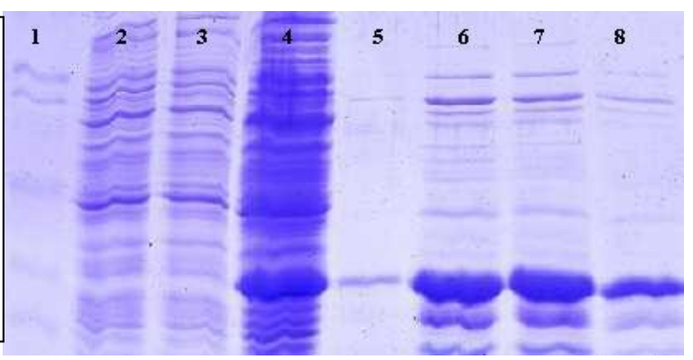
(3) Identification of the HIC1 target genes (ChIP on chip analyses-Nimblegen promoter array)

Currently I work on tools that I will use in my project.

Anti-β-catenin polyclonal antibodies

- PCR fragment of C-terminus (bp 1963-2650) of hβ-catenin was cloned into pET-28b vector (to obtain N-terminal His-tag)
- heat-shock transformation of *E.coli,* strain TOP 10 (to amplify the plasmid)
- production of protein in *E.coli,* strain BL21(DE3)
- isolation of the protein using TALON (via His-tag) in native (Hepes) and denaturated form (6M urea) (Figure 32.), purifying of the protein by dialysis in molecularporous membrane tube
- immunization of rabbits
- testing of ascites (in process)

Figure 32. SDS-PAGE of β-catenin
Lines: 1. Low range standard (Biorad)
2., 3. Before IPTG induction
4.After IPTG induction
5.-8. The first, second, third and fourth fraction of elution from TALON
β-catenin →



Anti-HIC1 monoclonal antibodies

- expression of hHIC1 from pET-28b- flHIC-1 construct in *E.coli*, strain BL21(DE3)
- isolation of the protein using Talon (via His-tag) in denaturated form (6M urea), purifying of the protein by dialysis in molecularporous membrane tube
- immunization of mice
- testing of ascites on COS-7 cells transfected with distinct HIC-1 constructs
- purifying of Ig on protein A/G and further testing on ELISA
- hybridomas (done by a lab assistent)
- screening of hybridomas and their subclones on ELISA, COS-7 transfected cells, western blots- total and/or nuclear lysates of cells with endogenous HIC-1 (WI38), inducible expression of HIC-1 (DLD-1/HIC-1 ARIAD), transfected cells (HEK 293) (Figure 33)
- so far 3 stable subclones producing specific IgG and 1 producing IgM

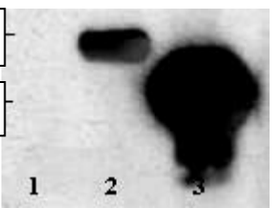


Figure 33. An IgG anti-HIC-1 antibody stained western blot
Lanes: 1- HEK 293 parental cells lysate
2- HEK 293 transfected with HIC-1-EYFPconstruct
3-HEK 293 transfected with HIC-1-FLAG construct
The anti-HIC1 antibodies were visualized by goat-anti-mouse/HRP conjugate (Biorad) and ECL detection system.

8. ACKNOWLEDGEMENTS

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