Instituto Juan March
de Estudios e Investigaciones

151 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Wnt Genes and Wnt Signalling

Organized by

J. F. de Celis, J. C. Izpisúa Belmonte and R. Nusse

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K. M. Cadigan
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Introduction
Roel Nusse

Over the past few years, there has been a surge in interest in the function of Wnt molecules in biological processes. Wnt proteins are secreted from cells, act as short range signaling molecules and are now recognized as one of the major families of developmentally important signaling molecules. Mutations in *Wnt* genes display remarkable phenotypes in the mouse, *C. elegans* and in *Drosophila*. Among functions provided by Wnt proteins are such intriguing processes as embryonic induction, the generation of cell polarity, and the specification of cell fate. One example of the role of Wnt signaling in body plan formation is axis formation and head induction in vertebrate embryos. Several inhibitors of Wnt signaling have been discovered and when those inhibitors are over-expressed or deleted, dramatic phenotypes ensue. Wnt signaling in vertebrates also plays a role in limb outgrowth. In addition, several components of Wnt signaling are implicated in the genesis of human cancer.

Until 5-6 years ago, our knowledge of the molecular mechanism of Wnt signaling was very limited, but over the past years, several major gaps have been filled. These include the identification of cell surface receptors and a novel mechanism of relaying the signal to the cell nucleus. These insights have come from different corners of the animal kingdom and have converged on a common pathway. Wnt signal transduction proceeds through a complex series of protein interactions. Signaling is initiated by binding of the Wnt protein to cell surface receptors, which then generate a signal to downstream components. There are two kinds of receptors: Frizzled and LRP; LRP is a general receptor, while the Frizzleds are specific for certain Wnts. After activation of Dishevelled, a key event in signaling is the regulation of the GSK3 protein kinase and its substrate beta-catenin. In the absence of a Wnt signal, GSK3 phosphorylates beta-catenin, which then becomes targeted for degradation. Beta-catenin and GSK are brought together by two scaffolding proteins: APC and Axin. The binding of Wnt to its receptors initiates a cascade of events that inhibits GSK3 and ultimately results in an accumulation of beta-catenin. Together with the DNA binding protein TCF, beta-catenin activates expression of Wnt target genes.

Remarkably, by studying this pathway, researchers have been able to predict which components could play a role in human cancer, and have indeed identified mutations in Wnt signaling components in such important human tumors such as colon carcinomas and melanomas. This component, beta-catenin, is now viewed as a major human oncogene, while negative regulators of Wnt signaling, in particular Axin and APC, are important tumor suppressor genes. At the same time, these molecule serve as an example of the power of the combinatorial approach that researchers have taken in this field: the Drosophila counterpart of beta-catenin (called armadillo) was first found in the fly as a component of the Wnt (or wingless) pathway.

The findings on the function of Wnt signaling in various organisms underscore the importance of bringing together researchers working in different areas. It was therefore extremely useful to have a meeting at the Juan March Foundation that was entirely devoted to Wnt signaling. The meeting resulted in extensive dialogues between researchers working on flies or worms and those working on human cancer.

Roel Nusse

Session 1: Wnt and Cancer
Chair: José F. de Celis

WNT signalling regulates axonal microtubule organization

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The formation of functional neuronal connections requires the proper navigation of axons to their target and the assembly of functional synapses. In recent years, a number of signalling molecules that regulate the behaviour of axons have been identified. These axon guidance molecules regulate the stability and organisation of microtubules and actin filaments. However, the mechanisms by which these molecules regulate the neuronal cytoskeleton are poorly understood. We have taken advantage of the well-characterised WNT signalling pathways to unravel the mechanisms that control microtubule stability and organisation in developing neurons.

We have previously showed that WNTs such as WNT-7a and WNT-3 induce profound effects on the morphology of axons. WNTs increase growth cone size, axon branching while decreasing axonal extension. Our studies of cultured neurons and the *Wnt-7a* mutant mouse have shown that WNT-7a regulates the morphological maturation of multisynaptic structures in the cerebellum. Our data is consistent with the view that WNT-7a acts as a retrograde signal that regulates pre-synaptic differentiation. Changes in axonal morphology induced by WNTs are associated with increased microtubule unbundling and the formation of looped microtubules at enlarged growth cones. To understand the mechanism by which WNT signalling regulates microtubules, we have examined Dishevelled, a downstream component of the WNT signalling pathway. Dishevelled is associated to axonal microtubules and increases microtubule stability. In the canonical WNT pathway, Dishevelled regulates ß-catenin-TCF transcriptional activation by inhibiting GSK-3ß, a serine/threonine kinase. We found that Dishevelled stabilises microtubules through the inhibition of GSK-3ß. However, Dishevelled signals to the microtubules through a ß-catenin- and transcriptional-independent pathway. More importantly, we found that axonal microtubules are stabilised by Dishevelled when this protein is localised to the axon. We also demonstrate that localisation of Dishevelled to axons results in local stabilisation of microtubules by regulating the phosphorylation of MAPs that directly control microtubule dynamics.

This work is funded by The Wellcome Trust.

BCL9-2, the homolog of BCL9/legless, is an essential component of the nuclear β–catenin/Lef-Tcf complex and acts in the Wnt signaling pathway *in vivo*

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The Wnt/β–catenin signaling pathway controls decisive steps in early development such as axis formation and patterning of mesoderm and neuroectoderm, and is involved in tumor progression in human.

Here we describe the identification and characterization of BCL9-2, the homolog of the putative oncogene BCL9, which is required for Wnt signaling *in vitro* and *in vivo*. In a search for novel proteins which modulate the transcriptional activity of the N-terminal half of β–catenin, we identified in a Yeast-2-Hybrid screen BCL9-2 as β–catenin binding protein. Both BCL9 and BCL9-2 share in total seven highly conserved clusters of homology, among them the β–catenin binding domain. Biochemical analysis demonstrates strong interaction of BCL9-2 with β–catenin and requires the first two armadillo repeats of β–catenin. Both, BCL9 and BCL9-2 activate the Lef/Tcf dependent transcription together with stabilized β–catenin. An N-terminal homology domain of BCL9-2 appears to be essential for transcriptional activity and particular localization within the nucleus. Cloning of the human, mouse and zebrafish orthologs of BCL9-2 and BCL9 shows that both proteins have an overall identity of approx. 35%. *In vivo* analysis of BCL9 and BCL9-2 in zebrafish development reveals different functions of the two homologs. By gene ablation studies using morpholino injections and RNA overexpression, we found that BCL9-2 acts downstream in the Wnt pathway.

We provide genetic evidence that BCL9-2 is an essential component in the Wnt cascade to specify patterning in early development.

Thus, our data reveal a specific function of BCL9-2 within the Wnt signaling pathway *in vitro* and *in vivo*.

GSK-3 binding proteins in Wnt signalling

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In Wnt signalling, GSK3 is recruited to a multiprotein complex via interaction with Axin, where it hyperphosphorylates -catenin, marking it for ubiquitination and destruction. We have now determined the crystal structure of GSK3 in complex with a minimal GSK3-binding segment of Axin, at 2.4Å resolution. The structure confirms the co-localisation of the binding sites for Axin and FRAT in the C-terminal domain of GSK3, but reveals significant differences in the interactions made by Axin and FRAT, mediated by conformational plasticity of the 285-299 loop in GSK3. Detailed comparison of the Axin and FRAT GSK3 complexes allows the generation of highly specific mutations, which abrogate binding of one or the other. Quantitative analysis suggests that the interaction of GSK3 with the Axin scaffold enhances phosphorylation of -catenin by >20,000 fold.

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Control of tissue growth by the bantam microRNA

Stephen Cohen

miRNAs are a class of short 21-23 nucleotide RNA molecules implicated in the control of gene expression. Large numbers of miRNAs have been identified, suggesting that they will constitute a significant proportion of the genetic information in plans and animal cells. To date functions have been assigned to very few miRNAs, but those studied regulate post-transcriptional expression. I will present recent results showing that the *bantam* gene of Drosophila encodes a 21-nucleotide miRNA that stimulates cell proliferation and simultaneously prevents apoptosis, thereby promoting tissue growth. *bantam* miRNA expression is temporally and spatially regulated in response to patterning cues that control cell proliferation during development, suggesting that *bantam* miRNA provides an essential link between pattern formation and growth control. One factor limiting the analysis of miRNA functions is the difficulty in identifying the genes that they regulate. I will present a computational method for predicting possible targets of miRNAs. Using this method we identify the anti-apoptotic gene *hid* as a target for regulation by the Drosophila *bantam* miRNA, providing an explanation for the anti-apoptotic activity of *bantam*.

Endocytosis regulates Wingless signaling

Elaine S. Seto and Hugo J. Bellen

In the developing *Drosophila* wing, Wingless spreads from the dorsal-ventral boundary to form a symmetric morphogen gradient that is essential for proper cell proliferation and cell fate specification. The mechanism of Wingless spread is still controversial, hypothesized to form through either diffusion or vesicle-mediated transport (transcytosis). We are studying the effects of intracellular trafficking on Wingless signaling by genetically altering the flow of endocytosis. When the production of Wingless is normal but internalization is blocked by the absence of functional Dynamin, we find that the Wingless gradient is expanded and the protein levels are increased, implying that Wingless spread can occur through diffusion. Although intracellular trafficking does not appear to be necessary for Wingless spread, our data indicate that endocytosis does play an important role in regulating Wingless signaling. When internalization is blocked, there is increased extracellular Wingless but decreased Distalless and Vestigial expression, therefore signaling must also occur in an intracellular vesicle compartment. Furthermore, when lysosomal degradation is impaired, proteins accumulate at the endosome and Wingless signaling is enhanced.

These findings indicate that Wingless signaling occurs at the endosome and is regulated by lysosomal degradation.

Session 2: Wnt receptors
Chair: Roel Nusse

Wnt-mediated transcriptional regulation in *Drosophila*

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In the resting state, it is thought that TCF family members bind to the promoter regions of Wnt target genes, where they often repress transcription via interactions with co-repressors. Upon Wnt stimulation, β-catenin (Arm in flies) is translocated to the nucleus, where it binds to TCF, converting it from a transcriptional repressor to an activator[1]. We are interested in this process and pursue this question using Wingless (Wg; a fly Wnt) signaling in *Drosophila* as a model.

While the Wnt signaling pathway is highly conserved throughout the animal kingdom, several important mechanistic differences have emerged between flies and vertebrates. The secreted antagonists of Wnt signaling identified in frogs and mammals (e.g., Frs, Dickkoph, Cerebus and WIF) do not appear to be present in flies. Likewise flies lack an obvious homolog for FRAT/GBP, which is thought to be essential for inactivating GSK-3 kinases, thus stabilizing β-catenin. Finally, the histone acetylases CBP and p300 have been shown to activate Wnt mediate transcription in vertebrates [2,3], but the lone fly CBP (dCBP) has been shown to be a negative regulator in flies[4]. The study of Wnt signaling in *Drosophila* continues to provide important challenges to the established model of how the pathway works [5,6]. Therefore, the issue of how deep the conservation goes between insects and vertebrates is not trivial.

My laboratory has been exploring the role of dCBP in fly Wnt signaling in flies and *Drosophila* cell culture. In vertebrates, CBP and p300 have been shown to bind to β-catenin and potentiate its transcriptional activation. Inhibition of CBP/p300 activity a viral protein could reduce Wnt signaling[2,3]. In flies, dCBP can bind and acetylate dTCF, reducing its affinity for Arm). Reduction of dCBP gene activity could elevate Wg signaling[4]. We find that overexpression of dCBP in fly imaginal discs blocks Wg signaling, consistent with the previously document negative role. However, in culture cells using reporter gene assays, we find that expression of dCBP can elevate Arm-mediated transcription. Moreover, inhibition of dCBP using RNAi causes a dramatic decrease in the ability of Arm to activate a reporter gene. Finally, we find that Arm robustly associates with dCBP *in vitro*. We are currently attempting to examine the effect of near complete removal of dCBP gene activity on Wg target gene expression in fly embryos and imaginal tissues.

We strongly suspect that dCBP plays a crucial positive role in regulating Wg targets genes. What other factors have been rigorously confirmed (i.e., genetically) to be necessary for dTCF/Arm activation of Wg target genes? Two such factors have been recently

identified, Legless (Lgl) and Pygopus (Pygo). Both genes are essential for Wg signaling in *Drosophila*[7-10] and vertebrate homologs of *pygo* are required in human cell culture [10]and Xenopus embryos[7]. Biochemical and genetic evidence supports a role for Lgl as an adapter that recruits Pygo to the dTCF/Arm complex[8]. Using anti-Pygo antibodies, we have shown that Pygo and Lgl can be co-immunoprecipitated, supporting the data of the Basler lab. We are currently attempting to see if this complex exists on Wg target promoter using Chromatin immunoprecipitation.

The genetic evidence is consistent with several models for Pygo biochemical action. Pygo could act to inactivate the dTCF co-repressors (i.e., as a derepressor). Alternatively, Pygo could be required more directly in transcriptional activation or chromatin access. We have data in cell culture that argues against the first model. RNAi inhibition of pygo message greatly reduces the ability of dTCF/Arm to activate a reporter gene. Pygo inhibition also cripples the ability of a Gal4-Arm fusion protein to activate a Gal4 reporter. Since the portion of Gal4 used is not thought to be bound by co-repressors, this suggests that Pygo acts downstream of corepressor inactivation.

An interesting aspect of many developmental signaling pathways is that they activate different genes in different cell types. The basis for this signaling diversity is not well understood. Another gene we identified in a genetic screen may shed some light on this phenomenon. The gene, called *split ends* (*spen*), encodes a large nuclear protein containing three RNA Recognition Motifs (RRM) [11,12] and a SPOC domain at the C-terminus. The human homolog of spen is called SHARP, which has been shown to act as a transcriptional co-repressor[13,14]. Loss of *spen* gene activity in the larval eye, leg and wing causes phenotypes consistent with a loss of Wg signaling. However, we can find no evidence for a requirement for spen in embryonic Wg signaling, though Spen is abundantly expressed there. Therefore, we think Spen is a tissue or promoter-specific factor involved in Wg transcriptional regulation. The crucial question now is: what is the difference between *spen*-dependent and *spen*-independent Wg targets.

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New components of the Wnt signalling pathway

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In vertebrate embryogenesis, Wnt/β-Catenin signalling is essential for body axis specification and mesoderm formation, and deregulation of the pathway induces tumor formation. We have used gene expression profiling of β-Catenin-deficient mouse embryos to characterize genetic networks that control early embryonic development. We define two distinct β-Catenin pathways that lead to axis and mesoderm formation, and isolate novel downstream components in each pathway. We find that Cripto is a major target of β-Catenin signalling in anterior-posterior axis specification and in colon cancer cells.

We also describe the vertebrate protein Diversin that interacts with two components of the Wnt/β-Catenin pathway, Casein kinase IεCKIε and Axin/Conductin. Diversin recruits CKIε to the β-Catenin degradation complex that consists of Axin/Conductin and GSK3β and allows efficient phosphorylation of β-Catenin at serine 45, thereby inhibiting β-Catenin signals. Morpholino-based gene ablation in zebrafish demonstrates that Diversin is crucial for axis formation during embryogenesis. Diversin is also involved in JNK activation and gastrulation movements. Our data demonstrate that Diversin is an essential component of the Wnt signaling pathway and acts as a molecular switch, which suppresses Wnt signals mediated by the β-Catenin pathway and stimulates Wnt signaling via JNK.

Regulation of organogenesis by LEF1 and Wnt signaling

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LEF1 is a nuclear mediator of Wnt signaling that activates transcription in association with β-catenin. This activation can be antagonized by interaction of LEF1 with PIASy, which mediates SUMO modification of LEF1 and sequesters LEF1 in nuclear bodies. In the mouse, LEF1 is required for the formation of organs that involve inductive interactions between epithelial and mesenchymal tissues. To examine whether all activities of LEF1 are mediated by interaction with β-catenin, we have generated a mutant allele of *Lef1* that carries point mutations in the β-catenin-interaction domain. In an initial analysis, the mutant mice were found to mimic the mice carrying a null mutation in *Lef1*, suggesting that most if not all activities of LEF1 *in vivo* require the interaction with β-catenin. Analysis of another mutant allele of Lef1, which generates a dominant negative form of LEF1 that interferes with the activity of other TCF proteins, indicated that Wnt/β-catenin signaling is not only involved in the generation of paraxial mesoderm but also in somitogenesis, apparently by affecting the expression of *Notch Dll1*.

In previous experiments, we found that the effects of LEF1 in the epithelium of developing tooth germs are cell non-autonomous and transferred to the subjacent mesenchyme. In search for the underlying mechanisms, we found that the *Fgf4* gene is a direct transcriptional target for LEF1, and we show that beads containing recombinant FGF4 can fully rescue the developmental arrest of *Lef1*-/- tooth germs. FGF4 beads rapidely induce expression of *Fgf3* in the mesenchyme, suggesting that Wnt signals are relayed via LEF-1 to induce sequentially expression of *Fgf4* in the epithelium and *Fgf3* in the mesenchyme. Finally, mesenchymal but not epithelial FGF proteins can induce *Shh* expression in the epithelium of *Lef1*-/- tooth germs. Thus, LEF1 mediates a cross-talk between Wnt and FGF signals as part of sequential and reciprocal communications between epithelium and mesenchyme during organ development.

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The beta-catenin/TCF complex imposes a crypt progenitor phenotype on colorectal cancer cells

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Mutations in the Wnt pathway components APC, beta-catenin and conductin all induce sustained complex formation of the co-activator beta-catenin with TCF transcription factors. The resulting transactivation of TCF target genes is believed to represent the primary transforming event in colorectal cancer (CRC). Yet, the consequence of the presence of mutationally activated beta-catenin/TCF in fully transformed CRC cells is unknown. We have constructed CRC cell lines carrying inducible dominant-negative TCF constructs. Inhibition of beta-catenin/TCF resulted in a rapid G1 arrest. DNA array analysis revealed the downregulation of a small set of transcripts. These genes were expressed in polyps, but also, physiologically, in the crypt progenitor compartments of the colon. By contrast, we observed the induction of multiple marker genes of intestinal differentiation upon inhibing beta-catenin/TCF in CRC cells. We provide evidence that p21 is responsible for this phenomenon. We conclude that beta-catenin/TCF inhibits differentiation and imposes a crypt progenitor phenotype on CRC cells. Moreover, inhibition of beta-catenin/TCF activity restores the differentiation program, despite the presence of multiple other mutations in CRC.

Session 3: Wnt and ß-catenin
Chair: Rudolf Grosschedl

Axin complex assembly and recruitment to the plasma membrane

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In the absence of Wnt ligand, β-catenin/Armadillo is phosphorylated by the Axin destruction complex, which earmarks it for rapid degradation by the ubiquitin system. Axin acts as a scaffold protein in this complex to assemble β-catenin/Armadillo substrate and kinases (casein kinase I and glycogen synthase kinase 3β). The activity of this complex in promoting the degradation of β-catenin is regulated in some way by the Adenomatous polyposis coli (APC) tumour suppressor which binds to Axin. On Wnt signalling, the Axin complex is inhibited. As a consequence, β-catenin accumulates and binds to TCF proteins to stimulate the transcription of Wnt target genes. Wnt-induced inhibition of the Axin complex depends on Dishevelled, a cytoplasmic protein that can bind to Axin, but the mechanism of this inhibition is poorly understood.

We have expressed Axin tagged with green fluorescent protein (GFP) in Drosophila embryos to study the regulation of the Axin complex. Our evidence suggests that (1) Drosophila E-APC is required for Axin complex assembly, (2) Dishevelled confers relocation of the Axin complex from the cytoplasm to the plasma membrane on Wingless signalling and (3) that recruitment of the Axin complex to the plasma membrane is sufficient for its inactivation. We propose a model, based on recycling endocytic vesicles, of how Dishevelled may convey the Axin complex to the plasma membrane in response to Wnt signalling to inhibit it.

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Wnt signalling and regional patterning of the embryonic zebrafish forebrain

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Our research is aimed at understanding the mechanisms that underlie the establishment of pattern within the vertebrate forebrain. During early development, the simple sheet of neuroepithelial cells that constitutes the neural plate is transformed into the highly complex and elaborate structures of the CNS. Nowhere is this transformation more dramatic than within the forebrain. This region gives rise to the telencephalon (which in humans forms the cerebral cortex and basal ganglia), the eyes, and the diencephalon (which includes the hypothalamus and thalamic nuclei). For correct function, neurons within these different domains of the forebrain must acquire their correct identities and make appropriate connections with target cells, often in distant regions of the CNS. For a neural cell to acquire appropriate positional identity within the CNS, it must know its position within two, and for some cells, within three axes. Thus, it must receive cues regarding its position along the antero-posterior (AP) and dorso-ventral (DV) axes, and in some cases, information on whether it is on the left or on the right side of the brain.

Recent work has implicated the Wnt signaling pathway in mediating AP positional identity within the forebrain neuroepithelium. A key finding for us was the demonstration that the *masterblind* (*mbl*) mutation is in the Wnt scaffolding protein, Axin1 (Heisenberg at al. 2001). *mbl* mutant embryos exhibit a transformation of the anterior neural plate (telencephalon and eye fields) into more posterior diencephalon. Axin functions as a negative regulator of Wnt signals and thus the AP transformation is likely due to enhanced Wnt activity. Further evidence that suppression of Wnt activity is required for establishment of anterior forebrain fates has come from the identification of a SFRP family secreted inhibitor of Wnt signaling which appears to be essential for induction of the telencephalon (Houart et al. 2002). Our more recent work is focusing upon roles for the Wnt pathway in induction and patterning of the eyes and hypothalamus. Both local suppression and local activation of canonical Wnt signalling can lead to a loss of eyes – this suggests that a low level of Wnt activity may be required for eye formation. We are currently exploring this possibility and have also begun to look at roles for non-canonical Wnt signalling in the early steps in eye formation.

Finally, we are examining if Wnt signalling plays role in patterning the ventral midline of the CNS. In the forebrain, ventral midline cells form hypothalamus whereas in caudal regions they differentiate as floorplate. The hypothalamus is unusual in that hypothalamic precursors originate near the organiser and migrate rostally to enter the forebrain territory. We have begun to explore whether Wnt signals influence the ability of axial signals (Nodals and Hhs) to induce hypothalamus versus floorplate. Our initial findings suggest that suppression of Wnt activity leads to axial tissue inducing hypothalamic identity.

Acknowledgements: Our work on the Wnt pathway has been done in collaboration with the groups of Masa Tada, Derek Stemple, Corinne Houart, Carl-Philipp Heisenberg, Trevor Dale and Robert Geisler.

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Role of Wnts in skeletal development

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The long bones of the vertebrate limbs are formed by a process referred to as endochondreal ossification, starting from mesenchymal condensations, which further differentiate into cartilagenous templates prefiguring the future skeletal elements [1]. The latter will be then be replaced by bone starting in the middle of the element (diaphysis) and proceeding towards the ends (epiphysis). In order for this to happen, cells within the cartilagenous template referred to as chondrocytes undergo a controlled differentiation program. The first sign of this differentiation is happening within the diaphysis whereby chondrocytes exit cell cycle and are now referred to as prehypertrohic chondrocytes expressing molecular markers such as Indian hedgehoge (Ihh) and Parathyroid-hormone-related-peptide receptor (PTHrP-R). Subsequently, the prehypertrophic chondrocytes mature into hypertrophic chondrocytes, which have a distinct morphological appearance and extracellular matrix, and express molecular makers such as Collagen X (ColX). Consequently, immature, proliferating chondrocytes are only found at each end (epiphysis) of the cartilage element, appearing as two morphologically distinct populations; proliferating chondrocytes in the articular region are small, round and closely packed, while adjacent to them is a zone of radially flattened proliferating chondrocytes. This maturation of chondrocytes is accompanied by characteristing changes within the population of flattened cells surrounding the cartilage template referred to as perichondrium. As the adjacent chondrocytes start to hypertrophy the perichondrium in the diaphysis differentiates into a structure known as the periosteum. The innermost periosteal cells adjacent to hypertrophic chondrocytes differentiate into osteoblasts, which secrete bone matrix (osteoid) that becomes progressively calcified, forming a membranous bone collar surrounding the diaphysis of the cartilagenous element [2].

Previously we, and others have shown that at least four different members of the Wnt-gene family, Wnt4, 5a, 5b, and 14, are expressed in distinct cell populations of the developing long bones in chick [3-7]. At early stages Wnt4 and 14 are predominantly expressed in cells of the joint region, while Wnt5a is expressed in the perichondrium / periosteum and its highly related family member Wnt5b in a subpopulation of prehypertrophic chondrocytes as well as in cells of the outer perichondrium [4, 5]. At later developmental stages expression of Wnt4 and Wnt5a can also be found in prehypertrophic chondrocytes [7].

Gain-of-function experiments in chick using a retroviral delivery system revealed that various Wnt-genes exhibit effects on limb skeletogenesis [3-5, 7, 8]. Overexpression of either Wnt4 or an N-terminal truncated form of β-catenin (CAβ-catenin) resulted in shortening of the infected skeletal elements. Histological and molecular marker analysis of the infected cartilage elements revealed that differentiation of hypertrophic chondrocytes, a maturated chondrocyte cell type, is accelerated in Wnt4 as well as CAβ-catenin infected skeletal elements [4]. The similarity of the phenotypes resulting from Wnt4 and CAβ-catenin overexpression in the skeletal elements suggests that Wnt4 signals in this context through the

β-catenin mediated canonical Wnt-signaling pathway. Correspondingly, skeletal elements infected with dominant-negative forms of members of the frizzled-receptors or the transcription factor Lef-1, as well as those infected with the Wnt-antagonist Frzb-1 display the opposite phenotype whereby maturation of hypertrophic chondrocytes is delayed [5, 6] (C. Hartmann unpublished observation). Interestingly, overexpression of either Wnt5a or 5b also results in shortening of the infected skeletal elements, however, histological and molecular marker analysis revealed that in contrast to Wnt4 this is due to a delay in chondrocyte differentiation [2, 7, 9]. It remains to be elucidated which signaling pathway is utilized by Wnt5a / 5b to exert the observed effects on chondrocyte maturation.

The mouse orthologs of Wnt4, 5a, 5b and 14 are expressed in the developing mouse skeleton in similar but not identical patterns [9, 10]. Recent gain-of-function studies suggested that the delay in chondrocyte differentiation caused by Wnt5a and Wnt5b Col2a1-driven transgene expression is due to differential effects on chondrocyte proliferation. Wnt4 and Wnt5a have been knocked-out, but surprisingly only mice lacking Wnt5a have a reported skeletal phenotype with reduced chondrocyte proliferation and a delay in maturation [10, 11].

Recently we generated a conditional knock-out allele of Wnt14. Wnt14 was previously implicated in playing a role in the induction process of synovial joints on the basis of gain-of-function experiments in chick [5]. Wnt14 mutant mice, generated by using germ-line deleter, are born but die within the first 24 hours. They do not display any defects in the formation of synovial joints, suggesting that Wnt14 is not exclusively required for joint induction in the mouse. However, Wnt14 mutants exhibit a transient chondrogenic differentiation phenotype, which is only apparent at early embryonic stages and not obvious at birth, suggesting that other Wnt-molecules could compensate for loss-of Wnt14 activity.

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ß-catenin function in mouse development

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ß-catenin is a central component of the E-cadherin cell adhesion complex and plays an essential role in the Wingless/Wnt signaling pathway. Wnts act as signaling molecules and are implicated in many developmental processes, including cell fate specification, polarity, migration and proliferation. A key player in the Wnt signaling cascade is ß-catenin, where it interacts with members of the LEF/TCF family of transcription factors and activates gene expression. In our attempts to identify target genes of the Wnt/ß-catenin signaling pathway in early mouse embryonic development we have used embryonic stem cells co-cultured are NIH3T3 fibroblasts expressing different Wnts as feeder cells. To decipher the role of ß-catenin as a mediator of Wnt signaling in mouse development, we have conditionally inactivated the ß-catenin gene in mouse development using the Cre/LoxP recombination system of bacteriophage P1.

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Factors and mechanisms affecting β-catenin phosphorylation

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The Wnt pathway controls numerous developmental processes via the β-catenin-TCF/LEF transcription complex. Deregulation of the pathway results in the aberrant accumulation of β-catenin in the nucleus, often leading to cancer. Normally, cytoplasmic β-catenin associates with APC and axin and is continuously phosphorylated by GSK-3β, marking it for proteasomal degradation. Wnt signaling is considered to prevent GSK-3β from phosphorylating β-catenin, thus causing its stabilization. However, the Wnt mechanism of action has not been fully resolved. Using mass spectrometry and phosphopeptide-specific antibodies, we have previously shown that a complex of axin and CKI induces β-catenin phosphorylation at a single site: serine 45 (S45)[1]. CKI-phosphorylation creates a priming site for GSK-3β and is both necessary and sufficient to initiate the β-catenin phosphorylation-degradation cascade. Wnt3A signaling and Dvl overexpression suppress S45 phosphorylation, thereby precluding the initiation of the cascade. However, once initiated, the progression of the phosphorylation cascade and the subsequent degradation of β-catenin can be further modulated by different mechanisms, in which PP2A and the two CKI isoforms, alpha and epsilon, play a major role. We will discuss the different factors and mechanisms involved in regulating β-catenin phosphorylation.

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Session 4: Wnt and development
Chair: Peter A. Lawrence

A canonical Wnt signaling pathway regulates the expression of the homeobox gene *mab-5* in the Q neuroblast lineage of *C. elegans*

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The Wnt family of secreted glycoproteins controls a wide variety of developmental processes, ranging from cell fate specification to determination of cellular polarity and migration. A key effector of the canonical Wnt signaling pathway is β-catenin, which activates the expression of specific Wnt target genes in collaboration with TCF transcription factors. In the absence of Wnt signaling, β-catenin is targeted for destruction by the proteasome and Wnt target gene expression is inhibited by the interaction of TCF with Groucho co-repressors.

To gain further insight into the complex mechanism and regulation of the canonical Wnt pathway, we are studying Wnt signaling in the relatively simple model organism *C. elegans*. We and others have found that a canonical Wnt pathway controls the expression of the homeobox gene *mab-5* in the Q neuroblast lineage (reviewed in [1]). The Q neuroblasts are born at similar anteroposterior positions on the left and right side of the animal and generate an identical set of daughter cells. The migration of the Q neuroblasts and their descendants is however strikingly different between the two sides of the animal: on the left side, the Q cell descendants migrate towards the posterior, whereas on the right side these cells localize in the anterior. This difference in migration is directed by the asymmetric expression of the homeobox gene *mab-5* [2]. Only QL and its daughter cells express *mab-5*, which induces migration towards the posterior; QR and its descendants do not express *mab-5* and migrate in the default anterior direction. The expression of *mab-5* in the Q neuroblast lineage is in turn regulated by a canonical Wnt pathway. Mutations in *egl-20*/Wnt, *lin-17*/Fz, *mig-1*/Fz, *mig-5*/Dsh, *bar-1*/β-catenin [3,4] and *pop-1*/Tcf [5] disrupt *mab-5* expression in QL and induce anterior migration of the QL daughter cells, whereas mutation of *pry-1*/Axin [4,6] and overexpression of *egl-20*/Wnt and *bar-1*/β-catenin results in ectopic expression of *mab-5* in QR and posterior migration of the QR daughter cells.

We are using the migration of the Q neuroblasts as an assay to study the canonical Wnt pathway; mutations that disrupt *egl-20*/Wnt signaling affect the direction of QL daughter cell migration, whereas mutations that inappropriately activate the Wnt pathway affect QR daughter cell migration. Using a genome-wide RNA interference (RNAi) based screen, we have identified 11 new genes that are required for correct Q daughter cell migration. These genes may function in *egl-20*/Wnt or *mab-5* signaling, or may affect the cell migration

process itself. We will present results on one gene that was identified in this RNAi screen, a homolog of the yeast retromer complex component VPS35. We find that a null mutation in *vps-35* closely phenocopies the *egl-20*/Wnt cell migration and polarity phenotypes. We speculate that *vps-35* may play a new role in Wnt signaling or Wnt gradient formation.

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Planar polarity and Wnts?

Casal, J., Struhl, G and Lawrence, P.A.

This talk concerns the adult abdomen of Drosophila as a model system for the study of pattern and polarity (Struhl et al., 1997a; Struhl et al., 1997b)

Although much is known of pattern formation in the parasegments and segments of the abdomen of the embryo, the adult abdomen is less well understood. The abdomen develops from histoblast nests and consists of alternating anterior (A) and posterior (P) compartments. These compartments form the engine that drives pattern formation. Hedgehog is produced by all the P cells and crosses over the compartment boundaries to form a morphogen gradient in the A compartment, the scalar concentration of Hedgehog at each point determining the differentiation of cells, including the development of bristles. We have evidence that the morphogen Wingless spreads back from a source at the rear of the A compartment and patterns the P compartment (Lawrence et al., 2002).

Many models have been put forward to explain planar polarity and (in our opinion) none, including our own, is satisfactory. One class of model asks that cells read the vector in a morphogen gradient we call "X". We have shown that Hh does not directly affect the orientation of the hairs and bristles (which indicate the planar polarity of the cells) but does act indirectly (Struhl et al., 1997a). One possibility is that Hh helps set up a gradient consisting of a self regulating system involving at least three genes, *dachsous, four-jointed* and *fat*, and that this gradient constitutes X (Casal et al., 2002; Yang et al., 2002). There is then a linked system that is downstream of X and is short-range. Its main function is to sense and respond to the X gradient in a way which ensures that all cells have a common polarity (Lawrence et al., 2002) . One element in this latter system appears to be the Wnt receptor, Frizzled (Adler et al., 1997; Vinson and Adler, 1987).

In my talk I will discuss our attempts to define the function of Wnts in the abdomen. The analysis is complicated because Wnts are needed in the dorsal abdomen to specify tergites: inactivation of the Wnt signalling pathway in clones of cells of the tergites changes them to ventral parts. Overexpression of the pathway in the ventral parts changes them to dorsal parts. But, also, polarity changes are induced by such clones. We have therefore made an attempt to see if any of the 7 Wnts defined in Drosophila can affect polarity directly, but the results were negative. We therefore have tentatively concluded that Wnts do not constitute a part of X (Lawrence et al., 2002). This conclusion raises the question: what is Frizzled doing?

Clones of *fz*$^{-}$ cells, as well as clones that overexpress the gene, have strong local polarity effects both inside and outside the clone. Attempts to understand this have occupied several groups but the mystery remains.

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An element of Wingless signalling as a potential target for Suppressor of Hairless Notch signalling

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The Notch gene of Drosophila encodes a receptor with a complex conserved structure consisting of an extracellular domain with 36EGFlike repeats involved in ligand binding and an intracellular domain, whose main feature is six cdc10/ANK repeats, involved in the transcution and processing of signals (1). Notch plays a central role in many cell fate decisions through a well characterized signalling event triggered by its interaction with either of two ligands, Delta and Serrate. This event leads to the cleavage of the intracellular domain of Notch and its translocation to the nucleus where it mediates transcriptional activation through its interaction with the transcription factor Suppressor of Hairless (2). Notch as well as all the features of this signalling event have been conserved and, with small modifications, can be found in vertebrates.

Genetic studies in Drosophila have also uncovered a second signalling activity of Notch which regulates signalling by Wingless. This activity has different structural requirements from that mediated by Suppressor of Hairless and is highlighted by an array of gain of function mutations in the extracellular and the intracellular domains of Notch which antagonize Wingless signalling (3 and reviewed in 4 and 5). In addition, and most importantly, in several instances loss of function of Notch (but not of Delta nor Suppressor of Hairless) rescues the phenotype of loss of function of Wingless or Dishevelled (reviewed in 6). This activity is likely to target the activity of Tcf (7). Thus, Notch encodes an activity, independent of Suppressor of Hairless which suppresses Wingless signalling.

Wingless can bind the extracellular domain of Notch (8, 9) and Dishevelled has been shown to interact with a specific region of the intracellular domain (3, 10). These interactions are likely to block the negative effect of Notch on Wingless signalling and to allow efficient Wingless signalling. Dosage dependent interactions between Notch and wingless support this possibility: Wingless signalling is generally enhanced by reduction in the dosage of Notch.

We have been interested in identifying the molecular linchpin of this interaction. We have recently identified an element of Wingless signalling which is targeted by Notch in a Suppressor of Hairless independent manner. We shall discuss these results and their implications for our understanding of the initial steps in Wingless signalling. Our

observations provide an explanation for many, but not all, of the genetic interactions that have been described between *Notch* and *wingless*.

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Anatomy of an inducible antagonist: Structure-function analysis of the Wnt antagonist naked cuticle (Nkd) defines Dishevelled binding and non-binding regions important for function *in vivo*

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Each signaling pathway employs feedback regulation as a means to ensure reproducibility and precision during development. For signaling by the Wingless/Wnt pathway, naked cuticle (nkd) is likely the only zygotically-acting feedback regulator active during early segmentation.

Nkd encodes a novel protein with a single EF hand motif. We recently demonstrated that Nkd binds to and can inactivate the positively acting Wnt signaling component, Dishevelled (Dsh), a key transducer of both canonical Wnt signals and planar cell polarity signals. Dsh is a multidomain protein that distinguishes between these two types of Wnt/Frizzled signals and, depending on the context, transmits them to largely non-overlapping sets of signaling-pathway specific components.

Despite the fact that Nkd and Dsh interact *in vitro*, the majority of Nkd and Dsh are apparently separated in signaling cells *in vivo*, perhaps because their association is transient or in competition with an interaction between Dsh and some of the seventeen reported Dsh-binding proteins. Astructure-function study shows that sequences in Nkd that bind Dsh, as well as an additional motif that doesn't bind Dsh *in vitro*, are important for nkd activity *in vivo*. Our data further indicate that Nkd may affect Dsh localization, accumulation, or phosphorylation state, all of which have been shown to be important for Dsh function. Future experiments will seek to discern the mechanisms by which Nkd antagonizes Dsh activity in Wnt signaling, as well as discover additional proteins that regulate the Nkd/Dsh interaction and its consequences for signaling.

Session 5: Wnt proteins
Chair: Alfonso Martinez Arias

Understanding Wnt signaling pathways in development and cancer

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Cell-cell communication plays a central role in animal development and tumor formation. Wnt signal transduction is a key part of this cell-cell communication network and is essential for establishing the basic vertebrate body plan and for maintaining human tissue homeostasis. Disruption of Wnt signal transduction causes abnormal embryogenesis and human cancers, including colorectal cancer and melanoma. The mechanism of Wnt signal transduction during embryogenesis and tumor formation is not fully understood.

Using a combination of molecular, biochemical and embryological techniques, we have been trying to elucidate the molecular mechanism of Wnt signal transduction in Xenopus embryos during their development and in human cells in culture. We particularly focus on elucidating how the Wnt receptor complex transduces Wnt signal from outside the cell into inside the cell, how the Wnt receptor complex specifies distinct transduction pathways to govern different aspects of embryogenesis, and the molecular composition and logic of these different transduction pathways. I will discuss aspects of the following areas:

1. Corroborating with genetic studies in Drosophila and mice, we demonstrated that LDL Receptor-Related Protein 5/6 (LRP5/6) functions as a co-receptor for seven-pass transmembrane Frizzled (Fz) receptors in Wnt signaling, and that LRP5/6 may form a complex with Fz upon Wnt stimulation (ref.1).

2. We found that Dickkopf-1 (Dkk-1), which is a secreted head inducer essential for head formation in Xenopus and mice, is a high affinity ligand for LRP5/6, and showed that Dkk-1 functions to promote head formation probably by blocking Wnt-induced Fz-LRP5/6 complex formation and Wnt/b-catenin signal transduction (ref.2).

3. Wnt signaling via the Fz-LRP5/6 receptor complex controls the protein level of oncogene product b-catenin. Deregulation of b-catenin is responsible for human colorectal cancer and several other malignancies. We found that b-catenin protein level is regulated by two protein kinases in the Axin complex, GSK-3 and CKIa, and elucidated the mechanism by which these two kinases controls sequential phosphorylation of b-catenin and its degradation (ref. 3).

4. Wnt signaling via the Fz receptor also controls vertebrate gastrulation, a process by which an embryo develops germ layers and positions its head versus tail in a correct order. We demonstrated that this novel Wnt signaling pathway, which does not require LRP5/6 (ref. 2) and is related to planar cell polarity (PCP) signaling in Drosophila, acts via governing

cytoskeleton through another sets of key cellular regulators, Rho and Rac GTPases (refs. 4 and 5). We also identified a novel protein called Daam1, which is specifically required for Wnt/Fz activation of Rho, but not Rac (refs. 4 and 5).

5. Dishevelled (Dvl) protein is a key molecule downstream of Fz in both b-catenin and PCP signaling. Its DIX domain is required for the b-catenin pathway activation, whereas the PDZ and DEP domains are involved in PCP signaling and Rho and Rac activation. In a collaborative NMR structural study with Dr. Overduin's group, we identified critical region and residues that mediate Dvl binding to actin filaments and vesicular membranes, and demonstrated that Dvl binding to actin may titrate it from Wnt/b-catenin signaling whereas Dvl binding to vesicular membranes is essential for its phosphorylation and Wnt/b-catenin signaling (ref. 6).

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Regulation of the Wnt signaling pathway by sumoylation

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The post-translational modifications of β-catenin and Tcf such as phosphorylation, ubiquitination, and acetylation are important for the regulation of the stability of β-catenin and the transcriptional activity of Tcf. SUMO is an ubiquitin related modifier. Many proteins including RanGAP, RanBP2, p53, Mdm2, and IkB have been shown to be sumoylated. Sumoylation of these target proteins is involved in mediating protein-protein interactions, subcellular compartmentalization, and protein stability.

Our analysis of various Wnt signaling molecules revealed that one of them, Tcf-4, is sumoylated. The sumoylation of Tcf-4 was enhanced by PIASy, a SUMO E3 enzyme, in intact cells and *in vitro*, whereas the sumoylation of Tcf-4 was not enhanced, but rather inhibited, by a PIASy RING mutant that does not bind to Ubc9, a SUMO E2 enzyme. Lys^{297} of Tcf-4 was a sumoylation site. Tcf-4, SUMO-1, and PIASy were colocalized in the nucleus, and were present in a complex in the PML body. Although PIASy did not affect the interaction of Tcf-4 with β-catenin or DNA, PIASy activated Tcf-4. Furthermore, β-catenin and PIASy activated Tcf-4 synergistically. The PIASy RING mutant inhibited Wnt-3a-, Dvl-1, or β-catenin-dependent Tcf-4 activation. These results suggest that sumoylation is involved in β-catenin-dependent and Tcf-4-mediated gene expression in the Wnt signaling pathway.

Taken together with our previous observations that Axam, a desumoylation enzyme, induces downregulation of β-catenin, sumoylation is an important post-translational modification of the regulation of the Wnt signaling pathway.

Kremens are novel Dickkopf receptors that regulate Wnt/β-catenin signalling

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Canonical Wnt signalling via the β-catenin pathway is transduced by two receptor families. Frizzleds and Lipoprotein receptor related proteins 5 and -6 (LRP5/6) bind Wnts and transmit their signal by stabilizing intracellular β-catenin. Wnt/β-catenin signalling is inhibited by the secreted protein Dickkopf1 (Dkk1), member of a multigene family, which acts as head inducer in the Spemann organizer of amphibian embryos. Recently, Dkk1 was shown to inhibit Wnt signalling by a novel mode of action, by binding to and antagonizing LRP5/6. We now describe the transmembrane proteins Kremen1 and -2 as novel high affinity Dkk1 receptors, which functionally cooperate with Dkk1 to block Wnt/β-catenin signalling. Kremen2 forms a ternary complex with Dkk1 and LRP6 and induces rapid endocytosis and removal of the Wnt receptor LRP6 from the plasma membrane. Unlike Dkk1, the related Dkk2 can function either as LRP6 agonist or antagonist, depending on the cellular context, suggesting that its activity is modulated by unknown co-factors. In human 293 fibroblasts transfected dkk2 activates LRP6 signaling. However, co-transfection of krm2 blocks the ability of Dkk2 to activate LRP6 and enhances inhibition of Wnt/Frizzled signaling. Krm2 also co-operates with Dkk4 to inhibit Wnt signaling, but not with Dkk3, which has no effect on Wnt signaling. Likewise, in Xenopus embryos, Dkk2 and Krm2 co-operate in Wnt inhibition leading to anteriorized embryos. These results suggest that Krm2 can function as a switch that turns Dkk2 from an activator into an inhibitor of Wnt/LRP6 signaling. Finally, we have investigated the role of Krm1 and Krm2 during early Xenopus embryogenesis. Consistent with a role in zygotic Wnt inhibition, overexpressed Krm anteriorises embryos and rescues embryos posteriorised by Wnt8. Antisense morpholino oligonucleotide (Mo) knockdown of Krm1 and Krm2 leads to deficiency of anterior neural development. In this process, Krm proteins functionally interact with Dkk1.The results indicate that Krm proteins function in a Wnt inhibition pathway regulating early AP patterning of the CNS.

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Signaling by isolated Wnt proteins

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While progress had been made in understanding the Wnt signaling pathway in target cells, Wnt proteins had never been isolated as active growth factors. The lack of active Wnt protein has been one of the most vexing problems in the field, and has led to a variety of indirect approaches to studying signaling. We have systematically tried to produce and isolate Wnt proteins and we have now purified several Wnts as biologically active molecules. An explanation for the unusual behavior of Wnt proteins and their insolubility came from establishing that active Wnt proteins are lipid modified. Using mass spectrometry, we mapped the modification site to one cysteine. Mutating that particular cysteine to an alanine results in a secreted form of Wnt3A that is not hydrophobic, and is not active. Furthermore, in a survey of *wingless* alleles in *Drosophila*, we found one natural allele containing a tyrosine replacing that same cysteine, providing additional evidence for the importance of this modification.

The finding that Wnts are lipid modified has several interesting implications. It provides a likely explanation for earlier observations regarding the role of the genes *porcupine* and *MOM*1 in Wnt signaling in *Drosophila* and *C. elegans*. These genes, required in Wnt producing cells have homology to acyl-transferases, enzymes that can attach lipids to substrates. These genetic similarities indicate that *porcupine* and *MOM-1* are enzymes that are dedicated to Wnt signaling, underscoring the significance of the lipid as an integral component of Wnt signaling.

Having purified Wnt proteins allowed us to test directly whether these signaling molecules can be used as growth or differentiation factors. In view of the evidence that Wnt signaling is important to maintain stem cell fate, we have concentrated our efforts on various kinds of stem or progenitor cells. While some of the results are preliminary, they are encouraging. For example, we have found that primary mesenchymal cells isolated from embryonic limb buds are profoundly influenced by added Wnt proteins. Normally, these cells can differentiate into various cell types, including chondrocytes. We found that Wnt protein inhibits differentiation, and acts as a mitogen for undifferentiated progenitor cells.

The notion that Wnts act as stem cell growth factors is substantiated by experiments done together with Irving Weissman at Stanford. Using his lab's extensive experience with hematopoietic stem cells (HSCs), we were able to promote expansion of undifferentiated, self-renewing HSCs with purified Wnt protein. The Wnt-exposed cells have the ability to reconstitute the entire hematopoietic system. Additional experiments have demonstrated that Wnt signaling is an essential pathway for HSC growth, and is perhaps the major regulator of these important cells *in vivo*.

POSTERS

Stat5a activation mediates the epithelial to mesenchymal transition induced by oncogenic RhoA

Salvador Aznar Benitah, Pilar F. Valeron, Hallger Rui and Juan Carlos Lacal

The involvement of Rho GTPases in signal transduction pathways leading to transcription activation is one of the major roles of this family of GTPases. Thus, the identification of transcription factors regulated by Rho GTPases as well as the understanding of the mechanisms of their activation and its biological outcome is of great interest. We provide here evidence that Rho GTPases modulate Stat5a, a transcription factor of the family of Signal Transducers and Activators of Transcription. RhoA triggers tyrosine phosphorylation (Y696) of Stat5a via a JAK2-dependent mechanism, and promotes DNA-binding activity of Stat5a. Tyrosine phosphorylation of Stat5a is also stimulated physiologically by LPA in a Rho-dependent manner. Simultaneously, RhoA reduces serine phosphorylation of Stat5a at both serine residues S726 and S780, resulting in a further increase of activity as defined by mutagenesis experiments. Furthermore, serine dephosphorylation of Stat5a by RhoA does not take place by downmodulation of either JNK1, MEK1 or p38 MAP kinases, as determined by transfection experiments or chemical inhibition of both MEK1, p38 and JNK serine kinases. Thus, RhoA regulates Stat5a via tyrosine phosphorylation, and by a yet to be determined novel downmodulating pathway that involves serine dephosphorylation. At last we provide evidence for a role of Stat5a in RhoA-induced epithelial to mesenchymal transition (EMT) with concomitant increase in vimentin expression, E-cadherin downregulation and cell motility.

The Wnt receptor LRP6/arrow can signal independently of Frizzled proteins

Keith Brennan, Jose M. Gonzalez Sancho, Louise R. Howe, Leslie A. Castelo-Soccio and Anthony M. C. Brown

The identification of the LRP5&6 and Arrow proteins as components of Wnt receptors has raised questions of the individual functions of the Frizzled and LRP5/6 proteins within the receptor. We have addressed these questions by performing a structure-function analysis of the Fzd1 and LRP6 proteins, which have previously been shown to be involved in transduction of the Wnt1 signal. Our results indicate that extracellularly deleted LRP6 or Arrow proteins are able to activate the intracellular Wnt signalling pathway in a ligand-independent and Frizzled-independent manner. In contrast, over expression of Frizzled proteins was unable to activate the Wnt/â-catenin pathway and can even attenuate signalling by Wnt1. Consequently we suggest that LRP5/6 or Arrow constitutes the principal signalling component of Wnt receptors for the Wnt/â-catenin pathway and that Frizzled acts as a ligand presenting co-receptor.

Identification and characterization of *Drosophila* Wingless cis-regulatory regions

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Wingless (wg) is expressed in many tissues (including ectodermal, neural, mesodermal and endodermal derivatives both in embryo and adult primordia) during *Drosophila* development, and therefore its function depends on a complex and dynamic regulation of its transcription. The cis-regulatory regions (CRR) controlling wg expression must lie in the large intergenic regions flanking wg (over 30kb on both 5' and 3' sides) and/or inside wg's introns. To date, two CRR have been clearly identified in the wg 5' untranslated region: one controlling embryonic expression, and other responsible of wg expression in the wing imaginal disc (Lessing and Nusse, 1998; Neumann and Cohen, 1997).

In order to identify and characterize the remaining wg CRR, we have resorted to three complementary approaches: reporter-gene analysis, molecular evolution and bioinformatics. So far, our results identify a number of regulatory functions located in the 3' untranslated region as well as in introns of the wg gene. In particular, we have narrowed down a head-specific enhancer to less than 500bp, and we are currently molecularly testing transcription factors that might be responsible for its function. In addition, we are performing a comparative/functional analysis of these CRR throughout a dense fly phylogeny to try to pinpoint changes in these sequences that might have relevance to the evolution of wg function.

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Wnts and the specification of the eye field within the anterior neural plate

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One of the earliest steps in the patterning of the vertebrate nervous system is the acquisition of regional identity by cells in different areas of the prospective central nervous system (CNS). During the last years, the mechanisms by which this regionalization is achieved within the anterior neural plate have started to be unraveled, and a number of signals involved in this process have been identified. However, the way by which the eye fate is specified is still unclear. Here, we make use of the zebrafish model system to study in detail the role of the Wnt pathway, one of the most important pathways in anterior neural plate patterning, during eye fate induction. By transplanting cells expressing different components of the Wnt pathway within the prospective eye field, we have locally modified the levels of Wnt activity in this region. We will show results indicating an unexpected qualitative difference in the way the different intracellular branches of the Wnt pathway affect the induction of the eye field. Thus, while activation of the canonical branch results in repression of eye fate, activation of the planar-cell-polarity (PCP) branch leads to activation of eye markers, and presumably induction of eye fate. We will discuss different interpretations of these results and build a model that explains the requirement for both the suppression of the canonical Wnt branch and for the activation of the PCP branch for eye field induction. This remains a working model that has to be tested, and we are currently undertaking a number of experiments to ascertain its validity.

FrzA overexpression in transgenic mouse reduces infarct size and modifies infarct healing

Laurent Barandon, Thierry Couffinhal, Pascale Dufourcq, Jérome Ezan, Pierre Costet, Philippe Alzieu, Lionel Leroux, Catherine Moreau, Danièle Daret, Cécile Duplàa

We have previously shown that FrzA/sFRP-1, a secreted Frizzled Related Protein, antagonist for the wnt/frizzled pathway, was expressed in the heart and vessel during mouse embryogenesis and adulthood. *In vitro*, FrzA is involved in cell cycle control of vascular cells.

Here, we showed an up-regulation of sFRP-1/FrzA and distinct wnt and fz member expression after myocardial infarction (MI). So, we assessed the hypothesis that FrzA could control the healing process after MI. To investigate the role of FrzA, we established transgenic mice (Tg) overexpressing the protein under a CMV promoter and developed a model of MI by ligation of the left anterior descending coronary artery. Analysis were realized in Tg mice backcrossed onto C57BL/6J strain for more than 6 generations. C57BL/6J mice were used as control group. FrzA reduced cardiac rupture after MI in Tg (6.5% vs 26.4% in control, n=165; $p<0.001$). MI was smaller in Tg at each time point (18±10.8% of LV circumference vs 30±14.2% in control at day 30; $p<0.001$; n=6 at each time point). Similar results were found when MI was created by cryolesion. The cardiac function was improved in Tg (3800±370 mmHg/s dp/dt max vs 2800±840 in control, -2800±440 dp/dt min vs -1800±211 in control at day 15; $p<0.001$; n=6). Early leukocyte infiltration was decreased in Tg within the first week (103±59 cells/mm2 vs 730±463 in control at day 7; $p<0.001$; n=6). Apoptosis index was decreased by 50% in Tg (1.1% vs 2.4% in control at day 7; $p<0.01$).

Cellular density was 2 fold most important in Tg at day 15 (4002±496 cells/mm2 vs 2075±359 in control, $p<0.001$). MMP-2 and -9 activity was reduced in Tg at day 4 (as assessed by zymography) and collagen deposition in the scar was increased in Tg. Capillary density in the scar was most important in Tg (290±103 vessels/mm2 vs 104±43 in control at day 15; $p<0.001$). Vessels were more muscularized and mean lumen area was 3 fold most important in Tg (952±902 µm2 vs 313±350 in control; $p<0.001$). In conclusion, overexpression of FrzA, through direct or indirect interaction with the different phases of the infarct healing, reduced the infarct size and improved cardiac function.

Lef-1 and Tcf-3 transcription factors mediate tissue-specific Wnt signalling during *Xenopus* development

Giulietta Roël, Fiona S. Hamilton, Yoony Gent, Andrew A. Bain, Olivier Destrée and Stefan Hoppler

Wnt signalling functions repeatedly during embryonic development to induce different but specific responses. What molecular mechanisms ensure that Wnt signalling triggers the correct tissue-specific response in different tissues? Early *Xenopus* development is an ideal model for addressing this fundamental question, since there is a dramatic change in the response to Wnt signalling at the onset of zygotic gene transcription: Wnt signalling components encoded by maternal mRNA establish the dorsal embryonic axis [1]; zygotically expressed Xwnt-8 causes almost the opposite, by promoting ventral and lateral and restricting dorsal mesodermal development [2-4].

Although Wnt signalling can function through different signal transduction cascades, the same b-catenin-dependent, canonical Wnt signal transduction pathway mediates Wnt signalling at both stages of *Xenopus* development [5, 6]. Here we show that while the function of the transcription factor XTcf-3 is required for early Wnt signalling to establish the dorsal embryonic axis, closely related XLef-1 is required for Wnt signalling to pattern the mesoderm after the onset of zygotic transcription [7]. Our results show for the first time that different transcription factors of the Lef/Tcf family function in different tissues to bring about tissue-specific responses downstream of canonical Wnt signalling.

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Wise, a novel modulator of the Wnt pathway by interacting with LRP6

Nobue Itasaki

Wise (Wnt modulator in surface ectoderm) was isolated by a functional screen assayed for activities that alter the antero-posterior (A-P) character of neuralised *Xenopus* animal caps. Wise encodes a secreted protein and is expressed in the surface ectoderm strongest near the neural tube. The embryonic phenotypes arising from ectopic expression of Wise or from injection of antisense morpholino oligonucleotide resemble those obtained when Wnt signalling is altered. Induction of neural posterior markers such as en2 in animal caps by Wise requires components of the canonical Wnt signalling pathway, indicating that in this context it activates the Wnt signalling cascade. Wise also induces nuclear localization of b-catenin in animal caps, suppoting activation of canonical Wnt pathway. In contrast, in other assays such as secondary axis induction, Wise antagonises the axis-inducing ability of Wnt8. Injection of Wise RNA into a ventral blastomere causes ectopic cement gland formation, mimicking a phenotype caused by Wnt signalling blockers. These results indicate that Wise can activate or inhibit the Wnt signalling pathway in a context-dependent manner. The Wise protein physically interacts with the Wnt co-receptor lipoprotein-receptor-related protein 6 (LRP6), and is able to compete with Wnt8 for binding to LRP6. These activities of Wise provide a new mechanism for modulating the balance of Wnt signalling.

Wnt signaling regulates early events of chondrogenic differentiation during chick limb development

Yasuhiko Kawakami and Juan Carlos Izpisua Belmonte

Wnt signaling is known to be involved in chondrogenic differentiation, such as controlling hypertrophic differentiation and joint formation, which are relatively late events during chondrogenesis. Vertebrate limb development provides an excellent model system for studying chondrogenic differentiation, leading to endochondral bone formation that comprises the majority of our skeleton. During limb development, undifferentiated mesenchymal cells undergo condensation to form a chondrogenic precursor, followed by differentiation into chondroblasts. These cells further differentiate into chondrocytes and form cartilage that acts as a template of endochondral bone formation.

Previous studies suggest a possible involvement of Wnt signaling in the early sages of chondrogenesis, however, the endogenous factor is not yet identified. We have isolated chick Wnt10a that is expressed in the entire ectoderm during limb development. Misexpression of Wnt10a by retrovirus vector results in a strong downregulation of chondrogenesis, both *in vitro* mesenchymal culture and in vivo. Analysis of molecular marker expression in the Wnt10a-misexpressed limb suggests that Wnt10a suppresses chondroblast formation, but not mesenchymal condensation. These data are consistent with a known embryological study, which have demonstrated that limb ectoderm provides inhibitory signaling for chondrogenesis.

Given the fact that chondrogenesis takes place in the core of limb mesenchyme, our data suggest that Wnt10a acts as an endogenous inhibitory factor of chondrogenesis during limb development to restrict a mass of mesenchymal condensation undergoing chondrogenesis.

Tyrosine phosphorylation of Axin by Glycogen Synthase Kinase-3beta

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Axin negatively regulates Wnt signaling by facilitating the phosphorylation of b-catenin by casein kinase-I and glycogen synthase kinase-3b (GSK-3b), marking it for proteosomal degradation (1). Axin is also phosphorylated by GSK-3b on serine and threonine residues, which increases its stability and its affinity for b-catenin (2). Here, we present evidence that axin undergoes GSK-3b-dependent tyrosine phosphorylation. Axin tyrosine phosphorylation is blocked by lithium, a GSK-3 inhibitor, but not by PP2, a tyrosine kinase inhibitor. Moreover, a mutant axin that cannot bind GSK-3b is not tyrosine-phosphorylated.

Axin is also phosphorylated on tyrosine by recombinant GSK-3b *in vitro*, suggesting that tyrosine phosphorylation *in vivo* is direct. Deletion and point mutation analyses suggest that Y309 and Y315 are the *in vivo* tyrosine phosphorylation sites. These residues lie in a sequence resembling the GSK-3b activation loop containing the GSK-3b autophosphorylation site. An unphosphorylated peptide containing the axin tyrosine phosphorylation sites inhibits GSK-3b tyrosine kinase activity *in vitro*, while a tyrosine-phosphorylated form of this peptide does not.

These observations are consistent with a model in which the GSK-3b pseudo-activation loop in axin inhibits GSK-3b, and tyrosine phosphorylation of axin relieves this inhibition. The tyrosine phosphorylation sites in axin have been mutated to phenylalanine and the activities of the mutants in the Wnt pathway will be discussed.

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Caudal Wnt8c is a target of FGF signalling and inhibits neuronal differentiation onset in chick spinal cord

Isabel Olivera- Martínez and Kate G. Storey

Wnt signalling promotes posterior versus anterior cell fates in the early neural plate and also mediates a gradient of proliferation in the dorso-ventral axis of the closed neural tube (for a review see Yamaguchi, 2001; Megason and McMahon, 2002). However, Wnt8c expression in the spinal cord primordia adjacent to regressing primitive streak, suggests an additional role for Wnt signalling in the maintenance of this neural precursor cell pool. We have shown recently that the onset of neuronal differentiation in the spinal cord requires attenuation of FGF signalling and that this is achieved by somite derived signals, which reduce Fgf8 transcripts in the spinal cord primordia (Diez del Corral et al. 2002).

Here we show that Wnt8c is induced by and requires MAPKinase mediated FGF signalling. In addition, we show that somite derived signals also repress Wnt8c in the primordia. Further, ectopic maintenance of Wnt8c in the developing neural tube inhibits neurogenesis onset and may therefore mediate the ability of FGF to repress neuronal differentiation. Wnt8c inhibits expression of Delta-1 and NeuroM, both markers of post-mitotic neurons, indicating that it acts early in the neurogenesis pathway, prior to cell cycle exit. However, ectopic expression of Wnt8c does not promote proliferation in the neural tube and appears to arrest neural cells in an undifferentiated state. On-going studies address the identity of the somite derived signal(s) that repress Fgf8 and Wnt8c, the requirement for canonical Wnt signalling as a mediator of FGF induced repression of neuronal differentiation and further characterisation of cells expressing ectopic Wnt8c.

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Insights into the molecular mechanisms of Wingless lysosomal degradation

Eugenia Piddini and Jean-Paul Vincent

The activity of Wingless is regulated in part by its degradation. Our lab has previously shown that in the embryonic epidermis of *Drosophila* the cells posterior to each stripe of wingless expression degrade Wingless more readily than the anterior cells, by a mechanism that targets Wingless for lysosomal degradation (Dubois et al., 2001).

We are interested in understanding the cell biological basis of this process. We are undertaking a candidate-based approach to try and identify the regulators involved.

We are establishing a cell-culture assay to assess the role of the *Drosophila* Wg receptors (Arrow and the Frizzled family) and their possible post-translational modifications in Wg degradation. Since ubiquitination is one important modification that regulates endocytic sorting of the receptors, we are investigating whether Wg degradation could be modulated by ubiquitination. We are also exploring the role of hrs (a gene involved in the formation of MVBs; Lloyd et al., 2002) in Wg trafficking.

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Wnt signaling regulates thalamic development

Chengji Zhou, Kathy I. Pinson, William C. Skarnes, Samuel J. Pleasure

Although it has been demonstrated that Wnt signaling pathways are crucial for normal development of the CNS, and several Wnt genes are expressed in the developing diencephalon, there has been no direct evidence for physiological functions of Wnts in the developing thalamus. Also, most studies on the effects of Wnt signaling in the spinal cord and cortex conclude that these effects are most likely to be due to alterations in precursor proliferation. Here we examine whether Wnts regulate diencephalic development by analyzing LRP6 mutant mice. Since LRP6 is a recently described Wnt co-receptor this would provide direct evidence for a role for Wnt signaling in these structures. In these mice, we have found that the majority of dorsal thalamic neurons (which strongly express several transcription factors Prox1, Tcf4, Gbx2 and Id4) do not develop.

This occurs despite the lack of any evidence for a proliferation defect in the dorsal thalamus. Also, projections from the dorsal thalamus to cortex failed to form properly. In addition, we noted the disruption of the boundary between ventral and dorsal thalamus (the zona limitans interthalamica), which has been proposed to be a local signaling center and the spread of ventral thalamic ventricular zone markers (Pax6 and Olig2) into the dorsal thalamic ventricular zone. Our results provide strong evidence for a function of the Wnt signaling pathway in thalamic development and furthermore, show that this is likely to be due to a loss of proper thalamic segmentation or cell fate regulation rather than effects on proliferation.

Dachsous, is required in the Wg receiving cells for wing-hinge specification

I. Rodríguez

In the wing imaginal disc of Drosophila, during the second larval instar, Wingless (Wg) signalling is essential to specify the wing blade and wing hinge. I have identified a new gen, dachsous (ds), that is required to facilitate the reception of Wg signalling in the receiving cells during this early proccess. ds encodes for a cadherin that is expressed in a restricted pattern during imaginal development. ds mutations show phenotypes which resemble to those caused by insuficient Wg signalling. These phenotypes can be rescued by increasing Wg. Genetic interactions with other components of wg signalling provide additional evidences that ds is new component of Wg signalling cascade during wing-hinge specification.

The transcriptional repressor brinker antagonizes Wingless signaling

E. Saller, A. Kelley, and M. Bienz

In the embryonic midgut of *Drosophila*, Wingless (Wg) signalling elicits threshold-specific transcriptional responses: low signalling levels activate target genes, whereas high signalling levels repress them.

Wg-mediated repression of the HOX gene Ultrabithorax (Ubx) is conferred by a response sequence within the Ubx B midgut enhancer, called WRS-R. It further depends on the Teashirt (Tsh) repressor which acts through the WRS-R without binding to it. Here, we show that Wg-mediated repression of Ubx B depends on Brinker which binds to the WRS-R. Furthermore, Brinker blocks transcriptional activation by ubiquitous Wg signalling. Brinker binds to Tsh *in vitro*, recruits Tsh to the WRS-R, and we find mutual physical interactions between Brinker, Tsh and the co-repressor dCtBP. This suggests that the three proteins may form a ternary repressor complex at the WRS-R to quench the activity of the nearby-bound dTCF/Armadillo transcription complex. Finally, brinker and tsh produce similar mutant phenotypes in the ventral epidermis, and double mutants mimic overactive Wg signalling in this tissue. This suggests that Brinker may have a widespread function in antagonizing Wg signalling.

Lineage-specific requirements of ß-catenin in neural crest stem cells

Hari L., Brault V., Kléber M., Lee H.Y., Ille F., Leimeroth R., Paratore C., Suter U., Kemler R., and Sommer L.

ß-Catenin plays a role both in Cadherin-mediated cell adhesion and in mediating Wnt signaling. To study the role of ß-catenin in neural crest development, we used the Cre/loxP system to ablate ß-catenin specifically in neural crest stem cells. While several neural crest-derived structures develop normally, mutant animals lack melanocytes and dorsal root ganglia (DRG). *In vivo* and *in vitro* analyses revealed that mutant neural crest cells emigrate but fail to generate an early wave of sensory neurogenesis that is normally marked by the transcription factor neurogenin2 (ngn2).

This indicates a role of ß-catenin in premigratory or early migratory neural crest and points to heterogeneity of neural crest cells at earliest stages of crest development. In addition, migratory neural crest cells lateral to the neural tube do not aggregate to form DRG and are unable to produce a later wave of sensory neurogenesis usually marked by the transcription factor ngn1. Culture experiments with neural crest stem cells in the presence of Wnt indicate that the requirement of ß-catenin for the specification of melanocytes and sensory neuronal lineages reflects roles of ß-Catenin both in Wnt signaling and in mediating cell-cell interactions.

Vertebrate *prickle1* regulates cell movements during gastrulation and neuronal migration

Filipa Carreira-Barbosa, Miguel L. Concha, Masaki Takeuchi, Naoto Ueno, Stephen W. Wilson and Masazumi Tada

During vertebrate gastrulation, mesodermal and ectodermal cells undergo convergent extension, a process characterised by prominent cellular rearrangements in which polarised cells intercalate along the medio-lateral axis leading to elongation of the antero-posterior axis. Recently, it has become evident that a non-canonical Wnt/Frizzled (Fz)/Dishevelled (Dsh) signalling pathway, related to the planar cell polarity (PCP) pathway in flies, regulates convergent extension during vertebrate gastrulation. We isolated and functionally characterised a zebrafish homologue *of Drosophila prickle (pk)*, a gene implicated in the regulation of PCP. Zebrafish *pk1* is expressed maternally and in migrating mesodermal precursors. Abrogation of Pk1 function by morpholino oligonucleotides leads to defective convergent extension movements, enhances the *silberblick (slb)/wnt11 and pipetail/wnt5* phenotypes and suppresses the ability of Wnt11 to rescue the *slb* phenotype. Gain-of-function of Pk1 also inhibits convergent extension movements and enhances the *slb* phenotype, most likely due to the ability of Pk1 to block the Fz7-dependent membrane localisation of Dsh. Finally, we show that *pk1* genetically interacts with *trilobite (tri)/strabismus* to mediate the caudally directed migration of cranial motor neurons as well as convergent extension. These results suggest that (1) during zebrafish gastrulation, Pk1 acts downstream of the non-canonical Wnt11/Wnt5 pathway to regulate convergent extension cell movements, but is unlikely to simply be a linear component of this pathway and that (2) Pk1 interacts with Tri to mediate posterior migration of branchiomotor neurons, probably independent of the non-canonical Wnt pathway.

Genetic and epigenetic changes in several components of the WNT signaling pathway in a large series of colorectal carcinomas

Lin Thorstensen, Tone Løvig, Guro E. Lind, Rolf I. Skotheim, Chieu B. Diep, Gunn I. Meling, Torleiv O. Rognum, Ragnhild A. Lothe

The WNT signaling pathway is important both in the embryogenesis and carcinogenesis. Approximately all colorectal carcinomas show alteration of this pathway, leading to accumulation of beta-catenin and transcription of downstream target genes like the c-MYC, cyclin D1 and the WISPs (WNT inducible signaling pathway proteins). The tumor suppressor gene, APC, is a key regulator of this pathway and is changed in the majority of colorectal tumors. Among 277 colorectal carcinomas, 37 were microsatellite instable-high (MSI-H), 33 MSI-low (L) and 207 microsatellite stable (MSS) (1). APC and TP53 mutations have previously been detected in 65% (144/220) and 46% (102/222) of these tumors, respectively (2, 3). Both mutations were more common in the MSI-L and MSS tumors than in the MSI-H tumors ($p<0.001$ and $p=0.003$, respectively), and TP53 mutations were found associated with aneuploid ($p<0.001$). Only 2/215 tumors screened for mutations in exons 3, 5, 6, 7 and 8 of beta-catenin showed alterations (2).

MSI tumors show a genome wide instability in repetitive sequences due to defect mismatch repair. Genes with repetitive sequences within their coding region are prone for mutations in these tumors. AXIN2, a component of the multiprotein complex leading to degradation of beta-catenin, was mutated in 6/32 (19%) of the MSI-H tumors. Interestingly, three cases, all with a specific frameshift mutation in AXIN2 leading to a late truncation in the protein, were concurrently mutated in APC. Mutations of the transcription factor TCF-4 ([A]9 in exon 17) and of the downstream target gene WISP-3 ([A]9 in exon 4) were observed in 42% (15/36) and 25% (9/36) of the MSI-H tumors, respectively.

Hypermethylation of the promoter region of APC was seen in 25% (13/53) of the tumors and E-cadherin was hypermethylated in 42% (15/36) of the cases.

Recently, we observed increased expression of gamma-catenin, a protein sharing functions with beta-catenin in cell adhesion and WNT signaling, in testicular germ cell tumors (TGCTs) (4). The fact that development of TGCT mimics the embryogenesis further strengthens the hypothesis that this pathway is involved in TGCT tumorigenesis.

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Enhancement of beta-catenin transactivation activity by the coactivator FHL2 on TCF-mediated transcription in epithelial cells

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We have identified the four and a half LIM-only protein 2 (FHL2) as a novel ß-catenin interacting protein in a yeast two-hybrid screen. We showed specific interaction of FHL2 with ß-catenin in yeast and mammalian cells, which requires the intact structure of FHL2 and armadillo repeats 1-9 of ß-catenin. FHL2 cooperated with ß-catenin to activate TCF/LEF-dependent transcription from a synthetic reporter and the cyclin D1 and IL-8 promoters in kidney and colon cell lines. In contrast, coexpression of ß-catenin and FHL2 had no synergistic effect on androgen receptor (AR)-mediated transcription, while each of these two co-activators independently stimulated AR transcriptional activity. Thus, the ability of FHL2 to stimulate the trans-activating function of ß-catenin might be dependent on the promoter context. Detection of increased FHL2 expression in hepatoblastoma, a liver tumor harbouring frequent ß-catenin mutations, suggests that FHL2 might enforce ß-catenin transactivation activity in cancer cells. We are currently investigating expression of TCF/ß-catenin target genes, such as cyclin D1 and c-myc, in hepatoblastoma.

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