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Molecular mechanisms of Wnt signalling in mammalian cells

Jan Lukáš

Supervisor: RNDr. Vladimír Kořínek, CSc.

Laboratory of Cell and Developmental Biology

Institute of Molecular Genetics
The Academy of Sciences of the Czech Republic

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I hereby declare that this thesis was written solely by me and that it has not been submitted, wholly or substantially, either for other academic award or for a qualification at any other institution. All sources of information used for the text compilation were properly referenced.

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LIST OF ABBREVIATIONS

ac	achaete
ADP	adenosine 5`-diphosphate
ALY	Ally of AML-1 and LEF1
AML-1	Acute myeloid leukaemia 1
AN	Angustifolia
AP-1	activator protein 1
AP	anterior-posterior
APC	Adenomatous polyposis coli
APR-1	APC-related 1
ArgBP2A	Abelson-related gene-binding protein 2A
arm	Armadillo
Atro	Atrophin
Axin	Axis inhibition
β_2AR	β_2-adrenergic receptor
βarr2	β-Arrestin 2
BARS	Brefeldin A-ADP-ribosylated substrate
Bcl-2, -6, -9	B-cell lymphoma 2, 6, 9
BCRL-2	Breakpoint cluster region-like 2
β-gal	β-galactosidase
Bkh	Brokenheart
BMP	Bone morphogenetic protein
Botv, botv	Brother of tout-velu
bp	base pair
BTB/POZ	Broad-complex, Tramtrack, Bric à brac/Poxvirus and Zinc finger
β-TrCP	β-transducin repeat-containing protein
C57MG	C57BL/6 mouse mammary gland - derived
CaM	Calmodulin
CaMKII	calcium/calmodulin-dependent protein kinase II
cAMP	cyclic 3`, 5`-adenosine monophosphate
CAP	c-Cbl-associated protein
Cbl	Casitas B-lineage lymphoma
CBP	CREB binding protein
cca	circa
cDNA	complementary DNA
ChIP	chromatin immunoprecipitation
Chrd	Chordin
Ci	Cubitus interruptus
CKIα, γ, δ, ε	Casein kinase Iα, γ, δ, ε
CKII	Casein kinase II
Cn	Calcineurin
CoA	coenzyme A
COS-7	CV-1 in origin, carrying SV40

CRD	Cystein rich domain (within SFRPs)
CRD	context-dependent regulatory/repression domain (within LEF/TCF factors)
CREB	cAMP response element-binding
CS	chondroitin sulphate
CtBP	C-terminal binding protein
CUL1	Cullin-1
DAG	1, 2-diacylglycerol
Dally, dally	Division abnormally delayed
DAZ	Deleted in azoospermia
DAZap2	Deleted in azoospermia-associated protein 2
DAZL1	DAZ-like 1
Dco	Discs overgrown
DCT	Dopachrome tautomerase
DEP	Dishevelled, Egl-10 and Pleckstrin
DFz	*Drosophila* Frizzled
D2-HDH	D-isomer-specific 2-hydroxyacid dehydrogenase
DIX	Dishevelled and Axin
DKK, Dkk	Dickkopf
DLD-1	D.L. Dexter-isolated colorectal adenocarcinoma 1
Dll, dll	Distalless
Dlp, dlp	Dally-like protein
Dly	Dally-like
DRM	detergent-resistant membrane microdomain
Ds	Dachsous
Dsh	Dishevelled (*Drosophila*)
dsRNA	double-stranded RNA
DV	dorso-ventral
Dvl, dvl	Dishevelled (vertebrates)
E1A	Early 1 adenovirus
E6-AP	E6-associated protein
E10.5	embryonic day 10.5 of development
EBNA	Epstein-Barr virus nuclear antigen
ECM	extracellular matrix
eIF4G	eukaryotic initiation factor 4G
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
ERK1	Extracellular signal-regulated kinase 1
ES cells	embryonic stem cells
ETS	E twenty-six
Evi	Evenness interrupted
Ext, EXT	Exostosin
Extl, EXTL	Exostoses (multiple)-like
FAP	familiar adenomatous polyposis
FH2	formin homology 2

Fj	Four-jointed
Fos	Finkel-Biskis-Jinkins (FBJ) murine osteosarcoma
Ft	Fat
Fz	Frizzled (*Drosophila*)
Fzd, fzd	Frizzled (vertebrates)
Fzd4S	Frizzled 4, splicing variant
Gal	galactose
GAG	glycosaminoglycan
GAP	GTPase-activating protein
GBP/Frat	GSK-binding protein/Frequently rearranged in advanced T-cell lymphomas
GDP	guanosine 5`-diphosphate
GEF	guanine nucleotide exchange factor
Glc	D-glucose
GlcA	D-glucuronic acid
GlcNAc	N-acetyl-D-glucosamine
GPCR	G protein-coupled receptor
GPI	glycosylphosphatidylinositol
Grg	Groucho-related gene
Gro	Groucho
GSK3β	Glycogen synthase kinase 3β
GST	Glutathione S-transferase
GTP	guanosine 5`-triphosphate
Gug	Grunge
HAT	histone acetyltransferase
HDAC	histone deacetylase
HDL	High-density lipoprotein
HECT	Homologous to E6-AP carboxyl terminus
HEK293	human embryonic kidney, clone 293
Hh	Hedgehog
HIC1	Hypermethylated in cancer 1
HIV-1	Human immunodeficiency virus-1
HMG	high mobility group
HMT	histone methyl transferase
HRG22	HIC1-related gene on chromosome 22
HS	heparan sulphate
HSPG	heparan sulphate proteoglycan
5-HT_{1c}	5-hydroxytryptamine receptor, class 1c
ICAT	Inhibitor of β-catenin and TCF
IdoA	L-iduronic acid
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor-binding protein
Ins(1,4,5)P_3	inositol-1,4,5-trisphosphate
int-1	integration 1
IP_3	inositol-1,4,5-trisphosphate

IP_3R	IP_3-gated Ca^{2+}-release channel
ISO	isoproterenol
JNK	c-Jun N-terminal kinase
kb	kilobase
kDa	kilodalton
Krm	Kremen
LDL	Low density lipoprotein
LEF	Lymphoid (lymphocyte) enhancer-binding factor
LIT-1	Loss of intestine 1
LRP	Low-density lipoprotein-related protein
MBOAT	membrane-bound O-acyl transferase
MDS	Miller-Dieker syndrome
MEK1	Mitogen-activated protein kinase kinase 1
MITF	Microphthalmia-associated transcription factor
MLL	Mixed lineage leukemia
MM	multiple myeloma
MMP7	matrix metalloproteinase 7
MMTV	mouse mammary tumour virus
mom	more mesoderm
mom-3/mig-14	more mesoderm 3/abnormal cell migration 14
MRLC	Myosin regulatory light chain
MSI-H	microsatellite instability-high
mSin3a	mammalian homologue of yeast SWI-independent 3A
MYC	myelocytomatosis
MyoII	Myosin II
NAD^+	nicotinamide adenine dinucleotide, oxidised form
NADH	nicotinamide adenine dinucleotide, reduced form
N-CoR	Nuclear receptor co-repressor
NDP	Norrie disease protein
NEDD4	Neural precursor cell-expressed developmentally downregulated gene 4
NEMO	NF-κB essential modulator
Net1	Neuroepithelial cell-transforming gene 1
NFAT	Nuclear factor of activated T-cells
NF-κB	Nuclear factor of kappa light chain gene enhancer in (activated) B-cells
NGF	Nerve growth factor
Nkd	Naked cuticle
NLK	NEMO-like kinase
NLS	nuclear localisation sequence/signal
nNOS	neuronal Nitric oxide synthase
NTR	Netrin
NuRD	Nucleosome remodelling and histone deacetylation
ORS	outer root sheath
OST	oligosaccharyl transferase
Pak1	p21-activated kinase 1

Pc	Polycomb
P/CAF	p300/CBP-associated factor
PCP	planar cell polarity
PCR	polymerase chain reaction
PDE	phosphodiesterase
PDZ	Post synaptic density, Disc large 1 and Zonula occludens 1
PE	primitive endoderm
PIASy	Protein inhibitor of activated STAT y
PIP_2	phosphatidyl inositol-4,5-bisphosphate
PKA, C, G	Protein kinase A, C, G
PLCβ	phospholipase Cβ
PLZF	Promyelocytic leukaemia zinc finger
PML	Promyelocytic leukaemia
POP-1	Posterior pharynx defect 1
Porc, porc	Porcupine
PP2A	Protein phosphatase 2A
PPARδ	Peroxisome proliferator-activated receptor δ
Ppt	Pipetail
Prtb	Proline codon-rich transcript, brain expressed
$PtdIns(4,5)P_2$	phosphatidyl inositol-4,5-bisphosphate
PTX	pertussis toxin
qRT-PCR	real-time quantitative RT-PCR
Rac	Ras-related C3 botulinum toxin substrate
Ras	Rat sarcoma
RGC	retinal ganglion cell
RGS	regulator of G protein signalling
Rho	Ras homologue gene family
RIBEYE	Retina synaptic ribbons-expressed
RNAi	RNA interference
ROCK	Rho-associated coiled-coil-containing protein kinase
Rok	Rho kinase
Rpd3	Reduced potassium dependency 3
RSPO, Rspo	R-spondin
RT-PCR	reverse transcription (reverse transcriptase) PCR
Ryk	Related to Y (tyrosine) kinase
SCF	SKP1, CUL1 and F-box
sens	Senseless
SET	Su(var)3-9, Enhancer of zeste, Trithorax SF-1 Steroidogenic Factor 1
Sfl, sfl	Sulfateless
SFRP	secreted Frizzled-related protein
SG	stress granule
sgl	Sugarless
SH2, 3	Src homology 2, 3
SHP-1	Src homology phosphatase 1

shRNA	short hairpin / small hairpin RNA
Sip1	Smad-interacting protein 1
siRNA	small interfering / short interfering / silencing RNA
Sirt1	Sirtuin 1
SKP1	S-phase kinase-associated protein 1
Slb	Silberblick
SMRT	Silencing mediator for retinoid and thyroid hormone receptors
Sost	Sclerostin
Sotv, sotv	Sister of tout-velu
Sox6	Sry box 6
Sqh	Spaghetti squash
Src	Sarcoma
Srt	Sprinter
SSEA	stage-specific embryonic antigen
STAT	Signal transducer and activator of transcription
SUMO	Small ubiquitin-related modifier
SWI/SNF	Switch(ing)-deficient/Sucrose non-fermenting
SYS	Symmetrical sisters
Szl, szl	Sizzled
T_3	3, 3`, 5-triiodo-L-thyronine
TBP	TATA-box binding protein
TCF	T-cell (specific) transcription factor
TCRα	T-cell receptor α chain
TGN	trans-Golgi network
Tld, tld	Tolloid
TLE	Transducin-like enhancer of split
tPA	tissue plasminogen activator
TSA	trichostatin A
TSPAN12	Tetraspanin 12
TSR1	thrombospondin repeat, type 1
Ttv, ttv	Tout-velu
Vps35	Vacuolar protein sorting 35
vs.	versus
Wdb	Widerborst
Wf	Wingful
Wg, wg	Wingless
WIF	Wnt inhibitory factor
Wls, wls	Wntless
Wnt, wnt	Wingless-type MMTV integration site family
WntD	Wnt inhibitor of Dorsal
WRM-1	Worm Armadillo 1
Xyl	D-xylose
Y2H	yeast two-hybrid
ZF	zinc finger

COMMENTS ON THE NOMENCLATURE, ABBREVIATIONS AND SYMBOL USAGE ADOPTED IN THE TEXT

Each species has its own conventions to name genes. The only common rule is that they are set in italics. For example, human genes are spelled out in capital letters (e.g. *RSPO1*) whereas mouse genes are with the first letter in upper case and the rest in lower case (*Rspo1*); in other species, as in zebrafish, all letters are in lower case (*rspo1*) while in others, e.g. *Drosophila*, with either upper or lower initial followed by lower case according to whether the first identified mutant allele gave dominant or recessive phenotype. Conventions for naming proteins or polypeptides are equally varied.

To adopt a unified style throughout the text, I have decided to set the conventions aside and follow a uniform rule: in all instances where a gene is referred to in a general sense without specifying human, mouse or other species, gene names are set in upper case initial followed by lower case letters and all printed in italics (*Wnt*). However, there is one exception to this adopted rule. Whenever a gene name comes from an acronym, all letters are in upper case (e.g. *APC* for Adenomatous polyposis coli, *EGFP* for Enhanced green fluorescent protein, *GSK* for Glycogen synthase kinase, *TCF* for T-cell factor). Whenever the species origin has to be emphasised, either the conventions typical for that organism are followed or a respective prefix precedes the name (**h** for human, **m** for mouse, **r** for rat, **ch** for chicken, **z** for zebrafish, **D** for *Drosophila*, **X** for *Xenopus*).

Gene and allele symbols are italicised while protein symbols are represented in a standard font. To distinguish between mRNA, genomic DNA, cDNA, siRNA or shRNA, a pertinent word or abbreviation is always appended to the symbol whenever it is not fully clear from the context. In the case of a fusion gene insert, a hyphen separates the symbols of the two genes (e.g. *EGFP-TCF4*). A corresponding protein, if it takes its name from the gene, is given the same name, with the first letter upper-case, but not in italics (Wnt, EGFP-TCF4). For proteins not named after genes but given names independently (such as actin, tubulin), the initial letter is not capitalised.

Allele symbols are superscripted. When necessary and to emphasise, the wild-type allele of a gene is indicated by a "+" mark in a superscript. Likewise, mutant alleles are indicated by a "-" sign (e.g. APC^{-}).

Any DNA that has been stably introduced into a germline of mice, rats, other animals or in a genome of a cell line is a transgene. Transgenes produced by a homologous recombination as targeted events at particular loci are given the symbol of the targeted gene with a superscript. Usually, mice with germline disruption of only one allele of a gene, i.e. heterozygotes, are distinguished from the homozygous knockouts ($APC^{+/-}$ *vs*. $APC^{-/-}$).

To use abbreviations and symbols concisely and in a unified manner throughout the whole text, the following rules have been adopted: at the first appearance in the text the full wording with the corresponding abbreviation appended in brackets are used; from there onwards, the abbreviation is used in all subsequent cases but at the beginning of a sentence. For these later appearances, a reader is referred to the list of abbreviations placed on page 1. Set aside this rule, standard one-letter symbols of amino acids and nucleotides are used throughout the text. These abbreviations are neither explained nor referenced in the list of abbreviations.

1 SUMMARY

Molecular signalling constitutes part of an intricate communication network that affects basic cellular activities and coordinates cell behaviour. The ability of cells to percieve and correctly respond to microenvironmental cues enables regulation of developmental processes, immune response, tissue repair as well as tissue homeostasis maintenance. In a multicellular organism, tight control of intercellular communication is essential, among other, not only for ensuring coordinated cell rearrangements but also as a precaution against its potential for getting out of hand. Incorrect signal processing or inappropriate activation can ultimately result in developmental defects, disorders such as autoimmune diseases, inflammatory diseases and cancer.

Communication among cells is often mediated by extracellular signalling molecules that bind to receptors and modulate activities of specific intracellular effectors. The Wnt (the name coined as a combination of ”wingless” and ”int-1”) family of secreted glycoproteins represents one group of signalling molecules that has been shown to control a variety of processes ranging from cell fate specification to tissue patterning. Moreover, deregulation of Wnt-mediated signalling is often implicated in the genesis of a number of cancers.

The presented thesis briefly introduces into basic molecular mechanisms underlying Wnt signalling. The experimental part focused mainly on the nuclear processes, the main aim having been an attempt to find novel proteins modulating the output of the whole signalling pathway.

The essence of the work is summarised in a collection of four articles with the unifying theme - a study of components and mechanisms that affect Wnt signalling. The main effort was put into finding interaction partners capable of influencing Wnt-induced transcriptional output. The second objective was an examination of the role of post-translational modifications in secretion, extracellular movement and signalling activity of mammalian Wnt1 and Wnt3a proteins. The aforementioned publications are as follows:

1. Valenta, T., **Lukas, J.** and Korinek, V. (2003): HMG box transcription factor TCF4's interaction with CtBP1 controls the expression of the Wnt target *Axin2*/*Conductin* in human embryonic kidney cells. *Nucleic Acids Res*, **31**: 2369-2380

2. Valenta, T., **Lukas, J.**, Doubravska, L., Fafilek, B. and Korinek, V. (2006): HIC1 attenuates Wnt signalling by recruitment of TCF4 and β-catenin to the nuclear bodies. *Embo J*, **25**: 2326-2337

3. **Lukas, J.**, Mazna, P., Valenta, T., Doubravska, L., Pospichalova, V., Vojtechova, M., Fafilek, B., Ivanek, R., Plachy, J., Novak, J. and Korinek, V. (2009): DAZap2 modulates transcription driven by the Wnt effector TCF4. *Nucleic Acids Res*, **37**: 3007-3020

4. Doubravska, L., Krausova, M., Gradl, D., Vojtechova, M., Tumova, L., **Lukas, J.**, Valenta, T, Pospichalova, V., Fafilek, B., Plachy, J., Sebesta, O. and Korinek, V. (2011): Fatty acid modification of Wnt1 and Wnt3a at serine is prerequisite for lipidation at cysteine and is essential for Wnt signalling. *Cell Signal*, **23**: 837-8

2 INTRODUCTION

The signalling pathways initiated by secreted Wnt glycoproteins provide important molecular means for cell fate specification (e.g. the decision to proliferate or differentiate) as well as for control of cell motility and polarity. They are involved in virtually every aspect of embryonic development and in control of homeostatic self-renewal in a number of adult tissues of multicellular organisms. Their importance is eloquently supported by the fact that they are conserved through evolution from *Hydra* to humans [9].

According to current knowledge, there exist at least three different signalling cascades initiated by Wnt proteins. The most studied and best understood is unarguably the so-called canonical (Wnt/β-catenin) pathway. It starts when Wnt ligand engages receptor complex of Frizzled/Low-density lipoprotein receptor-related protein (Fzd/LRP). This leads to a series of intricate intracellular events culminating in the stabilisation of a key protein β-catenin, which enters the nucleus and binds Lymphoid (lymphocyte) enhancer-binding factor/T-cell (specific) transcription factor (LEF/TCF) transcription factors to stimulate gene expression.

Some Wnt proteins trigger pathways collectively referred to as non-canonical. They are understood less well but so far appear to function in a β-catenin-independent manner. These non-canonical pathways have also been termed Wnt/calcium (Wnt/Ca^{2+}) [3; 9], Wnt/c-Jun N-terminal kinase (Wnt/JNK) [10; 11] and Wnt/planar cell polarity [Wnt/PCP; or Frizzled/planar cell polarity (Fzd/PCP)] pathways [12-15]. Their activation includes mechanisms that overlap with other signalling processes. In the Wnt/Ca^{2+} pathway, Wnt binding to a Fzd receptor leads to the release of intracellular Ca^{2+} and activation of enzymes such as calcium/calmodulin-dependent protein kinase II (CaMKII) and protein kinase C (PKC) in a heterotrimeric G protein-dependent manner [12]. The Wnt/JNK and PCP pathways appear to be similar to each other in utilising Fzd receptors, Dishevelled (Dsh/Dvl), JNK and Ras homologue (Rho) family GTPases. While Wnts are employed in the vertebrate Wnt/JNK signalling, no Wnt ligand has been identified for the *Drosophila* PCP pathway yet. Until now, the conservation of function of these pathways in *Caenorhabditis elegans* remains unclear.

On top of an existence of several modes of action for Wnt ligands, Norrin (NDP) and R-spondin (Rspo), two secreted proteins unrelated to Wnt, capable of binding to Fzd/LRP receptors and thus initiating the whole cascade, have been identified [17-20]. Furthermore, other molecules, revealed to function as alternative Wnt-binding receptors, have been described [21-24]. These include transmembrane tyrosine kinase receptors Receptor tyrosine kinase-like orphan receptor (Ror) [25; 26] and Related to receptor tyrosine kinases (Ryk) [27; 28] leading to alternative modes of Wnt signalling [21; 29-31]. All in all, it is evident that Wnt signalling should be viewed as a much more complex process with a network character rather than a set of isolated cellular pathways [13].

As our work has dealt with the canonical way of Wnt signalling, non-canonical pathways will be mentioned only in an extent enabling to complement the overall picture. A good starting point as a source of information for a reader interested in a more comprehensive view of Wnt pathways is provided on „The Wnt homepage“ curated by the Nusse Lab (http://www.stanford edu/group/nusselab/cgi-bin/wnt/).

2.1 An overview of signalling pathways triggered by Wnt proteins

2.1.1 Canonical Wnt/β-catenin signalling pathway

The canonical Wnt pathway represents one of the principal molecular mechanisms governing cell fate determination. It directs cell fate choices in both invertebrate and vertebrate embryogenesis. For instance, in *C. elegans* the Wnt/β-catenin signalling controls neuroblast progeny migration and ensures that vulval precursor cells adopt their fates correctly in the process of vulval induction. Among other developmental processes in *Drosophila*, it helps establish the segment polarity and controls formation of functional synapses during larval development. In vertebrates, canonical Wnt signalling is indispensable for anterior-posterior patterning, dorsal-ventral axis specification/formation in the developing embryo [14], neural crest induction and maintenance [15], body segmentation control as well as axon guidance during synapse formation [16]. Recently accumulated data point to the role in the maintenance of self-renewal potential of stem and progenitor cells in many different tissues [36-41]. Current evidence also supports an important involvement of Wnt/β-catenin pathway in some regeneration processes [42-45]. Misregulation of the Wnt signalling during embryogenesis leads to developmental defects while aberrant activation later in development is associated with degenerative diseases and a number of cancers [46-52].

As already mentioned above, the key event in the canonical Wnt signalling is the stabilisation of β-catenin in the cytoplasm of a target cell [53-58]. Beta-catenin is a protein that plays a dual role: it serves as a structural component of adherens junctions at the plasma membrane [17; 18] and functions as a transcriptional activator in the cell nucleus in response to Wnt signal [58]. Neither the mutual interconnection of these two pools of the protein nor the regulation of the β-catenin amount in these locations is well understood. The stability of β-catenin is controlled by a cytoplasmic multiprotein complex referred to as β-catenin degradation (destruction) complex [61-66]. This complex contains Axin, Adenomatous polyposis coli (APC), the serine/threonine kinases Casein kinase Iα (CKIα) and Glycogen sythase kinase 3β (GSK3β) together with the associated phosphatases, e.g. protein phosphatase 2A (PP2A). In the absence of a Wnt signal, β-catenin is continually targeted there and gets sequentially phosphorylated

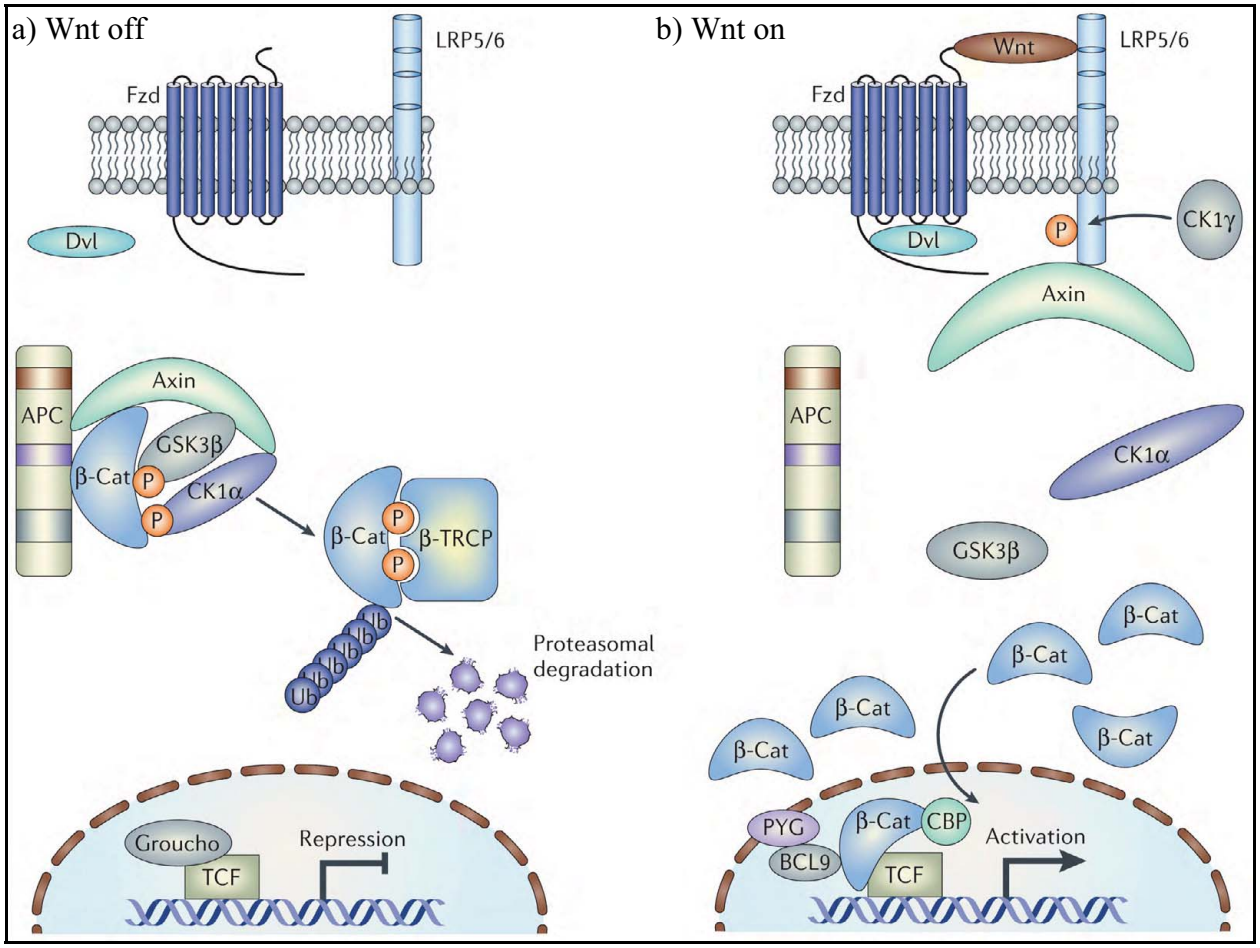

Fig. 1 The essence of canonical Wnt signalling

a) OFF-state. In the absence of Wnt, cytoplasmic β-catenin is targeted to the destruction complex. Phosphorylated by CK1 and subsequently by GSK3, β-catenin is recognised by β-TrCP, the subunit of E3 ubiquitin ligase and sent to proteasomal degradation. The Wnt target genes are repressed by TCF-TLE/Groucho and histone deacetylases (HDACs).
b) ON-state. In the presence of a Wnt ligand, a receptor complex forms between Fzd and LRP5/6. The signalling cascade continues through Dvl anchored by Fzd to LRP5/6 phosphorylation and Axin recruitment. This disrupts Axin-mediated phosphorylation/degradation of β-catenin, allowing it to accumulate and translocate to the nucleus where it acts as a coactivator of TCF-mediated transcription.
For details see the text. Adopted from [1], modified.

by the kinases of the destruction complex - first at S45 by CKIα, then by GSK3β at S33, S37 and T41 (amino acid residues are given in the standard one-letter code together with a number representing the position in a polypeptide) [67-71]. The interaction between the two kinases and β-catenin is facilitated by the scaffolding proteins Axin and APC [19]. The phosphorylation of β-catenin [70] earmarks it for binding to the F-box protein β-transducin repeat-containing protein (β-TrCP), a component of an E3 ubiquitin ligase. Subsequent ubiquitination by the SKP1, CUL and F-box protein (SCF) complex is followed by β-catenin degradation in the proteasome [20]. The outcome is that intracellular levels of free cytoplasmic β-catenin are kept low enough to prevent its transcriptional impact.

Upon Wnt ligand binding to the seven-pass transmembrane receptor Fzd and the co-receptor LRP, a cascade of signal relay is initiated that finally ends up in differential gene expression.

The signal is transmitted only when Fzd and LRP are complexed with Wnt [74-76]. Most Wnt proteins can bind to multiple Frizzleds and *vice versa* [21]. Recent work suggests that activation of Fzd/LRP causes two distinct events: binding of Axin to the cytoplasmic tail of LRP and Fzd-induced phosphorylation of Dvl. These two branches, i.e. LRP/Axin and Fzd/Dvl, then converge on β-catenin stability and nuclear localisation. The cytoplasmic tail of LRP contains two clusters of serines and threonines flanking five central PPP(S/T)P motifs that become phosphorylated following Wnt stimulation [78]. The prime-phosphorylation of the PPP(S/T)P sites by GSK3 is required for the subsequent phosphorylation of adjacent S/T sites by Casein kinase Iγ (CKIγ). Interestingly, CKIγ has a membrane anchor in the form of a palmitoyl tail. Casein kinase Iγ was shown to be essential for Wnt signalling to occur. Overexpression of *CKIγ* is sufficient to activate the pathway. Conversely, elimination of fatty acid anchor domain from CKIγ leads to halt in Wnt signalling [22]. A question of how these two kinases become activated by Wnt signal remains to be solved. Two obvious possibilities have been proposed so far. Either GSK3-dependent PPP(S/T)P motif phosphorylation is induced by Wnt [80] or it is the subsequent modification of the neighbouring S/T residues, catalysed by CKIγ, that is dependent on Wnt signal [79]. It has also been suggested that neither GSK3 nor CKIγ is activated by Wnt and instead, it is rather the Wnt-Fzd-LRP interaction allowing LRP to become prone to phosphorylation by the two kinases [23]. Anyway, LRP phosphorylation provides a docking site for Axin [82; 83], sequestering it away from the destruction complex. The recruitment of Axin to the plasma membrane leads to its inactivation and/or degradation and offers a mechanism for promoting β-catenin stability. Interestingly, Axin was shown to be present in cells in much lower concentrations than other proteins participating in β-catenin stability regulation [24]. Therefore, it seems very likely that Wnt signal influences β-catenin fate through fine-tuning Axin cytoplasmic levels. In such a model, Axin would play a dynamic role shuttling between the receptor and the β-catenin destruction complex rather than being a mere scaffolding protein [25]. Axin degradation at the plasma membrane should promote not only β-catenin stability but also its nuclear localisation because of the loss of cytoplasmic tethering [26]. Axin recruitment to the plasma membrane is the point where the second branch of the Wnt signal transmission seems to associate. It was shown that Wnt stimulation promotes Fzd-Dvl association [56; 87-90] and Fzd-dependent phosphorylation of Dvl [27]. The kinase responsible for Dvl phosphorylation during canonical Wnt signalling is thought to be Casein kinase Iε (CKIε) [68; 92-95]. Nevertheless, it is fair to note that there are other kinases able to phosphorylate Dvl following Wnt-mediated activation, among others Casein kinase Iδ (CKIδ) [96; 97] and Casein kinase II (CKII) [28]. Activated Dvl along with phosphorylated LRP participate in translocation of Axin to the plasma membrane. Thus, the activity of β-catenin destruction complex is inhibited and β-catenin escapes degradation. Accumulated β-catenin enters the nucleus where it binds to LEF/TCF (hereafter TCF) transcription factors [57; 99-105]. These DNA-binding proteins do

not have the ability to launch transcription *per se*. In the absence of a Wnt signal, TCF occupies target genes promoters and represses their transcription [29] through the recruitment of Transducin-like enhancer of split (TLE)/Groucho (Gro) and/or C-terminal binding protein (CtBP) co-repressors [106-109]. Beta-catenin brings a strong activation domain at its C-terminus [105] and recruits multiple chromatin-modifying and -remodelling complexes [reviewed in 30]. Thus, upon binding to TCF, β-catenin transiently converts it into a transcriptional activator. The formation of β-catenin/TCF bipartite transcription factor complexes stimulates the transcription of Wnt target genes. When Wnt signal ceases, β-catenin is removed from the nucleus, possibly by joined APC and Axin export activities [31-34], and subsequently degraded. As a consequence, TCF reverts back to a transcriptional repressor function.

In sum, the canonical pathway translates the Wnt signal into a transient transcription of target genes mediated by LEF/TCF factors. The whole pathway is a very complex mechanism tightly controlled at all its levels – extracellular, cytoplasmic as well as nuclear. Apart from the involved proteins that exert modulating or even blocking functions, the whole cascade contains many feedback regulatory loops (see further in the text). Genes coding for several proteins directly involved in the signal relay are also its transcriptional targets (feedback targets).

It should be stressed here that only the basic aspects of Wnt/β-catenin signalling (i.e. only the well established, core components of the pathway) were outlined and many so far non-resolved issues were not considered. Some of them will be discussed later in the text. It is also essential to always bear in mind that all the above sketched (as well as the next) represents just a model to fit the obtained experimental data as close as possible. Therefore, it reflects the present-day knowledge yet it is prone to revision.

2.1.2 Non-canonical Wnt signalling

The common feature of non-canonical Wnt signalling is an independence of β-catenin. The non-canonical pathways regulate processes as diverse as an establishment of hairs, bristles and ommatidia polarity in *Drosophila*, endoderm induction in *C. elegans*, cell polarity and morphogenetic movements (e.g. convergent extension during gastrulation and neurulation) in vertebrates.

The non-canonical Wnt pathway in *C. elegans* stands out in several aspects. It uses a β-catenin orthologue Worm armadillo-1 (WRM-1) and is distinct from the non-canonical pathways in vertebrates. Unlike in vertebrates, where the non-canonical Wnt signalling does not employ either β-catenin or LEF/TCF proteins, in *C. elegans* this pathway makes use of WRM-1/β-catenin, Posterior pharynx defect-1 (POP-1)/TCF and the Loss of intestine-1 (LIT-1)/Nemo-like kinase (Nlk). Other Wnt signalling components such as Wnt receptors, APC related-1

(APR-1)/APC, GSK-3/GSK3β and POP-1/TCF function in both types of pathways, but can behave differently according to the type of signalling. Furthermore, in *C. elegans* some variability exists in how the same components are utilised within non-canonical Wnt signalling pathways. For these peculiarities, which definitely lie far beyond the scope of this text, the non-canonical Wnt signalling in *C. elegans* will not be further discussed.

2.1.2.1 Planar cell polarity

In tissues, cells show more or less uniform properties and in addition to apical-basal polarity often require positional information in the plane. This type of polarisation in a field of cells is referred to as planar cell polarity (PCP).

Planar cell polarity provides a mechanism governing cell and tissue patterns and movements in both invertebrates and vertebrates. It is crucial for many developmental processes that require major cytoskeletal rearrangements. According to current knowledge, PCP integrates both local and global signals to orient both subcellular and multicellular structures with respect to body axes. Studies in *Drosophila*, mice, frogs and zebrafish have revealed that similarities as well as differences exist between *Drosophila* and vertebrates [e.g. 35-38].

One of the identified mechanisms responsible for controlling the organisation of repeating structures in a sheet of cells has been shown to utilise some of the proteins involved in the Wnt/β-catenin pathway. Therefore, the terms Wnt/PCP or Fzd/PCP were coined. In addition to providing polarising information to static cells, Fzd/PCP signalling establishes cell polarity also in dynamic tissues during their remodelling. Among the well-known examples belong the processes in *Drosophila* eye imaginal discs during ommatidial rotation of photoreceptor clusters and in vertebrates an elongation and closure of the neural tube or an orientation of the inner ear epithelium stereocilia [e.g. 39-41].

The initial findings regarding the cell polarisation within a tissue were gathered from insect cuticle transplantations and wound healing [42; 43]. The pioneering experiments that finally lead to the identification of PCP were conducted as early as in 1940s [44]. Planar cell polarity was first recognised in *Rhodnius prolixus* (blood-sucking triatomine bug), *Oncopeltus fasciatus* (large milkweed bug) and *Drosophila melanogaster* [122; 125; 126]. In *D. melanogaster* mutations of PCP genes cause disorganisation of cuticular structures and/or compound eye [127-131]. The wing hairs, for example, show swirls and wavy patterns instead of a characteristic proximal-distal orientation. Similarly, in the eye, PCP mutants show defects in the arrangement of photoreceptors. Based on these phenotypes, a set of genes responsible for PCP was discovered. Today it is obvious that many genes that control the establishment of planar polarity in *Drosophila* are evolutionarily conserved also in vertebrates [e.g. 132; 133].

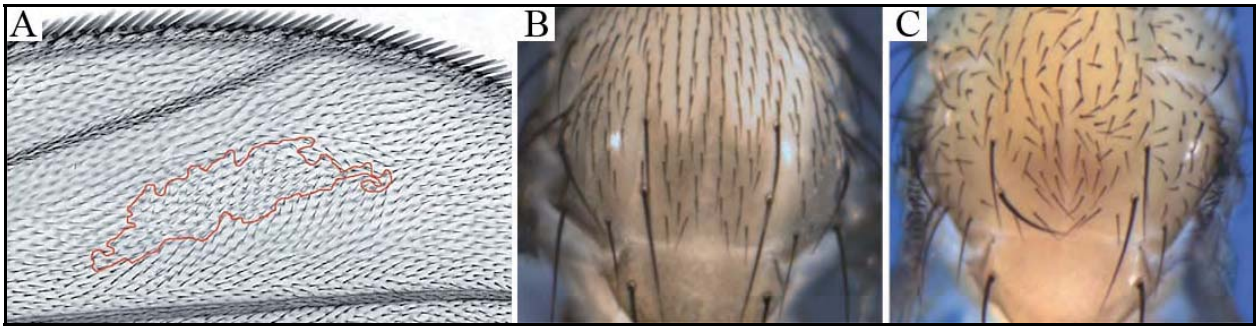

Fig. 2 Manifestation of epithelial planar polarity in *Drosophila* – examples

In *Drosophila*, mutations in several PCP genes cause re-polarisation of wild-type cells adjacent to the mutant clone. The effect is most obvious in the wing and the abdomen where the planar polarity is reflected in the regular arrangement of actin-based hairs.
A) $Fz^{-/-}$ clone (outlined in orange), identifiable by the expression of a marker causing wing cells to produce several wing hairs instead of one hair per cell, makes the neighbouring wild-type cells on the distal and lateral side re-orient themselves towards the mutant patch. The re-orientation is supposed to reflect the changes in the slope of the activity gradient of the polarizing factor. In a mutant clone, there is a local drop in the activity of the missing factor. Therefore, the wild-type cells surrounding the $Fz^{-/-}$ clone point their hairs towards cells with lower Fz activity, i. e. towards the clone. Clonal overexpression of PCP factors also causes non-autonomous phenotypes, but hairs point to the opposite direction as compared to the loss-of-function situation. Adopted from [2], modified.
B), C) PCP on thorax. Mechanosensory bristles on wild-type adult thorax (B) are uniformly patterned and oriented along the anterior-posterior axis of *Drosophila* body. In *DFz* mutant (C) the regular pattern is randomised. Adopted from [3], modified.

Frizzled/PCP signalling depends on complex interactions between core components which end up in their asymmetric distribution and ultimately polarised activity in a cell. Currently, no simple sequence of protein-protein interactions as in canonical pathway can be delineated. The whole mechanism can be viewed as a feedback regulatory loop rather than a linear signalling cascade. The core PCP pathway components in *Drosophila* are cell-surface proteins Frizzled (Fz), Strabismus (also known as Van Gogh; Stbm/Vang) and Flamingo (or Starry night; Fmi/ Stan) and the cytoplasmic proteins Dishevelled (Dsh), Prickle (also known as Spiny legs; Pk/Sple) and Diego (Dgo). The hallmark of the PCP process in *Drosophila* is the asymmetric and polarised membrane association of PCP proteins. At early fly pupal stages, at first PCP proteins are regularly distributed within the cytoplasm. Dishevelled and Dgo are recruited to the plasma membrane by their association with each other and the interaction of Dsh and membrane protein Fz. Flamingo, a seven-pass transmembrane protocadherin capable of mediating homophilic adhesion [45], recruits Fz and Stbm to the region of the adherens junctions [46]. Frizzled can associate with Dsh and Stbm binds Pk. Eventually, there are two distinct complexes localised to the apical cell surface, Fz/Dsh and Stbm/Pk. Once at the surface, the activity of all six core PCP proteins is required to sort out into proximal and distal domains to achieve a polarised planar distribution [47]. There is evidence that Pk can block the association between Fz and Dsh [137]. Conversely, Dgo can associate with Pk and Stbm and counteract Pk activity to allow Fz/Dsh complexes to form at the distal surface [138]. These interactions underlie the mechanism by which Stbm/Pk and Fz/Dsh complexes form in mutually exclusive cellular domains. It was observed in a live-imaging study that Fz-GFP gets transported along the microtubules in vesicles

that contain Fmi. Microtubule disorganisation results in the loss of Fz and Fmi from the surface and a disruption of Fz-GFP transport [139]. Therefore, it is likely that a microtubule network oriented in proximal-distal direction is active in establishing a polarised distribution of Fz in an apical plane of cells. The outcome is that Stbm and Pk localise to the proximal cell surface whereas Fz, Dsh and Dgo to the distal face of each cell. Flamingo localises to both proximal and distal faces. All of these proteins are under-represented on the remaining (i.e. anterior and posterior) faces. It appears that dynamic and persisting antagonism between PCP proteins at opposing cell surfaces serves to maintain their polarised distribution.

The experimental data strongly suggest that PCP proteins interact with each other either directly or indirectly not only at the surface of the same cell but also with PCP proteins at the surface of the adjacent cell [134; 140-143]. This is in agreement with the need for a mechanism that would spread the uneven distribution of proteins in the apical plane of a single cell to the whole plane of cells in a tissue. Currently, there are several models proposed to explain how directional information is propagated from cell to cell [144-146]. The „feedback“ model posits a heterophilic protein interactions between Fz and Stbm. Frizzled

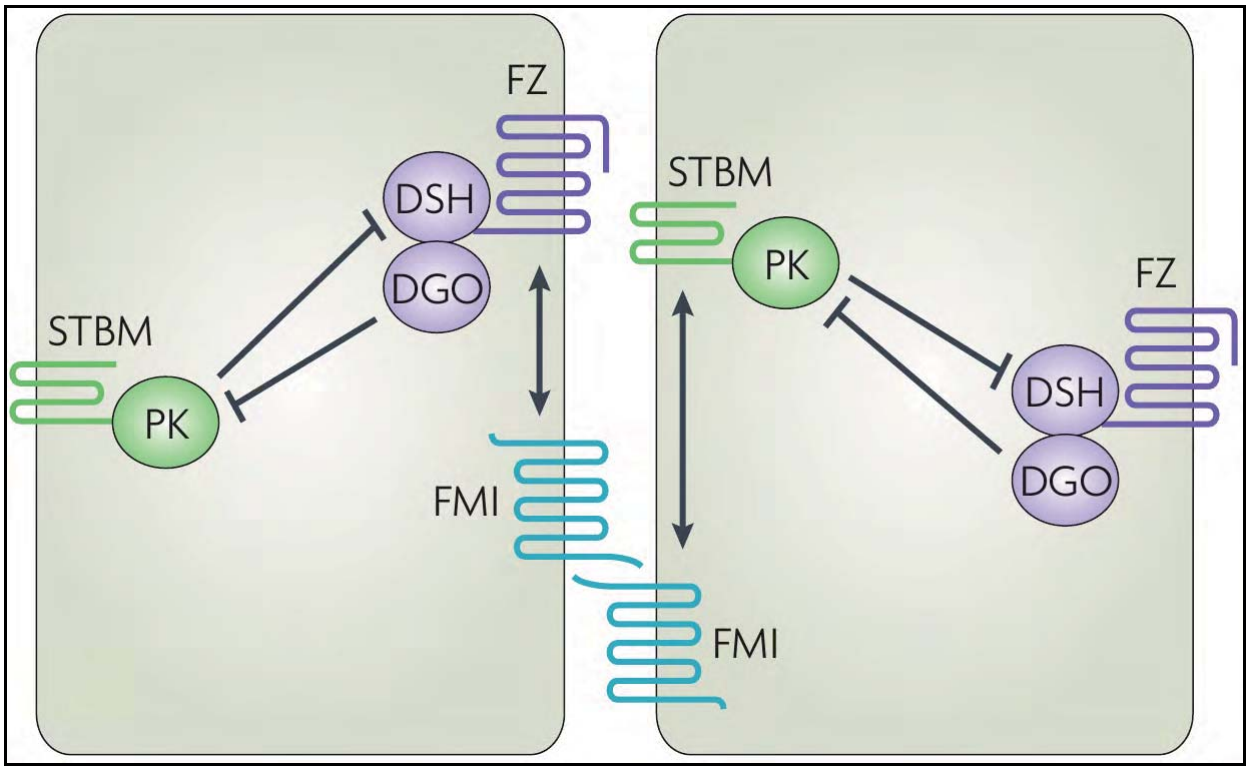

Fig. 3 Molecular interactions among the core Fz/PCP components in *Drosophila*

An apical view of two cells that have achieved asymmetric localisation of the Fz-Dsh-Dgo and Pk-Stbm complexes. Experimental data support the recruitment of Dsh to the cell membrane by Fz and the requirement of Stbm for the membrane localisation of Pk. It is assumed that initial weak bias in these membrane protein clusters trigger a process in which intrinsic aggregation-repulsion properties of PCP proteins underlie their redistribution within the apical plane. Prickle can interact with Dsh causing a reduction in Dsh membrane localisation, whereas Dgo can compete with Pk for Dsh binding and thus antagonise the inhibitory effect of Pk on Dsh. Genetic evidence places Fmi in both complexes, although confirmation of direct physical interactions is lacking. Adopted from [2].

accumulation at the distal surface of a cell would recruit or retain Stbm on the proximal surface of the adjacent cell. The complexes of PCP proteins, Fz/Dsh and Stbm/Pk, could then segregate into reciprocal domains in both cells through the antagonistic effect of proximally localised Pk on Fz-Dsh association. Although this model can do without a requirement for a gradient of Fz activity, it leaves unexplained the nature of the initial input that biases Fz localisation. Moreover, for the present, the interaction between Fz and Stbm across cell membranes remains purely speculative. An alternative model suggests that a gradient of Fz extracellular ligand and therefore Fz activity across the cell population accounts for both the local interactions between cells and global organisation of a tissue. However, in its original form this model could not explain the ability of cells to reorient at a distance from a local difference in Fz activity, such as at the boundary of a mosaic clone. So a refinement came in an assumption that cells respond to the gradient in Fz activity by adjusting their own Fz activity to equal the average of their neighbours [48]. This „averaging" mechanism would reinforce the global gradient in response to local variations and could generate required differences in Fz activity that propagate several rows of cells away from a mutant clone. Both models are plausible but postulate relationships that have not been identified. Direct interaction between Fz and Stmb has not been reported. Flamingo, proposed to bridge the interactions across cell boundaries, has not been shown to bind directly to any of the PCP proteins. In addition, it was shown that Pk is dispensable for the transmission of planar polarity signal in some cases [145; 147; 148], indicating that other proteins contribute to the feedback loop. Similarly, the mechanisms proposed to detect and respond to local differences in Fz activity have not been specified and no proof of the existence of a differential Fz activity has been obtained.

Genetic analyses have identified other genes in addition to the core PCP ones that regulate PCP signalling. One of them, Casein kinase Iε (also known as Discs overgrown; CKIε/Dco), is required for Dsh phosphorylation and positively regulates Fz-Dsh activity [149; 150]. Another protein, the $G\alpha_0$ subunit of heterotrimeric G proteins (also known as Brokenheart; Bkh), shows proximally enriched apical localisation during PCP generation in wing cells [49]. Another component, shared between *Drosophila* and vertebrate PCP pathways, is the regulatory subunit of Protein phosphatase 2A (PP2A), known as Widerborst (Wdb). In *Drosophila* wing epithelia, Wdb localises asymmetrically to the distal side of planar microtubules and polarises independently of the core PCP components. Widerborst positioning precedes the asymmetric localisation of the core Fz/PCP proteins and is required for polarised distribution of Fz and Fmi. Activation of PP2A through Wdb seems to be necessary for the maintenance of the planar microtubules. Expression of a dominant negative version of Wdb leads to the uniform dispersion of Fz/PCP proteins [152].

In vertebrate systems, further Fzd/PCP factors have been identified that might function upstream to establish the initial asymmetry. These include the Glypican 4 (also known

as Knypek; Kny), a transmembrane protein that likely serves as a Wnt co-receptor, and Wnt family members Wnt5 (also known as Pipetail; Ppt) and Wnt11 (also known as Silberblick; Slb). They do not seem to have a role in Fz/PCP signalling in *Drosophila* [50]. In addition, none of the Wnt family members in *Drosophila* shows an expression pattern suggestive of an activating function in Fz/PCP pathway.

In fact, the present-day data argue both in favour and against the idea of Wnts acting upstream of Fzd/PCP signalling. Experimental support for the involvement of Wnt ligands in the Fzd/PCP comes from the analysis of convergent extension (CE) cell movenment in vertebrates. The *wnt5*/*ppt* and *wnt11*/*slb* mutants in zebrafish *Danio rerio* disrupt CE [154-160]. The expression of Wnt5 or Wnt11 can restore the body axis elongation in *wnt11*/*slb* mutants [154; 155; 157]. The possible relationship of Wnt ligands to the Fzd/PCP pathway was further supported by the observation that overexpression of truncated variants of Dvl protein can bypass the requirement for Wnt11 activity [154; 155; 157].

The receptor for Wnt11 is thought to be Fzd7 [157; 161]. Zebrafish has two *Fzd7* paralogues, *fzd7a* and *fzd7b*. The second of the two, *fzd7b*, is expressed at the right time and place to be involved in early CE events. In *Xenopus*, Wnt11 can directly bind Fzd7 and loss of *Fzd7* function disrupts cell movements similarly to Wnt11 [161; 162].

In *Xenopus*, overexpression of a dominant negative variant of *dvl* that lacks either the DEP or the PDZ domains, which are essential for PCP in *Drosophila*, can disrupt CE [163-165].

In zebrafish, Wnt11 seems to affect the migration of cells during axis extension through subcellular localisation of E-cadherin by modulation of its recycling via GTPase Rab5 [51]. Interestingly enough, the *wnt11*/*slb* mutant phenotype can be partially suppressed by the expression of *Rho-associated kinase* (*Rok*) [52], a known downstream effector of Fz/PCP in *Drosophila* [168]. Congruently, the expression of a dominant negative form of *Rok* (*dnRok*) leads to CE defects [52]. Likewise, overexpression of either *RhoA* or its effector *Rok* in zebrafish is able to rescue CE defects in embryos lacking *Wnt5* function [53].

In mammals, *Wnt7a* is expressed in the so-called pillar cells during the differentiation of the organ of Corti, a vertebrate sensory structure in the inner ear. The addition of Wnt7a or Wnt antagonists affects the orientation of stereocilia in the organ tissue culture. However, no PCP phenotype was observed in *Wnt7a* null mice [54]. Nevertheless, it is possible that the addition of Wnt7a or Wnt antagonists has a dominant negative effect on other pathways required for PCP or that there exists a compensatory mechanism in the *Wnt7a*-knockout mice.

In zebrafish, a specific mesoderm cell population, the prechordal plate progenitors, migrates anteriorly to contribute to axis extension. This migration requires directed movements using oriented cell pseudopod-like protrusions, which enable cells to make contact and create traction by harnessing the actin cytoskeleton. These directed protrusions are lost in *wnt11*/*slb* zebrafish mutants. The directed movement is defective in that the progenitors migrate slower, often

straying from the normal path [154; 158; 166; 171]. However, the cells maintain the overall movement direction towards an animal pole, indicating that Wnt11 might provide a signal stabilising the movement direction rather than serving as a major directional cue.

Downstream of Fzd, the activity of Dsh/Dvl is essential for Fzd/PCP signalling. Dishevelled shows several differences in the behaviour in Fzd/PCP signalling from the canonical signalling, the most obvious being the required domains and downstream protein partners [154; 155; 163; 165; 172-174]. Dishevelled localises to the plasma membrane during non-canonical signalling [172; 163]. It was shown in *Xenopus* that the membrane Dvl localisation is crucial for activation of PCP downstream targets as well as for CE movements [175; 176]. Interestingly, *Fz* overexpression is sufficient for the recruitment of Dsh/Dvl in *Drosophila* as well as in both zebrafish and *Xenopus*. This suggests that under physiological conditions a mere Fzd enrichment in the plasma membrane might initiate the Fzd/PCP signalling cascade [172; 177-181].

Cumulatively, the above-mentioned examples imply a possible role for Wnt ligands in the PCP regulation in vertebrates while in *Drosophila* Wnts seem to be dispensable for the Fz/PCP signalling. Nevertheless, it should be mentioned that none of the Wnt family members in the fly genome is an orthologue of either *Wnt5* or *Wnt11*. Additionally, the common redundancy among Wnt proteins might mask a defect associated with a single-gene mutation. Also, the recent identification of Norrin, a non-Wnt ligand for vertebrate Fzd4 [18] indicates that a detection of another, non-Wnt Fz ligand is not beyond the realms of possibility.

In *D. melanogaster* there is a second group of candidates for the global spatial cue, which includes Fat (Ft) and Dachsous (Ds), two protocadherins that can interact across cell boundaries, and the type II transmembrane protein Four-jointed (Fj). The role of this group of proteins in vertebrate PCP has not been determined. The intracellular domain of Ft interacts with the nuclear factor Atrophin (also known as Grunge; Atro/Gug), indicating that the Ft-Ds interaction could function through the transcriptional regulation [182]. Genetic interactions indicate that Ft acts upstream of Fz signalling [55]. Therefore, it is tempting to speculate that Ft-Ds-Fj pathway might generate an initial assymetry. Regrettably, several observations are not consistent with this view. Although Ft and Ds are expressed in gradients in *Drosophila* eye, wing and abdomen, their expression gradients in these distinct contexts relative to the Fz/PCP activity gradient are different in every single case [141-143; 145; 153; 184-186]. These differences indicate a complicated relationship between these two PCP systems. Furthermore, a molecular link between the Ft activity and Fz signalling has not been identified. Therefore, although Ft, Ds and Fj are required for the establishment of PCP in all the tissues tested, they rather provide a distinct source of spatial information that might act in parallel to the Fz/PCP system [56].

Based on available data, it is possible to outline a plausible model of Fzd/PCP pathway downstream of Fzd receptor. Dishevelled seems to be the key player in Fzd/PCP downstream

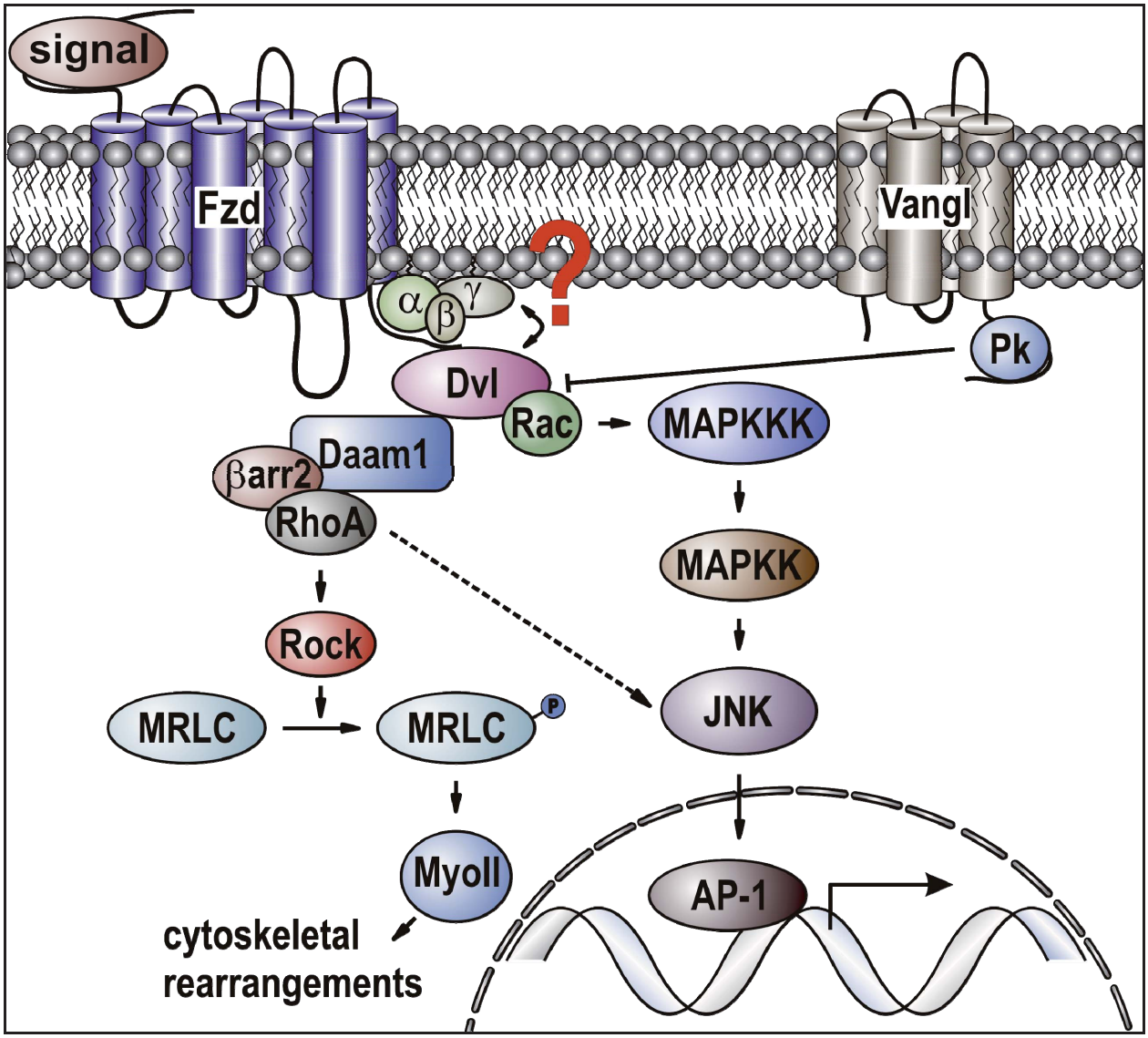

Fig. 4 The Fzd/PCP signalling

Generalised and rather speculative advance of molecular events during the Fzd/PCP signalling. The PCP proteins are presumed to be distributed in reaction to an as yet unidentified polarisation signal and a dynamic and persisting antagonism between PCP proteins themselves. The possible downstream cascades are indicated. For details, see the text. Vangl, mammalian homologue of *Drosophila* Stbm/Van Gogh.

signalling. It contains three conserved domains: the Dishevelled and Axin (DIX) domain at the N-terminus which is required for canonical Wnt signalling, the Post-synaptic density-95, Discs-large and Zonula occludens-1 (PDZ) domain in the midregion, and the Dishevelled, Egl-10 and Pleckstrin (DEP) domain located about midway between the PDZ domain and the C-terminus and required for Dsh/Dvl localisation during PCP signalling. Most recent observations suggest that Dsh/Dvl acts as scaffolding and provides docking sites for a diverse set of other proteins which together with Dsh/Dvl form multicomponent signalling complexes [reviewed in 57]. The PDZ domain of Dsh/Dvl provides a binding site for the cytoplasmic tail of Fzd [58; 59]. However, several observations indicative of an involvement of heterotrimeric G proteins in the signal relay have been published [16; 151; 188-195]. Regrettably, a region

of Dvl responsible for interaction with heterotrimeric G proteins has not been reported so far. Anyway, either binding directly to Fzd or via heterotrimeric G proteins, Dvl gets activated and then associates with a set of proteins, including Ras-related C3 botulinum toxin substrate (Rac) and Dishevelled-associated activator of morphogenesis 1 (Daam1). Dishevelled-associated activator of morphogenesis 1, a formin-homology protein, binds to both the DEP domain of Dsh/Dvl and to Ras homologue gene family, member A (RhoA) and thus mediates the formation of Dvl-RhoA complex, linking the core PCP apparatus to the cytoskeleton regulation [60]. Like other formins, Daam1 directs nucleation and elongation of new actin filaments. It was shown that for this mediation of cytoskeletal reorganisation a conserved formin-homology 2 (FH2) domain at its C-terminus is required. Daam1 exists in the cytoplasm in an autoinhibited state, which is caused by intramolecular interactions between the amino-terminal GTPase-binding domain (GBD), encompassing the diaphanous inhibitory domain (DID), and the carboxyl-terminal diaphanous autoregulatory domain (DAD). It has been proposed that the binding of Dvl within the DAD disrupts the intramolecular interactions between the DID and DAD domains and in this way Daam1 is released from autoinhibition. Removal of the DAD domain disrupts the interaction between Dvl and Daam1. Mutations within as well as removal of the DAD domain converts Daam1 into an active protein that can induce RhoA activation [61]. Neuroepithelial cell-transforming gene 1 (Net1), a RhoA-specific guanine nucleotide exchange factor (GEF) that associates with Dvl, may also facilitate RhoA activation during PCP signalling [62]. Another protein required for Dvl activation of RhoA, β-Arrestin 2 (βarr2), interacts with the N-terminal quarter of Daam1 and RhoA proteins, but not Rac, and regulates RhoA activation through Daam1 [199]. A downstream effector of RhoA, Rho-associated protein kinase (DRok in *Drosophila*, Rok or Rock in vertebrates), links Fzd/PCP signalling to actomyosin contractility via the regulation of the activity of Myosin II (MyoII) through nonmuscle Myosin regulatory light chain (MRLC or Sqh) phosphorylation [168]. A small regulatory GTPase Rac can form complexes with Dsh/Dvl independently of Daam1 and appears to act in parallel to RhoA downstream of Dsh/Dvl [200; 201]. The GTPase Rac activates the c-Jun N-terminal kinase (JNK) module, a known target of PCP signalling in both *Drosophila* and vertebrates [131; 173; 175; 200; 202-205]. RhoA has also been proposed to activate JNK independently of Rac [131; 173; 200; 206]. Protein kinase A (PKA) interferes with the PCP signalling, apparently by an inhibition of the Dvl-Daam1-RhoA complex formation required for RhoA activation [63]. Protein kinase A also acts downstream of heterotrimeric G proteins, crucial components of the Wnt/Ca^{2+} pathway [12; 64]. This might also imply that the two pathways, Fzd/PCP and Wnt/Ca^{2+}, interact.

2.1.2.2 Wnt/Ca^{2+} pathway

Some of the Frizzled receptors such as Fzd2 activate a cascade independent of β-catenin that leads to the release of intracellular Ca^{2+}. The first suggestion of an alternative pathway [3; 9; 208], as opposed to the Wnt/β-catenin signalling, was based on the results of a misexpression analysis in developing zebrafish and *Xenopus* embryos. Ectopic expression of *Xenopus Wnt5a* (*Xwnt5a*) or rat *Frizzled2* (*rFzd2*), but not *Xenopus Wnt8* (*Xwnt8*) or rat *Frizzled1* (*rFzd1*), induced Ca^{2+} fluxes in zebrafish embryos and activation of PKC in *Xenopus* [16; 209; 210]. Conversely, expression of *rFzd1*, but not *rFzd2*, can lead to the accumulation of Xwnt8 at the cell surface and activate Wnt/β-catenin target genes [177; 210]. Furthermore, the phenotype resulting from overexpression of *Wnt5a* in *Xenopus* embryos resembled the phenotype obtained by the *5-HT1c* serotonin receptor overexpression [209; 211]. At least some subclasses of serotonin receptors seem to mediate an increase of intracellular calcium levels in a G protein-dependent manner [65; 66].

According to their different biological activities in specific assays, vertebrate Wnt proteins were divided into two functional groups. It was observed that Wnt1, Wnt3a, Wnt8 and Wnt8b induce a secondary axis in early *Xenopus* embryos and transform C57MG mammary epithelial cells, whereas Wnt4, Wnt5a and Wnt11 are neither axis-inducing nor do they transform C57MG cells [214; 215]. These differences among Wnts are paralelled by the two functionally distinct classes of Frizzled proteins. Using the induction of Wnt/β-catenin target genes (*siamois*, *Xnr3*) and activation of PKC in *Xenopus* as assays for Fzd activity, it was demonstrated that expression of *Drosophila Fz* (*DFz*) and *Fz2* (*DFz2*), *Xenopus Fzd1* (*XFzd1*), rat *Fzd1* (*rFzd1*), mouse *Fzd7* (*mFzd7*) and *Fzd8* (*mFzd8*) activated Wnt/β-catenin targets, but not PKC. Conversely, expression of *rFzd2*, *mFzd3*, *mFzd4* and *mFzd6* activated PKC but not Wnt/β-catenin targets *siamois* or *Xnr3*. Markedly, the subgroupings of the different Fzd receptors follow their relative sequence identity [9; 210; 216].

As for the downstream events, the members of the „Wnt1 group“ of Wnt proteins activated the function of Fzd receptors leading to the stabilisation of β-catenin, while Wnt proteins of the „Wnt5a group“ altered cell movements and reduced cell adhesion when overexpressed in *Xenopus* embryos [215; 217; 218].

Taken together, all these observations raised a possibility that Wnt5a class of Wnt proteins might activate a similar pathway as does the serotonin receptor and that Wnts can activate two distinct signalling cascades, leading to different cellular and embryonic responses [3; 208].

Frizzled proteins display several features reminiscent of the G protein-coupled receptors (GPCRs). These include a seven-pass transmembrane structure [219-221], an exofacial and N-glycosylated N-terminal region and a cytoplasmic C-terminal tail with sites suitable for phosphorylation by protein kinase C [67]. Other cytoplasmic domains of Fzd receptors

contain potential sites for phosphorylation by PKA and CKII. Finally, they share a significant homology to other known GPCRs [68]. A calcium flux induced by ectopic expression of *Wnt5a* or *rFzd2* mRNA in zebrafish embryos is inhibited by pertussis toxin (PTX) [12], an ADP-ribosyltransferase known to target $G\alpha_i/G\alpha_o/G\alpha_t$ family of heterotrimeric G protein subunits [69]. Expression of the α subunit of transducin ($G\alpha_t$), which traps $\beta\gamma$ subunits to inhibit signalling [224], blocks the calcium mobilisation. These data strongly indicate that rFzd2 signals via the $\beta\gamma$ subunits of G proteins that are PTX-sensitive. The activity of CaMKII as well as the translocation of PKC to the plasma membrane are also sensitive to PTX and α-transducin [210; 216].

Yet another indirect evidence for the involvement of heterotrimeric G proteins comes from the observation that the intracellular calcium release stimulated by rFzd2 can be disturbed by L-690, 330, a specific inhibitor of cGMP-selective phosphodiesterases (PDEs). Cyclic GMP-selective phosphodiesterases participate in inositol phospholipids (phosphoinositides) signalling and are frequent downstream effectors of heterotrimeric G proteins. The rFzd2-dependent calcium flux suppression is overcome by injection of myo-D-inositol, a cyclic alcohol replenishing the inhibitor-induced decrease in an intermediate product [12]. Inhibitors of cGMP phosphodiesterase PDE6, dipyridamole and zaprinast, can block Wnt activation of Fzd2-mediated pathways [64].

Finally, expression of *rFzd2* in mouse F9 embryonic teratocarcinoma cells, followed by the co-culture with cells producing Wnt5a, but not Wnt8, induced formation of primitive endoderm (PE), as indicated by the expression of tissue plasminogen activator (tPA) and cytokeratin Endo-A (TROMA-I antigen) [225]. Consistent with the expression of these two prominent PE marker proteins was the parallel loss of the expression of the embryonic marker antigen SSEA1 (stage-specific mouse embryonic antigen) [68]. A functional chimera between an exofacial and transmembrane-spanning portions of the hamster β_2-adrenergic receptor (β_2AR), a prototypic GPCR, and the cytoplasmic loops of the rFzd2 was constructed. The β_2AR/ rFzd2 chimeric receptor induced PE in F9 cells in response to the β-adrenergic agonist isoproterenol (ISO) in a similar manner as did the rFzd2 activated by Xwnt5a. Moreover, microinjection of the *β_2AR/rFzd2* RNA into zebrafish embryos led to the activation of calcium transients within 20 minutes after stimulation with ISO [225]. Therefore, the β_2AR/rFzd2 chimera evoked a downstream response similar to that of Xwnt5a and rFzd2. Isoproterenol stimulation of endoderm formation was blocked by PTX and by oligonucleotides antisense to $G\alpha_o$, $G\alpha_{t2}$ and $G\beta_2$ [225]. Hence, this study established a requirement of specific G protein subunits for the Fzd-mediated β-catenin-independent signalling.

Presently, an alternative Wnt-induced pathway independent of β-catenin begins to emerge. It is assumed to begin with the stimulation of the Fzd receptor by the Wnt5a class of ligands. Currently, it is not clear whether other molecules serve as co-receptors as in the canonical

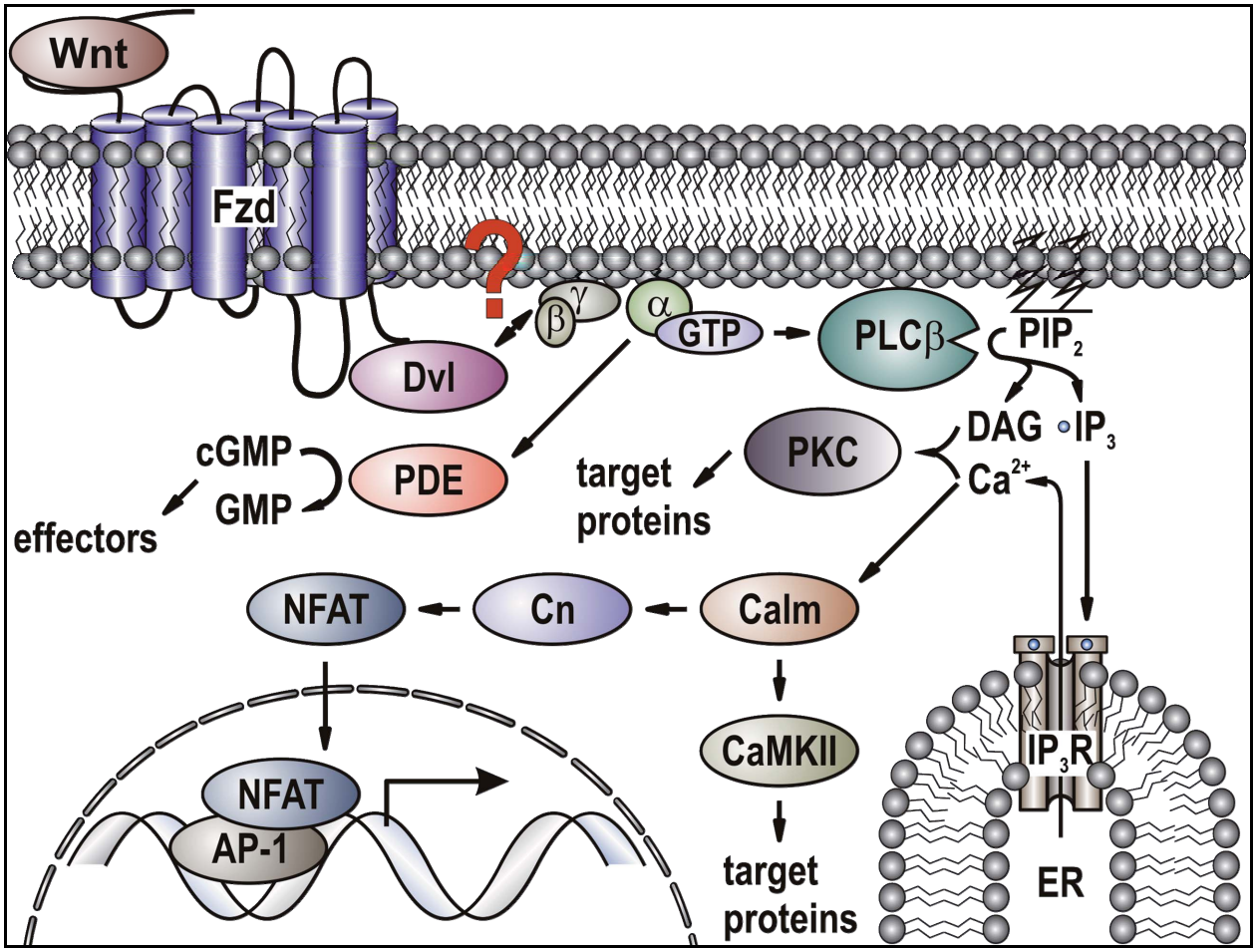

Fig. 5 The Wnt/Ca^{2+} pathway

The "Wnt5a class" of Wnt proteins presumably together with specific Fzd receptors induce Ca^{2+} intracellular fluxes. The involvement of heterotrimeric G proteins in the cascade is supposed but not definitely proved. The downstream effectors seem to be shared with other intracellular signalling pathways. AP-1, activator protein 1; IP_3R, IP_3-gated Ca^{2+}-release channel; PDE, cGMP-specific phosphodiesterase.

Wnt/β-catenin pathway. The exact mechanism by which the activated Fzd receptor relays the signal to the heterotrimeric G proteins is not known. A parallel is drawn from what is known about other GPCRs in this respect. Generally, the switch function of the receptor is considered to be linked with allosteric conformational changes of the 7-helix transmembrane bundle, which are passed on to the cytoplasmic loops of the receptor. These intracellular loops mediate the interaction with the α subunit of the heterotrimeric G protein.

Signal transmission via G proteins takes place in close association with the inner side of the plasma membrane. Both the α subunit and the βγ complex are membrane-tethered by lipid anchors that are introduced in the course of the posttranslational modification at the N-terminus of the α and at the C-terminus of the γ subunit. Like all regulatory GTPases, the heterotrimeric G proteins run through a cyclical transition between an inactive, GDP-bound and an active, GTP-bound form.

An inactive GDP-bound form of a heterotrimeric G protein is activated by the interaction

with a receptor. An exchange of GDP for GTP takes place in the binding site of the α subunit. Once the G protein is activated, it frees itself from the complex with the receptor, which either returns to its inactive ground state or activates further G proteins.

The βγ complex dissociates from the α subunit. The free α subunit with bound GTP represents the activated form of the G protein that transmits the signal further. As already mentioned above, one of the downstream effectors activated by the Gα is cGMP PDE. The activated Gα binds to the two inhibitory γ subunits of PDE dissociating them from the catalytic α and β subunits. Relieved of their inhibition, the catalytic subunits hydrolyse cGMP to GMP. This leads to the decrease of the intracellular cGMP levels and therefore to changes in the activity of cGMP-regulated effectors. One of the so far unanswered questions is what the downstream sensors for the changes in cGMP that follow the activation of Fzd receptors are. There are several prominent candidates including guanylylcyclases, cGMP-gated channels, protein kinase G (PKG) and phosphodiesterases.

The α subunit of the heterotrimeric G protein bears an intrinsic GTPase activity. Hydrolysis of GTP by the GTPase activity ends signal transmission at the level of G proteins. At this point, regulatory mechanisms may take effect in which proteins with the character of GTPase-activating proteins (GAPs) specifically stimulate the GTPase activity of the α subunit. Proteins known as regulators of G protein signalling (RGSs) can increase the GTPase activity by close to two orders of magnitude. Interestingly, some of the RGS family members have been implicated in the Wnt-sensitive processes during *Xenopus* embryonic development [70].

The β and γ subunits of the heterotrimeric G proteins released during activation remain as a tightly associated complex and perform a signal-mediating function. The Gβγ complex might activate the phospholipase Cβ (PLCβ) downstream of Fzd [71; 72]. Upon activation, PLC cleaves the phosphatidyl inositol-4,5-bisphosphate [PtdIns(4,5)P_2 or PIP_2], a relatively rare phospholipid in the inner leaf of the plasma membrane, into 1,2-diacylglycerol (DAG) and inositol-1,4,5-trisphosphate [Ins(1,4,5)P_3 or IP_3]. The products of the reaction, DAG and IP_3, function as second intracellular messengers. At this step, the signalling pathway splits into two branches. The IP_3 is a small water-soluble molecule that diffuses rapidly through the cytosol. There it releases Ca^{2+} from the endoplasmic reticulum (ER) by binding to IP_3-gated Ca^{2+}-release channels in the ER membrane. The channels are regulated by a positive feedback, in which the released Ca^{2+} can bind back to the channels to further increase the Ca^{2+} release.

Diacylglycerol activates a crucial serine/threonine Ca^{2+}-dependent protein kinase, PKC. The initial rise of the cytosolic Ca^{2+} induced by IP_3 is thought to alter PKC so that it translocates to the cytoplasmic face of the plasma membrane. There it is activated by the combination of Ca^{2+}, DAG and the negatively charged membrane phospholipid phosphatidylserine. When

activated, PKC phosphorylates specific serine or threonine residues on target proteins. In many cells, PKC is known to increase the transcription of specific genes. At least two mechanisms are known. In the first one, PKC activates a protein kinase cascade that leads to the phosphorylation and activation of a DNA-bound regulatory protein. In the second one, PKC phosphorylates an inhibitory protein, thereby releasing a cytoplasmic gene regulatory protein so that it can migrate to the nucleus and stimulate transcription of specific target genes. Currently, which of the above mechanisms is utilised in the pathway following Fzd activation remains an open question. In addition, the downstream effectors of PKC are not specified.

Calcium ions are not metabolised by the cell. Instead, they serve as a second messenger by forming ionic gradients. Cellular stimulation has been shown to induce either transient increase or oscillations of Ca^{2+} [73]. Much of the newly released Ca^{2+} is rapidly bound by Ca^{2+} binding proteins, including Calmodulin (CaM). Calmodulin is a conserved protein with four high-affinity Ca^{2+}-binding sites that undergoes a conformational change when it binds Ca^{2+}. In some cases, CaM serves as a permanent regulatory subunit of an enzyme complex, but in most cases, the allosteric activation of CaM by Ca^{2+} enables it to bind to various target proteins and thereby alter their activity. Most effects of Ca^{2+}/CaM are mediated by Ca^{2+}/CaM-dependent protein kinases (CaM kinases). One of the members of the family is Ca^{2+}/CaM-sensitive protein kinase II (CaMKII). This kinase can function as a molecular memory device, switching to an active state when exposed to Ca^{2+}/CaM and then remaining active even after the Ca^{2+} is withdrawn. This remarkable property is based on the autophosphorylation, which enables the kinase to stay active beyond the duration of the initial Ca^{2+} signal. The activity is maintained until phosphatases overwhelm the autophosphorylating activity of the enzyme and shut it off. Calcium/CaM-sensitive protein kinase II phosphorylates specific target proteins on serine or threonine residues and thus alters their activity. Particular CaMKII target proteins in the Wnt/Ca^{2+} pathway remain to be identified.

Another target of activated CaM is the protein serine phosphatase Calcineurin (Cn). Calcineurin is made up of a catalytic A subunit and a regulatory B subunit that shows similarity to CaM. A binding site for Ca^{2+}/CaM, required for activation of Cn, is found in the catalytic subunit. Upon Ca^{2+}/CaM association, Cn is activated and dephosphorylates a cytoplasmic Nuclear factor of activated T-cells (NFAT) transcription factor. In unstimulated cells, phosphorylated NFAT is present in the cytoplasm. Dephosphorylation of NFAT by Cn on key phosphate residues exposes a nuclear localisation sequence (NLS) that alows NFAT to enter the nucleus. Nuclear NFAT binds to DNA in cooperation with other transcription factors (e.g. AP-1) to activate target genes. Rephosphorylation of NFAT by an unknown priming kinase and GSK3 leads to NFAT inactivation and export from the nucleus [74; 75].

2.2 Proteins of the LEF/TCF family

2.2.1 Biochemical and functional characteristics of LEF/TCF proteins

In mammals, the LEF/TCF family consists of LEF1, TCF1, TCF3 and TCF4 that all share several common structural features [232; 233]. The extreme N terminus constitutes a binding domain for β-catenin [99; 100; 105; 234; 235]. The nearly identical HMG box, a DNA-binding domain, mediates recognition and occupation of specific DNA sequence motifs with an A/T A/T CAAA consensus [236]. All the mammalian LEF/TCF transcription factors associate with Gro/TLE co-repressors [109; 237; 238] by the aminoacid residues interspersed between the β-catenin-binding domain and the HMG box. The proteins Gro/TLE interact with the hypoacetylated N-terminus of histone H3 and also with HDAC1 [109; 239; 240]. This indicates that they have a role in establishing specialised repressive chromatin structure that prevents inappropriate activation of β-catenin/TCF target genes in the absence of a Wnt signal.

2.2.2 LEF/TCF expression patterns during development

During murine embryogenesis, LEF/TCF factors are expressed in partially overlapping patterns [235; 241; 242]. In a gastrulating embryo, *mTcf3* is expressed ubiquitously. On embryonic day 7.5 (E7.5), *mTcf3* expression is limited to the anterior part of the embryo and then gradually disappears over the next 2 to 3 days [235]. The genes of the other two LEF/TCF group members, *mTcf1* and *mLef1*, display an opposite pattern of expression with highest levels of both in the posterior part of the emryo [241], but nevertheless, all three expression regions do overlap. Moreover, *mTcf1* and *mLef1* virtually completely overlap in their early embryonic expression [241; 243] and postnatally, their parallel expression continues in the lymphocytes [76]. The expression of *mTcf4* is unique in that it commences much later in development than the other three family members and is essentially restricted to the diencephalon, mesencephalon and the intestinal epithelium. However, in the embryonic brain, again, *mTcf4* expression area overlaps with that of *mLef1* [235; 241; 245]. Lastly, the intestinal expression of *mTcf4* persists to the adult age and can be detected in the small intestine where it shows a gradient along the rostrocaudal axis. An analogous expression gradient, although at much lower levels, exists for the *mTcf3* mRNA [235].

Given the above-mentioned areas of shared expression, the common overall structural organisation and presumably identical DNA binding specificities of LEF/TCF group members, it is not surprising that several examples of possible functional redundancy were identified. The analyses of both single as well as compound gene targeting experiments in mice have

revealed conditions where a missing member of the LEF/TCF family can be functionally substituted by another one, additionally present in the same location. Mice mutant in *Lef1* lack skin appendages among other developmental defects but contrary to expectation the immune system seems unaltered [244; 245]. The healthy lymphoid cell populations can be explained by a substitutional role of *Tcf1* [246]. Likewise, *Tcf1* homozygous null mice are viable and display an early arrest in thymocyte differentiation as the only developmental defect [247]. The compensatory role of other LEF/TCF genes, e.g. *Lef1* replenishing function in limb development [241; 243], must be taken into account to describe the absence of other potential phenotypic abnormalities. The effect of the *Tcf4* loss which remains restricted to the small intestine and not expanded further to the whole intestinal tract in the $Tcf4^{-/-}$ mice can be attributed to the parallel and continuing *Tcf3* expression in the gut [77]. Besides, the possibly intact *Lef1* expression in *Tcf4* homozygous mutant mice might account for the normal midbrain development [235; 241; 245]. The fact that $Lef1^{-/-}/Tcf1^{-/-}$, but not $Tcf1^{-/-}/Tcf4^{-/-}$, embryos show an early loss of paraxial mesoderm, may be explained by the overlapping expression of *Tcf1* and *Lef1* in the presomitic mesoderm together with the exclusion of *Tcf4* expression in this compartment [243; 248]. Severe posterior truncations observed in $Tcf1^{-/-}/Tcf4^{-/-}$, but not in $Lef1^{-/-}/Tcf1^{-/-}$, embryos are likely to occur due to the lack of the *Tcf4* expression in the hindgut [248]. On the contrary, an untouched *Tcf4* expression in the gastro-intestinal tract can compensate for the missing Tcf1 in the compound $Lef1^{-/-}/Tcf1^{-/-}$ mice.

Together with the fact that single gene homozygous null mice did not display any defect in either paraxial mesoderm or caudal endoderm development, the results from the double knockouts indicate that LEF/TCF factors are at least partially interchangeable at the biochemical level. Furthermore, the data also point to the possibility that LEF1/TCF1 in the paraxial mesoderm and TCF1/TCF4 in the caudal endoderm most likely regulate overlapping sets of target genes [248].

2.2.3 Functional differences of individual LEF/TCF factors

Contrary to the initial view of LEF/TCFs as interchangeable factors (based on structural similarities, conserved DNA binding motif and overlapping expression patterns during development) it is now becoming increasingly apparent that not all members of the family are functionally equivalent. There are significant differences in the abilities of LEF/TCF proteins to regulate Wnt target genes [249-252].

The overall expression pattern of *Lef/Tcf* genes is overlapping only partially. Genetic data from knock-out and transgenic mice experiments manifest that different TCF family members perform distinct tasks in developmental processes [238; 248; 253-257]. For instance, the removal

of *LEF1* or *TCF1* gene generated partially non-redundant phenotypes in tissues where their expression patterns overlapped [243; 246]. Likewise, overexpression of dominant negative forms of LEF/TCFs produced non-identical phenotypes [258].

Recently, data indicative of LEF/TCFs`functional differences accumulate. For example, LEF1 appears to act mainly as a β-catenin-dependent transcriptional activator, whereas the repressor activities of TCF3 seem to predominate over its activating function [100; 258; 259]. In zebrafish, an amino-terminally truncated zTCF3 missing its β-catenin binding domain (ΔNTCF3) complements the headless phenotype caused by loss-of-function of wild-type *zTCF3* [78]. In ectopic expression experiments, injection of *mLEF1* into early *Xenopus* embryos induces the formation of a secondary axis [57; 99], mimicking the effect of Wnt activation [79]. By contrast, a dominant negative form of *XTCF3*, lacking the region required for β-catenin binding, blocks the axis duplication as well as the formation of the endogenous axis [100]. Even more strikingly, overexpression of wild-type *XTCF3* has no effect [100; 257]. Interestingly, XTCF4, the LEF/TCF family member most closely related to XTCF3, also fails in the assay [257]. In *Xenopus* renal epithelial A6 cells, LEF1 activates the Wnt target gene *fibronectin* (*FN*), whereas XTCF3 does not [261]. Similar to its *Xenopus* and zebrafish orthologues, mTCF3 was shown to function by repressing target genes in the early embryo where it seems to restrict induction of the anterior-posterior (AP) axis during the onset of gastrulation [262]. It was also reported that LEF1 and TCF3 have distinct effects on cell fate when transgenically expressed in the epithelial mouse skin stem cells. While LEF1 activates genes involved in hair cell differentiation in a β-catenin-dependent manner [245; 263; 264], TCF3 appears to act as a repressor to specify an alternative cell fate – it promotes stem cell maintenance and differentiation into the adjacent follicle outer root sheath (ORS) tissue [258]. Furthermore, the fact that ectopic expression of *ΔNTCF3* and *TCF3* produced similar phenotypes implies that in striking contrast to LEF1, the behaviour of TCF3 did not rely on Wnt signalling and β-catenin stabilization. Finally, the phenotype of *TCF3* mutant embryos is similar to those reported for the inhibitors of Wnt signalling, *Axin* or *APC*, as well as for ectopic expression of *Wnt8c* [262; 265-267].

Numerous isoforms capable of contributing to different developmental programs originate from *LEF*/*TCF* genes by the use of dual promoters and/or alternative splicing [238; 253; 255-257; 268]. One of the well-documented examples of specific isoforms with functional consequences are TCF1E and TCF4E, proteins with an alternative C-terminal „E“ tail. Several promoter studies have demonstrated that such isoforms play a role in regulation of specific target genes such as *CDX1* and *LEF1* [250-252]. The domain responsible for the unique activity of E-tail-containing TCF proteins is an auxiliary 30-amino-acid DNA interaction motif referred to as the „cysteine clamp“ (C-clamp). The E-tail is a result of an alternatively spliced exon in *TCF1* and *TCF4*, while in TCF3 half the C-clamp motif is missing and for LEF1, the E-tail is completely absent. The E-tail-containing isoforms of TCFs are more potent in their DNA

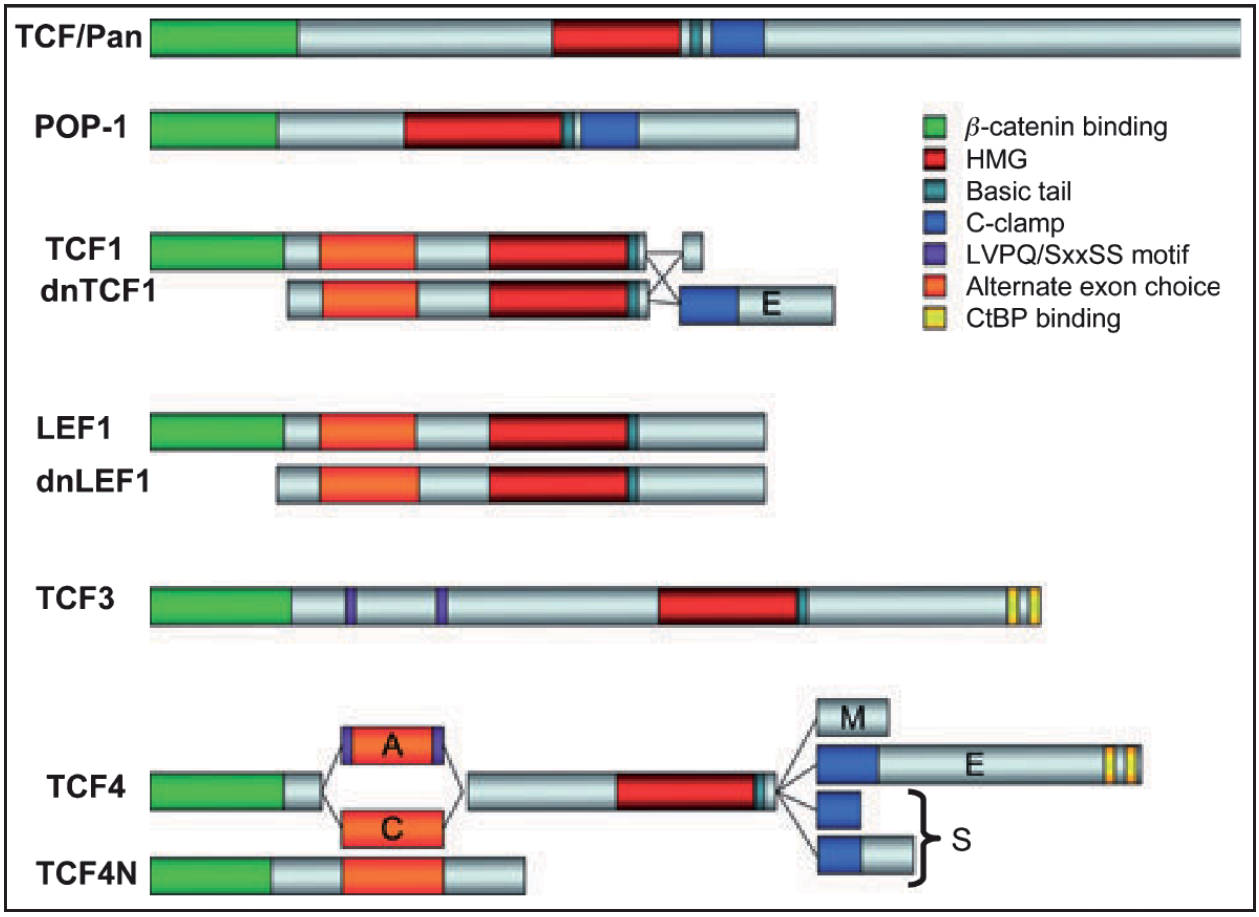

Fig. 6 Diversity of LEF/TCF factors

Invertebrates express a single TCF member containing the β-catenin binding (green), HMG (red), basic tail (turquoise) and C-clamp (blue) domains. In vertebrates, alternative promoter usage and splicing result in a plethora of TCF isoforms with diverse functional properties. Usage of alternative downstream promoters can give rise to isoforms (such as dnLEF1 or dnTCF1) lacking the β-catenin binding domain that function as natural dominant negatives. Alternative exon usage (orange) occurs in all of the family members except from TCF3. The LVPQ/SXXSS motif (purple), invariant in TCF3, confers repressive activity on isoforms containing it (e.g. TCF4A). The E-tail isoforms, TCF1E and TCF4E, encompass the intact C-clamp. The medium (M) isoforms lack it whereas the short (S) isoforms incorporate truncated C-clamp domains. Some TCF3 and TCF4 isoforms include CtBP-binding elements.
Adopted from [4].

binding activities because they encompass an additional DNA-binding region besides the usual HMG domain. Therefore, they may regulate a distinct set of Wnt target genes, including those with lower-affinity Wnt response elements (WREs). It is believed that LEF/TCF isoforms without the E-tail must achieve stable interactions through other mechanisms, such as co-operative associations with other factors [252].

Other *LEF*/*TCF* splice variants known to have biological function are those with alternatively used exon homologous to exon IVa of *hTCF1* [235; 238; 253; 268; 269]. This exon makes up part of the so-called context-dependent regulatory/repression domain (CRD) which separates the β-catenin-binding domain from the HMG DNA-binding domain. The internal exon in the CRD is obligatory in invertebrate TCFs and in vertebrate TCF3 and TCF4, but alternative in vertebrate LEF1 and TCF1. In vertebrate TCF3 and TCF4 exclusively, this exon is flanked by two short amino acid motifs, LVPQ and SXXSS (XX being LV in case of TCF3 or FL amino

acid residues in case of TCF4), also created by alternative splicing event [100; 238; 268]. In a thorough series of experiments carried out both *in vitro* and *in vivo* it was shown that the CRD central exon is an activating element, whereas the flanking LVPQ and SXXSS motifs bear repressive activities [238; 257]. The two short motifs are involved in post-translational modifications, which regulate the assembly of β-catenin/TCF complex and its activation capacity [238; 257]. The availability of SXXSS motif for phosphorylation directly affects TCF competence to form the ternary complex with β-catenin and DNA. Although a deletion of this motif allowed the resulting dephosphorylated TCF to form a complex with β-catenin, it was not sufficient to activate target genes. Transcriptional activation was achieved when both SXXSS and LVPQ motifs were absent, indicating an important regulatory role for the latter [238; 257].

Proteins of the LEF/TCF family are unable to activate transcription from reporters containing multimerised minimal binding motifs [270-272] implying that they do not behave as „classical" transcriptional factors and that they might be functionally neutral on their own. Rather, they are context-dependent transcription regulators that interact with other proteins, promoting the formation of transcription factor complexes with distinct properties and serving as molecules that facilitate interactions between various partners or dock them to proper chromosomal locations at their targets [273-275]. In concordance with this notion, LEF1 can activate gene expression when tethered to DNA in the context of the natural arrangement of other factors` binding sites, such as from the *T-cell receptor alpha chain* (*TCRα*) or the *Human immunodeficiency virus-1* (*HIV-1*) enhancers [274; 276-278].

A good example of LEF/TCFs` interactions with distinct partners and an assembly of specific higher-order complexes is illustrated by the regulation of the *TCRα* gene distal enhancer [274; 279]. Transcription stimulation by LEF1 in the context of *TCRα* depends on a ubiquitously expressed nuclear protein, Ally of AML-1 and LEF1 (ALY), that associates with the activation domains of LEF1 and Acute myeloid leukaemia 1 (AML-1) [280]. In melanocytes, LEF1 co-operates with the Microphthalmia-associated transcription factor (MITF) to transactivate the *Dopachrome tautomerase* (*DCT*) gene promoter [281]. In contradistinction to other members of the family, TCF3 was reported to interact with CK1ε and phosphorylation of TCF3 by this kinase increases TCF3 affinity for β-catenin [282]. Upon the interaction with the Protein inhibitor of activated STATy (PIASy), a nuclear matrix-associated Small ubiquitin-related modifier (SUMO) E3 ligase, LEF1 is targeted into nuclear bodies. This PIASy-mediated subnuclear sequestration leads to the repression of LEF1 activity [283]. On the contrary, PIASy was reported to enhance the sumoylation of TCF4, to target TCF4 to a subset of Promyelocytic leukaemia protein (PML) nuclear bodies and to stimulate the β-catenin-dependent transcriptional activity of TCF4 [80]. As it was reported that LEF1 had been sumoylated with SUMO-2 more efficiently than with SUMO-1 [283], whereas TCF4 was shown to be exclusively modified with SUMO-1 rather than SUMO-3 [285], it is tempting to speculate that modification

of LEF/TCFs with possibly functionally different SUMO isoforms might be responsible for the opposite effects of PIASy on LEF1 and TCF4.

In the absence of a Wnt signal the members of LEF/TCF family of transcription factors act as transcriptional repressors [29; 81-92]. Individual family members have acquired distinct mechanisms of transcriptional repression utilising different co-repressors. A repressive effect of LEF1 is achieved by its direct interaction with HDAC1 [91]. *Drosophila* TCF and hTCF1 require Gro family of transcriptional co-repressors for function [106; 107]. The XTCF3 uses the CtBP orthologue (XCtBP) together with the Groucho-related XGrg-4 [107; 108].

Upon binding, LEF/TCFs induce sharp bends in the DNA helix [290; 291]. Interestingly, the angle of DNA bend caused by individual LEF/TCFs differs quite significantly. Upon binding of LEF1, the DNA is bent at an angle of ~130°, whereas for TCF1 the bend angle of ~70° was reported [252; 290]. Differences in bending abilities certainly influence the architecture of higher-order, stereo-specific nucleoprotein complexes at particular promoters. Therefore, one can speculate that these differences might also at least partly account for the functional distinction between the LEF/TCF family members.

2.3 Wnt proteins

2.3.1 Intracellular processing of Wnt proteins

In contrast to transduction cascades initiated by Wnt ligands, the molecular mechanisms of Wnt processing and secretion from the producing cells had been almost overlooked for some time. With the initial identification of Porcupine (Porc) as one of the intracellular machinery members, a new research area has been opened. A closer examination of Wnt post-translational modifications, participating proteins, Wnt intracellular localisation and secretory routes give evidence of tight regulation of the whole process.

One of the factors recently recognised to be involved in Wnt secretion, called Wntless (Wls; also known as Evenness interrupted (Evi) or Sprinter (Srt) in *Drosophila* and mom-3/mig-14 in *C. elegans*), is a seven-pass transmembrane protein. It was found in the plasma membrane, endosomes and Golgi and is presumed to shuttle among these compartments [93-98]. Wntless is evolutionarily conserved from worm to man and so far seems to be specifically required for Wnt exocytosis as no other secreted protein has been found to be affected by *Wls* loss of function [99]. Many Wnt proteins in producing cells lacking *Wls* function remain trapped in the secretory pathway, unable to reach the plasma membrane. In *Drosophila* embryos such *Wls* mutations cause Wnt loss-of-function phenotypes and lead to accumulation of Wingless (Wg) in these cells

[99], mainly in the Golgi [97]. This observation together with the fact that Wls could be co-immunoprecipitated with Wnt3a [93] suggests that Wls might function in binding Wnt and thus concentrating it for export to the plasma membrane. Wntless is then recycled from the cell surface via clathrin-dependent endocytosis and is re-used for the next round of exocytosis [98; 100].

Recently, a protein complex called retromer, indispensable for Wnt secretion, was proposed to target Wls in the endosomal membrane and to route it to the trans-Golgi network (TGN). Retromer, first discovered in yeast as providing endosome-to-Golgi retrieval of receptors that mediate delivery of acid hydrolases to the prevacuolar compartment [101; 102], is highly conserved [103]. An analogous complex has been identified in mammals [104; 105]. A component of retromer, Vps35, was shown to co-immunoprecipitate with Wls and its mutation affected Wnt gradient formation as well as target genes expression [95; 103]. The role of retromer in Wnt secretion is conserved for different Wnt proteins in *Drosophila*, *C. elegans*, *X. tropicalis* and human cells [95]. The retromer function was shown by rescue experiments to be required in Wnt-producing rather than Wnt-receiving cells [103; 106]. In the absence of retromer function, Wls is degraded in lysosomes and consequently, Wnt secretion is impaired [95-98].

A segment polarity gene *porcupine* (*porc*) was identified in *Drosophila* in a screen for mutations that show staining pattern similar to embryo-lethal *wg* alleles producing mutant form of protein that is retained within cells [107]. *Porcupine* is evolutionarily well conserved and orthologues have been found in *C. elegans* (*mom-1*), *Xenopus* (*Xporc*), mouse (*Mporc*) and human (*MG61/PORC*) [108-112]. *Porcupine* function is required in Wnt-producing cells. Its loss results in Wnt retention in the ER [111] and blockage of Wnt secretion [107]. Ectopic expression of *Porc* in human cells increased the hydrophobicity of Wnt proteins (as judged by Triton X-114 phase-separation assay) as well as levels of cell-associated Wnt protein [113]. Porcupine is a multipass transmembrane protein residing in the ER [111] and shares a sequence homology with a family of membrane-associated O-acyltransferases [114; 115]. Although it is widely believed to mediate lipid modification of Wnt proteins, the actual mechanism remains elusive. Given that substrates for other enzymes from the membrane-bound O-acyl transferase (MBOAT) family represent either lipids or lipopolysaccharides, an attached carbohydrate rather than the protein core alone appears more likely to be the target. Examination of glycosylated and secreted ligands other than Wnt has indicated that *Porc* function might be Wnt-specific [111]. An unresolved question remains if *Porc* is active in the attachment of both acyl residues and/or whether additional acyltransferases are involved.

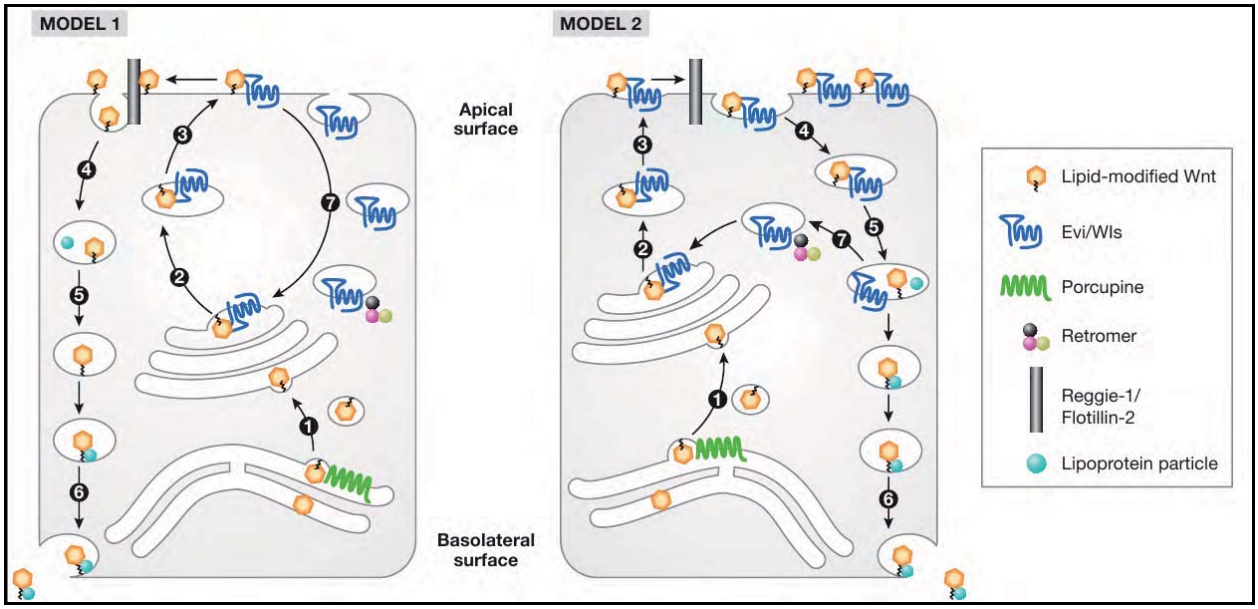

Fig. 7 Alternative models of Wnt intracellular processing and secretion

Wnt, lipid-modified by Porc in the ER (1), travels to the Golgi apparatus where it binds to Wls (2) that mediates its export to the apical plasma membrane (3). Model 1 assumes that Wnt dissociates from Wls on the plasma membrane where it is targeted to the Reggie/Flotillin-containing microdomains, gets internalised (4) and by an unknown mechanism loaded onto lipoprotein particles (5) in endosomal compartments. Next, Wnt is released from the basolateral surface for long-range signalling (6). Model 2 posits internalisation of Wnt together with Wls (4), their dissociation in endosomes (5) followed by loading of Wnt on lipoprotein particles and release from the basolateral surface (6). In both models, Wls is recycled to the Golgi apparatus in a retromer-dependent manner (7). Both models also presume apical membrane-bound Wnt signals to induce short-range targets, whereas the long-range concentration gradient forms on the basal surface.
Adopted from [5], modified.

2.3.2 Formation of Wnt extracellular morphogenetic gradients

Wnt proteins were observed to travel in the extracellular environment and to affect cell behaviour at a distance from the place of synthesis [116-119]. Wnts can also form gradients spreading from the producing cells. This graded mode of distribution has been shown to have biological consequences in Wnt pathway being activated to varying degrees and thus leading to slight differences in both repertoire and level of Wnt-dependent target genes expression. Apart from several contexts where the role of Wnt ligands as morphogens has been suggested and may be debatable [119-123], the morphogenetic properties of Wnts have been demonstrated e.g. in *Drosophila* developing wing, vertebrate embryonic neural tube and colonic crypts [116-118; 123-126]. It was reported that Wnt proteins can move over 20-25 cell diameters away from the source [116; 118; 127]. This contrasts markedly with their high hydrophobicity and poses a question of what machineries render them mobile and what enables them to reach such distances.

Based on experimental evidence, several mechanisms have been proposed for generation of morphogen gradients. A restricted or lateral diffusion model, where secreted morphogen molecules interact with their receptors, co-receptors and/or extracellular matrix (ECM)

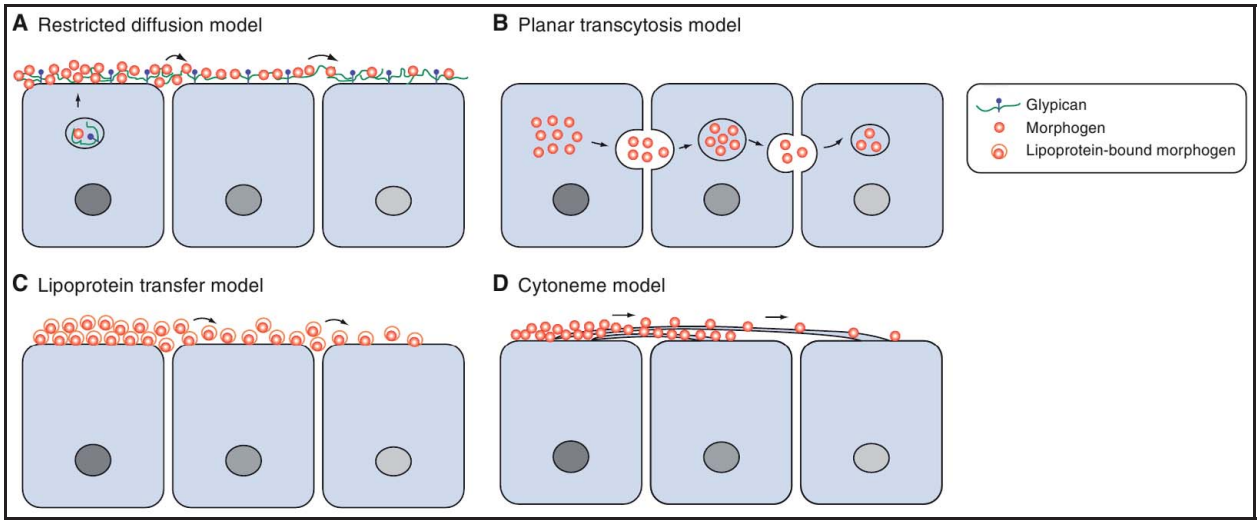

Fig. 8 Models of morphogen gradient formation

A) Restricted diffusion. Morphogens diffuse in the extracellular space, interacting with the ECM proteins, cell surface receptors and co-receptors.
B) Planar transcytosis. Morphogens get actively transported by repeated rounds of endocytosis and re-secretion.
C) Lipoprotein transfer. Morphogens, either associated with or incorporated into lipoprotein or other type of particles, are transferred long distances.
D) Cytonemes. Morphogens move along the apical outgrowths sent by the receiving cells towards the source.
Adopted from [6], modified.

components and by means of these interactions are succesively transfered from one cell to another, receives broad approval [327-330]. One of alternative models posits formation of a gradient by so-called planar transcytosis, an active process in which internalised morphogen travels through receiving cells and is re-released into extracellular space. Such repeated rounds of endocytosis followed by secretion towards more distant cells move morphogen away from its primary source [128-131]. Yet another model assumes dissemination in the form of extracellular particles (e.g. micelle-like multimers or high molecular weight aggregates) and either association with or incorporation into some kind of vehicles (e.g. various vesicles, exovesicular carriers, membrane fragments, membrane-coated lipophilic granules or lipoprotein particles) [9; 132-137]. In addition to the mentioned modes, some cells were reported to send actin-based long outgrowths, called cytonemes, towards morphogen-producing cells and make direct contact with them [138; 139]. Therefore, an interaction mediated by cytonemes or similar cellular extensions may also enable transfer of morphogens over long distances.

For morphogens that are not secreted and do not get dispersed extracelularly, alternative means exist in a cell displacement [140] or the so-called cell lineage transport [141]. In both scenarios, it is a cell population that acts as a morphogen carrier because of cell growth, proliferation and/or migration. Such a cell, after leaving a zone of expression, transports inherited pool of morphogen either as mRNA or protein or both entities over distances of several cell diameters [142]. The gradient formed in this way can be further shaped by molecular decay [143]. It should be emphasised that the described mechanisms need not necessarily be mutually exclusive. It is conceivable that some of them may act in parallel [142] and their usage might be dictated

by tissue and developmental context. Moreover, it has been observed that even for the same morphogen diverse mechanisms might be in operation in different developmental situations [117]. Most of them, acting either separately or in co-operation, have been proposed for Wnt gradient formation [e.g. 5; 7; 117; 129; 133; 136; 142; 144-158].

One class of cell surface and ECM-encompassed molecules that affect Wnt gradient is heparan sulphate proteoglycans (HSPGs) [159]. HSPGs are compound macromolecules whose long, unbranched chains of modified sugars called heparan sulphate (HS) glycosaminoglycans (GAG) are attached to a central protein core on a serine residue. The growing GAG chains start with a tetrasaccharide linkage region Xyl-Gal-Gal-GlcA that seems to be evolutionarily conserved in both vertebrates and invertebrates [160]. HSPGs are classified into several groups according to their protein core structure. Syndecans and glypicans are cell surface HSPGs linked to the plasma membrane by a highly conserved C-terminal transmembrane domain or a glycosylphosphatidylinositol (GPI) anchor, respectively. Perlecans are secreted HSPGs that carry HS side chains. Glypicans and perlecans contain exclusively HS GAG chains, but syndecans can be decorated with both HS and chondroitin sulphate (CS) [161]. The N-terminal globular domain of glypicans is stabilised by disulphide bonds. All three families of HSPGs are evolutionarily conserved among *Drosophila*, *C. elegans* and vertebrates [6; 162-165]. Mutations in genes coding for enzymes involved in the biosynthesis or modification of the HSPG side polysaccharide chains have been shown to cause defects in Wg gradient formation and signalling [153; 155; 158; 166-176]. In *Drosophila* wing disc, cells mutant in *sulfateless* (*sfl*), an orthologue of vertebrate heparan sulphate N-deacetylase/N-sulphotransferase involved in GAG modification, show reduced levels of extracellular Wg protein. At the same time, the expression as well as secretion of Wg in these cells remains normal [155]. It suggests that the entrapment of Wg at the surface of *sfl* mutant cells is impaired. This conclusion is further supported by the fact that in *Drosophila sfl* mutant wing imaginal discs both the expression of short-range

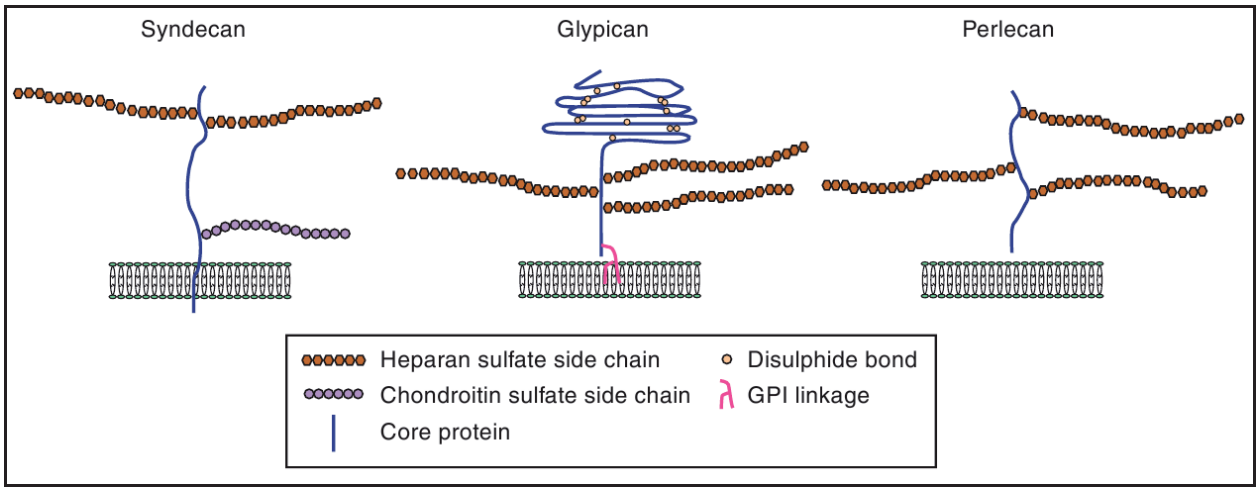

Fig. 9 Structural classes of heparan sulphate proteoglycans

For details see the text. Adopted from [6], modified.

and long-range Wg-dependent target genes is reduced or abolished [158]. Similar to *sfl*, mutation in *sugarless* (*sgl*), which encodes a *Drosophila* orthologue of UDP-glucose dehydrogenase, produces a segment polarity phenotype [166-168]. Ectopic expression of *wg* can rescue both *sfl* and *sgl* loss of function [166; 177] suggesting that by binding extracellular Wg, HSPGs might both limit its diffusion and make it available for its receptor. Absence of HSPGs on cell surface and in ECM possibly leads to a decrease of local Wg concentration under a certain threshold and thus reduction in Wg signalling. *Wingless* overexpression can compensate for the HSPG loss and therefore restore Wg signalling [166]. Other three genes required for HSPGs biosynthesis, *tout-velu* (*ttv*), *sister of tout-velu* (*sotv*) and *brother of tout-velu* (*botv*), code for HS co-polymerases and have their counterparts in vertebrate *Ext1*, *Ext2* and *Extl3*, respectively, members of the hereditary multiple exostoses (*Ext*) family of tumour suppressors. Interestingly, human *EXT1* and *EXT2* are associated with bone dysplasia characterised by multiple benign cartilage-capped tumours [178-180]. The marked reduction in HS GAG chains associated with mutation of any of the three *Drosophila Ext* genes is functionally manifested by decreased extracellular Wg protein levels and its signalling activities [153; 174; 175; 181; 182]. However, the impact of these mutations on Wg distribution and signalling varies among individual *Drosophila Ext* genes. In either *ttv*-, *sotv*- or *botv*-mutant clones in the wing discs, a region of a Wg long-range target gene *distalless* (*dll*) expression was reduced [153]. Strikingly, the Dll protein levels were significantly reduced only within the *botv*-mutant clones whereas in the case of *ttv* and *sotv* they remained comparable with those of the wild-type cells. Correspondingly, the extracellular Wg levels in *botv*-mutant clones were reduced more than in either *ttv*- or *sotv*- mutant clones. These observations suggested that *botv* mutation led to a decrease in both Wg distribution and its signalling activity but in cells mutant for either *ttv* or *sotv* only the range of Wg action was affected. Likewise, the expression of a Wg short-range target gene *senseless* (*sens*) was decreased in *botv*-mutant clones whereas it remained unaffected in clones mutant for *ttv* or *sotv* [153], confirming that Wg signalling was impaired only in *botv* mutants. However, in another study [174] it was shown that *ttv* and *sotv* do have a role in short-range Wg signalling. The expression of a Wg short-range target gene *achaete* (*ac*) was markedly reduced in *ttv*-, *sotv*- and *ttv-sotv* double mutant wing disc cells. To explain the apparent discrepancy observed between the two Wg short-range target genes, different sensitivity of *sens* and *ac* to Wg ligand together with distinct roles of individual *Ext* genes was proposed [153; 164]. In this model, *ac* would need higher Wg signalling activity than *sens* to be expressed. It was observed that the effect of either *ttv* or *sotv* mutation is much weaker than that of *botv* or *ttv-sotv* double mutation [153], suggesting that there remains a certain level of HS co-polymerase activity in the absence of either Ttv or Sotv. This assumption is also supported by biochemical data showing that Ttv and Sotv as well as their vertebrate orthologues Ext1 and Ext2 associate and form a complex *in vivo* [153; 183]. Biochemical studies also revealed that when expressed

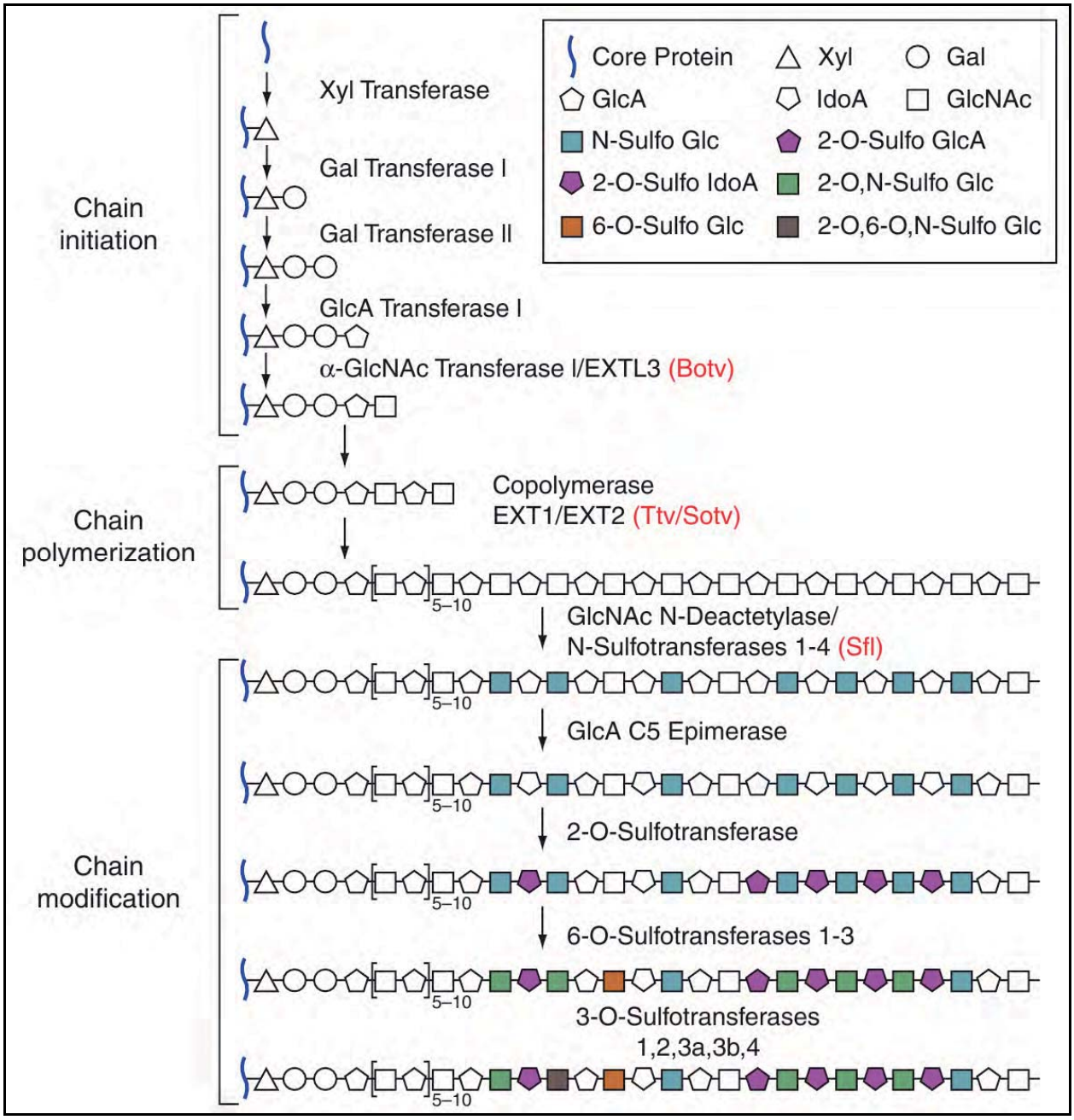

Fig. 10 Heparan sulphate chain biosynthesis

HS GAG chains are synthesised on a core protein by the sequential action of glycosyltransferases and modification enzymes in a three-step process involving chain initiation, polymerisation and modification. Four *Drosophila* enzymes, Botv, Ttv, Sotv and Sfl, the orthologues of Extl3, Ext1, Ext2 and N-deacetylase/N-sulphotransferase, respectively, are highlighted in red.
Gal, galactose; Glc, glucose; GlcA, glucuronic acid; GlcNAc, N-acetylglucosamine; IdoA, iduronic acid; Xyl, xylose.
Adopted from [6], modified.

separately, Ext1 and Ext2 have GlcNAc and GlcA transferase activities with Ext1 activity being stronger than Ext2 [183-185]. Nevertheless, *Ext1* and *Ext2* need to be expressed together for the glycosyltransferase to attain the maximal activity [183; 186]. Based on available biochemical and genetic data, it was proposed that Ttv and Sotv function in HS GAG polymerisation whereas Botv may be involved in initiation of HS GAG chain synthesis and possibly in elongation [153]. Therefore, when *botv* function is missing, the HS GAG chains are

not initiated and all their functions are abolished. However, in the absence of either *ttv* or *sotv*, HS GAG initiation ensued by residual chain synthesis would still occur thanks to the remaining HS co-polymerase activity. Shorter HS GAG chains might be able to assist Wg signalling as Wg co-receptors but might be insufficient in maintaining the extracellular levels of secreted Wg. Finally, when both *ttv* and *sotv* are removed in the double mutant, HS GAG chain synthesis is abrogated because of the lack of the glycosyltransferase activity even though *botv* is present. In an alternative model, reduced amount of intact HS GAG chains is synthesised in the absence of either Ttv or Sotv [153].

Further support for the role of HSPGs in Wnt signalling comes from the studies on *Drosophila* glypicans Division abnormally delayed (Dally) and Dally-like (Dlp; Dly). Both are able to bind Wg in a cell culture assay [147], but interestingly, only overexpression of *dlp* leads to an increased extracellular Wg accumulation whereas *dally* shows almost none or a very little ability in this respect [117; 147; 155; 187]. Rather paradoxically, ectopic expression of *dlp* also antagonises Wg signalling, possibly due to its ability to sequester Wg from its receptor Fz2 [155; 187].

In *Drosophila* wing imaginal discs, the larval precursors of wings in an adult fly, Wg ligand is produced by two stripes of cells demarkating the dorso-ventral (DV) compartment boundary that becomes a wing margin later on [188]. Dally is spread almost throughout the wing disc with two regions showing higher levels of the protein. The first is a stripe of cells at the AP boundary marked by a Hedgehog (Hh) signalling, the second runs along the DV boundary and opens wide towards both sides away from the AP boundary [7; 189; 190]. Dally-like is dispersed on almost all cells of the wing disc except from the zone of about 7-10 cell diameters wide at the DV boundary [7]. Wingless movement across a stripe of *dally-dlp* mutant cells is hampered, indicating that Wg cannot move by free diffusion and furthemore that HSPGs Dally and Dlp are involved [7]. However, mutations of *dally* and *dlp* result in different phenotypes [7; 147], suggesting that they fulfil distinct functions during Wg signalling. RNA interference

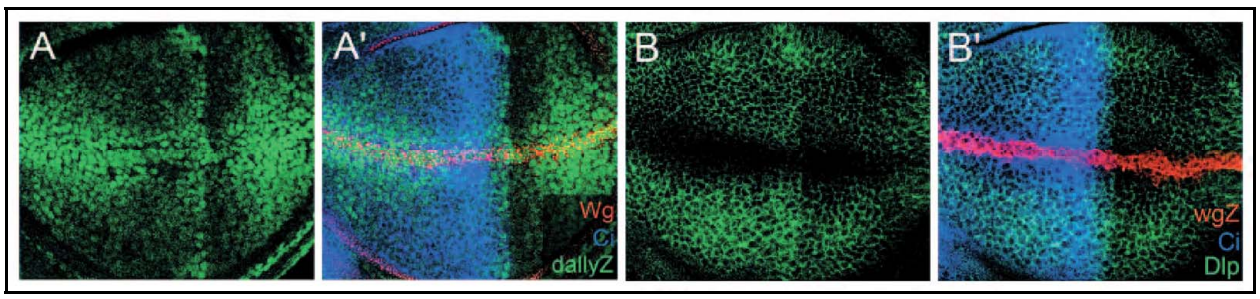

Fig. 11 Expression of *Dally* and *Dlp* in *Drosophila* third instar wing disc

(A, A`) *Dally* expression visualised by *Dally-lacZ* expression (green)
(B, B`) *Dlp* expression visualised by staining with anti-Dlp antibody (green)
The AP and DV compartment boundaries visualised by staining of Cubitus interruptus (Ci), a transcription factor involved in Hh signalling (blue in A`, B`), and *Wg* or *Wg-lacZ* expression (red in A`, B`), respectively.
All discs are oriented dorsal upwards, anterior leftwards. Adopted from [7], modified.

experiments show a genetic interaction between *dally* and *dlp* indicating that both glypicans may act synergically in shaping Wg gradient [155]. Oddly, *dlp* exerts opposite effects on Wg signalling depending on the distance from the Wg source. In a cell culture-based assay, a dsRNA directed at *dlp* up-regulated a Wg-reporter gene activity upon Wg induction. Conversely, under nonstimulated conditions, Dlp acted as a positive regulator as *dlp* dsRNA decreased the reporter activity [191]. Correspondingly, analogous dual impact of Dlp was observed also *in vivo*. Either loss or a decrease in *dlp* function in wing imaginal discs resulted in ectopic activation of Wg signalling and expanded expression domain of high-threshold target genes near the Wg source along with a reduction of low-threshold target genes expression further away from the Wg-producing cells [147; 150; 151]. Results from the experiments with *dlp* mutant clones and RNAi were complemented by *dlp* overexpression study showing exactly the opposite effect, i.e. a support of a long-range signalling at the expense of a short-range signalling [147]. Therefore, Dlp can reduce Wg activity near the Wg source where the ligand levels are high as well as promote Wg signalling far from the site of Wg production where the ligand levels are low. This seemingly disparate phenomenon might be well explained if one assumed that Dlp would block access of Wg to its receptor and rather favoured presentation of the ligand to the neighbouring cell [192]. In such a scenario, Wg would move by being handed over from Dlp on one cell to Dlp on the next. This transport of the ligand to more distant regions would be further facilitated by the existing Dlp gradient inverse to that of Wg [7].

Recent reports show that the levels and activity of HSPGs might be tightly regulated. In *Drosophila* wing imaginal discs, Notum (also known as Wingful; Wf), a secreted protein with structural homology to plant pectin acetylesterases, is expressed at the DV boundary where it closely follows the expression of Wg [187; 193]. Based on genetic and *in vitro* biochemical analysis, Notum was first proposed to cleave Dlp at the site of its GPI anchor and to release it from the membrane [150; 151; 187]. As Wg remains bound to Dlp that is shed off the cell surface, Notum may act as Wg antagonist by limitting the amount of Wg available for signalling. Recently, several observations led to a re-evaluation of the target of Notum activity. First, downregulation of *dlp* level at the DV boundary is not caused by Notum since in clones mutant for *Notum* Dlp levels do not change nor they are enhanced in *notum* homozygous wing discs. Second, contrary to *dlp*, *dally* is highly expressed in the same region where Notum is supposed to have the highest activity. Third, *dally*/*notum* double mutants exhibit phenotype similar to *dally* single mutants suggesting that *dally* acts downstream of *Notum* in shaping the Wg gradient. Therefore, it was reasoned that Dally seemed to be a more appropriate substrate for Notum activity than Dlp [7].

2.3.3 Factors and mechanisms affecting Wnt activity in the extracellular space

The activity of Wnt within its extracellular gradient is further modulated by additional factors. There are several classes of such molecules that employ different mechanisms.

Two groups of Wnt agonists have been identified so far. Norrin (alternatively named Norrie disease protein; NDP) is a product of a gene mutated in Norrie disease [194; 195], a condition characterised by a severe retinal hypovascularisation leading to blindness, which is in some patients accompanied by deafness and certain degree of mental retardation. It is a cysteine-rich secreted protein predicted to form oligomers composed of covalently cross-linked homodimers and to adopt a so-called cystine knot structure [196; 197]. Despite a complete lack of sequence homology and structural unrelatedness to Wnts, Norrin binds Fzd4 with an affinity in nanomolar range [198]. Rather surprisingly, Norrin shares several properties with Wnt proteins: its association to Fzd occurs within the CRD, it uses LRP as a co-receptor and in spite of being secreted it often stays attached to the cell surface [196; 198]. *In vitro*, Norrin activates canonical Wnt/β-catenin pathway in reporter cell lines only when *Fzd4* and either *Lrp5* or *Lrp6* are co-transfected [198]. The interaction with Fzd4 seems to be highly specific as no other Fzd receptor among the ten mammalian Frizzleds tested was found to bind Norrin [198; 199]. Remarkably, Fzd4 has got a soluble splicing variant denoted Fzd4S that contains the CRD [200]. Therefore, it seems very likely that this particular peptide represents a means of Norrin signal regulation in the extracellular space. Moreover, recently a member of the tetraspanin family, TSPAN12, has been identified as an auxiliary factor for Norrin receptor complex. Intriguingly, TSPAN12 selectively enhances Norrin/β-catenin signalling. Biochemical data further indicate that Norrin and TSPAN12 co-operatively promote Fzd4/LRP5 complex oligomerisation [201]. The requirement of TSPAN12 for Norrin/β-catenin and not Wnt/β-catenin signalling may allow only a subset of cells in a population to respond to Norrin and thus ensure spatiotemporal isolation of different modes of β-catenin signalling.

Mammalian R-spondin (Roof plate-specific spondin; Rspo) family of ligands comprises four members (Rspo1-4) that share a high degree of sequence identity [202]. Following the N-terminal signal peptide there are several structural domains – two furin-like Cys-rich domains, a thrombospondin type 1 repeat (TSR1) and C-terminal stretches of basic amino acids likely functioning as NLS. Rspo proteins are capable of stabilising β-catenin, inducing a TCF reporter activity as well as inducing known Wnt/β-catenin target genes. The two furin-like domains were found sufficient for these effects whereas the TSR1 and the basic C terminus were dispensable [203-208]. On the other hand, the TSR1 appears to be critical for the interaction with cell surface heparin sulphate proteoglycans and deletion of the TSR1 markedly diminishes Wnt signalling [205; 207]. Rather strangely, *R-spondins* do not induce axis duplication in *Xenopus* embryos unless their mRNA is co-injected with *LRP* or *Fzd* mRNA [205; 208]. Comparison of knockout

mice phenotypes, overlapping expression patterns of *Wnt* genes and *R-spondins* and other, mostly biochemical observations suggest that expression of *R-spondins* and their function depend on active Wnt signalling [203-207; 209]. Nowadays, it is widely accepted that R-spondins act as novel ligands that activate Wnt signalling but the exact mechanism of their action on cell receptors remains largely unresolved. Mutually inconsistent reports that Rspo proteins bind Kremen (Kringle domain-containing protein marking the eye and the nose; Krm) but not LRP [204], LRP and not Fzd [208], either Fzd or LRP [205] or none of them [203] have been published. Yet another model has been proposed according to which Rspo stimulates Wnt signalling by antagonising Dickkopf (Dkk)/Krm-mediated LRP endocytosis through the association with Krm [204]. However, this mode of action seems hardly plausible given that Rspo activates β-catenin signalling in cells lacking both *Krm* genes and that *Krm1*/*Krm2* double knockout mice do not show *Rspo* mutant defects in full [210]. Another rather unexpected observation added to this puzzle when Rspo proteins were reported to bind Wnt1 [205]. Clearly, further experimentation is needed to reconcile the seemingly disparate data. Alternatively, they may reflect processes accompanying activation of an as-yet-unidentified proper receptor for Rspo ligands.

Antagonists including Wnt inhibitory factor 1 (WIF1) [211] and Cerberus [212-214] bind Wnt proteins [211; 215] whereas members of the Dkk [216; 217] and Wise/Sclerostin (Sost) [218-221] families interfere with the signalling by association with the LRP5/LRP6 co-receptor [222-229].

Two substantially different models have been proposed for the Dkk-mediated inhibition of Wnt signalling. The first one, based on the analysis of Wnt1, Dkk1, Fzd8 and LRP6 proteins *in vitro*, posits that Wnt and Dkk binding to LRP is mutually exclusive and thus Dkk disrupts Wnt-induced Fzd/LRP complex formation by association with the co-receptor LRP [222]. The second model rests on overexpression studies in mammalian cells. It suggests that the single transmembrane-spanning protein Krm, a high-affinity receptor for Dkk [204; 230; 231] which is able to directly bind LRP [232], forms a ternary Dkk/Krm/LRP complex that triggers removal of LRP from the plasma membrane via endocytosis. In this way, a cell is made less sensitive or unresponsive towards the Wnt signal [230]. However, the results of recent biochemical and genetic studies [210; 233; 234] give evidence that Krm proteins are not essential for Dkk function and that Dkk inhibition of Wnt signalling is independent of LRP internalisation and degradation. Therefore, the model proposing direct competition between Dkk and Wnt for LRP binding seems more likely. Krm proteins may play a role in Dkk-mediated antagonism in specific tissues and contexts [210], particularly when the *LRP* expression level is high [210; 234].

In addition to Cerberus which binds to and inhibits Wnt as well as BMP and Nodal [215], other Wnt antagonists with multivalent abilities exist. Another member of the Cerberus/Dan/

Gremlin family of secreted inhibitors, named Coco, blocks signalling mediated by BMP, TGFβ and Wnt ligands [235]. Besides modulating insulin-like growth factor (IGF) signalling, insulin-like growth factor-binding protein 4 (IGFBP4) was reported to be an inhibitor of canonical Wnt signalling. Interestingly, several other members of the IGFBP family, namely IGFBP-1, -2, -4 and -6 were also found to antagonise Wnt signalling by binding to both Fzd and LRP6 [236].

The most prominent among secreted modulators are the group of secreted Frizzled-related proteins (SFRPs) [8; 237-256]. The N terminus of SFRPs bears the so-called cysteine-rich domain (CRD) encompassing ten cysteine residues at conserved positions [257] in a pattern identical to that of the extracellular part of Fzd [241; 242]. The C-terminal netrin (NTR) module of SFRPs contains several patches of conserved hydrophobic residues together with six cysteines [257; 258] and appears to confer heparin-binding ability [259]. In humans, five members of the SFRP family have been identified [238; 241; 243; 249; 251; 253; 254]. According to the sequence comparison and different genomic organisation, they form two clusters – *SFRP1*/*SFRP2*/*SFRP5* and *SFRP3*/*SFRP4*. The members within each of these two subgroups share a similar spacing between cysteine residues of the NTR module, which is distinct from that shared among the members from the other cluster [257]. It is tempting to speculate that the diversity in their cysteine patterns and the resulting spatial folding may provide a basis for the observed functional specificity of SFRP family members towards Wnt ligands. Additional differentiation may be further contributed by post-translational modifications. For example, SFRP1 is N-glycosylated at Asn172 [257] and sulphated at Tyr34 and Tyr36 [260] with both tyrosine residues conserved in SFRP5 but absent from other SFRPs [251]. The tyrosine sulphation of SFRP1, which was shown to partially destabilise the protein, was inhibited by heparin [260], pointing to the possibility that *in vivo* the stability of some SFRPs might be controlled by heparan sulphates.

Similar to the existence of many Fzd receptors that vary in ligand specificity, individual members of the SFRP family also seem to display preference or selectivity towards different Wnts. For example, SFRP1, SFRP2, SFRP3 and SFRP4 all bind directly to Wnt3a with affinities in the nanomolar range as measured by surface plasmon resonance, but only SFRP1 and SFRP2 are able to antagonise Wnt3a-induced accumulation of β-catenin in L cells [261]. Similarly, SFRP2 but not SFRP3 blocks Wnt3a signalling in an ES cell model of mesoderm differentiation [261]. The *SFRP3* mRNA is unable to suppress Wnt5a-induced head and/or tail malformations in *Xenopus* embryos even though Wnt5a co-immunoprecipitates with SFRP3 [262]. According to surface plasmon resonance, SFRP1 and SFRP2 bind to Wnt5a whereas SFRP3 and SFRP4 do not [261]. Instead, SFRP3 binds Wnt1 and Wnt8, reduces Wnt1-induced cytosolic β-catenin accumulation in HEK cells and inhibits development of ectopic body axis promoted by Wnt1 and Wnt8 in early *Xenopus* embryos [239; 240; 262]. Another example of differential effect was observed in the case of SFRP1, which rescues axial duplication phenotype in *Xenopus* embryos

caused by Wnt1, Wg, Wnt8 and Wnt3a. Interestingly, in each case SFRP1 exhibited distinct capacity to suppress Wnt-mediated dorsal axis bifurcation with the least potency shown towards Wnt3a [243]. Another remarkable phenomenon, a potentiation of Wg-mediated effect when the SFRP concentration was low and suppressing it when the SFRP concentration was high, was described with SFRP1 [259]. It remains to be determined whether this peculiar behaviour is unique to SFRP1 or whether it is a common feature of other SFRPs as well.

The debate on the mechanism(s) of Wnt signalling modulation by SFRPs is still unsettled. Since the identification of SFRPs, it has been widely accepted that SFRPs bind and sequester Wnt proteins preventing them from the interaction with their receptors. This opinion was based on the similarity in primary structure of the SFRP N-terminal domain to the extracellular CRD of Fzd [239; 240; 242; 262], which had been proposed to be the Wnt-binding element [67; 263]. However, data that are more recent point to a handful of new and previously unexpected possibilities. Rather surprisingly, the CRD of SFRP1 was dispensable for the observed direct interaction of SFRP1 and Wg as the SFRP1 mutant lacking this domain still exhibited binding similar to the wild-type SFRP1. On the contrary, SFRP1 deletion mutants lacking the whole or different portions of the C-terminal region (i.e. the NTR domain) showed either a loss or a significant reduction in Wg binding both in ELISA and co-immunoprecipitation experiments [259]. Moreover, these results corresponded well with the outcomes of Arm stabilisation assay in *Drosophila* Schneider (S2) cells that showed the CRD as unnecessary for the biological activity as well. However, the absence of CRD decreased the specific activity of SFRP1 [259]. On the other hand, partial deletions of SFRP3 CRD left its binding to Wnt1 unchanged but were deleterious for the SFRP3 ability to inhibit Wnt1-mediated axis duplication in *Xenopus* embryos. A complete CRD removal disrupted both the SFRP3/Wnt1 association and Wnt1 signalling modulation capability. By contrast, removal of the SFRP3 NTR domain left the truncated molecule capable of Wnt1 binding and preserved functional inhibition of Wnt1-mediated signalling, albeit less efficient [262]. Recently it was reported that both the cysteine-rich and netrin domains were required for optimal function of SFRP1. Particularly, the conserved tyrosine residue located in the second loop of the SFRP1 CRD together with the last 19 amino acid residues at the C-terminus played a critical role in the inhibitory activity [264]. All these apparently disparate observations might be a manifestation of an existence of multiple amino acid residues available for intermolecular contacts that are possibly distinct and specific for individual SFRPs. They might also point to the critical role of the above-mentioned differences among SFRPs in post-translational modifications. All these factors might lead to slight variances that govern individual SFRPs to discriminate between members of the Wnt family of ligands and result in distinct affinities with which one particular SFRP can bind and influence biological activity of several Wnts. Clearly, further work will show if it is possible to trace down common features or whether individual SFRPs possess unique properties

in binding Wnt ligands and modulating their activity. Based on results obtained so far, it appears that this effort will be further complicated by the fact that mere binding specificity determined biochemically *in vitro* need not necessarily correspond to functional interactions *in vivo*.

From the above-mentioned results it follows that SFRP proteins might either engage preferentially the CRD or the NTR domain or use both for binding to Wnt ligands. Furthermore, crystal structures of the CRDs from Fzd8 and SFRP3 together with biochemical analyses examining Wnt1, Wnt2, SFRP1, SFRP3, Fzd2, Fzd3 and Fzd6 suggest that the cysteine-rich domains can homodimerise or heterodimerise [265-268]. The latter case extends considerably the options for modulation of Wnt signalling. Besides acting as classical antagonists by sequestering Wnt ligands from their receptors [239; 240; 243; 269] the SFRP proteins may as well form inactive complexes with Fzd receptors through their homologous CRDs and block signal activation in this way [266]. Binding of SFRP to Fzd opens up possibility for an alternative scenario in which, in the absence of Wnt, SFRP might prove sufficient for activation of signal transduction. This is likely to be the case of SFRP1, which can act as a guidance cue and affect the behaviour of chicken and *Xenopus* retinal ganglion cell (RGC) axons. The SFRP1-mediated effect appears not to require Wnt inhibition and furthermore, interference with the *Fzd2* expression in RGCs abrogates the response of growth cones to SFRP1. The CRD of SFRP1 reportedly retains the activity of the whole protein molecule. Soluble SFRP1 co-immunoprecipitates with Fzd2 and binds specifically to the surface of cells overexpressing Fzd2. Therefore, at least in the context of neurite outgrowth, SFRP1 acts as ligand directly activating Fzd2 [268]. Another plausible mechanism resides in SFRP`s ability to use either of the two domains for binding to its partners. SFRPs might well act to favour Wnt-Fzd interactions by associating concurrently to both Wnt and Fzd and promote signal transduction by facilitating Wnt ligand presentation to a Fzd receptor [259]. SFRPs might also titrate out one another as in the developing kidney where SFRP1 blocks tubule formation and bud branching whereas SFRP2 opposes this effect. Binding of TCF to metanephric mesenchyme cell DNA is reduced by SFRP1 but not by SFRP2, indicating that SFRP2 does not antagonise Wnt4 signalling associated with the differentiation of the renal mesenchyme. Instead, it is likely that SFRP2 competes locally for binding to SFRP1 and thus promotes tubule differentiation and bud growth via Wnt4 signalling [270].

SFRPs are not solely Wnt-binding proteins but their manifold activities reach far beyond the Wnt-confined borders [8; 247]. Therefore, one of the attractive possibilities is that SFRPs may modulate Wnt signalling indirectly by affecting independently functioning pathways, e.g. BMP. Both Wnt and BMP signalling functionally participate in a number of biological processes and their crosstalk causes effects not achieved by any of them in isolation [271; 272]. Their interactions can be either synergistic [86; 273-279] or antagonistic [280-283], depending on cellular context. During *Xenopus* gastrulation, Sizzled (secreted frizzled; Szl), the anamniotic

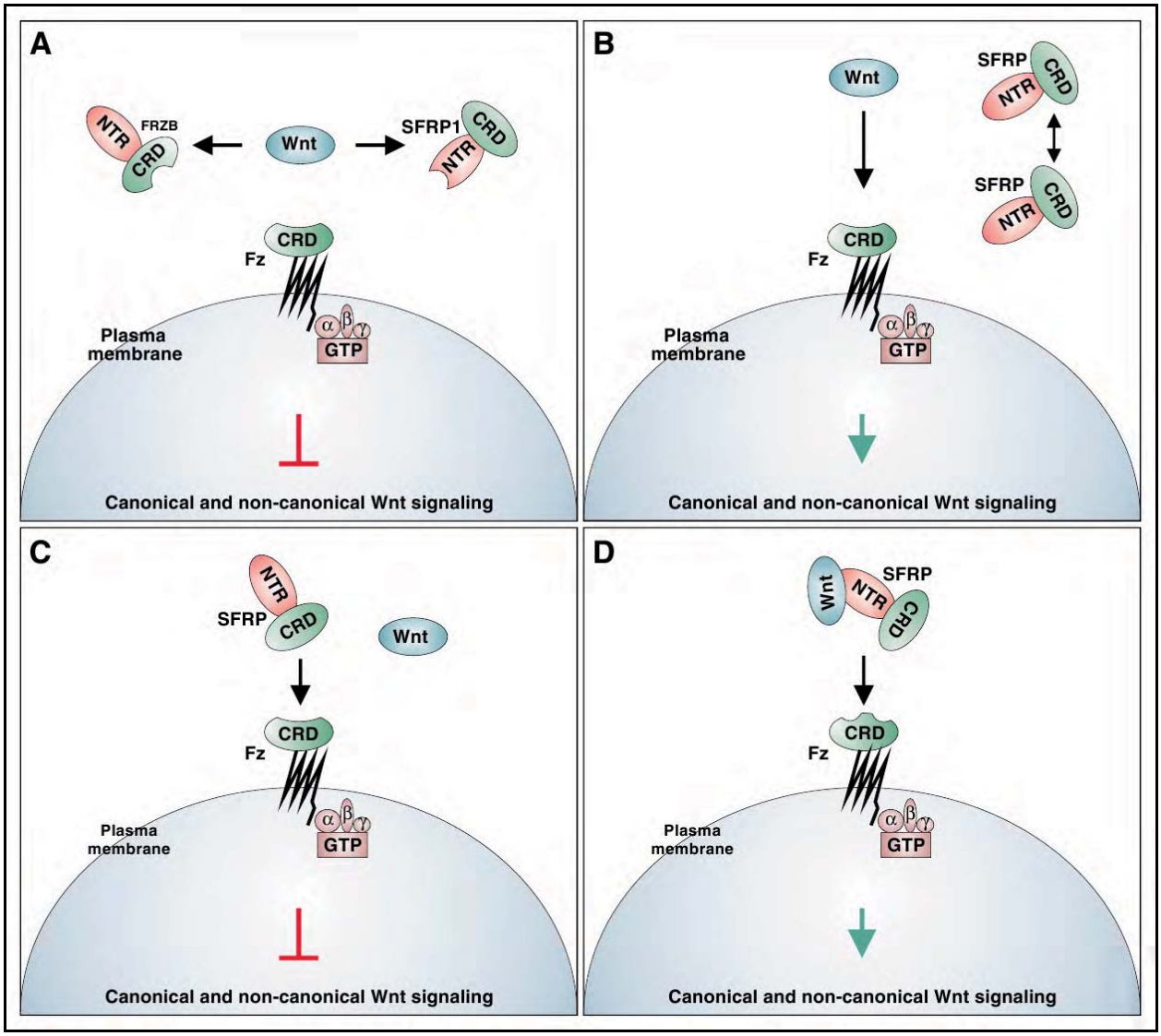

Fig. 12 Possible mechanisms by which SFRPs could modulate the Wnt signalling

A) Acting as antagonists by sequestering Wnt either by the CRD or NTR domain.
B) Favouring the Wnt signalling by titrating one another`s activity.
C) Preventing the Wnt signal transduction by forming signalling-inactive complexes with Fzd receptors.
D) Promoting signal transduction by simultaneously binding to both Wnt and Fzd.
Adopted from [8].

SFRP family member initially identified in *Xenopus* as a putative antagonist of XWnt8 [244], binds to BMP1/Tolloid (Tld) metalloprotease and prevents cleavage of Chordin (Chrd), a secreted protein that blocks binding of BMPs to their receptors [284-288]. By careful biochemical experimentation it was demonstrated that Szl and Chrd compete for the substrate binding site of Tld with similar affinities [285]. The protease inhibitory activity of Szl mapped to the CRD, pointing to a novel function of this domain. Likewise, SFRP2 also inhibited cleavage of Chrd by a Tolloid-family metalloprotease in a dose-dependent manner [285], indicating that SFRPs other than Szl might potentially act in an analogous way. Several additional studies examining influence of SFRPs on BMP signalling point also to situations other than dorso-ventral patterning during embryo gastrulation. For instance, in the embryonic chicken hindbrain, SFRP2 antagonises the programmed cell death [289] which was shown to be induced by BMP4

in rhombencephalon as well as in other sites and biological contexts [290-299]. Strikingly, inactivation of *Smad-interacting protein 1* (*Sip1*), a transcription factor involved in BMP signalling [300-304], in mouse cortical precursors, leads to a hippocampus loss in the adult, presumably due to increased apoptosis accompanied by ectopic activation of *Sfrp1*. *Smad-interacting protein 1* and *Sfrp1* show mutually exclusive expression pattern and moreover, Sip1 protein was detected on the *Sfrp1* promoter in cortical cells by ChIP assay, strongly suggesting that Sip1 negatively regulates *Sfrp1* expression [305]. In another study, SFRP1 and SFRP2 were found to play critical role in controlling the development of dorsal ectodermal tissues during early neural tube stages in vertebrate embryogenesis. By specifically inhibiting BMP4-mediated signalling, SFRP1 and SFRP2 maintain proper neural tube closure and ensure roof plate and neural crest cell fate segregation [306]. Recently, another novel role has been reported for SFRP2 in a study of heart defect in *Sfrp2*-null mice. SFRP2 was found to bind procollagen and enhance procollagen C-proteinase activity of BMP1/Tld [307]. The procollagen C-proteinase activity of BMP1/Tld is responsible for converting procollagen I-III into collagen fibrils that comprise an important fibrous component of the ECM [308].

2.3.4 Mechanisms supporting the reproducibility of Wnt morphogenetic gradients

Morphogen gradients play unique role in setting up coordinates for organ development. Therefore, any imperfection in an instructive ligand profile may lead to serious defects.

In addition to the already mentioned factors that affect biological activities of Wnt ligands in the extracellular space, recent years have offered a deeper insight into processes assisting in maintenance of Wnt morphogenetic gradients. Various controlling mechanisms have evolved to maintain stability, precision and robustness (i.e. buffering capacity) as well as to ensure reproducibility in case of perturbations caused by gene expression and environmental fluctuations. Tight regulations exist at the level of Wnt production and Wnt gradient establishment as well as a response to Wnt local concentration by the receiving cells.

Wnt morphogenetic gradient utilises a system of feedback self-regulatory mechanisms to protect against disruptive influences. Numerical analysis has revealed self-enhanced Wnt degradation as a general mechanism for achieving robustness towards fluctuations in Wnt production rates [309]. In *Drosophila* wing disc, active Wg stabilisation by DFz2 together with the downregulation of *DFz2* expression by Wg signalling leads to Wg gradient profile that drops rapidly close to the Wg source and more gradually further away from it [127; 188; 310; 311]. This gradient shape allows for efficient buffering of variations in Wg secretion rate as well as for Wg diffusion range extensive enough to enable patterning of the whole developmental field [309]. Besides the negative feedback based on reciprocal ligand-receptor interactions, other

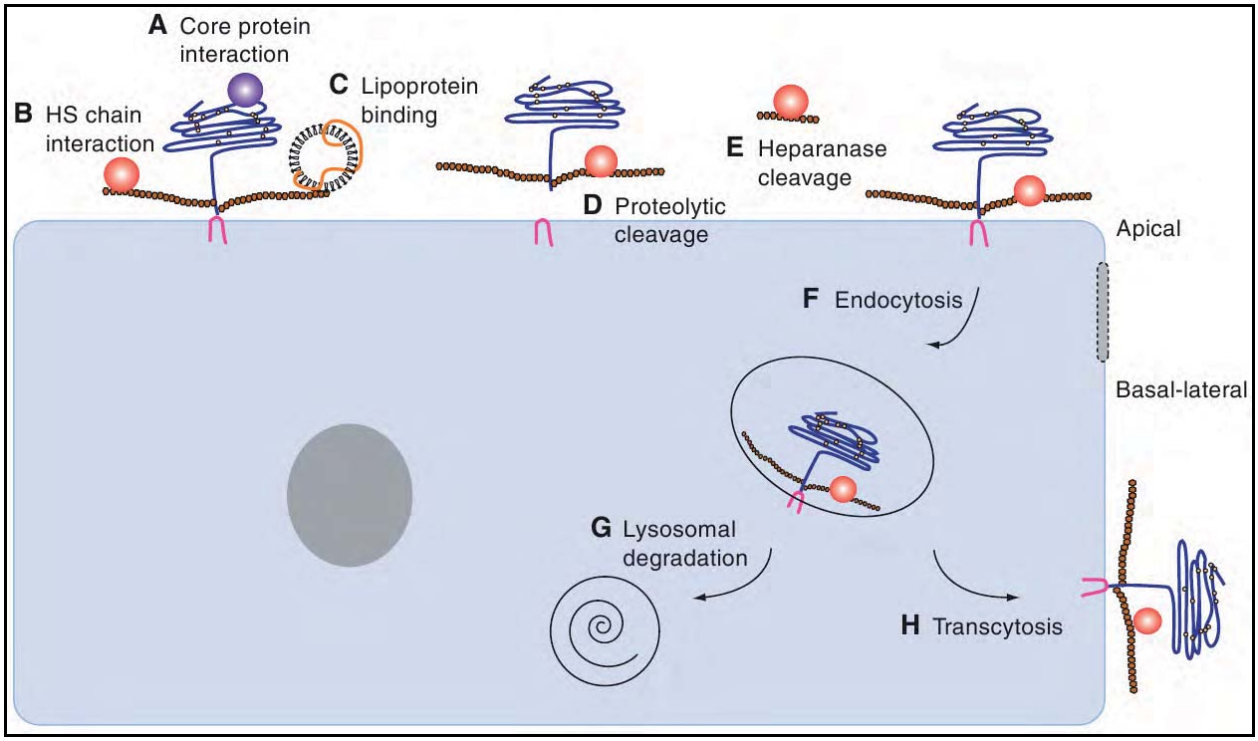

Fig. 13 Cell surface HSPGs could modulate morphogen gradients by several mechanisms

HSPGs can interact with morphogens either through their core proteins (A) or HS GAG chains (B). HS GAG chains can also recruit lipoprotein particle-associated morphogens (C). HSPG-bound morphogens could be released from the cell surface by HSPG shedding, assisted either by a protease (D) or heparanase (E) activity. Morphogens bound to HSPGs could also be endocytosed (F), targeted for lysosomal degradation (G) or transported from the apical to the basolateral membrane by transcytosis (H).
Adopted from [6].

means are likely to protect the local Wnt levels at the site of secretion from unavoidable biological variations. Intracellular regulatory mechanisms safeguarding Wnt secretion rates against the gene dosage variation and confined to the domain where the ligand is produced could represent one such possibility. Likewise, HSPGs restricted to the area near the Wnt source might modulate Wnt degradation either directly or by passing the ligand on to the receptors [147; 149; 150; 155; 158; 166; 173; 191].

Some of the above-mentioned mechanisms supporting robustness have been just proposed on the basis of numerical simulations and have not been investigated further in detail. Nevertheless, it is tempting to speculate that stability and reproducibility of the Wnt gradient as well as a response to Wnt signal might be ensured by regulatory mechanisms operating at several different levels.

In addition to the mechanisms buffering fluctuations in Wnt production/secretion, robustness to other parameters of the gradient such as the morphogen diffusion rate, receptor levels and morphoghen signalling activity should also be considered. Thus far, these possibilities have not been studied in detail. In this regard, circumstantial evidence points to HSPGs that might assist diffusion by actively transferring the ligand via their polysaccharide chains [7; 148; 191].

A number of transcriptional feedback circuits, whereby Wnt signalling directly influences expression of the internal components of its own signalling cascade as well as some of the extracellular modulators, was detected. Among those identified, hDkk1 [312-314], mSFRP2 [315], Wf/Notum [187; 193], hWnt11 [314], DFz3 [316], hFzd7 [317], naked cuticle (Nkd) [318-320], Axin2/Conductin [319; 321-323], hβ-TRCP [317; 324], the ubiquitin conjugating enzyme hUbc4/5E2 [317], DNlk [325], LEF1 [314; 326-329], hTCF1 [330] and hTLE [317] were upregulated, whereas Wg [331], DFz [332], DFz2 [127; 332] and Arrow/LRP [333] were downregulated. Some of the targets, such as Dally [173; 189] seem to be context-dependent. The extent of the list proves that Wnt signal-inducible feedback loops, both positive and negative, contribute to the precise regulation of the Wnt signalling, adding yet another level to mechanisms exerted by extrinsic, Wnt-independent factors.

The intracellular regulatory loops acting on signal transduction downstream of the receptor can not contribute to the buffering against perturbations in the extracellular morphogen profile unless the ligand modulates its own expression. This indeed was observed at the prospective wing margin in *Drosophila* third-instar wing imaginal discs [334].

Quantitative measurements revealed that Axin, a scaffolding protein of the β-catenin destruction complex, is present intracelularly in a several orders of magnitude lower concentration than other major components of the Wnt transduction cascade [335]. Consequently, a moderate increase in Axin concentration results in robust β-catenin degradation. In this way, a cell has got a powerful means of controlling the turnover of a key signalling element by a slight manipulation of the scaffolding protein concentration. The low Axin concentration has also other important consequences for keeping the intracellular homeostasis. As Axin interacts with components that are shared with other pathways, the result of a Wnt signal processing would be fluctuations of their concentrations. Due to low concentration of Axin, there are no measurable changes in GSK3β, Dvl or APC levels and so Wnt pathway is efficiently insulated from disturbing other systems and *vice versa* [335].

It was demonstrated that low concentration of Axin together with its increased instability, induced upon Wnt stimulation, directly affects the amplitude and duration of the response. It was also suggested that by regulation of Axin turnover a cell could substantially change its responsiveness towards transient and sustained Wnt signals. Such mechanism would even enable a cell to respond differently to the same signal quality under different developmental contexts [335].

Yet another feature of the Wnt pathway, namely that the degradation of Axin is APC-dependent, has been identified [335]. As mentioned above, even a subtle increase in Axin concentration instantly strengthens the degradation of β-catenin via promoting the formation of the destruction complex because both APC and GSK3β are in excess over Axin. Any drop

in APC concentration diminishes Axin breakdown so that continued β-catenin depletion is ensured. Without APC-Axin regulatory loop, β-catenin levels would rise sharply upon APC concentration fall. By making Axin turnover dependent on APC, accidental lowering of APC concentration is buffered within certain limits thanks to Axin counterbalance. It was proposed that the APC-mediated control of Axin degradation could serve as a precaution against environmental or genetic effects on APC [335]. By inference, specific susceptibility of a colonic epithelium to APC mutations could be viewed from the perspective of the pathway`s (in)ability to buffer decreased APC amounts in that tissue.

Even genetically identical cells in a population or within a tissue exhibit differences in individual protein levels due to an unavoidable stochastic fluctuations in gene expression [336-343]. It stands to reason that Wnt cascade constituents are not an exception in this respect. Yet the intrinsic noise does not seem to have any effect on the cell`s ability to respond reliably to Wnt stimulation. Experimentally perturbing the amounts of major intracellular Wnt pathway members and monitoring the response of the whole system has led to an unexpected finding that the Wnt-induced rise in β-catenin was quite insensitive to variation in most of the parameters when relative and not absolute β-catenin levels were considered [344]. Furthermore, it was shown that mathematically, there exists a region of values within which the fold-change in β-catenin amounts (i.e. the proportion of the Wnt-induced to the starting level) withstands variation of parameters. It was also demonstrated that cultured cells as well as whole organisms operate within these boundaries mantaining a relative change in β-catenin robust to naturally occurring noise in protein levels. The capability of a downstream transcriptional machinery to interpret two different amounts of β-catenin as identical, provided its fold-change remains constant, would serve well to filter the cell-to-cell variation in biochemical parameters. Another possible advantage of relative change detection over the absolute change detection might be that it enables to distinguish a signal in a noisy environment more reliably [344; 345].

In addition to the feedback regulation of receptor expression [127; 346], self-enhanced receptor-mediated active ligand protection or stabilisation [127; 346], signalling-induced self-inhibition [187; 193; 318] marginally mentioned above, there are other conceivable factors such as morphogenetic apoptosis [347-349], cell competition [347; 350-353], receptor-mediated endocytosis followed by regulated degradation [152; 346] and the like that might modify the shape of the Wnt extracellular gradient. However, for the sake of conciseness and in order to keep the text within reasonable limits they will not be described in detail.

3 RESULTS AND DISCUSSION

The Wnt family of glycoproteins controls numerous cell fate decisions in development by setting up new or altering the existing gene expression patterns through the activity of hetero-dimeric transcriptional activators. These consist of β-catenin and one of the LEF/TCF family members of DNA-binding proteins. In these bipartite transcription factor complexes β-catenin provides a transcriptional activation function, whereas a member of LEF/TCF family of DNA-binding proteins guides β-catenin to the promoter regions of specific target genes.

In reference to the lead-in on LEF/TCF characteristic features, it is evident that although these factors share a number of general properties and can be mutually interchangeable in some situations, in others they clearly exhibit unique behaviour. Wnt signalling pathway is used repetitively both in time and space throughout development of multicellular organisms to participate in numerous developmental programmes. Inevitably, β-catenin/TCF complexes induce only subsets of all potential target genes at any particular time and cellular background. How this differential tissue and stage-specific control over various subsets of target genes is achieved with such a limited number of nuclear effectors is not fully understood. It seems obvious that binding to various partners in distinct transcriptional complexes can render LEF/TCFs abilities required for launching context-dependent expression profiles. Therefore, we set out to find novel proteins interacting with LEF/TCF family of transcription factors. Our results are summarised in the three presented papers.

Since the discovery of Wnt1 [354], the first identified family member, in a screen for mutations caused by insertion of mouse mammary tumour virus (MMTV), much detailed knowledge has been gained on molecular events downstream of the Wnt receptors and their translation into differential target gene expression. On the contrary, our understanding of the Wnt maturation, secretion from cells and modes of distribution in extracellular environment is still rather sketchy. Therefore, we tackled experimentally the question of post-translational modifications and their contribution to secretion and biological functions of Wnt ligands. The resulting publication is inserted as the fourth commented paper of the thesis.

3.1 HMG box transcription factor TCF-4's interaction with CtBP1 controls the expression of the Wnt target *Axin2*/*Conductin* in human embryonic kidney cells

To find binding partners unique for individual members of the LEF/TCF family of transcription factors we have decided to start with the human TCF4. The aim was to find proteins interacting specifically with and thus affecting only the TCF4-mediated transcription. From the sequence analysis it is obvious that the C-terminus of hTCF4 is the most diverse part shared with other members of the family. Furthermore, some studies pointed to the possibility that the C-terminus might be important for differential regulation of some Wnt target genes by LEF/TCF factors [355; 356]. Therefore, we performed yeast two-hybrid (Y2H) screen of mouse 17-day embryo cDNA library using the C-terminus of *hTCF4* as bait. One of the obtained clones encoded the full length of mouse C-terminal binding protein 1 (mCtBP1), a transcriptional co-repressor.

The C-terminal binding protein was originally identified as a cellular phosphoprotein that interacts with the C-terminus of adenoviral E1A oncoprotein through a 5-amino acid motif, PLDLS [357; 358]. Other viral oncoproteins, EBNA3A and EBNA3C, were also shown to contain motifs similar to PLDLS and to bind CtBP [359; 360]. According to current knowledge, the general consensus for the CtBP-binding motif is P-X_1-D/N-S/T-X_2-K, where X_1 tends to be a bulky amino acid such as L or V and X_2 can be any amino acid residue. Although the 6-amino acid sequence is required for CtBP binding, the adjacent residues also influence the affinity of CtBP [361]. The motif is often flanked by charged residues, which may ensure its display on the protein surface. Additionally, CtBP binding can also be regulated by acetylation of residues adjoining the core motif. The adenoviral E1A protein was shown to be acetylated by p300 and P/CAF acetyltransferases at K239 which flanks the PXDLS motif and this acetylation blocks the interaction between CtBP and E1A [362].

The CtBP protein originally found as the E1A-binding partner is now designated CtBP1. Later, a highly related human and mouse proteins termed CtBP2 have been identified [363]. Whereas vertebrates contain two CtBP homologues, invertebrates code for a single CtBP gene. In *Arabidopsis*, a plant orthologue of CtBP, a product of gene *Angustifolia* (*AN*), controls polar elongation of leaf cells and trichome branching. Vertebrate CtBP2/RIBEYE (named in reference to the synaptic ribbon as the subnuclear organelle and the eye as the tissue of origin) is encoded by a bifunctional gene that uses alternative promoters together with RNA splicing to generate two distinct proteins. While the ribbon synapse protein RIBEYE is produced by the use of the tissue-specific promoter located within the first intron of the *CtBP2* gene, *CtBP2* is ubiquitously expressed from a different 5` promoter and the utilised alternative RNA splicing eliminates the RIBEYE-specific N-terminus [364; 365]. Similarly, the protein CtBP3/BARS (brefeldin A-ADP-ribosylated substrate), originally identified in rat and also expressed in human

cells, lacks the first 13 amino acid residues and almost certainly represents a product of an alternatively spliced CtBP1 transcript [366; 367].

The proteins of CtBP family are highly conserved in both invertebrates and vertebrates. They share a significant homology with NAD^+-dependent D-isomer-specific 2-hydroxyacid dehydrogenases (D2-HDH) [358]. All of the 15 conserved presumably catalytic residues of this dehydrogenase family are found in both CtBP1 and CtBP2. Besides, a signature Rossmann fold motif with its characteristic GXGXXG sequence (where X is any amino acid) required for binding of nicotinamide adenine dinucleotide (NAD) cofactors remains also conserved in CtBP1 and CtBP2. This domain serves also as a dimerisation interface allowing the formation of homo- or heterodimers between CtBP1 and CtBP2 [368-370]. Binding of NAD cofactors induces structural changes in CtBPs, which enhance dimerisation and may contribute to interactions with other proteins. In a cell, the redox status seems to play a role in modulation of CtBP function (repression *vs*. activation of transcription). Chemical treatments causing hypoxic environment that increase nuclear NADH levels were shown to stimulate binding of CtBP to target transcription factors and resulted in enhanced transcriptional repression [371].

The CtBP1 and CtBP2 proteins share 78% amino acid identity [363]. They appear to function interchangeably in many contexts [372], although some data point to possible differential regulation [373; 374]. In *Drosophila*, CtBP plays crucial roles during development [375]. Homozygous *dCtBP* mutations are embryonic lethal while reduced *dCtBP* dosage results in segmentation and patterning defects, phenotypes similar to those observed in repressor null embryos [375-377]. *Xenopus* orthologue XCtBP is expressed throughout development with transcripts localised to the head, central nervous system and tissues along the dorsal midline following neurulation [83]. By ectopic overexpression of CtBP protein fused to the GAL4 activation domain it was demonstrated that XCtBP participates in the regulation of head and notochord development employing several transcription factors [83]. In the mouse, *CtBP1* is expressed from embryo to adult while *CtBP2* expression remains rather restricted to the embryo [372]. Targeted inactivation of *CtBP1* yields animals that are about 30% smaller than wild-type and heterozygous littermates but viable and fertile. On the contrary, $CtBP2^{-/-}$ mice are embryonic lethal and exhibit numerous defects, including axial truncations, abnormal heart and brain development and extra-embryonic vascularisation failure [373]. The axial defects of CtBP2-null embryos correlate well with the reduced expression of *Brachyury*, a T-box transcription factor modulating mesodermal and neural cell fates during development and a known Wnt target [373; 378]. The axial truncation phenotype is also reminiscent of phenotypes caused by mutations in components of Wnt signalling pathway and suggests that CtBP2 may be a regulator of Wnt-mediated gene expression. However, in the case of *Brachyury*, rather surprisingly, the *in vivo* observed effect argues for CtBP2 being a transcription activator rather than a repressor.

Proteins of the CtBP family have been implicated in a startling number of transcriptional

regulation networks including cell cycle regulation and apoptosis. The CtBP-binding motifs have been found in numerous transcriptional regulators including zinc finger, basic helix-loop-helix, homeodomain, E twenty-six (ETS) domain, nuclear receptors and GATA family DNA-binding proteins. They have been shown to be involved in an assembly of multicomponent complexes and to act as transcriptional co-repressors both in a HDAC-dependent and HDAC-independent manner [379]. It is widely assumed that the building up of such multimeric complexes is facilitated by the CTPBs` ability to homo-/hetero-dimerise (or potentially form higher-order multimers), whereby each CtBP molecule can function as a bridging protein further associating with a PXDLS motif-containing partner. Furthermore, proteins other than CtBP can also support bridging function in these multiprotein complexes [380-383].

In some cases, CtBP-mediated repression was found insensitive or partially insensitive to HDAC inhibitors, suggesting that CtBP may utilise additional mechanisms [379; 383; 384]. It was reported, albeit vaguely, that CtBP is capable of directly binding to at least two components of the basal transcription machinery [379]. Therefore, direct inhibition of or interfering with transcriptional initiation might pose one of possible mechanisms. Other possible mechanisms might involve a compaction of chromatin structure as CtBP was reported to bind to the polycomb group proteins, *Xenopus* Polycomb (XPc) and human Polycomb 2 (hPc2) [368]. Polycomb proteins are thought to act through co-ordinating the formation of densely packaged heterochromatin. Therefore, CtBPs may act by recruitment into inactive chromatin regions. Another CtBP-associating protein, Ikaros, has also been reported to co-localise with centromeric heterochromatin and to mediate repression by recruitment of target genes into transcriptionally inactive nuclear domains [385]. Ikaros can also be found as a component of Nucleosome remodelling and histone deacetylation (NuRD) complexes, implicated in chromatin modifications. Alternatively, as histone methyltransferases (HMTs) together with HDACs were identified in CtBP-containing complexes, it was proposed that CtBP complexes might act by co-ordinating stepwise histone modifications resulting in a repressive chromatin environment [386]. Finally, CtBP might prevent histone acetylation (and subsequent transcriptional activation) by binding to histone acetyltransferase (HAT) co-activators such as p300/CBP and P/CAF and inhibiting their activity by blocking the accessibility to histones [387; 388].

Sequence analysis revealed that the C-terminus of TCF4 contained two variants of CtBP-binding motif shared with TCF3. Prior to our results, the requirement of these two motifs had been demonstrated for the interaction of *Xenopus* TCF3 and CtBP [83]. The experiments had also revealed the importance of XCtBP-mediated repressive effect of C-terminal XTCF3 domain for *Xenopus* development. Within the LEF/TCF family, the CtBP-interaction motif is fairly conserved in vertebrate TCF3 and TCF4 proteins [83; 85; 389; 390]. Its absence in other LEF/TCF family members implies that CtBPs might specifically regulate the transcription

mediated by these two TCF proteins, TCF3 and TCF4. Re-testing the interaction *in vitro* by glutathione S-transferase (GST) pulldown assay using different TCF4 deletion mutants confirmed that the C-terminus of TCF4 containing two putative CtBP-binding motifs was indispensable for the interaction with CtBP. Bacterially expressed CtBP interacted with *in vitro* translated full-length TCF4 and with its C-terminal fragment whereas C-terminally truncated proteins (either TCF4-LEF1 chimera or TCF4 N-terminus) did not. In this assay, CtBP1 and CtBP2 alike interacted with TCF4 (unpublished results), in agreement with already published functional similarity of these two proteins [373]. We also observed efficient CtBP1-CtBP2 heterodimerisation *in vitro*.

Further, to study effects of CtBP on TCF4-mediated transcription we used the synthetic reporter construct pTOPFLASH containing three copies of optimal DNA-binding motif for TCF upstream of a minimal *c-Fos* promoter driving luciferase expression [81; 389]. Upon transfection to human embryonic kidney 293 (HEK293) cells and co-cultivation of the transfectants with the *Wnt-1*-expressing feeder cells, the pTOPFLASH-driven luciferase expression was stimulated 6-7 times as compared to control feeders. Transfection of *CtBP*-expressing constructs repressed the *luciferase* expression in a concentration-dependent manner. To avoid the possible influence of adenoviral E1A oncoprotein constitutively expressed in HEK293 cells on the transcriptional properties of CtBP, we also used COS-7 cells as a parallel cell system. Analogously to HEK293 cells, co-transfection of TCF4, β-catenin and CtBP constructs together with pTOPFLASH into COS-7 cells resulted in decrease of *luciferase* expression proportional to the amount of *CtBP* in the transfection mixture. Additionally, the transactivation of synthetic reporter by C-terminally truncated TCF4 (the TCF4-LEF-1 chimera) remained unaffected by *CtBP* expression, highlighting the importance of TCF4 C-terminus for the interaction with CtBP. Altogether, the reporter assays have indicated that β-catenin/TCF-4 transcriptional activity is downregulated by CtBP and that the C-terminus of TCF4 is indispensable for this effect.

For further experiments we generated HEK293 cells with inducible expression of EGFP-tagged mCtBP1 (293-EGFP-CtBP1/Dox). The expression of *EGFP-CtBP* transgene (despite representing approximately one half of the endogenous CtBP protein levels) significantly decreased pTOPFLASH transcription in Wnt-1-stimulated cells compared to the activities of this reporter in 293-EGFP-CtBP1/Dox cells grown in the presence of doxycycline, i.e. with *CtBP1* repressed. We have also checked that this effect of CtBP can not be ascribed to decreased amounts of β-catenin/TCF4 complexes or to an interference with their formation. Next, we wanted to know if the mechanism of CtBP repression relies on HDACs. Treatment with trichostatin A (TSA), a HDAC inhibitor, completely neutralised the CtBP repressive effect both in 293-EGFP-CtBP/Dox cells and in transiently transfected HEK293 cells.

Proteins of the LEF/TCF family are known to interact with the Gro/TLE co-repressors [88;

391]. Hence, we were curious whether CtBP and Gro/TLE utilise the same inhibitory mechanisms. We examined the impact of TSA in conjunction with different TCF4 deletion mutants on the activity of pTOPFLASH reporter in a transient transfection luciferase assay. All deletion mutants repressed both basal and Wnt-1-induced pTOPFLASH *luciferase* gene transcription as compared to control empty expression vector. Rather surprisingly, the TCF4-LEF1 fusion lacking the C-terminal CtBP-interacting domain and thus associating only with Gro/TLE was more potent repressor than the full-length TCF4. Moreover, only the repression mediated by the full-length TCF4, i.e. by the protein encompassing both Gro/TLE and CtBP binding sites, was alleviated by trichostatin A treatment almost completely. These results suggest not only that CtBP and Gro/TLE proteins may use different mechanisms to repress TCF-mediated transcription but also that these two co-repressors may functionally interact. Both HDACs of class I and II associate with CtBPs, but in addition, CtBPs are known to act through HDAC-independent mechanisms [361; 379; 392; 393]. Almost complete release of the CtBP-mediated repression by TSA clearly demonstrates a HDAC-dependent mechanism for the full-length TCF4. Although little is known about the exact mechanism by which Gro/TLE proteins block transcription, it was demonstrated that Gro/TLE physically binds histone H3 and HDACs, namely Rpd3/HDAC1 [394-396]. The observed partial resistance of TCF4 deletion mutants comprising only the Gro/TLE interaction domain to TSA suggests that additional mechanisms of repression must be in operation. Given that Gro/TLE and CtBP sites of interaction lie far apart in the primary structure of TCF4, a steric competition for binding is unlikely, although without knowledge of spatial conformation it can not be ruled out completely. It was shown that *in vitro* dCtBP and Gro can bind simultaneously Hairy, another partner of these two co-repressors, despite being only some 11 amino acid residues apart [375; 384]. In addition, CtBP and Gro do not interact physically with each other both *in vitro* as well as *in vivo* [375; 384]. Therefore, it seems more probable that CtBP might antagonise the repressor activity of Gro similar to the CtBP interference of Gro-mediated transcriptional repression observed in the context of Hairy [384]. It has been proposed that CtBP, once bound to Hairy, may regulate its function by other regions of Hairy or by other Hairy-binding proteins [375]. According to this notion, a novel but further unspecified Hairy-interacting protein that could serve such function was mentioned to have been found in a Y2H screen [375]. A parallel model posits CtBP to attenuate Hairy-mediated repression by not allowing Gro to form as potent a repression complex [384]. Of interest, Brinker, another protein capable of interacting with CtBP and Gro, seems to repress target genes differentially – some by recruitment of either CtBP or Gro and some by fetching Gro alone [397; 398]. Anyway, the Gro/TLE-CtBP interplay in case of TCF factors (as well as Hairy and Brinker) is far from being fully understood and thus awaits further experiments.

We were able to detect the repressive effect of CtBP on β-catenin/TCF-mediated transcrip-

tion not only in synthetic promoter-driven gene reporter assays but also on a natural Wnt target gene in 293-EGFP-CtBP/Dox cells. Upon Wnt-1 stimulation of 293-EGFP-CtBP/Dox cells, we detected strong transcriptional activation of *Axin2*/*Conductin* using real-time quantitative RT-PCR (qRT-PCR). The induction of *EGFP-CtBP* in these cells significantly reduced *Axin2*/*Conductin* expression. However, we did not see an analogous effect on *Axin1*, a homologue of *Axin2*. The ability of CtBP to modulate endogenous *Axin2* but not *Axin1* promoter transcription implies that CtBP can selectively regulate the expression of other Wnt target genes.

The observation of Brannon *et al*. [83] and ours that CtBP is able to downregulate the Wnt-induced transcription was confirmed later by other laboratories. However, a question still remains whether CtBP-mediated repression is achieved by direct CtBP-TCF association. In spite of a clear proof of interaction *in vitro* we and others have not been able to show the existence of CtBP/TCF complex *in vivo*. Results from the M.Bienz laboratory [399] challenged the notion of CtBP as a direct co-repressor of TCF by stating that CtBP is neither directly associated with TCF *in vivo* nor does mutation of the CtBP binding motifs in TCF4 alter its transcriptional activity. Instead, they proposed an alternative mechanism of CtBP effect on β-catenin/TCF-mediated transcription. Their model is based on the finding of CtBP-binding motifs within APC protein and an ability to show the CtBP-APC association both *in vitro* and *in vivo*. Moreover, they were able to show that failure of CtBP-APC association led to increased levels of nuclear β-catenin/TCF complexes and the resulting TCF-mediated transcription. They question the previously proposed role of CtBP as a direct co-repressor of TCF. In their GST pulldown assay they obtained only a weak *in vitro* binding between CtBP and TCF and they also argue that the two proteins are not detectably associated in mammalian cells. It is known that CtBP binds nicotinamide adenine dinucleotides and that NAD^+ or NADH binding causes structural changes contributing to enhanced protein interaction [364; 369; 371]. It has been reported that the preferred ligand is NADH with over 100-fold higher affinity than NAD^+ [400]. Accordingly, in GST pulldown experiments we included NADH at a 20μM concentration (in contrast to [399]). Without NADH, we were not able to detect the interaction unless prolonged exposition was carried out (similarly to [399]). Although speculative, it is possible that the interaction *in vivo* is weak or transient or dependent upon a co-operative interaction(s) of other protein(s) present in the complex that is/are volatile. In either case it may not withstand the conditions of the co-immunoprecipitation experiment. On the contrary, the addition of NADH in the pull-down buffer promotes CtBP dimerisation and obviously stabilises the interaction with TCF and helps make it detectable. In addition, an inability to co-immunoprecipitate two proteins does not necessarily mean that they do not associate. In any case, we have detected clear *in vitro* interaction as well as a specific one in directed yeast two-hybrid assays. Hamada and Bienz [399] further show that LEF1, a protein that does not possess CtBP-binding motif, when co-transfected together with activated β-catenin (S33A), increased the pTOPFLASH transcription

in CtBP-deficient cells as compared to corresponding parental control cells. Strangely, based on this experiment, they draw a conclusion that LEF1-mediated transcription is more sensitive to CtBP loss than the transcription mediated by other LEF/TCF transcription factors. However, a parallel experiment with TCF4 to compare the activities of both these LEF/TCF family members under identical conditions is missing. So, under these circumstances, only the dependence of APC`s ability to antagonise TCF-mediated (or rather LEF-mediated) transcription on CtBP was tested. Consequently, their conclusion can not be valid. Similarly, they observed no difference in TCF4-mediated transcription between wild-type and the TCF4 construct where CtBP motif was mutated in three amino acid residues ($TCF4_{AxAxA}$) in transiently transfected COS cells. As we used different constructs lacking the whole C-terminal tails in our experiments, this might rather imply another, possibly spatial and yet unidentified element contained within the C-tail contributing to CtBP-mediated effect. It is imaginable that such element would not function on its own but in conjunction with the wild-type CtBP-binding motif. Finally, it should be pointed out that the results of [399] are not necessarily in contradiction to ours. It is not difficult to imagine that both CtBP-APC-β-catenin association as well as direct CtBP-TCF binding may serve to the downregulation of TCF-mediated transcription. The proposed model that CtBP lowers the availability of free nuclear β-catenin for binding to TCF by sequestering APC/β-catenin complexes can be easily supplemented by a direct CtBP-TCF interaction.

In another study, CtBP was observed transiently together with APC on the *c-Myc* enhancer at a time of a transcription shut-off overlapping the loss of β-catenin and the associated co-activators [401]. The complex containing CtBP and β-TrCP is thought to be recruited by APC and suggested to mediate an exchange of co-activator for co-repressor complexes at the enhancer. As this study was focused on LEF1, which does not possess CtBP-binding sites, the transient nature of CtBP occupation of the enhancer is understandable. It is also possible that CtBP-TCF4 direct interaction has only a supplementary role in downregulation of β-catenin/TCF4-mediated transcription and that CtBP exerts its repressive effect primarily by an APC-dependent mechanism. On the other hand, a partial co-localisation of CtBP and TCF4 isoforms containing CtBP binding motifs was reported. The co-localisation is lost in mismatch-repair deficient colorectal cancers (MSI-H) with a TCF4 frameshift mutation that leads to the selective loss of TCF4 isoforms with CtBP binding abilities [402]. In addition, in cell lines derived from such colorectal cancers CtBP is not able to repress TCF4-mediated transcription. These results clearly support the importance of a direct CtBP-TCF4 interaction for CtBP-mediated repressive activity.

3.2 HIC1 attenuates Wnt signalling by recruitment of TCF4 and β-catenin to the nuclear bodies

Until now, two distinct functions have been ascribed to CtBP proteins – a nuclear role in the regulation of transcription, and a cytoplasmic one in the membrane trafficking. The nuclear activities have been observed for both CtBP1 and CtBP2, whereas CtBP3/BARS is the only member of the family shown so far to participate in membrane dynamics [367; 368; 403]. Within a cell, both CtBP1 and CtBP2 can be found in the cytoplasm as well as in the nucleus [404]. Based on immunofluorescence microscopy of COS-7 cells overexpressing CtBP3/BARS and its high amino acid sequence identities to CtBP1 and CtBP2, CtBP3/BARS has also been proposed to have a nuclear function [370; 405]. This dual role of CtBP family proteins necessitates some kind of functional switch. It is becoming apparent that such a role can be fulfilled by a post-translational modification, an oligomerisation state or an interaction with different proteins [406; 407]. The regulation of CtBP1 shuttling between the nucleus and the cytoplasm quite likely controls the function of the protein. Several possibilities have been put forward. The phosphorylation of CtBP1 at S158 by p21-activated kinase (Pak1) triggers the change in CtBP1 cellular distribution from the nucleus to the cytoplasm and blocks the co-repressor functions [408]. The second modification, essential for the nuclear localisation and its co-repressor activity, is the SUMOylation of CtBP1 [374; 409]. An additional mechanism is the binding of CtBP1 to the PDZ domain of neuronal nitric oxide synthase (nNOS) which also blocks the nuclear accumulation of CtBP1 and favours its cytoplasmic localisation [374; 410]. Another, but so far not completely clarified mechanism is the mutually exclusive binding of nicotinamide adenine dinucleotides (NAD^+/NADH) and acyl-coenzyme A (CoA) to CtBPs. The competition between these cofactors for binding to the same domain is predicted to have consequential structural effects on CtBP proteins. While NAD^+/NADH promote oligomerisation and enhance the affinity for PXDLS-like motif-containing transcription factors, long-chain acyl-CoAs induce relaxation of the dimer and favour the open monomeric structure required for the cytoplasmic functions [370; 371; 400; 411-413]. However, the information of how the exchange between these cofactors is regulated is still missing. It has been proposed that the recruitment of CtBP near the membrane-encompassed acyl-CoAs and acyl-CoA-binding proteins might play a role. Interestingly, CtBP2 is not SUMOylated, it lacks the PDZ-binding motif and was shown to have a dominant nuclear pattern. The nuclear localisation pattern of CtBP2 is dictated by the unique N-terminal 20 amino acid domain containing three crucial lysine residues (K6, K8 and K10) that are subject to p300-mediated acetylation [404; 414; 415]. The single K10 residue seems to be critical for the nuclear retention of the protein [415]. In addition to SUMOylation, the formation of heterodimers between CtBP1 and CtBP2 as well as binding of CtBP1 to the PXDLS-like motif partner proteins was demonstrated to be the mechanism whereby CtBP1 can be recruited to the nucleus [404; 414].

We suspected that differential subcellular localisation of CtBP1 might influence its co-repressor activities towards TCF4. It had also been reported that one of the PXDLS variant motif-containing proteins, hypermethylated in cancer 1 (HIC1), associated with CtBP1 and recruited it to the specific subset of nuclear bodies [416]. Therefore, we focused on the effect of CtBP1-HIC1 interaction on β-catenin/TCF4 mediated transcription.

The tumour suppressor *HIC1* gene was identified because of its association with a CpG island at chromosome arm 17p13.3 that is aberrantly hypermethylated and transcriptionally inactivated in several common types of human cancer [417-419]. In addition, this locus is also frequently deleted in many cancers as well as in the Miller-Dieker syndrome (MDS), a severe form of lissencephaly [417; 420-428]. Mice with homozygous inactivation of *HIC1* display a reduction in overall size, embryonic and perinatal lethality together with other developmental anomalies resembling those found in MDS patients [429]. On the contrary, mice with only one *HIC1* allele disrupted develop different spontaneous gender-dependent malignant tumours with a predominance of epithelial cancers in males and lymphoid and mesenchymal tumours in females [430; 431]. Interestingly, in most of the tumour samples from the $HIC1^{+/-}$ animals examined, the functionality of the remaining *HIC1* wild-type allele was lost. The complete loss of *HIC1* function in the heterozygous mice involves dense methylation of the promoter of the remaining wild-type allele and seems to enhance the aggressiveness of the tumours [430].

There is a direct functional link of *HIC1* to another tumour suppressor gene, *TP53*. Interestingly, both genes reside closely on the same chromosome in human as well as in mouse and are frequently mutated or even deleted in cancers [424; 427; 432-435]. A double knockout approach was applied to study the relationship between these two tumour suppressor genes. The $HIC1^{+/-}$ $TP53^{+/-}$ mice were generated either in *cis* (*HIC1* and *TP53* alleles deleted on the same chromosome) or *trans* (*HIC1* and *TP53* alleles deleted on separate chromosomes) configuration. Intriguingly enough, each of these genetic arrangements yielded distinct phenotypes. The *cis* mice displayed accelerated tumour formation with the wild-type copies on the opposite chromosome increasingly deleted whereas *trans* mice developed tumours with epigenetic inactivation of the remaining *HIC1* allele and a deletion of the wild-type copy of *TP53* [431]. The distinct mechanisms acting in these two settings highlight the selection pressure for rapid tumorigenesis as the deletion can affect both *HIC1* and *TP53* genes simultaneously, while promoter hypermethylation inactivates only *HIC1*. For the first time, these findings provided an experimental evidence for a link between the epigenetic (*HIC1* promoter hypermethylation) and genetic (*TP53* deletion) silencing of tumour suppressor genes in cancer progression.

The *HIC1* gene is a direct target of p53, a product of the *TP53* gene [419; 436; 437]. The p53 protein is a transcription factor that integrates stimuli from a number of pathways in response to a wide range of cellular stresses [e.g. 438; 439-442]. Stresses such as DNA damage, hypoxia or oxidation lead to hyperacetylation of p53 by p300/CBP [e.g. 443; 444].

Upon acetylation, p53 is stimulated and restricts cell growth by triggering cell cycle arrest, senescence or apoptosis [445-452]. The HIC1 protein is involved in a complex regulation of p53 tumour suppression activity. The HIC1 transcriptional repression complex contains the class III histone deacetylase Sirtuin 1 (Sirt1). In a regulatory loop, HIC1-Sirt1 complex binds to and represses transcription from the *Sirt1* promoter [453]. The Sirt1 protein is a NAD^+-dependent deacetylase [454] that couples the hydrolysis of NAD^+ to the deacetylation reaction [454-456]. The transcriptional activity of p53 can be reversed by Sirt1-mediated deacetylation of p53 leading to p53 inactivation [453]. Thus, Sirt1 protein is under the direct control of HIC1 and affects the p53 transcriptional function in a complex transcriptional and post-transcriptional regulation loop. Adding to the complex regulation network, HIC1 has recently been identified as a target of Sirt1-mediated deacetylation, which influences its transcriptional repression activity [457].

Under physiological conditions, actively expressed HIC1 represses *Sirt1* transcription [453]. Acetylation of p53 enhances its function to control growth arrest and apoptosis in response to stress conditions [445; 450; 451]. In the course of aging or during the early stages of tumour progression, the *HIC1* promoter undergoes hypermethylation [417; 419; 430; 453; 458]. Gradual loss of *HIC1* function results in upregulation of *Sirt1*. According to one of proposed models, deacetylation of p53 may also compromise its ability to activate the *HIC1* promoter and over time this might promote further silencing of the gene [453]. Silenced *HIC1* may undermine p53 functions through the constitutive activation of *Sirt1* and increase the risk for neoplastic transformation. Such cells are able to bypass control mechanisms (e.g. disrupt cell cycle control), allow mutations to accumulate and survive DNA damage. The ultimate result might be an uncontrolled proliferation of tumorigenic cells.

HIC1 is a transcriptional repressor belonging to the Broad complex, Tramtrack, Bric à brac/ Poxvirus and Zinc finger-zinc finger (BTB/POZ-ZF) family of proteins [459]. The N-terminal BTB/POZ domain, a highly conserved segment of approximately 100 amino acids, mediates protein-protein interactions, often in the context of multiprotein transcriptional complexes. In contrast to other proteins of the BTB/POZ-ZF family such as B-cell lymphoma 6 (BCL-6), Promyelocytic leukemia zinc finger (PLZF) and Kaiso, the BTB/POZ domain of HIC1 fails to interact with commonly used members of repressing complexes Silencing mediator for retinoid and thyroid hormone receptors/Nuclear receptor co-repressor (SMRT/N-CoR), mammalian homologue of yeast SWI-independent 3A (mSin3A) or HDAC1 [459]. The five Krüppel-like Cys_2-His_2 (C_2H_2) zinc finger motifs in its C-terminal part mediate HIC1 binding to the specific DNA sequence 5`-$^{\mathbf{C}}/_{\mathbf{G}}$NG$^{\mathbf{C}}/_{\mathbf{G}}$GGGCA$^{\mathbf{C}}/_{\mathbf{A}}$CC-3`. The mutational analyses have pointed to the functional importance of the GGCA core motif bound by zinc fingers 3 and 4. The analysis of DNA-binding properties also revealed that the BTB/POZ domain hampers the association of HIC1 to a single site but mediates cooperative binding of HIC1 to the concate-

nated binding sites [460]. The BTB/POZ domain and the central region function as autonomous repressive modules when tethered to DNA by fusion with the GAL4 DNA-binding domain [416; 459]. While the repression mediated by the BTB/POZ domain alone is TSA-insensitive, the HIC1 central region-GAL4 chimera-mediated repression is TSA-sensitive showing that full length HIC1 can mediate transcriptional repression by both HDAC-dependent and HDAC-independent mechanisms [416; 459]. The central proline-rich region contains conserved GLDLSKK motif highly related to the consensus CtBP interaction motif and recruits HDAC1 through CtBP [416]. The dimerisation of HIC1 supported by BTB/POZ domain is required for the interaction with CtBP [416].

A human homologue of HIC1, located on the long arm of the chromosome 22 in a region subject to translocations (22q11.2) and known as Breakpoint cluster region-like 2 (BCRL-2), has been identified [461]. This gene was named *HRG22* (*HIC1-related gene on chromosome 22*). The highly similar genomic organisation of *HRG22* and *HIC1* strongly suggests that these genes may have evolved from a common ancestor through gene duplication. Together with *HIC1*, *HRG22* gene defines a subgroup of BTB/POZ domains unable to recruit HDACs-containing complexes. The *HRG22* gene codes for a zinc finger factor that shares high sequence homology (> 80%) as well as many functional properties with HIC1. A short GLDLSKK/R motif responsible for the CtBP interaction is perfectly conserved among HIC1 and HRG22 proteins from various species. The BTB/POZ domain of HRG22 can homodimerise and also heterodimerise with the related HIC1 BTB/POZ domain. Furthermore, both proteins completely co-localise into punctuated nuclear substructures [461].

In the extension to our previous work showing repression of β-catenin/TCF-mediated transcription by CtBP, we studied the role of CtBP-interacting protein HIC1. We pursued the possibility that other proteins, interacting with CtBP and influencing its subcellular localisation may further modulate Wnt-mediated β-catenin/TCF-dependent transcription. Using confocal microscopy, we observed re-location of otherwise diffuse CtBP into subnuclear structures upon co-transfection with *HIC1*. In full agreement with the previously published results [416], this ability was dependent on the presence of the CtBP-binding motif of HIC1. We also noted that a small fraction of CtBP remained in another kind of subnuclear punctate structures distinct from the HIC1 bodies. The HIC1 mutant missing the BTB/POZ oligomerisation domain (HIC1-ΔPOZ) which lost the ability to concentrate into nuclear dots, still retained the competence to draw CtBP from the cytoplasm to the nucleus. When co-transfected together with HIC1 and CtBP, TCF4 was efficiently recruited to the HIC1 bodies. As both HIC1 and TCF4 had been shown to interact with CtBP, we expected that CtBP might be the mediator of the TCF4 recruitment to the HIC1 bodies. This possibility was tested and confirmed by using the TCF4 mutant incapable of CtBP binding (TCF-4mutCtBP). Surprisingly, further experiments

showed that CtBP was dispensable for the HIC1-mediated re-location of TCF4 to the nuclear bodies. However, the nuclear sequestration of TCF4 by HIC1 is less effective in CtBP(-/-) than in CtBP(+) cells, suggesting an important structural role for CtBP in the process. The results from the confocal microscopy were confirmed by the co-immunoprecipitation and GST pulldown assays. We were able to show that TCF4 associates directly with HIC1 both *in vitro* and *in vivo* and that this interaction involves several regions in both proteins rather than a single delimited domain. Co-immunoprecipitation of HIC1 and TCF4 from the nuclear extracts of CtBP(-/-) cells further supported that CtBP is not essential for the HIC1-TCF4 association.

Full-length HIC1 substantially reduced the β-catenin/TCF-mediated transcription. The inhibitory effect of HIC1 was demonstrated on synthetic LEF/TCF-responsive (pTOPFLASH) as well as on natural (*Axin2*) promoter-driven reporter. Furthermore, HIC1 also repressed the selected Wnt target genes in several cell lines. The antagonising effect of HIC1 was also confirmed by an experiment with primary WI38 cells. Knockdown of endogenous HIC1 levels via RNA interference in these cells led to an effective HIC1 downregulation as monitored by Western blotting, confocal microscopy and qRT-PCR analysis. Upon endogenous HIC1 downregulation, the partial disappearance of subnuclear HIC1-specific structures was accompanied by an increase in basal promoter activity of *Axin2*, a well-established target of Wnt signalling pathway. When WI38 cells were stimulated by Wnt3a, the transcriptional response of *Axin2* was further enhanced as compared to parallel control siRNA-treated cells. Intriguingly, we observed that activation of Wnt target genes was dependent on cellular background. While in HEK293 cells the Wnt3a ligand stimulated transcription of *Sp5*, *Axin2* and *Cyclin D1*, in WI38 cells only *Axin2* transactivation was detected. In colon carcinoma DLD-1 cells, a cell line with constitutively active Wnt signalling, ectopic expression of the dominant negative (i.e. blocking) TCF4 reduced the transcription of *Tenascin C* but not *Axin2* or *Cyclin D*. These seeming discrepancies possibly reflect different inputs from various cellular pathways converging on a given promoter.

Having observed that HIC1 represses Wnt-dependent transcription, we wanted to elucidate the mechanism of this HIC1-mediated inhibition. It was shown that HIC1 forms a transcriptional repression complex with Sirt1 deacetylase that binds to and represses transcription from the *Sirt1* promoter [453]. Another negative regulator of the canonical Wnt signalling from the BTB/POZ family of transcription factors, Kaiso, inhibits β-catenin-mediated transactivation of mouse and human matrix metalloproteinase 7 (also known as *Matrilysin*; *MMP7*) and *Siamois* in *Xenopus* [462-464]. Presence of a species-conserved Kaiso consensus sequence has been reported also for β-catenin/TCF target genes such as *PPARδ*, *c-MYC* and *Cyclin D1* [462; 463]. Interestingly, Kaiso exhibits dual-specificity DNA binding. It recognises a specific 5`-TCCTGCNA-3` consensus (where N represents any nucleotide) as well as methylated CpG dinucleotides [464; 465]. Zinc fingers 2 and 3 seem to be necessary and

sufficient for DNA binding in either case [464]. The exact mechanism is not fully understood but it appears obvious that Kaiso functions as a HDAC-dependent transcriptional repressor recruiting macromolecular complexes that include mSin3A, SMRT or N-CoR [462; 466; 467]. It also remains possible that Kaiso might regulate some Wnt/β-catenin target genes by recognition of methylated CpGs rather than the sequence-specific binding sites.

Contrary to the already published direct binding of HIC1 to the *Sirt1* promoter, our ChIP analysis revealed that HIC1 did not bind either directly or indirectly (i.e. via TCF4) to the promoters of two selected TCF-regulated genes. Yet, *HIC1* ectopic expression was able to diminish endogenous TCF4 occupancy of the *SP5* promoter in both non-stimulated and Wnt-stimulated HEK293 cells transfected with *β-catenin* and *HIC1*. In addition, β-catenin binding to the promoter region was completely eliminated. On the contrary, transfection of *HIC1-ΔPOZ* mutant into HEK293 cells left both β-catenin and TCF4 binding to the TCF-specific DNA element of *SP5* promoter virtually unaffected. These results were confirmed by an analogous ChIP experiment using DLD/HIC1 cells with regulated expression of the *EGFP-HIC1* transgene. In these cells, *HIC1* expression efficiently blocked transcription from the promoter of *Tenascin C*, a recently identified β-catenin/TCF target gene in human colorectal tumours [468]. Clear association of both β-catenin and TCF4 with the proximal TCF-dependent element of *Tenascin C* promoter in parental DLD-1 cells was substantially reduced upon *HIC1* induction in DLD/HIC1 cells as monitored by ChIP. Importantly, although shown not to bind directly to either *SP5* promoter in HEK293 or *Tenascin C* promoter in DLD-1 cells, EGFP-HIC1 was fully capable of binding its recognition DNA element in the *Sirt1* promoter. This indicates a potentially novel mechanism of HIC1 transcriptional repression.

Collectively, the data reveal a mechanism by which HIC1 influences β-catenin/TCF-dependent transcription. The repressive HIC1 effect is likely based on the recruitment of β-catenin/TCF complexes to the specific subnuclear structures that are spatially and functionally separated from the transcription of Wnt target genes. The HIC1-mediated sequestration secludes TCF4 from association with its target genes` promoters. The simultaneous targeting of β-catenin to the HIC1 bodies appears to be indirect and mediated by the interaction with TCF4. However, based on the obtained data, an alternative participation of an unknown factor involved in re-location of β-catenin to the HIC1 bodies can not be completely excluded.

Altogether, the results suggest that the insulation of β-catenin/TCF complexes from promoters by HIC1 can uncouple the target genes from Wnt signalling inputs but at the same time leaves them responsive to other regulatory signals.

Impaired interplay of tumour suppressor HIC1 and the effectors of Wnt signalling may play a role in tumorigenesis. Recently, it was demonstrated that HIC1-mediated transcriptional repression of *Sirt1* might be influenced by the NADH concentration affecting the formation of the HIC1-CtBP repressive complex through the redox-sensing ability of CtBP [469].

An increase in intracellular NADH levels stimulates CtBP binding to its partners and potentiates CtBP-mediated repression [371; 400]. These conditions could be relevant in diseased states such as malignancy, where the relative hypoxia of cancer cells could contribute to metastasis and an escape from the mechanisms leading to cell death. Hypoxia, which increases free nuclear NADH levels, leads to a decrease of CtBP interaction with HIC1 and consequently to *Sirt1* derepression, independent of HIC1 status. Therefore, not only epigenetic inactivation via methylation of *HIC1* promoter but also functional impairment through blockage of HIC1-CtBP complex formation can (partially) eliminate HIC1 repressive activity. Inactivation of HIC1 (potentially connected to compromised p53 activities) together with deregulation of Wnt signalling may increase cellular risk for neoplastic transformation.

3.3 DAZap2 modulates transcription regulated by the Wnt effector TCF4

Pursuing further our notion that functional differences identified among LEF/TCF factors possibly stem from the interactions of individual members of the family with distinct partners, we performed another screen to identify such TCF-associating proteins. This time, we focused on the N-terminus of TCF4 outside the highly conserved β-catenin- and DNA-binding domains. Having experienced that the placement of the GAL4 domain in the fusion protein functioning as bait might influence the outcome of the Y2H screening procedure, we used two baits, with GAL4 DNA-binding domain appended either to the N- or C-terminus. As expected, the two parallel experiments yielded two distinct, non-overlapping sets of clones. By re-testing of all the isolated clones found in the primary screen in a directed yeast minimating assays for the specificity of interaction the number of potential interactors has dropped from 128 to 20 that were sequenced. One of the resultant clones, that showed specific interaction with TCF4, contained a plasmid insert coding for the full-length Deleted in azoospermia-associated protein 2 (DAZap2).

Mammalian DAZap2 was originally identified as an interacting protein of the germ cell-specific RNA-binding proteins Deleted in azoospermia (DAZ) and DAZ-like 1 (DAZL1) in a two-hybrid screen of a human testis cDNA library with DAZ used as bait [470]. Later, DAZap2 became recognised as the most significantly downregulated transcript in the genome-wide screen of bone marrow mononuclear cells from patients with multiple myeloma (MM), a malignant plasma cells disorder [471].

The mouse homologue, *Proline codon-rich transcript, brain expressed* (*Prtb*), was first cloned from a screen for genes expressed in the developing inner ear using a gene trap strategy [472; 473]. The *lacZ* reporter gene inserted by this approach to the *Prtb* locus showed expression in cultured embryonic stem (ES) cells upon a spontaneous differentiation as well as upon addition of thyroid hormone (T_3) and nerve growth factor (NGF), i.e. factors shown previously to affect the inner ear development. In the resultant chimeric embryos, at E12.5 the expression was restricted to the developing heart and later, at E13.5, in the prospective sensory region of the cochlear epithelium [473]. At E15.5, the embryos displayed a broad, but weak β-galactosidase (β-gal) activity in most tissues except for the brain. Interestingly, at this developmental stage the β-gal expression in the gut was reported to be slightly higher than in other tissues. In addition, a very strong activity was observed in the brain of adult mice, suggesting a role of *Prtb* in the brain functions. Intriguingly, mice homozygous for the *Prtb* null allele were viable and fertile displaying no obvious abnormalities [472]. In a study searching for genes that play a role in osteoblast adhesion, *Prtb* isolation was based on its two-fold serum-induced mRNA upregulation during early stages of adhesion and spreading [474]. Likewise, *Prtb* mRNA levels increased substantially in primary rat astrocytes when exposed to ammonia or hypoosmolarity

[475]. In embryonal carcinoma P19CL6 cells, a pluripotential mouse cell line able to differentiate efficiently into cardiac myocytes upon DMSO treatment, Prtb was shown to interact with Sry box 6 (Sox6) transcription factor and to regulate the expression of the α_{1c} subunit of the cardiac L-type Ca^{2+} channel [476]. Of particular interest, Sox6 belongs to the family of HMG transcription factors that act on DNA by inducing a sharp bend in the double helix [477; 478]. Hence, it is tempting to speculate that Prtb/DAZap2 might help Sox as well as LEF/TCF proteins to perform their architectural role in which they organise local chromatin structure and assemble other DNA-bound factors into biologically active multiprotein complexes [479; 480]. Recently, another interaction partner for Prtb has been found in eukaryotic initiation factor 4G (eIF4G) [481]. Under cellular stress, Prtb together with eIF4G seem to participate in formation of discrete cytoplasmic structures called stress granules (SGs), where translation initiation factors and 40S ribosomal subunits are sequestered and in this way translation is halted. The above-mentioned examples suggest that Prtb plays important roles in physiological responses to various environmental conditions. Finally, the stability of Prtb in cells is regulated by the interaction with Neural precursor cell-expressed developmentally downregulated gene 4 (NEDD4), an E3 ubiquitin ligase with a catalytic domain of the homologous to E6-AP carboxyl terminus (HECT) class [482].

Altogether, these as well as other [483] interactions point to possibly diverse roles of Prtb/DAZap2. Potential involvement of DAZap2 in different processes and thus a necessity to preserve certain arrangements for interactions with partner proteins might also account for the observed high conservation throughout the evolution. Human *DAZap2* has a highly related orthologues in three distant species, *Ciona intestinalis* (sea squirt), *Apis mellifera* (honey bee) and *Tribolium castaneum* (red flour beetle), suggesting an early origin and/or vital cellular functions. The *DAZap2* gene four-exon structure is also well preserved – a rat, a mouse and a frog have the same gene organisation as fish and human. It is ubiquitously expressed in normal tissues and cell lines as a single and abundant 1.9 kb transcript. In human, in addition to the functional *DAZap2* located on chromosome 12, there is a pseudogene located on chromosome 2. The pseudogene contains no introns, but two cca 300-bp insertions; the inactivating nonsense mutation (a TAG for TAC substitution) resides some thirty nucleotides downstream from the start codon. The *DAZap2* gene encodes a 17-kDa protein containing several potential Src homology SH2 and SH3 domain binding motifs as well as a proline-rich region at the C-terminus [484].

Having isolated DAZap2 as TCF4-binding protein, we further delineated the minimal region on TCF4 responsible for the interaction by directed yeast minimating assays using a set of TCF4 deletion constructs. A quite short stretch of amino acid residues (aa 214 to 251) proximal to the TCF4 DNA-binding domain turned out to be indispensable for the association. Although

the homologous regions in other members of the LEF/TCF family are not conserved completely, they still contain amino acids that display similar biochemical properties. This might reflect the common structural element requisite for the interaction. Another HMG-containing transcription factor, Sox6, has recently been shown to bind DAZap2. Regrettably, the segment on Sox6 accountable for the contact of these proteins has not been mapped [476]. Co-immunoprecipitation assays revealed that all LEF/TCF family members associate with DAZap2 with similar affinities.

Besides co-immunoprecipitations of ectopically expressed proteins, we also performed co-immunoprecipitations of endogenous proteins from several cell lines. Intriguingly, in DLD-1, human adenocarcinoma cells with constitutively active Wnt signalling due to mutations in tumour suppressor APC, β-catenin was present in the precipitate obtained with the DAZap2 antibody. We also observed the opposite, i.e. a specific co-immunoprecipitation of DAZap2 on the β-catenin antibody. Since we did not detect any association between β-catenin and DAZap2 (in contrast to DAZap2-TCF4 direct binding) as assessed by a GST pull-down assay, we conclude that these proteins do not interact directly but are brought together by a common partner, TCF4. The interaction was also examined by a confocal microscopy of cells transfected with *EGFP*-tagged *DAZap2* and *TCF4* alone and in combination. In single-transfected cells, TCF4 was nuclear while EGFP-DAZap2 had predominantly cytoplasmic and partly nuclear distribution. Upon co-transfection of *TCF4*, the observed subcellular EGFP-DAZap2 distribution changed markedly into a nuclear one. Similar effects, i.e. translocation of DAZap2 by TCF4 into the nucleus, were seen in two cell lines examined as well as with a *MYC*-tagged *DAZap2* construct. The sequestration of DAZap2 into the nucleus clearly indicates a DAZap2-TCF4 interaction and supports other data.

To examine functional consequences of DAZap2-TCF4 association, we first assessed its influence on β-catenin/TCF-mediated transcription in luciferase reporter assays. We did not observe any effect of ectopically expressed *DAZap2* on Wnt signalling either in cells stimulated with recombinant Wnt3a or in DLD-1 cells (Wnt signalling constitutively active). Then, we turned to downregulation of *DAZap2* via RNA interference (RNAi). When endogenous *DAZap2* was silenced by four different siRNA duplexes, the TCF-driven transcription from either integrated or ectopic reporter was diminished. Interestingly, the reduction in transcriptional activity corresponded well with the efficiency of the individual siRNAs. These experiments were further confirmed by analogous results obtained with three different cell lines (HEK293, DLD-1 and C57MG) stably transduced by *DAZap2* shRNAs cloned into a retro- or lentiviral vectors. Again, the cells expressing *DAZap2* shRNAs that downregulated endogenous DAZap2 levels more efficiently, displayed also more pronounced reduction in TCF-dependent reporter activity.

To test whether *DAZap2* knockdown has any effect on transcription of endogenous Wnt target genes, we first assessed mRNA levels of several putative β-catenin/TCF targets by qRT-

PCR. Somewhat surprisingly, while *DAZap2* silencing resulted in significant decrease of the *c-MYC* gene expression, other putative Wnt target genes *Dkk1*, *Axin2* and *Cyclin D1* remained virtually unaffected. Subsequent ChIP analysis revealed that decreased DAZap2 levels lead to lower β-catenin and TCF4 occupancy of *c-MYC* promoter contrary to the β-catenin/TCF-responsive element of the *Cyclin D1* promoter where TCF4 binding was not affected. As the levels of β-catenin and TCF-4 remained unchanged by *DAZap2* downregulation, we conclude that DAZap2 possibly regulates a subset of Wnt target genes in a context-dependent manner.

3.4 Fatty acid modification of Wnt1 and Wnt3a at serine is prerequisite for lipidation at cysteine and is essential for Wnt signalling

Wnt glycoproteins constitute a conserved family of secreted molecules that play important roles in formation of tissues and organs during development and also mediate cell-to-cell signalling in adult tissues. Consistent with their involvement in many key processes (cell polarity control, cell fate determination, cell migration, tissue patterning, stem cell maintenance) [e.g. 485; 486-492], mutations in *Wnt* genes and/or evasion of Wnt signalling from tight regulation contribute to developmental disorders, degenerative diseases and cancer [e.g. 125; 493; 494-508].

Wnt genes have been identified in animals from sponges (*Porifera*), placozoans (*Placozoa*), ctenophores (*Ctenophora*) and cnidarians (*Cnidaria*) to human but appear to be missing in prokaryotes, unicellular eukaryotes such as yeast, and plants [509-525]**.** Most of Wnt proteins are about 40 to 50 kDa in size, with the exception of dWnt3/5 and dWnt4, which are 113 kDa and 59 kDa, respectively. Due to their insolubility no Wnt has been crystallised yet, therefore little is known about their precise molecular structure. Based on the conserved pattern and spacing of 23 or 24 cysteine residues it was proposed that the proper folding of Wnt proteins might be dependent on the intramolecular disulphide bonds [509; 526].

Apart from the conserved cysteine residues, one of a few common features of Wnt proteins appears to be the N-terminal signal sequence, which targets them into the secretion pathway. Owing to high hydrophobicity, Wnt ligands stick to the cell surface and the ECM [107; 159; 527-531]. Their hydrophobicity stems from modification with fatty acid residues. The first identified was a palmitate attached to the conserved N-terminal cysteine (C77) of mWnt3a [532]. Palmitoylation at the corresponding cysteine residue was then experimentally confirmed for chWnt1 and chWnt3a, mWnt1 and mWnt5a as well as for *Drosophila* Wg (the Wnt1 ortho-logue) [113; 533; 534]. Recently, a second adduct in the form of palmitoleic acid was detected in mWnt3a on the internal serine (S209) residue [535]. Interestingly, this serine together with its surrounding residues is also evolutionarily well conserved among members of the Wnt family. Thus, it is widely believed that other Wnt proteins might be modified similarly. So far, such acylation has been reported for Wg and chWnt1 [113; 534].

Another common characteristic of Wnts is an asparagine-linked glycosylation at multiple sites [528; 536-538]. These residues seem to show different degree of conservation among the Wnt family with some of them shared and others specific for the individual Wnt molecules [526; 539]. Except from several Wnts, glycosylations have not been systematically analysed and their physiological role is not fully understood. As yet, contradictory reports have been published about whether glycosylation is essential for secretion and activity of Wnt proteins. In one of the pioneering studies on this theme it was shown that mutations of predicted

glycosylation sites in mWnt1 did not eliminate its activity in cultured cells [538]. On the contrary, when glycosylation sites were mutated in mWnt3a or mWnt5a, the secretion of these ligands was impaired [533; 539]. Interestingly, loss of glycosylation seems not to be essential for mWnt5a activity [533]. Currently, it is difficult to decide whether a common requirement of glycosylations for Wnt function exists until more experimental data are available. In other proteins glycosylation serves as a sorting signal for apical secretion [540]. It is therefore very likely that this modification plays similar role in those Wnt-secreting cells that display apical-basal polarity.

To investigate the role of post-translational modifications in secretion, extracellular movement and signalling activity of mammalian Wnt1 and Wnt3a proteins, we first tested all potential residues predicted for N-glycosylation or acylation. Mutational analysis combined with the use of N-glycosylation inhibitor tunicamycin and metabolic labelling of cultured cells with tritiated palmitate revealed that murine Wnt1 and Wnt3a are both N-glycosylated and acylated. Namely, Wnt1 is triply N-glycosylated at asparagines 29, 316 and 359 (N29, N316 and N359) and doubly acylated (palmitoylated at C93 and possibly palmitoleylated at S224) whereas Wnt3a is N-glycosylated at asparagine residues 87 and 298 (N87 and N298), palmitoylated at C77 and palmitoleylated at S209 (the numbering includes the residues comprising putative signal peptides). Thus, our results were in agreement with the already published analyses [113; 532; 535-537; 539; 541]. Interestingly, we observed that when *Wnt1* with S224 mutated to alanine [*Wnt1(S224A)*] had been transfected into cultured cells, a completely acyl-free Wnt1 protein was produced. Moreover, an identical effect was also detected in case of corresponding S209 in mWnt3a. This strongly suggests that acylations at S224 in mWnt1 and S209 in mWnt3a are somehow essential for further palmitoylation of N-terminal cysteines in the respective proteins. Such notion is also supported by the recently characterised *Drosophila* Wnt inhibitor of Dorsal (WntD) [542; 543] where the particular position corresponding to S209 in Wnt3a and also conserved in other Wnt ligands that undergo acylation is occupied by glutamine. Even despite the presence of the potentially modifiable cysteine corresponding to C77 in Wnt3a, the mature WntD protein remains free of any fatty acyl attachment [544].

Remarkably, Wnt acylation mutants Wnt1(C93A), Wnt1(S224A), Wnt1(C93A,S224A), Wnt3a(C77A), Wnt3a(S209A) and Wnt3a(C77A,S209A) remained fully N-glycosylated, indicating that this post-translational modification of Wnt ligands is independent of their acylation status. Intriguingly, in both Wnt1 and Wnt3a, mutations interfering with N-glycosylation led to reduced fatty acyl content. Moreover, a regressive trend was noticed with singly mutated forms exhibiting higher degree of acylation than those bearing combinations of mutated sites. Clearly, results from the metabolic labelling experiment suggest that glycosylations occur earlier and somehow condition Wnt ligands for subsequent modification with fatty acids. This

conclusion adds another piece to the still open debate on the roles of and a mutual relationship between glycosylation and acylation of Wnt proteins. Considerable amount of available data provide evidence that Porc, an ER membrane-resident acyltransferase, is involved in the processing of Wnt [107; 110; 111; 113; 534; 545-548]. The dependence of the Wnt hydrophobicity degree on *Porc* expression level [113; 534; 535] implies Porc role in acylation. Moreover, Porc is required for covalent fatty acyl attachment on S209 in Wnt3a [535]. Curiously enough, Porc was also reported to stimulate glycosylation of Wg and several murine Wnt proteins [110; 547]. However, no obvious change in glycosylation status was detected in Wnt3a(S209A) mutant when compared to the wild-type protein and furthermore, down-regulation of *Porc* by siRNA did not have any effect on Wnt3a glycosylation [535]. Therefore, not all Wnt ligands appear to require Porc for their efficient glycosylation. Nevertheless, Porc still may promote Wnt glycosylation by tethering Wnt to the ER membrane and making it accessible to the oligosaccharyl transferase (OST) complex [547]. Biochemical analysis suggests that Porc binds twenty-four N-terminal amino acids of Wg highly conserved among *Drosophila* and vertebrate Wnt family members and containing the palmitoylated cysteine as well as the glycosylated asparagine [547]. Thus, Porc might represent a point of convergence for N-glycosylation and acylation for at least some Wnt ligands.

Several tests, namely reporter gene assays, qRT-PCR analysis, β-catenin staining in Wnt-responsive mammalian cells and ectopic axis induction in *Xenopus* embryos, were used to compare the signalling activities of wild-type and mutant Wnt constructs. In all these experiments, the non-acylated forms [Wnt1(S224A), Wnt1(C93A,S224A), Wnt3a(S209A) and Wnt3a(C77,S209A)] failed to function, strongly suggesting that fatty acyl attachment to Wnt1 and Wnt3a at their respective serine residues (i.e. S224 in Wnt1, S209 in Wnt3a) is essential for signalling competence. Strikingly, the activities of the non-palmitoylated Wnt1(C93A) and Wnt3a(C77A) as compared to those of the wild-type proteins proved strong dependence on the applied testing system. In *Xenopus* embryos, Wnt1(C93A) reached approximately two-thirds of the Wnt1 activity whereas Wnt3a(C77A) was as active as Wnt3a. In the mammalian cells, both Wnt1(C93A) and Wnt3a(C77A) showed much more limited functionality. The discrepancy observed between the results obtained from experiments with cultured cells versus those with *Xenopus* embryos might stem from the much more complex situation of a *Xenopus* embryo where many different Wnt ligands, receptors and/or extracellular modifiers are produced. The experimentally monitored phenotype, i.e. the duplication of the body axis, is the result of the intricate interplay among all these factors. Therefore, it may be possible even for a mutant classified in a cell culture-based assay as signalling-incompetent to push the equilibrium of the whole system towards the secondary axis formation, possibly by interaction(s) unaffected by the acyl absence. Alternatively, *Xenopus* cells may express a distinct set of receptors that remains efficiently stimulated even by the non-palmitoylated Wnt1 and Wnt3a mutants.

We noticed differences in behaviour of the Wnt1 and Wnt3a glycosylation-deficient variants in the performed tests. Wnt1(N29,316,359Q) showed highest (even higher than the wild-type Wnt1) activities of all tested Wnt1 proteins when both autocrine and paracrine signalling abilities were assessed. However, in co-culture experiment evaluating fitness in paracrine signalling, Wnt1(N29,316,359Q) activity was much reduced. In all instances, Wnt3a(N87,297Q) did not reach levels achieved by the wild-type protein. This fact corresponds well with the observed slower rate of secretion of this particular variant.

From our analyses, several conclusions can be reached as regards the role of post-translational modifications of Wnt1 and Wnt3a ligands in their secretion and biological activity. The fact that non-glycosylated variants of Wnt1 and Wnt3a remain capable of signalling and also retain biochemical features similar to their respective wild-type counterparts indicates that N-glycosylation is not essential for the secretion, movement in the extracellular space or signalling abilities. Importantly, all signalling-active Wnt3a forms [i.e. Wnt3a, Wnt3a(C77A), Wnt3a(N87,298Q)] not only accumulated in the ECM but were also detected in the conditioned media. On the contrary, we were unable to find any Wnt1 variant in cell supernatants. This is in good agreement with previously published reports showing that radioactive metabolic labelling together with immunoprecipitation and long time exposure must be used in order to get Wnt1 traced in the cell culture medium [529]. In the fractionation experiment, wild-type Wnt1 and its non-glycosylated form Wnt1(N29,316,359Q) was found in the ECM. This association was markedly decreased by the absence of palmitoyl in Wnt1(C93A) and almost undetectable in case of acyl-deficient mutants Wnt1(S224A) and Wnt1(C93A,S224A). The amounts of Wnt1(C93A), Wnt1(S224A) and Wnt1(C93A,S224A) were reduced correspondingly also in membrane fractions. In case of Wnt3a the situation was analogous with the exception that Wnt3a(C77A) lacking palmitoyl moiety did not show any decrease in association with the ECM and was released into the culture medium as efficiently as the wild-type protein. Therefore, it appears that Wnt1 and Wnt3a acylation is linked to efficient transport of mature proteins onto the cellular surface and their movement in the extracellular space but it is not indispensable for these processes. Recently, it was reported that the S209-linked palmitoleate is essential for Wnt3a interaction with the carrier protein Wls [549]. Given that acyl-deficient mutants of both Wnt1 and Wnt3a still reach the plasma membrane, it seems plausible to propose an existence of yet another, a Wls-independent route of secretion.

Intriguingly and contrary to the previously published report [534], the absence of modification with acyl chains does not impair targeting to lipid rafts (DRMs). This observation suggests that Wnt proteins reach DRMs irrespective of their modification by fatty acyls. It further implies that there need not necessarily be a functional relationship between the presence of Wnt proteins in DRMs and Wnt signalling. We offer a protein-protein interaction as the alternative mechanism responsible for targeting Wnts to DRMs. In support of this notion, such a mechanism has already

been proposed for several other proteins including a splice variant of SHP-1 protein phosphatase, c-Cbl-associated protein (CAP), Abelson-related gene-binding protein 2A (ArgBP2A) and vinexin α [550; 551].

Acylations of Wnt1 and Wnt3a have proved significant for the ligand movement in the extracellular space. Absence of a single fatty acyl in Wn1 impaired and in Wnt3a significantly reduced paracrine signalling. This finding directly conflicts former notion that fatty acyl(s) attachment limits Wnt distribution owing to potential interactions with components of cell membranes and extracellular matrix [107; 113; 527; 528; 531; 532]. The opposite view, i.e. that fatty acyl adducts promote rather than restrict the spread of Wnt outside the cells, appears more likely. In this respect, several supportive observations have been published. First, *Drosophila* Wg, a Wnt1 orthologue, associates via its acyl chains with the lipoprotein particles called lipophorins and RNAi of the *apolipophorin* narrows Wg signalling range in wing discs. Lowering the lipophorin levels affects Wg long-range but not short-range signalling, suggesting that lipophorins may act specifically as vehicles for Wg spreading over longer distances from the source [136]. Second, Wnt3a also appears to be released from mammalian cells onto lipoprotein particles, namely high-density lipoproteins (HDLs) and the C77-attached palmitate seems crucial for this association. Furthermore, Wnt3a(C77S), a construct missing its palmitate moiety, is secreted normally into culture medium but it loses association with lipoproteins and its signalling activity markedly decreases [137]. Third, artificially prepared liposomes enhance and sustain biological activity of purified Wnt3a both *in vitro* and *in vivo* [552].

We also experimentally proved that all tested Wnt1 and Wnt3a proteins regardless of presence or absence of their post-translational modification(s) showed binding to the Fzd/LRP receptor complex. This observation is in stark contrast to the published claims that either mutation of C77 or a secondary enzymatic removal of the C77-bound palmitate from Wnt3a ligand abrogates the interaction of Wnt3a with Fzd and LRP [539; 553]. However, in those reports the interactions were tested by a pulldown assay using recombinant fragments of Fzd and LRP instead of co-immunoprecipitation of full-length proteins. Therefore, a possible reason for the apparent discrepancy might lie in the experimental conditions.

From the present day perspective, the Wnt family of ligands rather than being a unified group appears to be composed of several clusters of proteins with more or less similar properties. Therefore, one should be very circumspect in an attempt to generalise conclusions drawn from our observations to other Wnt proteins. Further experimentation is needed in order to classify individual ligands according to their shared biochemical properties and/or biological activities.

4 CONCLUSION

Wnt signalling governs cell fate decisions in many physiological conditions and its diverse effects on cells are tissue- and context-dependent. Wnt ligands induce only subsets of all potential target genes at any particular time and cellular background. Yet all the different and highly specific outcomes result from function of a limited number of nuclear effectors. Although the stability of β-catenin is pivotal to the activation of Wnt target genes, LEF/TCF proteins also play significant roles, further adjusting transcriptional output patterns. They can be interchangeable in some but unique (i.e. can perform specific, non-redundant functions) in other contexts. We believe that this functional plasticity of LEF/TCFs is to a great extent due to distinct combinations of other co-regulators interacting with these proteins in transcriptional complexes.

We have described three different proteins that interact with LEF/TCFs and affect β-catenin/TCF-dependent transcription. Two of the proteins repress the TCF4-mediated transcription, yet by distinct mechanisms. While CtBP seems to directly bind to TCF4 and in conjunction with APC also diminish the availability of β-catenin, HIC1 associates with TCF4 and recruits it together with β-catenin into a specialised set of nuclear bodies. We have also identified DAZap2 as a novel protein that might participate in modulating the LEF/TCF-mediated transcription. But at the moment, the exact DAZap2 mode of action remains rather speculative.

One of the aims of our studies was to examine mechanisms modulating the activity of Wnt signalling in the nucleus. The main results can be summarised as follows:

1. We have identified CtBP protein to attenuate the Wnt signalling by suppressing the transcription mediated by β-catenin/TCF4 complexes. We have also shown that HDACs are required for this CtBP inhibitory effect.

2. We have established a relation between the tumour suppressor HIC1 protein and the Wnt signalling. We presume that HIC1 acts by diverting β-catenin/TCF4 complex from the target gene promoters to a specified class of nuclear bodies. In this mechanism, CtBP seems to play an additional, structural role to enhance the HIC1-mediated effect.

3. We have identified DAZap2, a highly conserved 17kDa protein, as a novel modulator of Wnt signalling. We were not able to detect any effect (either stimulatory or inhibitory) on β-catenin/TCF-mediated transcription when *DAZap2* was ectopically overexpressed but *DAZap2* knockdown via RNAi clearly reduced Wnt-dependent transcription. A short stretch of amino acids proximal to the DNA-binding domain of TCF was mapped as indispensable

for the interaction with DAZap2. Intriguingly, although this region shows only a partial conservation among LEF/ TCF factors, all LEF/TCFs were observed to interact with DAZap2 with a similar affinity. Moreover, as not all of the tested putative Wnt target genes seem to be affected by changes in DAZap2 levels, we conclude that DAZap2 might function as a context-dependent regulator of specific Wnt-responsive genes.

We are still far from understanding what lies behind the distinct functional abilities of LEF/ TCF factors as well as differential regulation of Wnt target genes in a particular context. We are just beginning to unveil some of the mechanisms possibly still not knowing their actual relevance. The presented work is just a little contribution to the endeavour to understand these questions better.

Our second concern was the role of post-translational modifications, namely N-glycosylation and acylation, in secretion, extracellular movement and signalling activity of mammalian Wnt1 and Wnt3a proteins. Our study on this subject has yielded the following findings:

1. N-glycosylation of mWnt1 and mWnt3a primes these Wnt proteins for the ensuing acylation. N-glycosylation is not essential for their secretion, movement in the extracellular space or signalling abilities.

2. Acylations at serine residues (S224 in mWnt1 and S209 in mWnt3a) precede and are vital for the subsequent palmitoylation of N-terminal cysteines in the respective proteins to occur.

3. Wnt1 and Wnt3a acylation as such is not indispensable for the export of the synthesised Wnt1 or Wnt3a protein to the cell surface nor does it influence their targeting to lipid rafts. However, it seems to be of prime importance when the association with the ECM is considered.

4. The double fatty acylation of mouse Wnt1 and Wnt3a have proved significant for the ligand movement in the extracellular space and for correct signalling functionality in mammalian cells. Contrary to the broadly accepted view we propose that acylation of Wnt ligands supports rather than hinders their distribution in the extracellular space.

5. Rather surprisingly and contrary to the published claims, neither acylation nor N-glycosylation of Wnt 1 or Wnt3a are vitally important for the interaction with the Fzd receptor or LRP co-receptor.

Although our experimental data enabled us to draw several conclusions concerning the role of post-translational modifications of Wnt1 and Wnt3a ligands, there are still many questions remaining unanswered. Recently, it was reported that the S209-linked acylation is required for the Wnt3a binding to the carrier protein Wls [549]. Does our observation that the acyl-deficient Wnts still reach the cell surface together with the existence of WntD, a ligand that is naturally devoid of any fatty acyl modification, indicate another, yet to be identified secretion mechanism? Can differential Wnt acylation assist in control of the extracellular gradient formation? Obviously, more experimental work is needed to answer these and many other arising questions.

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7 SUPPLEMENTS

Supplements contain reprints of publications 7.1 – 7.4

7.1 Supplement No. I

HMG box transcription factor TCF-4's interaction with CtBP1 controls the expression of the Wnt target *Axin2*/*Conductin* in human embryonic kidney cells

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HMG box transcription factor TCF-4's interaction with CtBP1 controls the expression of the Wnt target Axin2/Conductin in human embryonic kidney cells

Tomas Valenta, Jan Lukas and Vladimir Korinek*

Institute of Molecular Genetics Academy of Sciences of the Czech Republic, Videnska 1083, 142 20 Prague 4, Czech Republic

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ABSTRACT

Members of the Tcf/Lef family of the HMG box transcription factors are nuclear effectors of the Wnt signal transduction pathway. Upon Wnt signaling, TCF/LEF proteins interact with β-catenin and activate transcription of target genes, while, in the absence of the Wnt signal, TCFs function as transcriptional repressors. All vertebrate Tcf/Lef transcription factors associate with TLE/Groucho-related co-repressors, and here we provide evidence for an interaction between the C-terminus of the TCF-4 HMG box protein and the C-terminal binding protein 1 (CtBP1) transcriptional co-repressor. Using Wnt-1-stimulated human embryonic kidney 293 cells, we show that CtBP1 represses the transcriptional activity of a Tcf/β-catenin-dependent synthetic promoter and, furthermore, decreases the expression of the endogenous Wnt target, Axin2/Conductin. The CtBP1-mediated repression was alleviated by trichostatin A treatment, indicating that the CtBP inhibitory mechanism is dependent on the activity of histone deacetylases.

INTRODUCTION

Members of the Wnt family of growth factors control numerous developmental processes and aberrant activation of Wnt genes, and Wnt signaling is also implicated in deregulated cell growth and cancer (reviewed in 1,2). Insights into the mechanisms of the Wnt signaling pathway have emerged from genetic studies in *Drosophila*, biochemical experiments in cell culture, ectopic gene expression in early *Xenopus* embryos and from target gene inactivation in mice (3). A striking characteristic of Wnt signaling is its conservation during evolution. Components of the Wnt signaling pathway have been found in such evolutionary distant species as slime mould *Dictyostelium*, diploblastic metazoan Hydra, *Caenorhabditis elegans* and in higher vertebrates, including mammals (4–6). This indicates that the Wnt signaling cascade is one of the primary regulatory mechanisms controlling cell fate decisions in multicellular organisms.

A brief outline of the canonical Wnt pathway is the following (for more detailed information, refer to the Wnt homepage at http://www.stanford.edu/~rnusse/wntwindow.html). The interaction of extracellular Wnt ligands with the Frizzled/LRP receptor complex results in increased intracellular levels of β-catenin in the target cell (7–9). In unstimulated cells, free cytoplasmic β-catenin is marked on N-terminal serine and threonine residues by phosphorylation, and then ubiquitinated and rapidly degraded by the proteasome pathway (10–13). The β-catenin degradation machinery includes casein kinase Iα (CKIα) and a multiprotein cytoplasmic complex containing serine–threonine kinase GSK-3, Axin1 (or its homolog Axin2/Conductin) and the APC tumor suppressor (14–20). When cells are stimulated by Wnt proteins, the cytoplasmic phosphoprotein Dishevelled is recruited to the plasma membrane and, by a so far unknown mechanism, inhibits the function of the GSK-3/Axin/APC complex (21,22). The unphosporylated stable β-catenin molecules accumulate in the cytoplasm and also translocate into the nucleus. Nuclear β-catenin forms heterocomplexes with TCF/LEF proteins (23–25). The TCF/β-catenin heterodimers act as bipartite transcription factors and activate the expression of the specific Wnt responsive genes (26,27). Approximately 50 Wnt targets have been identified to date, and several of these genes encode proteins related to cell cycle regulation, e.g. c-myc, Cyclin D1 and Pitx2 (28–31).

Relay and final processing of the Wnt signal in the target cell is controlled at several levels. In the extracellular space, proteins of the Dkk, WIF and SFRP families interact directly with Wnt ligands or their receptors and inhibit transmission of the signal into the cell (32–37). In the cytoplasm, LIT-1/Nemo-like kinase phosphorylates TCFs and regulates the DNA binding and subcellular distribution of TCF/LEF factors (38–40). β-Catenin activity in the nucleus may be promoted by interaction with a number of proteins including Pontin52, Brg-1, Pygopus, p300 and Lines (41–45). β-Catenin-mediated transcription is repressed by Reptin52 (a Pontin52 homolog), ICAT, XSox17α/β and XSox-3 protein (46,47). Other interacting partners modulate the function of TCF/LEF proteins.

In mammals, the Tcf/Lef family consists of four genes: *Tcf-1*, *Lef-1*, *Tcf-3* and *Tcf-4*. All TCF/LEF proteins display several common structural features (48,49). They contain a nearly identical DNA-binding domain, the HMG box, recognizing the consensus sequence A/T A/T CAAA. The

*To whom correspondence should be addressed. Tel: +4202 41062471; Fax: +4202 44472282; Email: korinek@biomed.cas.cz

extreme N-terminus harbors the β-catenin interacting domain. Although capable of binding to DNA, TCF/LEF proteins possess only limited ability to activate transcription. They might rather be viewed as nuclear vehicles targeting other auxiliary proteins to a specific set of promoters. β-Catenin possesses a strong transcription activation domain, so it is an example of an activating interacting partner. In contrast, all mammalian TCFs also associate with TLE/Groucho co-repressors and, in the absence of the Wnt signal, repress the transcription of the Tcf-dependent synthetic reporter or endogenous Tcf targets (50). TLE/Groucho proteins interact with the hypoacetylated N-terminal tail of histone H3 and also with human histone deacetylase 1 (HDAC 1) and its *Drosophila* homolog Rpd3 (51,52). This suggests that the function of TLE/Groucho is to form a specialized repressive chromatin structure that prevents the inappropriate activation of β-catenin/TCF target genes.

The participation of Wnt signaling in multiple developmental programs leads to the question of how β-catenin/TCF complexes discriminate between different subsets of all potential Wnt target genes at a given time and cellular background. Functional differences between individual TCF/LEF proteins could be one possible explanation. Although *Tcf/Lef* mRNAs are expressed in complex and often overlapping patterns during embryogenesis and in adult tissues, gene targeting experiments in mice revealed that Tcf-1, Lef-1 and Tcf-4 execute different developmental programs and their functions are only partially redundant (53–55). Additionally, several observations indicated that TCFs interact with distinct partners and show individual context-dependent DNA-binding and transactivation properties. For example, it has been reported that LEF-1 cooperates with microphthalmia-associated transcription factor (MITF) to up-regulate the dopachrome tautomerase gene promoter in melanocytes (56), and that activation of the T-cell receptor alpha enhancer by LEF-1 depends on ALY, a nuclear protein that specifically associates with LEF-1 and AML-1 (57). The activity of LEF-1 is suppressed by its association with PIASy, a nuclear matrix-associated SUMO E3 ligase, which sequesters LEF-1 into nuclear bodies (58) and, furthermore, direct interaction of LEF-1 with HDAC 1 mediates a repressive effect of LEF-1 in the absence of Wnt signaling (59). Another Tcf family member, TCF-3, interacts with casein kinase 1ε (CK1ε), and phosphorylation of TCF-3 by CK1ε stimulates its binding to β-catenin (60). In *Xenopus laevis*, XTCF-3 requires XCtBP to function as a transcriptional repressor (61). Recently, Hecht and Stemmler showed that TCF-4 and LEF-1 exhibit significant differences in regulating the promoter of two Wnt-responsive genes, *Cdx-1* and *Siamois*, in human embryonic kidney cells (62). Cdx-1 activation strictly depended on the promoter-specific transactivation domain located at the TCF-4 C-terminus. In addition, multiple isoforms are generated from *Tcf/Lef* mRNAs by way of alternative splicing or by use of dual promoters, and these isoforms display diverse functional properties (63–65). Thus, selective interaction of members of the Tcf/Lef family with various partners and their intrinsic DNA-binding properties can support the execution of different developmental programs.

To study the regulatory mechanisms of Wnt signaling, we screened for proteins interacting with human TCF-4. In this report, we show that the C-terminal binding protein 1 (CtBP1) associates with the TCF-4 C-terminus and that CtBP1 overexpression in Wnt-1-stimulated 293 cells represses transcription from the Wnt-responsive reporter and also decreases the activity of the endogenous Axin2 promoter. The CtBP1-mediated repression is alleviated by trichostatin A treatment, indicating that the CtBP inhibitory mechanism is dependent on the action of histone deacetylases.

MATERIALS AND METHODS

Plasmids

Constructs encoding proteins containing N-terminal EGFP were generated in pEGFP/C vector (BD Clontech). Myc-tagged cDNAs were prepared in pK-Myc vector. This vector was created by replacing the EGFP region in pEGFP/C with a T7 promoter and Myc tag coding sequence. Full-length cDNAs without N-terminal tags contained either natural initiator or a PCR-introduced consensus Kozak sequence. The expression plasmids for Tcf-4 were derived from human *Tcf-4E* cDNA (GenBank accession No. NM_030756) using naturally occurring restriction enzyme sites: *Sma*I (position 236 bp from ATG), *Nsi*I (995) and *Nde*I (1159). Plasmids encoding full-length or truncated forms of mCtBP1 were prepared in an analogous way using *Bgl*II, *Pvu*II, *Kpn*I and *Pst*I internal restriction enzyme sites. Detailed information about the constructs is available upon request. The mouse *Lef-1* cDNA was obtained from a mouse testis cDNA library (a gift from Stoil Dimitrov, IMG AS CR, Prague, Czech Republic). The TCF4–LEF-1 chimeric construct was generated by an exchange of the 3′ coding region in *Tcf-4* with the C-terminal coding sequence of mouse *Lef-1* using the *Apa*LI restriction enzyme site conserved in both genes. Human β-*catenin* cDNA was obtained from a bone marrow MATCHMAKER cDNA library (BD Clontech). Human *CtBP1* cDNA was amplified from the cDNA of 293 cells, mouse *CtBP2* was cloned from day-19 mouse embryo cDNA. Mouse *Wnt-1* cDNA was a gift from Marc van Dijk (University Hospital Utrecht, Utrecht, The Netherlands). For retroviral transduction, *Wnt-1* or EGFP-tagged *mCtBP1* were ligated into pLNIT (generously provided by Fred H. Gage, The Salk Institute, La Jolla, CA) or pLNCX vector (BD Clontech). Platinum Pfx DNA Polymerase (Invitrogen) was used for all PCR amplifications. PCR-derived constructs were verified by sequencing.

Yeast two-hybrid screen

A pre-transformed mouse 17-day embryo MATCHMAKER cDNA library (Clontech) was screened with the last 185 amino acids of the human TCF-4 protein fused to the GAL-4 DNA-binding domain (DBD) in pGBKT7 as bait. Positive clones were subjected to the specificity test using the GAL-4 DBD or GAL-4 DBD–lamin fusion protein as bait. Those clones that interacted specifically with the GAL-4 DBD–TCF-4 bait were sequenced.

Cell culture, transfections and retrovirus infection

Human embryonic kidney (HEK) cell lines 293, monkey kidney cells COS-7, rat fibroblast cell line Rat2, EcoPack-293 and AmphoPack-293 (Clontech) cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented

with 10% fetal bovine serum (Hyclone). Transfections were performed using the lipofectamine reagent (Invitrogen), as described by the manufacturer. For retrovirus production, ecotropic EcoPack-293 or amphotropic AmphoPack-293 packaging cells were seeded in 6-well plates 12 h prior to transfection and then transfected with 3 μg of a particular retroviral expression construct. After 24 h, the growth medium was replaced with 2 ml of fresh medium. After a further 24 h incubation, the supernatant containing non-replicating forms of the virus was harvested. Target cell lines were infected in 6-well plates with the virus supernatant in the presence of 5 μg of polybrene (Sigma) per ml. Twenty-four hours later, the medium was replaced by complete DMEM supplemented with either G418 (concentration 1 mg per ml) or hygromycin B (0.5 mg per ml; both antibiotics were purchased from Invitrogen) and doxycycline (Sigma), concentration 1 μg per ml [expression of the transduced cDNAs in pLNIT is regulated by doxycycline (66)]. After a 10 day selection, the growing (resistant) cells were washed extensively with DMEM and subsequently cultivated in fresh selective medium without doxycycline. The expression of the transferred genes was confirmed by western blot analysis.

Reporter gene assays, trichostatin A treatment and Wnt-1 stimulation experiments

For reporter gene assays, 293 cells were seeded into 24-well plates (approximately 10^5 cells per well) and transfected 2 h later with a lipofectamine mixture containing 50 ng *Renilla* pRL-SV40 plasmid (Promega) as an internal control, 400 ng luciferase reporter plasmid and up to 1 μg of the particular expression vector. The total amount of DNA was kept constant by adding empty expression vector where necessary. The firefly luciferase reporter constructs pTOPFLASH and pFOPFLASH, containing either three copies of the optimal Tcf motif GATCAAAGG or three copies of the mutant motif GGCCAAAGG, respectively, were described previously (27). The lipofectamine–DNA mixtures for reporter gene assays in 293-EGFP-CtBP1/Dox contained 50 ng *Renilla* pRL-SV40 internal control plasmid and 400 ng luciferase reporter plasmid only, i.e. no empty vector was added. COS-7 cells were seeded in 12-well plates and transfected by lipofection with 50 ng *Renilla* pRL-SV40 plasmid, 400 ng luciferase reporter plasmid and various combinations of the following plasmids (see Fig. 5A): TCF-4 expression vector (400 ng), β-catenin expression vector (800 ng) and CtBP1 expression vector (0.5–2 μg). Additional empty pK-Myc plasmid was added when necessary to make the total amount of DNA equivalent. Firefly and *Renilla* luciferase activities in cell lysates were determined 15 h post-transfection using the dual luciferase system (Promega), according to the protocol supplied by the manufacturer, and a single tube luminometer Sirius (Berthold). All reporter gene assays were done in triplicate. The reporter gene activities shown are average values, along with the standard deviations from at least three independent experiments after normalization against the *Renilla* luciferase activities. Treatments with the deacetylase inhibitor trichostatin A (Sigma) were performed using the drug at 300 nM concentration for 12 h. Wnt-1 stimulation experiments were performed as follows. 3×10^5 293 cells were seeded into 6-well plates and transfected by lipofectamine–DNA mixtures. The amounts of individual DNAs in the mixtures were proportionally increased by a factor of three as compared to the experiments in 24-well plates. Four hours post-transfection, the cells were washed extensively with DMEM and then 1×10^5 Rat2-Wnt-1/Const cells expressing stable levels of Wnt-1 protein were plated over the target cells. Parental Rat2 cells were used as a negative control. After co-cultivation for 3–30 h, the cells were harvested together and further processed for the reporter gene assay, RNA isolation, electrophoretic-mobility shift assay (EMSA) or immunoblotting. When feeders with inducible *Wnt-1* expression (Rat2-Wnt-1/Dox) were used, the experimental procedure was modified as follows. We regularly grew the feeder cells in a complete medium supplemented with 2 ng of doxycycline per ml. This low concentration of the antibiotic was sufficient to suppress Wnt-1 production, and, additionally, Wnt-1 protein started to appear almost instantly upon the removal of doxycycline. Transfected 293 cells were covered with Rat2-Wnt-1/Dox cells and co-cultivated in medium without doxycycline. Cell cultures growing in the presence of doxycycline (5 ng per ml) were used as negative controls. The regulation of CtBP1 expression in 293-EGFP–CtBP/Dox cells was rather 'leaky', thus we cultivated these cells in higher concentrations of Dox (1 μg/ml). The full induction of the CtBP1 transgene was then achieved during 15 h growth in the absence of doxycycline.

GST interaction assays

Full-length GST–mCtBP1 and GST–mCtBP2 fusion proteins were expressed in the BL21 (DE3) strain of *Escherichia coli* using the pET-42b vector (Novagen). Full-length or truncated TCF-4 and full-length mCtBP1 protein were produced *in vitro* using the TNT Coupled Reticulocyte System (Promega) and corresponding pK-Myc constructs. All coupled transcription–translations were performed in a total volume of 50 μl using 10 μl of [^{35}S]methionine (ICN Biomedicals) per reaction. Twenty microliters of radiolabeled TCFs or mCtBP1 were incubated with GST–mCtBP1 or GST–mCtBP2 proteins bound to glutathione–Sepharose 4B beads (Amersham Pharmacia Biotech) in GST binding buffer [phosphate-buffered saline, pH 7.4, 0.5% (v/v) Nonidet P-40, 20 μM NADH (Sigma) and Protease Inhibitor Cocktail (Sigma)] for 1 h at 4°C. Beads were collected by centrifugation and washed five times in GST binding buffer. Bound proteins were separated by SDS–PAGE and analyzed by autoradiography.

Antibodies and immunoblotting

The following commercially available antibodies were used: β-catenin, CtBP, mouse monoclonal (Santa Cruz); EGFP, mouse monoclonal (BD Clontech); Myc tag, mouse monoclonal 9E10 (Roche Molecular Biochemicals); TCF-4, mouse monoclonal (Sigma); Wnt-1, rabbit polyclonal (Santa Cruz); and LEF-1, goat polyclonal (Santa Cruz). To monitor protein expression, 2×10^5 293 cells, seeded into 6-well plates, were transfected with 3 μg of expression vector. Twenty-four hours later cells were harvested and disrupted directly in SDS–PAGE sample buffer. The cell lysates were cleared of cell debris and chromosomal DNA by ultracentrifugation at 200 000 *g*, then loaded onto SDS–PAGE gels, transferred to polyvinylidene fluoride membranes (Pall Gelman Laboratory)

and immunoblotted with the appropriate antibodies. Proteins were visualized with an enhanced chemiluminiscence system (Pierce).

EMSA

The nuclear extracts were prepared according to Dignam *et al.* (67), snap frozen in liquid nitrogen and stored at –70°C. The final buffer composition was 20 mM HEPES, 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF and 20% glycerol. Extracts were prepared from intact nuclei that were washed three times to avoid contamination with cytoplasmic β-catenin. As the optimal Tcf/Lef probe, we used a double-stranded 19-nucleotide oligomer AGAACCCTTTGATCTT-AGG; the control probe was AGAACCCTTTGGCCTTAGG. The oligonucleotides were end-labeled using T4 polynucleotide kinase (Fermentas) and [γ-^{32}P]ATP (3000 Ci/mmol; ICN Biomedicals). A typical binding reaction contained 8 µg of nuclear protein, 1 ng of radiolabeled probe and 300 ng of poly-(deoxyinosic–deoxycytidylic) acid (poly [dIdC]; Sigma) in 25 µl of binding buffer (60 mM KCl, 1 mM EDTA, 1 mM DTT, 10 mM HEPES, pH 6.9 and 10% glycerol). Samples were incubated for 30 min at room temperature, an antibody was added and the samples were incubated for a further 30 min. The binding reactions were loaded onto 5% polyacrylamide gels with 0.5× Tris–borate–EDTA running buffer. Electrophoresis was carried out at 15°C at 220 V constant voltage. Gels were transferred onto Whatman paper, dried and exposed to the BAS-phosphoimager screen (Fuji).

Real-time quantitative RT–PCR

One hundred micrograms of total RNA from Wnt-1-stimulated 293 or control cells isolated by the guanidine thiocyanate (Fluka) method (68) were further purified using the Micro-to-Midi total RNA isolation system (Invitrogen). Random or oligo dT-primed cDNA was prepared in a 20 µl reaction from 2.5 µg of total RNA using Superscript II RNAseH$^-$ reverse transcriptase (Invitrogen). One percent of the resulting cDNA was used for one quantitative PCR reaction. The 10 µl reaction mixtures also contained 1× Platinum Quantitative PCR SuperMix-UDG (Invitrogen), acetylated BSA (final concentration 3 µg per ml; New England Biolabs), Sybr Green I (final dilution 1:40 000; Amresco) and 5 pmol of each specific primer. All primers were calculated using Primer 3 computer services at http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi/primer3_www.cgi. The primer pairs were designed and tested to be specific for human genes, i.e. the primers did not prime on rat or mouse cDNA. We used primers for the following human genes (the primers are written in the 5′ to 3′ direction, the first primer is derived from the plus and the second primer from the minus DNA strand): *Axin1*, CCTGTGGTCTACCCGTGTCT, GCTATGAGGAGTGGTCCAGG; *Axin2*, CTGGCTTTGGT-GAACTGTTG, AGTTGCTCACAGCCAAGACA; *GAPDH*, CACCACACTGAATCTCCCCT, CCCCTCTTCAAGGGG-TCTAC; *CyclinD1*, CCATCCAGTGGAGGTTTGTC, AGC-GTATCGTAGGAGTGGGA; *SDHA*, AGATTGGCACCT-AGTGGCTG, ACAAAGGTAAGTGCCACGCT; *CtBP1*, CCTTCGCGTTCCTCGTTA, AAGAACGTTCATGGGAG-AATAA; *Tcf-1*, CCTCTGCCTCCCTAGCTTTT, ATGGGG-GAGATGGGTAGAGA; *Lef-1*, CTGCTAGAGACGCT-GATCCA, TGGCTCTTGCAGTAGACGAA. cDNAs were produced from at least two independent RNA isolations, and the PCR reactions were performed in triplicate for each primer set. The specificity of the PCR products was verified by sequencing. Two housekeeping genes, *Glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) and *Succinate dehydrogenase complex, subunit A* (*SDHA*), were used as internal control genes to standardize the quality of different cDNA preparations (69). The cycling was performed in a LightCycler instrument (Roche Molecular Biochemicals). The results were analyzed using the LightCycler 3 software package. The relative abundance of *Axin1* and *Axin2* mRNA in 293 cells stimulated by Rat-2-Wnt-1/Const cells versus control 293 cells (co-cultivated with the parental Rat2 cell line) was derived from the average CT values of each triplicate after normalizing against the levels of *SDHA* cDNA. Results of a representative experiment are shown.

RESULTS

TCF-4 interacts with the transcriptional co-repressors CtBP1 and CtBP2

To gain insight into the molecular function of TCF-4 protein, we started a search for novel TCF-4-interacting proteins using a yeast two-hybrid system. We selected a 180 amino acid region distal to the HMG box covering the C-terminus of TCF-4 as bait. This region has a unique primary structure within the Tcf/Lef family and displays only limited homology to the C-terminus of TCF-3, the closest relative of TCF-4 (Fig. 1). One strong positive obtained from a day-17 mouse embryo cDNA library corresponded to *CtBP1*, a widely expressed transcriptional co-repressor. The cDNA encoded the entire 430 amino acid CtBP1 polypeptide. The CtBP1–Gal4 activation domain fusion protein interacted specifically with the TCF-4 bait and not with the Gal-4 DBD alone or with the Gal-4 DBD–lamin fusion protein, respectively (see Materials and Methods).

We further attempted to delineate the minimal region of CtBP1 required for association with TCF-4. We generated a series of N- and C-terminal deletions of CtBP1 fused to the Gal4 activating domain and tested their interaction with Gal4 DBD–TCF-4 in a yeast two-hybrid system. Each truncation of CtBP1 displayed a dramatically reduced ability to bind TCF-4 (data not shown). This indicates the complex multidomain mode of interaction between CtBP1 and TCF-4.

Human CtBP1 was originally discovered during a screen for cellular proteins that complex with the C-terminal region of the adenoviral E1A protein (70). Subsequently, a highly homologous human polypeptide termed CtBP2 was identified by analysis of EST database sequences (71). Other vertebrates such as rodents and *Xenopus* also contain two *CtBP* homologs, while invertebrates have a single *CtBP* gene. The CtBP proteins bind to a short sequence motif PLDLS conserved among the E1A proteins of all human and primate adenoviruses. Different variants of this motif are also present in other CtBP-interacting partners that function mainly as negative regulators of transcription (reviewed in 72). Two putative CtBP-binding sequences are also present in the C-termini of TCF-3 and TCF-4 proteins (Fig. 1); Brannon and others showed that simultaneous mutations in both these sites abolished the association of XTCF-3 with XCtBP, a *Xenopus* homolog of CtBP1, *in vitro* (61).

Figure 1. Amino acid comparison of the C-terminal regions of human TCF-4 and TCF-3. Amino acid identities are indicated by corresponding letters; amino acid similarities are indicated by double dots (closely related residues) or single dots (distantly related residues). Sequences were aligned with CLUSTALW. Overlining indicates highly conserved HMG box sequences; two putative CtBP binding sites are boxed. The arrows depict the amino acid sequence used in a yeast two-hybrid screen.

Given the putative CtBP-binding motifs in the C-terminus of TCF-4, we tested the interaction using bacterially purified GST–CtBP1 or GST–CtBP2 and various forms of TCF-4 translated *in vitro* (Fig. 2A). As shown in Figure 2B, a GST fusion protein containing complete CTBP1 interacted with [^{35}S]methionine-labeled full-length TCF-4 (FLTCF-4) or with its C-terminal fragment (ΔN_{Nde}TCF-4). Truncated proteins that lacked either sequences distal to the HMG box (TCF-4–LEF-1 chimera) or the entire C-terminal region (ΔC_{Nsi}TCF-4) failed to interact with mCtBP1. GST-tagged CtBP2 showed an identical mode of interaction (not shown), and, additionally, we observed efficient heterodimerization of CtBP1 and CtBP2 *in vitro* (Fig. 2B). Taken together, these data indicate that TCF-4 associates with CtBP1 and CtBP2 proteins and that the TCF-4 C-terminus is indispensable for the interaction.

Wnt signaling is activated in 293 cells co-cultivated with Wnt-1 protein-producing feeder cells

By retrovirus infection we generated several polyclonal rat fibroblast cell lines with constitutive (Rat2-Wnt-1/Const) or doxycycline-repressible (Rat2-Wnt-1/Dox) expression of the mouse *Wnt-1* gene (Fig. 3A). Because Wnt molecules possess only limited solubility and mostly stay attached to the cell surface of the producing cell, we activated Wnt signaling in 293 cells by co-cultivation with *Wnt-1*-expressing feeder cells. The human embryonic kidney 293 cells selected for the study activate the Tcf/β-catenin-dependent reporter if transiently transfected with *Wnt-1* expression plasmid, and, additionally, express high levels of *Tcf-4* mRNA and produce predominantly a longer variant, i.e. the TCF-4E form, of TCF-4 protein, which contains both CtBP-binding motifs [(27); Fig. 3B].

As shown in Figure 3C, Wnt-1-producing feeders stimulated the Tcf-reporter pTOPFLASH 6–7-fold as compared to control feeders. Co-transfection of an expression construct encoding dominant negative TCF-4, which lacks the N-terminal β-catenin interacting domain (ΔN_{Sma}TCF-4), completely abrogated the reporter gene activity, whereas a β-catenin expression plasmid strongly enhanced transcription from the pTOPFLASH reporter. The activity of the negative control reporter pFOPFLASH did not display any significant changes. We also employed a gel retardation supershift assay to determine whether Wnt signaling induces a nuclear TCF-4/β-catenin complex in stimulated 293 cells. We did not detect TCF–DNA complexes in nuclear extracts prepared from parental Rat2 fibroblast cells or Rat2-Wnt-1-transfected cells (not shown), therefore we performed gel retardations with compound nuclear extracts isolated from 293 and feeder cells. Using an optimal Tcf-motif as probe, we readily obtained two types of TCF–DNA complexes from parental and from both unstimulated and stimulated 293 cells; one of these complexes could be supershifted with a monoclonal antibody recognizing human TCF-4 (Fig. 3D). Although 293 cells express all four *Tcf/Lef* genes, previous studies showed that TCF-1 and TCF-3 remain associated with insoluble nuclear components and are not eluted from nuclei by conventional extraction procedures (73,74). Thus, we concluded that the second gel-retarded complex contained endogenous LEF-1 protein and we attempted to supershift this complex with an antibody recognizing LEF-1. Addition of the LEF-1-specific antibody to the binding reaction led to almost complete diminishing of the original protein–DNA complex while the TCF-4–DNA complex stayed intact (Fig. 3D). We assumed that the second TCF–DNA complex indeed contains LEF-1 protein, and that the interaction between LEF-1 and the DNA probe was destabilized by the antibody added. Stimulation of 293 cells by Rat2-Wnt-1 cells resulted in the appearance of additional bands that could be further supershifted with a β-catenin antibody (Fig. 3D). Taken together, these data provide clear proof that the exogenous Wnt signal delivered by the Wnt-1-producing feeder cells, is effectively transmitted into human 293 cells, stimulates the formation of TCF/β-catenin complexes and subsequently activates the transcription of Wnt-responsive promoters. We further used this cellular system to study the effects of CtBP1 on Wnt-induced transcription.

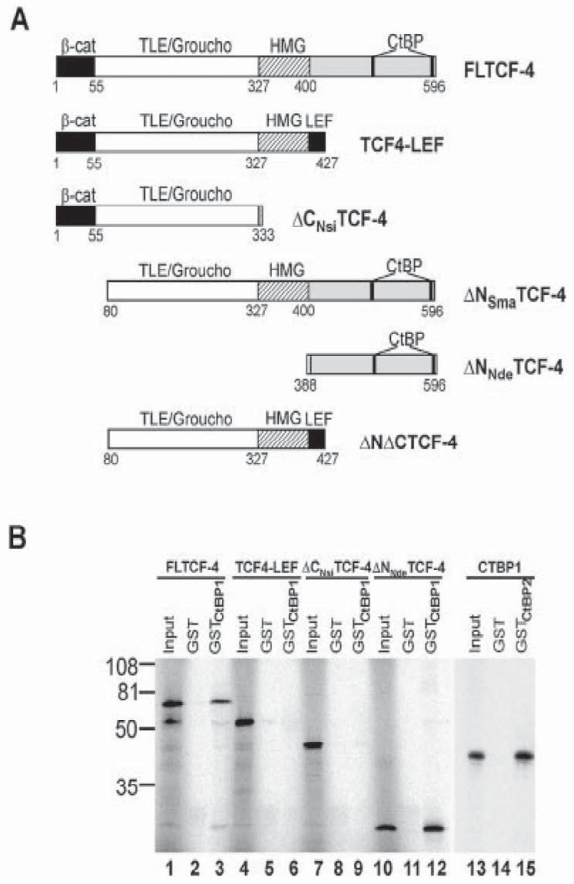

Figure 2. The C-terminus of TCF-4 interacts with CtBP1. (**A**) A schematic representation of the human TCF-4 deletion constructs used in this study. All constructs contained the N-terminal Myc-tag (not depicted). β-cat, β-catenin interaction domain; TLE/Groucho, TLE/Groucho binding domain; CtBP, CtBP-binding sites; HMG, DNA-binding domain; LEF, LEF-1 C-terminus. (**B**) *In vitro* interaction of TCF-4 with CtBP1. GST–CtBP1 and GST–CtBP2 fusion proteins were conjugated to glutathione–Sepharose beads and incubated with the indicated ^{35}S-labeled TCF-4 proteins translated *in vitro*. After washing and recovery of the beads, associated proteins were resolved by SDS–PAGE and analyzed by autoradiography. In lanes 1, 4, 7, 10 and 13, 10% of the input labeled proteins was applied directly onto the gel. Full-length TCF-4 (lane 3) and the TCF-4 C-terminal fragment (lane 12) bind to GST–CtBP1. TCF4–LEF-1 chimera (line 6) or the N-terminal fragment of TCF-4 lacking CtBP-binding sites (lane 9) do not interact with GST–CtBP1. *In vitro* labeled CtBP1 associates with GST–CtBP2 (lane 15). None of the proteins bind to GST-bound Sepharose beads (lanes 2, 5, 8, 11 and 14). The positions of molecular weight markers in kDa are indicated at the left.

CtBP1 represses TCF/β-catenin-dependent transcription by a trichostatin A-sensitive mechanism

CtBP proteins function as short-range transcriptional co-repressors, therefore we examined the functional relevance of the TCF-4–CtBP1 interaction in reporter assays. It has been reported previously that N-terminal modification of the CtBP1 protein may change its association with histone deacetylases (75); for that reason, we tested the activity of three different CtBP1 constructs encoding mCtBP1 with an N-terminal Myc-tag, EGFP-tagged mCtBP1 and, finally, full-length human CtBP1 without N-terminal modification. All three constructs repressed transcription from the Tcf-dependent pTOPFLASH reporter in a concentration-dependent manner in both unstimulated and stimulated 293 cells, whereas the activity of the control reporter pFOPFLASH was not affected by CtBP1 (Fig. 4A, only data for human CtBP1 are shown). CtBP1 was originally described as a protein interacting with the adenoviral oncogene E1A. Since 293 cells express E1A, we used an alternative cell system to avoid the possibility that the observed effects are dependent on E1A expression. We utilized the previously established transient β-catenin–Tcf reporter assay. In this assay, co-transfection of TCF-4 and β-catenin into COS-7 cells results in transactivation of the Tcf-dependent luciferase reporter gene (Fig. 4B). Upon addition of increasing amounts of CtBP1, the luciferase activity decreases proportionally only when the construct encoding full-length TCF-4 (FLTCF-4) capable of binding to CtBP was co-transfected. Transactivation mediated by β-catenin and C-terminally truncated TCF-4 (TCF4–LEF) was not affected by CtBP1. The reporter gene assays indicated that Tcf/β-catenin transcriptional activity is down-regulated by TCF-4–CtBP1 interaction. To study the mechanisms of CtBP1 action in greater detail, we generated 293 cells with inducible expression of EGFP-tagged mCtBP1. We obtained four polyclonal cell lines (293-EGFP–CtBP1/Dox) producing EGFP–mCtBP1 protein in the quantities representing approximately one-half of the amount of endogenous CtBPs (Fig. 5A). For further analysis we utilized a mixture of these cell lines. We further compared EGFP–CtBP1 production from the *CtBP1* transgene with the EGFP–CtBP1 levels obtained in transient transfections. Results of a western blot analysis showed that transfection of 0.5 μg of the CtBP1 expression construct generated cellular amounts of EGFP–CtBP1 protein similar to the levels obtained in 293-EGFP–CtBP1/Dox cells upon removal of doxycycline. Transfection of a larger quantity of the construct produced even more EGFP–CtBP1 protein than the amount of endogenous CtBPs (Fig. 5A). Nevertheless, the relatively moderate expression of the *EGFP–CtBP1* transgene significantly decreased pTOPFLASH transcription in Wnt-1-stimulated cells, whereas the activities of this reporter in 293-EGFP–CtBP1/Dox cells growing in the presence of doxycycline, i.e. with repressed CtBP1, were comparable to values obtained in the parental cell line (Fig. 5B). We readily obtained almost identical amounts of TCF/β-catenin complexes in Wnt-1-stimulated 293-EGFP–CtBP1/Dox cells independently of the expression status of the *CtBP1* transgene (not shown). This indicates that CtBP levels do not interfere directly with the formation of such complexes or with the upstream steps of Wnt signaling. We further determined whether the mechanism of CtBP1 repression relies on histone deacetylases. As shown in Figure 5C, treatment with trichostatin A, a histone deacetylase inhibitor, alleviated the repressive effect of CtBP1 in 293-EGFP–CtBP1/Dox cells. The trichostatin A treatment also completely neutralized the repressive effect of CtBP1 in transient transfection assays (Fig. 5D). We conclude that CtBP1 acts as a repressor of Wnt-mediated transcription via recruitment of histone deacetylases to the target promoter.

Besides CtBP, 293 cells express other TCF-4-interacting proteins, the TLE/Groucho co-repressors (52). Therefore, we asked next whether both types of negative regulators utilize the same inhibitory mechanisms. We examined the impact of different TCF-4 deletions on the activity of the pTOPFLASH reporter in unstimulated and stimulated 293 cells (see Fig. 2A

for details about the constructs used). TCF-4 constructs, when overexpressed in 293 cells, decreased the basal and the Wnt-1-primed activity of pTOPFLASH but, strikingly, TCF4-LEF, which interacts only with TLE/Groucho and lacks the C-terminal CtBP-binding domain, appeared to be more potent repressor than full-length TCF-4 (Fig. 5E). In addition, only repression mediated by full-length TCF-4, i.e. by a protein containing both TLE/Groucho and CtBP interaction regions, has been almost completely alleviated by the trichostatin A treatment. These results indicate that the TLE/Groucho proteins may utilize different mechanisms than do CtBPs to repress the Tcf-responsive promoter and/or that the action of these repressors might be mutually exclusive.

CtBP1 down-regulates the expression of Axin2, a Tcf/β-catenin target in 293 cells

We examined the expression levels of several human genes related to Wnt signaling in Wnt-1-stimulated 293 cells. We designed sets of human-specific primers that selectively amplified cDNA produced from 293 mRNA and did not function with rat or mouse cDNAs, respectively (see Materials and Methods). This allowed us to assess the level of expression of the selected human genes even in 293/Rat2 mixed RNA samples. Upon Wnt-1 stimulation, we noticed significant changes in expression of several genes: (i) we detected a moderate up-regulation of presumptive Wnt targets *Cyclin D1*, *Tcf-1* and *Lef-1* (see Supplementary Material) and (ii) we also detected strong transcriptional activation of *Axin2/Conductin*. Axin2 was recently identified by DNA-array technology as a gene positively regulated by TCF/β-catenin complexes in colorectal and liver tumors and in rat RK3E epithelial cells (76,77). We tested the time course of induction of the *Axin2* transcript in stimulated cells (Fig. 6A). We detected an 8-fold increase in *Axin2* mRNA as early as 3 h after stimulation (a 3 h interval was a minimal time period necessary for attachment of feeder cells onto the 293 cells). The maximal quantity of *Axin2* mRNA was reached after 9 h of co-cultivation. Following this time point, *Axin2* levels started to decrease but, nevertheless, after 15 h still remained approximately six times higher than in control 293 cells. Expression of the *Axin1* gene, an *Axin2* homolog, did not change significantly at any time point tested. We further

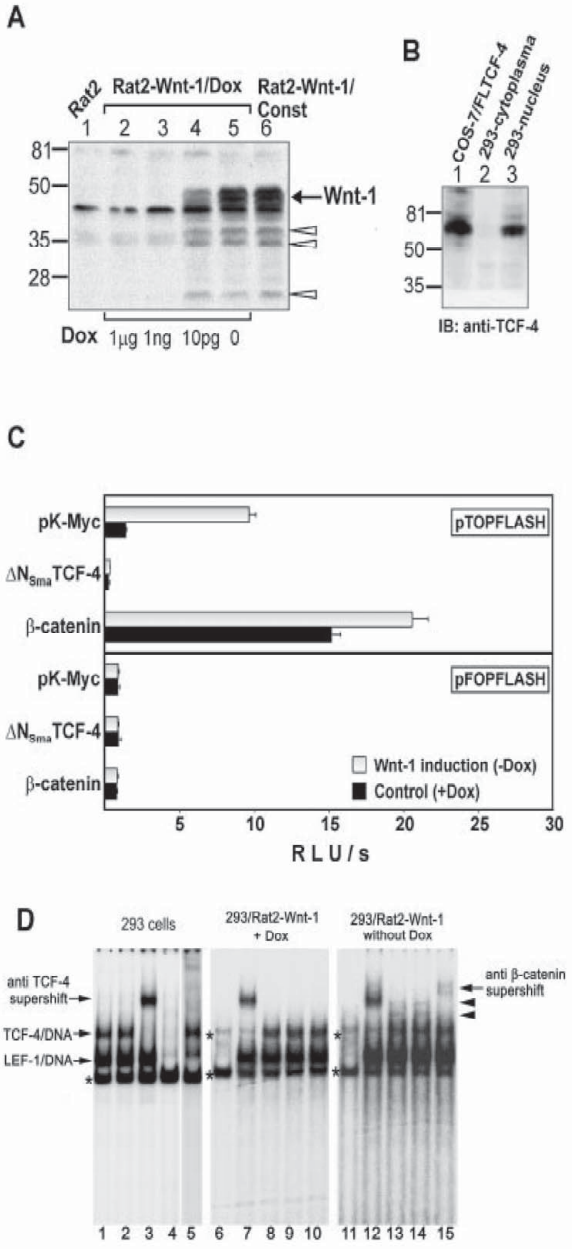

Figure 3. Wnt-1 protein-producing Rat2 feeder cells activate Wnt signaling in 293 cells. (**A**) Wnt-1 expression is strictly regulated in Rat2 fibroblasts by doxycycline. Western blot analysis of total cell lysates from parental Rat2 cells (lane 1), Rat2 cells with doxycycline-regulated Wnt-1 expression (Rat2-Wnt-1/Dox, lanes 2–5) and Rat2 cells with constitutive production of Wnt-1 (Rat2-Wnt-1/Const, lane 6). The Rat2-Wnt-1/Dox cells were grown at different concentrations (1 μg, 1 ng and 10 pg per ml) or in the absence of doxycycline, as indicated at the bottom. SDS–PAGE and immunoblotting with Wnt-1 antibody resolved cell lysates. The arrow shows the position of the putative Wnt-1 protein; the positions of shorter degradation products are indicated by open arrowheads. Molecular weight markers in kDa are at the left. (**B**) 293 cells express a longer form of TCF-4 protein. A total cell lysate from COS-7 cells transfected with expression vector encoding full-length TCF-4E protein (lane 1), and from cytoplasmic and nuclear fractions prepared from 293 cells (lanes 2 and 3, respectively) were resolved by SDS–PAGE and immunoblotted with an anti-TCF-4 antibody recognizing an epitope proximal to the HMG box. Molecular weight markers in kDa are at the left. (**C**) Wnt-1 expressing Rat2 cells induce the activation of the Tcf reporter in 293 cells. The human embryonic kidney 293 cell line was co-transfected with the expression plasmids indicated on the *y*-axis and the Tcf reporter construct pTOPFLASH (containing wild-type Tcf-binding sites) or pFOPFLASH (with mutated Tcf motifs) as a negative control. Four hours post-transfection, DNA mixtures were removed and Rat2-Wnt-1/Dox fibroblasts were subsequently plated over the 293 cells. Cultures were further grown either in the absence (Wnt-1 induction) or presence of doxycycline (control) for 15 h, then the cells were harvested together and processed to assay the reporter gene activities. Luciferase activities [shown on the *x*-axis as relative light units per second (RLU/s)] were corrected for the efficiency of transfection using the internal control *Renilla* pRL-SV40 expression plasmid. Average values and their standard deviations from three independent experiments are shown. (**D**) Wnt-1 induces nuclear TCF–β-catenin complexes in 293 cells. A gel retardation assay performed with nuclear extracts from parental 293 cells (left) and from 293 cells co-cultivated with Rat2-Wnt-1/Dox cells growing in the presence (control, middle) or absence (Wnt-1 induction, right) of doxycycline. Samples in lanes 1, 8 and 13 were incubated under standard conditions. Anti-β-catenin antibody was added to the samples in lanes 2, 10 and 15. Anti-TCF-4 antibody was added to the samples in lanes 3, 7 and 12. Anti-LEF-1 antibody was added to the sample in lane 5. Filled arrowheads indicate the positions of the TCF/β-catenin complexes. A control antibody (anti-HA tag) was added to the samples in lanes 9 and 14. Asterisks indicate non-specific bands also observed with a probe mutated in the Tcf-binding site (lanes 4, 6 and 11).

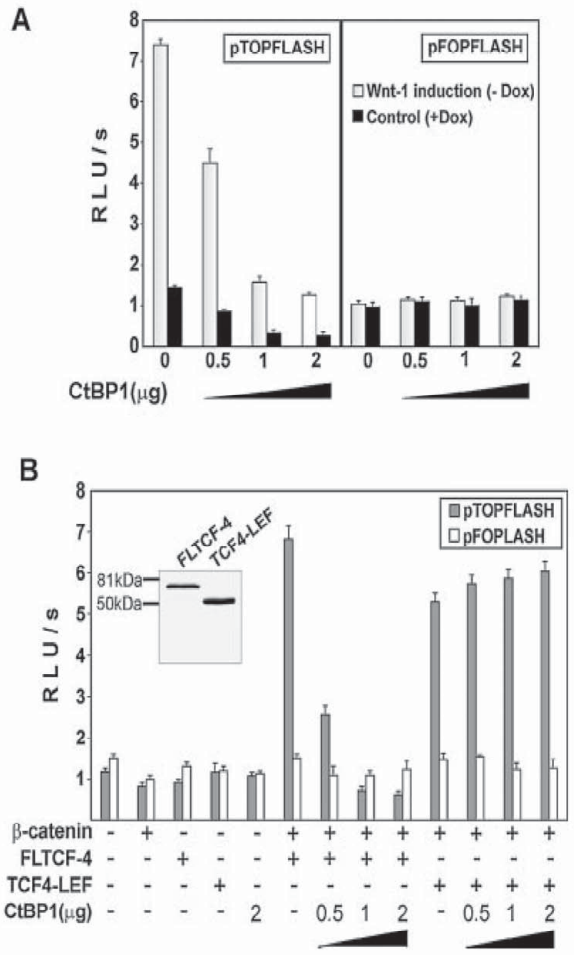

Figure 4. CtBP1 represses TCF/β-catenin transcription. (**A**) CtBP1 represses TCF/β-catenin transcription in 293 cells. Human 293 embryonic kidney cells were co-transfected with the indicated amounts of CtBP1 expression plasmid and the Tcf reporter construct pTOPFLASH or the negative control reporter pFOPFLASH using the lipofectamine reagent. Four hours post-transfection, DNA–lipofectamine mixtures were removed and 293 cells were covered with Rat2-Wnt-1/Dox fibroblasts containing the *Wnt-1* gene driven by the doxycycline-repressed promoter. The cultures were further grown in the presence (control) or absence of doxycycline (Wnt-1 stimulated). Following co-cultivation for 15 h, the cells were harvested, and luciferase (firefly) and *Renilla* luciferase activities were determined in cell lysates. (**B**) CtBP1 can repress transactivation mediated by TCF-4 and β-catenin in COS-7 cells. COS-7 cell line was co-transfected with the Tcf reporter constructs and a specific TCF-4 construct, β-catenin and with the indicated amount of CtBP1 plasmid. Luciferase and *Renilla* luciferase activities were determined in cell lysates 15 h following transfection. Whole cell extracts were analyzed by western blotting with TCF-4 monoclonal antibody (inset). All transfections were done in triplicate. Relative luciferase light units per second (RLU/s) are average values corrected for the efficiency of transfection by determining the luciferase/*Renilla* ratio.

examined the influence of CtBP1 overexpression on the activation of the *Axin2* gene. *Axin2* mRNA in 293-EGFP–CtBP1/Dox cells stimulated by Rat2-Wnt-1/Const feeder cells and with the repressed *EGFP–CTBP1* transgene was up-regulated 6-fold (compared to the same cells co-cultivated with parental Rat2 fibroblasts). However, the induction of *CtBP1* in Wnt-1-activated cells significantly down-regulated *Axin2* expression (Fig. 6B). These results indicate that a cellular dose of CtBPs modulates the activity of the Axin-2 promoter and, by implication, might selectively regulate the expression of other Wnt target genes.

DISCUSSION

In this report we provide evidence for an interaction between the TCF-4 protein, a member of the Tcf/Lef family of nuclear mediators of Wnt signaling, and the transcriptional co-repressor CtBP1. Using Wnt-1-stimulated human embryonic kidney 293 cells, we demonstrate that CtBP1 represses the transcriptional activity of a Tcf/β-catenin-dependent synthetic promoter and, furthermore, decreases the expression of the endogenous Wnt target Axin2. We further show that the inhibitory effect of CtBP is neutralized by the histone deacetylase inhibitor trichostatin A.

TCF/LEF proteins represent a specific type of transcription factor. They harbor β-catenin and TLE/Groucho interaction domains that determine whether, under given circumstances, the protein will act as a transcriptional activator or repressor. TCFs display essentially identical DNA-binding specificities, thus they have been considered as equivalent nuclear components of the Wnt signaling pathway. However, recent studies revealed functional differences among TCF family members. The most obvious feature of individual TCFs is their ability to associate with various cellular proteins. From that point of view, LEF-1 and TCF-3 proteins have been the most thoroughly studied Tcf/Lef family members (see Introduction). The main focus of this study is TCF-4 protein.

The limited solubility of Wnt proteins is a critical obstacle to studying the biological function of these molecules *in vitro*. We developed a dual cell system for Wnt pathway activation in which the target cells are stimulated by cells producing Wnt factors and growing in close proximity. This type of stimulation presumably corresponds well to the situation occurring *in vivo* in solid tissues and organs. At first, we generated rat fibroblast cell lines with constitutive or inducible Wnt-1 expression, and, using a Tcf/β-catenin-dependent reporter assay and a β-catenin supershift assay, we showed that these cell lines evidently activate Wnt signaling in neighboring 293 cells. β-Catenin overexpression from a transiently transfected construct resulted in a robust activation of the Tcf/β-catenin-dependent reporter, which was further augmented if the cells were simultaneously stimulated by Wnt-1-producing feeders (Fig. 3C). This indicates that β-catenin is indeed a key and also a limiting molecule in Wnt signaling. We further utilized this cell system to test the function of CtBP1 in the context of a Wnt-responsive promoter. Since 293 cells express E1A protein and CtBP1 was originally described as a protein interacting with this adenoviral oncoprotein, we used the COS-7 cell system in parallel to avoid the possibility that the observed effects are dependent on E1A expression. The repressive effect of CtBP1 depended directly on the expression levels obtained from either transiently or steadily transfected CtBP1 plasmids. This dosage-dependent repression corresponds well to the phenotype of mice harboring various combinations of *CtBP1* and *CtBP2* mutant alleles. *CtBP1*-null mice are small but viable, whereas *CtBP2* mutants die by E10.5 due to aberrant extraembryonic development. Different *CtBP1/CtBP2* compound mutant mice display additional dose-sensitive defects in a wide range of developmental processes (78). It is surprising that although 293 cells produce an abundance of LEF-1 (79), which is a protein that does not interact with CtBP, higher amounts of CtBP1 efficiently suppressed transcription from the Wnt-responsive

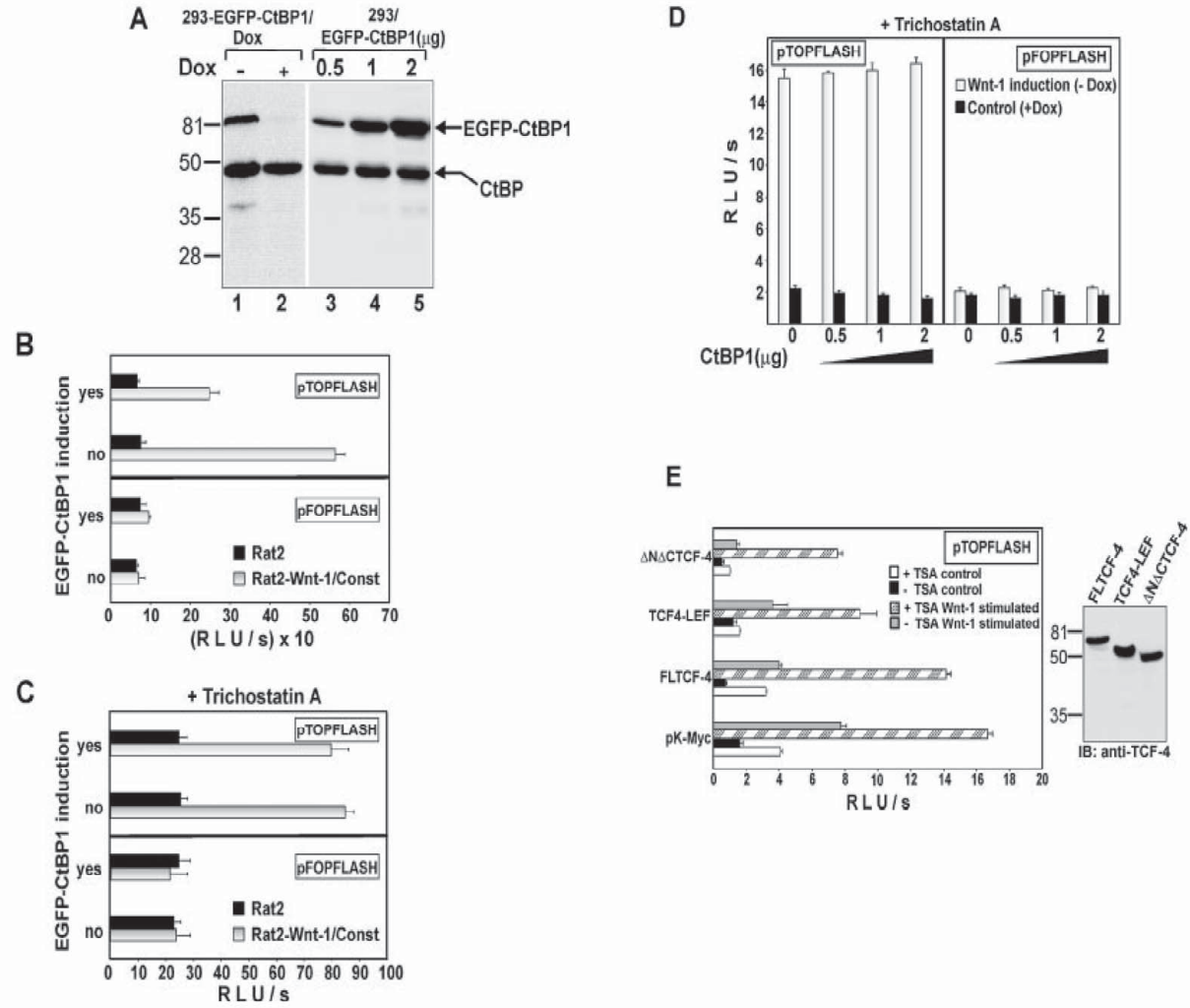

Figure 5. Histone deacetylases inhibitor trichostatin A alleviates the repressive effect of CtBP1 on the Wnt-responsive promoter. (**A**) 293-EGFP–CtBP1/Dox cells produce EGFP tagged mCtBP1 in quantities that represent one half of the amount of endogenous CtBPs. Total cell lysates from 293 cells expressing EGFP–CtBP1 from a doxycycline-repressive promoter growing in the absence (EGFP–CtBP1 induced, lane 1) or presence (EGFP–CtBP1 repressed, lane 2) of doxycycline (1 μg/ml) and from the parental 293 cell line transiently transfected with different amounts of plasmid encoding EGFP-tagged CtBP1 (lanes 3–5, micrograms of transfected construct are indicated on the top) were analyzed by western blot analysis. The anti-CtBP monoclonal antibody used to visualize proteins on the blots recognizes both human CtBP1 and CtBP2 proteins. The positions of molecular weight markers in kDa are indicated at the left. (**B**) Expression of the *EGFP–CtBP1* transgene down-regulates activity of the Wnt-resposive promoter in 293-EGFP–CtBP1/Dox cells. 293-EGFP–CtBP1/Dox cell line was transfected with the indicated Tcf reporter constructs. Four hours post-transfection, DNA mixtures were removed, the cells washed extensively and Rat2-Wnt-1/Const fibroblasts (steadily producing the Wnt-1 protein) or parental Rat2 cells were subsequently plated over the 293 transfectants. Cultures were further grown for 15 h either in the absence (EGFP–CtBP induction: yes) or presence (EGFP–CtBP induction: no) of doxycycline (1 μg/ml). (**C**) Trichostatin A treatment releases the repressive function of EGFP–CtBP1. The experiment was performed as described above in (B) except that trichostatin A (final concentration 300 nM) was added simultaneously with the feeder cells. (**D**) Trichostatin A treatment alleviates the CtBP1-mediated repression in transient transfection assay. Human 293 embryonic kidney cells were co-transfected with the indicated amounts of CtBP1 expression plasmid and the Tcf reporter constructs using the lipofectamine reagent. Four hours post transfection, DNA lipofectamine mixtures were removed and 293 cells were covered with Rat2-Wnt-1/Dox fibroblasts. The cultures were further grown in the presence (control) or absence of doxycycline (Wnt-1 stimulated). Trichostatin A (300 mM final) was added simultaneously with the feeder cells. Following co-cultivation for 15 h, the cells were harvested and luciferase and *Renilla* luciferase activities were determined in cell lysates. (**E**) Tcf-mediated repression is partially insensitive to trichostatin A. Human 293 embryonic kidney cells were co-transfected with empty expression vector pK-Myc or TCF-4 deletion constructs indicated on the *y*-axis and the Tcf reporter plasmid pTOPFLASH. TCF-4 proteins were produced at comparable levels in the transfected cells as shown by western blot analysis (right panel). Four hours post-transfection, cells were covered with Rat2 cells (control) or Rat2-Wnt-1/Const cells (Wnt-1 stimulated), and half of the samples were further treated with trichostatin A (TSA, final concentration 300 nM). Following an additional 12 h, the cells were harvested, and luciferase and *Renilla* luciferase activities were determined in cell lysates. Transfections were done in triplicate. Average luciferase light units per second (RLU/s) corrected to *Renilla* luciferase activities and their standard deviations are shown.

reporter to basal levels (Fig. 4A). This fact might be explained by the greater ability of TCF-4 to form a ternary complex with DNA and β-catenin (80).

TCF-4 (and TCF-3) resembles two other repressors, Hairy and Brinker, which also interact with both TLE/Groucho and CtBP proteins (81,82). The precise mechanisms of how TLE/Groucho and CtBP contribute to gene repression remain to be elucidated. In *Drosophila*, Groucho functionally interacts with the histone deacetylase Rapd3 (51). Recruitment of Rapd3 to target promoters would result in the formation of a more compact chromatin structure followed by a transcriptionally repressed state. The recruitment of histone deacetylases is likely to be the main function of the TLE/Groucho proteins, although only the partial release of TLE/Groucho-Rapd3

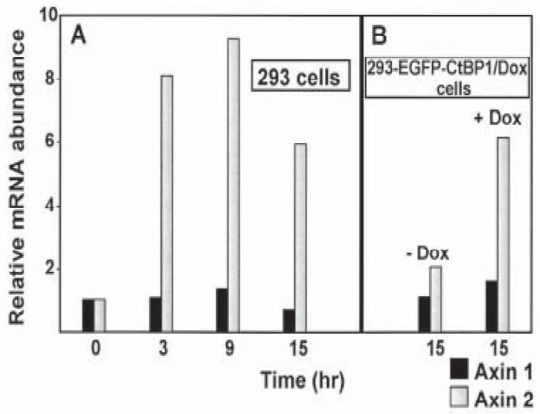

Figure 6. CtBP1 down-regulates the expression of Axin2 in 293 cells. (**A**) Wnt-1 activates *Axin2* mRNA in 293 cells. The results of quantitative real-time PCR performed with cDNA generated from 293 cells stimulated by Wnt-1-producing feeder cells (Rat2-Wnt-1/Const) or by control Rat2 cells are shown. The 293 and feeder cells were co-cultivated for the indicated period of time, then harvested and random primed cDNA was prepared from total RNA. The PCR reactions were performed for each primer set in triplicate using cDNAs produced from at least two independent RNA isolates. (**B**) EGFP–CtBP1 decreases responsiveness of the *Axin2* gene to the Wnt-1 stimulation but has no effect on the expression of the *Axin1* gene. 293-EGFP–CtBP1/Dox cells were co-cultivated with feeder cells stably producing Wnt-1 protein (Rat2-Wnt-1/Const) or control Rat2 fibroblasts as a negative control. Cells were co-cultivated for 15 h in the presence (EGFP–CtBP1 repressed) or absence (EGFP–CtBP1 overexpressed) of doxycycline (1 μg/ml). The random primed cDNAs generated from the relevant RNAs were analyzed. The PCR reactions were performed for each primer set in triplicate using cDNAs produced from at least two independent RNA isolates. The results of a representative experiment are shown. The results were analyzed using the LightCycler 5.1 software package, and the values of a representative experiment are shown. The relative abundance of *Axin1* and *Axin2* mRNA in Wnt-1-stimulated versus control cells was derived from the average CT values of each triplicate after normalizing to the levels of *SDHA* cDNA.

repression by the histone deacetylase inhibitor trichostatin A indicates that additional mechanisms of repression may exist. CtBP also induces transcriptional silencing in a histone deacetylase-dependent or -independent manner. CtBP1 associates with class I and class II histone deacetylases, and CtBP-related down-regulation of certain promoters has been reported to be sensitive to trichostatin A (83). In contrast, repression of several other promoters was insensitive to trichostatin A treatment (75). We detected a complete release of the CtBP-mediated repression by trichostatin A. This clearly indicates the involvement of the histone deacetylases in the repression mechanism. Since 293 cells express both TLE/Groucho and CtBP proteins (52 and this manuscript), we further evaluated the contribution of the TLE/Groucho and CtBP co-repressors to the repression of the activity of the pTOPFLASH reporter. Overexpression of TCF-4–LEF deletion containing only the TLE/Groucho interaction domain significantly inhibited the basal and Wnt-1-dependent activity of the Tcf reporter. This is in agreement with the observation that the C-terminally truncated forms of XTCF-3 keep their repressive potential in *Xenopus* embryos, even if they lack the CtBP-interacting domains (61). Surprisingly, the putative repressive action of TLE/Groucho was only partly suppressed by trichostatin A, and a greater sensitivity to this chemical was only revealed by the full-length TCF-4-mediated repression (Fig. 5E). This implies the possibility of parallel repressive action of CtBP and TLE/Groucho, which might contribute to the regulation of different Wnt targets. Since the TLE/Groucho and CtBP-binding sites are quite far apart in TCF-4 and TCF-3 proteins, we assume that there is no steric competition for these sites as in Hairy protein (82).

We took advantage of the different origin of human 293 and rat feeder cells and used real-time quantitative RT–PCR to analyze the changes in expression of selected genes upon Wnt stimulation. The strongest up-regulation was observed for *Axin2* mRNA. This finding was rather expected because Axin2 was recently identified as a gene activated by β-catenin overexpression in the related rat RKE3 cells (76). We further evaluated the impact of CtBP1 overexpression on Axin2 activation and detected a strong reduction in the levels of *Axin2* mRNA. Such a robust effect was surprising because the quantities of the ectopically expressed CtBP1 protein represented approximately one-half of the amount of endogenous CtBPs. 293 cells express all four TCF/LEF proteins, although at different levels, TLE/Groucho, and both CtBP1 and CtBP2 co-repressors (V. Korinek, unpublished data). TCF-4 associates with TLE/Groucho proteins and also with CtBPs, and these proteins also form homo- or heterodimers. The strength of the interaction between CtBPs and their partners can be further potentiated by the levels of nuclear NAD(H) (84,85). We propose that the composition of various Wnt signaling pathway components and their expression levels dictate the primary sensitivity of a given cell to the Wnt signal. The balance in such a system can be disturbed by changing the levels of only one component. Likewise the defects in other negative regulators of Wnt signaling (e.g. in the tumor suppressor APC), mutations that inactivate CtBP proteins could also lead to inappropriate activation of the Wnt pathway. On the other hand, non-physiological increases in the expression levels of CtBPs might by implication result in a significant reduction in Wnt signaling output in the stimulated cell.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

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SUPPLEMENTARY MATERIAL

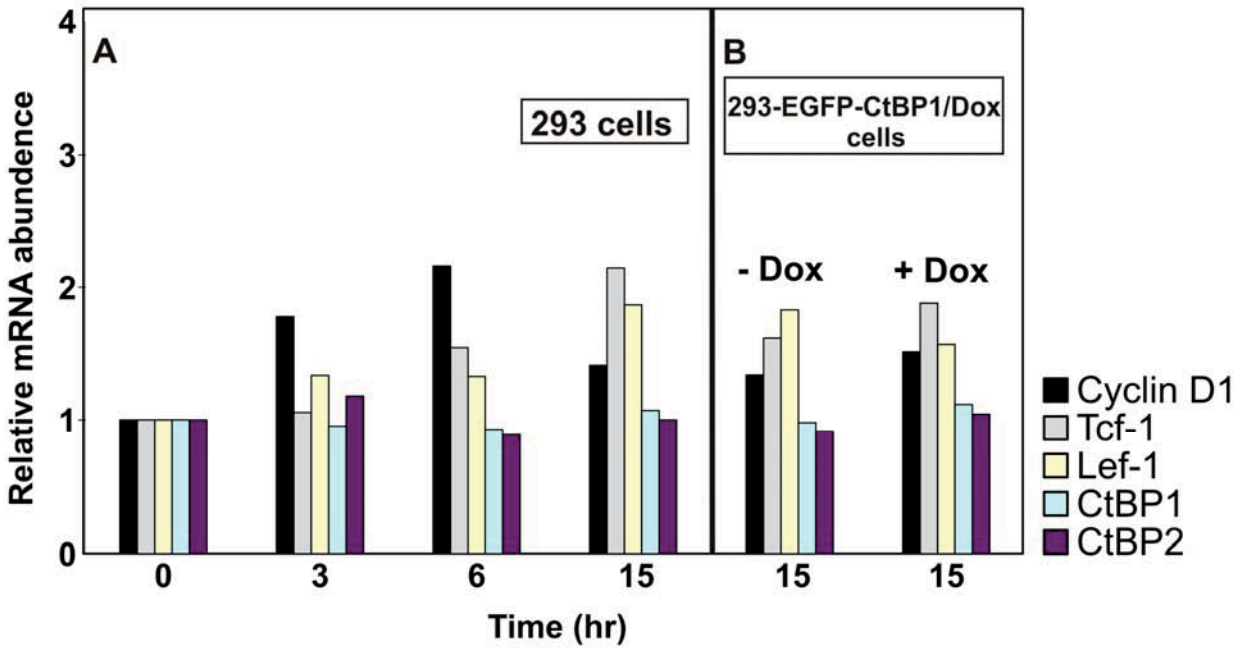
Wnt-1 upregulates Wnt-targets *Cyclin D1*, *Lef-1* and *Tcf-1* mRNA in 293 cells

A, Results of quantitative real-time PCR performed with cDNA generated from 293 cells stimulated by feeder cells stably producing Wnt-1 protein (Rat2-Wnt-1/Const) or by control Rat2 cells are shown. The 293 and feeder cells were co-cultivated for the indicated period of time, then harvested, and random primed cDNA was prepared from total RNA.

B, CtBP1 overexpression has no obvious effect on the levels of *Cyclin-1*, *Lef-1*, *Tcf-1*, *CtBP1,* and *CtBP2* RNA in Wnt-1-stimulated 293 cells. 293-EGFP-CtBP1/Dox cells were co-cultivated with Rat2-Wnt-1/Const or control Rat2 fibroblasts as a negative control for 15 hours in the presence (EGFP-CtBP1 repressed) or absence (EGFP-CtBP1 over-expressed) of Doxycycline (1 µg/ml). The random primed cDNAs generated from the relevant RNAs were analysed. The picture shows the relative abundance of *Cyclin D1, Lef-1*, *Tcf-1*, *CtBP1* and *CtBP2* mRNAs in Wnt-1-stimulated 293-EGFP-CtBP1/Dox cells versus control 293-EGFP-CtBP1/Dox in two situations: (1) when EGFP-CtBP1 is expressed (-Dox) or (2) repressed (+Dox).

The results were analyzed using the LightCycler 5.1 software package, and the values of a representative experiment are shown. The relative abundance of *Cyclin-1*, *Lef-1*, *Tcf-1*, *CtBP1* and *CtBP2* mRNA in Wnt-1-stimulated versus control cells was derived from the average CT values of each triplicate after normalizing to the levels of *SDHA* cDNA.

Figure 6



HIC1 attenuates Wnt signalling by recruitment of TCF4 and β-catenin to the nuclear bodies

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HIC1 attenuates Wnt signaling by recruitment of TCF-4 and β-catenin to the nuclear bodies

Tomas Valenta, Jan Lukas, Lenka Doubravska, Bohumil Fafilek and Vladimir Korinek*

Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague, Czech Republic

The hypermethylated in cancer 1 (*HIC1*) gene is epigenetically inactivated in cancer, and in addition, the haploinsufficiency of *HIC1* is linked to the development of human Miller–Dieker syndrome. *HIC1* encodes a zinc-finger transcription factor that acts as a transcriptional repressor. Additionally, the HIC1 protein oligomerizes via the N-terminal BTB/POZ domain and forms discrete nuclear structures known as HIC1 bodies. Here, we provide evidence that HIC1 antagonizes the TCF/β-catenin-mediated transcription in Wnt-stimulated cells. This appears to be due to the ability of HIC1 to associate with TCF-4 and to recruit TCF-4 and β-catenin to the HIC1 bodies. As a result of the recruitment, both proteins are prevented from association with the TCF-binding elements of the Wnt-responsive genes. These data indicate that the intracellular amounts of HIC1 protein can modulate the level of the transcriptional stimulation of the genes regulated by canonical Wnt/β-catenin signaling.

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Introduction

The Wnt signaling pathway plays essential roles in different developmental processes, including cell determination, stem cell survival and organogenesis. In addition, mutational activation of this pathway is implicated in deregulated cell growth and cancerogenesis (reviewed in Logan and Nusse, 2004; Reya and Clevers, 2005). The stabilization of β-catenin is central to the canonical Wnt pathway. In the absence of Wnt signals, β-catenin is phoshorylated by a complex of proteins, including adenomatous polyposis coli (APC), glycogen synthase kinase-3β and axin. Phosphorylation of β-catenin results in its ubiquitylation and degradation by the proteasome. Wnt factors inhibit the APC complex. The result of such inhibition is the stabilization of β-catenin, which accumulates in the cell and translocates into the nucleus where it associates with transcription factors of the TCF/LEF family. TCF/LEF proteins function as nuclear effectors of the Wnt signaling pathway. The DNA-binding specificity of these polypeptides is defined by the HMG box, an 80 amino-acid domain whose primary sequence is virtually identical in all TCF/LEF family members (reviewed in Clevers and van de Wetering 1997). TCF/LEF factors possess only a limited ability to activate transcription. They act as 'connectors' linking other polypeptides to a distinct set of promoters. β-Catenin contains a strong transcription activation domain, thus its interaction with TCFs results in transcription of the Wnt-responsive genes. Many of these genes execute Wnt-mediated cell specification during development or regulate cell proliferation (for more detailed information, refer to the Wnt homepage http://www.stanford.edu/~rnusse/wntwindow.html). In contrast, mammalian TCFs also bind to TLE/Groucho corepressors and, in the absence of the Wnt signal, repress the transcription of TCF-specific promoters (Roose *et al*, 1998). Additionally, two TCF/LEF family members, TCF-3 and TCF-4, associate with C-terminal binding proteins (CtBPs) (Brannon *et al*, 1999; Valenta *et al*, 2003).

The CtBP proteins bind to a short sequence motif PLDLS conserved among the E1A proteins of all human and primate adenoviruses. Different variants of this motif are also present in many other CtBP-interacting partners that function mainly as sequence-specific DNA-binding transcription factors.

In this study, we focused on one of the CtBP-associating proteins, hypermethylated in cancer 1 (HIC1), which redirects CtBP to a specific set of nuclear dot-like structures called HIC1 bodies (Deltour *et al*, 2002). The *HIC1* gene was identified as a candidate tumor suppressor gene frequently epigenetically silenced or deleted in different types of solid tumors (Herman and Baylin, 2003). *HIC1* encodes a zinc-finger transcription factor that belongs to a group of proteins known as the BTB/POZ family (Broad-Complex, Tramtrack, Bric à brac/poxvirus, and zinc finger) (reviewed in Albagli *et al*, 1995). A 714 amino-acid human HIC1 polypeptide contains the N-terminal BTB/POZ domain involved in dimerization and in protein–protein interactions. The C-terminal region interacts with a specific DNA sequence; the GLDLSKK motif responsible for the interaction with the CtBP proteins is located in the central part. Gene inactivation experiments in mice recently confirmed that HIC1 is a genuine tumor suppressor. Heterozygous $Hic1^{+/-}$ mice develop malignant spontaneous tumors after a year of life (Chen *et al*, 2003, 2004). These tumors show dense methylation of the remaining wild-type *Hic1* allele promoter accompanied by a complete absence of *Hic1* expression in the cancer tissue. *HIC1* gene resides within a 350 kb region on chromosome 17p13.3, deleted in most patients with Miller–Dieker syndrome (MDS) (Dobyns and Truwit, 1995). This links (in addition to tumorigenesis) the haploinsufficiency of *HIC1* to the development of MDS.

In the present study, we show specific binding between HIC1 and a principal Wnt signaling pathway component, TCF-4. We further demonstrate that overexpression of HIC1

*Corresponding author. Institute of Molecular Genetics AS CR, Videnska 1083, 142 20 Prague 4, Czech Republic. Tel.: +4202 41062471; Fax: +4202 44472282; E-mail: korinek@biomed.cas.cz

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suppresses the TCF-mediated transcription, and *vice versa*, the inactivation of endogenous HIC1 by RNA interference (RNAi) increases the basal expression of the *Axin2* gene and elevates the transcriptional response of this Wnt signaling target to Wnt stimulation. A deletion mutant of HIC1 lacking the oligomerization BTB/POZ domain can neither form the nuclear bodies, nor antagonize Wnt signaling, nor interact with TCF-4 *in vivo*. This clearly indicates that the HIC1 inhibitory function depends on the ability to form nuclear bodies and to recruit TCF-4 into these structures. Interestingly, β-catenin is also relocated by HIC1, but this sequestration seems to be indirect and mediated via its interaction with TCF-4. In addition, we provide evidence that CtBP1 increases the efficiency of recruitment of the TCF-4 into the HIC1 bodies and further strengthens the suppressive effect of the HIC1 protein on Wnt signaling. Finally, using chromatin immunoprecipitation (ChIP), we show that as a consequence of the relocation into the HIC1 speckles, TCF-4 and β-catenin are prevented from binding to the promoters of the TCF-responsive genes.

Results

HIC1 sequesters TCF-4 into nuclear bodies

Exogenous CtBP1 is distributed in the cytoplasm and in the nucleus in a mostly diffused pattern. Only a fraction of nuclear CtBP1 is localized into distinct structures, described previously as CtBP bodies (Sewalt *et al*, 1999). Cotransfection of the full-length HIC1 construct results in complete relocation of CtBP1 into the nuclear HIC1 bodies (Figure 1B). This relocation is dependent on the direct interaction between HIC1 and CtBP1, as the HIC1-ΔCtBP polypeptide lacking the CtBP-interacting motif loses the translocative properties of the wild-type HIC1 protein. The HIC1 mutant with a deletion encompassing the N-terminal oligomerization BTB/POZ domain still preserved the ability to 'pull' CtBP1 into the nucleus. However, the localization of both proteins was diffuse and no formation of nuclear punctuated structures was observed.

Previously, we have shown an interaction between CtBP1 and TCF-4 in yeast and *in vitro* (Valenta *et al*, 2003). However, we and others in following studies were unable to detect the association of these proteins in mammalian cells. We took advantage of the clear nuclear targeting of CtBP1 by HIC1 and visualized TCF-4 and CtBP1 using confocal microscopy.

In CtBP-positive COS-7 cells, TCF-4 was efficiently sequestered into the HIC1 bodies (Figure 2B). Triple staining and the overlay of the images showed that in these bodies TCF-4, HIC1 and CtBP1 co-localized. CtBP interacts with both, TCF-4 and HIC1 proteins, thus we supposed that the TCF-4 recruitment was mediated by CtBP. As expected, mutated TCF-4 (TCF-4mutCtBP), which is unable to bind CtBP, was not sequestered into the HIC1 bodies even in the *CtBP1* background (Figure 2C). Surprisingly, in *CtBP(−/−)* cells (these cells were derived from $CtBP1^{-/-}CtBP2^{-/-}$ embryos (Hildebrand and Soriano, 2002)), wild-type HIC1 and TCF-4 still partially colocalized in the nuclear dots. We examined next, whether variants of HIC1 differ in their ability to concentrate TCF-4 into the HIC1 bodies in *CtBP1(+)* cells. Whereas wild-type proteins perfectly colocalized in nuclear dots, the HIC1-ΔCtBP mutant displayed only limited capability to sequester nuclear TCF-4.

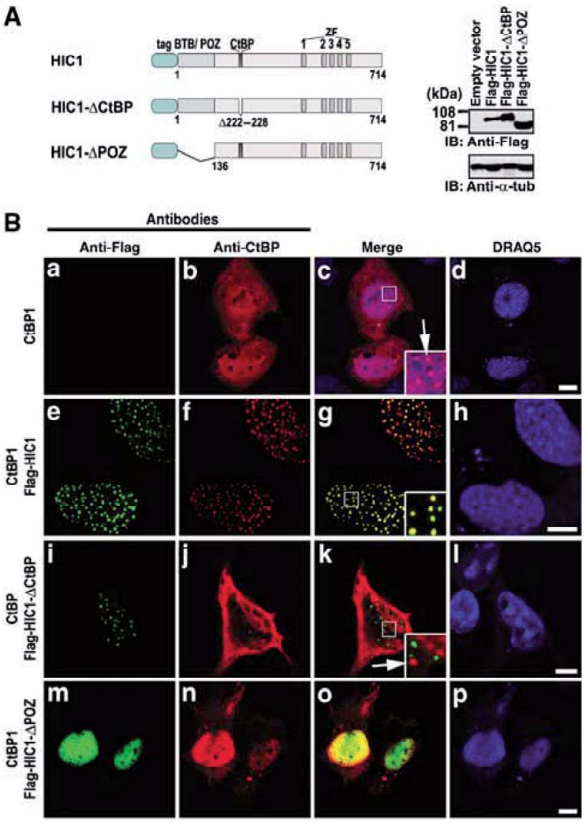

Figure 1 HIC1 targets CtBP into the nuclear bodies. (**A**) A schematic representation of the human HIC1 constructs used in this study. tag, Flag or EGFP tag; BTB/POZ, the BTB/POZ domain; CtBP, CtBP-binding site; ZF, five C_2H_2 Krüppel-like zinc fingers. Right, Western blots of total cell extracts after transfection with the Flag-HIC1 constructs, probed with anti-Flag or with anti-α-tubulin as internal control. (**B**) Confocal microscopy images of *CtBP(−/−)* cells trasfected with constructs indicated on the left and subsequently stained with mouse anti-Flag and rabbit anti-CtBP antibody. The DRAQ5 nuclear stain was gained in the blue channel. Mutant HIC1-ΔCtBP polypeptide lacking the CtBP-interacting motif displays the punctuated expression of the wild-type HIC1 protein (compare (e) and (i)) but does not influence the distribution of CtBP (compare (b) and (j)). As seen in (j, k), a fraction of nuclear CtBP is still localized in the CtBP bodies (arrows in insets (c, k)), which evidently differ from the HIC1 bodies. Bar, 10 μm.

Taken together, these results suggested that TCF-4 and HIC1 could form nuclear aggregates even in the absence of CtBP; nevertheless, CtBP mediates more efficient recruitment of TCF-4 into the HIC1 bodies.

TCF-4 binds directly to HIC1

The existence of HIC1/TCF-4 complexes in mammalian cells was evidenced using co-immunoprecipitation of HIC1 with endogenous TCF-4. By using anti-TCF-4 antibody, a robust coisolation of TCF-4 with wild-type HIC1, and with HIC1-ΔCtBP, was obtained from lysates of human 293 cells; the truncated HIC1-ΔPOZ protein did not co-immunoprecipitate with TCF-4 (Figure 3A). TCF-4 was also isolated from the Flag-tagged HIC1 using anti-Flag or anti-HIC1 antibodies. Furthermore, we immunoprecipitated the endogenous HIC1/TCF-4 complexes from whole-cell lysates prepared from mouse embryos on day 12.5 p.c. We also performed coimmunoprecipitation experiments in mouse *CtBP(−/−)*

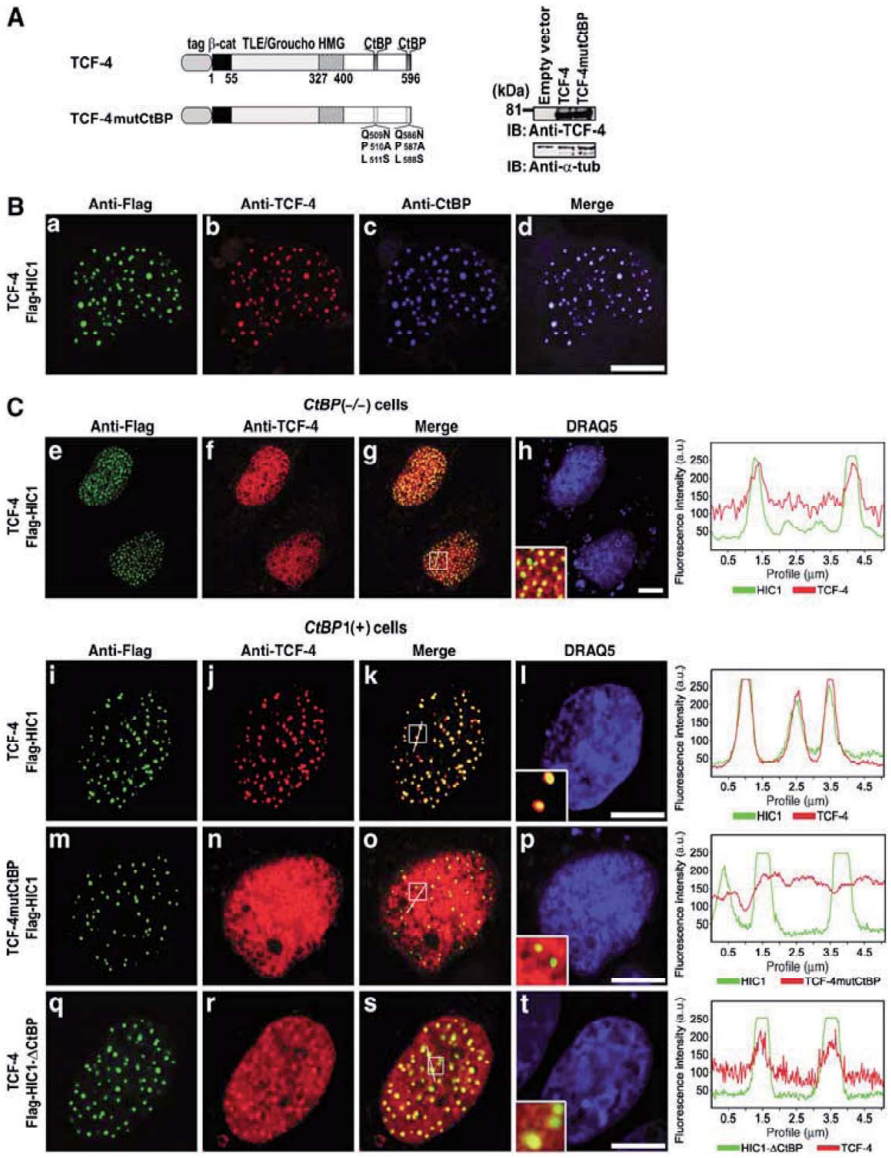

Figure 2 TCF-4 and HIC1 form nuclear protein complexes in mammalian cells. (**A**) TCF-4 expression constructs. The TCF-4mutCtBP polypeptide has a triple amino-acid substitution of each CtBP-binding motif as indicated. tag, myc or EGFP tag; β-cat, β-catenin interaction domain; TLE/Groucho, TLE/Groucho binding domain; CtBP, CtBP binding sites; HMG, DNA-binding domain. Right, Western blots of total cell extracts after transfection with the indicated TCF-4 constructs, probed with anti-TCF-4 or with anti-α-tubulin. (**B**) CtBP, HIC1 and TCF-4 colocalize in COS-7 cells. (**C**) Simultaneous interaction between CtBP, TCF-4 and HIC1 is essential for the efficient nuclear sequestration of TCF-4 into the HIC1 bodies. Confocal microscopy images of *CtBP(−/−)* and *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. The nuclear sequestration of TCF-4 by HIC1 is less efficient in *CtBP(−/−)* than in *CtBP(+)* cells (compare (e, f, g, h) to (i, j, k, l)). The formation of the TCF-4/HIC1 bodies in *CtBP(+)* cells strictly depends on the presence of the intact CtBP-binding sites in TCF-4 (m, n, o, p). Notice only a partial colocalization of TCF-4 and HIC1-ΔCtBP (q, r, s, t). Bar, 10 μm.

and *CtBP1(+)* cells, and we detected association of EGFP-tagged HIC1 and TCF-4 in both *CtBP*-negative and *CtBP1*-positive cells. The coimmunoprecipitation was specific for HIC1, because a parallel assay did not show any binding of a control, EGFP-nls protein to TCF-4 (Figure 3B, only results from *CtBP(−/−)* cells are shown). These results implied a direct interaction between TCF-4 and HIC1.

The direct binding between TCF-4 and HIC1 was studied *in vitro* by pull-down assays between bacterially expressed GST-tagged TCF-4 and *in vitro* translated HIC1. GST-TCF-4 associated only with *in vitro* translated full-length HIC1 and not with the N-terminally truncated HIC1-ΔPOZ (Figure 4B). As was shown by others, the deletion of the BTB/POZ domain involved in the homo- and heteromeric interactions often prevents the association of HIC1 with other partners, although this domain is not directly included in the protein–protein interaction (Deltour *et al*, 2002). Due to extensive degradation of GST-HIC1 in bacterial lysates, we could not test the binding of the full-length HIC1 immobilized on the glutathione-Sepharose beads to *in vitro* translated TCF-4. Nevertheless, we prepared GST-fusion proteins containing partly overlapping N-terminal, internal, and C-terminal HIC1 fragments (Figure 4A), and used these more stable proteins in pull-down assays. As shown in Figure 4B, all three

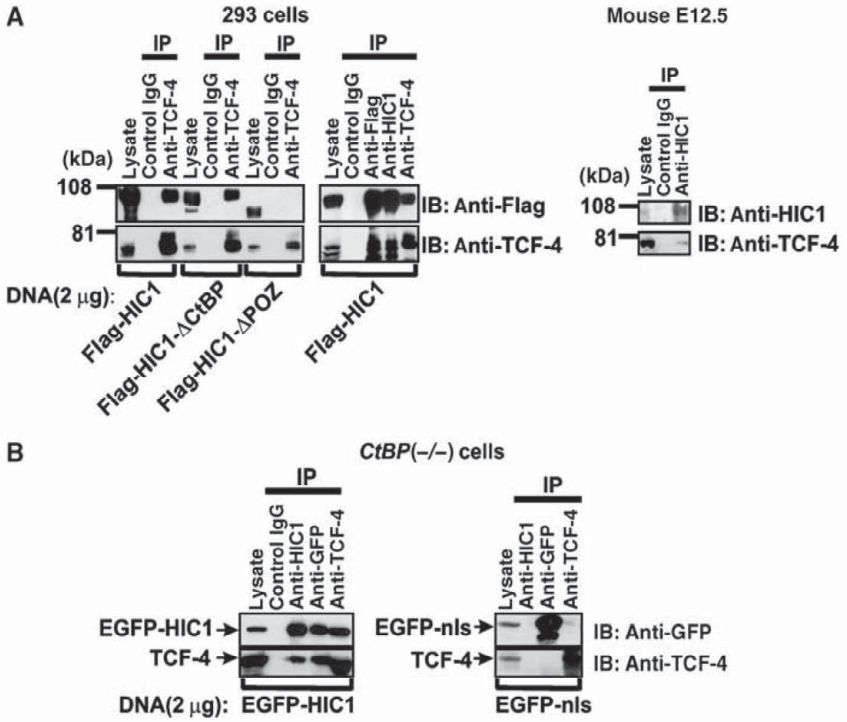

Figure 3 Association between TCF-4 and HIC1 *in vivo*. (**A**) Left, coimmunoprecipitations between endogenous TCF-4 and various exogenous HIC1 proteins in human 293 cells. Right, coimmunoprecipitations of endogenous HIC1 and TCF-4 in cells derived from mouse embryos on day 12.5 p.c. IP, immunoprecipitation; IB, immunoblotting; in lanes denoted 'lysate' five percent of the total sample were loaded. (**B**) TCF-4 associates with HIC1 in *CtBP(−/−)* cells. Coimmunoprecipitation of endogenous TCF-4 with EGFP-HIC1 is specific for HIC1 as evidenced by no detectable interaction between TCF-4 and EGFP-nls in a control experiment (right).

GST-HIC proteins interacted with full-length TCF-4; however, the strongest interacting domain was localized in the internal and C-terminal region of HIC1. Importantly, TCF-4mutCtBP binds to GST-HIC1 proteins equally well as the wild-type protein. Two nonoverlapping TCF-4 fragments were used to delineate domains involved in binding to HIC1. Whereas the C-terminus of TCF-4 interacted strongly with the GST-linked C-terminal part and the HIC1 internal fragments, a substantially less avid interaction was observed between the TCF-4 C-terminus and the GST-HIC1 N-terminus. Finally, the N-terminal part of TCF-4 did not show any affinity to HIC1. TCF-4 and HIC1 bind specific DNA sequences and their interaction domains were mapped in part to the DNA-binding regions of these proteins. To exclude the possibility that the association between TCF-4 and HIC1 is indirect and might be mediated by a DNA bridge from contaminating DNA, we performed a GST-pull-down with translated HIC1-mutZF3 protein containing a single amino acid exchange in the third zinc finger (Figure 4A). This mutation abolishes the DNA binding of the mutated protein to its recognition motif (Pinte *et al*, 2004). We also treated translated TCF-4 and glutathione-Sepharose bound GST-HIC1 with DNase I prior to the pull-down. As shown in Figure 4C, the HIC1-ZF3 associates with GST-TCF-4 with a comparable avidity to the wild-type protein and, moreover, the DNase I treatment even slightly improved *in vitro* binding of TCF-4 and HIC1.

Altogether, the data reported here indicated that HIC1 interacts directly with TCF-4 in a complex multidomain mode of interaction. These data also implied that this interaction is not dependent on the presence of the intact CtBP-binding sites in TCF-4.

HIC1 inhibits TCF/β-catenin-driven transcription

To examine whether HIC1-mediated sequestration of TCF-4 affects the Wnt-dependent transcription, pTOPFLASH was cotransfected with each of the HIC1 constructs into 293 cells, the cells were subsequently stimulated by the Wnt ligand and the levels of the TCF-mediated transcription were determined. In 293 cells, Wnt3a-containing medium induced robust 25-five fold activation of the Tcf reporter pTOPFLASH as compared to the control medium (Figure 5). The cotransfection of wild-type HIC1 resulted in a substantial decrease of the pTOPFLASH activity, the HIC1-ΔCtBP was less than half as effective as wild-type HIC1, and finally, the HIC1-ΔPOZ mutant appeared to be completely inefficient in the downregulation of the pTOPFLASH-driven transcription. We did not observe any effect whatsoever on the pTOPFLASH reporter in the nonstimulated cells. Nevertheless, HIC1 repressed the Wnt-induced transcription of a luciferase reporter containing a 5 kb promoter region of the well-established Wnt target *Axin2* gene (Figure 5) (Jho *et al*, 2002). Conversely, the HIC1 overexpression did not disturb the transcription from the negative-control reporter pFOPFLASH, or from the synthetic reporter G1E1B-Luc activated by Gal4-DBD-VP16 fusion protein (data not shown).

HIC1 contains five Krüppel-like C_2H_2 zinc fingers in its C-terminal part. Recently, Pinte *et al* (2004) investigated the DNA binding properties of the isolated zinc finger domain and defined a specific DNA motif recognized by HIC1. Full-length HIC1 binds probes with a single recognition site poorly; however, the wild-type protein interacts cooperatively with complex probes containing multiple HIC1-specific

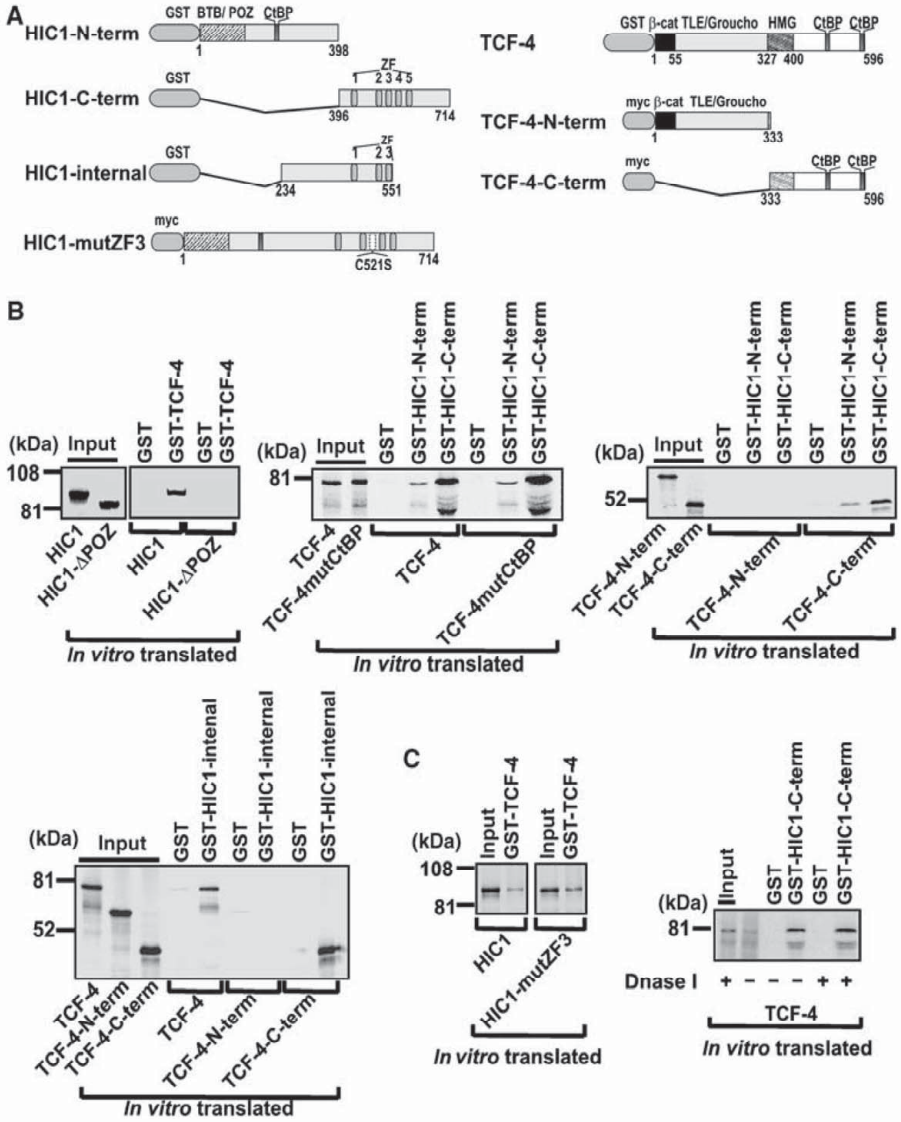

Figure 4 *In vitro* interaction of TCF-4 and HIC1. (**A**) Structures of HIC1 and TCF-4 proteins used in the *in vitro* pull-down assays (see also the diagrams of the additional HIC1 and TCF-4 constructs depicted in Figures 1A and 2A). (**B**) Pull-down assays between bacterially expressed GST-fusion and *in vitro* translated proteins, as indicated. Ten percent of the total reactions were loaded in lanes denoted 'input'. (**C**) Right, TCF-4/HIC1 interaction is resistant to DNase I treatment; left, the intact DNA-binding domain of HIC1 is not essential for the interaction with TCF-4.

sequences. In contrast, the N-terminally truncated HIC1-ΔPOZ, lacking the BTB/POZ domain, interacts preferentially with a probe containing a single HIC1-binding site. We performed an electrophoretic mobility-shift assay (EMSA) with *in vitro* translated TCF-4 and HIC1. HIC1-ΔPOZ, and with a lower efficiency, full-length HIC1, bound to a simple HIC1-specific probe, but these proteins did not interact directly with probes containing single or multiple TCF recognition motifs (Supplementary Figure 1).

The inhibitory effect of HIC1 on the transcriptional activation of pTOPFLASH was also found in DLD-1 cells, that is, cells with a mutant *APC* gene. The lack of functional APC protein in these adenocarcinoma cells results in the accumulation of β-catenin and in constitutive activation of the TCF-dependent target genes (van de Wetering *et al*, 2002; Rosin-Arbesfeld *et al*, 2003). By retroviral transduction, we generated DLD/HIC1 cells containing the *HIC1-EGFP* regulated by AP21967, a synthetic dimerizer (Ariad). We examined the levels of the TCF/β-catenin-dependent transcription simultaneously in three independent DLD/HIC1 cell lines at three different levels of HIC1-EGFP expression (dimerizer concentrations: 0, 0.25 and 25 nM). The result of a representative experiment is shown in Figure 6A. At maximum induction when HIC1 was produced in amounts comparable to the physiological levels of endogenous HIC1 in primary human WI38 cells (Figure 9C), the pTOPFLASH activity decreased to approximately 40% when compared to the DLD/HIC1 cells growing without the inducer (Figure 6A). Transcription from the negative control reporter pFOPFLASH did not change during the experiment. Since DLD-1 cells express high amounts of both TCF-4 and β-catenin (Korinek *et al*, 1997), we asked first whether HIC1 can function by decreasing the intracellular levels of β-catenin and/or TCF-4. Using anti-TCF-4 and β-catenin antibodies, we performed immunoblotting of cell lysates prepared from DLD/HIC1 growing with dimerizer for 5 days or without HIC1 induction.

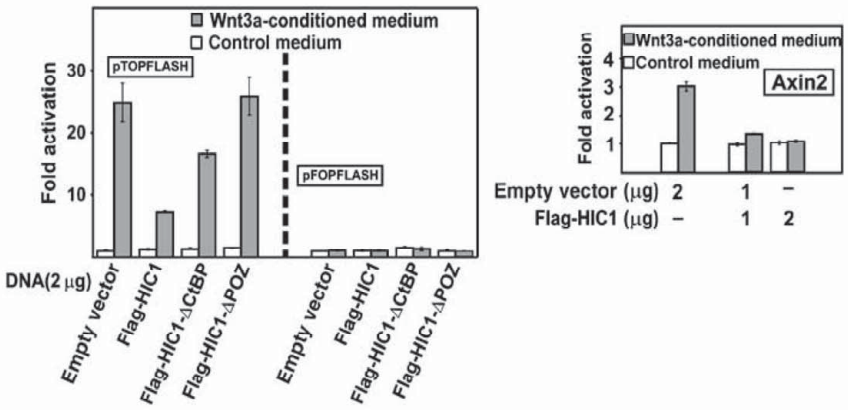

Figure 5 HIC1 represses the Wnt-stimulated transcription. Reporter gene assay with the Wnt-responsive promoters. 293 cells were cotransfected with the reporters and the HIC1 constructs as indicated and stimulated for 24 h with Wnt3a-conditioned or control medium. Luciferase (firefly) activities were corrected for the efficiency of transfection using the internal control *Renilla* luciferase expression plasmid. The reporter activity in unstimulated mock-transfected cells was arbitrarily set to 1. The histograms represent mean values of triplicate experiments and SDs (standard deviations) are shown by error bars.

The analysis revealed that HIC1 overexpression does not reduce the overall levels of the endogenous TCF-4 and β-catenin proteins in the cells (Figure 6A). However, HIC1 sequestered endogenous β-catenin and TCF-4 into the nuclear HIC1 bodies (Figure 6B and Supplementary Figure 2). Using anti-GFP antibody, we coimmunoprecipitated β-catenin, HIC1 and TCF-4 in one complex from 293 cells (Figure 6C). As we did not observe a direct interaction between β-catenin and HIC1 *in vitro* using pull-down assays (Supplementary Figure 3), we concluded that β-catenin associates with HIC1 indirectly, possibly by binding to TCF-4.

In summary, these data indicated that HIC1 specifically represses TCF-mediated transcription. Intriguingly, the repression is dependent on the recruitment of the TCF-4 to the HIC1 bodies rather than on the direct interaction of HIC1 with the promoters of the repressed genes.

HIC1 regulates Axin2 transcription

The *HIC1* gene is silenced by promoter methylation in most of the tumor-derived cell lines tested so far; nevertheless, we detected *HIC1* mRNA expression in human medulloblastoma DAOY cells, human primary fibroblast WI38 cells and also in mouse embryo STO cells. Using anti-HIC1 antibody, we visualized the nuclear HIC1 bodies in all these cells (see higher magnification insets in Figures 7B and 8A; staining of STO cells is not shown). Treatment of DAOY cells with 5-aza-2′-deoxycytidine (5-aza-2′-dCyt), that is, with an agent blocking DNA methylation, resulted in two-fold increase in the levels of *HIC1* mRNA (Figure 7A). This increased *HIC1* expression was also detected at the protein level and the 5-aza-2′-dCyt-treated cells contained a higher amount of larger HIC1 bodies than untreated cells (Figure 7B, insets). We further tested the effect of HIC1 knockdown on Wnt signaling. We found that in WI38 cells the *HIC1* mRNA level was reduced to 20% upon transfection with HIC1 short inhibitory RNAs (siRNAs), compared to an irrelevant control (a mixture of anti-GFP and anti-luciferase siRNAs) (Figure 8A). The effective downregulation of HIC1 in the transfected cells was confirmed by Western blotting and confocal microscopy (Figure 8A, inset). Recently, it was shown by Chen *et al* (2005) that HIC1 binds the SIRT1 promoter and directly represses its transcription. Thus, as expected, upon HIC1 knockdown we observed increased levels of the *SIRT1* mRNA; we also noted a 60% increase of basal transcription of *Axin2*, the Wnt signaling pathway target gene (Figure 8A). Interestingly, upon Wnt stimulation, the HIC1 siRNAs remarkably (almost two-fold) elevated only the transcriptional response of the Axin2 promoter, while the expression of the *SIRT1* and two housekeeping genes remained unchanged (Figure 8B). Such robust activation of *Axin2* was quite astonishing as the HIC1 siRNAs treatment already increased the levels of the *Axin2* mRNA in unstimulated cells. These data, obtained with physiological amounts of HIC1 and with the endogenous Wnt signaling target, supported our observations about HIC1 antagonizing Wnt signaling.

HIC1 diverts TCF-4 and β-catenin from the Wnt-responsive promoters

The mechanism of the HIC1 action on the endogenous TCF-responsive promoters was first studied by ectopic expression of full-length HIC1 in 293 cells. At the mRNA level, HIC1 overexpression partly blocked the increase in abundance of three Wnt signaling responsive genes *Axin2*, *Sp5* and *Cyclin D1* (Shtutman *et al*, 1999; Leung *et al*, 2002; Weidinger *et al*, 2005) observed in control cells upon 12- or 24-h stimulation with Wnt3a (Figure 9A). The inhibition appeared to be incomplete because about 50% transfection efficiency was routinely achieved for the 293 cells (data not shown). Thus, although HIC1 obviously inhibited the *Axin2*, *Sp5* or *CyclinD1* stimulation in the transfected cells, the overall amounts of the corresponding mRNAs were moderately elevated in Wnt3a-treated cell cultures. The mRNA abundance of *GAPDH* and *CtBP2*, that is genes with no direct relationship to Wnt signaling, did not change in the experiment.

To clarify the mechanisms of the HIC1-mediated repression, ChIP was performed on a cluster of TCF-binding sites in the Sp5 promoter (Supplementary Figure 4) (Takahashi *et al*, 2005). The ChIP assay showed that HIC1 did not associate with the Sp5 promoter; furthermore, the binding of endogenous TCF-4 to this promoter was decreased by wild-type HIC1 overexpression and not by HIC1-ΔPOZ in both stimulated and nonstimulated 293 cells (Figure 9B, only data for Wnt3a-

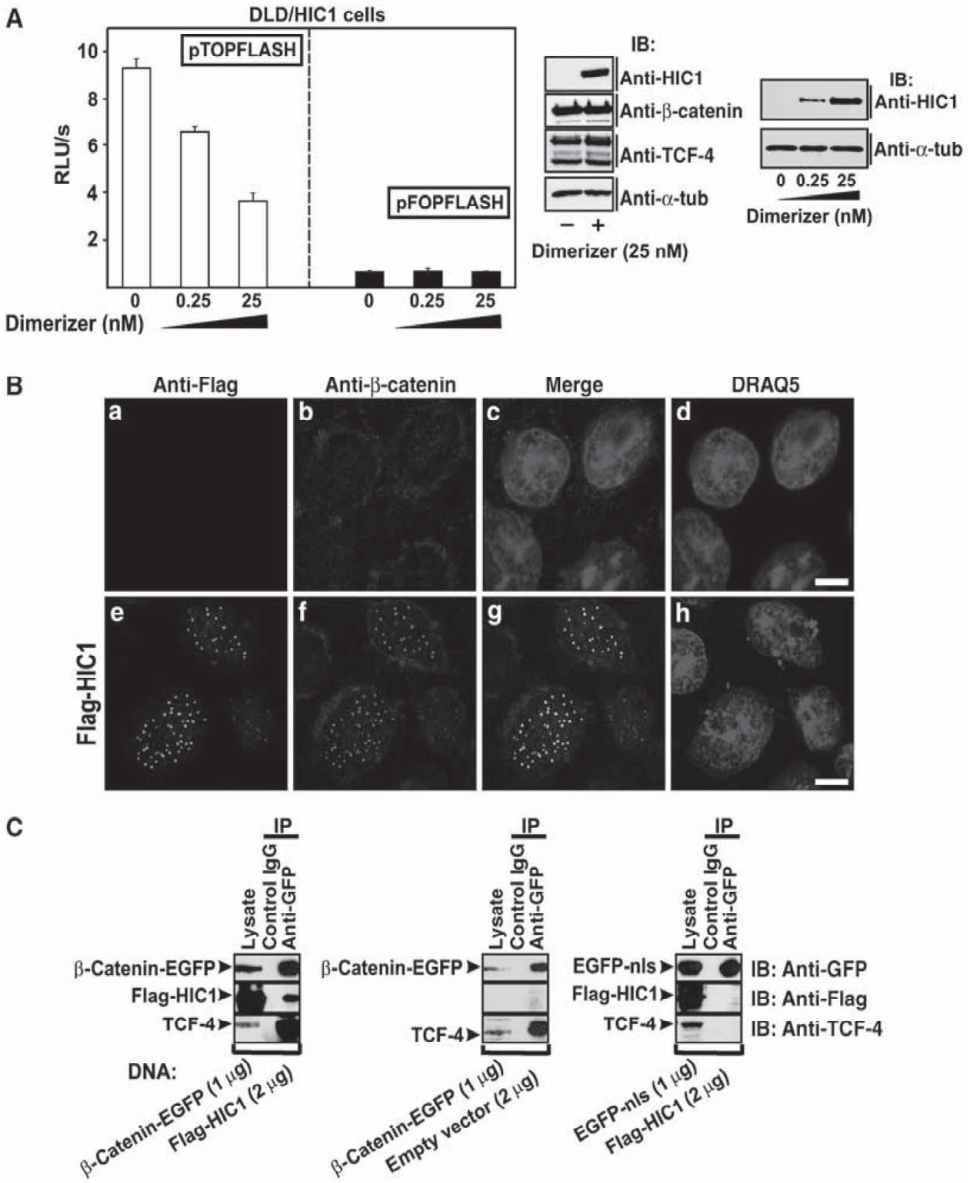

Figure 6 HIC1 represses TCF/β-catenin signaling in DLD-1 adenocarcinoma cells. (**A**) Right, transgenic DLD/HIC1 cells growing at higher concentrations of a synthetic compound AP21967 (dimerizer) contain increasing amounts of the HIC1 protein as evidenced by Western blots of total cell extracts probed with anti-HIC1 antibody. Middle, HIC1 expression does not influence the protein levels of TCF-4 and β-catenin. Left, the constitutive activity of the TCF-dependent reporter pTOPFLASH is suppressed by increasing amounts of the HIC1 protein. Average luciferase light units per second (RLU/s) corrected for the efficiency of transfection determined as the luciferase/*Renilla* ratio from five experiments are given (right, pFOPFLASH values). (**B**) Colocalization of HIC1 with endogenous β-catenin. Confocal micrographs of DLD-1 cells transfected with the full-length Flag-HIC1 construct stained with anti-Flag and anti-β-catenin antibody. Bar, 10 μm. (**C**) HIC1 expression does not disrupt the binding between TCF-4 and β-catenin. Coimmunoprecipitation of endogenous TCF-4 with ectopically expressed β-catenin is not affected by co-expression of HIC1 (compare left and middle panel). The coimmunoprecipitation is specific for β-catenin as indicated by a control experiment using the EGFP-nls instead of β-catenin-EGFP fusion protein (right).

induced cells are shown). We then co-transfected HIC1 and β-catenin, and performed ChIP with an antibody directed against this Wnt effector protein. The results unambiguously showed the presence of β-catenin on the Sp5 promoter in control cells (transfected with an empty vector or with the HIC1-ΔPOZ mutant which is deficient in TCF binding). In contrast, wild-type HIC1 completely eliminated association of exogenous β-catenin with the TCF-responsive element of the *Sp5* gene (Figure 9B).

In DLD-1 cells, we did not detect any *Sp5* mRNA and, moreover, expression of *Axin2* and *Cyclin D1* seemed to be independent of the TCF/β-catenin as the transient transfection of a dominant-negative form of TCF-4 (ΔN-TCF-4) did not reduce transcription of these Wnt signaling target genes. However, ΔN-TCF-4 inhibited production of *Tenascin C*, a recently identified TCF/β-catenin target gene active in colon carcinoma cells growing at the invasive front of the tumors (data not shown) (Beiter *et al*, 2005). For the ChIP assay we used DLD/HIC1 cells with regulated expression of the HIC1-EGFP transgene. Predictably, HIC1 expression did not inhibit the activity of the in DLD-1 cells TCF-independent Axin2 and Cyclin D1 promoters (Figure 9C). This was not caused by

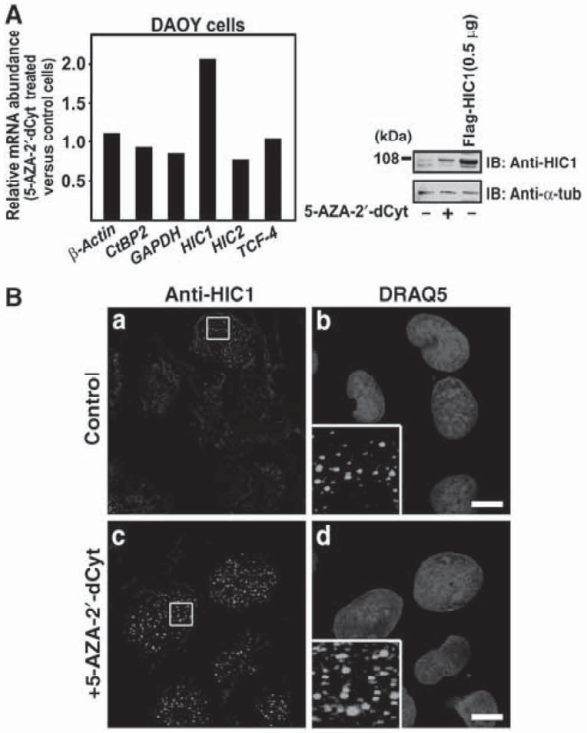

Figure 7 The nuclear HIC1 bodies in DAOY cells. (**A**) Increased expression of *HIC1* mRNA and protein in the cell line DAOY after treatment with 1 μM 5-aza-2′-deoxycytidine for 6 days. The expression was analyzed by qRT–PCR (left) or by Western blotting using anti-HIC1 antibody. In lane 3, a lysate from 293 cells transfected with 0.5 μg of the Flag-HIC1 construct was loaded. (**B**) Confocal micrographs of DAOY cells treated with 5-aza-2′-deoxycytidine (c, d) or with a vehicle (a, b) stained with the affinity purified anti-HIC1 antibody. Bar, 10 μm.

a dysfunction of the HIC1-EGFP fusion protein as HIC1-EGFP efficiently attenuated pTOPFLASH transcription (Figure 6A) and the ectopically expressed HIC1-EGFP construct displayed the same activity as wild-type HIC1 in suppressing. Nevertheless, HIC1 efficiently blocked transcription from a promoter of the *Tenascin C* gene (Figure 9C). ChIP analysis of the proximal TCF-dependent DNA element in the *Tenascin C* promoter (Supplementary Figure 4) revealed a clear binding of TCF-4 and β-catenin in parental DLD-1 cells. In DLD/HIC1, upon induction with the dimerizer HIC1 significantly decreased the association of both TCF-4 and β-catenin with the *Tenascin C* promoter, but no interaction of this promoter, with HIC1 was observed (Figure 9D). Simultaneously, the functionality of HIC1-EGFP to bind its cognate DNA motif was confirmed by ChIP of the HIC1-recognition element in the SIRT1 promoter (Figure 9D). These data indicate that as a result of the recruitment to the HIC1 bodies, TCF-4 and β-catenin are prevented from associating with TCF target genes.

Discussion

Since the canonical Wnt/β-catenin signaling alone is unlikely to regulate multiple developmental programs initiated by the Wnt ligands, we searched for additional proteins that could modulate the function of the TCF/LEF factors. In the present study, we identified tumor suppressor HIC1 as a new nuclear modulator of the Wnt signaling pathway.

Nuclear sequestration of TCF-4 into the HIC1 bodies

HIC1 encodes a zinc-finger transcription factor that acts as a transcriptional repressor. Additionally, the HIC1 protein binds CtBP and via its N-terminal BTB/POZ domain forms nuclear aggregates known as HIC1 bodies. Here, we present convincing data based on confocal microscopy showing the colocalization of the Wnt signaling effector protein, TCF-4, HIC1 and CtBP1 in these nuclear bodies. We further show that HIC1 directly associates with TCF-4 *in vitro* and *in vivo*. However, the efficient sequestration of TCF-4 into the HIC1 bodies depends on the presence of the CtBP in the TCF-4/HIC1 complex. Strikingly, in CtBP-positive cells, wild-type HIC1 and the TCF-4mutCtBP protein (this variant lacks the CtBP-binding motifs but is still able to interact with HIC1 *in vitro* (Figure 4A and B)) do not display an overlapping localization (Figure 2C). We speculate that CtBP induces a specific spatial arrangement of the HIC1 bodies. Such arrangement possibly favors the recruitment of TCF-4 via the interaction with CtBP. This conclusion is supported by a different morphology of the HIC1 aggregates, which in the presence of CtBP show more compact appearance than the HIC1/TCF-4 bodies without CtBP (Figure 2C). The absence of HIC1 and TCF-4mutCtBP colocalization argues against the obvious objection that overexpressed proteins aggregate and sequester other proteins that they do not normally interact with.

Using *in vitro* pull-down assays, we mapped the main regions of the interaction to the C-terminal parts of HIC1 and TCF-4. Surprisingly, whereas GST-HIC C-terminal and GST-HIC internal fragments interacted with *in vitro* translated TCF-4, the N-terminally truncated HIC-ΔPOZ variant failed to interact with full-length GST-TCF-4. We assume that the anchoring of the HIC1 protein fragments to GST-beads possibly prevents the incorrect folding induced by the deletion of the structurally essential BTB/POZ domain.

Many nuclear factors involved in pre-mRNA splicing, regulation of transcription, apoptosis or cell cycle progression are localized in distinct structures called speckles or nuclear bodies (reviewed in Lamond and Spector, 2003). Using confocal microscopy we visualized HIC1 bodies at endogenous expression levels in the nuclei of three different cell types. Is the physical sequestration of the transcription factors main function of the HIC1 bodies or are there some other physiological roles for these structures? What is the proportion between the 'free' HIC1 protein (i.e. HIC1 associated with promoters or other factors) and HIC1 aggregated in the bodies? More experimental work needs to be carried out to answer these questions.

HIC1 represses TCF-mediated transcription

The results presented here show that full-length HIC1 substantially reduced the levels of TCF-mediated transcription of two different TCF/β-catenin-regulated reporters (Figure 5), and furthermore, several selected endogenous Wnt signaling target genes were also affected by HIC1 expression (Figures 8 and 9). Importantly, HIC1 knockdown in normal cells enhanced the levels of the transcriptional stimulation induced by the Wnt3a ligand (Figure 8B). Thus, HIC1 specifically repressed transcription dependent on TCF and this repression occurred at physiological levels of the HIC1 protein. Interestingly, we observed that the activator role of Wnt signaling was dependent on the particular cellular back-

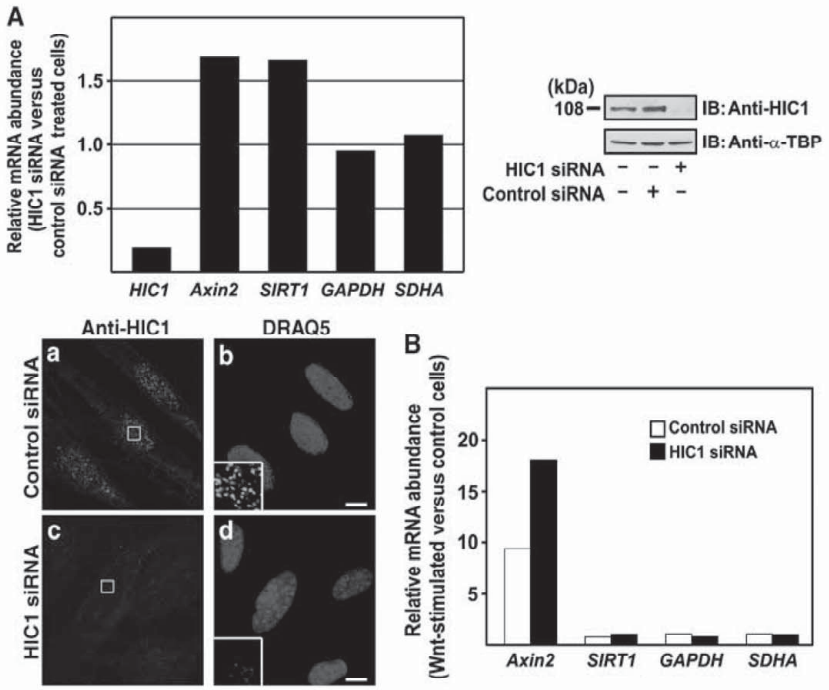

Figure 8 HIC1 knockdown increases the TCF-mediated transcription. (**A**) Human primary cells WI38 were transfected with HIC1 siRNAs or control siRNAs and the changes in the levels of *HIC1* mRNA or protein were tested 24 h post-transfection. Left, results of the qRT–PCR analysis. The relative abundance of the given mRNA in HIC1 siRNA versus control siRNA-transfected cells was derived from the average CT values of four independent experiments after normalizing to the levels of β-*actin* cDNA. Right, Western blots of nuclear extracts prepared from the indicated cells. Bottom, confocal micrographs of WI38 cells transfected with the indicated siRNAs and stained with the affinity purified anti-HIC1 antibody. Bar, 10 μm. (**B**) The activity of the Wnt-dependent promoter of the *Axin2* gene is increased by HIC1 knockdown. Results of qRT–PCR analysis performed with cDNA generated from WI38 cells transfected with the indicated siRNAs upon 24-h stimulation with Wnt3a. Six PCR reactions were done for each primer set. The relative abundance of the indicated mRNA in Wnt3a-stimulated versus control cells was derived from the average CT values after normalizing to the levels of β-*actin* cDNA.

ground. For example, the stimulation by Wnt3a activated transcription of *Sp5*, *Axin2* and *Cyclin D1* in 293; in primary fibroblast WI38 cells, only robust transactivation of the *Axin2* gene was detected, while the expression levels of *Sp5* and *Cyclin D1* remained under detection limits. In colon carcinoma DLD-1 cells, that is, in cells with constitutive active Wnt signaling, ectopic expression of a dominant-negative (blocking) form of TCF-4 inhibited expression of *Tenascin C* but not *Axin2* and *Cyclin D1* mRNA. This clearly indicates that gene expression is in general regulated by inputs from various cellular pathways that integrate in the regulatory regions of a particular gene. Nevertheless, HIC1 regulated the transcriptional response of all tested genes showing the reactivity to the Wnt signal in the given cell type.

Mechanisms of HIC1-mediated inhibition

It was well documented that Kaiso, a member of the BTB/POZ protein family, interacts with sequence-specific elements in several Wnt target genes (Park *et al*, 2005). In *Xenopus*, Kaiso and TCF act in concert on the *siamois* promoter. The Kaiso's general role is not completely understood, but it is likely to also include a direct binding and the recruitment of corepressors such as N-CoR to a subset of Wnt targets. Recently, Chen *et al* (2005) showed that HIC1 forms a transcriptional repression complex with SIRT1 deacetylase. This complex directly binds and represses transcription from the promoter of the *SIRT1* deacetylase gene.

ChIP analysis revealed that HIC1 does not bind directly or indirectly (i.e. via TCF-4) the regulatory elements in the TCF-responsive genes. In contrast, HIC1 partly reduces the occupancy of the promoter of the *Sp5* gene by endogenous TCF-4. This partial sequestration was expected as only a fraction of the cells expressed exogenous HIC1. In addition, HIC1 eliminated binding of ectopically expressed β-catenin to the *Sp5* promoter.

ChIP analysis of the TCF-binding element in the *Tenascin C* promoter in DLD/HIC1 cells showed a substantial decrease in association of TCF-4 and β-catenin upon HIC1 induction. Taken together, these data indicate that HIC1-mediated sequestration prevents TCF-4 from binding its target promoter. Although β-catenin targeting to the HIC1 bodies seems to be indirect and mediated via its interaction with TCF-4, we cannot completely exclude participation of an unknown factor involved in relocation of β-catenin into the HIC1 bodies (Figure 10). These results imply that HIC1-mediated sequestration can uncouple the TCF/β-catenin-regulated promoters from various inputs related to Wnt signaling, but still may leave such promoters responsive to other regulatory signals.

Materials and methods

Plasmids and RNAi

All constructs were made by standard molecular biology techniques. Triple amino-acid substitutions (Q_{509} to N_{509}, P_{510} to A_{510}, L_{511} to S_{511}, Q_{586} to N_{586}, P_{587} to A_{587}, L_{588} to S_{588}) were introduced into

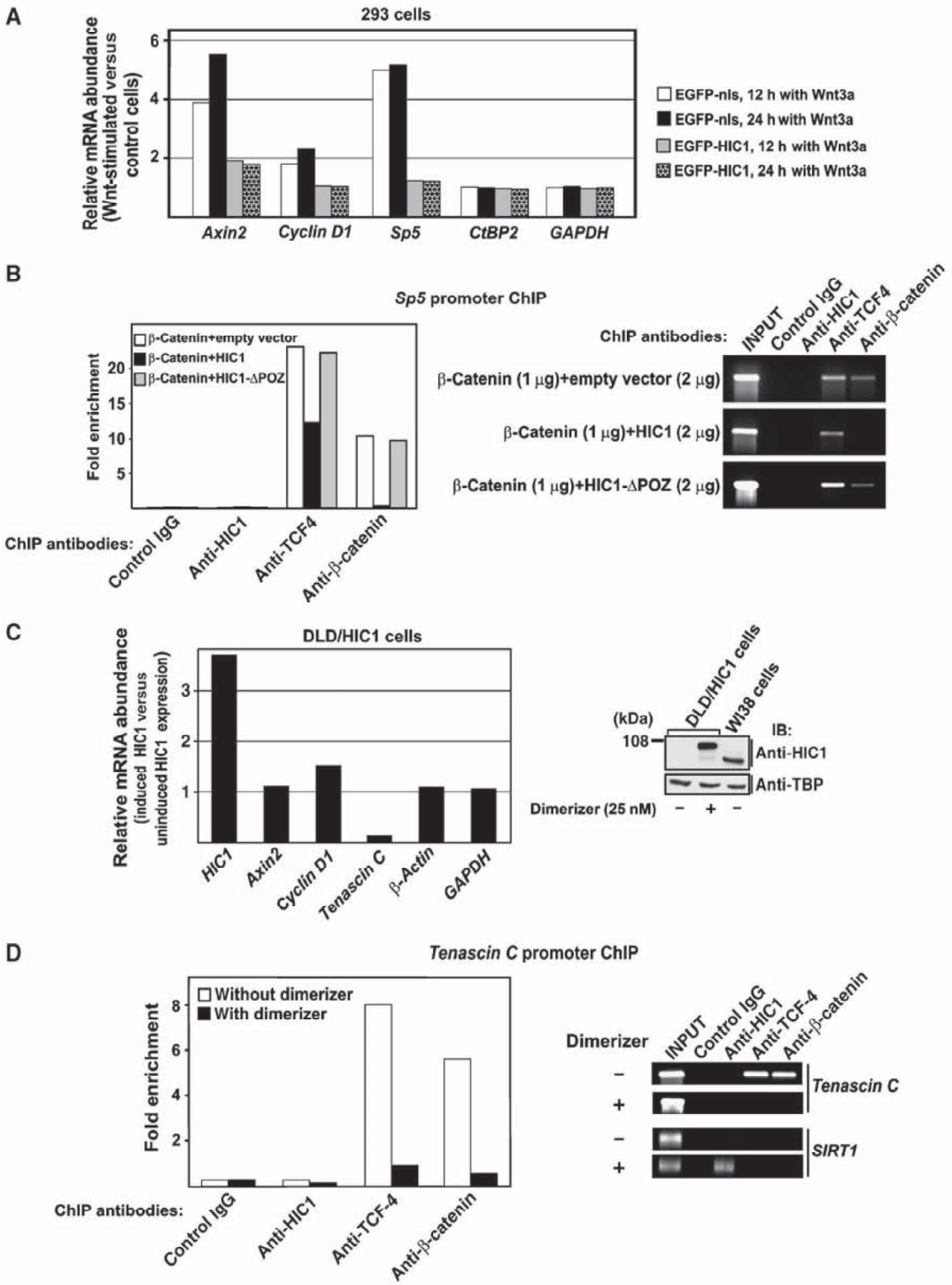

Figure 9 HIC1 sequesters TCF and β-catenin from the TCF-dependent promoters. (**A**) HIC1 blocks transcriptional activation of the Wnt signaling target genes. Results of qRT–PCR analyses performed with cDNA generated from 293 cells transfected with the indicated constructs upon 12- or 24-h stimulation with Wnt3a. Four to six PCR reactions were performed for each primer set. The relative abundance of the indicated mRNA in Wnt3a-stimulated versus control cells was derived from the average CT values after normalizing to the levels of β-*actin* cDNA. (**B**) HIC1 is not associated with the Sp5 promoter but sequesters TCF-4 and β-catenin from the TCF-specific DNA element of this promoter. ChIP analysis of chromatin isolated from 293 cells transfected with the indicated constructs. The diagram at the left represents real-time PCR values obtained with primers spanning the respective DNA element, normalized to the inputs. The image on the right depicts relevant PCR products after 29 cycles of amplification. (**C**) HIC1-EGFP blocks transcription of the Tenascin C promoter in DLD-1 cells. Left, results of qRT–PCR analyses performed with cDNA prepared from DLD/HIC1 cells growing in the presence of the dimerizer (25 nM; HIC1 induction) or without induction. Right, Western blot analysis of nuclear extracts isolated from DLD/HIC1 and WI38 cells. (**D**) HIC1 sequesters TCF-4 and β-catenin from the *Tenascin C* promoter. Left, ChIP analysis of chromatin isolated from DLD/HIC1 cells prior to and upon HIC1-EGFP induction. The diagram at the left represents real-time PCR values obtained with primers spanning the proximal TCF-binding element in the *Tenascin C* promoter, normalized to the inputs. The image on the right depicts relevant PCR products after 29 cycles of amplification. Right bottom, although HIC1-EGFP is not associated with the *Tenascin C* promoter, it binds its recognition element in the SIRT1 promoter.

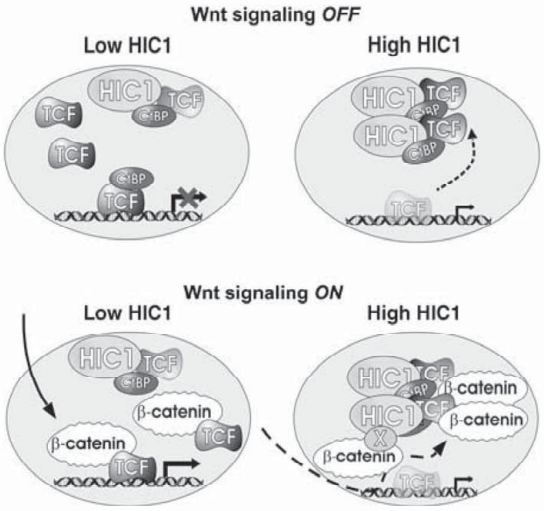

Figure 10 A model for HIC1 suppression of the transcriptional response induced by Wnts. The regulation of a complex promoter integrating inputs from the Wnt and other signaling pathways is depicted. At low levels of HIC1 the activity of the promoter depends mainly on the Wnt signaling components. High levels of HIC1 uncouple the promoter from Wnt signaling. X depicts a hypothetical factor mediating besides TCF-4 the interaction between β-catenin and HIC1.

each of the CtBP-binding sites in TCF-4mutCtBP by using a site-directed mutagenesis kit (Stratagene). For gene knockdowns HIC1 siRNAs were purchased from Ambion. See the Supplementary data for more detailed description of the plasmids used in this study.

Cell culture and transfections

Cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Hyclone) and antibiotics. All cell lines were regularly checked for the presence of mycoplasma. Transfections were performed using the Lipofectamine reagent (Invitrogen) as described by the manufacturer. DLD/HIC1 cells were produced using ARGENT Regulated Transcription Retrovirus Kit (Ariad). CtBP(−/−) cells derived from the $CtBP1^{-/-}CtBP2^{-/-}$ embryos were kindly provided by J Hildebrand.

Production of Wnt3a-conditioned medium

Mouse Wnt3a-producing L cells (L Wnt-3A; ATCC No. CRL-2647) were cultured in complete DMEM supplemented with G418 (0.5 mg per ml; Alexis). Control and Wnt3a-conditioned medium was prepared according to the protocol provided by the supplier.

Luciferase assays

Reporter gene assays were performed as described previously (Valenta *et al*, 2003). To assay TCF-mediated transcription, firefly luciferase pTOPFLASH and pFOPFLASH (Korinek *et al*, 1997) and the *Axin2* promoter reporter constructs (Jho *et al*, 2002) (a gift from F Costantini) were used. The G1-E1B-Luc reporter and the Gal4-DBD-VP16 construct were kindly provided by C Svensson.

EMSA

Full-length TCF-4, full-length human HIC1 and HIC1-ΔPOZ protein were produced *in vitro* using the Quick TNT Coupled Reticulocyte System (Promega). The assay was performed as reported previously (Valenta *et al*, 2003).

GST interaction assays and DNase I treatment

GST-TCF-4, GST-β-catenin, GST-IIIC1-N-term (aa 1–398), GST-IIIC1-C-term (aa 396–714) and GST-HIC1-internal (aa 234–551) fusion proteins were expressed in the BL21 (DE3) strain of *Escherichia coli* using the pET-42b vector (Novagen). Relevant proteins were produced *in vitro* using the Quick TNT Coupled Reticulocyte System (Promega). The detailed protocol for the GST pull-downs was described previously (Valenta *et al*, 2003). DNase I treatment of GST-bound or *in vitro* translated proteins was performed in 1 × DNase I buffer (Invitrogen), for 30 min at RT with 0.2 U of DNase I (Invitrogen) per 1 μl of the reaction mixture.

Antibodies

Antisera to TCF-4, HIC1, CtBP1, β-catenin and EGFP were produced by immunization of rabbits with bacterially expressed proteins; mouse mAbs to TCF-4 and HIC1 were prepared using standard techniques from splenocytes of mice immunized with a bacterially produced TCF-4 fragment (aa 31–333) and HIC1 fragment (aa 230–404) respectively. The following commercially available mouse monoclonal antibodies were used: anti-β-catenin (Santa Cruz Biotechnology), anti-dephospho-β-catenin (Alexis), anti-CtBP (Santa Cruz Biotechnology), anti-GFP (BD Clontech), anti-Myc 9E10 (Roche Molecular Biochemicals), anti-Flag M2 (Sigma), anti-Flag (Exbio Praha).

Immunofluorescent microscopy

Cells grown on coverslips were fixed 24 h after transfection in cold methanol (−20°C, 5 min) and then briefly in acetone (−20°C). Fluorochromes were ALEXA 488, 594, and 680 dyes (dilution 1:500; Invitrogen), Cy5 dye (1:500, Amersham Pharmacia Biotech). The samples were mounted in MOWIOL (Calbiochem) containing nuclear staining dye DRAQ 5 (1:750; Alexis). Immunofluorescences were visualized using a confocal laser scanning microscope (TCS SP; Leica). All images were scanned separately in the 'sequential scanning mode' for the green, red and blue channels using a × 100/1.40 oil-immersion objective. The ratio of colocalization was quantified by measuring the overlap in the fluorescence intensities of corresponding channels along selected profiles using Leica confocal software. Image files were processed with Adobe Photoshop.

RNA purification and real-time qRT–PCR

Standard procedures were used for RNA purification and reverse transcription. The primers used are listed in Supplementary Table S1 (Supplementary data). The cycling was performed in an Mxp3000 instrument (Stratagene).

ChIP

293 cells stimulated with Wnt3a-conditioned or control medium, DLD-1 and DLD/HIC1 cells were subjected to the ChIP assays according to Kirmizis *et al* (2004). See the Supplementary data for the primer sequences.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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SUPPLEMENTARY INFORMATION

Materials and Methods

Plasmids and mutagenesis

Full-length HIC1, HIC1-ΔCtBP (deleted aa 222-228) and HIC1-ΔPOZ (aa 136-714) constructs encoding proteins of human origin containing N-terminal or C-terminal EGFP were generated by standard cloning procedures in pEGFP-C1 and pEGFP-N3 vector (BD Clontech), respectively. Constructs encoding HIC1 with an N-terminal Flag-tag (in pcDNA3) (Deltour et al., 2002) were provided by D. Leprince. Plasmids encoding HIC1 tagged with a C-terminal Flag were generated by subcloning into pCMV-Flag-5a vector (Sigma). Myc- and EGFP-tagged constructs encoding full-length mouse or human CtBP1 and human TCF-4 were described previously (Valenta et al., 2003). Plasmids encoding the N-terminal (TCF-4-N-term; aa 1-333) or C-terminal fragment of human TCF4 (TCF-4-C-term; aa 333-596) were prepared in the pK-myc vector (Valenta et al., 2003) by standard cloning techniques. For retroviral transduction, EGFP-tagged human HIC1 was ligated into the pLHIT variant of pLNIT vector (provided by F.H.Gage); untagged mouse CtBP1 was expressed from pBABE-puro retroviral plasmid (a gift from J. Hildebrand). Details of plasmids and sequences of primers used for the mutagenesis are available on request.

Primers for ChIP

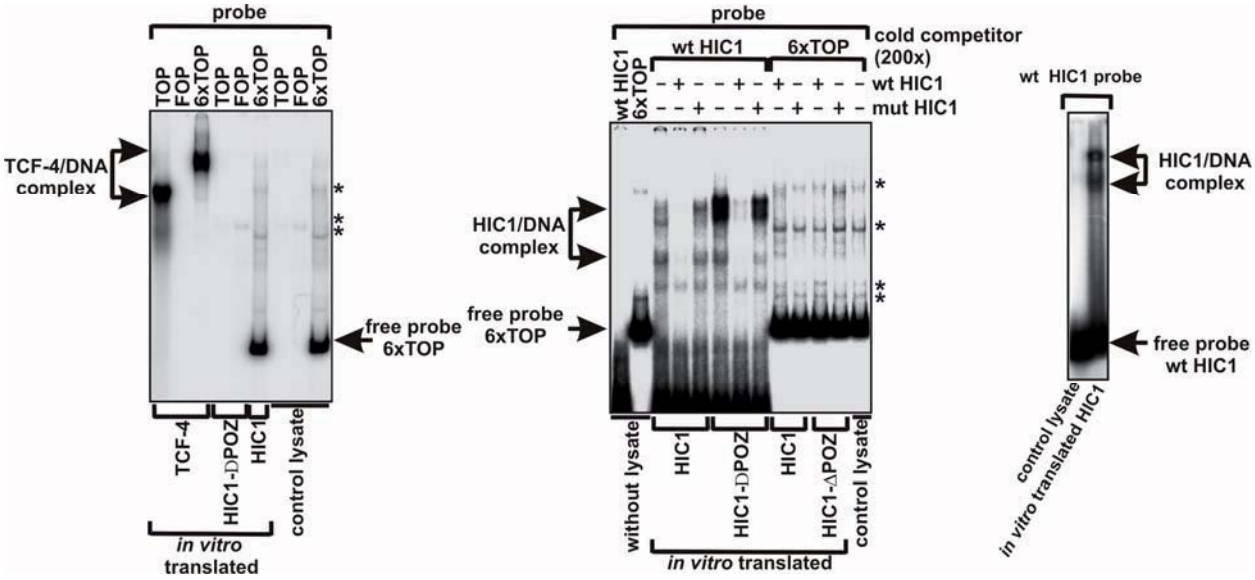
The primers used for the PCR amplification were: *Sp5* promoter, sense: 5'-TCCAGACCAACAAA CACACC-3' and antisense 5'-GCTTCAGGATCACCTCCAAG-3'; *Tenascin C* promoter, sense: 5'-ACTGGGGCTGGAACAAAGAT-3' and antisense 5'AGCGAGTACAGGGACTGAGC-3'; *SIRT1* promoter, sense: 5'-GATAGAAACG CTGTGCTCCA-3' and antisense 5'-CCTTCCTTTCT AGCGTGAGC-3'; negative control regions were derived from the distal exon of the *Axin2* gene, sense: 5'-CTGGCTTTGGTGAACTGTTG-3' and antisense 5'-AGTTGCTCACAGCCAAGACA-3', or from the distal exon of the *Tenascin C* gene, sense: 5'-CAGTAGAGGCAGCCCAGAAC-3' and antisense 5'- AGAGAGAGGGGTTGTGCTGA-3'.

Supplementary Table S1 A set of oligonucleotide primers used for RT-PCR

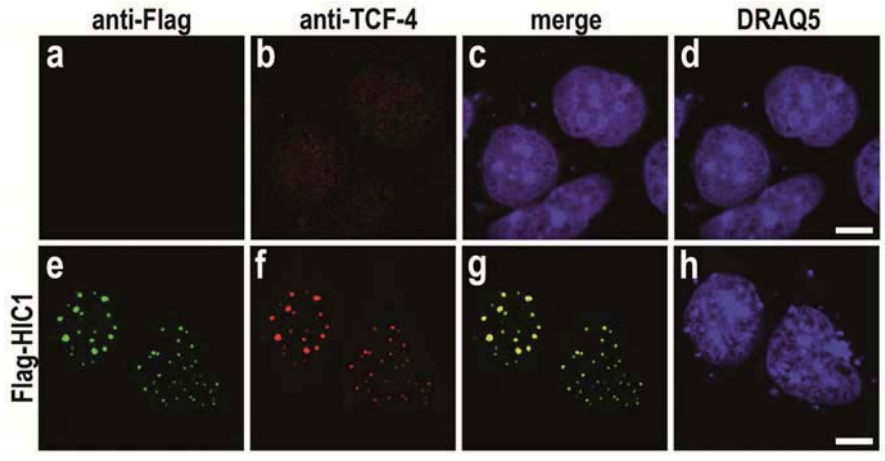
gene	origin		sequence
Axin1	human	forward	5'-CCTGTGGTCTACCCGTGTCT-3'
		reverse	5'-GCTATGAGGAGTGGTCCAGG-3'
	mouse	forward	5'-ACCCAGTACCACAGAGGACG-3'
		reverse	5'-CTGCTTCCTCAACCCAGAAG-3'
Axin2	human	forward	5'-CTGGCTTTGGTGAACTGTTG-3'
		reverse	5'-AGTTGCTCACAGCCAAGACA-3'
	mouse	forward	5'-TAGGCGGAATGAAGATGGAC-3'
		reverse	5'-CTGGTCACCCAACAAGGAGT-3'
beta-actin	human	forward	5'-GGCATCCTCACCCTGAAGTA-3'
		reverse	5'-AGGTGTGGTGCCAGATTTTC-3'
	mouse	forward	5'-GATCTGGCACCACACCTTCT-3'
		reverse	5'-GGGGTGTTGAAGGTCTCAAA-3
CtBP2	human	forward	5'-CCATCCAGTGGAGGTTTGTC-3'
		reverse	5'-AGCGTATCGTAGGAGTGGGA-3'
Cyclin D1	human	forward	5'-CCATCCAGTGGAGGTTTGTC-3'
		reverse	5'-AGCGTATCGTAGGAGTGGGA-3'
GAPDH	human	forward	5'-CACCACACTGAATCTCCCCT-3'
		reverse	5-CCCCTCTTCAAGGGGTCTAC-3'
	mouse	forward	5'-AACTTTGGCATTGTGGAAGG-3'
		reverse	5-ATCCACAGTCTTCTGGGTGG-3'
HIC1	human	forward	5'-CGACGACTACAAGAGCAGCA-3'
		reverse	5'-TGCACACGTACAGGTTGTCA-3'
	mouse	forward	5'-CAACCTGTACGTGTGCATCC-3'
		reverse	5'-ACGTGTGCATTCAGCTGTTC-3'
HIC2	human	forward	5'-CTCCACAGTGTTCCAGCAGA-3'
		reverse	5'- CAGCAGCTTGCCTGTGTAGA-3'
SDHA	human	forward	5'-AGATTGGCACCTAGTGGCTG-3'
		reverse	5'-ACAAAGGTAAGTGCCACGCT-3'
	mouse	forward	5'-AAGGCAAATGCTGGAGAAGA-3'
		reverse	5'-TGGTTCTGCATCGACTTCTG-3'
SIRT1	human	forward	5'-GCAGATTAGTAGGCGGCTTG-3'
		reverse	5'-AGCGCCATGGAAAATGTAAC-3'
Sp5	human	forward	5'-ACTTTGCGCAGTACCAGAGC-3'
		reverse	5'-ACGTCTTCCCGTACACCTTG-3'
TCF-4	human	forward	5'-TAGCTTTAGCGTCGTGAACC-3'
		reverse	5'-GGGGGCAAATTAAGAAAAGTG-3'
Tenascin C	human	forward	5'-GTCACCGTGTCAACCTGATG-3'
		reverse	5'-GTTAACGCCCTGACTGTGGT-3'

Supplementary figures

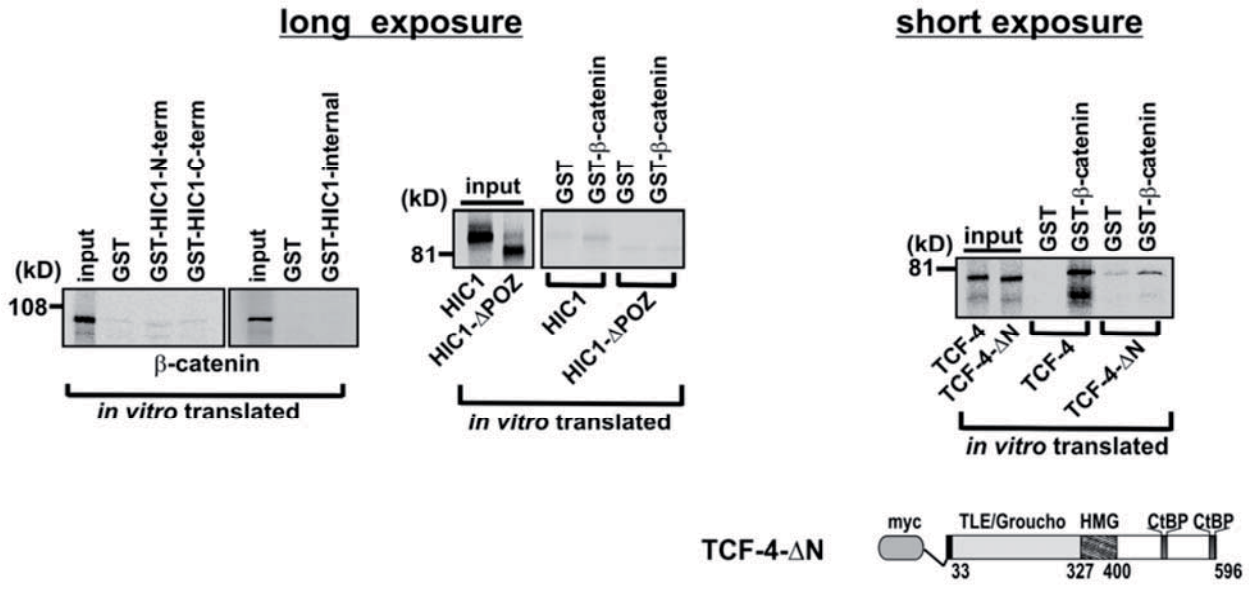
Supplementary Figure S1 TCF-4 and HIC1 differ in the DNA-binding specificities. A gel-retardation assay performed with the TCF-4 and HIC1 proteins translated *in vitro*. As the optimal Tcf/Lef probe, a double-stranded 12-nucleotide oligomer 5'-ACCCTTTGATCT-3' was used; the control probe was 5'-ACCCTTTGGCCT-3'. A PCR fragment amplified from pTOPFLASH (Upstate Biotechnology) containing 6 copies of the Tcf/Lef interaction motif was used as the multiple TCF-specific (6xTOP) probe. The wild-type HIC1 probe was double-stranded oligonucleotide 5'-GGGC-CTGGGGGCAACCCAATCAC-3', the negative control HIC1mut was 5'-GGGCCTGGGGATGA-CCCAATCAC-3'. TCF-4/DNA and HIC1/DNA complexes and the positions of the free probes are indicated by arrows. Stars indicate non-specific bands.



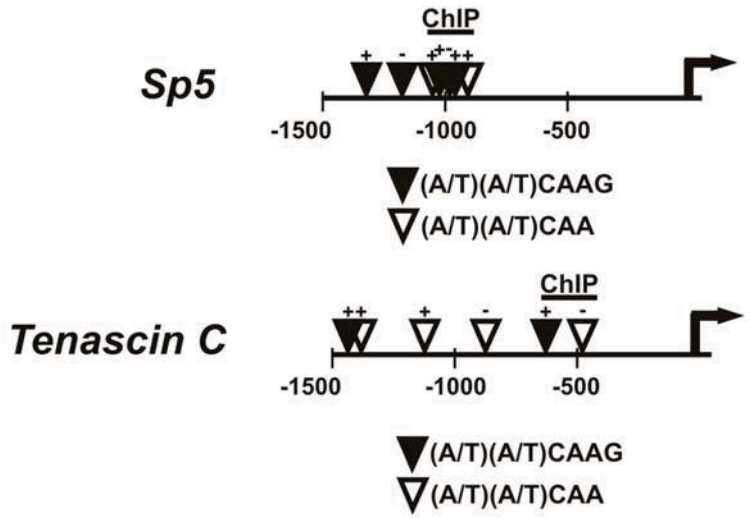
Supplementary Figure S2 HIC1 bodies in DLD-1 adenocarcinoma cells contain endogenous TCF-4. Colocalization of HIC1 with endogenous TCF-4. Confocal micrographs of DLD-1 cells transfected with full-length Flag-HIC1 construct stained with anti-Flag and anti-TCF-4 . Bar, 10μm.



Supplementary Figure S3 HIC1 and β-catenin do not interact directly**.** Pull-down assays between bacterially expressed GST-fusion and *in vitro* translated proteins as indicated. Ten percent of the total reactions were loaded in lanes denoted "input". The gel images marked "long exposure" were obtained after a three-day exposition to the BAS-phosphoimager screen, the image named "short exposure" was gained after a standard overnight exposition. No obvious interaction was detected between β-catenin and HIC1, although the same isolate of GST-β-catenin clearly interacted with full-length TCF-4. A structure of the dominant negative TCF-4-ΔN construct lacking the main β-catenin interaction domain (used as a negative control) is also shown (bottom).



Supplementary Figure S4 Schematic representation of the human *Sp5* and *Tenascin C* promoters. The TCF-binding sites are depicted by open (basic) or filled (optimal) triangles. The orientation of these sites is indicated by (+) or (-). Areas used for ChIP are shown above the relevant promoter.



DAZap2 modulates transcription regulated by the Wnt effector TCF4

Lukas, J., Mazna, P., Valenta, T., Doubravska, L., Pospichalova, V., Vojtechova, M., Fafilek, B., Ivanek, R., Plachy, J., Novak, J. and Korinek, V.

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Dazap2 modulates transcription driven by the Wnt effector TCF-4

Jan Lukas, Petr Mazna, Tomas Valenta, Lenka Doubravska, Vendula Pospichalova, Martina Vojtechova, Bohumil Fafilek, Robert Ivanek, Jiri Plachy, Jakub Novak and Vladimir Korinek*

Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Videnska 1083, 142 20 Prague 4, Czech Republic

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ABSTRACT

A major outcome of the canonical Wnt/β-catenin-signalling pathway is the transcriptional activation of a specific set of target genes. A typical feature of the transcriptional response induced by Wnt signalling is the involvement of Tcf/Lef factors that function in the nucleus as the principal mediators of signalling. Vertebrate Tcf/Lef proteins perform two well-characterized functions: in association with β-catenin they activate gene expression, and in the absence of Wnt ligands they bind TLE/Groucho proteins to act as transcriptional repressors. Although the general characteristics of Tcf/Lef factors are well understood, the mechanisms that control their specific roles in various cellular backgrounds are much less defined. In this report we reveal that the evolutionary conserved Dazap2 protein functions as a TCF-4 interacting partner. We demonstrate that a short region proximal to the TCF-4 HMG box mediates the interaction and that all Tcf/Lef family members associate with Dazap2. Interestingly, knockdown of Dazap2 not only reduced the activity of Wnt signalling as measured by Tcf/β-catenin reporters but additionally altered the expression of Wnt-signalling target genes. Finally, chromatin immunoprecipitation studies indicate that Dazap2 modulates the affinity of TCF-4 for its DNA-recognition motif.

INTRODUCTION

The Wnt-signalling pathway is essential during different developmental processes for determining cell fate. In addition, aberrant activation of this pathway has been implicated in cellular transformation and cancer [see some recent reviews (1–3)]. Transcription factors of the Tcf/Lef family are important downstream effectors of the so-called canonical Wnt/β-catenin-signalling pathway. In vertebrates the family consists of four members: Tcf-1, Tcf-3, Tcf-4 and Lef-1 (4). All vertebrate Tcf/Lef proteins (further referred to as Tcfs) contain virtually identical DNA-binding domains, a high mobility group (HMG) box, and a highly conserved β-catenin-interacting region. In the absence of the Wnt signal, Tcf/Lef factors interact with Transducin-like enhancer of split (TLE)/Groucho co-repressors to mediate the transcriptional repression of Tcf-bound genes (5–7). Alternatively, upon initiation of Wnt signalling the constitutive degradation of β-catenin is inhibited allowing this protein to accumulate both in the cytoplasm and nucleus, with the nuclear form able to displace TLE/Groucho co-repressors from Tcfs (8). Since β-catenin contains a strong transactivation domain, Tcf/β-catenin heterocomplexes function as transcriptional activators of specific Wnt-responsive genes such as *c-myc* (9), *Cyclin D1* (10,11), *Axin2* (12) and *CD44* (13). For a more comprehensive survey on Wnt signalling, please refer to the Wnt signalling home page at http://www.stanford.edu/%7ernusse/wntwindow.html.

Although the general function of Tcfs as transcriptional repressors or co-activators is well understood, their specific roles in Wnt signalling or cell physiology are much less defined. Besides β-catenin and TLE/Groucho co-repressors several other proteins associate with the HMG box of Tcfs. Such factors include proteins containing the I-mfa domain that mask the DNA-interacting region of Tcf-3, thereby preventing Tcf-3/β-catenin heterodimers from activating transcription (14). Likewise, RUNX3 forms a ternary complex with β-catenin and Tcfs to attenuate the transactivation potential of Tcf/β-catenin complexes by decreasing their DNA-binding activity (15).

Expression of mouse *Tcf/Lef* genes during embryogenesis and in adult tissues often overlaps. Nevertheless, gene-targeting experiments have demonstrated that individual Tcf members control their own cell biological

*To whom correspondence should be addressed. Tel: +4202 4106 3146; Fax: −4202 4447 2282; Email: korinek@img.cas.cz

programs (16–19). This observation implies that throughout evolution the functions originally executed by a single Tcf polypeptide have been distributed in more complex organisms among several family members. A plausible explanation for the functional diversity among Tcfs would be their selective interaction with distinct partners as the amino-acid sequences outside the highly conserved DNA- and β-catenin-binding domains are less homologous. Indeed, it has been reported that LEF-1 activates some promoters together with ALY, a nuclear protein that specifically binds LEF-1 and AML-1 (20). Additionally, LEF-1 cooperates with the Microphthalmia-associated transcription factor (MITF) to activate the expression of melanocyte-specific genes (21). Interestingly, although the activity of LEF-1 is suppressed by association with PIASy (a nuclear matrix-associated SUMO E3 ligase), this interaction results in increased TCF-4-regulated transcription (22,23). Two Tcf/Lef family members, Tcf-3 and Tcf-4, contain binding motifs for C-terminal-binding proteins (CtBPs) at their C-termini (24–26). As CtBPs operate as short-distance transcriptional repressors, interaction with such factors strengthens the repressive potential of these Tcfs in the absence of Wnt signalling (27). Besides CtBP, TCF-4 also binds the Hypermethylated in cancer 1 (HIC1) tumour suppressor. This interaction leads to the recruitment of TCF-4 into nuclear 'speckles' called HIC1 bodies. Upon association with HIC1, TCF-4 is unable to bind Wnt-responsive gene promoters. Thus, HIC1 functions as a nuclear TCF-4-specific Wnt pathway inhibitor (27). Finally, to add another layer of complexity to the regulation of Wnt target genes it has also been demonstrated that alternative promoters and/or alternative splicing of Tcf/Lef mRNAs occurs (28,29). A mechanism by which distinct Lef/Tcf isoforms may acquire individual properties is illustrated by their interaction with Hic-5 (hydrogen peroxide-induced clone 5). Hic-5 has been shown to bind a highly conserved and alternatively spliced exon of Lef/Tcf proteins and this results in the formation of a Lef/Tcf subtype-specific repressive complex that prevents target gene activation (30).

Mammalian Dazap2, also known as Proline codon-rich transcript, brain expressed (Prtb), was originally isolated in a mouse gene trap screen as a transcript expressed in the inner ear (31). This gene encodes a small 17 kDa protein that is highly conserved throughout evolution. The protein does not share significant sequence homology with any protein family and its most notable feature is a high content of prolines (17%) and several potential Src homology 2 (SH2)- and SH3-binding motifs (32). The *Dazap2* gene is broadly expressed during mouse embryonic development and in adult mouse and human tissues (31,33–35). Interestingly, *Dazap2* mRNA and protein are frequently down-regulated in multiple myeloma patients (36) whilst *Dazap2* mRNA is known to increase in adhering mouse osteoblasts or in rat astrocytes grown in high ammonia or hypo-osmotic conditions (37). In humans, Dazap2 interacts with RNA-binding testes-specific proteins DAZ and DAZL1 (35). In addition, Dazap2 also binds the Sox6 transcription factor to regulate L-type Ca^{++} channel α_{1c} expression during cardiac myocyte development (33). Recently, Kim and colleagues (38) described the interaction of Dazap2 with the Eukaryotic initiation factor 4G (eIF4G) which is essential for the formation of discrete cytoplasmic foci, named stress granules (SGs). SGs are formed upon translation inhibition and contain translation initiation factors and 40S ribosomal subunits (34,38). Finally, the protein level of Dazap2 is regulated by its interaction with NEDD4, an E3 ubiquitin ligase (39). Taken together, the aforementioned data indicate that Dazap2 functions in diverse roles in cell biology and physiology.

In this study we have used a yeast two-hybrid screen to identify Dazap2 as a TCF-4 interacting partner. Furthermore, we show that a short region proximal to the TCF-4 HMG box mediates this interaction. Interestingly, although this region is only partially conserved among Tcfs, all Tcf/Lef family members associate with Dazap2 in mammalian cells. Upon interaction with TCF-4 the subcellular distribution of Dazap2 is dramatically shifted from the cytoplasm into the nucleus. Upon knockdown of *Dazap2* a reduction in the activity of Tcf/β-catenin reporters was observed along with the expression of several Wnt-signalling target genes. Chromatin immunoprecipitation experiments performed in cells with down-regulated Dazap2 expression revealed a remarkable decrease in TCF-4 binding to Tcf-responsive elements in the promoters of genes tested. We propose that Dazap2 modulates the affinity of Tcfs to their recognition motifs.

MATERIALS AND METHODS

Plasmid constructs

Constructs encoding proteins fused at the N-terminus to EGFP were prepared using the pEGFP-C vector (Clontech); plasmids encoding Myc-tagged proteins were generated using the pK-Myc vector (26) and plasmids expressing Flag-tagged polypeptides were constructed in the vector pFlag-CMV-5a (Sigma). cDNAs encoding human *TCF-4* (GenBank accession number NM_030756), human *TCF-1* (NM_003202), and mouse *Lef-1* (NM_010703) were described previously (26,40,41). Full-length cDNA encoding human *TCF-3* (NM_031283) was purchased from Open Biosystems whilst the cDNA encoding human *β-catenin* (NM_205081) was kindly provided by B. Vogelstein (The Johns Hopkins Kimmel Cancer Center); cDNA encoding the full-length human DAZAP2 protein (NP_055579) was cloned by RT–PCR using template mRNA isolated from DLD-1 cells. PCR amplification steps were performed with Phusion High-Fidelity DNA Polymerase (Finnzymes). Mouse cDNA encoding *Wnt-1* [a gift from M.van Dijk (University Hospital Utrecht)] was subcloned into the mammalian expression vector pXJ41 (kindly provided by L. Andera, IMG, Prague, Czech Republic). Mouse cDNA encoding *Grg4* [a TLE/Groucho repressor (NM_011600)] was kindly provided by Z. Kozmik (IMG). Human *TAB1* (U49928), mouse *Tak1* (D76446) and mouse *Nlk* (NM_008702) cDNAs were obtained from T. Ishitani (Nagoya University). PCR-derived constructs were verified by sequencing; details of plasmid constructs are available on request.

Yeast two-hybrid screen

The cDNA encoding the N-terminal part of TCF-4 lacking the β-catenin-interacting domain [amino acids (aa) 31–333] was subcloned into the vector pGBKT7 (Clontech) and introduced into yeast AH109 cells by the standard lithium acetate transformation protocol. Expression of the TCF-4 fragment and GAL-4 DNA-binding domain (DBD) bait protein was tested in cell lysates by immunoblotting using anti-Myc and anti-TCF-4 monoclonal antibodies. A pre-transformed mouse 17-day embryo Matchmaker cDNA library amplified in the yeast strain Y187 (Clontech) was used for the screen according to the manufacturer's instructions. The 'library' and 'bait' cells were first mated in liquid cultures before subsequent plating on selective agar plates. After incubation at 30°C for 7–10 days, clones of growing cells were picked and streaked onto fresh selective plates and subjected to β-galactosidase filter lift assays. Plasmids isolated from positive clones were transformed into the yeast strain Y187 and their specificity tested by mini-mating with AH109 yeast cells that expressed the GAL-4 DBD or GAL-4 DBD-Lamin fusion proteins as bait. Clones that specifically interacted with the GAL-4 DBD-TCF-4 bait were sequenced. For the interaction domain mapping experiment cDNAs encoding corresponding fragments of TCF-4 (see legend to Figure 1 for details) were subcloned into the pGBKT7 vector and the resulting constructs were transformed into AH109 yeast cells. Individual yeast clones were mated with Y187 cells containing the Dazap2 prey and the growth of diploid yeast was tested on agar plates under selective conditions.

Cell lines, transfections, retrovirus production and infection

Human embryonic kidney (HEK) 293, human HeLa, DLD-1 and U2OS cells and mouse Wnt3a-producing L cells were purchased from ATCC. Mouse C57MG cells were kindly provided by R. Nusse and K. Willert (Stanford University). HEK 293 FT cells used for production of retroviral stocks were obtained from Invitrogen. pSuperTOPFLASH HEK 293 (STF 293) cells containing the integrated variant of the Wnt/β-catenin-responsive luciferase reporter pSuperTOPFLASH (42) were a kind gift from Q. Xu and J. Nathans (Johns Hopkins University). Cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Hyclone) and antibiotics. Cells in cultures were regularly checked for the presence of mycoplasma and only mycoplasma-free cells were used in experiments. Transfections of human cells were performed using the Lipofectamine RNAiMAX [small inhibitory RNAs (siRNA) transfections] or Lipofectamine 2000 reagent (plasmid or combined plasmid and siRNAs transfections). Both reagents were purchased from Invitrogen. C57MG cells were transfected using Fugene HD (Roche). Retroviral stocks were produced as described previously (26). Lentiviral stocks were prepared using the Trans-Lentiviral Packaging System (Open Biosystems) according to the manufacturer's protocol. Retroviral (or lentiviral) infections have been described previously (26); puromycin (Alexis; final concentration 5 μg/ml) resistant cells were selected without subcloning for 10 days and used in subsequent experiments.

GST interaction assays

Constructs expressing Glutathione S-transferase (GST)-TCF-4 fusion proteins were prepared using the pET-42b vector (Novagen). GST-TCF-4 (full-length), GST-TCF-4-N-term (aa 1–333), GST-TCF-C-term (aa 333–596), GST-TCF-4 (aa 1–214), GST-TCF-4 (aa 1–228), GST-TCF-4 (aa 214–310), GST-TCF-4 (aa 228–310) and GST-β-catenin (full-length) fusion proteins were expressed in the BL21 (DE3) strain of *Escherichia coli*. The mouse Dazap2 and human TCF-4 proteins were produced *in vitro* using the Quick TNT Coupled Reticulocyte System (Promega). A detailed protocol describing GST pull-down assays was reported previously (26).

Antibodies, co-immunoprecipitation and western blotting

Antiserum to Dazap2 was produced by immunization of rabbits or hens with a bacterially expressed mouse full-length polypeptide. An anti-β-catenin rabbit polyclonal antibody was produced by immunization with a bacterially expressed C-terminal fragment (aa 585–781) derived from the human polypeptide; an anti-β-catenin mouse monoclonal antibody was prepared in collaboration with Exbio Praha (Czech Republic) using standard techniques from the splenocytes of mice immunized with a bacterially produced β-catenin fragment. The anti-TCF-4 monoclonal and anti-TCF-4 and anti-EGFP rabbit polyclonal antibodies were reported previously (27). The following commercially available mouse monoclonal antibodies were used: anti-Myc 9E10 (Roche), anti-Flag M2 (Sigma), anti-Flag (Exbio), anti-α-tubulin TU-01 (Exbio). A detailed protocol describing the immunoblotting procedure can be found in the Supplementary Data.

Immunofluorescent microscopy

For immunofluorescence studies, Dazap2 polyclonal antibodies were purified by affinity chromatography using the GST-Dazap2 antigen coupled to glutathione Sepharose 4B (Amersham Pharmacia Biotech) (43). The purified antibodies were subsequently stored at 4°C in PBS supplemented with 1% BSA [(w/v), Sigma, molecular biology grade]. Monoclonal antibodies were used as hybridoma cell culture supernatants without dilution. Cells grown on coverslips were fixed (when cells were transfected prior to staining, the fixation was performed 18 h after transfection) in cold methanol (−20°C, 5 min) followed by a brief incubation in acetone (−20°C, 30 s). Alternatively, fixation was performed for 10 min at room temperature using a 4% (v/v) solution of paraformaldehyde (Electron Microscopy Sciences) diluted in PBS before cells were subsequently permeabilized with 0.2% (v/v) Triton X-100 (Sigma; room temperature, 15 min) diluted in PBS. After washing with PBS, cells were pre-incubated in 1% BSA (Fraction V; Sigma) for 20 min at room temperature. The cells were then stained with primary GST-Dazap2-purified polyclonal antibodies (5 μg/ml in PBS) or with an anti-TCF-4 monoclonal antibody (undiluted hybridoma culture supernatant; room

temperature, 60 min). The samples were washed three times with PBS, and consecutively incubated with a relevant fluorescently labelled secondary antibody. The ALEXA 488 dye conjugated to goat anti-chicken or goat anti-rabbit antibodies and ALEXA 594 conjugated to a goat anti-mouse antibody (dilution 1:500; Molecular Probes) were used. To pre-block the Dazap2 antibody, antigen-purified antibodies (20 μg/ml) were incubated with the bacterially expressed Dazap2 or EGFP (negative control) proteins (overnight at 4°C; the concentration of recombinant protein in each sample was 50 μg/ml), before being diluted in PBS (final concentration 5 μg of the antibody per ml) and used for staining. Finally, the samples were washed three times in PBS, incubated with DAPI nuclear stain (Molecular Probes; 1 min, room temperature, final concentration 1 μM), washed and mounted in MOWIOL (Calbiochem). Immunofluorescence was visualized using a confocal laser scanning microscope (TCS SP5; Leica). The system was carefully tested for overlaps between individual optical channels and the microscopic images were taken separately for each fluorescence channel using the sequential scanning mode.

RNA purification and real-time quantitative RT–PCR (qRT–PCR)

Standard procedures were used for RNA purification and reverse transcription. Briefly, total RNAs were isolated from cells using the Trizol reagent (Invitrogen); random or oligo dT-primed cDNA was prepared in a 20 μl reaction from 1 μg of total RNA using Superscript II RNAseH$^-$ reverse transcriptase (Invitrogen). cDNAs were produced from at least two independent RNA isolations and the PCR reactions were performed in triplicate for each primer set. Two percent of the resulting cDNA was used for one quantitative PCR reaction. Control reactions (containing corresponding aliquots from cDNA synthesis reactions that were performed without reverse transcriptase; minus RT controls) were run in parallel duplicates. PCR reactions were run using the LightCycler 480 Real-Time PCR System (Roche). Typically, a 5 μl reaction mixture contained 2.5 μl of LightCycler 480 SYBR Green I Master mix (Roche), 0.5 μl of primers (final concentration 0.5 μM) and cDNA diluted in 2 μl of deionized water. Crossing-threshold (CT) values were calculated by LightCycler® 480 Software (Roche) using the second-derivative maximum algorithm. The specificity of each PCR product was analysed using the in-built melting curve analysis tool for each DNA product identified; additionally, some selected PCR products were verified by sequencing. All primers were calculated using Primer 3 computer services at http://frodo.wi.mit.edu/. Two housekeeping genes, *β-actin* and *ubiquitin C* (*Ubc*) were used as internal control genes to standardize the quality of different cDNA preparations (44). Primer sequences are listed in Supplementary Data.

Wnt3a purification

Recombinant mouse Wnt3a ligand was isolated from the culture medium of Wnt3a-producing L cells according to a detailed protocol published on the Internet (http://www.stanford.edu/%7ernusse/assays/W3aPurif.htm). The activity of individual batches of purified Wnt3a protein were tested using Wnt3a-stimulated and control (Wnt3a storage buffer added only) STF 293 cells and luciferase assays.

Knockdown of Dazap2

For gene knockdowns of human *DAZAP2*, four duplex siRNAs were purchased from Dharmacon. The target sequences (on the plus DNA strand) were as follows: #1 5′-GGA GCC AAC GUC CUC GUA A-3′, #2 5′-CAC CAU GUC AGC CGC AUU U-3′, #3 5′-UCA GAG CUC UAU CGU CCG A-3′, #4 5′-CUU CAU GGG UGG UUC AGA U-3′. Control EGFP siRNA (Dharmacon) target sequence was: 5′-GCG ACG TAA ACG GCC ACA AGT TC-3′. Cells transfected with duplex siRNAs at a concentration of 30 nM were grown for 24–72 h before further analysis. To generate a stable knockdown of *Dazap2* in mouse C57MG cells, the cells were transduced with retroviruses (purchased from Open Biosystems) that express *Dazap2* shRNA (shRNA #1 code: TRCN0000085966, shRNA #2 code: TRCN0000085965). Non-silencing lentiviral shRNAmir (pGIPZ; Open Biosystems) were used as a control. The constructs were packaged and transduced into the target cells as described by the manufacturer. The cells were selected without subcloning using appropriate antibiotics before they were used for further analysis.

Reporter gene assays and Wnt stimulation

To assay TCF-mediated transcription, firefly luciferase pTOPFLASH and pFOPFLASH reporters containing either three copies of the optimal Tcf motif GATCAAAGG or three copies of a mutant motif GGCCAAAGG, respectively were used (45). Additionally, *Cyclin D1* reporter constructs containing one copy of the Tcf-interacting motif (designated 163CD1LUC) or its mutated variant (163mtLefCD1LUC) (10) (a gift from A. Ben-Ze'ev, The Weizmann Institute of Science) and the *Axin2* promoter reporter (46) (a gift from F. Costantini, Columbia University Medical Center) were used. Reporter gene assays were performed as described previously (26). Briefly, cells were seeded into 24-well plates (~10^5 cells per well, depending on the cell type) and transfected 2 h later with a Lipofectamine mixture containing 100 ng *Renilla* pRL-SV40 plasmid (Promega) as an internal control, 500 ng luciferase reporter plasmid, and up to 1 μg of the particular expression vector. The total amount of DNA was kept constant by adding empty expression vector where necessary. For transfection into STF 293 cells (containing an integrated TCF-dependent reporter pSuperTOPFLASH) (42,47), a mixture that included 50 ng *Renilla* plasmid and up to 1.5 μg of a specific expression or stuffer vector was prepared. Two independent systems were utilized to activate Wnt signalling: (i) co-transfection of cells with a *Wnt1*-expressing plasmid. Cells were analysed 24 h post-transfection; (ii) stimulation of cells with purified recombinant Wnt3a ligand. Cells were transfected with corresponding constructs and 15 h post-transfection recombinant Wnt3a (only the vehicle

was used in control experiments) was added and the cells cultured for additional 16 h before their harvest and lysis. The activity of firefly and *Renilla* luciferase in cell lysates were determined using the Dual luciferase system (Promega) and a single tube luminometer Sirius (Berthold). All reporter gene assays were done in triplicate. Reporter gene activities shown are average values plus standard deviations calculated from at least three independent experiments after normalization against the activity of *Renilla* luciferase.

Chromatin immunoprecipitation (ChIP)

For ChIP experiments, STF 293 cells were grown in 10-cm culture dishes and transfected either with siRNA #2 targeting *Dazap2* or control siRNA (non-silencing siRNA set; Dharmacon). Two days later the cells were transferred into 15-cm culture dishes and subsequently stimulated with Wnt3a or Wnt3a storage buffer alone for 16 h. The cells were fixed directly in the dishes using formaldehyde [1% (v/v); Sigma] then harvested and subjected to ChIP analysis according to Kirmizis (48). Usually, chromatin isolated from cells grown in one 15-cm culture dish was used for immunoprecipitation with one specific antibody. Rabbit anti-Dazap2, TCF-4 and β-catenin polyclonal antibodies were used for ChIP; the negative control experiments were performed with a rabbit anti-EGFP polyclonal antibody. The amount of precipitated DNA was analysed using the LightCycler 480 Real-Time PCR System (Roche) in an analogous manner to real-time qRT–PCR. Half a percent of decrosslinked and purified (by phenol extraction) input chromatin (chromatin that was not subjected to ChIP) was analysed in control PCR reactions. The primers used for the PCR amplification are listed in the Supplementary Data.

RESULTS

Dazap2, a small evolutionary conserved protein, interacts with the TCF-4 N-terminus

It is well known that the function of the nuclear effectors of Wnt signalling, the Tcf/Lef transcription factors, can be modulated by various interacting partners. As such, we decided to perform a yeast two-hybrid screen (Y2H) to search for novel TCF-interacting proteins. An N-terminal part of human TCF-4 protein (aa 31–333) was used as bait. This truncated protein lacks its very N-terminus that encodes the main β-catenin-interacting domain (8,49) as well as the C-terminal DNA-binding HMG box (Figure 1A). From a cDNA library collected from a Day 17 mouse embryo we obtained seven yeast colonies (out of $\sim 5 \times 10^7$ diploid yeast cells) growing in selective broths. Of these clones only one encoded a protein that specifically interacted with the TCF-4 bait and not the Gal-4 DNA-binding domain (DBD) alone, Gal-4 DBD-Lamin and Gal-4 DBD-p53 fusion proteins or with the C-terminal part of TCF-4 used as bait in our previous study (26) (Figure 1A and data not shown). The resulting plasmid DNA isolated from the yeast cells encoded a full-length 168 aa polypeptide described previously as Proline codon-rich transcript, brain expressed (Prtb) or

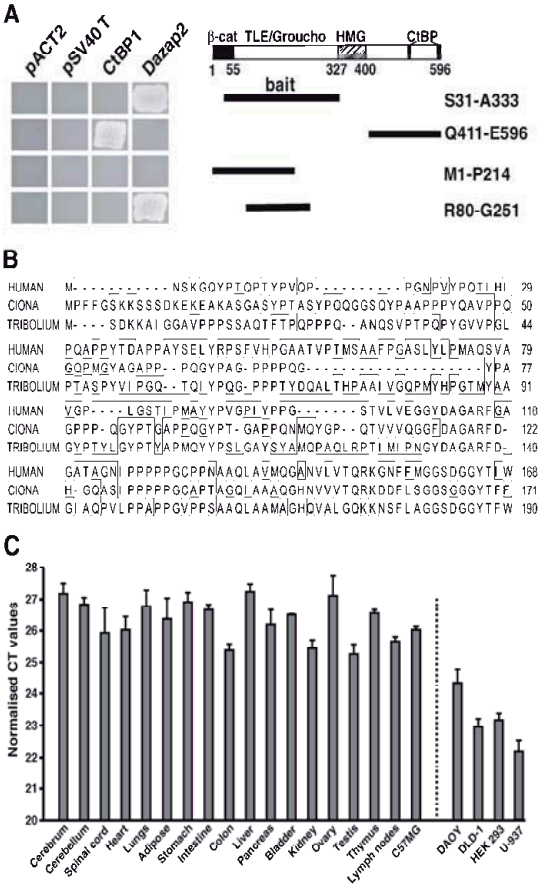

Figure 1. Interaction between Dazap2 and TCF-4 in a yeast two-hybrid screen. (**A**) Deletion mutants of human TCF-4 (schematically represented on the right as thick black lines) were tested in an Y2H mini-mating assay for interaction with full-length mouse Dazap2. The left panel shows the growth of clones of yeast cells on selective agar plates. The yeast cells contain plasmids as indicated above and express TCF-4 deletion mutants that are depicted on the right. None of the TCF-4 proteins binds to the separate GAL4 activation domain (AD) encoded by 'empty' library vector pACT2 or to the fusion protein GAL4 AD-SV40 T large antigen (pSV40 T). β-cat, β-catenin interaction domain; TLE/Groucho, TLE/Groucho-binding domain; CtBP, CtBP-binding sites; HMG, DNA-binding domain. (**B**) Amino-acid comparison of human, sea squirt (*Ciona*) and red flour beetle (*Tribolium*) Dazap2. Protein sequences were aligned by the ClustalV program. The amino-acid differences are boxed. GenBank accession numbers: *Homo sapiens*, NP_055579; *Tribolium castaneum*, XP_973572; *Ciona intestinalis*, NM_001032667. (**C**) The *Dazap2* gene is broadly expressed in tissues and cell lines. Results of qRT–PCR analyses performed with Dazap2-specific primers on cDNA generated from adult mouse tissues, mouse mammary epithelium C57MG cells, human medulloblastoma DAOY, human embryonic kidney HEK 293, human adenocarcinoma DLD-1 and human lymphoma U-937 cells. The reactions were performed in triplicate. The results shown are from one representative experiment from a total of two. The expression levels of *Dazap2* mRNA in the indicated tissues or cell lines are presented as average CT values and the corresponding standard deviations (SD) after normalization to the levels of *β-actin* cDNA.

DAZ-associated protein 2 (Dazap2) (31,35). Although Dazap2 does not share significant homology with any known protein family, Dazap2 orthologues isolated from various species display remarkable sequence similarity (there is only one amino acid change between human and mouse proteins), especially within the C-terminal half (Figure 1B and Supplementary Figure 1). To determine

the expression pattern of *Dazap2* mRNA in adult mouse tissues and several different cell lines we performed quantitative real-time RT–PCR analysis (qRT–PCR), which revealed virtually ubiquitous expression (Figure 1C). We further delineated the minimal region in TCF-4 required for association with Dazap2. We generated several deletions in the TCF-4 bait used for the primary screen and tested their ability to interact with full-length Dazap2 via Y2H. As shown in Figure 1A, truncated bait containing aa 80–251 was able to bind Dazap2, however, a protein spanning aa 1–214 could not. Taken together, these results reveal that the relatively short sequence in TCF-4 spanning aa 214–251 is essential for the interaction.

All Tcf/Lef family members associate with Dazap2 in mammalian cells

Direct binding between Dazap2 and TCF-4 was evaluated *in vitro* by pull-down assays that utilized bacterially expressed GST-tagged TCF-4 and *in vitro* translated Dazap2. Dazap2 associated both with full-length GST-TCF-4 and with the truncated TCF-4 N-terminal fragment. No interaction was detected between Dazap2 and GST alone or with the C-terminal part of TCF-4 (TCF-4-C-term) that encompassed the DNA-binding HMG box domain (Figure 2A and data not shown). Furthermore, we performed a detailed mapping of the putative interaction domains in both proteins using pull-down assays. In agreement with Y2H the Dazap2-binding region was mapped to aa 214–228 in the TCF-4 N-terminus (Figure 2A). On the other hand, any truncation of Dazap2 abolished its association with TCF-4 (Supplementary Figure 2 and data not shown), indicating that non-adjacent parts of the Dazap2 polypeptide participate in the interaction interface. The interaction of Dazap2 with TCF-4 was further confirmed in mammalian cells using co-immunoprecipitations. Experiments involving HEK 293 cells double-transfected with constructs expressing Dazap2-Flag and TCF-4-Myc demonstrated that Flag-tagged Dazap2 could be co-isolated with Myc-tagged TCF-4 when an anti-Myc monoclonal antibody was used for precipitation; conversely, Myc-tagged TCF-4 was present in the anti-Flag precipitates (Figure 2B). The interaction of these two proteins is specific since parallel single-transfection assays did not reveal any binding of Dazap2 and TCF-4 to the anti-Myc- or anti-Flag-tag antibodies, respectively (Figure 2B). In addition, we analysed the ability of endogenous TCF-4 and Dazap2 to interact by performing co-immunoprecipitation assays with rabbit polyclonal antibodies raised against these polypeptides. Analysis of a variety of cell types (DLD-1, HEK 293, C57MG) by co-immunoprecipitation using the anti-Dazap2 or anti-TCF-4 antibodies confirmed our earlier observations and demonstrated that endogenous Dazap2 and TCF-4 do associate. Negative control reactions using an anti-EGFP rabbit polyclonal antibody failed to pull down any proteins again confirming that the interaction is specific (Figure 2C and data not shown). The human colon adenocarcinoma cell line, DLD-1 harbours a mutation in the tumour suppressor

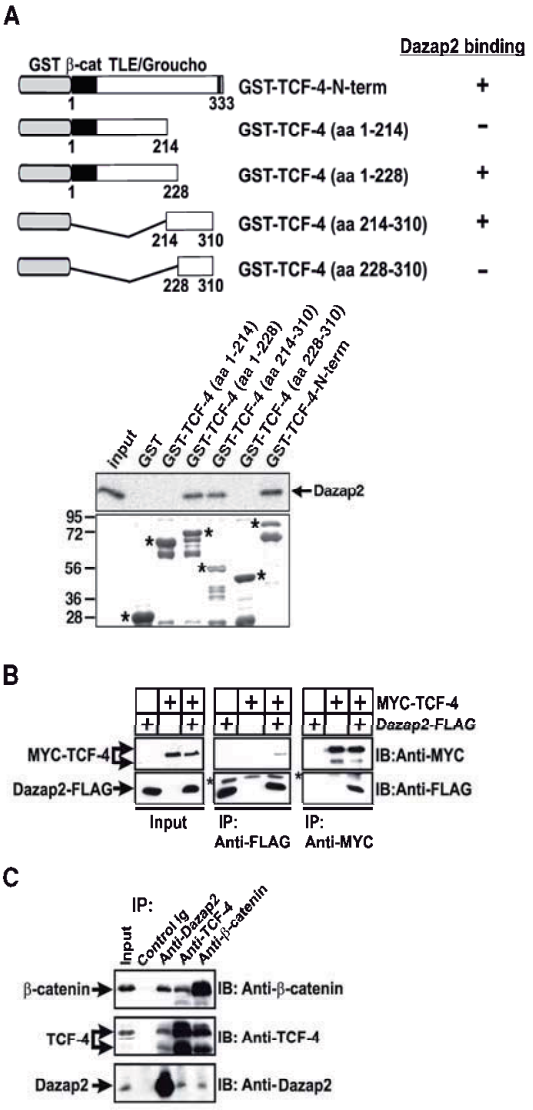

Figure 2. Association between Dazap2 and TCF-4 *in vitro* and in mammalian cells. (**A**) Interaction of Dazap2 with TCF-4 in GST pull-down assays. The top panel represents a schematic diagram of the TCF-4 proteins used in the *in vitro* pull-down assays. The bottom panel displays the pull-down assay results between the different bacterially expressed GST fusion TCF-4 proteins and *in vitro* translated [^{35}S]-labelled Dazap2. Ten percent of the total reaction was loaded in the lane denoted 'Input'. Under the autoradiograph is a Coomassie Blue-stained gel that shows the amount of GST-tagged TCF-4 proteins used in the experiment. The putative intact forms of the recombinant proteins are labelled by asterisks; the faster migrating bands result from a partial degradation of the corresponding GST fusion proteins. Molecular weight markers in kDa are indicated on the left. (**B**) Co-immunoprecipitation of FLAG-tagged Dazap2 and MYC-tagged TCF-4. Cell lysates prepared from HEK 293 cells transfected with constructs as indicated were precipitated using anti-MYC and anti-FLAG monoclonal antibodies. The asterisks indicate the light chains of immunoglobulins used in the experiments. (**C**) Endogenous complexes of Dazap2, TCF-4 and β-catenin in human cells. Lysates prepared from DLD-1 cells were subjected to immunoprecipitation with anti-Dazap2, anti-TCF-4, anti-β-catenin or anti-EGFP (Control Ig) rabbit polyclonal antibodies. Blots were probed by either anti-TCF-4, anti-β-catenin mouse monoclonal or anti-Dazap2 chicken polyclonal antibodies. In lanes denoted 'Input', 10% of the total lysate used for one immunoprecipitation was loaded; IP, immunoprecipitation; IB, immunoblotting.

APC that results in the accumulation of nuclear TCF-4/β-catenin complexes. Interestingly, Dazap2 was present in the precipitates obtained by incubating DLD-1 cell lysates with an anti-β-catenin antibody and similarly, β-catenin was isolated using an anti-Dazap2 antibody (Figure 2C). Since we did not detect any association between Dazap2 and β-catenin in GST pull-down assays (Supplementary Figure 3) we conclude that these proteins do not interact directly but are instead brought to one heterocomplex via association with their common partner, the TCF-4 factor. We also noted that the endogenous TCF-4 protein extracted from various mammalian cells migrates in the denaturing gels as a double band representing polypeptides of the apparent molecular weight 65 and 85 kDa, respectively (Figures 2C, 5A, 6A and data not shown). Interestingly, both these bands showed immunoreactivity with various monoclonal antibodies recognizing different epitopes in TCF-4 (data not shown). Moreover, ectopic expression of TCF-4 generated two different protein forms similar to their endogenously produced counterparts (Figure 2B and Supplementary Figure 5). As the expression of the both putative TCF-4 proteins was specifically down-regulated by *TCF-4* shRNA (Supplementary Figure 4) and the predicted M_w of TCF-4 is 65.3 kDa, we concluded that the slower migrating and mostly more prominent band represents the TFC-4 polypeptide, possibly modified by sumoylation (23).

To visualize the subcellular distribution of endogenous DAZAP2 protein we selected the human lymphoma U-937 cell line as it exhibits a relatively high level of *DAZAP2* mRNA expression (Figure 1C). Analysis of Dazap2-stained cells by confocal microscopy revealed a predominantly cytoplasmic distribution with some additional nuclear staining (diffuse or in distinct dots or 'puncta') (Figure 3A). The staining of putative Dazap2 protein was specific as both polyclonal antisera showed a virtually identical subcellular distribution. Moreover, the observed reactivity was completely abolished by pre-incubation of the primary antibodies with Dazap2 (and not with control EGFP) recombinant protein (Figure 3A and data not shown). Finally, we tested the co-localization of ectopically expressed TCF-4 and Dazap2 (either EGFP- or Myc-tagged) in HeLa cells. In single-transfected cells expressing either TCF-4 or Dazap2, TCF-4 was clearly nuclear whilst Dazap2 (visualized by either EGFP or Myc antibodies) displayed a primarily cytoplasmic and partly nuclear localization. When both proteins were co-expressed, Dazap2 was sequestered to the nucleus in a dose-dependent manner unlike the control EGFP-only protein, which remained uniformly distributed between the cytoplasm and nucleus irrespective of the presence or absence of the TCF-4 factor (Figure 3B and data not shown). Altogether, the data reported here indicate that Dazap2 interacts directly with TCF-4 and that this interaction results in its subcellular redistribution to the nucleus.

As the essential region in TCF-4 required for binding to Dazap2 displays some sequence homology with the corresponding sequences in other Tcf/Lef family members (Figure 4A), we decided to further analyse whether Dazap2 can interact with additional Tcf/Lef proteins.

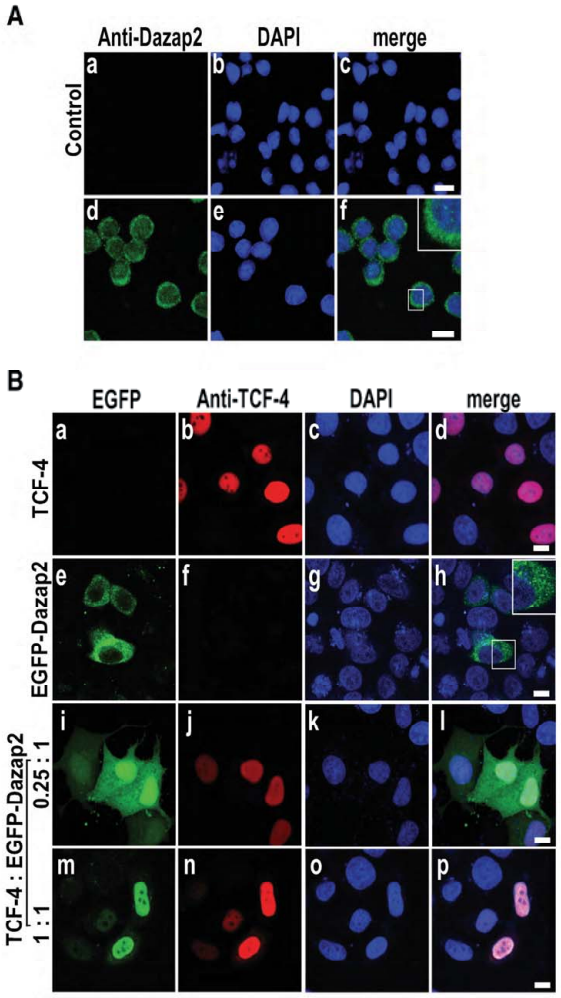

Figure 3. TCF-4 translocates Dazap2 into the nucleus. (**A**) Endogenous Dazap2 protein shows a mostly cytoplasmic distribution in human cells. Laser scanning confocal micrographs of U-937 cells stained with an antigen-purified chicken anti-Dazap2 polyclonal antibody. The merged images (c) and (f) were generated by an overlay of endogenous Dazap2 captured in the green channel and the DAPI nuclear stain captured in the blue channel. The first row, labelled 'Control', shows the cells stained with the primary antibody pre-blocked with Dazap2 recombinant protein as described in the 'Materials and methods' section; inset in (f) shows a magnified image as indicated. Bar, 10 μm. (**B**) Nuclear co-localization of ectopically expressed EGFP-Dazap2 and TCF-4 proteins. Laser scanning confocal microscopy images of HeLa cells transfected with constructs (as indicated on the left) and subsequently stained with the mouse anti-TCF-4 monoclonal antibody. The images (i, j, k, l) show cells transfected with TCF-4 and EGFP-Dazap2 constructs at a ratio 0.25 (TCF-4) to 1 (EGFP-Dazap2); the images (m, n, o, p) were obtained at a ratio 1 to 1. The merged images (d, h, l, p) were generated by an overlay of the corresponding images gained either in the green input channel detecting EGFP-Dazap2, red input channel detecting TCF-4 or blue channel to detect the DAPI nuclear stain. Inset in (h) shows a magnified image as indicated. Bar: 10 μm.

Constructs expressing Myc-tagged TCF-1, -3, -4, Lef-1 and Flag-tagged Dazap2 were either single- or double-transfected into HEK 293 cells and their ability to interact was tested by co-immunoprecipitation using anti-Myc or anti-Flag antibodies as described above. All TCF/Lef proteins analysed were isolated from cell lysates containing Flag-Dazap2 using the anti-Flag antibody.

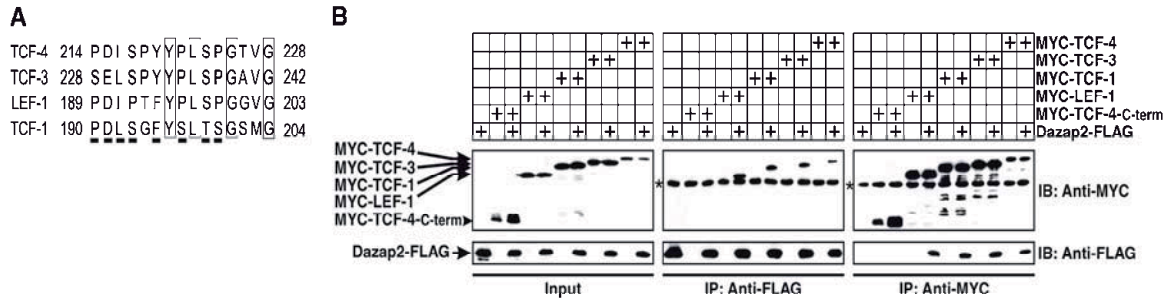

Figure 4. Dazap2 interacts with all members of the Tcf/Lef family. (**A**) The region of TCF-4 required for interaction with Dazap2 as identified by GST pull-down assays (aa 214–228) is partly conserved in other Tcf/Lef family members. The corresponding sequences of the human proteins were aligned using the ClustalV program. Fully conserved amino-acid residues are boxed whilst amino-acid residues with similar biochemical properties are underlined. GenBank accession numbers: TCF-4, NP_110383; TCF-3, NP_112573; LEF-1, NP_057353; TCF-1, NP_003193. (**B**) Co-immunoprecipitation of Dazap2-FLAG and MYC-tagged TCF/LEF transcription factors in human HEK 293 cells transfected with various Tcf expression constructs as indicated. Co-immunoprecipitation of TCF/LEF proteins using an anti-FLAG antibody is also shown. In the gel denoted 'Input', 10% of total lysates was loaded. The asterisks indicate the heavy chains of immunoglobulins used in the experiments.

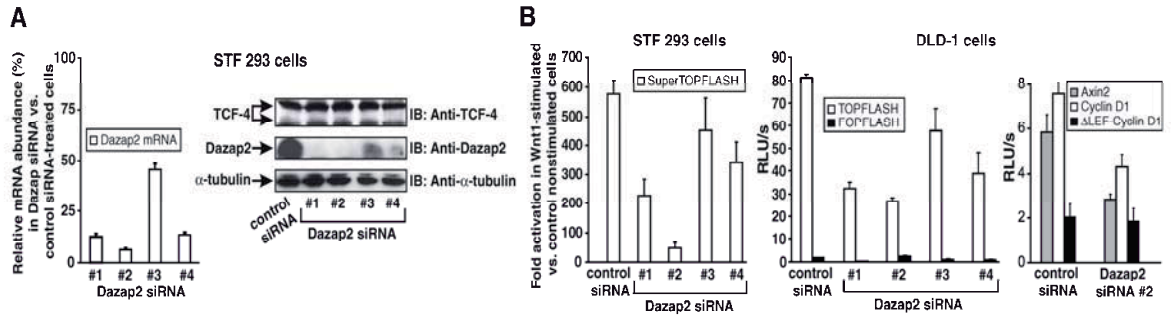

Figure 5. The activity of the Wnt/β-catenin-dependent reporters positively correlates with the amounts of Dazap2 in the cells. (**A**) HEK 293 cells containing the integrated reporter pSuperTOPFLASH (STF 293 cells) were transfected with four different *Dazap2*-specific siRNAs marked 1 to 4 or with control siRNA (*EGFP*-specific) and the changes in the levels of *Dazap2* mRNA or protein were tested 24 h post transfection. The left panel shows the results obtained by qRT–PCR analysis. The relative abundance of *Dazap2* mRNA in *Dazap2* siRNA- versus control siRNA-transfected cells (the level of *Dazap2* mRNA in control cells was set to 100%) was derived from the average CT values after normalizing to the levels of *β-ACTIN* cDNA. Data from two independent experiments performed in triplicate were combined in the graph. SDs (standard deviations) are shown by error bars. The right panel shows western blots of whole cell extracts prepared from STF 293 cells treated with siRNAs as indicated. Blots were probed with anti-Dazap2, anti-TCF-4 or anti-α-tubulin (as an internal control) antibodies. (**B**) Results of the reporter gene assays in human cells. STF 293 and DLD-1 adenocarcinoma cells were transfected with *Dazap2* or control siRNAs. To stimulate the integrated pSuperTOPFLASH in STF 293 cells the siRNA transfection mixtures additionally contained a *Wnt1*-expression construct; mixtures including an empty vector were used in control transfections. DLD-1 cells were co-transfected with siRNA and the Tcf/β-catenin-responsive reporter pTOPFLASH or pFOPFLASH as a negative control. After 24 h, cells were harvested and luciferase (firefly) activities were determined in the lysates. The histograms represent mean values of triplicate experiments with SDs. The reporter activity in unstimulated (empty vector) STF 293 cells was arbitrarily set to 1. For DLD-1 cells the average luciferase light units per second (RLU/s) are given; the values were corrected for the efficiency of transfection using the internal control *Renilla* luciferase expression plasmid. The results of one representative experiment from two in total are shown.

Reciprocally, Dazap2 was similarly pulled down from cells co-expressing Myc-tagged TCF/Lef proteins with the anti-Myc antibody (Figure 4B). In agreement with previous results Dazap2 did not co-immunoprecipitate with the C-terminal part of TCF-4 (TCF-4-C-term) lacking the Dazap2-interaction domain (Figure 4B). Since the short stretch of amino acids mediating the Dazap2 binding is in close proximity to a region which is alternatively spliced in most of Tcf/Lef family members (alternative splicing involves exon IVa in Tcf-1, exon VI in Lef-1 and exon VIII in Tcf-4) (50–52), we generated a TCF-4 variant lacking exon VIII and tested its ability to bind Dazap2 in GST pull-down and co-immunoprecipitation assays. As this variant associated with Dazap2 to the same extent as the non-spliced form (Supplementary Figure 5), the assays further confirmed the importance of the already defined interaction motif.

Dazap2 knockdown decreases Wnt/β-catenin-mediated transcription

To examine whether Dazap2 overexpression has any effect on Wnt-mediated transcription we co-transfected a TCF/β-catenin-responsive luciferase reporter, pTOPFLASH or its mutated variant, pFOPFLASH into HEK 293 cells in conjunction with a Dazap2 expression construct. The cells were subsequently stimulated by recombinant Wnt3a and the luciferase activities were determined from cell lysates. The ectopic expression of Dazap2 displayed neither an inhibitory nor stimulatory effect on Wnt signalling. Furthermore, analyses of Wnt-regulated transcription in DLD-1 cells that should exhibit constitutive Wnt signalling or U2OS cells that are activated by the Wnt3a ligand were not affected by *Dazap2* overexpression (data not shown). Finally, RNA interference (RNAi) was used to

down-regulate the expression of *Dazap2* and the resulting Tcf/β-catenin-dependent transcription was analysed. First, we utilized four distinct siRNA duplexes and tested their efficiency towards *Dazap2* mRNA using qRT–PCR and western blotting. As shown in Figure 5A, all four siRNAs reduced the levels of *Dazap2* mRNA and protein to different extents, with siRNA #1 and #2 showing the highest efficiency (as compared to the control EGFP siRNA). Subsequently, we introduced by lipofection the siRNAs into cells containing the genome-integrated variant of the pTOPFLASH reporter, pSuper TOPFLASH [the resultant cells were termed pSuper TOPFLASH (STF) 293]. To stimulate Wnt signalling the cells were co-transfected with a *Wnt1* expression construct. Twenty-four hours post-transfection the cells were harvested, cell lysates prepared and luciferase activities measured. In a parallel analogous experiment, DLD-1 cells were co-transfected with siRNA duplexes and a DNA mixture containing the pTOPFLASH or pFOPFLASH (negative control) reporter in addition to a *Renilla* luciferase-expressing plasmid that acted as an internal control. The next day cells were harvested and processed as described for STF 293 cells. Surprisingly, Tcf-driven transcription measured from the integrated or ectopic reporter pTOPFLASH was substantially reduced in the *Dazap2* siRNA-treated cells. Of importance, the extent of signalling reduction corresponded to the efficiency of the *Dazap2* knockdown as documented for the individual *Dazap2*-specific siRNAs (Figure 5B). Virtually identical results were obtained with Wnt-stimulated U2OS cells (data not shown). Furthermore, Dazap2 protein knockdown also negatively affected the transcription of two additional well-defined Tcf/β-catenin-dependent reporter constructs that contained either a 5-kb promoter region of the *Axin2* (46) gene or a 163-nt long enhancer element proximal to the transcription start of the *Cyclin D1* gene (10) (Figure 5B). Importantly, when a single Tcf/Lef-binding site in the *Cyclin D1* reporter was mutated, the resulting plasmid (designated as ΔLEF-Cyclin D1) not only lost its responsiveness to Wnt signalling but its basal expression did not change in cells treated with *Dazap2* or control siRNA (Figure 5B). To ensure the two *Dazap2*-specific siRNAs used did not induce any non-specific 'off-target' effects, two lentiviral constructs containing shRNA against mouse *Dazap2* were purchased and introduced into mouse mammary epithelium C57MG cells. Since both shRNAs showed a similar efficiency to down-regulate *Dazap2* expression (Figure 6A) and the Wnt stimulation had no effect on Dazap2 expression (Figure 6A and B), the polyclonal cell cultures were mixed and luciferase reporter assays performed. As expected, the cells with down-regulated *Dazap2* displayed a decrease in Wnt-stimulated transcription as compared to control cells containing non-silencing shRNAs (generated in parallel experiments) (Figure 6C). Therefore, these experiments confirm our previous results obtained from transient transfection assays performed in human cells.

To analyse whether *Dazap2* knockdown has any effect on the transcription of endogenous Wnt-signalling target genes, C57MG cells expressing *Dazap2* or control shRNAs were stimulated with Wnt3a ligand and the

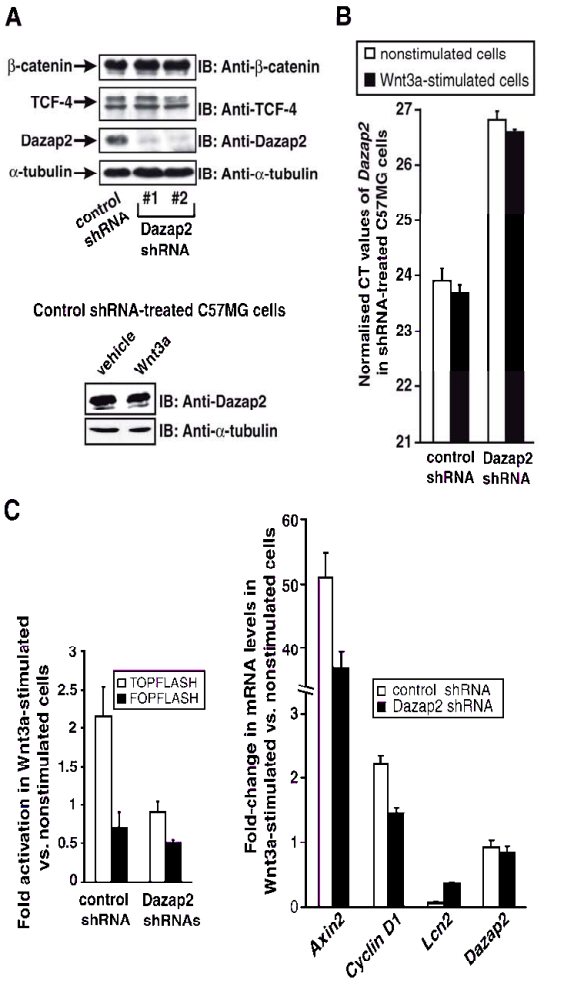

Figure 6. Down-regulation of *Dazap2* reduces the responsiveness of C57MG cells to Wnt3a stimulation. (**A**) The top panel shows a series of western blots from whole cell extracts prepared from mammary gland epithelium C57MG cells transduced with retroviral vectors expressing either a non-silencing control shRNA or two different *Dazap2* shRNAs. Prior to harvesting, cells were stimulated with Wnt3a ligand for 16 h. The blots were probed with antibodies as indicated. The bottom panel shows the western blot of whole cell extracts prepared from non-silencing control shRNA-treated cells incubated with Wnt3a or vehicle for 16 h. (**B**) The relative abundance of *Dazap2* mRNA in control or *Dazap2* shRNAs-expressing (a mixed culture of cells containing *Dazap2* shRNA 1 and 2 was used) cells as measured by qRT–PCR. The cells were cultured without activation or stimulated with Wnt3a for 16 h. The expression of the *Dazap2* gene is indicated by average CT values obtained by qRT–PCR assay on the corresponding mRNA after normalization to the levels of *β-actin* cDNA. (**C**) The left panel depicts control or *Dazap2* shRNA #1 and #2-producing C57MG cells that were transfected with the indicated reporters. Twenty-four hours post-transfection, cells were either stimulated with Wnt3a ligand or grown without stimulation. After additional 16 h, the cells were harvested and luciferase activities were determined in lysates. These activities were corrected for the efficiency of transfection by determining the luciferase/*Renilla* ratio. Values in unstimulated cells were arbitrarily set to 1. Transfections were done in triplicates and the results from one experiment out of two in total are presented with SDs. The right panel shows cDNAs prepared from Wnt3a-treated or control cells expressing non-silencing or *Dazap2* shRNAs analysed by qRT–PCR. The relative abundance of the indicated mRNAs in given cells (the levels of the tested mRNAs in unstimulated cells was set to 1) was derived from the average CT values after normalizing to the levels of *β-actin* cDNA. Data shown are from one representative experiment from a total of two.

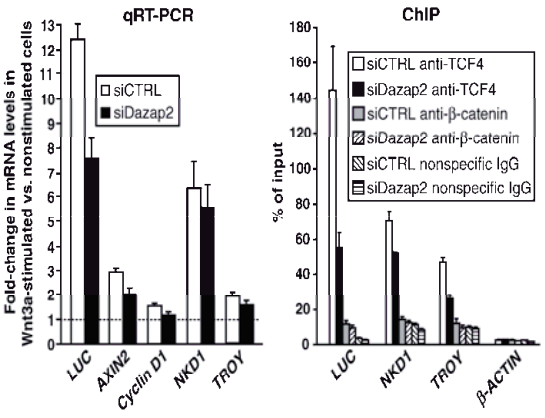

Figure 7. *Dazap2* knockdown decreases binding of TCF-4 to the promoters of the Wnt-signalling target genes. STF 293 cells were transfected with *Dazap2* siRNA #2 (siDazap2) or control siRNA (siCTRL). After a 2-day expansion the cells were transferred to large culture dishes and stimulated with either Wnt3a ligand or Wnt3a storage buffer only. After 16 h, the cells were harvested and used for mRNA isolation and cDNA preparation. Alternatively, the cells were fixed directly in the dishes and utilized for chromatin immunoprecipitation (ChIP). The left panel shows the results from qRT–PCR analysis. The relative abundance of the indicated mRNAs in Wnt3a-stimulated versus control cells was derived from the average CT values after normalizing to the levels of *β-ACTIN* cDNA. The right panel shows the ChIP analysis of chromatin isolated from STF 293 cells transfected with control or *Dazap2* siRNA #2 duplexes. Only results for Wnt3a-stimulated cells are shown. The diagram represents the relative amounts of the respective DNA element pulled-down by the indicated antibodies. The amount of input non-immunoprecipitated DNA (evaluated separately for each primer set) was arbitrarily set to 100%. Data from one representative experiment from two in total are given.

expression of several putative Tcf/β-catenin targets was assessed by qRT–PCR. As shown in Figure 6C, *Dazap2* down-regulation resulted in the lower stimulation of the *Cyclin D1* and *Axin2* genes by Wnt3a than observed in control shRNA-producing cells. Interestingly, the reduced level of Dazap2 protein partly relieved the Wnt-mediated repression of the *lipocalin 2* (*Lcn2*) gene (Figure 6C). Additionally, we performed qRT–PCR analysis in STF 293 cells transiently transfected with control or *Dazap2* siRNA. As described above, STF 293 cells contain a stably integrated luciferase reporter that is under the control of eight Tcf/Lef-binding sites. This endogenous reporter, which we named *Luciferase* (*LUC*), was activated at the mRNA level up to 13-fold upon stimulation with Wnt3a for 16 h (Figure 7). In agreement with the reporter gene assays, *Dazap2* knockdown resulted in an approximately forty percent reduction in transcription of this gene after treatment with Wnt3a. A similar although less robust decrease in Wnt-activated expression was observed in several other Wnt-signalling target genes including *AXIN2*, *CYCLIN D1*, *naked cuticle homologue 1* (*NKD1*; 53,54) and *tumor necrosis factor receptor superfamily, member 19* [(*TNFRSF19*), known also as *TROY*; (55,56) (Figure 7)]. To clarify the possible mechanism behind Dazap2 function we wanted to perform ChIP analysis. Since we did not succeed to perform ChIP on the promoters of the *Axin2* and *Cyclin D1* genes in any mouse or human cells (including C57MG and STF 293 cells) we extended our analysis to the TCF-responsive enhancers of the *Luciferase*, *NKD1* and *TROY* genes. We did not observe ChIP using our anti-Dazap2 antibody and furthermore, we only detected a limited pull-down of the *Luciferase* transgene (the most extensively up-regulated gene in the assay) by the anti-β-catenin antibody. Instead we used our anti-TCF-4 antibody to immunoprecipitate DNA elements that spanned the promoter regions of the tested genes (Figure 7). Interestingly, reduction of Dazap2 lowered the binding of TCF-4 to the promoters of these Wnt-signalling target genes. To verify the ChIP results, two control experiments were carried out. First, we performed ChIP with a non-specific antibody (rabbit anti-EGFP). Second, we used an anti-TCF-4 antibody in an attempt to immunoprecipitate irrelevant chromosomal DNA that concealed the open reading frames of the *β-ACTIN* or *LUC* genes. In these experiments we never noted any effect of the cellular levels of Dazap2 on the quantity of precipitated DNA (Figure 7 and data not shown). As *Dazap2* knockdown did not alter the amount of TCF-4 and β-catenin in the cell (Figures 5A, 6A and data not shown) these results imply that the Dazap2/TCF-4 interaction might influence the efficiency of TCF-4 binding to the promoters of the genes regulated by the canonical Wnt-signalling pathway.

DISCUSSION

In this report we provide evidence for an association between the nuclear Wnt-signalling pathway effectors, Tcf/Lef proteins, and a small evolutionary conserved protein Dazap2. Dazap2 was isolated in a Y2H screen that utilized the N-terminal part of the TCF-4 factor as bait. Although the Dazap2-interacting domain in TCF-4 is only partially preserved in other Tcf/Lef proteins, co-immunoprecipitation assays carried out in mammalian cells clearly demonstrated that all Tcf/Lef family members bind to Dazap2 with similar affinities (Figure 4B). We further delineated a short region in TCF-4 spanning aa 214–228 as the interaction domain essential for Dazap2 binding (Figure 2A). Interestingly, the homologous region in all Tcfs contains amino acids that are not identical but display similar biochemical properties (Figure 4A). This might indicate a common structural basis for the association of Tcf/Lef proteins with Dazap2.

Dazap2 was originally identified as a transcript expressed in the mouse inner ear with its expression further observed in the embryonic heart and developing and adult mouse brain (31). This report is in stark contrast to our finding that illustrates ubiquitous expression of *Dazap2* mRNA in various mouse tissues (Figure 1C). Nevertheless, our data are in agreement with northern blot analyses that indicate broad expression of the *Dazap2* gene in different human and mouse tissues and cell lines (32–34). The most remarkable feature of Dazap2 is the conservation of its DNA and protein sequence throughout evolution. The identity between human and mouse protein orthologues is virtually 100% (there is only one aa change from 168 aa in total) (Supplementary Figure 1) and human Dazap2 aligns well, especially at the C-terminus, with the putative Dazap2 proteins identified in the invertebrates *Ciona*

intestinalis and *Tribolium castaneum* (Figure 1B). Given that Dazap2 was identified as a binding partner of many cellular proteins (57), the necessity to preserve these interactions might possibly explain the low mutational rate of the Dazap2 sequence throughout evolution. With respect to the high sequence homology of Dazap2 in various species it is rather striking that *Dazap2* mutant mice do not display any remarkable phenotype and are born and bred as their wild-type littermates (31). The *Dazap2*$^{-/-}$ mice were generated from ES cells via gene trap technology. Although we cannot exclude that the modified *Dazap2* locus still produces an intact protein, the insertion site of the reporter gene just several nucleotides downstream of the putative translation start site and a complete absence of *Dazap2* mRNA expression indicate that the mutant animals are really *Dazap2* null. There are two possibilities that might explain an absence of phenotype in *Dazap2*$^{-/-}$ mice. First, the phenotype may be very subtle and/or the mice have to be challenged in some way to display any phenotype and second, there is another *Dazap2* homologue in the mouse genome that can functionally replace the damaged gene. Indeed, a sequence database search in the mouse genome revealed a *Dazap2* pseudogene localized on chromosome 4 and one gene similar to *Dazap2* on chromosome 13. This *Dazap2-like* gene comprises several exons and introns and encodes a putative 168 aa polypeptide that is highly homologous to Dazap2 (Supplementary Figure 6). Therefore, we speculate that these genes are redundant and *Dazap2-like* can compensate for the absence of the *Dazap2* gene. Interestingly, we have not been able to detect the expression of the Dazap2-like product in any of the cell lines tested (data not shown). Thus, to settle this matter definitively, the possible redundancy of these two genes should be tested directly in the *Dazap2* mutant animals. Another noticeable feature of the Dazap2 protein is its subcellular localization. Several authors have used confocal or fluorescent microscopy to visualize ectopically expressed Dazap2 as a wild-type untagged protein or as a variant fused to different tags (mostly N-terminal FGFP). These authors described the subcellular distribution of Dazap2 as diffuse in the cytoplasm and nucleus (32,36), in nuclear puncta (39) or in the nucleus and cytoplasmic SG bodies (38). These experiments were predominantly performed in HeLa cells. Interestingly, we noted in HeLa (and Cos-7) cells that both tagged or untagged Dazap2 principally localizes in the cytoplasm and partly in the nucleus but this distribution can differ in a limited fraction of the cells possibly as a consequence of the cell cycle. Nevertheless, Dazap2 was efficiently translocated to the nuclei (or retained in the nuclei) in cells expressing TCF-4, thus further confirming the interaction between Dazap2 and TCF-4 (Figure 3B).

To assess the biological significance of the association between Dazap2 and Tcfs, we first ectopically expressed Dazap2 together with three different Tcf/β-catenin-responsive reporters in various cells. The cells were further stimulated with purified Wnt3a ligand and the activities of the reporters were determined in lysates. Surprisingly, we never observed changes in the transcriptional activity of the reporters depending on the increased levels of Dazap2. Subsequently, we utilized RNAi technology to test the influence of *Dazap2* knockdown on Wnt-dependent transcription. In cells with reduced levels of *Dazap2* mRNA and protein the activities of the Tcf/β-catenin reporters were significantly decreased and the extent of reduction correlated well with the ability of each particular siRNA to down-regulate *Dazap2* (Figure 5A and B). The observed results were not related to the non-specific 'off-target' effects of the siRNA duplexes used since control non-silencing siRNA did not show any impact on the transcriptional activity of the tested constructs. Additionally, similar effects on Wnt signalling were observed in cells stably expressing *Dazap2* shRNAs (Figure 6C). Importantly, we also demonstrated by qRT–PCR that *Dazap2* knockdown also negatively influenced the transcriptional activation of endogenous Wnt-signalling target genes, although to a lesser extent than observed for the reporter genes (Figures 6C and 7). There are several possibilities that could explain the partial discrepancy between the results obtained from the reporter gene assays and the qRT–PCR analysis of endogenous genes. First, the transcriptional regulation of the reporter genes is less complex than that of the endogenous promoters and possibly more dependent on the activity of the Wnt pathway. Second, in the transient siRNA transfections we noted fast 'exhaustion' of *Dazap2* siRNA followed by rapid return of *Dazap2* mRNA and protein to the original levels (data not shown). This in fact could be responsible for the less pronounced effects of *Dazap2* knockdown especially in experiments where prolonged treatment with *Dazap2* siRNA was needed (Figure 7). Finally, the reporters used in the study encode a 'standard' luciferase protein that is quite stable and accumulates in the cells. This presumably would also explain the differences between luminometric measurements and the qRT–PCR analysis.

A possible explanation for the negative impact of *Dazap2* down-regulation on Wnt-stimulated transcription is that Dazap2 is a stabilizing component of TCF-4/β-catenin heterocomplexes (Figure 2C). To evaluate this possibility we tried to examine whether reduced levels of Dazap2 could disrupt these complexes. However, co-immunoprecipitation experiments from shRNA-producing cells did not reveal any difference in the amounts of β-catenin pulled down by TCF-4 in cells with normal or decreased *Dazap2* expression (data not shown). We also excluded the possibility that Dazap2 is important for the production or stability of TCF-4 or β-catenin which could have accounted for the decrease in transcriptional activation from the reporters as *Dazap2* knockdown did not influence the expression of either of these Wnt-signalling effectors (Figures 5A, 6A and data not shown). The Dazap2 interacting domain in Tcfs partly overlaps with a region that *in vivo* is bound by TLE/Groucho proteins (5,6,8). Therefore, we tested the hypothesis that Dazap2 binding might block the association of Tcfs with these co-repressors and would in turn increase the activating function of the Tcf/Lef transcription factors. We transfected pTOPFLASH into control siRNA- or *Dazap2* siRNA-treated HEK 293 cells together with Grg4, a mouse TLE/Groucho homologue.

The cells were subsequently stimulated with Wnt3a, harvested and reporter activities measured in cell lysates. In a parallel experiment, control siRNA- or *Dazap2* siRNA-treated cells were lipofected with pTOPFLASH and cDNA encoding either a negative regulator of Wnt signalling, nemo-like kinase (NLK) alone or in combination with its upstream activating kinases TAB1 and TAK1. The TAB/TAK/NLK cascade is a downstream component of a negative feed-back loop that is activated by Wnt signalling (58). Active NLK phosphorylates Tcfs and importantly, this phosphorylation prevents the binding of Tcf/β-catenin complexes to DNA and consequently leads to Tcf ubiquitylation and degradation (59–61). Interestingly, the NLK phosphorylation sites in Tcfs are located just proximal to the region indispensable for Dazap2 binding (59). Intriguingly, Winkel and colleagues (62) recently reported that Dazap2 (referred to as PRTB) interacts with TAK1 kinase and enhances its enzymatic activity. Nevertheless, we did not observe any of the repressive effects of Grg4 or NLK on Wnt signalling (using the ectopic or integrated reporter) and thus we did not notice any enhancement of this repression in cells with reduced *Dazap2* expression. Based on these observations we excluded the possibility that Dazap2 functions as a blocker of the negative functions of TLE/Groucho and NLK. Finally, we performed a ChIP experiment utilizing chromatin isolated from STF 293 cells with *DAZAP2* down-regulated. The assay revealed a remarkable decrease in the association of TCF-4 to the Tcf-responsive sites in the promoters of Wnt-signalling target genes (Figure 7). These data imply that the Tcf/Lef interacting partner Dazap2 can modulate *in vivo* the affinity of Tcfs for their recognition motifs. Interestingly, in mouse mammary gland epithelium C57MG cells, decreased levels of Dazap2 partly relieved the repression on the *Lcn2* gene mediated by Wnt3a treatment (Figure 6C). As the repressive effect of active Wnt signalling on *Lcn2* does not depend on the direct binding of Tcf/β-catenin complexes to the *Lcn2* promoter (63), this result suggests that Dazap2 levels might modulate the complex output of the Wnt-signalling pathway. To prove this hypothesis, we tested the expression of genes that respond differentially to the Wnt signal in C57MG cells, *Stromelysin-1* (*Sl-1*/*Mmp3*) and *mesothelin* (*Msln*) (64). However, qRT–PCR analysis revealed that the transcription of these two genes depends on the plating density of cells in culture rather than specifically on Wnt signalling (P.M. and V.K., unpublished data). Nevertheless, preliminary cDNA microarray data obtained from mRNAs isolated from Wnt-stimulated C57MG cells expressing *Dazap2*-specific or control shRNAs indicate that indeed, both the activating and also the inhibitory function of Tcf/Lef proteins might be influenced by the amounts of Dazap2 (P.M., R.I. and V.K., unpublished data).

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Supplementary data

Supplementary Materials and Methods

Coimmunoprecipitation and western blotting

To obtain whole cell lysates, human or mouse cells were incubated in lysis buffer [50 mM Tris, pH 7.8, 400 mM NaCl, 0.5% (v/v) Triton X-100] supplemented with Complete (EDTA free) protease inhibitor cocktail (Roche) for 30 min at 4°C (rotating platform). The lysates were cleared by centrifugation (14 000 x g, 15 min, 4°C) and used for coimmunoprecipitation with an appropriate antibody coupled to protein A/G Sepharose beads (Amersham Pharmacia Biotech). For one assay 20-30 micro litres of beads loaded with 1 μg of purified (on protein A Sepharose) monoclonal antibody were used; alternatively, beads were loaded with 10 μl of unpurified antiserum. After elution (SDS sample buffer) the precipitates were separated by SDS PAGE and transferred onto polyvinylidene fluoride membranes (Millipore). The membranes were blocked with 5% (w/v) non-fat milk in PBS containing 0.05% (v/v) Tween-20 (Sigma) and incubated with specific primary antibodies at a final concentration of 1 μg/ml (the unpurified chicken anti-Dazap2 antiserum was diluted 500 times). After several washes with PBS/Tween the blots were incubated with the appropriate peroxidase-conjugated anti-rabbit (BioRad), anti-mouse (BioRad) or anti-chicken secondary antibodies (Sigma) diluted according to the manufacturer's recommendation. The proteins were visualised with an enhanced chemiluminescence system (Pierce).

qRT-PCR analysis

Primers for the following human and mouse genes were used (first primer is derived from the plus and the second primer from the minus DNA strand): human *AXIN2* (*hAXIN2*), 5'-TGA GGT CCA CGG AAA CTG TTG ACA GT-3', 5'-CCC TCC CGC GAA TTG AGT GTG A-3'; mouse *Axin2* (*mAxin2*), 5'-TAG GCG GAA TGA AGA TGG AC-3', 5'-CTG GTC ACC CAA CAA GGA GT-3'; *hCYCLIN D1*, 5'-CCA TCC AGT GGAGGT TTG TC-3', 5'-AGC GTA TCG TAG GAG TGG GA-3'; *mCyclin D1*, 5'-AGT GCG TGC AGA AGG AGA TT-3', 5'-CTC TTC GCA CTT CTG CTC CT-3'; *Dazap2* (priming on both human and mouse cDNAs), 5'-ACC CTA TAC CGA TGC TCC AC-3', 5'-CAG AGA GGC TCC AGG AAA TG-3'; *hTROY*, 5'-CTA TGG GGA GGA TGC ACA GT-3', 5'-TCT CCA CAA GGC ACA CAC TC-3'; *hNKD1*, 5'-CGC CGG GAT AGA AAA CTA CA-3', 5'-GCA TTG AGC TGA CAC GAA AA-3'; *mLcn2*, 5'-GCC CAG GAC TCA ACT CAG AA-3', 5'-GAC CAG GAT GGA GGT GAC AT-3'; *firefly luciferase* (*LUC*), 5'-TCA AAG

AGG CGA ACT GTG TG-3', 5'-CGC TTC CGG ATT GTT TAC AT-3'; *h*β-*ACTIN*, 5'-ATG GCC ACG GCT GCT TCC AGC-3', 5'-GGG TGT AAC GCA ACT AAG TCA T-3'; *m*β-*Actin*, 5'-GAT CTG GCA CCA CAC CTT CT-3', 5'-GGG GTG TTG AAG GTC TCA AA-3'; *hUBIQUITIN C* (*UBC*), 5'-GCT TTG TTG GGT GAG CTT GT-3', 5'-TCA CGA AGA TCT GCA TTT TGA-3'; *mUbc* (*Ubc*), 5'-ATG TGA AGG CCA AGA TCC AG-3', 5'-TAA TAG CCA CCC CTC AGA CG-3'.

Primers for ChIP

Primers derived from the Tcf/Lef-dependent enhancer of the integrated pSuperTOPFLASH reporter [designed as the *Luciferase* gene (*LUC*)], sense: 5'-AGT GCA GGT GCC AGA ACA TT-3' and reverse 5'-AAC AGT ACC GGA ATG CCA AG-3'; control region downstream this enhancer (*CTRL1*), sense: 5'-ATC CAT CTT GCT CCA ACA CC-3' and reverse 5'-TCG CGG TTG TTA CTT GAC TG-3'; *NKD1* promoter, 5'-GAC CTC CCC AGA CAA AAC AA-3', 5'-TCA GCC AGT CTC TGG GAT CT-3'; *TROY*, 5'-TTT CAT CTC CCT GCT CGT CT-3', 5'-TGC GAA AAA TGC AGT GAA AG-3'; β-ACTIN (open reading frame), 5'-ATG GCC ACG GCT GCT TCC AGC-3', 5'-GGG TGT AAC GCA ACT AAG TCA T-3'.

Supplementary Figure Legends

Supplementary Figure 1 Evolutionary conservation of the vertebrate Dazap2 proteins

Protein sequences were aligned using the ClustalV program. The amino acid residues that differ from the "consensus" (defined by at least three proteins species matching for a given position) are boxed. GenBank accession numbers: *Homo sapiens*, NP_055579; *Mus musculus*, NP_036003; *Danio rerio*, NP_956087; *Xenopus laevis*, NP_001086003; *Tribolium castaneum*, XP_973572; *Ciona intestinalis*, NM_001032667. The sequence of chicken (*Gallus gallus*) Dazap2 was assembled from three independent ESTs: BB630813, BY097208, CJ065215.

Supplementary Figure 2 The truncation of Dazap2 disrupts its binding to TCF-4

The pull-down assay results between the bacterially expressed GST fusion TCF-4 N-terminus and different variants of the *in vitro* translated [^{35}S]-labelled Dazap2 protein. Ten percent of the total reaction was loaded in the lane denoted "Input". A Coomassie Blue-stained gel under the auto-

radiograph shows the amount of GST-tagged TCF-4 protein and GST (control) used in the individual pull-downs. The putative intact form of the fusion protein is labelled by an asterisk; the faster migrating bands result from a partial degradation of the recombinant polypeptide. Molecular weight markers in kDa are indicated on the left.

Supplementary Figure 3 Association of Dazap2 with β-catenin is indirect and mediated by TCF-4

Dazap2, the full-length TCF-4 protein and its truncated variant lacking the main β-catenin interaction domain (TCF-ΔN; used as a negative control) were produced *in vitro* using the Quick TNT Coupled Reticulocyte System (Promega). Two separate TNT reactions were set up to produce both [^{35}S]-la belled (total volume 10 μl) and non-labelled TCF-4 (50 μl) proteins. One half (5 μl) of the labelling reaction was mixed with corresponding non-labelled protein and pre-incubated with GST-β-catenin bound to Glutathione Sepharose 4 for 1 hour at 4°C. The excess of the unbound TCF-4 proteins was washed off three times with GST binding buffer. Then, [^{35}S]-labelled Dazap2 was added to the mix-ture and incubated for 1 hour at 4°C. The beads were collected by centrifugation and washed three times in GST binding buffer. Bound proteins were eluted, separated by SDS-PAGE and analysed by autoradiography. Both autoradiograph (top) and Coomassie-stained gel (bottom) are shown. In the lane denoted "Input" one tenth of *in vitro* produced Dazap2 was loaded. To estimate the translational efficiency of the individual TCF-4 constructs, a second half of the reaction containing labelled TCF-4 polypeptide was loaded in the line denoted "Transl". Note that Dazap2 was specifically retained on GST-β-catenin only when the latter was preincubated with the full-length TCF-4 protein. On the contrary, Dazap2 was not detectable when incubated with GST-β-catenin-bound beads either alone or in combination with the N-terminally truncated TCF-4 variant.

Supplementary Figure 4 The TCF-4 protein is produced in mammalian cells in two different forms

Results of western blotting of the cell extracts prepared from DLD-1 cells stably transduced with lentiviral vectors expressing either *TCF-4* or a control non-silencing shRNA (purchased from Open Biosystems). The blots were probed with the anti-TCF-4 and anti-α-tubulin (a loading control) monoclonal antibody. Molecular weight markers in kDa are indicated on the left.

Supplementary Figure 5 The TCF-4 protein lacking exon VIII retains its ability to bind Dazap2

Left, pull-down assays between [^{35}S]-labelled Dazap2 and the GST fusion proteins containing the complete TCF-4 N-terminal part (GST-TCF-4-N-term; aa 1-333) or its variant lacking the amino acids encoded by exon VIII (GST-TCF-4-N-term Δ exon VIII). Ten percent of the total reaction was loaded in the lane denoted "Input". A Coomassie Blue-stained gel under the autoradiograph shows the amount of the GST-tagged TCF-4 protein used in the experiment; the putative intact forms of the recombinant proteins are labelled by asterisks. (**B**) Co-immunoprecipitation of FLAG-tagged Dazap2 and MYC-tagged TCF-4 full-length (MYC-TCF-4) or a variant lacking the amino acids encoded by exon VIII (MYC-TCF-4 Δ exon VIII). Cell lysates prepared from HEK 293 cells transfected with constructs as indicated were precipitated using anti-MYC and anti-FLAG monoclonal antibodies. In lanes denoted "Input", ten percent of the total lysate used for one immunoprecipitation were loaded; IP, immunoprecipitation; IB, immunoblotting.

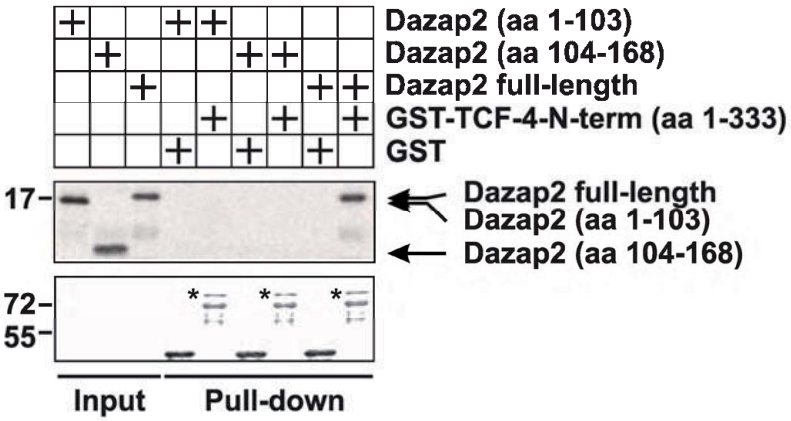
Supplementary Figure 6 The mouse genome contains two homologous Dazap2 proteins

Alignment of Dazap2 (NP_036003) derived from the gene on chromosome 15 with the putative Dazap2-like protein (XP_001473666) encoded on chromosome 13. The sequences were aligned using the ClustalV program. The amino acid residues that differ are boxed.

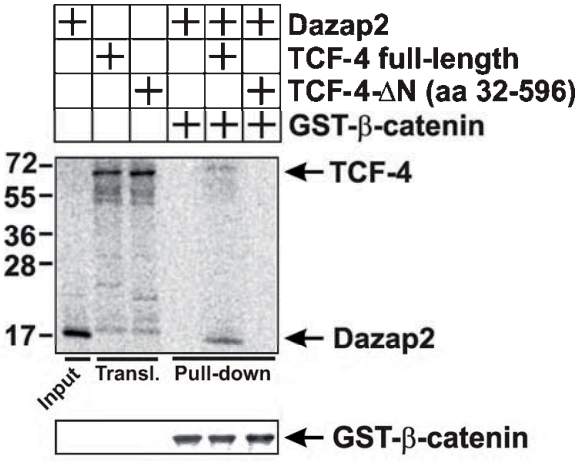
Supplementary Figure 1

```
HUMAN    MNSKGQYPTQPTYPVQPPGNP-VYPQTLHLP-QAPPYTDAPPAYSELYRP  48
MOUSE    MNSKGQYPTQPTYPVQPPGNP-VYPQTLHLP-QAPPYTDAPPAYSELYRP  48
CHICKEN  MNGKGQYPTQPPYPVQSAANPPVYPQTVPLP-QPPPYTDAPPAYSELYRP  49
XENOPUS  MNNKGQYPSAPAYPTQAPNSQSVYPPTMHLP-QAPSYTDAPPAYSELYRA  49
DANIO    MNNKGSYPQQAVYPQQSTA--PVYPPAMQVPAQVSQYPDAPPPYSEVYQP  48

HUMAN    SFV--HPGAATVPTMSAAFPGASLYLPM-AQSVAVGPLGSTIPMAYYPVG  95
MOUSE    SFV--HPGAATVPTMSAAFPGASLYLPM-AQSVAVGPLGSTIPMAYYPVG  95
CHICKEN  SFV--PLGAATVPTMSAAYPGASVFLPV-AQSVAVGPIGSSVPMAYYPVG  96
XENOPUS  AYM--QQAAAANMSALSAHYPSTSMYLPM-AQPMQVAQMSSQVPMAYYPIG  96
DANIO    RYMAPPPAPGQMPQMTSAYPGTQMYMPMHAQTVPMGAMASSVPMAYYPMG  98

HUMAN    PIYPPGSTVLVEGGYDAGARFGAGATAGNIPPPPPGCPPNAAQLAVMQGA  145
MOUSE    PIYPPGSAVLVEGGYDAGARFGAGATAGNIPPPPPGCPPNAAQLAVMQGA  145
CHICKEN  PVYPPGSTVLVEGGFDAGARFGAGGTAS-IPPPPPGCPPNAAQLAVMQGA  145
XENOPUS  PVYPPGSTVLVDGGYDAGARFGVGNSPS-VPPPPTGCPPNAAQLAAMQGA  145
DANIO    PVYPPGSTVMVDGGFDAGARFGPG-TGSSIPPPPPGHLPNAAQMAAMQGA  147

HUMAN    NVLVTQRKGNFFMGGSDGGYTIW                              168
MOUSE    NVLVTQRKGNFFMGGSDGGYTIW                              168
CHICKEN  NVLVTQRKGNFFLGGSDGGYTI                               167
XENOPUS  NVLVTQRKGNYFMGGSDGGYTIW                              168
DANIO    NVVMTQRKGNFFMGGSSGGYTIW                              170
```

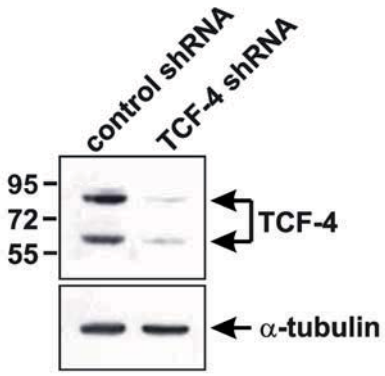
Supplementary Figure 2



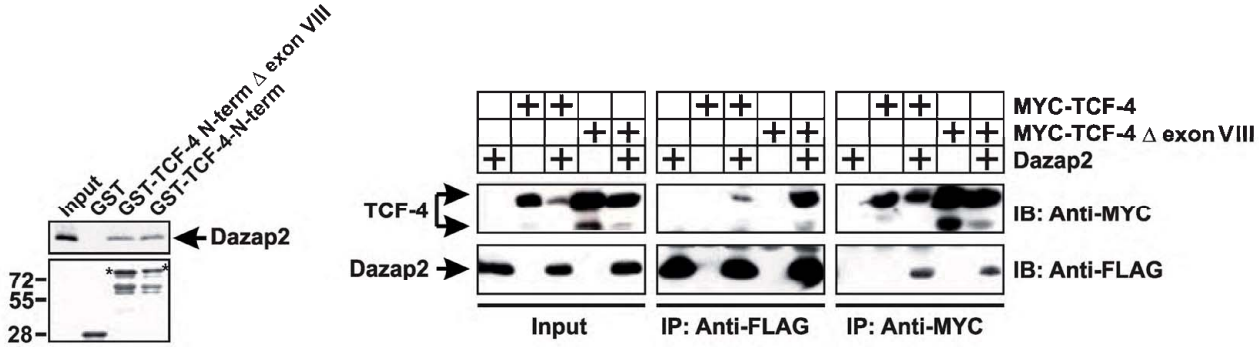
Supplementary Figure 3



Supplementary Figure 4



Supplementary Figure 5



Supplementary Figure 6

```
Dazap2        MNSKG-QYPTQPTYPVQPPGNPVYPQTLHLPQAPPYTDAPPAYSELYRPS  49
Dazap2-like   MNSKGGQYPTQPTYPVQPPGNPVYPQTLHLPQAPPYTDAPPAYSELYRPS  50

Dazap2        FVHPGAATVPTMSAAFPGASLYLPMAQSVAVGPLGSTIPMAYYPVGPIYP  99
Dazap2-like   FVHPGAATVPTMSAAFPGASLYLPMAQSVAVGPLGSTIPMAYYAVGPNYS 100

Dazap2        PGSAVLVEGGYDAGARFGAGATAGNIPPPPPGCPPNAAQLAVMQGANVLV 149
Dazap2-like   PGSAVLVEGGHDAGARFGAGATAGNIPPPPPGCPPNTAQLAVMQGANVPV 150

Dazap2        TQRKGNFFMGGSDGGYTIW                                 168
Dazap2-like   TQLKGNFFMGGT-GGYTMW                                 168
```

Fatty acid modification of Wnt1 and Wnt3a at serine is prerequisite for lipidation at cysteine and is essential for Wnt signalling

Doubravska, L., Krausova, M., Gradl, D., Vojtechova, M., Tumova, L., Lukas, J., Valenta, T., Pospichalova, V., Fafilek, B., Plachy, J., Sebesta, O. and Korinek, V.

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Fatty acid modification of Wnt1 and Wnt3a at serine is prerequisite for lipidation at cysteine and is essential for Wnt signalling

Lenka Doubravska [a,1], Michaela Krausova [a,1], Dietmar Gradl [b], Martina Vojtechova [a], Lucie Tumova [a], Jan Lukas [a], Tomas Valenta [a], Vendula Pospichalova [a], Bohumil Fafilek [a], Jiri Plachy [a], Ondrej Sebesta [c], Vladimir Korinek [a,*]

[a] Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Videnska 1083, 142 20 Prague 4, Czech Republic
[b] Zoologisches Institut II, Universität Karlsruhe, Kaiserstrasse 12, 76131 Karlsruhe, Germany
[c] Department of Genetics and Microbiology, Faculty of Science, Charles University in Prague, Vinicna 7, 128 43 Prague 2, Czech Republic

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ABSTRACT

The Wnt family of proteins is a group of extracellular signalling molecules that regulate cell-fate decisions in developing and adult tissues. It is presumed that all 19 mammalian Wnt family members contain two types of post-translational modification: the covalent attachment of fatty acids at two distinct positions, and the N-glycosylation of multiple asparagines. We examined how these modifications contribute to the secretion, extracellular movement and signalling activity of mouse Wnt1 and Wnt3a ligands. We revealed that O-linked acylation of serine is required for the subsequent S-palmitoylation of cysteine. As such, mutant proteins that lack the crucial serine residue are not lipidated. Interestingly, although double-acylation of Wnt1 was indispensable for signalling in mammalian cells, in *Xenopus* embryos the S-palmitoyl-deficient form retained the signalling activity. In the case of Wnt3a, the functional duality of the attached acyls was less prominent, since the ligand lacking S-linked palmitate was still capable of signalling in various cellular contexts. Finally, we show that the signalling competency of both Wnt1 and Wnt3a is related to their ability to associate with the extracellular matrix.

1. Introduction

The body of a multi-cellular organism is a highly organized structure of cells, tissues and organs. A handful of evolutionarily conserved cell signalling pathways is responsible for generating this structural complexity, both during development and its maintenance in adulthood. The Wnt pathway, initiated by secreted Wnt proteins, controls a remarkably diverse array of processes that include cell proliferation, differentiation, cell migration and cell polarity. Deregulation of Wnt signalling is implicated in a number of human disorders and cancer (reviewed in [1–4]). Currently, several different modes of the pathway have been recognized, with the majority of Wnt-dependent cascades requiring the seven-pass transmembrane Wnt receptor, Frizzled (Fz), the co-receptor low-density lipoprotein receptor-related protein (Lrp) and cytoplasmic protein, Dishevelled (Dvl). In canonical Wnt signalling the association of Wnt ligand with its corresponding receptors leads to the stabilization and accumulation of β-catenin protein via Dvl-dependent inhibition of the Axin, glycogen synthase kinase 3 (Gsk-3), and adenomatous polyposis coli (Apc) multi-protein complex. Subsequently, β-catenin enters the cell nucleus and together with the T-cell factor (Tcf)/Lymphoid Enhancer Factor (Lef) transcriptional regulators activates the expression of Wnt target genes [5] (a detailed summary on Wnt signalling can be found at the Wnt homepage http://www.stanford.edu/~rnusse/wntwindow.html).

The mammalian genome encodes 19 Wnt proteins of approximately 350–400 amino acids in length that contain an invariant pattern of 23–24 cysteines. It is presumed that many of these cysteine residues participate in the formation of intra-molecular disulphide bonds that stabilize proper folding of the polypeptide [6]. Although the degree of sequence identity between some Wnt family members is only 18%, it is thought that all Wnt proteins form a similar three-dimensional structure [7].

Mouse Wnt3a is post-translationally acylated by the attachment of two fatty acid adducts [8,9]. The modification by palmitic acid occurs at the first cysteine residue (C77) of the mature secreted protein, whilst the linkage of palmitoleic acid occurs at serine 209. In many Wnt ligands, the regions containing the acylated amino acids are homologous; thus it is thought that the majority of Wnt proteins are doubly-acyl-modified (reviewed in [10,11]). Indeed, S-acylation at the corresponding "prototype" C77 in Wnt3a was experimentally verified

* Corresponding author. Institute of Molecular Genetics, AS CR, Videnska 1083, 142 20 Prague 4, Czech Republic. Tel.: +420 241063146; fax: +420 244472282.
E-mail address: korinek@img.cas.cz (V. Korinek).
[1] These authors contributed equally to this work.

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in chicken Wnt1 and Wnt3a, mouse Wnt5a, *Drosophila* Wingless (Wg; the Wnt1 orthologue) and Wnt8 [9,12–14]. The presence of the O-acyl moiety at positions homologous to S209 in Wnt3a has been studied in less detail; however, it was confirmed in Wg and chicken Wnt1 [12,15]. To date, *Drosophila* WntD is the only known non-lipidated member of the Wnt family [16]. Another common biochemical feature of Wnt ligands is N-linked glycosylation. The N-glycosylation status of approximately 11 different Wnts has been examined with all proteins displaying a distinct pattern of N-linked oligosaccharide attachment at multiple positions [6,17–20].

It has been well documented in both *Drosophila* and *C. elegans* that Wnts act as morphogens, initiating specific responses in relation to the amount of a particular Wnt protein in the extracellular space [21,22]. Thus, within a given tissue, the concentration of Wnt molecules can provide positional information to cells influencing their developmental fate. The effect of each post-translational modification on the Wnt "morphogenic" behaviour is rather elusive (reviewed in [23]). Interestingly, ectopically expressed mammalian Wnts are not easily diffusible and remain tightly associated with the cell surface [18,24,25]. It was suggested that the fatty acid moieties are responsible for this "stickiness" and limit Wnt long-range signalling [11]. However, the concentration gradient of Wg can be mediated by membranous exovehicles, so-called argosomes [26], or by lipoprotein particles [27]. These findings would rather support a positive role of lipid adducts in long-range signalling of Wg in *Drosophila* tissues.

In cultured mammalian cells, Wnt3a lacking the palmitic adduct is normally secreted, but its signalling activity is considerably perturbed [9,12,17]. This reduction is caused by a decreased affinity for the receptors, Frizzled or Lrp [17,28]. Similar results were obtained using corresponding mutants of Wnt5a and Wg [13,15]. However, the same Wg mutant was not functional in the *Drosophila* wing imaginal disc as it was retained in the endoplasmic reticulum [15]. Other discrepancies were also observed in studies involving O-acyl-linked and N-glycosylated modifications. For example, mutation of the acylated S209 residue of Wnt3a resulted in an inefficiently secreted protein, whereas the equivalent mutation (S239A) in Wg was released from *Drosophila* S2 cells with the same efficiency as wild-type ligand [8,15]. In Wnt1, the absence of N-linked oligosaccharide chains did not impair its activity and was properly secreted and induced transformation of Wnt-sensitive mouse mammary epithelial cells [19,25,29]. In contrast, N-glycosylation of Wnt3a and Wnt5a ligands was necessary for their efficient secretion. Finally, purified and subsequently enzymatically deglycosylated Wnt5a preserved its activity, but a non-glycosylated mutant form of Wnt3a was less active than its wild-type counterpart [13,17,30].

In the present study, we thoroughly examined the N-glycosylation and acylation status of mouse Wnt1 and Wnt3a ligands to determine how these post-translational modifications affect the secretion and signalling activities of these polypeptides. The activity tests included secondary axis formation in *Xenopus* embryos, reporter gene assays and real-time quantitative RT-PCR (qRT-PCR) analysis. Additionally, β-catenin stabilization, the hallmark of canonical Wnt signalling, was visualized by confocal microscopy in Wnt-producing cells. We demonstrated that fatty acid modification at the serine residue precedes and conditions subsequent palmitoylation of cysteine. Wnt ligands without any lipidic adducts were still N-glycosylated and secreted. In contrast, non-N-glycosylated Wnts displayed a decreased rate of secretion. This phenomenon could account for the lower activity of non-N-glycosylated ligands in paracrine signalling. Although we observed some discrepancies between the outcome of the experiments performed in *Xenopus* embryos and in cultured cells, both testing systems showed that the non-lipidated Wnts were completely inactive. In addition, our studies revealed functional and biochemical differences between Wnt1 and Wnt3a proteins. Particularly, the relationship between fatty acid content and activity was less stringent in Wnt3a since S-acyl-deficient ligand [Wnt3a(C77A)] – contrary to the corresponding Wnt1 variant [Wnt1(C93A)] – retained a substantial signalling activity. Moreover, Wnt3a(C77A) was deposited on the extracellular matrix (ECM) and released to culture medium with the same efficiency as the wild-type polypeptide. Interestingly, we never detected any Wnt1 in cell supernatants, which indicates that the majority of the extracellular protein binds to the cell surface or ECM. However, the ability of acyl-deficient Wnt1 mutants to adhere to ECM was severely impaired.

2. Materials and methods

2.1. Plasmids and lentiviral constructs

Constructs encoding mouse wild-type and mutant Wnt1 [31] and Wnt3a (kindly provided by O. Machon) proteins were generated in the mammalian lentiviral vector, pCDH1 (System Biosciences). Single or multiple amino acid substitutions were introduced into corresponding cDNA using a site-directed mutagenesis kit (Stratagene). Wnt1 and Wnt3a lacking the signal peptide (ΔNWnt1 and ΔNWnt3a, respectively) were generated by PCR and cloned into the pCDH1 vector [31]. PCR amplification steps were performed with Phusion High-Fidelity DNA Polymerase (Finnzymes). The truncated proteins were fused at the N-terminus to the Myc-tag (ΔNWnt1) or HA-tag (ΔNWnt3a). EGFP-tagged mouse Frizzled (Fz) 4, cloned into the pCS2 vector, was obtained from V. Bryja. The expression construct encoding Flag-tagged mouse Lrp5 was a kind gift from M. Semenov and X. He [32]. PCR-derived constructs were verified by sequencing; details of plasmid constructs are available on request.

2.2. Software and statistical analysis of data

Protein sequence alignments were performed using the MegAlign program (DNASTAR Lasergene 7). Signalling peptides and N-glycosylation sites were predicted using the Expert Protein Analysis System (ExPASy) at http://www.expasy.ch. Fisher's exact test was used to analyse the statistical significance of the results of the double axis formation assay. Data sets obtained in the gene reporter and qRT-PCR analyses were evaluated by Student's *t*-test.

2.3. Cell culture, transfections and generation of stable cell lines

Human HeLa, human embryonic kidney (HEK) 293, mouse L_{TK-}, mouse 3T3 and mouse Wnt3a-producing L cells were purchased from ATCC. Rat2 and mouse Wnt1-transduced Rat2 fibroblasts (Rat2Wnt1) were kindly provided by A. Brown [33]. Rat2 cells producing wild-type mouse Wnt3a were described previously [34]. HEK 293 FT cells utilized for packaging lentiviral stocks were purchased from Invitrogen. Super-TOPFLASH HEK 293 (STF 293) cells containing the genome-integrated Wnt/β-catenin-responsive luciferase reporter, SuperTOPFLASH [35] were obtained from Q. Xu and J. Nathans. All cell lines were maintained in Dulbecco's modified Eagle's medium [(DMEM; purchased from Biochrom AG)] supplemented with 10% fetal bovine serum (Hyclone), penicillin, streptomycin and gentamicin (Invitrogen). Transient transfections were performed using Fugene HD (Roche). Lentiviruses were prepared using the Trans-Lentiviral Packaging System (Open Biosystems). Rat2 cells transduced with the corresponding recombinant lentiviruses were selected without subcloning using puromycin (Alexis; 5 μg/ml).

2.4. Antibodies, co-immunoprecipitations, western blotting and tunicamycin treatment

cDNAs encoding N-terminally His-tagged mouse Wnt1 [amino acids (aa) 225–370] and Wnt3a (aa 190–355) were subcloned into the pET28b vector (Novagen). Recombinant proteins were purified from bacterial [*E. coli*, strain BL-21 (DE3)] cell lysates by TALON affinity

resins (Clontech) and utilized for immunizing rabbits or chickens. The preparation of rabbit anti-EGFP polyclonal antibody was described previously [36]. For co-immunoprecipitation experiments, HEK 293 FT cells were transfected overnight with an appropriate combination of constructs. The cells were harvested, washed with ice-cold phosphate-buffered saline (PBS) and disrupted in lysis buffer [1% NP-40, 20 mM Tris (pH 7.5), 100 mM NaCl, 10 mM EDTA, protease inhibitor cocktail Complete (Roche)] for 30 min on ice. The samples were centrifuged (16,000×*g*, 10 min, 4 °C) and the resulting supernatants transferred to fresh tubes and incubated for 3 h at 4 °C with Wnt1 or Wnt3a rabbit polyclonal antibodies bound to protein A/G Sepharose beads (Pierce). The beads were washed three times using lysis buffer and the retained proteins were eluted in Laemmli sample buffer [37] and immunoblotted. A detailed protocol describing the immunoblotting procedure can be found elsewhere [38]. To detect immunoprecipitated Wnt proteins, Wnt-specific polyclonal antisera from chickens were utilized. The following commercially available rabbit polyclonal and mouse and rabbit monoclonal antibodies were used: anti-α-tubulin (TU-01; Exbio), anti-β-catenin (610154; BD Transduction Laboratories and EM-22; Exbio), anti-Flag (M2; Sigma), anti-GFP (JL8; Clontech), and anti-Wnt3a (C64F2; Cell Signalling Technology). Peroxidase-conjugated anti-chicken, anti-mouse or anti-rabbit secondary antibodies were purchased from Sigma. Tunicamycin (Sigma) treatment was performed overnight at a final concentration of 1 μg/ml.

2.5. Metabolic labelling with [^{3}H] palmitate

HEK 293 cells grown on a 15 cm culture dish were transfected with the relevant Wnt-expression constructs. The next day cells were starved for 1 h in serum-free DMEM and incubated for an additional 3 h in DMEM supplemented with 5% dialysed foetal bovine serum and [^{3}H] palmitate (Perkin Elmer, final concentration 0.5 mCi/ml). Cell lysates were immunoprecipitated using rabbit anti-Wnt1 or anti-Wnt3a polyclonal antibodies. Precipitated proteins were separated in two SDS–PAGE gels. One gel was blotted and stained with chicken anti-Wnt1 or anti-Wnt3a polyclonal antisera. The second gel replica was fixed, soaked in Amplify Solution (Amersham Biosciences) and subsequently dried and exposed to Hyperfilm MP (Amersham Biosciences) for up to 3 months at −80 °C.

2.6. Immunofluorescent microscopy

For immunofluorescence studies, Wnt1- and Wnt3a-specific rabbit polyclonal antibodies were purified by affinity chromatography using Glutathione S-transferase (GST)-Wnt1 or GST-Wnt3a proteins coupled to glutathione Sepharose 4B (Amersham Pharmacia Biotech) [39]. The recombinant GST-fusion proteins (same sequences of Wnt1 or Wnt3a used for immunizations) were expressed from the pET42b vector (Novagen) in the bacteria *E. coli* BL-21/DE3. To distinguish extracellular and intracellular pools of Wnt proteins mammalian cells grown on coverslips were transfected with the relevant Wnt-expression construct. The next day antigen-purified anti-Wnt1 or anti-Wnt3a antibody was added to the culture medium. After 30 min at 37 °C, the cells were washed three times with PBS and fixed (10 min at 4 °C) in 4% (w/v) paraformaldehyde (Electron Microscopy Sciences) solution made in PBS. The samples were subsequently permeabilized in 0.2% Triton X-100/PBS solution (5 min, 4 °C), rinsed 3 times with PBS and stained for 1 h using a goat anti-rabbit antibody conjugated with the Alexa 488 dye (Molecular Probes). After another round of excessive washing using PBS, the specimens were incubated (1 h, 4 °C) with the same Wnt1- or Wnt3a-specific rabbit antibody. The coverslips were washed with PBS and stained using the Alexa 594 dye conjugated to a goat anti-rabbit antibody (Molecular Probes). For double staining of mouse L and 3T3 cells, the specimens were washed three times with PBS and fixed (10 min at 4 °C) in 4% paraformaldehyde. The samples were subsequently permeabilized in a 0.2% Triton X-100/PBS solution (5 min, 4 °C), rinsed 3 times with PBS and incubated consecutively with Wnt antibodies (1 h, 4 °C) and with an anti-β-catenin monoclonal antibody (additional 12 h, 4 °C). The coverslips were washed with PBS and stained using the Alexa 594 dye conjugated to a goat anti-rabbit antibody and Alexa 647 goat anti-mouse antibody (Molecular Probes). Finally, the samples were washed three times in PBS, incubated with DAPI nuclear stain (Molecular Probes; 1 min, room temperature, final concentration 1 μM), washed and mounted in MOWIOL (Calbiochem). Immunofluorescence was visualized using a confocal laser scanning microscope (TCS SP5; Leica) and analysed by ImageJ software (NIH freeware). Rat2 fibroblasts stably expressing various Wnt proteins were seeded on coverslips and processed as described for transiently transfected cells, except that the same secondary antibody (Alexa 488 conjugated to a goat anti-rabbit antibody) was used to visualize both the natively labelled and fixed pool of Wnt protein.

2.7. Double axis formation assay

cDNAs encoding wild-type, lipid- or N-glycosylation-deficient Wnt1 and Wnt3a proteins were subcloned into the pCS2 vector (Addgene). Capped mRNAs were synthesized from the Not I restriction-linearized constructs using the mMessage mMachine kit (Ambion). Eggs obtained from *Xenopus laevis* were fertilized by a standard method [40]. Twenty pg (in 4 nl) of each mRNA was microinjected into the marginal zone of the ventral blastomeres of 4-cell stage embryos. Embryos were kept as described previously [41] and at the neurulae stage scored for the double axis phenotype.

2.8. RNA purification and qRT-PCR

Total RNAs were isolated from cells using the Trizol reagent (Invitrogen). A detailed description of the qRT-PCR procedure was given previously [38]. All primers were calculated using the Primer 3 computer service at http://frodo.wi.mit.edu/. Two housekeeping genes, *Glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) and *Ubiquitin b* (*Ubb*) were used as internal control genes to standardize the quality of different cDNA preparations [42]. cDNAs were produced from at least two independent RNA isolations and the PCR reactions were performed in triplicate for each primer set. The primers are written in the 5′ to 3′ direction; the first primer is derived from the plus and the second primer from the minus DNA strand:

rAxin1, ACCCAGTACCACAGAGGATG, CTGCTTCCTCATCCCAGAAG;
rAxin2, GCTGGAGAAGCTGAAACTGG, GACAGGTGGTCGTCCAAGAT;
rGAPDH, AAACCCATCACCATCTTCCA, GTGGTTCACACCCATCACAA;
rUbb, TCTTCGTGAAGACCCTGACC, CAGGTGCAGGGTTGACTCTT;
mWnt1, ATCGATT TTGGTCGCCTCTT, CTTGGCGCATCTCAGAGAAC;
mWnt3a, GCGGCTGTAGTGAGG ACATT, GCACTTGAGGTGCATGTGAC.

2.9. Autocrine and paracrine reporter gene assays

A detailed protocol of the reporter gene assay was described previously [38]. Briefly, to assay autocrine Wnt signalling, HEK 293 and 3T3 cells were transfected with firefly luciferase TOPFLASH and FOPFLASH reporters containing either multiple copies of the optimal Tcf motif GATCAAAGG or multiple copies of mutant motif GGCCAAAGG, respectively [43]. DNA mixtures further included the particular Wnt expression construct and as an internal control, *Renilla* pRL-SV40 plasmid (Promega). STF 293 cells that contain an integrated TCF-dependent reporter, SuperTOPFLASH [35,44], were lipofected with *Renilla* and Wnt-expressing plasmids only. Cells were harvested 24 and 48 h after transfection. To assess paracrine signalling, STF 293 cells were transfected "in batch" with the *Renilla*-expressing plasmid. The next day the cells were mixed at a ratio of 1:1 with either parental

Rat2 cells or Rat2 cells stably expressing wild-type or mutant Wnts. Twenty-four and 48 h later the activity of firefly and *Renilla* luciferase in cell lysates was determined using the Dual-Glo Luciferase Assay System (Promega) and EnVision 2100 Multilabel Reader (Perkin Elmer). Reporter gene activities were normalized against the activity of *Renilla* luciferase. All reporter gene assays were performed in triplicate. The results of a representative experiment from three in total are presented.

2.10. Recombinant Wnt3a purification

Mouse Wnt3a ligand was isolated from the culture medium of Wnt3a-producing L cells without the heparin purification step according to a detailed protocol of Willert and colleagues [9].

2.11. Density gradient ultracentrifugation

The plasma membranes of Rat2 fibroblasts stably expressing wild-type or mutant forms of Wnt1 or Wnt3a proteins were solubilized on ice in membrane lysis buffer (1% Brij 98; 20 mM Tris (pH 8.2), 100 mM NaCl, 10 mM EDTA, 50 mM NaF, 1 mM Na_3VO_4, Complete protease inhibitor cocktail) for 30 min. Lysates were then mixed at a 1:1 ratio with ice-cold 80% (w/v) sucrose diluted in membrane lysis buffer. The samples were transferred into 5 ml ultracentrifugation tubes and overlaid initially with 3.5 ml of ice-cold 30% sucrose diluted in membrane lysis buffer and then 0.5 ml of membrane lysis buffer. Fractions were collected upon centrifugation (268,000×*g*, 20 h at 4 °C) in the MLS-50 rotor (Beckman Coulter) from the top to the bottom of the tubes. Prior to Western blotting, proteins were precipitated by chloroform and methanol [45].

2.12. Isolation of the ECM and plasma membranes

Cells grown in 10 cm culture dishes for 72 h to 90% confluence were washed twice in Ca^{2+}, Mg^{2+}-free PBS and incubated for 10 min at 37 °C in Ca^{2+}, Mg^{2+}-free PBS supplemented with 5 mM EGTA. Cells were detached by gentle pipetting and the cell-free surfaces of the culture dishes were subsequently washed three times using ice-cold Ca^{2+}, Mg^{2+}-free PBS and once with distilled water. The ECM was then harvested by scraping the surface of the dish in Laemmli sample buffer. To isolate plasma membranes, the detached cells were washed twice in ice-cold PBS and resuspended in hypotonic buffer [10 mM Hepes (pH 7.5), 10 mM KCl, 10 mM $MgCl_2$, Complete protease inhibitor cocktail]. Cells were disrupted by passing them 10 times through a 25 gauge needle. Nuclei were removed from lysates by centrifugation (400×*g*, 5 min, 4 °C). Supernatants were transferred to fresh tubes and centrifuged at 18,000×*g* (10 min, 4 °C) to pellet the plasma membranes. To prepare whole-cell lysates (WCL), cells grown in one 10 cm culture dish were washed twice in PBS and subsequently harvested in Laemmli sample buffer.

3. Results

3.1. N-glycosylation and fatty acid modification of Wnt1 and Wnt3a ligands

To investigate the possible function of N-glycosylation or lipidation of Wnt proteins, we generated mutant Wnt1 and Wnt3a polypeptides containing single or multiple amino acid substitutions at the putative modification position(s) (Fig. 1A). Four or two asparagine residues were mutated to glutamine in mouse Wnt1 and Wnt3a, respectively. Expression constructs encoding wild-type or mutant proteins were lipofected into HEK 293 cells; the resulting cells were then treated either with the N-glycosylation inhibitor, tunicamycin or vehicle alone. Cell lysates were prepared and subjected to immunoblotting using an anti-Wnt1 or Wnt3a antibody. As shown in Fig. 1B, wild-type Wnt1 migrates in SDS-PAGE gels as a quadruplet, whilst Wnt3a was detected as a double band. Tunicamycin-treated cells expressed only one protein species, indicating that Wnt1 is N-glycosylated at three and Wnt3a at two residues. Further analysis of each mutant ligand revealed that Wnt1 is N-glycosylated at asparagine residues 29, 316 and 359, whilst Wnt3a is N-glycosylated at asparagine 87 and 298 [(Fig. 1B); (the numbering accounts for the signalling peptide)].

In addition to N-glycosylation, Wnt3a is palmitoylated on cysteine 77 and the palmitoleoyl modification occurs at serine 209 [8,9].The aforementioned amino acids and the homologous residues in Wnt1 (cysteine 93 and serine 224, respectively; see Fig. 1A) were mutated to alanines. The acylation status of wild-type and mutant polypeptides were tested in cells metabolically labelled with tritiated palmitate. Interestingly, substitution at the critical serine residue resulted in the expression of an entirely non-acylated form (Fig. 2).

3.2. Non-acylated Wnts do not induce ectopic axis in developing Xenopus embryos

We next compared the ability of wild-type and mutant Wnt proteins to dorsalize *Xenopus* embryos. Equal amounts of capped mRNA encoding different variants of Wnt1 or Wnt3a were injected into ventral blastomeres. Each embryo was then scored for the formation of the secondary axis. The analysis (results are summarized in Fig. 3) demonstrated that whilst Wnt1 and Wnt3a ligands display some similarities, there are also quite striking differences. For instance, the putative non-acylated forms of Wnt1(S224A), Wnt1 (C93,S224A), ΔNWnt1, Wnt3a(S209A) and Wnt3a(C77,S209A) failed to induce double axis. However, Wnt1(N29,316,359Q) lacking any N-glycosylation sites appeared to be the most potent Wnt. Interestingly, Wnt1(C93A) retained approximately two thirds of the activity of wild-type Wnt1, whilst the equivalent mutant Wnt3a(C77A) functioned as its wild-type Wnt3a counterpart. Finally, Wnt3a(N87,298Q) ligand lacking N-glycosylated residues displayed a significantly reduced ability (approximately one third) to produce *Xenopus* embryos with two body axes.

3.3. Mutant Wnts lacking the lipid-modified cysteine residue display decreased functionality in mammalian cells

We examined the effect of N-glycosylation or fatty acid modification on the signalling properties of Wnt ligands in mammalian cells. Expression constructs encoding wild-type or N-glycosylation/acylation mutants of Wnt1 and Wnt3a were introduced into SuperTOPFLASH 293 (STF 293) cells. These HEK 293-derived cells can be used as a sensitive cellular system to quantify Wnt signalling as they contain the genome-integrated TCF/β-catenin-dependent luciferase reporter, SuperTOPFLASH [35,46]. Constructs encoding Wnt1 and Wnt3a without a signalling peptide (ΔNWnt1 and ΔNWn3a) were used as negative controls in this and subsequent assays as these truncated forms remain intracellular (see e.g. Fig. 5B and C). In a parallel experiment, parental HEK 293 cells were transfected with the corresponding Wnt construct together with the "original" TCF/β-catenin-reporter, TOPFLASH [43]. Twenty-four hours post-transfection the cells were harvested and luciferase activities were determined in cell lysates. Contrary to our findings in the *Xenopus* embryo readout, the single-acyl mutant Wnt3a (C77A) was less stimulatory than wild-type Wnt3a. Moreover, the luciferase activities in STF 293 cells transfected with the expression constructs encoding analogous Wnt1(C93A) ligand or the acyl-deficient Wnt1 variants [Wnt1(S224A), Wnt1(C93, S224A)] were as low as in control cells transfected with the "empty" vector. Thus, we concluded that all theses mutant proteins appeared to be "signalling-dead". In agreement with the results of the double axis formation assay, non-N-glycosylated Wnt1(N29,316,359Q) was more functional than wild-type Wnt1; however, N-glycosylation-depleted Wnt3a(N87,298Q) protein

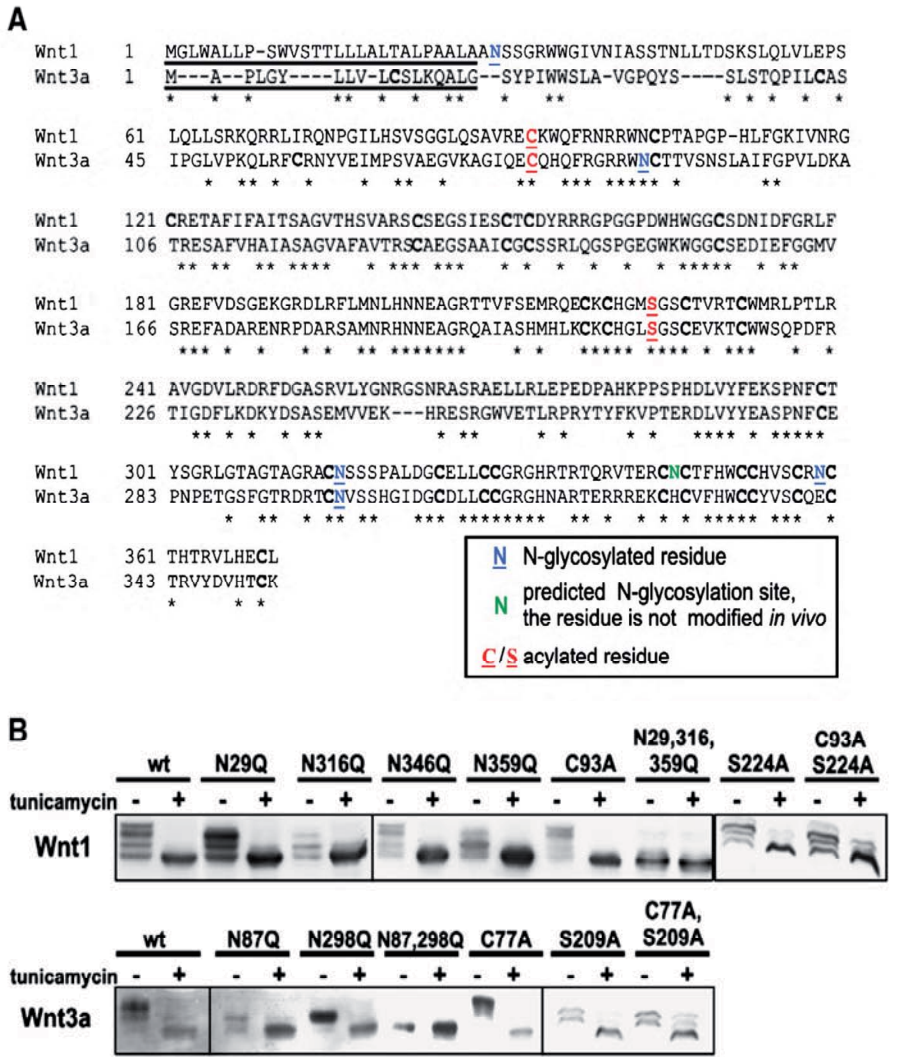


Fig. 1. N-glycosylation of Wnt1 and Wnt3a proteins. (A) Comparison of mouse Wnt1 and Wnt3a polypeptide sequences displaying the predicted and experimentally confirmed N-glycosylation sites. The putative signal peptides are underlined. The amino acid residues conserved in both Wnt1 and Wnt3a are marked by asterisks. The acylated cysteine and serine residues are also highlighted and the unmodified cysteines are typed in bold. (B) N-glycosylation status of Wnt1 and Wnt3a proteins. Human HEK 293 cells were transfected with constructs encoding either wild-type (wt) or mutant Wnt1 or Wnt3a polypeptides. Mutations were made at the putative N-glycosylated asparagines [(N); replaced by glutamine (Q)] or acylated cysteine (C) or serine (S) [both replaced by alanine (A)] residues. Eight hours post transfection either the N-glycosylation inhibitor, tunicamycin (1 μg/ml final concentration) or vehicle (DMSO) was added to cells. Cultures were incubated for an additional 18 h before being harvested and lysates prepared. Cell lysates were subjected to immunoblotting using either anti-Wnt1 or anti-Wnt3a antibodies.

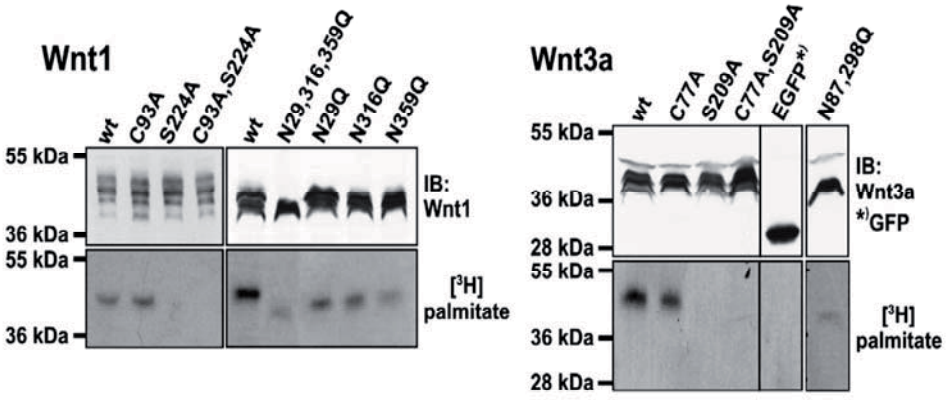


Fig. 2. In both Wnt1 and Wnt3a, fatty acid modification of serine is essential and precedes palmitoylation at the cysteine residue. HEK 293 cells metabolically labelled with [^{3}H] palmitate were transfected with the indicated Wnt1 or Wnt3a expression constructs or negative control EGFP-producing plasmid. The ectopic proteins were immunoprecipitated from cell lysates 20 h post-transfection using the corresponding rabbit polyclonal antisera. The precipitates were resolved in two SDS-PAGE gels. One of these gel replicas was dried and exposed directly to Hyperfilm MP (bottom panels); the second gel was blotted and stained with chicken anti-Wnt1 or anti-Wnt3a polyclonal antibodies or with a mouse anti-GFP monoclonal antibody (upper panels). IB, immunoblotting.

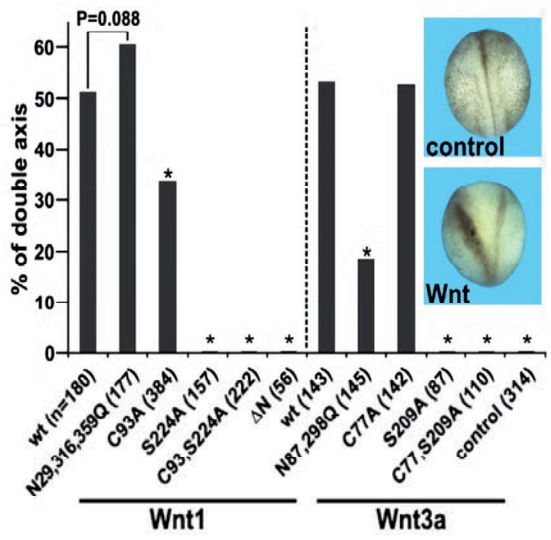


Fig. 3. Wnt1 and Wnt3a without fatty acyl adducts do not induce double axis formation in *Xenopus* embryos. Embryos at the 4-cell stage were microinjected with 20 pg of the indicated mRNA in the marginal zone of ventral blastomeres and scored for secondary axis induction at the neurulae stage (see the inset). Differences between wild-type and mutant ligands were compared by the Fisher exact probability test. *$P < 0.001$. ΔN, truncated Wnt1 lacking signalling peptide; control, non-injected embryos.

showed a remarkably reduced ability to stimulate Wnt-responsive reporters (Fig. 4A; only results for STF cells are shown). We attempted to elucidate the observed discrepancies between the outcome of the tests performed in *Xenopus* embryos and in mammalian cells. At first, we injected decreased amounts of Wnt-encoding mRNAs into ventral blastomeres and scored for the formation of the secondary axis. Next, we extended the reporter gene assay and measured the luciferase activities in lysates of transfected cells 48 h post-transfection. Nevertheless, in these experiments all Wnt variants performed in a similar way as in the previous setup, confirming the initial results (Fig. 4A). Importantly, in all cellular backgrounds tested, the negative control reporter FOPFLASH never responded to any of the expressed Wnt proteins (not shown).

Subsequently, an analogous study was conducted in Wnt-responsive Rat2 fibroblasts [34]. In this study, lentiviral vectors were utilized to generate a pool of polyclonal Rat2 cell cultures that stably expressed either wild-type or mutant Wnt ligands. Although we did not succeed in preparation of Rat2 cells producing ΔNWnt1 and Wnt1(C93A,S209A) in levels comparable to the other forms (Fig. 6A and Supplementary Fig. S1), we used all types of transduced cells for following analyses. Total RNAs isolated from these Rat2 cells were utilized to examine the effect of each Wnt1 or Wnt3a variant on the transcription of the endogenous Wnt signalling target gene, *Axin2*. As a negative control, the Wnt-insensitive *Axin1* was analysed. In addition, mRNA levels of two housekeeping genes, *GAPDH* and *Ubb*, were assessed. The data generated from our qRT-PCR analysis agreed well with the results obtained in our reporter gene assay (Fig. 4B). *Axin2* mRNA was strongly up-regulated by wild-type proteins and the Wnt1(N29,316,359Q) mutant. Wnt3a(C77A) and Wnt3a(N87,298Q) were less stimulatory whilst the non-acylated forms of Wnt were not functional. Interestingly, Wnt1(C93A) showed a small level of activity in Rat2 fibroblasts (Fig. 4B).

Given that the previous experiments were unable to differentiate between autocrine and paracrine Wnt signalling, we decided to assess paracrine signalling only by co-culturing two different (one emitting and the other receiving the Wnt signal) cell types. We mixed "reporter" STF 293 cells with Rat2 fibroblasts stably expressing Wnt1 and Wnt3a variants. Cells were harvested 24 and 48 h after plating and SuperTOPFLASH activities were determined in cell lysates. Remarkably, "N-glycosylation-minus" Wnt1(N29,316,359Q) displayed reduced signalling capacity when paracrine only signalling was measured versus when both autocrine and paracrine signalling was determined (Fig. 4C).

To verify the results of the reporter gene assays we searched for a suitable cellular system that would allow us to directly visualize Wnt signalling. We noted that, in contrast to the majority of cells of human origin, mouse 3T3 and L cells robustly stabilize β-catenin upon addition of recombinant Wnt3a and accumulate β-catenin in the nuclei of stimulated cells (Fig. 5A). As such, constructs expressing Wnt1 or Wnt3a variants were lipofected into L or 3T3 cells grown on coverslips. The cells were fixed 24 h later and stained with anti-β-catenin and Wnt-specific antibodies (Fig. 5B and C; only data for L cells are shown). By visualizing the stabilization of β-catenin, we could detect Wnt signalling affected by wild-type, N-glycosylation-deficient and "single-acyl" mutant Wnt3a(C77A) proteins. In contrast, S-acyl-deficient Wnt1(C93A), non-acylated Wnt3a(S209A) and Wnt1 (S224A) and signalling peptide-devoid forms (ΔNWnt1, ΔNWnt3a) were inactive. Interestingly, the control ΔNWnt1 protein was localized to the cell nucleus [Fig. 5B; see also Supplementary Fig. S2C, panels o, o']. We do not have any plausible explanation for this rather peculiar behaviour.

3.4. Non-functional Wnt proteins are secreted

An obvious explanation for the limited functionality of mutant Wnt proteins is their impaired secretion due to possible misfolding and retention within an intracellular compartment. To capture the extracellular pool of individual Wnt, relevant Wnt1- or Wnt3a-expression constructs were transfected into HEK 293, HeLa, 3T3 and L cells grown on coverslips. Twenty-four hours post-transfection, growing cells were briefly incubated with an anti-Wnt1 or anti-Wnt3a antibody. To visualize extracellular and membrane-associated Wnt (further referred to as "surface" Wnt) the cells were washed, fixed, permeabilized and the retained anti-Wnt immunoglobulins were directly stained with a fluorescently-conjugated secondary antibody. To detect intracellular Wnt, the samples were further subjected to another round of staining using the same primary antibody in combination with a differentially-labelled secondary antibody. The second staining also detected surface Wnt molecules whose epitopes were not fully saturated with the first-round staining [Supplementary Figs. S2A, panels l, l'; S2B, panels p, p']. Approximately 20 cells of each cell type expressing particular Wnt were scanned using a confocal microscope. Virtually identical results were obtained for all utilized cell types (Supplementary Fig. S2). Importantly, we never observed surface labelling of intracellular ΔNWnt1 and ΔNWnt3a mutant proteins [Supplementary Fig. S2C, panel n]. Typically, single- or double-acyl-deficient ligands displayed a similar cell surface and intracellular distribution as wild-type Wnts. A somewhat different picture was observed for proteins lacking N-glycosylation. The surface localization of Wnt1(N29,316,359Q) was reduced whilst extracellular Wnt3a(N87,298Q) was almost undetectable. Nevertheless, the results from these assays exclude the possibility that limited secretion can explain the lack of activity demonstrated by the acyl-deficient Wnt proteins.

This observation led us to test the distribution of wild-type and mutated Wnt1 and Wnt3a in Rat2 cells stably expressing Wnt proteins. The cells (grown to 90% confluence in 10 cm dish) were detached by EGTA-treatment, and the surface of each dish was thoroughly washed and SDS-PAGE sample buffer used to yield any proteins associated with the ECM. To obtain the plasma membrane fractions, the detached cells were harvested, washed and disrupted under hypotonic conditions. Finally, whole-cell lysates (WCL) were obtained by direct lysis of cells growing in a parallel "replica" dish using SDS-PAGE sample buffer. Analysis of the ECM, membrane and WCL samples by immunoblotting revealed that wild-type and non-glycosylated polypeptides have a strong affinity for the ECM. The same biochemical feature was

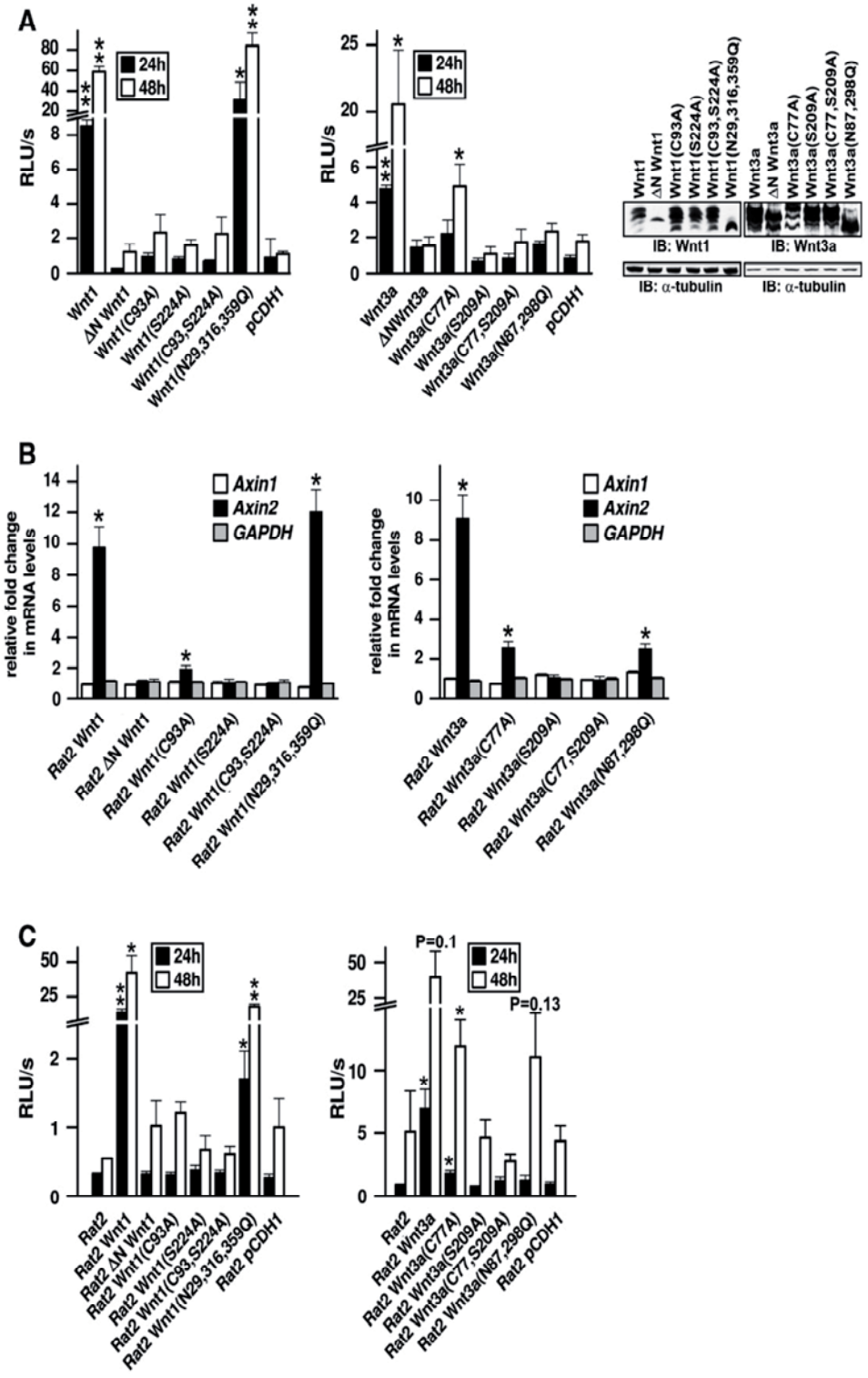


Fig. 4. Wnt3a lacking the palmitoylated cysteine residue retains the capacity to activate Wnt-dependent transcription in mammalian cells. (A) Activation of Wnt signalling in transiently transfected cells. HEK 293 cells containing the integrated reporter, SuperTOPFLASH (STF 293 cells), were transfected with constructs expressing wild-type or mutated variants of mouse Wnt**1** (the left panel) or Wnt3a (the right panel) proteins. pCDH**1** denotes cells transfected with an "empty" vector. Cells were harvested 24 or 48 h later and luciferase activities were determined in cell lysates. The histograms represent average luciferase light units per second (RLU/s) of a triplicate corrected for the efficiency of transfection using the internal control *Renilla* luciferase expression construct. SDs are shown by error bars. The results from one representative experiment out of three in total are shown. Western blots of whole-cell extracts prepared from STF 293 cells 24 h post-transfection are shown at the right. Blots were probed with anti-Wnt1, anti-Wnt3a or anti-α-tubulin [used as a "loading" control] antibodies. (B) qRT-PCR analysis of mRNAs isolated from Rat2 fibroblasts with retroviral constructs expressing wild-type or mutant Wnt1 or Wnt3a ligands. The resulting cells were used in triplicate experiments to determine the expression levels of *Axin2* (a Wnt signalling target gene), *Axin1* (negative control) and *Gapdh* (a "housekeeping" gene). The relative abundance of corresponding mRNAs was derived from the average CT values after normalizing to the levels of *ubiquitin B* (*Ubb*). The expression level of the genes in Rat2 cells transduced with the empty retroviral vector was set as 1. (C) Reduced activity of non-glycosylated Wnt1 protein in the paracrine signalling assay. STF 293 cells transfected with the *Renilla* luciferase expression construct were plated together with control cells [parental Rat2 fibroblasts or cells transduced with the empty lentivirus (denoted Rat2 pCDH1)] or with Rat2 cells stably producing the indicated Wnt constructs. The cells were harvested and lysed 24 or 48 h after plating. The average luciferase activities determined in cell lysates upon normalization to the *Renilla* levels are given. Results of three independent experiments performed in triplicates were combined to the final diagrams. Differences in values obtained in cells transfected/transduced with empty vector and in cells expressing given Wnt ligand was calculated by Student's t-test. *$P<0.05$; **$P<0.01$.

displayed by Wnt3a(C77A). The other ligands with reduced acyl content or completely without lipidation behaved differently and were preferentially co-isolated with membranes (Fig. 6A). Moreover, we attempted to evaluate how different forms of Wnt ligands are released from cells to culture medium (CM). We directly precipitated the proteins from CM using corresponding antibodies or, alternatively, we employed the purification protocol including Blue-Sepharose beads (see Materials and methods). The isolation was insensitive to any type of modification since all Wnt1 and Wnt3a variants could be quantitatively isolated from cell lysates (Fig. 6A). None of the Wnt1 forms was detected in CM. This was in stark contrast to Wnt3a, where all signalling-competent ligands were released to CM (Fig. 6A and data not shown).

Additionally, we examined the possible deposition of Wnt proteins on the ECM directly by confocal microscopy. Since the Wnt3a-specific signal was too low to obtain a good quality images, we used Rat2 cells expressing wild-type Wnt1 and Wnt1(C93A). The cells were seeded at low density onto coverslips and allowed to grow for 72 h. The specimens were stained using a protocol to capture both native extracellular/membrane-associated and intracellular Wnt (see Materials and methods). Strikingly, only wild-type Wnt1 displayed a remarkable deposition on the surface of the slip. This "deposit" was absent in the cells expressing mutated Wnt1(C93A), where the distribution of the proteins was localized to the membrane and intracellular compartments (Fig. 6B).

3.5. Non-acylated Wnt1 and Wnt3a are targeted to membrane microdomains and interact with the Frizzled receptor and Lrp co-receptor

Many palmitoylated proteins are associated with specialized detergent-resistant membrane microdomains (DRMs). These domains, also called lipid rafts, are thought to possibly serve as assembly sites for membrane signalling complexes [47]. Interestingly, it was previously shown that Wg is associated with DRMs and that ligands produced in cells grown in the presence of 2-bromopalmitate (an inhibitor of O-acyltransferases) lose their localization with lipid rafts [14]. We isolated membranes from cells transiently or stably expressing wild-type or mutant Wnt1 or Wnt3a. Cells were fractionated by ultracentrifugation according to density in a sucrose concentration gradient. Interestingly, all Wnts were located in the caveolin-containing, low-density fractions (presumable DRMs; Supplementary Fig. S3). This observation indicates that the presence of Wnts in lipid rafts is not dependent on their fatty acid content (or level of N-glycosylation). Furthermore, these results imply that there is no functional connection between Wnt signalling and the association of Wnt ligands with DRMs.

Finally, these results prompted us to examine the ability of wild-type and mutant Wnts to bind to their receptor, Fz, and co-receptor, Lrp. We performed a series of co-immunoprecipitation assays that showed no significant differences between the abilities of wild-type or signalling-deficient Wnt proteins to associate with both Fz4 and Lrp5 (Supplementary Fig. S4). In summary, although we noted that the signalling function of the tested Wnt ligands is related to their capacity to adhere to the ECM, the other biochemical features, including receptor complex binding and membrane distribution to lipid rafts, do not differ between wild-type and non-functional Wnt1 and Wnt3a proteins.

4. Discussion

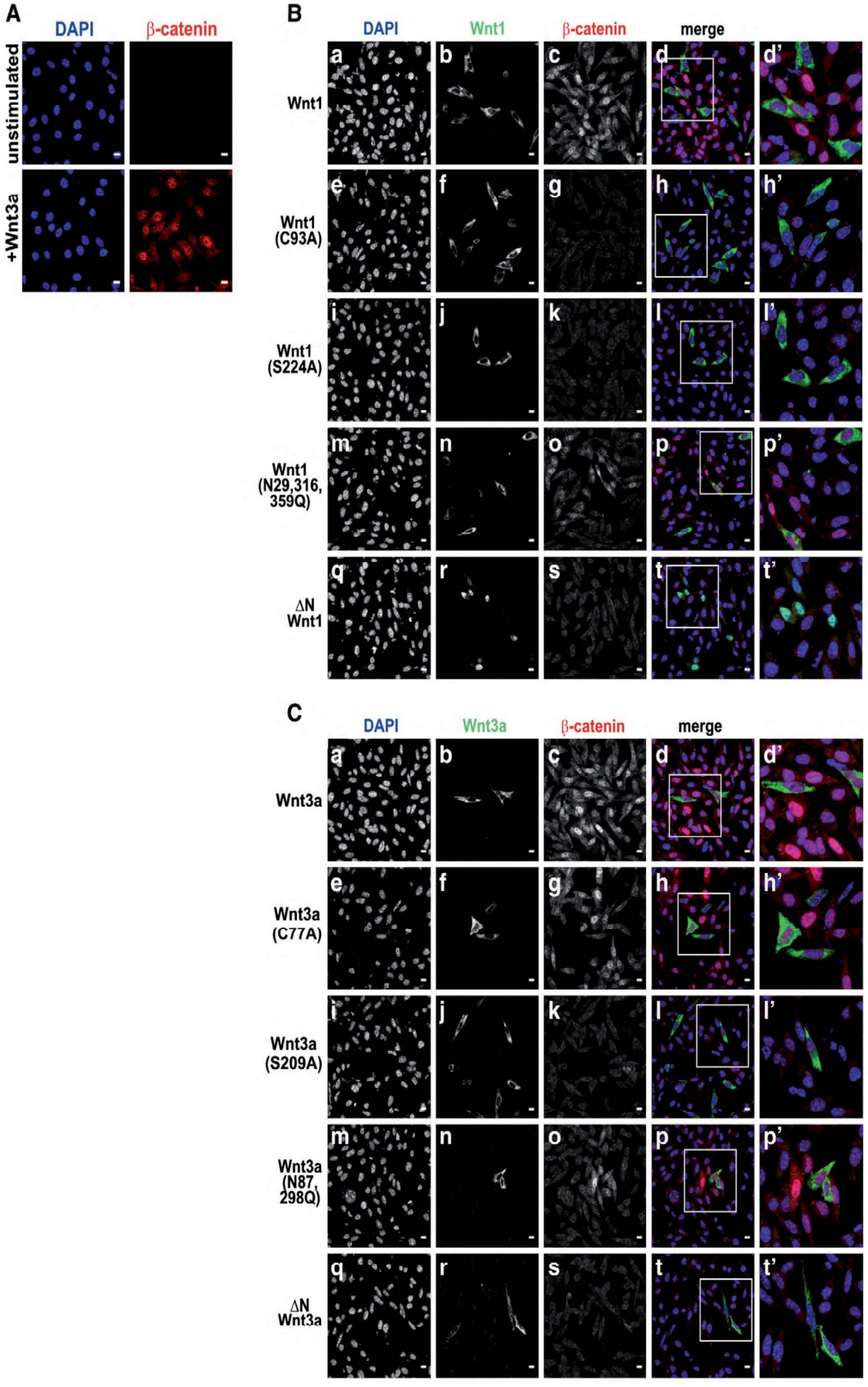
Mouse Wnt1 and Wnt3a are two mammalian members of the Wnt family of extracellular signalling proteins that are modified post-translationally by N-glycosylation and fatty acid addition. The aim of this study was to define the relationship between post-translational modification and the signalling activities of these proteins. We demonstrated that for signalling in mammalian cells, the attachment of two fatty acid moieties is crucial. Originally it was presumed that the lipidic adducts mediate a tighter association of the ligand with the cell surface. However, our results reveal that the opposite is true and acylation promotes Wnt release into the extracellular space.

In agreement with previously published results [17,19], Wnt1 is modified at three out of the four predicted N-linked oligosaccharide sites, whilst Wnt3a is modified twice (Fig. 1A and B). As both Wnts showed a similar pattern of N-glycosylation in human, rodent and *Xenopus* cells (Supplementary Fig. S5), it seems that the positions of the N-glycosylated residues are invariant and independent of the cellular context [17,19,20,48]. Analogous to Wnt3a, the Wnt1 polypeptide includes two acyl additions located at different positions (Fig. 1). Strikingly, mutations preventing the attachment of O-linked fatty acid in both Wnt1 and Wn3a completely abolished palmitoylation at the N-terminal cysteine residue (Fig. 2). This implies that the initial lipidation of serine is essential for subsequent fatty acid modification. Such a conclusion is supported by the recent characterization of WntD. This *Drosophila* Wnt lacks the conserved serine that is contained in other Wnt ligands subjected to the acyl modification. Although WntD contains the potentially S-acylated site, the mature protein does not carry any lipidic adducts [16].

Fatty acid-deficient Wnts were still fully N-glycosylated, indicating that such modifications are not influenced by the presence or absence of fatty acyl moieties. The attachment of acyl adducts to Wnt molecules is presumably catalysed by O-acyltransferase Porcupine (Porc) [12,14,49]. Interestingly, the ectopic expression of Porc increased N-glycosylation of Wg and of several other mouse Wnt ligands [6,50]. Nevertheless, Wnt3a N-glycosylation is not affected in cells treated with Porc-specific siRNAs [8]. Interestingly, N-glycosylation-deficient Wnts displayed apparently reduced acyl content (Fig. 2). This would imply that N-glycosylation precedes and conditions Wnts for efficient acylation.

To compare the signalling properties of wild-type and post-translationally modified-deficient Wnts, several Wnt activity tests were performed. These tests included double axis formation in *Xenopus*, a reporter gene assay, qRT-PCR analysis and direct β-catenin staining in Wnt-responsive mammalian cells. In each assay performed, the acyl-deficient forms of Wnt1 [Wnt1(S224A), Wnt1(C93A, S224A)] and Wnt3a [Wnt3a(S209A), Wnt3a(C77, S209A)] were not able to function, implying that the presence of O-linked fatty acids is indispensable for correct signalling. Interestingly, we noted that non-palmitoylated Wnt1(C93A) and Wnt3a(C77A) exhibited different signalling capabilities depending upon the testing system used. In

Fig. 5. β-catenin stabilization test in mammalian cells. (A) Wnt signalling induces robust accumulation of β-catenin in mouse L cells. Laser scanning confocal microscopy images of L cells treated for 24 h either with recombinant Wnt3a or Wnt storage buffer. The cells were stained with an anti-β-catenin monoclonal antibody (red channel) or DAPI nuclear stain (blue channel). (B) The signalling properties of Wnt1 variants in L cells. Cells (grown on coverslips) were transfected with the indicated Wnt1-expression construct. Twenty-four hours later the cells were fixed, permeabilized and washed with PBS. Subsequently, the specimens were incubated with rabbit anti-Wnt1 polyclonal and mouse anti-β-catenin monoclonal antibodies. Rabbit immunoglobulins were detected using the ALEXA 488 dye conjugated to a goat anti-rabbit antibody [shown in greyscale in panels b, f, j, n, r; or depicted in green in the merged images (d, h, l, p, t)]. β-catenin-specific staining was visualized with the ALEXA 594 dye conjugated to a goat anti-mouse antibody [panels c, g, k, o, s; or red in the merged images]. The merged pictures were generated by overlaying the corresponding images gained in each appropriate input channel with the image obtained in the blue channel to capture DAPI nuclear staining [shown in greyscale in panels a, e, i, m, q; in blue in the merged images]. Boxed areas in (d), (h), (l), (p) and (t) are magnified in (d′), (h′), (l′), (p′), and (t′), respectively. (C) The β-catenin stabilizing activity of Wnt3a and its variants. L cells transfected with Wnt3a constructs were processed as in panel B using antigen-purified anti-Wnt3a polyclonal antiserum and anti-β-catenin monoclonal antibodies. Bar, 10 μm.


A
DAPI
β-catenin
unstimulated
+Wnt3a
B
DAPI
Wnt1
β-catenin
merge
Wnt1
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Wnt1 (N29,316, 359Q)
ΔN Wnt1
C
DAPI
Wnt3a
β-catenin
merge
Wnt3a
Wnt3a (C77A)
Wnt3a (S209A)
Wnt3a (N87, 298Q)
ΔN Wnt3a

Xenopus embryos Wnt3a(C77A) was active as wild-type ligand and related Wnt1(C93A) retained approximately one third of the signalling capacity of wild-type Wnt1 (Fig. 3). On the other hand, in mammalian cells, the relationship between lipidation and function is apparently more stringent as we observed only limited signalling from single-acylated Wnt3a(C77A) and Wnt1(C93A) (Figs. 4 and 5). In the double axis formation assay the exogenous proteins function in the presumably complex cellular environment of developing embryo that contains various endogenous Wnt ligands and Wnt signalling inhibitors. The resulting phenotype (secondary axis) is generated by interplay among these proteins. Therefore, in the case that mutant Wnt retains some capacity to bind and block the inhibitors, the secondary axis might be induced even by signalling-deficient ligand. Alternatively, *Xenopus* cells can produce a different set of the Frizzled receptors than mammalian cells that can be effectively stimulated even by S-acyl-deficient Wnt1 or Wnt3a.

We observed that non-glycosylated Wnt3a was always less active than its wild-type counterpart (Figs. 3 and 4). This observation is consistent with the slower rate of secretion of non-glycosylated Wnt3a (Supplementary Fig. 2B). Similarly, N-glycosylation-deficient Wnt1 displayed decreased surface expression and reduced paracrine signalling (Fig. 4C and Supplementary Fig. S2A and C). Paradoxically, Wnt1(N29,316,359Q) was the best performing Wnt in all tests that included autocrine signalling (e.g. formation of artificial body axis).

Our results demonstrate that for intracellular transport to the cellular surface, fatty acid modification is not essential. Interestingly, the absence of lipidic adducts does not influence Wnt targeting to DRMs (lipid rafts; Supplementary Fig. S3). This observation is contradictory to the previous finding of Zhai and colleagues [14]. These researchers showed that 2-bromopalmitate, a systemic inhibitor of O-acyltransferase activity, abolishes the fractionation of Wg with DRMs. To our knowledge, the localization of these mutant Wnt

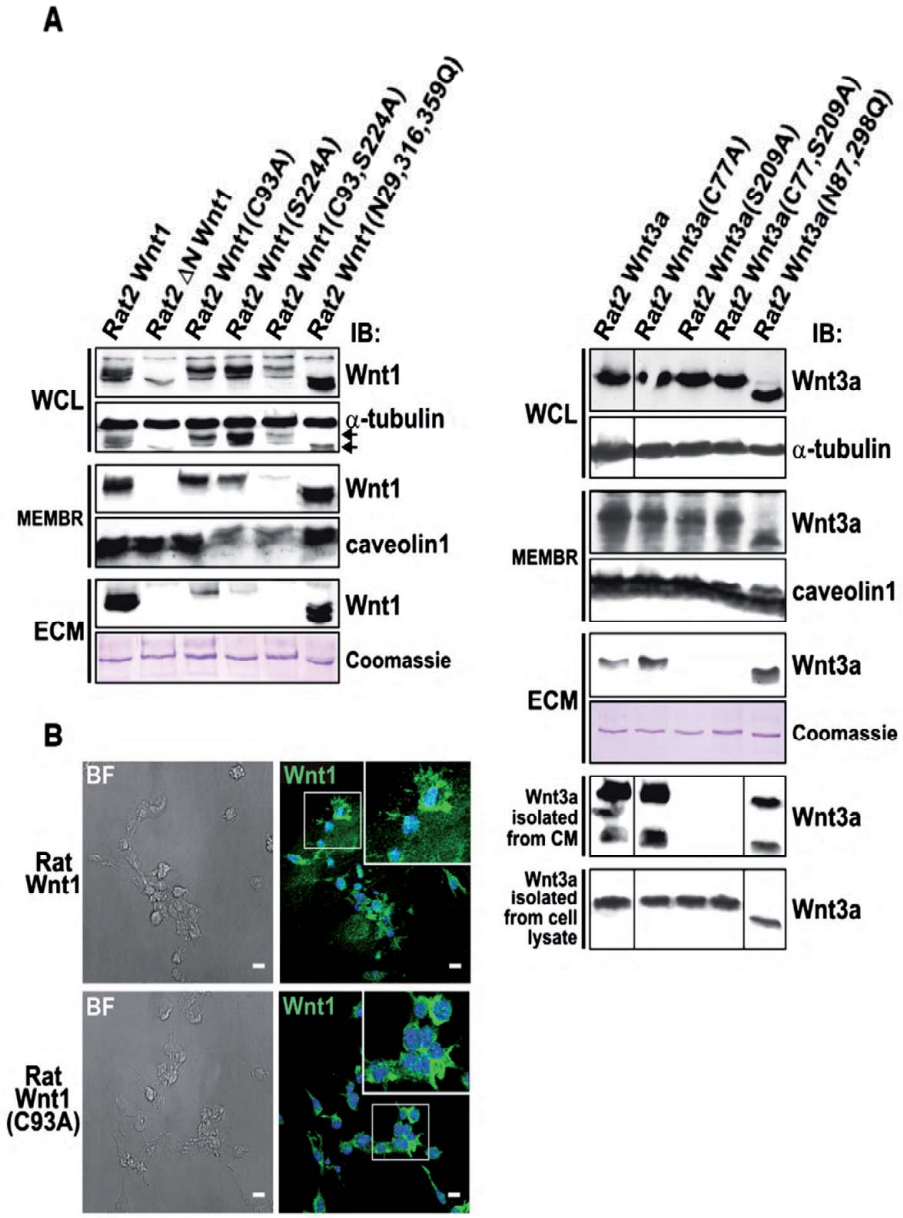

Fig. 6. Acylation promotes deposition of Wnt1 and Wnt3a on the ECM. (A) Cellular distribution of Wnt1 and Wnt3a variants expressed in Rat2 fibroblasts. Western blot analysis of indicated Wnt proteins present in whole-cell lysates (WCL) or co-isolated with plasma membranes (MEMBR) or the ECM. As loading controls, blots were re-probed with anti-α-tubulin and anti-caveolin1 antibodies, or the gel replicas were stained with Coomassie Brilliant Blue (Coomassie). Notice the remnants of the Wnt1 signal on the anti-α-tubulin-stained blot (arrows).The bottom two panels on the right show immunoblots of Wnt3a purified from conditioned media (CM) or cell lysates using Blue Sepharose. (B) Laser scanning confocal microscopy images of Rat2 fibroblasts stably producing wild-type Wnt1 or Wnt1(C93A) mutant polypeptides. Right, both extracellular and intracellular Wnt1 were stained using a rabbit anti-Wnt1 antibody followed by a goat anti-rabbit ALEXA 488 dye-conjugated secondary antibody. Bright field (BF) images are shown on the left. Bar, 10 μm.

proteins to lipid rafts has not been tested. Thus, we suggest that the association of Wnts with DRMs might be mediated via a protein–protein interaction that is not dependent on the presence of any acyl modification. In favour of this theory, protein targeting to DRMs via this mechanism was recently proposed for a variant of the SHP-1 protein phosphatase [51]. Further, we have shown that the interaction of Wnts with the Fz receptor or Lrp co-receptor is independent of the acylation or N-glycosylation status of Wnts (Supplementary Fig. S4). This result is somewhat controversial, as two previous publications indicated that Wnt3a containing no S-linked acyl moiety is unable to bind to Lrp or Fz [17,28]. Both studies, however, demonstrated this phenomenon via pull-down assays that utilized Wnt3a interacting with only recombinant fragments of Fz and Lrp, rather than co-immunoprecipitations using full-length proteins. Therefore, the variation in experimental procedure may explain the different outcomes.

Finally, we tested the distribution of wild-type and mutated Wnt1 and Wnt3a in Rat2 cells stably expressing Wnt proteins. In contrast to Wnt3a, we have never observed the release of any form of Wnt1 into culture medium. This is in agreement with previous studies demonstrating that only a minimal portion of biosynthetically labelled Wnt1 can be detected in cell supernatants [18,25]. Strikingly, we noted that only wild-type Wnt1 and non-glycosylated Wnt1 (N29,316,359Q) protein strongly associate with the ECM. This association was dramatically reduced by the absence of the S-acyl group and was almost completely abolished in acyl-deficient ligand. Instead, both these mutant proteins were preferentially co-isolated with the membrane fraction (Fig. 6A). This observation is consistent with previous findings that indicated that the interaction of Wg with extracellular matrix proteins helps it to spread in the extracellular milieu [52]. The nature of the deposits visualized by staining of native Wnt1 ligand (Fig. 6B), possible involvement of heparan sulfate proteoglycans (HSPGs) in Wnt1/Wnt3a movement, and the mechanisms mediating the Wnt1 (and Wnt3a) interaction to the ECM or plastic surface of the culture dish are unclear. Since the non-glycosylated forms of both Wnt1 and Wnt3a are signalling-competent and display similar biochemical features as wild-type protein, it would seem that N-glycosylation is less important than acylation for the transport of Wnts into the extracellular space. Interestingly, all Wnt3a variants that displayed signalling activity [wild-type Wnt3a, Wnt3a(C77A), Wnt3a(N87,298Q)] were not only deposited on the ECM, but they were also found in culture supernatants. Recently, Neumann and colleagues have discovered that Wnt3a is released from mammalian cells as lipoprotein particles. Wnt3a lacking its palmitate moiety is still secreted; nevertheless, its activity is reduced [53]. Remarkably, liposomal packaging of recombinant Wnt3a using exogenous lipids potentiates its signalling function [54]. In conclusion, the role of the N-terminal palmitate is to mediate lipoprotein packaging that subsequently enhances the action of Wnt3a ligand. Very recently, Coombs and co-authors reported that Wnt3a binding to the carrier protein wntless (WLS) requires lipid modification of Wnt3a at serine 209 [30]. Since acyl-deficient Wnts still reach the cell surface, we presume that mutant Wnts, similarly to non-lipidated WntD, might utilize yet another route of secretion that is not dependent on WLS. Given the results outlined above, we wonder why acylation is essential for Wnt activity. One plausible explanation recently proposed by Bazan and de Sauvage would be that only lipidated Wnt can productively engage with the Fz receptor [55].

Despite a number of revealing facts about the role of acylation and N-glycosylation for Wnt signalling, our study still raises a number of important questions. We still do not know whether differential acylation of Wnt ligands can regulate their gradients in various tissues. Further, although it was thought that Wnt1 and Wnt3a were redundant in the mouse [56], we observed their distinct features in some of our biochemical and functionality tests. Clearly more studies are needed to answer these and other critical questions regarding the intriguing role of Wnt signalling in living organisms.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.cellsig.2011.01.007.

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Supplementary Figures

Supplementary Figure S1 Comparison of expression levels of ectopic Wnt1 and Wnt3a mRNAs in Rat2 cells

qRT-PCR analysis of total RNA isolated from Rat2 fibroblasts transduced with retroviral constructs expressing wild-type or mutant Wnt1 or Wnt3a ligands. The abundance of corresponding mRNA is given as the average CT values after normalizing to the *Ubb* levels.

Supplementary Figure S2 Non-acylated and non-N-glycosylated Wnt1 and Wnt3a are secreted

(**A**) Laser scanning confocal microscopy images of HeLa cells transfected with the indicated Wnt1-expression constructs grown overnight on coverslips. To visualize extracellular Wnt1, living cells were incubated with fresh culture medium containing antigen-purified anti-Wnt1 polyclonal antiserum (30 min at 37°C). Cells were then washed, fixed in paraformaldehyde and treated with Triton X-100. The retained rabbit immunoglobulins were directly stained using the ALEXA 488 dye conjugated to a goat anti-rabbit antibody. Subsequently, the specimens were carefully rinsed with PBS and incubated with the same anti-Wnt1 primary antibody. The final staining was performed with ALEXA 594 conjugated to a goat anti-rabbit antibody. The merged images (d, h, l, p, t) were generated by an overlay of the corresponding scans gained in the green input channel detecting extracellular or endocytosed Wnt/immunoglobulin complexes [panels (b, f, j, n, r)], red input channel (c, g, k, o, s) detecting mainly intracellular and partly extracellular [see e.g. the panel (l)] Wnt and blue channel capturing the DAPI nuclear stain (a, e, i, m, q). Boxed areas in (d), (h), (l), (p) and (t) are magnified in (d'), (h'), (l'), (p'), and (t'), respectively. (**B**) HeLa cells were transfected with Wnt3a constructs and processed as in (A) using antigen-purified anti-Wnt3 polyclonal antiserum. (**C**) Laser scanning confocal microscopy images of L cells transfected with the indicated Wnt1-expression constructs. The cells were processed as in (A); greyscale images are presented only. Boxed areas in (b), (e), (h), (k) and (o) are magnified in (b'), (e'), (h'), (k'), and (o'), respectively. Bar, 10 μm.

Supplementary Figure S3 The absence of acyl adducts does not influence Wnt1 targeting to lipid rafts

Density gradients of the membrane fractions isolated from Rat2 cells stably expressing wild-type Wnt1 or mutant non-acylated Wnt1(S224A). Six fractions were taken from top (T) to bottom (B) of sucrose gradient and subjected to immunoblotting (IB) with the antibody as indicated.

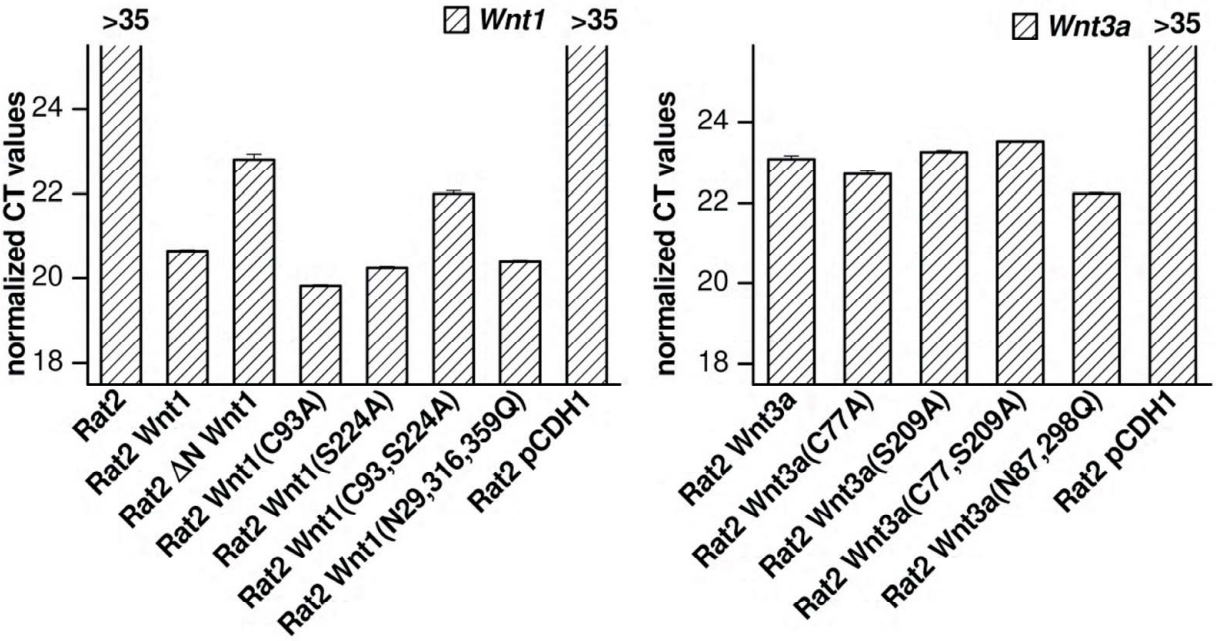
Supplementary Figure S4 All secreted variants of Wnt1 or Wnt3a interact with the Frizzled receptor and Lrp co-receptor

Co-immunoprecipitation of EGFP-tagged mouse Frizzled 4 (Fz4-EGFP) and Flag-tagged mouse Lrp5 (Lrp5-Flag) with different Wnt1 and Wnt3a proteins. Cell lysates prepared from HEK 293 cells transfected with constructs as indicated were precipitated using anti-Wnt1 or anti-Wnt3a rabbit polyclonal antibodies. Precipitated Wnt proteins were detected using the appropriate chicken anti-Wnt antisera. Anti-Flag or anti-EGFP monoclonal antibodies were used to detect Lrp5 and Fz8, respectively. In lanes denoted “input”, ten percent of the total lysate used for one immunoprecipitation were loaded. Notice negligible association of Lrp5 or Fz8 with intracellular ΔNWnt3a and ΔNWnt1 proteins. IB, immunoblotting; IP, immunoprecipitation.

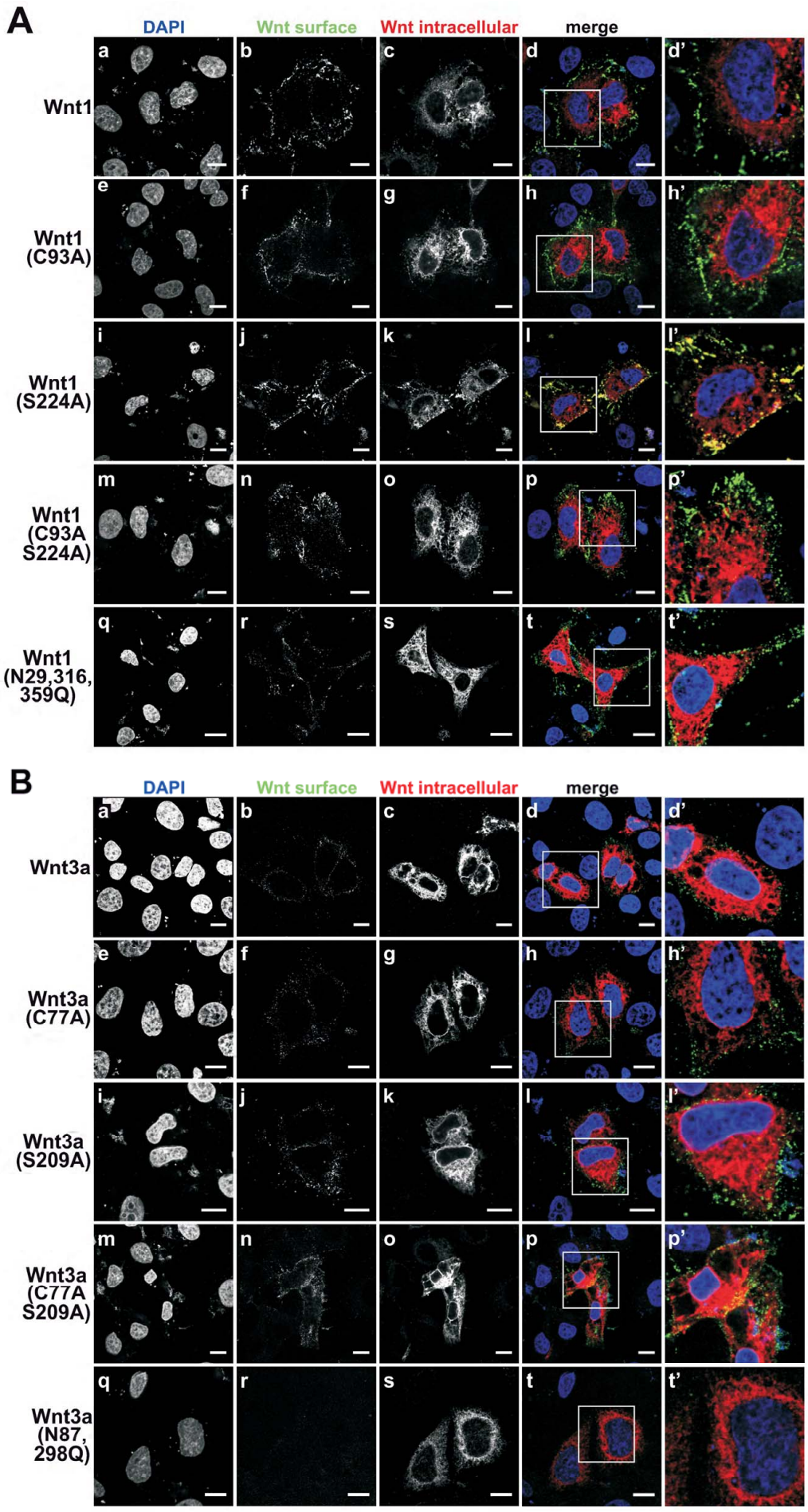
Supplementary Figure S5 The N-glycosylation status of Wnt1 and Wnt3a in Xenopus cells

Xenopus A6 cells were lipofected with the indicated Wnt-expression constructs. Transfected cells were processed as described in the legend to Fig. 1B.

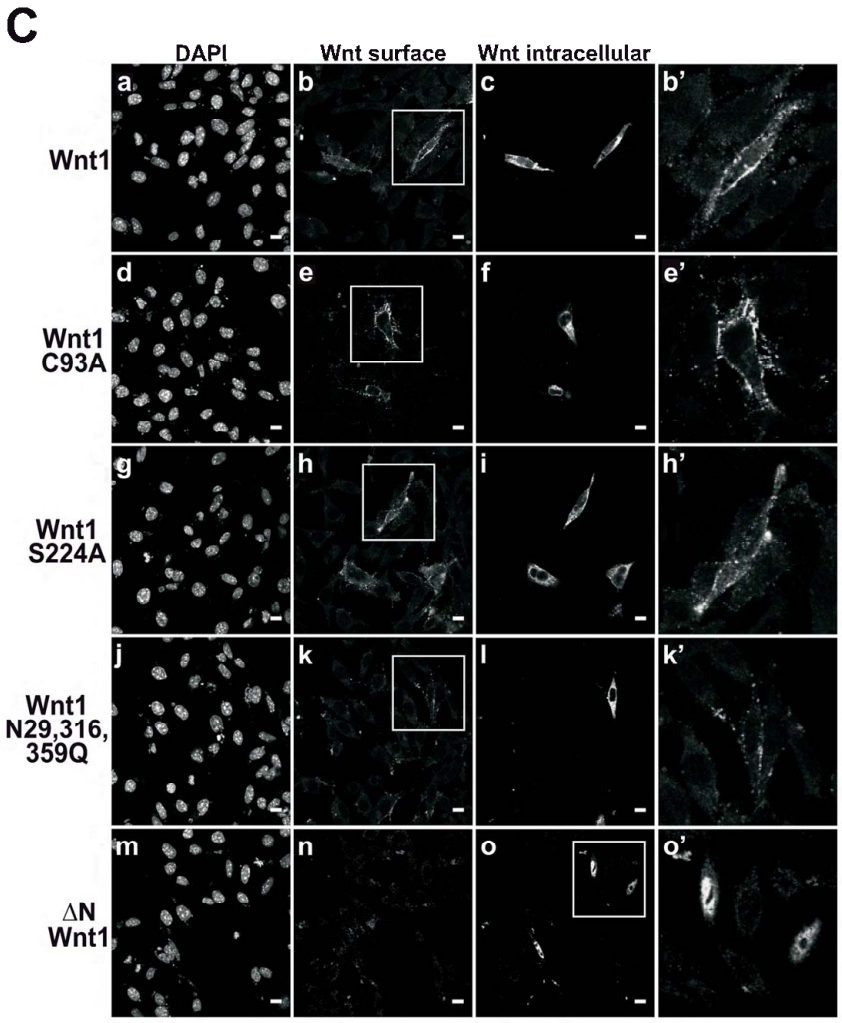
Supplementary Figure S1



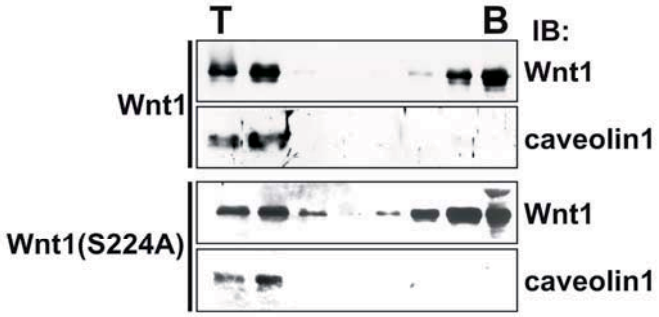
Supplementary Figure S2



Supplementary Figure S2



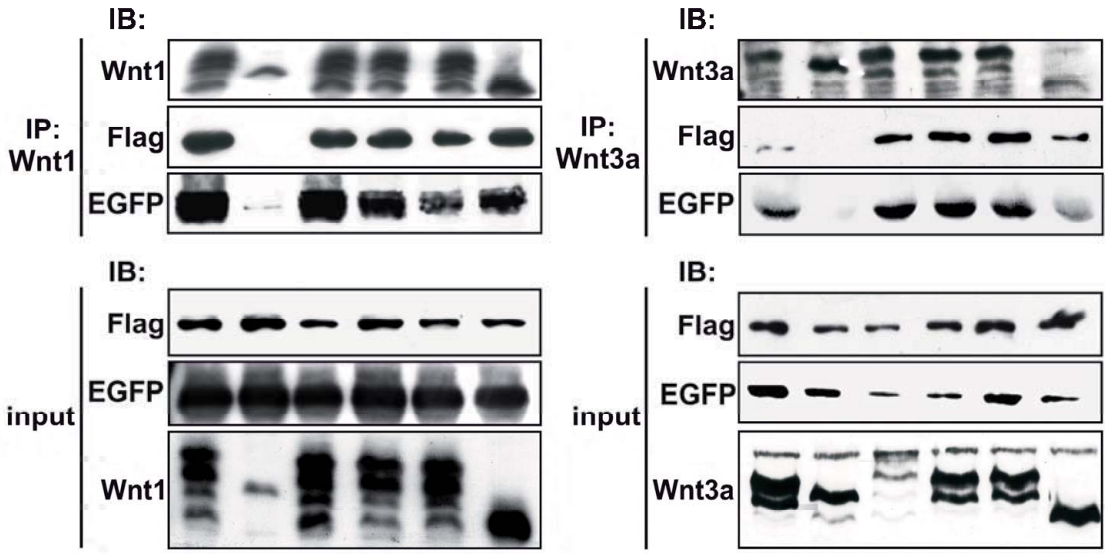
Supplementary Figure S3



Supplementary Figure S4

Wnt1 wt	+	–	–	–	–	–
ΔN	–	+	–	–	–	–
C93A	–	–	+	–	–	–
S224A	–	–	–	+	–	–
C93A,S224A	–	–	–	–	+	–
N29,316,359Q	–	–	–	–	–	+
Lrp5-Flag	+	+	+	+	+	+
Fz4-EGFP	+	+	+	+	+	+

Wnt3a wt	+	–	–	–	–	–
ΔN	–	+	–	–	–	–
C77A	–	–	+	–	–	–
S209A	–	–	–	+	–	–
C77A,S209A	–	–	–	–	+	–
N87,298Q	–	–	–	–	–	+
Lrp5-Flag	+	+	+	+	+	+
Fz4-EGFP	+	+	+	+	+	+



Supplementary Figure S5