Faculty of Natural Sciences, Charles University in Prague
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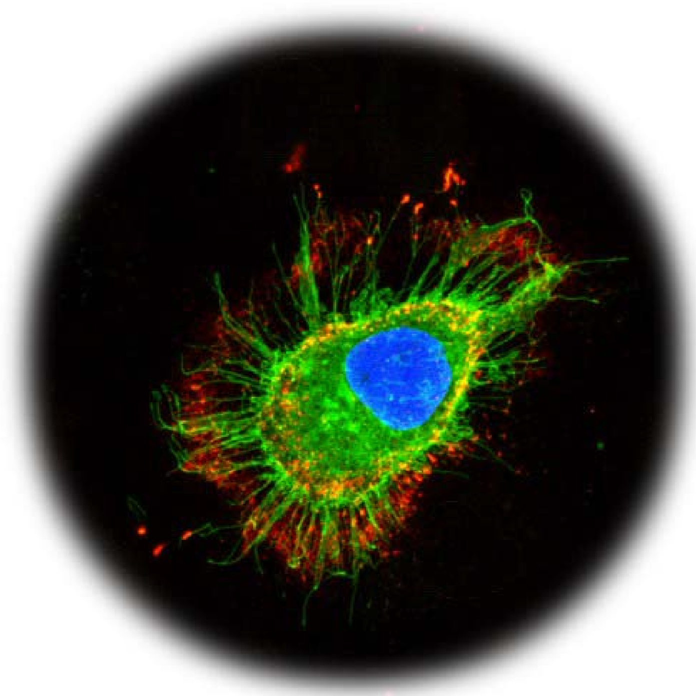
Wnt signaling inside out



Doctoral Ph.D. thesis

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Prague, 2011

Announcement

I declare that I wrote this doctoral thesis by myself and that all the used literature sources are cited. I also pronounce the thesis not being proposed for any other academic title award.

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Signiture:

∗ Cover image: **"HELA cell sitting on the Wnt protein carpet"**
Confocal microphotograph of HELA cell transfected by Fz4-EGFP and Wnt1 and next day stained without fixation against Wnt1 protein for 30 minutes. After that paraformaldehyde fixation and secondary antibody staining followed. Wnt1 stained in red (rabbit Wnt1 polyclonal, goat anti rabbit - Alexa 594), Frizzled4-EGFP (green), nucleus (blue, DAPI).

"I have been to hell and back
And let me tell you it was wonderful."

Louis Bourgeois

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Abbreviations list

APC	Adenomatous Polyposis Coli
Asef	APC-stimulated guanine exchange factor
β-TrCP	β-Transducing repeat Containing Protein
BMP	Bone Morphogenetic Protein
Brg1	Brahma-related gene 1
CBP	CREB (cyclic AMP response element binding protein) Binding Protein
Cdk	Cyklin-dependent kinase
CK1	Casein Kinase 1
CRD	Cysteine Rich Domain
CtBP	C-terminal Binding Protein
Dazap	Deleted in azoospermia (DAZ)-associated protein
DEP	Dishevelled, Egl-10, Pleckstrin
DISC	Death-inducing death domain
DIX	Dishevelled-Axin
Dkk	Dickkopf
Dlp	Dally like protein
DR	Death Receptor
DSCR1	Down Syndrome Critical Region Protein 1
Dsh/Dvl	Dishevelled
ECM	Extracellular matrix
EGF	Epidermal Growth Factor
EMT	Epithelial-Mesenchymal Transition
ER	Endoplasmatic Reticulum
FADD	Fas-associated death domain
FAP	Familial Adenomatous Polyposis
Fz	Frizzled
GBP/Frat	GSK3-Binding Protein/Frequently rearranged in T-cell lymphomas
GEF	Guanine nucleotide Exchange Factor
GPCR	G-protein Coupled Receptor
GPI	Glycosylphosphatidylinositol
GRK5/6	G-protein Coupled Receptor Kinase 5/6
GSK3	Glycogen Gynthase Kinase 3
HAT	Histone Acetylase
HDAC	Histone Deacetylase
HDL	High Density Lipoprotein
HEAT	Huntingtin, Elongation factor 3, Protein Phosphatase 2A, TOR1
HECT	Homologous to E6-AP C-Terminus
Hh	Hedgehog
HIC1	Hypermethylated In Cancer 1
HiRE	HIC1 Responsive Element
HMG	High Mobility Group
HMT	Histone Methyltransferase
HSPG	Heparan Sulphate Proteoglycan
ICAT	Inhibitor of β-Catenin and Tcf4
IGFBP4	Insulin-like Growth Factor Binding Protein 4

IKK	IκB Kinase
Int-1	Integrated-1
JNK	c-Jun N-terminal Kinase
KLF4	Krüppel-Like Factor 4
Krm	Kremen
LA	LDLR type A
LDLR	Low Density Lipoprotein Receptor
LEF-1	Lymphoid Enhancer Factor-1
LRP5/6	Low Density Lipoprotein Related Protein 5/6
MACF1	Microtubule Actin Crosslinking Factor 1
MBOAT	Membrane Bound O-Acyl Transferase
MCR	Mutation Cluster Region
MLL1/2	Mixed-Lineage Leukemia 1/2
NES	Nuclear Export Sequence
NLK	Nemo-Like Kinase
NLS	Nuclear Localizing Signal
NPC	Niemann-Pick type C protein
NTR	Netrin
PAF1	Polymerase-Associated Factor 1
PCP	Planar Cell Polarity
PDGF	Platelet-Derived Growth Factor
PDZ	PSD95, DlgA, ZO1
PI4KIIα	Phosphatidylinositol 4-Kinase type II α
PIASy	Protein Inhibitor of Activated STAT y
PIP5KI	Phosphatidylinositol-4-Phosphate 5-Kinase type I
Porc	Porcupine
PPPS/TPxS	$(Proline)_3$-Serine/Tyrosine-Proline-any amino acid-Serine
PP1/2A	Protein Phosphatase 1/2A
Prtb	Proline-rich transcript of the brain
Pygo	Pygopus
RanBP3	Ran Binding Protein 3
RGS	Regulators of G-protein Signaling
Rspo 1	R-spondin 1
Ryk	Related to Y (tyrosine) kinase
SCF	SKP1-Cul1-Fbox protein complex
SFRP	Secreted Frizzled Related Protein
SKI	Skinny Hedgehog
SMRT/N-CoR	Silencing Mediator of Retinoid and Thyroid Hormone Receptors/ Nuclear Receptor Co-Repressor
S/TxV	Serine/Tyrosine-any amino acid-Valine
SUMO	Small Ubiquitin-related Modifier
SWI/SNF	Switch/Sucrose Non Fermentable
TBL1	Transducin β-Like Protein 1
TBLR1	TBL1-Related Protein 1
TCF	T-Cell Factor
TLE	Transducin-Like Enhancer
TRAIL	Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand
TRRAP	Transformation/Transcription Domain-Associated Protein
VEGF	Vascular Endothelial Growth Factor
Wg	Wingless

Wif	Wnt inhibitory factor
Wls	Wntless
WRE	Wnt Responsive Element
YWTD	tyrosine, tryptophan, threonine, aspartic acid

1. Summary

Signaling pathways function as molecular instruments mediating cellular response to intrinsic and extrinsic inputs, which can consequently lead to cell division or differentiation on one side and cell death on the other. Molecular network of different pathways enables the intercellular communication and hence the whole organism can exist and function coordinately.

The Wnt signaling pathway belongs among evolutionarily old and conserved molecular pathways and acts in many different processes during development [1-5]. Moreover, it is necessary for maintenance of adult tissues as it participates in regeneration [6-12]. Diverse malignancies, where repressive components of the Wnt pathway are non-functional, represent seamy side of the scope [13-15].

This thesis is based on 4 publications covering Wnt signaling on very multifarious levels. Firstly, I focus on processing of Wnt protein which stands at the beginning of the cascade as extracellular morphogen. Secondly, survival effect of Wnt producing fibroblasts on leukemia cells after induction of apoptosis by ligand TRAIL is discussed. The third issue shows novel components of the Wnt signaling pathway and introduces us into nucleus – "bottom" level of the pathway.

1. Fatty acid modification of Wnt1 and Wnt3a at serine is prerequisite for lipidation at cysteine and is essential for Wnt signalling. **Doubravská L**, Krausova M, Gradl D, Vojtechova M, Tumova L, Lukas J, Valenta T, Pospichalova V, Fafilek B, Plachy J, Sebesta O, Korinek V. Cell Signal. 2011 May;23(5):837-48. Epub 2011 Jan 16.

2. Wnt-expressing rat embryonic fibroblasts suppress Apo2L/TRAIL-induced apoptosis of human leukemia cells. **Doubravská L**, Símová S, Cermak L, Valenta T, Korínek V, Andera L. Apoptosis. 2008 Apr;13(4):573-87. Epub 2008 Mar 18.

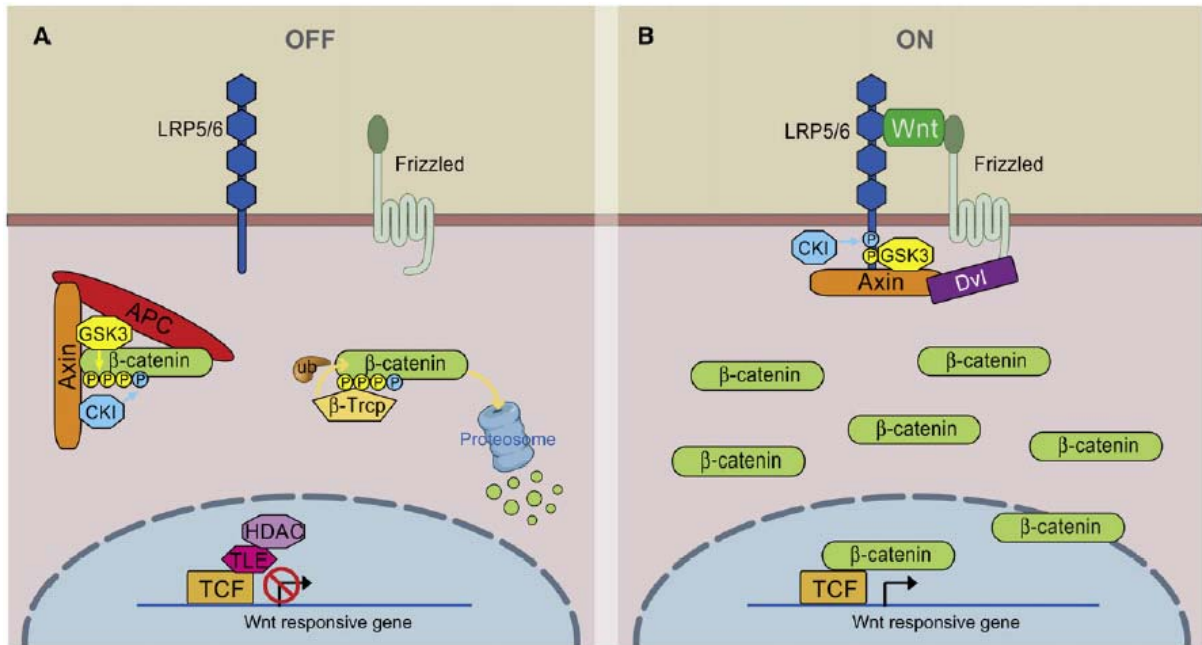
3. Dazap2 modulates transcription driven by the Wnt effector TCF-4. Lukas J, Mazna P, Valenta T, **Doubravska L**, Pospichalova V, Vojtechova M, Fafilek B, Ivanek R, Plachy J, Novak J, Korinek V. Nucleic Acids Res. 2009 May;37(9):3007-20. Epub 2009 Mar 20.

4. HIC1 attenuates Wnt signaling by recruitment of TCF-4 and beta-catenin to the nuclear bodies. Valenta T, Lukas J, **Doubravska L**, Fafilek B, Korinek V. EMBO J. 2006 Jun 7;25(11):2326-37. Epub 2006 May 25.

2. "Wnt digest"- introduction

Signaling pathways use many common molecules which serve as crossing points in the molecular network of the cells. The Wnt pathways are divided into canonical and non-canonical ones according to their ability to stabilize β-catenin, which is the key molecule for the canonical Wnt signaling. In this thesis we deal with the canonical β-catenin-dependent signalization (Wnt/β-catenin signaling). It is important to perceive that thinking of Wnt signaling in terms of linear pathways, either intracellularly or extracellularly is already surpassed. It is necessary to view the Wnt signal transduction as a complex network of protein interactions, with multiple outcomes, cross-talk and regulatory inputs at practically all cellular levels.

The central mechanism of Wnt/β-catenin signaling rests in constant degradation of β-catenin in the absence of Wnt ligand. The degradation complex is composed of scaffolding protein axin, the tumor suppressor *adenomatous polyposis coli* gene product (APC), casein kinase 1 (CK1), and glycogen synthase kinase 3 (GSK3). Kinases CK1 and GSK3 sequentially phosphorylate the amino terminal region of β-catenin, resulting in its recognition by β-Trcp, which is E3 ubiquitin ligase subunit. Subsequent β-catenin ubiquitination leads to the proteasomal degradation of the protein that prevents it from entering the nucleus. The Wnt target genes are thereby repressed by the DNA bound T cell factor/lymphoid enhancer factor (TCF/LEF) family of proteins. Wnt/β-catenin signaling is turned on when Wnt ligand binds to Frizzled (Fz) receptor and its co-receptor low-density lipoprotein receptor-related protein 5 or 6 (LRP5/6). The membrane triplex recruits scaffolding protein Dishevelled (Dvl) to the cytoplasmic part of Fz. This results in LRP phosphorylation and binding of the axin complex to the receptor. Sequentially axin-mediated β-catenin phosphorylation is inhibited, β-catenin is stabilized and reaches the nucleus to form complexes with TCF/LEF transcription factors and activates the Wnt target gene expression. For more comprehensive information the reader is referred to "the Wnt home page" (http://www.stanford.edu/group/nusselab/cgi-bin/wnt/) or to recent reviews [13, 16, 17].



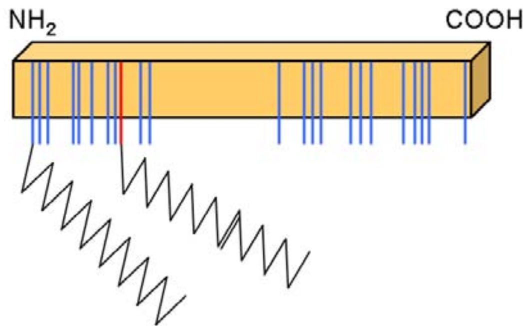
Wnt/β-catenin signaling.
(A) In the absence of Wnt, cytoplasmic β-catenin forms a complex with axin, APC, GSK3, and CK1, and is phosphorylated by CK1 (blue) and subsequently by GSK3 (yellow). Phosphorylated β-catenin is recognized by the E3 ubiquitin ligase β-Trcp, which targets β-catenin for proteasomal degradation. The Wnt target genes are repressed by TCF-TLE/Groucho and histone deacetylases (HDAC).
(B) In the presence of Wnt ligand, a receptor complex forms between Fz and LRP5/6. Dvl anchored by Fz leads to LRP5/6 phosphorylation and axin recruitment. This disrupts axin-mediated phosphorylation/degradation of β-catenin, allowing β-catenin to accumulate in the nucleus where it acts as a co-activator for TCF to switch the Wnt-responsive genes on. (Adopted from [17])

2.1 The membrane level

2.1.1 Wnt ligands

Wnt proteins are hydrophobic molecules with a high number of conserved cysteines. They obtained their name “Wnt” from a combination of *Drosophila melanogaster* segment polarity gene *wingless* and its mouse orthologue *Int1* [18]. The *Wnt* genes appear in sea anemone *Nematostella vectensis* earliest in evolution and persist to humans [19]. All 19 mammalian Wnt proteins share signal peptide that targets them to the endoplasmatic reticulum and through the secretory pathway to be consequently extruded out of cells. Posttranslational modifications such as multiple N-glycosylations are attached during processing [20-22]. Porcupine (Porc), which belongs to the family of membrane-bound O-acyltransferases (MBOATs) [23-25], is located in endoplasmatic reticulum membrane to

mediate palmitoyl and palmitoleoyl binding to conserved cysteine and serine, respectively [26-29]. The acylations are proposed to be necessary for secretion and active Wnt signaling [30].



The Wnt1 protein scheme.
Blue lines indicate a set of conserved cysteines while red line represents the position of serine modified by palmitoleoyl. First cysteine of Wnt1 corresponds to Wnt3a C77 which was identified to be palmitoylated [26]. Both acyls are shown.

Protein Wntless (Wls, also known as Evenness interrupted or Sprinter) is necessary for Wnt secretion [31-34]. It is located in endoplasmatic reticulum, Golgi network, endosomes and the plasma membrane and seems to direct the Wnts through the secretory machinery to plasmatic membrane. After the Wnt extracellular release Wls is endocytosed and returned back to trans-Golgi by a retromer complex [35-41]. Wnt3a acyl modification on serine 209 seems to be required for Wls binding and moreover vacuolar acidification plays a role in the release of palmitoylated Wnt3a from Wls [42]. Recently, Wls was shown to act as a versatile carrier that guides Wingless to presynaptic terminals of motor neurons and then escorts it across the synaptic cleft [43]. According to these characteristics Wls protein could be called a Wnt chaperone.

Wnt proteins are associated with so-called lipid rafts, specialized detergent-resistant membrane microdomains which are believed to serve as docks for many signaling molecules such as receptors, and their immediate downstream partners [27]. Reggie-1/Flotillin-2 is one of the major lipid raft components and apparently, it has influence on the extent of extracellular Wnt [and also of another morphogen Hedgehog (Hh)] concentration gradient released by producing cells. It somehow mediates higher mobility of Wnts and supports long-range gradient formation [44]. It is supposed that Reggie-1/Flotillin-2 creates specialized rafts

similar to caveolae, which are able to recruit multiprotein complexes [45]. The process of the Wnt traffic outside cells still remains poorly understood.

Recently characterized *Drosophila* Wnt family member WntD (Wnt inhibitor of Dorsal) behaves differently compared to the Wnt features mentioned above. It does not require Porcupine for its secretion or signaling activity since it is not lipidated. Even the Wls function is not necessary for WntD secretion [46]. It is interesting that WntD does not contain particular conserved serine which was shown to be acylated in Wnt3a. In spite of different biochemical properties WntD plays a role in dorso-ventral patterning in *Drosophila* embryos and also regulates the Toll/Dorsal–mediated antimicrobial response in adult flies [47] [48].

2.1.1.1 Lipoparticles as extracellular Wnt vehicles

In *Drosophila* imaginal discs secreted Wingless is attached to lipoparticles called argosomes that also serve as vehicles for other morphogens like Hedgehog [49, 50]. Association of Wnt3a with high-density lipoprotein (HDL) but not low-density lipoprotein (LDL) particles in mammalian cell culture medium has been published recently [51]. This implies that acyl modifications could participate in this kind of traffic in extracellular millieu. HDL specific scavenger receptors SR-BI/II function possibly in loading of Wnt3a to the HDL particles. Since SR-BI/II are found in lipid rafts, the membrane microdomains probably represents ideal environment for the Wnt-scavenger receptors interaction. There is also a possibility that endocytosis is prerequisite for Wnt loading into lipoparticles as observed in *Drosophila* S2 cells [52] or in mammalian fibroblasts [53]. Recycling lipoprotein particles could also encounter Wnt protein in the endosomal system leading to the secretion of the more mobile Wnt pool [54].

The negatively charged sulfate and carboxyl groups of heparan sulfate proteoglycans of the extracellular matrix (ECM) can interact with positively charged residues on lipoproteins and recruit them [55]. Subsequently, morphogens retained in lipoparticles attached to the ECM can be more accessible to their receptors.

2.1.1.2 The extracellular matrix in Wnt signaling

The second way of extracellular movement is connected to ECM components namely heparan sulphate proteoglycans (HSPGs, glypicans) to which Wnt proteins tightly bind [56]. HSPGs are linked to the cell surface via glycosylphosphatidylinositol (GPI) anchor and possess the cysteine rich domain (CRD) next to the stalk-like domain with the heparan sulphate attachment sites in its core protein structure. They are qualified as receptors for several heparin-binding proteins including various morphogens [Wnt/Wingless, Hedgehog, transforming growth factor-β (TGFβ), fibroblast growth factor (FGF)] and participate in generation of concentration gradients of these proteins [57-61].

There are six HSPGs called glypican 1–6 (GPC1-6) in vertebrates and two HSPGs - Dally and Dally-like (Dlp) proteins - in *Drosophila*. It was shown that particularly heparan sulphate chains are important for Wg interaction and it is mainly their sulfation status that influences the strength of binding [62-64]. HSPGs play obviously a major role in morphogen linkage but also chondroitin sulfate side chains of syndecans can be also involved [65]. As for glypicans, GPC3 was shown to regulate Wnt signaling independently of its heparan sulfate chains [66, 67] and acts as a modulator of both canonical and non-canonical Wnt pathways [68].

Pieces of knowledge obtained in *Drosophila* help to depict the HSPGs function in Wg signaling. Dally acts as a typical co-receptor, which positively influences Wg signaling by presenting the ligand to its receptors. On the contrary, Dlp reduces short range in benefit of long range signaling [69, 58, 59]. Dong Yan and colleagues show that the crucial role of Dlp is maintaing Wg on the cell surface where it competes with Fz2 receptor – whether the competition ends by activation or inhibition of Wg signaling depends on the ratio of all the three players, Wg, Dlp and Fz2 [70]. Dlp activates Wg signaling faraway from Wg producing cells where concentration of Fz2 is high compared to Wg levels and *vice versa* [71, 58, 59]. Interestingly, Dlp core protein directly interacts with Wg while heparan sulfate chains only increase the strength of the interaction [70]. These observations indicate that HSPGs-dependent diffusion is the mechanism of the Wg distribution and gradient formation.

2.1.1.3 Comparison of Wnt and Hedgehog modifications´role

To recapitulate: there is a pool of extracellular Wnt associated with the ECM, a soluble pool found in cell culture medium and moreover, a pool of Wnt associated with plasmatic membranes. Hedgehog (Hh) and Wnt ligands share similar characteristics; nevertheless, the roles of Hh modifications were elucidated in more detail.

Hh is auto-catalytically cleaved by the intein-like mechanism and cholesterol is covalently linked to its C-terminus [72-74]. Hh is also palmitoylated on a cysteine residue near its N-terminus by the endoplasmatic reticulum protein Skinny Hedgehog (SKI) [75]. Interestingly, SKI acyltransferase modifies another soluble protein Spitz, which is epidermal growth factor receptor (EGFR) ligand [76]. Cholesterol tethers Hh molecules to the plasmatic membrane and increases the Hh local concentration. If cholesterol is missing, Hh spreads easily and dilutes out. Thereby Hh devoid of cholesterol is able to activate low-threshold target genes only [77].

Palmitate and cholesterol are essential for the Hh function and for the production of soluble multimers, which serve as the extracellular Hh pool functioning in long-range signaling [78-80]. When Hh lysine 132 is mutated, no oligomerization happens, no interaction with HSPGs is possible and monomeric Hh signals only in an autocrine manner [81]. In *Drosophila* imaginal discs Hh is transported via lipoparticles called lipophorins [50]. However there are no data showing Hh association with the vertebrate lipophorin homolog apolipoprotein B [82]. It is clear that the lipophorin-deficient discs reduce Hh long-range spreading and leave short-range target genes unaffected [50, 83]. Hh multimers and lipoparticles are associated with HSPGs [84, 85, 77], which can regulate Hh endocytosis [86, 87].

Different modifications drive the secretion of Hh in various forms (multimers, lipoparticles, membrane or ECM associated), thus regulating the protein activity in particular tissue.

2.1.2 Frizzled receptors

Wnt proteins interact with their receptors Frizzleds and co-receptors LRP5/6 on the plasmatic membrane and bring them in close proximity which leads to downstream signaling

events. The Frizzled family represents ten individual G-protein coupled receptors (GPCRs) in human genome including a typical structure of seven transmembrane helixes. The extracellular N-terminal part of Fz receptors contains the cysteine rich domain (CRD) named according to its invariant pattern of ten cysteine residues. The CRD interacts with Wnt proteins with high affinity [88-92]. Frizzleds possess a hydrophobic pocket according to their structural similarity to NPC (Niemann-Pick type C) protein, which uses it for cholesterol uptake [93]. There is a hypothesis that Fz receptors might employ the NPC-like pocket to associate with acyl modifications of the Wnts and modulate the strength of signaling [94].

The cytoplasmic C-terminal part of Fz receptor mediates several different signalizations. C-terminal motif Ser/Thr-X-Val ("X" indicates any amino acid residue) constitutes a binding motif for proteins containing the PDZ domain (originally described in PSD, Disc-large and ZO1 protein) (hFz3, 6 and 9 do not possess this motif) [95]. There is the crossing among canonical/β-catenin-dependent and non-canonical pathways which resides in the interaction of particular Wnt ligand with the specific Fz receptor. Fz receptor cellular context dictates the Wnt signaling output. Both ligand and receptor behave promiscuously which is nicely demonstrated by Wnt5a known to be mainly a non-canonical activator but after its interaction with exactly Fz4 and LRP5 in human HEK293 cells, β-catenin is stabilized [96]. Another example of receptor signaling specificity is shown in *Drosophila*; Fz is involved in the establishment of planar cell polarity (PCP, one of the non-canonical pathways) as it has a lower affinity for Wingless than Fz2 does, which is involved in β-catenin signaling rather than PCP [97-99]. Exchanging the C-terminal sequences of Fz and Fz2 reverses their behavior [100]. But it is not that simple to view the receptor as the sole determinant of Wnt signaling activation. *Xenopus* Fz7 can mediate multiple intracellular responses. It affects convergent extension movements (the non-canonical pathway) that involve Dishevelled, Syndecan 4 and Cdc42, but it is also required for dorso-ventral mesoderm specification upstream of β-catenin [101-103].

A very special role for *Drosophila* Fz2 was described in developing synapse. Wg is secreted from the pre-synaptic terminals to be bound to Fz2 on the post-synaptic membranes, which induces synaptic growth and differentiation. Upon ligand-receptor interaction, Fz2 is endocytosed, cleaved and its C-terminal part translocates to the nucleus, where it potentially regulates transcription [104].

As Frizzleds are G protein-coupled receptors, there is a question whether G proteins are used for signaling. It was shown that $G\alpha_o$ subunit of G protein complex is necessary for canonical and non-canonical (PCP) signaling [105], which corresponds to bioinformatics

analysis of Fz cytoplasmatic sequences [95]. Another evidence for G protein necessity in the Wnt-stimulated canonical pathway is its sensitivity to pertussin toxin, an ADP-ribosyltransferase that inactivates only members of the Gi/Go family of G proteins [106]. Additionally, within minutes of cell stimulation by Wnt3a, trimeric G proteins mediate the dissociation of the GSK3-axin protein complex [107], which corresponds to recently published data that *Drosophila* axin is a direct interaction partner of Gαo-GTP in the Wnt signal transduction [108]. The binding occurs via axin RGS (regulators of G protein signaling) domain known in other proteins to bind Gα-subunits of trimeric G proteins [109].

2.1.3 The novel class of Wnt receptors

A single-pass receptor tyrosine kinase Ror2 possesses the CRD domain mediating interaction with the Wnt proteins. Ror2 dimerizes upon Wnt5a binding and triggers a non-canonical pathway resulting in cell migration and inhibition of β-catenin dependent pathway [96, 110-113]. On the other hand Ror2 potentiates Wnt1 or Wnt3a mediated gene expression [114-116] but it does not appear to require Ror2 tyrosine kinase activity, raising the possibility that Ror2 could function as a co-receptor presenting the Wnts to the Frizzleds.

Ryk, an atypical tyrosine kinase receptor without kinase activity [117], contains the Wnt inhibitory factor (Wif) domain and forms a complex with Wnt1 and Fz8, which serves as a prerequisite for TCF-mediated activation of Wnt1 target genes in mammalian neurites [118]. However, whether Ryk mediates Wnt signaling in concert with Fz-LRP5/6 or independently is not clear but it is able to recruit directly Dishevelled via its PDZ binding motif. Ryk activates also non-canonical pathways [119].

2.1.4 LRP co-receptors

LRP5/6 are type I single-transmembrane proteins with the large extracellular part consisting of YWTD (tyrosine, tryptophan, threonine, aspartic acid) β-propellers interleft by EGF-like domains. LDLR (low-density-lipoprotein receptor) type A domains (LA domains) are close to transmembrane region which continues with short cytoplasmic C-end where five PPP(S/T)P (proline 3x, serine/threonine, proline) sites are phosphorylated by different kinases after Wnt bridges LRP to Frizzled. LRP5/6 are classified as members of a subfamily of the

LDLR (low-density-lipoprotein receptor) family according to their extracellular structure but the proteins behave differently as for their activity. The Wnt proteins interact with N-terminal β-propellers of LRP5/6 together with Mesd1 (mesoderm development) chaperon which brings co-receptors from a secretory pathway to the plasmatic membrane [120-122]. Both LRPs act partially redundantly but LRP6 deletion leads to prenatal lethality, a defect similar to this seen in Wnt1, Wnt3 or Wnt7A knocked-out mice [123], while LRP5 deletion reveals normal embryogenesis but later osteoporosis and metabolic disorders [124, 125].

Wnt recruitment of LRP co-receptors appeared to be a prerequisite for inducing β-catenin signaling [126-128]. However, LRP6 takes also part in non-canonical pathways leading to convergent extension movements and tissue polarity establishment in vertebrates [129, 130]. It seems that signaling context can be modulated by other interacting molecules such as single-pass transmembrane receptor Kremen (Krm, Kringle-coding gene marking the eye and the nose) [131] or soluble secreted Dickkopfs (Dkks) [132]. Dkk family includes Dkk1, 2, 3 and 4, which exhibit distinct and dynamic expression patterns and may have distinct properties as Dkk3, which does not affect Wnt signaling [133, 134]. Neither of Dkk or Kremen family is present in *Drosophila* and *Caenorhabditis elegans* but both can be found in cnidarians or bilaterians.

Dkk1 functions by preventing Fz/LRP6 complex formation via LRP6 recruitment [135] and the other possible mechanism is different but not mutually incompatible with this one. Dkk-1 interacts with Krm1 or 2 in addition to LRP5/6 [136] with high affinity and promotes internalization of this ternary complex. Kremen behaves in a duplicate manner. In the presence of Dkk it helps with internalization, while being alone Kremen promotes the cell surface localization of LRP [137]. Dkk2 can activate or inhibit Wnt signaling depending on the cellular context [138, 139, 134]. It could be regulated by Krm2 but not Krm1, which invariably turns Dkk2 into a Wnt inhibitor [134].

R-spondin1 (Rspo1) was found to interfere with Dkk1/Krm-mediated internalization of LRP6 through the interaction with Krm [140], indicating that Rspo1 may participate in a Wnt/β-catenin pathway through increased levels of cell surface LRP6. The whole R-spondin family of secreted proteins (Rspo1-4) is able to amplify Wnt/β-catenin signaling in the presence of Wnt ligands and LRP6 receptor and the mechanism probably resides in Dkk inhibition [141].

Other LRP6 binding proteins belong to the Wise/SOST family, which also reveal both stimulatory and inhibitory activities depending on the cell context [142]. Wise proteins are secreted and include members that bind and antagonize bone morphogenetic proteins

(BMPs) [143]. SOST/sclerostin shares 36% identity with Wise and also binds LRP proteins to disrupt Fz/LRP complex and to inhibit β-catenin signaling [144, 145]. The other way of Wise action is the recruitment of LRP6 by endoplasmatic reticulum retained Wise thereby the cell surface LRP6 is decreased [146].

2.1.5 Other ligands in Wnt signaling pathway

A Wnt inhibitory protein (Wif) binds to the Wnts extracellularly and inhibits their activity. It may also influence Wnt stability and diffusion. Similarly secreted Frizzled-related proteins (SFRPs) are Wnt interacting antagonists, which act via their CRD domains. The SFRP family consists of five members in humans with different expression profiles in particular tissues during development and adulthood but the proteins can function partially redundantly [147]. SFRPs possess except of N-terminal CRD domain (with 10 conserved cysteines like Fz or Ror receptors) also a NTR (netrin) modul with positively charged residues to bind extracellular matrix [148]. The interesting feature of the CRD domains is their ability to mutually interact, so that homo- or hetero-dimers are formed [90]. This property provides different explanations of SFRPs operation. The proteins can not only interact with the Wnt ligands, but also with each other to inhibit themselves [149] or directly with the Frizzled receptors [150]. There are also many other SFRPs interacting partners such as components of extracellular matrix or members of the BMP family. SFRPs may also act as novel Wnt regulators - they help Wnt spreading inside extracellular space in *Xenopus* embryos rather than inhibit Wnt function [151].

Norrin [152, 153] - named after Norrie, a Dutch ophthalmologist, who firstly described a rare disease of blindness, deafness and mental retardation - is a secreted protein, which forms dimers that further aggregate to oligomers which are attached to extracellular matrix [154]. It was shown that Norrin directly interacts with specifically Fz4 and LRP5/6 to trigger β-catenin signaling and is able to stand in for Wnt ligands [155, 156]. This kind of signaling happens particularly in retina and other parts of brain during development and results in proper angiogenesis. Recently tetraspanin Tspan12 has been described to play a key role in the Norrin-receptor complex function as it interacts with the complex and increases its activity via promoting multimerization of Fz4 [157].

Another ligand of Wnt receptors is IGFBP4 (insulin-like growth-factor-binding protein 4) acting during cardiogenesis. This protein interacts with and modulates the action of insulin-

like growth factors (IGFs) but IGFBP4 also binds to Fz8 and LRP6 to prevent Wnt3a from switching the canonical pathway on [158]. IGFBP4 thus enhances cardiomyocytes differentiation *in vitro* and *in vivo*.

Shisa protein represents a distinct family of Wnt antagonists. It traps Frizzled receptors in the endoplasmic reticulum (ER) and prevents them from reaching the cell surface, thereby inhibiting Wnt signaling [159]. Such like mechanism serves also to regulate Wnt secretion. A widely expressed ER-resident Oto protein is involved in adding atypical glycophosphatidyl anchors to Wnt1 and Wnt3a, which results in their ER-retention [160].

Above mentioned ligands and receptors influencing the Wnt pathway indicate how a complicated and complex network of regulation leads to the proper level of signaling in particular tissue. The boarder between correct and aberrant signaling can be modulated by many different mechanisms not only on the plasmatic membrane level.

2.2 The cytoplasmatic level

2.2.1 Wnt-off state

In the absence of Wnt ligand, a cytoplasmic protein conglomerate called the degradation complex carries on phosphorylation of β-catenin which is consequently degraded in the 26S proteasome. Axin, the scaffolding protein with binding sites for GSK3 (glycogen synthase kinase 3*)*, CK1α (casein kinase 1α), APC (adenomatous polyposis coli), and β-catenin coordinates sequential phosphorylation of β-catenin at serine 45 by CK1α, and then at threonine 41, serine 37, and serine 33 by GSK3 [161-163]. The E3 ubiquitin ligase $SCF^{\beta\text{-TrCP}}$ (SKP1-Cul1-Fbox protein complex) binds to phosphorylated serine 33 and 37 and prepares β-catenin for degradation [164]. In many cancers β-catenin with these mutated serines or threonine escapes ubiquitination and so degradation is changed to permanent activation of the canonical pathway. APC, a large multifunctional scaffolding protein, also interacts directly to β-catenin and together with axin creates the core of the degradation complex. APC and axin are tumor suppressors and their mutations are often found in different cancers (about 80% of colorectal cancers reveal both APC alleles inactivated [165]).

GSK3 and CK1 phosphorylate, besides the phosphorylation of β-catenin, also axin and APC. It increases their binding properties to β-catenin and efficiency to degrade it [166]. Homeostasis is maintained by serine/threonine phosphatases PP1 and PP2A, which counteract the action of GSK3 and CK1 and are associated to the axin complex. PP1 dephosphorylates axin which leads to the disassembly of the complex, whereas PP2A has β-catenin as its substrate. On the other hand, APC protects β-catenin from PP2A dephosphorylation by removing PP2A from axin [167]. It results in β-catenin ubiquitination and it also increases axin availability for a further round of β-catenin phosphorylation [162].

2.2.2 Wnt-on state

Upon Wnt bridging Fz and LRP together, Dvl is recruited to Fz via its PDZ binding motif (S/TxV) and brings axin with bound GSK3 to close proximity of five LRP C-end PPPS/TPxS motifs. These dually phosphorylated motifs are docking sites for the axin complex [168-170, 128]. GSK3 phosphorylation of PPPS/TP primes xS phosphorylation by CK1γ, which is the only one of the CK1 family with a palmitoyl membrane anchor [171]. GSK3 counts for most of PPPS/TP modifications as experiments in cells without GSK3α/β indicate [172, 170]. Interestingly other kinases also take part in LRP phosphorylation. G-protein coupled receptor kinases GRK5 and GRK6 are membrane associated proteins known to phosphorylate and desentize seven-transmembrane G-protein-coupled receptors. GRKs directly phosphorylate the PPPSP motifs of LRP6 and regulate canonical Wnt signaling [173]. Also phosphatidylinositol 4-kinase type II α (PI4KIIα) and phosphatidylinositol-4-phosphate 5-kinase type I (PIP5KI) were shown to phosphorylate LRP6 on threonine 1479 and serine 1490 upon Wnt3a activation in mammalian and *Xenopus* cells [174].

The Dvl protein acts as a platform for LRP clustering via axin after Wnt activation. Forced oligomerization of LRP6 is sufficient to induce Wnt signaling and this oligomerization bypasses the need for Dvl [128]. The existence of „LRP6 signalosomes“, ribosome-sized complexes of LRP6 aggregates cumulated after Wnt activation, has been shown recently [175, 176]. Dvl and axin each harbor the DIX domain that exhibits dynamic polymerization [177], which could explain the mechanism of clustering. In unstimulated cells Dvl forms cytoplasmic aggregates or “punctuates” via its DIX modul containing also axin and CK1ε. After Wnt stimulus aggregates are recruited to the plasmatic membrane where Dvl-axin interaction behaves very dynamically [178].

The described membrane protein complex formation leads to inhibition of β-catenin phosphorylation as the same players of the destruction complex are recruited to Fz-LRP receptors. Phosphorylated LRP6 PPPS/TPxS motifs directly and specifically stop GSK3 phosphorylation of β-catenin serine 33, 37 and threonine 41 but CK1 phosphorylation on serine 45 continues [179, 180]. It is consistent with the observation of dephosphorylated β-catenin at the plasma membrane closed to active LRP6 and axin quickly upon Wnt stimulation [181]. Other mechanisms such as axin-GSK3 dissociation as a consequence of axin PP1 dephosphorylation [182] or axin degradation can also influence β-catenin stabilization [107, 183].

Many other proteins were shown to somehow regulate or affect cytoplasmic level of the Wnt pathway. For example, MACF1 (microtubule actin crosslinking factor 1) protein of the spectraplakin family known to connect cytoskeleton components to membrane junctional complexes [184] was shown to interact with the axin complex and LRP6 and to help their binding at the membrane [185]. Opposite function was addressed to novel Wnt antagonist called Bili, which inhibits the recruitment of axin to LRP6 during pathway activation [186]. Tankyrase 1/2 are poly-ADP-ribosylating enzymes binding axin and stimulating its degradation through the ubiquitin-proteasome system. As axin is concentration-limiting component of the Wnt pathway, tankyrase regulation plays a crucial step during signaling [187].

2.2.2.1 Dishevelled

The scaffolding protein Dishevelled (Dsh in *Drosophila*, Dvl1-3 in mammals) is a common element for canonical and non-canonical pathways. Dvl interacts with Frizzled receptors and with axin at once through one of its three domains - DIX, PDZ and DEP - PDZ is used for Fz binding [188] and DIX serves as an axin interactor [189].

Casein kinases CK1α, β, δ and ε bind to and phosphorylate Dvl [190, 191] and act as potent activators of β-catenin signaling. CK2 phosphorylates Dsh/Dvl too [192, 193]. Both kinases are constitutively active and it is interesting how evolution played with their number – in *Drosophila* there are eight of them, mammals possess seven CK1s and eighty seven CK1s exist in *Caenorhabditis elegans* [194]. Dvl phosphorylation in PDZ and DEP domains can modulate their binding properties. For example, Dvl recruits a GBP/Frat (GSK3 binding

protein/Frequently rearranged in T-cell lymphoma) protein to the destruction complex and CK1ε activity increases Dvl binding of GBP [195, 196].

β-arrestin was described as a Dvl binding partner, which interacts with CK1 phosphorylation sites in the PDZ domain [197]. It also binds axin and so a link is created, which positively stimulates canonical signaling [198]. β-arrestin competes with CK1/2 for Dvl interaction sites and the balance among them determines different non-canonical pathways [199].

2.2.2.2 The role of endocytosis

Although endocytosis has traditionally been considered to mediate the down-regulation of receptors and attenuate signaling, it can also be a mechanism that might activate signaling. Wnt receptors Fz5 and LRP6 were shown to internalize upon Wnt3a adding by caveolin-mediated endocytosis in HEK293 cells [200]. Caveolin directly binds to LRP6, which is endocytosed and phosphorylated at once and this leads to subsequent steps of the Wnt canonical pathway. Interestingly, Dkk1 induces clathrin-dependent LRP6 endocytosis and thereby counteracts caveolin-dependent endocytosis which directs to inhibition of β-catenin accumulation [201]. On the contrary, Wg or Wnt3a were described to internalize via a clathrin-mediated pathway in L cells [202].

The influence of endocytosis on Wingless gradient formation and distribution in *Drosophila* was studied by Jean-Paul Vincent group. They clearly demonstrate that endocytic trafficking modulate Wg distribution in epithelia by providing a degradation route [203]. For degradation of the Wg/Fz2 complex, *Drosophila* LRP homolog Arrow must be also present [204, 205]. Inhibition of endocytosis by different approaches generates controversial data on attenuating or increasing Wnt signaling. It can be probably explained by general stopping of endocytosis which can influence many other processes in cells [206, 207].

Above mentioned Dvl binding partner β-arrestin, a well-known clathrin adaptor, could play a possible role in endocytosis during Wnt signaling [208, 209]. In the absence of β-arrestin in *Xenopus*, β-catenin is not stabilized in response to Wnt [198]. β-arrestin also acts in non-canonical signaling endocytosis where it induces Wnt11/Fz7/Dsh complex internalization upon receptor tyrosine kinase Ryk stimulus [119].

Another important point represents cell surface proteoglycans endocytosis. An extracellular matrix (ECM) is internalized by a mechanism dependent on flotilin and dynamin

but not on clatrin or caveolin [210]. Many ECM ligands next to Wnt proteins can be endocytosed while bound to ECM without any other receptors. Diverse Wg internalizations occur on apical or basal side in *Drosophila* wing disc – the Wg/Arrow/Fz2 complex is endocytosed apically while Wg without receptors is internalized on the basal side [211], so that ECM might serve as a receptor here.

2.2.2.3 APC

The adenomatous polyposis coli (APC) tumor suppressor is a multifunctional protein, which has been detected at several unexpected intracellular locations, implicating its regulation of cell migration, apoptosis or DNA repair.

There are two APC genes in mammals (APC, APC2) widely expressed during development [212]; containing many different domains for all types of functions but as for Wnt signaling mainly β-catenin and axin binding sequences are important. They serve for the creation of the destruction complex, where APC plays as a scaffold protein and a tumor suppressor [213]. Nevertheless, APC influences the Wnt pathway on many other levels. It promotes export of β-catenin from nucleus via its nuclear export sequence (NES) and via its nuclear localization signal (NLS) APC comes to the nucleus and regulates Wnt-induced transcription [214, 215]. The mechanism of regulation rests in binding β-catenin and blocking its interaction with TCF/LEF transcription factors [216] and direct association with the nuclear repressor complex (βTrCP, CtBP, TLE-1, HDAC) inhibiting β-catenin/TCF transcription [217, 218].

Germ line mutations in one allele of APC give rise to the intestinal polyp disorder, familial adenomatous polyposis (FAP), a heritable predisposition to colorectal cancer [219, 220]. Mutations of both alleles lead to tumorigenesis. APC cancers have in majority mutations targeted at a centrally-located ~300 amino acid sequence known as the mutation cluster region (MCR), which introduces premature stop codons, resulting in the set of C-terminal truncated APC proteins with increased mobility and altered functions [221, 222]. A common feature of shortened APC mutants is the retention of the armadillo repeat sequences that bind protein Asef (APC-stimulated guanine exchange factor), an exchange factor for the Rac and Cdc42 GTPases. Stimulation of those GTPases is connected with membrane ruffling, a formation of lamellipodia, and cell migration via actin cytoskeleton [223, 224]. Usually

truncated APC loses a binding site for axin and partly for β-catenin, which results in "just-right β-catenin signaling" that is crucial for relevant tumor formation [225].

APC has other functions in addition to controlling the Wnt pathway such as taking part in adherens junctions, where it provides the link between E-cadherin and α-catenin, which in turn binds actin [226]. APC further associates with the growing end of microtubules and microtubule-interacting proteins and influences their stability [227]. Moreover, APC proteins are present in mitotic spindles, centrosomes or kinetochores and it implies possible control of mitosis and chromosome segregation. In fact, APC-mutant cells reveal chromosomal abnormalities of two types: quantitive changes and chromosomal translocations [222, 228, 229].

APC has a central role in initiation and promotion of colorectal cancer because its inactivation provides a strong selective advantage concerning cell proliferation, migration and genetic instability.

2.2.2.4 β-TrCP

Phosphorylated β-catenin is recognized by Fbox protein β-TrCP1/2 (β-Transducin repeat Containing Protein 1/2), which acts as a SCF E3 ligase subunit [230, 231]. These ligases possess the RING-finger domain allowing direct transfer of ubiquitin (7.5 kDa) to their substrates in contrast to the HECT-domain (Homologous to E6-AP C-Terminus) E3 ligases, which are charged with ubiquitin by E2 enzym to transfer it to the substrate. The SCF complex composes of adaptor protein SKP1 (S-phase kinase-associated protein 1), scaffold protein Cul1 (cullin) and one of many Fbox proteins (69 in humans) serving as substrate recognizers. Besides phosphorylated β-catenin Fbox protein β-TrCP distinguishes also cell cycle or pro-apoptotic regulators as substrates. The SCF complex is always active and plays a prominent role during the G1/S phase of cell cycle.

Polyubiquitinylated β-catenin is degraded in the proteasome system. β-catenin can potentially escape degradation through mutations in the N-terminal phosphorylated sites [232].

2.2.2.5 β-catenin

β-catenin belongs to the armadillo protein family, which is characterized by the armadillo (arm) domain consisting of a repeating 42 amino acid motif. This repeat has been found in other proteins such as plakoglobin (γ-catenin) and p120. A structural feature of the arm domain is a long positively charged groove serving as a versatile protein binding interphase and β-catenin function is completely influenced by proteins binding to this groove in mutually exclusive fashion [233, 234]. Phosphorylation of these interacting partners introduces negative charges that enhance their binding affinity to β-catenin and results in dynamic balance between β-catenin pools either in transcription or in cell adhesion.

β-catenin is a central component of cadherin-mediated adherens junctions [235, 236], which are particularly important in formation and maintenance of intercellular adhesion [237] and also coordinate cellular movements within epithelia together with transmitting information from the cell surface to the cell interior [238]. E-cadherins physically link neighboring cells through its single-pass extracellular domains [239] by creating calcium-dependent homotypic interactions. The cytoplasmic tail of E-cadherin is linked to the arm repeat family of catenins, namely p120-catenin and β-catenin, while plakoglobin might act redundantly if β-catenin is missing [240]. p120-catenin interacts with the membrane-proximal cadherin tail involved in lateral clustering and cadherin stabilization, in contrast, β-catenin interacts with the distal part of the cadherin tail and also with α-catenin, an unrelated catenin that can bind directly to filamentous actin cytoskeleton as well as to actin-binding proteins [241, 242]. The cadherin/β-catenin interaction can be influenced by CK1, GSK3β and CK2 kinases, which play key roles in the regulation of the nuclear signaling form of β-catenin, thereby there is a possibility that canonical Wnt signals can influence cell adhesion [243-246]. Over-expression of cadherins leads to an increased β-catenin adhesion pool with subsequently decreased β-catenin transcription and vice versa showing that distinct β-catenin pools are dynamically connected and influenced by the competition among interacting proteins [247-249].

As mentioned above, β-catenin plays a central role in canonical Wnt signaling. It is constitutively degraded upon its N-terminus phosphorylation in the Wnt-off state, while after switching the pathway on β-catenin is no more phosphorylated but it is stabilized in the cytoplasm and translocates to the nucleus where it regulates transcription [250]. It seems that nuclear compartment is gained independently of classical nuclear localization sequences

(NLSs) [251] since the arm repeat region of β-catenin resembles HEAT (Huntingtin, Elongation factor 3, Protein Phosphatase 2A, TOR1) repeats of the nuclear import factor importin-β and so it can probably mediate its own import via direct interaction with nucleoporins [252, 215]. On the other hand, β-catenin export from the nucleus was shown to be dependent on APC [253, 216], axin [254], menin [255] or RanBP3 (Ran Binding Protein 3), which binds β-catenin in the Ran/GTP manner [256]. On the contrary, Bcl9 is a β-catenin binding partner accountable for its active import to the nucleus [257]. Thus β-catenin is thought to continually shuttle in and out of the nucleus, and interaction with either cytosolic or nuclear proteins ultimately influences its distribution by retention [258].

Recently, Wu and colleagues showed that Wnt-induced β-catenin stabilization is not enough for nuclear translocation, but small GTPase Rac1 has to be triggered in parallel [259]. Rac1 activates JNK2 and facilitates the formation of a complex with β-catenin, with the last mentioned being phosphorylated on serines 191 and 605 by JNK2, which results in β-catenin nuclear import. Interestingly, Rac1 participates also in non-canonical Wnt pathways (PCP or convergent extension) [260, 261]. Moreover, Rac1 and its GEFs (Guanine nucleotide Exchange Factors) are connected with the regulation of the nuclear β-catenin/TCF complex as well as the axin/APC cytoplasmic complex [262, 263].

Very different molecules can affect the destruction complex and allow β-catenin to escape from degradation. For example, p68 RNA helicase binds β-catenin N-terminus and prevents it from GSK3 phosphorylation [264]. This happens upon PDGF (Platelet-Derived Growth Factor) signaling inducing the phosphorylation of p68 by the c-Abl kinase, which is necessary for PDGF-induced epithelial-mesenchymal transition (EMT) – a developmental process during which epithelial cells lose polarity, cell contacts and acquire characteristics typical of mesenchymal cells such as increased motility. EMT is critical for gastrulation and organogenesis, and is likely to be involved also in cancer invasion [265].

Other proteins such as IKKα (IκB Kinase α) or Cdks (Cyclin dependent kinases) are able to phosphorylate β-catenin and other proteins of the destruction complex and influence their function [266-268].

2.3 The nuclear level

Once in the nucleus, β-catenin interacts with the TCF/LEF (T Cell Factor/Lymphoid Enhancer Factor) family of DNA-bound transcription factors [269-272]. TCFs are associated

with the Groucho/TLE (Transducin-like enhancer of Split) gene repressor complex in the absence of β-catenin, which promotes histone deacetylation and chromatin compaction [270-276]. Upon Wnt signaling, β-catenin recruits other nuclear co-activators and converts TCFs from repressors to transcriptional activators [277-279].

2.3.1 TCF/LEF

There are four TCF genes in mammals – TCF1, LEF1, TCF3 and TCF4 – compared to only one gene in *Drosophila* or *Caenorhabditis*. TCF proteins belongs to a high-mobility group (HMG) of DNA-binding factors recognizing consensus sequence referred to as the Wnt responsive element (WRE), CCTTTGWW (W represents either T or A), and upon DNA binding they bend it up to 130° to alter a local chromatin structure [280, 281]. The highly conserved HMG domain consists of a HMG box recognizing WRE and bending DNA followed by a 9 amino acids long linker and NLS of the same length, which also helps to bind DNA via its basic character.

Wnt target genes are frequently flanked with multiple WREs, most of which are located at large distances from transcription start sites [282]. Another GC element downstream of the typical WRE can be recognized by the second DNA binding domain called C-clamp (cysteine clamp, ~30 amino acids; formerly known as CRARF), which is present in some TCF splicing variants (E tailed isoforms), allowing regulation of different sets of target genes [283-285]. Interestingly, all major *Drosophila* TCF isoforms contain C-clamp that binds to a seven-base-pair motif ("helper site") near the classic WRE and this bipartite mechanism promotes DNA recognition to respond to Wg signaling [286-288].

An invertebrate TCF consists of four domains: an N-terminal β-catenin-binding domain (1-55 amino acids), a central domain, a well-conserved and already mentioned HMG domain and a long C-terminal tail. This generalized picture of TCF structure is conserved in vertebrate LEF/TCFs, with vertebrate TCF1E isoforms being similar in overall domains to invertebrate ones. Other vertebrate TCF isoforms have lost parts of these domains and/or include novel peptide motifs.

Tcf1 and *Lef1* genes possess alternative promoters which, when transcribed, result in short isoforms lacking β-catenin-binding domains (ΔN-TCF1/ΔN-LEF1). These isoforms act as endogenous dominant-negative TCF/LEF or constitutive repressors of the Wnt signaling pathway [289, 290]. Alternative splicing in the central domain of TCF1 and LEF1 produces

transcripts with or without a central alternative exon [291, 292]. This exon is always included in TCF3 and TCF4, however, it is differently spliced in TCF4 so that alternative exon can be but does not have to be flanked by small motifs LVPQ and SxxSS [293-295], which are always present in TCF3 [271]. LVPQ and SxxSS motifs regulate the formation of a DNA/TCF/β-catenin complex and act as transcription repressive elements [295, 296]. Because the central domain is bound by the repressor complex Groucho/TLE or a set of activators depending on the cell context, it is called a context-dependent regulatory domain (CRD).

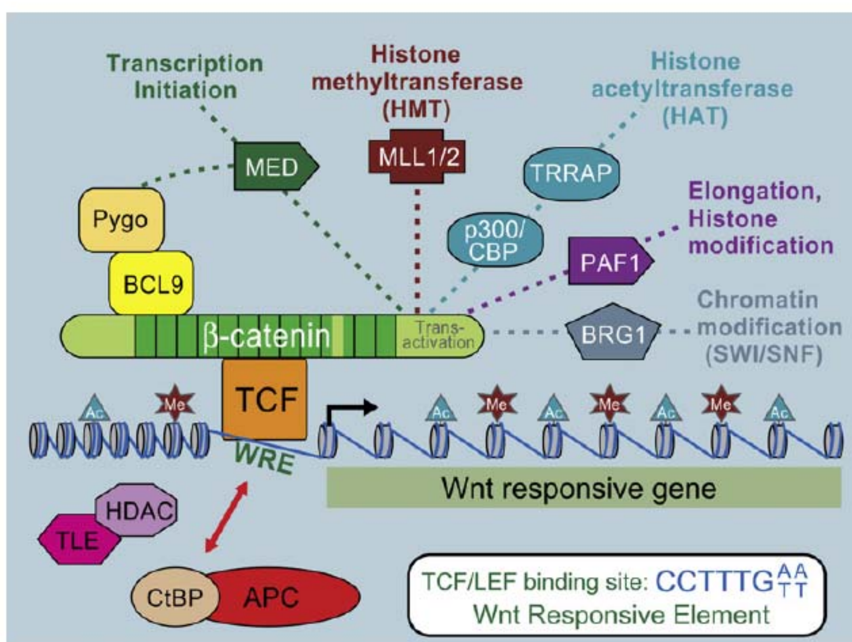
Alternative splicing at the C-terminus in TCF1 and TCF4 gives rise to isoforms that have a variety of C-tails with or without three conserved regions: the CRARF motif (C-clamp), the RKKKCIRY motif and binding sites for the CtBP (C-terminus binding protein) cofactor [291-294, 297]. The long TCF4 isoforms contain all three regions, TCF1E has only the CRARF and RKKKCIRY motifs, LEF1 has none of them and TCF3 has only the proposed CtBP-binding sites [298].

Some general rules appear to describe LEF/TCFs functions approximately: TCF1 and TCF4 act as both repressors and activators, and LEF1 is often an activator whereas TCF3 is mostly a repressor but sometimes an activator [298, 299]. Specific LEF/TCFs isoforms can activate different genes [283] and moreover, posttranslational modifications of LEF/TCFs can further modulate their activities. The small ubiquitin-related modifier (SUMO) modification (sumoylation) of LEF1 and TCF4 by the SUMO ligase PIASy represses LEF1 activity by targeting it to nuclear bodies but enhances TCF4/β-catenin transcription [300, 301]. LEF1 sumoylation is located to the β-catenin-binding domain and protects LEF1 against trans-activator binding. On the contrary, TCF4 is sumoylated in the Groucho/TLE region, which reverses the balance toward activation status. The *Drosophila* acetyltransferase CREB-binding protein (dCBP) has been shown to regulate wingless signaling negatively by acetylating TCF [302]. The acetylation inhibits the binding of TCF to Armadillo/β-catenin. In contrast, acetyltransferases p300 and CBP serve as co-activators for β-catenin in vertebrates [277]. Phosphorylation is specific to individual LEF/TCF proteins conferring different regulation. For example, TCF3 phosphorylation by CK1ε and LEF1 phosphorylation by CK2 enhance their binding to β-catenin and diminish LEF1 binding to Groucho/TLE [195, 303]. On the other hand, LEF1 and TCF4 phosphorylation in the central domain by Nemo-like kinase (NLK) leads to lower TCF/LEF/β-catenin complex binding to DNA and contributes to the down-regulation of LEF/TCF transcriptional activity [304, 305].

2.3.2 Nuclear modulators of Wnt signaling

There are many proteins binding to β-catenin and/or TCF/LEF proteins, which somehow modulate their interaction and therefore act as antagonists or agonists of the Wnt signaling pathway. Plenty of these proteins can target components of the basal transcription machinery to regulate gene expression or alter the chromatin structure.

One of the well-known mechanisms involves removal or addition of an acetyl group on lysine in N-terminal tails of histones through the recruitment of histone deacetylases (HDACs) or histone acetylases (HATs), respectively. Deacetylation of histones causes chromatin condensation restricting the access of transcriptional factors to DNA and conversely, acetylation is a well-established chromatin modification in active genes. Mono, di or trimethylation of lysines or arginines in histone tails by histone methyltransferases (HMTs) also correlates with active (H3K3me3; histone 3, lysine 3 trimethylation) or repressed (H3R2me1; histone 3, arginine 2 monomethylation) transcription, respectively. This "histone code" specifies chromatin interactions with different protein complexes at various stages of the transcriptional activation of a target gene [17].



Nuclear TCF/β-catenin complexes.
Dotted lines represent interaction between complexes or with β-catenin. During active Wnt target gene transcription, APC promotes the exchange between β-catenin/co-activators with co-repressors CtBP, TLE, and HDAC in a cyclic manner (double-headed red arrow) while TCF remains bound to the WRE. Ac and Me symbolize histone modi-fications, i.e. acetylation and methylation, respectively.
(Adopted from [17])

2.3.2.1 Nuclear repressors

Groucho/TLE (*Drosophila*/humans; Transducin-Like Enhancer of split) repressors do not bind to DNA directly but interact with TCF/LEF transcription factors in the absence of Wnt signaling [273]. The repressing effect is mediated by interactions with the hypoacetylated N-terminal tail of histone H3 and with human HDAC1 [306-308].

CtBP (C-terminal Binding Protein) represses Wnt targets by direct interaction with some TCFs [309-312] or by binding to APC and diverting β-catenin away from TCF [310, 311, 217, 218]. Nuclear antagonists Chibby and ICAT (Inhibitor of β-Catenin and TCF4) bind to β-catenin, disrupt diverse β-catenin interactions and promote its nuclear export [313, 314]. Another transcriptional repressor HIC1 (Hypermethylated in cancer 1) creates nuclear bodies by its oligomerization and recruits there β-catenin/TCF4 to avoid Wnt-induced transcription [315]. KLF4 (Krüppel-Like Factor 4), a transcription factor critical for intestine differentiation, interacts directly with the β-catenin/TCF4 complex and inhibits histone acetyltransferase p300/CBP recruitment by β-catenin. KLF4 protects β-catenin as well as histones on Wnt target genes from p300/CBP (CREB [Cyclic AMP Response Element-Binding Protein] Binding Protein) acetylation [316, 317].

Many other proteins were described to repress Wnt signaling on the nuclear level using the mechanisms mentioned above [17].

2.3.2.2 Nuclear co-activators

Plenty of β-catenin-associated co-activators exist. They are recruited through β-catenin N-terminal and C-terminal trans-activation domains. The β-catenin N-terminal trans-activation domain interacts with the adaptor protein BCL9/Legless, which in turn recruits Pygopus (Pygo) [318-322]. Unlike most co-activators that have general roles in transcription, BCL9 and Pygo in *Drosophila* are specifically required for β-catenin-dependent transcription [323]. Pygo is a permanently nuclear protein, which recruits β-catenin/BCL9 upon Wnt signaling [324, 325, 257]. If the pathway is off, Pygo also co-occupies chromatin, and may help capture β-catenin/BCL9 for TCF at the onset of signaling [326]. Pygo preferentially binds to dimethylated H3K4 (histone 3 tail, lysine 4) upon interaction with BCL9 [327],

which may provide a separate β-catenin anchor on chromatin, thereby freeing TCF for interaction with Groucho to pause or terminate transcription [323].

The C-terminal domain of β-catenin recruits p300/CBP [328, 329, 277]. p300 and CBP are closely related proteins that promote transcriptional activation through several mechanisms, including the recruitment of the basal transcriptional machinery and acetylation of widespread (up to 30kb) histones surrounding the WRE in response to triggering the Wnt pathway [330]. By contrast, CBP was shown as a negative regulator of Wnt signaling in flies, where it acetylates the HMG domain of TCF and thereby reduces its ability to bind to β-catenin [302]. Generally, these HATs regulate in two modes; they act as a buffer to regulate TCF/β-catenin interaction, but have an additional role as β-catenin binding transcriptional co-activators [331].

Besides p300/CBP, another histone acetyltransferases TRRAP/p400/TIP60 and histone methyltransferases (HMTs) MLL1/2 take part [332, 333, 218]. The SWI/SNF (Switching-defective/Sucrose Nonfermenting) family of ATPases acts to remodel chromatin structure – the SWI2/SNF2 family protein Brg1/Brahma directly binds to β-catenin and is suggested to regulate nucleosome arrangements in Wnt-responsive target genes [278]. And the PAF1 (Polymerase-Associated Factor 1) complex helps transcription elongation and histone modifications via its interaction with β-catenin [334, 335, 323, 330].

Under Wnt-stimulated conditions, β-catenin and its co-activators cycle on and off the WRE with an hour periodicity and are replaced by Groucho/TLE [218, 303]. Thus Groucho/TLE also act as repressors during Wnt-responsive transcription. This cycling may employ other repressors such as CtBP [336] and two related components of the SMRT/N-CoR co-repressor complex, TBL1 (transducin β-like protein 1) and TBLR1 (TBL1-Related Protein 1), which are also β-catenin co-activators for at least some Wnt-responsive genes [337].

2.3.3. Wnt target genes

Because of Wnt signaling involvement in many cellular processes during development, Wnt target genes depend on the tissue context and the time point after activation [338, 339]. However, *Axin2* represents an almost universal Wnt target gene similarly to the Wingless-induced pathway antagonist *Naked cuticle* in *Drosophila* [340, 341, 16].

Wnt signaling can promote the expression of Wnt pathway components, which indicates regulation via feedback control. The levels of Frizzleds, LRP or HSPG are controlled, providing fine-tuning of Wg activity on the cell surface [342, 343127]. Wnt signaling also influences cell proliferation by direct induction of cell cycle regulators such as c-Myc and CyclinD1 [344, 345]. Proliferation [346-348], apoptosis [349, 350], differentiation [351-353], cell adhesion [354, 355] or immune response [356, 357] are affected by Wnt induced target genes too. Nowadays, there are about 100 identified direct Wnt signaling target genes and the full overview is upgraded on the Wnt home page (http://www.stanford.edu/group/nusselab/cgi-bin/wnt/target_genes).

It is necessary to mention that next to the direct target genes, which are controlled via the known TCF/LEF binding motif, there are plenty of indirect target genes. For example, Wnt signaling direct target VEGF (vascular endothelial growth factor) [347] acts as a pathway regulator because it switches on the VEGF receptor tyrosine kinase pathway leading to its own target genes (e.g. DSCR1, Down syndrome critical region protein 1) [358]. Similarly, c-Myc is a transcription regulator which only triggers effectors (e.g. p21) [359, 338].

Besides the activation of target genes by TCF/LEF:β-catenin complexes there is also evidence of their direct repression. β-catenin is recruited by TCF/LEF to a traditional WRE (CTTTGWWS) and possibly another sequence element (AGAWAW) directs the supression mode [287]. As an example serves *Drosophila* UbxB (Ultrabithorax B) transcription factor whose activity is increased by weak Wg signaling but suppressed by strong Wg signals [360] [361] and other examples of Wnt/Wg-mediated repression have reference to osteocalcin [362], E-cadherin [354] or Decapentaplegic [363].

3. Results and discussion

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

Wnt proteins are characterized by a high number of conserved cysteines, which are supposed to generate disulphide bonds and determine protein structure. Hydrophobic disposition of the Wnts was explained by discovering lipid modifications, namely cysteine palmitoylation [26] and serine palmitoleoylation [28]. Because both acylated residues are highly conserved among Wnt proteins across different species it is believed that they are generally lipid-modified. The acylations are mediated by the membrane bound O-acyltranspherase Porcupine [26, 28, 29] and have already been evidenced for murine Wnt3a (cysteine 77), *Drosophila* Wnt homolog Wingless (Wg) or Wnt5a [27, 21].

Palmitic acid attached to cysteine in the amino-terminal part of Wnt3a relates to signaling activity because non-palmitoylated protein reveals decreased function despite the fact that it is normally secreted outside cells [26]. Also non-palmitoylated Wnt5a or Wg are found in the extracellular space in cell cultures but *in vivo* studies in the *Drosophila* imaginal discs non-palmitoylated Wg seems to be retained in the endoplasmic reticulum and so not secreted [21, 30]. As mentioned above, the loss of palmitate leads to lower Wnt activity which can be caused by increased dilution of the Wnts in the extracellular space [54] or by disrupted interaction with Frizzled receptors [128, 21, 22, 30].

The second acyl, mono-unsaturated palmitoleic acid, modifies conserved serine 209 of Wnt3a and was dedicated to correct intracellular targeting [28]. Similarly, Coombs et al. shows that serine acylation is required for interaction of the Wnts with Wls protein which serves as a chaperone and brings Wnt proteins through a secretory pathway to the plasmatic membrane [42]. On the other hand, Wg without acyl on equivalent serine 239 is normally secreted extracellularly and associates with the plasmatic membrane [30]. For over-all view, cysteine palmitoylation is not necessary for Wls binding [31].

Thus the function of lipid modifications has not been fully unraveled yet. However, it is obvious that acyls are connected to signaling activity and proper secretion. Not only the Wnts but also other morphogens, such as Hedgehog, use lipid adducts to attach lipoparticles, which serve as vehicles, and facilitate long range signaling so much necessary during the development [49-51, 364]. Lipids can simply anchor Wnt proteins to the plasmatic membrane

or to special regions of plasmatic membrane called lipid rafts, where signaling complexes assemble. Incidentally, Wg was detected in these detergent-resistant membrane rafts in dependence on the palmitoylation status [27]. Forming of multimers is another possibility how to overcome Wnt hydrophobicity and potentiate signaling capacity at once [365, 44].

Besides hydrophobic modifications, Wnt proteins are posttranslationally N-glycosylated on multiple asparagines. There are mutually contradicting data about the role of N-glycosylations in Wnt secretion [366, 21, 22] but it is easy to imagine that oligosaccharide chains modulate interaction of the Wnts with extracellular matrix components mainly heparan sulphate proteoglycans (HSPGs). The role of Porcupine in N-glycosylation of the Wnts is speculative. In the absence of Porc, the glycosylation status of Wg is impaired and *vice versa* – Porc overexpression results in enhanced N-glycosylation [20]. On the contrary, Wnt3a N-glycosylations are not changed when Porc is missing [28]. Thus, if there is any connexion between lipid adducts and N-glycosylations is not clear but a possible scenario would be that lipids anchor Wnt protein to the endoplasmatic reticulum membrane and help N-glycosylation machinery to proceed.

The forementioned knowledge of Wnt modifications role still raises many questions and we decided to contribute to its elucidation. We focused on murine Wnt1 and Wnt3a, which possess three and two N-glycosylations respectively and both of them two acyls. Mutations of particular amino acids allowed us to prepare a set of mutant Wnt proteins which were supposed to have a single acyl adduct or were absolutely unglycosylated. Using radioactively labeled palmitate we showed that serine acylation precedes and conditions cysteine acylation. This result corresponds to the data about the only found non-lipidated Wnt, *Drosophila* WntD [46], which lacks conserved serine in its sequence and which is not palmitoylated on the second modification site that is conserved cysteine.

Wnt activity relies on lipid modifications according to our results. Non-lipidated Wnt1 and Wnt3a do not induce a double-axis phenotype in *Xenopus* embryos in comparison to wild type proteins. Interestingly, single acyl on serine allows to both proteins almost comparable activity to wild type Wnts in *Xenopus*. In cell cultures there is a different situation, one acyl left on Wnt3a facilitates only partial activity measured by a reporter assay or target gene axin2 upregulation or by β-catenin stabilization. And the same testing of single acylated Wnt1 showed no or nearly no activity similarly to non-lipidated forms of the Wnts. All the mutant Wnts even without any lipid adducts were stained in the extracellular site of the plasmatic membrane, showing that the secretory pathway is not disrupted. But the crucial difference between nonfunctional Wnt proteins and functional ones was their absence from

isolated extracellular matrix. Thus, in our opinion, acyl modifications allow Wnt proteins to attach HSPGs either by themselves or as lipoparticles' components. Besides, it has been already shown in *Drosophila* that lipophorin, which forms lipoparticles bearing Wg or Hh, directly interacts with HSPGs [85]. We excluded the possibility that nonfunctional Wnts do not interact with Frizzled or LRP receptors according to our data and the environment of lipid rafts itself does not account for Wnt activity either.

Although Wnt1 and Wnt3a are partially redundant [367], their N-glycosylations probably manage different abilities. An unglycosylated form of Wnt1 behaves as the most potent activator of a Wnt signaling pathway unlike unglycosylated Wnt3a that signals only lightly in all tested assays. The only exception was observed when paracrine activation was detected. The cells producing unglycosylated Wnt1 were co-cultured with receiving cells that contained a reporter vector and these circumstances showed lower activity of unglycosylated Wnt1 than the wild type protein. We conclude that in case of unglycosylated Wnt1, which is normally secreted outside the cells, N-glycosylations help to spread for longer distances. In case of Wnt3a, the unglycosylated protein is less secreted outside the cells and this can be one explanation of lower activity taken together with the fact, that unglycosylated forms of both Wnts revealed a lower acylation status. On the contrary, fatty acid-deficient Wnts were still fully N-glycosylated, indicating that such modifications are not influenced by the presence or absence of acyl moieties. In summary, our data suggest that N-glycosylation precedes and conditions Wnts for efficient acylation.

In fine, our part in solving the Wnt modification puzzle inheres mainly in two original results. Firstly, acyl modification on conserved serine precedes and conditions the palmitoylation of conserved cysteine. Secondly, lipid adducts are related to the presence of Wnt proteins on extracellular matrix, which is connected to their ability to signal.

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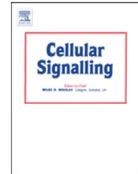

Fatty acid modification of Wnt1 and Wnt3a at serine is prerequisite for lipidation at cysteine and is essential for Wnt signalling

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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.

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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

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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. SuperTOPFLASH 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)].

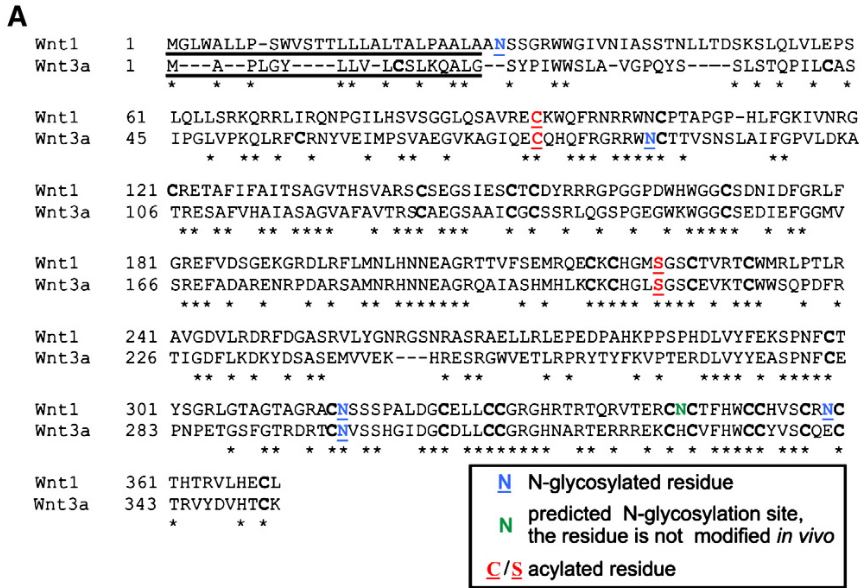
In addition to N-glycosylation, Wnt3a is palmitoylated on cysteine 77 and the palmitoleoyl modification occurs at serine 209 [8,9].The aforementioned amino acids and the homologous residues in Wnt1 (cysteine 93 and serine 224, respectively; see Fig. 1A) were mutated to alanines. The acylation status 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



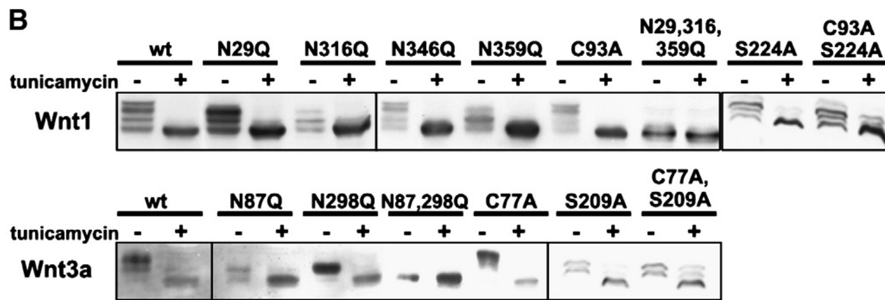


Fig. 1. N-glycosylation of Wnt1 and Wnt3a proteins. (A) Comparison of mouse Wnt1 and Wnt3a polypeptide sequences displaying the predicted and experimentally confirmed N-glycosylation sites. The putative signal peptides are underlined. The amino acid residues conserved in both Wnt1 and Wnt3a are marked 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.

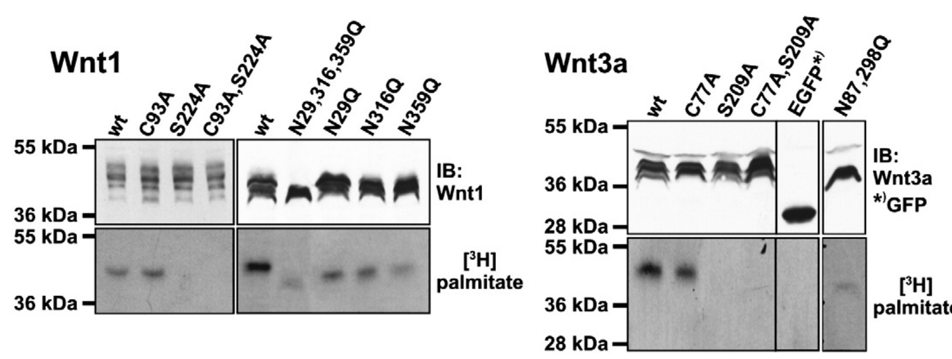


Fig. 2. In both Wnt1 and Wnt3a, fatty acid modification of serine is essential and precedes palmitoylation at the cysteine residue. HEK 293 cells metabolically labelled with [^{3}H] palmitate were transfected with the indicated Wnt1 or Wnt3a expression constructs or negative control EGFP-producing plasmid. 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.

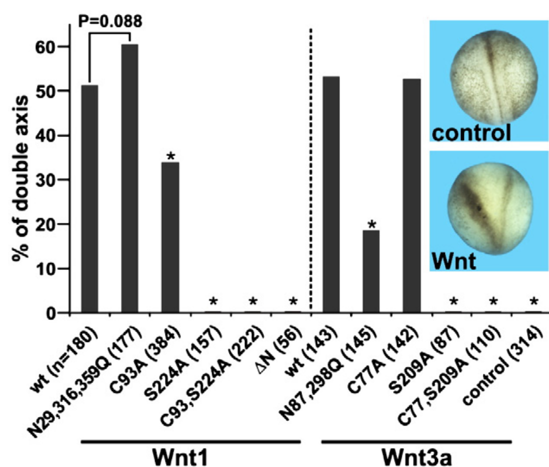


Fig. 3. Wnt1 and Wnt3a without fatty acyl adducts do not induce double axis formation in *Xenopus* embryos. Embryos at the 4-cell stage were microinjected with 20 pg of the indicated mRNA in the marginal zone of ventral blastomeres and scored for secondary axis induction at the neurulae stage (see the 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

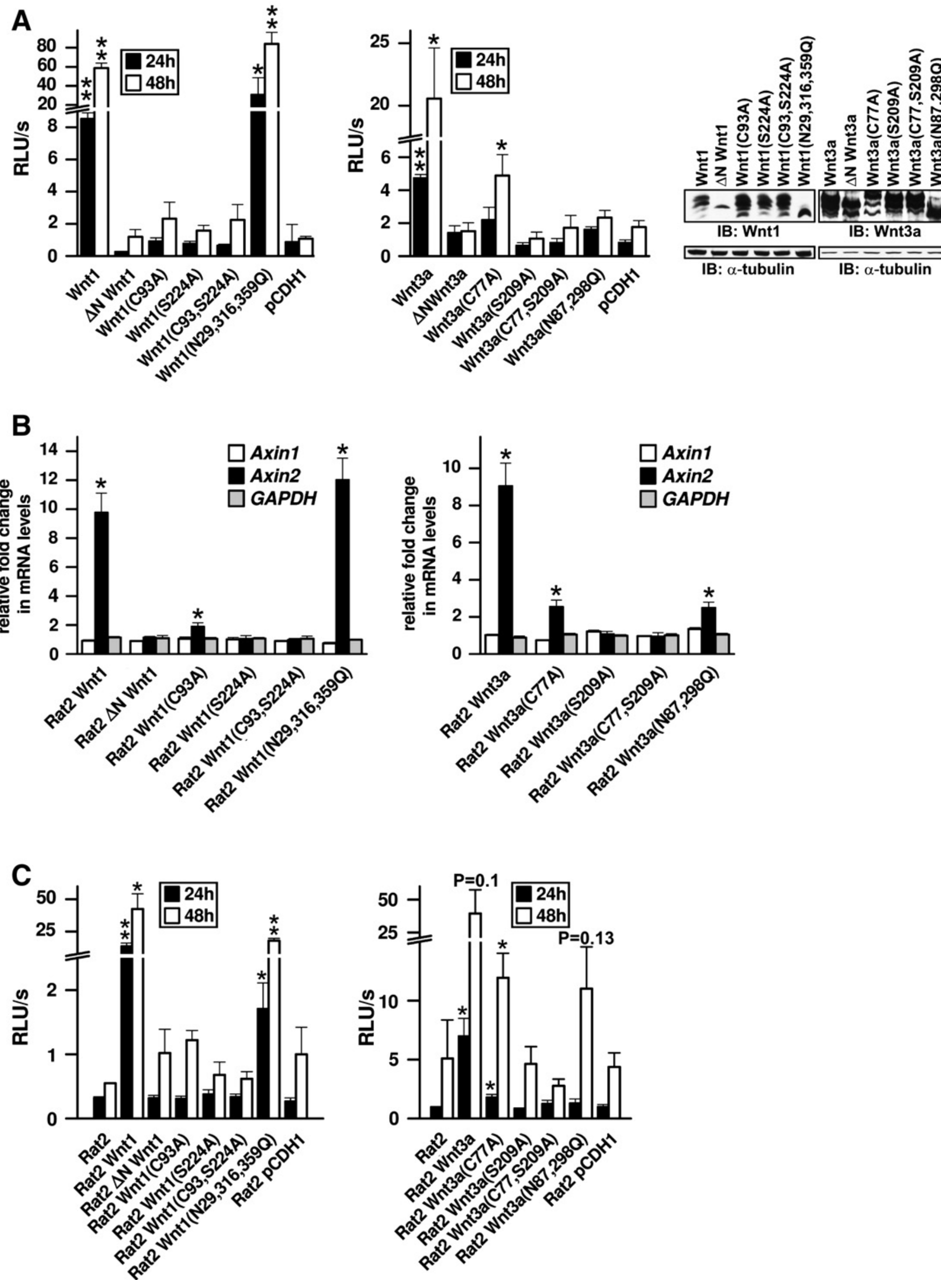


Fig. 4. Wnt3a lacking the palmitoylated cysteine residue retains the capacity to activate Wnt-dependent transcription in mammalian cells. (A) Activation of Wnt signalling in transiently transfected cells. HEK 293 cells containing the integrated reporter, SuperTOPFLASH (STF 293 cells), were transfected with constructs expressing wild-type or mutated variants of mouse Wnt1 (the left panel) or Wnt3a (the right panel) proteins. pCDH1 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

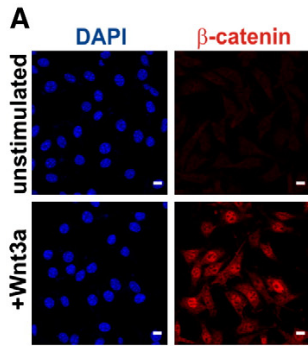
Mouse Wnt1 and Wnt3a are two mammalian members of the Wnt family of extracellular signalling proteins that are modified post-translationally by N-glycosylation and fatty acid addition. The aim of this study was to define the relationship between post-translational modification and the signalling activities 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
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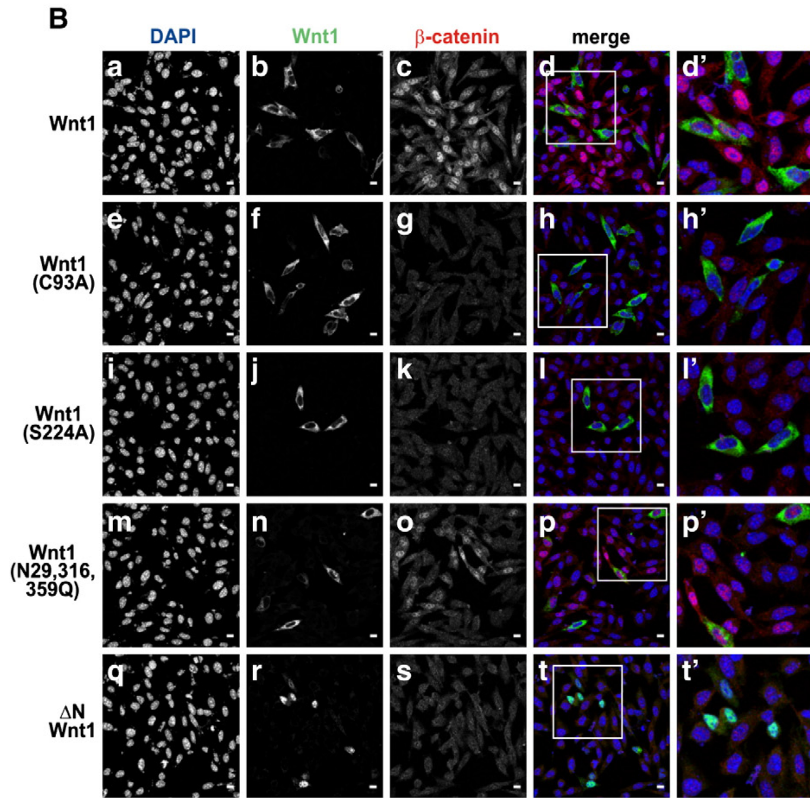
B
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Wnt1 (C93A)
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Wnt1 (N29,316, 359Q)
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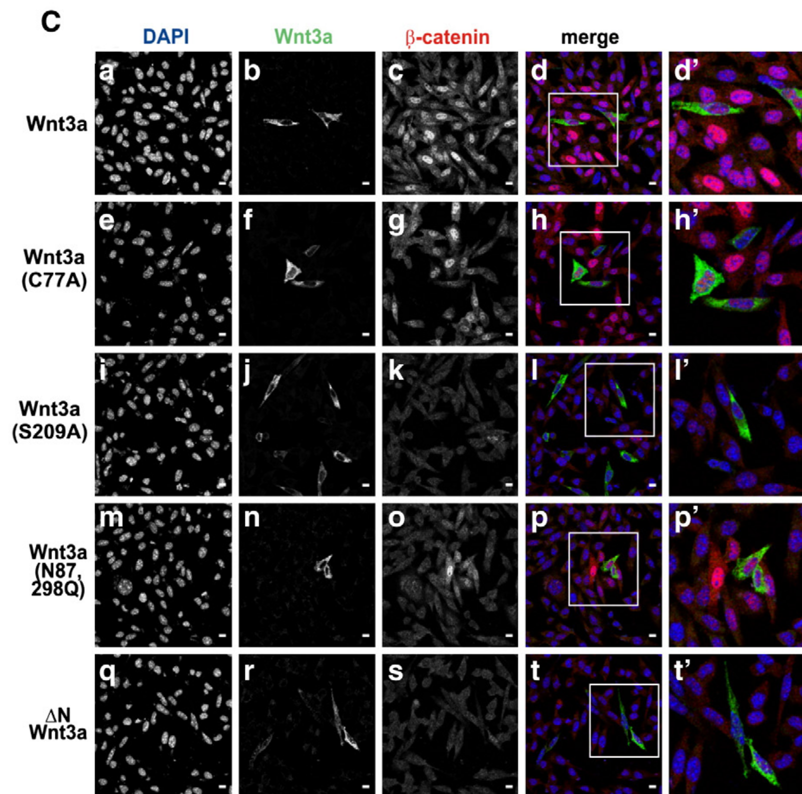
C
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Wnt3a (C77A)
Wnt3a (S209A)
Wnt3a (N87, 298Q)
ΔN Wnt3a

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

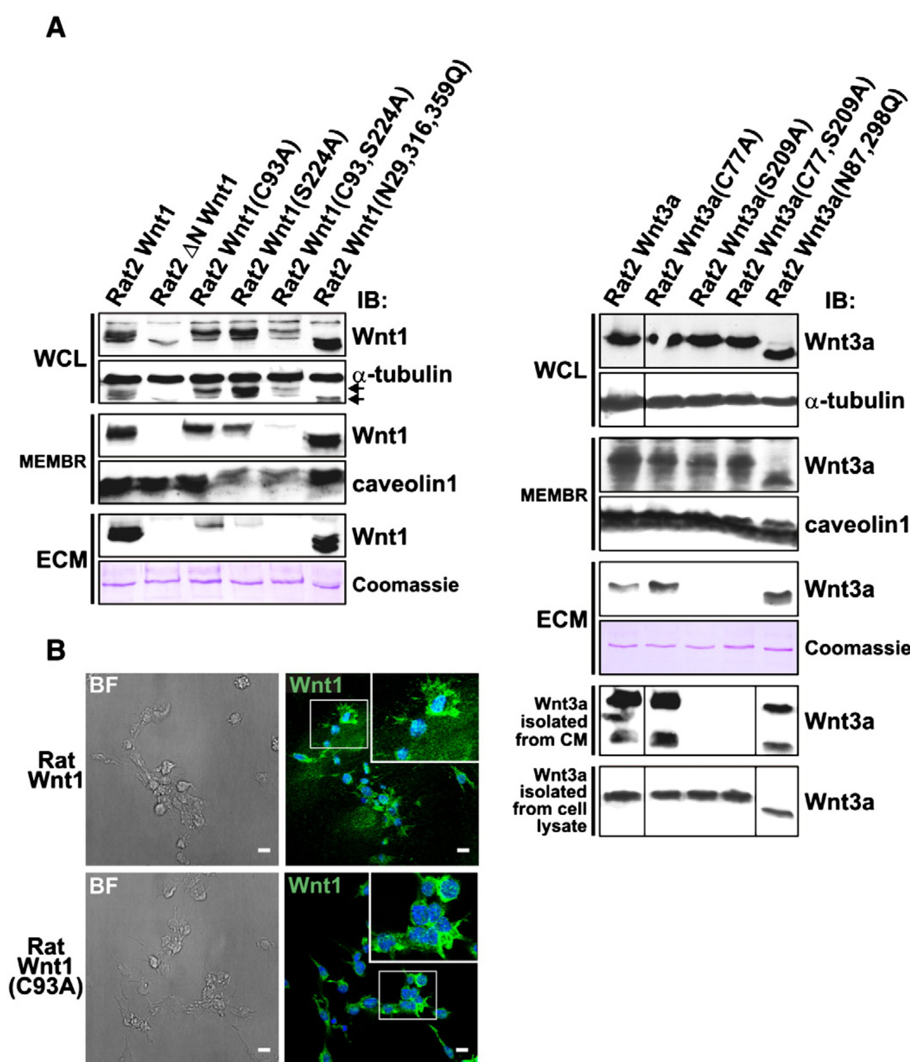

Fig. 6. Acylation promotes deposition of Wnt1 and Wnt3a on the ECM. (A) Cellular distribution of Wnt1 and Wnt3a variants expressed in Rat2 fibroblasts. Western blot analysis of indicated Wnt proteins present in whole-cell lysates (WCL) or co-isolated with plasma membranes (MEMBR) or the ECM. As loading 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.

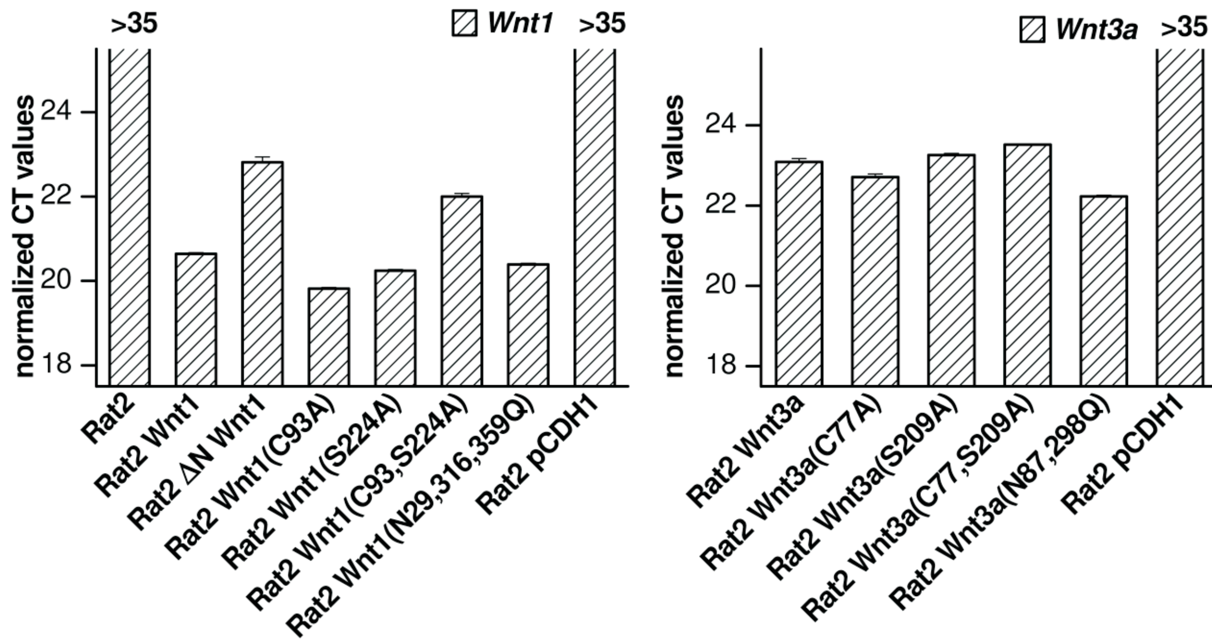
Supplementary Figure S4 All secreted variants of Wnt1 or Wnt3a interact with the Frizzled receptor and Lrp co-receptor

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.

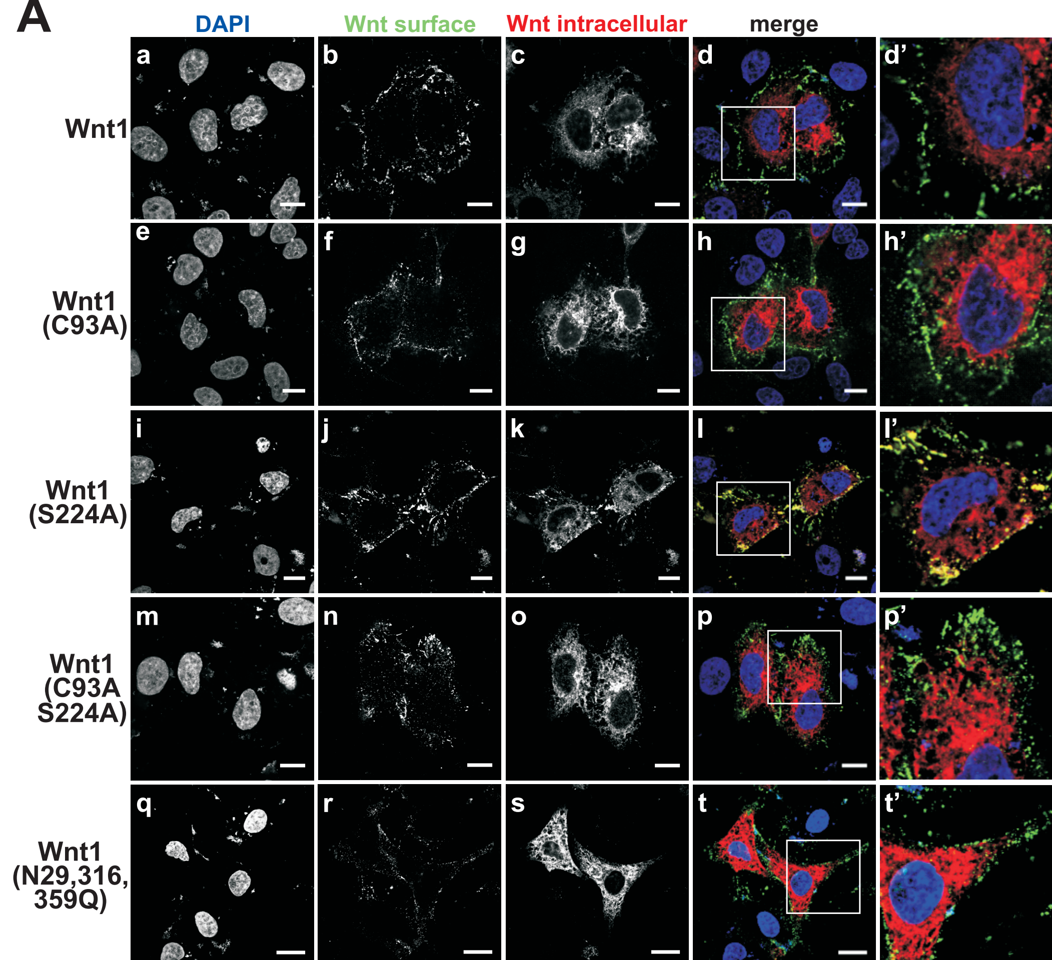
Supplementary Figure S5 The N-glycosylation status of Wnt1 and Wnt3a in Xenopus cells

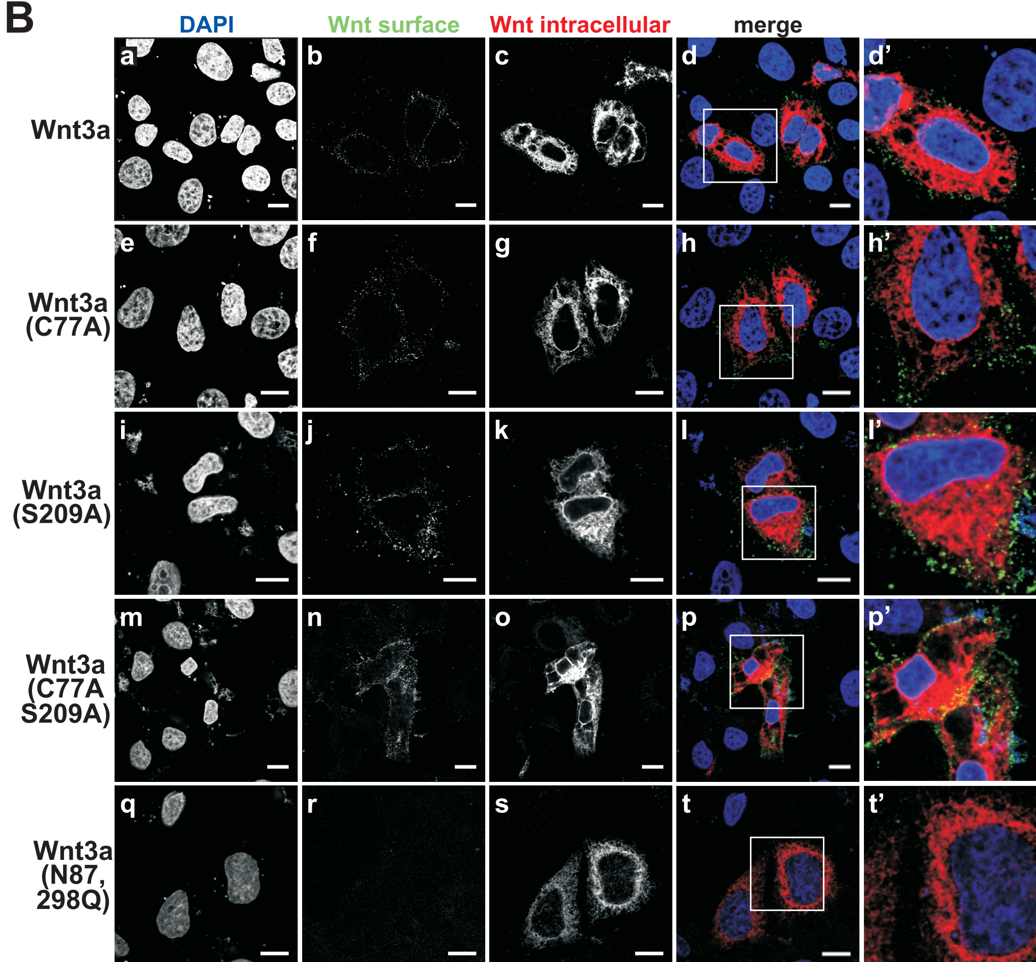
Xenopus A6 cells were lipofected with the indicated Wnt-expression constructs. Transfected cells were processed as described in the legend to Fig. 1B.

Supplementary Figure S1

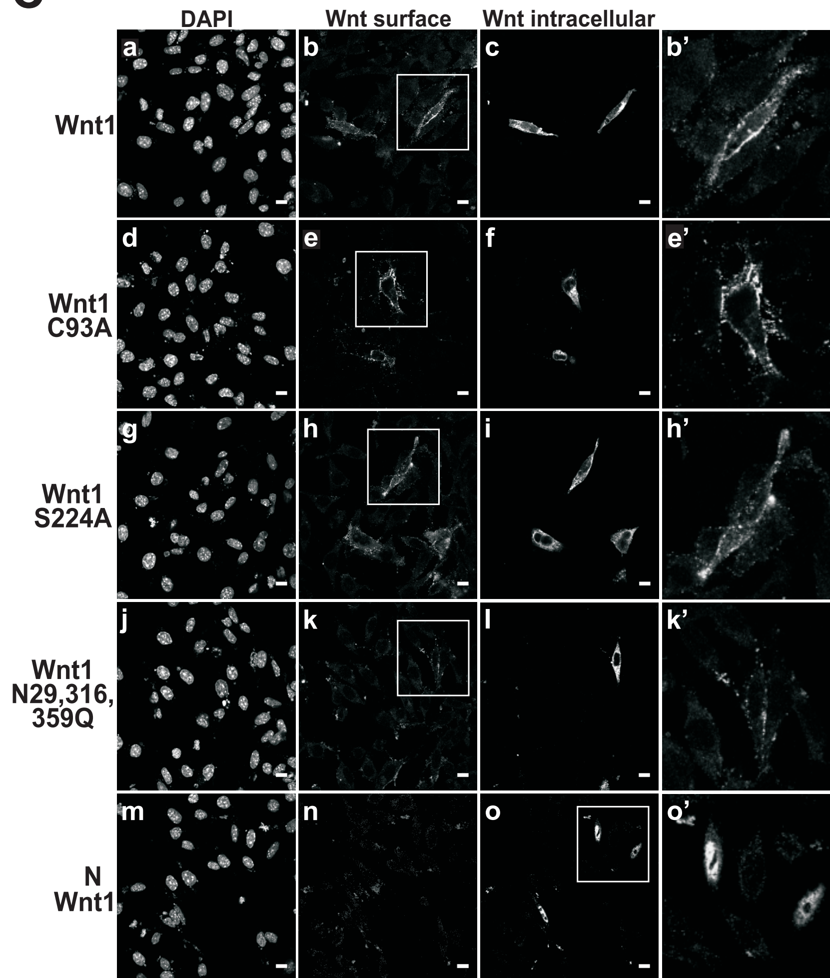


Supplementary Figure S2

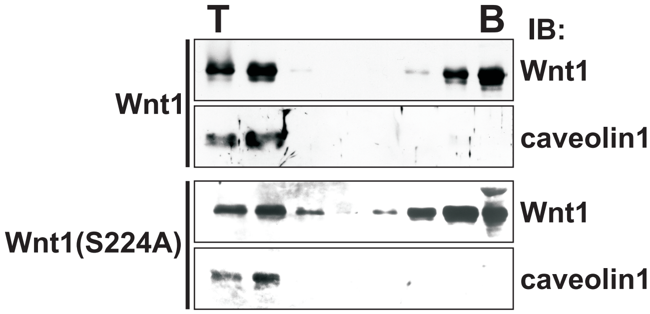




Supplementary Figure S2



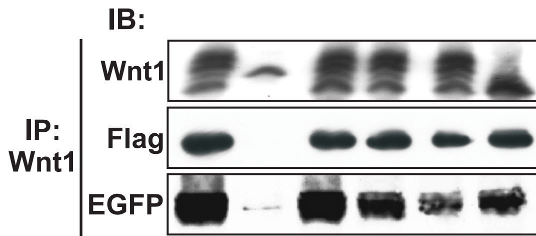
Supplementary Figure S3

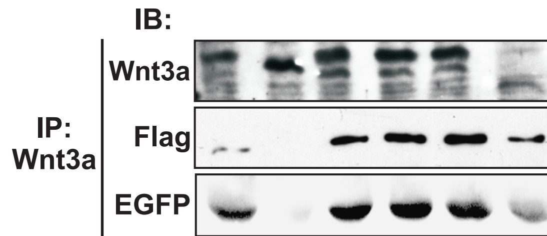


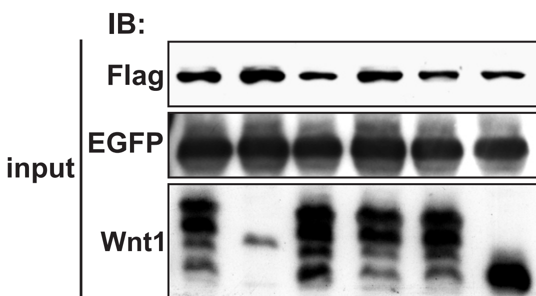
Supplementary Figure S4

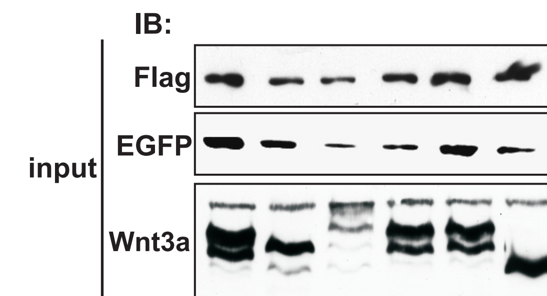
Wnt1 wt	+	−	−	−	−	−
N	−	+	−	−	−	−
C93A	−	−	+	−	−	−
S224A	−	−	−	+	−	−
C93A,S224A	−	−	−	−	+	−
N29,316,359Q	−	−	−	−	−	+
Lrp5-Flag	+	+	+	+	+	+
Fz4-EGFP	+	+	+	+	+	+

Wnt3a wt	+	−	−	−	−	−
N	−	+	−	−	−	−
C77A	−	−	+	−	−	−
S209A	−	−	−	+	−	−
C77A,S209A	−	−	−	−	+	−
N87,298Q	−	−	−	−	−	+
Lrp5-Flag	+	+	+	+	+	+
Fz4-EGFP	+	+	+	+	+	+

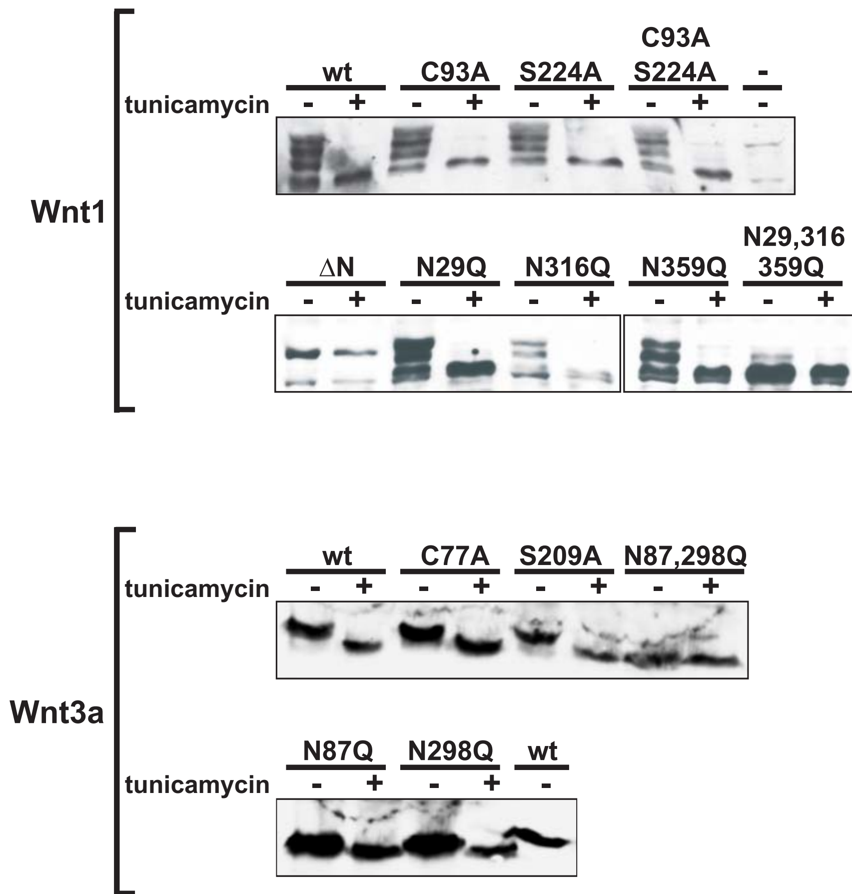








Supplementary Figure S5



3.2 Wnt-expressing rat embryonic fibroblasts suppress Apo2/TRAIL-induced apoptosis of human leukemia cells

Wnt signals provide maintenance of a number of stem cells including hematopoietic ones by self-renewal and play a crucial role in the most immature stages of T cell development [368-374, 26]. Similarly, Wnt proteins allowed proliferation of maturing B cells via Lef1-mediated transcription during B lymphopoiesis [375, 376]. The same pathway, when dysregulated, is connected to leukemias of both myeloid and lymphoid lineages. For example, in the case of chronic myeloid leukemia, granulocyte-macrophage progenitors display a long-term renewal ability in dependence on triggered Wnt signaling and thus these progenitors mimic hemotopoietic stem cells [377]. Moreover, leukemias of different origin have in common stabilized β-catenin that is not mutated [378, 371]. It rather seems that Wnt pathway antagonists may be epigenetically silenced [379] and highly expressed Wnt proteins keep the signaling in a set of leukemias on [380-382]. The theory of cancer stem cells (CSCs) consists in the idea that limitless growth of tumors is maintained by a reservoir of cells with stem cell characteristics such as Wnt/β-catenin pathway being essential [383, 384]. These CSCs continuously supply leukemic clones and are believed to be responsible for recurrence of the disease after anticancer therapy. The selfrenewal mechanism is used by both stem cells on one side and cancer cells on the other side. How to treat only leukemia cells without destroying rare stem cells, which are essential for maintaining all the blood cell lineages?

There is a possibility of Apo2 ligand or tumour necrosis factor-related apoptosis-inducing ligand (Apo2L/TRAIL), which predominantly kills cancer cells [385]. TRAIL is a transmembrane protein that forms the homotrimer with extracellular parts being proteolytically cleaved from the cell surface and so it acts like a soluble ligand [386]. There are five death receptors (DR) binding TRAIL but only two of them (DR4 and DR5) are able to trigger apoptosis. Within minutes upon TRAIL binding to DRs, a death-inducing signaling complex (DISC) emerges. It consists of an adaptor protein FADD (Fas-associated death domain) and apoptosis initiator caspase-8 and caspase-10 [387, 388]. Initiator caspases autocleave and activate effector caspases such as caspase-3, which in turn cleaves numerous cellular proteins leading thereby to programmed cell death. Caspase-8 may cleave the pro-apoptotic Bcl-2 family member Bid that translocates to mitochondria and triggers so called intrinsic apoptotic signaling via dissipating mitochondrial transmembrane potential [389, 390]. Unsuprisingly, many proteins can regulate or invert the apoptotic process such as cFLIP (cellular FLICE-inhibitory protein), which competes for the recruitment of initiator caspases

in the DISC [391]. Bcl family members behave predominantly antiapoptotically [392-394] and IAPs (inhibitors of apoptosis) have an analogous effect [395-397]. Also, the proportion of pro- and anti-apoptotic death receptors may establish signaling output. Likewise, different pathways triggered by TRAIL can modulate a cellular answer, especially anti-apoptotic NF-κB signalling or MAPK and PKB/Akt pathways [398-400]. Another protein kinase CK2 was shown to suppress TRAIL-induced apoptosis on various levels of signaling [401].

Taken together, TRAIL is able to induce programmed cell death, especially to malignant cells. These cells often use Wnt signaling as a proliferation instrument which also imparts them "stemmness" character. Here we show that human pre-B leukemia cells co-cultured with rat embryonic fibroblasts expressing Wnt1 or Wnt3a (Rat2Wnt1/Wnt3a) are resistant to TRAIL-triggered apoptosis. Our study excluded the Wnt-only scenario meaning that Wnt itself is not a sufficient anti-apoptotic agent in this model. It rather seems that rat fibroblasts transduced by Wnt1 or Wnt3a themselves have an anti-apoptotic effect, probably by providing secondary stimulus caused by the original Wnt signaling .

More closely, a set of multilineage leukemia-derived cells were subjected to TRAIL while being co-cultured with Rat2Wnt1 or empty Rat2 fibroblats. The tested leukemia cell lines were sensitive to TRAIL and differed in their ability to respond to feeder Rat2Wnt1 cells by means of protection against TRAIL apoptosis. There are cell lines coming under death stimulus independently of Rat2Wnt1 presence (Ramos, ML-1, HL-60, THP-1, NK 3.3, TF-1), cells partially protected from TRAIL (Jurkat, CEM) and finally the most interesting group of cell lines, which are almost TRAIL insensitive upon Rat2Wnt1 co-culture (KM3, REH, Molt-4). Therefore, Rat2Wnt1 feeder cells protect rather immature than mature lymphocyte-derived leukemia cell lines such as pre-B cells (KM3, REH) or lymphoblast-related cells (Molt-4). It is worth of note that Wnt signaling is essential for maturing of normal lymphocytes of both T and B lineages by giving proliferation and pro-survival instructions.

But what is the mechanism? We observed apoptotic process being attenuated in pre-B cell lines incubated with Rat2Wnt1 compared to control cells incubated with Rat2. Initial caspase-8 is less processed and thus subsequent events such as Bid cleavage or caspase-3 action are less efficient. It should be explained by a partial decrease in cell surface DR4 and DR5 expression in pre-B cells co-cultured with Rat2Wnt1, moreover, quantification of receptors mRNA showed significant down-regulation. A range of pro- and anti-apoptotic genes which might be regulated by Wnt signaling in pre-B cells were analysed by qRT-PCR, however, expression of BCL-2, BCL-XL, SURVIVIN and other potential molecules was not

changed [402, 403]. These data imply that proximal events of TRAIL-induced signaling may be targeted by the Rat2Wnt1 anti-apoptosis activity.

Wnt/β-catenin signaling was evidenced to prevent apoptosis in several models [402, 349, 404, 405]. To elucidate whether the above mentioned phenomenon is caused directly by Wnt signaling, another “canonical” Wnt ligand was employed. The Wnt3a protein is easy to aplicate in comparison to Wnt1 because it is secreted to the media of producing cells and thus a conditioned medium or an isolated protein are available. Secreted Wnt1 is tightly associated with the extracellular matrix and this is the reason why we co-cultured pre-B cells with feeder Rat2Wnt1 fibroblasts to trigger Wnt1 signaling. Unfortunately, Wnt3a itself was not able to rescue leukemia cells from apoptosis and even its expression in different fibroblasts than Rat2 did not act anti-apoptotically. However, transduced Rat2Wnt3a recapitulated Rat2Wnt1 functional efficiency to suppress TRAIL-induced apoptosis in pre-B cells. These results point to another signal which is produced exactly in Rat2 fibroblasts, which themselves respond to expressed canonical Wnt1 or Wnt3a [405]. This unknown factor(s) mediates the anti-apoptotic protection of lymphoid malignant cells. The only clue about the mysterious molecule is bound to the embryonic origin of rat fibroblasts in comparison to other ones used, but not anti-apoptotic fibroblasts $L_{TK,}^{-}$ which originated from adult mice. Because pre-B cells were in direct contact with feeder cells, the wanted molecule could be either a component of rat fibroblasts extracellular matrix or a kind of secreted ligand that would easily travel toward the pre-B cells.

Comparison of expression profiles of thouse fibroblast lines transduced by canonical Wnts would help to find the key molecules responsible for induction of TRAIL insensivity. Anyway, our approach was different – using a set of pro-survival pathways inhibitors we found out that NFκB and ERK1/2 pathways are active in pre-B cells and obviously deliver general prosurvival stimuli. NFκB signaling was triggered in both KM3 and REH cells already without any treatment and ERK1/2 kinase activity started after TRAIL addition independently of feeder cells quality. These results show that the anti-apoptotic signal coming from Rat2Wnt1/Wnt3A fibroblasts remains covert.

Soluble recombinant TRAIL together with agonistic monoclonal antibodies against DR4 and DR5 are undergoing clinical trials to establish efficient anticancer therapy. Although many cancer cell lines are sensitive to TRAIL-induced cell death, on the other hand, many primary tumor cells are not, despite the expression of death receptors [406, 407]. Our study might help to slightly uncover one of the causes of TRAIL insensitivity in tumors. We observed an anti-apoptotic effect of Wnt expressing cells on pre-B leukemia-derived cell lines

upon TRAIL induction. The pro-survival signal is not caused directly by Wnt signaling but rather another molecule induced in Wnt expressing embryonic fibroblasts takes part.

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ORIGINAL PAPER

Wnt-expressing rat embryonic fibroblasts suppress Apo2L/TRAIL-induced apoptosis of human leukemia cells

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Abstract Wnt signaling enhances cell proliferation and the maintenance of hematopoietic cells. In contrast, cytotoxic ligand Apo2L/TRAIL induces the apoptosis of various transformed cells. We observed that co-culture of human pre-B leukemia cells KM3 and REH with Wnt1- or Wnt3a-producing rat embryonic fibroblasts efficiently suppressed Apo2L/TRAIL-induced apoptosis of the lymphoid cells. This suppression occurs at the early stages of the Apo2L/TRAIL apoptotic cascade and, interestingly, the activation of the Wnt pathway alone in human leukemia cells is not sufficient for their full anti-apoptotic protection. We hypothesize that a stimulus emanating specifically from Wnt1- or Wnt3a-expressing rat fibroblasts is responsible for the observed resistance to Apo2L/TRAIL. This anti-apoptotic signaling was significantly hampered by the inhibition of the MEK1/ERK1/2 or NFκB pathways in KM3 and REH cells. Our results imply that paracrine Wnt-related signals could be important for the survival of pre-B cell-derived malignancies.

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Abbreviations

ActD	Actinomycin D
CHX	Cycloheximide
HSC	Hematopoietic stem cells
PARP	PolyADP-ribose polymerase
PBS	Phosphate buffered saline
PI	Propidium iodide
RT-qPCR	Quantitative real-time RT PCR
SD	Standard deviation

Introduction

The regulation of apoptosis is interrelated with cell proliferation, and a number of pro-proliferative signals have been shown to influence the apoptotic status of the affected cell. A large family of secreted lipid-modified glycoproteins, Wnt ligands, participates in the regulation of cell proliferation, differentiation and survival [1, 2]. The interaction of Wnt proteins with their receptor complex—transmembrane polypeptides of the Frizzled and LRP families—results in the activation of so-called canonical and non-canonical signaling in the target cell [3–5]. β-catenin is the key player of the canonical Wnt pathway. In non-stimulated cells β-catenin is marked for ubiqutinylation and degradation by the action of a large multiprotein complex that includes Ser/Thr kinase GSK3β and two "scaffold" proteins, Axin1/2 and APC. Canonical signaling inhibits the activity of the GSK3β/Axin1/2/APC complex, and stabilized β-catenin accumulates in the cytoplasm and also in the cell nucleus. Nuclear β-catenin associates with the transcription factors of the Tcf/Lef family and activates expression of their target

genes. Non-canonical Wnt signaling leads to the mobilization of intracellular calcium or to the activation of kinases of the JNK family and small GTPases of the Rho family [6]. In the hematopoietic system Wnt proteins participate in the maintenance and differentiation of hematopoietic stem cells (HSC) and progenitors [7]. Wnt signaling is also required during the maturation of thymic T cells [8, 9] and for the survival of B cells at the pre-B-cell stage [10, 11]. Furthermore, the active Wnt pathway suppresses the apoptosis of serum-deprived hematopoietic CD34+ progenitors. The pro-proliferative and anti-apoptotic function of Wnt signaling was observed not only in hematopoietic cells, but also in other cell types. It was reported that Wnt-activated signaling inhibits serum withdrawal-induced apoptosis of osteoblasts, preadipocytes, and fibroblasts [12–15]. The potential anti-apoptotic function of Wnt signaling was also evidenced by the inhibition of Wnt1 or Wnt2 signaling with monoclonal antibodies or with the Wnt antagonist Dickkopf-1. These treatments induced the apoptosis of malignant melanoma or mesothelioma cells [16–18].

In contrast to Wnt proteins, death ligands of the TNFα family induce mainly the apoptosis of target cells [19–21]. Among them, TNFα-Related Apoptosis Inducing Ligand (Apo2L/TRAIL) preferentially induces the apoptosis of tumor cells and is considered to be a new promising anti-tumor agent [22–24]. Apo2L/TRAIL interacts with its pro-apoptotic receptors TRAIL-R1 (DR4) or TRAIL-R2 (DR5), induces the formation of the intracellular Death-Inducing Signaling Complex (DISC) and subsequently activates initiator caspase-8. Activated caspase-8 cleaves Bid, a pro-apoptotic BH3-only member of the BCL-2 family. Truncated Bid (tBid) translocates to mitochondria where it activates mitochondrial apoptotic signaling [25]. Apo2L/TRAIL-induced apoptosis can be affected at a number of regulatory check points, for example at the level of the expression and cellular localization of pro- (TRAIL-R1, TRAIL-R1) and anti-apoptotic (TRAIL-R3, TRAIL-R4) receptors and, furthermore, by the production of the caspase-8 competitor FLIP, the post-translational modification of Bid or the enhanced expression of anti-apoptotic proteins from the Bcl-2 and IAP families. Receptor-mediated activation of the PI3K/Akt, ERK1/2 or protein kinase C signaling pathways or tumor elevated activity of casein kinase II can also negatively influence the outcome of Apo2L/TRAIL-induced apoptosis [26–30].

Here we report that Apo2L/TRAIL–induced apoptosis of human pre-B leukemia cells can be suppressed by rat embryonic fibroblasts (Rat2) transduced with retroviruses expressing Wnt1 or Wnt3a proteins. The efficient suppression of apoptosis requires a direct contact of the leukemia cells with Wnt-producing feeder cells, and, strikingly, the anti-apoptotic action of the feeders cannot be substituted by either recombinant Wnt3a, conditioned medium containing Wnt3a or by Wnt3a-expressing mouse L cells. This indicates that not active Wnt signaling in pre-B leukemia cells *per se* but rather a specific stimulus induced in the Wnt-responsive rat cells confers the resistance against Apo2L/TRAIL.

Materials and methods

Cells, reagents and antibodies

Human hematopoietic cell lines KM3, REH (pre-B cell origin); Ramos (B-cells); ML-1, HL-60, THP-1 (myeloid cells); Jurkat, CEM, MOLT-4 (T cells); NK3.3 (NK cells) and TF-1 (erythromyeloid cells) were cultured in RPMI medium supplemented with 10% FCS. Mock-infected (Rat2MV7) and mouse Wnt1-transduced Rat2 fibroblasts (Rat2Wnt1) were kindly provided by A. Brown [31]. Rat2Wnt3a cells were generated by the transduction of Rat2MV7 cells with pLHCX retrovirus (BD Clontech) containing the mouse *Wnt3a* gene. Upon transduction the resistant cells were selected using hygromycin (Invitrogen; final concentration 300 µg/ml). Wild-type mouse L cells (ATCC No. CRL-2648) and L cells stably transfected with the mouse *Wnt3a* gene (LWnt3a; ATCC No. CRL-2647) were grown in DMEM medium supplemented with 10% FCS. All cells were mycoplasma negative. Control (derived from parental L cells) and Wnt3a-conditioned media (CM) were prepared according to the protocol provided by the supplier. The MEK1/2 inhibitors PD98059 (final concentration 50 µM) and UO126 (10 µM), the PI3 kinase inhibitors Wortmannin (1 µM) and LY294002 (10 µM), and cisplatin (50 µM) were purchased from Sigma, while the NFκB inhibitor 6-Amino-4-(4-phenoxyphenylethylamino)quinazoline was obtained from Calbiochem. Recombinant mouse Wnt3a and Wnt5a ligands were purchased from R&D Systems. Human N-terminally His-tagged recombinant Apo2L/TRAIL (amino acids 95–281) was affinity-purified in our laboratory from cell lysates of producer bacteria (*E. coli*, strain BL-21) and contaminating bacterial endotoxins were removed by Endotrap chromatography (Profos AG). Alternatively, commercially available Apo2L/TRAIL was used (Alexis, R&D Systems). AnnexinV-FITC (final concentration 2.5 µg/ml) was donated by Apronex Ltd., Czech Republic. Hoechst 33258 (0.2 µg/ml) was purchased from Molecular Probes. Antibodies were purchased from the following suppliers: mouse monoclonal anti-Myc tag (9E10; final dilution 1 µg/ml, Roche Applied Science), mouse monoclonal anti-phospho-ERK1/2 (final dilution 1:2000, Cell Signaling), mouse monoclonal anti-α-tubulin (1 µg/ml, Exbio), rabbit polyclonal anti-caspase-8 and anti-caspase-3 (final dilution for both antibodies was 1:1000, BD Biosciences), rabbit

polyclonal anti-Bid (1:1000, Santa Cruz) and anti-Wnt1 (1:1000, Santa Cruz), mouse monoclonal anti-β-catenin (1:500, Santa Cruz), rabbit polyclonal anti-polyADP-ribose polymerase (PARP) (1:1000, Santa Cruz), rabbit polyclonal anti-ERK1/2 (1:1000, Cell Signaling). Anti-Apo2L/TRAIL receptors (TRAIL-R1, TRAIL-R2, both 10 μg/ml) flow cytometry set and mouse monoclonal anti-cFlip$_L$ (1:500) antibody were purchased from Alexis. Phycoerythrin conjugated mouse anti-rat MHC class I (RT1A) antibody were obtained from BD Pharmingen and used at 2 μg/ml. NFκB Family Transcription Factor Assay Kit was obtained from Active Motif.

Co-culture experiments

Rat2MV7, Rat2Wnt1, Rat2Wnt3a, L and LWnt3a cells were seeded onto 12- or 6-well plates coated with poly-D-lysine hydrobromide (Sigma) and grown until the cells reached 80–90% confluence. Then, tested hematopoietic cells (10^6 per one well of the 12-well plate) were spread onto the feeder fibroblasts and co-cultured for 8 h. Hematopoietic cells were then either harvested by gentle pipetting and analyzed or the appropriate treatment was applied, cells were co-cultured for an additional time and finally harvested and analyzed.

Proliferation assays

KM3 cells (3×10^5) were spread in triplicates onto 90% confluent layers of feeder cells in 24-well plates, and after 8 h [^{3}H]thymidine (Amersham Biosciences) was added to the cultures (2 μCi per each well). Fifteen hours later all cells in the well were harvested by scraping, and after washing with phosphate buffered saline (PBS) the incorporated radioactivity was measured by liquid scintillation counting. Feeder cells growing without KM3 cells were treated under the same conditions and used to determine the background [^{3}H]thymidine incorporation.

Western blotting

Cell lysates were separated by SDS PAGE and transferred onto nitrocellulose membranes (Amersham Biosciences). The membranes were blocked with 5% (w/v) nonfat milk in PBS containing 0.05% (v/v) Tween-20 (Sigma) and incubated with specific primary antibodies. After several washes with PBS/Tween the blots were incubated with appropriate peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies (BioRad) diluted according to the manufacturer's recommendation. The proteins were visualized with an enhanced chemiluminescence system (Pierce).

Flow cytometry and apoptosis assays

Hematopoietic cells were separated from the layer of feeder cells by gentle pipetting, washed with ice-cold PBS and incubated with the blocking solution (PBS containing 20% human serum, 1% gelatin and 0.1% azide) on ice for 15 min. Cells were washed once with wash buffer (PBS containing 1% gelatin and 0.1% azide), incubated with the appropriate primary monoclonal antibodies (see the "Cell lines, reagents and antibodies" subsection), washed twice with ice-cold wash buffer and finally incubated with the secondary goat anti-mouse antibody coupled to phycoerythrin (anti-IgG1-PE, 2 μg/ml; BD Biosciences). After two final washes the surface expression of the receptors on living cells (negatively stained with Hoechst 33258) was analyzed by LSRII flow cytometer (BD Biosciences). For the apoptosis assays, cells were either mock-treated or treated with recombinant Apo2L/TRAIL (200 ng/ml) for corresponding time periods. After the treatment, cells were harvested, washed with ice-cold PBS and stained with AnnexinV-FITC and/or propidium iodide (PI). Stained cells were analyzed by flow cytometry.

RNA isolation and quantitative real-time PCR

Total RNA was prepared using Trizol Reagent (Invitrogen) according to the manufacturer's instructions. Isolated RNAs were treated with DNAse I (Fermentas; 1 U per 2 μg of total RNA, 30 min at 37°C) and then finally purified using the Micro-to-Midi total RNA isolation system (Invitrogen). Random-primed cDNA was prepared in a 20 μl reaction from 0.5 to 1 μg of total RNA using Superscript II RNAseH$^-$ reverse transcriptase (Invitrogen). Samples containing all components except reverse transcriptase were prepared in parallel and used as negative controls. One per cent of the resulting cDNA was amplified using SYBR-Green PCR Master Mix (Roche Applied Science) and human specific primers (Suppl. Table ST1) in a LightCycler® 480 System (Roche Applied Science). All primers were calculated using Primer 3 computer services at http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi/primer3_www.cgi. cDNAs were produced from at least two independent RNA isolations, and the PCR reactions were performed in triplicate for each primer set. A housekeeping gene, *Succinate dehydrogenase complex, subunit A* (*SDHA*), was used as the internal control gene to standardize the quantity of different cDNA preparations. The relative abundance of the tested genes in Wnt-stimulated versus control cells (co-cultivated with parental Rat2MV7 or L cells, or with CM obtained from these "empty" cells) was derived from the average crossing threshold (CT) values of each triplicate after normalizing

against the levels of *SDHA* cDNA. Results of a representative experiment are shown using TM4 software [32].

Cycloheximide (CHX) and Actinomycin D (ActD) treatment

KM3 cells were treated for 1 h with CHX or ActD (final concentration 5 and 1 μM, respectively; both drugs were purchased from Sigma). The drug-containing medium was replaced with fresh drug-free medium, and the cells were seeded on feeder fibroblasts. After 8 h Apo2L/TRAIL was added (200 ng/ml), and after an additional 15 h the KM3 cells were harvested and analyzed. In a parallel experiment, feeder cells were pre-incubated with CHX (5 μM) or ActD (1 μM) for 1 h; the drug-containing medium was then washed and untreated KM3 cells were seeded onto the feeders and the cultures processed as indicated above.

Results

Rat2 fibroblasts expressing Wnt1 protein suppress Apo2L/TRAIL-induced apoptosis of a subset of leukemia cells

The cytotoxic ligand Apo2L/TRAIL efficiently induces the apoptosis of various tumor-derived cell lines [22–24], and, in contrast, the aberrant activation of the Wnt signaling pathway is often tumorigenic and ultimately leads to cancer in many tissues including the brain, gastrointestinal tract, skin, ovaries and the hematopoietic system (reviewed in [2, 33]).

As Wnt signaling is also required for the normal growth of HSC in vitro and in vivo, and furthermore for B- and T-cell development [10, 34], we tested the effect of Wnt1 protein on the survival of different hematopoietic cell lines treated with the soluble recombinant form of Apo2L/TRAIL ligand. Secreted Wnt glycoproteins strongly adhere to the extracellular matrix, thus the most efficient way to deliver the Wnt signal to the target cells was to culture the studied hematopoietic cells with a layer of feeder cells producing Wnt ligand [35]. In the initial experiments adherent Rat2 fibroblasts transduced with retroviruses expressing the mouse *Wnt1* gene (Rat2Wnt1) were utilized [31], and Rat2 cells infected with the “empty” retroviruses (Rat2MV7) were used as negative control feeder cells. Human leukemia cells of various origin were seeded on a monolayer of feeders for 8 h, then Apo2L/TRAIL was added to the culture medium and the cells cultured together for an additional 15 h. Finally, the human cells were carefully harvested, and the ratio of living vs. dead cells was determined using fluorescence-activated cell sorting (FACS) analysis of PI-positive cells. Since Rat2 fibroblasts are completely resistant to Apo2L/TRAIL, the “contamination” of the human cells by detached feeder cells (determined by staining with anti-rat MHC class I antibody) never exceeded one percent of the analyzed cells (data not shown) and was neglected in the analysis. Representative results are summarized in Table 1—only human cell lines sensitive to Apo2L/TRAIL were included.

Table 1 Wnt1-expressing Rat2 fibroblasts suppress, to various degrees, Apo2L/TRAIL-induced apoptosis of co-cultured human leukemia cell lines

Cell line	Origin	Propidium iodide-positive cells (%)			
		Untreated cells	Plastic dish	Rat2MV7	Rat2Wnt1
KM3	Pre-B cell	3 ± 2	43 ± 3	53 ± 4	5 ± 3
REH	Pre-B cell	2 ± 2	42 ± 4	49 ± 3	4 ± 3
Ramos	B cell	5 ± 3	68 ± 4	74 ± 5	72 ± 5
ML-1	Myeloid	3 ± 1	53 ± 3	48 ± 2	43 ± 3
HL-60	Myeloid	5 ± 2	34 ± 3	31 ± 5	35 ± 3
THP-1	Myeloid	2 ± 1	25 ± 2	30 ± 3	23 ± 4
Jurkat	T cell	3 ± 1	43 ± 4	41 ± 3	23 ± 2
CEM	T cell	5 ± 2	70 ± 4	73 ± 3	43 ± 3
Molt-4	T cell	4 ± 2	46 ± 4	21 ± 2	7 ± 2
NK 3.3	NK cell	4 ± 1	38 ± 3	54 ± 4	46 ± 3
TF-1	Erythromyeloid	3 ± 2	54 ± 4	43 ± 3	39 ± 3

The indicated leukemia-derived cells (10^6/well of 12-well plate) were grown either without feeder cells (plastic dish) or co-cultivated on a cushion of Rat2MV7 or Rat2Wnt1 fibroblasts for 8 h, then Apo2L/TRAIL was added to a concentration of 200 ng/ml and the cultivation continued for an additional 15 h. Cells were gently released from the feeder fibroblasts by pipetting, stained with PI and the portion of dead, i.e. PI-positive cells, determined by flow cytometry. Average values and standard deviations (SDs) from triplicates are shown. As both Rat2MV7 and Rat2Wnt1 cells are completely resistant to Apo2L/TRAIL, the detached feeders never exceeded 1% of the total amount of the analyzed cells (quantified by anti-rat MHC class I staining; not shown) and were neglected in the FACS analysis

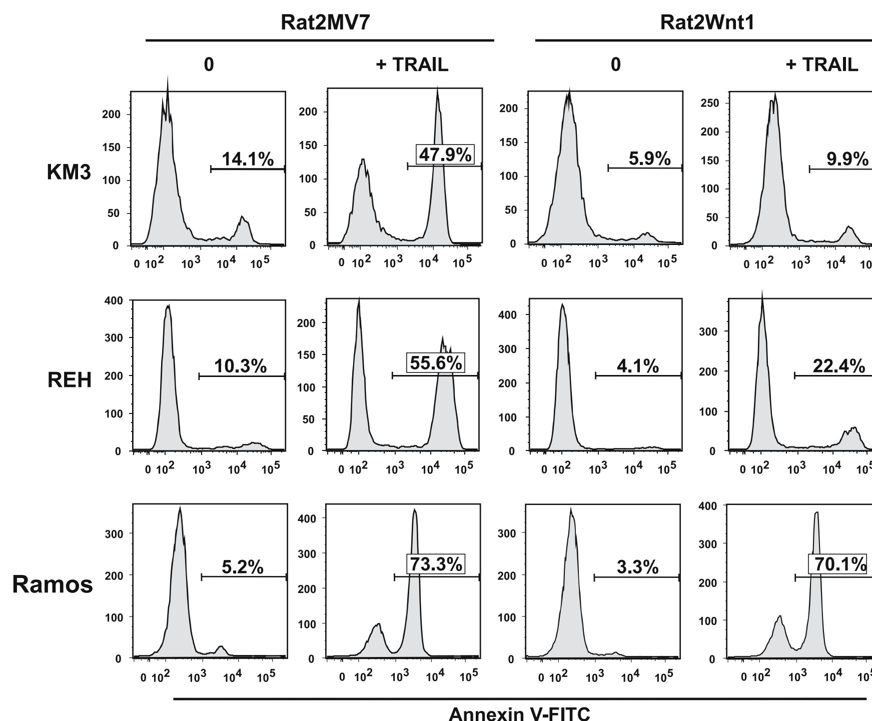


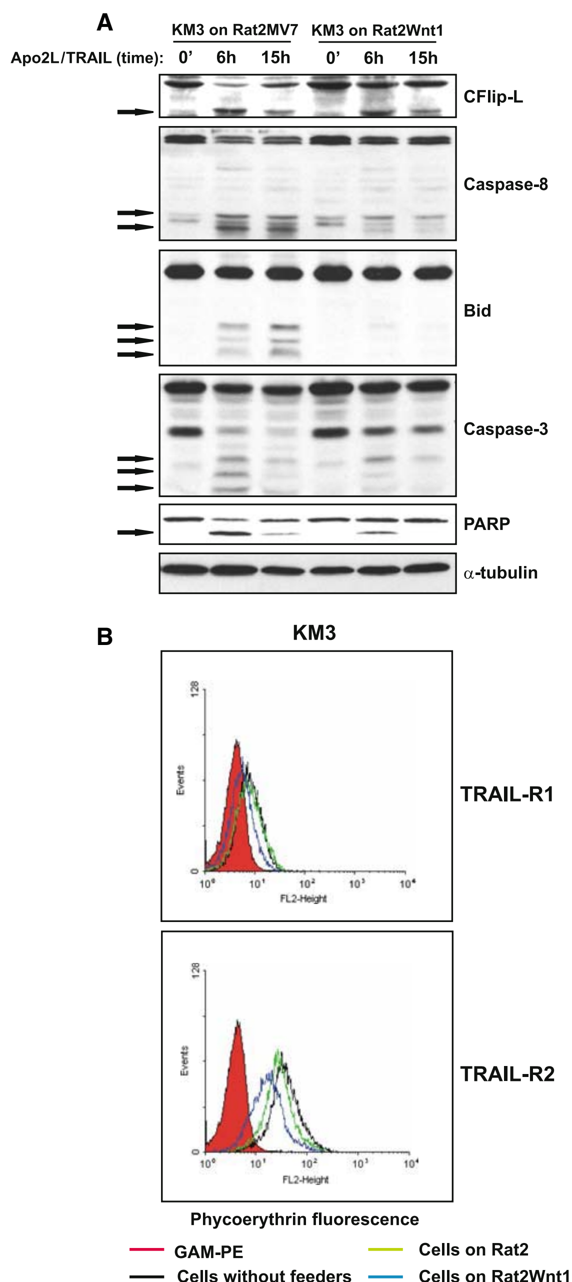
Fig. 1 The Rat2Wnt1-mediated inhibition of Apo2L/TRAIL-induced apoptosis of pre-B leukemia KM3 and REH cells. The leukemia cells were processed as described in the legend to Table 1 except that Annexin V-FITC staining was used to distinguish the apoptotic cells. Representative histograms from three independent experiments are shown

According to the effect of Rat2Wnt1 fibroblasts, human leukemia cell lines have been divided into three groups: (1) cells virtually unaffected by Rat2Wnt1 feeders: Ramos, ML-1, HL-60, THP-1, NK 3.3, TF-1; (2) cells partially protected against Apo2L/TRAIL (up to a 43% decrease in the number of apoptotic cells): Jurkat, CEM; (3) virtually all cells rescued by Rat2Wnt1 fibroblasts: KM3, REH, Molt-4. Comparable results were obtained when early apoptotic cells were detected by Annexin V-FITC conjugates, although, as expected, the fraction of positive cells was to some extent higher than in the experiment in which PI staining was used (Fig. 1; plots only for KM3, REH and Ramos cells are shown). In summary, in contrast to mature lymphocyte-derived leukemia cells, leukemia-derived immature lymphocytes were substantially protected against Apo2L/TRAIL-induced apoptosis by incubation with Rat2Wnt1 fibroblasts. The T lymphoblastic cell line Molt-4 lost responsiveness to Apo2L/TRAIL already while growing on control Rat2MV7 feeders. Therefore, just the pre-B cells KM3 and REH were selected to study how Wnt signaling can affect Apo2L/TRAIL-induced apoptosis.

Rat2Wnt1 feeder cells suppress the proximal events in Apo2L/TRAIL apoptotic signaling in KM3 and REH pre-B cells

The initial event in Apo2L/TRAIL-induced apoptosis is the DISC-mediated activation of caspase-8 followed by caspase-8-mediated processing of the pro-apoptotic BH3-only sentinel Bid to its active truncated form (tBid) [25]. Caspase-8 activation and Bid processing were strongly inhibited in KM3 cells cultured on Rat2Wnt1 fibroblasts (Fig. 2a). Similarly, the processing of effector caspase-3 and the cleavage of its target proteins PARP and cFlip were, at the 6-h time point, significantly attenuated and at the later time point (15 h after the addition of Apo2L/TRAIL) fully suppressed. Furthermore, we detected Rat2Wnt1-induced downregulation of the Apo2L/TRAIL receptors TRAIL-R1 and TRAIL-R2 (Fig. 2b and data not shown).

Rat2Wnt1-induced downregulation of TRAIL-R1 and TRAIL-R2 cell surface expression might be of a transcriptional and/or post-transcriptional origin. Using quantitative real-time RT PCR (RT-qPCR), we examined Rat2Wnt1-induced changes in the transcription of the *TRAIL-R1* and *TRAIL-R2* genes. As shown in Fig. 3, the levels of both *TRAIL-R1* and *TRAIL-R2* mRNAs were reduced to some extent in KM3 (20% reduction for both receptors) and also in REH (20% decrease for *TRAIL-R1* and 30% for *TRAIL-R2*) cells. Furthermore, the transcription of several other apoptosis- and proliferation-related genes changed minimally in KM3 and REH cells cultured on Rat2Wnt1 feeders (Fig. 3). Although the specificity of the response to Wnt ligands depends on the cellular context and is not well understood, we detected upregulation of the known target of the Wnt/β-catenin pathway, *c-MYB* [36],



◀ **Fig. 2** Rat2Wnt1 fibroblasts suppress caspase-8 and Bid processing and lower the surface expression of TRAIL-R1 and TRAIL-R2 in KM3 cells. (**a**) KM3 cells were cultured on Rat2MV7 or Rat2Wntl fibroblasts for 8 h; subsequently, Apo2L/TRAIL (200 ng/ml) was added and the cells were grown for an additional 0, 6 or 15 h, then harvested and lysed. The cell lysates were analyzed by Western blotting with the corresponding antibodies. Arrows indicate cleaved forms of c-Flip, caspase-8, Bid, caspase-3 and PARP. Staining with anti-tubulin was used as an internal loading control. (**b**) KM3 cells were cultured on Rat2MV7 or Rat2Wntl fibroblasts for 8 hrs; cells were gently harvested by pipetting and analyzed for the cell surface expression of TRAIL-R1 and TRAIL-R2 by flow cytometry

in Rat2Wnt1-stimulated KM3 and REH cells. Moreover, we also observed a substantial increase in the transcription of *EPAS1* (*HIF-2α*), a gene that we have recently identified as Wnt/β-catenin-responsive (V.K. unpublished data). The expression of additional genes activated by canonical Wnt signaling in various cell types—*Cyclin D1*, *AXIN-2*, and *SURVIVIN*—was unaffected by the Wnt1-producing feeder cells (Fig. 3 and data not shown).

Canonical Wnt/β-catenin signaling is not sufficient to attenuate Apo2L/TRAIL-induced apoptosis of pre-B leukemia cells

Direct Wnt-induced signaling was shown to provide protection against serum withdrawal or drug-induced apoptosis in osteoblasts and fibroblasts [12, 14]. These effects were examined either in Wnt1-transformed cells or using exogenous recombinant Wnt3a protein. Because our results implicated a potential role of Wnt signaling in the anti-apoptotic protection of pre-B leukemia cells, we evaluated this role using additional Wnt reagents. As the source of the Wnt signal we employed murine Wnt3a-producing L cells (Wnt3a belongs to canonical Wnt ligands as Wnt1 protein) and conditioned medium (CM) prepared from these cells. Furthermore, we utilized commercially available recombinant Wnt3a and Wnt5a polypeptides; parental L cells and CM medium prepared from these "empty" cells were used as negative controls. Strikingly, the canonical (Wnt3a-related) signaling conferred significantly less protection to KM3 and REH cells against Apo2L/TRAIL than did Rat2Wnt1 fibroblasts, and non-canonical Wnt5a-mediated signaling was entirely ineffective (Fig. 4a; Supplementary Fig. S1). To resolve this apparent discrepancy, we transduced Rat2MV7 cells with retroviral constructs expressing the mouse *Wnt3a* gene and used these cells as feeders in co-culture assays. The production of Wnt3a protein endowed the rat fibroblasts with similar functional properties as the Rat2Wnt1 feeders, thus Rat2Wnt3a cells efficiently suppressed Apo2L/TRAIL-induced apoptosis of KM3 and REH cells (Fig. 4b). These results imply that effective anti-apoptotic protective function depends on the origin of the feeder cells and, in addition, is only marginally linked to direct canonical Wnt signaling in the target cells. To exclude the possibility that Rat2Wnt1 (and Rat2Wnt3a) fibroblasts enhance cell proliferation and in this indirect way counterbalance the pro-apoptotic effect of Apo2L/TRAIL, proliferation assays employing thymidine incorporation were performed (Table 2). The assays showed that neither Wnt1- nor Wnt3a-expressing Rat2 fibroblasts enhanced the proliferation of co-cultured KM3 and REH cells.

Rat2Wnt1 vs. Rat2MV7	KM3	REH
PCNA	1.2 ± 0.2	0.8 ± 0.1
MNSOD	0.8 ± 0.1	1.0 ± 0.1
DAXX	0.8 ± 0.1	1.2 ± 0.1
SURVIVIN	1.1 ± 0.1	1.0 ± 0.0
DIABLO	0.8 ± 0.1	0.9 ± 0.2
CYLD	1.2 ± 0.1	0.7 ± 0.1
HTRA2	1.0 ± 0.1	1.0 ± 0.1
DAPK1	0.8 ± 0.2	0.7 ± 0.3
MCL1	1.0 ± 0.1	1.0 ± 0.1
BCL-XL	1.3 ± 0.1	1.1 ± 0.2
BCL-2	1.3 ± 0.3	1.2 ± 0.1
BNIP3	0.8 ± 0.1	0.9 ± 0.1
BAX	0.9 ± 0.1	1.0 ± 0.3
TIGAR	0.7 ± 0.3	0.8 ± 0.2
CDKN1A	0.7 ± 0.2	0.7 ± 0.1
FLIP	1.2 ± 0.1	0.7 ± 0.3
CASP10	1.0 ± 0.1	1.0 ± 0.1
TRAIL-R1	0.8 ± 0.1	0.7 ± 0.2
TRAIL-R2	0.8 ± 0.1	0.8 ± 0.1
DRAM	1.0 ± 0.2	0.9 ± 0.2
c-MYC	1.2 ± 0.1	0.8 ± 0.2
c-MYB	6.2 ± 2.9	2.1 ± 0.1
EPAS1	18.4 ± 4.5	4.4 ± 1.6

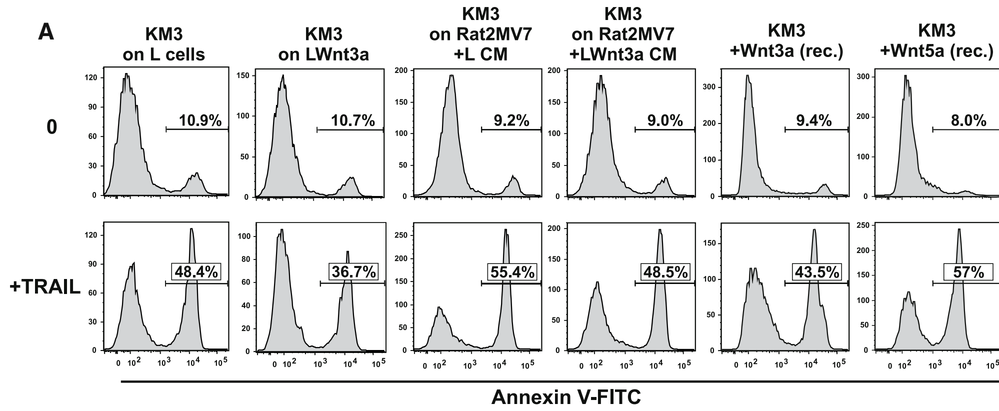
Fig. 3 Rat2Wnt1 cells upregulate the transcription of the putative Wnt/β-catenin target genes *c-Myb* and *EPAS1* in KM3 and REH cells. Total RNAs extracted from KM3 or REH cells upon 8-h co-culture with Rat2MV7 or Rat2Wnt1 feeders were analyzed by real-time RT-qPCR. The PCR reactions were performed for each primer set in triplicate using random-primed cDNAs produced from at least two independent RNA isolates. Results of one from two representative experiments are shown. The results demonstrate the relative abundance of the indicated mRNAs in Wnt1-stimulated (grown on Rat2Wnt1 feeders) versus control leukemia cells (grown on Rat2MV7 feeders) upon normalization to the levels of *SDHA* cDNA. SDs are shown in superscript

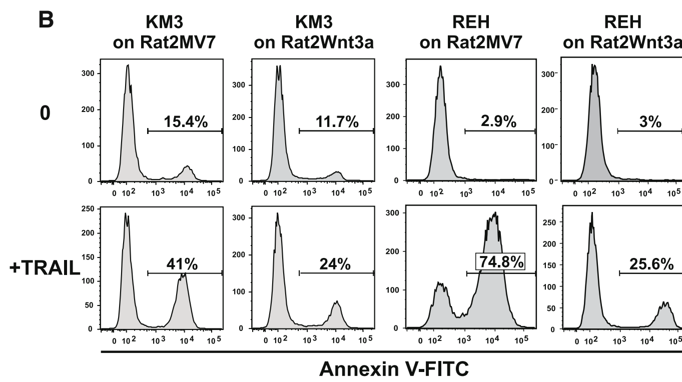
The hallmark of the activation of the canonical Wnt pathway is the post-translational stabilization and accumulation of β-catenin. The incubation of KM3 and REH cells with Wnt1-and Wnt3a-producing cell lines or treatment with recombinant Wnt3a ligand or with CM containing Wnt3a protein induced the stabilization of β-catenin. As expected, co-culture with the parental cell lines (L and Rat2MV7), CM collected from these cells or recombinant non-canonical Wnt5a protein did not cause an increase in β-catenin levels (Fig. 4c). Moreover, RT-qPCR analysis showed a similar effect of the Wnt3a-related stimuli in KM3 and REH cells as when Rat2Wnt1 feeder cells were used, i.e. a decrease in the expression of the *TRAIL-R1* and *TRAIL-R2* receptors and a clear upregulation of the putative Wnt signaling targets *c-Myb* and *EPAS1* (Fig. 4d and data not shown).

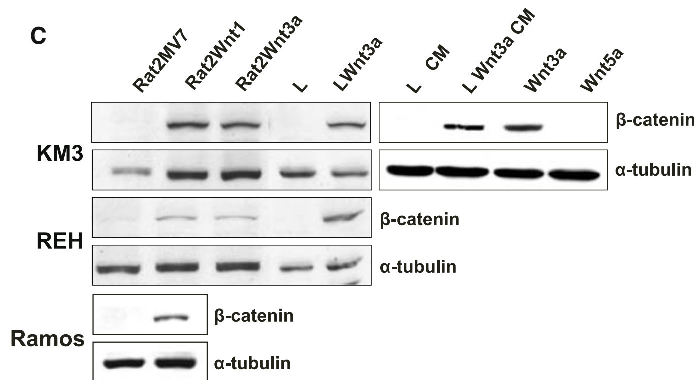
Thus, apparently, the activation of the canonical Wnt signaling pathway does not endow pre-B leukemia cells with resistance against Apo2L2/TRAIL; some unknown factor(s) produced specifically by Wnt1- or Wnt3a-transformed rat fibroblasts are mediating the anti-apoptotic protection of lymphoid cells. This conclusion was further supported experimentally using the Apo2L/TRAIL-sensitive B cell line Ramos (Burkitt lymphoma-derived). Co-cultivation of these cells on Rat2Wnt1 fibroblasts resulted in a substantional accumulation of β-catenin (Fig. 4c). However, in contrast to pre-B leukemia cells, neither Rat2Wnt1 nor Rat2Wnt3a feeders protected Ramos cells against Apo2L/TRAIL-induced apoptosis (Fig. 1, Suppl. Fig. S1 and not shown), thus confirming that activated Wnt/β-catenin signaling per se does not suffice for full anti-apoptotic protection. Moreover, it indicates that the anti-apoptotic effect of Rat2Wnt1 or Rat2Wnt3a feeders is pre-B leukemia cell specific.

Inhibitors of transcription and translation and MEK1/ERK1/2 or NFκB pathways block anti-apoptotic effect of Rat2Wnt1 fibroblasts

General transcription or translation inhibitors such as ActD or CHX sensitize many initially Apo2L/TRAIL-resistant cells to Apo2L/TRAIL-induced apoptosis, e.g. via down-regulation of the competitive caspase-8 inhibitor FLIP [37]. KM3 cells were treated for 1 h with CHX or ActD. The drug-containing medium was replaced with fresh drug-free medium, and the cells were seeded on feeder fibroblasts and after additional 8 h incubated with Apo2L/TRAIL. As shown in Fig. 5a, treatment of KM3 cells with either CHX or ActD virtually blocked the anti-apoptotic effect of Rat2Wnt1 feeder cells; moreover, CHX and, to a lesser extent, ActD increased the overall sensitivity of KM3 cells to Apo2L/TRAIL. This indicates that the pro-survival stimuli are at least partially transmitted via short-lived proteins. Significantly more striking data emerged when ActD- or CHX-treated feeders were used. One-hour pre-incubation of Rat2Wnt1 with the drugs entirely inhibited their anti-Apo2L/TRAIL action (Fig. 5a). Furthermore, although CHX and ActD provoked a substantial decrease in the production of Wnt1 protein, the remaining amounts of Wnt1—most probably adhered to the extracellular matrix—were sufficient to stabilize β-catenin in KM3 cells to the levels comparable to cells grown on untreated Rat2Wnt1 fibroblasts (Fig. 5b). Consequently, Rat2Wnt1 feeders upon the addition of CHX or ActD still activated Wnt/β-catenin transcription in co-culture experiments (data not shown). Taken together, these results confirm that the anti-apoptotic stimulus emanating from the rat feeder cells does not rely on the activation of the Wnt signaling pathway in the target pre-B cells.







D

	Rat2Wnt3a vs. Rat2MV7	LWnt3a vs. L cells	recomb. Wnt3a vs. untreated
PCNA	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.0
BCL-2	1.1 ± 0.0	1.3 ± 0.0	1.0 ± 0.1
BAX	0.8 ± 0.0	0.9 ± 0.1	1.0 ± 0.1
CIAP2	1.2 ± 0.0	1.1 ± 0.0	1.2 ± 0.1
TRAIL-R1	0.5 ± 0.1	0.7 ± 0.3	0.6 ± 0.3
TRAIL-R2	0.6 ± 0.2	0.8 ± 0.2	0.7 ± 0.3
c-MYC	0.7 ± 0.0	0.9 ± 0.3	0.8 ± 0.0
c-MYB	3.2 ± 0.3	6.0 ± 0.0	1.9 ± 0.2
EPAS1	24.1 ± 1.3	21.1 ± 3.4	5.0 ± 0.5

◀**Fig. 4** Activation of canonical Wnt/β-catenin signaling is not sufficient to suppress Apo2L/TRAIL-induced apoptosis of KM3 and REH cells**.** (**a**) KM3 and REH cells (see also Suppl. Fig. S1) were incubated with the indicated feeder cells, CM, recombinant Wnt3a (50 ng/ml) or Wnt5a (0.5 µg/ml) for 8 h and then treated with recombinant Apo2L/TRAIL for an additional 12 h. Apo2L/TRAIL-induced apoptosis was analyzed by Annexin V-FITC surface staining and flow cytometry. (**b**) Cells were processed as in (**a**) except that Rat2MV7 cells transduced with a retroviral construct expressing mouse Wnt3a were used as feeders (referred here as Rat2Wnt3a). (**c**) KM3, REH and Ramos cells were incubated with the indicated feeders, CM or recombinant proteins for 8 h, then the cells were harvested and lysed. The total cell lysates were analyzed by Western blotting using anti-β-catenin or anti-α-tubulin (loading control) antibody. (**d**) KM3 cells were co-cultured with Rat2Wnt3a, Rat2MV7, LWnt3a, L feeders, or with/without recombinant Wnt3a. After 8 h cells were harvested and purified total RNAs were analyzed by RT-qPCR as described in the legend to Fig. 3

The MEK1/ERK1/2 and PI3K/Akt pathways are implicated in the inhibition of apoptosis induced by various stimuli [38, 39]; therefore, we examined whether Rat2Wnt1-related signaling might possibly trigger these pathways in KM3 and REH cells. In contrast to the PI3 kinase inhibitors Wortmannin and LY294002 that were unable to hamper the anti-apoptotic function of Rat2Wnt1 fibroblasts (data not shown), suppressing MEK1/ERK1/2 activity in human pre-B leukemia cells using the inhibitor PD98059 significantly compromised their viability even when co-cultured with Rat2Wnt1 fibroblasts (Fig. 6a and Suppl. Fig. S2A). However, we were unable to observe RatWnt1-induced activation of ERK1/2 phosphorylation, and thus the MEK1/ERK1/2-related protection was either linked to constitutive ERK1/2 signaling or was connected with its Apo2L/TRAIL-induced activation (Suppl. Fig. S2B). The pro-apoptotic function of Apo2L/TRAIL is, in addition to MEK1/ERK1/2 signaling, negatively affected by NFkB-triggered signaling, and the final fate of the target cell depends on the balance between these pro- and anti-apoptotic stimuli [40–42]. Taking into account the importance of NFkB signaling in regulating Apo2L/TRAIL-induced apoptosis, we tested the influence of an NFκB activation inhibitor [43] on the apoptosis of KM3 and REH cells. As shown in Fig. 6b and Suppl. Fig. S2C), the NFκB activation inhibitor blocked anti-apoptotic signaling derived from Rat2Wnt1 feeder cells. Nevertheless, we did not observe—using readouts for both nuclear (DNA binding assay) and cytoplasmic (IκB phosphorylation) markers—any quantitative differences in the activity of the NFkB pathway. In fact, NFκB signaling was already active in untreated KM3 and REH cells (data not shown). Additionally, we detected no significant changes in the transcription of several NFκB putative target genes (*MNSOD*, *MCL1*, *FLIP*, *CIAP2*) in cells primed with various stimuli (Figs. 3 and 4d). Thus, up to now the nature of the anti-apoptotic signal derived from the Rat2Wnt1/Wnt3a cells is elusive and will require additional investigation.

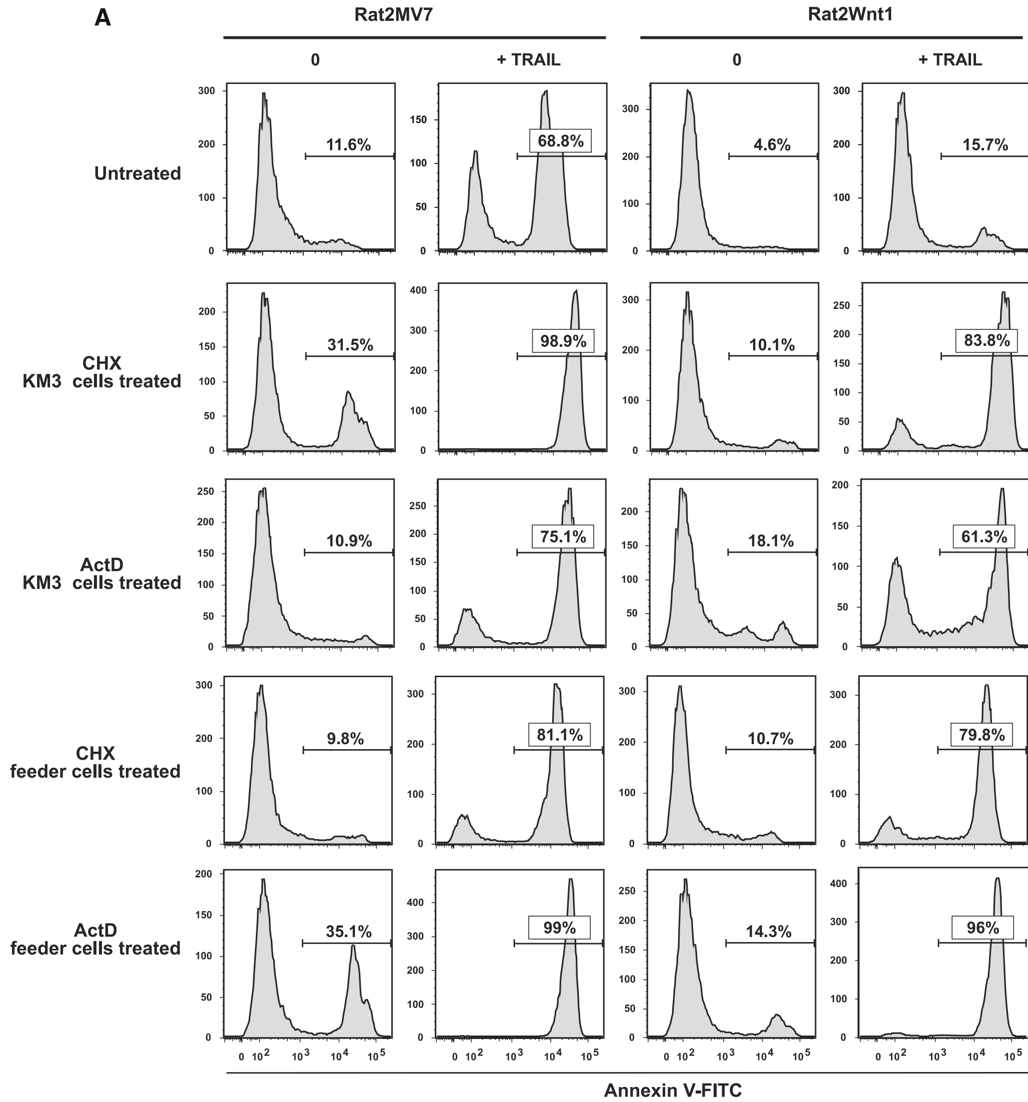
Table 2 Feeder cells of various origin have limited effect on the proliferation of co-cultured KM3 pre-B cells

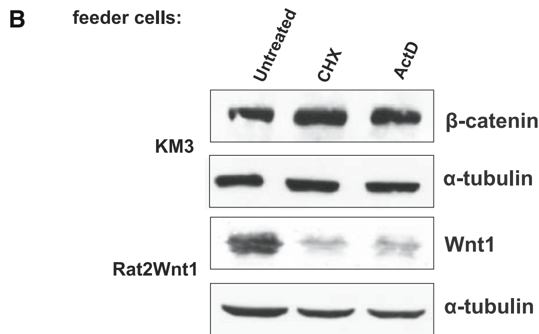
KM3 on	Relative proliferation (%)
Plastic	100 ± 14
Rat2MV7	93.2 ± 8.2
Rat2Wnt1	77.8 ± 4
Rat2Wnt3a	77.4 ± 20.4
L_{TK-}	74.2 ± 15.1
LWnt3a	77.3 ± 11.8

KM3 cells were cultured without feeder cells (plastic) and on Rat2MV7 or Rat2Wnt1, L or LWnt3a fibroblasts for 8 h, then [^{3}H]thymidine was added and the cells grown for an additional 15 h before harvesting. The incorporation of [^{3}H]thymidine was quantified by liquid scintillation. Two independent experiments were performed in triplicate. The combined results of all, i.e. six, replicates for each set up are shown with the corresponding SDs. The results are expressed as the proliferation ([^{3}H]thymidine incorporation) of KM3 cells grown with the indicated feeders (upon subtraction of background radioactivity retained in the feeders cells) relative to the proliferation of KM3 cultured without feeders (set to 100%)

Discussion

Wnt signaling is essential for the proliferation and survival of lymphocyte progenitors and HSC; furthermore, it has been well documented that the activation of the Wnt signaling pathway might be one of the fundamental mechanisms linked to various types of leukemia [15, 44–48]. As transcription induced by Wnt1 protein protects various adherent cells against cancer therapy-mediated apoptosis [12, 49, 50], we tested whether active Wnt signaling might block the apoptosis of human leukemia cells induced by Apo2L/TRAIL, the death ligand of the TNFα family. Wnt proteins strongly adhere to the extracellular matrix, thus Rat2Wnt1 feeder cells expressing the *Wnt1* gene were utilized as a source of the Wnt signal in co-culture experiments. In the initial screen Rat2Wnt1 fibroblasts attenuated Apo2L/TRAIL-induced apoptosis of several immature T- and B-cell-derived cell lines but not cells of myeloid, NK or mature B lymphocyte origin (Table 1). This observation is in agreement with the proposed role of Wnt signaling in the regulation of pre-B cell and T-cell development [9, 10]. It should be noted that T-cell lines behaved to some extent in a similar manner as pre-B cells (Table 1), nevertheless the anti-apoptotic effect of Rat2Wnt1 feeders exerted on REH and KM3 cells was much more robust. Therefore, in the subsequent study we focused only on these cell lines.


A
Rat2MV7
Rat2Wnt1
0
+ TRAIL
0
+ TRAIL
Untreated
11.6%
68.8%
4.6%
15.7%
CHX
KM3 cells treated
31.5%
98.9%
10.1%
83.8%
ActD
KM3 cells treated
10.9%
75.1%
18.1%
61.3%
CHX
feeder cells treated
9.8%
81.1%
10.7%
79.8%
ActD
feeder cells treated
35.1%
99%
14.3%
96%
Annexin V-FITC


B
feeder cells:
Untreated
CHX
ActD
KM3
β-catenin
α-tubulin
Rat2Wnt1
Wnt1
α-tubulin

◀**Fig. 5** Cycloheximide- or Actinomycin D-treated RatWnt1 feeder cells retain the ability to stimulate Wnt/β-catenin signaling but do not suppress Apo2L/TRAIL-induced apoptosis of KM3 cells. (**a**) KM3, Rat2MV7 or Rat2Wnt1 cells were pre-treated with CHX (final concentration 5 μM) or ActD (1 μM) for 1 h; the medium was replaced with fresh drug-free medium and the cells used for subsequent experiments. Pre-treated Rat2MV7/Wnt1 fibroblasts were used as feeder cells for untreated KM3 cells and vice versa—pre-treated KM3 cells were cultivated on untreated rat fibroblasts as indicated. After 8-h co-cultivation, Apo2L/TRAIL was added, and the cells were cultured for an additional 12 h, harvested by gentle pipetting and the apoptotic cells analyzed by Annexin V-FITC surface staining and flow cytometry. (**b**) Rat2Wnt1 were treated as indicated in (**a**) for 1 h, then KM3 cells were added to the culture dishes. After 8 h the leukemia cells were harvested and lysed. The total cell lysates were analyzed by Western blotting using anti-β-catenin or anti-α-tubulin (loading control) antibody. The results of one from two representative experiments are shown

We observed the partial inhibition of cFLIP-L, caspase-8, caspase-3 and Bid processing in KM3 cells grown with Rat2Wnt1 feeders; moreover, we detected Rat2Wnt1-induced downregulation of the Apo2L/TRAIL receptors TRAIL-R1 and TRAIL-R2. These data suggest that Wnt signaling might affect the proximal receptor- and DISC-mediated events in Apo2L/TRAIL-mediated apoptosis. However, Rat2Wnt1 cells also partly suppressed the apoptosis of KM3 or REH cells induced by CHX or by the DNA crosslinking drug cisplatin (see Fig. 5a and Suppl. Fig. 3). As both reagents preferentially trigger intrinsic apoptotic signaling, these results indicate that the anti-apoptotic signals emanating from Rat2Wnt1 fibroblasts might be of a complex nature and possibly affect not only extrinsic but also intrinsic apoptotic mechanisms. Moreover, since we were unable to detect significant changes in the expression of anti-apoptotic genes such as *BCL-2* or *BCL-XL* (see below), we hypothesize that Rat2Wnt-induced signaling mediates a post-translational modification of some key component(s) of the pro- or anti-apoptotic pathways. Using co-immunoprecipitation, we did not observe any differences in the assembly of DISC in Rat2Wnt1- versus Rat2MV7-stimulated KM3 cells (data not shown); this indicates that molecular components downstream of DISC might be affected by feeder-derived signaling.

Wnt1 represents the canonical, i.e. TCF/β-catenin-dependent, member of the Wnt family, and thus we also focused on the possible changes in transcription in human cells cultured on Rat2Wnt1 vs. cells grown on control Rat2MV7 fibroblasts. The RT-qPCR analysis of *TRAIL-R1* and *TRAIL-R2* genes showed a moderate but clear and reproducible downregulation of these genes, indicating that Wnt1-stimulation influences the stability or expression of the corresponding mRNAs (Fig. 3). The canonical Wnt pathway mediates anti-apoptotic signaling in Rat-1 fibroblasts, osteoblasts and rat intestinal RIE cells [12, 49, 50]. There are partly conflicting data about the Wnt-dependent activation of the anti-apoptotic proteins Bcl-2 and Bcl-XL. Wnt1 expression does not stimulate the expression of Bcl-2/Bcl-XL in RIE cells, but it was reported that Wnt3a upregulates Bcl-2 production in osteoblasts [12, 50]. The results of the quantitative real-time PCR analysis of mRNA isolated from Rat2Wnt1-stimulated KM3 or REH cells did not confirm the apparent Wnt-mediated upregulation of *BCL-2* or *BCL-XL* expression. Additionally, although *SURVIVIN* was described as a direct target gene of the Wnt pathway in colorectal cells [51], we did not observe upregulation of this gene in cells stimulated with Rat2Wnt1 fibroblasts (Fig. 3). Furthermore, the expression of several Wnt signaling-related, proliferative or pro- and anti-apoptotic protein-encoding genes did not respond to the type of feeder cells used (Fig. 3). Nevertheless, we detected increased expression of two putative targets of the Wnt/β-catenin pathway, *c-MYB* and *EPAS1*. EPAS1 function in lymphoid cells has not yet been documented; however, the transcription factor and proto-oncogene *c-MYB* is expressed mainly in hematopoietic tissues. *c-MYB* is the cellular homologue of *v-MYB*, the oncogene transduced by avian leukemia viruses [52]. Expression of c-MYB is required for the development of various hematopoietic lineages including T- and B-lymphocytes [53–56]. Experiments performed in vitro have shown that hematopoietic cells lacking c-MYB do not multiply [57], and, conversely, constitutive overexpression of *c-Myb* blocks the differentiation of myeloid and erythroid cells [58, 59]. We explored various stimuli activating the Wnt signaling pathway and, although all so-called canonical stimuli stabilized β-catenin and activated *c-MYB* and *EPAS1* transcription and inhibited the expression of *TRAIL-R1* and *TRAIL-R2* mRNAs, we detected only a limited protective effect against Apo2L/TRAIL (approximately a 5–10% decrease in the number of apoptotic cells; Fig. 4a, c, d; Suppl. Fig. S1 and data not shown). Therefore, it seems improbable that c-MYB and/or EPAS1 expression and the downregulation of TRAIL-R1 and TRAIL-R2 receptors blocks Apo2L/TRAIL-induced apoptosis in pre-B KM3 and REH cells. On the other hand, the effects of canonical Wnt signaling—especially downregulation of the pro-apoptotic Apo2L/TRAIL receptors—could be at least partly responsible for the protective effect of the Wnt-expressing feeder cells. In addition, these results (Fig. 4a) further imply that the decisive anti-apoptotic protection is provided by additional signals emanating specifically from Wnt1/Wnt3a-transduced rat fibroblasts. Yamane and colleagues described a similar phenomenon of paracrine signaling between B lineage cells and Wnt3a-stimulated stromal cells [60]. This assumption is further supported by the fact that Wnt signaling is activated in rat embryonic fibroblasts expressing the ectopic *Wnt1* gene [49]. To exclude the possibility that the anti-apoptotic features of Rat2Wnt1 are dependent on randomly gained

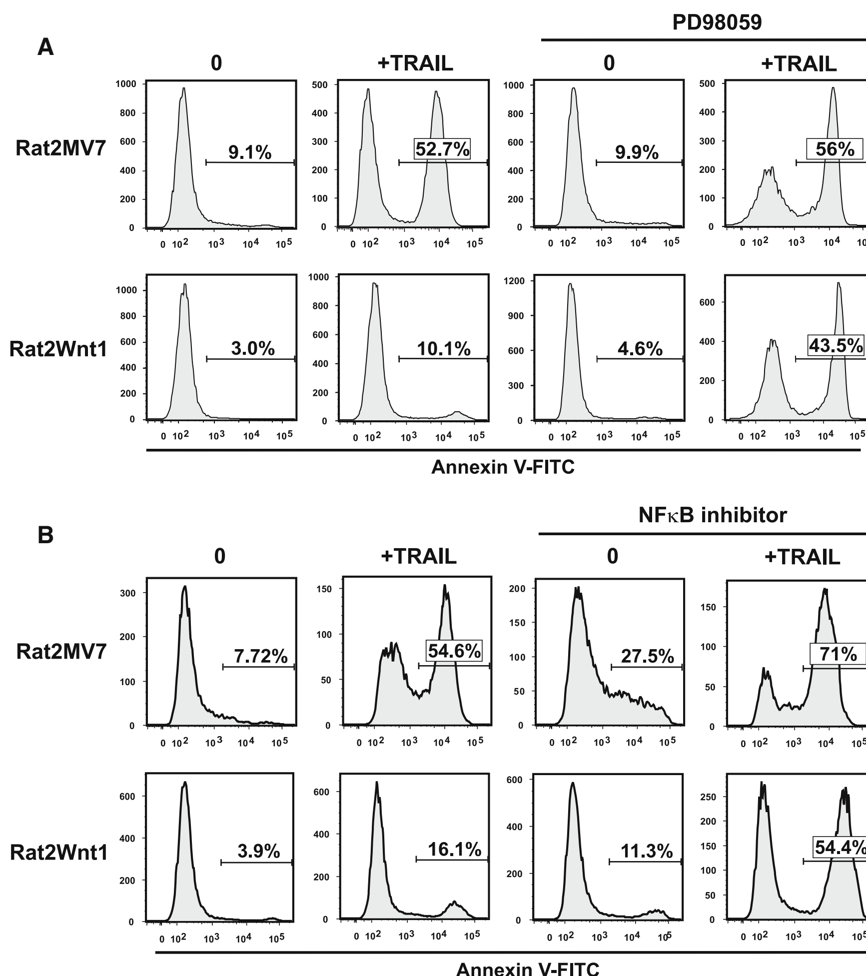


Fig. 6 Anti-apoptotic effect of Rat2Wnt1 fibroblasts is blocked by inhibitors of the MEK1/ERK1/2 or NFκ-B pathways. (**a**) KM3 cells were treated as described in Fig. 1 except that 90 min before Apo2L/TRAIL addition, the MEK1 inhibitor PD98059 was added to the culture media (final concentration 50 μM). The cells were analyzed by Annexin V-FITC staining and flow cytometry. (**b**) KM3 cells were treated as described in Fig. 1 except 1 h before Apo2L/TRAIL addition, the NFκ-B activation inhibitor 6-Amino-4-(4-phenoxyphenylethylamino) quinazoline was added to the culture media (final concentration 10 μM). The cells were analyzed by Annexin V-FITC staining and flow cytometry. The results of one from two representative experiments are shown

clonal characteristics, we transduced Rat2MV7 cells with the *Wnt3a* gene and used the resulting Rat2Wnt3a cell line in co-culture assays. These feeders displayed similar properties as did Rat2Wnt1 cells, thus confirming our assumption.

There are conflicting data describing the influence of the Wnt pathway on the proliferation rate of pre-B cell lines and B-cell progenitor acute lymphoblastic leukemia (ALL) cells. Whereas Khan and colleagues [61], using recombinant Wnt3a protein or Wnt3a CM, noted the stabilization of β-catenin and increased growth and survival in pre-B ALL cells and also of various (including REH) pre-B cell lines, surprisingly, Nygren and co-workers observed the opposite effect, i.e. the growth inhibitory function of Wnt3a ligand on primary pre-B ALL cells and on REH and other pre-B leukemia cell lines [62]. We performed an thymidine incorporation assay, and although we noticed the decreased proliferation of KM3 cells cultured on Rat2Wnt1 or Rat2Wnt3a cells (if compared to cells growing without feeders or on Rat2MV7 fibroblasts), virtually the same growth inhibitory effect of LWn3a and parental L cells was also detected (Table 2 and data not shown). This implies that first, the decreased proliferation is not related to the status of the Wnt signaling pathway in human cells and second, that enhanced cell proliferation does not replenish the apoptotic cells. What is responsible for the difference between "anti-apoptotic" Rat2Wnt1(Rat2Wnt3a) and the significantly less efficient LWnt3a cells, particularly if both parental cell lines are Wnt-responsive [63, 64]? Rat2 fibroblasts were derived from rat embryos whereas LWnt3a cells originate from adult mice. We can speculate that the different somatic origin might account for the unequal response to Wnt signaling, resulting in differences in the expression of a yet-unknown anti-apoptotic molecule in Rat2Wnt1/Wnt3a. Additionally, as LWnt3a are in fact monoclonal, another plausible explanation would be that

this individual clone lost some cell biology properties during the selection procedure.

We presumed that Wnt-transformed Rat2 fibroblasts produce an identified anti-apoptotic, membrane or extracellular matrix-associated factor. The treatment of the feeder cells with inhibitors of transcription or translation, ActD and CHX respectively, indicated that the anti-apoptotic stimulus originating in the Rat2Wnt1 fibroblasts is a short-lived molecule. However, it is rather peculiar that a 1-h incubation with the given inhibitor continues to function for an additional 8 h upon inhibitor withdrawal, i.e. during co-culture with human cells. We speculate that some residual amount of the inhibitors retained inside the cells can enhance their overall action. Another possibility would be that Wnt1 ligand deposited on the extracellular matrix can induce β-catenin stabilization in KM3 but that the reduced levels of Wnt1 are not sufficient for the autocrine stimulation of the rat feeder cells. Nevertheless, the experiments with the transcription/translation inhibitors support the idea that active Wnt signaling in pre-B cells is not the main pathway providing the resistance to Apo2L/TRAIL.

In our search for potential anti-apoptotic signals derived from the Rat2Wnt1 feeder cells, we used an array of chemical drugs blocking various pro-survival pathways in KM3 and REH cells. Thus far we succeeded with inhibitors of the MEK1/ERK1/2 and NFκB pathways. Surprisingly, the activity of both pathways (i.e. ERK1/2 kinase and NFκB cascade) was not dependent on the type of feeder cells used. We detected the activation of ERK1/2 kinase by Apo2L/TRAIL itself (Fig. Suppl. Fig. S2B) and, moreover, we also observed that NFκB signaling is already active in untreated KM3 cells growing without feeder layers. Therefore, these pathways possibly deliver general pro-survival stimuli, and the nature of the anti-apoptotic signal emanating from the Rat2Wnt1 or Rat2Wnt3a fibroblasts still remains obscure. We expect that expression analysis using hybridization of total RNAs isolated from the different feeder cells onto DNA microarrays will help to resolve this puzzle.

In summary, the results presented in this study indicate that in the target cells, Wnt signaling can trigger a molecular response that might effectively influence the behavior of "by-stander" cells. The unraveling of the molecular mechanisms underlying the anti-apoptotic action of the Wnt pathway might be important for the development of efficient anti-tumor strategies.

Conclusions

In this communication we show that embryonic rat fibroblasts producing canonical Wnt1 or Wnt3a ligands can efficiently suppress Apo2L/TRAIL-induced apoptosis of human leukemia pre-B cells in co-culture experiments. The observed effect is not primarily induced by activated Wnt signaling in the target hematopoietic cells, but rather is apparently transmitted by a secondary stimulus originating from the Wnt-transformed rat feeder cells. This Rat2Wnt-related anti-apoptotic signaling is partially suppressed through the inhibition of the MEK1/ERK1/2 or NFκB pathways. Such an indirect effect of the activation of the Wnt pathway might account for the enhanced survival of some hematopoietic malignancies.

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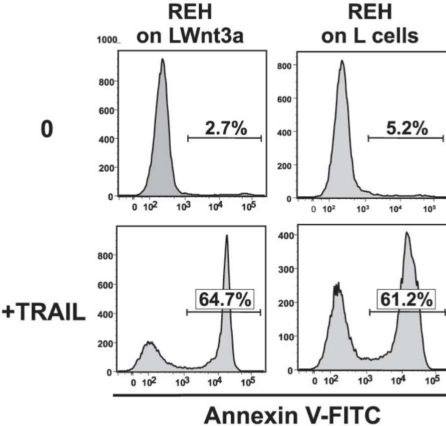
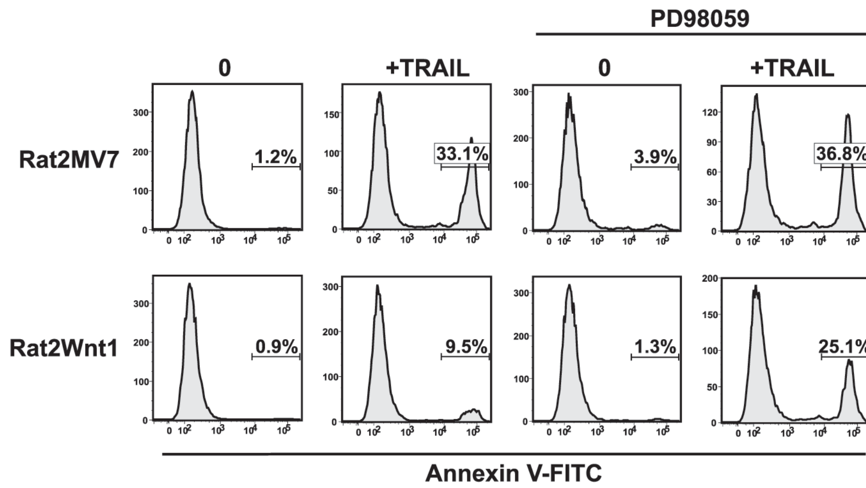
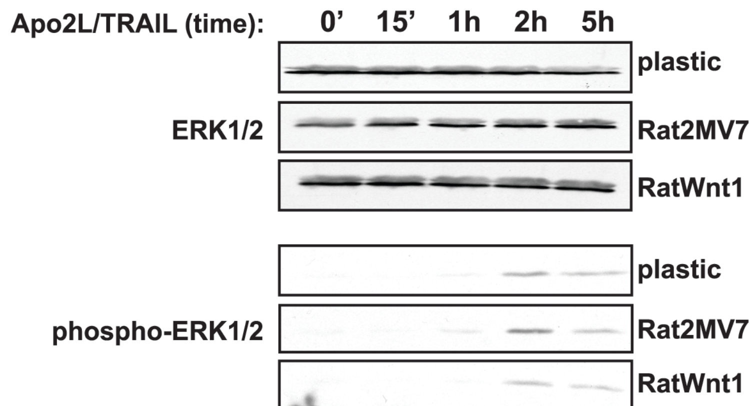
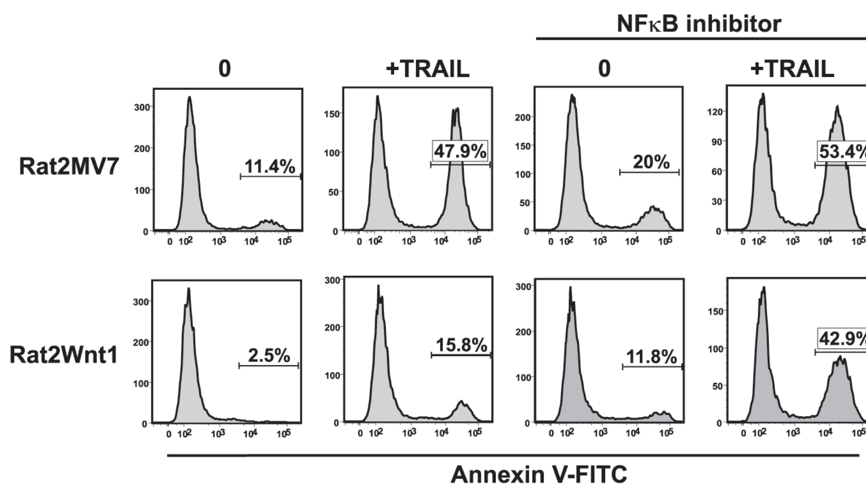
Supplementary Figure S1



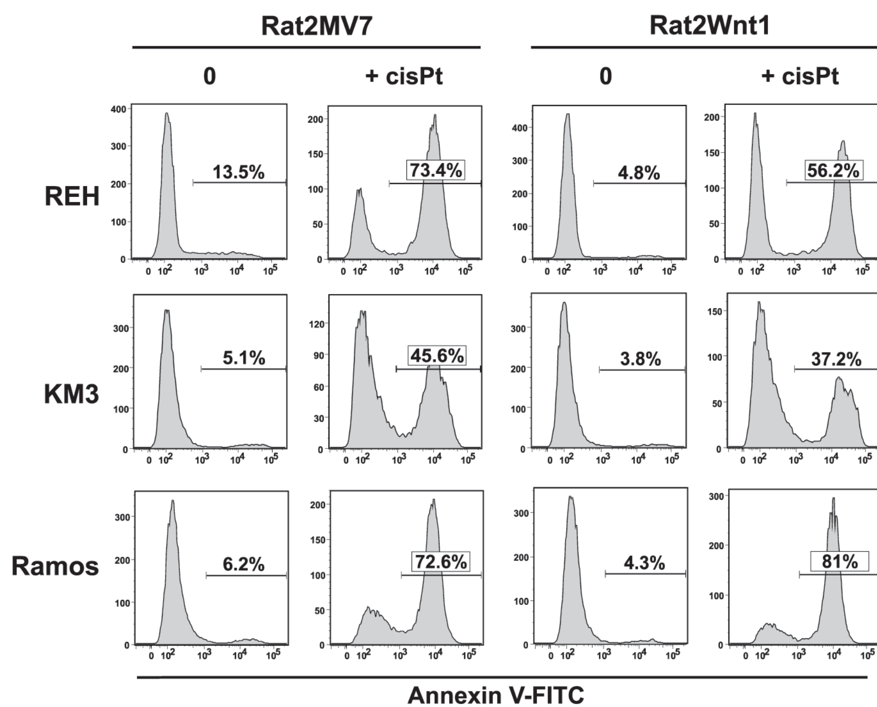
Supplementary Figure S2







Supplementary Figure S3



Supplementary Table ST1 Primers for real-time PCR

BAX.FWD	GCTGGACATTGGACTTCCTC
BAX.REV	GTCTTGGATCCAGCCCAAC
BCL-2.FWD	CAAAGCTGCAGGCTGTTTAAG
BCL-2.REV	TGCCTGAAGACTGTTAATTGT
BCL-XL.FWD	CAGTAAAGCAAGCGCTGAGG
BCL-XL.REV	TGGGATGTCAGGTCACTGAA
BNIP3.FWD	ACCCTCAGCATGAGGAACAC
BNIP3.REV	AGCAGCAGAGATGGAAGGAA
Casp10.FWD	TCCCTGCAGGACAGTATTCC
Casp10.REV	TGGCCAGACCAAGTAGGAAG
CDKN1A.FWD	ATGAAATTCACCCCCTTTCC
CDKN1A.REV	AGGTGAGGGGACTCCAAAGT
CIAP2.FWD	TTGTTGAACACTTGAAGCCATC
CIAP2.REV	ACCAAAGCAGACTAGAACATGAA
C-MYB.FWD	TGGTGGAACAGAATGGAACA
C-MYB.REV	TCGATTCGGGAGATAATTGG
C-MYC.FWD	AGCGACTCTGAGGAGGAACA
C-MYC.REV	CTCTGACCTTTTGCCAGGAG
CYCLIN D1.FWD	CCATCCAGTGGAGGTTTGTC
CYCLIN D1.REV	AGCGTATCGTAGGAGTGGGA
DAPK1.FWD	AAGACAGGCACGGCAATACT
DAPK1.REV	TGCAGTTTGCTTCACAGAGG
DAXX.FWD	GCCGCTAGGAAACAGCTATG
DAXX.REV	ATCTTCCACCCACTGTCCTG
DIABLO.FWD	GTGAGTGCCTCCTGTGGTCT
DIABLO.REV	AGTGCCCAAGGGCTAAGAAC
DRAM.FWD	CCTTAGGGTGACACTCTGGAA
DRAM.REV	GAGTGCGATCTGTGAATGGA
EPAS1.FWD	GAACAGCAAGAGCAGGTTCC
EPAS1.REV	CGACAGGCTGTAGTCCTGGTA
FLIP.FWD	CGAGGCAAGATAAGCAAGGA
FLIP.REV	TCTGGGGCAACCAGATTTAG
HTRA2.FWD	CCGAGACAGAGGGTTAAATGA
HTRA2.REV	AGGAGTCAGTGCTGGTGGTT
MCL1.FWD	CCATGGGTGCTGTGACACTA
MCL1.REV	AAGGGACTTCCTCCCACCT
MNSOD.FWD	CAAATTGCTGCTTGTCCAAA
MNSOD.REV	ACACATCAATCCCCAGCAGT
PCNA.FWD	TTGCACTGAGGTACCTGAACT
PCNA.REV	TCACCGTTGAAGAGAGTGGA
SDHA.FWD	TACAAGGTGCGGATTGATGA
SDHA.REV	GGTGTGCTTCCTCCAGTGTT
SURVIVIN.FWD	CTGCCTGGTCCCAGAGTG
SURVIVIN.REV	GTGGCACCAGGGAATAAACC
TIGAR.FWD	CACTCCCTTCAGAGGCTCAA
TIGAR.REV	TGGACTGACTGAAACTCGCTA
TRAIL-R1.FWD	AACATCGTGCCCTTTGACTC
TRAIL-R1.REV	TACCAGCTCTGACCACATCG
TRAIL-R2.FWD	CCAGCAAATGAAGGTGATCC
TRAIL-R2.REV	GCACCAAGTCTGCAAAGTCA

3.3 Dazap2 modulates transcription driven by the Wnt effector TCF-4

LEF/TCF transcription factors are downstream players of the canonical Wnt signaling pathway which associate with Groucho/TLE transcriptional repressors if the pathway is turned off [273, 275]. Upon activation, cytoplasmic β-catenin is no more degraded but translocates into the nucleus where it joins DNA binding LEF/TCFs displacing Groucho/TLE and together they mediate responsive gene transcription [250]. LEF/TCFs proteins act as bimodal factors with interacting partners establishing transcriptional activation or inhibition [276]. Besides that, other interacting proteins influence LEF/TCFs function. For example, HIC1 recruits TCF4 to nuclear bodies and protects it from transcription [315] while CtBP binds TCF3 and TCF4 in the absence of Wnt signaling and helps to suppressive mode [309, 311, 312]. Further, LEF/TCFs are also regulated by alternative splicing, alternative promoters or posttranslational modifications which fulfill a complicated mosaic of Wnt signaling control [289, 290, 292, 294, 297, 300, 301, 303, 195].

Proline-rich transcript of the brain (Prtb) called also Dazap2 is a small 17kDa protein that is highly conserved across different species. Mouse Prtb shares 99% amino acid sequence identity with its human homologue but it is not related to any other known protein family. High content of prolines and possible Src homology 2 (SH2) and SH3 binding motifs characterize Dazap2 which is expressed particularly in a developing heart and especially in an adult brain. However a Dazap2-null mutant is viable and fertile without functional abnormalities [408]. There are two Dazap proteins [Deleted in azoospermia (DAZ)-associated proteins] both interacting with DAZ, which is a germ-cell-specific RNA binding protein responsible for proper spermatogenesis [409]. Interestingly, Dazap1 is expressed predominantly in testis, compared to Dazap2, which reveals ubiquitously, and functions probably in mRNA transcription and transport [410]. Dazap2 was identified as a nuclear substrate for ubiquitin ligase (E3) Nedd4, which targets Dazap2 for rapid degradation [411]. It localizes besides cytoplasm also into nuclear speckles characterized by the presence of the splicing protein SC35, suggesting that Dazap2 may have a role in the transcription or splicing of RNA transcripts [412]. Dazap2 expression is reduced in most patients with multiple myelomas on both the mRNA and the protein levels [413]. The Sox6 (Sry related HMG box) transcription factor is a Dazap2 interactor and both proteins are necessary for proper cardiac myocyte development [414]. Further, the eIFG4 (eukaryotic initiation factor G4) scaffold protein, which has a pivotal role in the initiation of translation, binds also Dazap2 and they

both together participate in the stress granule formation upon environmental disturbances [415]. Surprisingly, Dazap2 was identified as a novel and essential branch of FGF-induced neural patterning [416]. However, all the above mentioned phenomenons linked to Dazap2 have in common the fact, that their molecular basis remains to be elucidated.

In this study, Dazap2 was identified as a TCF4 binding partner. A relatively short sequence in TCF4 N-terminus aa 214-228 is essential for the interaction as we mapped by yeast two-hybrid screen and confirmed by the pull-down assays *in vitro*. Even endogenous levels of both proteins were able to co-immunoprecipitate in variety of cell lines extruding the false interaction. Moreover, Dazap2 is able to interact with all other members of the LEF/TCF family – TCF1, TCF3 and LEF1 – despite the fact that the interaction motif is in close proximity to a alternatively spliced region in most of those transcription factors. Interestingly, the binding motif is not entirely conserved in LEF/TCF proteins, but it consists of amino acids with similar biochemical properties connoting common structural features.

Remarkably, in the DLD-1 adenocarcinoma cell line, which reveals the Wnt pathway permanently triggered through APC tumor suppressor mutation, Dazap2 was found in one complex with β-catenin. Although no direct interaction was detected, it is easy to imagine that TCF4 which interacts with both Dazap2 and β-catenin can mediate their co-immunoprecipitation.

Cellular distribution of the endogenous Dazap2 protein in U-937 monocytes displays an already described pattern although previous data were based on an ectopically expressed protein [415]. Dazap2 locates predominantly into the cytoplasm and some dot-like structures together with a diffuse mode are visible in the nucleus. Anyway, Dazap2 translocates to the nuclei when ectopically co-expressed with TCF4 and that confirms their mutual interaction.

To unravel Dazap2 function in the Wnt signaling pathway, it was transfected together with a TCF/β-catenin reporter gene construct in different cell lines. After pathway triggering by soluble Wnt3a and subsequent measuring of reporter gene activities we found neither increased nor decreased transcription. The opposite strategy, down-regulation of Dazap2 by RNA interference, was performed with all four distinct small interfering RNAs (siRNAs) being able to reduce not only Dazap2 mRNA but also the protein level to different extent. siRNAs were introduced into STF cells containing an endogenous Wnt reporter gene system and upon Wnt1 transfection, TCF-driven transcription was measured. Dazap2 down-regulation leads to decreased reporter gene activity in the distinct siRNA-dependent manner. The less Dazap2 expression observed, the less TCF transcription measured. Similar results were obtained in DLD-1 or U2OS cells. Furthermore, two additional TCF/β-catenin reporter

gene constructs containing regulatory sequences of either Wnt target gene *Axin2* or *Cyclin D1* revealed the same results. Except transient transfections of siRNAs, lentiviral constructs containing shRNAs (small hairpin RNAs) against mouse Dazap2 were introduced into the mammary epithelium C57MG cell line to achieve stable Dazap2 down-regulation. As supposed, a decreased amount of Dazap2 leads to reduced Wnt responsive transcription particularly of *Axin2* and *Cyclin D1* target genes [340, 345]. Interestingly, another responsive gene *Lipocalin 2* (*Lcn2*) [417] was conversely relieved from Wnt-induced repression. Briefly again, an increased amount of Dazap2 protein in the cells does not influence Tcf/β-catenin transcription while down-regulated Dazap2 particularly reduces Wnt target gene expression.

But what is the mechanism of Dazap2 function? We focused directly on the Wnt-induced target genes' promoters where a TCF4 transcription complex binds and performed chromatin immunoprecipitation (ChIP) in normal and lowered Dazap2 conditions. STF cells, which contain a luciferase reporter gene under the control of eight LEF/TCF binding sites, were used. Unfortunately, we did not succeed to perform ChIP analysis on promoters of classical Wnt target genes *Axin2* and *Cyclin D1* in any used mouse and human cell lines (including STF and C57MG cells). Interestingly, decrease in Dazap2 expression leads to reduced binding of TCF4 to the promoters of other observed TCF-responsive genes, *NKD1* (naked cuticle homologue 1) [418, 419] and *TROY* (tumor necrosis factor receptor superfamily, member 19) [420, 421] and particularly luciferase. These results correspond to mRNAs quantification of the mentioned genes from the cells treated in the same conditions. Because Dazap2 knockdown does not influence expression level of either TCF4 or β-catenin, our data indicate that Dazap2 affects the TCF4 binding ability to the promoters of Wnt-responsive genes.

In summary, Dazap2 was identified as a novel LEF/TCF family interactor, which modulates TCF4 affinity for its specific DNA sequence.

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Dazap2 modulates transcription driven by the Wnt effector TCF-4

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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

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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.

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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

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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 GATCAAA GG 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

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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

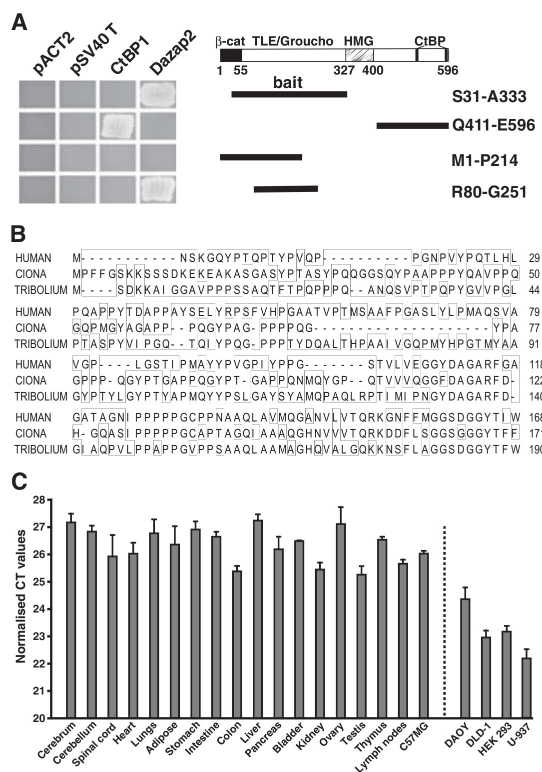

Figure 1. Interaction between Dazap2 and TCF-4 in a yeast two-hybrid screen. (**A**) Deletion mutants of human TCF-4 (schematically represented on the right as thick black lines) were tested in an Y2H mini-mating assay for interaction with full-length mouse Dazap2. The left panel shows the growth of 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

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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

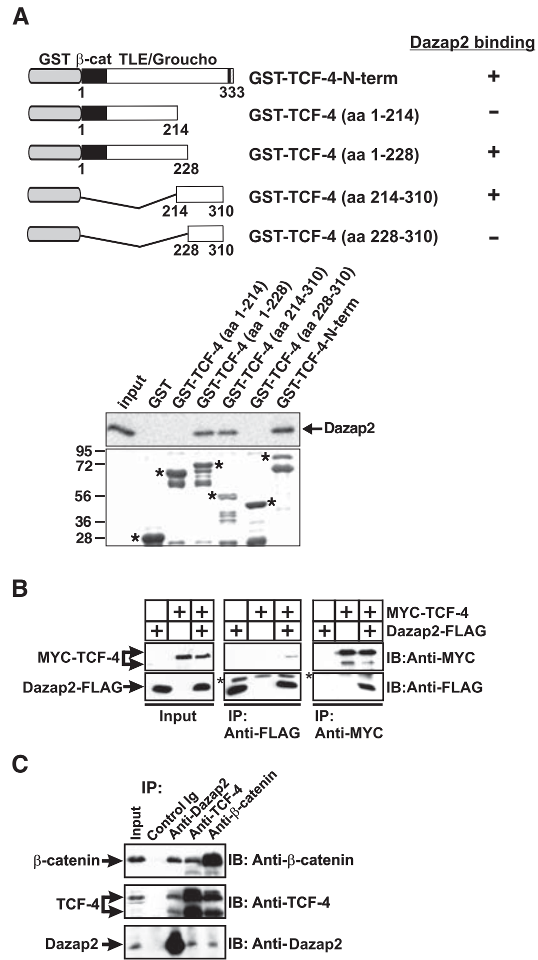

Figure 2. Association between Dazap2 and TCF-4 *in vitro* and in mammalian cells. (**A**) Interaction of Dazap2 with TCF-4 in GST pull-down assays. The top panel represents a schematic diagram of the TCF-4 proteins used in the *in vitro* pull-down assays. The bottom panel displays the pull-down assay 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.

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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 preincubation 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.

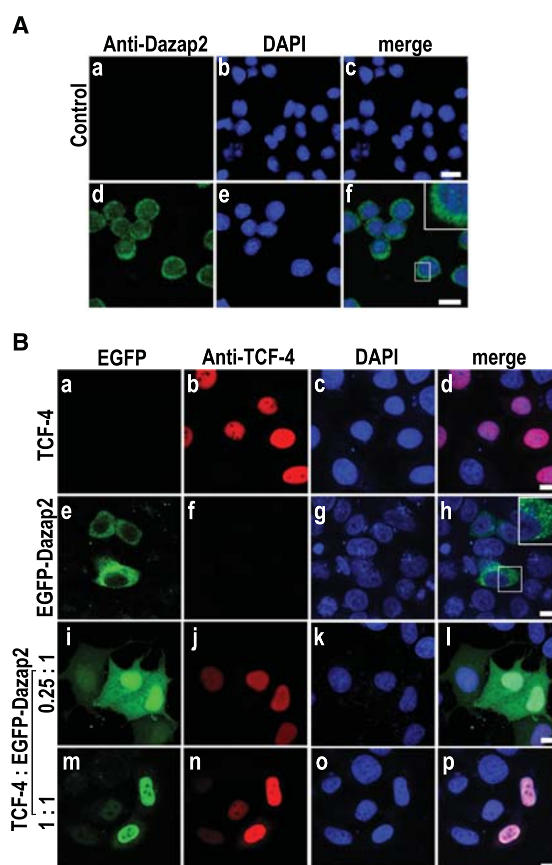

Figure 3. TCF-4 translocates Dazap2 into the nucleus. (**A**) Endogenous Dazap2 protein shows a mostly cytoplasmic distribution in human cells. Laser scanning confocal micrographs of U-937 cells stained with an antigen-purified chicken anti-Dazap2 polyclonal antibody. The merged images (c) and (f) were 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 preblocked 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.

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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

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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

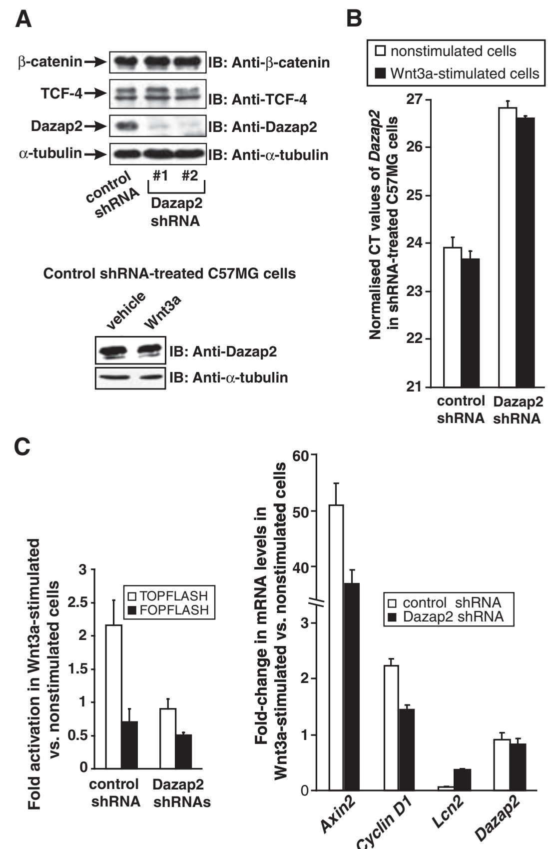

Figure 6. Down-regulation of *Dazap2* reduces the responsiveness of C57MG cells to Wnt3a stimulation. (**A**) The top panel shows a series of western blots from whole cell extracts prepared from mammary gland epithelium C57MG cells transduced with retroviral vectors expressing either a non-silencing 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.

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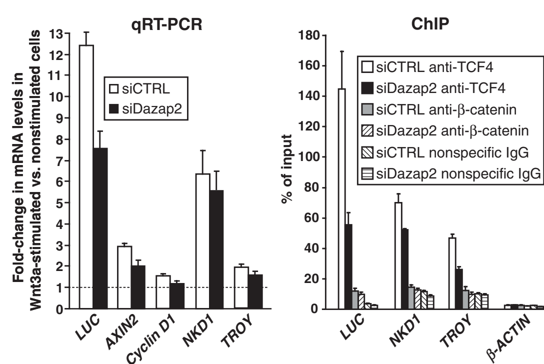

Figure 7. *Dazap2* knockdown decreases binding of TCF-4 to the promoters of the Wnt-signalling target genes. STF 293 cells were transfected with *Dazap2* siRNA #2 (siDazap2) or control siRNA (siCTRL). After a 2-day expansion the cells were transferred to large culture dishes and stimulated with either 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*

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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 EGFP). 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.

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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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Conflict of interest statement. None declared.

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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’; *hβ-ACTIN*, 5’-ATG GCC ACG GCT GCT TCC AGC-3’, 5’-GGG TGT AAC GCA ACT AAG TCA T-3’; *mβ-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 autoradiograph 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]-labelled (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 mixture 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.

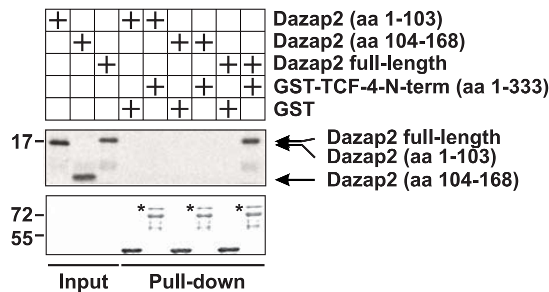
Supplementary Figure 6 The mouse genome contains two homologous Dazap2 proteins

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.

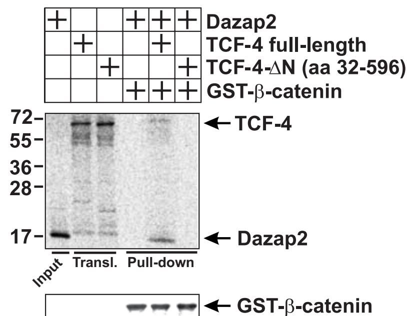
Supplementary Figure 1

```
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--QQAAANMSALSAHYPSTSMYLPM-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
```

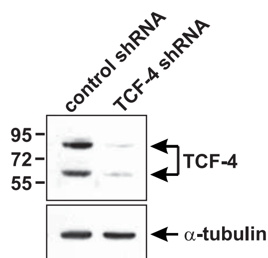
Supplementary Figure 2



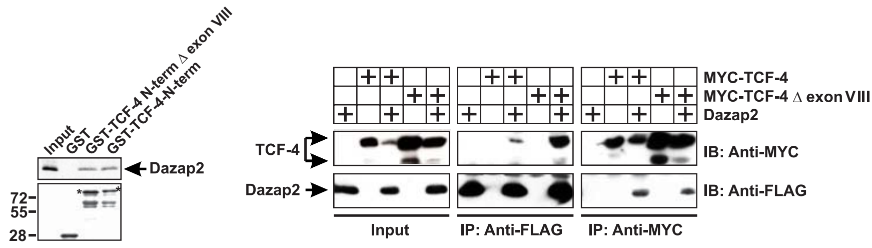
Supplementary Figure 3



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

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

Hypermethylated in cancer 1 (HIC1) was identified as a new potential tumor suppressor according to its frequent deletion or epigenetic silencing in various types of leukemias and solid tumors [422]. It is located at the chromosomal region 17p13.3 close to another tumor suppressor p53, which may act as a transcriptional activator of HIC1 [423]. Five Krüppel-like zinc fingers in the C-terminus, a BTB/POZ interaction domain (Broad complex, Tramtrack and Bric à brac/Poxviruses and Zinc finger) in the N-terminus and central region with a GLDLSKK/R motif recruiting CtBPs (C-terminal binding proteins) compose together HIC1 protein [424, 425, 311]. C-terminal part is responsible for sequence-specific DNA-binding on HiRE (HIC1-responsive element) and transcriptional repression of target genes [426] mediated by both histone deacetylase-dependent and -independent co-repressor complexes [425, 427, 428]. BTB/POZ domains generally arrange homo- and hetero-oligomerization which is in case of HIC1 visible as dot-like structures called HIC1 nuclear bodies. Moreover, dimerization of HIC1 provides co-repressor CtBP1 binding [425]. The HIC1 BTB/POZ domain also autonomously represses transcription [429, 430] via formation of a complex with histone deacetylase SIRT1 (sirtuin 1) [427]. The HIC1 central region appears to be a second repression domain exhibiting CtBP-dependent repression mechanisms [431]. There is also located lysine 314 targeted by two competing posttranslational modifications – SUMOylation and acetylation – which cause recruitment of distinct co-repressors leading to binding of different target gene promoters [428, 432]. On the contrary O-glycosylation posttranslational modifications are attached to a HIC1 DNA-binding domain and do not impress recognition of HiRE [433].

The CtBP co-repressor is a non-DNA-binding protein that interacts with a subset of sequence-specific transcription factors to bring about repression [425]. The mechanism by which CtBP affects transcription is probably related to its interaction with several histone deacetylases [434, 435] and also CtBP distribution within the cell could be crucial. While sumoylation targets CtBP into the nucleus, its phosphorylation means relocation to the cytoplasm. Besides, binding of different proteins contributes to CtBP shuttling between nuclear and cytoplasmic compartments and it is HIC1 that pulls CtBP into the nucleus by contrast to a nNOS protein that holds it in the cytoplasm [425, 436]. Transcription factors

TCF3 and TCF4 were evidenced to bind CtBP and to repress Wnt target genes in dependence on histone deacetylases [312, 309].

Because HIC1 can manipulate CtBP distribution and at the same time CtBP co-repressor influences TCF/β-catenin driven gene transcription we focused on a possible chain of events among the mentioned proteins. We show that HIC1 recruits TCF4 into HIC1 bodies and CtBP even strenghtens this recruitment. This new repression mechanism of HIC1 subsists in draging a TCF4/β-catenin complex away of Wnt responsive elements and inhibiting so Wnt induced transcription instead of affecting directly DNA via HiRE.

In more detail, we confirmed that wild type HIC1 forms nuclear bodies and the BTB/POZ domain is crucial for existence of these structures. CtBP also forms distinct structures in nuclei next to the diffused pattern [437] but when co-transfected with HIC1, it is completely retracted into the HIC1 bodies. If the GLDLSKK/R motif in HIC1 central region is missing, CtBP preserves its indigenous nuclear phenotype. TCF4 stained in the same cells co-localizes with both HIC1 and CtBP in nuclear bodies. Surprisingly, in cells completely lacking CtBPs [438], TCF4 still partially co-localizes with HIC1 in nuclear dot-like structures. To answer whether HIC1 directly binds with TCF4, several co-immunoprecipitations, both *in vivo* and *in vitro,* were done and these experiments confirmed that TCF4 straightly interacts with HIC1 independently on the GLDLSKK/R motif. Following mapping suggested a complex multidomain mode of HIC1/TCF4 interaction.

Using the luciferase reporter gene system which contains several Wnt responsive elements enables easy measuring of TCF/β-catenin mediated transcription and HIC1 strongly attenuates Wnt induced signaling depending on the presence of the BTB/POZ domain. HIC1 lacking the CtBP binding motif causes only partial suppression of reporter gene expression which nicely corresponds with microscopical observations of TCF4 entirely partially drawn into HIC1 bodies in CtBP^{-}/$^{-}$ cells. Similarly lowered expression of HIC1 by RNA interference in non-tumor cell lines lead to HIC1 bodies' disappearance and loss of HIC1 suppressor activity as we also show by increased expression of Wnt target gene *Axin2*.

We asked whether HIC1 can function by disabling the interaction between TCF4 and β-catenin or by decreasing their intracellular levels. HIC1 introduced into DLD-1 cells that reveal constitutively active TCF/β-catenin transcription does not modify endogenous levels of either TCF4 or β-catenin. Wnt target gene *Tenascin C* expression is lowered in these cells although HIC1 withdraws also β-catenin via TCF4 into nuclear bodies and so another mechanism must be employed.

Kaiso, another BTB/POZ containing a transcription factor, binds sequence specific elements in Wnt responsive genes [439] but any similar interaction of HIC1 was excluded. On the other hand, chromatin immunoprecipitation clearly exhibited that the amount of TCF4 and β-catenin present on Wnt target gene *Tenascin C* promoter is strongly reduced in DLD-1 cells over-expressing HIC1. Similarly in 293 cells co-transfected with HIC1 and β-catenin, HIC1 completely eliminates association of exogenous β-catenin with the Wnt responsive elements of the *SP5* gene. These results denote the idea that HIC1 represses Wnt signaling by removal of TCF4/β-catenin away from specific sequences in favour of nuclear bodies which seem to serve as a kind of protein depository.

HIC1 transcriptionally regulates genes controlling cell growth or cell death in response to DNA damage [440]. It is lost in plenty of solid tumors and leukemias, which makes HIC1 interesting for therapeutic approaches as well as for revealing the mechanisms of its function.

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

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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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Subject Categories: signal transduction
Keywords: β-catenin; nuclear HIC1 bodies; TCF-4; Wnt signaling

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

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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.

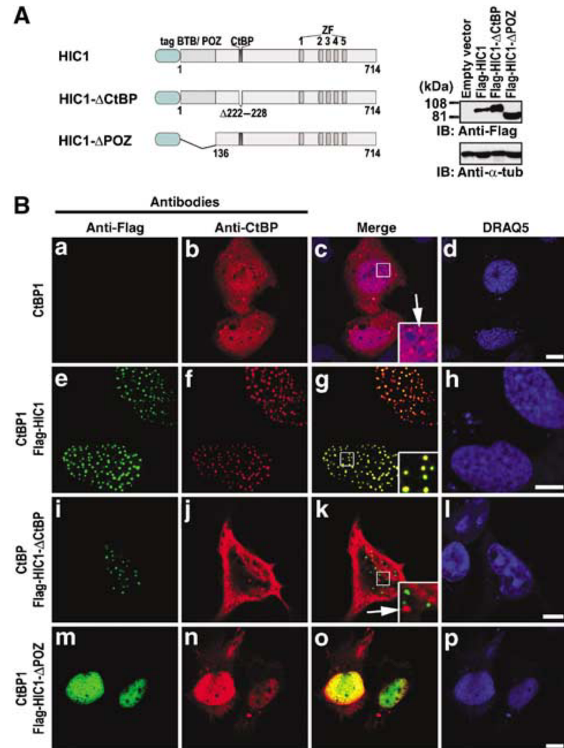

Figure 1 HIC1 targets CtBP into the nuclear bodies. (**A**) A schematic representation of the human HIC1 constructs used in this study. tag, Flag or EGFP tag; BTB/POZ, the BTB/POZ domain; CtBP, CtBP-binding site; ZF, five C_2H_2 Krüppel-like zinc fingers. Right, Western blots of total cell extracts after 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(−/−)*

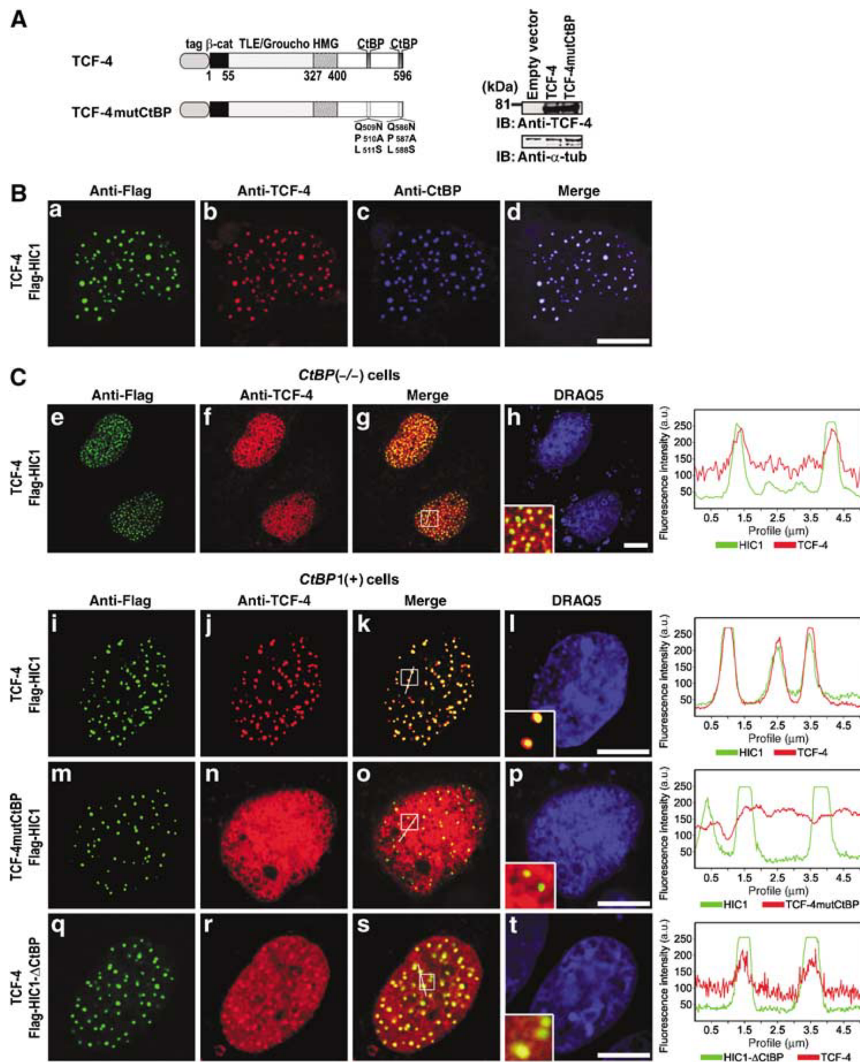

Figure 2 TCF-4 and HIC1 form nuclear protein complexes in mammalian cells. (**A**) TCF-4 expression constructs. The TCF-4mutCtBP polypeptide has a triple amino-acid substitution of each CtBP-binding motif as indicated. tag, myc or EGFP tag; β-cat, β-catenin interaction domain; TLE/Groucho, TLE/Groucho 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

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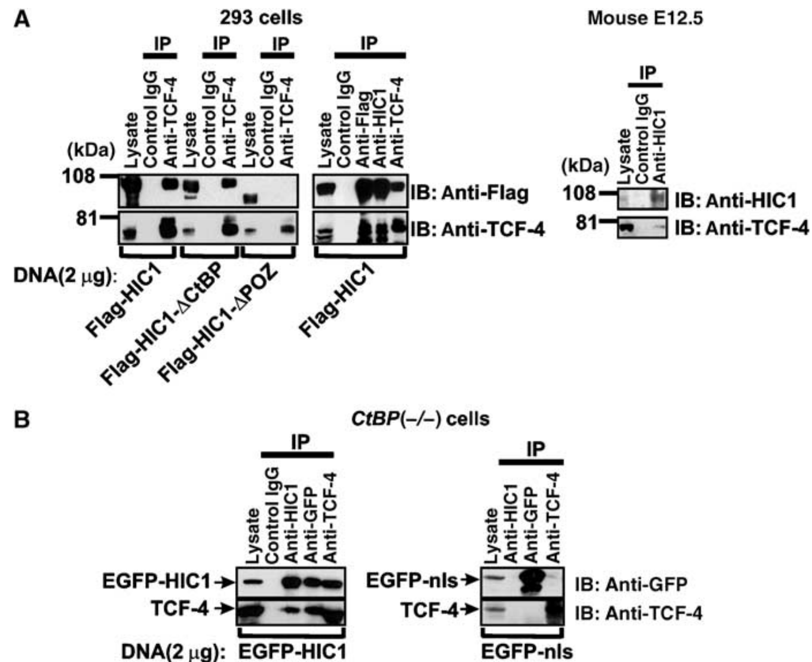

Figure 3 Association between TCF-4 and HIC1 *in vivo*. (**A**) Left, coimmunoprecipitations between endogenous TCF-4 and various exogenous HIC1 proteins in human 293 cells. Right, coimmunoprecipitations of endogenous HIC1 and TCF-4 in cells derived from mouse embryos on day 12.5 p.c. IP, immunoprecipitation; 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

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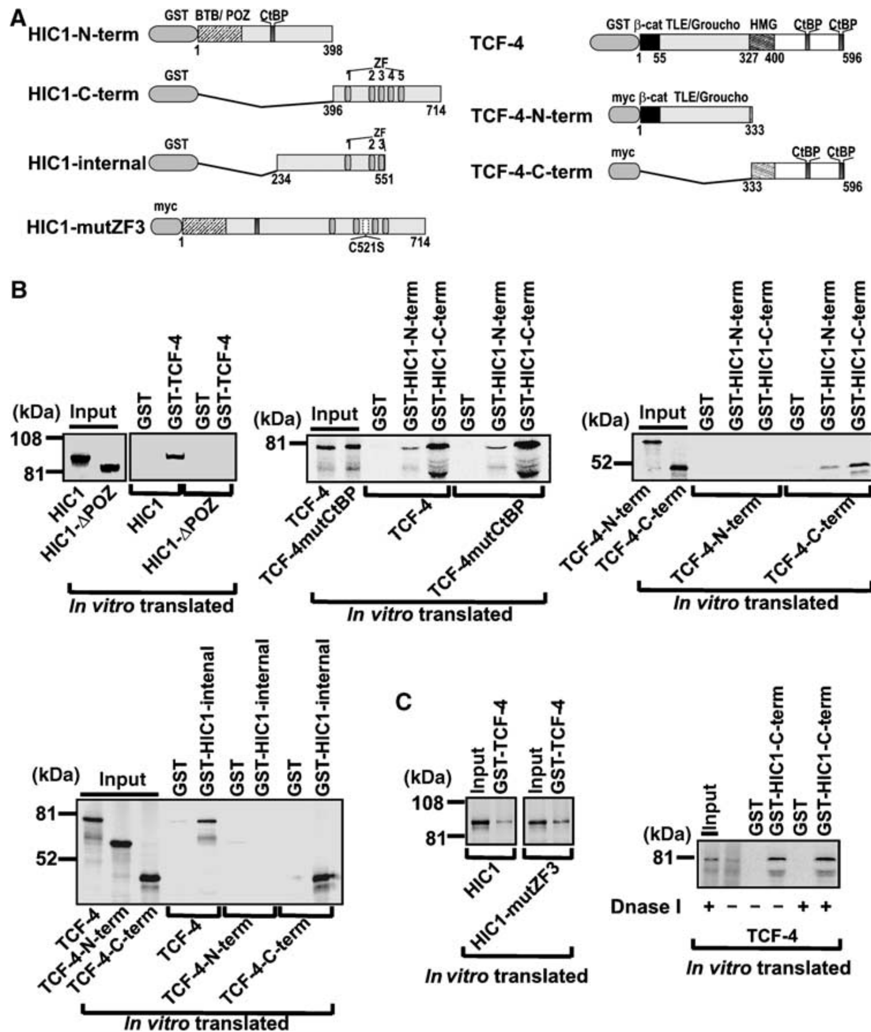

Figure 4 *In vitro* interaction of TCF-4 and HIC1. (**A**) Structures of HIC1 and TCF-4 proteins used in the *in vitro* pull-down assays (see also the diagrams of the additional HIC1 and TCF-4 constructs depicted in Figures 1A and 2A). (**B**) Pull-down assays between bacterially expressed GST-fusion 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.

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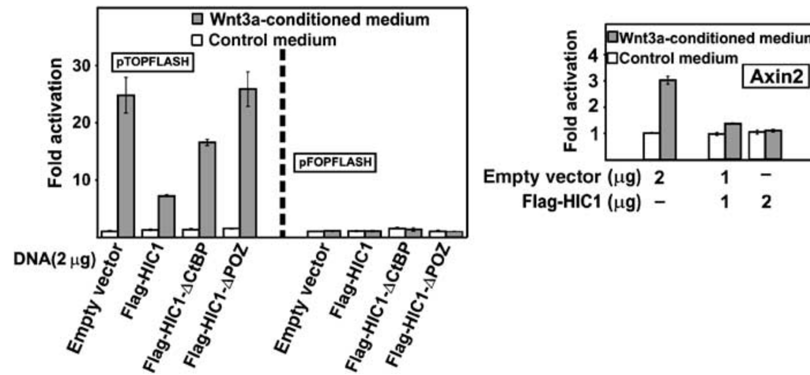

Figure 5 HIC1 represses the Wnt-stimulated transcription. Reporter gene assay with the Wnt-responsive promoters. 293 cells were cotransfected with the reporters and the HIC1 constructs as indicated and stimulated for 24 h with Wnt3a-conditioned or control medium. Luciferase (firefly) activities were 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-

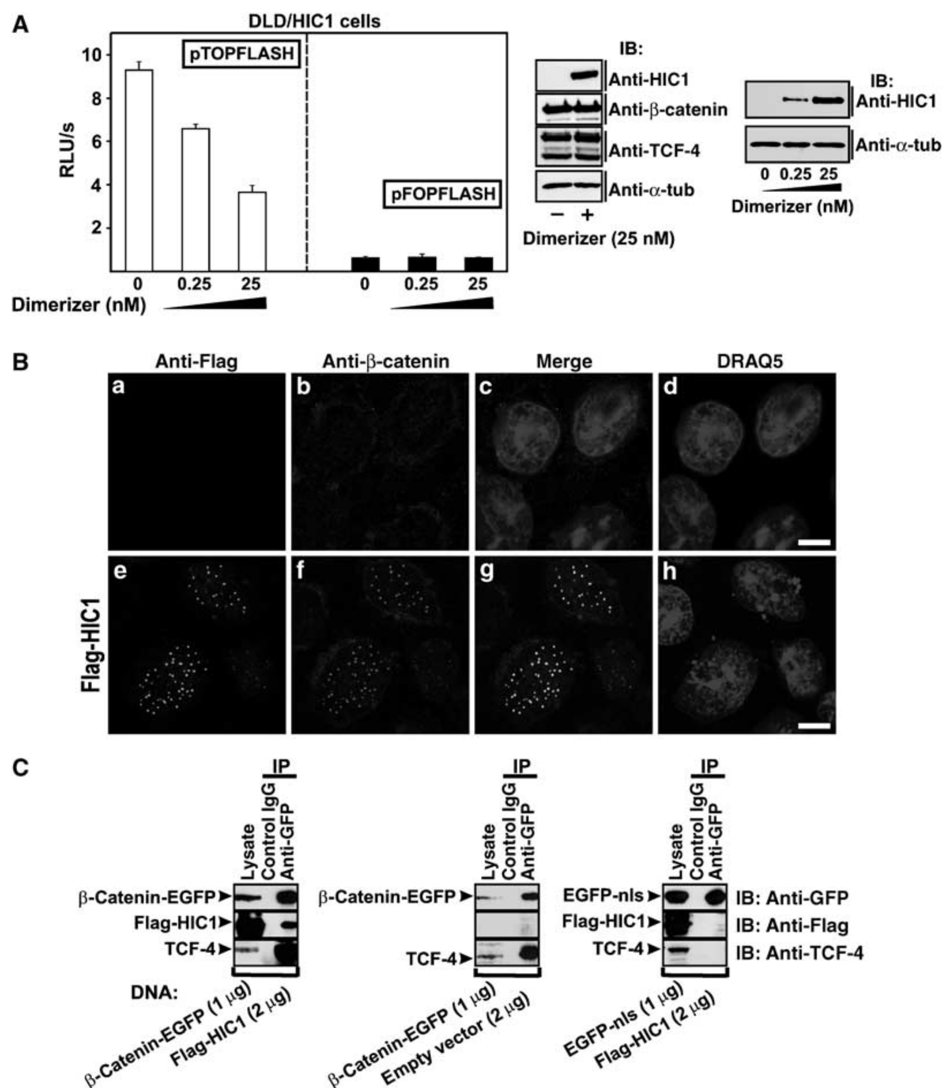

Figure 6 HIC1 represses TCF/β-catenin signaling in DLD-1 adenocarcinoma cells. (**A**) Right, transgenic DLD/HIC1 cells growing at higher concentrations of a synthetic compound AP21967 (dimerizer) contain increasing amounts of the HIC1 protein as evidenced by Western blots of total cell extracts probed 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

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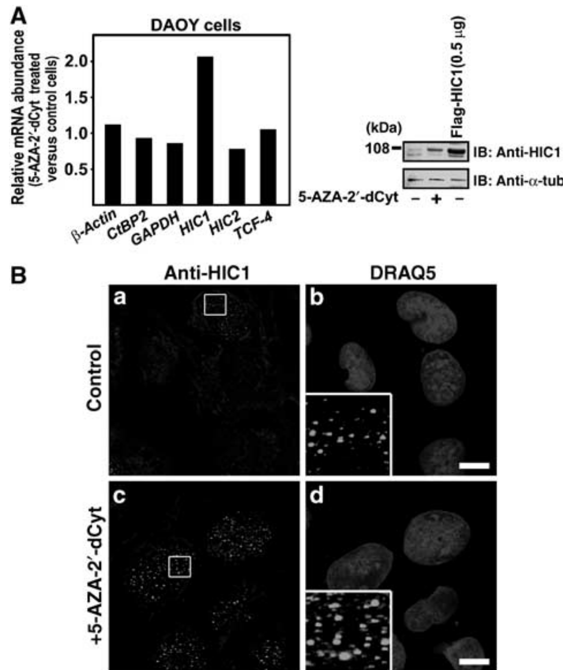

Figure 7 The nuclear HIC1 bodies in DAOY cells. (**A**) Increased expression of *HIC1* mRNA and protein in the cell line DAOY after treatment with 1 μM 5-aza-2′-deoxycytidine for 6 days. The expression was analyzed by qRT–PCR (left) or by Western blotting using anti-HIC1 antibody. In lane 3, a lysate 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-

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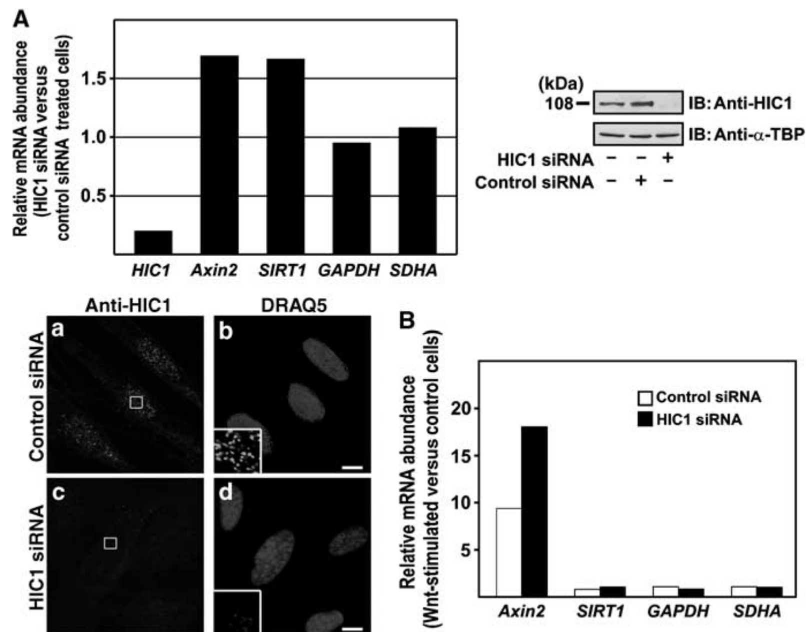

Figure 8 HIC1 knockdown increases the TCF-mediated transcription. (**A**) Human primary cells WI38 were transfected with HIC1 siRNAs or control siRNAs and the changes in the levels of *HIC1* mRNA or protein were tested 24 h post-transfection. Left, results of the qRT–PCR analysis. The relative abundance 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

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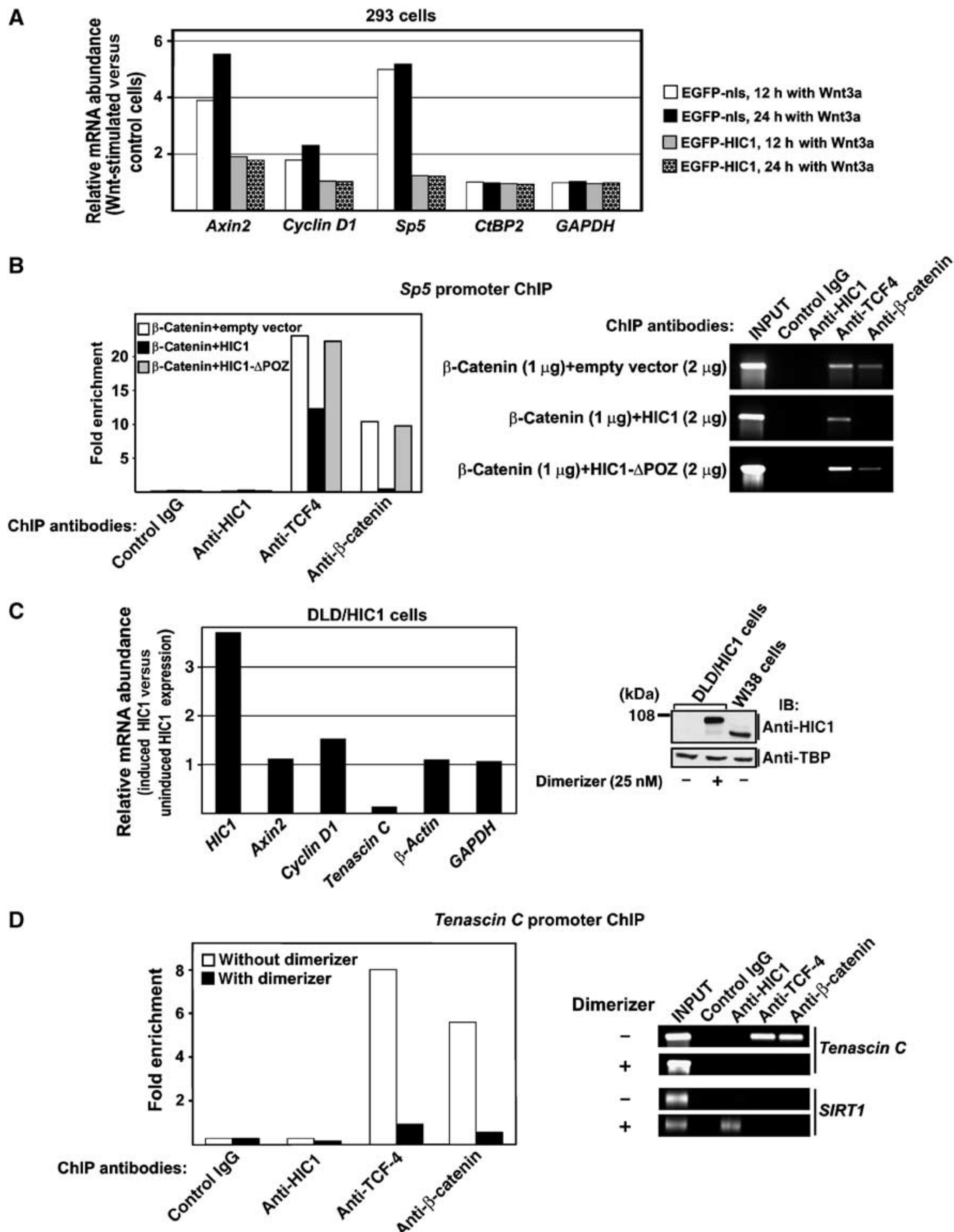

Figure 9 HIC1 sequesters TCF and β-catenin from the TCF-dependent promoters. (**A**) HIC1 blocks transcriptional activation of the Wnt signaling target genes. Results of qRT–PCR analyses performed with cDNA generated from 293 cells transfected with the indicated constructs upon 12- or 24-h stimulation 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.

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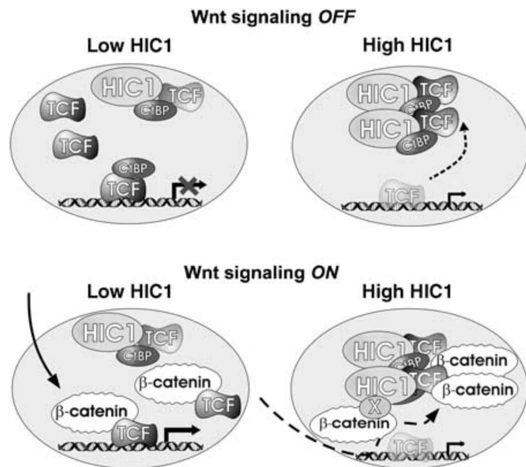

Figure 10 A model for HIC1 suppression of the transcriptional response induced by Wnts. The regulation of a complex promoter integrating inputs from the Wnt and other signaling pathways is depicted. At low levels of HIC1 the activity of the promoter depends mainly on the Wnt signaling components. High 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-HIC1-N-term (aa 1–398), GST-HIC1-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 TCF-4 (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’-TCCAGACCAACAAACACACC -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’- CCTTCCTTTCTAGCGTGAGC-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

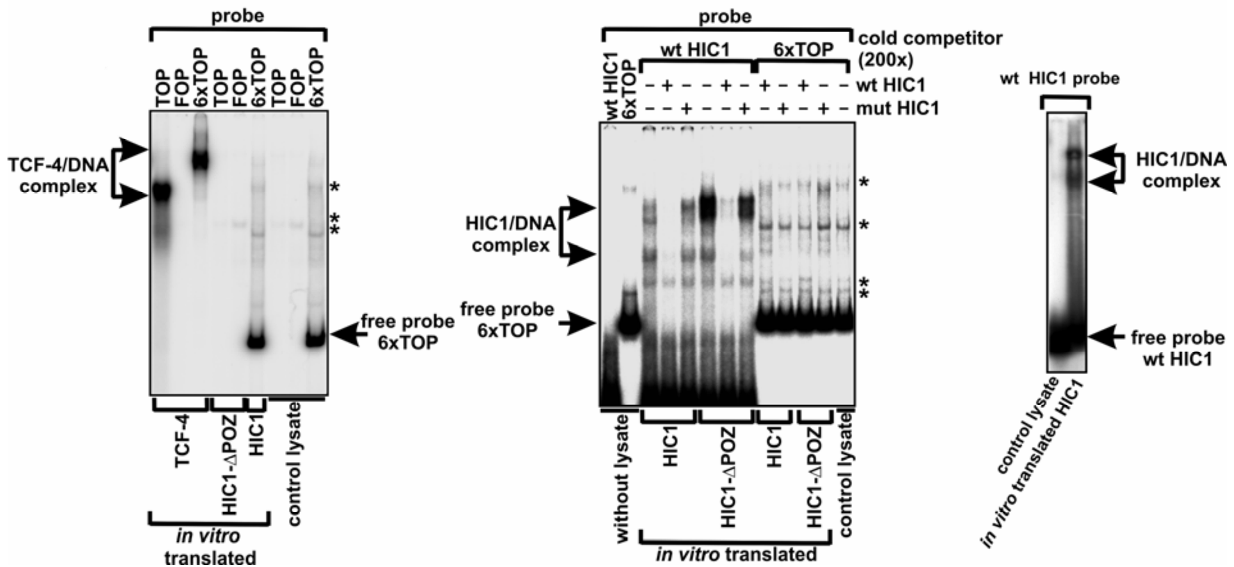
gene	origin	sequence
Axin1	human	forward 5'-CCTGTGGTCTACCCGTGTCT-3'
		reverse 5'-GCTATGAGGAGTGGTCCAGG-3'
	mouse	forward 5'-ACCCAGTACCACAGAGGACG-3'
		reverse 5'-CTGCTTCCTCAACCCAGAAG-3'
Axin2	human	forward 5'-CTGGCTTTGGTGAACTGTTG-3'
		reverse 5'-AGTTGCTCACAGCCAAGACA-3'
	mouse	forward 5'-TAGGCGGAATGAAGATGGAC-3'
		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'

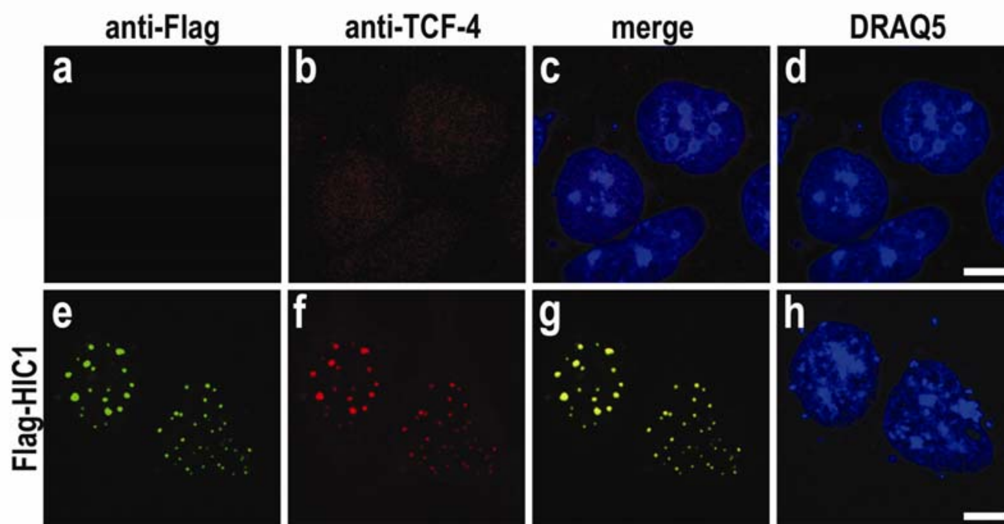
Supplementary figures

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’-GGGCCTGGGGGCAACCCAATCAC-3’, the negative control HIC1mut was 5’-GGGCCTGGGGATGACCCAATCAC-3’. TCF-4/DNA and HIC1/DNA complexes and the positions of the free probes are indicated by arrows. Stars indicate non-specific bands.



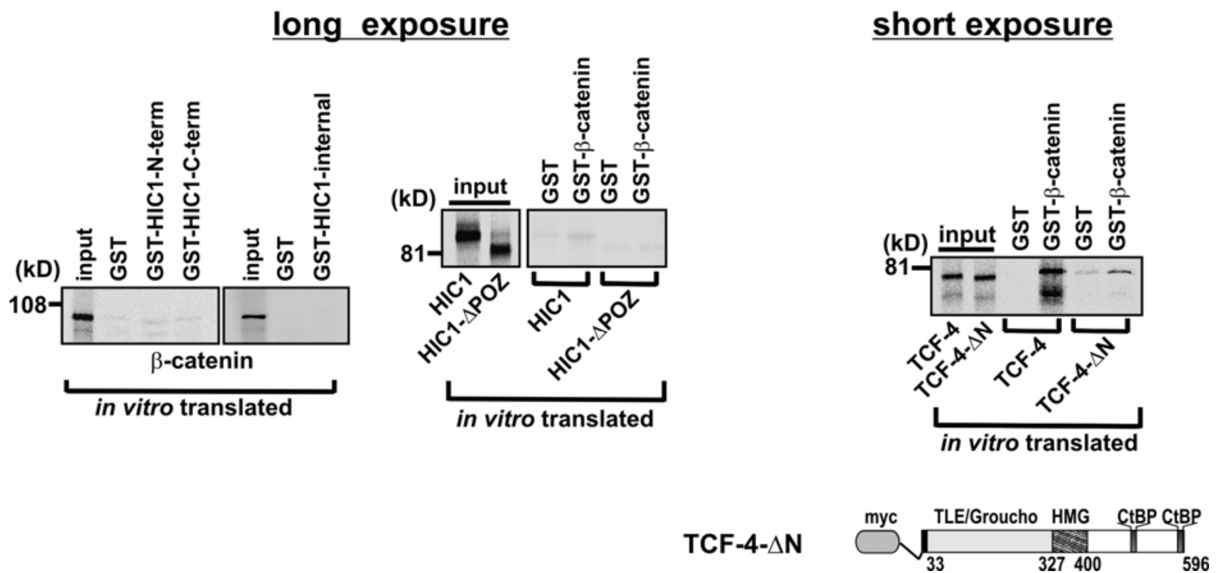
Supplementary Figure S2 HIC1 bodies in DLD-1 adenocarcinoma cells contain endogenous TCF-4. Colocalization of HIC1 with endogenous TCF-4. Confocal micrographs of DLD-1 cells transfected with full-length Flag-HIC1 construct stained with anti-Flag and anti-TCF-4 . Bar, 10μm.



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

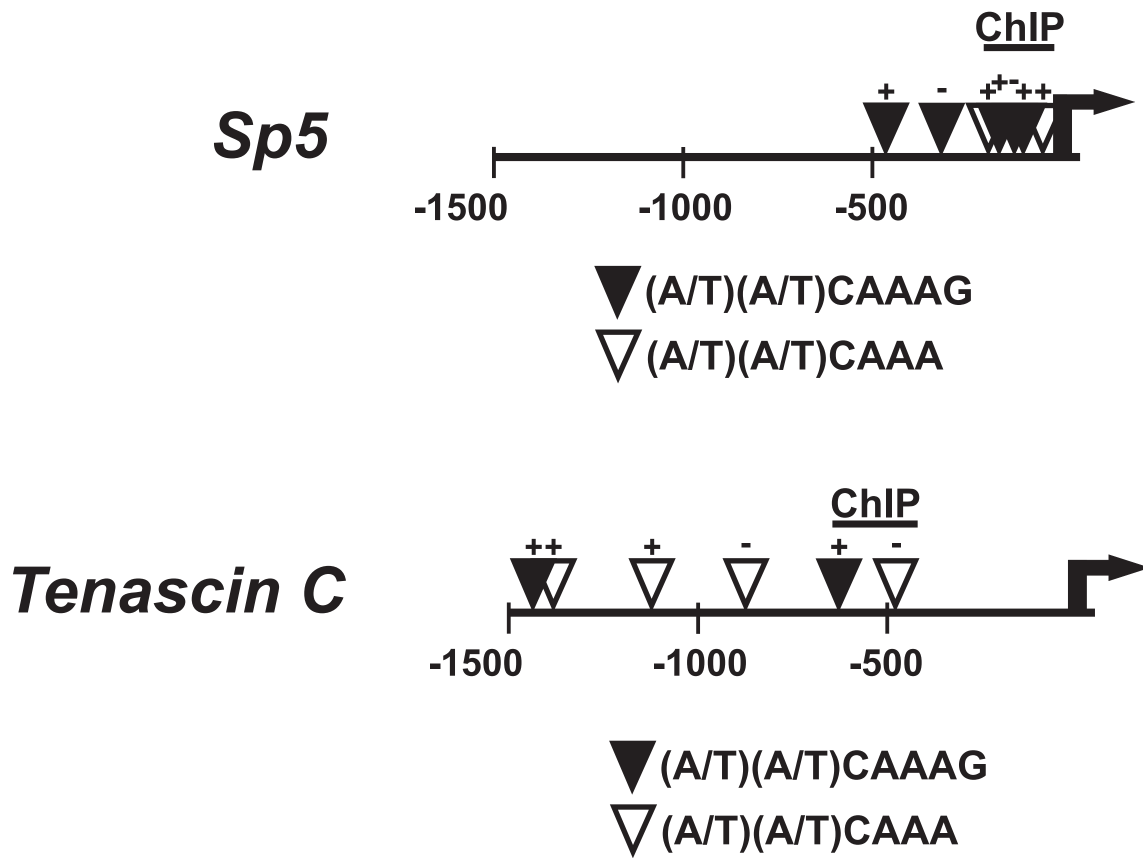
TCF-4-ΔN construct lacking the main β-catenin interaction domain (used as a negative control) is also shown (bottom).

Supplementary figure 3



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.

Supplementary Figure 4



4. Conclusion

This thesis is based on four publications that aim to increase the knowledge about Wnt signaling pathway. Our results are summed up below.

I. Firstly, at the level of plasmatic membrane, there are Wnt ligands which trigger the signaling cascade. The ligands are double acylated and we provide the evidence of sequential acyl adding with serine palmitoleoylation being the condition of cysteine palmitoylation. Moreover, lipid adducts are related to the presence of Wnt proteins on the extracellular matrix, which is connected to their ability to signal.

II. Secondly, we observed an anti-apoptotic effect of the Wnt expressing cells on pre-B leukemia-derived cell lines upon TRAIL induction. The pro-survival signal is not caused directly by Wnt signaling but rather another molecule induced in the Wnt expressing embryonic fibroblasts takes part. Nevertheless, Wnt-induced decrease of pro-apoptotic TRAIL receptors expressed by leukemia cells may be related to observed phenomenon. Because many primary tumors are TRAIL-insensitive, our data may touch the mechanism that cancer cells use.

III. Further, focusing on the nucleus we describe a novel TCF4 interacting protein called Dazap2 which modulates TCF4 affinity for its specific DNA sequence.

IV. And lastly we found a new repression mechanism of another TCF4 binding partner - HIC1. It subsists in draging the TCF4/β-catenin complex into the nuclear HIC1 bodies, away of Wnt-responsive elements, and inhibiting so Wnt induced transcription.

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