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Structure, function and disease relevance of Wnt inhibitory factor 1, a secreted protein controlling the Wnt and hedgehog pathways

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ABSTRACT

Wnts and Hedgehogs (Hh) are large, lipid-modified extracellular morphogens that play key roles in embryonic development and stem cell proliferation of Metazoa. Both morphogens signal through heptahelical Frizzled-type receptors of the G-Protein Coupled Receptor family and there are several other similarities that suggest a common evolutionary origin of the Hh and Wnt pathways. There is evidence that the secreted protein, Wnt inhibitory factor 1 (WIF1) modulates the activity of both Wnts and Hhs and may thus contribute to the intertwining of these pathways. In this article, we review the structure, evolution, molecular interactions and functions of WIF1 with major emphasis on its role in carcinogenesis.

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Cancer; epigenetic silencing; evolution; hedgehog; promoter hypermethylation; Wnt; Wnt inhibitory factor 1

Introduction

Cell proliferation of embryonic and adult tissues of animals is controlled by diverse signaling pathways including the Notch, epidermal growth factor (EGF), transforming growth factor β (TGFβ), Hedgehog (Hh) and Wnt pathways. Thanks to recent advances in comparative genomics and phylogenomics it has become clear that the Wnt and Hh signaling pathways are evolutionarily related and that Wnt inhibitory factor 1 (WIF1) may have a role in the control of both the Wnt and Hh signaling.

The Wnt signaling pathways

Wnt signaling plays essential roles in the control of cell proliferation, aberrant activation of Wnt signaling is responsible for the development of several types of cancer. Wnts exert their effects through the activation of three distinct but interconnected signaling pathways (Boutros et al. 1998; Kestler and Kühl 2008; Nusse et al. 2008; van Amerongen, Mikels, and Nusse 2008; van Amerongen and Nusse 2009; Clark, Nourse, and Cooper 2012).

The canonical Wnt/β-catenin pathway, the non-canonical Wnt/planar cell polarity (PCP) and Wnt/calcium pathways have been shown to mediate signaling through interaction of Wnts with heptahelical Frizzled-type receptors. Disheveled proteins play a central role both in canonical and non-canonical Wnt signaling: by binding to the cytoplasmic C-terminal tails of Frizzled receptors they transduce the Wnt signal to down-stream effectors (Figure 1).

Canonical Wnt signals are transmitted through Frizzled receptors which form co-receptor complexes with the low-density lipoprotein receptor related proteins 5/6 (LRP5/6) (Figure 1(A)). The key player of the canonical Wnt signaling pathway is β-catenin. In the absence of a Wnt stimulus the transcriptional coactivator β-catenin is degraded by a multiprotein destruction complex that includes axin, adenomatous polyposis coli (APC), the Ser/Thr kinases glycogen synthase kinase 3β (GSK3β) and casein kinase 1 (CK1) and the ubiquitin ligase β-TrCP. β-Catenin is phosphorylated by GSK3β and CK1, subsequently ubiquitinated and degraded in the proteasomes (Stamos and Weis 2013). Binding of Wnt proteins to Frizzled-Lrp5/6 receptor complexes leads to GSK3β inactivation, resulting in the release of unphosphorylated β-catenin from the multiprotein destruction complex, β-catenin is then translocated into the nucleus where it binds to the transcription factor,

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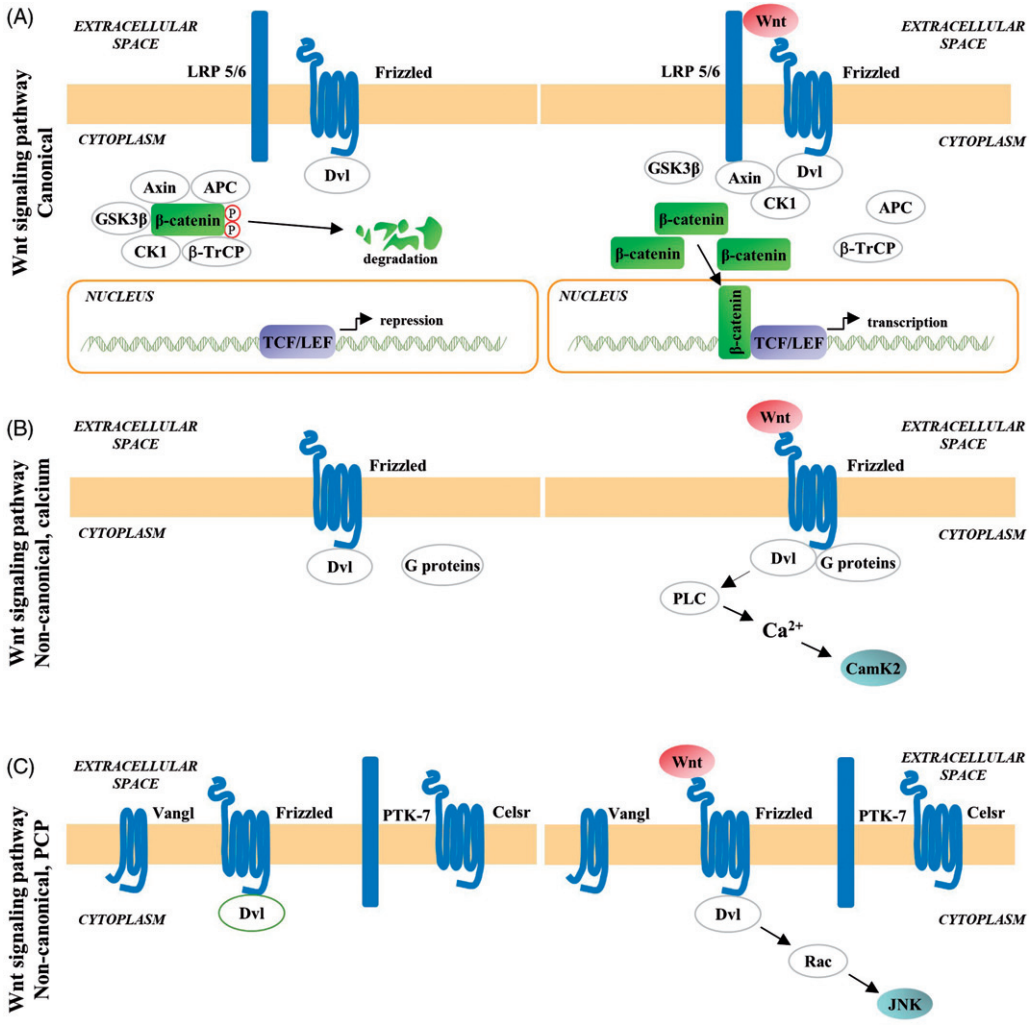

Figure 1. Frizzled-dependent Wnt signaling pathways. Wnt ligands transduce their signal through three Frizzled-dependent pathways: the canonical Wnt/β-catenin pathway, the non-canonical Wnt/calcium and Wnt/planar cell polarity pathways. Disheveled proteins (Dvl) play a central role both in canonical and non-canonical Wnt signaling. (A) The canonical pathway requires the co-receptor LRP5/6. In the absence of a Wnt stimulus, the transcriptional coactivator β-catenin is phosphorylated by a multiprotein complex composed of adenomatous polyposis coli (APC), axin, glycogen synthase kinase 3β (GSK3β) and casein kinase 1 (CK1), modified by the ubiquitin ligase β-TrCP and subsequently degraded in the proteasomes. Wnt binding leads to the accumulation of unphosphorylated β-catenin that can enter the nucleus and together with the transcription factor TCF/LEF induces the expression of Wnt target genes. (B) In the calcium pathway binding of Wnt to Frizzled results in activation of phospholipase C (PLC), leading to an increase of cytosolic calcium (Ca^{2+}) concentration and the subsequent activation of calcium-dependent kinase 2 (CamK2). (C) In the PCP pathway, activation of the receptor complexes containing Frizzled and transmembrane proteins Vangl, Celsr and PTK7 lead to activation of RacGTPases (Rac) and JNK1, promoting cytoskeleton remodeling.

T-cell factor/lymphoid enhancer-binding factor (TCF/LEF) and induces the expression of Wnt target genes.

In the case of the Wnt/calcium pathway (Figure 1(B)), binding of Wnts to Frizzled receptors leads to an increase in intracellular calcium concentration and activation of calmodulin-dependent protein kinase type 2 (CamK2). In the Wnt/ PCP pathway, Wnt activation of receptor complexes containing Vang-like transmembrane proteins (Vangl), Cadherin Egf Lag Seven-pass G-type Receptor 1 (Celsr) and tyrosine kinase 7 (PTK7) leads to activation of Rac/Rho GTPases and subsequently to activation of kinases such as c-jun N-terminal kinase (JNK), promoting cytoskeleton remodeling (Figure 1(C)).

Frizzled-type receptors, however, are not the only membrane proteins that bind Wnts. Domains closely related to the ligand-binding Fz domain of Frizzled receptors have been identified in Ror- and MuSK-type receptor tyrosine kinases (Masiakowski and Yancopoulos 1998; Saldanha, Singh, and Mahadevan 1998; Xu and Nusse 1998) and these receptor tyrosine kinases were also found to modulate Wnt signaling cascades (Hikasa et al. 2002; Oishi et al. 2003; Green, Kuntz, and Sternberg 2008; Burden, Yumoto, and

Zhang 2013; Martinez et al. 2015; Stricker, Rauschenberger, and Schambony 2017; Roy, Halford, and Stacker 2018). Inactive tyrosine-protein kinase 7, a receptor tyrosine kinase with an extracellular region composed of seven immunoglobulin-like domains, has also been shown to bind Wnts (Peradziryi et al. 2011).

Furthermore, the extracellular part of receptor tyrosine kinases of the Ryk family was shown to contain a domain related to the WIF domain of WIF1 (Patthy 2000) and these receptor tyrosine kinases were also shown to be involved in Wnt-binding (Yoshikawa et al. 2003; Lu et al. 2004; Inoue et al. 2004; Cadigan and Liu 2006; Green, Nusse, and van Amerongen 2014; Roy, Halford, and Stacker 2018).

The tyrosine kinase domains of the Wnt-binding Ryk-, Ror- and PTK7 receptor tyrosine kinases, however, deviate from the consensus tyrosine kinase sequence. They have atypical amino acid residues at multiple sites essential for kinase activity, therefore, they are predicted to be pseudokinases (Hovens et al. 1992; Stacker et al. 1993; Yoshikawa et al. 2001; Mendrola et al. 2013; Murphy et al. 2014). In the absence of kinase activity, the Wnt-binding receptor pseudokinases may function as co-receptors or competitive inhibitors of Frizzled receptor-mediated Wnt signaling. Interestingly, Ryk undergoes sequential proteolytic cleavage to release the extracellular region into the extracellular space. A metalloprotease cleaves between the WIF domain and transmembrane helix in the Ryk extracellular region to shed the extracellular Wnt-binding WIF domain (Halford et al. 2013). The shedding of the extracellular WIF domain of Ryk raises the possibility of a Wnt sequestration role: these soluble fragments may bind Wnts to prevent them from stimulating membrane-bound receptors (Roy, Halford, and Stacker 2018).

The activity of Wnts is regulated by a variety of secreted extracellular proteins that interfere with the formation of the Wnt-receptor complexes. Members of the Dickkopf family bind to LRP5/6 and prevent association of the receptor with Wnt, whereas Cerberus, WIF1 and members of the secreted frizzled-related protein (SFRP) family bind directly to Wnts and prevent their binding to the receptor complex (Kawano and Kypta 2003; Filipovich et al. 2011; Cruciat and Niehrs 2013; Malinauskas and Jones 2014).

Wnt signaling is also modulated by a variety of other proteins that may act as Wnt inhibitors and activators (Malinauskas and Jones 2014). Prominent among these are heparan sulfate proteoglycans, such as glypicans (Yan and Lin 2009; Yan et al. 2009).

The key importance of the canonical Wnt/β-catenin pathway in stem cell control may be illustrated by the fact that mutations that inactivate the tumor suppressors APC and axin or activate β-catenin, resulting in constitutive activation of the Wnt signaling pathway, are among the most frequent causes of carcinogenesis (Zhan, Rindtorff, and Boutros 2017). The genes for these proteins (*APC*, *AXIN1* and *CTNNB1*) are included in the list of the 125 most important genes whose mutation drives carcinogenesis (Vogelstein et al. 2013). Additional genes of the Wnt pathways (e.g. *BTRC, DKK3*, *DKK4*, *LEF1*, *PTK7, ROR1*, *ROR2*, *RYK*, *SFRP4* and *WIF1*) are included in the Cancer Gene Census (Sondka et al. 2018, https://cancer.sanger.ac.uk/census) and/or in the list of cancer genes (Candidate Cancer Gene Database, Abbott et al. 2015, http://ccgd-starrlab.oit.umn.edu/about.php) identified by forward genetic screens in mice (see Supplementary Table S1).

Furthermore, the crucial importance of the canonical Wnt/β-catenin pathway in carcinogenesis is underlined by the fact that epigenetic changes (DNA methylation, histone modification and interference by noncoding RNAs) of practically all key protein components of the canonical Wnt signaling pathway (Wnt ligands, Frizzled receptors, LRP5/6, Dickkopfs, SFRPs, WIF1, β-catenin, axin, APC, casein kinase 1, TCF/LEF transcription factor) have been associated with cancer initiation and progression (Wils and Bijlsma 2018). The importance of Wnt-antagonists in the control of cell proliferation may be illustrated by the fact that epigenetic silencing of the genes for WIF1, SFRPs and Dickkopf proteins is associated with aberrant activation of the Wnt-β-catenin pathway in a variety of cancers, whereas restoration of their expression inhibits tumor progression (Filipovich et al. 2011).

The hedgehog signaling pathways

Hh signaling is also essential for the control of cell proliferation in embryonic and adult tissues and aberrant Hh signaling activity may also lead to the development of cancers. Hhs also exert their effects through the activation of several different intracellular signal transduction pathways (Figure 2).

In the canonical smoothened-dependent signaling pathway (Figure 2(A)), Hh regulates the activity of GLI transcription factors. In this pathway, in the absence of an Hh ligand, Patched (PTCH), a 12-pass transmembrane Hh receptor, inhibits the activity of the 7-pass transmembrane protein Smoothened

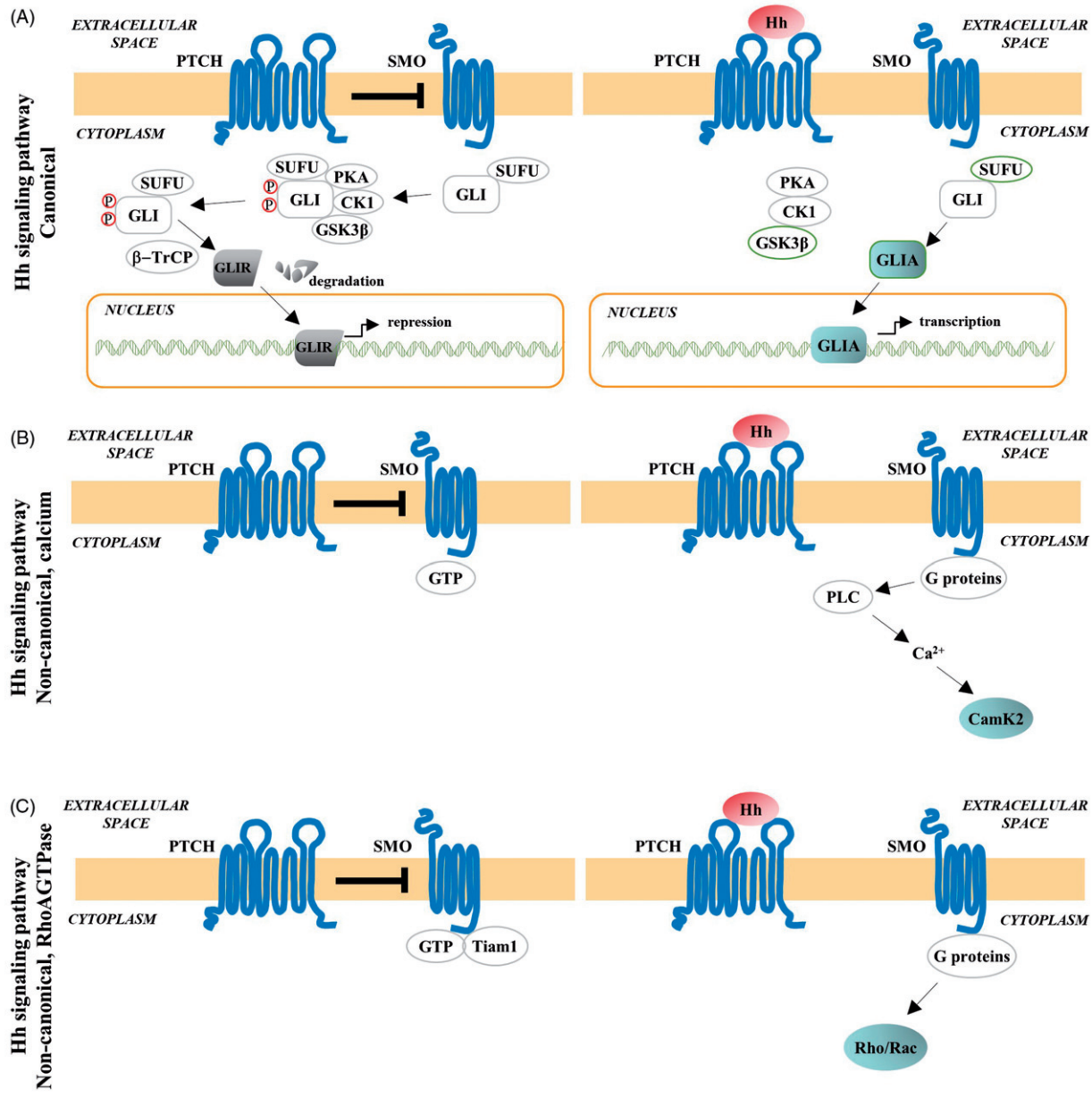

Figure 2. Smoothened-dependent Hh signaling pathways. Hh ligands transduce their signal through three Smoothened-dependent pathways: the canonical pathway, the non-canonical Hh/calcium and Hh/RhoAGTPase signaling pathways. (A) In the canonical Smoothened-dependent pathway signaling is mediated by the GLI zinc finger transcription factors. In the absence of the Hh ligand, the 12 transmembrane Hh receptor Patched (PTCH) inhibits the function of the 7 transmembrane protein Smoothened (SMO). In this inactive state full-length GLI is modified by protein kinase A (PKA), glycogen synthase kinase-3β (GSK3β), casein kinase 1 (CK1) and by the ubiquitin ligase β-TrCP and is subsequently cleaved into the transcriptional repressor form, GLIR. The repressor form GLIR translocates to the nucleus and inhibits the expression of Hh target genes. Binding of Hh to Patched (PTCH) relieves the inhibition of Smoothened (SMO) and as a result of the activity of SMO full-length GLI bypasses the phosphorylation by PKA, GSK3β and CK1, leading to the formation of activated GLI (GLIA). Activity of SMO also results in the inhibition of Suppressor of fused homolog (SUFU), the suppressor of GLIA, permitting the formation of GLIA. GLIA then translocates to the nucleus where it induces the expression of Hh target genes. (B) In the non-canonical Hh/Calcium pathway binding of ligand to SMO results in activation of phospholipase C (PLC), leading to an increase of cytosolic calcium (Ca^{2+}) concentration and the subsequent activation of calcium-dependent kinase 2 (CamK2). (C) In the non-canonical Hh/RhoAGTPase signaling pathway, Hh signals through the activity of SMO via the stimulation of Rac1 and RhoAGTPases.

(SMO), blocking the signaling activity of the pathway. In this inactive state full-length GLI is modified by protein kinase A (PKA), GSK3β, CK1 and by the ubiquitin ligase β-TrCP and is subsequently cleaved into the transcriptional repressor form, GLIR. The repressor form GLIR translocates to the nucleus and inhibits the expression of Hh target genes. Binding of Hh to PTCH relieves the inhibition of SMO and as a

result of the activity of SMO full-length GLI bypasses the phosphorylation by PKA, GSK3β and CK1, leading to the formation of activated GLI (GLIA). Activity of SMO also results in the inhibition of suppressor of fused homolog (SUFU), the suppressor of GLIA, permitting the formation of GLIA. GLIA then translocates to the nucleus where it induces the expression of Hh target genes (Ingham and McMahon 2001; Wilson and Chuang 2010; Robbins, Fei, and Riobo 2012; Briscoe and Thérond 2013; Sari et al. 2018).

There are several GLI-independent non-canonical Hh signaling cascades that respond to Hh and elicit cellular responses, ranging from Ca^{2+} signaling, cytoskeletal rearrangement to apoptosis. Some of them are SMO-dependent others do not require the activity of this transmembrane protein (Robbins, Fei, and Riobo 2012; Teperino et al. 2012, 2014). In one of the SMO-dependent non-canonical pathways, SMO activation is coupled to a rapid Ca^{2+} influx and subsequent activation of CamK2 (Figure 2(B)). In another SMO-dependent, GLI-independent non-canonical Hh signaling pathway the activity of SMO leads to stimulation of Rac1 and RhoA small GTPases (Polizio et al. 2011) (Figure 2(C)). There is also evidence for a SMO-independent, GLI-independent Hh signaling pathway that operates via PTCH and inhibits the activation of caspase-3 and promotes cell survival by inactivating the PTCH pro-apoptotic activity (Chinchilla et al. 2010).

The various Hh signaling pathways are modulated by interactions of Hh with multiple membrane-associated protein partners including cell adhesion molecule-related/down-regulated by oncogenes (CDO), Brother of CDO (BOC), growth arrest-specific protein 1 (GAS1), Hh interacting protein (HHIP) and glypicans (GPCs) (Beachy et al. 2010). CDO and BOC proteins are single-pass type I transmembrane proteins, their extracellular parts contain multiple immunoglobulin and fibronectin type III repeats. Both CDO and BOC bind Hh proteins and positively regulate Hh signaling (Tenzen et al. 2006; Yao, Lum, and Beachy 2006). The GPI-anchored cell surface associated protein GAS1 also binds and positively regulates Hh signaling (Allen, Tenzen, and McMahon 2007; Martinelli and Fan 2007). On the other hand, Hh interacting protein (HHIP) that binds Hhs with high affinity, functions as an inhibitor of Hh signaling (Chuang and McMahon 1999; Chuang, Kawcak, and McMahon 2003). The heparan sulfate proteoglycans, glypicans have also been shown to play an important role in the modulation of the signaling activity of Hhs (Gallet, Staccini-Lavenant, and Therond 2008; Capurro et al. 2008; Kim et al. 2011).

Interestingly, the secreted protein Shifted (Shf), the *Drosophila* ortholog of vertebrate WIF1, was also shown to bind and control the distribution and movement of Hh (Glise et al. 2005; Gorfinkiel et al. 2005).

The importance of Hh signaling in the control of cell proliferation is underlined by the observation that mutations of SMO and PTCH1 that constitutively activate Hh signaling are among the major causes of carcinogenesis (Riobo 2012; Cannonier and Sterling 2015; Wu et al. 2017; Girardi et al. 2019). The genes of SMO (*SMO*) and PTCH1 (*PTCH1*) are included in the list of genes whose mutation drives carcinogenesis (Vogelstein et al. 2013). Several other genes of the Hh pathways (e.g. desert Hh *DHH*, sonic Hh *SHH*, *BTRC, GLI1, SUFU*) are also included in the Cancer Gene Census (Sondka et al. 2018, https://cancer.sanger.ac.uk/census) and/or in the Candidate Cancer Gene Database (Abbott et al. 2015, http://ccgd-starrlab.oit.umn.edu/about.php; see Supplementary Table S1).

The role of the canonical Hh pathway in cancer initiation and progression is also supported by the fact that epigenetic changes of practically all key components of this signaling pathway (genes of Hh ligands, *BOC*, *BTRC*, *GAS1*, *GLI1*, *GLI2*, *GLI3*, *HHIP*, *PTCH1*, *SMO* and *SUFU*) have been associated with carcinogenesis (Wils and Bijlsma 2018).

Similarities of Wnt and hedgehog pathways suggesting their common evolutionary origin

It has been pointed out previously that there are several similarities of the Hh and Wnt pathways that suggest their common evolutionary origin (Kalderon 2002; Nusse 2003).

The features they share include the use of GSK3β, CK1 and the ubiquitin ligase β-TrCP to regulate the proteolysis of the key transcriptional effectors of their canonical pathways (β-catenin in the Wnt pathway, GLI in the Hh pathway, see Figures 1(A) and 2(A)).

SUFU, originally identified as a protein that binds to GLI and antagonizes its activity in the canonical Hh pathway, was also found to bind to β-catenin, export it from the nucleus thereby negatively regulating β-catenin-dependent transcription (Meng et al. 2001). Thus, there is evidence that SUFU may act as a common regulator of both the Hh and Wnt pathways and contribute to intertwining the two canonical pathways (Taylor et al. 2004; Min et al. 2011).

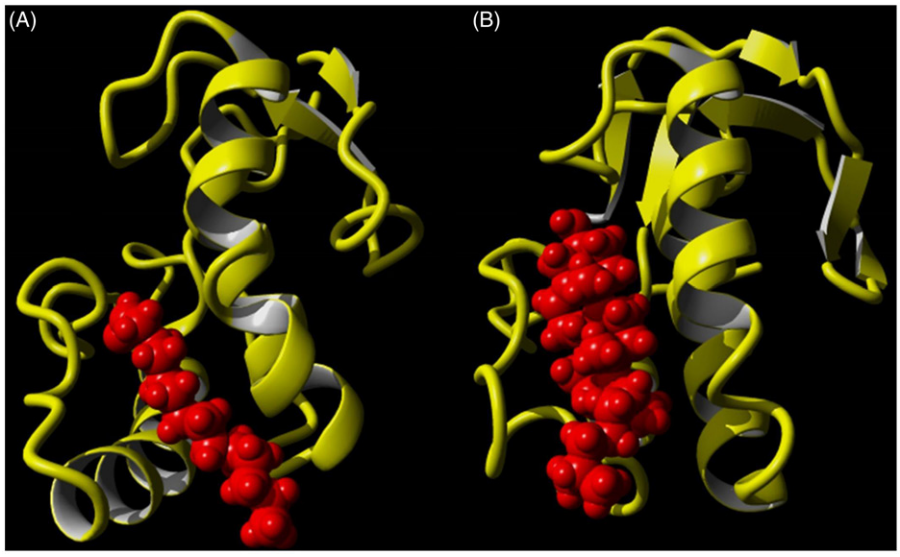

Figure 3. Lipid binding sites of the Fz domains of Frizzled and Smoothened receptors. (A) Structure of the Fz domain of Frizzled-8 in complex with the palmitoleic acid side-chain of Wnt8 ligand (4f0a.pdb). The backbone structure of the Fz domain is shown in yellow, the palmitoleic acid side-chain of Wnt8 is highlighted in red. (B) Structure of the Fz domain of Smoothened in complex with cholesterol ligand (6D35.pdb). The backbone structure of the Fz domain is shown in yellow, the cholesterol ligand is highlighted in red.

Recent studies suggest that there is also some similarity of the non-canonical Frizzled-Wnt and the non-canonical SMO-dependent Hh cascades in that both the Wnt/calcium and the Hh/calcium pathways mediate Ca^{2+} influx and subsequent activation of CamK2 (see Figures 1(B) and 2(B)). The similarity of Hh and Wnt signaling also holds for the cascades in which activation of Frizzled or SMO leads to stimulation of Rac and RhoA small GTPases, resulting in rearrangements of the cytoskeleton (see Figures 1(C) and 2(C)).

A further similarity of the Hh and Wnt pathways is that both Hhs and Wnts are lipid-modified and that the lipid modification of these morphogens is crucial for their signaling activity.

Wnts and Hhs are palmitoleoylated and palmitoylated by porcupine and Hh acyltransferase, respectively; both enzymes are members of the membrane bound O-acyltransferase (MBOAT) family (Buglino and Resh 2012). Wnt proteins are palmitoleoylated in their N-terminal parts and this modification is essential for the signaling activity of these morphogens (Willert et al. 2003; Doubravska et al. 2011). Analysis of the structure of Wnt8 in complex with the ligand binding cysteine-rich Fz domain of the receptor, Frizzled-8, has provided an explanation for the importance of this lipid modification: the ligand-receptor interaction was found to be dominated by the palmitoleic acid side-chain of Wnt8 that is inserted into a deep groove of the ligand-binding Fz domain of the receptor (Janda et al. 2012; Figures 3(A) and 4(A)).

The precursors of Hh proteins are modified by palmitic acid at the very N-terminal end of the proteins after their signal sequence has been removed. The C-terminal protease domain of the Hh precursor cleaves the precursor in an autocatalytic manner to release the active N-terminal signaling domain and during this cleavage, the C-terminus of the signaling domain becomes covalently modified by a cholesterol molecule (Porter et al. 1996; Porter, Young, and Beachy 1996; Pepinsky et al. 1998).

N-terminal palmitoylation of Hh proteins is indispensable for their signaling activity: fatty-acylated Hh is far more active than the unacylated ligand, blocking Hh palmitoylation blocks Hh signaling (Williams et al. 1999; Kohtz et al. 2001; Petrova et al. 2013; Tukachinsky et al. 2016). Recent studies on "native" palmitoylated Hh (i.e. the ligand that has both a C-terminal cholesterol and an N-terminal fatty-acid modification) in complex with its receptor, PTCH1, have provided an explanation for the importance of the palmitoylation of the Hh ligand. These studies have revealed that the palmitoylated N terminus of the extended N-terminal part of the ligand inserts into a cavity between the extracellular domains of PTCH1 and dominates the receptor-ligand interface (Qi et al. 2018; Figure 4(B)).

It should be pointed out that, due to their lipidation, Wnts and Hhs are highly hydrophobic, a property expected to cause their retention in the plasma membrane, close to the site of their synthesis. Nevertheless, Wnts and Hhs can act at both long

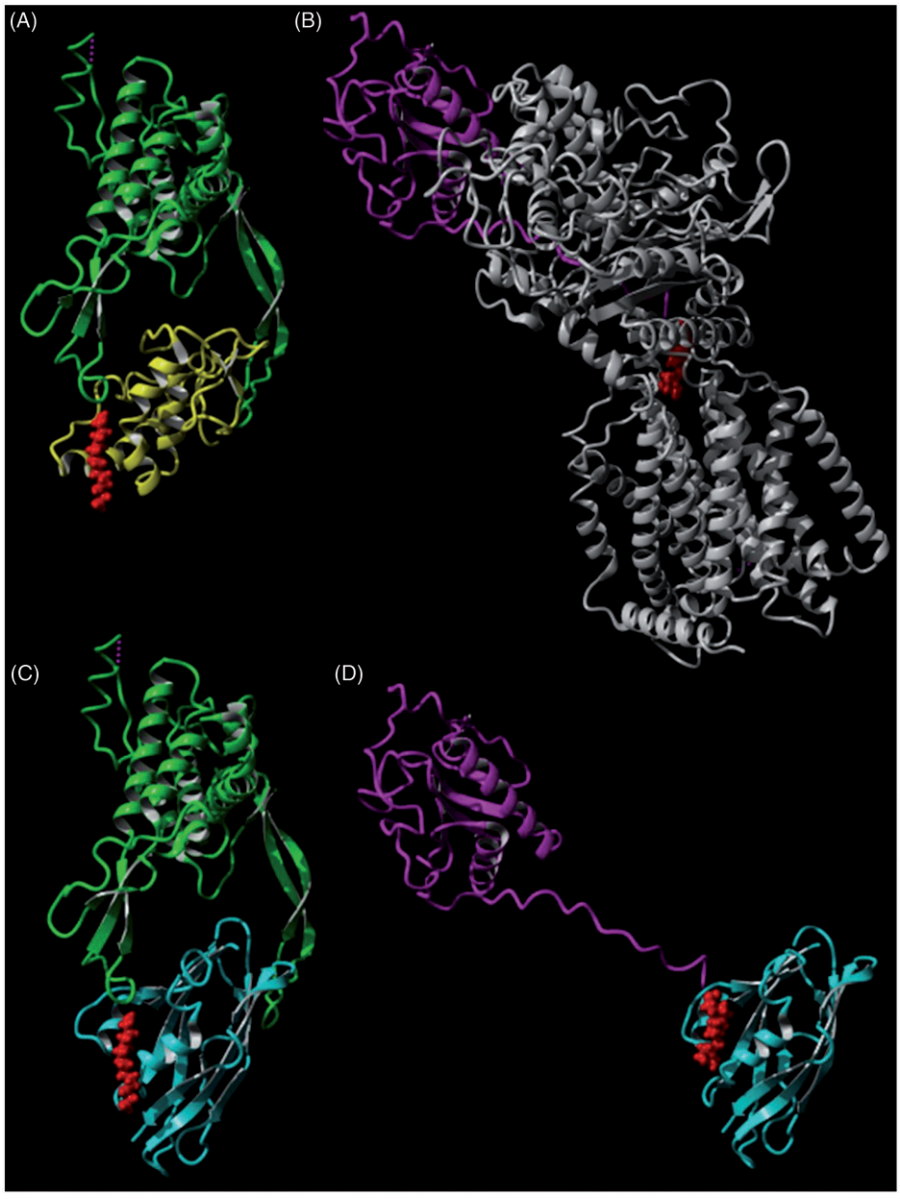

Figure 4. Complexes of Wnt and Hedgehog with their receptors and with Wnt Inhibitory Factor 1. (A) Structure of Wnt8 in complex with the Fz domain of the receptor, Frizzled-8 (4f0a.pdb). The backbone structure of Wnt is shown in green, the Fz domain is shown in yellow, the palmitoleic acid side-chain of Wnt8 is highlighted in red. Note that Wnt8 grasps the Fz domain at two distinct sites and in one of these sites the ligand-receptor interaction is dominated by the palmitoleic acid side-chain of Wnt8. (B) Structure of hedgehog in complex with its receptor, Patched1 (6d4j.pdb). The backbone structure of Patched1 is shown in gray, the palmitoylated N-terminal domain of Hedgehog is shown in magenta, the palmitoyl moiety is highlighted in red. Note that that the palmitoylated N terminus of the extended N-terminal part of the ligand inserts into a cavity between the extracellular domains of Patched1. (C) Model of the complex formed by interaction of Wnt (4f0a.pdb) with the WIF domain of Wnt Inhibitory factor 1 (2ygn.pdb). The backbone structure of palmitoleoylated Wnt is shown in green, the WIF domain is shown in blue. Note that in this model the palmitoleic acid moiety of Wnt (highlighted in red) inserts into the alkyl-binding site of the WIF domain of Wnt Inhibitory factor 1. (D) Model of the complex formed by interaction of the palmitoylated N-terminal domain of Hedgehog (6d4j.pdb) with the WIF domain of Wnt Inhibitory factor 1(2ygn.pdb). The backbone structure of the N-terminal domain of Hedgehog is shown in magenta, the WIF domain is shown in blue. Note that in this model the palmitoyl moiety of Hh (highlighted in red) inserts into the alkyl-binding site of the WIF domain of Wnt Inhibitory factor 1.

and short range to control growth and patterning during development raising questions as to the mechanisms that permit their movement (Panáková et al. 2005).

The constraints that lipidation imposes on the solubility and spread of Wnts and Hhs within tissues suggests that, in order to diffuse and transfer from secreting to receiving cells, they need to shield their

lipid moiety. Several models have been proposed: one possibility is that they travel on lipoprotein particles. In harmony with this model, both Wnts and Hhs were found to copurify with lipoprotein particles, suggesting that they may act as vehicles for the movement of lipid-linked morphogens (Panáková et al. 2005; Langton, Kakugawa, and Vincent 2016).

Wnts and Hhs may be rendered soluble and mobile in the extracellular space by forming complexes with Wnt- and Hh-binding proteins. For example, the secreted Wnt-interacting molecule Swim facilitates Wnt diffusion through the extracellular matrix and promotes long-range Wnt signaling by maintaining the solubility of Wnt (Mulligan et al. 2012).

Recent studies have provided some insight as to how interactions of Hhs may aid their transport from secreting to receiving cells (Hall, Cleverdon, and Ogden 2019). The transporter-like protein Dispatched (Disp) serves to mobilize Hh ligands to travel toward distant cellular targets. In the absence of Disp function, Hhs fail to release from signal-producing cells; although juxtacrine signaling to cells directly adjacent to a Hhs source is maintained without Disp, long-range targets do not receive ligand.

Disp is a homolog of the Hh receptor PTCH; they are both members of a family of bacterial efflux pumps. These proteins have a characteristic topology with 12 transmembrane domains, arranged in two halves each containing six transmembrane helices and one large extracellular globular domain (see PTCH in Figure 2). Based on the homology of Disp and PTCH, it is plausible to assume that Hh binds to Disp in a way similar to that observed in the case of PTCH (Figure 4(B)). Hh is transferred from the Disp-Hh complex to the Hh-binding protein secreted protein Scube2 and/or heparan-sulfate proteoglycans, allowing the travel of Hh to more distant cellular targets.

A further similarity of the Wnt and Hh pathways is that heparan-sulfate proteoglycans play a significant role in the transport of both Wnts and Hh proteins. Heparan-sulfate proteoglycans are proteins with branched sugar side chains that are expressed on the cell surface and form complexes with a variety of signaling molecules, including Wnts and Hhs (Xie and Li 2019). Glypicans are among the most important groups of heparan sulfate proteoglycans. These proteins contain an N-terminal α-helical fold followed by a stalk region that is attached to the outer cell membrane *via* a glycosylphosphatidyl-inositol anchor. The stalk regions contain a number of attachment sites for heparan sulfate chains (Kim et al. 2011). The heparan sulfate moieties of glypicans play a key role in their ability to modulate the signaling activity of a wide variety of heparin-binding growth factors, such as fibroblast growth factor, Hhs, Wnts and members of the TGFβ families. All these morphogens elicit concentration-dependent responses in target cells and their interaction with heparan sulfate moieties of glypicans is required to establish their proper distribution (Yan and Lin 2009). Recent work, however, has demonstrated a role for the N-terminal protein domain that lacks heparan sulfate modifications in mediating responsiveness to Wnt and Hh signals, the protein cores may thus mediate functions that are more specific to particular glypican-morphogen interactions (Capurro et al. 2008; Yan et al. 2009; Williams et al. 2010; Yan et al. 2010).

Finally, the most striking feature shared by the Wnt and Hh pathways is that signaling by the ligands is mediated by closely related heptahelical transmembrane receptors (Frizzled and SMO, respectively) that belong to the same family of G-protein coupled receptors (GPCRs). GPCRs are usually grouped into 6 classes based on sequence homology and functional similarity. Frizzled and SMO belong to the F group, their domain architectures are identical: they have seven transmembrane helices and an N-terminal extracellular cysteine-rich ligand-binding domain, the Fz domain. There is now compelling evidence that this unique domain architecture evolved in Eukaryotes before the origin of animals (Prabhu and Eichinger 2006; Harwood 2008; Nordström et al. 2011; Krishnan et al. 2012; Yan et al. 2014).

Despite the structural similarity of the Frizzled and SMO receptors, their role in morphogen signaling shows major differences. First, the morphogen proteins initiating signaling of the Wnt and Hh pathways are unrelated. Studies on the structure of Wnts have revealed that these large secreted morphogens actually consist of two distinct structural domains: an N-terminal saposin-related domain and a C-terminal cystine knot cytokine-related domain (Janda et al. 2012; Bazan, Janda, and Garcia 2012). Based on these structural similarities it has been suggested that the common ancestor of Wnts was formed in Metazoa by fusion of a saposin-like protein with a cystine-knot cytokine (Bazan, Janda, and Garcia 2012).

Hh proteins also consist of two domains: an N-terminal ligand domain (the HH-signal domain) that gets released from the Hh precursor through the action of the C-terminal intein domain (the Hint domain). Studies on the evolutionary history of the constituent domains suggest that the domain architecture

characteristic of Hhs arose only in Metazoa (Adamska et al. 2007b; Matus et al. 2008; Roelink 2018).

Another major difference between the two pathways is that, whereas Wnt proteins bind to the Fz domain of Frizzled receptors, SMO receptors are not thought to interact with Hh proteins or any other extracellular protein ligand. Hh activates SMO indirectly by binding to PTCH, a 12-transmembrane protein that inhibits the activity of SMO. As a consequence of the PTCH-Hh interaction, SMO is released from the inhibitory activity exerted by PTCH (McCabe and Leahy 2015; Figure 2).

Nevertheless, recent studies have revealed some interesting similarities in the activation of Frizzled and SMO receptors as a consequence of the action of Wnt and Hh. Frizzled is activated by binding of Wnt to the extracellular Fz domain of the receptor. As discussed above, analysis of the structure of Wnt8 in complex with the Fz domain of Frizzled-8 has revealed that the two domains (the saposin-like and cytokine-like domains) of the Wnt morphogen grasp the Fz domain at two distinct binding sites. The palmitoleic acid side-chain of the N-terminal saposin-like domain of Wnt8 is inserted into a deep groove of the Fz domain of the receptor, whereas the C-terminal cytokine-related domain of Wnt8 forms contacts with a site on the opposite side of the Fz domain (Janda et al. 2012; Figures 3(A) and 4(A)).

SMO is activated by binding of cholesterol to the extracellular cysteine-rich Fz domain of the receptor. Recent studies on the crystal structures of SMO have shown that upon binding of cholesterol to the Fz domain, the Fz domain undergoes a dramatic reorientation and causes the transmembrane domain to adopt a conformation similar to active G-protein-coupled receptors (Huang et al. 2018). Significantly, the residues involved in binding of sterol ligands by the Fz domain of SMO are structurally equivalent with those contacting the Wnt lipid adduct in the lipid-binding groove of the homologous Fz domains of Frizzled receptors (Myers et al. 2013; Nachtergaele et al. 2013; Huang et al. 2018; Figure 3(A,B)).

In view of the common evolutionary origin of Frizzled and SMO receptors, the structural equivalence of their binding site for lipid-moieties of Wnts or cholesterol is probably more than a coincidence. Since Wnts and Hhs arose only in Metazoa (Nichols et al. 2006; Adamska et al. 2007a; Matus et al. 2008; Roelink 2018), it is plausible to assume that in lower eukaryotes the common ancestor of Frizzled and SMO receptors functioned as lipid- and sterol-sensing receptors. According to this view, with the divergence of the Frizzled and SMO families, SMO receptors retained their specificity for low molecular weight lipid ligands (such as cholesterol), whereas Frizzled receptors evolved to bind lipid molecules attached to the large, newly formed and expanding family of Wnt proteins. Although the lipid moieties of Wnts retained a critical role in their interaction with the Fz domain of Frizzled receptors, their protein–protein interactions with the saposin-like and cytokine-like domains of Wnts became of increasing importance. It is noteworthy in this respect that there is a striking asymmetry in the evolution of the SMO and Frizzled families in Metazoa. Whereas the Frizzled family expanded parallel with the expansion of the Wnt family, in the majority of Metazoa there is a single *SMO* gene (Yan et al. 2014; Schenkelaars et al. 2015).

Recent studies have also provided an insight into the molecular function of PTCH1 that explains how it inhibits the activity of SMO. Zhang et al. (2018) have shown that the structure of the PTCH1 protein shows striking transmembrane domain similarities to prokaryotic transporters. A central hydrophobic conduit with cholesterol-like contents resembles that used by other transporters, suggesting that PTCH has cholesterol transport activity and that PTCH1 inhibits SMO by controlling the availability of cholesterol for SMO. According to this model, binding of Hh to PTCH1 blocks its transporter activity, thereby relieving SMO from the inhibitory control of PTCH1.

Evolution of the Wnt and hedgehog pathways

Comparative genomics of various groups of Metazoa lend support to the view that key constituents of the three major Frizzled/SMO-dependent pathways evolved in early multicellular animals, concurrent with the evolution of multicellularity.

So far no Wnts or Hhs have been described from fungi, plants or unicellular eukaryotes, suggesting that the fusion of their constituent domains occurred in Metazoa (Adamska et al. 2007b; Bazan, Janda, and Garcia 2012). Although the Wnt and Hh signaling pathways appear to be inventions of Metazoa it is possible to identify some components of these signaling pathways in protozoa (Holstein 2012). Several Frizzled/SMO-like genes have been described in the social ameba *Dictyostelium discoideum*. Sixteen of these encode cysteine-rich Fz domain, indicating that the domain architecture typical of Frizzled/SMO receptors arose prior to the emergence of Metazoa (Harwood 2008). Orthologs of the Wnt co-receptor proteins LRP5/6, however, are apparently missing

from these slime molds (Eichinger et al. 2005; Prabhu and Eichinger 2006).

Of the main components of the canonical Wnt signal transduction cascade, GSK3β, CK1 and a protein related to β-catenin, aardvark, are present in *Dictyostelium*, but other key components of the Wnt signal transduction cascade, such as disheveled, axin and APC proteins, or orthologs of the TCF/LEF transcription factors have not been found, suggesting that these are metazoan-specific innovations (Harwood et al. 1995; Grimson et al. 2000; Coates et al. 2002; Schilde et al. 2004).

In the case of the simple metazoa, Porifera a basal set of Wnts, Wnt receptors and cytoplasmic transducers have been identified. The genome of the sponge *Amphimedon queenslandica* was shown to contain the main components of the canonical Wnt/β-catenin pathway: besides β-catenin, Frizzled receptor and GSK3β, three Wnt genes and genes for the coreceptor Lrp5/6, disheveled, axin, APC, transcription factor TCF/LEF and SFRPs have been identified (Adamska et al. 2007a, 2010; Srivastava et al. 2010). Studies on the sponge *Oscarella carmela* have also revealed that it expresses core components of the Wnt pathway (Wnts, Frizzled receptors, dickkopfs, β-catenin and disheveled) and the receptor tyrosine kinase MuSK. These analyses have also provided evidence for components of the Hh pathway (PTCH, SUFU), suggesting that key elements of both the Wnt and Hh pathways were in place early in animal evolution, before the divergence of sponge and eumetazoan lineages (Nichols et al. 2006). More recent studies on transcriptomes of eight sponge species belonging to four classes of porifera (*Hexactinellida*, *Demospongiae*, *Homoscleromorpha* and *Calcarea*) have shown that all sponge classes share an unexpectedly large complement of genes with other metazoans. Surprisingly, hexactinellid, calcareous and homoscleromorph sponges were found to share more genes with bilaterians than with nonbilaterian basal metazoans, suggesting loss of genes in the latter groups (Riesgo et al. 2014). Most components of the Hh signaling pathway were present in all sponge classes. Significantly, there is a *SMO* gene that clearly lies within the SMO family and does not cluster with the closely related Frizzled genes. The other key components of the pathway, PTCH, GSK3β, CK1, SUFU, GLI, β-TrCP and HHIP were all found in the poriferan transcriptomes. Apparently, most key components of the Wnt signaling pathways are also present: Wnt, Frizzled receptor, LRP5/6, disheveled, axin, APC, β-catenin, TCF/LEF, SFRPs, dickkopf and WIF1 were identified in at least some of the porifera classes. Although earlier studies on *Amphimedon queenslandica* have concluded that genes for constituents of the noncanonical Wnt pathways are missing (Adamska et al. 2007a, 2010) more recent studies on genomes and transcriptomes from all poriferan lineages confirmed that members of the PCP pathway, including Vangl and Celsr orthologs are present in one sponge lineage, suggesting that the full PCP pathway may have arisen in the last common ancestor of animals (Schenkelaars et al. 2016).

The genome of the Ctenophore *Mnemiopsis leidyi* also contains orthologs of the main components of Wnt signaling: four Wnt ligands, β-catenin, disheveled, two Frizzled receptors, SFRP, transcription factor TCF/LEF, LRP5/6, GSK3β, CK1, APC (Pang et al. 2010), but *Mnemiopsis leidyi* was found to lack most of the major genes necessary for Hh signaling, such as a Hh ligand, the SMO receptor and SUFU (Ryan et al. 2013).

The genome of the placozoan, *Trichoplax adhaerens*, was found to have three Wnt genes and genes for Frizzled receptors, GSK3β, β-catenin, disheveled, axin and transcription factor TCF/LEF (Srivastava et al. 2008). The *Trichoplax* genome also contains Vangl and Celsr orthologs, supporting the existence of a functional PCP pathway (Schenkelaars et al. 2016). However, Srivastava et al. (2008) did not find evidence for a functioning Hh pathway in *Trichoplax,* since there was no evidence for genes of Hh ligand, PTCH or SMO receptor, or GLI-like transcription factors.

The genomes of the Cnidarians *Nematostella* (Putnam et al. 2007) and *Hydra* (Chapman et al. 2010) contain members of all bilaterian Wnt gene subfamilies (Kusserow et al. 2005; Lee et al. 2006; Lengfeld et al. 2009) that act in the canonical and the PCP pathways, and all core components of the Wnt receptor and the β-catenin destruction complex are present. The number of Frizzled receptors was found to be lower than that of the ligands, suggesting that the radiation of Wnt genes was followed by diversification of the receptors. Orthologs of proteins involved in PCP signaling (Vangl, RhoA and RAC1) as well as components of the Ca^{2+} signaling pathway (PLC, PKC, CamK2 and Calcineurin) are also present in Cnidaria. Furthermore, several secreted Wnt antagonists have been identified. e.g. SFRP, WIF, Cerberus and dickkopf proteins (Technau et al. 2005; Augustin et al. 2006; Guder, Philipp, et al. 2006, Guder, Pinho, et al. 2006; Lee et al. 2006; Holstein, Watanabe, and Ozbek 2011; Technau and Steele 2011). Genomic surveys indicate that *Nematostella vectensis* possesses essentially the full repertoire of Hh signaling pathway

components found among bilaterian model organisms (Matus et al. 2008). These studies on *Nematostella vectensis* have identified two typical *Hh* genes encoding both ligand domain and an autocatalytic intein domain, a *PTCH* gene, two *SMO* genes, genes for two glypicans, a CDON/BOC/related Hh-binding type I receptor and orthologs of HHIP and GAS1 which bind extracellular Hh ligands and regulate their activity and range of movement. *Nematostella vectensis* was also shown to possess genes encoding GLI3 and core components of the GLI cleavage complex including SUFU, two β-TRCPs as well as associated GLI kinases (PKA, GSK3β and CK1). Conversely, orthologs of WIF1 were apparently missing from *Nematostella vectensis,* leading the authors to suggest that it evolved in bilateria after the divergence of cnidarians and bilaterians (Matus et al. 2008).

In summary, comparative genomic studies indicate that key elements of both the Wnt and Hh pathways were in place early in animal evolution, before the divergence of sponge and eumetazoan lineages, suggesting that Wnt and Hh signaling diverged at a very early stage of metazoan evolution.

Evolution of Wnt inhibitory factor 1

Since there is some controversy in the literature as to the presence or absence of WIF1 proteins in basal metazoan, we have reexamined the evolutionary history of this protein.

Hsieh et al. (1999) first characterized WIF1 from fish, amphibia and mammals as a secreted protein with an N-terminal WIF domain and five EGF-like domains (Figure 5(A)). Subsequently, it was shown that an ortholog of vertebrate WIF1s is also present in *Drosophila* (Glise et al. 2005; Gorfinkiel et al. 2005). The domain architecture of the *Drosophila* Shf protein is identical with that of vertebrate WIF1 orthologs in as much as it also has an N-terminal WIF module and five EGF-like domains (Figure 5(A)). In addition to Vertebrates and Arthropods, WIF domains orthologous with the WIF domains of WIF1 are also present in Porifera, Cnidaria, Annelida, Brachiopoda, Mollusca, Echinodermata and Hemichordata (see Supplementary Table S2 and Figure 5(A)), indicating that this domain-type was present prior to the divergence of metazoan phyla. It is noteworthy that the architecture of the protein identified in the sponge, *Oopsacas minuta*, has significant similarity to bilaterian WIF1 proteins: it has an N-terminal WIF domain, three EGF domains and a C-terminal immunoglobulin domain, suggesting that WIF domains and EGF domains were fused in the first Metazoa, to yield the common ancestor of WIF1 proteins (Figure 5(A)).

Cnidaria also have secreted proteins containing WIF domains orthologous with those of WIF1 proteins, these proteins, however, lack EGF domains. They have C-terminal NTR domains, a WIF-NTR domain architecture reminiscent of the Fz-NTR architecture of SFRPs (see Supplementary Table S2 and Figure 5(B)).

It should be emphasized that WIF domains distantly related to those of WIF1 proteins are also present in Ryk-type receptor kinases (Patthy 2000). Proteins with domain architectures characteristic of Ryk-type receptor kinases are found in Placozoa, Cnidaria and all groups of Bilateria, but have not been found in Porifera and Ctenophora (see Supplementary Table S2 and Figure 5(C)). Since Placozoa belong to the same clade as Cnidaria and Bilateria (Ryan et al. 2013) this pattern may suggest that the domain architecture characteristic of Ryks was formed in this clade after its divergence from Porifera and Ctenophora.

Molecular functions of Wnt inhibitory factor 1

Using Wnt-dependent axis induction in early *Xenopus* embryos Hsieh et al. (1999) have found that human WIF1 blocks the activity of *Xenopus* Wnt8 in a dose-dependent manner. Importantly, in this assay, the N-terminal WIF domain of human WIF1 was as effective as full-length human WIF1, suggesting that Wnt-binding activity is associated primarily with this domain.

The molecular function of *Drosophila* Shf, however, was found to be strikingly different from that of vertebrate WIF1 proteins. In flies carrying mutations of the *Shf* gene, Hh does not accumulate normally and the range of Hh movement and signaling is strongly reduced (Glise et al. 2005; Gorfinkiel et al. 2005). Conversely, Shf had no detectable roles in Wnt signaling: its overexpression did not generate Wnt-related defects. Furthermore, although overexpression of human WIF1 inhibits Wnt signaling in *Drosophila*, it failed to rescue the *shf* phenotype, indicating that the functions of vertebrate and *Drosophila* WIF1 proteins diverged significantly (Glise et al. 2005; Gorfinkiel et al. 2005; Han and Lin 2005).

Shf protein has been shown to be required for the stability and normal levels of Hh protein and to control the distribution and diffusion of lipid-modified Hh in the extracellular matrix of the Hh-producing

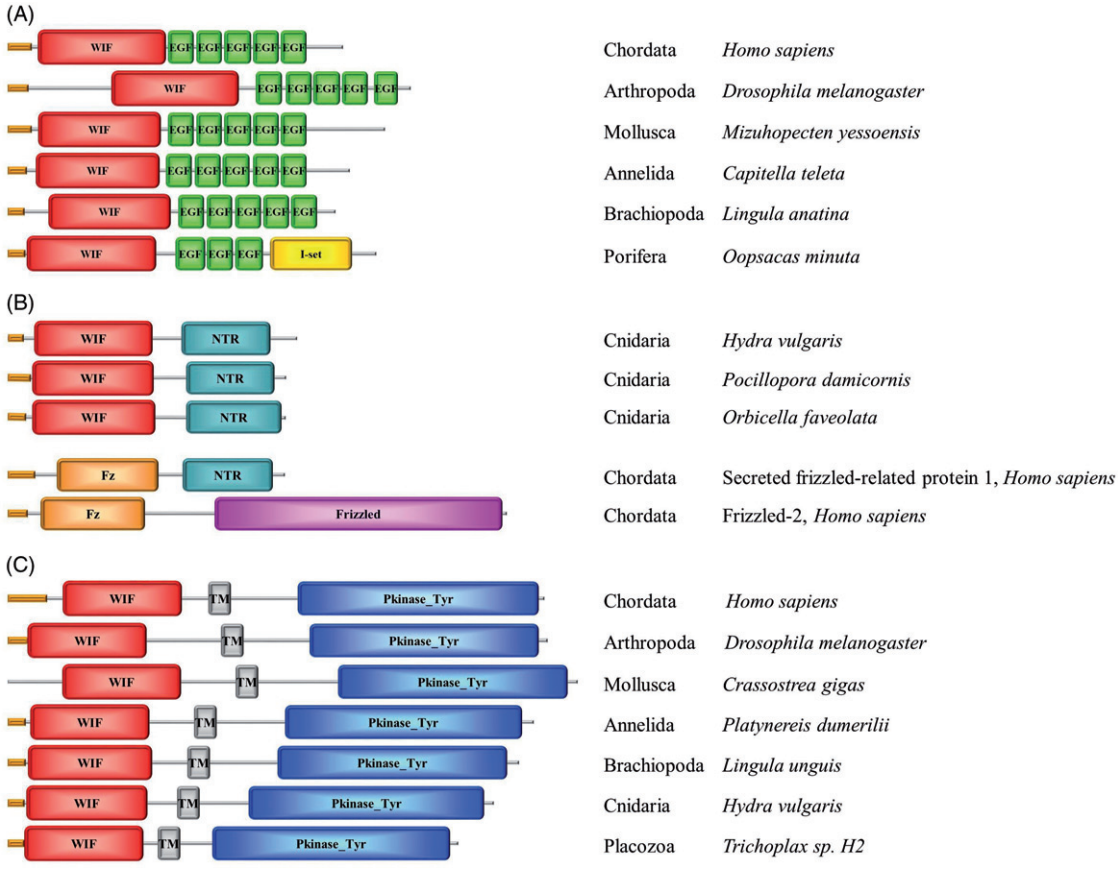

Figure 5. Domain architectures of proteins containing WIF domains. (A) WIF domains orthologous with the WIF domain of human Wnt inhibitory factor 1 are present in proteins of Porifera and various Bilaterian phyla. The domain architectures of these proteins are also similar in that they contain multiple C-terminal EGF domains (for details, see Supplementary Table S2). (B) WIF domains orthologous with the WIF domains of Wnt inhibitory factor 1 are also present in Cnidaria, these proteins, however, lack EGF domains and have C-terminal NTR domains. This domain architecture is reminiscent of secreted frizzled related proteins in which Fz domains are fused to NTR domains (for details, see Supplementary Table S2). (C) WIF domains related to the WIF domains of Wnt inhibitory factor 1 are present in Ryk-type receptor kinases. Ryk proteins are found in Placozoa, Cnidaria and all groups of Bilateria (for details, see Supplementary Table S2).

cells (Glise et al. 2005; Gorfinkiel et al. 2005). Significantly, the authors have demonstrated (Glise et al. 2005; Gorfinkiel et al. 2005) that Hh proteins lacking cholesterol- or palmitoyl moieties can diffuse into nonexpressing cells in the absence of Shf activity, suggesting that Shf only interacts with dually lipid-modified Hh and that the Hh–Shf interaction is primarily through the lipid moieties of Hh. Shf was also shown to interact with heparan sulfate proteoglycans stabilizing the interaction between Hh and the proteoglycans. *Drosophila* Shf thus serves as an extracellular proteoglycan-dependent modulator of Hh signaling, whereas vertebrate WIF1 proteins have been considered to function only to bind and inhibit the activity of Wnts.

To investigate which parts of the *Drosophila* and human WIF1 proteins are responsible for their Hh and Wnt specificity, Sánchez-Hernández et al. (2012) analyzed the activity of chimeric constructs during wing development. The authors have shown that in the domain swap chimeras, the WIF domain confers the specificity for Hh or Wnt morphogen, whereas the EGF repeats are important for the interaction of WIF1 proteins with the extracellular matrix. Avanesov et al. (2012) have shown that, just as Shf reinforces interactions between Hh and glypican heparan sulfate proteoglycans, WIF1 strengthens interactions between Wnt and glypicans, modulating the action of glypicans toward Wnt inhibition. They have also shown that interaction of glypicans and the glypican-binding EGF-like domains of WIF1 are required for WIF1's full Wnt-inhibiting activity. These authors (Avanesov et al. 2012) have also used chimeric constructs of WIF1 and Shf to investigate the structural basis of the effects of these proteins on Wnt and Hh signaling. Full Wnt inhibition required the WIF domain of vertebrate WIF1 and the heparan sulfate proteoglycan-binding EGF-like domains of either

vertebrate WIF1 or *Drosophila* Shf. Full promotion of Hh signaling was found to require both the EGF-like domains of Shf and the WIF domains of either WIF1 or Shf. The observation that the WIF domain of vertebrate WIF1 can increase the Hh promoting activity of EGF domains of Shf suggests that it is capable of interacting with Hh. In fact, full-length vertebrate WIF1 affected distribution and signaling of Hh in *D. melanogaste*r, albeit weakly, suggesting a possible role for WIF1 as a modulator of vertebrate Hh signaling.

These data suggest that whereas the WIF domains of both *Drosophila* Shf and vertebrate WIF1 are capable of interacting with Hh, only the WIF domain of WIF1 has high affinity for Wnt. A possible explanation for this asymmetry in the cross-talk of the two pathways is that in the case of the interaction of Hh with WIF domains the interactions are dominated by the lipid moieties of the morphogen, whereas in the case of the interaction of Wnts with WIF domains specific protein-protein interactions are of greater importance.

There is some further evidence for WIF1-mediated cross-talk between the Hh and Wnt pathways. It has been shown that Shf maintains short-range Hh signaling via a mechanism that does not require the presence of or binding to the *Drosophila* glypicans Dally and Dally-like protein (Avanesov and Blair 2013). These authors have presented evidence that Shf binds to the CDO/BOC family Hh co-receptors Interference Hh (Ihog) and Brother of Ihog, suggesting that Shf regulates short-range Hh signaling through interactions with the receptor complex. They have also shown that Ihog can increase the Wnt-inhibitory activity of vertebrate WIF1, raising the possibility that interactions between WIF1 and vertebrate CDO/BOC family members may be involved in Hh signaling.

Studies on human WIF1 proteins have provided some information on the structural basis of the interactions of WIF1 proteins with Wnt, Hh and with glypicans that may explain the functional similarities and differences of the *Drosophila* and human WIF1 proteins.

The three-dimensional structure of the WIF domain of WIF1 was first determined in solution by NMR spectroscopy (Liepinsh et al. 2006). Unexpectedly, the detergent (Brij-35) used in the refolding protocol of recombinant WIF domain was found to bind tightly via the alkyl chain to a unique site of the WIF domain, suggesting that this site serves as a high affinity alkyl-binding site. Since the activity of Wnts depends on the presence of a palmitoleoylated residue in their amino-terminal saposin-like domain, it was suggested that this alkyl-binding site of the WIF domain might be involved in binding Wnts activated by fatty acid modification. The high affinity of WIF domains for palmitoleoylated residues would also explain the remarkably high affinity of human WIF1 not only for different mammalian Wnts, but also for Wnts from *Xenopus* and *Drosophila*.

The importance of the alkyl-binding site of the WIF domain for the WIF1-Wnt interaction has been tested by mutagenesis of the residues of the alkyl-binding site (Bányai, Kerekes, and Patthy 2012). These studies have confirmed that the alkyl-binding site is critical for Wnt-binding: substitution of residues known to interact with the alkyl-moiety of Brij-35 has diminished the affinity of the WIF domain for Wnts. Nevertheless, none of the mutations resulted in complete elimination of the binding of the WIF domain to Wnts, suggesting that – in analogy with the Wnt-Fz interaction – the N-terminal and C-terminal domains of Wnts may bind to the WIF domain at distinct sites. This hypothesis was tested using the C-terminal cytokine-like domains of Wnts and arginine-scanning mutagenesis of the WIF domain. The results of these analyses suggested that, whereas the N-terminal, lipid-modified saposin-like domains of Wnts interact with the alkyl-binding site of the WIF domain, the cytokine-related domains of Wnts bind to a surface on the opposite side of the WIF domain (Kerekes, Bányai, and Patthy 2015; Figure 4(C)).

Although the structure of the WIF domain of Shf or its complex with lipid-modified Hh has not yet been determined, it seems likely that the palmitoylated N-terminal region of Hh binds to the alkyl-binding site of the WIF domain in a way similar to that proposed in the case of the WIF–Wnt complex (see Figure 4(C,D)). Since the palmitoyl group attached to the long, extended N-terminal arm of Hh may be distant from the globular part of the ligand-binding domain, the relative contribution of protein–protein interactions may be less significant in the case of the Hh-WIF interaction than in the case of the Wnt-WIF interaction. This interpretation would also explain the observation that the WIF domains of both *Drosophila* and vertebrate WIF1 proteins are capable of binding Hh (with moderate affinity), but only vertebrate WIF1 proteins bind Wnts with high affinity (Sánchez-Hernández et al. 2012; Avanesov et al. 2012).

Malinauskas et al. (2011) have studied both full-length WIF1 protein and isolated WIF domain to map areas involved in Wnt-recognition using biophysical, biochemical and cellular assays. Their studies

also suggest that the putative Wnt-binding site of WIF1 covers a discontinuous but discrete area of the WIF domain surface that includes the surface surrounding the alkyl-binding site identified by the NMR studies. Studies on the WIF1 protein have also implicated the five EGF domains in Wnt binding. The crystal structure of WIF1 revealed that the EGF domains are wrapped back, interfacing with the WIF domain. It has been suggested that EGFs 2-5 of WIF1 contain a heparan sulfate proteoglycan-binding site, consistent with a model assigning a major role of the proteoglycan interactions in the localization of WIF1 type proteins within morphogen gradients.

The suggestion that the C-terminal cytokine-like domains of Wnts bind to a surface of the WIF domain that is opposite the alkyl-binding site (Kerekes, Bányai, and Patthy 2015) raises the possibility that in the ancestor of WIF1 proteins this surface may have served to bind some members of the cystine-knot cytokine superfamily. It is noteworthy in this respect that Surmann-Schmitt et al. (2012) have found that WIF1 binds the cystine knot domain of connective tissue growth factor (CTGF) and inhibits CTGF activity, leading the authors to suggest that the biological function of WIF1 may not be confined to the modulation of Wnt signaling but may also include the regulation of other signaling pathways.

Tumor suppressor function of Wnt inhibitory factor 1

In order to explore the physiological functions of WIF1 *in vivo*, Kansara et al. (2009) have generated transgenic mice in which the WIF1 coding sequence was disrupted. *Wif1*$^{-/-}$ mice were born at the expected Mendelian ratios and adult mice lacked obvious phenotype in any tissues in which WIF1 was expressed. The animals were able to breed successfully, however, *Wif1*$^{-/-}$ mice were found to be more susceptible to spontaneous and radiation-induced osteosarcoma than wild type littermates, suggesting a tumor suppressor role for WIF1.

The significance of WIF1 as a tumor suppressor is also supported by the work of Wei et al. (2015). These authors have studied a large family with strong familial susceptibility to multiple early onset cancers including prostate, breast, colon and several other cancers. They have shown that the presence of a variant in the regulatory region of HNRNPA0 is associated with elevated cancer incidence in this family and that a second rare Cys294Phe mutation of WIF1 interacted with the HNRNPA0 variant resulting in extremely high risk for cancer in carriers of mutations in both genes.

The Cys294Phe mutation of WIF1 identified in this family disrupts a conserved disulfide bond of the fourth EGF domain of the WIF1 protein, yielding a mutant protein with an unpaired cysteine. The misfolded protein is unlikely to fulfill its function as a negative regulator of various Wnts and this may lead to aberrant activation of Wnt signaling pathways. According to this interpretation, it is the inactivation of WIF1 as a tumor suppressor that predisposes carriers of this WIF1 mutation to multiple early onset cancers.

There is also evidence that rearrangement and loss of *WIF1* gene may contribute to carcinogenesis. Chromosome rearrangements involving 12q13–15 are frequent among several tumors, including pleomorphic adenomas. The common molecular target for these aberrations is the *HMGA2* gene, but various fusion partners of *HMGA2* have been reported in tumors. These fusion transcripts yield either truncated HMGA2 proteins lacking the acidic COOH-terminal tail, or chimeric forms of HMGA2 proteins in which the COOH-terminal tail is replaced by distinct ectopic sequences. *WIF1* also maps to this chromosome region, approximately 700 kb proximal to *HMGA2*. Queimado, Lopes, and Reis (2007) were the first to report the identification of the *WIF1* gene as a novel *HMGA2* fusion partner in a salivary gland pleomorphic adenoma. The observed HMGA2/WIF1 fusion transcript results in the replacement of the COOH-terminal tail of HMGA2 with nine amino acids coded by frame-shifted WIF1 exon 10. In the pleomorphic adenoma expressing the HMGA2/WIF1 fusion transcript the authors observed upregulation of HMGA2 and downregulation of WIF1 supporting the hypothesis that *WIF1* is a salivary gland tumor suppressor gene and that *WIF1* downregulation might contribute to the development and/or progression of pleomorphic adenomas. Persson et al. (2009) studying salivary gland pleomorphic adenoma have also identified tumors that had *HMGA2-WIF1* gene fusions with amplification of the fusion oncogene, demonstrating that *WIF1* is a recurrent and frequent fusion partner of *HMGA2* in pleomorphic adenoma. Analysis of the various chimeric transcripts revealed that none of them encoded an intact WIF domain therefore the fusion proteins cannot function as Wnt inhibitors. Accordingly, the *HMGA2-WIF1* fusions observed in salivary tumors may contribute to carcinogenesis in two different ways: by converting *HMGA2* into a

fusion oncogene and by depriving the tumor suppressor WIF1 of its Wnt inhibitory activity.

Nevertheless, a survey of the mutation spectrum of the *WIF1* gene in cancer tissues indicates that it does not qualify as a Mut-driver gene as defined by Vogelstein et al. (2013). The authors defined Mut-driver genes as those that contain a sufficient number and type of driver gene mutations to unambiguously distinguish them from other genes, whereas Epi-driver genes are expressed aberrantly in tumors but not frequently mutated. Three major epigenetic mechanisms are known: DNA methylation, histone modification, and interference by noncoding strands of RNA (ncR) of different lengths, such as microRNA (miRNA or miR) and long non-coding RNA (lncR) (Dawson and Kouzarides 2012).

The data compiled in Supplementary Tables S3 and S4 provide ample evidence to indicate that the *WIF1* gene belongs to the category of Epi-driver genes. Silencing of *WIF1* by methylation (Supplementary Table S3) and targeting of *WIF1* by noncoding RNAs (Supplementary Table S4) contributes to a large variety of tumors since these epigenetic mechanisms lead to downregulation of the expression of the *WIF1* gene.

Wnt inhibitory factor 1 as a biomarker and a therapeutic target

In view of the overwhelming evidence that inactivation or epigenetic silencing of *WIF1* contributes to a large variety of tumors, there is increasing interest in the use of *WIF1* as an epigenetic biomarker for early detection, diagnosis, staging and prognosis of cancer, usually as part of biomarker panels containing other components of the Wnt pathway (Urakami, Shiina, Enokida, Kawakami, Kawamoto, et al. 2006, Urakami, Shiina, Enokida, Hirata, et al. 2006; Qi et al. 2007; Lee et al. 2009; Yoshino et al. 2009; Zhang et al. 2014, 2016; Garrigou et al. 2016; Guo et al. 2017; Liu et al. 2017; Hu et al. 2018; Beggs et al. 2019). For example, Urakami and coworkers compared the combined methylation status of a panel of Wnt antagonist genes (*DKK3*, *SFRP1*, *SFRP2*, *SFRP4*, *SFRP5* and *WIF1*) in renal cell carcinoma and the corresponding normal renal tissue. The methylation levels of all Wnt antagonists were significantly higher in carcinoma than in normal renal tissue: the multigene methylation score of this panel of Wnt antagonist genes proved to have a high sensitivity and specificity as an epigenetic biomarker panel for detection of renal cell carcinoma (Urakami, Shiina, Enokida, Hirata, et al. 2006). In a recent study, the Enhanced Neoplasia Detection and Cancer Prevention in Chronic Colitis (ENDCaP-C) trial has used a multiplex methylation marker panel (*SFRP1*, *SFRP2*, *SFRP4*, *SFRP5*, *WIF1*, *TUBB6*, *SOX7*, *APC1A*, *APC2*, *MINT1*, *RUNX3*) to compare the methylation status of these genes in patients with cancer, with dysplasia and without neoplasia in order to test the value of these genes as epigenetic markers to identify colitis associated cancer. These studies have shown that for neoplastic mucosa a five marker panel (SFRP2, SFRP4, WIF1, APC1A and APC2) was accurate in detecting pre-cancerous and invasive neoplasia and dysplasia (Beggs et al. 2019).

Furthermore, as part of the efforts to develop cancer therapies that exploit inhibitors of the Wnt pathway (Baarsma, Königshoff, and Gosens 2013; Anastas and Moon 2013; Pez et al. 2013), several studies assessed the possible use of WIF1 as a therapeutic target in cancer (Kawano and Kypta 2003; Filipovich et al. 2011; Malinauskas and Jones 2014).

There are two major types of therapeutic approaches targeting *WIF1*. One of these aims to restore expression of the epigenetically silenced *WIF1* gene, the other uses recombinant WIF1 protein or vectors to express recombinant WIF1 protein.

In the case of the first approach, DNA methyltransferase inhibitors represent promising new drugs for cancer therapies. The most widely used DNA methyltransferase inhibitors include 5-azacytidine, 5-aza-2'-deoxycytidine, zebularine, procaine, MG98, epigallocatechin-3-gallate and RG108 (Stresemann et al. 2006; Linnekamp et al. 2017).

Several studies have shown that 5-aza-2′-deoxycytidine can reverse the hypermethylation of the *WIF1* gene and restore its expression (Mazieres et al. 2004; Taniguchi et al. 2005; Urakami, Shiina, Enokida, Kawakami, Tokizane, et al. 2006; Qi et al. 2007; Yang et al. 2010; Wu et al. 2011; Hu, Dai, and Zeng 2013; Varol et al. 2014; Fotouhi et al. 2014; Huang et al. 2016). Similarly, procaine, procainamide and epigallocatechin-3-gallate have been reported to cause demethylation and reactivation of methylation-silenced *WIF1* gene, restore WIF1 expression and downregulate the Wnt canonical pathway, suggesting that they may have a potential use for preventing the development of cancer (Gao et al. 2009a, 2009b; Hu, Dai, and Zeng 2013).

Curcuminoids have also been reported to be potential hypomethylation agents. The *WIF1* promoter region is efficiently demethylated by treatment with bisdemethoxycurcumin, restoring expression of epigenetically silenced *WIF1* gene, causing inhibition of

Wnt signaling (Liu, Yang, Gong, et al. 2011, Liu, Yang, Zhou, et al. 2011; Xu et al. 2015). Norcantharidin treatment has also been shown to promote demethylation and reactivation of the *WIF1* gene, leading to downregulation of the canonical Wnt signaling pathway (Xie et al. 2015, 2016). Recent studies also suggest that genistein inhibits the cell invasion and migration of colon cancer cells by inducing demethylation and recovery of the activity of WIF1 and downregulation of Wnt signaling (Zhu, Ren, and Tang 2018). Similarly, triptolides, that were shown to decrease proliferation, migration and invasion of lung cancer cells, were found to exert their anti-tumor effects by epigenetic changes restoring WIF1 expression (Reno et al. 2016; Nardi et al. 2018; Mao et al. 2018).

The therapeutic use of DNA methyltransferase inhibitors to restore activity of epigenetically silenced *WIF1* gene, however, has an obvious limitation: their action is not specific for the *WIF1* gene. To overcome undesirable side effects, several attempts have been made to test vectors that express recombinant WIF1 protein. The therapeutic potential of this approach may be illustrated by the work of Lin et al. (2007). These authors have shown that nonviral gene transfer of the *WIF1* gene with a pcDNA3.1 expression vector resulted in the downregulation of Wnt signaling and inhibition of melanoma cell growth.

Treatment of established hepatocellular carcinoma tumors with adenoviral vectors carrying WIF1 fused with Fc fragment of human IgG1 also resulted in significant inhibition of tumor growth and prolonged animal survival, indicating that the Wnt antagonist WIF1-Fc inhibits Wnt signaling and exerts potent antitumor activity (Hu et al. 2019). The pVAX-WIF1 expression plasmid has been used to explore the antitumor effect of WIF1 on A549 lung cancer cells *in vitro* and on A549 subcutaneous xenografts *in vivo* (An et al. 2015). These studies have revealed that transfection with pVAX-WIF1 significantly inhibited proliferation and promoted apoptosis in A549 cells, moreover, pVAX-WIF1 significantly inhibited the tumor growth of the A549 subcutaneous xenograft *in vivo*. Rubin et al. (2010) have used the PCDNA3.1-WIF1 expression plasmid to study the potential tumor suppressor function of WIF1 in osteosarcoma. These authors have found that WIF1 overexpression significantly decreased tumor growth rate in nude mice and markedly reduced the number of lung metastasis *in vivo* in a mouse model of osteosarcoma.

Huang et al. (2016) have demonstrated that, in gallbladder cancer, WIF1 generally exhibited low levels of expression as a result of gene promoter methylation. Transfection of the GBC-SD gallbladder cell line with WIF1-expression plasmid resulted in significant decrease in cell proliferation, invasion and metastasis. A nude mouse tumor transplantation experiment showed that the oncogenicity of the GBC-SD cells expressing WIF1 was substantially lower compared with that of the untransfected GBC-SD cells, suggesting that WIF1 may be an effective treatment target for gallbladder cancer.

Kim et al. (2007) have shown that WIF1 is downregulated in non-small cell lung cancer. To test the therapeutic potential of WIF1, lung cancer cell lines A549 and H460 were transfected with the mammalian expression vector pcDNA3.1 containing the *WIF1* gene and recombinant WIF1 protein was also used to treat H460 cells. The authors have shown that colony formation of WIF1-transfected cells was decreased and recombinant WIF1 protein was also able to inhibit H460 cell proliferation. Furthermore, WIF1 significantly inhibited the growth of H460 tumor xenografts in nude mice. These data suggest that WIF1 is able to inhibit lung cancer cell growth both *in vitro* and *in vivo* and argue for the potential use of WIF1 in the treatment of lung cancer.

In human primary glioblastoma multiforme tumors WIF1 expression is significantly lower than in normal brain tissue. Transfection of U251 human glioblastoma-derived cells with the pcDNA3.1-WIF1 vector resulted in the inhibition of cell growth, suggesting that WIF1 is a potent inhibitor of glioblastoma multiforme tumors growth (Wu et al. 2012).

Song et al. (2015) have studied six hepatocellular carcinoma cell lines of HepG2, Hep3B, Huh7, PLC/PRF/5, SMMC-7721 and MHCC97 and the immortalized human liver cell line THLE-3. They have found that the expression of WIF1 in hepatocellular carcinoma cell lines was lower than that in the normal liver cell lines, while there was basically no expression of WIF1 in the highly metastatic cell line MHCC-97 and moderate expression in HepG2 and SMMC-7721. Transfection of hepatocellular carcinoma cell lines HepG2 and SMMC-7721 with the eukaryotic expression vector pcDNA3.1-WIF1 resulted in a significant reduction in the viability and invasion ability in the carcinoma cells, indicating that up-regulation of WIF1 expression can significantly inhibit the invasion and metastasis of HepG2 and SMMC-7721 of hepatocellular carcinoma cell lines.

Yee et al. (2010) have studied the effect of WIF1 on the growth and cellular invasiveness of a bone metastatic prostate cancer (PCa) cell line, PC3.

The authors have shown that the *WIF1* gene promoter was hypermethylated and its expression was down-regulated in the majority of PCa cell lines. 5-Azacytidine induced re-expression of WIF1 or transfection with pcDNA3.1-WIF1 vector significantly reduced tumor growth in a xenograft mouse model, suggesting that blocking Wnt signaling in PCa by WIF1 may represent a novel strategy to reduce metastatic disease burden in PCa patients.

In summary, there is increasing evidence that WIF1 is a valuable epigenetic biomarker for the detection, diagnosis and characterization of various types of cancer and is a promising therapeutic target in cancers in which the *WIF1* gene is epigenetically silenced.

Concluding remarks

The fact that proteins with WIF domains orthologous with those of vertebrate WIF1 proteins are present in Porifera, Cnidaria and various groups of Bilateria argues for an ancient Metazoan origin and an important biological function of these proteins. Very little is known, however, about the function of these proteins in basal metazoa since studies on WIF1-related proteins are limited to vertebrates and *Drosophila*. Furthermore, these studies have led to some apparent controversies as to the function of WIF1 proteins: whereas research on vertebrate WIF1 emphasizes their role as negative regulators of the Wnt pathway, studies on the *Drosophila* ortholog have revealed that it functions primarily as a positive modulator of the Hh pathway.

These apparently different functions of vertebrate and arthropod proteins, however, have a surprisingly similar structural basis: lipid moieties of both morphogens appear to play a crucial role in their binding to WIF1 proteins and the stability of both the Wnt-WIF1 and Hh-WIF1 complexes is reinforced by interaction with the heparan sulfate proteoglycans, glypicans. One of the differences distinguishing Wnt-WIF1-glypican and Hh-WIF1-glypican ternary complexes is that the WIF1-Wnt complex is characterized by extensive protein-protein interactions, whereas in the case of the WIF1-Hh complex the contribution of the protein-protein interactions are probably less significant.

Future work on WIF1 is needed to elucidate the contribution of WIF1 proteins to the control of Wnt and/or the Hh pathways and to decide whether genetic, epigenetic and therapeutic changes in the activity of human WIF1 protein affect both the Wnt and Hh pathways. The significance of this work is that it may help decide whether loss of WIF1 activity promotes carcinogenesis only through activation of the Wnt-pathways or perturbations of other pathways also contribute to carcinogenesis. Another reason why the characterization of the interaction of WIF1 with Hh is important is that therapeutic application of WIF1 may have significant, undesirable side effects if WIF1 has important functions distinct from its role as an antagonist of Wnts.

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