

Secreted and Transmembrane Wnt Inhibitors and Activators

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Signaling by the Wnt family of secreted glycoproteins plays important roles in embryonic development and adult homeostasis. Wnt signaling is modulated by a number of evolutionarily conserved inhibitors and activators. Wnt inhibitors belong to small protein families, including sFRP, Dkk, WIF, Wise/SOST, Cerberus, IGFBP, Shisa, Waif1, APCDD1, and Tiki1. Their common feature is to antagonize Wnt signaling by preventing ligand–receptor interactions or Wnt receptor maturation. Conversely, the Wnt activators, R-spondin and Norrin, promote Wnt signaling by binding to Wnt receptors or releasing a Wnt-inhibitory step. With few exceptions, these antagonists and agonists are not pure Wnt modulators, but also affect additional signaling pathways, such as TGF- β and FGF signaling. Here we discuss their interactions with Wnt ligands and Wnt receptors, their role in developmental processes, as well as their implication in disease.

Wnt signaling is regulated at different levels by a wide range of effectors. These effectors function as agonists or antagonists and act either intracellularly to modulate components of the signal transduction machinery or extracellularly to modulate ligand–receptor interactions. Antagonists and agonists are of great importance, as they control the fine-tuning of Wnt signaling and inhibit or activate Wnt-regulated developmental processes, such as anterior–posterior (AP) axial patterning, somitogenesis, angiogenesis, vasculogenesis, and limb, bone, tooth, and eye formation, and they are implicated in pathological events, including cancer and bone disease. Six families of secreted and four families of transmembrane Wnt antagonists are

known to date: the Dickkopf proteins (Dkks), secreted Frizzled-related proteins (sFRPs), Wnt-inhibitory factor 1 (WIF-1), Wise/SOST, Cerberus, insulin-like growth-factor binding protein 4 (IGFBP-4), Shisa, Wnt-activated inhibitory factor 1 (Waif1/5T4), adenomatous polyposis coli down-regulated 1 (APCDD1), and Tiki1, the latter four being transmembrane. Among them, the Dkk protein family is best characterized. Two families of growth factors are known to activate Wnt signaling besides Wnts, Norrin, and R-spondins (Rspo). These protein families are not related to each other, and some of them act specifically on canonical Wnt signaling, whereas others affect both canonical and noncanonical Wnt pathways. This

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review describes the individual families and their members, with emphasis on their structure, mode of action, and role in development and disease. Related reviews examining Dkks (Niehrs 2006), sFRPs (Jones and Jomary 2002; Kawano and Kypta 2003; Bovolenta et al. 2008), Cerberus (Belo et al. 2009), and Rspo family members (Yoon and Lee 2012) are available.

SECRETED WNT INHIBITORS

Dkk Protein Family

Physical Properties and Structure

Dkks represent a small family of evolutionarily conserved secreted glycoproteins. The founding member of the family, Dkk1, was identified as embryonic head inducer and Wnt antagonist in *Xenopus* (Glinka et al. 1998). Since then, Dkks were identified in other vertebrates, including humans, as well as in invertebrates such as *Dictyostelium*, cnidarians, urochordates, and ascidians, but not in *Drosophila* and *Caenorhabditis elegans*. For a more comprehensive overview on the topic, the reader is referred to Niehrs (2006). In vertebrates, the Dkk family comprises four members, Dkk1–4. They consist of 255–350 amino acids and share two conserved cysteine-rich domains (CRDs) (Fig. 1). Whereas the amino-terminal CRD, DKK_N, is unique to the Dkks, the carboxy-terminal CRD shows homology with the colipase fold, a domain found in a wide range of functionally unrelated proteins, including, for example, colipases, toxins, protease inhibitors, and prokineticins (Niehrs 2006). In addition to the two CRDs, an sgy domain is found only in Dkk3 (see below).

Dkk3 appears to be a divergent member of the Dkk family. In contrast to *Dkk1*, -2, and -4, which are more related to each other than they are to *Dkk3* (Glinka et al. 1998), *Dkk3* shares sequence homology with *soggy* (*sgy*), a distant *Dkk* family member, also called Dickkopf-like protein 1 (Krupnik et al. 1999). In addition, Cnidaria, *Hydra*, and *Nematostella* each have only two *Dkk* genes, one related to vertebrate *Dkk1*, -2, and -4 (Guder et al. 2006) and one related to vertebrate *Dkk3* (Fedders et al. 2004). Furthermore, human *Dkk1*, -2, and -4 are locat-

ed on the same chromosome 4/5/8/10 paralogy group, genes of which duplicated early in vertebrate evolution (Pollard and Holland 2000; Luke et al. 2003), but *Dkk3* is not part of this group.

Little is known about *Soggy* outside of a potential role in spermatogenesis and its homology with *Dkk3* (Kaneko and DePamphilis 2000; Kohn et al. 2005).

Mechanism of Action

Of the various signaling pathways activated by Wnts, Dkks specifically inhibit the Wnt/ β -catenin signaling cascade. Dkk1 and Dkk2 bind to low-density lipoprotein receptor-related protein (LRP) 5/6 with high affinity and an apparent K_d in the range of 0.3 to 0.7 nM (Bafico et al. 2001; Mao et al. 2001; Semenov et al. 2001). As for Dkk1 and Dkk2, a functional interaction with LRP6 has been shown for Dkk4. In contrast, Dkk3 does not bind LRP6 and does not affect Wnt signaling (Mao et al. 2001; Mao and Niehrs 2003), but rather regulates transforming growth factor- β (TGF- β) signaling (Pinho and Niehrs 2007). Within LRP6, the last two YWTD–epidermal growth factor (EGF) repeat domains mediate binding to Dkk1 (Mao et al. 2001). Within Dkk1, the colipase fold, but not the DKK_N domain, is sufficient for LRP6 binding and Wnt inhibition (Brott and Sokol 2002; Li et al. 2002; Mao and Niehrs 2003), and mutation of the conserved Cys-220 of the colipase fold abolishes the interaction (Semenov et al. 2001).

Genetic evidence for a Dkk1–LRP6 interaction is provided by the rescue of severe developmental defects of *LRP6*- and *Dkk1*-null mice observed in *LRP6/Dkk1* double-knockout mice (MacDonald et al. 2004).

In addition to LRP5/6, Dkks bind with high affinity to another class of receptors, Kremen1 and 2 (*Krm1/2*), evolutionarily conserved single-pass transmembrane proteins. They contain a Kringle, WSC, and CUB domain, which are all required for Dkk1 interaction, and an intracellular domain without obvious sequence motifs (Mao et al. 2002). Kremens bind both Dkk1 and Dkk2 but not Dkk3 with an apparent K_d in the nanomolar range (Mao et al. 2002). Similar to

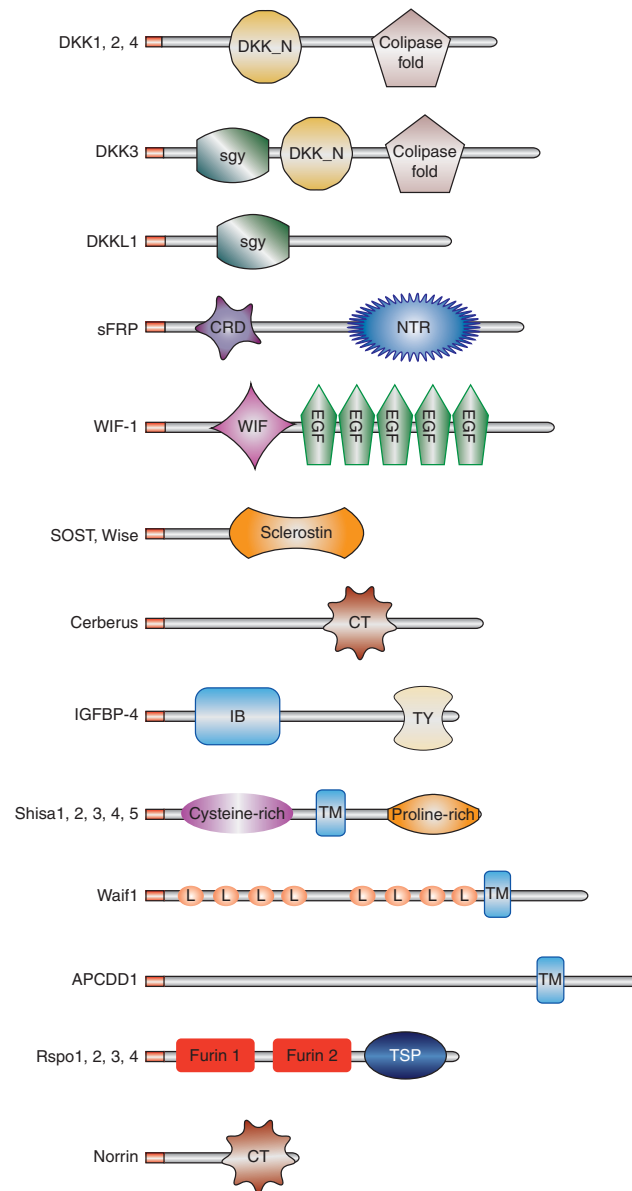


Figure 1. Domain structure of Wnt antagonists and agonists. Signal peptides are shown in red. NTR, Netrin-related motif; WIF, Wnt-inhibitory factor 1 domain; EGF, epidermal growth factor-like domain; CT, cystine knot-like domain; IB, insulin growth factor binding protein domain; TY, thyroglobulin type-1 domain; TM, transmembrane domain; L, leucine-rich repeats; TSP, thrombospondin type-1 domain.

the Dkk1–LRP6 interaction, it is the colipase fold of Dkk1, which is necessary and sufficient for Kremen binding (Mao and Niehrs 2003). Krm1/2 greatly potentiate the ability of Dkk1 to inhibit Wnt signaling, and membrane attachment of the Krm2 extracellular domain is critical for its function. Evidence for a functional interaction of these proteins *in vivo* is provided by loss-of-function experiments. Morpholino-mediated knockdown of Krm1/2 in *Xenopus* embryos induces head defects, which can be rescued by *Dkk1* mRNA overexpression. In

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addition, there is strong enhancement of head defects when both *Dkk1* and *Krm1/2* are inhibited (Davidson et al. 2002). Furthermore, in mice, these proteins genetically interact during limb development, as triple-mutant *Krm1*^{-/-}/*Krm2*^{-/-}/*Dkk1*^{+/-} mice show enhanced formation of ectopic digits (Ellwanger et al. 2008).

The dominant mode by which *Dkk1* acts is to prevent the Wnt–LRP6 interaction and disrupt the Wnt-induced Fz8–LRP6 complex formation (Fig. 2A) (Semenov et al. 2001). In addition, when Kremen is present, *Dkk1* can form a ternary complex with *Krm2* and LRP6 and induce rapid endocytosis and removal of LRP6 from the plasma membrane (Fig. 2B) (Mao et al. 2002). Recent biochemical studies (Semenov et al. 2008; Wang et al. 2008) and the characterization of *Krm1*^{-/-}/*Krm2*^{-/-} double-mutant mice (Ellwanger et al. 2008) indicate that Kremens are not universally required for *Dkk1* function, but rather this interaction is physiologically relevant only in certain tissues, and that the ability of *Dkk1* to prevent Wnt–LRP6 interaction may be sufficient for effective Wnt antagonism in many cells.

Dual Role of *Dkk2*

Unlike *Dkk1*, which is a pure inhibitor of Wnt/ β -catenin signaling, *Dkk2* is able to act either as inhibitor or activator of the pathway, depending on the cellular context. When overexpressed in *Xenopus*, *Dkk2* synergizes with coexpressed Fz8 (Wu et al. 2000) or LRP6 (Brott and Sokol 2002) to induce Wnt/ β -catenin signaling. In contrast, in HEK293T or NIH3T3 cells, *Dkk2* inhibits Wnt/ β -catenin signaling when cotransfected with Wnt and Fz, and activates the pathway when cotransfected with LRP5/6 (Wu et al. 2000; Li et al. 2002; Mao and Niehrs 2003). The colipase fold of *Dkk2* is sufficient for this synergy (Li et al. 2002). In the presence of *Krm2*, though, *Dkk2* acts as Wnt inhibitor (Mao and Niehrs 2003), indicating that receptor context may determine its action.

Structure–function analysis revealed that the isolated colipase fold of *Dkk1* is sufficient to synergize with LRP6 in activating Wnt signaling, whereas its DKK_N domain is important

to impart the inhibitory effect. The DKK_N domain of *Dkk2* is neutral in this regard (Brott and Sokol 2002). Thus, whereas the colipase fold of *Dkk1/2* mediates binding of Dkks to LRPs, the DKK_N domain modulates the outcome of this interaction (Brott and Sokol 2002).

Role of Dkks in Embryonic Development and Disease

Given the paramount role that Wnts play during embryonic development, it is not surprising that one main function of Dkks is to control cell fate in vertebrates as they show highly regionalized expression (Grotewold et al. 1999; Monaghan et al. 1999; Hashimoto et al. 2000; Shinya et al. 2000; Chapman et al. 2004; Diep et al. 2004; Idkowiak et al. 2004; Fjeld et al. 2005; Nie 2005; Nie et al. 2005). *Dkk1*, -2, and -3 mouse mutants are available and a summary of their phenotypes is presented in Table 1.

***Dkk1* and AP Axial Patterning.** Wnt/ β -catenin signaling plays an important role in AP patterning of the primary embryonic axis. Together with bone morphogenetic protein (BMP) and Nodal signaling, Wnt/ β -catenin signaling inhibits anterior development in general, and in particular the anterior central nervous system. This inhibition is prevented by the Spemann organizer in *Xenopus*, the zebrafish shield, and the mouse anterior mesendoderm, which secretes a cocktail of growth factor antagonists (De Robertis and Kuroda 2004; Niehrs 2004, 2010).

Dkk1 was initially identified as a gene conferring Spemann's head organizer activity in *Xenopus* embryos. *Dkk1* is specifically expressed in the anterior mesendoderm and induces entire ectopic heads when coexpressed with BMP inhibitors, whereas injection of anti-*Dkk1* antibodies yields microcephalic or even headless *Xenopus* embryos (Glinka et al. 1998). In zebrafish, *Dkk1* is also expressed in organizer derivatives of gastrulating embryos (Hashimoto et al. 2000; Shinya et al. 2000) and promotes, when overexpressed, anterior neural development (Hashimoto et al. 2000). These data support the model that Wnt/ β -catenin signaling inhibits anterior embryonic development, which is prevented by *Dkk1*.

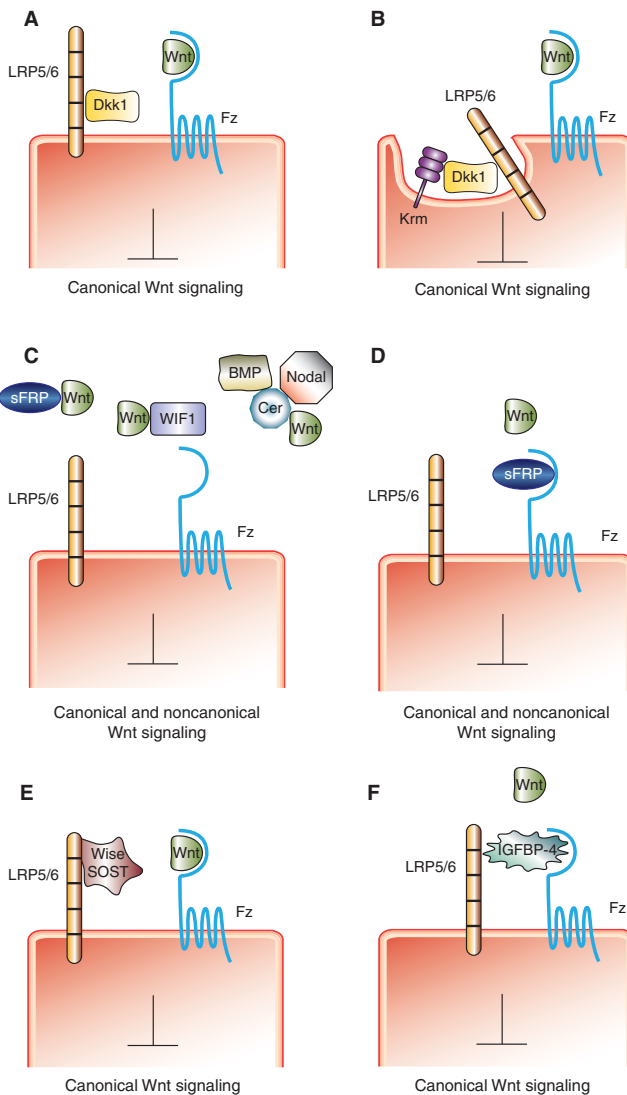


Figure 2. Models of Wnt signaling inhibition. (A,B) Dkk1 binding to LRP6 disrupts the Wnt-induced Fz–LRP6 complex formation (A) and/or induces LRP6 endocytosis in the presence of its coreceptor Kremen (B). (C) sFRPs, WIF-1, and Cerberus sequester Wnt, thereby inhibiting both canonical and noncanonical Wnt signaling. (D) sFRPs may also inhibit canonical and noncanonical Wnt signaling by binding to Fz. (E) Wise/SOST binding to LRP6 blocks Wnt-induced Fz–LRP6 complex formation. (F) IGFBP-4 binds to LRP6 and Fz, thereby preventing signal transduction by Wnt.

In mouse embryos, *Dkk1* is expressed from gastrula to neurula in the anterior visceral endoderm, anterior mesendoderm, and foregut endoderm, respectively, tissues which are all associated with anterior specification. Mice mutant for *Dkk1* lack head structures anterior to the mid-hindbrain boundary, and already at embry-

onic day 7.5 (E7.5) the anterior marker *Hex1* fails to be expressed (Mukhopadhyay et al. 2001). In the transgene-induced mouse mutant *doubleridge*, which harbors a hypomorphic *Dkk1* allele (*Dkk1^d*) with 10% normal expression levels, head development is normal. However, *Dkk1^{d/-}* compound heterozygotes on a

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Table 1. Mouse mutant phenotypes

Mouse mutant	Phenotype	References
<i>Dkk1</i> ^{-/-}	Embryonic lethal Absence of anterior head structures Postaxial polysyndactyly Fused vertebrae	Mukhopadhyay et al. 2001
<i>Dkk1</i> ^{d/d}	Viable and fertile Postaxial polysyndactyly	MacDonald et al. 2004
<i>Dkk2</i> ^{-/-}	Viable and fertile Osteopenia Blindness, cornea transformation	Li et al. 2005a; Mukhopadhyay et al. 2006
<i>Dkk3</i> ^{-/-}	Viable and fertile Hyperactive Reduced lung ventilation and elevated hematocrit Elevated IgM and natural killer cell levels	Barrantes Idel et al. 2006
<i>Sfrp1</i> ^{-/-}	Viable and fertile Normal and healthy	Satoh et al. 2006
<i>Sfrp2</i> ^{-/-}	Viable and fertile Hindlimb syndactyly at lower frequency	Satoh et al. 2006
<i>Sfrp5</i> ^{-/-}	Viable and fertile Normal and healthy	Leaf et al. 2006; Satoh et al. 2006
<i>Sfrp1</i> ^{-/-} / <i>Sfrp2</i> ^{-/-}	Embryonic lethal Severe shortening of the thoracic region Craniofacial defects Limb outgrowth defects, extra digits Defects in gonad morphology, gonad positioning, and reproductive tract maturation	Satoh et al. 2006; Warr et al. 2009
<i>Wif-1</i> ^{-/-}	Normal development Susceptible to spontaneous and radiation-induced osteosarcoma	Kansara et al. 2009
<i>Wise</i> ^{-/-}	Viable and fertile Defects in tooth development	Kasai et al. 2005; Yanagita et al. 2006; Ahn et al. 2010
<i>Sost</i> ^{-/-}	Viable and fertile Increased bone density, bone volume, bone formation, and bone strength	Li et al. 2008a
<i>Cer1-1</i> ^{-/-}	Viable and fertile	Simpson et al. 1999; Belo et al. 2000; Shawlot et al. 2000; Stanley et al. 2000
<i>Igfb4</i> ^{-/-}	Viable and fertile Modest growth deficit	Ning et al. 2006
<i>Shisa</i> ^{-/-}	Viable and fertile Dwarf (in two-thirds of mutants)	Furushima et al. 2007
<i>Shisa3</i> ^{-/-}	Normal and fertile	Furushima et al. 2007
<i>Shisa4</i> ^{-/-}		
<i>Shisa5</i> ^{-/-}		
<i>Wai1/5T4</i> ^{-/+}	Viable Hydrocephalus	Southgate et al. 2010
<i>Rspo1</i> ^{-/-} XX	Viable Partial sex-reversed phenotype Masculinized features	Chassot et al. 2008; Tomizuka et al. 2008

Continued

Table 1. *Continued*

Mouse mutant	Phenotype	References
<i>Rspo2</i> ^{-/-} <i>Rspo2</i> ^{Tg/Tg}	Mortality Defects: craniofacial, skeleton, limb, digits, tail, renal, cardiovascular, respiratory, reproductive	Nam et al. 2007a; Aoki et al. 2008; Bell et al. 2008; Yamada et al. 2009; Jin et al. 2011
<i>Rspo3</i> ^{-/-}	Embryonic lethal Defective angiogenesis in placenta and yolk sac	Aoki et al. 2007; Kazanskaya et al. 2008
<i>Norrin</i> ^{-/-}	Abnormal vasculature in cochlea and retina	Richter et al. 1998; Rehm et al. 2002

C57BL/6J genetic background show a variety of head defects, from hydrocephaly with micrognathia to loss of anterior head structures and eyes (MacDonald et al. 2004). Whereas *Dkk1* heterozygous mice are normal and fertile, mice double heterozygous for *Dkk1* and the BMP inhibitor *Noggin* show head defects similar to those of *Dkk1* homozygous mutants (del Barco Barrantes et al. 2003), supporting the model that double inhibition of BMP and Wnt signals is required for head development (Glinka et al. 1997).

Dkk1 plays also a role in pregastrulation at E5.5–E6, when the initial proximal–distal axis of the mouse embryo is converted to AP polarity, by a process involving coordinated cell migrations (Kimura-Yoshida et al. 2005). Similarly, *LRP5*^{-/-}/*LRP6*^{-/-} double mutants fail to induce and/or maintain the precursors of the primitive streak in the proximal epiblast of the pregastrula embryo (Kelly et al. 2004).

Role of *Dkk1* in Limb, Bone, and Eye Formation. During early limb development, Wnt/ β -catenin signaling by *Wnt3a* is required for formation of the apical ectodermal ridge (AER), a signaling center that controls limb growth (Barrow et al. 2003; Soshnikova et al. 2003). Similarly, overexpression of *Dkk1* in chicks induces limb truncation, and this is accompanied by apoptosis (Mukhopadhyay et al. 2001; Grotewold and Ruther 2002). Conversely, overactivation of Wnt signaling by expression of a gain-of-function mutation of β -catenin results in expansion of the AER (Soshnikova et al. 2003). An expansion of the AER is observed in both *Dkk1*-null and hypomorphic *Dkk1*^{d/d} mutants, suggesting a genetic interaction with *Wnt3a* (Mukhopadhyay

et al. 2001; Adamska et al. 2004; MacDonald et al. 2004). In addition to AER expansion, *Dkk1*-null and *Dkk1*^{d/d} mutants display postaxial polysyndactyly in the forelimbs (Mukhopadhyay et al. 2001; MacDonald et al. 2004), and normal digit numbers are restored in *Dkk1*^{d/d}/*LRP6*^{+/-} mice (MacDonald et al. 2004). Polydactyly in *Dkk1* mutants can be reversed by the simultaneous loss of *Wnt7a* (Adamska et al. 2004). Thus, *Dkk1* can control different steps involving *Wnt3* or *Wnt7a* signaling during mouse limb development and digit patterning.

Wnt/ β -catenin signaling plays a central role in mammalian bone density regulation (Krishnan et al. 2006). Loss-of-function mutations of *LRP5* are associated with the recessive familial osteoporosis–pseudoglioma syndrome (Gong et al. 2001), whereas gain-of-function mutations of *LRP5* are associated with diseases of high bone mass (Boyden et al. 2002). Interestingly, for one particular *LRP5* gain-of-function mutation, G171V, decreased inhibition of *LRP5* by *Dkk1* was suggested to account for increased *LRP5* signaling (Boyden et al. 2002; Ai et al. 2005). Similar to humans, heterozygous *Dkk1*^{+/-} mice show an increase in bone mineral density (Morvan et al. 2006; MacDonald et al. 2007). Conversely, transgenic mice overexpressing *Dkk1* in bone develop osteopenia (Li et al. 2006), suggesting that *Dkk1* antagonizes Wnt–*LRP5* signaling to regulate physiological levels of bone mass.

Wnt/ β -catenin signaling plays an important role during eye development, and *Dkk1*, -2, and -3 are highly expressed in this organ (for details, see Niehrs 2006). The severity of head defects in *Dkk1*-null mutants precludes analysis of the role that *Dkk1* may play in the

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developing eye. In contrast, *Dkk2* knockout mice are viable, but blind, because of the development of a keratinized corneal epithelium and skin appendages as a result of constitutive Wnt/ β -catenin signaling in the cornea (Mukhopadhyay et al. 2006).

Dkks and Disease. A number of studies have implicated Dkk family members in human disease based on transcriptional profiling and epigenetic studies (for details, see Niehrs 2006). In particular, alterations of Dkk expression have been observed in a number of cancer models, which is not surprising given the importance of Wnt signaling in cancer biology. In colon cancer, for example, the observed epigenetic silencing of *Dkk1* and other *Dkk* family genes is likely to contribute further to the activation of the Wnt/ β -catenin pathway (Aguilera et al. 2006; Sato et al. 2007). *Dkk1* expression itself can be directly up-regulated by Wnt/ β -catenin signaling in a T-cell factor (TCF)-dependent manner, but this mechanism appears to be lost through silencing in colon cancer (Gonzalez-Sancho et al. 2005).

Dkk1 has also been implicated in neurodegenerative processes and induction of apoptosis after neuronal injury (Caricasole et al. 2004; Cappuccio et al. 2005).

sFRP Protein Family

Physical Properties and Structure

The sFRPs represent the largest family of secreted Wnt inhibitors and resemble the ligand-binding CRD domain of the Frizzled family of Wnt receptors. The founding member of the family, Frzb (for Frizzled motif associated with bone development), was first purified as a chondrogenic factor from bovine cartilage extracts (Hoang et al. 1996). Shortly after, Frzb was isolated during a differential screen for cDNAs enriched in the Spemann organizer of *Xenopus* embryos (Bouwmeester et al. 1996) and shown to act as a Wnt antagonist (Leyns et al. 1997; Wang et al. 1997). Subsequently, additional members of the family were identified (Finch et al. 1997; Melkonyan et al. 1997; Rattner et al. 1997).

In humans, the sFRP family comprises five members, sFRP1–5, with sFRP3 being the or-

tholog of Frzb, and orthologous genes have been identified in all vertebrates analyzed so far, as well as in invertebrates, but not in *Drosophila* (Bovolenta et al. 2008). On the basis of sequence homology and phylogenetic analysis, sFRP1, -2, and -5 form a subgroup that diverges from the one formed by sFRP3 and -4 (Jones and Jomary 2002; Kawano and Kypta 2003; Bovolenta et al. 2008). This clustering also reflects a different genomic organization. Similar to human *Dkk1*, -2, and -4, sFRP1, -2, and -5 are located within the same chromosome 4/5/8/10 paralogy group, whereas sFRP3 and -4 are not part of this group. A third subgroup of sFRPs has been identified in *Xenopus*, chick, and zebrafish, but not in mammals. Members of this subgroup are *Sizzled*, *Sizzled2*, and *Crescent*, and they share sequence similarities with the sFRP1, -2, and -5 subgroup (Pfeffer et al. 1997; Salic et al. 1997; Bradley et al. 2000; Pera and De Robertis 2000; Houart et al. 2002).

Human sFRPs consist of 295–346 amino acids and share at the amino terminus a CRD domain (Fig. 1). The CRDs of sFRPs show 30%–50% sequence similarity with those of Fz receptors and contain 10 conserved cysteine residues (Rehn et al. 1998), which form a pattern of disulfide bridges (Chong et al. 2002). The carboxy-terminal part of sFRPs contains a Netrin-related motif (NTR), which is found in a number of unrelated proteins, including the axon guidance protein netrin 1, tissue inhibitors of metalloproteinases (TIMPs), type-1 procollagen C-proteinase enhancer proteins (PCOLCEs), and complement proteins (Fig. 1) (Banyai and Patthy 1999). The NTR domain is characterized by segments of positively charged residues that appear to confer heparin-binding properties (Uren et al. 2000) and by six cysteine residues that form three disulfide bridges (Chong et al. 2002).

Mechanism of Action

Initial biochemical and functional analysis using *Xenopus* embryos and cultured cells showed that Frzb/sFRP3 binds to Wnt1 and XWnt8 and inhibits Wnt/ β -catenin signaling (Leyns et al. 1997; Lin et al. 1997; Wang et al. 1997). These results favor a model wherein sFRPs inhibit Wnt



signaling by sequestering Wnts away from active receptor complexes (Fig. 2C). The CRD domain of Frzb seemed to be necessary and sufficient for both activities, Wnt binding and inhibition (Lin et al. 1997). In contrast, a mutant of sFRP1 lacking the CRD domain retained the ability to bind to Wingless, the *Drosophila* Wnt homolog (Uren et al. 2000). Another study of sFRP1 structure and function using Wnt reporter assays indicates that both protein domains, CRD and NTR, are important for optimal Wnt inhibition (Bhat et al. 2007). Furthermore, a recent study on sFRP1 combining biochemical and functional assays in cell culture and medaka fish embryos shows that its NTR domain mimics the function of the entire molecule in binding Wnt8 and inhibiting Wnt signaling (Lopez-Rios et al. 2008). These conflicting results might imply that sFRPs have multiple Wnt-binding sites and/or sFRP–Wnt pairs associate with different affinities. Indeed, whereas sFRP1–4 have been shown to bind to Wnt3a with affinities in the nanomolar range, sFRP1 and -2, but not sFRP3 and -4, bound to Wnt5a. Moreover, only sFRP1 and -2 could block Wnt3a signaling in L cells (Wawrzak et al. 2007). Several other studies have described possible biochemical and/or functional specificity of the sFRPs–Wnt interactions in different developmental models, including the neural tube (Galli et al. 2006), somites (Lee et al. 2000), vascular endothelium (Dennis et al. 1999), and developing heart (Schneider and Mercola 2001), as well as during AP axial patterning of *Xenopus* embryos (Bradley et al. 2000; Pera and De Robertis 2000).

The crystal structures of the CRDs from mouse Fz8 and mouse sFRP3 have been determined and revealed the potential for the CRDs to dimerize (Dann et al. 2001). This possibility has been supported by the ability of sFRPs and Fz proteins to form homo- and heteromeric complexes through the CRD domain (Bafico et al. 1999; Rodriguez et al. 2005). These findings suggest an alternative model of how sFRPs could inhibit Wnt signaling, by forming nonfunctional complexes with Fz receptors (Fig. 2D).

Unlike Dkks, which specifically inhibit Wnt/ β -catenin signaling, sFRPs can also inhi-

bit noncanonical Wnt/PCP (planar cell polarity) signaling (Li et al. 2008b; Satoh et al. 2008; Matsuyama et al. 2009; Sugiyama et al. 2010), which is not surprising, as they bind to both types of Wnts. Inhibition of PCP signaling, which antagonizes Wnt/ β -catenin signaling (Yan et al. 2001; Schwarz-Romond et al. 2002; Simons et al. 2005; Li et al. 2011), may also explain why sFRPs can activate Wnt/ β -catenin signaling (Swain et al. 2005). Interestingly, in *Xenopus*, Frzb and Crescent can activate canonical Wnt signaling by promoting the diffusion of Wnt8 and Wnt11 (Mii and Taira 2009). In addition to Wnt signaling, sFRPs can regulate other signaling cascades. sFRP1, for example, binds to RANKL, a member of the tumor necrosis factor family, thereby inhibiting osteoclast formation (Hausler et al. 2004). sFRP1 also interacts with and inhibits the metalloprotease ADAM10, thus acting as a negative modulator of Notch signaling to regulate retinal neurogenesis (Esteve et al. 2011). Sizzled binds to and inhibits the activity of BMP1/Tolloid, a metalloprotease that cleaves the BMP antagonist chordin, thereby inhibiting BMP signaling (Lee et al. 2006).

Role of sFRPs in Embryonic Development and Pathological Events

The expression pattern of several sFRPs has been analyzed in different developmental systems, including medaka fish, *Xenopus*, chick, and mouse embryos (for details, refer to Jones and Jomary 2002; Bovolenta et al. 2008). sFRPs show a dynamic, partly overlapping, but also distinct expression pattern. To examine their roles in embryogenesis, single *Sfrp1*, *Sfrp2*, and *Sfrp5* knockout mice (Leaf et al. 2006; Satoh et al. 2006), as well as different combinations of double- and triple-mutant mouse lines carrying homozygous or heterozygous mutations of two or all three genes, have been generated (Table 1) (Satoh et al. 2008). Apart from *Sfrp1*^{-/-}/*Sfrp2*^{-/-} double-mutant and *Sfrp1*^{-/-}/*Sfrp2*^{-/-}/*Sfrp5*^{-/-} triple-mutant mice, the other mutant mice are viable and fertile and have no obvious abnormalities in gross morphology, indicative of the redundant role of *Sfrps*

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in embryonic development. In contrast, inactivation of *Sfrp1* plus *Sfrp2* is embryonic lethal as a result of severe shortening of the AP axis and incomplete somite segmentation, and this phenotype becomes more severe when *Sfrp5* is also inactivated. Genetic analysis combining *Sfrp* triple-knockout mice and *Loop-tail* mice, which carry a mutation in *Stbm/Vangl2*, revealed the involvement of sFRPs in convergent extension through the regulation of the PCP pathway (Sato et al. 2008). In addition, *Dkk1*-deficient embryos carrying *Sfrp1* homozygous and *Sfrp2* heterozygous mutations display irregular somites and indistinct intersomitic boundaries, indicating that sFRP-mediated inhibition of Wnt/ β -catenin signaling is required for somitogenesis (Sato et al. 2008).

In addition to the role in regulating AP-axis elongation and somitogenesis during mouse development, *Sfrp1* and *-2* exert redundant roles also in embryonic organogenesis, as both genes are required for normal male sexual development in mice (Warr et al. 2009).

In line with their function as Wnt signaling antagonists, sFRPs appear to act as tumor suppressor genes, as loss or down-regulation of sFRP expression has been observed in a variety of invasive carcinomas. Epigenetic silencing of sFRP1 and *-2* by hypermethylation seems to occur in basically all tumor types, and the *sFRP1* promoter methylation status has been proposed to serve as a biomarker for cancer detection and progression (for details, see Bovolenta et al. 2008; Esteve and Bovolenta 2010). sFRPs are also implicated in other pathological events, such as osteolysis and heterotopic ossification (Gordon et al. 2007) and during photoreceptor degeneration (Hackam 2005).

WIF-1

Wnt-inhibitory factor 1 was first identified as an expressed sequence tag from the human retina (Hsieh et al. 1999), and is present in fish, amphibians, and mammals. *Wif-1* is expressed in a variety of tissues, being most abundant in brain, lung, retina, and cartilage (Hsieh et al. 1999; Hunter et al. 2004; Hu et al. 2008; Surmann-Schmitt et al. 2009). WIF-1 is a protein of 379

amino acids with a unique and highly conserved WIF domain, five EGF-like repeats, and a hydrophilic tail (Fig. 1). Interestingly, the WIF domain is also found in the extracellular domain of RYK receptor tyrosine kinase. The phenotype induced by overexpression of WIF-1 in *Xenopus* embryos, namely, induction of a secondary axis and abnormal somitogenesis, together with its ability to block XWnt8 activity, suggested it plays a role in Wnt signaling (Hsieh et al. 1999). Indeed, WIF-1 binds to XWnt8 and Wingless and inhibits the interaction between XWnt8 and *Drosophila* Fz2 (Hsieh et al. 1999). This result suggests that similar to sFRPs, WIF-1 prevents Wnt from binding to its receptors, thus affecting canonical and noncanonical pathways (Fig. 2C). Recently, WIF-1 has been shown to bind to both types of Wnts, canonical and noncanonical, including Wnt3a, Wnt4, Wnt5a, Wnt7a, Wnt9a, and Wnt11, and to regulate Wnt activity during cartilage development (Surmann-Schmitt et al. 2009).

The mechanism of how WIF-1 regulates Wnt signaling is not completely understood, but the observed silencing of WIF-1 in different tumors suggests that like Dkks and sFRPs, this secreted factor plays an important role in cancer and other biological processes related to Wnt signaling (Chien et al. 2009; Kansara et al. 2009; Elston and Clifton-Bligh 2010).

Wise and SOST

Wise, also known as SOSTDC1 (sclerostin domain-containing 1), Ectodin, and USAG-1 (uterine sensitization-associated gene-1), has been isolated by a functional screen for activities that alter the AP character of neuralized *Xenopus* tissues (Itasaki et al. 2003). Sequence and structural analysis revealed that Wise forms a cystine knot (Ellies et al. 2006; Lintern et al. 2009) and belongs together with SOST to the CAN subfamily of cystine knot-containing BMP antagonists (Avsian-Kretschmer and Hsueh 2004). In *Xenopus* Wise appears to be a context-dependent regulator of Wnt signaling; it may inhibit or activate Wnt signaling in different assays (Itasaki et al. 2003). In cell culture Wise blocks Wnt1 and Wnt3a activities in reporter



assays (Yanagita et al. 2004; Blish et al. 2008). It interacts with LRP6 through one of the three loops formed by the cystine knot (Lintern et al. 2009) and is able to compete with Wnt8 for binding to LRP6 (Fig. 2E) (Itasaki et al. 2003). Not only secreted Wise is able to inhibit Wnt signaling, but endoplasmic reticulum (ER)-retained Wise is also able to do so by reducing cell surface LRP6 (Guidato and Itasaki 2007). Wise also binds the LRP4 receptor (Ohazama et al. 2008), which is able to modulate Wnt signaling mediated by LRP5 and -6 (Ohazama et al. 2008; Li et al. 2010).

Wise is expressed in various tissues, including the surface ectoderm of the posterior axis, branchial arches, dermal papilla in hair follicles, vibrissae, tooth cusps, rat endometrium, developing testis, and kidney (Lintern et al. 2009). Wise-null mice are viable and fertile and apparently healthy, except that they exhibit tooth abnormalities, including supernumerary incisors and molars, fused molars, and cusp defects (Table 1) (Kassai et al. 2005; Yanagita et al. 2006; Ahn et al. 2010). This abnormal tooth development seems to be caused by elevated Wnt/ β -catenin signaling. Indeed, in *Wise*^{-/-}/*LRP5*^{-/-}/*LRP6*^{+/-} triple-mutant mice, most of the different tooth defects of *Wise*-null mutants are rescued, indicating that inhibition of Wnt/ β -catenin by Wise controls tooth development (Ahn et al. 2010).

Wise shares 38% identity and a cystine knot domain with SOST (Fig. 1). SOST is highly expressed in osteoblasts and osteocytes, and loss-of-function mutations or down-regulation of SOST are responsible for two rare forms of autosomal recessive severe craniofacial hyperostoses, sclerosteosis (Balemans et al. 2001; Brunkow et al. 2001), and van Buchem disease (Balemans et al. 2002; Staehling-Hampton et al. 2002), which are characterized by overgrowth of bone tissue. Similarly, *SOST*-null mutant mice have a high-bone-mass phenotype characterized by increase in bone mass density, bone volume, bone formation, and bone strength (Table 1) (Li et al. 2008a).

SOST antagonizes Wnt signaling by binding to the first two YWTD-EGF repeat domains of LRP5 and LRP6 (Li et al. 2005b; Semenov et al. 2005). Like Dkk1, SOST has the ability to disrupt

Wnt1-induced Fz8-LRP6 complex formation, suggesting a potential mechanism of action (Fig. 2E) (Semenov et al. 2005). Thus, the loss of SOST function likely leads to the hyperactivation of Wnt signaling that underlies bone overgrowth seen in sclerosteosis patients. Interestingly, sclerosteosis shares remarkable similarities with diseases of high bone mass caused by gain-of-function mutations in the *LRP5* gene, such as the G171V point mutation (Boyden et al. 2002; Little et al. 2002), and this mutation as well as an analogous mutation in LRP6 (G158V) blocks binding of SOST to LRP5 and LRP6 (Ellies et al. 2006; Semenov and He 2006). Evidence for a genetic interaction between SOST and LRP6 is provided by rescue experiments. In *SOST*^{-/-}/*LRP6*^{-/-} double-mutant mice, the hand and foot defects observed in *LRP6*^{-/-} mutant mice are rescued (Collette et al. 2010).

Wise and SOST belong to a subfamily of cystine knot-containing proteins, members of which inhibit BMP signaling (Avsian-Kretschmer and Hsueh 2004). In cell culture models Wise and SOST inhibit BMP signaling (Laurikkala et al. 2003; Winkler et al. 2003); however, the physiological role of this inhibition is still unclear.

Cerberus

Cerberus was isolated from *Xenopus* as an abundant organizer-specific gene, capable of inducing ectopic heads, when overexpressed in *Xenopus* embryos (Bouwmeester et al. 1996). Cerberus-related proteins have been identified in other vertebrates, such as zebrafish, chick, and mouse (Belo et al. 2009). They share a cystine knot domain at the carboxyl terminus (Fig. 1) and undergo proteolytic cleavage after secretion (Piccolo et al. 1999). *Xenopus* Cerberus binds to Nodal, BMP, and Wnt proteins via independent sites and inhibits all three signaling pathways, which leads to simultaneous head formation and trunk inhibition (Piccolo et al. 1999). Conversely, Morpholino-mediated knockdown of Cerberus in *Xenopus* embryos impairs head induction (Silva et al. 2003; Kuroda et al. 2004).

Unlike its *Xenopus* counterpart, mouse Cerl-1 does not bind Wnt and inhibit Wnt

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signaling (Belo et al. 2000) and is not essential for mouse head formation (Table 1) (Simpson et al. 1999; Belo et al. 2000; Shawlot et al. 2000; Stanley et al. 2000). Rather, Cerberus-related proteins are key regulators of Nodal signaling and play an important role in the establishment of left–right asymmetry in the vertebrate embryo (Rodriguez Esteban et al. 1999; Yokouchi et al. 1999; Zhu et al. 1999; Hashimoto et al. 2004; Marques et al. 2004; Tavares et al. 2007; Vonica and Brivanlou 2007).

IGFBP-4

IGFBP-4 belongs to the family of insulin-like growth factor binding proteins (IGFBPs), which modulate the actions of insulin-like growth factors (IGFs) (Firth and Baxter 2002). IGFBP-4 was identified in a screen for factors able to induce cardiomyocyte differentiation of P19CL6 cells (Zhu et al. 2008a). It promotes cardiogenesis in an IGF-independent fashion, namely, by antagonizing Wnt/ β -catenin signaling (Zhu et al. 2008a). It binds directly to LRP6 and Fz8 via the carboxy-terminal thyroglobulin domain and blocks binding of Wnt3a to the receptors (Fig. 2F). Morpholino-mediated knockdown of IGFBP-4 in *Xenopus* embryos leads to cardiac defects, similar to those induced by overexpression of XWnt8. These defects were rescued by coexpression of a dominant–negative form of LRP6, suggesting that the cardiogenic effect of IGFBP-4 is mediated by inhibition of Wnt/ β -catenin signaling.

Of the six IGFBP protein family members, IGFBP-1, -2, and -6, but not IGFBP-3 and -5, are also able to bind directly to LRP6 and Fz8 and to inhibit Wnt/ β -catenin signaling, albeit with a lower efficiency compared with IGFBP-4 (Zhu et al. 2008a). Thus, the lack of cardiac phenotypes in *Igfb-4*-null mice or *Igfb-3*, -4, and -5 triple-knockout mice (Ning et al. 2006) may be caused by genetic redundancies between IGFBP-4 and other IGFBPs.

IGFBP-4-mediated inhibition of Wnt/ β -catenin signaling might have some implications for cancer biology. Treatment with IGFBP-4 reduces cell proliferation in some cancer cell lines in vitro, and overexpression of IGFBP-4 atten-

uates the growth of prostate cancer in vivo (Durai et al. 2006).

TRANSMEMBRANE WNT INHIBITORS

Shisa Protein Family

The Shisa protein family comprises five subfamilies in vertebrates (Furushima et al. 2007). Recently, in a search for sequence similarity to Shisa proteins, four additional subfamilies were found in vertebrates (Pei and Grishin 2012). Shisa proteins share a signal peptide, an amino-terminal CRD, a predicted transmembrane segment, and a carboxy-terminal proline-rich region (Fig. 1) (Pei and Grishin 2012).

The founding member of this family, *Xenopus Shisa1*, was identified as a gene specifically expressed in the prospective head ectoderm and the Spemann organizer (Yamamoto et al. 2005). In *Xenopus* embryos, overexpressed Shisa1 expands anterior neural structures, and together with a BMP inhibitor induces a secondary head. Conversely, knockdown of Shisa1 by Morpholino injection in *Xenopus* embryos suppresses anterior neural marker expression and head structures, indicating a requirement for head formation. Shisa1 does not inhibit BMP and Nodal signaling, but functions in head formation as an inhibitor of Wnt and fibroblast growth factor (FGF) signaling. It functions cell-autonomously in the ER, where it traps Fz and the FGF receptor and prevents their maturation (Yamamoto et al. 2005).

In *Xenopus*, three paralogous genes to *XShisa1* were identified: *XShisa2*, -3, and -4 (Nagano et al. 2006; Silva et al. 2006). *XShisa2* is expressed in somitic mesoderm and like Shisa1 inhibits both Wnt and FGF signaling, thereby regulating segmental patterning during somitogenesis (Nagano et al. 2006). *Shisa* homologs were also reported in zebrafish, rat, mouse, chicken, and human (Katoh and Katoh 2005; Furushima et al. 2007; Hedge and Mason 2008; Zhu et al. 2008b).

In mouse, four Shisa family members are present: mShisa and mShisa3, -4, and -5 (Furushima et al. 2007). The expression of *mShisa* is similar to that of *XShisa1* and -2. It is expressed



in anterior visceral endoderm, anterior mesoderm, anterior neuroectoderm, and somitic mesoderm (Furushima et al. 2007). Like *XShisa1* and *-2*, overexpressed *mShisa* inhibits Wnt signaling in *Xenopus* and mammalian cells. However, *mShisa*^{-/-} mutant mice had no phenotype in either head development or somitogenesis, and double-knockout mutants of *mShisa* with *mShisa3*, *-4*, or *-5* displayed no additional phenotype (Furushima et al. 2007). These results suggest that Shisa loss-of-function in mouse head and somites development may be compensated by other Wnt antagonists, such as Dkk, sFRP, and Cerberus.

Waif1/5T4

Waif1a was identified in gene expression profiling of zebrafish embryos as a novel direct Wnt/ β -catenin target and functionally characterized in zebrafish, *Xenopus*, mouse, and mammalian cells (Kagermeier-Schenk et al. 2011). *Waif1a* is a single-pass plasma membrane protein with several leucine-rich repeats in the extracellular part and a short carboxyl terminus without conserved motifs (Fig. 1). In zebrafish and *Xenopus*, *Waif1a* is expressed during early embryogenesis in regions of active Wnt/ β -catenin signaling, in particular the dorsolateral marginal epiblast during gastrulation, and its expression is directly regulated by Wnt8. *Waif1a* inhibits Wnt/ β -catenin signaling in zebrafish and *Xenopus*. Likewise, mouse and human *Waif1/5T4* antagonizes Wnt signaling in cultured cells. Zebrafish *Waif1a* acts as a direct feedback inhibitor of Wnt8-mediated mesoderm and neuroectoderm patterning during zebrafish gastrulation. At the molecular level *Waif1a* binds to LRP6 and inhibits both Wnt3a- and Dkk1-induced LRP6 internalization without affecting Wnt3a-induced LRP6 phosphorylation, by a mechanism that needs further elucidation. At the same time *Waif1a* activates noncanonical Wnt/PCP signaling in zebrafish embryos and *Xenopus* explants by promoting the ability of Dkk1 to activate Wnt/PCP signaling.

The elevated expression of *Waif1/5T4* in human carcinomas, which correlates with poor survival prognosis, highlights its relevant role

in cancer biology (Starzynska et al. 1994; Nagamura et al. 2002).

APCDD1

Adenomatosis polyposis coli down-regulated 1 (APCDD1) was initially isolated in a search for genes that were down-regulated after induction of APC in SW480 colon cancer cells (Takahashi et al. 2002). APCDD1 is a membrane-bound glycoprotein, conserved throughout vertebrate evolution. In mouse embryos, *APCDD1* is expressed in the nervous and vascular system, the inner ear, and the mesenchyme of several developing organs (Jukkola et al. 2004). Abundant expression of *APCDD1* is observed in mouse and human hair follicles (Jukkola et al. 2004; Shimomura et al. 2010). A point mutation (L9R) in *APCDD1* causes hereditary hypotrichosis simplex, a rare autosomal dominant form of hair loss (Shimomura et al. 2010). This mutation is located in the signal sequence and impairs protein transport to the plasma membrane. The finding that APCDD1 is a direct Wnt/ β -catenin target (Takahashi et al. 2002), and its similarity in expression with Wise (O'Shaughnessy et al. 2004), encouraged Shimomura et al. to analyze if APCDD1 may function as a Wnt inhibitor. They found that soluble APCDD1 binds to Wnt3a and to the extracellular domain of LRP5 in vitro, and it inhibits Wnt/ β -catenin signaling in cultured cells, possibly by preventing Fz from binding to Wnt. In vivo, APCDD1 inhibits Wnt signaling during the generation of neurons from progenitors in the developing chick nervous system and during axis specification in *Xenopus* embryos. Given its broad expression in various cell types, APCDD1 may regulate several other biological processes controlled by Wnt signaling.

Tiki1

Tiki1 was identified by functional cDNA screening as an organizer-specific gene, required for anterior neural development in *Xenopus* (Zhang et al. 2012). *Tiki1* encodes an evolutionary conserved transmembrane metalloprotease that inhibits Wnt signaling by removing eight

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amino-terminal residues from Wnt itself, thereby leading to formation of oxidized Wnt oligomers with impaired receptor-binding capability (Zhang et al. 2012).

WNT ACTIVATORS

R-Spondin Protein Family

Physical Properties and Structure

R-Spondins (Rspo1 to -4) are a small family of four secreted growth factors, which in addition to Wnts potently activate β -catenin signaling. The first identified member of this family was human R-spondin 3 (Chen et al. 2002). Subsequently, mouse *R-spondin1* (roof plate-specific spondin) was discovered as a gene specifically expressed in the roof plate of the neural tube and the dorsal part of telencephalon (Kamata et al. 2004), and *Xenopus R-spondin2* was isolated in a functional screen for its property to activate Wnt/ β -catenin signaling (Kazanskaya et al. 2004). Rspos are evolutionarily conserved and are present also in invertebrates, such as hemichordates, chordates, and echinoderms, but not in *Drosophila* and *C. elegans* (Kim et al. 2006; Yoon and Lee 2012). In mammals, Rspo1 to -4 show about 60% overall sequence homology and share a signal peptide, two amino-terminal furinlike CRDs, followed by a thrombospondin type-1 domain and a positively charged carboxy-terminal region (Fig. 1) (Kazanskaya et al. 2004).

Although Rspos contain an amino-terminal signal peptide, secreted proteins are barely detectable in the medium of transfected cells (Kazanskaya et al. 2004). Treatment of cells with soluble heparin or sodium chlorate, an inhibitor of sulfation, increases the level of Rspo proteins in the conditioned medium, suggesting that Rspos may bind to heparin sulfate proteoglycans, including syndecans and glypicans (Nam et al. 2006). Indeed, Rspo3 binds syndecan 4 (Sdc4) (Ohkawara et al. 2011) (see below).

Mechanism of Action

The characteristic feature of all four Rspos is their ability to activate canonical Wnt signaling. Rspos synergize with Wnts (Kazanskaya et al.

2004; Kim et al. 2005; Nam et al. 2006; Wei et al. 2007; Kim et al. 2008) and require the presence of Wnts to activate β -catenin signaling (Binnerts et al. 2007). The precise mechanism of Rspo-driven β -catenin activation is poorly understood, yet recent discoveries started to shed light on it.

For a couple of years the Rspo receptor turned out to be a matter of controversy. Initially, it was suggested that Rspos bind to Fz8 (Nam et al. 2006), but this interaction was not confirmed (Wei et al. 2007; Ohkawara et al. 2011). In another study Kremen was reported to bind Rspo1 (Binnerts et al. 2007), but this interaction may not be physiologically relevant, because *Kremen1* and -2 double-knockout mice are viable, unlike most *Rspo* single-mutant mice, and fibroblasts isolated from these *Kremen*-deficient mice respond normally to Rspo (Ellwanger et al. 2008). Alternatively, LRP6 has been proposed as Rspo receptor (Wei et al. 2007), but this interaction also was not confirmed by others (Binnerts et al. 2007; Glinka et al. 2011). Of note, none of these studies addressed whether Fz8, LRP6, or Kremen was required for Rspo signaling.

Recently, several groups showed that the leucine-rich repeat containing G-protein-coupled receptor 5 (LGR5) and its homolog LGR4 are Rspo receptors. LGR5 marks proliferative stem cells in several Wnt-dependent compartments, such as small intestine, colon, stomach, and hair follicle, whereas LGR4 expression is much broader (Barker and Clevers 2010). LGR4 and -5 are required for Rspo signaling, and they synergize with Rspo and Wnt3a in Wnt/ β -catenin signaling. Rspo1 to -4 bind with high affinity to LGR4 and -5 and their apparent K_d was determined to be in the nanomolar range (Carmon et al. 2011; de Lau et al. 2011; Glinka et al. 2011). Both Rspo binding to LGR4/5 and its function are mediated by the furin domains (Kazanskaya et al. 2004; Glinka et al. 2011). Moreover, Rspo3-LGR4 signaling requires clathrin-mediated endocytosis, which is essential for β -catenin activation (Glinka et al. 2011). Requirement of Rspo1/Wnt3a-induced clathrin-mediated endocytosis of LGR5 for Wnt/ β -catenin signaling was not observed by others (Carmon

et al. 2012), which might be due to artificial over-expression of LGR5 together with LRP6 and Fz5.

What is the mechanism by which Rspo and LGR4/5 potentiate Wnt/ β -catenin signaling? Cong and his coworkers provide insight into this mechanism. In a search for novel negative Wnt regulators, they identified transmembrane E3 ubiquitin ligase ZNRF3 and its homolog ring finger 43 (RNF43) (Hao et al. 2012). ZNRF3 inhibits Wnt signaling by promoting the turnover of Fz receptors and LRP6. Rspo binds to the extracellular part of ZNRF3 and promotes the interaction between ZNRF3 and LGR4, which results in ZNRF3 clearance from the

membrane. These results suggest a model wherein Rspo activates Wnt signaling by inhibiting ZNRF3 in an LGR4-dependent manner, resulting in the accumulation of Wnt receptors at the cell surface (Fig. 3A). The ZNRF3-mediated suppression of Wnt/ β -catenin signaling is crucial for lens development in mice (Hao et al. 2012).

Apart from their function in canonical Wnt signaling, Rspos also amplify Wnt/PCP signaling. In particular, Rspo3 binds Sdc4 through its thrombospondin type-1 domain, and they functionally interact during *Xenopus* gastrulation and head cartilage morphogenesis, two

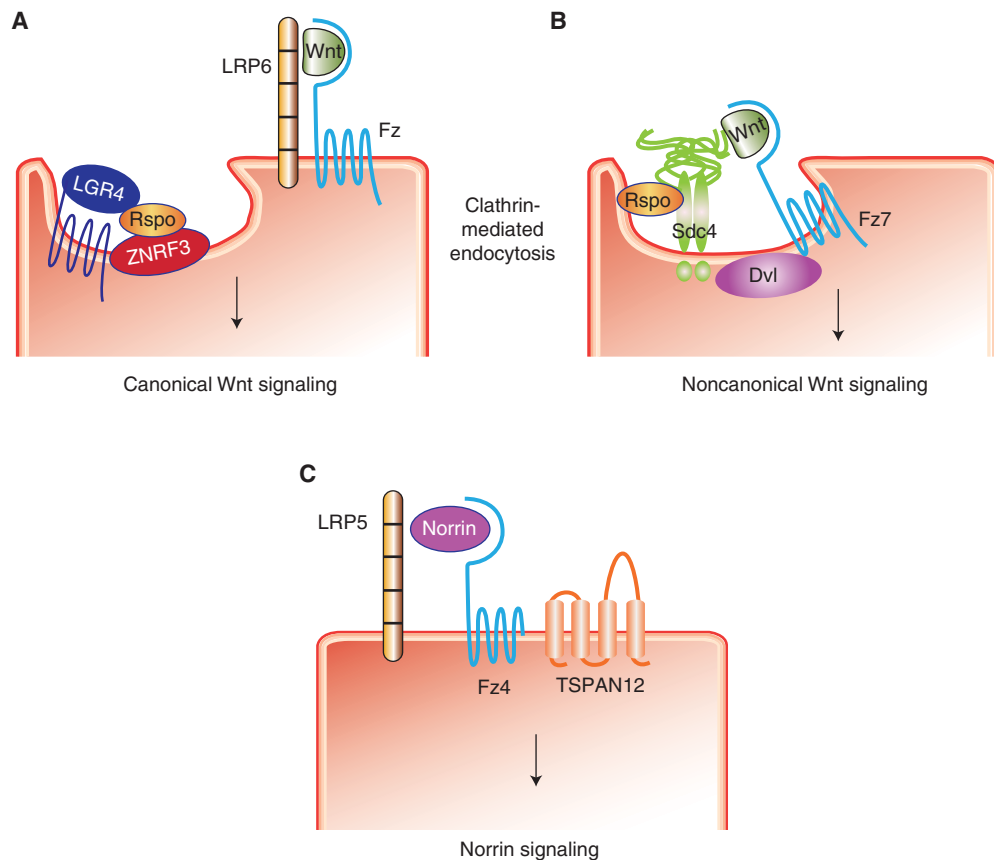


Figure 3. Models of Wnt signaling activation. (A) Rspo binds to ZNRF3 and LGR4 and induces clathrin-mediated receptor endocytosis. Internalized ZNRF3 is unable to ubiquitinate and target Wnt receptors for degradation. As a consequence, Fz and LRP6 accumulate on the plasma membrane and transmit canonical Wnt signals. (B) Rspo3 binding to Sdc4 promotes clathrin-mediated endocytosis of the Wnt receptor complex and thereby activates noncanonical Wnt signaling. (C) TSPAN12 is part of the Norrin/Fz4/LRP5 signaling complex and promotes Norrin/ β -catenin signaling.

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Wnt/PCP-driven processes (Ohkawara et al. 2011). Rspo3/Sdc4 signaling requires Wnt5a, Fz7, and Dvl and activates JNK (Ohkawara et al. 2011). Moreover, Rspo3 binding to Sdc4 promotes clathrin-mediated endocytosis, which is essential for PCP signal transduction (Fig. 3B). Not surprisingly, LGR4/5 are also essential for Rspo3-induced Wnt/PCP signaling in *Xenopus* (Glinka et al. 2011), and the interaction of ZNRF3 with Rspo and LGR4 suggests that this complex functions in Wnt/PCP signaling as well. Further studies are needed to clarify the interplay between Rspo/Sdc4 and Rspo/LGR/ZNRF3 in PCP signaling, and to determine the factor(s) that confer specificity of Rspo to signal either by β -catenin or the PCP pathway.

Role of Rspos in Embryonic Development and Disease

Loss-of-function analyses in *Xenopus* and mice as well as recent genetic studies in humans underline the role of Rspos in embryonic development and disease. In *Xenopus* and mouse, Rspo family members show differential expression in a broad range of embryonic tissues, such as dorsal neural tube, somites, tailbud, AER of the limb, and developing brain (Kazanskaya et al. 2004; Nam et al. 2007b). They are coexpressed with several Wnt genes and their expression is regulated by Wnts (Kazanskaya et al. 2004).

Role of Rspo1 in Female Sex Determination.

Mutations in human *Rspo1* were identified in individuals with female-to-male sex reversal (Parma et al. 2006) or XX true hermaphroditism (Tomaselli et al. 2008), a disorder of gonadal development characterized by the presence of both ovarian and testicular tissue. These mutations result either in a stop codon immediately after the signal peptide or in a truncated protein lacking the first furinlike domain, and thus are linked to the inability of Rspo1 to activate Wnt signaling. The potential role of *Rspo1* as a candidate female-determining gene is confirmed in *Rspo1* knockout XX mice, which display masculinized ovaries (ovotestis) with epididymis and vas deferens-like structures (Chassot et al. 2008; Tomizuka et al. 2008). The observed reduction

of *Wnt4* expression and the impaired Wnt/ β -catenin activity in these mice, as well as the sex reversal phenotype observed in *Wnt4*^{-/-} animals (Vainio et al. 1999), suggest a crucial role for Rspo1/Wnt4/ β -catenin signaling in ovary development.

Mutations in human *Rspo1* are also responsible for palmoplantar hyperkeratosis and predisposition to squamous cell carcinoma of the skin (Parma et al. 2006), suggesting that *Rspo1* might act as a tumor suppressor gene. Indeed, suppression of *Rspo1* expression by hypermethylation of its promoter is found in leukemia cell lines and leukemia patients (Kuang et al. 2008).

Role of Rspo2 in Limb, Craniofacial, and Lung Morphogenesis and Myogenesis.

Footless is a mouse mutant carrying a hypomorphic allele of *Rspo2* (*Rspo2*^{Tg/Tg}) (Bell et al. 2008). *Rspo2* knockout mice were generated (Nam et al. 2007a; Aoki et al. 2008; Bell et al. 2008; Yamada et al. 2009; Jin et al. 2011). All these animals immediately die after birth and display hind-limb defects, severe laryngeal and tracheal malformations, lung hypoplasia, and branching defects. In particular, abnormal limb development is manifested through a delayed maturation of the AER. Expression of *Axin2* and the *TopGAL* transgene is significantly reduced in the AER of *Rspo2* mutant mice, indicating that Rspo2 activity in the AER is mediated by Wnt/ β -catenin signaling (Nam et al. 2007a; Bell et al. 2008). Furthermore, double-knockout mutants of *Rspo2* and *LRP6* (*Rspo2*^{Tg/Tg}/*LRP6*^{-/-}) showed much more severe limb defects than the single-knockouts, indicating that both genes functionally interact to regulate limb development (Bell et al. 2008).

In *Xenopus*, Rspo2 through Wnt/ β -catenin signaling is required for the expression of myogenic marker genes, *myf5* and *myoD*, and later muscle development (Kazanskaya et al. 2004). Consistently, limb-specific *myf5* expression is down-regulated in *Rspo2* knockout mice (Han et al. 2011), supporting a role of Rspo2 in myogenesis.

Role of Rspo3 in Vasculogenesis and Angiogenesis. In *Xenopus* embryos, Rspo3 regulates the balance between hematopoietic and endo-



thelial differentiation by promoting angioblast specification and inhibiting blood cell specification. It does so by promoting Wnt/ β -catenin signaling, which is required for expression of *VEGF* (Kazanskaya et al. 2008). In mice, targeted deletion of *Rspo3* is embryonic lethal because of angiogenesis defects in placenta and yolk sac (Aoki et al. 2007; Kazanskaya et al. 2008). As in *Xenopus*, mouse *Rspo3* is required for Wnt/ β -catenin-mediated induction of vascular endothelial growth factor (VEGF). Moreover, recombinant *Rspo3* promotes proliferation and angiogenesis in endothelial cell lines (Kazanskaya et al. 2008).

Role of *Rspo4* in Nail Development. Mutations in *Rspo4* cause onychia (Bergmann et al. 2006; Blaydon et al. 2006; Bruchle et al. 2008; Ishii et al. 2008), an autosomal recessive disorder characterized by partial or complete loss of finger- and toenails (Baran and Kechijian 2001). *Rspo4* mutations are found either in the furin-like domains, or predict truncated versions lacking any feature of an *Rspo* protein.

Role of *Rspos* in Cancer. In addition to the possible role of *Rspo1* as tumor suppressor, *Rspo2* and *-3* promote tumorigenesis in vivo. *Rspo2* was identified as a common integration site for the mouse mammary tumor virus (MMTV) in mouse mammary tumors (Lowther et al. 2005), as well as a candidate colorectal cancer gene (Starr et al. 2009). In a similar screen for MMTV insertion sites, *Rspo3* was identified as a candidate breast cancer gene, which can strongly enhance oncogenicity (Theodorou et al. 2007).

Norrin

The Norrie disease pseudoglioma (*NDP*) or *Norrin* gene encodes a small secreted factor containing a cystine knot motif (Fig. 1) and a tertiary structure similar to that of TGF- β (Meitinger et al. 1993). Mutations in *Norrin* cause Norrie disease (Berger et al. 1992; Chen et al. 1992; Meindl et al. 1992), an X-linked disorder characterized by vascular abnormalities in the eye and blindness, often accompanied by progressive hearing loss and mental retardation (Berger 1998).

The nearly identical vascular defects in the retina and inner ear observed in *Fz4*^{-/-} mice (Xu et al. 2004) and in *Ndp* knockout mice (Richter et al. 1998; Rehm et al. 2002) encouraged Nathans and his coworkers to analyze whether Norrin and *Fz4* constitute a ligand-receptor pair. Indeed, Norrin is a high-affinity ligand for *Fz4*. It binds to the CRD of *Fz4* with nanomolar affinity and cooperates with *Fz4* to activate lymphoid enhancer factor (LEF)/TCF-mediated transcription in an LRP5/6-dependent manner (Xu et al. 2004). Thus, although structurally unrelated to Wnts, Norrin functions like a Wnt. Norrin function requires three pairs of cysteines that form the conserved trio of disulfide bonds shared among all cystine knot proteins (Smallwood et al. 2007).

Evidence for a functional interaction between *Norrin*, *Fz4*, and *LRP5* in vivo is provided by the finding that in humans, mutations in each gene cause familial exudative vitreoretinopathy (Chen et al. 1993; Robitaille et al. 2002; Toomes et al. 2004a,b), an inherited disease characterized by peripheral retinal avascularity (Warden et al. 2007). Consistent with the findings in human patients, targeted inactivation of *Norrin*, *Fz4*, or *LRP5* in mice causes similar alterations in the retinal vascularization (Xu et al. 2004; Luhmann et al. 2005a; Xia et al. 2008).

Among the 10 mammalian *Fzs*, *Fz4* is the only Norrin receptor (Smallwood et al. 2007). Yet *Fz4* can also transduce the signal of Wnts, raising the question of how *Fz4*/*LRP5* can respond to different types of ligands to activate β -catenin-dependent transcription. The identification of TSPAN12, a tetraspanin family member, as a component of the Norrin/*Fz4*/*LRP5* complex provides an answer to this question. TSPAN12 specifically promotes *Fz4*/*LRP5* signaling induced by Norrin, but not by Wnt ligands, to regulate retinal vascular development in mice (Fig. 3C) (Junge et al. 2009).

The expression of mouse Norrin in the developing retina, neural tube, and brain and its expression in the adult central nervous system, uterus, and decidua (Luhmann et al. 2005b; Ye et al. 2011) suggest that Norrin may have developmental functions beyond those described in the eye.

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CONCLUDING REMARKS

Considerable progress has been made in our effort to understand the molecular mechanism by which Wnt inhibitors and activators regulate Wnt signaling. These molecules have emerged as key regulators of Wnt signaling—controlled processes during embryogenesis, including head, limb, bone, heart, somites, and vasculature development, and have implications for pathological events, including cancer and bone disease. It is not surprising that agents targeting Dkk1 and sclerostin are currently in clinical trials. BHQ880, an anti-Dkk1 neutralizing antibody, is being investigated as an osteoblastogenesis stimulator for the potential treatment of bone lesions in multiple myeloma patients (Fulciniti et al. 2009), and anti-sclerostin monoclonal antibody AMG 785 is being examined for the potential treatment of diseases associated with bone loss, including osteoporosis (Lewiecki 2011; Padhi et al. 2011). Moreover, because of its potent proliferative effect on the intestinal epithelium in vivo and in vitro, Rspo1 holds promise as therapeutic target for gastrointestinal diseases and regenerative medicine (Kim et al. 2005; Ootani et al. 2009; Sato et al. 2009).

It has become evident that beyond the regulation of Wnt signaling, Wnt inhibitors and activators do modulate other signaling cascades as well, and we will benefit from future research to fully understand their Wnt-dependent and -independent roles.

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