

The functions and possible significance of Kremen as the gatekeeper of Wnt signalling in development and pathology

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Abstract

Kremen (Krm) was originally discovered as a novel transmembrane protein containing the kringle domain. Both Krm1 (the first identified Krm) and its relative Krm2 were later identified to be the high-affinity receptors for Dickkopf (Dkk), the inhibitor of Wnt/ β -catenin signalling. The formation of a ternary complex composed of Krm, Dkk, and Lrp5/6 (the coreceptor of Wnt) inhibits Wnt/ β -catenin signalling. In *Xenopus* gastrula embryos, Wnt/ β -catenin signalling regulates anterior-posterior patterning, with low-signalling in anterior regions. Inhibition of Krm1/2 induces embryonic head defects. Together with anterior localization of Krms and Dkks, the inhibition of Wnt signalling by Dkk-Krm action seems to allow anterior embryonic development. During mammalian development, *krm1* mRNA expression is low in the early stages, but gradually and continuously increases with developmental progression and differentiation. In contrast with the wide, strong expression of *krm1* mRNA in mature tissues, expression of *krm1* is diminished in a variety of human tumor cells. Since stem cells and undifferentiated cells rely on Wnt/ β -catenin signalling for maintenance in a low differentiation state, the physiological shutdown of Wnt/ β -catenin signalling by Dkk-Krm is likely to set cells on a divergent path toward differentiation. In tumour cells, a deficit of Krm may increase the susceptibility to tumorigenic transformation. Both positive and negative regulation of Wnt/ β -catenin signalling definitively contributes to diverse developmental and physiological processes, including cell-fate determination, tissue patterning and stem cell regulation. Krm is quite significant in these processes as the gatekeeper of the Wnt/ β -catenin signalling pathway.

Keywords: axis formation • β -catenin • cancer • Dickkopf (Dkk) • Frizzled (Fz) • Kremen (Krm) • Kringle domain • low-density lipoprotein (LDL) receptor-related protein (Lrp) • stem cell • Wnt

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Introduction

The Wnt family of signalling proteins participates definitively in diverse developmental and physiological processes, including cell-fate determination, tissue patterning, cell proliferation/differentiation and stem cell regulation. The mouse *Wnt* gene, formerly called *Int-1*, was originally identified by Nusse and Varmus as a proto-oncogene that induced tumours when the mouse mammary tumour virus was integrated into a preferential integration site [1]. Subsequently the *Drosophila* homologue of *int-1* was identified as the segment polarity gene *wingless*, and thus the integrated name 'Wnt' was created.

In *Drosophila*, mutations in *disheveled* and *armadillo* genes displayed abnormalities similar to *wingless*, whereas mutations in *shaggy/zeste-white3* caused the opposite phenotype, suggesting a new signal transduction pathway. In vertebrate *Xenopus*, duplication of the body axis was observed in the early stage of embryos after injection of *wnt1* mRNA [2], and this observation provided a convenient assay to study components of the Wnt signalling pathway. Axis duplication was also induced by *disheveled*, β -catenin (the vertebrate homolog of *armadillo*), and a dominant-negative form of glycogen synthase kinase 3 (GSK3) (the vertebrate homolog of *shaggy/zeste-white3*). Independent of these studies, the *adenomatous polyposis coli* (*APC*) gene was identified in a hereditary human cancer and APC protein was thereafter found to interact with β -catenin. These studies provided not only a highly conserved signalling pathway activated by Wnt proteins, but also the connection between the Wnt signalling pathway and human cancer.

Kremen (Krm) was originally discovered as a novel transmembrane receptor-like protein, containing an extracellular kringle domain [3]. Later, Krm was shown to be the receptor for Dickkopf (Dkk) protein [4], which is the inhibitor of the Wnt signalling pathway. Together with Dkk, Krm constitutes machinery, functioning as a cell surface gatekeeper for the entry of Wnt signalling. Although biological and physiological studies of Krm have largely remained open, this article reviews structural and functional characteristics of Krm and discusses potential roles of Krm in development, diseases, and cancer, based on its expression and function.

Identification and structure of Krm

Krm was originally cloned through an approach to identifying novel proteins that contain the kringle domain. The kringle domain, a unique structural motif composed of triple-disulfide-linked peptides (Fig. 1A), was first identified in the blood coagulation factor prothrombin. It was subsequently identified in a variety of proteins that play diverse roles in biological and physiological processes. For instance, the kringle domain is conserved in serine proteases that are involved in blood coagulation and fibrinolysis (e.g. plasminogen, tPA, etc), hepatocyte growth factor [5], and Ror transmembrane receptor tyrosine kinase [6]. Therefore, molecular cloning of novel kringle-containing proteins was thought to provide a clue to understanding hitherto unrevealed molecular mechanisms that underlie complex biological processes.

The strategy undertaken to clone cDNAs for kringle-containing proteins was based on the structure of a unique stretch of 16 or 17 amino acids in the kringle domain (Fig. 1A and B). This is characterized by a 5 or 6 amino acid stretch in which amino acid sequences are variable and unique to each kringle, and this variable region is surrounded by highly conserved sequences. Using degenerate primers especially designed for the two conserved sequences, reverse transcriptase RT-PCR generated short cDNA sequences with variable regions that were highly unique to each kringle (Fig. 1B). Through large-scale sequence analyses of concatemers composed of amplified short cDNA fragments (Fig. 1C), one short cDNA fragment encoding a part of a novel kringle-protein was identified and used to clone full-size cDNA. The full-size cDNA contained a 1422 bp open reading frame and encoded a putative protein composed of 473 amino acid residues. Since it was a novel kringle-containing protein and its expression pattern marked the facial structures in embryos, this molecule was named Krm (Kringle-coding gene marking the eye and the nose) [3].

Krm is a type-I transmembrane protein composed of an extracellular region of 389 amino acids, a transmembrane domain and a cytoplasmic region of 64 amino acids. The extracellular region of Krm has a kringle domain, a WSC domain and a CUB domain (Fig. 1D). The CUB domain, originally identified in complement subcomponent C1r/C1s, embryonic sea

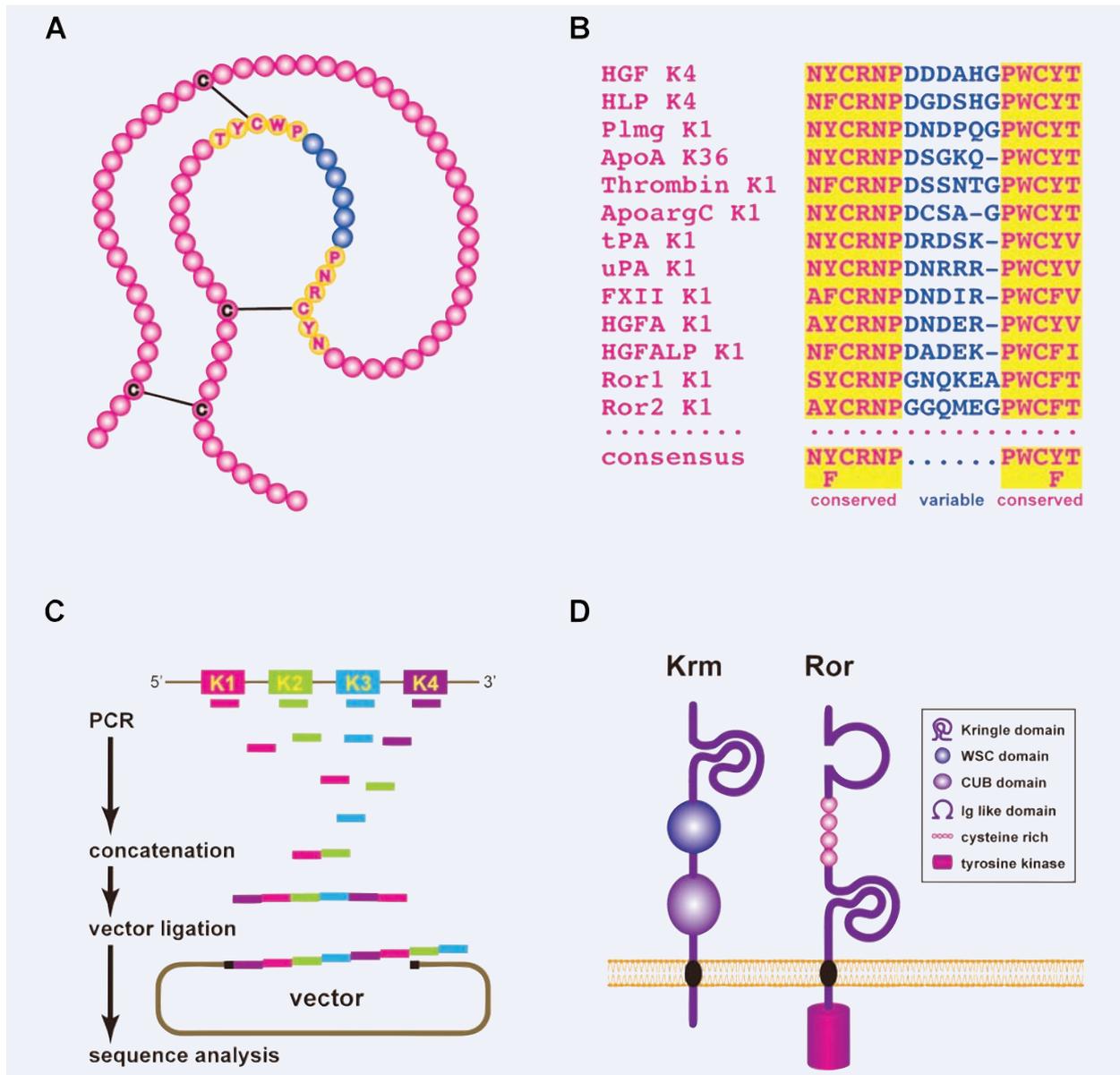


Fig. 1 Schematic structures of kringle domain and Krm. **(A)** The kringle domain and localization of a variable/unique stretch of 5 or 6 amino acids (blue circles) surrounded by two highly conserved sequences (yellow circles). **(B)** Multiple alignments of amino acid sequences of two highly conserved sequences that surround a variable region of the kringle domain. Two highly conserved sequences are boxed with yellow. **(C)** Outline for comprehensive analysis of partial cDNA fragments for kringle-containing proteins. PCR-amplified kringle tags are concatenated and ligated into the cloning plasmid. For details, see Nakamura *et al.* [3]. **(D)** Schematic representation of the two transmembrane proteins containing kringle domain, Krm and Ror.

urchin protein Uegf, and bone morphogenetic protein-1, is characterized by a spanning sequence motif with an antiparallel L-barrel structure [7, 8]. The CUB domain has been identified in proteins regulating

development, including tolloid (a dorso-ventral patterning molecule), Bp10 and Span (blastula specific proteins) and neurophilin (a specific receptor for semapholin/collapsin) [7, 9, 10]. A stretch of 77 amino

acid residues located between the kringle and CUB domains is homologous with the WSC domain. The WSC domain was originally identified in WSC1/SLG1 yeast protein involved in cell wall integrity and stress response [11]. The intracellular region of Krm is not homologous with other proteins, including typical motifs for intracellular signal transduction. In vertebrates, two *krm* genes have been identified, *krm1* and *krm2* [4]. Although amino acid sequence homologies between vertebrate Krm1 and Krm2 are only 35–40%, both the occurrence and the order of their domains are conserved in all orthologs [12].

In addition to Krm, it is notable that the kringle domain is also found in the Ror receptor tyrosine kinase. Although both Krm and Ror are kringle-containing transmembrane proteins involved in the Wnt signalling pathway, their functions are different. Ror is a receptor for Wnt-5a and is involved in the development of the heart and limbs in mice and the guidance of migrating cells, asymmetric cell division and axonal outgrowth (Fig. 1D) [13–17].

Krm as a negative regulator in Wnt signalling

Wnt family proteins are secreted, cysteine-rich glycoproteins and are composed of 19 *Wnt* genes that share 27% to 83% amino acid sequence identity in humans [18]. Wnts are highly hydrophobic, a characteristic not predicted from their primary sequence; this property is due to fatty acylation. In Wnt3a, two conserved cysteine and serine residues (Cys77 and Ser209) are acylated. A mutant form of Wnt3a, in which the palmitoylated Cys77 is substituted with Ala, cannot activate Wnt signalling [19]. Fatty acid modification at Ser209 is regulated for intracellular transport and secretion [20]. It is highly likely that the hydrophobic property defines graded and/or restrictively localized distribution of Wnts as unique signalling molecules required for body axis specification, cell fate determination, stem cell maintenance, etc. [21–23].

Wnts trigger at least three pathways through Wnt receptors of the Frizzled (Fz) seven transmembrane class. These are (1) the canonical, or Wnt/ β -catenin pathway [24], (2) the planar cell polarity (PCP) pathway, which recruits small GTPases of the rho/cdc42

family to activate Jun kinase (JNK) and (3) the Wnt/ Ca^{2+} cascade [25]. In the Wnt/ β -catenin pathway, the best understood Wnt signalling pathway, Wnt proteins released from or presented on the surface of effector cells act on neighbouring target cells by binding to the Fz and low-density lipoprotein (LDL) receptor-related protein 5/6 (Lrp5/6) complex at the cell surface (Fig. 2A). These receptors transduce a signal to several intracellular proteins that include Dishevelled (Dv), glycogen synthase kinase-3 β (GSK-3 β), Axin, APC and β -catenin. The GSK-3 β /APC/Axin complex controls cytoplasmic β -catenin levels through regulation of proteasome-mediated degradation. Low-cytoplasmic β -catenin levels are normally maintained by continuous degradation, but when cells receive Wnt signals, the degradation pathway is inhibited, and consequently β -catenin accumulates in the cytoplasm and nucleus (Fig. 2A). Nuclear β -catenin interacts with transcription factors such as lymphoid enhancer-binding factor 1/T cell-specific transcription factor (LEF1/TCF).

Wnt/ β -catenin signalling is inhibited by the secreted protein Dickkopf1 (Dkk1), which consists of four main members in vertebrates (Dkk1~Dkk4). Dkk1, the original member of the Dkk family, was identified as a head inducer and Wnt antagonist in *Xenopus* [26]. Dkks are glycoproteins of 255–350 amino acids, with calculated molecular weights between 24 and 29 kD for Dkk1, Dkk2 and Dkk4, and 38 kD for Dkk3. Dkks bind to Lrp5/6 and this binding inhibits the functional complex formation between Wnts, Fz and Lrp5/6, thereby inhibiting the Wnt/ β -catenin signalling pathway (Fig. 2B) [27, 28]. Although the inhibition of the Wnt/ β -catenin pathway is the primary action of Dkks, recent publications have indicated that Dkks facilitate the Wnt/PCP pathway [29]. In this case, association of Dkk with Lrp5/6 inhibited the Wnt/ β -catenin pathway, which was associated with switching from the Wnt/ β -catenin to the Wnt/PCP pathway.

Krm1 and Krm2 are the second set of high-affinity receptors for Dkk, and Krm proteins strongly cooperate with Dkk to inhibit Wnt/ β -catenin signalling (Fig. 2B) [4]. Because *Drosophila* does not have Dkk or Krm homologs, but does have an Lrp homologue, the functional association of Krm and Dkk has been analysed in *Drosophila* [4]. Ectopic co-expression of vertebrate *dkk* and *krm* together, but neither of these genes alone, results in inhibition of Wg/Wnt signalling [4]. Upon binding to Dkk1, Krm proteins (both Krm1 and Krm2) are recruited into a complex with

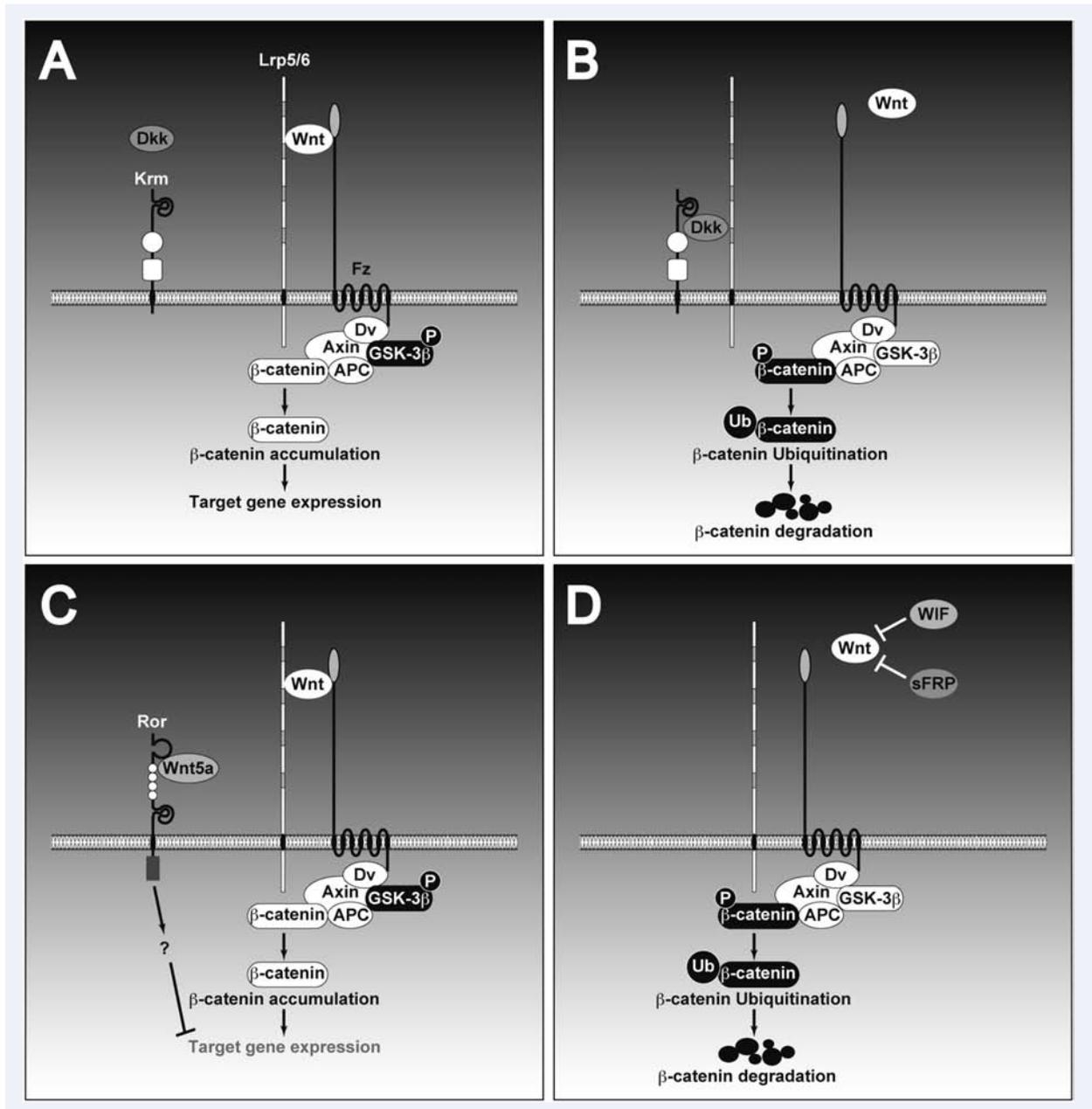


Fig. 2 Regulation of Wnt/β-catenin signalling pathway. **(A)** Wnt forms a ternary complex with Fz and Lrp5/6, which promotes stabilization of β-catenin, thereby activating the pathway. **(B)** Dkk1 forms a ternary complex with Lrp5/6 and Krm, which blocks Wnt signal transduction by causing endocytosis of Lrp5/6. **(C)** Ror is the receptor for Wnt5a and blocks Wnt/β-catenin signalling by inhibiting β-catenin-dependent gene transcription. **(D)** WIF and sFRP antagonize the binding of Wnt to Fz.

Lrp5/6, which leads to rapid endocytosis and removal of Lrp5/6 from the plasma membrane. The inhibitory function of Dkks depends on the presence

of appropriate Krm proteins. Dkk2 requires Krm2 in order to inhibit Wnt signalling and cannot function with Krm1 to down-regulate the Wnt signal [30]. In

particular, Krm2 regulates Dkk2 to inhibit Wnt signalling; however, Dkk2 can function as either a Wnt agonist or antagonist depending on the cellular context [30]. Krm1 and Krm2 bind both Dkk1 and Dkk2, but not Dkk3, with an apparent K_d in the nM range. A recent publication indicates that R-Spondin1 interferes with Dkk1/Krm-mediated internalization of Lrp6 through an interaction with Krm [31], suggesting that R-Spondin1 may participate in Wnt/β-catenin signalling through increased levels of cell surface Lrp6.

The specific interaction of Krm with Dkk depends on its extracellular domains [4]. Deletion of any of the extracellular domains in Krm, including a kringle, WSC or CUB domain, abolishes the association with Dkk1. In contrast, deletion of the intracellular domain of Krm does not significantly affect its binding to Dkk1 and inhibition of Wnt/β-catenin signalling. Attachment of the extracellular region of Krm to the plasma membrane is critical for its function, because a GPI-anchored extracellular region of Krm confers Dkk1 binding and Wnt inhibition, but a secreted form of the extracellular region of Krm is inactive. This suggests that the Krm cytoplasmic domain is of minor importance to the inhibition of Wnt signalling. However, a possibility that the cytoplasmic domain may also play a role in the biological function of Krm cannot be excluded, because amino acid sequence homology between humans and *Xenopus* reaches approximately 70% for the cytoplasmic region of Krm.

In addition to Dkk and Krm, there are other negative cell-surface modulators for the Wnt/β-catenin pathway. The kringle-containing receptor tyrosine kinase Ror inhibits the transcriptional activity of β-catenin (Fig. 2C). The Wnt inhibitory factor (WIF) and the secreted Fz-related protein (sFRP) act as Wnt antagonists through their competitive binding to the Wnt receptor Fz (Fig. 2D) [32].

Expression of Krm

During embryonic development, both *krm1* and *krm2* mRNAs are expressed at early developmental stages of the mouse embryo (Figs 3 and 4A) [3, 12]. During the progression of embryogenesis, these expression levels gradually increase (Fig. 4A). *krm1* mRNA is detectable in the mouse embryo in some sensory organs (optic vesicle, otic vesicle and nasal pit, etc.), and in the lungs, heart, apical ectodermal

ridge (AER) and somites (Fig. 3A–C) [3, 12, 33, 34]. The expression patterns of *krm2* are similar to those of *krm1* [12]. *krm1* mRNA is also expressed in the floor plate, which is the region important for commissural neurons to establish the correct neuronal network connections (Fig. 3D–G). *krm1* mRNA expression is detectable at both the rostral and lumbar levels of the floor plate of the neural tube, suggesting a possible role for Krm in the regulation of Wnt/β-catenin dependent neural network connections. In the adult mouse, relatively strong expression of *krm1* mRNA is seen in various tissues (Fig. 4B).

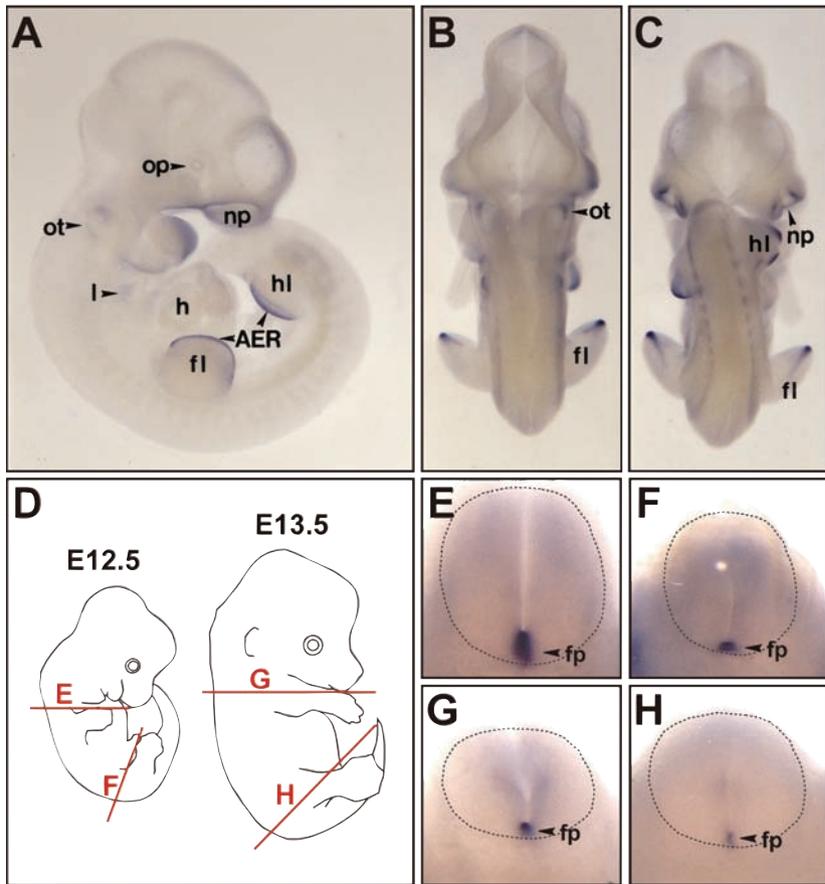
In *Xenopus*, *krm* mRNAs are present throughout embryogenesis. Zygotic expression starts in early (*krm2*) and late gastrulation (*krm1*), and it remains relatively constant throughout neurulation and organogenesis [12]. *krm2* expression is observed in the gastrula marginal zone, except for the Spemann organizer. At the early neurulation stage, *krm1* and *krm2* expression is seen in the anterior mesoderm. In mid-neurulae neural tubes, expression is seen in the dorsal midline, in two longitudinal stripes, and in the prechordal plate. In tailbud embryos, expression is seen in the hatching gland, the branchial arch, the dorsal otic vesicle, the fin mesenchyme and the pronephric duct. At the tailbud stage, *krm1* exhibits additional expression in the notochord and somites, compared with the expression of *krm2* [12].

Krm in development

The Wnt signalling pathway is known to be important for embryogenesis, through both *in vivo* and *in vitro* studies. Likewise, many studies have pointed out the importance of negative regulation of this pathway for successful cell-fate determination and organogenesis. Introduction of loss-of-function mutations and impaired negative regulation of the Wnt/β-catenin signalling pathway results in developmental abnormality in experimental animals (Table 1).

During early patterning of the vertebrate central nervous system (CNS), neural inducers and modifiers establish a crude anterior-posterior (AP) pattern before and during gastrulation, which becomes refined at later stages. During *Xenopus* gastrulation, AP patterning of the entire neural plate is regulated by Wnt/β-catenin signalling. This signalling is higher in posterior regions of the embryo and lower in

Fig. 3 Spatial expression patterns of *krm1* mRNA in mouse embryo. (A–C) *In situ* hybridization of E10.5 mouse whole embryo. Lateral (A), dorsal (B) and ventral (C) view of embryo. (D) Schematic representation of E12.5 and E13.5 mouse embryos. Lines indicate the level of the sections shown in E–H, respectively. (E–H) *In situ* hybridization of E12.5 and E13.5 mouse transverse sections. E–H show the rostral and the lumbar level sections of E12.5 mouse embryo and the rostral and the lumbar level sections of E13.5 mouse embryo, respectively. AER, apical ectodermal ridge; fl, forelimb bud; h, heart; hl, hindlimb bud; l, lung; np, nasal pit; op, optic vesicle; ot, otic vesicle; fp, floor plate.



anterior regions, probably as a consequence of Wnt and Wnt inhibitor expression domains being predominantly posterior and anterior, respectively. Indeed, one distinguishing feature of organizing centres involved in anterior neural induction in vertebrates is the expression of Dkks and Krms. Inactivation of the *Dkk1* in *Xenopus* embryos and targeted deletion of the *dkk1* gene in mouse embryos results in microcephalic embryos [26, 58, 64]. In *Xenopus* embryos, inhibition of *Krm1/2* induces embryonic head defects. In addition, there is strong enhancement of head defects when both *Dkk1* and *Krm1/2* are inhibited [12]. These data support the model that inhibition of Wnt/ β -catenin signalling by Dkk-Krm action allows anterior embryonic development.

In the developing heart, Wnt signalling is critical during the very early stages of differentiation to mesoderm lineages, but it is repressive during later stages of differentiation to cardiac myocytes [65–67]. Thus, inhibition of the Wnt pathway seems to be permissive for differentiation of cardiac myocytes. In *Xenopus*, inhibition of Wnt signalling by *Dkk1* leads

to enhanced cardiac development, while in mice, sFRP leads to repair after myocardial infarction [68–71]. Expression of *krm1* mRNA is low in the embryonic heart but high-level *krm1* expression is seen in the adult heart. In an *in vitro* skeletal muscle differentiation model, the expression of *krm1* mRNA is detectable earlier than that of *myogenin* and gradually increases during the progression of differentiation [3]. Inhibition of Wnt signalling by Dkk-Krm cooperation may support myocardial development and differentiation.

Appropriate spatio-temporal inhibition of the Wnt/ β -catenin pathway plays a critical role in lung development and morphogenesis. In the developing lung, Wnt/ β -catenin signalling is dynamic and active throughout the epithelium and in the proximal smooth muscle cells until E12.5. However, from E13.5 onward, the Wnt signal is no longer present in the mesenchyme and the activity in the epithelium is reduced distally, concomitant with the onset of expression of *dkk1* in the distal epithelium [34]. Constitutive activation of the Wnt/ β -catenin pathway

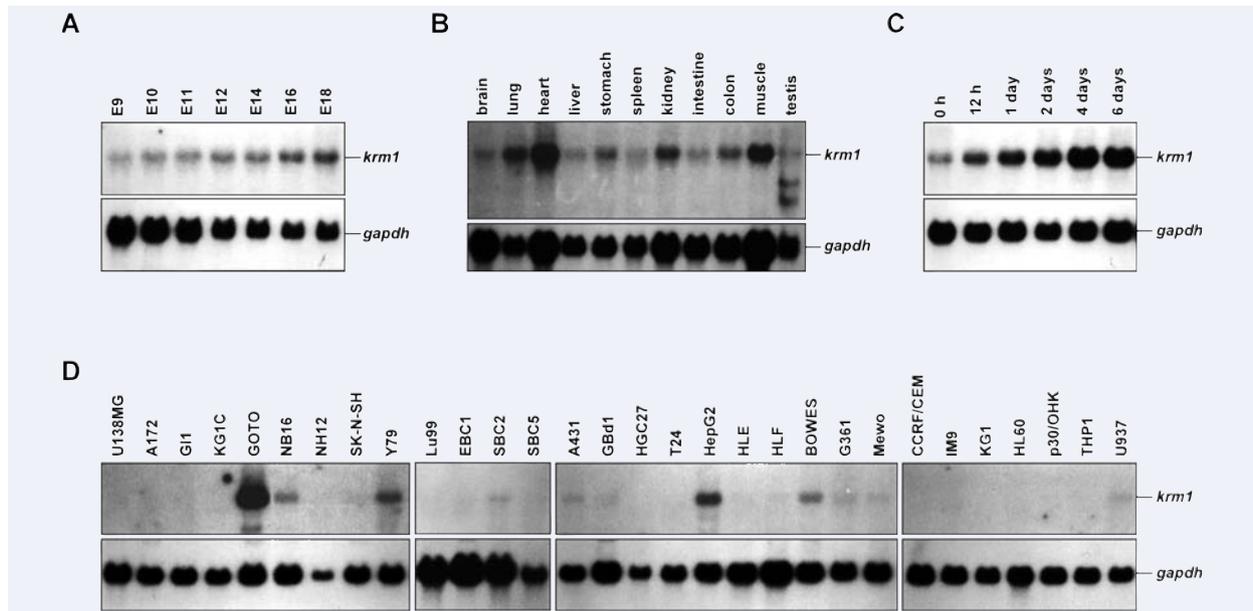


Fig. 4 (A) Expressions of *krm1* mRNA in whole mouse embryos at serial developmental stages. (B) Tissue distribution of *krm1* mRNA in adult mouse. (C) Change in the expression of *krm1* mRNA during myogenic differentiation in C2C12 cells. Expression of *krm1* mRNA in C2C12 cells cultured for various periods under conditions permissible for differentiation was analysed. (D) Expressions of *krm1* mRNA in human cancer cell lines.

in lung progenitor cells consistently results in a lack of alveoli and a reduction in lung size [72]. On the other hand, depletion of β -catenin in both epithelium and mesenchyme in lung explants results in increased branching morphogenesis [73]. Thus, Wnt/ β -catenin signalling appears to have a repressive function on lung branching morphogenesis during late stages of lung development. Along with Dkk1 expressed in the distal epithelium, the involvement of Krm in alveolar formation is significant.

Commissural neurons in the mammalian dorsal spinal cord send axons ventrally toward the floor plate, where they cross the midline and turn anteriorly toward the brain; a gradient of chemoattractant(s) inside the spinal cord controls this change in direction. Several Wnt proteins stimulate the extension of commissural axons after crossing the midline. *wnt4* mRNA is expressed in a decreasing anterior-to-posterior gradient in the floor plate, and a directed source of Wnt4 protein attracts after crossing commissural axons. Commissural axons in mice lacking the Wnt receptor Fz3 displayed anterior-posterior guidance defects after crossing the midline [51]. Thus, in the floor plate, Wnt-Fz signalling guides commissural axons along the anterior-posterior axis

of the spinal cord. *krm1* shows distinct expression in the floor plate from the rostral to the lumbar level (Fig. 3D–G). This raises the question of the role of Krm in the floor plate. Cells in the floor plate accomplish a specialized mission, to precisely regulate anterior/posterior turning of commissural axons. Since the regulatory role and microenvironment of the floor plate for axonal extension, turning, or repulsion are defined by floor plate cells, changes in the biological characteristics of floor plate cells should be avoided. Cells that accept Wnt signalling may be commissural neurons but not floor-plate cells. Therefore, Krm in the floor-plate cells may play a role in shutting down Wnt signalling to acquire and maintain the specialized characteristics of floor-plate cells and the related microenvironment.

Wnt signalling in stem cell regulation and diseases

Tissue homeostasis and regeneration are supported by two distinct systems, the stem cell system and the

Table 1 Abnormalities associated with loss-of-function in Wnt signalling molecules

| Gene | Stimulus | Species | Phenotypes | References |
|---------------|------------------|----------------|---|-------------|
| <i>Wnt1</i> | Knockout | Mouse | Loss of portion of the midbrain and cerebellum. Deficiency in dorsal neural tube derivatives in double knockout with <i>Wnt3a</i> | [35–38] |
| <i>Wnt3</i> | Knockout | Mouse | Defect in early gastrulation. Perturbations in establishment and maintenance of the apical ectodermal ridge (AER) in the limb | [39, 40] |
| <i>Wnt3a</i> | Knockout | Mouse | Defect in somite and tailbud development. Deficiency in dorsal neural tube derivatives in double knockout with <i>Wnt1</i> | [38, 41–43] |
| <i>Wnt4</i> | Knockout | Mouse | Defect in kidney development. Defect in sex determination | [44, 45] |
| <i>Wnt5a</i> | Knockout | Mouse | Truncated limbs and antero-posterior axis. Defects in distal lung morphogenesis. Chondrocyte differentiation defects | [46, 47] |
| <i>Wnt7a</i> | Knockout | Mouse | Abnormal development of the oviduct and uterus | [48] |
| <i>Wnt7b</i> | Knockout | Mouse | Defect in placental development. Respiratory failure, defects in early mesenchymal proliferation leading to lung hypoplasia | [49] |
| <i>Wnt11</i> | Knockout | Mouse | Ureteric branching defects and kidney hypoplasia | [50] |
| <i>Fz3</i> | Knockout | Mouse | Defect in major axon tracts within the forebrain. Perturbed anterior-posterior guidance of commissural axon | [51,52] |
| <i>Fz4</i> | Knockout | mouse | Cerebellar, auditory, optical, and oesophageal defects | [53–55] |
| <i>Fz5</i> | Knockout | mouse | Essential for yolk sac and placental angiogenesis | [56] |
| <i>Fz7</i> | Antisense oligo | <i>Xenopus</i> | Depletion of maternal <i>Xfz7</i> disrupts dorsal anterior development | [57] |
| <i>Dkk1</i> | Knockout | Mouse | Deletion of anterior head structures and limb. Postaxial polysyndactyly. Fused vertebrae Increase in bone formation and bone mass | [58] |
| <i>Dkk2</i> | Knockout | Mouse | Increase in bone formation and bone mass. Blindness, cornea transformation | [59, 60] |
| <i>Lrp5</i> | Knockout | Mouse | Osteopenia | [61, 62] |
| <i>Lrp6</i> | Knockout | Mouse | Defect in caudal somites and limb. Truncation of the axial skeleton | [62, 63] |
| <i>Krm1/2</i> | Morpholino oligo | <i>Xenopus</i> | Defect in anterior neural development | [12] |
| <i>Ror1/2</i> | Knockout | Mouse | Truncated limbs and antero-posterior axis. Lung hypoplasia | [16, 17] |

simple duplication system. In tissues such as nervous tissue, in which differentiated cells lack the ability to duplicate, stem cells proliferate and differentiate into specific cell types. By contrast, in the simple duplication system, differentiated cells retain the ability to duplicate in response to tissue injury. The two systems cooperate in tissue homeostasis and regeneration, and the balance between the stem cell system and the simple-duplication system depends on the tissue type. For instance, liver regeneration largely depends on the simple-duplication system of differentiated hepatocytes, while hepatic stem cells participate in the renewal of hepatocytes, which occurs over a few hundred days.

Since physiological attenuation or suppression of Wnt signalling is necessary for cell fate determina-

tion and differentiation, stem cells are likely to rely on the Wnt signalling pathway for maintenance at the required low-differentiation level, even in adult tissues largely composed of differentiated cells [74]. Multipotent epidermal stem cells reside in the bulge region of hair follicles, and the Wnt signalling pathway plays a key role in the activation and maintenance of bulge stem cells for progression toward hair formation and regeneration of hair follicles. Conditional deletion of the β -*catenin* gene, or transgenic expression of dominant-negative Lef1/Tcf4, in established hair follicles disrupts the process by which bulge stem cells provide hair lineage precursors [75]. In one study, hair follicles were newly formed after cutaneous wounding and a stem cell population was established in the regenerated hair

follicles. In this *de novo* hair folliculogenesis, inhibition of Wnt signalling abrogated wound-induced hair folliculogenesis, whereas overexpression of the Wnt ligand increased the number of regenerated hair follicles in which stem cells were re-populated in adult mouse skin [76]. In addition, Wnt/ β -catenin signalling regulates self-renewal of haematopoietic stem cells in an autocrine and paracrine manner, as Wnts are produced by haematopoietic stem cells as well as by bone marrow stromal cells [74, 77].

In intestinal epithelium, the proliferating crypt precursors and differentiated villus cells form a contiguous epithelial sheet of cells that is in perpetual upward motion. Stem cells reside near the bottom of the crypt and produce the transit-amplifying progenitor cells that differentiate into villus cells. Wnt/ β -catenin signalling is a dominant force in cell fate determination along the crypt-villus axis [78]. In neonatal mice lacking *Tcf4*, the crypt progenitor compartment is absent [79], implying that physiological Wnt signalling is regulated for the establishment of this progenitor compartment. Inhibition of Wnt signalling by transgenic expression of *Dkk1* induces complete loss of the crypt in adult mice. Thus, Wnt signalling plays an important role in the maintenance of the stem cell niche, a specific environment in which stem cells maintain their capacity to self-renew and generate differentiated progeny. Instead, attenuation or inhibition of the Wnt pathway regulates subsequent differentiation of daughter cells that escape from the stem cell niche. *Dkk-Krm* co-operation may play roles not only in the homeostasis of the stem cell niche, in which characteristics of stem cell and non-stem cell populations are maintained, but also in the differentiation of cells that arise from the stem cell population.

A variety of abnormalities caused by genetic and functional inhibition of parts of the Wnt signalling pathway in experimental animals imply that impaired function and dysregulation in the Wnt signalling pathway may also cause both non-neoplastic and neoplastic disorders in humans. Human diseases caused by aberrant regulation of Wnt/ β -catenin signalling are listed in Table 2 [80]. In bone formation, Wnt/ β -catenin signalling plays an important role in increasing bone mass, particularly through biological activities in the ectoblastic cell lineage, including renewal of stem cells, stimulation of pre-osteoblast replication, induction of osteoblastogenesis, and inhibition of osteoblast and osteocyte apoptosis [103]. Thus, dysregulation of Wnt/ β -catenin signalling leads to abnormal bone formation. Osteoporosis-

pseudoglioma syndrome (OPPG), characterized by low bone mass, is caused by a loss-of-function mutation in the *Lrp5* gene, whereas a gain-of-function mutation in *Lrp5* is associated with a high bone mass phenotype [85]. It is notable that knockout of the *Lrp5* gene results in the development of osteopenia characterized by low bone mass in mice. Deletion of a single allele of the *dkk1* gene leads to an increase in bone formation and bone mass [104]. Likewise, deletion of the Wnt antagonist sFRP1 enhances bone formation, and target genes of β -catenin signalling have been implicated in bone formation [105, 106]. It can be predicted that a *krm* defect might result in a high bone mass phenotype due to excess Wnt signalling.

There is another human bone disorder caused by dysregulation of Wnt signalling. Transmembrane tyrosine kinase *Ror2*, the Wnt5a receptor, down-regulates β -catenin-mediated transcription and is involved in the development of the skeletal, cardiovascular and genital systems. Mutations in *ror2* have been shown to cause two distinct human disorders, autosomal recessive Robinow syndrome and dominantly inherited Brachydactyly type B [88, 89, 102]. Both disorders show symptoms of skeletal dysplasia, characterized by short stature, mesomelic limb shortening, brachydactyly, spinal segmental abnormalities, genital hypoplasia and dysmorphic facial appearance [90, 91]. The recessive form of Robinow syndrome is a disorder caused by loss-of-function mutations in *ror2*, whereas Brachydactyly type B is a dominant disease and is presumably caused by gain-of-function mutations in *ror2*, which is possibly associated with a constitutive active *Ror2*-mediated signalling. The reason both loss-of-function and gain-of-function mutations in *ror2* result in the same symptoms is not yet known. *Ror2* knockout newborn mice (*ror2*^{-/-}) exhibit dwarfism, short limbs and tail, facial malformations, cyanosis and ventricular septal defects, resembling the Robinow syndrome phenotype [107, 108].

The Wnt/ β -catenin pathway contributes not only to cardiac development, but also to cardiac physiology and pathology [109]. Cardiac hypertrophy is an adaptive response of the heart to sustained pressure overload, caused by pathological stimuli, such as myocardial infarction. Because cardiac myocytes are terminally differentiated, they can only respond by hypertrophic growth [110]. In mice lacking *Dv-1*, the onset of pressure-overload-induced cardiac hypertrophy was attenuated when compared with wild type mice [111]. In addition, conditional overexpression of constitutively

Table 2 Human diseases caused by abnormality in Wnt signalling

| Diseases/Phenotypes | Gene | Nature of miscues | References |
|--|------------------|----------------------------|--------------|
| Non-neoplastic | | | |
| Schizophrenia | <i>Wnt1</i> | Increased expression | [81] |
| Tetra-amelia phenopype | <i>Wnt3</i> | Homozygous mutation | [82] |
| Mullerian-duct regression and virilization. Intersex phenotype | <i>Wnt4</i> | Gene duplication | [83] |
| Familial exudative vitreoretinopathy | <i>Fz4</i> | Loss of function | [84] |
| Osteoporosis-pseudoglioma syndrome | <i>Lrp5</i> | Loss of function | [85] |
| A high bone mass phenotype | <i>Lrp5</i> | Gain of function | [86, 87] |
| Robinow syndrome | <i>Ror2</i> | Reduce or loss of function | [88--91] |
| Brachydactyly B | <i>Ror2</i> | Gain of function | [88, 91, 92] |
| Acute lymphoblastic leukaemia | <i>Wnt5a</i> | Loss of function | [93] |
| Lung cancer | <i>WIF</i> | Hypermethylation | [94] |
| Colorectal cancer | <i>sFRP</i> | Hypermethylation | [95] |
| Non small cell lung cancer | <i>Dv3</i> | Overexpression | [96] |
| Familial adenomatous polyposis Colorectal cancer | <i>APC</i> | Loss of function | [97, 98] |
| Hepatocellular carcinoma | <i>Axin1</i> | Loss of function | [99] |
| Colorectal cancer. Hepatocellular carcinoma. Melanoma. Prostate cancer | <i>β-catenin</i> | Oncogenic mutation | [100, 101] |
| Colorectal cancer | <i>Dkk1</i> | Hypermethylation | [102] |

active GSK-3 β led to regression of established pressure overload-induced hypertrophy [112]. Therefore, in cardiovascular medicine, regulation of the Wnt/ β -catenin pathway is thought to provide novel therapeutic targets for antihypertrophic therapy.

Wnt signalling, Krm and cancer

Given the definitive roles of the Wnt/ β -catenin pathway in cell fate specification, embryogenesis and maintenance of stem cell characteristics and niche, it is conceivable that defects or deregulation of the Wnt signalling pathway may lead to the development of tumours. Although a partial list of malignant diseases associated with aberrant Wnt signalling is provided in Table 2, abnormalities in the Wnt signalling pathway have been noted in a wide variety of human malignancies [78, 113, 114].

Involvement of the Wnt/ β -catenin pathway in malignant disease was first noted through the identification

of the *APC* gene as a tumour suppressor gene in patients with a hereditary cancer syndrome termed familial adenomatous polyposis (FAP) [115, 116]. FAP patients inherit one defective *APC* allele, and as a consequence develop large numbers of benign colon polyps in early adulthood. Inactivation of the second *APC* allele permits progression of polyps into malignant adenocarcinoma. Mutational inactivation of *APC* leads to the inappropriate stabilization of β -catenin, and loss of both *APC* alleles occurs in a majority of sporadic colorectal cancers [117]. In cases of colorectal cancer where *APC* is not mutated, either *Axin2* is mutated [118], or an activating (oncogenic) mutation occurs in the *β-catenin* gene [119]. Although the importance of β -catenin in tumourigenesis has been highlighted in colon cancer, oncogenic mutation in *β-catenin* has been noted in a variety of cancers, including colorectal cancer, hepatocellular carcinoma, melanoma and prostate cancer.

Based on the information that impaired Wnt/ β -catenin signalling results in the accumulation of β -catenin, it is conceivable that aberrant expression

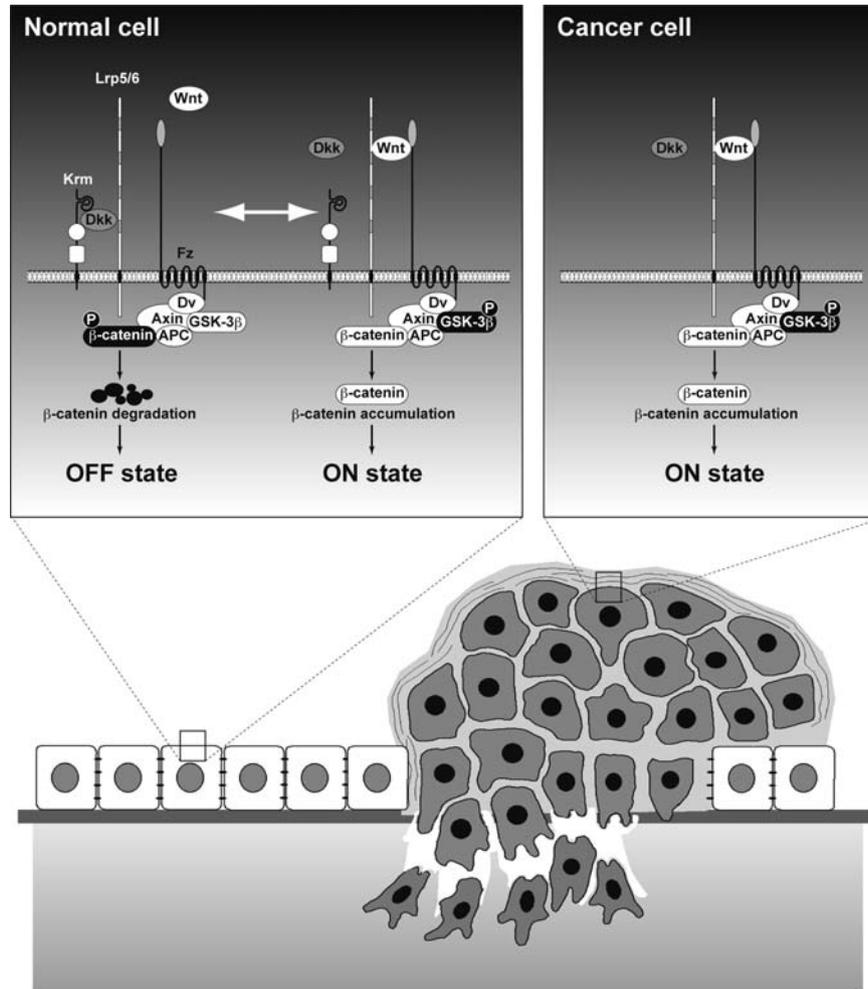


Fig. 5 Putative model involved in constitutively active Wnt/ β -catenin signalling in cancer cells, from the aspect of decreased or diminished Krm expression. In normal cells, cytoplasmic β -catenin level is bi-directionally regulated by the complex formations between Lrp5/6-Wnt-Fz and Lrp5/6-Dkk-Krm. In contrast, in cancer cells, the decreased or diminished expression of Krm allows constitutive accumulation of β -catenin, leading to abnormal Wnt/ β -catenin signalling.

and/or function of Krm as a negative regulator of Wnt/ β -catenin signalling may contribute to the development of cancer. Figure 4D shows gene expression of *krm1* in a variety of human tumour cell lines. *krm1* is expressed in various mouse normal tissues, and relatively strong expression is seen in the lung, heart, stomach, kidney, colon and skeletal muscle (Figure 4B), suggesting that Krm1 may play a role in suppression of Wnt/ β -catenin signalling in differentiated cell types. In contrast, among 30 different human tumour cell lines, the expression of *krm1* has disappeared in many lines, its expression is low in several lines, and high-level expression is seen in only a few tumour cell lines.

It is interesting that *krm1* mRNA is hardly detectable in human lung cancer cell lines (Lu99, EBC1, SBC2 and SBC5), though species difference between human and mice must be taken into consideration. Mutation of genes that relate to activation of

Wnt signalling has been found in several human lung cancer cells [120, 121]. Instead, inhibition of Wnt1 or Wnt2 by siRNA or monoclonal antibodies results in apoptosis of non-small-cell lung cancer (NSCLC) cells [120, 121]. Introduction of the WIF gene or protein inhibits H460 lung cancer cell growth both *in vitro* and *in vivo* [122]. These results suggest that inhibition of Wnts-dependent activation of the Wnt/ β -catenin pathway may be a therapeutic approach.

The mechanism by which Krm1 expression decreases or disappears is unknown. However, the lack of Krm1 expression may allow Wnt-dependent activation of the Wnt/ β -catenin pathway, thereby increasing the susceptibility to tumourigenic transformation of cells and malignant progression, not only in lung cancer but in a variety of human malignant tumours (Fig. 5). Nevertheless, in some cancer cells the inhibition of the Wnt/ β -catenin pathway was associated with activation of the β -catenin independent cascade.

This result suggests that Dkk-Krm may modulate Wnt signalling from the β -catenin dependent to the β -catenin independent pathway in some cancer cell types, rather than simply shutting down the Wnt/ β -catenin signalling. The significance of the lack of, or the decrease in, Krm expression in cancer cells remains to be addressed.

Perspectives

The decrease or lack of *krm* expression in a variety of human tumour cells led our group to consider that the understanding of transcriptional regulation of the *krm* gene provides a new mechanism by which cancer cells escape the gatekeeper function of Dkk-Krm to acquire constitutively active Wnt/ β -catenin signalling. At the same time, the understanding of transcriptional regulation of Krm provides a mechanism by which stem cells or undifferentiated progenitor cells achieve a competent state that allows subsequent activation of a series of genes (*e.g.* tissue-specific transcription factors and their target genes) for cellular differentiation. Shutdown of Wnt/ β -catenin signalling does not in itself specify the direction of cell-fate determination, while cellular competence for diverse differentiation requires an appropriate shutdown of Wnt/ β -catenin signaling. Dkk-Krm co-operation is likely to set the stage for undifferentiated progenitor cells or stem cells to progress toward differentiation, prior to activation of a series of genes that specify cellular differentiation. In other words, Krm expression may set cells on a divergent path toward differentiation, whereas down-regulation of Krm directs them toward tumorigenic transformation. Identification of a unique transcription factor for *krm* gene expression and micro RNA that regulates the turnover of *krm* mRNA is an approach to be considered.

Compared with the overwhelming body of knowledge on the important roles of the Wnt/ β -catenin signalling pathway in development and pathology, the biological and physiological roles of Krm have remained largely unaddressed. The use of single and double *krm1* and *krm2* knockout mice is significant. Expression of Krms in human pathology, including malignant tumours, must be analysed to understand the pathogenic roles of aberrant expression of Krms. There are multiple sites of single nucleotide polymorphism (SNP) in the genomic sequences of

krm1 and *krm2*, in both the 5' upper stream and mRNA transcriptional regions [Ensembl: *krm1* (<http://www.ensembl.org/HomoSapiens/geneview?gene=ENSG00000183762;db=core>), *krm2* (<http://www.ensembl.org/HomoSapiens/geneview?gene=ENSG00000131650;db=core>)]. The relationship of these SNPs to human diseases is of interest. Finally, addressing the questions described above would provide opportunities for drug discovery, targeting *krm* gene expression and function.

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