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Themed Section: WNT Signalling: Mechanisms and Therapeutic Opportunities

REVIEW ARTICLE

The Dickkopf1-cytoskeleton-associated protein 4 axis creates a novel signalling pathway and may represent a molecular target for cancer therapy

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Dickkopf 1 (DKK1) is a secreted protein and antagonizes oncogenic Wnt signalling by binding to the Wnt co-receptor, low-density lipoprotein receptor-related protein 6. DKK1 has also been suggested to regulate its own signalling, associated with tumour aggressiveness. However, the underlying mechanism by which DKK1 promotes cancer cell proliferation has remained to be clarified for a long time. The cytoskeleton-associated protein 4 (CKAP4), originally identified as an endoplasmic reticulum membrane protein, was recently found to act as a novel DKK1 receptor. DKK1 stimulates cancer cell proliferation when CKAP4 is expressed on the cell surface membrane. Although there are no tyrosine residues in the intracellular region of CKAP4, CKAP4 forms a complex with PI3K upon the binding of DKK1, leading to the activation of Akt. Both DKK1 and CKAP4 are frequently expressed in pancreatic and lung tumours, and their simultaneous expression is negatively correlated with prognosis. Knockdown of CKAP4 in cancer cells and treatment of mice with the anti-CKAP4 antibody inhibit Akt activity in cancer cells and suppress xenograft tumour formation, suggesting that CKAP4 may represent a therapeutic target for cancers expressing both DKK1 and CKAP4. This review will provide details of the novel DKK1-CKAP4 signalling axis that promotes cancer proliferation and discuss the possibility of targeting this pathway in future cancer drug development.

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Abbreviations

AAH, atypical adenomatous hyperplasia; APC, adenomatous polyposis coli; APF, anti-proliferating factor; CKAP4, cytoskeleton-associated protein 4; CRD, cysteine-rich domain; DKK, Dickkopf; ER, endoplasmic reticulum; HCC, hepatocellular carcinoma; ICC, intrahepatic cholangiocarcinoma; LRP6, low-density lipoprotein receptor-related protein 6; MM, multiple myeloma; SP-A, surfactant protein A; TCF, T-cell factor; tPA, tissue plasminogen activator; VSMC, vascular smooth muscle cell; WIF-1, Wnt inhibitory factor-1

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Introduction

Mutations and aberrant activities of the Wnt pathway are frequently observed in a wide variety of diseases (Moon *et al.,* 2004; Clevers and Nusse, 2012), which is not surprising given the importance of Wnt signalling in various cellular processes, including cell proliferation, differentiation, motility and polarization. The diseases associated with abnormalties of Wnt signalling occur in tissues that normally depend on Wnt, most notably in the intestine. Germline mutations in the *adenomatous polyposis coli* (APC) gene, a Wnt signalling component, cause familial adenomatous polyposis coli and additional mutations in *kras*, *p53* and *smad4* lead to the progression of polyps to malignant tumours, that is, colorectal cancer (Kinzler and Vogelstein, 1996). Loss of APC functions leads to the stabilization of **β-catenin** and the constitutive complexes between β-catenin and T-cell factor 4 (TCF4) by disrupting the Axin complex function, which degrades cytoplasmic β-catenin, resulting in the expression of various Wnt target genes, some of which are critical for cell proliferation and migration (Kikuchi, 2003; Polakis, 2007; Kikuchi *et al.,* 2011). More than 75% of sporadic colorectal cancer patients carry the *APC* loss of function mutations and about 15% of patients have β-*catenin* oncogenic mutations (Kinzler and Vogelstein, 1996). Mutations in the β-*catenin* and *Axin1* genes are also observed in a variety of solid tumours, and these mutations cause the stabilization of β-catenin (Walther *et al.,* 2009).

The synthetic compounds that disrupt the interaction of β-catenin and TCF4, including PKF115-854, CPG049090 and ICG-001 (Emami *et al.,* 2004; Lepourcelet *et al.,* 2004), and inhibit β-catenin stabilization, such as IWR1, XAV939, JW67 and JW74 (Chen *et al.,* 2009; Huang *et al.,* 2009; Waaler *et al.,* 2011), may be of potential in the development of new anti-cancer drugs. However, although some analogues of these compounds have been advanced to clinical testing, these approaches do not always guarantee future success. Therefore, in addition to the development of the inhibitors that target Wnt signal molecules directly, other target molecules involved in Wnt signalling need to be identified.

Dickkopf1 (DKK1), a secreted protein, is a direct target molecule whose expression is induced by a β-catenin/TCF4 complex and essential for animal development (Niehrs, 2006). As DKK1 inhibits Wnt signalling, DKK1 basically acts as a tumour suppressor (Table 1). Interestingly, DKK1 is frequently and highly expressed in certain types of human cancers, and its expression is associated with tumour aggressiveness, suggesting that DKK1 can also function as an oncogenic protein (Table 1). Therefore, DKK1-dependent cancer signalling may be a novel target, but how DKK1 promotes tumour formation has remained to be clarified for a long time. In this review, we describe details of a novel receptor of DKK1 in cancer cells and discuss a possible cancer therapy that would target the new DKK1 signalling pathway.

DKK1 as a negative regulator of Wnt signalling

There are at least six secretory antagonistic proteins that modulate Wnt signalling. Soluble frizzled-related proteins (Kawano and Kypta, 2003), frizzled-related protein (also known as Crescent) (Pera and De Robertis, 2000), Cerberus (Piccolo *et al.,* 1999) and **Wnt inhibitory factor-1 (WIF-1)** (Hsieh *et al.,* 1999) bind to Wnt proteins and sequester them from Wnt cell surface receptors, thereby inhibiting the Wnt pathway. Wnt modulator in surface ectoderm (also known as sclerostin domain containing 1) causes Wnt signalling inhibition by competing with Wnts for the binding to the Wnt co-receptor low-density lipoprotein receptor-related proteins 6 (LRP6) (Itasaki *et al.,* 2003). DKK1 also binds to LRP6 and sequesters it from the cell surface membrane, resulting in the inhibition of the Wnt pathway (Mao *et al.,* 2001; Semënov *et al.,* 2001; Brott and Sokol, 2002; Yamamoto *et al.,* 2008; Sakane *et al.,* 2010).

DKK1 was originally identified as an embryonic head inducer in *Xenopus* embryos and shown to be a secreted protein that antagonizes Wnt signalling (Glinka *et al.,* 1998; Niehrs, 2006). Subsequently, the DKK protein family was shown to be comprised of four members (DKK1, DKK2, DKK3 and DKK4), and DKKs were identified in other vertebrates including humans and in invertebrates. From the phenotypes of DKK1 knockout mice, DKK1 is essential for various developmental processes, including anterior–posterior patterning, limb development, somatogenesis and eye formation (Mukhopadhyay *et al.,* 2001; Niehrs, 2006). Heterozygous DKK1 mutant mice are variable but show a high bone mass due to increased bone formation (Morvan *et al.,* 2006), whereas transgenic expression of DKK1 causes osteopenia and suppresses cell proliferation in the intestines with architectural degeneration (Pinto *et al.,* 2003; Li *et al.,* 2006). Thus, DKK1 is involved in many biological phenomena in the development and adult life of animals.

Of the multiple Wnt signalling pathways including the β-catenin-dependent pathway (β-catenin pathway) and the β-catenin-independent pathway, DKK1 has been thought to modulate mainly the former pathway. DKK1 contains two characteristic cysteine-rich domains (CRD-1 and CRD-2) (Glinka *et al.,* 1998) and binds to LRP6 through CRD-2, thereby suppressing the β-catenin pathway. In the 'two endocytic routes and two outputs model', LRP6 is predominantly present in the lipid-raft (detergent-resistant) membrane fractions, and Ror2, a Wnt5a receptor, is found in the non-lipid raft membrane fractions (Yamamoto *et al.,* 2006; Kikuchi *et al.,* 2009; Sato *et al.,* 2010a) (Figure 1). **Wnt3a** induces LRP6 internalization in a caveolin-dependent manner, which is required for the activation of the β-catenin pathway, and **Wnt5a** induces the internalization of **Ror2** with **frizzled** in a clathrin-dependent manner, resulting in the activation of the β-catenin-independent pathway (Yamamoto *et al.,* 2006; Sato *et al.,* 2010a; Jiang *et al.,* 2012; Demir *et al.,* 2013), whereas DKK1 induces LRP6 internalization through a clathrin-mediated route, resulting in removal of LRP6 from the plasma membrane (Mao *et al.,* 2001; Yamamoto *et al.,* 2008; Sakane *et al.,* 2010) (Figure 1). **Kremen1/2** has also been suggested to be a cell surface receptor of DKK1 (Mao *et al.,* 2002), but the role of Kremen1/2 may be marginal in DKK1-dependent Wnt signal inhibition (Ellwanger *et al.,* 2008; Wang *et al.,* 2008). As DKK1 is one of the direct

Table 1

Functions of DKK1 in various cancer cells

Cancer types (References)	Cell lines	Phenotypes	Clinical samples	Functions of DKK1
Renal cell carcinoma (Hirata *et al.*, 2011)	A498	DKK1 overexpression inhibited cell proliferation and migration and induced apoptosis.	–	Tumour suppressive
Colon cancer (Aguilera *et al.*, 2006; Qi *et al.*, 2012)	HCT116	DKK1 overexpression inhibited cell proliferation, migration and invasion.	Among 217 cases, 131 cases (60.4%) showed positive for DKK1.	Tumour suppressive
	DLD-1	DKK1 overexpression inhibited cell proliferation.	Among 54 cases, DKK1 was hypermethylated in nine cases (17%).	Tumour suppressive
Breast cancer (Mikheev *et al.*, 2008, Qiao *et al.*, 2008, DiMeo *et al.*, 2009)	MCF-7	DKK1 knockdown promoted cell proliferation.	–	Tumour suppressive
	SUM1315 and MDA-MB-231	DKK1 overexpression inhibited cell proliferation.	–	Tumour suppressive
Melanoma (Mikheev *et al.*, 2007)	MDA-MB435	DKK1 overexpression inhibited cell proliferation and induced apoptosis.	–	Tumour suppressive
Placental choriocarcinoma (Peng *et al.*, 2006)	JAR	DKK1 overexpression inhibited cell proliferation and induced apoptosis.	–	Tumour suppressive
Mesothelioma (Lee *et al.*, 2004)	H28, MS-1	DKK1 overexpression inhibited cell proliferation and induced apoptosis.	–	Tumour suppressive
Cervical carcinoma (Mikheev *et al.*, 2004)	HeLa	DKK1 overexpression inhibited cell proliferation and induced apoptosis.	–	Tumour suppressive
Gliobalastoma (Shou *et al.*, 2002)	U87MG	DKK1 induced apoptosis.	–	Tumour suppressive
Hepatcellular carcinoma (Chen *et al.*, 2013)	Bel7402	DKK1 knockdown inhibited cell migration and invasion.	Metastatic tumours showed much higher DKK1 expression in comparison with that in the primary tumours.	Oncogenic
Lung cancer (Sato *et al.*, 2010a; Kimura *et al.*, 2016)	A549	Anti-DKK1 Ab or DKK1 knockdown inhibited cell proliferation, migration and invasion.	Among 128 cases, 98 cases (76.6%) showed positive for DKK1.	Oncogenic
Pancreas cancer (Takahashi *et al.*, 2010; Kimura *et al.*, 2016)	S2-CP8	DKK1 knockdown inhibited cell proliferation, migration and invasion.	Among 23 cases, 17 cases (73.9%) showed positive for DKK1. Among 59 cases, 45 cases (76.3%) showed positive for DKK1.	Oncogenic
	SUIT-2	DKK1 knockdown inhibited cell migration.	–	Oncogenic
Oesophageal cancer (Makino *et al.*, 2009; Li *et al.*, 2011)	EC9706	DKK1 overexpression promoted cell proliferation and invasion.	Among 138 cases, 115 cases (83.4%) showed positive for DKK1.	Oncogenic

continues

Table 1 (Continued)

Cancer types (References)	Cell lines	Phenotypes	Clinical samples	Functions of DKK1
	–	–	Among 170 cases, 72 cases (42.4%) showed positive for DKK1. Patients with DKK1-positive tumours had poorer DFS than those with negative oesophageal cancer (squamous cell carcinoma type).	Oncogenic
Multiple myeloma (Yaccoby *et al.*, 2007; Fulciniti *et al.*, 2009)	Primary multiple myeloma cell	Anti-DKK1 Ab inhibited cell proliferation.	–	Oncogenic
Prostate cancer (Hall *et al.*, 2008; Hall *et al.*, 2010; Rachner *et al.*, 2014)	PC-3	DKK1 knockdown inhibited tumour formation.	High DKK1 levels in tumours were associated with shorter overall survival. High DKK1 levels in the serum were associated with a significantly shorter overall and disease specific survival.	Oncogenic

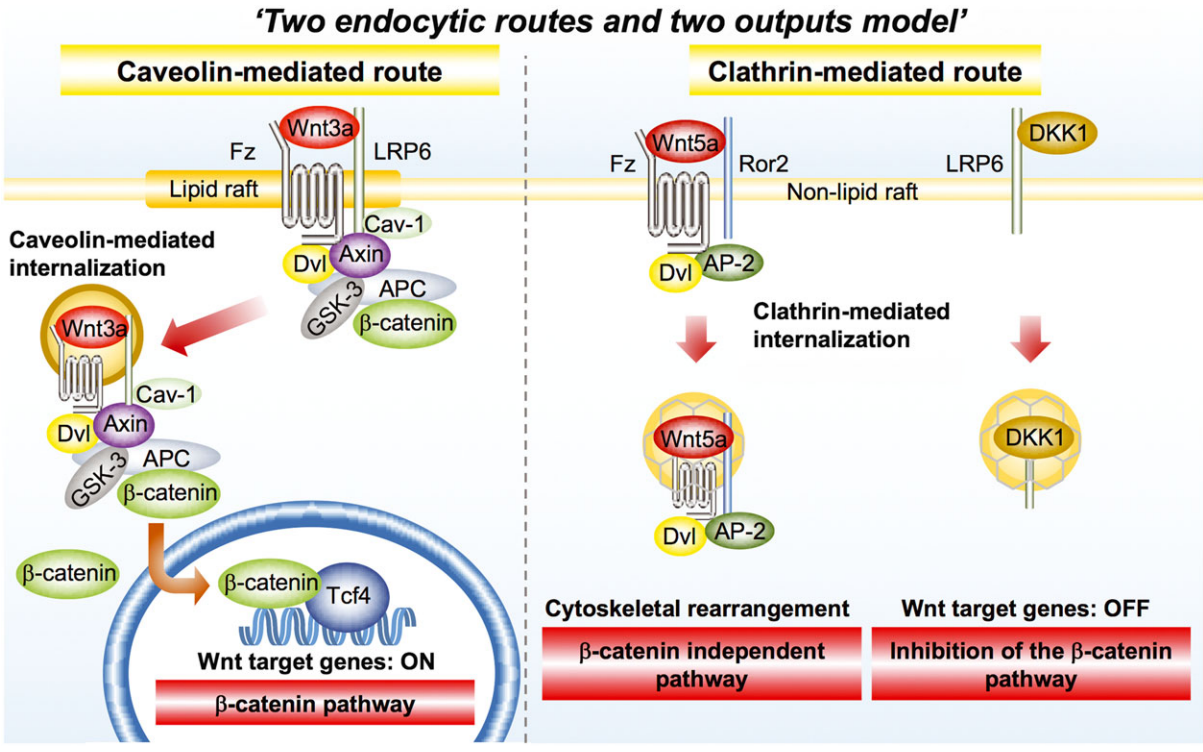

Figure 1

'Two endocytic routes and two outputs model' in the Wnt signalling pathways. See details in the text. AP-2, adaptor protein complex 2; Cav-1, caveolin-1; Fz, frizzled; Ror2, receptor tyrosine kinase-like orphan receptor 2; Dvl, dishevelled; **GSK-3**, glycogen synthase kinase 3.

target molecules expressed by the β-catenin pathway (Gonzalez-Sancho *et al.,* 2005), DKK1 creates a negative feedback loop for Wnt signalling.

DKK1 has dual actions on tumour formation

Given that DKK1 acts as a negative regulator of Wnt signalling that potentially functions in oncogenic signalling, it would be reasonable that DKK1 has tumour-growth inhibitory activity (Table 1). Indeed, DKK1 is down-regulated in colorectal cancer and melanoma probably due to DNA hypermethylation and this observation is associated with advanced stages of tumourigenesis (Gonzalez-Sancho *et al.,* 2005; Aguilera *et al.,* 2006; Kuphal *et al.,* 2006). DKK1 has shown growth inhibition of various cancer cell lines *in vitro* and *in vivo*, including MCF-7, MDA-MB-231 and SUM-1315 breast cancer, HCT116 and DLD-1 colon cancer and Hela cervical cancer cells (Mikheev *et al.,* 2008; Qiao *et al.,* 2008; DiMeo *et al.,* 2009; Qi *et al.,* 2012). In addition, DKK1 has been reported to induce or increase sensitivity to apoptosis in H28 and MS-1 mesothelioma, U87MG glioblastoma, A498 renal cell carcinoma, MDA-MB435 melanoma, JAR placental choriocarcinoma and HeLa cells (Shou *et al.,* 2002; Lee *et al.,* 2004; Mikheev *et al.,* 2004; Peng *et al.,* 2006; Mikheev *et al.,* 2007; Hirata *et al.,* 2011), but it is not clear if the apoptosis-inducing effects of DKK1 are through the inhibition of Wnt signalling.

In parallel with reports that DKK1 functions as a tumour suppressor, it has been shown that DKK1 expression levels are increased in a variety of cancers, including oesophageal, lung, pancreas, prostate and breast cancers, multiple myeloma, hepatoblastoma, hepatocellular carcinoma (HCC) and Wilms' tumour and that its high expression in tumour tissue is associated with cancer progression and aggressiveness (Tian *et al.,* 2003; Wirths *et al.,* 2003; Forget *et al.,* 2007; Yaccoby *et al.,* 2007; Yamabuki *et al.,* 2007; Hall *et al.,* 2008; Makino *et al.,* 2009; Takahashi *et al.,* 2010; Li *et al.,* 2011; Chen *et al.,* 2013; Rachner *et al.,* 2014; Kimura *et al.,* 2016) (Table 1). Serum DKK1 levels are also significantly higher in lung, oesophageal and prostate cancer patients than in healthy controls (Yamabuki *et al.,* 2007; Rachner *et al.,* 2014). By ELISA, more than 70% of DKK1-positive cases were diagnosed as lung and oesophageal cancers and less than 5% of healthy controls were DKK1 positive, suggesting that DKK1 may be useful as a diagnostic and prognostic marker (Yamabuki *et al.,* 2007). It is not surprising that DKK1 is increased in multiple myeloma with osteolytic lesions, because DKK1 inhibits osteoblast proliferation and induces osteopenia by antagonizing Wnt signalling (Krishnan *et al.,* 2006). Interestingly, DKK1 supports prostate cancer growth independent of Wnt signalling through the reduction in p21 expression (Hall *et al.,* 2010).

DKK1 elevation in cancers may be explained as a result of aberrant activation of Wnt signalling, because DKK1 is a direct target molecule of Wnt signalling. However, as there are cancers where Wnt signalling is not activated aberrantly, it is hard to explain why DKK1 expression increases in these cancers in the context of Wnt signalling. The mechanism of Wnt signal-independent expression of DKK1 needs to be clarified. In addition, the molecular mechanism underlying DKK1-dependent cancer progression remains unclear. It could be hypothesized that DKK1 binds to an unknown cell surface receptor to stimulate cell proliferation and that DKK1 has distinct functions, independent of Wnt signalling. To support this hypothesis, a novel receptor of DKK1 had to be identified in cancer cells.

Identification of CKAP4 as a novel DKK1 receptor

Wnt proteins are secreted apically and basolaterally and in both ways in polarized epithelial cells (Figure 2). For instance, Wnt3a, Wnt5a and Wnt5b are secreted basolaterally in polarized MDCK cells and rat intestinal epithelial IEC6 cells, whereas **Wnt11** is secreted apically (Gon *et al.,* 2013; Yamamoto *et al.,* 2013; Yamamoto *et al.,* 2015). **Wnt1** and Wingless (Wnt1 homologue in *Drosophilla*) are secreted both apically and basolaterally in MDCK cell and *Drosophilla* wing disc cells (Yamazaki *et al.,* 2016; Yamamoto *et al.,* in press). Wnt receptor Frizzled2 is localized to the basolateral membrane, and frizzled7 is localized to both apical and basolateral membranes (Yamamoto *et al.,* 2015). Wnt co-receptors, such as LRP6 for Wnt3a and Ror2 for Wnt5a, are localized to the basolateral membrane, whereas

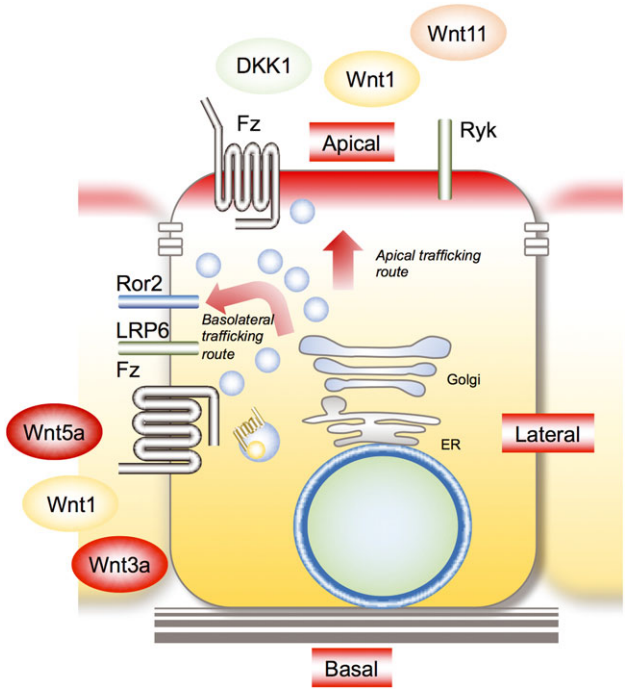

Figure 2

Polarized trafficking of Wnt proteins and receptors. Wnt3a, Wnt5a and Wnt5b are secreted basolaterally, and Wnt11 is secreted apically. Wnt1 is secreted in both ways. Wnt receptor frizzled2 is localized to the basolateral membrane, and Frizzled7 is localized to both apical and basolateral membranes. Wnt co-receptors are localized to specific membrane domains (e.g. basolateral membrane, LRP6 and Ror2; apical membrane, Ryk). DKK1 is secreted apically. Fz, Frizzled; Ryk, receptor-like tyrosine kinase.

receptor-like tryosine kinase (Ryk) for Wnt11 is present in the apical membrane (Gon *et al.,* 2013; Yamamoto *et al.,* 2015). Thus, it is likely that ligands and receptors in Wnt signalling trafficked to the same direction for efficient signalling.

DKK1 is predominantly secreted apically in polarized MDCK cells, and the addition of recombinant DKK1 to the apical side results in cell proliferation, but addition to the basolateral side shows no effect (Kimura *et al.,* 2016). Because LRP6 is localized to the basolateral membrane of MDCK cells, it is thought that DKK1 acts on the putative receptor that is localized to the apical membrane and stimulates cell proliferation. To identify the receptor that mediates DKK1-dependent cell proliferation, biotinylated cell surface proteins were precipitated with DKK1 and the precipitated proteins analysed by mass spectrometry (Kimura *et al.,* 2016). Among the DKK1-binding proteins, a 63-kDa protein was identified as cytoskeleton-associated protein 4 (CKAP4, also known as P63, CLIMP-63 and ERGIC-63).

Structure and functions of CKAP4

CKAP4 is a type II transmembrane protein that is not glycosylated but is reversibly palmitoylated (Schweizer *et al.,* 1993a; Schweizer *et al.,* 1994; Klopfenstein *et al.,* 1998; Vedrenne and Hauri, 2006). CKAP4 was originally discovered as a protein largely confined to the endoplasmic reticulum (ER). CKAP4 has an intracellular region (aa 1–105), a transmembrane region (aa 106–127) and an ER luminal region containing stretches of heptad repeats (aa 128–602) (Klopfenstein *et al.,* 2001) (Figure 3A). CKAP4's luminal region consists of predicted coiled-coil domains (aa 130–214 and aa 256–460) with a leucine zipper (LZ) (aa 468–503) followed by a coiled-coil domain (aa 533–602). The coiled-coil domain has the propensity to oligomerize and tends to be involved in intermolecular interactions. Indeed, CKAP4 runs as bands of 63 and 120 kDa and a poorly resolved smear higher than at 310 kDa in SDS-PAGE under non-reducing conditions (Schweizer *et al.,* 1993a). In addition, the recombinant luminal region forms a helical 91 nm long rod-like structures, and its sedimentation equilibrium is 25.7 S (Klopfenstein *et al.,* 2001). Fluorescence recovery after photobleaching analysis revealed that wild-type CKAP4 has a very low recovery rate and that the CKAP4 mutant with deletion of the luminal region shows a rapid rate (Klopfenstein *et al.,* 2001). Thus, the CKAP4 luminal region seems to form higher-order oligomers. These structural characteristics may explain the specific localization of CKAP4 to the rough ER, where it is excluded from high-curvature regions (Shibata *et al.,* 2010).

Another approach to clarify the mechanism by which ER sheets are formed identified CKAP4 as an abundant integral membrane protein enriched in ER sheets (Shibata *et al.,* 2010). Knockdown of CKAP4 in COS cells did not affect ER morphology, but the ER sheets were spread throughout the cytoplasm. In addition, the luminal width was reduced in CKAP4-depeleted cells. Therefore, CKAP4 could function in segregating ER sheets close to the nucleus and maintaining the luminal width by the binding of CKAP4 localizing to opposing cisternal membranes in an antiparallel or parallel manner.

In the transmembrane and intracellular regions, there are two cysteine residues (Cys100 and Cys126) (Figure 3A). CKAP4 with Mr of 120 kDa in non-reducing SDS-PAGE may be a dimer of CKAP4 through a disulfide bond. Although Cys126 is a target of the disulfide bond, it is reported that Cys100 is modified with palmitate by palmitoyl acyltransferase DHHC2 (Schweizer *et al.,* 1995; Zhang *et al.,* 2008). Palmitoylation of CKAP4 is detected in the mitotic phase but minimally or not in interphase of CHO cells (Mundy and Warren, 1992), and palmitoylation is enhanced by brefeldin A, which blocks transport of proteins out of the ER and results in the disassembly of the Golgi apparatus (Misumi *et al.,* 1986), in interphase of Vero cells (Schweizer *et al.,* 1993b). Wild-type CKAP4 is present on the cell surface and perinuclear membranes in addition to the ER, whereas CKAP4 C100S, in which Cys100 is mutated to Ser, is confined to the ER (Planey *et al.,* 2009). Furthermore, anti-proliferating factor (APF) induces nuclear translocation of CKAP4 in wild-type HeLaS3 cells but not in DHHC2 knockdown cells (Planey *et al.,* 2009). Thus, palmitoylation may be required for the trafficking of CKAP4 from the ER to the cell surface membrane and nucleus.

CKAP4 functions as a cell surface membrane protein

Although the predominant localization of CKAP4 is to the ER, CKAP4 has been shown to be present on the cell surface membrane of vascular smooth muscle cells (VSMCs), type II pneumocytes and bladder epithelial cells, where it functions as a receptor for **tissue plasminogen activator (tPA)**, surfactant protein A (SP-A) and APF, respectively (Razzaq *et al.,* 2003; Conrads *et al.,* 2006; Gupta *et al.,* 2006) (Figure 3B). The ER luminal region of CKAP4 is equivalent to its extracellular region and functions to bind ligands when CKAP4 is localized to the cell surface membrane.

tPA catalyses the conversion of plasminogen to plasmin, a major enzyme responsible for clot degradation (Clowes *et al.,* 1990). tPA binds to CKAP4 on VSMC surface membrane and anti-CKAP4 antibody inhibits the binding of tPA to VSMC (Razzaq *et al.,* 2003). Expression of an N-terminally truncated CKAP4 mutant that is localized to the plasma membrane in COS cells results in more than sevenfold increase in plasminogen activity, which is reversed with an anti-CKAP4 antibody (Schweizer *et al.,* 1994; Razzaq *et al.,* 2003).

SP-A binds to type II pneumocytes in the lung *via* calcium-dependent (specific) binding and calcium-independent (non-specific) binding. Knockdown of CKAP4 results in an inhibition of SP-A specific binding (Bates *et al.,* 2008) and anti-CKAP4 antibody blocks SP-A's specific binding and inhibits SP-A's ability to inhibit surfactant secretion (Gupta *et al.,* 2006; Bates, 2010), supporting CKAP4's ability to bind to SP-A. Although the mechanism is not clear, it is reported that SP-A induces CKAP4 translocation from the ER to cell surface membrane through **Akt** activation (Kazi *et al.,* 2010).

APF is a sialoglycopeptide elevated in the urine of patients with interstitial cystitis, a chronic, painful bladder disease (Keay *et al.,* 2000). APF inhibits the proliferation of normal bladder epithelial cells and bladder cancer cells through binding to CKAP4. Knockdown of CKAP4 or treatment with

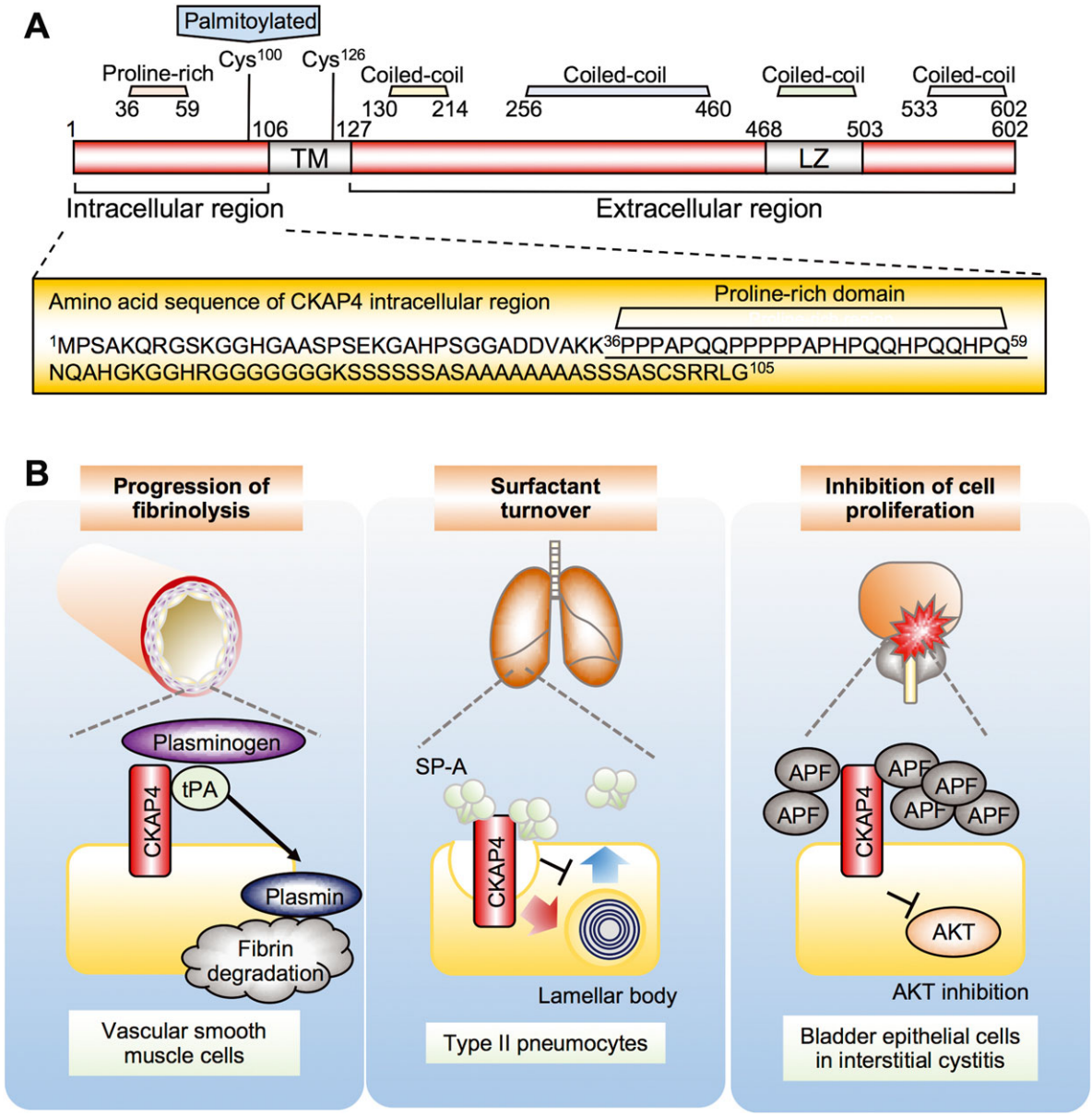

Figure 3

Structure and functions of CKAP4 in the cell surface membrane. (A) Schematic diagram of CKAP4. Amino acid residues in the CKAP4 intracellular region are enlarged, showing no tyrosine residues. The proline-rich motif that interacts with the p85 subunit of PI3K is underlined. Cys, cysteine; TM, transmembrane. (B) Possible functions of CKAP4 in the cell surface membrane. Left panel, tPA binds to CKAP4 and regulates plasmin production and progression of fibrinolysis in vascular smooth muscle cells. Middle panel, SP-A binds to CKAP4 and induces CKAP4 internalization and regulates secretion of lamellar body. Right panel, APF binds to CKAP4 and inhibits cell proliferation through the suppression of Akt in bladder epithelial cells.

anti-CKAP4 antibody inhibits APF's effects on cell proliferation and gene expression (Conrads *et al.*, 2006). It has also been shown that changes in **MMP2**, p53 and **CCN2** protein expression and Akt phosphorylation in response to APF are abrogated following CKAP4 knockdown in T24 bladder carcinoma cells (Shahjee *et al.*, 2010; Matika *et al.*, 2012). These results indicate that CKAP4 is essential for mediating the signalling from three ligands, tPA, SP-A and APF.

CKAP4 functions as a receptor of DKK1 and regulates cell proliferation

CKAP4 is localized to the apical membrane of polarized MDCK cells (Kimura *et al.*, 2016). Therefore, the addition of DKK1 to the apical region stimulates MDCK cell proliferation. DKK1 induces the internalization of CKAP4 from the cell surface membrane through a clathrin-mediated route. DKK1 directly binds to the extracellular region of

CKAP4 with a Kd of 0.42 nM which is comparable with the Kd (0.34–0.5 nM) of DKK1 binding to LRP6 (Mao *et al.,* 2001; Semënov *et al.,* 2001). The binding of DKK1 to CKAP4 activates Akt through **PI3K** (Figure 4).

It is well known that as soon as growth factor receptors, such as **EGF** receptor and PDGF receptor, are tyrosine phosphorylated in the intracellular region by ligand binding, the SH2 domain of the p85 regulatory subunit of PI3K is recruited to the receptor, resulting in the production of phosphatidylinositol-3,4,5-triphosphate and then activating Akt (Wymann and Pirola, 1998). However, as there are no tyrosine residues in the cytoplasmic region of CKAP4 (Figure 3A), it is theoretically impossible to bind to PI3K by the same mechanism as growth factor receptors. Instead, the intracellular region of CKAP4 has the proline-rich motif to which the SH3 domain of p85 binds, depending on DKK1. The proline-rich motif of CKAP4 may be masked in the absence of DKK1, and the closed region would be opened by the binding of CKAP4 and DKK1, which results in the recruitment of p85. This is the novel mechanism by which a cell surface receptor activates Akt through PI3K in response to an extracellular ligand. Thus, when CKAP4 is present on the cell surface membrane, DKK1 can promote cell proliferation through the activation of the PI3K-Akt signal cascade although it remains to be clarified whether DKK1-dependent CKAP internalization is involved in the regulation of signalling.

DKK1 and CKAP4 are required for cancer cell proliferation

Because CKAP4 was discovered as an ER residual protein (Schweizer *et al.,* 1993a), it is reasonable that CKAP4 is expressed in many cancer and non-cancer cells lines. However, whether CKAP4 is localized to the cell surface membrane depends on cell type. For instance, CKAP4 is detected on the cell surface membrane of A549 lung, S2-CP8 pancreatic, HeLaS3 cervical and TE-8 oesophageal cancer cells and HepG2 hepatoblastoma cells (unpublished observations). DKK1 expression levels in cultured cell lines vary depending on cell type: DKK1 is well expressed in some cancer cell lines, including A549, S2-CP8, TE-8, HepG2 and KKLS gastric cancer cells; DKK1 is less expressed in HeLaS3 and AGS gastric cancer cells; and DKK1 is minimally detected in non-tumour cell lines, including MDCK, X293T kidney epithelial and Eph4 mammary cells.

p85 forms a complex with CKAP4 in S2-CP8 cells, and complex formation is suppressed by DKK1 knockdown (Kimura *et al.,* 2016). Akt activity and cell proliferation are

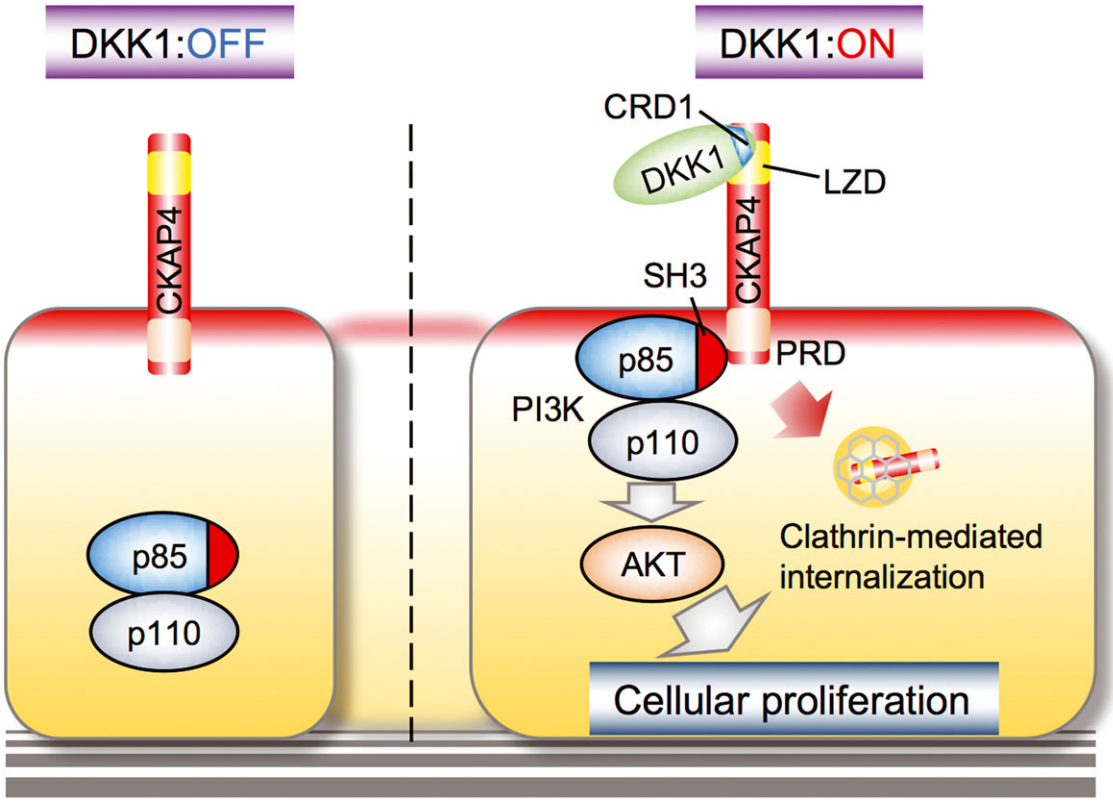

Figure 4

The DKK1 and CKAP4 signalling axis. DKK1 binds to the extracellular region of CKAP4. CRD-1 of DKK1 and LZD of CKAP4 are required for their interaction. PRD of CKAP4 binds to the SH3 domain of the p85 subunit upon DKK1 binding to CKAP4, resulting in the activation of the PI3K and Akt signalling pathway and the promotion of cell proliferation. CRD, cysteine-rich domain; LZD, leucine-rich domain; PRD, proline-rich domain.

suppressed in DKK1- or CKAP4-depleted S2-CP8 and A549 cells, and inhibition is rescued by expression of DKK1 or CKAP4. In contrast, CKAP4 knockdown in HeLaS3 cells does not affect cell proliferation, because HeLaS3 cells express CKAP4 but little express DKK1. Thus, expression of both DKK1 and CKAP4 is necessary for cancer cell proliferation, and CKAP4 acts as an oncogene product when it is expressed on the cell surface membrane. The formation of the xenograft tumours derived from CKAP4-depeleted S2-CP8 and A549 cells is less than that of control tumours (Kimura *et al.,* 2016). Under the conditions where expression of wild-type DKK1 rescues the phenotypes induced by DKK1 knockdown, DKK1ΔCRD-1, which fails to bind to CKAP4, does not rescue tumour formation, supporting the notion that DKK1 functionally interacts with CKAP4 in tumour formation *in vivo*. Therefore, CKAP4 may be a potential molecular target for cancer therapy.

APF inhibits Akt activity and proliferation in bladder cells through CKAP4 (Shahjee *et al.,* 2010). In HCC cells, CKAP4 associates with EGF receptors and inhibits EGFR signalling, resulting in decreased proliferation and invasion capabilities (Li *et al.,* 2014a). It is unknown whether other CKAP4 ligands, such as SP-A and tPA, are involved in the proliferation of these cancer cells. Thus, the downstream signalling and binding proteins of CKAP4 in cancer cells may vary depending on cell type.

CKAP4 expression is related with prognosis of cancer patients

In line with the observations that high expression levels of DKK1 in tumour tissue are associated with aggressiveness in various types of cancers, CKAP4 is also expressed in pancreatic, lung and oesophageal tumours and is minimally detected in non-tumour tissues (Kimura *et al.,* 2016) (unpublished observations) (Figure 5A). Importantly, patients positive for both DKK1 and CKAP4 show poor prognosis and

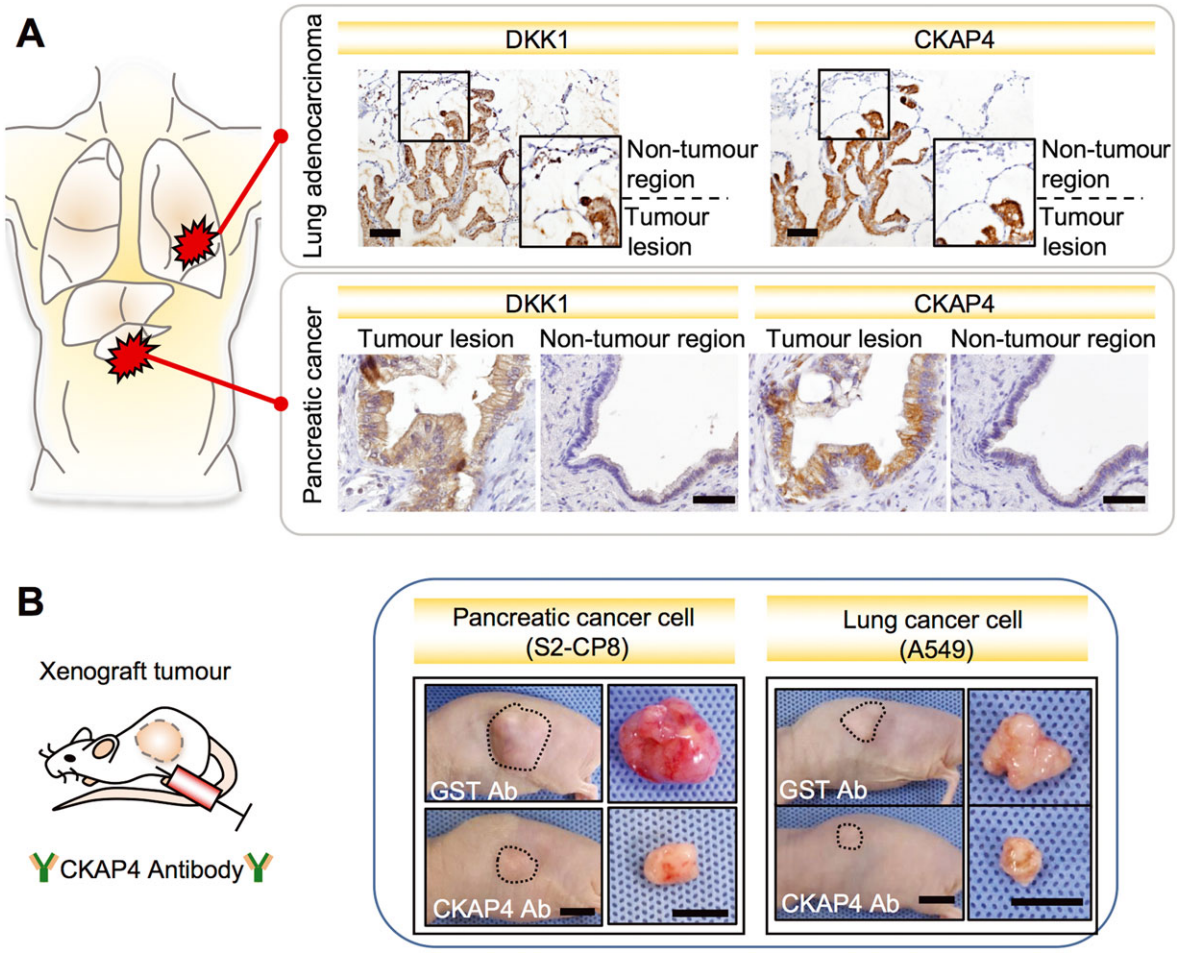

Figure 5

Simultaneous expression of DKK1 and CKAP4 in cancers and inhibition of cancer cell proliferation by anti-CKAP4 antibody. (A) In lung adenocarcinoma and pancreatic cancer tissues, DKK1 and CKAP4 are expressed in tumour lesions and minimally detected in non-tumour regions. Scale bars, 100 μm in lung adenocarcinoma and 50 μm in pancreatic cancer. (B) In the xenograft tumour model using nude mice subcutaneously implanted with pancreatic cancer cells (S2-CP8 cells, left panels) and lung cancer cells (A549 cells, right panels), anti-CKAP4 antibody suppressed tumour formation. Anti-Glutathione S-transferase (GST) antibody was used as a control. Representative appearance of one mouse (left pictures) and extirpated xenograft tumours (right pictures) are shown. Scale bars, 10 mm (Kimura *et al.,* 2016).

reduced relapse-free survival than do patients positive for either DKK1 or CKAP4 or negative for both. Thus, simultaneous expression of DKK1 and CKAP4, a ligand and a receptor, plays critical roles in cancer aggressiveness. It is notable that DKK1 but not CKAP4 is detected in lung atypical adenomatous hyperplasia (AAH). AAH cells proliferate along the pre-existing alveolar epithelium and show increased cell size and prominent nucleoli. The grade of atypia in lung AAH is usually milder than that in adenocarcinoma (Mori *et al.,* 2001). Therefore, CKAP4 expression may be involved in the transition from the precancerous state to the cancerous state.

There are opposing reports for intrahepatic cholangiocarcinoma (ICC) and HCC (Li *et al.,* 2013; Li *et al.,* 2014b). CKAP4 expression is associated with tumour size and lymph node metastasis of ICC, but CKAP4 is expressed less in lymph node metastatic lesions rather than in primary lesions. ICC patients with low CKAP4 expression have a shorter overall survival and higher recurrence, suggesting that CKAP4 acts as tumour suppressor and is a favourable prognostic marker. HCC patients with high expression of CKAP4 show a favourable overall survival and a longer disease-free survival compared with low expression. Since DKK1 expression levels in ICC and HCC have not been examined in these studies, whether the tumour suppressor activity of CKAP4 in ICC and HCC is related to DKK1 remains unclear.

DKK1-CKAP signalling axis is a molecular target for cancer therapy

A series of experiments concerning the roles of DKK1 and CKAP4 in cancers suggested that both proteins are molecular targets for cancer therapy. A SCID-hu mouse model, where DKK1-producing multiple myeloma (MM) cells are injected in the implanted human bone, developed osteoporosis and bone lesions with growth of MM cells and increased DKK1 in murine blood (Fulciniti *et al.,* 2009). Injection with a humanized anti-DKK1 monoclonal antibody (BHQ880) increased and decreased the numbers of osteoblast and MM cells, respectively, in human bone. Since BHQ880 has no direct effect on MM cells but inhibits MM cell growth in the presence of bone marrow stromal cells, the inhibition by BHQ880 would not affect Wnt signalling directly in MM cells. Anti-DKK1 polyclonal antibody also suppressed xenograft tumour formation by DKK1-overexpressing A549 cells (Sato *et al.,* 2010b). This antibody was generated by immunizing with the N-terminal 120 amino acids of DKK1, including CRD-1 which binds CKAP4 but not LRP6. Therefore, it is possible that growth inhibition by the antibody is due to the inhibition of the binding of DKK1 and CKAP4.

A humanized anti-DKK1 monoclonal therapeutic antibody (DKN-01) underwent phase I evaluation in relapsed oesophageal squamous carcinoma (five patients) and gastro-oesophageal junctional cancer (four patients) in combination with paclitaxel (Bendell *et al.,* 2016). Three patients had a partial response, and three other patients showed stable disease. Another phase I evaluation was done in advanced biliary cancer (21 patients) in combination with gemcitabine and cisplatin. Among 18 patients who were evaluable for responses, three patients had a partial response, five showed stable disease and one had progressive disease (see http://www.leaptx.com/news/). Further investigation for both evaluations is ongoing. However, the underlying mechanism of DKN-01 effects on cancer growth is not known at present.

As an anti-DKK1 antibody has possible side effects, including effects on bone homeostasis and cell proliferation by activating Wnt signalling, the anti-CKAP4 antibody may be more specific for anti-proliferative effects by blocking the DKK1-CKAP4 signalling axis. Anti-CKAP4 polyclonal antibody inhibits the *in vitro* binding of DKK1 to CKAP4, DKK1-induced Akt activation in MDCK, S2-CP8 and A549 cells and xenograft tumour formation caused by S2-CP8 and A549 cells (Kimura *et al.,* 2016) (Figure 5B). In contrast, the antibody did not affect tumour formation caused by HeLaS3 cells. In the cells, CKAP4 is expressed on the cell surface membrane but DKK1 expression is marginal, confirming that simultaneous expression of DKK1 and CKAP4 plays a role in cancer promotion. Thus, CKAP4 could be a molecular target for cancers expressing both DKK1 and cell surface CKAP4.

Future perspectives

In this article, the novel DKK1-CKAP4 signalling axis in cancer proliferation and a possible cancer drug using anti-CKAP4 antibody have been reviewed (Figure 6). Identification of CKAP4 as a DKK1 receptor expands the understanding of the DKK1 signalling mechanism and defines a previously unrecognized input to the PI3K and Akt pathway. There are several questions to be addressed in further studies.

The first question is how subcellular localization of CKAP4 is regulated. CKAP4 is largely confined to the ER, and only a marginal part (a few percent) is present on the cell surface membrane. Clarifying the trafficking mechanism of CKAP4 is important because the DKK1-CKAP4 signalling axis is operational only when CKAP4 is localized to the cell surface membrane. When CKAP4 is overexpressed in the cell surface membrane of cancer cells, DKK1-dependent inhibition of Wnt signalling may be suppressed by competing with LRP6 for the binding to DKK1. In addition, it has been reported that CKAP4 is palmitoylated at Cys100 by DHHC2 (Schweizer *et al.,* 1995; Zhang *et al.,* 2008) and APF-mediated nuclear localization of CKAP4 requires the Cys100 residue. Thus, palmitoylation could be important for subcellular localization of CKAP4. Palmitoylated transmembrane proteins are recruited to the lipid raft microdomains of the cell surface membrane (Levental *et al.,* 2010). LRP6 has been shown to be palmitoylated at the juxtamembrane cysteine residues (Abrami *et al.,* 2008) and localized to both the detergent-resistant membrane (DRM) and non-DRM of the cell surface (Yamamoto *et al.,* 2006). DKK1 induces the translocation of LRP6 from DRM to non-DRM and internalizes LRP6 in a clathrin-dependent manner, thereby suppressing Wnt signalling (Yamamoto *et al.,* 2008). Therefore, it is tempting to speculate that DKK1 regulates the localization of CKAP4 in the microdomains of the cell surface membrane, as well as that of LRP6.

The second issue is whether other DKK family members also act as a ligand for CKAP4. The DKK family comprises four

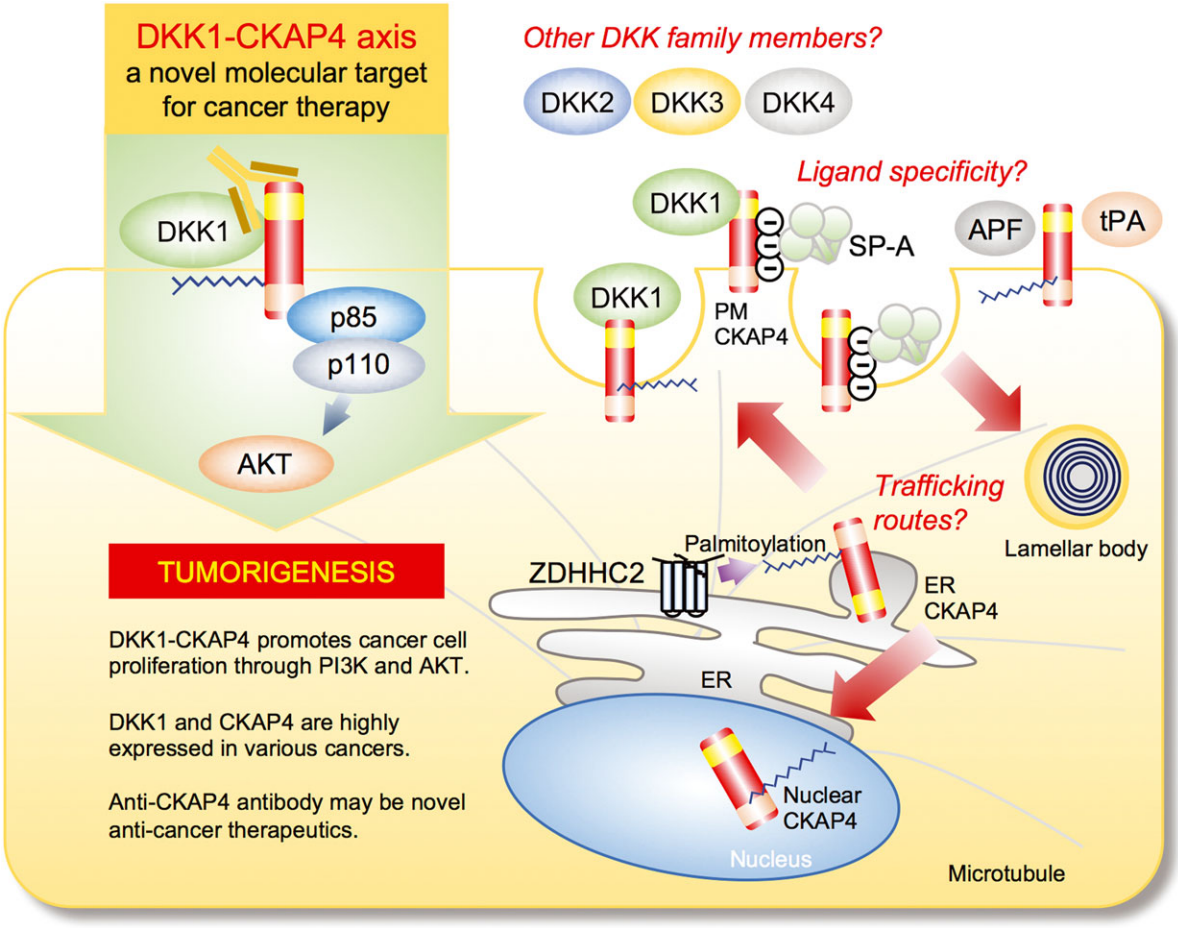

Figure 6

The DKK1–CKAP4 axis as a novel molecular target for cancer therapy and future perspectives. Upon binding to CKAP4, DKK1 promotes cancer cell proliferation through the activation of PI3K and Akt. Anti-CKAP4 antibody inhibits the binding of DKK1 to CKAP4 and xenograft tumour formation. There are questions to be addressed. (1) 'Trafficking routes': it is unclear how subcellular localization of CKAP4 is regulated. Although CKAP4 functions as a receptor, CKAP4 is mainly localized to the ER. Post-translational modification, such as palmitoylation at Cys100 by DHHC2, could be involved in the subcellular localization of CKAP4. (2) 'Other Dkk family members': it is unknown whether other DKK family members also act as a ligand for CKAP4. Since CRD-1 is conserved among the four *DKK* genes, DKK2, 3 and 4 would interact with CKAP4. (3) 'Ligand specificity': Although several ligands for CKAP4 other than DKK1 have been identified, the specificity of the binding between CKAP4 and ligands has not been addressed. See details in the text.

conserved proteins, DKK1, 2, 3 and 4. However, the functions of DKK family members other than DKK1 are less well characterized. DKK2 and DKK4 antagonize Wnt signalling, similar to DKK1, but DKK2 can also activate Wnt signalling depending on the cell (Niehrs, 2006). Physiological role of DKK3 is controversial and pleiotropic. While DKK3 positively regulates Wnt signalling in Müller glia and HEK293 cells (Nakamura *et al.*, 2007), it is also reported that DKK3 has no effect on Wnt signalling (Veeck and Dahl, 2012). However, CRD-1 is highly conserved among the four *DKK* genes, raising the possibility that DKK2, 3 and 4 could also interact with CKAP4.

The third one is specificity of the binding between CKAP4 and ligands. The LZ domain of CKAP4 is required for binding to DKK1. Although the region of CKAP4 that interacts with other ligands is not known, it has been hypothesized that the negatively charged amino acid cluster region of CKAP4 (the region of amino acid 318–328 containing five negatively charged amino acids) binds to the positively charged region of SP-A (Bates, 2010). There are 165 charged residues over the entire 474-amino acid extracellular region of CKAP4. These charged residues may be involved in the binding to other ligands through hydrophilic interactions. It is important to clarify whether these ligands share a common 3D structural domain that binds to CKAP4.

Lastly, humanized anti-CKAP4 monoclonal antibody absolutely needs to be generated for use in human cancer therapy, and the antibody must be tested in mouse cancer models other than xenograft tumours derived from cancer cell lines. To examine the adverse effects of anti-CKAP4 antibody *in vivo*, the analyses of phenotypes of CKAP4 knockout mice would provide information on expected side effects and contribute to the understanding of CKAP4 functions. It is also important to develop diagnostic methods to pick out cancer patients who would respond effectively to treatment with anti-CAKP4 antibody. As an

ELISA to detect serum DKK1 is already available, it would be necessary to determine the presence of cell surface CKAP4 in cancer patients. If CKAP4 was secreted with microvesicles from cancer cells into the serum, exosomes could be a good candidate to examine. Thus, much further work is needed to understand the whole picture of the novel DKK1-CKAP4 signalling axis in tumourigenesis.

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (Alexander *et al.*, 2015a,b,c).

Conflict of interest

The authors declare no conflicts of interest.

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