Dynamic palmitoylation controls the microdomain localization of the DKK1 receptors CKAP4 and LRP6

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Dickkopf1 (DKK1) was originally identified as an antagonist of Wnt signaling that binds to and induces the clathrin-mediated endocytosis of the Wnt coreceptors low-density lipoprotein receptor–related proteins 5 and 6 (LRP5/6). DKK1 also binds to cytoskeleton-associated protein 4 (CKAP4), which was originally identified as an endoplasmic reticulum (ER) protein but also functions at the plasma membrane as a receptor for various ligands. The DKK1-CKAP4 pathway is activated in several human cancers and promotes cell proliferation by activating signaling through the kinases PI3K and AKT. We found that both CKAP4 and LRP6 primarily localized to detergent-resistant membrane (DRM) fractions of the plasma membrane in a palmitoylation-dependent manner and that palmitoylation of CKAP4 was required for it to promote cell proliferation. DKK1 induced the depalmitoylation of both CKAP4 and LRP6 by acylprotein thioesterases (APTs), resulting in their translocation to the non-DRM fractions. Moreover, DKK1-dependent depalmitoylation of both receptors required activation of the PI3K-AKT pathway. DKK1 simultaneously bound CKAP4 and LRP6, resulting in the formation of a ternary complex. LRP5/6 knockdown decreased DKK1-dependent AKT activation and cancer cell proliferation through CKAP4, whereas CKAP4 knockdown did not affect DKK1-dependent inhibition of Wnt signaling through LRP5/6. These results indicate that the palmitoylation states of CKAP4 and LRP6 play important roles in their signaling and that LRP5/6 enhance DKK1-CKAP4 signaling.

INTRODUCTION

Wnt signaling is essential for organ formation during development and for organ homeostasis in postnatal life (1). Of the multiple signaling pathways downstream of Wnt ligands, the β-catenin–dependent pathway is extensively studied (1–3). In certain types of cells, low-density lipoprotein receptor–related proteins 5 and 6 (LRP5/6) act as Wnt coreceptors at the plasma membrane, are localized to the detergent-resistant membrane (DRM) fractions, which could reflect lipid rafts (4), and undergo Wnt-dependent internalization in a caveolin-dependent manner to activate the β-catenin pathway (5–8). Dickkopf1 (DKK1) was originally identified as a secreted protein that antagonizes Wnt signaling in Xenopus laevis embryos (9, 10). DKK1 binds to LRP6, transfers it to the non-DRM fractions, and induces its internalization through a clathrin–mediated route, resulting in removal of LRP6 from the cell surface (11, 12). How DKK1 translocates LRP6 from the DRM to the non-DRM fractions remains to be clarified.

It has been shown that DKK1 also recognizes cytoskeleton-associated protein 4 (CKAP4, also known as p63, CLIMP-63, and ERGIC-63), a cell surface receptor that activates signaling through the kinases phosphatidyl inositol-3 kinase (PI3K) and AKT, stimulating proliferation in both normal epithelial cells and cancer cells (13–17). Although evidence is accumulating that DKK1-CKAP4 signaling is important for cancer cell proliferation, whether the membrane microdomain localization of CKAP4 is regulated in response to DKK1, like that of LRP6, has not yet been elucidated.

Palmitoylation is an important posttranslational modification in which palmitate is covalently linked to specific cysteine residues through thioester bonds (S-palmitoylation) (18–20). This modification is reversible and mediated by a family of DHHC (Asp-His-His-Cys) motif–containing palmitoyl acyltransferases (PATs) (21, 22) and acyl protein thioesterases (APTs) (23) or palmitoyl protein thioesterases (24). Whereas palmitoylation of soluble proteins allows them to traffic to membrane domains, as shown for Ras at the plasma membrane (25), the roles of palmitoylation of integral membrane proteins remain incompletely understood (26).

Palmitoylation of LRP6 at Cys1394 and Cys1399 occurs in the endoplasmic reticulum (ER), and the absence of LRP6 palmitoylation leads to the retention of LRP6 in the ER (27), suggesting that palmitoylation of LRP6 is necessary for its trafficking from the ER to the cell surface. CKAP4 has also been shown to be modified with palmitate at Cys100 by the PAT DHHC2 (28, 29). Wild-type CKAP4 is present on the cell surface and perinuclear membranes in addition to the ER membrane, whereas CKAP4C100S, in which Cys100 is mutated to Ser, is confined to the ER membrane in HeLa cells (30). These findings suggest that palmitoylation is also required for the proper trafficking of CKAP4. Whether palmitoylation of either receptor may have some additional roles after trafficking to the plasma membrane, including in DKK1 signaling, and whether both receptors may mutually interact remain to be clarified.

Here, we show that DKK1 induced depalmitoylation of both CKAP4 and LRP6, resulting in their translocation from DRM fractions to non-DRM fractions, and that palmitoylation of CKAP4 was necessary for DKK1-CKAP4 signaling. In addition, activation of the PI3K-AKT pathway was required for depalmitoylation of both CKAP4 and LRP6. Furthermore, the formation of a ternary complex between LRP6, CKAP4, and DKK1 enhanced DKK1-dependent cancer cell proliferation. Thus, dynamic cycling of CKAP4 and LRP6 palmitoylation and depalmitoylation and the association of the two receptors regulate DKK1 signaling.

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RESULTS

CKAP4 localizes to DRM fractions and moves into non-DRM fractions in response to DKK1

Because DKK1 moves LRP6 out of lipid rafts (12), we examined the distribution of CKAP4 in the DRM and non-DRM fractions of wild-type S2-CP8 human pancreatic cancer cells separated by sucrose density gradient ultracentrifugation (31). Endogenous CKAP4 was primarily localized to the non-DRM fractions, but some was also present in the DRM fractions (Fig. 1A). Because the largest fraction of CKAP4 is localized to the ER membrane, we also examined the localization of CKAP4 at the plasma membrane by labeling cell surface proteins with biotin before sucrose density gradient ultracentrifugation and isolating the biotinylated proteins from each fraction. Most of the endogenous CKAP4 at the cell surface was found in the DRM fractions (Fig. 1A).

Because DKK1 is highly abundant in S2-CP8 cells (13), we knocked out DKK1 in S2-CP8 cells using the CRISPR-Cas9 system [S2-CP8/DKK1 knockout (KO) cells] (32) to eliminate the potential influence of endogenous DKK1 on CKAP4 localization (fig. S1A). Recombinant DKK1 was purified, and its ability to inhibit Wnt3a-dependent accumulation of β-catenin and to activate AKT was confirmed (fig. S1, B to D). In Western blots, DKK1 was detected as a broad band between 25 and 35 kDa, consistent with its estimated molecular mass (25 kDa) plus N-linked and O-linked glycans that are added posttranslationally (33). The amount of CKAP4 in the plasma membrane of S2-CP8/DKK1 KO cells decreased in the first 60 min after stimulation with DKK1, and then CKAP4 reappeared on the cell surface at 120 to 180 min after DKK1 stimulation (Fig. 1B). Treatment with monodansylcadaverine (MDC), which inhibits clathrin-mediated endocytosis (34), or knockdown of clathrin or μ-adaptin suppressed DKK1-induced CKAP4 internalization and caused CKAP4 to be retained on the cell surface (Fig. 1B and fig. S1, E and F).

However, it is possible that CKAP4 that was not detectable in the non-DRM fractions at 15 to 30 min after stimulation (Fig. 1C). At 120 to 180 min after stimulation, CKAP4 reappeared in the DRM fractions. When the cells were pretreated with MDC, CKAP4 was nearly undetectable in both DRM fractions in Rab5-depleted cells, and CKAP4 was not recycled in Rab11-depleted cells (fig. S1, E and F). In addition, DKK1-induced internalization of CKAP4 was inhibited in Rab5-depleted cells, and CKAP4 KO was not recycled in Rab11-depleted cells (fig. S1, E and F). Thus, DKK1 induced CKAP4 internalization in a clathrin- and Rab11-dependent manner, followed by the Rab11-dependent recycling to lipid rafts.

When S2-CP8/DKK1 KO cells were stimulated with DKK1, the amounts of CKAP4 in the plasma membrane were reduced in the DRM fractions and were only faintly detected in the non-DRM fractions at 15 to 30 min after stimulation. CKAP4 was nearly undetectable in both fractions at 60 min (Fig. 1C). After S2-CP8/DKK1 KO cells were recycled in Rab11-depleted cells, and CKAP4 reappeared in the DRM fractions. When the cells were pretreated with MDC, CKAP4 was retained in the non-DRM fractions at 60 to 180 min after DKK1 stimulation (Fig. 1C). A DKK1 mutant lacking the cysteine-rich domain 1 (DKK1ΔCRD1), which binds to LRP6 but not to CKAP4 (13), inhibited Wnt3a-dependent accumulation of β-catenin but did not activate AKT (fig. S1, C and D).

When cells were stimulated with recombinant DKK1ΔCRD1, CKAP4 at the cell surface did not move to the non-DRM fractions (fig. S1G). Because the DRM fractions of the plasma membrane could reflect lipid rafts (4), these results suggest that CKAP4 in lipid rafts moved to nonlipid rafts in response to DKK1. However, it is possible that CKAP4 that
Fig. 2. APTs, PI3K, and AKT mediate DKK1-induced palmitoylation of CKAP4. (A) Top panels: 17-ODYA–labeled S2-CP8/DKK1 KO cells were pretreated with the clathrin-mediated endocytosis inhibitor MDC and then stimulated with DKK1. CKAP4 immunoprecipitates (IP) from the membrane fractions and total lysates were subjected to the click chemistry assay, affinity purified [AP (click)], and immunoblotted for CKAP4. β-Actin is a loading control. The amounts of palmitoylated CKAP4 were quantified and are expressed as arbitrary units compared with values for cells without DKK1 simulation. (B) S2-CP8/DKK1 KO cells pretreated with MDC were stimulated with DKK1 or DKK1(ΔCRD1). The cells were biotinylated and subjected to the APEGs assay. Reactions in which hydroxylamine (HAM) was omitted are negative controls. Total lysates and biotinylated precipitates (membrane) were immunoblotted for CKAP4. The amounts of palmitoylated CKAP4 were quantified and are expressed as relative to the total CKAP4 abundance. (C and D) After S2-CP8/DKK1 KO cells pretreated with MDC were treated with PalB (G) or transfected with a scrambled control siRNA or a set of siRNAs targeting APT1/2 (APT1#1 + APT2#1) (D), the cells were stimulated with DKK1 followed by APEGs assay of the biotinylated membrane proteins. The amounts of palmitoylated CKAP4 were quantified and are expressed as relative to the total CKAP4 abundance. (E) Wild-type (WT) S2-CP8, S2-CP8/CKAP4 KO, and S2-CP8/DKK1 KO cells were pretreated with the amine-to-amine cross-linker DSP. Total protein lysates (input) and CKAP4 IP (membrane) were immunoblotted for CKAP4 and APT1.

(F and G) 17-ODYA–labeled S2-CP8/DKK1 KO cells pretreated with MDC were treated with wortmannin (F) or AKT inhibitor VIII (G) and then were stimulated with DKK1. Total lysates (input) and CKAP4 IP were subjected to the click chemistry assay [AP (Click)] and immunoblotted for CKAP4 and clathrin. Clathrin is a loading control. The amounts of palmitoylated CKAP4-HA were quantified and are expressed as arbitrary units compared with values for control cells without DKK1 simulation.

(H) APT1 activity assays. Lysates of 17-ODYA–labeled S2-CP8/CKAP4 KO/CKAP4-HA cells were immunoprecipitated with an antibody specific for HA, and the IP were used as substrates in APT1 assays. S2-CP8/DKK1 KO/APT1-FLAG cells treated with or without AKT inhibitor VIII were stimulated with DKK1 conditioned medium and lysed. FLAG immunoprecipitates of the lysates were used as the enzyme source for APT1 assays. Substrates and enzyme sources were mixed as indicated and subjected to the APT1 assay. The reactions were immunoblotted for HA. The amounts of palmitoylated CKAP4-HA were quantified and are expressed as arbitrary units compared with values for time 0, and statistical analysis was performed. The values for DKK1-treated (DKK1) cells at each time point were used as controls. All blots are representative of three independent experiments, and results are shown as means ± SD of three independent experiments. *P < 0.05, **P < 0.01 (Student’s t test).
resides in the ER or Golgi directly translocates to the non-DRM fractions by DKK1 stimulation.

**CKAP4 is efficiently palmitoylated at the cell surface**
CKAP4 is modified with palmitate at Cys\(^{100}\) (28, 29). To examine the roles of CKAP4 palmitoylation, we generated a CKAP4 mutant in which Cys\(^{100}\) was mutated to Ser (CKAP4\(^{100S}\)), and stably expressed it or wild-type CKAP4 (CKAP4\(^{WT}\)) in S2-CP8/CKAP4 KO cells and in another cell line, X293T/CKAP4 KO cells (fig. S2, A and B). X293T cells, a noncancerous epithelial cell line, was used to confirm the phenotypes of CKAP4\(^{WT}\) and CKAP4\(^{100S}\). The amounts of CKAP4\(^{WT}\) and CKAP4\(^{100S}\) and cell surface localization in both cell types were comparable to that of endogenous CKAP4 in the respective parental cells (fig. S2, A and B).

We confirmed palmitoylation of CKAP4 at Cys\(^{100}\) in these cells using three methods: acyl-biotinylation exchange (ABE) assay (36, 37), click chemistry assay (38), and acyl-PEGyl exchange gel shift (APEGS) assay (39). In the ABE assay, endogenous palmitoylated CKAP4 was biotinylated in a hydroxyamine-dependent manner; ectopically expressed CKAP4\(^{WT}\), but not CKAP4\(^{100S}\), was also biotinylated comparably to that of endogenous CKAP4 (fig. S2C). In the click chemistry assay, wild-type S2-CP8 cells were metabolically labeled with the palmitate analog 17-octadecynoic acid (17-ODYA), which was then modified with azide biotin and used to precipitate palmitoylated proteins with NeutrAvidin agarose beads. This assay also showed cell surface endogenous CKAP4 to be modified with palmitate (fig. S2D).

In the APEGS assay using maleimide polyethylene glycol (mPEG), the ratio of palmitoylated to nonpalmitoylated CKAP4 was calculated (fig. S2E). In the total lysates of S2-CP8 cells, about 50% of endogenous CKAP4 was modified with mPEG (fig. S2E). Similarly, about 50% of ectopically expressed CKAP4\(^{WT}\) present in the total lysates was modified with mPEG, but CKAP4\(^{100S}\) was not (fig. S2E). In contrast, in membrane preparations, about 80% of endogenous and ectopically expressed CKAP4\(^{WT}\) was modified with mPEG (fig. S2E), indicating that palmitoylation of CKAP4 occurred efficiently at the plasma membrane rather than at intracellular membranes.

Ectopically expressed FLAG-tagged DKK1 (DKK1-FLAG) formed complexes with CKAP4\(^{WT}\) and CKAP4\(^{100S}\) in S2-CP8/CKAP4 KO cells to similar extents as did endogenous CKAP4 in wild-type S2-CP8 cells (fig. S2F), and internalization and recycling of CKAP4\(^{WT}\) and CKAP4\(^{100S}\) were similarly induced by DKK1 treatment in S2-CP8/CKAP4 KO cells (fig. S2G), suggesting that palmitoylation of CKAP4 was not required for the trafficking of CKAP4 from the ER to the cell surface, for the binding of CKAP4 to DKK1, or for the internalization of CKAP4.

Among the 23 members of the ZDHHC family of palmitoyltransferases, ZDHHC2 localizes to the ER and Golgi and palmitoylates various substrates including CKAP4, and ZDHHC5 is the plasma membrane–localized PAT protein (21, 22). Consistent with previous observations (28, 40), biochemical and immunocytochemistry assays in S2-CP8, HeLa, and X293T cells showed that ZDHHC2 and ZDHHC5 were primarily localized to the ER and the plasma membrane, respectively (fig. S3, A and B). Although knockdown of ZDHHC2 in S2-CP8 cells did not affect the amount of cell surface CKAP4 that palmitoylated CKAP4 (~80%), knockdown of ZDHHC5 reduced that to ~55% (fig. S3, C and D). We transiently expressed FLAG-tagged CKAP4 (FLAG-CKAP4) along with hemagglutinin (HA)–tagged ZDHHC2 (HA-ZDHHC2) or HA-ZDHHC5 in X293T cells (fig. S3E). In control X293T cells expressing only FLAG-CKAP4, the amount of mPEG modification of ectopically expressed CKAP4 at the cell surface was about 50%, which was lower than that of endogenous CKAP4 in S2-CP8 cells, likely due to differences between the cell types (fig. S3F).

**DKK1 induces depalmitoylation of CKAP4**
In the click chemistry and APEGS assays, DKK1 did not affect the proportion of CKAP4 that was palmitoylated in total lysates of S2-CP8/DKK1 KO cells, whereas wild-type DKK1 (DKK1\(^{WT}\)), but not DKK1\(^{100S}\), reduced the palmitoylation of CKAP4 at the cell surface (fig. 2, A and B). These results suggest that DKK1 induces depalmitoylation of CKAP4 at the cell surface.

Palmostatin B (PalB) is an inhibitor of depalmitoylases, including APT1, APT2, and palmitoyl protein thioesterases (41, 42). PalB suppressed the DKK1-induced decreases in palmitoylated CKAP4 (fig. 2C), and similar effects were observed in cells in which both APT1 and APT2 (APT1/2) were knocked down using two different sets of small interfering RNAs (siRNAs) (fig. 2D and fig. S4, A and B). Moreover, CKAP4 in the plasma membrane formed a complex with APT1 in wild-type S2-CP8 cells but not in S2-CP8/DKK1 KO cells (fig. 2E). Thus, DKK1 induced depalmitoylation of CKAP4 at the plasma membrane through APT1/2.

Because DKK1-CKAP4 signaling activates the PI3K-AKT pathway (13), we treated S2-CP8/DKK1 KO cells with inhibitors of PI3K (wortmannin and LY294002) or AKT (AKT inhibitor VIII) before the DKK1 treatment. These inhibitors suppressed DKK1-induced depalmitoylation of CKAP4 (fig. 2, F and G, and fig. S4C). To determine whether the PI3K-AKT pathway was involved in depalmitoylation of CKAP4 downstream of DKK1, we examined AKT activity in vitro using FLAG-tagged AKT1 (APT1-FLAG) immunoprecipitated from S2-CP8/DKK1 KO cells and 17-ODYA–labeled CKAP4. When cells were stimulated with DKK1, AKT was activated, and treatment with AKT inhibitor VIII completely inhibited this (fig. S4D). The depalmitoylation activity of APT1-FLAG from cells treated with DKK1 was enhanced compared with that from untreated cells, and treatment cells with AKT inhibitor VIII prevented DKK1-mediated enhancement of the depalmitoylation activity of APT1-FLAG (fig. 2H). AKT1/2 does not contain putative AKT phosphorylation sites (RXXXS/T) (43), and AKT did not phosphorylate AKT1 or AKT2 in vitro (fig. S4E). Therefore, PI3K and AKT were likely to be indirectly involved in DKK1-induced depalmitoylation of CKAP4 through AKT1/2.

PalB inhibited DKK1-induced CKAP4 translocation from the DRM fractions to the non-DRM fractions (fig. 3A), and treatment with PalB or knockdown of AKT1/2 inhibited the DKK1-induced internalization of CKAP4 at the cell surface (fig. 3, B and C). Furthermore, treatment of S2-CP8/DKK1 KO cells with inhibitors of PI3K or AKT inhibited DKK1-induced CKAP4 movement to the non-DRM fractions (fig. 3, D and E). These results suggest that DKK1-induced depalmitoylation of CKAP4 was required for CKAP4 translocation and internalization at the cell surface.

**Palmitoylation is required for localization of CKAP4 to the DRM fractions**
Ectopically expressed CKAP4\(^{WT}\) showed a distribution pattern similar to that of endogenous CKAP4 in the total lysates and at the plasma
membrane, whereas CKAP4C100S localized only to the non-DRM fractions (Fig. 4A). Both mPEG-modified and mPEG-unmodified CKAP4WT were detected in the DRM fractions of the plasma membrane, but little CKAP4WT was present in the non-DRM fractions of the plasma membrane (Fig. 4B). In contrast, CKAP4C100S localized only to the non-DRM fractions and showed no mPEG modification in the plasma membrane (Fig. 4B), suggesting that palmitoylation was required for the localization of CKAP4 to lipid rafts of the cell surface. The amount of ectopically expressed CKAP4WT modified with mPEG in the DRM fractions of the plasma membrane was reduced to about 60%, which was lower than that of ectopically expressed CKAP4WT in the plasma membrane (the DRM plus the non-DRM fractions) of S2-CP8/CKAP4 KO cells (Fig. S2E). This may be an artificial reduction due to the long assay procedure, which includes multiple steps in the localization of CKAP4 to lipid rafts of the cell surface. The amounts of CKAP4 in the DRM and non-DRM fractions at each time point were quantified and are expressed relative to the values for the sum of DRM and non-DRM fractions. In all panels, results are shown as means ± 5D of three independent experiments. *P < 0.05 (Student's t test).

**Palmitoylation states of CKAP4 determine DKK1 signaling**

We next examined the role of CKAP4 palmitoylation in DKK1-CKAP4 signaling. DKK1 promotes the formation of a complex between CKAP4 and the p85α regulatory subunit of PI3K (13). The majority of p85α was found in the non-DRM fractions of total lysates of S2-CP8 cells that ectopically expressed p85α (Fig. 5A), whereas p85α complexed with biotinylated surface proteins was primarily found in the DRM fractions of the plasma membrane (Fig. 5A) as previously shown in Caco-2 cells (44). p85α was also present in the DRM fractions of the plasma membrane of X293T cells that ectopically expressed p85α (Fig. S5A). Complex formation between CKAP4 and p85α was observed in the DRM fractions
Fig. 4. Palmitoylated and nonpalmitoylated CKAP4 interact and mutually influence one another’s localization in membrane microdomains. (A) S2-CP8/CKAP4 KO/CKAP4 WT-HA (WT-HA) and S2-CP8/CKAP4 KO/CKAP4 C100S-HA (C100S-HA) cells were biotinylated, and cell lysates were fractionated. Total lysates and biotinylated membrane proteins were immunoblotted for CKAP4, clathrin, and flotillin-2. (B) The DRM fractions and the non-DRM fractions of the membrane proteins in (A) were subjected to the APEGS assay and immunoblotted for CKAP4. (C) S2-CP8/CKAP4 KO cells transiently expressing CKAP4 WT-FLAG alone or both CKAP4 WT-FLAG and CKAP4 C100S-HA were biotinylated and fractionated. Total lysates and biotinylated membrane proteins were blotted and probed with the indicated antibodies. (D) Lysates of X293T/CKAP4 KO cells expressing CKAP4 WT-FLAG and CKAP4 WT-HA (D) or CKAP4 WT-FLAG and CKAP4 C100S-HA (E) were immunoprecipitated with antibodies specific for FLAG or HA. Total lysates (input) and immunoprecipitates (IP) were probed with antibodies specific for FLAG or HA. All blots are representative of three independent experiments.

not in the non-DRM fractions of S2-CP8 and X293T cells (Fig. 5B and fig. S5B). Moreover, CKAP4 WT, but not CKAP4 C100S, formed a complex with p85α when expressed in S2-CP8/CKAP4 KO (Fig. 5C). Thus, CKAP4 interacted with PI3K in lipid rafts.

AKT activity in S2-CP8 cells was reduced by KO of CKAP4, and this decrease was rescued by expression of CKAP4 WT but not CKAP4 C100S (Fig. 5D). Treatment of S2-CP8/DKK1 KO cells with MDC did not affect DKK1-dependent AKT activation, whereas treatment with MβCD completely suppressed that activation (Fig. 5E). When S2-CP8/DKK1 KO cells were treated with PalB or transfected with siRNAs targeting APT1/2, activation of AKT was still detected at 60 to 90 min after stimulation with DKK1 (Fig. 5, F and G), indicating that AKT activation was prolonged. Therefore, palmitoylation of CKAP4 enhanced DKK1-dependent activation of the PI3K-AKT pathway.

CKAP4 KO decreased the proliferation of S2-CP8 cells in both two-dimensional (2D) and 3D cultures, and this phenotype was rescued by expression of CKAP4 WT, but not by expression of CKAP4 C100S (Fig. 6, A and B). In xenograft assays, tumors induced by S2-CP8/CKAP4 KO cells or S2-CP8/CKAP4 KO cells expressing CKAP4 WT were smaller than those induced by wild-type S2-CP8 cells or S2-CP8/CKAP4 KO cells expressing CKAP4 WT (Fig. 6C). In A549 lung cancer cells, in which DKK1-CKAP4 signaling is activated (13), expression of CKAP4 WT, but not CKAP4 C100S, suppressed AKT activation and cell proliferation induced by CKAP4 KO (Fig. 6, D and F). Together, these results indicate that palmitoylation of CKAP4 was necessary for DKK1-dependent activation of the PI3K-AKT pathway and cell proliferation.

DDK1-induced depalmitoylation of LRP6 out of the DRM fraction

We compared the roles of LRP6 palmitoylation with those of CKAP4 palmitoylation. The click chemistry assay revealed that palmitoylation of endogenous LRP6 in S2-CP8 cells was suppressed and enhanced by treatment with 2-bromopalmitate (2-BP), which is a general inhibitor of palmitoylation (45), and the depalmitoylase inhibitor PalB, respectively (Fig. 7A). In S2-CP8/DKK1 KO cells, DKK1 induced depalmitoylation of LRP6 in the plasma membrane, and PalB inhibited it (Fig. 7B). DKK1 induced the movement of endogenous LRP6 from the DRM to the non-DRM fractions at the cell surface in a dose-dependent manner (fig. S6A), and PalB inhibited this movement (Fig. 7C). Treatment with PalB or knockdown of APT1/2 suppressed DKK1-induced internalization of LRP6 from the cell surface (Fig. 7D and E). Treatment with wortmannin inhibited the DKK1-induced depalmitoylation of LRP6 at the cell surface (Fig. 7F), and DKK1-induced depalmitoylation of LRP6 was attenuated in X293T/CKAP4 KO cells (Fig. 7G), suggesting that DKK1-dependent LRP6 depalmitoylation required the CKAP4-PI3K-AKT pathway. PalB treatment did not significantly affect the inhibitory activity of DKK1 on Wnt signaling in S2-CP8/DKK1 KO cells (fig. S6B). Therefore, depalmitoylation of LRP6 is unlikely to be essential for the inhibitory action of DKK1 on Wnt signaling. Together, these results suggest that DKK1 induces depalmitoylation of LRP6 through the CKAP4-PI3K-AKT pathway, leading to the removal of LRP6 from the DRM fractions, which is followed by the internalization of LRP6 from the non-DRM fractions.

Simultaneous binding of DKK1 to CKAP4 and LRP5/6 enhances DKK1-CKAP4 signaling

We examined whether CKAP4 and LRP5/6 mutually affect DKK1 signaling when they were simultaneously localized to the cell surface in the same cells. In X293T cells, the formation of a complex between ectopically expressed FLAG-LRP6 and CKAP4-HA was observed when DKK1-FLAG was simultaneously expressed, but not when DKK1-FLAG and DKK1-FLAG were expressed (Fig. 8A). Recombinant DKK1-FLAG, which binds to CKAP4 but not LRP6, activated AKT but did not inhibit Wnt3a-dependent accumulation of β-catenin (fig. S1, C and D). In addition, in the plasma membrane of X293T cells, CKAP4-HA and FLAG-LRP6 formed a complex in the presence of recombinant DKK1-FLAG,
Fig. 5. Palmitoylation-dependent localization of CKAP4 to the DRM fraction is necessary for DKK1-CKAP4 signaling. (A) S2-CP8 cells transiently expressing p85α were biotinylated and fractionated. Total lysates and precipitates were immunoblotted for the indicated proteins. p85α that cofractionated with biotinylated plasma membrane proteins is indicated as “Pull down.” (B) The DRM fractions and the non-DRM fractions in (A) were immunoprecipitated with control IgG or an antibody (Ab) against CKAP4. Total protein lysates (input) and immunoprecipitates (IP) were immunoblotted for p85α and CKAP4. A closed triangle indicates a blank lane. (C) Total lysates (input) of S2-CP8/CKAP4 KO, S2-CP8/CKAP4 KO/CKAP4WT-HA (WT-HA), and S2-CP8/CKAP4 KO/CKAP4ΔCRD1-HA (ΔCRD1-HA) cells were immunoprecipitated with an antibody recognizing HA and immunoblotted for p85α and CKAP4. (D) Lysates of WT S2-CP8, S2-CP8/CKAP4 KO, S2-CP8/CKAP4 KO/CKAP4WT-HA, and S2-CP8/CKAP4 KO/CKAP4ΔCRD1-HA cells were immunoblotted for phosphorylated AKT (pAKT), total AKT, and clathrin. (E) After S2-CP8/DKK1 KO cells were pretreated with or without MDC, the cells were stimulated with DKK1 for the indicated time periods. Cell lysates were blotted and probed with antibodies for the indicated proteins. (F and G) After S2-CP8/DKK1 KO cells were pretreated with PalB (F) or transfected with a scrambled siRNA or siRNAs APT1#1 and APT2#1 (G), the cells were stimulated with DKK1 for the indicated time periods, and cell lysates were immunoblotted for the indicated proteins. The band intensities of phosphorylated AKT at each time point were quantified and are expressed as arbitrary units compared with the values at time 0. All results are representative of three independent experiments, and data in (F) and (G) are shown as means ± SD of three independent experiments. *P < 0.05 (Student’s t test).
Fig. 6. Palmitoylation of CKAP4 is required for cell proliferation. (A) WT S2-CP8, S2-CP8/CKAP4 KO, S2-CP8/CKAP4 KO/CKAP4 WT-HA (WT-HA), and S2-CP8/CKAP4 KO/CKAP4 WT-1000–HA (C100S-HA) cells were subjected to a fluorescent dye–based 2D cell proliferation assay. The fluorescence intensity was measured at the indicated time points and was expressed as arbitrary units compared with the values for WT cells at day 7. n = 3 independent experiments. Results are shown as means ± SD of five replicates for each day. (B) Phase-contrast images of the indicated S2-CP8 cells cultured for 5 days in 3D Matrigel and quantification of the total area of spheres per field. Results are expressed as arbitrary units compared with WT cells. n = 3 independent experiments. Results are shown as means ± SD of five replicates for each day. (C) The indicated S2-CP8 cells were subcutaneously implanted into immunodeficient mice. The volumes of xenograft tumors were measured at day 21. Representative mice and extirpated xenograft tumors are shown. Dotted lines show the outline of the tumors. Results are plotted as box and whiskers diagrams, where the median is represented with a line, the box represents the 25th to 75th percentile, and error bars show the 5th to 95th percentile. n = 8 [WT S2-CP8 (Control)] and 4 (S2-CP8/CKAP4 KO, S2-CP8/CKAP4 KO/CKAP4 WT-HA, S2-CP8/CKAP4 KO/CKAP4 WT-1000–HA). Scale bars, 10 mm. (D) WT A549, A549/CKAP4 KO, A549/CKAP4 KO/CKAP4 WT-HA (WT-HA), and A549/CKAP4 KO/CKAP4 WT-1000–HA (C100S-HA) cells were biotinylated, and the cell surface proteins were precipitated. Total lysates and biotinylated proteins (membrane) were immunoblotted for the indicated proteins. Results are representative of three independent experiments. (E) The indicated A549 cells were subjected to the fluorescent dye–based 2D cell proliferation assay. The fluorescence intensity was measured at the indicated time points and was expressed as arbitrary units compared with WT cells at day 7. n = 3 independent experiments. Results are shown as means ± SD of five replicates for each day. (F) Phase contrast images of the indicated A549 cells S2-CP8 cells cultured for 5 days in 3D Matrigel and quantification of the total area of spheres per field. Results are expressed as arbitrary units compared with WT cells. n = 3 independent experiments. Results are shown as means ± SD of five replicates per culture. Scale bar, 100 μm. *P < 0.05; **P < 0.01 (Student’s t test) (A, B, E, F) or Wilcoxon rank sum test (C).

the inhibitor of Wnt production 2 (IWP-2), which inhibits the secretion of Wnt ligands (46), attenuated the expression of AXIN2 and inhibited the proliferation of S2-CP8 cells (fig. S8, A to C), suggesting that Wnt secreted from S2-CP8 cells was required for cell proliferation. Therefore, to evaluate the contribution of LRP5/6 to DKK1–CKAP4 signal-dependent cell proliferation, cells that are dependent on DKK1–CKAP4 signaling because LRP5/6 knockdown showed reduced AKT activity and cell proliferation, although the extent was partial compared with that in cells in which CKAP4 was knocked down (fig. S8, G and H). Together, these results suggest that LRP5/6 enhanced DKK1–CKAP4 signaling independently of Wnt–β-catenin signaling.

According to the Cancer Genome Atlas, expression of CKAP4, DKK1, LRP5, and LRP6 mRNAs increased in many cancers compared with normal tissues (table S1). It is intriguing to speculate that LRP5/6 promotes aggressiveness of cancer cells by cooperating with DKK1–CKAP4 signaling because LRP5, DKK1, and CKAP4 mRNAs are highly expressed in pancreatic cancer (fig. S9). Higher expression of LRP5/6 in pancreatic cancer may be involved in the activation of not only the Wnt–β-catenin pathway but also the DKK1–CKAP4 pathway, which is activated in several human cancers and promotes cell proliferation (13–16).
DISCUSSION

In this study, we examined the roles of palmitoylation in CKAP4 and LRP6 in DKK1 signaling. CKAP4 palmitoylation was necessary for DKK1-induced activation of the PI3K-AKT pathway, and LRP6 palmitoylation enhanced DKK1-CKAP4 signaling. Although DHHC2 knockdown prevents the nuclear translocation of CKAP4 in response to antiproliferating factor (30), it is not clear whether palmitoylation of CKAP4 is involved in this process. We found that palmitoylation of CKAP4 was not necessary for the trafficking of CKAP4 from the ER to the plasma membrane. This is in contrast to LRP6 because palmitoylation of LRP6 is necessary for it to exit from the ER (27). In addition, palmitoylation of CKAP4 is unlikely to be involved in a conformational change that may affect its binding to ligands because DKK1 induced the internalization of CKAP4C100S similarly as it did...
Capan-1

CRD1-FLAG

IP: IgG
IP:HA Ab

in lipid rafts when simultaneously expressed with CKAP4 WT, endogenous CKAP4 in the plasma membrane of S2-

CRD1-FLAG, and

CRD2-FLAG (ΔCRD2), and then cell surface CKAP4-HA was immunoprecipitated. Total lysates (input) and immunoprecipitates (IP) were immunoblotted for LRP6, HA, and DKK1. The open arrowhead indicates IgG. (B) WT X293T cells ectopically expressing FLAG-LRP6 with or without CKAP4-HA were stimulated with purified DKK1 WT (WT), DKK1 CRD1 (ΔCRD1), or DKK1 CRD2 (ΔCRD2), and then cell surface CKAP4-HA was immunoprecipitated. Total lysates (input) and immunoprecipitates (IP) were immunoblotted for FLAG and HA. (C) S2-CP8/DKK1 KO cells were transfected with scrambled siRNA (Control) or siRNAs specific for LRP5 and LRP6 (LRP5#1 and LRP6#1) and then stimulated with the indicated concentration of DKK1. Lysates were immunoblotted for phosphorylated AKT (pAKT), total AKT, and HSP90. HSP90 is a loading control. The band intensities of phosphorylated AKT were quantified and HSP90. HSP90 is a loading control. The band intensities of phosphorylated AKT (pAKT), total AKT, and HSP90. HSP90 is a loading control. The band intensities of phosphorylated AKT (pAKT) were expressed as arbitrary units compared with the values for WT cells.

The open arrowhead indicates IgG. (D) WT X293T cells ectopically expressing FLAG-LRP6 with or without CKAP4-HA were stimulated with purified DKK1 WT (WT), DKK1 CRD1 (ΔCRD1), or DKK1 CRD2 (ΔCRD2), and then cell surface CKAP4-HA was immunoprecipitated. Total lysates (input) and immunoprecipitates (IP) were immunoblotted for FLAG and HA. (E) S2-CP8 cells were subjected to the fluorescent dye–based 2D cell proliferation assay. The fluorescence intensity of the dye was measured each day and expressed as arbitrary units compared with the values for WT cells at day 5. (F) Lysates of WT S2-CP8 cells transiently expressing p85α and transfected with scrambled siRNA or siRNAs specific for LRP5/6 (LRP5#1 and LRP6#1) were immunoprecipitated with an antibody specific for CKAP4. Total lysates and immunoprecipitates were immunoblotted for p85α, CKAP4, and LRP6. The band intensities for the coimmunoprecipitated p85α were expressed as arbitrary units compared with the values forcontrol cells. (G) Lysates of WT Capan-1 cells transfected with scrambled siRNA and Capan-1/DKK1-FLAG cells transfected with scrambled siRNA for scramble or siRNA specific for CKAP4 (CKAP4#1) or LRP5/6 (LRP5#1 and LRP6#1) were probed with the indicated antibodies. The band intensities for pAKT were expressed as arbitrary units compared with the values for WT cells. (H) WT Capan-1 and Capan-1/DKK1-FLAG cells were subjected to the 2D cell proliferation assay, and results are expressed as arbitrary units compared with the values for WT cells at day 5. Results are representative of three independent experiments (A and B), shown as means ± 5D of three independent experiments (C, D, F, and G) and shown as means ± SD of four biological replicates at each day (E and H). *P < 0.05; **P < 0.01 (Student’s t test).

CKAP4 WT. However, palmitoylation was required for the incorporation of CKAP4 into lipid rafts because CKAP4 C110S was exclusively localized to nonlipid rafts.

Most of the endogenous CKAP4 in the plasma membrane of S2-CP8/DKK1 KO cells was found to be palmitoylated, and CKAP4 was found in lipid rafts. The extracellular region of CKAP4 contributes to oligomer formation (47). Because CKAP4 WT and CKAP4 C110S formed complexes with one another and CKAP4 C110S was observed in lipid rafts when simultaneously expressed with CKAP4 WT, endogenous nonpalmitoylated CKAP4 is likely to be present in lipid rafts. DKK1 stimulation reduced the proportion of CKAP that was palmitoylated from around 80% to around 50% and caused most of the CKAP4 to move out of lipid rafts. Those results indicate that both palmitoylated and nonpalmitoylated CKAP4 was located in nonlipid rafts when the one-half of CKAP4 was not palmitoylated. These observations suggest that proper balance between palmitoylated and nonpalmitoylated forms of CKAP4 and their complexes is important for regulating their microdomain localization, and DKK1-dependent CKAP4 palmitoylation state would induce move-out of CKAP4 from lipid rafts to nonlipid rafts.

Our results also showed that DKK1-induced depalmitoylation of CKAP4 was mediated by APT1/2 in a manner that depended on
PI3K-AKT signaling. The detailed mechanism by which AKT regulates APT1/2 activity is still unclear, but it is intriguing to speculate that DKK1-CKAP4 signaling–dependent activation of the PI3K-AKT pathway stimulates APT1/2, contributing to a reduction in DKK1-CKAP4 signaling. We propose that DKK1-CKAP4 signaling is regulated by the dynamic control of CKAP4 palmitoylation and de-palmitoylation, which determines CKAP4 localization to lipid rafts and downstream PI3K-AKT pathway activity, and there may be a negative feedback mechanism in which the PI3K-AKT pathway enhances the de-palmitoylation activity of APT1 (fig. S10).

We did not directly show that palmitoylation is necessary for the localization of LRP6 to lipid rafts because LRP6<sup>11394S/C1395S</sup>, a mutant form of LRP6 that cannot be palmitoylated, is never trafficked to the plasma membrane (27). However, most of the endogenous LRP6 at the plasma membrane in S2-CP8 cells was localized to the DRM fractions, and DKK1 induced de-palmitoylation of LRP6 and its translocation to nonlipid rafts. Therefore, palmitoylated LRP6 should be primarily present in lipid rafts. Note that the PI3K-AKT pathway was also involved in DKK1-induced de-palmitoylation of LRP6. Thus, palmitoylation turnover of CKAP4 and LRP6 by DKK1 could be regulated by a common mechanism. The inhibition of LRP6 de-palmitoylation did not affect the inhibitory action of DKK1 on Wnt signaling, suggesting that translocation of LRP6 to nonlipid rafts may not be essential for it but that DKK1-induced de-palmitoylation of LRP6 may be necessary for desensitizing Wnt signaling.

Turnover of CKAP4 and LRP6 palmitoylation may be a common mechanism for controlling their distribution in cell surface microdomains. Because it is thought that lipid rafts are thicker than other parts of the plasma membrane due to the presence of cholesterol (48), changes in the conformation of the transmembrane domain due to palmitoylation may alter the effective lengths of hydrophobic segments of CKAP4 and LRP6, causing the protein to partition into lipid rafts.

One of the important roles of lipid rafts on the cell surface might be the clustering of signal transduction machinery, including components of T cell receptor, endothelial growth factor, Ras, and Hedgehog signaling (4). The association between CKAP4 and the p85α subunit of PI3K was observed in cells expressing DKK1; however, interaction between CKAP4<sup>1C105S</sup> and p85α was not observed, suggesting that p85α resides close to the cytoplasmic side of lipid rafts and is recruited to CKAP4 by DKK1 stimulation, resulting in the efficient activation of signaling cascades. The initial signaling events in lipid rafts are important because depletion of cholesterol inhibits the DKK1-dependent activation of AKT, and CKAP4<sup>1C105S</sup> does not rescue DKK1-dependent S2-CP8/CKAP4 KO cell proliferation either in vitro or in vivo, supporting the notion that DKK1-CKAP4 signaling is activated in lipid rafts. DKK1-dependent AKT activation was prolonged by treatment with PalB, but not with MDC, suggesting that removal of CKAP4 from lipid rafts is more critical for desensitization of DKK1 signaling than for CKAP4 internalization through clathrin-mediated endocytosis.

One of the unanticipated findings of this study is that LRP5/6 enhanced DKK1-CKAP4 signaling. DKK1 binds to CKAP4 and LRP6 through its CRD1 and CRD2 domains, respectively (9, 13), probably in lipid rafts. DKK1 activated the pathways downstream of CKAP4 (the DKK1-CKAP4 pathway) and inhibited that of LRP6 (the β-catenin pathway) simultaneously in the same cell, and the binding of DKK1 to LRP5/6 enhanced the CKAP4-PI3K-AKT pathway. DKK1 may preferentially activate AKT through DKK1-CKAP4 signaling over inhibiting Wnt-β-catenin signaling and, as a result, stimulate cell proliferation when CKAP4 and LRP6 are both present. In addition, palmitoylation of LRP6 would contribute to the activation of DKK1-CKAP4 signaling by keeping LRP6 localized to lipid rafts. Further studies, including immunohistochemical analyses of human cancer specimens to evaluate the coexpression of DKK1, CKAP4, and LRP5/6 with clinical outcome assessments or investigation about the mechanism in which DKK1 and CKAP4 proteins are overexpressed in human cancers, are needed to gain a better understanding of how DKK1-CKAP4 signaling is regulated in human cancers and how LRP5/6 are involved in it.

**MATERIALS AND METHODS**

**Sucrose density gradient ultracentrifugation for the preparation of DRM fractions**

Preparation of the DRM fractions was performed as previously described (6, 12). S2-CP8 or X293T cells grown in 100-mm-diameter dishes were lysed in 850 μl of ice-cold buffer A [25 mM Hepes-NaOH (pH 7.4), 150 mM NaCl, and 5 mM EDTA] containing 0.2% Triton X-100 for S2-CP8 cells or containing 0.4% Triton X-100 for X293T cells supplemented with protease inhibitors [leupeptin (10 μg/ml), aprotinin (10 μg/ml), and phenylmethylsulfonyl fluoride (PMSF; 50 μg/ml)]. Next, cell lysates were further homogenized using a Potter-Elvehjem homogenizer (40 strokes) and subsequently passed through a 26-gauge needle. Lysates (0.625 ml) were mixed with an equal volume of 80% (w/v) sucrose in buffer A and overlaid with 2.5 ml of 35% sucrose in buffer A, followed by 1.25 ml of 5% sucrose in buffer A and 0.1% bovine serum albumin (BSA) and 100 μM EDTA. Fractions (500 μl per fraction) were harvested from the top of the gradient. Starting from the top of the gradient, fractions 2, 3, and 4 were obtained as the DRM fractions, and fractions 8, 9, and 10 as the non-DRM fractions. To isolate the plasma membrane–associated proteins, plasma membrane proteins were biotin labeled (see the method of APEGS) before cell lysis. After sucrose density gradient ultracentrifugation, each fraction was precipitated using NeutrAvidin agarose beads, and the precipitates were probed with the indicated antibodies. Endogenous flotillin-2 and clathrin indicate the positions of the DRM and non-DRM fractions, respectively.

**Click chemistry palmitoylation assay**

Click chemistry assay was performed as previously described (38). S2-CP8 cells were grown in 100-mm dishes and serum starved for 24 hours. The medium was switched to serum-free medium containing 0.1% bovine serum albumin (BSA) and 100 μM 17-ODYA, and the cells were incubated for 24 hours. The cells were lysed in 500 μl of buffer B [50 mM Heps-NaOH (pH 7.4), 150 mM NaCl, and 1% SDS] supplemented with EDTA-free protease inhibitors. Lysates were reacted with biotin-azide using the Click-IT Protein Reaction Buffer Kit according to the manufacturer's protocol. Biotinylated proteins were precipitated with NeutrAvidin agarose beads, and the precipitates were probed with the indicated antibodies.
with 60 µl of buffer B supplemented with EDTA-free protease inhibitors and subjected to click chemistry assay. Carrier proteins were added before chloroform/methanol precipitation (CM ppt).

**APEGs assay**

The APEGs assay was performed as previously described (39) with modification. Subconfluent S2-CP8 cells (in a 100-mm culture dish) were serum starved for 24 hours. The cells were washed three times with ice cold phosphate-buffered saline (PBS) containing 1 mM CaCl\(_2\) and MgCl\(_2\) and then incubated with sulfo-N-hydroxysuccinimide-biotin (0.5 mg/ml) for 30 min at 4°C. Quenching was performed by washing the cells three times with ice-cold PBS containing 50 mM NH\(_4\)Cl and then washing them once with ice-cold PBS buffer. The cells were then lysed in 1 ml of buffer C (PBS containing 5 mM EDTA and 4% SDS) supplemented with protease inhibitors. After sonication and centrifugation at 20,000g for 15 min at room temperature (RT), the soluble proteins (0.4 to 0.5 mg/ml, 1 ml) were reduced with 25 mM TCEP [tris(2-carboxyethyl)phosphine] for 1 hour at 55°C, and free cysteine residues were alkylated with 50 mM N-ethylmaleimide for 3 hours at RT. After CM ppt, the precipitates were suspended in 250 µl of buffer C supplemented with pepstatin A (10 µg/ml) and then centrifuged at 20,000g for 10 min at RT to completely remove the undissolved protein pellet. The supernatant (125 µl) was mixed with 375 µl of either buffer D [1.33 M hydroxylamine (pH 7.0), 0.2% Triton X-100, and 5 mM EDTA] or buffer E [1.33 M tris-HCl (pH 7.0), 0.2% Triton X-100, and 5 mM EDTA], and the mixtures were incubated for 1 hour at 37°C (buffer E was used as a control). After CM ppt, the precipitates were resuspended in 150 µl of buffer C supplemented with pepstatin (10 µg/ml). Soluble proteins (0.5 to 0.75 mg/ml, 100 µl) were PEGylated with 20 mM mPEG-5k in the presence of 10 mM TCEP for 1 hour so that newly exposed cysteinyten thiol groups could be labeled with mPEG-5k. After CM ppt, the precipitates were resuspended in 500 µl of buffer A containing 0.1% SDS and 1% Triton X-100 and centrifuged at 20,000g for 10 min at 4°C. To isolate plasma membrane proteins, the supernatants were precipitated using NeutrAvidin agarose beads, and the precipitates were probed with indicated antibodies. When necessary, cells were pretreated with 50 µM PalB for 48 hours at 37°C or with 50 µM 2-BP for 24 hours at 37°C indicated antibodies. When necessary, cells were pretreated with 50 µM 2-BP for 24 hours at 37°C and homogenates were immunoprecipitated with anti-FLAG M2 agarose affinity gels. Precipitates were suspended in buffer F and were used as an enzyme (on beads). After S2-CP8 cells stably expressing CKAP4-HA were labeled with 100 µM 17-ODYA for 48 hours, cell lysates were immunoprecipitated with anti-HA antibody. Precipitants were suspended in buffer F, separated in equal volumes, and supplied as substrates (on protein G beads). Substrates (50 µl) were mixed with 50 µl of enzyme sources and then incubated for indicated time periods at 37°C. Reaction was quenched by adding SDS (up to final concentration 1%), and palmitoylation levels of substrates in supernatants were evaluated in click chemistry palmitoylation assay.

**Immunoprecipitation**

To immunoprecipitate the plasma membrane–localized proteins, cells were treated with 25 µM MDC for 1 hour, and then cell surface proteins were labeled with antibodies for 30 min while rocking at 4°C in the binding buffer [Dulbecco’s modified essential medium containing 20 mM Hepes-NaOH (pH 7.4) and 0.1% BSA]. Cells were harvested, and lysates were immunoprecipitated with SureBeads Protein G Magnetic Beads (80 µl/ml; Bio-Rad, Marnes-la-Coquette, France) for 1 hour at 4°C. When necessary, cells were pretreated with 25 µM MDC for 1 hour and then treated with 4 mM dithiobis-(succinimidyl propionate), and with DSP (an amine-to-amine cross-linker) for 2 hours while rocking at 4°C in PBS buffer (pH 8.0). Beads should be washed with distilled water at the final wash if CKAP4 proteins were precipitated.

**Binding of CKAP4 to the PI3K regulatory subunit in the DRMs**

Lysates of S2-CP8 cells expressing p85α or X293T cells expressing CKAP4-HA, DKK1-FLAG, and p85α were fractionated into the DRM and non-DRM fractions, and aliquots of the non-DRM fractions were diluted with buffer A to adjust the sucrose density equal to that of the DRM fractions. Next, the DRM fractions and diluted non-DRM fractions were immunoprecipitated with anti-CKAP4 polyclonal antibody or control rabbit immunoglobulin G (IgG; 2 µg/ml) for 30 min at 4°C, and the precipitates were further incubated with SureBeads Protein G Magnetic Beads (80 µl/ml; Bio-Rad, Marnes-la-Coquette, France) for 30 min at 4°C.

To examine the binding of CKAP4-HA or CKAP4-C100S-HA to p85α, S2-CP8/CKAP4 KO cells stably expressing CKAP4-HA or CKAP4-C100S-HA and overexpressing p85α (confluent in three 100-mm diameter dishes) were lysed in 1.5 ml of buffer G [25 mM tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 60 mM n-octyl-β-glucoside] containing protease inhibitors [leupeptin (10 µg/ml), aprotinin (10 µg/ml), and 1 mM PMSF]. The lysates were then incubated with 25 µl of anti-HA-tag mAb-Magnetic Agarose (Medical and Biological Laboratories, Nagoya, Japan) for 1 hour at 4°C.

**Protein kinase assay**

X293T cells or X293T cells transfected with pCGN/HA-AKT wild type or pCGN/HA-AKT\(^{K179M}\) were lysed in buffer H [20 mM tris-HCl (pH 7.5), 150 mM NaCl, and 1% Triton X-100] containing 1 mM EDTA, 1 mM EGTA, protease inhibitors, and phosphatase inhibitors.
Lysates (500 μg of protein) were immunoprecipitated with the anti-HA antibody, and the precipitates were washed four times with buffer H to be used as an enzyme. The HA-AKT precipitates were incubated with glutathione S-transferase (GST)–APT1, GST-APT2 (5 μg of protein), or GST-GSK3β (2 μg of protein) in 30 μl of kinase reaction mixture [20 mM tris–HCl (pH 7.5), 10 mM MgCl$_2$, 1 mM dithiothreitol, 20 μM [γ-32P] adenosine triphosphate (1000 to 2000 cpm/pmol)] for 15 min at 30°C. GST-GSK3β was used as a positive control for the substrate of AKT.

**Clinical data analyses using open sources**

The data of DKK1, CKAP4, LRPS, and LRPS6 mRNA expression in 33 types of human cancers were obtained from the Gene Expression Profiling Interactive Analysis (GEPIA) online database (http://geopia.cancer-pku.cn/). Tumors and normal samples in the GEPIA database were derived from The Cancer Genome Atlas and Genotype-Tissue Expression projects. Differential analysis was performed using one-way analysis of variance (ANOVA). P values below 0.05 were considered statistically significant.

**Others**

The lists of antibodies, materials and chemicals, plasmids, and cell lines used in this study are described in tables S2 to S5. Generation of KO cells by the CRISPR-Cas9 system was performed as previously described (32, 49) with modification. The target sequences for human CKAP4 (5′-gggtgagccaccccttgcgca-3′) and human DKK1 (5′-ttaccgg-ggcttgcggctgag-3′) were designed with the help of CRISPR Genome Engineering Resources (http://crispr.mit.edu/) (50). To generate S2-CP8, A549, X293T, and Capan 1 cells stably expressing proteins, parental cells (5 × 10⁴ cells per well in a 12-well plate) were transduced with lentivirus and selected with Blasticidin S.

For the assays for endocytosis, ABE assay, 3D Matrigel culture, and xenograft tumor formation were performed as previously described (5, 13, 51–54). Cell proliferation assays were performed using a CyQUANT NF Cell Proliferation Assay Kit according to the manufacturer’s instruction. The relative fluorescence intensities of counted cells at each day were expressed as arbitrary units when compared with the values for control cells at the final day. When necessary, IWP-2 was added into culture medium. Knockdown of protein expression by siRNA and quantitative polymerase chain reaction (PCR) was performed as previously described (5, 13, 52, 53). The lists of target sequences for siRNA and primer sequences for quantitative PCR are described in tables S6 and S7, respectively. Quantification and analysis of band intensities of Western blot were performed by ImageJ software. In Western blotting, clathrin, HSP90, β-actin, β-tubulin, and glycogen synthase kinase–3β were used as loading controls.

**Study approval**

The protocol for obtaining human specimens was approved by the Ethical Review Board of the Graduate School of Medicine, Osaka University, Japan (no. 13455). All protocols used for animal experiments in this study were approved by the Animal Research Committee of Osaka University, Japan (no. 21-048-1).

**Statistics**

All experiments were repeated at least three times, and results are expressed as means ± SD. Statistical analyses were performed using the JMP software (SAS Institute, Inc., Cary, NC). The mean values of continuous outcome variables were tested with Student’s t test. P values less than 0.05 were considered statistically significant. For Fig. 6, Wilcoxon rank sum test was used. Western blotting data are representative of results obtained from at least three independent experiments, and the results are shown as means ± SD of three independent experiments.

**SUPPLEMENTARY MATERIALS**

ske.scienmag.org/cgi/content/full/12/608/eaat9519/DC1

Fig. S1. DKK1 induces endocytosis of CKAP4.

Fig. S2. Palmitoylation of CKAP4 at Cys⁵ is not essential for ER exit or binding to DKK1.

Fig. S3. ZDHHC5 palmitoylates CKAP4 in the cell surface.

Fig. S4. Knockdown of APTs or inhibition of PI3K-AKT signaling inhibits DKK1-induced depalmitoylation of CKAP4.

Fig. S5. CKAP4 interacts with PI3K in the detergent-resistant fractions of the plasma membrane.

Fig. S6. Depalmitoylation of LRPS6 does not affect DKK1-dependent inhibition of Wnt–β-catenin signaling.

Fig. S7. CKAP4 is not involved in the DKK1-induced inhibition of Wnt–β-catenin signaling.

Fig. S8. Wnt–β-catenin signaling is not activated in Capan-1 cells.

Fig. S9. DKK1, CKAP4, and LRP6 are highly expressed in human pancreatic cancer tissues.

Fig. S10. Schematic model of DKK1-CKAP4 signaling.

Table S1. Expression of CKAP4, DKK1, LRPS, and LRPS6 in tumor and normal samples from public datasets.

Table S2. List of antibodies used in this study.

Table S3. List of materials and chemicals used in this study.

Table S4. List of plasmids used in this study.

Table S5. List of primers used in this study.

Table S6. List of target sequences for siRNAs.

Table S7. List of primer sequences for quantitative PCR.

View/request a protocol for this paper from Bio-protocol.

**REFERENCES AND NOTES**


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Dynamic palmitoylation controls the microdomain localization of the DKK1 receptors CKAP4 and LRP6

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**DKK1 promotes receptor depalmitoylation**

Dickkopf1 (DKK1) inhibits Wnt signaling by promoting internalization of the Wnt coreceptor LRP6. DKK1 also binds to the cell surface receptor CKAP4 and stimulates signaling through the kinases PI3K and AKT, which promotes cell proliferation. Using cultured cancer cell lines, Sada et al. found that CKAP4 and LRP6 were palmitoylated and localized to lipid rafts. Palmitoylation of CKAP4 was required for DKK1-stimulated cell proliferation. DKK1 stimulated depalmitoylation of both receptors, causing them to move out of lipid rafts in a PI3K- and AKT-dependent manner, and removal of CKAP4 from lipid rafts desensitized cells to DKK1. DKK1, CKAP4, and LRP6 formed a ternary complex, and depalmitoylation of both receptors, causing them to move out of lipid rafts in a PI3K- and AKT-dependent manner, localized to lipid rafts. Palmitoylation of CKAP4 was required for DKK1-stimulated cell proliferation. DKK1, CKAP4, and LRP6 formed a ternary complex, and LRP6 enhanced PI3K-AKT activation downstream of DKK1 and CKAP4. Thus, dynamic palmitoylation of DKK1 receptors plays an important role in both mediating the response to DKK1 and in controlling the sensitivity of cells to DKK1.