The A-Kinase Anchoring Protein (AKAP) Glycogen Synthase Kinase 3β Interaction Protein (GSKIP) Regulates β-Catenin through Its Interactions with Both Protein Kinase A (PKA) and GSK3β*

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The A-kinase anchoring protein (AKAP) GSK3β interaction protein (GSKIP) is a cytosolic scaffolding protein binding protein kinase A (PKA) and glycogen synthase kinase 3β (GSK3β). Here we show that both the AKAP function of GSKIP, i.e., its direct interaction with PKA, and its direct interaction with GSK3β are required for the regulation of β-catenin and thus Wnt signaling. A cytoplasmic destruction complex targets β-catenin for degradation and thus prevents Wnt signaling. Wnt signaling causes β-catenin accumulation and translocation into the nucleus, where it induces Wnt target gene expression. GSKIP facilitates control of the β-catenin stabilizing phosphorylation at Ser-675 by PKA. Its interaction with GSK3β facilitates control of the destabilizing phosphorylation of β-catenin at Ser-33/Ser-37/Thr-41. The influence of GSKIP on β-catenin is explained by its scavenger function; it recruits the kinases away from the destruction complex without forming a complex with β-catenin. The regulation of β-catenin by GSKIP is specific for this AKAP as AKAP220, which also binds PKA and GSK3β, did not affect Wnt signaling. We find that the binding domain of AKAP220 for GSK3β is a conserved GSK3β interaction domain (GID), which is also present in GSKIP. Our findings highlight an essential compartmentalization of both PKA and GSK3β by GSKIP, and ascribe a function to a cytosolic AKAP-PKA interaction as a regulatory factor in the control of canonical Wnt signaling. Wnt signaling controls different biological processes, including embryonic development, cell cycle progression, glycogen metabolism, and immune regulation; deregulation is associated with diseases such as cancer, type 2 diabetes, inflammatory, and Alzheimer’s and Parkinson’s diseases.

A-kinase anchoring proteins (AKAPs) are a family of about 50 scaffolding proteins. Their conserved function is the compartmentalization of protein kinase A (PKA). PKA holoenzyme consists of a dimer of regulatory (RIα, RIβ, RIλ, or RIβ) and two catalytic subunits each bound to one R subunit. AKAPs directly interact with R subunits and tether the kinase to defined cellular compartments such as vesicles, the sarcoplasmic reticulum, or the cytoskeleton. This compartmentalization confers a tight spatiotemporal control to PKA signaling, and enables PKA to elicit a specific cellular response to each of the many stimuli that cause cAMP elevation and thereby lead to activation of this ubiquitous kinase. AKAPs directly interact with further signaling proteins, thus mediating crosstalk between signaling systems: phosphatases, dephosphorylating PKA-phosphorylated substrates, adenylyl cyclases, synthesizing cAMP, and phosphodiesterases (PDEs), hydrolyzing cAMP. Several AKAPs bind further kinases such as protein kinase C (PKC), which are activated by signals other than cAMP, e.g., Ca2+. AKAPs and their interactions play key roles in a variety of physiological processes such as vasopressin-mediated water reabsorption in renal principal cells and cardiac myocyte contractility (1–5, 7, 8).

A new example of an AKAP that mediates crosstalk is the cytoplasmic GSK3β interaction protein (GSKIP). It binds PKA and GSK3β (9). The ubiquitously expressed serine/threonine protein kinase GSK3β is a component of multiple signaling systems such as canonical Wnt, insulin, Hedgehog, Notch, and TGFβ signaling. GSK3β is constitutively active. It can be inactivated by phosphorylation of Ser-9 by multiple kinases including protein PKA (10), PKB (11), and p38 MAPK (12). GSK3β is involved in the regulation of different biological processes, e.g., embryonic development, cell cycle progression, glycogen metabolism, and immune regulation. Deregulation of GSK3β is associated with pathologies such as cancer, type 2 diabetes, bipolar disorder, cardiac hypertrophy inflammatory, and Alzheimer’s and Parkinson’s diseases (13–16). The diversity of GSKβ functions is also reflected by its presence in different cellular compartments; GSK3β is located in the cytosol, at the plasma membrane, in the nucleus, and in mitochondria (17, 18).

In canonical Wnt signaling, GSK3β assembles with Axin, β-catenin, adenomatous polyposis coli (APC), and casein of variance; 8-AHA-cAMP, 8-(6-aminohexyl)aminoadenosine-3’,5’-cyclic monophosphate; IP, immunoprecipitation.
kinase 1 (CK1) in the destruction complex in the cytosol. In the absence of a Wnt signal, GSK3β phosphorylates β-catenin at Thr-41, Ser-33 and Ser-37 (19–23). This targets β-catenin for ubiquitination and proteasomal degradation and inhibits Wnt signaling (24). Wnt signaling is activated by binding of Wnt ligands to receptor complexes at the plasma membrane, consisting of LRPs/FRZ single-pass transmembrane protein and G protein-like receptors of the Frizzled (Fz) family. The β-catenin destruction complex is recruited to the LRPs/FRZ receptor, accompanied by an inhibition of degradation of β-catenin. Then β-catenin accumulates and translocates into the nucleus where it induces expression of Wnt target genes (25, 26).

GSKIP seems able to induce β-catenin accumulation in the cytoplasm and the nucleus and to activate transcription through direct interaction with GSK3β when overexpressed in HeLa and neuroblastoma SH-SY5Y cells (27, 28). In SH-SY5Y cells, the GSKIP overexpression blocked neurite outgrowth during retinoic acid-mediated differentiation. Although the data were obtained in cell systems overexpressing GSKIP, they suggested that GSKIP acts as a regulator in canonical Wnt signaling.

We observed that GSKIP facilitates the phosphorylation of GSK3β by PKA at Ser-9 and thereby its inhibition (9), and that GSKIP deficiency modulates this phosphorylation and thus GSK3β activity during development (29). The loss of GSKIP in mice causes a cleft palate, resembling the one in Gsk3β−/− mice (30), and perinatal lethality through respiratory distress (29). Knock-out of any of the subunits of PKA does not cause a cleft palate. Thus GSKIP and GSK3β are crucial for normal craniofacial development. This most likely does not involve Wnt signaling, as Wnt signals apparently do not influence phosphorylation of Ser-9 of GSK3β (31).

Here we show that GSKIP affects specific phosphorylations of β-catenin and thus Wnt signaling through its direct interactions with PKA and GSK3β. To our knowledge, this is the first time that a physiological function can be ascribed to a cytosolic AKAP-PKA interaction.

Results

Endogenous GSKIP Regulates Wnt Signaling through Phosphorylation of β-Catenin by PKA—The scaffolding protein assembling the destruction complex is Axin. Axin exists in two isoforms, Axin1 and Axin2; Axin1 is constitutively expressed, and Axin2 expression is regulated through Wnt signaling (32, 33). GSK3β phosphorylates Axin-bound β-catenin within the destruction complex at Ser-33/Ser-37/Thr-41, targeting it for proteasomal degradation. We initially examined whether endogenous GSKIP influences the composition of the destruction complex. We knocked down GSKIP in HEK293 cells using siRNA and immunoprecipitated endogenous GSK3β (Fig. 1). As expected, GSK3β co-immunoprecipitated with Axin1 in the presence of a control non-targeting siRNA (siNT). The siRNA-mediated reduction of GSKIP increased the co-immunoprecipitation of GSK3β and Axin1, indicating that GSKIP affects the composition of the complex by recruiting GSK3β.

Next, we performed siRNA experiments to assess the role of endogenous GSKIP in coordinating PKA in Wnt signaling. PKA phosphorylates β-catenin at Ser-675 and thereby stabilizes the protein (34, 35). Cytosols from HEK293, A549, and HeLa cells were analyzed by Western blotting. In the absence of a Wnt signal, the level of β-catenin, unphosphorylated or phosphorylated at Ser-675, was low (Fig. 2, A–C). Wnt3a stimulation increased β-catenin expression, which was enhanced by the knockdown of GSKIP. Thus the reduction of the scaffold GSKIP apparently sets free the kinase, which then phosphorylates its substrate. Inhibition of PKA with the stearate-coupled and thus membrane-permeant specific heat-stable PKA inhibitor peptide (PKI) or H89, either alone or in combination with the GSKIP siRNA, inhibited the increase in Ser-675 phosphorylation.

To measure Wnt signaling activity, we performed Wnt reporter assays using the TOPflash/FOPflash reporter system (36). HEK293 cells were transfected with siRNAs together with the reporter vectors and a vector encoding Renilla luciferase, used as an internal standard. Luciferase activity was measured 48 h later. The measurements showed that the knockdown of GSKIP and the associated increase in Ser-675 phosphorylation caused an increase in luciferase activity, indicative of an increase in β-catenin-mediated transcription (Fig. 2D).

Binding of Wnt to its receptor causes phosphorylation of LRPs at Ser-1490 by GSK3β, and other kinases. This phosphorylation was not affected by the knockdown of GSKIP (Fig. 2E). The knockdown also did not result in a significant change of the level of Ser(P)-9 GSK3β, indicating that Ser-9 is not involved in control of GSK3β over Wnt signign if GSKIP is reduced (Fig. 2E). In addition, GSKIP knockdown did not alter the phosphorylation of β-catenin at Ser-33/Ser-37/Thr-41 in the presence of the proteasome inhibitor, MG-132 (Fig. 2F). MG-132 was used to obtain signals in the Western blot as the phosphorylation marks β-catenin for proteasomal degradation. The actual enrichment of cytosolic proteins in the cytosolic fraction used for the experiments such as those depicted in Fig. 2, A–C, was confirmed by Western blotting (Fig. 2G).

Direct Interactions of GSKIP with Both PKA and GSK3β Are Required for Control of Wnt Signaling under Resting Conditions—To confirm the requirement of the direct interaction between GSKIP and PKA for the control of β-catenin, we

![FIGURE 1. GSKIP controls β-catenin expression](image)
GSKIP Controls β-Catenin
generated a PKA-binding-deficient (GSKIP-N42I) variant of GSKIP, and as a control, the previously described GSK3β-binding-deficient GSKIP variant, GSKIP-L130P (27, 37). We initially analyzed the interaction of the GSKIP variants with PKA by cAMP-agarose precipitation. cAMP-agarose precipitates regulatory subunits of PKA and the associated AKAPs. When compared with the wild type and GSKIP-L130P, little GSKIP-N42I was detectable in the precipitates, confirming its inability to interact with PKA (Fig. 3A). To analyze binding of the GSKIP variants to endogenous GSK3β, tagged proteins were expressed in HEK293 cells. Endogenous GSK3β was immunoprecipitated, and Western blotting was used to detect GSK3β or the variants (Fig. 3B). Wild type GSKIP and GSKIP-N42I co-precipitated with GSK3β, whereas GSKIP-L130P did not. Similar experiments where GSKIP was immunoprecipitated via its FLAG tag were also performed (Fig. 3C).

Only the wild type GSKIP triggered a significant increase in basal β-catenin-induced transcription, whereas no changes were observed in the presence of the PKA-binding-deficient or GSK3β-binding-deficient variants of GSKIP (Fig. 3D). These findings show that the interactions of GSKIP with both PKA and GSK3β are required for β-catenin-dependent transcription under resting conditions.

Interactions of GSKIP with Both PKA and GSK3β Are Required for Wnt-stimulated Transcriptional Activation through β-Catenin—HEK293 cells were transfected with GSKIP, GSKIP-L130P, or GSKIP-N42I together with TOPflash/FOPflash. Stimulation for 24 h with Wnt3a-conditioned medium increased Wnt signaling in the presence of wild type GSKIP (Fig. 4A). Ablation of binding to either PKA or GSK3β prevented the Wnt-induced transcription. We further examined whether the PKA-binding-deficient GSKIP-N42I would affect the PKA phosphorylation of β-catenin (Fig. 4, B and C). Surprisingly, and in contrast to the increase in Ser-675 phosphorylation if GSKIP was down-regulated, the loss of PKA binding to GSKIP did not alter the phosphorylation of Ser-675 in the presence of Wnt. We also investigated the influence of GSKIP on Wnt signaling following stimulation with recombinant Wnt3a in a concentration-dependent manner for 24 h. Wnt3a and GSKIP expression activated Wnt signaling additionally (Fig. 4D).

We inhibited the proteasomal degradation of proteins with MG-132 using HEK293 cells that expressed the GSKIP variants. MG-132 increased the levels of β-catenin phosphorylated at Ser-33/Ser-37/Thr-41 (19, 38) and of β-catenin (Fig. 5A). However, only in the presence of wild type GSKIP, Ser(P)-33/Ser-37/Thr-41 β-catenin was reduced significantly and cytosolic β-catenin increased (Fig. 5, A and B).

Thus collectively, it appears that GSKIP must interact with both kinases to regulate Wnt-dependent transcription through β-catenin. It involves phosphorylation of Ser-33/Ser-37/Thr-41 and Ser-675 of β-catenin by GSK3β and PKA, respectively. When GSKIP expression is knocked down, i.e. probing the endogenous role of GSKIP, the mode of action is independent from the previously observed Ser-9 phosphorylation of GSK3β by PKA, which GSKIP facilitates (9). GSKIP and β-catenin did not co-immunoprecipitate, indicating that stable complex formation of the two proteins is not required for the observed GSKIP-mediated control of β-catenin phosphorylation (Fig. 5C).

GSKIP-dependent Regulation of β-Catenin Is Specific for this AKAP—Besides GSKIP, two other AKAPs, MAP2 and AKAP220 (AKAP11), bind both PKA and GSK3β (9, 39–41). AKAP220 and GSKIP both facilitate phosphorylation of GSK3β at Ser-9 by PKA and thus inhibition of GSK3β (9, 39). The binding sites for PKA are conserved within the AKAP family. The site in AKAP220 was previously mapped (41). We examined whether AKAP220 binds GSK3β in a similar way as other GSK3β-interacting proteins, namely by a GSK3β interaction domain (GID). AKAP220 binds GSK3β directly in a region that spans amino acid residues 1017–1386 (39). We performed protein sequence alignment of mammalian Axin1/2, GSKIP, and AKAP220, which suggested a putative GID in this region, because they show strong conservation among the homologs and contain central FLL motifs that are identical at the critical positions (Fig. 6A). In addition, the motif was predicted to form an α-helix, as is true of other GIDs (42). We spot-synthesized this region of AKAP220 as 25-mer overlapping peptides and investigated interactions of the peptides with recombinant GST-GSK3β that had been pre-exposed to peptides representing the GSK3β-binding domain of GSKIP (GSKIPtide) or with the GSK3β-binding-deficient variant GSTPtide-L130P (9, 27) (Fig. 6B). Remarkably, binding of GSKIP to spots A8, A9, and A10 of human AKAP220 was observed in the presence of GSTPtide-L130P, but binding was strongly reduced in the presence of GSKIPtide. We performed alanine scans to identify single amino acid residues of the AKAP220 GID that contribute to GSK3β binding (Fig. 6C). Substitutions revealed that Phe-1162, Leu-1166, and Leu-1170 were each essential for binding. This shows that as in Axin1/2 and GSKIP, an FLL motif within the GID of AKAP220 is required for the interaction with GSK3β.

GSKIP, Axin, and AKAP220 Compete for GSK3β Binding—Differently tagged GSK3β, AKAP220, Axin1, GSKIP, and combinations thereof were produced in HEK293 cells, followed by co-immunoprecipitation and Western blotting. GSK3β precipitated in the cytosolic fractions of HEK293 cells that were used in A were confirmed by Western blotting. The cells were treated with Wnt3a-enriched or control medium. Shown are representative blots from n = 4 experiments.

FIGURE 2. Endogenous GSKIP enhances Wnt signaling through controlling PKA phosphorylation of β-catenin at Ser-675. HEK293, A549, and HeLa cells were left untreated or treated as indicated with siRNA to knock down GSKIP (siGSKIP), control siRNA (siNT), Wnt3a-enriched medium, or control medium in the absence or presence of the PKA inhibitors, PKA inhibitor peptide (PKI) or H89, or the proteosome inhibitor MG-132. A–C, cytosolic fractions were purified from the three cell types by and analyzed by Western blotting (WB) for expression of the indicated proteins. The signals were densitometrically analyzed, and ratios of β-catenin/GAPDH and Ser(p)675-β-catenin/GAPDH were calculated; n = 6, mean ± S.E., ANOVA, **p < 0.01, ***p < 0.001. D, TOPflash/FOPflash (TOP/FOP) luciferase assay. HEK293 cells were stimulated with Wnt-enriched or control medium; n = 4, mean ± S.E., ANOVA, **p < 0.01. E, cell lysates were purified from HEK293 cells, and the indicated proteins were detected by Western blotting. Semi-quantitative densitometric analysis of Western blots is depicted as ratios of Ser(P)1490LRP6/GAPDH and Ser(P)19 GSK3β/GAPDH; n = 6, mean ± S.E., ANOVA, *p < 0.05, **p < 0.001, F, cytosolic fractions were purified, and the indicated proteins were detected by Western blotting. Semi-quantitative densitometric analysis of Western blots shows the ratios of β-catenin/GAPDH and (Ser(P)133/ Ser[37/Thr-41])β-catenin/GAPDH; n = 6, mean ± S.E., ANOVA, ***p < 0.001, p533/537/T41, Ser(P)133/Ser[37/Thr-41]. G, the enrichment of the indicated proteins in the cytosolic fractions of HEK293 cells that were used in A was confirmed by Western blotting. The cells were treated with Wnt3a-enriched or control medium. Shown are representative blots from n = 4 experiments.
GSKIP Controls β-Catenin

**FIGURE 3. Elevated levels of GSKIP cause up-regulation of Wnt signaling under resting conditions through interactions with both PKA and GSK3β.**

A. HEK293 cells transiently expressing an empty control vector or the indicated GSKIP variants were subjected to cAMP-agarose precipitations. GSKIP, regulatory RIIα subunits of PKA, and HSP90 (as a control) were detected by Western blotting (WB). Semi-quantitative densitometric analysis of results is shown as the ratio of the GSKIP pulldown (PD) to GSKIP in the input. The interaction of regulatory RIIα subunits of PKA with GSKIP-N42I is strongly reduced, but not that with the GSK3β-binding-deficient mutant CFP-GSKIP-L130P; n = 5, mean ± S.E., ANOVA, ***, p < 0.01.

B and C, endogenous GSK3β (B) or FLAG-tagged GSKIP versions (C) were immunoprecipitated (IP; IgG, negative control immunoprecipitation). Precipitated proteins were detected by Western blotting, and signals were analyzed by semi-quantitative densitometry. Depicted are the ratios of the GSKIP IP to the GSK3β IP (B) and of the GSK3β IP to the GSKIP IP (C); n = 3, mean ± S.E., ANOVA, *, p < 0.05, ***, p < 0.01.

D, TOPflash/FOPflash (TOP/FOP) luciferase assays using HEK293 cells expressing the indicated proteins in the absence of a Wnt signal (Wnt; n = 4, mean ± S.E., ANOVA, *, p < 0.05, **, p < 0.01.

The level of AKAP220 was reduced upon co-expression of Axin1, and GSKIP co-expression strongly diminished the AKAP220 level. Co-immunoprecipitated AKAP220 was then calculated as the ratio to the input of the competitors, which was reduced when cells co-expressed Axin1 and even more so with GSKIP (Fig. 7A). We then examined whether GSKIP competes with Axin1/2 for GSK3β binding by co-immunoprecipitation and Western blotting, as GSKIP and Axin1/2 share a common motif for binding GSK3β (Fig. 7B) (27). In cells expressing Axin1 or Axin2, the amount of GSKIP bound to GSK3β was significantly reduced (Fig. 7B). These results indicate that AKAP220, GSKIP, and Axin1 compete for the binding of GSK3β. As these proteins may assemble with GSK3β in different cellular compartments, Axin, AKAP220, and GSKIP might establish functionally different pools of GSK3β.
**GSKIP and AKAP220 Play Different Roles in Wnt Signaling**

We compared the influence of GSKIP and AKAP220 on β-catenin-dependent transcription. Fig. 3D shows that only GSKIP activated the luciferase reporter system in HEK293 cells under resting conditions. In HEK293 cells stimulated for 4 h with Wnt, only GSKIP enhanced the reporter system but not AKAP220 (Fig. 4A). The cytosolic fractions of untreated HEK293 cells and cells treated with Wnt3a were enriched, and β-catenin levels were monitored by Western blotting (Fig. 8). These levels were strongly increased in the Wnt-stimulated samples. The effect of Wnt on β-catenin levels significantly increased in the presence of GSKIP, but not in the presence of AKAP220. As in the presence of elevated levels of GSKIP (Fig. 4), the elevation of AKAP220 levels did not affect the phosphorylation of Ser-675 of β-catenin (Fig. 8). Axin1 up-regulation was observed upon expression of wild type GSKIP in unstimulated cells but not in the presence of AKAP220. This was not seen upon Wnt stimulation. Thus GSKIP and AKAP220 exert different effects on β-catenin, although both bind GSK3β and PKA. This suggests that they control different pools of GSK3β and PKA that influence the Wnt signaling destruction complex in different ways.

### FIGURE 4

**The interaction of GSKIP with both GSK3β and PKA is required for enhancing Wnt signaling in the presence of a Wnt stimulus.**

A, HEK293 cells expressing the indicated proteins were subjected to TOPflash/FOPflash (TOP/FOP) luciferase assays to detect Wnt signaling activity in the presence of Wnt-conditioned medium. n = 4, mean ± S.E., ANOVA, *, p < 0.05. B, cytosolic fractions were purified, and the indicated proteins were detected by Western blotting. C, the signals were semi-quantitatively analyzed by densitometry; n = 5, mean ± S.E., ANOVA, *, p < 0.05, **, p < 0.01, ***, p < 0.001. D, HEK293 cells expressing an empty vector control or wild type CFP-GSKIP were stimulated with increasing amounts of recombinant Wnt3a, and TOPflash/FOPflash luciferase assays were performed to determine Wnt signaling activity; n = 4, mean ± S.E., ANOVA, ***, p < 0.001.
**Discussion**

Our findings demonstrate that GSKIP regulates Wnt signaling through its direct protein-protein interactions with both GSK3β and PKA. The two GSKIP-bound kinases differentially modulate the phosphorylation and thereby the stability of β-catenin (Fig. 9). GSKIP apparently has a “scavenger” activity for GSK3β and PKA that is independent of a direct interaction of GSKIP with the destruction complex. Instead, GSKIP establishes an independent GSK3β/PKA pool. We observed a role of GSKIP in Wnt signaling in three different cell lines. The ubiqui-
FIGURE 6. GSK3β binds AKAP220 at a conserved GID. A, alignment of the GIDs from the indicated human (Hs), rat (Rn), and mouse (Mm) proteins, and prediction of AKAP220 three-dimensional structure in the 1162–1176 region through secondary structure analysis using PSIPRED at the University College London (UCL) Department Of Computer Science. aa, amino acid. B, amino acid residues 1017–1386 of AKAP220 were spot-synthesized as 25-mer overlapping peptides and overlaid with GST alone or with GST-GSK3β that was pre-incubated with either the inactive control peptide GSKIPtide-L130P or the peptide GSKIPtide, which blocks the interaction of GSK3β with spot-synthesized peptides. Interaction of GSK3β with peptides was detected using anti-GSK3β antibodies. The sequences of the GSK3β-binding peptides in positions A8–A10 of the peptide array are shown. The sequence common in all three peptides is highlighted. C, the GID of AKAP220, amino acids 1155–1179, was spot-synthesized as 25-mer peptides (wild type (wt)) or as peptides where each amino acid was substituted by an alanine. The peptides were overlaid with recombinant GSK3β, and interaction was detected using anti-GSK3β antibodies.
co-immunoprecipitation of YFP-GSK3 ubiquitously expressed GSKIP (9), and the interaction presumably solicits AKAP-PKA interaction. Based on the conservation and time, our data ascribe a physiological function to a strictly cytoplasmic elevation of cAMP (9), or Wnt stimulation. Thus for the first time, its location is not altered, for example, by retinoic acid (28), an effect that requires a competition of LRP5/6 by PKA (49), and PKA-dependent phosphorylation of osteoblast differentiation, a process that involves phosphorylation of LRP5/6 by PKA (49), and PKA-dependent phosphorylation and thus inhibition of GSK3β (50). Whether such processes require an interaction between PKA and AKAPs, and specifically with GSKIP, remains to be determined.

We asked whether the effects observed for GSKIP are specific for this one AKAP, or whether an effect on Wnt signaling can also be achieved through a simple scaffolding of PKA and GSK3β by another AKAP such as AKAP220. GSKIP and AKAP220 differ in their cellular location: GSKIP is cytoplasmic (9, 27), whereas AKAP220 is predominantly found at the plasma membrane and on peroxisomes (41, 51–53). GSKIP is cytosolic (Fig. 2G) (9), and there is no evidence of a stimulus-dependent redistribution to another compartment. Its location is not altered, for example, by retinoic acid (28), an elevation of cAMP (9), or Wnt stimulation. Thus for the first time, our data ascribe a physiological function to a strictly cytosolic AKAP-PKA interaction. Based on the conservation and ubiquitous expression of GSKIP (9), the interaction presumably plays a role in Wnt signaling in many types of cells. Because GSKIP is an RII-specific AKAP (9), Wnt signaling must be controlled by PKA type II, the PKA that contains RII subunits. PKA is involved in the control of Wnt signaling in various processes. For example, parathyroid hormone stimulation promotes osteoblast differentiation, a process that involves phosphorylation of LRP5/6 by PKA (49), and PKA-dependent phosphorylation and thus inhibition of GSK3β (50). Whether such processes require an interaction between PKA and AKAPs, and specifically with GSKIP, remains to be determined.

We asked whether the effects observed for GSKIP are specific for this one AKAP, or whether an effect on Wnt signaling can also be achieved through a simple scaffolding of PKA and GSK3β by another AKAP such as AKAP220. GSKIP and AKAP220 differ in their cellular location: GSKIP is cytoplasmic (9, 27), whereas AKAP220 is predominantly found at the plasma membrane and on peroxisomes (41, 51–53). GSKIP does not interact with β-catenin, whereas AKAP220 forms a complex with β-catenin and cadherin at the plasma membrane, which competes for β-catenin in Wnt signaling (51, 54). However, a direct competition would affect Wnt signaling activity and cytosolic β-catenin levels. This effect does not seem to explain our observations and emphasizes the neutrality of AKAP220 in Wnt signaling. This is also in line with observations that the role of the AKAP220-cadherin complex in endo-

FIGURE 7. GSKIP, Axin1, Axin2, and AKAP220 compete for GSK3β binding. A, immunoprecipitation of YFP-GSK3β from HEK293 cells and detection of the indicated proteins by Western blotting (WB). The semi-quantitative analysis of co-immunoprecipitated HA-AKAP220 is depicted as the ratio of precipitated HA-AKAP220 and precipitated GSK3β; n = 6, mean ± S.E., ANOVA, *p < 0.05. B, co-immunoprecipitation of YFP-GSK3β and the indicated proteins from HEK293 cells. The proteins were detected by Western blotting. Semi-quantitative densitometric analysis of the signals is depicted as the ratio of precipitated GSK3β to precipitated GSK3β; n = 5, mean ± S.E., ANOVA, **p < 0.01.
Some effects due its broad involvement in several of these pathways. Targeting AKAP-dependent protein-protein interactions whose hydrophobic face docks into a hydrophobic groove on the surface of GSK3β. Like Axin 1/2 and GSKIP, the GID of human AKAP220 contains an FLL motif and thus is likely to interact with GSK3β in a similar way. The presence of the GID domain explains the competition between Axin1/2, AKAP220, and GSKIP for binding to GSK3β and provides further evidence that the scaffolding proteins define distinct pools of this kinase. An additional motif was recently discovered in a nearby region in AKAP220, identifying Thr-1132, which can be phosphorylated by GSK3β itself to permit binding (41). However, a previous study demonstrated binding between kinase-dead GSK3β and AKAP220 (39), which suggests the existence of further points of contact between AKAP220 and GSK3β (41), likely the FLL motif we identified.

By adding a new level to our understanding of Wnt signaling control, our findings may have important clinical implications. Increased Wnt signaling plays a role in various cancers, neurological diseases, fibrosis, and other diseases (55, 56). A duplication of the chromosomal region encoding GSKIP predisposes to myeloid lymphoma, presumably due to increased GSKIP protein, inhibition of GSK3β, and increased Wnt signaling (57). A general challenge in targeting the Wnt signaling pathway for therapeutic purposes is its complex crosstalk with MAPK, bone morphogenetic protein (BMP), Hedgehog, Notch, cAMP/PKA, and other pathways (50, 56, 58, 59). Targeting GSK3β itself is equally challenging as it would likely cause side effects due to its broad involvement in several of these pathways. An option might be to displace a specific pool of GSK3β from a cellular compartment where it has specific, undesirable effects on Wnt signaling. GSKIP, AKAP220, and Axin establish different pools of GSK3β, but GSK3β interacts with further proteins including MAP2, FRAT1, -2, and -3, and DISC1. Our work opens a door on understanding the interplay of these GSK3β interactions with regard to Wnt signaling. The interaction of GSKIP with GSK3β in the cytosol increases Wnt signaling. It would be worthwhile to evaluate the therapeutic potential of pharmacological interference with this interaction, because GSKIP is up-regulated in breast cancer, melanoma, and other forms of cancer that also exhibit a deregulation of Wnt signaling. Targeting AKAP-dependent protein-protein interactions is feasible and may be a novel approach in the treatment of various diseases (7, 45, 60).
Experimental Procedures

**GSKIP Variants**—Mutagenesis was carried out using a pECFP-GSKIP plasmid (9) with the QuickChange II Site-Directed Mutagenesis Kit (Agilent) according to the manufacturer’s recommendation. To prevent the interaction of GSKIP with PKA, asparagine 42 was replaced by isoleucine. To generate a GSKIP variant deficient for binding of GSK3β, leucine 130 was replaced by proline (37). The following primers were used: N42IFwd, CTC GAA GCT GAA GCA GTT GTA AAT GAT GTT CTC TTT GCT; N42IRrev, TCG TTT CTC TTG TAG TAA ATG TTG ACG AAG TCG AAG CTC; L130PFwd, CCG ACT GCT TCA AAG ACC GGA AGC TTT GAA AAG AG; and L130PRrev, CTC TTT TCA AAG CTT CCG GTC TTT GAA GCA GTG CG, thus generating the plasmids pECFP-GSKIP-N42I and pECFP-GSKIP-L130P. The cDNAs were cloned into pCMV6ev (OriGene) to generate the vectors pCMV6-GSKIP-FLAG, pCMV6-GSKIP-L130P-FLAG, and pCMV6-GSKIP-N42I-FLAG.

**Cell Culture, Transfection, and Fractionation**—HEK293, A549, and HeLa cells were grown in DMEM (GlutaMAX, 10% FCS, Thermo Fisher Scientific) and transfected with the above mentioned plasmids, the vectors pECFP, pEYFP, pEYFP-GSK3β, pΔECFP-Myc-GSKIP (CFP deleted), pCDNA3.1-FLAG-Axin1, pCDNA3.1-FLAG-Axin2, TOPflash, FOPflash, pRL-SV40, and pCGN-HA-APK220 (kindly provided by K. Taskén, The Biotechnology Centre of Oslo, University of Oslo, Norway), SMARTpool siGENOME GSKIP siRNA, and siGENOME Non-Targeting siRNA #2 (GE Healthcare, Chalfont St Giles, UK) using Lipofectamine2000 (Thermo Fisher Scientific) (9).

For cell fractionation and cytosol purification, a digitonin semi-permeabilization protocol was adapted (61). Briefly, HEK293 cells were treated with WNT-enriched or control medium for 4 h, washed twice with PBS, and semi-permeabilized with Digitonin (61). The lysis buffer containing the cytosol was cleared by centrifugation (supernatants; 5,000 × g, 5 min). The adherent, permeabilized cells were washed twice with PBS and lysed in radioimmunoprecipitation assay buffer. Purity of the cytosolic fractions was confirmed by Western blotting with antibodies directed against pan-Cadherin, LaminA/C and GAPDH.

**Immunoo- and cAMP-Agarose Precipitations, and Western Blotting**—Immunoprecipitations were carried out as described (9, 46). Cells were lysed in standard lysis buffer (10 mM K2HPO4, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 1% Triton X-100, 0.2% sodium deoxycholate, pH 7.4) containing protease inhibitors (cOmplete; Roche Applied Science, Rotkreuz, Switzerland) and phosphatase stops (PhosSTOP, Roche Applied Science). The lysates were cleared by centrifugation (20,000 × g, 4 °C, 10 min). Proteins were immunoprecipitated with anti-GFP (62, 63) or anti-GSK3β (L-17, Goat, Santa Cruz Biotechnology, Dallas, TX) antibodies, and protein A-conjugated agarose (Sigma-Aldrich) or anti-FLAG-coupled magnetic beads (Sigma-Aldrich).

As described previously (9), for cAMP-agarose precipitations, the lysates were incubated with 8-AHA-cAMP-agarose (4 °C, 3 h; BioLog, Bremen, Germany). Agarose-bound proteins were washed four times with lysis buffer and eluted with Laemmli sample buffer.

Western blotting was carried out as described (9, 46). The following antibodies were used: HSP90 mouse (AC88; Enzo Life Sciences, Lörrach, Germany); GFP mouse (IL-8; Takara Bio Inc., Shiga, Japan); GFP-01, rabbit (custom-made (62)); GSKIP, rabbit, (custom-made (9)); LaminA/C, goat (N-18; Santa Cruz Biotechnology); HA High Affinity, rat (3F10; Roche Applied Science); FLAG (M2), mouse and pan-Cadherin, mouse (Sigma-Aldrich); Axin1 (C7B12), rabbit; Myc tag (9B11), rabbit; GAPDH (H-12), rabbit; GSK3β (27C10), rabbit; phospho-GSK3β (S9), rabbit; phospho-β-catenin (S675), rabbit; β-catenin (#9562), rabbit; and phospho-β-catenin (Ser-33/Ser-37/Thr-41), rabbit (all from Cell Signaling, Cambridge, UK); and RIIα antibody (mouse, BD Biosciences). Signals were detected using EMD Millipore Immobilon Western Chemiluminescent HRP Substrate (Thermo Fisher Scientific) and an Odyssey Fc Imaging System (LI-COR Biotechnology, Lincoln, NE).

**Peptide Spot Synthesis and Overlay with Recombinant GSK3β**—25-mer peptides were spot-synthesized as described using an Intavis ResPep SL spot synthesizer (Intavis, Cologne, Germany) and incubated with a recombinant fusion protein of GSK3β and glutathione S-transferase (GST; Cell Signaling; 1 μg/ml) or GST alone (9, 43, 64, 65).

**Luciferase Assays**—HEK293 cells were transfected with plasmids encoding the GSKIP variants together with pRL-SV40 and TOPflash or FOPflash vectors and stimulated with recombinant human Wnt3a (R&D Systems, Minneapolis, MN) or Wnt3a-conditioned medium 24 h after transfection (6). The cells were lysed 24 h later, and luciferase activity was measured using the Dual-Luciferase Assay Kit (Promega, Fitchburg, WI) and a Centro XS3 LB 960 luminoimeter (Berthold Technologies, Bad Wildbad, Germany). Luciferase activity was induced by binding of β-catenin to the reporter system. Activity of the TOPflash and FOPflash vectors was normalized to activity of the pRL-SV40 vector, and normalized TOPflash activity was divided by the normalized FOPflash activity to obtain a FOPflash/TOPflash ratio. For siRNA experiments, 24 h after knockdown of GSKIP, the cells were transfected a second time with the reporter vectors. After an additional 24 h, the cells were stimulated for a further 24 h with Wnt3a-conditioned medium.

**Statistical Analysis**—GraphPad Prism 5.0 (GraphPad Software) was used to perform statistical analyses.

**Author Contributions**—A. D. and M. F. S. carried out cloning, immunoprecipitations, and Wnt signaling activity measurements. E. P., M. C. M., and V. A. D. carried out immunoprecipitations. P. S. performed peptide spot and overlay experiments and immunoprecipitations. W. B. provided plasmids for Wnt signaling analysis and contributed to writing the manuscript. M. F. S., A. D., and E. K. designed experiments and wrote the manuscript.

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