THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 286, NO. 17, pp. 14870–14880, April 29, 2011
© 2011 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.

Protein Kinase A-mediated 14-3-3 Association Impedes Human Dapper1 to Promote Dishevelled Degradation*[S]

Received for publication, December 11, 2010, and in revised form, January 23, 2011 Published, JBC Papers in Press, January 24, 2011, DOI 10.1074/jbc.M110.211607

Hua Chen[‡§1], Linhua Liu[‡§1], Benyu Ma[‡§1], Ting Martin Ma[‡§], Jun-Jie Hou[¶], Guo-Ming Xie[‡§], Wei Wu[§], Fu-Quan Yang[¶], and Ye-Guang Chen[‡§2]

From the [‡]State Key Laboratory of Biomembrane and Membrane Biotechnology and [§]School of Life Sciences, Tsinghua University, Beijing 100084 and the [¶]Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China

Wnt signaling regulates embryo development and tissue homeostasis, and its deregulation leads to an array of diseases, including cancer. Dapper1 has been shown to be a key negative regulator of Wnt signaling. However, its function and regulation remain poorly understood. In this study, we report that 14-3-3β interacts with human Dapper1 (hDpr1). The interaction is dependent on protein kinase A (PKA)-mediated phosphorylation of hDpr1 at Ser-237 and Ser-827. 14-3-3β binding attenuates the ability of hDpr1 to promote Dishevelled (Dvl) degradation, thus enhancing Wnt signaling. We further provide evidence that PKA-mediated Dpr1 phosphorylation may contribute to growth and tumor formation of colon cancer Caco2 cells. Finally, we show that cyclooxygenase-2 expression and PKA activation are positively correlated with Dvl protein levels in colon cancer samples. Together, our findings establish a novel layer of regulation of Wnt signaling by PKA via the 14-3-3-Dpr1-Dvl axis.

Both the canonical and noncanonical Wnt signaling pathways are evolutionarily conserved and play key roles in development and disease (1–4). The canonical Wnt/β-catenin pathway is initiated by Wnt ligands binding to their receptors Frizzled and low density lipoprotein receptor-related protein 5/6 (LRP5/6), which leads to the cytosolic accumulation of β-catenin by disrupting the β-catenin destruction complex consisting of Axin, adenomatous polyposis coli (APC),[3] and glycogen synthase kinase 3β (GSK3β). Accumulated β-catenin translocates into the nucleus and activates the transcription of Wnt target genes in collaboration with the transcription factor T cell factor/lymphoid enhancer factor (5, 6). In addition to the planar cell polarity (PCP) pathway, which regulates cell polarity and convergent extension, noncanonical Wnt signaling can also be mediated by Ca^{2+} and other signaling molecules (7, 8).

Dishevelled (Dvl) is the central player in both the canonical and noncanonical Wnt signaling pathways, serving as a scaffold protein bridging Frizzled and downstream components (8–10). Dvl can also function in the nucleus (11), where it cooperates with c-Jun, β-catenin, and TCF to regulate Wnt target gene transcription (12). Therefore, like many other signaling proteins, Dvl activity is tightly regulated. It can be phosphorylated by casein kinase 2 (13). The stability of Dvl proteins is regulated by an array of proteins, including Dapper1/Dact1, Inversin, NEDL1, Prickle-1, and KLHL12 (14–17). Our recent work also showed that Dvl protein is ubiquitinated by pVHL-containing E3 ubiquitin ligase and can be regulated by autophagy (18). In addition, altered Dvl expression has been implicated in oncogenesis. Dvl is overexpressed in prostate cancer, non-small cell lung cancer, mesothelioma, and other cancers, and its up-regulation has been indicated to be correlated with activation of Wnt/β-catenin signaling (19–21).

Dapper1 (Dpr1), also called Dact1, as a Dvl-interacting protein, is a negative regulator of both canonical and noncanonical Wnt signaling (14, 22). Three Dpr family members, Dpr1, Dpr2, and Dpr3, have been described in mammals (23), and they appear to have distinct functions. Human Dpr1 as well as Dpr3 can inhibit Wnt signaling by promoting Dvl degradation (14, 24, 25). Dpr1 can also disrupt the formation of a complex between β-catenin and LEF1 in the nucleus (26). Zebrafish Dpr1 and Dpr2, as well as the *Xenopus* homolog Frodo, have also been reported to positively regulate Wnt signaling; Dpr1 functions in the canonical Wnt/β-catenin pathway although Dpr2 acts in Wnt/Ca^{2+}-PCP signaling (27, 28). We have also demonstrated that zebrafish and mouse Dpr2 inhibit transforming growth factor-β (TGF-β)/Nodal signaling by promoting degradation of their type I receptors (25, 29). Studies with knock-out mouse models revealed that Dpr2 functions in re-epithelialization of skin wounds by attenuating TGF-β signaling (30), whereas Dpr1 plays a critical role in PCP signaling during development of mice (31, 32). Dpr1 regulates morphogenesis of the primitive streak by controlling Vangl2 activity (31) or modulates PCP signaling in early embryos by controlling the level and the cellular localization of Dvl proteins (32). In addition, Dpr1 and Dpr3 have been reported to be down-regulated in human hepatocellular carcinoma and colorectal cancer, respectively (24, 33).

The 14-3-3 protein family consists of seven members that are highly conserved, ubiquitously expressed, and capable of modulating diverse biological processes through protein-protein interactions (34). All 14-3-3 isoforms recognize three phosphor-

* This work was supported by National Natural Science Foundation of China Grants 30930050 and 30921004 and the 973 Program Grants 2006CB943401 and 2010CB833706 (to Y.-G. C.) and 2010CB833703 (to F.-Q. Y.).

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental "Experimental Procedures" and Figs. S1–S6.

[1] These authors contributed equally to this work.

[2] To whom correspondence should be addressed. Tel.: 86-10-62795184; Fax: 86-10-62794376; E-mail: ygchen@tsinghua.edu.cn.

[3] The abbreviations used are: APC, adenomatous polyposis coli; Dvl, Dishevelled; PCP, planar cell polarity; PGE2, prostaglandin E2; hDpr1, human Dpr1; CREB, cAMP-response element-binding protein; qRT-PCR, quantitative RT-PCR.

ylation-dependent binding motifs: RS*X*(pS/T)*X*P, pSX_{1-2}-COOH, and R*XXX*(pS/T)*X*P, where pS/T represents a phosphoserine or phosphothreonine (35, 36). Both 14-3-3ζ and -ϵ have been reported to interact with β-catenin and can either positively or negatively regulate Wnt signaling depending on the cellular context (37, 38).

Cyclic AMP-dependent protein kinase (protein kinase A (PKA)) has been shown to modulate Wnt signaling. PKA can phosphorylate and stabilize β-catenin, facilitating Wnt/β-catenin signaling (39, 40). The PKA-mediated phosphorylation of GSK3α and GSK3β leads to inhibition of their kinase activity (41). Cyclooxygenase-2 (COX-2) is involved in prostaglandin E_2 (PGE_2) synthesis and highly expressed in colon cancer (42, 43). PGE_2, through PKA and protein kinase B (PKB/AKT) activation, has also been shown to promote β-catenin signaling (44, 45).

In this study, we report that human Dpr1 (hDpr1) interacts with 14-3-3, and this interaction is dependent on the PKA-mediated phosphorylation of hDpr1 at Ser-237 and Ser-827. 14-3-3 binding impairs the ability of hDpr1 to promote Dvl degradation and enhances Wnt/β-catenin signaling. Furthermore, we find a positive correlation between PKA activation and Dvl protein levels in colon tumors. Our findings suggest a molecular interaction between PKA, hDpr1, and Dvl and a possible contribution of this interaction to tumorigenesis.

EXPERIMENTAL PROCEDURES

Expression Plasmids—The mammalian expression plasmids for HA-tagged hDpr1 and FLAG-Dvl2 were described previously (14). The 14-3-3 from pBluescript (kindly provided by Dr. Jianguo Chen, Peking University) was subcloned into pCMV-Myc. Human Dpr1(1–420) was amplified from the full-length hDpr1 using PCR and cloned into pGBKT7 as a bait for yeast two-hybrid screening. EYFP-difopein, PKI, and PKA plasmids were kind gifts from Dr. Haian Fu (Emory University), Dr. Yuan Zhang (Peking University), and Dr. G. Stanley McKnight (University of Washington), respectively. SiRNA oligonucleotides against Dvl1/2/3 and c-Jun were synthesized from Shanghai GenePharma based on the sequences described previously (12).

Yeast Two-hybrid Screen—The yeast two-hybrid screen system (Matchmaker 3) and the human embryonic brain library were purchased from Clontech. Yeast two-hybrid screening was performed according to the manufacturer's instructions.

Cell Culture, Transfection, Immunoprecipitation, Immunoblotting, and Luciferase Reporter Assays—All the cells were maintained in DMEM (Invitrogen) supplemented with 10% FBS except Caco2 cells that were maintained in DMEM plus 20% FBS. Transfection, immunoprecipitation, immunoblotting, and reporter assays were performed as described previously (14, 26). The sources of the antibodies are listed as follows: anti-14-3-3, and JNK (Santa Cruz Biotechnology); HA and Myc (made in-house); phosphoserine and FLAG (Sigma); Dvl2, CREB, PCREB, and nonphospho-β-catenin (Cell Signaling Technology); secondary anti-mouse antibodies conjugated to horseradish peroxidase (Amersham Biosciences). The human Dpr1 antibody was described previously (14).

Mass Spectrometry Analysis—GST-hDpr1 was sliced out from SDS-polyacrylamide gels and subjected to in-gel digestion as described previously (37). Protein reduction and alkylation were performed with DTT and iodoacetamide, respectively. Digestion was performed with trypsin overnight at 37 °C. Peptides were extracted with 0.1% formic acid and subjected to liquid chromatography-tandem MS (LC-MS/MS) analysis.

Quantitative RT-PCR— qRT-PCR was performed using total RNA isolated from the indicated cells and repeated at least three times for each gene with a Stratagene MX3000PTM system using a SYBR Green assay. Expression values were normalized to GAPDH expression. The primer sequences are as follows: c-*myc*, 5′-TCTCCTTGCAGCTGCTTAG-3′ and 5′-GTCGTAGTCGAGGTCATAG-3′; *Axin2*, 5′-AGTGTGAGGTCCACGGAAAC-3′ and 5′-CTTCACACTGCGATGCATTT-3′; *cyclin D1*, 5′-CTGGCCATGAACTACCTGGA-3′ and 5′-CTCCGCCTCTGGCATTTTGG-3′; and *GAPDH*, 5′-GAAGGTGAAGGTCGGAGTC-3′ and 5′-GAAGATGGTGATGGGATTTC-3′.

Immunohistochemistry—The colon cancer tissue slides were obtained from Xianfeng Biotech (Xi'an, China). The slides were dewaxed in xylene and rehydrated according to a standard protocol, and the procedure was performed as described previously (12).

Colony Formation Assay—To generate hDpr1 (wild-type and the AA mutant) stable cell lines, transfected Caco2 cells were selected in the presence of G418 (0.5 μg/μl). Cells were resuspended in DMEM (1 ml with 40% FBS and 0.33% agar) and plated over a layer of solidified DMEM containing 40% FBS, 0.66% agar (2 ml). Plates were incubated at 37 °C for 2 weeks, and colonies were stained with crystal violet (0.005%; Sigma).

Tumor Formation in Nude Mice—Caco2 cells (5×10^6 cells in 200 μl of PBS) were subcutaneously injected into 4–6-week-old female mice. Six animals were used in each group. Mice were sacrificed when tumors appeared 4–6 weeks later for measurement of tumor weight. The animal study was approved by the Animal Care Committee, Tsinghua University.

RESULTS

Human Dpr1 Interacts with 14-3-3—To identify hDpr1-interacting proteins, the N terminus of hDpr1(1–420) was used as a yeast two-hybrid bait to screen a human fetal brain library. One of the positive clones encoded the full-length 14-3-3β. To validate the interaction between hDpr1 and 14-3-3β, HEK293T cells were co-transfected with Myc-tagged hDpr1 and HA-tagged 14-3-3β. Anti-HA immunoprecipitation and anti-Myc immunoblotting revealed that hDpr1 was co-precipitated with 14-3-3β (Fig. 1*A*). Reverse co-immunoprecipitation assay also confirmed this interaction (Fig. 1*B*).

We found that all the seven members of mammalian 14-3-3, when ectopically expressed in HEK293T cells, were able to interact with hDpr1 (supplemental Fig. S1*A*). The interaction between 14-3-3 and hDpr1 could also be detected at endogenous levels in HeLa cells (Fig. 1*C*). Furthermore, difopein, a peptide binding to the substrate-binding site of 14-3-3 (46), impaired the interaction between 14-3-3β and hDpr1 (Fig. 1*D*). Domain mapping showed that all the truncated mutants of hDpr1 bound to 14-3-3β, but the C-terminal region (amino acid 611–836) had higher affinity (Fig. 1*E*). These results

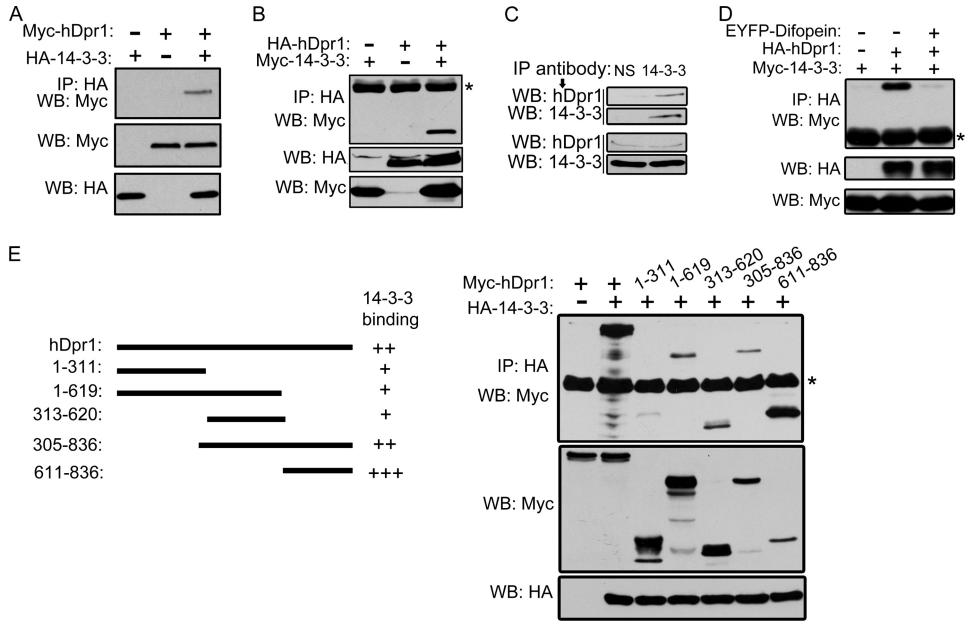

FIGURE 1. **Identification of 14-3-3β as a human hDpr1-interacting protein.** *A* and *B*, 14-3-3β interacts with human hDpr1. HEK293T cells were transfected with HA-tagged 14-3-3β (1.5 μg) and Myc-hDpr1 (1.5 μg) or HA-hDpr1 and Myc-14-3-3β. At 40 h post-transfection, cell lysates were harvested for anti-HA immunoprecipitation (*IP*) and anti-Myc immunoblotting (*upper panels*). Protein expression was confirmed with total cell lysates (*middle* and *lower panels*). *C*, interaction between hDpr1 and 14-3-3β at the endogenous levels. HeLa cell lysates were subject to immunoprecipitation with control mouse IgG or anti-14-3-3β antibody. 14-3-3β-associated hDpr1 is revealed by anti-hDpr1 immunoblotting (*upper panel*). *D*, difopein impairs the 14-3-3 and hDpr1 interaction. HEK293T cells were transfected with the indicated plasmids, and protein interaction was examined. *E*, mapping the 14-3-3β-interacting domain of hDpr1. HEK293T cells were transfected with indicated plasmids and their interaction was examined. *Asterisk* indicates IgG heavy chain (*B* and *E*) or light chain (*D*). *WB*, Western blot.

together demonstrated that 14-3-3β is a novel interacting protein of hDpr1.

PKA Enhances the Interaction between 14-3-3β and hDpr1—The fact that 14-3-3 proteins typically bind to phosphoserine/phosphothreonine-containing proteins prompted us to ask whether 14-3-3β associates with hDpr1 in a phosphorylation-dependent manner. As shown in Fig. 2*A*, treatment of immunoprecipitated hDpr1 with calf intestine alkaline phosphatase dramatically reduced its interaction with 14-3-3β. Several positively charged amino acids in a conserved amphipathic groove of 14-3-3 proteins, for instance Lys-51 in the case of 14-3-3β, have been found to be involved in the binding with the phosphorus moiety of its binding partners (47). Substitution of this lysine with glutamic acid (K51E) greatly impaired the binding of 14-3-3β with hDpr1 (Fig. 2*B*), supporting that phosphorylation plays a critical role in mediating hDpr1–14-3-3 interaction.

To identify the kinase mediating hDpr1 phosphorylation, which is responsible for the recognition by 14-3-3, we inspected all the consensus kinase phosphorylation sequences in hDpr1 with the 14-3-3 binding sequences and found the possible involvement of AKT and PKA. We then examined whether the hDpr1 and 14-3-3β interaction was influenced by AKT or PKA. As shown in Fig. 2*C*, overexpression of the catalytic subunit of PKA markedly enhanced the interaction between 14-3-3β and hDpr1. PKA could also enhance hDpr1 association with 14-3-3ζ (supplemental Fig. S1*B*) and other isoforms (data not shown). Consistently, hDpr1 associated with PKA (Fig. 2*D*). In contrast, we did not observe any significant effect of AKT on the 14-3-3β-hDpr1 interaction (supplemental Fig. S1*C*).

To confirm that PKA kinase activity *per se* is important for the 14-3-3β-hDpr1 interaction, we stimulated PKA with the adenylate cyclase activator forskolin or the cyclic AMP analog 8-Br-cAMP and found that the 14-3-3β-hDpr1 interaction was augmented by these activators at overexpressed (Fig. 2*E*) and endogenous (Fig. 2*F*) protein levels. It is well established that COX-2-produced PGE2, can activate PKA via its receptor EP2/4. Next, we tested if PGE_2 has any effect on the 14-3-3β-hDpr1 interaction. The data in Fig. 2*G* indicated that PGE2, could also enhance the interaction, and celecoxib, a nonsteroidal anti-inflammatory drug that inhibits COX-2 (42, 48), decreased the interaction (Fig. 2*H*). Consistent with this, the PKA inhibitor PKI inhibited the PKA-induced binding of hDpr1 with 14-3-3β (Fig. 2*I*). These data strongly suggest that the PKA-mediated phosphorylation of hDpr1 plays a critical role in the 14-3-3-hDpr1 interaction.

PKA Phosphorylates hDpr1 at Ser-237 and Ser-827—The requirement of PKA kinase activity for the 14-3-3-hDpr1 interaction led us to test whether hDpr1 is a direct substrate of PKA. To this end, we generated a GST-tagged hDpr1 without interfering with its inhibitory effect on Wnt signaling (supplemental Fig. S2*A*). GST-hDpr1 was purified from HEK293T after treating with 8-Br-cAMP for 1 h (supplemental Fig. S2*B*) and subjected to mass spectrometry analysis. Two serine residues (Ser-237 and Ser-827) were identified to be phosphorylated (Fig. 3*A*). Both residues match well with the consensus PKA phosphorylation motif R*XX*(S/T) and are conserved among the Dpr1 from different species (Fig. 3*B*). Substitution of them with alanine abolished 8-Br-cAMP-induced serine phosphorylation of hDpr1 (Fig. 3*C*), indicating that both Ser-237 and Ser-827 are PKA phosphorylation sites. Consistently, PGE2 also induced hDpr1 phosphorylation (Fig. 3*D*).

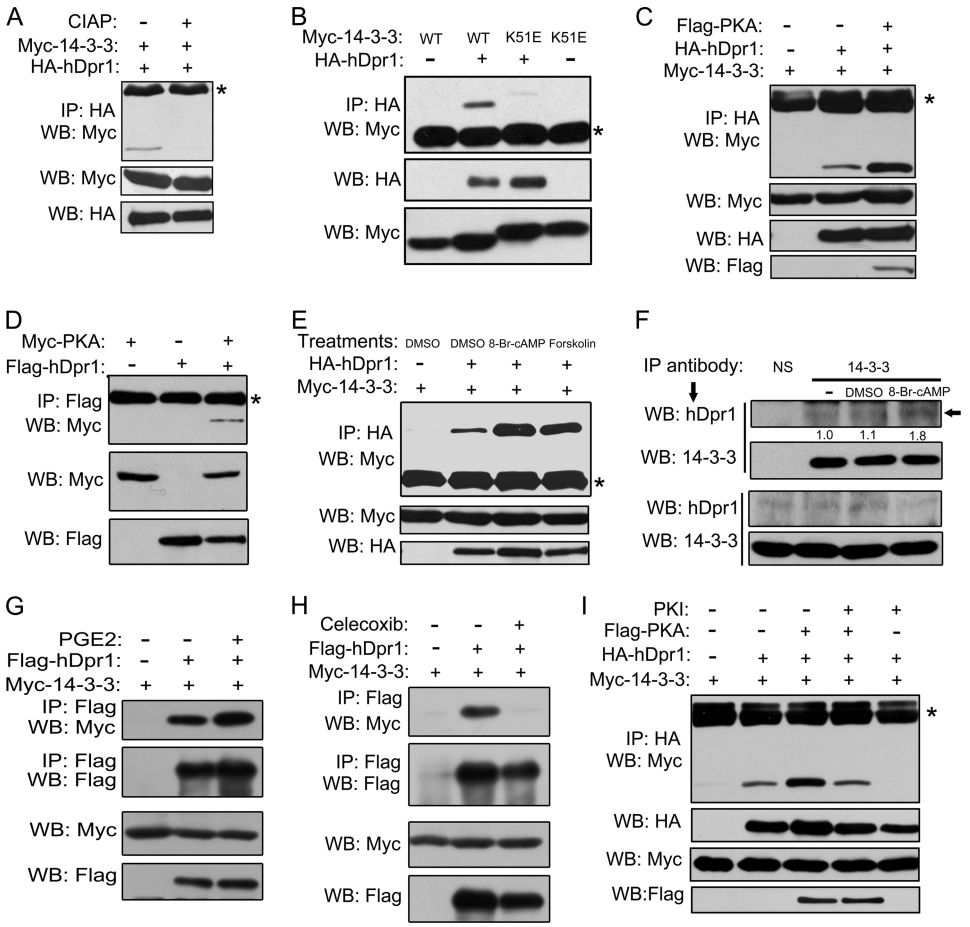

FIGURE 2. **Protein kinase A enhances 14-3-3β and hDpr1 interaction.** *A,* 14-3-3β and hDpr1 interaction depends on phosphorylation. HA-hDpr1 expressed in HEK293T cells was immunoprecipitated (*IP*) using anti-HA antibody, treated with or without calf intestinal alkaline phosphatase (*CIAP*), and then mixed with an equal amount of total cell lysates from HEK293T cells expressing Myc-14-3-3β. hDpr1-associated 14-3-3β was revealed by anti-Myc immunoblotting (*upper panel*). Protein expression was confirmed with total cell lysates (*middle* and *lower panels*). *WB*, Western blot. *B,* K51E mutation of 14-3-3β impairs the 14-3-3β-hDpr1 interaction. Anti-HA immunoprecipitation and anti-Myc immunoblotting were performed to detect protein interaction in transfected HEK293T cells. *C–E,* PKA enhances 14-3-3β and hDpr1 association and interacts with hDpr1. Transfected HEK293T cells were treated with 8-Br-cAMP or forskolin for 1 h prior to harvesting (*E*). *F,* 8-Br-cAMP enhances endogenous 14-3-3β and hDpr1 interaction. HeLa cells were treated with 8-Br-cAMP (25 μM) for 1 h before harvesting, and cell lysates were subject to immunoprecipitation with control mouse IgG or anti-14-3-3 antibodies. 14-3-3-associated hDpr1 was revealed by anti-hDpr1 immunoblotting (*upper panel*). *Arrow* indicates the hDpr1 band. *G,* PGE_2 enhances the 14-3-3β-hDpr1 interaction. HEK293T cells were transfected with indicated plasmids. The cells were treated with PGE_2 (10 μM) for 6 h before harvesting. Anti-FLAG immunoprecipitation and anti-Myc immunoblotting were performed to detect protein interaction. *H,* celecoxib decreases the 14-3-3β-hDpr1 interaction. The transfected HEK293T cells were treated with celecoxib (10 μM) for 6 h before harvesting. *I,* PKI inhibits PKA-induced 14-3-3β-hDpr1 interaction. *Asterisk* indicates IgG heavy chain (*A, C, D,* and *I*) or light chain (*B* and *E*).

To further confirm that the PKA-mediated phosphorylation contributes to the 14-3-3-hDrp1 interaction, hDpr1 mutants with a single or double mutation to alanine or aspartic acid of Ser-237 and Ser-827 were tested for their ability to interact with 14-3-3. Without PKA stimulation, all of these mutants showed a comparable binding affinity to 14-3-3β, except for decreased affinity in S237A and the double alanine mutants (AA) (Fig. 3*E*). However, the PKA-enhanced 14-3-3β-hDpr1 interaction was greatly impaired by alanine substitution (Fig. 3*F*). Mutation of either Ser-237 or Ser-827 had a similar effect as mutation of both suggested that both are important for 14-3-3 binding. Collectively, the above results demonstrate that PKA promotes the 14-3-3-hDpr1 interaction by phosphorylating Ser-237 and Ser-827 of hDpr1.

PKA-mediated hDpr1 Phosphorylation Positively Modulates Wnt Signaling—To determine the functional significance of the 14-3-3-hDpr1 interaction on Wnt signaling, we examined whether 14-3-3β influences Wnt signaling using Wnt-responsive luciferase reporters. As reported previously (14), hDpr1 inhibited Wnt1-induced expression of the reporter TOPFlash (Fig. 4*A*). Overexpression of 14-3-3β could partially reverse the inhibitory effect of hDpr1 in a dose-dependent manner. Co-expression of 14-3-3β with PKA increased the basal expression of the Wnt-responsive LEF luciferase (Fig. 4*B*), and this cooperation was further observed to reverse the inhibitory effect of hDpr1 (Fig. 4*C*).

To confirm the importance of PKA-mediated Dpr1 phosphorylation in the modulation of Wnt signaling, we compared the activities of wild-type and AA mutant hDpr1 in inhibiting 8-Br-cAMP-induced Wnt reporter expression. The data in Fig. 4*D* showed that the AA mutant was more potent than wild-type hDpr1 to block 8-Br-cAMP-induced Wnt reporter expression. Furthermore, both PKI and difopein inhibited forskolin-enhanced Wnt reporter expression (Fig. 4*E*). 14-3-3β (K51E), which cannot bind to hDpr1, also functioned in a dominant negative manner to impair

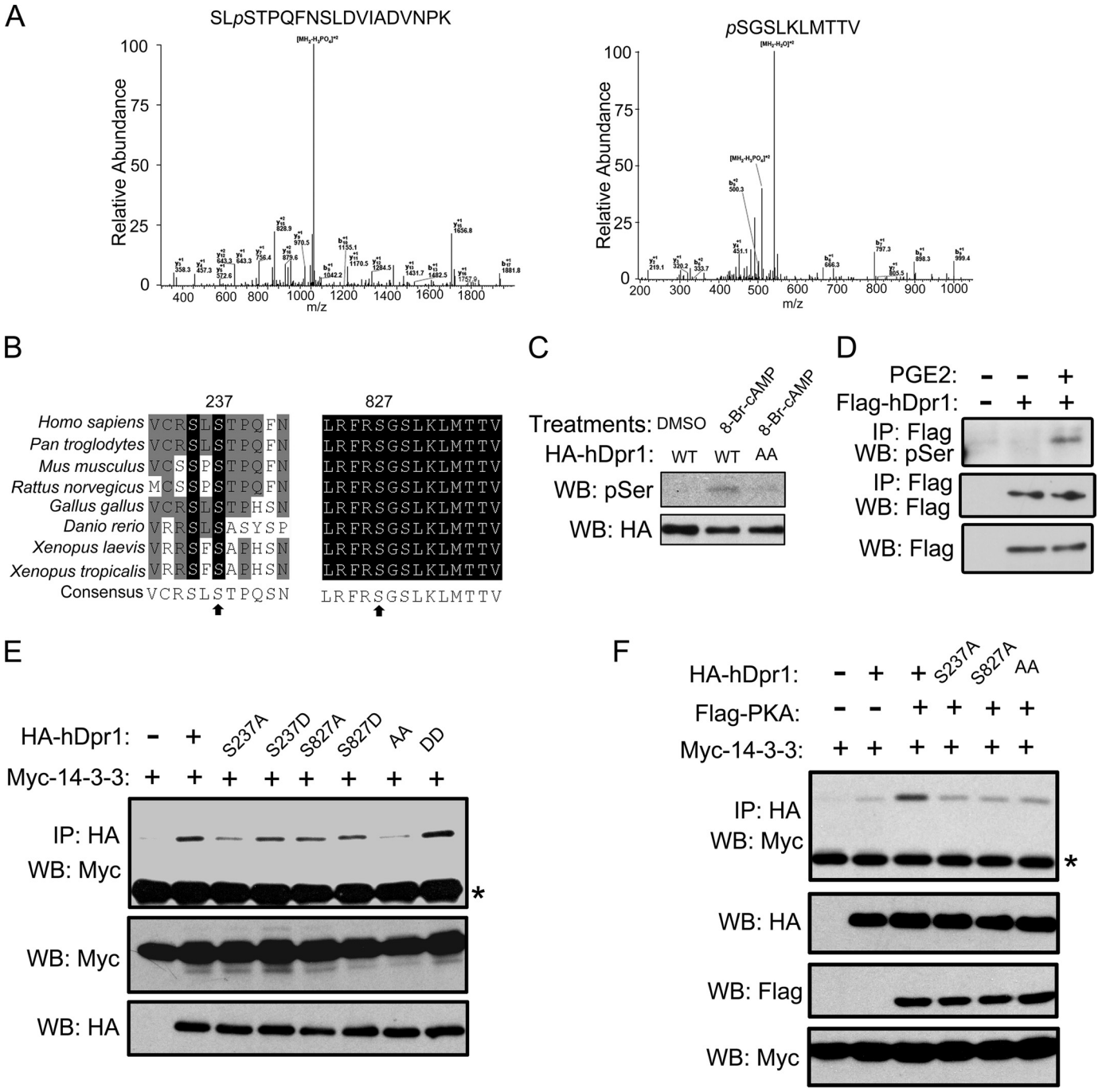

FIGURE 3. **PKA phosphorylates hDpr1 at Ser-237 and Ser-827.** *A,* GST-tagged hDpr1 was harvested from transfected HEK293T cells after 1 h of 8-Br-cAMP treatment and subjected to mass spectrometry analysis. *B,* sequence alignment of Dpr1 proteins from different species. Identical amino acids are *shaded black* and conserved amino acids *shaded gray*. Ser-237 and Ser-827 are conserved in all the species. *C,* HEK293T cells were transfected with WT and AA (S237A and S827A) HA-hDpr1. The cells were treated with 8-Br-cAMP for 1 h before harvested for anti-HA immunoprecipitation and then anti-phosphoserine immunoblotting (*upper panel*). The membrane were stripped and re-probed with anti-HA antibody (*lower panel*). *WB*, Western blot. *D,* PGE_2 induces hDpr1 phosphorylation. HEK293T cells were transfected with FLAG-hDpr1. The cells were treated with PGE_2 for 6 h before harvesting for anti-FLAG immunoprecipitation (*IP*) and then anti-phosphoserine immunoblotting. The membrane were stripped and re-probed with anti-FLAG antibody. *E* and *F,* HEK293T cells were transfected with indicated plasmids for 40 h, and cell lysates were harvested for anti-HA immunoprecipitation and anti-Myc immunoblotting (*upper panel*). Protein expression was confirmed by immunoblotting (*middle* and *lower panels*). *Asterisk* indicates IgG light chain (*E* and *F*).

reporter expression. These data strongly suggest that PKA enhances Wnt signaling through phosphorylating hDpr1 and modulating the 14-3-3-hDpr1 association. The conclusion is further supported by the observation that the mRNA levels of Wnt targets *Axin2* and c-*myc* in SW480 cells and *Axin2* in HEK293T cells were elevated upon treatment of 8-Br-cAMP or forskolin (Fig. 4, *F* and *G*). 8-Br-cAMP also activated a JNK-responsive reporter (supplemental Fig. S3*A*), which can be used as an indicator of noncanonical Wnt signaling (22). Consistent with this observation, both 8-Br-cAMP and forskolin induced the phosphorylation of JNK (supplemental Fig. S3*B*).

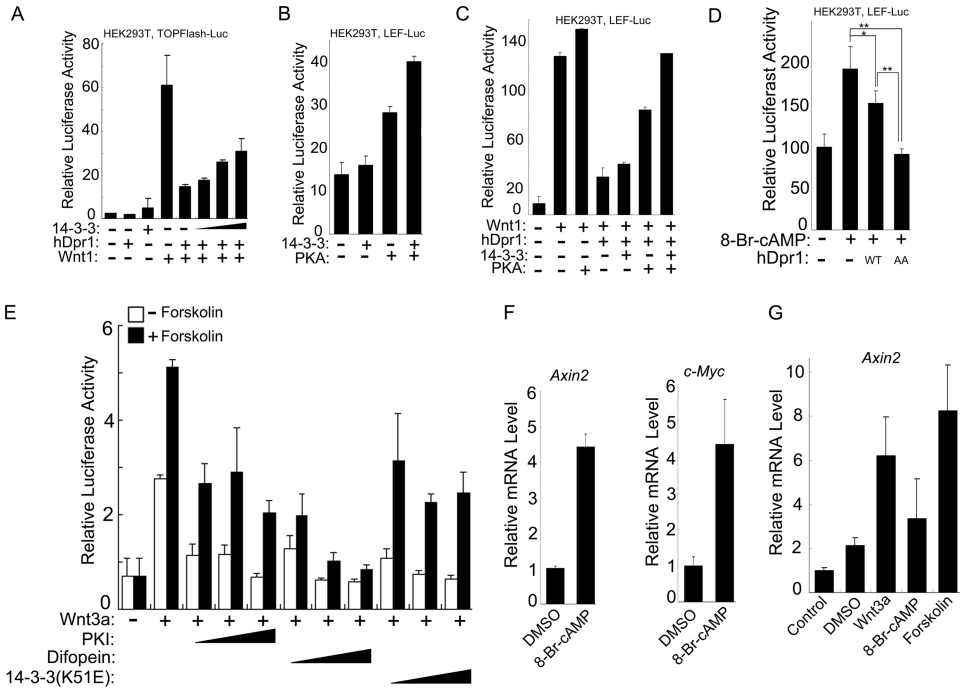

FIGURE 4. **PKA-mediated phosphorylation of hDpr1 positively modulates Wnt signaling.** *A,* 14-3-3β reverses the inhibitory effect of hDpr1 on TOPFlash reporter expression. HEK293T cells were transfected with TOPFlash-luciferase (0.3 μg), Wnt1 (0.2 μg), hDpr1 (0.2 μg), and increasing amounts of 14-3-3β plasmids (0.1, 0.2, and 0.3 μg). The cells were harvested at 48 h post-transfection for luciferase measurement. *B* and *C,* PKA cooperates with 14-3-3β to increase the expression of LEF-luciferase reporter. *D,* WT and AA mutant hDpr1 inhibits 8-Br-cAMP-activated LEF-luciferase activity. HEK293T cells were transfected with indicated plasmids and treated with or without 8-Br-cAMP for 12 h before harvested for luciferase assay. *E,* PKI, difopein, and 14-3-3β (K51E) inhibit TOPFlash expression. HEK293T cells were transfected with indicated plasmids, treated with Wnt3a conditioned medium and forskolin (25 μM) for 20 h, and then harvested for luciferase assay. *F,* 8-Br-cAMP increases the expression of *Axin2* and c-*myc* in SW480 cells. After 8-Br-cAMP treatment for 12 h, total RNA was isolated for qRT-PCR to determine the expression of *Axin2* and c-*myc*. The fold change in mRNA level of genes was normalized to GAPDH. *G,* 8-Br-cAMP and forskolin increase *Axin2* expression in HEK293T cells. After treatment with Wnt3a conditioned medium, 8-Br-cAMP, or forskolin for 12 h, total RNA was isolated for qRT-PCR to determine *Axin2* expression. GAPDH was used as a control. In the reporter assay, pRL-tk *Renilla* reporter (20 ng) was co-transfected to normalize transfection efficiency. All quantitative data were derived from three independent experiments and expressed as mean ± S.D. The *asterisk* indicates a statistically significant difference (**, $p < 0.01$; *, $p < 0.05$).

Interaction with 14-3-3 Impedes hDpr1 to Degrade Dvl—We previously demonstrated that Dpr1 could inhibit Wnt signaling by promoting Dvl degradation (14). We thus asked whether PKA activation influences the stability of Dvl proteins. Indeed, both 8-Br-cAMP and forskolin increased Dvl levels in HEK293T (Fig. 5*A*), Caco2 cells (Fig. 5*B*), and HeLa cells (supplemental Fig. S4*A*). Consistently, ectopic expression of PKA increased the Dvl2 protein level, whereas both wild-type and AA mutant hDpr1 induced Dvl2 degradation (Fig. 5*C*). Moreover, overexpression of PKA inhibited wild-type hDpr1-induced Dvl2 degradation but had a minimal effect on AA mutant activity, indicating that hDpr1 phosphorylation is important for PKA to stabilize Dvl proteins. Both forskolin and 8-Br-cAMP elevated the Dvl protein levels in $Dpr1^{+/+}$ but not in $Dp1^{-/-}$ mouse embryonic fibroblasts (Fig. 5*D*), further supporting the indispensable role of Dpr1 in PKA-induced Dvl stabilization.

Next we examined whether PKA-induced Dvl stabilization depends on the interaction between Dpr1 and 14-3-3. As shown in Fig. 5*E*, PKA was unable to stabilize Dvl protein when difopein was co-expressed, indicating that PKA-induced Dvl stabilization depends on the 14-3-3-hDpr1 interaction. We then determined how 14-3-3 binding interferes with hDpr1 induced Dvl degradation. 14-3-3 has been shown to regulate the activities of its interacting proteins by changing their subcellular localization (49) or modulating their interaction profiles (50). But PKA activators had neither effect on hDpr1 subcellular distribution, its co-localization with Dvl2 (supplemental Fig. S4*B*), nor the binding of hDpr1 with Dvl2 (supplemental Fig. S4*C*).

PKA Enhances the Activity of Dvl in the Nucleus—Colon cancer cells usually exhibit a high canonical Wnt/β-catenin signaling activity due to loss-of-function mutations of APC or gain-of-function mutations of β-catenin (1, 3, 6, 7). Consistent with previous reports, inhibition of GSK3β by lithium chloride could increase the active β-catenin in HEK293T, but it had no apparent effect in these colon cancer cells, including Caco2 and SW480 (supplemental Fig. S5). PKA activation further enhances the expression of Wnt targets in SW480 cells (Fig. 4*F*), whereas inhibition of PKA activity decreased the expression of the Wnt reporter LEF-luciferase and its targets in Caco2 cells (Fig. 6, *A* and *B*). These observations suggest that PKA may promote Wnt signaling independent of downstream signaling of GSK3β in these colon cancer cells.

It has been reported that Dvl can cooperate with c-Jun to stabilize the β-catenin-TCF interaction in the nucleus and thus enhance Wnt signaling (12). Our findings raised the possibility that PKA may enhance Dvl stability to potentiate Wnt signaling in the nucleus. To test this possibility, we examined the subcellular localization of Dvl upon PKA activation or inhibition. As shown in Fig. 5, *F* and *G*, 8-Br-cAMP and forskolin treatments resulted in nuclear accumulation of Dvl, whereas Rp-cAMP and celecoxib decreased the Dvl level in nucleus. Overexpres-

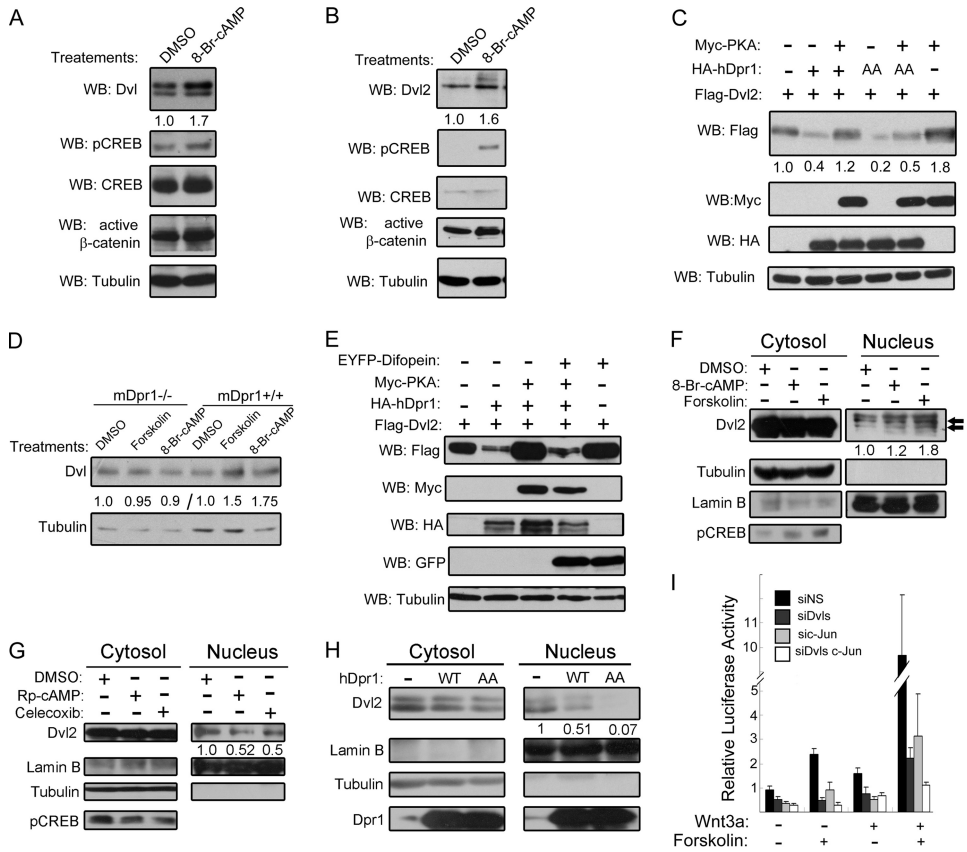

FIGURE 5. **PKA-induced 14-3-3-hDpr1 interaction stabilizes Dvl proteins.** *A* and *B*, 8-Br-cAMP stabilizes Dvl2 in HEK293T (*A*) and Caco2 cells (*B*). HEK293T and Caco2 cells were treated with carrier DMSO or 8-Br-cAMP for 4 h and then harvested for anti-Dvl immunoblotting. *C*, PKA antagonizes hDpr1-induced Dvl degradation. HEK293T cells were transfected with indicated plasmids. Dvl level was examined by anti-FLAG immunoblotting at 40 h post-transfection. *D*, PKA-induced Dvl stabilization requires hDpr1. Wild-type or Dpr1$^{-/-}$ mouse embryonic fibroblasts were treated with 8-Br-cAMP or forskolin for 4 h and then harvested for anti-Dvl immunoblotting. *E*, difopein attenuates the ability of PKA to stabilize Dvl. HEK293T cells were transfected with indicated plasmids. Dvl2 level was determined at 40 h post-transfection. *F* and *G*, Caco2 cells were treated with forskolin, 8-Br-cAMP, Rp-cAMP, or celecoxib for 12 h. Cytosolic and nucleic fractions were used to determine Dvl2 levels by anti-Dvl2 immunoblotting. Tubulin and lamin B are cytosol and nucleus markers, respectively. *H*, overexpression of hDpr1 decreases Dvl level in the nucleus. HEK293T cells were transfected with the indicated plasmid. Dvl levels were determined at 48 h post-transfection. *I*, knockdown of Dvl and c-Jun attenuates Wnt responsive reporter expression in HEK293T cells. HEK293T cells were transfected with indicated plasmids and harvested at 48 h post-transfection for luciferase assay. The *number below* the gel indicates the relative Dvl band densities after normalizing against tubulin or lamin B. *WB*, Western blot.

sion of Dpr1 also led to a decrease of Dvl in the nucleus, and importantly AA Dpr1 is more potent than WT Dpr1 on this effect (Fig. 5*H*). Consistent with these, knockdown of c-Jun and Dvl1/2/3 impeded the forskolin-induced reporter expression (Fig. 5*I*). Collectively these data support that PKA potentiates Wnt signaling by enhancing Dvl activity in the nucleus.

COX-2 Stabilizes Dvl Proteins—COX-2 has been reported to be up-regulated in certain cancers, including colorectal cancers, and can activate PKA via PGE2 (51, 52). This prompted us to examine whether COX-2 inhibition affects Wnt signaling via the PKA-Dvl axis. We found that the COX-2 inhibitor celecoxib could decrease Wnt-responsive reporter activity in Caco2 cells (Fig. 6*A*), which express a high level of COX-2 (data not shown). Interestingly, PKA could rescue the inhibitory effect of celecoxib on the Wnt-responsive reporter (supplemental Fig. S6*A*). The PKA inhibitor Rp-cAMP also inhibited reporter expression (Fig. 6*A*). Consistent with this, both inhibitors reduced the expression of the Wnt target genes *Axin2* and *cyclin D1* (Fig. 6*B*). Importantly, ectopic expression of Dvl could rescue the inhibitory effect of Rp-cAMP and celecoxib on the Wnt-responsive reporter in Caco2 cells (Fig. 6*C*).

In line with the above results, both celecoxib and Rp-cAMP decreased the Dvl protein levels in Caco2 cells (Fig. 6*D*). It is worth noting that these inhibitors did not change the active β-catenin levels, consistent with a high basal β-catenin activity in Caco2 cells (53). Taken together; these data suggest that COX-2 functions via PKA to stabilize Dvl proteins and thus activate Wnt signaling in Caco2 cells.

PKA-mediated Dpr1 Phosphorylation Plays a Role in Tumorigenesis—It is well established that deregulation of Wnt signaling is associated with colon cancer development. To test whether PKA-induced hDpr1 phosphorylation may contribute to tumorigenesis, we generated Caco2 clones that stably expressed wild-type and the AA mutant hDpr1 (supplemental Fig. S6*B*). As expected, expression of the Wnt target gene *Axin2* was significantly reduced in the hDpr1-expressing cells with the lowest level in the AA mutant cells (supplemental Fig. S6*C*). Colony formation assay showed that hDpr1 decreased colony formation, and the colony number was even lower in AA mutant cells (Fig. 6*E*). Consistent with this, hDpr1 significantly suppressed tumor growth in nude mice, and the AA mutant was more effective (Fig. 6*F*). These data together suggest that hDpr1

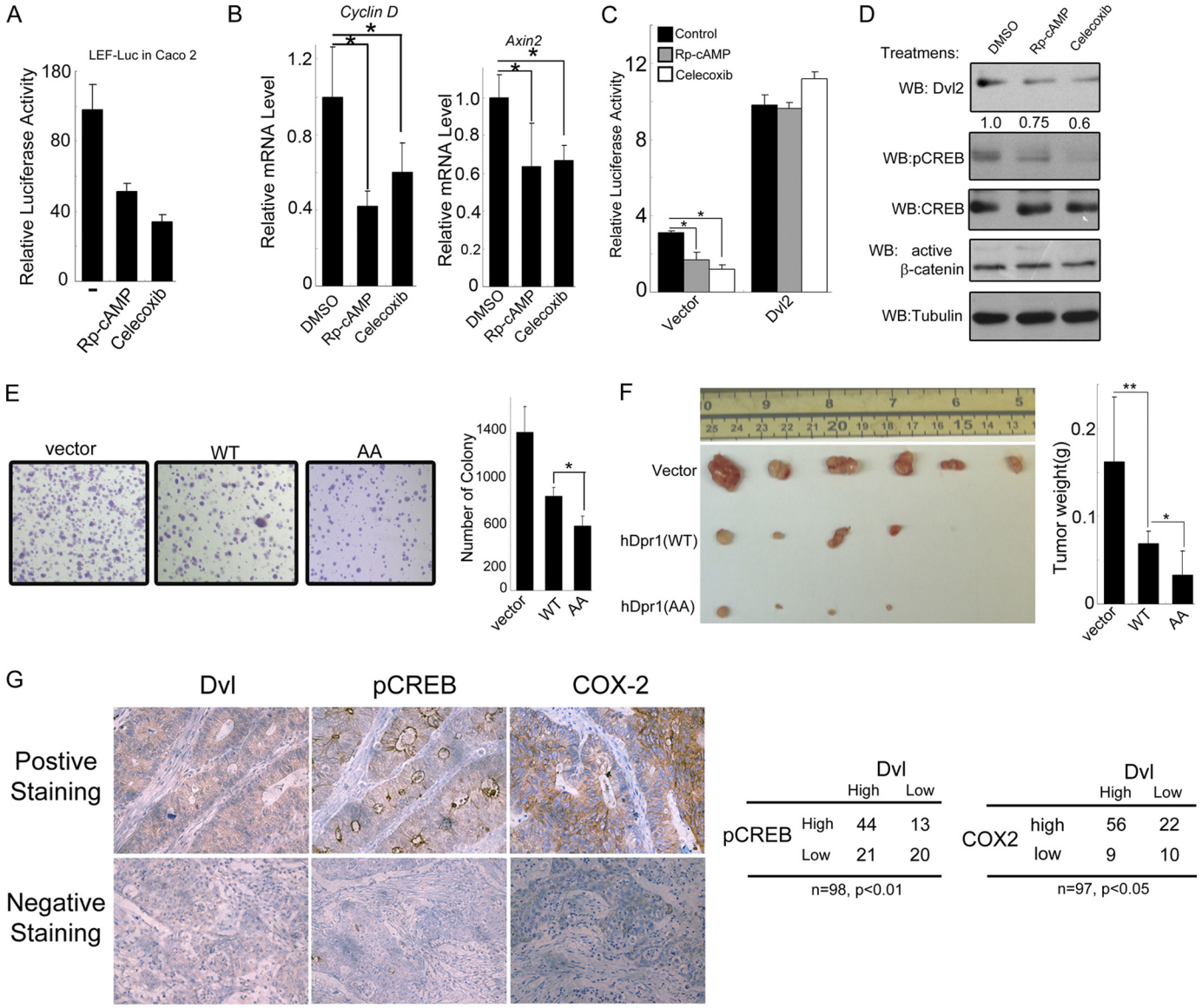

FIGURE 6. **PKA-mediated Dvl stabilization is correlated with colon cancer development.** *A,* celecoxib and (R_p)-cAMP decrease LEF-luciferase expression. Caco2 cells transfected with indicated plasmids were treated with celecoxib (10 μM) or (R_p)-cAMP (10 μM) for 12 h and then harvested for luciferase assay. *B,* celecoxib and (R_p)-cAMP decrease the expression of *cyclin D1* and *Axin2*. Caco2 cells were treated with celecoxib or (R_p)-cAMP for 4 h. Total RNA was isolated for qRT-PCR to determine the expression of *cyclin D1* and *Axin2*. The fold change in mRNA levels was derived from three independent experiments and expressed as means ± S.D. after normalizing against GAPDH. *C,* Dvl2 rescues the inhibitory effects of celecoxib and (R_p)-cAMP on LEF1-luciferase expression. Caco2 cells were transfected with the reporter or the reporter plus Dvl2 and were then treated with celecoxib or (R_p)-cAMP for 24 h before harvesting of the cell for luciferase assay. *D,* celecoxib and (R_p)-cAMP decrease Dvl2 level in Caco2 cells. After celecoxib or (R_p)-cAMP treatment for 4 h, Caco2 cells were harvested for immunoblotting. The *number below* the gel indicates the relative band densities after normalizing against tubulin. *WB,* Western blot. *E,* ectopic expression of hDpr1 inhibits colony formation. Caco2 cells stably expressing empty vector, wild-type hDpr1, or AA mutant were cultured in soft agar for 14 days in the presence of 0.5 μg/μl G418 and then stained with crystal violet. The colony number was counted and expressed as mean ± S.D. from triplicate experiments. *F,* hDpr1 suppresses tumor formation in nude mice. Nude mice at 4–6 weeks of age were injected subcutaneously with indicated Caco2 stable cells. Tumors were dissected and weighed 4 weeks later. *G,* Dvl protein levels are positively correlated with COX-2 and pCREB in colon tumor samples. Immunohistochemical analysis was carried out with anti-Dvl, anti-COX-2, or anti-pCREB antibodies and revealed by staining with diaminobenzene. *Left panels,* a representative of Dvl, pCREB, and COX-2 staining. *Right panels,* summary of the expression correlation between Dvl, pCREB, and COX-2. The correlation between Dvl and pCREB levels or Dvl and COX-2 levels was analyzed with χ test. The *asterisk* indicates a statistically significant difference (**, $p < 0.01$; *, $p < 0.05$).

negatively regulates cell growth, and phosphorylation-defective mutation enhances the antiproliferative effect of hDpr1.

The above results demonstrate that COX-2 stabilizes Dvl and activates Wnt signaling via PKA in Caco2 cells. To further establish the link among COX-2, PKA, and Dvl in tumorigenesis, we examined the protein expression of COX-2, pCREB, and Dvl in human colon tumor samples and found that COX-2 expression and CREB phosphorylation significantly correlated with Dvl expression (Fig. 6*G*). These data suggest that PKA-induced Dvl stabilization may contribute to COX-2-mediated colon cancer development.

DISCUSSION

How Dpr1 regulates Wnt signaling is still largely unclear. In this study, we provide evidence that 14-3-3β interacts with hDpr1, and its interaction attenuates the inhibitory effect of

hDpr1 on Wnt signaling. We found that the 14-3-3β-hDpr1 association depends on PKA-mediated phosphorylation of hDpr1 at the residues Ser-237 and Ser-827. The substitution of these serine residues with alanine eliminates PKA-enhanced 14-3-3β-hDpr1 interaction. Furthermore, PKA-induced 14-3-3-hDpr1 interaction impairs the ability of hDpr1 to promote Dvl degradation, consequently promoting Wnt signaling. We further found that PKA-mediated hDpr1 phosphorylation may play a role in tumorigenesis. These results demonstrated that PKA positively regulates Wnt signaling by attenuating Dpr1 to degrade Dvl.

PKA Facilitates the Interaction between 14-3-3 and hDpr1—In a yeast two-hybrid screening, we identified 14-3-3β, a phosphoserine-binding protein (34, 36), as an interactor of hDpr1. Their interaction was verified by immunoprecipitation, and all of the seven 14-3-3 isoforms could interact with hDpr1 when overexpressed in HEK293T. Furthermore, we demonstrated that the 14-3-3-hDpr1 association requires the conserved amphipathic groove of 14-3-3 proteins involved in the binding with the phosphorus moiety of its binding partners (47), as 14-3-3β K51E mutant lost its binding with hDpr1, and difopein, a peptide binding to the substrate-binding site of 14-3-3 (46), impaired the association. The interaction also depends on the PKA-mediated phosphorylation of hDpr1 at Ser-237 and Ser-827 as PKA overexpression or activation enhanced it, although PKI or mutation of these residues to alanine decreased it.

A recent study showed that *Xenopus* Dpr1 can be phosphorylated by casein kinase 1δ/ε (54). This phosphorylation is promoted by the interaction of Dpr1 with Dvl, and it in turn decreases this interaction. Interestingly, the casein kinase 1-mediated phosphorylation attenuates the ability of Dpr1 to promote β-catenin degradation. Although the underlying mechanism is unclear and the phosphorylation sites remain to be identified, this observation is in line with our finding that phosphorylation negatively modulates Dpr1 activity.

PKA Regulates Wnt Signaling via Dpr1 and Dvl—We found that PKA can positively modulate the Wnt/β-catenin pathway by phosphorylating hDpr1 and therefore stabilizing Dvl indirectly. PKA can physically interact with hDpr1 and augments the 14-3-3-hDpr1 association. We further identified Ser-237 and Ser-827 in hDpr1 as the phosphorylation sites of PKA. Phosphorylation of these sites is functionally important as the alanine substitution of these residues abrogated the 8-Br-cAMP-enhanced 14-3-3β-Dpr1 interaction and PKA-mediated Dvl stabilization. We noticed that the mutation of Ser-237 and Ser-827 to aspartic acid could not enhance the 14-3-3β-hDpr1 association. It could be explained by the previous observation that aspartic acid and glutamic acid residues are poor substitutes for phosphoserine or phosphothreonine in mediating 14-3-3 binding (55).

PKA can facilitate Wnt/β-catenin signaling through inactivation of GSK3 or stabilization of β-catenin (39–41). However, in colon cancer cells, which usually exhibit high canonical Wnt signaling activity due to loss-of-function mutations in APC or gain-of-function mutations of β-catenin (1, 3, 6, 7, 53), we found that forskolin can further increase Wnt3a-stimulated reporter activity and the expression of Wnt target genes in SW480 and Caco2 cells. Inhibition of GSK3 by LiCl could not further increase the active form of β-catenin in these cells and other colon cancer cells we tested. These data suggest that PKA may potentiate Wnt signaling independently of GSK3 inhibition and/or β-catenin stabilization in these colon cancer cells.

Previous studies showed that 14-3-3ζ can stabilize AKT-phosphorylated β-catenin and therefore enhance β-catenin transcriptional activity (37) or cooperate with Chibby to sequester β-catenin in the cytoplasm to antagonize the signaling (38), thus modulating the Wnt/β-catenin pathway in both positive and negative manners. We found that 14-3-3β can positively regulate Wnt signaling by reversing the inhibitory effect of hDpr1. We found that PKA stabilizes Dvl by promoting 14-3-3-hDpr1 association. PKA-induced Dvl stabilization depends on the presence of 14-3-3 and hDpr1 as depletion of Dpr or blocking 14-3-3 binding impeded PKA-induced Dvl stabilization. It has been reported that 14-3-3 binding affects the subcellular localization of various proteins (38, 49, 56), and hDpr1 shuttles between the cytoplasm and the nucleus (26). But PKA influenced neither the subcellular localization of hDpr1 nor its interaction with Dvl. How 14-3-3 binding influences hDpr1 to control Dvl stability is still an open question. It has been reported that 14-3-3 binding could induce a conformation change of its binding proteins without changing their interaction profile (57, 58). A similar mechanism could work here, and it awaits future experimental confirmation.

PKA activators increased Dvl protein levels and the expression of Wnt target genes in SW480 cells. Consistently, inhibition of PKA activity by (R_p)-cAMP decreased Dvl levels and impaired Wnt signaling in Caco2 cells. How does Dvl up-regulate Wnt signaling in these colon cancer cells, which have a high level of active β-catenin due to APC mutation (53)? Dvl has been shown to up-regulate the transcriptional activity of Wnt in the nucleus by cooperation with c-Jun (12). In accordance with this, we observed that knockdown of c-Jun or Dvl expression decreases forskolin-stimulated Wnt reporter expression. These data support the assumption that PKA could promote Wnt signaling at the level of Dvl, independently of GSK3 inhibition or direct β-catenin stabilization in colon cancer cells. Moreover, we found that PKA activation resulted in an increase of Dvl levels in the nucleus, whereas PKA inhibition decreased its nuclear accumulation. Overexpression of Dpr1 has the same effect as PKA inhibition. These data indicate that PKA can modulate the Wnt pathway by regulating the nuclear accumulation of Dvl proteins.

PAK-mediated Dvl Stabilization Plays a Role in Tumorigenesis—Up-regulation of COX-2 and its product PGE_2 have been associated with the development of colorectal cancer (43). It has been documented that the activation of Wnt signaling by PGE_2 occurs primarily through PKA (44, 59). Consistent with these observations, we found that PGE_2 enhances hDpr1 phosphorylation and thus augments 14-3-3β-hDpr1 association, whereas inhibition of COX-2 with celecoxib decreases the 14-3-3β-hDpr1 interaction. Both PKA and COX-2 inhibitors decrease Wnt-responsive reporter expression and Dvl levels in Caco2 cells. These data implied that COX-2 promotes Wnt signaling by activating PKA in these cells. Using Caco2 cells stably expressing wild-type and AA mutant

hDpr1, we found that hDpr1 suppresses anchor-independent growth of cells in soft agar and tumor formation in nude mice. Moreover, the AA mutant is more potent in these assays, indicating that PKA-mediated hDpr1 phosphorylation may promote tumor formation.

Dvl has been shown to be overexpressed in several types of tumors (19–21). Our recent study also showed that Dvl expression positively correlates with colon cancer development (18). To explore the possible link among COX-2, PKA, and Dvl in tumorigenesis, we examined the levels of COX-2, pCREB (as an indicator of PKA activity), and Dvl in colon cancer samples, and we found that COX-2 expression and PKA activity are positively correlated with Dvl expression. These data together suggest that the elevated PKA activity as a result of COX-2 upregulation may suppress hDpr1 activity and subsequently increase Dvl protein levels in colorectal cancer. Indeed, Dpr1 and Dpr3 are down-regulated in human hepatocellular and colorectal cancers, respectively (24, 33). Our results establish Dpr1 as another node for the cross-talk between PKA and Wnt signaling. Our findings also suggest a new mechanism underlying the role of COX-2 in colon cancer development and provide a molecular basis for the possible intervention of colon cancer.

Acknowledgments—We thank Drs. Jianguo Chen, Lin Li, Randall T. Moon, Yuan Zhang, Haian Fu, and G. Stanley McKnight for plasmids. We are grateful to the members of Chen laboratory for valuable discussions.

REFERENCES

1. MacDonald, B. T., Tamai, K., and He, X. (2009) *Dev. Cell* **17,** 9–26
2. Moon, R. T., Kohn, A. D., De Ferrari, G. V., and Kaykas, A. (2004) *Nat. Rev. Genet.* **5,** 691–701
3. Klaus, A., and Birchmeier, W. (2008) *Nat. Rev. Cancer* **8,** 387–398
4. Nusse, R. (2008) *Cell Res.* **18,** 523–527
5. Angers, S., and Moon, R. T. (2009) *Nat. Rev. Mol. Cell Biol.* **10,** 468–477
6. Clevers, H. (2006) *Cell* **127,** 469–480
7. Logan, C. Y., and Nusse, R. (2004) *Annu. Rev. Cell Dev. Biol.* **20,** 781–810
8. Gao, C., and Chen, Y. G. (2010) *Cell. Signal.* **22,** 717–727
9. Wharton, K. A., Jr. (2003) *Dev. Biol.* **253,** 1–17
10. Malbon, C. C., and Wang, H. Y. (2006) *Curr. Top. Dev. Biol.* **72,** 153–166
11. Itoh, K., Brott, B. K., Bae, G. U., Ratcliffe, M. J., and Sokol, S. Y. (2005) *J. Biol.* **4,** 3
12. Gan, X. Q., Wang, J. Y., Xi, Y., Wu, Z. L., Li, Y. P., and Li, L. (2008) *J. Cell Biol.* **180,** 1087–1100
13. Willert, K., Brink, M., Wodarz, A., Varmus, H., and Nusse, R. (1997) *EMBO J.* **16,** 3089–3096
14. Zhang, L., Gao, X., Wen, J., Ning, Y., and Chen, Y. G. (2006) *J. Biol. Chem.* **281,** 8607–8612
15. Simons, M., Gloy, J., Ganner, A., Bullerkotte, A., Bashkurov, M., Krönig, C., Schermer, B., Benzing, T., Cabello, O. A., Jenny, A., Mlodzik, M., Polok, B., Driever, W., Obara, T., and Walz, G. (2005) *Nat. Genet.* **37,** 537–543
16. Chan, D. W., Chan, C. Y., Yam, J. W., Ching, Y. P., and Ng, I. O. (2006) *Gastroenterology* **131,** 1218–1227
17. Angers, S., Thorpe, C. J., Biechele, T. L., Goldenberg, S. J., Zheng, N., MacCoss, M. J., and Moon, R. T. (2006) *Nat. Cell Biol.* **8,** 348–357
18. Gao, C., Cao, W., Bao, L., Zuo, W., Xie, G., Cai, T., Fu, W., Zhang, J., Wu, W., Zhang, X., and Chen, Y. G. (2010) *Nat. Cell Biol.* **12,** 781–790
19. Mizutani, K., Miyamoto, S., Nagahata, T., Konishi, N., Emi, M., and Onda, M. (2005) *Tumori* **91,** 546–551
20. Uematsu, K., He, B., You, L., Xu, Z., McCormick, F., and Jablons, D. M. (2003) *Oncogene* **22,** 7218–7221
21. Uematsu, K., Kanazawa, S., You, L., He, B., Xu, Z., Li, K., Peterlin, B. M., McCormick, F., and Jablons, D. M. (2003) *Cancer Res.* **63,** 4547–4551
22. Cheyette, B. N., Waxman, J. S., Miller, J. R., Takemaru, K., Sheldahl, L. C., Khlebtsova, N., Fox, E. P., Earnest, T., and Moon, R. T. (2002) *Dev. Cell* **2,** 449–461
23. Fisher, D. A., Kivimäe, S., Hoshino, J., Suriben, R., Martin, P. M., Baxter, N., and Cheyette, B. N. (2006) *Dev. Dyn.* **235,** 2620–2630
24. Jiang, X., Tan, J., Li, J., Kivimäe, S., Yang, X., Zhuang, L., Lee, P. L., Chan, M. T., Stanton, L. W., Liu, E. T., Cheyette, B. N., and Yu, Q. (2008) *Cancer Cell* **13,** 529–541
25. Su, Y., Zhang, L., Gao, X., Meng, F., Wen, J., Zhou, H., Meng, A., and Chen, Y. G. (2007) *FASEB J.* **21,** 682–690
26. Gao, X., Wen, J., Zhang, L., Li, X., Ning, Y., Meng, A., and Chen, Y. G. (2008) *J. Biol. Chem.* **283,** 35679–35688
27. Waxman, J. S., Hocking, A. M., Stoick, C. L., and Moon, R. T. (2004) *Development* **131,** 5909–5921
28. Gloy, J., Hikasa, H., and Sokol, S. Y. (2002) *Nat. Cell Biol.* **4,** 351–357
29. Zhang, L., Zhou, H., Su, Y., Sun, Z., Zhang, H., Zhang, L., Zhang, Y., Ning, Y., Chen, Y. G., and Meng, A. (2004) *Science* **306,** 114–117
30. Meng, F., Cheng, X., Yang, L., Hou, N., Yang, X., and Meng, A. (2008) *J. Cell Sci.* **121,** 2904–2912
31. Suriben, R., Kivimäe, S., Fisher, D. A., Moon, R. T., and Cheyette, B. N. (2009) *Nat. Genet.* **41,** 977–985
32. Wen, J., Chiang, Y. J., Gao, C., Xue, H., Xu, J., Ning, Y., Hodes, R. J., Gao, X., and Chen, Y. G. (2010) *J. Biol. Chem.* **285,** 11023–11030
33. Yau, T. O., Chan, C. Y., Chan, K. L., Lee, M. F., Wong, C. M., Fan, S. T., and Ng, I. O. (2005) *Oncogene* **24,** 1607–1614
34. Fu, H., Subramanian, R. R., and Masters, S. C. (2000) *Annu. Rev. Pharmacol. Toxicol.* **40,** 617–647
35. Coblitz, B., Wu, M., Shikano, S., and Li, M. (2006) *FEBS Lett.* **580,** 1531–1535
36. Yaffe, M. B. (2002) *FEBS Lett.* **513,** 53–57
37. Tian, Q., Feetham, M. C., Tao, W. A., He, X. C., Li, L., Aebersold, R., and Hood, L. (2004) *Proc. Natl. Acad. Sci. U.S.A.* **101,** 15370–15375
38. Li, F. Q., Mofunanya, A., Harris, K., and Takemaru, K. (2008) *J. Cell Biol.* **181,** 1141–1154
39. Hino, S., Tanji, C., Nakayama, K. I., and Kikuchi, A. (2005) *Mol. Cell. Biol.* **25,** 9063–9072
40. Taurin, S., Sandbo, N., Qin, Y., Browning, D., and Dulin, N. O. (2006) *J. Biol. Chem.* **281,** 9971–9976
41. Fang, X., Yu, S. X., Lu, Y., Bast, R. C., Jr., Woodgett, J. R., and Mills, G. B. (2000) *Proc. Natl. Acad. Sci. U.S.A.* **97,** 11960–11965
42. Steinbach, G., Lynch, P. M., Phillips, R. K., Wallace, M. H., Hawk, E., Gordon, G. B., Wakabayashi, N., Saunders, B., Shen, Y., Fujimura, T., Su, L. K., and Levin, B. (2000) *N. Engl. J. Med.* **342,** 1946–1952
43. Oshima, M., Dinchuk, J. E., Kargman, S. L., Oshima, H., Hancock, B., Kwong, E., Trzaskos, J. M., Evans, J. F., and Taketo, M. M. (1996) *Cell* **87,** 803–809
44. Fujino, H., West, K. A., and Regan, J. W. (2002) *J. Biol. Chem.* **277,** 2614–2619
45. Castellone, M. D., Teramoto, H., Williams, B. O., Druey, K. M., and Gutkind, J. S. (2005) *Science* **310,** 1504–1510
46. Masters, S. C., and Fu, H. (2001) *J. Biol. Chem.* **276,** 45193–45200
47. Gardino, A. K., Smerdon, S. J., and Yaffe, M. B. (2006) *Semin. Cancer Biol.* **16,** 173–182
48. Sinicrope, F. A., and Gill, S. (2004) *Cancer Metastasis Rev.* **23,** 63–75
49. Brunet, A., Kanai, F., Stehn, J., Xu, J., Sarbassova, D., Frangioni, J. V., Dalal, S. N., DeCaprio, J. A., Greenberg, M. E., and Yaffe, M. B. (2002) *J. Cell Biol.* **156,** 817–828
50. Zha, J., Harada, H., Yang, E., Jockel, J., and Korsmeyer, S. J. (1996) *Cell* **87,** 619–628
51. Phelps, R. A., Broadbent, T. J., Stafforini, D. M., and Jones, D. A. (2009) *Cell Cycle* **8,** 2549–2556
52. Castellone, M. D., Teramoto, H., and Gutkind, J. S. (2006) *Cancer Res.* **66,** 11085–11088

53. Ilyas, M., Tomlinson, I. P., Rowan, A., Pignatelli, M., and Bodmer, W. F. (1997) *Proc. Natl. Acad. Sci. U.S.A.* **94,** 10330–10334
54. Teran, E., Branscomb, A. D., and Seeling, J. M. (2009) *PLoS One* **4,** e5522
55. Manning, B. D., and Cantley, L. C. (2007) *Cell* **129,** 1261–1274
56. Hermeking, H. (2003) *Nat. Rev. Cancer* **3,** 931–943
57. Tzivion, G., Luo, Z., and Avruch, J. (1998) *Nature* **394,** 88–92
58. Obsil, T., Ghirlando, R., Klein, D. C., Ganguly, S., and Dyda, F. (2001) *Cell* **105,** 257–267
59. Goessling, W., North, T. E., Loewer, S., Lord, A. M., Lee, S., Stoick-Cooper, C. L., Weidinger, G., Puder, M., Daley, G. Q., Moon, R. T., and Zon, L. I. (2009) *Cell* **136,** 1136–1147