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RIPK4 phosphorylates Dishevelled proteins to regulate canonical Wnt signaling

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Abstract

Receptor interacting protein kinase 4 (RIPK4) is required for epidermal differentiation (1–4) and is mutated in Bartsocas-Papas syndrome (5, 6). While RIPK4 binds protein kinase C (5, 6), RIPK4 signaling mechanisms are largely unknown. We show that ectopic RIPK4 induces cytosolic β -catenin accumulation and a transcriptional program similar to Wnt3a, whereas kinase-defective or

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Bartsocas-Papas syndrome *RIPK4* mutants do not. Ectopic *ripk4* synergized with Wnt family member *xwnt8* in *Xenopus*, whereas *ripk4* morpholinos or kinase-defective *RIPK4* antagonized Wnt signaling. Mechanistically, *RIPK4* interacted constitutively with the Wnt adaptor protein DVL2 and, after Wnt3a stimulation, with the co-receptor LRP6. Phosphorylation of DVL2 at Ser²⁹⁸ and Ser⁴⁸⁰ by *RIPK4* favored canonical Wnt signaling. Growth of a Wnt-dependent N-Tera2 xenograft tumor model was suppressed by *RIPK4* knockdown, suggesting that *RIPK4* overexpression may contribute to the growth of certain tumor types.

Keywords

LRP6; β -catenin; *Xenopus*

Mice lacking *RIPK4* die at birth with fused external orifices due to defective epidermal differentiation. Mutation of human *RIPK4* in autosomal recessive Bartsocas-Papas syndrome also causes severe defects in face, skin, and limb development (5, 6). To better define the *RIPK4* signaling pathway, we used a Human Signal Transduction PathwayFinder PCR Array to determine gene expression changes in HEK293T cells after transfection with *RIPK4*. Canonical Wnt target genes such as *CCND1*, *LEF1*, *JUN*, *Myc*, and *TCF7* were upregulated (fig. S1), prompting us to compare the effect of *RIPK4* to Wnt3a treatment in responsive PA-1 teratocarcinoma cells (7). Transfected *RIPK4*, but not the related kinases *RIPK1*, *RIPK2*, and *RIPK3*, caused similar transcriptional changes to Wnt3a (Fig. 1A). Examples of Wnt3a target genes (8) that also were upregulated by *RIPK4* include *AXIN2*, *APCDD1*, *GADI*, and *NKX1-2* (fig. S2). Consistent with there being overlap between *RIPK4* and Wnt signaling, *RIPK4* activated a Wnt-dependent TOPbrite luciferase reporter gene in HEK293 cells (9, 10), and exogenous Wnt3a did not increase the signal further (Fig. 1B).

Wnt signaling stabilizes β -catenin in the cytosol, thereby facilitating its interaction with TCF transcription factors to drive Wnt-dependent gene expression (11). Transfected *RIPK4*, but not *RIPK1*, *RIPK2*, or *RIPK3*, caused β -catenin to accumulate in the cytosol of HEK293T cells even in the absence of exogenous Wnt3a (Fig. 1C). *CTNNB1* mRNA encoding β -catenin was not increased by *RIPK4*, which suggests that β -catenin accumulation was not due to transcriptional upregulation (Fig. 1C), but probably resulted from protein stabilization as seen during Wnt signaling. The kinase activity of *RIPK4* appears to be important for *RIPK4* mimicry of Wnt signaling, because kinase mutant *RIPK4* K51R was expressed at the same level as WT *RIPK4*, but failed to activate the TOPbrite luciferase reporter system (fig. S3A) and did not promote cytosolic β -catenin accumulation (fig. S3B). By contrast, the interaction of *RIPK4* with PKC- δ or PKC- β (5, 6) appears to be dispensable for β -catenin stabilization because *RIPK4* K51R still binds both PKC isoforms (fig. S4A), and knockdown of both PKC isoforms does not block *RIPK4*-induced β -catenin accumulation (fig. S4B). Consistent with these data, PKC family inhibitor Gö6983 had no effect on *RIPK4*-induced β -catenin accumulation (data not shown). Significantly, two Bartsocas-Papas syndrome *RIPK4* point mutants (I81N, I121N) and one truncation mutant (S376X) were expressed as well as WT *RIPK4*, but did not cause β -catenin accumulation (Fig. 1D), implying that *RIPK4* signaling to β -catenin may be relevant to mammalian development.

Mutants p.T184I and p.R260TfsX14 did not express in this system. Quite strikingly, *RIPK4* knockdown in A2780 and COV434 ovarian cancer cells, as well as HEK293T cells inhibited β -catenin accumulation, TOPbrite luciferase activation, as well as transcription of Wnt3a target genes *AXIN2* and *APCDD1*, in response to Wnt3a (Fig. 1E, fig. S5), suggesting a critical role for RIPK4 in Wnt3a signaling in these particular cells. It is worth noting, however, that reliance on RIPK4 may be specific to certain cell types, because RIPK4 knockdown in pancreatic PANC1 cells, kidney 786-O cells, and breast HCC38 or HS578T cells, had no effect on Wnt3a-induced β -catenin accumulation (data not shown).

Ectopic expression of Wnt pathway agonists in *Xenopus* ventral-vegetal cells results in axis duplication during early embryogenesis (12–16). Overexpression of *Xenopus ripk4* alone did not produce a secondary axis, but *ripk4* did synergize with sub-threshold levels of *xwnt8*, such that a full secondary axis developed in approximately 25% of embryos (Fig. 2A). To test whether *ripk4* was necessary for Wnt signaling *in vivo*, we used the animal cap system to assay the expression of two direct Wnt targets, *xnr3* and *siamois* (17, 18). Translation-blocking morpholinos to *ripk4* reduced the expression of *xnr3* and *siamois* induced by *Xwnt8* overexpression (Fig. 2B and fig. S6). These experiments suggest that *ripk4* can modulate Wnt signaling in the developing *Xenopus* embryo. *ripk4* overexpression in dorsal-marginal cells, similar to Wnt agonists (19), broadened the region expressing the dorsal organizer gene *chordin*, whereas kinase-dead *ripk4*-K52R caused the region to contract (Fig. 2B). While morpholinos to *ripk4* had no effect on dorsal-ventral axis specification (data not shown), they did anteriorize the neural tube (Fig. 2C), a phenotype linked to reduced Wnt signaling in the gastrula (20), as the hindbrain markers *engrailed2* and *krox20* were shifted posteriorly (Fig. 2D). Furthermore, the expression of *engrailed2* was reduced when *ripk4* was knocked down (Fig. 2D). This phenotype was unlikely to be a non-specific, off-target effect of the *ripk4* morpholinos, because both morpholinos gave identical phenotypes, and it was reversed by co-injection of non-targeted human *RIPK4* (Fig. 2D; column 3). In addition, *ripk4*-K52R overexpression in dorsal-anterior cells elicited similar changes to the *ripk4* morpholinos (Fig. 2D; column 4), consistent with the kinase dead mutant interfering with signaling by endogenous *ripk4*. Finally, *ripk4* or *xwnt8* overexpression had the opposite effect of the *ripk4* morpholinos, causing posteriorization of the neural tube as hindbrain markers shifted anteriorly, and *engrailed2* expression was expanded (Fig 2D; column 5–6). Collectively, these experiments support the notion that RIPK4 regulates Wnt signaling *in vivo*.

We investigated how RIPK4 regulates Wnt signaling by looking at RIPK4-induced cytosolic β -catenin accumulation and TOPbrite reporter gene activation after siRNA knock down of canonical Wnt pathway components (fig. S7A–B). Knockdown of either the Wnt co-receptor LRP6 or the three cytoplasmic Dishevelled adaptors (DVL1–3) blocked cytoplasmic β -catenin accumulation (fig. S7A) and decreased TOPbrite luciferase activity (fig. S7B) after RIPK4 overexpression. These data prompted us to explore whether RIPK4 interacts with LRP6 and/or DVL proteins. Endogenous RIPK4 co-immunoprecipitated with endogenous DVL2 from HEK293T cells, irrespective of whether the cells were treated with Wnt3a (Fig. 3A, fig. S7C), and a direct interaction seems likely because *in vitro* translated DVL2 and RIPK4 interacted too (Fig. 3B). While RIPK4 associated with DVL2

constitutively, we found that Wnt3a treatment promoted LRP6 interaction with RIPK4, albeit not until 15 minutes after treatment (Fig. 3C). Regardless, these findings are consistent with RIPK4 acting at the level of the Wnt receptor complex.

Given that RIPK4 kinase activity is required for cytoplasmic β -catenin accumulation (fig. S3), we investigated whether RIPK4 phosphorylates LRP6, DVL, or their associated proteins upon recruitment to the Wnt receptor complex. Proteomic analyses failed to reveal any evidence of LRP6 phosphorylation (data not shown) so next we explored DVL proteins as potential RIPK4 substrates. RIPK4 overexpression in HEK293T cells mimics Wnt signaling (Fig. 1) so initially we co-expressed DVL2-FLAG with either wild-type or K51R kinase mutant RIPK4-FLAG. Stable isotope labeling (21) of the cells transfected with wild-type RIPK4 allowed us to specifically identify RIPK4 kinase-dependent DVL2 phosphopeptides after the wild-type and kinase mutant lysates were pooled and the FLAG-tagged proteins affinity purified (fig. S8). Mass spectrometry revealed that phosphopeptides from the PDZ (GDGGIYIGS²⁹⁸IMK) and DEP (KYAS⁴⁸⁰GLLK) domains of DVL2 were more abundant with wild-type RIPK4 than K51R RIPK4 (fig. S8A). Significantly, both sites appear conserved being found in DVL isoforms from xenopus, zebrafish, mouse, and human (fig. S8B).

To explore whether DVL2 is phosphorylated directly by RIPK4, we performed *in vitro* kinase assays using RIPK4 kinase domain (amino acids 1–300) purified from Sf9 insect cells and FLAG-tagged DVL2 PDZ or DEP domains expressed using wheat germ extract. Wild-type RIPK4 kinase domain, but not the corresponding K51R mutant, phosphorylated both the PDZ and DEP domains of DVL2 (Fig. 3D), indicating that these domains can be phosphorylated directly by RIPK4. Mutation of Ser298 and Ser480 to alanine blocked phosphorylation of the DVL2 PDZ and DEP domain, respectively (Fig. 3D), which provided independent validation of the phosphorylation sites identified by mass spectrometry. Rabbit antibodies recognizing phosphorylated Ser298 and phosphorylated Ser480 in DVL2 were able to detect by western blotting wild-type DVL2 overexpressed in HEK293T cells, and ablation of the signal by mutation of the relevant serine in DVL2 to alanine confirmed the specificity of each antibody (fig. S8C). Consistent with DVL2 being a substrate of RIPK4, the extent of DVL2 phosphorylation increased dramatically upon co-transfection of RIPK4 (fig. S8D). As a further control for the phospho-specificity of the antibodies, the phospho-DVL2 bands disappeared when the cell lysates were treated with calf intestinal alkaline phosphatase (CIP; fig. S8D).

Next, we examined whether Wnt3a stimulated DVL2 phosphorylation in HEK293T cells. Phosphorylation of DVL2 Ser298 and Ser480 increased transiently after 10 minutes of Wnt3a treatment and was no longer detectable at 20 minutes (fig. S8E). This Wnt3a-induced DVL2 phosphorylation was prevented by RIPK4 knockdown (Fig. 3E and fig. S8E), implying a critical role for RIPK4 in DVL2 phosphorylation. To interrogate whether DVL2 phosphorylation is necessary for Wnt3a signaling, we generated DVL-null mouse embryo fibroblasts (MEFs) by knocking down *Dvl3* in *Dvl1*^{-/-}*Dvl2*^{-/-} MEFs (22), and then reconstituted them with either wild-type DVL2-FLAG or phospho-site mutant DVL2-S298A/S480A-FLAG. Wnt3a-induced accumulation of β -catenin in the cytosol was reduced

in cells expressing the DVL2 phospho-site mutant (Fig. 3F), suggesting that phosphorylation of S298 and/or S480 is necessary for maximal Wnt3a signaling.

DVL proteins are reported to enter large signaling complexes in response to Wnt3a (23, 24), coalescing into punctate structures by immunofluorescence microscopy (25). We found that approximately 20% of HeLa cells transfected with DVL2-FLAG contained puncta when stained with anti-FLAG antibodies, while the rest exhibited a more uniform cytoplasmic distribution of DVL2 (Fig. 3G). Co-transfection of a RIPK4-GFP fusion increased the percentage of cells containing DVL2 puncta to more than 75% (Fig. 3G and fig. S9), suggesting that RIPK4 facilitates DVL2 signalosome formation. Consistent with this notion, mutation of RIPK4 target residues Ser298 and Ser480 in DVL2 to alanine negated the ability of RIPK4 to promote the formation of DVL2 puncta (Fig. 3G).

Mutation of *WNT* genes or other components in the Wnt pathway are found in various human cancers (26, 27). Our data that RIPK4 enhances Wnt signaling by phosphorylating DVL proteins led us to examine whether any human tumors overexpress *RIPK4*. Microarray data suggested that *RIPK4* transcription is significantly upregulated in many human ovarian, skin, and colorectal tumors (Fig. 4A). By western blotting, we observed elevated RIPK4 and cytosolic β -catenin accumulation in several human ovarian adenocarcinomas, while RIPK4 was less abundant and cytosolic β -catenin not detected in most non-cancerous ovarian tissue samples (Fig. 4, B and C). To determine whether RIPK4 contributes to tumor development by enhancing Wnt signaling, we used a lentivirus expressing a *RIPK4* short hairpin RNA (shRNA) to knock down RIPK4 in a Wnt-dependent N-Tera2 xenograft tumor model (8, 28). As a control, we also infected HCT116 cells, which harbor a stabilizing and activating β -catenin mutation (29) and are predicted to be unaffected by RIPK4 deficiency. We achieved significant, albeit incomplete knockdown of RIPK4 in cultured N-Tera2 and HCT116 cells (Fig. 4D) and, remarkably, this was sufficient to suppress the growth of N-Tera2 tumor xenografts in athymic nude mice (Fig. 4, E and F). By contrast, RIPK4 knockdown had no effect on the growth of HCT116 tumor xenografts. RIPK4 knockdown appeared to suppress Wnt signaling in the N-Tera2 tumors because expression of two Wnt-responsive genes, *GAD1* and *AXIN2*, was decreased relative to N-Tera2 tumors expressing control shRNAs (Fig. 4G). As predicted, RIPK4 knockdown had no effect on *GAD1* and *AXIN2* expression in HCT116 tumors. These data are further evidence that RIPK4 promotes Wnt signaling, and imply that RIPK4 overexpression might have a significant impact on the growth of Wnt-dependent tumors.

In summary, we have identified a novel mechanism of action for RIPK4 that might explain why its mutation in mammals causes severe developmental defects. Collectively, our data suggest that RIPK4 is recruited to the LRP6 co-receptor and phosphorylates DVL proteins following Wnt stimulation, leading to maximal stabilization of β -catenin and transcription of Wnt-responsive genes (fig. S10). Strong support for this model comes from the fact that mutant forms of RIPK4 associated with human Bartsocas-Papas syndrome appear compromised in this signaling ability. We speculate that RIPK4 modulation of Wnt signaling is restricted to specific cell types since RIPK4 deficiency in mice does not phenocopy loss of an essential Wnt pathway component such as β -catenin (29xxxxx). Furthermore, RIPK4 expression seems restricted to vertebrates; therefore, phosphorylation

of DVL proteins in lower organisms might be compensated by a different kinase. Finally, inappropriate exacerbation of Wnt signaling by RIPK4 may be relevant for cancer since we observed RIPK4 overexpression in some human tumors and RIPK4 was critical for tumor growth in a Wnt-dependent xenograft model.

Materials/Methods

Plasmids, antibodies, recombinant proteins, and reagents

RIPK4-Myc cDNA was synthesized (Blue Heron) and cloned into pEF2 (Invitrogen). DVL2-FLAG-Myc, DVL3-FLAG-Myc, DVL2-GFP, RIPK4-GFP plasmids were from Origene. All mutants were generated using a QuickChange Site-Directed Mutagenesis kit (Stratagene). Antibodies recognized RIPK4 (Novus clone 2G3), actin, PKC- δ , DVL2, DVL3, phospho-LRP6-Ser1490, LRP6, β -catenin, GSK3- β , or AXIN1 (Cell Signaling Technology), GSK3 (Millipore), HA or FLAG (Sigma), Myc (US Biologicals), DVL1 or DVL2 (Santa Cruz Biotech), and PKC- β (BD Biosciences). DVL2 phospho-Ser298 and DVL2 phospho-Ser480 rabbit polyclonal antibodies were generated by Prosci, and monoclonal antibodies were then derived by Epitomics and Genentech. Other reagents were Wnt3a (Genentech and R & D systems), anti-FLAG and anti-HA beads (Sigma), calf intestinal alkaline phosphatase and all restriction enzymes (New England Biolabs).

Cell culture and transfection

HEK293 cells transfected stably with a TOPbrite firefly luciferase Wnt reporter and pRL-SV40 Renilla luciferase (Promega) were maintained in DMEM media supplemented with 10% fetal bovine serum (Sigma). Luciferase assays were performed as described (10). MEFs, HEK293T, and HeLa cells were cultured in DMEM containing 10% FBS. PA-1 cells were grown in 50:50 high-glucose DMEM and Ham's F12 with 10% FBS. COV434 and A2780 cells were cultured in RPMI-1640 with 10% FBS. Plasmids were transfected with Fugene 6 (Roche) or Lipofectamine 2000 (Invitrogen). siRNAs listed in Supplemental Table 1 were from Dharmacon or Ambion and transfected using Lipofectamine RNAiMax (Invitrogen).

Xenopus

Xenopus laevis eggs were collected and fertilized *in vitro* and animal caps prepared and cultured according to standard protocols (30), or processed for whole mount *in situ* hybridization according to standard protocols (31). RNA for injection was transcribed for β Gal, *Xwnt8*, and *ripk4* *in vitro* from restriction enzyme-digested plasmids with the mMessage Kit (Ambion) using SP6 polymerase. *X. laevis ripk4* clone BC043634 was from Open Biosystems. Morpholinos for Ripk4 (GeneTools LLC) were *ripk4*-MO1: 5' GGA CGC GCC CTC CTT ATC CAC CAT A (injected at 30ng/blastomere), and *ripk4*-MO2: 5' TAA TGT CAC CGC CGC GCC CCC GAG T (injected at 60ng/blastomere). Antisense digoxigenin probes were synthesized *in vitro* from plasmids for *engrailed2*, *krox20*, and *chordin*. RT-PCR primers were *xnr3*: 5'GTT TAT CTC CCC ACT GAT GGC GAT G, 5'GCT TTG GAC GGT ATC AGA TTC CTG; *epidermal keratin*: 5'CAC CAG AAC ACA GAG TAC, 5'CAA CCT TCC CAT CAA CCA; *xbra*: 5'GGA TCG TTA TCA CCT CTG, 5'GTG TAG TCT GTA GCA GCA; *ornithine decarboxylase (ODC)*: 5'GGG CTG GAT

CGT ATC GTA GA, 5'TGC CAG TGT GGT CTT GAC AT; and *siamois*: 5'GAA ACC ACT GAT TCA GGC AGA, 5'TTG CAA TAC GGC ATC TGT TC.

Western blotting and immunoprecipitations

Western blot and immunoprecipitations were performed as described (32). Cytosolic β -Catenin levels were determined after depleting cadherin-associated β -catenin with ConA-Sepharose (GE Healthcare) (33). To detect phosphorylated DVL2, cells were lysed in NP40 buffer (1% NP-40, 120 mM NaCl, 50 mM Tris-HCl pH 7.4, 1 mM EDTA pH 7.4) containing 6 M urea, plus protease and phosphatase inhibitors. Whole cell lysates were immunoprecipitated with DVL2 antibody (Cell Signaling Technology) overnight and then anti-rabbit IgG beads (Ebioscience) for 2 h. The beads were washed extensively with high salt buffer (20 mM HEPES pH 7.9, 1.5 mM MgCl₂, 420 mM NaCl, 0.2 mM EDTA, 25% glycerol) and then low salt buffer (20 mM Tris pH 7.4, 300 mM NaCl, 0.2 mM EDTA, 20% glycerol, 0.1% NP40). Eluted DVL2 protein was probed with phospho-specific DVL2 antibodies and HRP-conjugated anti-rabbit trueblot antibody (eBiosciences).

Recombinant RIPK4 kinase domain

Human RIPK4 residues 2–300 (wild-type or mutant K51R) were cloned into a modified pAcGP67 baculovirus transfer vector (BD Pharmingen) containing an N-terminal His6 tag followed by a Tobacco Etch Virus (TEV) protease site and a C-terminal FLAG tag. Both constructs include a fortuitous mutant (G²⁶⁸R) in the C-terminal lobe of the kinase away from the active site that does not affect kinase activity. RIPK4 protein expressed in Sf9 cells was purified at 4°C. Cells from a 5 L culture were re-suspended in lysis buffer (50mM Tris-HCl pH 8.0, 0.3M NaCl, 0.5mM TCEP, 5% glycerol, 50mM NaF, 5mM imidazole, plus Roche complete protease inhibitors cocktail EDTA-free), microfluidized, and centrifuged. Soluble RIPK4 was affinity purified with Ni-NTA Superflow resin (Qiagen). The column was washed in buffer A (25mM Tris-HCl pH 8, 1M NaCl, 5% glycerol, 0.5mM TCEP, 20mM imidazole) and RIPK4 proteins were eluted with buffer A containing 250mM imidazole. The His6 tag was removed by overnight dialysis with TEV protease in buffer B (20mM Tris-HCl pH 8.0, 0.2 M NaCl, 0.5 mM TCEP, 5% glycerol). Uncleaved material was removed by passage over a Ni-NTA Superflow column. The cleaved material was further purified using size exclusion chromatography with an S-75 column (GE Healthcare) in buffer C (20mM Tris-HCl pH 8.0, 150mM NaCl, 0.5mM TCEP, 5% glycerol).

In vitro kinase assay

DVL2-PDZ-FLAG (DVL2 residues 267–353, either wild-type or mutant S298A) and DVL2-DEP-FLAG (DVL2 residues 436–505, either wild-type or mutant S480A) were expressed using high-yield wheat germ extract (Promega) and then immunoprecipitated with anti-FLAG M2 beads overnight. The washed beads were used for *in vitro* kinase assays. Briefly, beads (50 μ L) were mixed with 0.1 μ g of RIPK4 kinase domain (wild-type or mutant K51R), 40 μ M ATP (Promega), and 30 μ Ci γ -³²P-ATP (Perkin Elmer) in kinase buffer (Cell Signaling Technology). Reactions were performed at room temperature for 1 h.

RNA sequencing and TaqMan

RNA was extracted with a Qiagen RNeasy kit. All TaqMan primers and probes were from Applied Biosystems. Reactions were performed in an ABI 7500 Real Time PCR System (Applied Biosystems). Data were analyzed with Sequence Detection Software v1.4 (Applied Biosystems). *GAPDH* mRNA levels were used to normalize *AXIN2*, *GAD1*, *SAX1*, *APCDD1* mRNA levels.

Quantitative analysis of DVL2 phosphorylation sites by RIP4

A SILAC experiment was performed as described (21). Briefly, HEK293 cells were cultured in DMEM SILAC media (Thermo) supplemented with 10% dialyzed FBS (Invitrogen), plus 50 µg/mL lysine +8, 40 µg/mL arginine +10 (Sigma) (heavy media), or regular 50 µg/mL lysine, 40 µg/mL arginine (Sigma) (light media) for more than 10 days. Labeling efficiency was verified by mass spectrometry. 500 µg of DVL2-FLAG-Myc was transfected into 2×10^8 HEK293T cells, along with 500 µg of RIPK4-FLAG (heavy media), or 500 µg of RIPK4-K51R-FLAG (light media) for 48 h. Cells were lysed in NP40 buffer. Equal amounts of the two lysates were mixed and immunoprecipitated with FLAG beads as described (34). Samples from the immunoprecipitation were digested with trypsin at 37°C overnight and phosphopeptides enriched using TiO₂. The resulting phosphopeptide mixture was injected onto a 0.1 × 100 mm column packed with 1.7 µm BEH-130 C18 using a NanoAcquity UPLC (Waters) and introduced to a LTQ-Orbitrap mass spectrometer (ThermoFisher Scientific) through an ADVANCE electrospray ionization source. Peptides were analyzed in data dependent top8 mode, with a full MS scan collected in the FTMS at 60,000 resolution and MS/MS collected in the ion trap on the top 8 most abundant species.

Generation of DVL2-expressing MEFs

DVL2-FLAG (wild-type and mutants) was cloned into pLenti6.3/V5-TOPO (Invitrogen). 5 µg of expression plasmid was mixed with 8.9 and VSVG at a molar ratio of 1:2.3:0.2, and transfected into HEK293T cells for 48 h. Filtered virus particles were used to infect *Dvl1*^{-/-} *Dvl2*^{-/-} *Dvl3*^{+/+} MEFs. The cells were then infected with lentiviral particles expressing *Dvl3* shRNA (Open Biosystems). The resulting cells were maintained in DMEM media containing 10% FBS, 2 µg/mL puromycin, and 10 µg/mL blasticidin.

Immunofluorescence microscopy

HeLa cells were transfected with DVL2-FLAG and RIPK4-GFP for 24–36 h. The cells were washed with PBS, fixed in 4% paraformaldehyde at room temperature for 25 min, washed again with PBS, and permeabilized with 0.3% Triton X-100 for 10 min. After blocking in 10% horse serum for 30 min, cells were stained with anti-FLAG antibody in 10% normal horse serum/0.3% Triton X-100 for 1 h. Bound antibody was revealed with Alexa568-conjugated anti-mouse antibody for 45 min. Cover slips were washed with PBS and mounted on microscope slides. Images were acquired on a Zeiss AxioImager.Z1 microscope (Intelligent Imaging Innovations) equipped with a CoolSnapHQ-cooled CCD camera (Roper Scientific) and a 63× PlanApochromat, NA 1.4 objective. Between 10–16 z sections at 0.3 µm intervals were acquired. Images were processed with Slidebook 4.2 software (Intelligent Imaging Innovation). Contrasts were adjusted identically for each series of panels.

Xenografts

N-Tera2 and HCT116 cells were cultured in McCoy's medium with 15% FBS. MMTV-Wnt1 tumor cells were plated in mammary cell culture media (Epicut; Stemcell Technology). Cells were incubated with lentiviral particles (Sigma-Aldrich) at multiple of infection (MOI) of 2–3. Infected cells were cultured in 1 µg/mL puromycin the next day. Resistant cells were trypsinized, washed, resuspended in HBSS: matrigel (1:1), and injected subcutaneously into the left flank of nude mice (~ 0.5 million cells per mouse). Tumor volumes were measured weekly.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. RIPK4 stimulates the canonical Wnt pathway

A. Heat map representation of RNA sequencing data from PA1 teratocarcinoma cells transfected with empty vector, RIPK1, RIPK2, RIPK3, or RIPK4 for 48 h, or treated with 200 ng/mL Wnt3a for 16 h.

B. HEK293 cells carrying a TOPbrite luciferase reporter were transfected in the absence or presence of Wnt3a-conditioned media for 5 h. Error bars represent the s.e.m. of triplicate luciferase measurements. Results are representative of 3 independent experiments.

C. HEK293T cells transfected for 48 h. Transcripts levels were based on the same experiment.

D. HEK293T cells transfected for 48 h.

E. A2780 or COV434 cells were transfected with control or *RIPK4* siRNAs for 60 h and then given vehicle or Wnt3a for 2 h.

Fig. 2. *ripk4* modulates Wnt signaling in *Xenopus*

A. Secondary axis formation in *Xenopus* embryos injected ventral-vegetally with *ripk4* (1 ng), *xwnt8* (1 pg), or both. A total of 269 embryos were examined in 3 independent experiments. Dorsal views of representative embryos are shown.

B. Semi-quantitative RT-PCR of animal caps derived from embryos injected with Xwnt8 alone or together with *ripk4*-MO1 and assayed for expression of direct Wnt target genes *xnr3* and *siamois*.

C. Length of *chordin* expression along the blastopore lip. Embryos were injected in 2 dorsal cells at 4-cell stage. Bars represent the mean $\pm$ s.e.m of 108 embryos. Vegetal views of representative embryos are shown.

D. Hindbrain marker gene expression (*engrailed2* and *krox20*, blue) following single blastomere injection at the two-cell stage (*ripk4*-MO1, *ripk4*-MO2, sorted by fluorescence of co-injected fluorescein tracer) or the four-cell stage (all others). Injected side is on the right with anterior on top. Red staining is due to nuclear β -galactosidase tracer.

Fig. 3. RIPK4 phosphorylates DVL to promote canonical Wnt signaling

A. HEK293T cells were transfected with control (Ctrl) or *RIPK4* siRNAs for 72 h and then given vehicle or 200 ng/mL Wnt3a for 30 min.

B. RIPK4-Myc and DVL2-FLAG were expressed with rabbit reticulocyte and wheat germ *in vitro* translation systems, respectively. The proteins were mixed and immunoprecipitated with anti-FLAG beads.

C. HEK293T cells treated with 200 ng/mL Wnt3a.

D. *In vitro* kinase assays using the RIPK4 kinase domain (wild-type or K51R mutant) and FLAG-tagged DEP or PDZ domains of DVL2.

E. HEK293T cells transfected with control or *RIPK4* siRNA for 72 h, and then given vehicle or 200 ng/mL Wnt3a for 10 min. Non-specific bands (*).

F. *Dvl*-null MEFs reconstituted with empty vector or DVL2 (wild-type or mutant S298A S480A) and treated with Wnt3a.

G. Immunofluorescence microscopy of HeLa cells transfected with RIPK4-GFP and DVL2-FLAG for 36 h. Cells containing DVL2 puncta (signalosome) were enumerated by counting 250 cells per condition. Scale bars: 10 μm.

Fig. 4. Knockdown of *RIPK4* delays Wnt-dependent xenograft tumor growth

A. Microarray analysis of *RIPK4* transcription in human colorectal, ovarian, and melanoma samples. N: non-cancerous tissues; C: cancer.

B. Western blotting of human ovarian adenocarcinomas and non-cancerous ovarian tissue samples.

C. Box and whisker plots indicate *RIPK4* and cytoplasmic β -catenin levels after quantitation by densitometry of 4B. Error bars represent the s.e.m. (n=6 for non-cancerous, and n=9 for tumors).

D–G. N-Tera2 and HCT116 cells were transduced with lentiviral particles encoding *RIPK4* or control (Ctrl) shRNAs (C) and injected subcutaneously into athymic nude mice (D–F).

E. Representative tumor-bearing mice and dissected tumors after 47 days.

F. Average tumor volumes. Error bars represent the s.e.m. (n=10).

G. *GAD1* and *AXIN2* expression in the xenograft tumors. Error bars represent s.e.m. (n=8).