Loss of Dact1 Disrupts Planar Cell Polarity Signaling by Altering Dishevelled Activity and Leads to Posterior Malformation in Mice*

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Wnt signaling plays a key role in embryogenesis and cancer development. Dvl (Dishevelled) is a central mediator for both the canonical and noncanonical Wnt pathways. Dact1 (Dapper, Dpr), a Dvl interactor, has been shown to negatively modulate Wnt signaling by promoting lysosomal degradation of Dvl. Here we report that Dact1-deficient mice have multiple physiological defects that resemble the human neonate disease congenital cystic adenomatoid malformation, renal agenesis/dysplasia, fused kidneys, and loss of bladder. These urogenital defects can be traced to impaired hindgut formation starting at embryonic day 8.25. Examination of morphological changes and Wnt target gene expression revealed that the planar cell polarity (PCP) signaling is deregulated, whereas the canonical Wnt/β-catenin pathway is largely unaffected in mutant embryos. Consistently, the activity of the PCP signal mediators Rho GTPase and c-Jun N-terminal kinase is altered in Dact1−/− mouse embryonic fibroblasts. We further observed alterations in the protein level and the cellular distribution of Dvl in the primitive streak of mutant embryos. An increased amount of Dvl2 tends to be accumulated in the cortical regions of the cells, especially at the primitive streak ectoderm close to the posterior endoderm that laterly forms the hindgut diverticulum. Together, these data suggest that Dact1 may regulate vertebrate PCP by controlling the level and the cellular localization of Dvl protein.

Wnt signaling is crucial to determine the fates of cells during embryogenesis and for tissue homeostasis after birth. The alteration of Wnt signaling results in human congenital diseases and carcinogenesis (1–3). Wnt signaling is instigated by binding of extracellular Wnt ligands to Fz (Frizzled) transmembrane receptors, leading to membrane recruitment and activation of Dvl. From Dvl, Wnt signals converge into at least two branches: the β-catenin-dependent canonical pathway and the β-cate

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The abbreviations used are: PCP, planar cell polarity; JNK, c-Jun N-terminal kinase; MEF, mouse embryonic fibroblast; En, embryonic day n; GFP, green fluorescent protein; CMV, cytomegalovirus; RT, reverse transcription; ES cell, embryonic stem cell.
in zebrafish (28), similar to the function of Dact1 homolog Frodo in Xenopus (32). Our studies demonstrated that zebrafish and mouse Dact2 inhibit transforming growth factor-β/Nodal signaling during mesoderm induction by promoting lysosomal degradation of transforming growth factor-β type I receptors (27, 33). Zebrafish Dact2 may be also involved in the Wnt/PCP pathway because it is required for normal convergence extension movements in embryos (28). Human Dact3 has been shown to act as a negative regulator of Wnt/β-catenin signaling and may be associated with colorectal cancer formation (34). Consistent with the role of Dact3 in tumorigenesis, human Dact1 is down-regulated in hepatocellular carcinoma, and this down-regulation was correlated with the cytoplasm accumulation of β-catenin (35).

In the present study, we generated Dact1-deficient (Dact1<sup>−/−</sup>) mice. Embryonic analyses revealed severe posterior malformations resulting from impaired hindgut development at E8–8.5. Furthermore, we found that PCP signaling was disrupted, whereas the canonical Wnt signaling remains largely normal in this region of Dact1<sup>−/−</sup> mice. The inactivation of Dact1 resulted in the enhanced expression and altered cellular distribution of Dvl2 in the malformed ectoderm of the primitive streak region. Consistent with the above findings, Dvl protein levels were also increased in Dact1<sup>−/−</sup> mouse embryonic fibroblasts (MEFs) in compared with wild-type cells. Our findings suggested that the dysfunctions of PCP signaling mediated by the elevated activity of Dvl might account for the posterior malformations in Dact1<sup>−/−</sup> mice.

**EXPERIMENTAL PROCEDURES**

**Generation of Dact1<sup>−/−</sup> Mice**—All of the mice used in this study were bred and maintained on a normal 12-h light/12-h dark cycle and provided regular mouse chow and water ad libitum in an Association for Assessment and Accreditation of Laboratory Animal Care-accredited specific pathogen-free facility (Model Animal Research Center, Nanjing University). A 3.5-kb sequence containing 3′ sequence of the Dact1 gene was amplified by PCR from a 129/Sv mouse genomic library using two primers (5′-CTCGAGATTATCTGGTTTGACATA-GCTC-3′ and 5′-GGGCGCGATGGTATATACCGAGACC-3′), which contain Xhol/EcoRV and NotI restriction sites, respectively. The amplified fragment was inserted into the Xhol-NotI sites of the vector ploxP (36) to generate ploxP-Dact1<sup>−/−</sup> targeting vector. The Southern probe was prepared by digesting the plasmid with Hpal and EcoRV and detected a 16-kb fragment from the wild-type allele as well as a 5.5-kb fragment from the targeted allele (see Fig. 1).

**Genotypic Analysis**—The mice were genotyped by Southern blot as described above or by PCR. For PCR analysis, the primers GDAPIF1 (5′-TAACGAGGGCAACAGGAG-3′) and GDAPIR1 (5′-AAAGCTTATCTGCGATC-3′) were amplified from the Dact1 genomic sequence amplified a 400-bp fragment from the wild-type allele (see Fig. 1). The combinatory use of primer GDAPIF1 and primer Neo1 (5′-TTCTGGATTCATCGAC-GTGG-3′) amplified a 1-kb fragment from the mutant allele.

**Histology and Immunohistochemistry**—Tissues were dissected and fixed in 4% paraformaldehyde at 4 °C overnight, dehydrated, embedded in paraffin, sectioned at 8 μm, and stained with hematoxylin and eosin. Photographs were taken using an Olympus light microscope with a digital camera. Immunofluorescence assays were performed on frozen sections. α-Dvl2 was from Cell Signaling, and rhodamine phalloidin was from Invitrogen. E8–8.5 embryo transverse sections (15 μm) were examined under a Zeiss LSM 710 laser scanning microscope at identical illumination conditions and further processed with ZEN 2009 Software (Carl Zeiss). Integral optical density of the ectoderm area was measured using Image-Pro Plus 6.0 software (Media Cybernetics), and equal-sized squares along the ectoderm were measured in wild-type or Dact1<sup>−/−</sup> embryos.

**Skeletal Staining and Whole Mount in Situ Hybridization**—Skeletal staining with Alcian Blue and Alizarin Red and whole mount in situ hybridization were performed as described (38, 39). The Dact1 in situ probe corresponding to its coding sequence (1250–1601 nucleotides) was cloned as described (30). The Axin2 probe was kindly provided by Drs. F. Costantini and E.-H. Jho (40), and the probes of Shh (sonic hedgehog), Pax1 (paired box gene 1), and Pax3 (paired box gene 3) were described (41).

**Cell Culture and Lentiviral Constructs**—All of the cell lines were maintained in Dulbecco’s modified essential medium supplemented with 1% penicillin-streptomycin, 10% fetal calf serum at 37 °C in a humidified, 5% CO₂ incubator. Mouse Wnt3a-conditioned medium was produced as previously described (31). Primary MEFs were isolated and cultured as previously described (39). The Dact1 overexpression in MEFs was reintroduced by a lentiviral system, using GFP as control. pHR<sup>−</sup>-CMV-GFP lentiviral constructs were described previously (42). The Dact1 cDNA was inserted into the BamHI-Xhol sites of pHR<sup>−</sup>-CMV-GFP to replace the GFP fragment. The vesicular stomatitis virus glycoprotein pseudotyped lentiviral vector was generated by transient cotransfection of the vector construct pHR<sup>−</sup>-CMV-GFP or pHR<sup>−</sup>-CMV-Dact1 (12 μg) with the vesicular stomatitis virus glycoprotein-expressing construct pMD.G (6 μg) and the packaging construct pCMV-DAR8.9 (9 μg) into 293T cells. The medium was replaced with fresh medium at 12 h after transfection. The lentivirus was harvested at 36 and 60 h after transfection. The cells were harvested at 72 h after lentiviral infection.

**Glutathione S-Transferase-Rho-binding Domain Binding Assay and Immunoblotting**—Glutathione S-transferase-Rho-binding domain binding assay for detection of GTP binding activity of RhoA was performed as described (43). For immunoblotting, the cells in 100% confluency were lysed at 4 °C for 10 min with lysis solution (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM...
EDTA, 0.5% Nonidet P-40, 10 mM NaF, 20 mM sodium β-glycerophosphate, 1 mM dithiothreitol, and protease inhibitors), and the lysates were analyzed by SDS-PAGE, immunoblotted with various antibodies, and detected with an enhanced chemo-luminescent substrate (Pierce). The antibodies used were as follows: α-Dvl2 (Cell Signaling), α-activated β-catenin (Millipore), α-β-catenin (Zymed Laboratories Inc.), α-β-actin (Sigma), α-Dvl3 (kindly provided by Dr. Lin Li), α-Dpr1 (described in Ref. 29). Other antibodies were from Santa Cruz Biotechnology.

Luciferase Assays—MEFs were seeded in 24-well dishes in a primary density of 8 × 10^4 cells/well for 18 h prior to transfection. Transfection was performed with Lipofectamine 1D and RT-PCR (not shown).

Majority of Dact1^−/− Neonates Were Born Runt and Perinatally Lethal—Dact1^+/− mice were first bred in a mixed B6;129/SV background and then backcrossed for more than five generations to C57BL/6 background before use in this study. Strikingly, there were 25 Dact1^+/+, 50 Dact1^+/−, and only one Dact1^−/− in 76 adult mice from F2 Dact1^+/− intercrosses, which was dramatically distinctive from the expected Mendelian segregation, suggesting that Dact1^−/− mouse died either at embryonic stages or before weaning. Indeed, isolation of E18.5 embryos was verified by whole mount in situ hybridization (Fig. 1A). Southern blot (Fig. 1B) and genotyping PCR (Fig. 1C) were employed to identify the targeted allele in ES cells, mice, and embryos. The absence of Dact1 transcripts in Dact1^−/− embryos was verified by whole mount in situ hybridization (Fig. 1D) and RT-PCR (not shown).

RESULTS

Generation of Dact1-deficient Mice—We generated constitutive Dact1 knock-out mice by conventional knock-out approaches using 129/SV ES cells. A 2-kb fragment including a part of the promoter region, entire exon 1 containing the translation initiation code ATG and partial intron 1 was replaced by a reversed neomycin resistance cassette (Fig. 1A). Southern blot (Fig. 1B) and genotyping PCR (Fig. 1C) were employed to identify the targeted allele in ES cells, mice, and embryos. The absence of Dact1 transcripts in Dact1^−/− embryos was verified by whole mount in situ hybridization (Fig. 1D) and RT-PCR (not shown).

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FIGURE 1. Targeted inactivation of the Dact1 gene. A, diagrams of the wild-type Dact1 allele (top), the construct used for generating the targeted allele (middle), and the inactivated gene after homologous recombination (bottom). A PGKneo cassette was inserted in the opposite orientation relative to Dact1 transcription, and the promoter region, exon 1, and partial intron 1 were deleted to generate targeted allele (Fig. 1A). Southern blot analysis of ES cells. Using the 3′-flanking probe indicated in Fig. 1A, a 5.5-kb EcoRV fragment appears in the targeted clone in addition to the 16-kb EcoRV wild-type fragment. C, PCR genotyping of genomic tail DNA. Using the PCR primer pairs indicated in A, wild-type and knock-out loci generate 400- and 1000-bp fragments, respectively. D, whole mount in situ hybridization shows the absence of Dact1 transcripts in Dact1^−/− embryos. Scale bar, 5 mm.
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FIGURE 2. Dact1−/− mice are perinatal lethal with posterior malformation associated with severe urogenital defects. A, Dact1−/− mutants are born runt, with a short body and a short tail (arrowhead), compared with its wild-type and heterozygous siblings. B, double staining of newborn skeletons. E18.5 embryos were stained with Alizarin Red (bone) and Alcian Blue (cartilage). Most of the skeletons are the same between wild type and mutants, except for the caudal vertebrae (red arrowheads). The mutant exhibited malformed sacral vertebrae and condensed lumbar vertebrae (the region between the red arrows). More than four embryos were examined. C, the urogenital system outline of E17.5 embryos. The mutant (lower panels) showed abnormal fused hydromeophic kidneys (k), lack of bladder (b), unidentified ureters (ur), misconnected blind-ended ureters (female, ut) or van deferens (male, vd), compared with the heterozygous siblings (upper panels). In contrast, the adrenal glands (a), ovaries (o), and testes (t) of mutant embryos were in normal size and right position. D, phenotypes of Dact1−/− surviving female adult. The mutant 10-month-old female had mild cystic kidneys (k), lack of vagina (v) (left panel), and uterine (ut) hydrometra (upper right panel). The lower right panel showed no vagina open (vog) adjacent to the anal canal (ac). E, hematoxylin- and eosin-stained mid-sagittal section of E13.5 Dact1−/− (left panel) and Dact1+/− (right panel) specimens. The mutant showed a mass of undifferentiated urogenital tissues, instead of developing urogenital tract seen in its heterozygous sibling, ac, anal canal; b, bladder; gt, genital tubercle; uc, umbilical cord; v, urethral plate; udus, undifferentiated urogenital tissues. More than four embryos were examined. F, Shh expression in developing caudal region of wild-type (upper panel) and Dact1−/− (lower panel) embryos at E8.25 (6 somites) (left panel, ventral view), E9 (13 somites) (middle panel, lateral view), and E10.5 (right panel, lateral view), as shown by whole mount in situ hybridization. Shh mainly expressed in notochord (nc), and gut epithelia, including midgut (mg) and hindgut (hg). The mutant showed truncated and no dilated hindgut and shorter notochord (left and middle panels). At E10.5, no cloaca (Ca) swelled in mutant embryos. More than five mutant embryos have been employed for each stage. st, somites; scale bar, 5 mm in A, B, and D, and 0.5 mm in C, E, and F.

that the death of most of Dact1−/− mice occurred perinatally. The only surviving female adult of Dact1−/− was viable but infertile.

To understand what caused the death of Dact1−/− mice, newborn pups were examined. Dact1−/− pups were born with a short body and a short tail (Fig. 2A), compared with Dact1+/+ and Dact1−/+ siblings, indicating causal developmental defects as observed in Wnt3a, Wnt5a, low density lipoprotein receptor-related protein 6, and Ltap-deficient mice (9, 13, 44, 45). Scrutiny of litters at birth showed that newborn Dact1−/− pups died within 24 h. To investigate the short and somewhat curly body of Dact1−/− embryos, we examined bone and cartilage development using Alizarin Red and Alcian Blue staining at E18.5. Major defects were localized caudally in the coccyges (Fig. 2B). Consistent to the observation of the loss of most caudal coccyges, we also found the caudal lumbar vertebrae and the ilia were often malformed. However, the sacral vertebrae remained intact. Total lumbar numbers of Dact1−/− embryos were equivalent to that of wild type, but the intervals between lumbar vertebrae were slightly decreased in Dact1−/− (Fig. 2B), implying mild defects of somitogenesis and segmentation at late stages in the last few somites. The numbers and shapes of thoracic vertebrae, ribs, and limbs were grossly normal.

Dact1−/− Lethality Is Due to Urogenital Defects—In addition to short tails in Dact1−/− neonates, the external genitalia and anuses, as well as the outlet of urethrae, were absent (15 of 15) (Fig. 2E). The severity of these defects could contribute to the perinatal lethality of Dact1−/− newborn pups (44). Inside, Dact1−/− embryos exhibited severe defects on kidneys and the urogenital tracts (Fig. 2C). Renal malformations were observed from E12.5 to postnatal day 0. 84% of Dact1−/− embryos showed mid-fusion (16 of 19), 11% showed unilateral agenesis (2 of 19), and 5% had no kidney (1 of 19) under the most severe circumstances. 11 of 12 Dact1−/− embryos at E17.0 or later stages showed typical hydromeophrosis, whereas the one without hydromeophrotic symptoms had one kidney and entire ureter-bladder tract. 18 of 19 Dact1−/− embryos mentioned above had no bladders. The
No Cloaca Is Formed at the Early Developmental Stages of Dact1−/− Embryos—After defining the urogenital defects in Dact1−/− embryo at E18.5, we decided to trace back to the earlier stages when the defects emerged in mutant embryos. First it was confirmed that there were no developing genital tubercles and the total sets of bladder, urethra, and anal canal in Dact1−/− embryos (46). We then observed the morphologic changes in embryos (46). We then observed the morphologic changes in earlier stages when the defects emerged in mutant embryos. Earlier stages when the defects emerged in mutant embryos. Earlier stages when the defects emerged in mutant embryos. Earlier stages when the defects emerged in mutant embryos.

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Defects in the hindgut of Dact1−/− embryos (Fig. 3B, C). Although Dact1−/− embryos appeared disorganized at the caudal end, as indicated by the length of the presomitic mesoderm, no somites were affected at this stage. These results suggested that paraxial mesoderm fate is unaffected in Dact1−/− embryos.

To further confirm that there was no obvious anomaly for the canonical Wnt/β-catenin signaling in Dact1−/− embryos, we isolated MEFs and determined the active β-catenin level (Fig. 3E). The canonical Wnt signaling showed no significant differences in Dact1−/− MEFs in comparison with wild-type cells. However, the increased level of Dvl2 protein was clearly noticed in Dact1−/− embryos (Fig. 3D, E). We also noted that the increased level of Dvl2 protein was clearly noticed in Dact1−/− embryos (Fig. 3D, E). We also noted that the increased level of Dvl2 protein was clearly noticed in Dact1−/− embryos (Fig. 3D, E). We also noted that the increased level of Dvl2 protein was clearly noticed in Dact1−/− embryos (Fig. 3D, E). We also noted that the increased level of Dvl2 protein was clearly noticed in Dact1−/− embryos (Fig. 3D, E). We also noted that the increased level of Dvl2 protein was clearly noticed in Dact1−/− embryos (Fig. 3D, E). We also noted that the increased level of Dvl2 protein was clearly noticed in Dact1−/− embryos (Fig. 3D, E). We also noted that the increased level of Dvl2 protein was clearly noticed in Dact1−/− embryos (Fig. 3D, E). We also noted that the increased level of Dvl2 protein was clearly noticed in Dact1−/− embryos (Fig. 3D, E). We also noted that the increased level of Dvl2 protein was clearly noticed in Dact1−/− embryos (Fig. 3D, E). We also noted that the increased level of Dvl2 protein was clearly noticed in Dact1−/− embryos (Fig. 3D, E). We also noted that the increased level of Dvl2 protein was clearly noticed in Dact1−/− embryos (Fig. 3D, E). We also noted that the increased level of Dvl2 protein was clearly noticed in Dact1−/− embryos (Fig. 3D, E). We also noted that the increased level of Dvl2 protein was clearly noticed in Dact1−/− embryos (Fig. 3D, E). We also noted that the increased level of Dvl2 protein was clearly noticed in Dact1−/− embryos (Fig. 3D, E). We also noted that the increased level of Dvl2 protein was clearly noticed in Dact1−/− embryos (Fig. 3D, E). We also noted that the increased level of Dvl2 protein was clearly noticed in Dact1−/− embryos (Fig. 3D, E). We also noted that the increased level of Dvl2 protein was clearly noticed in Dact1−/− embryos (Fig. 3D, E). We also noted that the increased level of Dvl2 protein was clearly noticed in Dact1−/− embryos (Fig. 3D, E). We also noted that the increased level of Dvl2 protein was clearly noticed in Dact1−/− embryos (Fig. 3D, E). We also noted that the increased level of Dvl2 protein was clearly noticed in Dact1−/− embryos (Fig. 3D, E). We also noted that the increased level of Dvl2 protein was clearly noticed in Dact1−/− embryos (Fig. 3D, E). We also noted that the increased level of Dvl2 protein was clearly noticed in Dact1−/− embryos (Fig. 3D, E). We also noted that the increased level of Dvl2 protein was clearly noticed in Dact1−/− embryos (Fig. 3D, E). We also noted that the increased level of Dvl2 protein was clearly noticed in Dact1−/− embryos (Fig. 3D, E). We also noted that the increased level of Dvl2 protein was clearly noticed in Dact1−/− embryos (Fig. 3D, E). We also noted that the increased level of Dvl2 protein was clearly noticed in Dact1−/− embryos (Fig. 3D, E). We also noted that the increased level of Dvl2 protein was clearly noticed in Dact1−/− embryos (Fig. 3D, E). We also noted that the increased level of Dvl2 protein was clearly noticed in Dact1−/− embryos (Fig. 3D, E). We also noted that the increased level of Dvl2 protein was clearly noticed in Dact1−/− embryos (Fig. 3D, E). We also noted that the increased level of Dvl2 protein was clearly noticed in Dact1−/− embryos (Fig. 3D, E).

The PCP Signaling Is Deregulated in Dact1−/− Embryos—Because Dact1 is a negative regulator of both canonical and non-canonical Wnt signaling (25, 29), we speculated that PCP signaling might be altered in Dact1−/− mutants. Indeed, the
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neural tube in Dact1−/− embryos was significantly broader than in wild-type siblings (Fig. 4A), suggesting defects in neural tube elongation. In agreement with this, we observed significantly broadened neural tubes in E9.5 Dact1−/− embryo tail buds (Fig. 4B), indicating that Dact1 plays a role in neural plate convergent extension and neural tube closure (21). These data together suggest that PCP signaling was deregulated in the absence of Dact1, which was further supported by the data that RhoA GTPase and JNK activities, markers of PCP signaling, were changed with less active RhoA but more active JNK in Dact1−/− MEFs (Fig. 4, C and D). Consistent with the increased level of Dvl2 in Dact1−/− MEFs, Dvl3 was also increased (Dvl1 was undetectable in MEFs) (Fig. 4D). The effect of Dact1 on PCP signaling was further confirmed by reintroduction of Dact1 into Dact1−/− MEFs, and Dact1 expression restored Dvl2/3, p-JNK, and active RhoA levels in Dact1−/− MEFs similar to the ones in wild-type cells (Fig. 4, C and D).

Then we investigated whether there are any alterations in the level and distribution of Dvl2 protein in the hindgut region, where the morphologic abnormalities occurred at E8.25 Dact1−/− embryos (Fig. 4E; note the malformed endoderm). Immunofluorescence staining revealed an increased expression of Dvl2 protein in the primitive streak area, where hindgut starts to form, in Dact1−/− embryos, especially in the ectoderm region (Fig. 4, E and F). Interestingly, remarkable intensified staining of Dvl2 was also observed in the cortical regions of the ectodermal cells in Dact1−/− embryos, whereas the intensity tended to evenly distribute in the corresponding region of wild type (Fig. 4E). The disrupted cell polarity, signed by phalloidin-stained F-actin, was also observed in the ectoderm of Dact1−/− embryos at E8.5 (7–8 somites) (Fig. 4G), albeit not in E8.25 (5 somites) (Fig. 4E). Together, these data suggest that the altered Dvl protein level and distribution by inactivation of Dact1 may lead to deregulation of PCP signaling in the primitive streak, which contributes to the posterior malformation in Dact1−/− embryos.

**DISCUSSION**

Dact1 functions as a negative regulator of Wnt signaling by promoting Dvl degradation. In this study, we showed that inactivation of the Dact1 gene leads to posterior malformation and perinatal lethality of mice. The urogenital defects are due to impaired hindgut formation in the primitive streak at E8.25. Further analyses revealed that the PCP signaling, but not the canonical Wnt signaling, is deregulated, which most likely results from the altered protein level and distribution of Dvl2 in the ectoderm region of the primitive streak.

The phenotypes of Dact1 null mice resemble the human neone-date disease congenital caudal regression syndrome, and the defective phenotypes include caudal vertebral agenesis, anorectal malformation, renal agenesis/dysplasia, fused kidneys, as well as bladder loss (49–51). However, only a small percentage of Dact1−/− embryos mimic the sacral agenesis symptom of congenital caudal regression syndrome, suggesting a more specified syndrome spectrum in Dact1−/− embryos. Developmental defects of kidneys in Dact1−/− mice could be traced back to E11.5 when the ureteric bud branches into metanephric mesenchyme and begins to generate the definitive renal collecting system and to induce formation of renal vesicles (52, 53). Despite the loss of cloaca, the Wolffian duct elongates and the ureteric bud develops normally in Dact1−/− embryos. However, the two metanephric rudiments derived from the ureteric bud are much closer to each other than in wild type. Furthermore, no Dact1 mRNA expression was detected in the metanephros. This suggested that the severe kidney abnormalities may occur secondarily, which can be partly attributed to the small volume of the caudal parts. Dact1−/− embryos display an intact paraxial mesoderm, an abnormally closed posterior neuroectoderm, but a twisted notochord and a significantly impaired caudal endoderm, but Dact1 mRNA is mainly expressed in the posterior ectoderm and mesoderm (30) (data not shown). Therefore, the abnormal phenotypes are not well correlated with Dact1 mRNA expression. This may be due to the discordant protein and mRNA expression patterns (54). Unfortunately, the lack of specific antibodies against Dact1 protein prohibits us from examining Dact1 protein expression. Another possibility is that the cells in the posterior ectoderm and mesoderm may produce signals that are required for proper development of not only the neuroectoderm and notochord but also the adjacent endoderm. Posterior endoderm started to become malformed at the site where notochord is adjacent to the gut, indicating that the molecular changes in notochord possibly influence hindgut formation, as previously reported (55).

Our findings suggested that the phenotypes observed in Dact1−/− embryos result from the altered expression and distribution of Dvl protein. Previous work has linked the abnormalities in cardiac outflow tract, coelom, and neural tube closure in Dvl knock-outs to impaired PCP signaling (24). The defective neural tube closure and posterior malformation were also observed in Dact1−/− embryos, indicating that PCP signaling was disrupted by Dact1 inactivation. Both gain-of-function and loss-of-function of Dsh/Dvl have been reported to result in convergent extension movement defect in Xenopus embryos (56–59). We have also observed altered Dvl2 distribution in the cortical regions of the cells in the Dact1−/− E8.25 primitive streak ectoderm. The subcellular distribution of Dvl may be directly associated to its physiological functions in regulating PCP. It was reported that the DEP domain, which plays an essential role in Dsh/Dvl-mediated PCP signaling in Drosophila and Xenopus (21, 56–59), was required for Dvl2 to regulate neurulation in mouse embryos (23). Consistent with the role of Dact1 in PCP signaling, the alteration of RhoA and JNK activity was observed in Dact1−/− MEFs. The change of RhoA and JNK activities in opposing directions was reported in disrupted PCP signaling related to mouse neural tube closure (60, 61). However, the defects of Dact1−/− mice might also be due to other mechanisms because Dact1 has been reported to regulate Wnt signaling independent of the Dvl pathway (31, 62, 63).

During the preparation of this manuscript, Suriben et al. (64) reported a Dact1−/− mutant with a deletion of exon 2. The phenotypes of that mutant, including perinatal lethality, caudal regression, absent bladder, and the fused hydronephric kidneys, are very similar to those of our mutant. They also found that PCP signaling, but not canonical Wnt signaling, is responsible for the posterior malformation in hindgut. The difference
FIGURE 4. **Deregulation of the PCP signaling is associated with altered Dvl activities in Dact1−/− mice.**

A, E15.5 wild-type and Dact1−/− embryos transversely sectioned through the lumbar. The neural tube was significantly wider in mutants than in wild-type siblings. At the E15.5 and postnatal day 0 stages, ratios of medial-lateral to dorsal-ventral axis were statistically analyzed in Dact1−/− and Dact1+/− mice, respectively. **, p < 0.01; *, p < 0.05. More than four embryos were examined.

B, malformed neural tube closure (arrowhead) is shown in E9.5 Dact1−/− embryos in the last several somites by whole mount in situ hybridization staining of Pax3 (dorsal view).

C, decreased level of RhoA-GTP in Dact1−/− MEF as shown by pulldown assay. Glutathione S-transferase-Rho-binding domain, which specifically binds to RhoA-GTP (50 μg each), was added in equal cell lysates of Dact1+/− and Dact1−/− MEF with or without Dact1 overexpression. After incubation in 4 °C for 1 h, RhoA was examined by anti-RhoA immunoblotting. Inputs (10% of total lysates) are shown in the lower panels. The endogenous protein levels of Dact1 were not detected because of the limited antibody sensitivity. The band density was quantitated by BandScan software (Glyko), and the relative density of pulled down RhoA was normalized to the total RhoA (input) in the same sample.

D, MEF lysates (the same batch of cells prepared as in C) were subjected to immunoblotting with the indicated antibodies, and tubulin was used as a loading control. The relative density of Dvl2/3 in each lane was normalized to tubulin in the same sample, and the relative density of p-JNK was normalized by total JNK.

E, E8.25 (5 somites) embryos were transversely sectioned at the primitive streak region, and indirect anti-Dvl2 immunofluorescence (green) was performed. Phalloidin stains F-actin (red). The images were taken by a Zeiss LSM 710 laser scanning microscope under identical illumination conditions and processed with Zen2009 software. Insets, a higher magnification highlights Dvl2 distribution patterns in ectoderm cells. A straight line indicates the direction from basal to apical side of two adjacent cells (designated as 1 and 2 sequentially), and signal intensity of Dvl2 (green) or phalloidin (red) along the line was shown in the right graphs. The yellow and blue arrows point to the cell boundary, and they correspond to the points shown as lines of the same colors in the right graphs. ec, ectoderm; en, endoderm. F, integral optical density of nine equal-sized squares along the ectoderm was measured in wild-type or Dact1−/− embryos, respectively, with Image-Pro Plus 6.0 software. Dvl2 signal average intensity = (integral optical density sum)/area. **, p < 0.01. G, phalloidin staining of F-actin in the primitive streak region of E8.5 (7–8 somites) embryos. Scale bars, 20 μm in E and G; 0.5 mm in A and B.
between these works is the possible molecular mechanism underlying these defects. They reported that Dact1 is genetically interacted with a Pcp component Vangl2, whereas our data showed that alteration of Dvl activity may account for Dact1 loss-induced defects. We found that the increase of Dvl2 protein expression was restricted in the ectoderm and adjacent mesoderm in the primitive streak regions. Nonetheless, these works together demonstrate an essential role for Dact1 in the modulation of posterior development in mouse embryos.

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REFERENCES

1. Moon, R. T., Kohn, A. D., De Ferrari, G. V., and Kaykas, A. (2004) Nat. Rev. Genet. 5, 691–701
2. Clevers, H. (2002) Cell 109, 469–480
3. Logan, C. Y., and Nusse, R. (2004) Annu. Rev. Cell Dev. Biol. 20, 781–810
4. Angers, S., and Moon, R. T. (2009) Nat. Rev. Mol. Cell Biol. 10, 468–477
5. Wang, J., and Wynshaw-Boris, A. (2004) Curr. Opin. Genet. Dev. 14, 533–539
6. Huang, H., and He, X. (2008) Trends Biochem. Sci., in press
7. Zallen, J. A. (2007) Curr. Opin. Genet. Dev. 17, 482–488
8. Seifert, J. R., and Mlodzik, M. (2007) Nat. Rev. Genet. 8, 126–138
9. Wang, Y., and Nathans, J. (2007) Development 134, 647–658
10. Simons, M., and Mlodzik, M. (2008) Annu. Rev. Cell Dev. Biol. 24, 517–540
11. Hamblet, N. S., Lijam, N., Ruiz-Lozano, P., Wang, J., Yang, Y., Luo, Z., Mei, L., Chien, K. R., Sussman, D. J., and Wynshaw-Boris, A. (2002) Development 129, 5827–5838
12. Wallingford, J. B., and Harland, R. M. (2002) Development 129, 5815–5825
13. Wang, J., Mark, S., Zhang, X., Qian, D., Yoo, S. J., Radde-Gallwitz, K., Zhang, Y., Lin, X., Collazo, A., Wynshaw-Boris, A., and Chen, P. (2005) Nat. Genet. 37, 980–985
14. Wang, J., Hamblet, N. S., Mark, S., Dickinson, M. E., Brinkman, B. C., Segil, N., Fraser, S. E., Chen, P., Wallingford, J. B., and Wynshaw-Boris, A. (2006) Development 133, 1767–1778
15. Etheridge, S. L., Ray, S., Li, S., Hamblet, N. S., Lijam, N., Tsang, M., Greer, J., Kardos, N., Wang, J., Sussman, D. J., Chen, P., and Wynshaw-Boris, A. (2008) PLoS Genet. 4, e1000259
16. Cheyette, B. N., Waxman, J. S., Miller, J. R., Takemaru, K., Sheldahl, L. C., Khebitsova, N., Fox, E. P., Earnest, T., and Moon, R. T. (2002) Dev. Cell 2, 449–461
17. Katoh, M., and Katoh, M. (2003) Int. J. Oncol. 22, 907–913
18. 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
19. Waxman, J. S., Hocking, A. M., Stoick, C. L., and Moon, R. T. (2004) Development 131, 5909–5921
20. Zhang, L., Gao, X., Wen, J., Ning, Y., and Chen, Y. G. (2006) J. Biol. Chem. 281, 8607–8612
21. Fisher, D. A., Kivimäie, S., Hoshino, J., Suriben, R., Martin, P. M., Baxter, N., and Cheyette, B. N. (2006) Dev. Dyn. 235, 2620–2630
22. Gao, X., Wen, J., Zhang, L., Li, X., Ning, Y., Meng, A., and Chen, Y. G. (2008) J. Biol. Chem. 283, 35679–35688
23. Waxman, J. S., Hocking, A. M., Stoick, C. L., and Moon, R. T. (2004) Dev. Cell 6, 2620–2630
24. Etheridge, S. L., Ray, S., Li, S., Hamblet, N. S., Lijam, N., Tsang, M., Greer, J., Kardos, N., Wang, J., Sussman, D. J., Chen, P., and Wynshaw-Boris, A. (2008) PLoS Genet. 4, e1000259
25. Cheyette, B. N., Waxman, J. S., Miller, J. R., Takemaru, K., Sheldahl, L. C., Khebtsosova, N., Fox, E. P., Earnest, T., and Moon, R. T. (2002) Dev. Cell 2, 449–461
26. Katoh, M., and Katoh, M. (2003) Int. J. Oncol. 22, 907–913
27. 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
28. Waxman, J. S., Hocking, A. M., Stoick, C. L., and Moon, R. T. (2004) Development 131, 5909–5921
29. Zhang, L., Gao, X., Wen, J., Ning, Y., and Chen, Y. G. (2006) J. Biol. Chem. 281, 8607–8612