Frizzled 4 Regulates Arterial Network Organization Through Noncanonical Wnt/Planar Cell Polarity Signaling

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Rationale: A growing body of evidence supports the hypothesis that the Wnt/planar cell polarity (PCP) pathway regulates endothelial cell proliferation and angiogenesis, but the components that mediate this regulation remain elusive.

Objective: We investigated the involvement of one of the receptors, Frizzled4 (Fzd4), in this process because its role has been implicated in retinal vascular development.

Methods and Results: We found that loss of fzd4 function in mice results in a striking reduction and impairment of the distal small artery network in the heart and kidney. We report that loss of fzd4 decreases vascular cell proliferation and migration and decreases the ability of the endothelial cells to form tubes. We show that fzd4 deletion induces defects in the expression level of stable acetylated tubulin and in Golgi organization during migration. Deletion of fzd4 favors Wnt noncanonical AP1-dependent signaling, indicating that Fzd4 plays a pivotal role favoring PCP signaling. Our data further demonstrate that Fzd4 is predominantly localized on the top of the plasma membrane, where it preferentially induces Dvl3 relocalization to promote its activation and α-tubulin recruitment during migration. In a pathological mouse angiogenic model, deletion of fzd4 impairs the angiogenic response and leads to the formation of a disorganized arterial network.

Conclusions: These results suggest that Fzd4 is a major receptor involved in arterial formation and organization through a Wnt/PCP pathway. (Circ Res. 2012;110:47-58.)

Key Words: blood vessels ▪ imaging ▪ ischemia ▪ transgenic mice ▪ vascular biology

During development, blood vessel formation ensures tissue growth and organ function in the entire organism. The essential role of Wnt/Frizzled signaling in the development of the vascular network was established when it was demonstrated that deletion of distinct Wnt genes caused embryonic lethality with severe phenotypes. A growing body of evidence supports the hypothesis that the Wnt/planar cell polarity (PCP) pathway regulates endothelial cell proliferation and angiogenesis,1-3 but the components that mediate this regulation remain elusive. Embryo-specific deletion of Wnt7b/7a, which bypassed early lethality because of Wnt7b effect on placenta formation, demonstrated a role of Wnt7a/7b ligands in blood–brain barrier formation through Wnt canonical signaling.4 These models also indicated that Fzd4 is a prominent receptor involved in vascular formation. Fzd4 has been linked to genetic diseases altering retinal vascular development in Norrie disease, familial exudative vitreoretinopathy,5,6 and osteoporosis–pseudoglioma.7 In mice, Fzd4 controls retinal vascular growth and organization,8 and blood–brain barrier formation in the cerebellum.9 Moreover, Fzd4 is linked to sterility.10 We have previously demonstrated that the action of sFRP1, a secreted regulator of the Wnt pathway, is mediated in part by Fzd4 in endothelial cells.2 The sFRP1 stimulates angiogenesis in vivo and in vitro11 via a noncanonical Wnt-dependent mechanism and activates downstream signaling factors such as GSK3β and Rac1. There is growing evidence of a link between noncanonical Wnt/PCP signaling and angiogenesis.12 Studies using Dvl2 mutants deficient in PCP signaling but capable of inducing the Wnt/β-catenin pathway have been shown to alter endothelial cell properties.13 The formin dishevelled-associated activator of morphogenesis-1, an essential player in cytoskeletal reorganization, regulates gastrulation in Xenopus embryogenesis through noncanonical Wnt signaling14 and has been reported to coordinate a PCP pathway in endothelial cells.3

In the current study, we explore the link between Fzd4 through a PCP pathway and angiogenesis. By investigating the expression and function of Fzd4 in postnatal vessel formation, we demonstrate that Fzd4 reduces arterial vessel formation in...
multiple organs. Fzd4 impairs vascular cell proliferation and migration and performs this function through a noncanonical Wnt/PCP-dependent pathway altering golgi organization and tubulin acetylation. Among the Dvl members, we identified Dvl3 as a preferential target of Fzd4, because Fzd4 relocates and activates Dvl3 on the apical cell membrane. We report that Fzd4 expression is polarized in cells and organizes with Dvl3 to create a signaling platform favoring α-tubulin recruitment and reorganization. Moreover, in posts ischemic neoangiogenesis, fzd4 deletion impairs the angiogenic process. In control mice, after surgery, an arterial network is generated that extends directionally toward the extremity of the leg during the revascularization process, where as depletion of fzd4 induces formation of a disorganized nondirectional and nonfunctional arterial network.

Materials and Methods
An expanded Methods section describing all procedures and protocols is available in the Online Data Supplement (http://circres.ahajournals.org/).

Results
Impairment of Arterial and Arteriolar Formation in the Absence of Fzd4
To elucidate the function of Fzd4 in postnatal development, we studied its vascular expression pattern in fzd4 Δ/Δmice, which allows visualization of Fzd4-expressing cells using the β-galactosidase reporter driven by the fzd4 promoter. Our results demonstrated differential expression in the adult vasculature with expression in the aorta, carotid artery, and other large arteries, and no expression in large veins (online Figure 1). Frequent fzd4 promoter activity was detected in the endothelial cell (EC) and smooth muscle cell (SMC) layers of small arterioles and in various vascular beds, including the heart, kidney, and skeletal muscle (online Figure 1).

Microcomputed tomography three-dimensional quantitative analysis of the cardiac and renal vasculature was performed in fzd4−/− mice. Comparison between fzd4−/− and fzd4Δ/Δ adult mice revealed a striking arterial phenotype with a quantitative reduction in arterial branching and density (Figure 1A, B). In the heart of fzd4Δ/Δ mice, large coronary arteries enclose the heart and divide to give rise to an arterial network toward the apex that penetrates and perfuses the heart wall. In fzd4−/− mice, the large coronary arteries were present, but the arterial network was severely reduced, most notably in the apex and interventricular septum. In the kidney of fzd4Δ/Δ mice, the main renal artery courses in the renal hilum, dividing into segmental arteries that branch into the lobar arteries, supplying a dense capillary network in the cortex. In contrast, the kidney of fzd4−/− mice showed a reduction of the smaller arteries, particularly in the renal cortex while the primary arterial architecture in the renal hilum was conserved. Quantitative polymerase chain reaction analysis of heart and kidney from fzd4−/− and fzd4Δ/Δ pups demonstrated a significant decrease in the arterial marker Ephrin B2 mRNA expression in both the heart (0.37±0.24-fold) and kidney (0.35±0.20-fold) of fzd4−/− compared to fzd4Δ/Δ pups (P<0.05), whereas mRNA for the venous marker Eph B4 was not statistically different between these groups (Figure 1C). Western blot analysis of kidney and heart tissues also confirmed a significant decrease in expression of Ephrin B2 (Figure 1D). These data support the notion that Fzd4 is required for the development of arterioles and capillaries, but is not required for the formation of larger vessels.

Because the loss of fzd4 induced an impressive vascular phenotype described, we next examined the functional impact of fzd4−/− on cardiac and renal function. No difference in diastolic or systolic heart function was observed in any group (Tau and contractile index dp/dt minimum and maximum under basal or dobutamine treatment), and we did not identify any water, acid–base, or electrolyte imbalance when mice were studied under basal conditions (online Table I).

Loss of fzd4 Altered Vascular Cell Proliferation and Migration
To gain further insight into the cell-autonomous function of Fzd4, we isolated ECs, SMCs, and murine embryonic fibroblasts (MEFs) from wild type and fzd4Δ/Δ mice (Figure 2A, online Figure II). Primary fzd4Δ/Δ EC, SMC, and MEF proliferation, analyzed by counting cells positive for BrdUrd, was significantly reduced compared to fzd4Δ/Δ EC and MEF, respectively (Figure 2B, C, D). Consistent with these findings, fzd4 knockout (kDa) of fzd4Δ/Δ MEF and human microvascular EC reduced their proliferation as early as 2 days after culture, confirming an important impact of Fzd4 on EC and MEF proliferation (online Figure III). We next investigated the branching capability of fzd4Δ/Δ EC in an in vitro three-dimensional matrigel assay. Consistent with results of the proliferation assay, fzd4−/− EC had decreased ability to form branches in matrigel assay (Figure 2C). Additional comparisons showed that fzd4−/− EC had a reduced migration compared to control EC using scratch and transwell assays under serum, vascular endothelial growth factor A, or fetal growth factor 2 activation (Figure 2F). These data suggest that Fzd4 controls cell migration under indistinct stimuli. Similarly, wounding assays demonstrated that fzd4−/−/SMC and MEF have a significantly reduced capacity to migrate compared to, respectively, that of fzd4Δ/Δ SMC and MEF (Figure 2G, H).

Loss of fzd4 Altered Microtubule Stabilization and Cell Polarization During Migration
Because cell proliferation and migration are affected by fzd4 depletion, we analyzed the level of acetylated tubulin in MEF.

Non-standard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>AP1</td>
<td>activator protein 1</td>
</tr>
<tr>
<td>DEP domain</td>
<td>Dvl, EgI-10, Pleckstrin domain</td>
</tr>
<tr>
<td>DIX</td>
<td>dishevelled, Axin domain</td>
</tr>
<tr>
<td>Dvl</td>
<td>dishevelled</td>
</tr>
<tr>
<td>EC</td>
<td>endothelial cell</td>
</tr>
<tr>
<td>Fzd4</td>
<td>Frizzled4</td>
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<tr>
<td>MEF</td>
<td>murine embryonic fibroblast</td>
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<tr>
<td>PCP</td>
<td>planar cell polarity</td>
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<td>PDZ domain</td>
<td>postsynaptic density 95, discs large, zonula occludens-1 domain</td>
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<tr>
<td>sFRP1</td>
<td>soluble frizzled-related protein 1</td>
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<td>SMC</td>
<td>smooth muscle cell SMC</td>
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Cell extracts were examined by Western blotting. The absence of $fzd4$ resulted in decreased $\alpha$-tubulin acetylation without a change in the protein level of $\alpha$-tubulin (Figure 3A). Because cell migration requires cell polarization, we directly measured cell polarity when stimulated by a directional stimulus by evaluating microtubule organization and by calculating the organization and volume of the Golgi in EC, SMC, and MEF migrating out of a wounded monolayer. The $fzd4$ MEF display a filamentous network of $\alpha$-tubulin oriented toward the wound healing. Suppression of Fzd4 decreased and altered the organization of the tubulin in migrating MEF (Figure 3B).

Quantitative analysis of fixed immunostained samples showed that in migrating cells, Golgi assembled in front of the nucleus in a dense and compact structure (Figure 3C). We estimated the average labeling intensity of the Golgi along the line crossing the center of the nucleus with the center of the Golgi and showed that the Golgi labeling intensity was reduced in the front of the nucleus in $fzd4^{-/-}$ EC, SMC, and MEF compared to control wild-type cells (Figure 3C). Moreover, in $fzd4^{-/-}$ cells, the volume of the Golgi was increased compared to control cells, revealing that the Golgi was less organized and largest at the front of the nucleus in $fzd4^{-/-}$ cells compared to control cells (Figure 3).

**Cooperation of Fzd4 With Dvl3 Is Required for Noncanonical Signaling Pathway**

Because Dvl has been shown to play a role in cell polarity, we next investigated whether Fzd4 may shuttle Dvl away from the Wnt/β-catenin pathway to favor a noncanonical pathway. We found that in $fzd4^{-/-}$ compared to $fzd4^{+/+}$ MEF, the basal level in the active β-catenin fraction (ABC, the form of...
β-catenin dephosphorylated on Ser37 and Thr41 was significantly increased; under Wnt3a activation, both fztd4/−/− and fztd4+/+ MEF were able to induce the active β-catenin fraction (Figure 4A). To determine if this increase in active β-catenin is associated with transcriptional activity, we transfected the cells with the topflash β-catenin construct. A significant increase in reporter activity was found in fztd4/−/− compared to fztd4+/+ MEF. To test if Fzd4 can control the Wnt canonical pathway, we analyzed the modulation by Fzd4 of the top reporter activity. In HEK293T cells, we showed that expression of Fzd4 alone represses Wnt3a activation of the topflash reporter. Dvl3 overexpression induces strong top reporter activity. Coexpression of Fzd4 and Dvl3 blocks Dvl3-dependent activation of β-catenin transcriptional activity (Figure 4C).

We next addressed the requirement of Fzd4 to induce noncanonical signaling. We have previously demonstrated that in EC, sFRP1 via Fzd4 was able to induce a noncanonical pathway via a dependent activation of downstream components of the Wnt–PCP pathway such as GSK3β and Rac1. Because it was reported that in Wnt/PCP signaling, Dvl activates JNK via a Rac-dependant pathway, we analyzed JNK signaling in fztd4/−/− and fztd4+/+ MEF transfected with an AP1-dependent transcriptional luciferase reporter and analyzed its activation under Wnt5A activation. We found an increase in AP1 reporter transcriptional activity (Figure 4C).
Figure 3. Loss of fzd4 impairs vascular cell polarization. The fzd4 deletion leads to decreased acetyl tubulin expression and organization. A, The fzd4<sup>−/−</sup> and fzd4<sup>+/−</sup> murine embryonic fibroblast (MEF) extracts were prepared and analyzed by Western blotting. The membranes were probed with antibodies recognizing α-tubulin either indiscriminately (α-tubulin) or only when in acetylated form (acetyl tubulin), and relative expression of acetyl tubulin was reported on the graph. **P<0.01. B, Wound-edge fzd4<sup>−/−</sup> and fzd4<sup>+/−</sup> MEFs were stained with anti-α-tubulin to visualize the microtubule network. Loss of fzd4 revealed an alteration of oriented tubulin organization. C, Golgi organization in migrating fzd4<sup>−/−</sup> primary endothelial cells (ECs), smooth muscle cells (SMCs) and MEFs was established by measuring the fluorescence labeling of the Golgi (red) and the nucleus (DAPI; blue) along a line drawn through the nucleus toward the migrating edge and the volume in each cell (as shown in fzd4<sup>−/−</sup> and fzd4<sup>+/−</sup> MEF image on the top). Note a marked flattened Golgi profile in front of fzd4<sup>−/−</sup> cells (white dots) compared to fzd4<sup>−/−</sup> cells (black dots) showing that fzd4 deletion induces an altered Golgi organization in migrated cells. Values represent intensity distribution of fluorescence (arbitrary units) ±SD (n=50). The result of one of two representative experiments is shown. Migration direction was schematized by an arrow. D, To quantify the Golgi volume, the surface reconstruction of the Golgi was designed using Surpass software (Bitplane) as imaged with SMCs. For the three cell types, note a marked increase of Golgi volume in front of fzd4<sup>−/−</sup> cells compared to fzd4<sup>+/−</sup> cells, showing that fzd4 deletion induces an altered Golgi organization in migrated cells. Values represent Golgi volume ±SEM. **P<0.005 and ***P<0.001 vs fzd4<sup>+/−</sup>.
interrogate the ability of Fzd4 to interact preferentially with one of the Dvl isoforms to induce the Wnt noncanonical pathway. We transfected HEK293T cells with the AP1 luciferase reporter and measured activity of cells transfected with Dvl1, Dvl2, and Dvl3 in the absence or presence of Fzd4 (Figure 4E). Transfections with plasmid coding for either Dvl isoforms or Fzd4 alone do not activate the reporter. However, cotransfection with Fzd4 and Dvl1 or Fzd4 and Dvl3 lead to an activation of the AP1 reporter, which is not found when Fzd4 and Dvl2 are cotransfected.
Fzd4 Preferentially Directs Relocalization and Activation of Dvl3 and, to a Lesser Extent, Dvl1

We examined, on a cell-by-cell basis, colocalization of Dvl isoforms with Fzd4. Transient expression of Dvl1, Dvl2, and Dvl3 tagged with green epitope (respectively, Dvl3G, Dvl2G, and Dvl3G) showed punctate staining throughout the cytoplasm (Figure 5A), which correlates with large multiprotein complexes, as previously reported.17,18 Fzd4 tagged with the HA epitope (Fzd4-HA) showed preferential localization at the apical side of the cytoplasmic membrane. Cotransfection with Fzd4-HA revealed a major change in the distribution of Dvl3G and Dvl1G with relocalization to the cytoplasmic membrane and an even appearance throughout the cytoplasm in 95% and 70% of the cells, respectively. We did not find relocalization of Dvl2G when cotransfected with Fzd4-HA (Figure 5A). Quantification of Fzd4-induced relocalization of Dvl to the cytoplasmic membrane was performed by merging images and using Imaris software confocal image analysis. Through this method, we established that Fzd4-HA was able to preferentially relocalize Dvl3G to the membrane as compared to Dvl1G (28.4% of Dvl3G vs 2.7% of Dvl1G). Together, these results imply that Fzd4 interacts preferentially with Dvl3 and, to a lesser extent, with Dvl1, but not with Dvl2 to induce the noncanonical Wnt pathway.

We confirmed the ability of Fzd4 to associate with the endogenous Dvl1 and Dvl3 isoforms from mammalian cell lysates by immunoprecipitation and immunoblotting assays. Cell extracts were prepared from HEK293T cells transfected with Fzd4-GFP fusion protein and immunoprecipitated with antibodies against GFP. Immunoprecipitation of Fzd4-GFP and colocalized at the plasma membrane with \(\alpha\)-tubulin. F, NIH 3T3 cells were transfected either with Fzd4-GFP or with Dvl3G plasmid. Lysates were immunoprecipitated with anti-GFP antibody and then analyzed by Western blotting with the indicated antibodies.
led to coprecipitation of endogenous Dvl1 and Dvl3 (Figure 5B). We next examined the capacity of Fzd4 to activate Dvl3 and found that cotransfection of HEK293T with Fzd4-HA and Dvl3G led to strong phosphorylation of Dvl3G, as shown by a mobility shift of the protein on SDS-PAGE (Figure 5C). To confirm that the observed signaling is specific to Fzd4, cells were cotransfected with a plasmid coding for the CRD domain of Fzd4 (Fzd4 CRD). Fzd4 CRD overexpression did not induce exogenous Dvl3G phosphorylation. Because we show that Fzd4 affects Dvl3 localization and interacts physically with Dvl3, we next wanted to confirm that the activation of Dvl3 was localized at the cytoplasmic membrane. HEK293T were transfected with a plasmid coding for Fzd4-GFP; then, membrane and cytoplasmic fractions were separated using ultracentrifugation steps. Western blot analysis revealed that Fzd4-GFP was exclusively detected in the membrane fraction and not in the cytosolic fraction, although endogenous Dvl3 is evenly distributed in both fractions. These results indicate that Fzd4 leads to an endogenous Dvl3 shift in Fzd4 transfected cells, but not in control membrane fractions. In the cytosolic fractions, we did not detect any modification of endogenous Dvl3 profile both in Fzd4-GFP transfected and control conditions (Figure 5D).

We found that Fzd4 accumulates in a polarized way at the cytoplasmic membrane on the top of the cell (Figure 5A). When cells were transfected with Fzd4-GFP, we found an increase in α-tubulin in membrane fractions, although the overall level of α-tubulin detected in the cytoplasm was not modified in Fzd4 transfected versus control conditions (Figure 5D). Because Fzd4 affects tubulin organization (Figure 3B), we then investigated whether Fzd4-based molecular signals might drive the selective localization of Dvl3 and α-tubulin in polarizing migrating cells. When we overexpressed Fzd4-HA with DVL3G and then visualized it with immunofluorescence, we found that both colocalized in an oriented manner driven partially by α-tubulin at the cytoplasmic membrane (Figure 5E). Using immunoprecipitation, we found that Fzd4-GFP interacts with α-tubulin, and when Fzd4-GFP and Dvl3G were cotransfected, Fzd4 and Dvl3G form a complex that enhances α-tubulin recruitment (Figure 5F).

We have performed experiments to analyze whether re-expression of Fzd4 with Dvl3 in siFzd4-treated EC might restore their ability to proliferate and form tubes. We show that double expression of Fzd4 along with Dvl3 was able to significantly rescue the impact of fzd4 deletion on EC proliferation, but expression of Fzd4 or Dvl3 alone did not have this effect (Figure 6A). Tube formation assays revealed that siFzd4 treatment reduced the ability of cells to form capillary networks and that Dvl3 with Fzd4 was able to rescue the fzd4 deletion phenotype, significantly increasing branch lengths and branch numbers per branch point (Figure 6B). It is noteworthy that Dvl3 alone is able to significantly increase capillary network formation. Collectively, these results suggest that Fzd4 and Dvl3 interact to promote the Wnt-dependent pathway in vascular cells.

Our genetic analysis together with a biochemical analysis have identified Fzd4 with Dvl3, and to a lesser extent Dvl1, as crucial partners for PCP pathway induction in vascular cells. These results confirmed that Fzd4 expression is polarized in cells and organizes a platform with Dvl3 to enhance α-tubulin recruitment.

**Dvl3 DEP Domain Is Crucial for Fzd4-Induced Relocalization**

Next, we sought to identify the domain of Dvl3 that interacts with Fzd4. It has been previously shown that Dvl-mediated activation of the Wnt/β-catenin or the Wnt/PCP signaling pathway is dependent on the availability of specific domains DIX, PDZ, and DEP. Although the DIX and PDZ domains are necessary for Wnt canonical signaling, the DEP domain is essential for PCP signaling21–23 and linked to Rac 1 activation.20,24 To identify the Dvl domain necessary for Fzd4 interaction, we used myc-tagged truncated Dvl constructs lacking the DIX, the PDZ, the DEP Dvl3, and a construct containing DEP domain (DEP). Distribution of the constructs was examined after transfection of HEK293T cells. Any one of the myc-tagged Dvl3 deleted constructs were detected in the cytoplasm in a punctate pattern mimicking the Dvl3-tagged myc pattern. It should be noted that the DEP Dvl3 construct also was detected in the nucleus (data not shown). The constructs were then cotransfected with a plasmid coding for HA-tagged Fzd4 in HEK293T cells. Only deletion of DEP domain impeded Dvl3 relocalization at the membrane. It is worth noting that DEP mutants strongly accumulate and colocalize with Fzd4 into the cytoplasmic membrane, confirming the major role of the DEP domain for interaction with Fzd4 (online Figure IV). In combination, these results indicate that Fzd4 regulates the phosphorylation and polarized cellular localization of Dvl3, and that Fzd4/Dvl3 interaction functions in noncanonical Wnt signaling.

**Fzd4 Deletion Induces Arterial Disorganization in Ischemia-Induced Angiogenesis**

To understand the importance of Fzd4 during vascular formation, we evaluated in vivo whether Fzd4 gene disruption impairs adult angiogenesis. It is noteworthy that under nonischemic conditions, quantitative analysis of the arterial network by microcomputed tomography showed a reduction in both arterial density and branching in the hind limb of fzd4−/− compared to control mice (Figure 7A, C, D). These results were confirmed with a lower capillary density in fzd4−/− versus control mice, evaluated by immunohistochemistry (online Table I). We then explored the capacity of fzd4−/− mice to restore a functional arterial network after ischemia using a femoral artery ligation model. Blood flow recovery in the hind limb was reduced in fzd4−/− compared to control mice after 2 weeks (Figure 7B). This was consistent with a reduction in both the arterial density and branching in fzd4−/− mice after ischemia, evaluated by quantitative analysis of microcomputed tomography images (Figure 7C, D). Interestingly, three-dimensional images revealed that in fzd4−/− mice compared to controls, neovasculature structures were severely disorganized. In control mice, angiogenesis induces the formation of vascular structures oriented directionally toward the extremity of the hind limb. In fzd4−/− mice, we found a modification of the organization with the formation of few clusters of...
arterioles with a radial organization (Figure 7A). These data demonstrate that Fzd4 deletion impairs arterial formation and vessel-oriented branching in peripheral organ. These data support the concept that Fzd4 plays a role major in vessel organization via Wnt-dependent PCP polarity pathway.

**Discussion**

Fzd4 is expressed in different vascular beds throughout the organism. Genetic defects of fzd4 have been linked to familial exudative vitreoretinopathy, a progressive retinal vascular disease. The fzd4 deletion has been shown in mice to impair fertility, to alter vessel formation in the cerebellum, and to interact with Norrin to form retinal vessel network. In addition, our group has previously demonstrated that Fzd4 can interact with sFRP1, a potent angiogenic factor. All these data taken together indicate that Fzd4 signaling may play an important role in vascular formation. In this article, we extend and strengthen this notion by providing evidence that Fzd4 is required for formation and branching of the vascular arterial network in peripheral organs (with regard to their vascular system) through a noncanonical Wnt/PCP-dependent mechanism.

![Figure 6](http://circres.ahajournals.org/)

**Figure 6.** Re-expression of Fzd4 with Dvl3 in sifzd4-treated endothelial cells (ECs) restored their ability to proliferate and form tube. **A.** ECs were transfected and after 48 hours were counted per well. The sifzd4-treated ECs reduced their proliferation compared to control (siCt). Cotransfection with Fzd4-HA and Dvl3-myc plasmids rescue significant sifzd4-EC proliferation compared to Fzd4 or Dvl3 plasmid transfected cells. *P<0.05, n=3. **B.** ECs were seeded on matrigel after transfection with siRNA and plasmid as indicated. After 6 hours, ECs were fixed, photographed, and mean length and branch number per branch point were counted. The sihfzd4 treatment reduced cell ability to form capillary network compared to siCt treatment. The sifzd4 EC transfection with Dvl3 plasmid alone (sifzd4/Dvl3) and with Dvl3 and Fzd4 plasmids (sifzd4/Dvl3/Fzd4) significantly increased branch lengths and branch numbers per branch point compared to siCt conditions. *P<0.05; **P<0.001, n=3.
Fzd4 Deletion Selectively Impairs Small Artery and Capillary Formation in Peripheral Organs

We have demonstrated that Fzd4 is expressed throughout the vasculature, and in EC and SMC of arteries, small arterioles, and capillaries, but not in large veins, and that fzd4 depletion results in a drastic reduction in the number of small-caliber arteries and in their branching. This defective vessel development is restricted to the arterial system consistent with the observation of reduced arterial, but not venous, marker expressions. Collectively, these data suggest the importance of properly regulating Fzd4 activity during arterial development because Fzd4 controls induction and branching of small arterioles and capillary beds at the distal end of tissue. Despite these marked arterial defects and a reduction in body size, other aspects of heart and kidney vascular development, such as formation and branching of large arteries, are not affected.

The formation of new vessels requires both vascular cell proliferation and migration to enable sprout growth and the formation of a branched network. We have demonstrated that reducing expression of Fzd4 mitigated EC and SMC proliferation, migration, and EC tube formation. In a wound model, the Golgi is oriented in front of the nucleus around the centrosome, the organizing center for cytoplasmic microtubules. In this model, we showed that fzd4 deletion impairs both tubulin and Golgi reorganization and capillary-like network formation ex vivo, which support a critical role of Fzd4 in vessel branching.

Thus, we hypothesize that Fzd4 can control vascular formation by a noncanonical-dependent mechanism in peripheral organs.
How Is the Wnt/Frizzled Signaling Pathway Involved in the Vascular System?

In vitro studies have demonstrated a functional role of the canonical β-catenin–dependent pathway in EC.27 It has been proposed that during development, the Wnt/β-catenin pathway was only functional during angiogenesis in the central nervous system but not in peripheral tissue as in heart and kidney;28 after birth, the activation of the Lef/Tcf Wnt reporter system is only restricted to a few vascular beds in the central nervous system.29 Analysis of β-catenin conditional depletion demonstrated a major role of β-catenin in blood–brain barrier maturation.30 Consistent with these findings, in responder top gal transgenic mice, we could not detect any β-gal–positive EC, outside of the central nervous system, in heart and kidney at postnatal day 5 or in adults (data not shown). Additionally, we have demonstrated that sFRP1 was also able to activate a noncanonical Rac1-dependant signaling pathway in EC.2 Recent studies demonstrated a role of PCP in endothelial cells through Daam1-forming family members. Daam1 has been shown to be involved in polarity of embryonic development in Xenopus and Drosophila acting downstream of frizzled receptors via an interaction with Dvl.31 In EC, activated Daam1 would control proliferation and migration,3 affecting both cell polarity and cell movement,31 regulating the microtubule network.32

Our data corroborate the role of PCP in EC and disclose the role of Fzd4 in vascular cells in Wnt/PCP signaling pathway, because we showed that Fzd4 regulates microtubule network. Using in vitro assays, we report that Fzd4 was able to repress the Wnt canonical pathway and activate the noncanonical pathway. These results demonstrate that Fzd4 is localized in a polarized manner on the top of the cell membrane, and it is able to recruit and preferentially activate Dvl3. Three isoforms of Dvl proteins have been identified and share high-sequence homology. Genetic approaches have previously shown that Dvl has been involved in PCP signaling during gastrulation and that it has a functionally redundant role during development.33,34 Interestingly, Dvl3 mutants exhibited craniorachischisis, which is often associated with PCP signaling disruption. Our data further demonstrate that Fzd4 is one of the major receptors of the Wnt/PCP pathway in angiogenesis and facilitates cell polarization through tubulin network reorganization.

Fzd4 and Pathogenesis

Our results suggest that absence of Fzd4 leads to impaired vascular cell polarization. To test the impact of Fzd4 deletion on angiogenesis, we used a mouse model of vessel formation after hind limb ischemia. These data revealed that the absence of Fzd4 impairs adult neoangiogenesis and results in the formation of a disorganized network. Given the fact that the noncanonical Wnt cascade is involved in the growth and guidance of neurons,35 it may be speculated that the directional growth of the peripheral vascular network is a feature of PCP signaling involving Fzd4 receptors.

This study extends our understanding of the role of Fzd4 in adult peripheral organs and during postischemic angiogenesis, and implicates Fzd4 as an important regulator in the directional development and branching morphogenesis of small-caliber arteries and arterioles through a Wnt/PCP pathway in vascular cells.

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Disclosures

None.

References


Novelty and Significance

What Is Known?

- The frizzled/planar cell polarity (PCP) pathway is a highly conserved signaling cascade that coordinates cell behavior at the tissue level in a two-dimensional plane. Frizzled receptor has been shown to participate in planar cell polarity.
- Frizzled 4 is a prominent receptor involved in retinal vascular growth and organization, although its role in angiogenesis remains unknown.

What New Information Does This Article Contribute?

- Global deletion of frizzled 4 receptor in mice alters arteriolar network in heart, kidney, and hindlimb.
- The absence of frizzled 4 impairs vascular properties and dysregulates vascular cell polarization through a noncanonical planar cell polarity pathway in cell assays.
- Frizzled 4 deletion impairs angiogenesis after hindlimb ischemia.

Frizzled/planar cell polarity (PCP) pathway is involved in cell polarization during tissue remodeling and cell motility. Although it was first recognized in the epithelium, it is also observed in other cells, as well. During angiogenesis, coordination of vascular cell growth and migration is preponderant, and as core PCP components as frizzled4 (Fzd4) receptors are expressed in vascular cells, we investigated the role of Fzd4 in arterial network formation and organization. We show that deletion of fzd4 impairs arterial and arteriolar formation in several organs as heart, kidney, and skeletal muscle. At the cell level, fzd4 deletion impaired vascular cell proliferation, migration, and polarization. Fzd4 interacts preferentially with Dvl3 to shuttle Dvl away from the Wnt/beta catenin canonical pathway to favor a noncanonical planar cell polarity-dependent pathway. After hindlimb ischemia, fzd4 deletion impaired arterial neo-formation and led to severe neo-vessel disorganization. Our findings reveal a crucial role of Fzd4 in global arterial formation and angiogenesis through the noncanonical PCP pathway. Our results show for the first time that frizzled signaling pathway plays a fundamental role in vascular remodeling. These findings could facilitate the development of new PCP pathway targets through frizzled 4 for the treatment of ischemic disease.
Frizzled 4 Regulates Arterial Network Organization Through Noncanonical Wnt/Planar Cell Polarity Signaling

Betty Descamps, Raj Sewduth, Nancy Ferreira Tojais, Béatrice Jaspard, Annabel Reynaud, Fabien Sohet, Patrick Lacolley, Cécile Allières, Jean-Marie Daniel Lamazière, Catherine Moreau, Pascale Dufourcq, Thierry Couffinhal and Cécile Duplàa

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SUPPLEMENTAL MATERIAL

DETAILED METHODS

Experimental Animals

$fzd4^{+/-}\text{Luc}Z$ heterozygous knock-in-mice were bred in C57BL6 (8 backcross) and CBA (7 backcross) background and then interbred to generate $fzd4^{-/-}$ and control (wild-type) littermate mice. Genotyping of $fzd4$ mutant mice were performed as previously described. A description of the hindlimb ischemia model and laser Doppler perfusion imaging can be found in the Supplemental section.

We produced $fzd4^{+/-}$ hets during the breeding as $fzd4^{-/-}$ mice are sterile and could only be generated using $fzd4^{+/-}$ mice crossbreeding. The $fzd4^{+/-}$ mice are healthy and fertile and are grossly indistinguishable from their littermate wild-type. They displayed no gross alteration in the morphology (there is no significative differences in weight and size compared to wild type littermate). However a careful examination showed that $fzd4^{+/-}$ mice have a vascular phenotypic alteration of the arterial network in the hindlimb after ischemia, compared to wild-type littermates but not likely at the $fzd4^{+/-}$ level.

For microCT scanning

The vasculature was imaged with a high-resolution micro-CT imaging system (GE eXplore Locus SP), set to a 0.016-mm effective detector pixel size as previously described. The apparatus used for imaging the vessels was the eXplore Locus Micro-CT scanner from General Electric Healthcare® with spatial resolutions of 36 to 7 μm, and used with the 3D image reconstruction software (Digisens®). Data were acquired in an axial mode covering a single hindlimb as described. The quantification parameters were obtained using the Microview ABA® program. Bones of the hindlimb were delimited and digitally deleted and the density of contrast medium was used as a reference for the program.
Immunohistochemistry

Staining with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was performed with heart, kidney, skeletal muscle and large arteries and veins as described². Tissues were then embedded in paraffin and sectioned. Nuclear counterstaining was carried out with nuclear red.

Five-micron, paraffin-embedded sections were cut transversely from the mouse muscle. After immunolabelling with antibodies against CD-31 (BMA biomedical), the capillary density was recorded as the number of CD31 positive capillaries per mm² as described ⁷. A minimum of 30 randomly chosen pictures were recorded at 40X magnification under a microscope for each animal at each time point with a camera connected to a PC. Positive cells were manually counted on captured images and the data were analyzed with the help of Sigma Plot software. All analysis was performed by an investigator blinded to treatment groups.

Cell isolation from mutant and wild-type mice:

Primary endothelial cells (EC) and smooth muscle cells (SMC) from adult mouse kidneys and aorta, were isolated as previously described ³⁴. MEF were isolated from 12.5 day post coïtum mouse embryo ⁵.

- Primary Renal Endothelial Cell Isolation

Mice were sacrificed, and kidneys were removed, minced in slices and incubated with collagenase (Type I, 2mg/ml; Sigma) for 45 min at 37°C with agitation. The suspensions were triturated with a canule and filtered with 70µm filters (BD) followed by a second filtration with 30µm filters (Miltenyi). After centrifugation and washings, the pellets were resuspended and plated in growth media (MEM supplemented with D-valine). After five days, cells were trypsinized and incubated with 12.5µg/ml of anti-CD31 antibody (Pharmingen) and 5µg/ml of
anti-endoglin antibody (Santacruz) for 30min at room temperature. Next, the cell solution was incubated with 20µl of Goat anti-rat IgG microbeads (Miltenyi) for 15min, at 4°C. After washing, magnetic separation with MS MACS columns (Miltenyi) was performed and isolated cells were plated in growth media (EBM-2). Greater than 85% of the cells stained positively for CD31.

- Primary Aortic Smooth Muscle Cells Isolation

Aortas were removed and dissected away from fat and extraveneous tissue in sterile, ice-cold, phosphate-buffered saline (PBS) plus CaCl₂/MgCl₂ (Gibco). Each aorta was incubated in dissecting media consisting of PBS plus CaCl₂/MgCl₂, 1mg/ml collagenase (Type IV, Sigma), and 125 µg/ml elastase (Sigma), for 30 min at 37°C. After removal of the adventitia, aortas were minced and shaken gently at 37°C for 2h in dissecting media. The dissociated tissues were subsequently rinsed with PBS plus CaCl₂/MgCl₂, centrifuged, resuspended and plated in growth media. Greater than 95% of the cells stained positively for α-smooth muscle actin, and myosin heavy chain. All assays were performed with cells before passage four.

- Mouse Embryonic Fibroblast (MEF) Isolation

Primary MEFs were derived from 13.5-day-old embryos. Following removal of the head and organs, embryos were rinsed with PBS, minced, and digested with trypsin (0.05% solution containing 25µg/ml DNase) for 90 min at 37°C. Trypsin was inactivated by addition of 100% FBS and cells were plated in growth media. All assays were performed with cells before passage five.

Proliferation assay
Standard procedures were employed. The bromodeoxyuridine (BrdU) assay was conducted as follows, cells were grown on glass slides (Labtek-II system, Nalge Nune International Corp) and incubated with a BrdU solution (10µM, Sigma) for 30 min, and revealed with an anti-BrdU antibody (Oxford Biotechnologies) after incubation with hydrochloric acid and neutralization using sodium tetraborate as described \textsuperscript{6}. Nuclei cells were stained with Vectashield mounting medium with DAPI (Vector). BrdU labelling cells were counted, and a ratio BrdU labelling /total cell per field was expressed.

**Migration (Wound) Assay**

To study cell migration, we used Culture-Inserts (Ibidi). Cells were plated on each well of Culture-Inserts and allowed to reach confluence. Cells were serum starved for 24h, then Culture-Inserts were gently removed, and cells were stimulated or not with FGF2 (20ng/ml). The subsequent gaps were imaged at baseline and 20 hr later.

**Chemotaxis Assay**

Transwell cell culture chambers containing polycarbonate membrane with 8-µm pore size (Becton Dickinson.) were coated with 0.2% gelatin for MEF and 2% for endothelial cells. Primary and siRNA treated cells were serum starved for 24 hr then trypsinized and plated at $10^5$ cells/insert in a Transwell chamber. After 2 hr, the bottom compartment was filled with 800 µl DMEM plus 1% or 10% FBS, or DMEM plus FGF2 (20ng/ml) or VEGF-A (40ng/ml), and the assembly was incubated at 37°C for 16 h, to allow cell migration. In the negative controls, bottom chambers were filled with DMEM serum-free. After incubation, the membranes were washed with PBS, and cells were fixed in 2% paraformaldehyde, permeabilized with 0.2% Triton X-100 and stained with Mayer’s Hematoxylin solution (Merck) according to the manufacturer’s protocols. Cells, that did not migrate, were gently
removed from the top surface, and cells, that had migrated to the bottom of the membrane, were counted in seven random fields using a Nikon inverted microscope.

**Capillary-like tube formation on Matrigel**

Tube formation assays were set up using μ-slide angiogenesis (Ibidi) following manufacturer instructions. Human Umbilical Vein cells (HUVEC), purchased from Lonza, were transfected with scramble siRNA or anti-hfzd4 siRNA (si hfzd4) (30 nM) and transfected either with control plasmid or with plasmids coding for Dvl3 tagged myc or Fzd4 tagged HA. 48 hours after transfection, 15,000 cells per well were plated on top of Matrigel® (Becton Dickinson) in growth medium (EBM-2). After 6 hr at 37 °C, tube formation was examined under an inverted light microscope (Olympus). Branch lengths were measured and branch numbers per branch point were counted. siRNAs were transfected using Interferin (Ozyme) and plasmid with jetprime (Ozyme), following the manufacturer's protocol. The oligonucleotides used were either a validated negative control (Eurogentec) or designed by Qiagen for hfzd4: 5’-GAA UGA UAG UGC CUU UAA Att -3’ (#SI02639105) and Ambion for mfzd4: 5’- GGA UGU GCA AUA AUU UUC Utt -3’ (#61777). The plasmids encoding for Dvl3 tagged myc were generated in the laboratory and for Fzd4 tagged HA were kindly provided by Tomas Kirchhausen (CBR Institute for biomedical research, Boston).

**Golgi polarization assay**

This assay was conducted in a migration assay using Culture-Inserts (Ibidi) (see above). 24hrs before experiments, confluent monolayers were serum starved and then activated with FGF2 (20ng/ml). Anti-Golgin97 antibody (Invitrogen), pericentrine and FITC phalloidin (Sigma) were used to stain the Golgi, Microtubule Organizing Center (MTOC) and actin cytoskeleton, respectively.
For the analysis, all images were taken with the same intensity parameters and only cells located in the first row of the wound edge were measured. For determination of the Golgi profile, a line was drawn along the cell and passing by the nucleus, in the direction of the migration edge. For each fluorochrome, the intensity profile was measured along this line using the Axiovision 4.7 program. To quantify Golgi volume, golgi and nucleus surface reconstructions were assigned and cell surface area/cell volume analysis was performed using the Surpass program (Imaris) after 3D deconvolution with autoQuant deconvolution (Mediacybernetics). For each cellular type, at least 50 cells were examined.

**Cell culture**

Wnt3a and Wnt5a producing mouse L cells (American Type Culture Collection ATCC) were cultured in DMEM medium, 10% fetal bovine serum (FBS) and penicillin-streptomycin. Conditioned medium was harvested as described in ATCC protocol. Primary renal endothelial cells were cultured in MEM supplemented with 100µg/ml Heparin, 50µg/ml ECGF, 3.5g/L Glucose, 50mg/L D-valine, 20% FBS (Hyclone) and penicillin-streptomycin, and plated on a 0.2% gelatin-coated dish. After purification, they were cultured in EBM-2 complete medium with EGM2 single coat (Clonetics), and plated on a 5µg/ml fibronectin-coated dish. Mouse smooth muscle cells, embryonic fibroblasts (MEF), NIH3T3 and HEK293T were cultured in DMEM supplemented with 10% FBS (Hyclone) and penicillin-streptomycin, and plated on a 0.1% gelatin-coated dish. Human microvascular endothelial cells (HMEC-1) were cultured in EBM complete medium with EGM-MV single coat (Clonetics), and plated on a 2% gelatin-coated dish. All cells were serum starved with DMEM plus 1% FBS/0.25%BSA.

**Analysis of mRNA expression by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)**
For RT-PCR analysis 1µg of total RNA from mouse tissue or cell extracts was reverse-transcribed: PCR was performed using IQ SYBR Green supermix (Bio-Rad) ². An MJ Research Opticon and the following parameters were used for real-time PCR: 95°C for 5 minutes followed by 34 cycles of 95°C for 30 seconds, 60°C for 1 minute, 72°C for 30 seconds, 76°C for 1 minute and 80°C for 1 minute. Negative controls without RT were prepared in parallel for each RNA sample. All experiments were performed in triplicate and differences in cDNA input were compensated by normalization to expression of P0 or α-actin.

qPCR Primer list

Primers used are as follows: P0 forward primer: 5′-GCGACCTGGAAGTCAA-3′, and P0 reverse primer: 5′-CCATCAGCACCACAGCCTTC-3′, beta-actin forward primer: 5′-GGAGGAAGAGGATGCAGCA-3′, and beta-actin reverse primer: 5′-GAAGCTGTGCTATGTTGCTCTA-3′; ephrinB2 forward primer: 5′-AAGCCAAGGCAACACCCAG-3′, and ephrinB2 reverse primer: 5′-CAGGCGATAAACAAACAAAC-3′; ephB4 forward primer: 5′-AATGCCACAGCCTTAGACAG-3′, and ephB4 reverse primer: 5′-AATGCCACAGCCTTAGACAG-3′; mfzd4 forward primer: 5′-TGACAACTTTCACGCCTGCTC-3′, and mfzd4 reverse primer: 5′-TGACAACTTTCACGCCTGCTC-3′; hfzd4 forward primer: 5′-TACAAGCCACATCGTAGCAACAACTC-3′; and hfzd4 reverse primer: 5′-TACAAGCCACATCGTAGCAACAACTC-3′.

Western Blot analysis and immunoprecipitation
Organ tissues or cells were lysed for 30 minutes in RIPA buffer (1% Nonidet P-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulphate, 50mmol/L Tris, pH 8.0, 2µg/ml aprotinin, 10µg/ml leupeptin, 200µM AEBSF, 1mM benzamidine, 1µM orthovonadate). Cell lysates were cleared by centrifugation at 14,000 g for 20 min. Supernatant fractions were used for Western blot or immunoprecipitation. For Western blot analysis, cell lysates were resolved by SDS-PAGE and probed with indicated antibodies. For immunoprecipitation analysis, 500 µg of lysates were incubated overnight with the primary antibody at +4°C with gentle agitation. After this 20 µl of protein G-Sepharose slurry was added and samples were incubated for 1 hr at +4°C. Beads were washed three times and subsequently resuspended and boiled in 20 µl of loading buffer for SDS-PAGE, followed by western blotting.

Antibodies used were: anti EphrinB2 and EphB4 antibodies from Santa Cruz, anti phosphorylated β catenin (anti-ABC) clone 8E7 monoclonal antibody from Upstate, anti β catenin, acetylated tubulin and α tubulin from sigma, anti Dvl3 and Dvl1 from Santa Cruz, anti GFP from Molecular probes and anti HA from Roche. Binding of antibodies to the blots was detected using Odyssey Infrared Imaging System (LI-COR Biosciences).

**Luciferase reporter assay**

For the canonical pathway, MEF and HEK 293T cells grown in 24-well culture plates were transiently transfected at 60-70% confluence with 0.2 µg of 8XTOPflash reporter, 0.1 µg of β galactosidase reporter (to evaluate efficiency of transfection) and various plasmids as indicated for individual experiments. The total quantity of DNA in the transfection mixes was adjusted to equal amounts with empty vector. 24 hr after transfection, cells were activated with Wnt3a or Wnt5a conditioned medium as indicated. Luciferase activity was measured using the Luciferase Reporter Assay system (Promega) and a luminometer according to the
manufacture’s specifications. The firefly luciferase activity was normalized to β galactosidase activity.

For the non canonical pathway, MEF and HEK 293T cells were transiently transfected with 0.2 μg of AP1 reporter, 0.1 μg of β galactosidase reporter (to evaluate efficiency of transfection) and various plasmids as indicated for individual experiments.

**Immunofluoresence microscopy**

For tubulin staining, cells were fixed in and stained with monoclonal anti α tubulin. For Fzd4 and Dvl3 co-localization, HA-tagged Frizzled 4 was detected using anti-HA antibodies (Roche) followed by Alexa Fluor 568 conjugated goat anti-mouse IgG. The DNA was counterstained by 4’,6-diamidino-2-phenylindole (DAPI). For co-localization studies, cells were analyzed with a Nikon laser scanning confocal microscope using multichannel scanning (Nikon PCM 2000) using a 60x/1.4 ApoPlan oil immersion objective, and EZ200 software (Nikon). Single XY scans had an optical slice thickness of 0.4μm. Three-dimensional projections were digitally reconstituted from stacks of confocal optical slices by Imaris software (Bitplane).

**Mouse model of unilateral hind limb ischemia**

Unilateral hind limb ischemia was operatively induced as previously described with the excision of the femoral and saphenous arteries 6. This study was conducted in accordance with both institutional guidelines and those in force in the European community for experimental animal use (L358-86/609/EEC). Seven days after the surgery, perfusion using Laser Doppler perfusion imaging (LPDI) vascular density was used to record serial blood flow measurements, and connectivity was analyzed using microCT analysis on serial tissue sections.
Statistical analysis

Results are expressed as means ± SD. All analyses were performed with appropriate software (Statview 5-1, Abacus). Comparison of continuous variables between two groups was performed by a one-way ANOVA and subsequently, if statistical significance was observed, by a two-sided paired t test. A value of $p < 0.05$ was considered to be statistically significant.

SUPPLEMENTAL REFERENCES


Online Figure I: Fzd4 vascular expression pattern in fzd4<sup>+/-</sup>lacZ mice.
Xgal staining in (A) coronary (B) skeletal muscle and (C) kidney arterioles, (D) a carotid artery, although not in large veins, (E) an aorta, and (F) an artery in kidney hileum. In A and in E, high magnification insert (100X) of coronary artery and aorta, respectively, revealing Xgal stained EC. V: Vein; A: artery. Xgal staining is shown with black arrows. Nuclei are counterstained with nuclear red.

Online Figure II:

Online Figure II: CD31 immunolabelling of isolated mouse endothelial cell

After isolation of mouse endothelial cells from wild-type (fzd4 +/+ ) and mutants (fzd4 +/-) mice, CD31 immunolabelling (Red) was used to confirm endothelial origin and establish the purity of the selected population. DAPI (Blue) revealed the nuclei.
Online Figure III: Effect of Fzd4 knock down on endothelial cell (HMEC-1) and MEF proliferation:

Knock-down of Fzd4 expression was quantitatively analyzed using real-time PCR in HMEC-1 (A) and in wild-type murine MEF (B) transfected with siRNA control versus siRNA against Fzd4. Note siRNA against human Fzd4 (hfzd4) and mouse Fzd4 (mfzd4) induce 75% of inhibition of endogen Fzd4 expression in HMEC-1 and in MEF. Values are means ± SD, P<0.001 vs control. Knock-down of Fzd4 induced a significant reduction of proliferation of HMEC-1 (A) and MEF (B) after cell counting per well. Values correspond to total cell numbers per well ± SD; n=3. **, P>0.005 and ***, P>0.001 vs control.
Online Figure IV:

A

1

DIX

PDZ

DEP

716

Myc

ΔDIX Dvl3-Myc

1

218

320

716

ΔPDZ DVL3-Myc

1

377

ΔDEP Dvl3-Myc

412

716

DEP-Myc

B

DEP-Myc + Fzd4-HA

ΔDEP DVL3-Myc + Fzd4-HA

ΔDIX DVL3-Myc + Fzd4-HA

ΔPDZ DVL3-Myc + Fzd4-HA
Online Figure IV: DEP domain is essential for Dvl3 re-localization by Fzd4:

(A) A schematic diagram of mDvl3 deletion mutants. Full length (716 aa) mDvl3 was tagged with myc at the C terminus and contained 3 domains, DIX, PDZ and DEP; ΔDix Dvl3-myc represents mDvl3 sequence deleted of the Dix domain; ΔPDZ Dvl3-myc represents Dvl3-myc sequence deleted of the PDZ domain; ΔDEP Dvl3-myc represents Dvl3-myc sequence deleted of the DEP domain; DEP-myc represents Dvl3 sequence deleted of the DIX and PDZ domain (B). NIH 3T3 cells were transfected with plasmid coding for HA-tagged Fzd4 (Fzd4-HA, red labeling) and with myc-tagged mDvl3 deletion mutants (green labeling) as indicated. Co-localization of Fzd4 and Dvl3 mutants was analyzed by confocal. Note that deletions of DIX and PDZ domains did not impair Dvl3 re-localization by Fzd4 although DEP domain deletion blocks mutant recruitment at the cytosolic membrane. Mutants containing DEP domains (DEP-myc mutant) show a strong co-localization with Fzd4 at the migrating edge of the cells (white arrow). The high magnification photos at right and bottom represent perpendicular views of the confocal acquired images. Yellow/white staining indicates co-localization of Fzd4-HA with Dvl3-myc mutants at the cytoplasmic membrane but not with ΔDEP-Dvl3-myc mutant maintained in the cytoplasm.
Online Table I:

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<th>(fzd4^{+/+})</th>
<th>(fzd4^{-/-})</th>
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<td>17.2 ± 1.5**</td>
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<td>Aldosteron excretion (/day/g)</td>
<td>599 ±157</td>
<td>913 ± 656</td>
<td>NS</td>
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**Capillary number**

Non ischemic muscle  436,09 ± 110,90  179,39 ± 57,07  <0,01

Ischemic muscle

<p>| | | | |</p>
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<td>D7</td>
<td>183,05 ± 98,75</td>
<td>62,53 ± 26,34</td>
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<td>119,2 ± 76,28</td>
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<td>D21</td>
<td>774,75 ± 181,28</td>
<td>200,07 ± 84,16</td>
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Kidney  143,02 ± 90,27  10,24 ± 16,72  <0,01

Despite this microvasculature phenotype, there was no significant difference in the ratio heart or kidney weight/total weight in adult. As loss of \(fzd4\) induced an impressive vascular phenotype described above, we have examined the functional impact of \(fzd4^{-/-}\) on cardiac, renal and vascular
functions. No difference in diastolic or systolic heart function was observed in any group (Tau and contractile index $dP/dt$ min and max under basal or dobutamine treatment) (Table 1). We next examined the impact of $fzd4$ deletion on renal function. Glomerular filtration rate, assessed by the measure of creatinine clearance, was not effected when the difference in body size was taken into account. We then hypothesized that renal hypoperfusion could promote secondary hyperaldosteronism as observed in bilateral renal artery stenosis but neither plasma renin concentration nor aldosterone excretion was significantly increased in the mutated animals when compared to controls. Further, we did not identify any water, acid-base or electrolyte imbalance when mice were studied under basal conditions. Altogether, these results show that $fzd4$ deletion reduced drastically the arterial network formation in both the heart and kidney, it did not lead to significant impairment in overall cardiac or renal function.