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TSPAN12 Regulates Retinal Vascular Development by Promoting Norrin- but Not Wnt-Induced FZD4/β-Catenin Signaling

Harald J. Junge,[1] Stacey Yang,[1,5] Jeremy B. Burton,[1,5] Kim Paes,[4] Xiao Shu,[1,6] Dorothy M. French,[2] Mike Costa,[3] Dennis S. Rice,[4] and Weilan Ye[1,*]
[1]Tumor Biology and Angiogenesis Department
[2]Pathology Department
[3]Department of Cancer Targets
Genentech Inc., 1 DNA Way, South San Francisco, CA 94080, USA
[4]Ophthalmology Department, Lexicon Pharmaceuticals Inc., 8800 Technology Forest, The Woodlands, TX 77381-1160, USA
[5]These authors contributed equally to this work
[6]Present address: Department of Nutritional Sciences and Toxicology, 119 Morgan Hall, University of California, Berkeley, Berkeley, CA 94720-3104, USA
*Correspondence: loni@gene.com
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SUMMARY

Mutations in the genes encoding the Wnt receptor Frizzled-4 (FZD4), coreceptor LRP5, or the ligand Norrin disrupt retinal vascular development and cause ophthalmic diseases. Although Norrin is structurally unrelated to Wnts, it binds FZD4 and activates the canonical Wnt pathway. Here we show that the tetraspanin *Tspan12* is expressed in the retinal vasculature, and loss of *Tspan12* phenocopies defects seen in *Fzd4*, *Lrp5*, and *Norrin* mutant mice. In addition, *Tspan12* genetically interacts with *Norrin* or *Lrp5*. Overexpressed TSPAN12 associates with the Norrin-receptor complex and significantly increases Norrin/β-catenin but not Wnt/β-catenin signaling, whereas *Tspan12* siRNA abolishes transcriptional responses to Norrin but not Wnt3A in retinal endothelial cells. Signaling defects caused by Norrin or FZD4 mutations that are predicted to impair receptor multimerization are rescued by overexpression of TSPAN12. Our data indicate that Norrin multimers and TSPAN12 cooperatively promote multimerization of FZD4 and its associated proteins to elicit physiological levels of signaling.

INTRODUCTION

Vascular diseases of the retina are a major cause of impaired vision and blindness. Retinas in the majority of mammals are perfused by three layers of vascular networks that include two intraretinal capillary beds. The formation of intraretinal capillaries is linked to the activity of the canonical Wnt pathway (Fruttiger, 2007) that promotes accumulation of β-catenin and stimulates LEF/TCF-mediated transcriptional programs (Gordon and Nusse, 2006). In humans, mutations in the Wnt-receptor *Frizzled-4* (*Fzd4*) and the coreceptor *Lrp5* cause familial exudative vitreoretinopathy (FEVR) (Warden et al., 2007), an inherited disease characterized by incomplete vascularization of the peripheral retina (Berger and Ropers, 2001). Mutations in the gene encoding the cysteine knot protein Norrin cause Norrie disease, FEVR, retinopathy of prematurity (ROP), or Coat's disease. The retinal vasculature is altered in each of these diseases (Berger and Ropers, 2001). Consistent with the findings in human patients, targeted inactivation of *Norrin, Lrp5,* or *Fzd4* in mice results in similar retinal phenotypes characterized by a dramatic reduction of intraretinal capillaries (Luhmann et al., 2005; Xia et al., 2008; Xu et al., 2004). Norrin is a high-affinity ligand for FZD4 that signals via stabilizing β-catenin and activates LEF/TCF-mediated transcription in an LRP5/6-dependent manner (Xu et al., 2004). FZD4 is the only Norrin receptor among 10 Frizzleds (Smallwood et al., 2007), and FZD4 can also mediate the function of Wnts (Lobov et al., 2005). Norrin is a cysteine knot protein that lacks the structural characteristics of Wnts, and it is unclear how FZD4/LRP5 can respond to two types of structurally distinct ligands and elicit similar intracellular changes (i.e., the accumulation of β-catenin and activation of LEF/TCF-mediated transcription). Here we report the finding that FZD4/LRP5 signaling induced by Norrin, but not by Wnt ligands, depends on an additional membrane protein TSPAN12.

TSPAN12 is a member of the phylogenetically ancient tetraspanin family (Serru et al., 2000). All tetraspanins share common structural features that distinguish them from other four transmembrane domain proteins (Garcia-Espana et al., 2008). Investigation of several prototypic tetraspanins has led to a model in which tetraspanins organize specialized tetraspanin-enriched microdomains (TEMs) that act as signaling platforms in the plasma membrane (Boucheix and Rubinstein, 2001; Hemler, 2005). Through a genetic screen, we discovered that *Tspan12* is expressed in the retinal vasculature and *Tspan12* mutant mice phenocopy a multitude of defects observed in *Norrin*,

Fzd4, and *Lrp5* mutant mice. Through a series of in vivo and in vitro analyses, we demonstrated that Tspan12 specifically regulates Norrin/β-catenin but not Wnt/β-catenin signaling by modulating FZD4 multimerization.

Since the canonical β-catenin signaling pathway regulates a plethora of important biological processes, one of the key questions in the field is how spatiotemporal control of signal activation is achieved (van Amerongen et al., 2008). In this study, we show that *Tspan12* expression is restricted to the vasculature within the retina, whereas *Norrin* (Hartzer et al., 1999), *Frizzled-4* (Wang et al., 2001), and *Lrp5* (Figueroa et al., 2000) are more broadly expressed in the retina. The requirement of TSPAN12 for Norrin/β-catenin but not Wnt/β-catenin signaling may enable only a subset of *Frizzled-4*-expressing cells to respond to Norrin, thereby providing a hitherto unrecognized mechanism to generate specific spatiotemporal patterns of β-catenin signaling.

RESULTS

Targeting of the *Tspan12* Gene

We carried out a large-scale reverse genetic screen in mice to identify disease-related phenotypes by knocking out 475 genes that encode putative transmembrane or secreted proteins (T. Tang et al., personal communication). Through this effort, we identified a line of mutant mice exhibiting retinal vascular defects in the fluorescein angiography test (Figure S2A available online). The gene mutated in this line encodes a previously uncharacterized tetraspanin named TSPAN12 (synonyms: TM4SF12 and Net-2). The murine *Tspan12* gene (refseq NM_173007) contains nine exons (Figure S1A) and is located on chromosome 6 (human chromosome 7). The gene product is a highly conserved 4TM protein with a predicted molecular weight (MW) of 35 kDa and whose electrophoretic mobility is slightly higher than predicted (Figure S1F and data not shown). The predicted topology features two extracellular loops, with both N and C termini localized intracellularly (Figure S1B). Of the 136 bp in exon 3, a 3′ portion of 97 bp and additional intronic sequences were eliminated by our targeting strategy (Figure S1C). Thus, the start codon and a major portion of the first membrane-spanning region were replaced by the targeting cassette (an IRES element followed by the lacZ derivative BGeo as a reporter of TSPAN12 expression, and the puromycin resistance gene driven by a PGK promoter). Gene targeting was confirmed by Southern blot and PCR (Figures S1D and S1E). Loss of the start codon abolished translation of TSPAN12 in the homozygous knockout (KO or −/−) mice, as evident by immunoprecipitation (IP) followed by western blot from lysates of embryos using a TSPAN12 polyclonal antibody (Figure S1F).

Vascular Defects in the *Tspan12* Mutant Mice

Tspan12⁻/⁻ mice were viable and fertile. Analysis of LacZ expression on P15 retinal sections of *Tspan12*⁻/⁻ or *Tspan12*⁺/⁻ mice (Figures 1B and S3B) and in situ hybridization using *Tspan12* riboprobe on wild-type P15 retinal sections (Figure S3A) revealed expression of *Tspan12* in the neonatal retinal vasculature. LacZ expression was also detected in the neonatal meningeal vasculature (Figure S3C) and in nonvascular cell types, such

as the smooth muscle cells in the neonatal intestine (Figure S3D). The alteration of retinal vessels (Figures 1B and S2A) prompted us to analyze the retinal vasculature further. In the murine retina, a superficial vascular plexus in the nerve fiber layer (NFL) develops from the central artery through a combination of sprouting, migration, and remodeling between P0 and P8. Subsequently, vessels sprout vertically into the retina and ramify in the outer plexiform layer (OPL) and inner plexiform layer (IPL), where two capillary beds are established, resulting in a three-layered vascular architecture (Fruttiger, 2007). We examined retinas between P5 and P12 and found that the centrifugal outgrowth of the NFL vasculature was moderately delayed in *Tspan12*⁻/⁻ retinas (Figures 1C and 1D and data not shown). At P11, vertical sprouts and OPL capillaries appeared in *Tspan12*⁺/⁺ mice, whereas both were completely absent in *Tspan12*⁻/⁻ mice (Figures 1E and 1F). The fact that *Tspan12* expression was detected in the vasculature but not other retinal tissues, together with the observation that the retinal histology appeared normal at P11 by hematoxylin and eosin (H&E) staining (Figures 1G and 1H), suggests that the vascular defect is primary. In adult *Tspan12*⁻/⁻ mice, the OPL remains avascular, confirming that the defect is not transient (Figures 1I and 1J). The thickness of the outer nuclear layer in *Tspan12*⁻/⁻ retinas was consistently reduced in adult but not neonatal mice (compare Figures 1E, 1F, 1I, and 1J), indicating that neural cells were secondarily affected by the vascular defects.

The phenotypes in *Tspan12*⁻/⁻ mice described above are similar to those reported for *Fzd4* (Xu et al., 2004), *Lrp5* (Xia et al., 2008), and *Norrin* (Luhmann et al., 2005) mutant mice. We therefore examined *Tspan12*⁻/⁻ mice for additional characteristics observed in *Norrin or Fzd4* mutant mice. Microaneurisms extending from the NFL toward the inner nuclear layer were found in *Norrin* mutant mice at P15 (Luhmann et al., 2005). P16 retinas of *Tspan12*⁻/⁻ mice contained microaneurisms that were remarkably similar to those described in *Norrin* mutant mice (Figure 2A). Strikingly, these lesions develop at similar time points in *Norrin*⁻/⁻ and *Tspan12*⁻/⁻ mice. Since aberrant formation of retinal vascular fenestrations is prominent in *Fzd4* mutant mice (Xu et al., 2004), we examined expression of the fenestrated endothelial marker MECA-32 (also called PLVAP) in *Tspan12*⁻/⁻ mice. Consistent with the fact that normal retinal vessels are not fenestrated, MECA-32 was not detected in *Tspan12*⁺/⁺ retinal endothelial cells (ECs) (Figure 2B, top panel). However, strong MECA-32 signal was observed in *Tspan12*⁻/⁻ retinal ECs (Figure 2B, bottom panel). Delayed hyaloid vessel regression was reported in *Norrin* (Luhmann et al., 2005), *Fzd4* (Xu et al., 2004) and *Lrp5* (Lobov et al., 2005) mutant mice. We found that hyaloid vessel regression was also significantly delayed in *Tspan12*⁻/⁻ mice at several developmental stages examined (Figure 2C). Three additional parallels between *Tspan12* and *Fzd4* and/or *Norrin* mutant mice were observed: focal hemorrhage in the adult retinas was found in *Tspan12* (Figure S2B) and *Fzd4* (Xu et al., 2004) mutant mice; retinal glial cell activation was reported in *Norrin* mutant mice (Luhmann et al., 2005) and was also observed in *Tspan12*⁻/⁻ retinas (Figure S2C); vessel enlargement in the stria vascularis of the inner ear was observed in *Tspan12* (Figure S2D), *Fzd4* (Xu et al., 2004), and *Norrin* (Rehm et al., 2002) mutant mice.
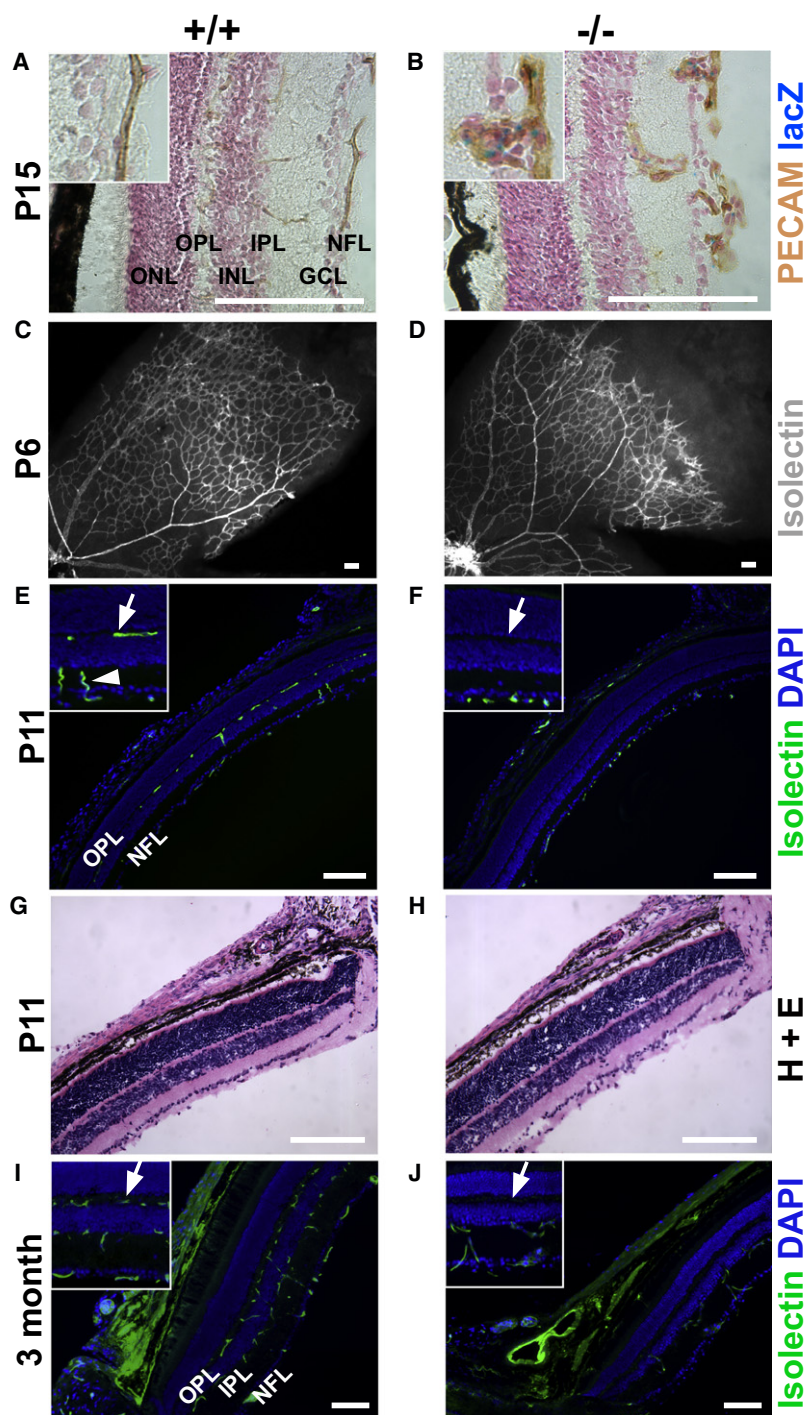
+/+

-/-

PECAM lacZ

Isolectin

Isolectin DAPI

H + E

Isolectin DAPI

P15 / A / B

OPL IPL NFL
ONL INL GCL

P6 / C / D

P11 / E / F

OPL NFL

P11 / G / H

3 month / I / J

OPL IPL NFL

Figure 1. *Tspan12* Is Required for the Development of Intraretinal Capillaries

(A and B) Double staining of PECAM (brown) and β-galactosidase (blue) on postnatal day 15 (P15) retinal sections. β-galactosidase-positive cells are only detected in the vasculature of −/− animals.

(C and D) IsolectinB4 (IB4) staining of whole-mount P6 retinas.

(E and F) P11 retinal sections stained with IB4 (green) and DAPI (blue).

(G and H) P11 retinal sections stained with hematoxylin (blue) and eosin (pink).

(I and J) Sections of adult retinas stained with IB4 (green) and DAPI (blue). Arrows: location of the OPL. Arrowhead: vertical sprout.

ONL = outer nuclear layer, INL = inner nuclear layer, GCL = ganglion cell layer. OPL, IPL, and NFL are defined in the text. Scale bars = 100 μm.

MECA-32 in the *Tspan12*$^{-/-}$ but not $^{+/+}$ retina (Figure 2B) suggests that Tspan12 is required for β-catenin signaling in retinal ECs.

Tspan12 Genetically Interacts with *Norrin* or *Lrp5*

To further investigate if TSPAN12 is functionally linked to Norrin/β-catenin signaling, we generated compound mutant mice that lacked one allele each of *Tspan12* and *Norrin* or *Tspan12* and *Lrp5*. Because the *Norrin* and *Lrp5* alleles reported here (Figure S4) have not been previously described, we examined if our *Norrin* or *Lrp5* homozygous mutants displayed the documented phenotypes. We found that both *Norrin*$^{-/y}$ and *Lrp5*$^{-/-}$ mice indeed had a leaky and aberrant retinal vasculature (Figure S5A), lacked the IPL and OPL capillary beds, and strongly upregulated *MECA-32* (Figure S5B and data not shown). We then crossed *Tspan12*$^{+/-}$ mice with either *Lrp5*$^{+/-}$ or *Norrin*$^{-/y}$ mice and analyzed postnatal retinal vascular development in resulting wild-type, single-gene heterozygous, or transheterozygous offspring. First, we quantified the number of vertical sprouts extending from the NFL vasculature into the OPL (method described in Figure S6) because lack of vertical sprouts appears to be an early and possibly primary defect in *Norrin, Lrp5, Fzd4*, and *Tspan12* mutant mice. Loss of one allele of *Tspan12* or *Lrp5* or *Norrin* caused

Since Norrin and FZD4 are a ligand/receptor pair that, in conjunction with the coreceptor LRP5, activate the canonical Wnt pathway and promote accumulation of cytoplasmic β-catenin, our phenotypic characterization of *Tspan12*$^{-/-}$ mice suggests that TSPAN12 may be required for Norrin/β-catenin signaling mediated by FZD4 and LRP5. In addition, since MECA-32 upregulation is reported to be a readout of impaired canonical β-catenin signaling (Liebner et al., 2008), the strong vascular expression of
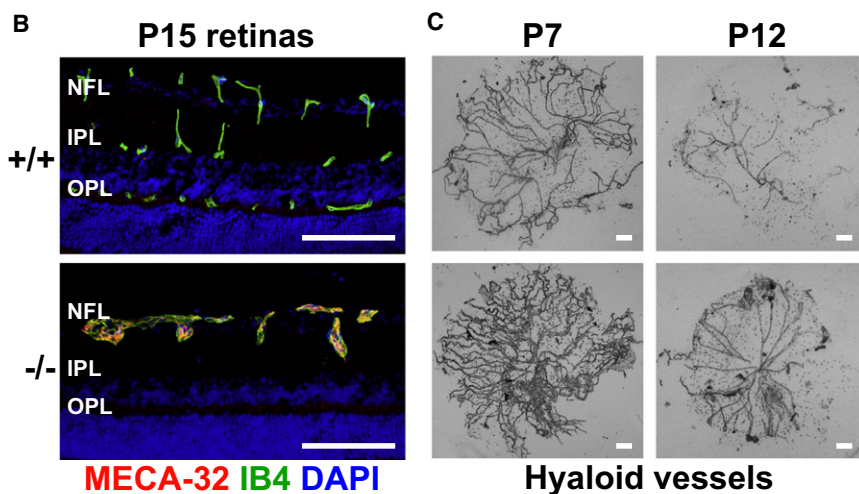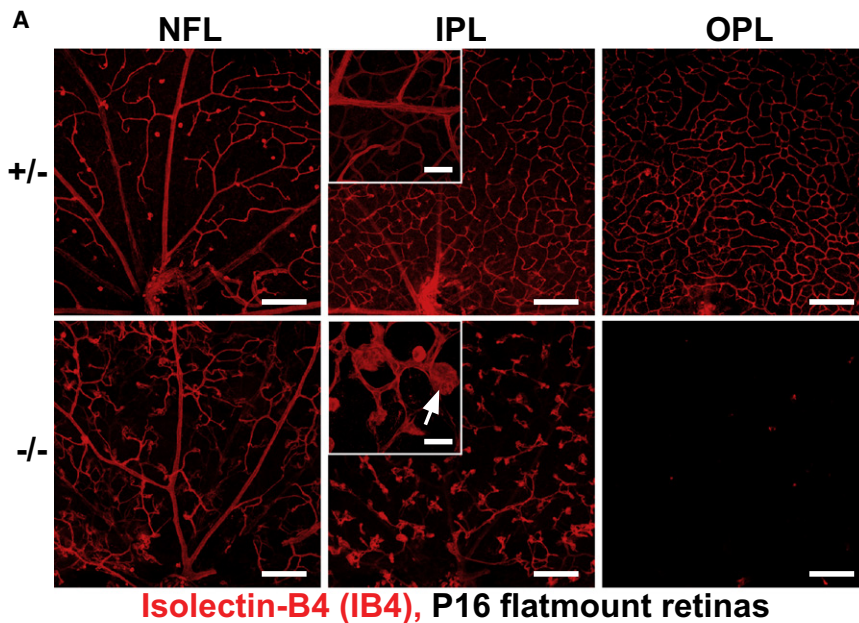
minimal to moderate (0%–15.5%) reduction of vertical sprouts, whereas concurrent loss of one *Tspan12* allele with either one *Norrin* or one *Lrp5* allele significantly reduced sprouting by 40%–50% (Figures 3A and 3B). The strong statistical significance of the differences (p < 0.0001, Figures 3A and 3B) between the *Tspan12*$^{+/-}$;*Lrp5*$^{+/-}$ or *Tspan12*$^{+/-}$;*Norrin*$^{+/-}$ transheterozygous mice and their single-gene heterozygous littermates indicates that *Tspan12* genetically interacts with *Lrp5*

A

NFL IPL OPL

+/-

-/-

Isolectin-B4 (IB4), P16 flatmount retinas

B

P15 retinas

NFL
IPL
OPL

+/+

NFL
IPL
OPL

-/-

MECA-32 IB4 DAPI

C

P7 P12

Hyaloid vessels

Figure 2. Formation of Microaneurisms, Aberrant Fenestration, and Delayed Hyaloid Vessel Regression in *Tspan12*$^{-/-}$ Mice

(A) Confocal projections of IB4-stained NFL (left), IPL (middle), and OPL (right) vasculatures in P16 whole-mount retinas.

(B) P15 retinal sections stained with MECA-32 (red), IB4 (green), and DAPI (blue).

(C) Hyaloid vessels isolated from P7 and P12 neonatal eyes.

In all panels, *Tspan12* genotypes are indicated on the left. Scale bars = 100 μm (main panels) and 10 μm (insets). Arrows in (A) and (B): microaneurisms.

TSPAN12 Enhances Norrin/β-Catenin Signaling

In order to determine the role of TSPAN12 in Norrin/β-catenin signaling, we performed Topflash reporter assays in HEK293 cells. Cells were transfected with reporter constructs and plasmids encoding FZD4 and LRP5, as well as either a plasmid encoding TSPAN12 or a negative control plasmid. Transfected cells were then cultured in the presence or absence of 10 nM recombinant Norrin. In FZD4 and LRP5 cotransfected cells, Norrin induced a 6.85- ± 0.037-fold activation of reporter activity compared to cells expressing the receptors but not stimulated with Norrin. The addition of TSPAN12 strongly increased the response to Norrin and resulted in a 19.78- ± 2.57-fold activation (Figure 4A), whereas the negative control reporter Fopflash was not activated under these conditions (Figure S7A). Activity of the internal control (Renilla luciferase under a constitutive promoter) was virtually identical in cells expressing TSPAN12 or vector control (not shown). Overexpression of TSPAN12 alone, or coexpression of TSPAN12 and LRP5 in the absence of FZD4, was not sufficient to allow activation with Norrin. Coexpression of FZD4 and TSPAN12 in the absence of exogenous LRP5 led only to a very weak activation (Figure 4A). Thus, exogenous TSPAN12 strongly enhances Norrin/β-catenin signaling only when FZD4, LRP5, and Norrin are present, and it can do so over a wide range of Norrin concentrations (Figure S7B).

We then investigated if TSPAN12 can enhance signaling induced by other FZD ligands by transfecting cells with human Wnt3a cDNA in the presence or absence of FZD4 and LRP5 cDNAs. Intriguingly, TSPAN12 did not enhance Wnt3a/β-catenin signaling through endogenous Frizzleds and LRPs or overexpressed FZD4 and LRP5 (Figure 4B). We also examined if TSPAN12 could enhance signaling initiated by Wnt5a, 7a, or 7b because Wnt7b is a FZD4 ligand that functions during hyaloid

or *Norrin* in the regulation of intraretinal capillary development. In a different analysis, MECA-32 staining on retinal sections from the same mice analyzed above revealed only sporadic weak expression in *Tspan12*$^{+/-}$, *Norrin*$^{+/-}$, or *Lrp5*$^{+/-}$ retinas (arrows, Figures 3C and 3D) but significant and broad expression in the *Tspan12*$^{+/-}$;*Norrin*$^{+/-}$ or *Tspan12*$^{+/-}$;*Lrp5*$^{+/-}$ transheterozygous retinal vasculatures (Figures 3C and 3D). These data argue that *Tspan12* genetically interacts with *Norrin* or *Lrp5* with regard to the transcriptional response to Norrin/β-catenin signaling.

Interestingly, the transheterozygous mice did not manifest the full spectrum of phenotypes found in the homozygous single-gene knockouts, suggesting that they are hypomorphic in nature.

Taken together, our in vivo analyses provide strong genetic evidence that TSPAN12 may be involved in Norrin/β-catenin signal transduction.

A
Retinal vertical sprouts in offspring of *Tspan12* +/- mated with *Lrp5* +/-

*p<0.0001

NS

NS

| Tspan12: | +/+ | +/- | +/+ | +/- |
| Lrp5: | +/+ | +/+ | +/- | +/- |

Genotypes

● P15
✕ P14

Vertical sprouts (% of wild-type average)

B
Retinal vertical sprouts in offspring of *Tspan12* +/- mated with *Norrin* -/y

*p<0.0001

NS

*P<0.001

| Tspan12: | +/+ | +/- | +/+ | +/- |
| Norrin: | +/+ | +/+ | +/- | +/- |

Genotypes

● P15
○ P30

Vertical sprouts (% of wild-type average)

C

Tspan12 +/+ Lrp5 +/+ *Tspan12 +/- Lrp5 +/+* *Tspan12 +/+ Lrp5 +/-* *Tspan12 +/- Lrp5 +/-*

NFL
IPL
OPL

P15 retinal sections Isolectin B4 MECA-32 DAPI

D

Tspan12 +/+ Norrin +/+ *Tspan12 +/- Norrin +/+* *Tspan12 +/+ Norrin +/-* *Tspan12 +/- Norrin +/-*

NFL
IPL
OPL

100 μm

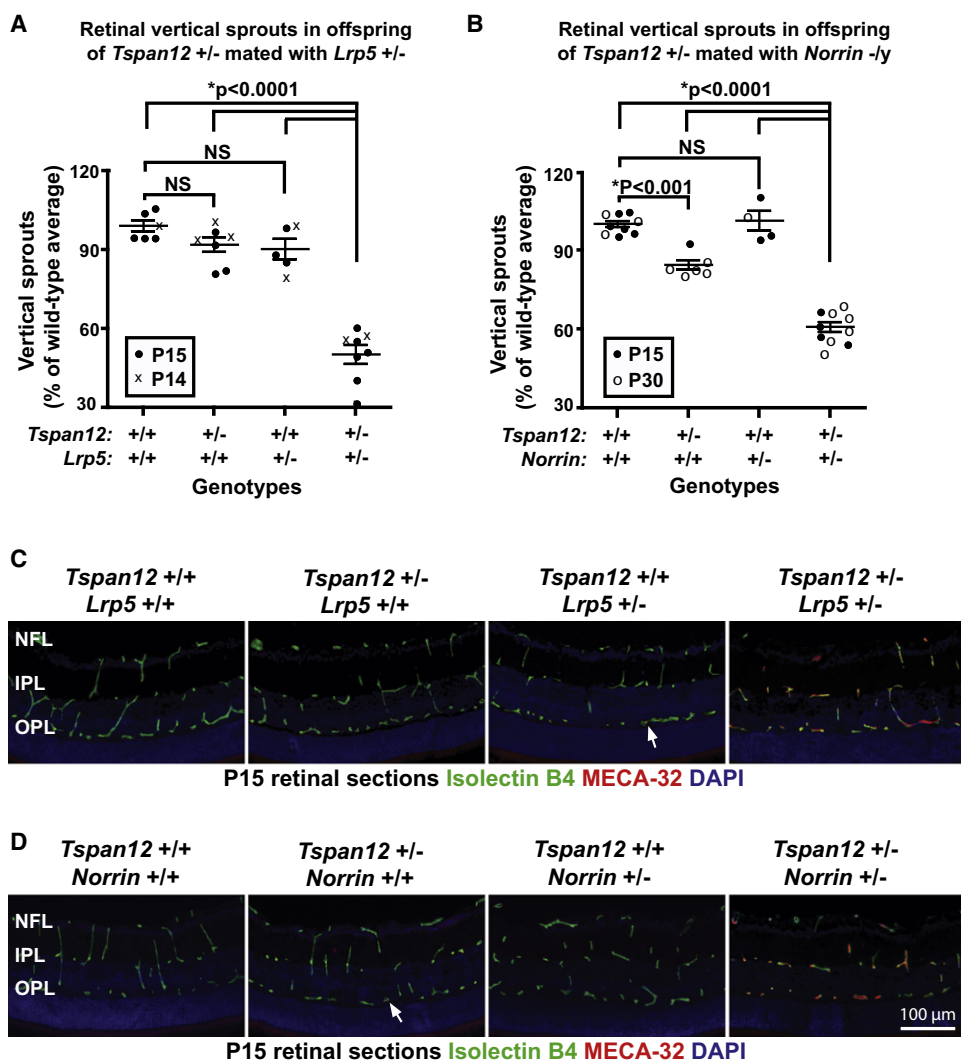**P15 retinal sections Isolectin B4 MECA-32 DAPI**

Figure 3. *Tspan12* Genetically Interacts with *Norrin* and *Lrp5*

(A and B) Quantitative analysis of vertical sprouts in the retinas of postnatal mice from two mating setups indicated on top of the graphs. Each symbol represents one animal. Group averages and standard errors are overlaid over individual data points.

(C and D) Immunofluorescence staining of IB4 (green), MECA-32 (red), and DAPI (blue) on retinal sections. White arrows indicate sporadic weak expression of MECA-32.

vessel regression (Lobov et al., 2005), and Wnt7a and 7b are required for the vascularization of the spinal cord (Daneman et al., 2009; Stenman et al., 2008). Again we found that TSPAN12 enhanced only Norrin/β-catenin signaling but not signaling induced by several Wnts (Figures S8A and S8B). In agreement with the ligand specificity observed in vitro, we found that loss of *Tspan12* did not affect spinal cord vascular development (Figure S9), a process regulated by Wnt7a and 7b (Daneman et al., 2009; Stenman et al., 2008). Although we cannot formally rule out that TSPAN12 enhances signaling initiated by other Wnt ligands, our in vitro and in vivo analyses suggest that TSPAN12 functions selectively in Norrin/β-catenin signaling.

We further analyzed the specificity of the tetraspanin-mediated enhancement of Norrin/β-catenin signaling by substituting TSPAN12 with CD9, CD63, CD151, TSPAN7, TSPAN8,

TSPAN11, and TSPAN13. None of these TSPANs enhanced Norrin/β-catenin signaling (Figure 4C and data not shown), confirming that TSPAN12 has specific functions that are distinct from other tetraspanins. Finally, we coexpressed TSPAN12 with multiple Frizzleds that do not bind Norrin (Smallwood et al., 2007). As expected, Norrin did not activate these Frizzleds, and TSPAN12 also had no effect on them (Figures 4C and S8C). When Wnt3a was used to activate this set of Frizzled receptors, TSPAN12 did not enhance their activity either (Figure S8C).

The ability of TSPAN12 to enhance Norrin/β-catenin signaling was verified by examining β-catenin stabilization in 293 cells overexpressing FZD4 and LRP5 with or without TSPAN12. Whereas recombinant Norrin increased β-catenin in the absence of TSPAN12, coexpression of TSPAN12 led to further increase of β-catenin levels (Figure 4D). Again, TSPAN12 had no effect on
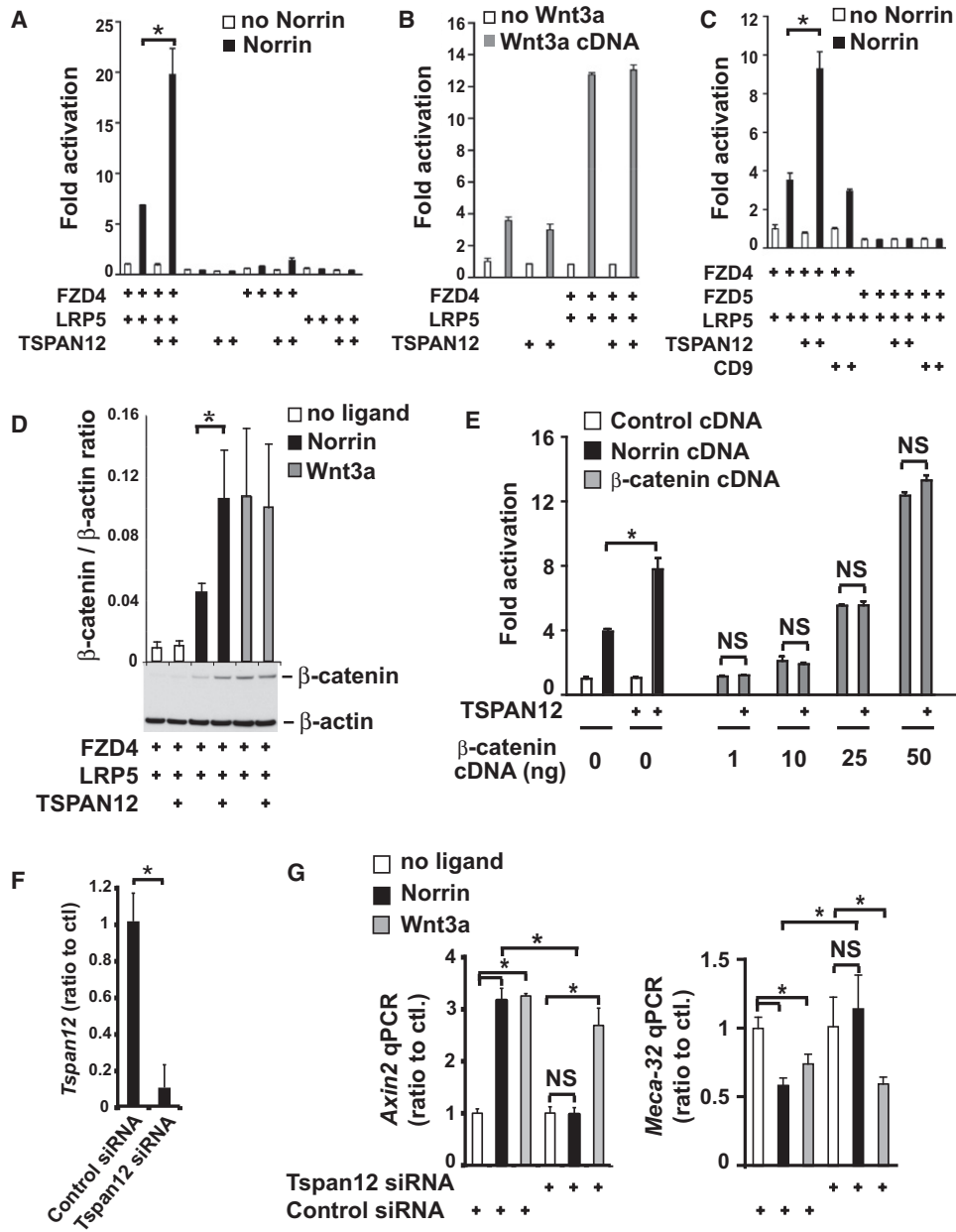
Figure 4. TSPAN12 Enhances Norrin/β-Catenin but Not Wnt/β-Catenin Signaling

(A–C) Topflash assay in 293 cells transfected with plasmids indicated below the graph and stimulated with ligands indicated in each panel.

(D) Lysates of 293 cells expressing the indicated proteins and stimulated with Norrin or Wnt3a were probed with anti-β-catenin and anti-β-actin antibodies. The density of each band was quantified, and the ratios between the β-catenin and β-actin band densities for each condition were calculated and plotted in the top panel.

(E) Topflash reporter activities induced with (black bars) and without Norrin (white bars) in 293 cells transfected with FZD4 and LRP5 or stimulated by expressing increasing concentrations of β-catenin in the presence or absence of TSPAN12 (gray bars).

(F) Quantitative real-time PCR of the *Tspan12* message in human retinal ECs transfected with a control or *Tspan12* siRNAs.

(G) Quantitative real-time PCR of *Axin2* (left) and *Meca-32* (right) messages in human retinal ECs treated with the indicated ligands and transfected with a control or *Tspan12* siRNAs. In (F) and (G), *Tspan12, Axin2*, and *Meca-32* messages were normalized to the housekeeping gene *Gapdh* in all samples then calculated as ratios to the averages of untreated cells.

Bars in all plots represent the mean of triplicate samples; error bars represent standard deviations (SD). Asterisks indicate a significant difference between a pair of samples with a p value < 0.05.

β-catenin levels induced by Wnt3a (Figure 4D). When we activated the Topflash reporter directly by β-catenin overexpression, coexpression of TSPAN12 had no effect (Figure 4E). Taken together, our findings suggest that TSPAN12 specifically enhances Norrin signaling through FZD4 and LRP5, and that it acts upstream of β-catenin stabilization.

TSPAN12 Is Required for Norrin/β-Catenin Signaling in Retinal Endothelial Cells

Because we detected TSPAN12 expression in the retinal vasculature (Figures 1B, S3A, and S3B), and activity of the β-catenin pathway is documented in several types of central nervous system (CNS) ECs including those of the retina (Phng et al., 2009), brain (Liebner et al., 2008), and spinal cord (Daneman et al., 2009; Stenman et al., 2008), we asked whether TSPAN12 is required for Norrin/β-catenin signaling in retinal ECs. We used human retinal microvascular endothelial cells (HRMVECs) to investigate the function of endogenous TSPAN12. PCR analysis showed that *Tspan12*, *Fzd4*, and *Lrp5* were expressed in HRMVECs (data not shown), and siRNA-mediated silencing efficiently reduced the endogenous *Tspan12* message (Figure 4F). Stimulation of HRMVECs with either Norrin or Wnt3a significantly increased levels of *Axin2* mRNA, a target gene of the canonical β-catenin pathway in many cell types, and significantly decreased levels of *Meca-32*, a target gene suppressed by canonical β-catenin signaling in CNS ECs in vitro and in vivo (Liebner et al., 2008). Transfection with a pool of *Tspan12* siRNA (Figure 4G) or an independent single *Tspan12* siRNA (data not shown) in HRMVECs completely abolished the regulation of *Axin2* and *Meca-*32 by Norrin but had no effect in cells stimulated with Wnt3a (Figure 4G). Our observation that *Meca-*32 is upregulated in the retinal vasculature of *Norrin*, *Lrp5*, and *Tspan*12 mutant mice (Figures 2B and S5B) in vivo, in conjunction with the finding that *Meca-*32 upregulation is a direct consequence of losing β-catenin signaling in CNS ECs (Liebner et al., 2008) and HRMVECs (Figure 4G), provides strong evidence that TSPAN12 is required for Norrin/β-catenin signaling in retinal ECs.

TSPAN12 Is a Component of the Norrin-Receptor Complex

Given that TSPAN12 is a transmembrane protein and acts upstream of β-catenin stabilization, it may function in the plasma membrane by interacting with components of the Norrin-receptor complex. In order to test this possibility, we first asked if TSPAN12 colocalizes with FZD4 on the cell surface. To this end, we transfected HeLa cells with N-terminally flag-tagged FZD4 (flag is extracellular) and HA-tagged TSPAN12 (HA is intracellular). Plasma membrane FZD4 was detected with a flag antibody on nonpermeabilized live cells on ice to prevent internalization, and TSPAN12 was detected subsequently after fixation and permeabilization. This staining protocol revealed abundant FZD4 expression on the surface of HeLa cells within numerous punctae, and TSPAN12 significantly colocalized with FZD4-positive punctae in the plasma membrane (Figures 5A and 5D). In contrast, CD9 was not found in FZD4-positive structures (Figure 5B). When FZD4 was substituted with FZD5 and coexpressed with TSPAN12 we found that TSPAN12 and FZD5 were mostly separated (Figures 5C and 5D). A fraction of overex-

pressed TSPAN12 was also observed in the endoplasmic reticulum (ER) (colocalized with Calnexin in separate experiments; data not shown). These data suggest that when TSPAN12 is transported to the plasma membrane, it colocalizes with FZD4 in punctate structures.

Next we asked if TSPAN12 is physically associated with the Norrin-receptor complex in the plasma membrane. We coexpressed FZD4, LRP5, and TSPAN12 in 293 cells and used FZD5 and CD9 as specificity controls for FZD4 and TSPAN12, respectively. After incubation of these cells with conditioned medium containing flag-Alkaline Phosphatase (AP)-Norrin, Norrin-associated membrane proteins were mildly crosslinked before Norrin was immunoprecipitated (IPed) with a flag antibody (Figure 5E). Norrin was bound to cells expressing FZD4/LRP5 with and without TSPAN12 but was washed off from cells expressing FZD5/LRP5/TSPAN12 or TSPAN12/LRP5 (Figure 5E, 2nd panel), hence Norrin efficiently pulled down FZD4 but not FZD5 (Figure 5E, top panel). TSPAN12 was co-IPed with Norrin/FZD4 but not when FZD4 was substituted with FZD5 or when no Frizzled was present. In contrast, CD9, although expressed at a similar level as TSPAN12, was not co-IPed with Norrin/FZD4 (Figure 5E, 3rd panel). LRP5 was present in the FZD4 complex pulled down by Norrin (Figure 5E, bottom panel). Thus, TSPAN12 is physically associated with the Norrin/FZD4/LRP5 ligand-receptor complex. TSPAN12 also co-IPed with Norrin when only FZD4 but no LRP5 was present (Figure S10A). When TSPAN12 and LRP5 were coexpressed in the absence of FZD4, and TSPAN12 was IPed, no association with LRP5 was detected (Figure S10B). Another LRP family member VLDLR was absent from the complex containing Norrin, FZD4, and TSPAN12 (Figure S10C), confirming that LRP5 is a specific component of the ligand-receptor complex. Taken together, our data suggest that TSPAN12 is a component of the Norrin-receptor complex, and FZD4 is a pivotal molecule that mediates the association of all other elements in this complex.

TSPAN12 Does Not Alter Ligand-Receptor Binding

The colocalization and association of TSPAN12 with the Norrin-receptor complex prompted us to consider three possible mechanisms by which TSPAN12 can enhance Norrin signaling: (1) TSPAN12 binds Norrin directly and brings more ligands into the receptor complex; (2) TSPAN12 enhances the ability of FZD4 to bind Norrin; (3) TSPAN12 increases the surface concentration of FZD4. To test these possibilities, we carried out the following experiments.

Figure 5E shows that the first possibility is not supported by our results; nonetheless, we tested it further by incubating HeLa cells overexpressing FZD4, LRP5, or TSPAN12 with conditioned medium containing flag-AP-Norrin and subsequently detecting the receptor-bound flag-AP-Norrin by AP substrate staining. Consistent with previous reports (Xu et al., 2004), we found that flag-AP-Norrin efficiently bound to cells expressing FZD4 but not LRP5. Importantly, Norrin did not bind to cells expressing TSPAN12 alone (Figure 5F), indicating that TSPAN12 does not bind to Norrin directly.

Next, HeLa cells overexpressing FZD4/LRP5, together with TSPAN12 or vector control, were probed with several dilutions of flag-AP-Norrin conditioned medium (Figure 5G). Binding of
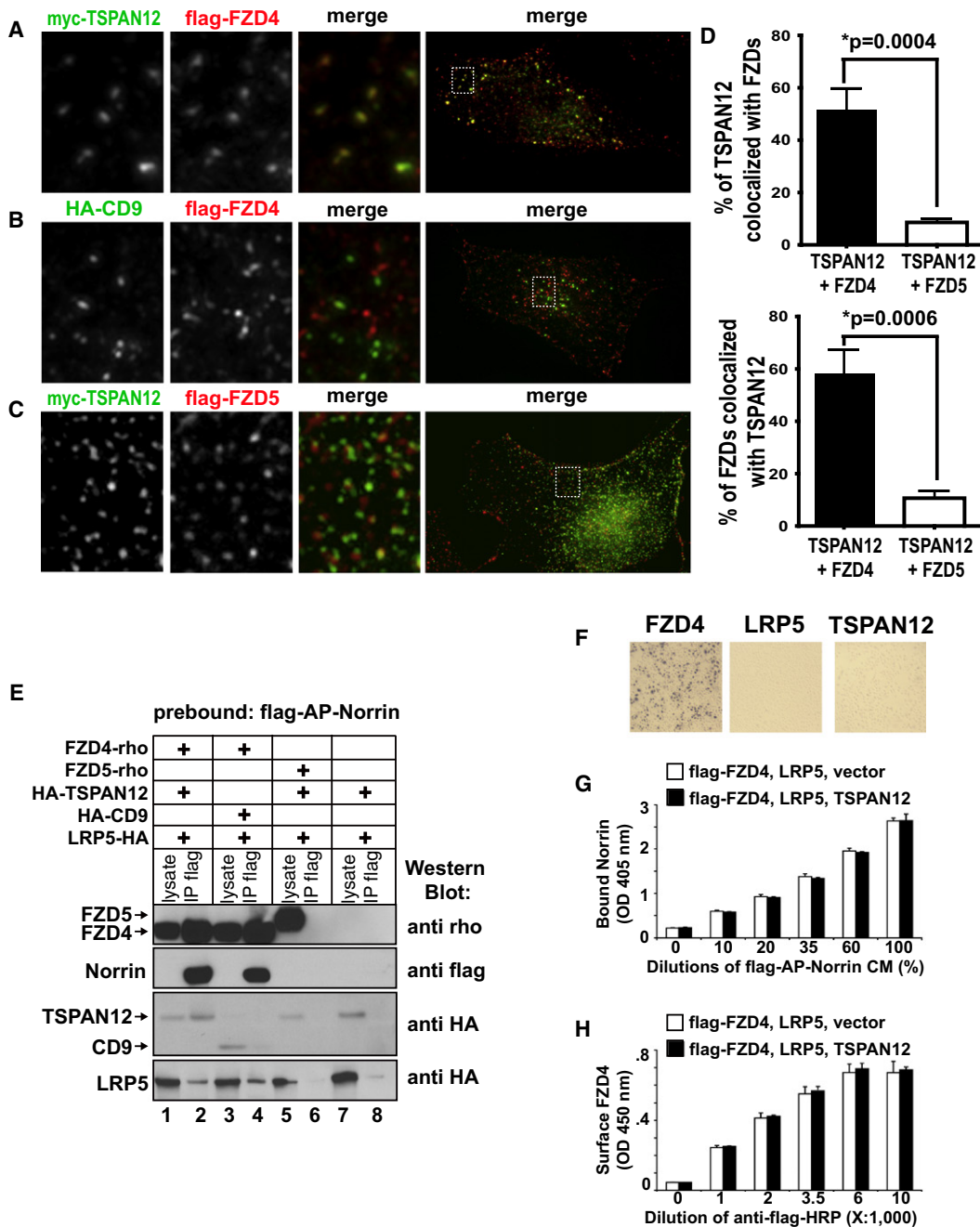
Figure 5. TSPAN12 Is a Component of the FZD4-Receptor Complex but Does Not Alter Norrin/FZD4 Binding

(A–C) Deconvolution microscopic images of HeLa cells expressing the indicated proteins. White frames in the far right panels outline the areas shown in the three panels on the left.

(D) Percentages of green voxels colocalized with red voxels (top panel) or red voxels colocalized with green voxels (bottom panel) in cells transfected with either FZD4 + TSPAN12 (black bars, n = 6) or FZD5 + TSPAN12 (white bars, n = 6). Quantification was done using three-dimensional images of cells obtained by deconvolution microscopy. Bars represent the mean of six samples; error bars represent SD.

(E) 293 cells expressing the indicated proteins were incubated with flag-AP-Norrin conditioned medium (CM), washed, and mildly crosslinked. Extracts were IPed with anti-flag antibody. Total cell lysates (in lanes labeled with "lysate") or proteins that were coprecipitated with Norrin (in lanes labeled with "IP flag") were detected with anti-epitope tag antibodies indicated on the right. Proteins detected by each epitope tag antibody are indicated on the left.

(F) Binding of flag-AP-Norrin to HeLa cells expressing the indicated proteins was detected after formation of the purple AP reaction product.

(G) HeLa cells transfected with the indicated plasmids were incubated with several dilutions of flag-AP-Norrin CM, and the bound Norrin was quantified by an AP substrate assay.

(H) In an experiment parallel to that in panel G, surface FZD4 was measured with several dilutions of an HRP-conjugated anti-flag antibody and quantified with an HRP substrate assay. Bars in (G) and (H) represent the mean of triplicate samples; error bars represent SD (see Figure S11).
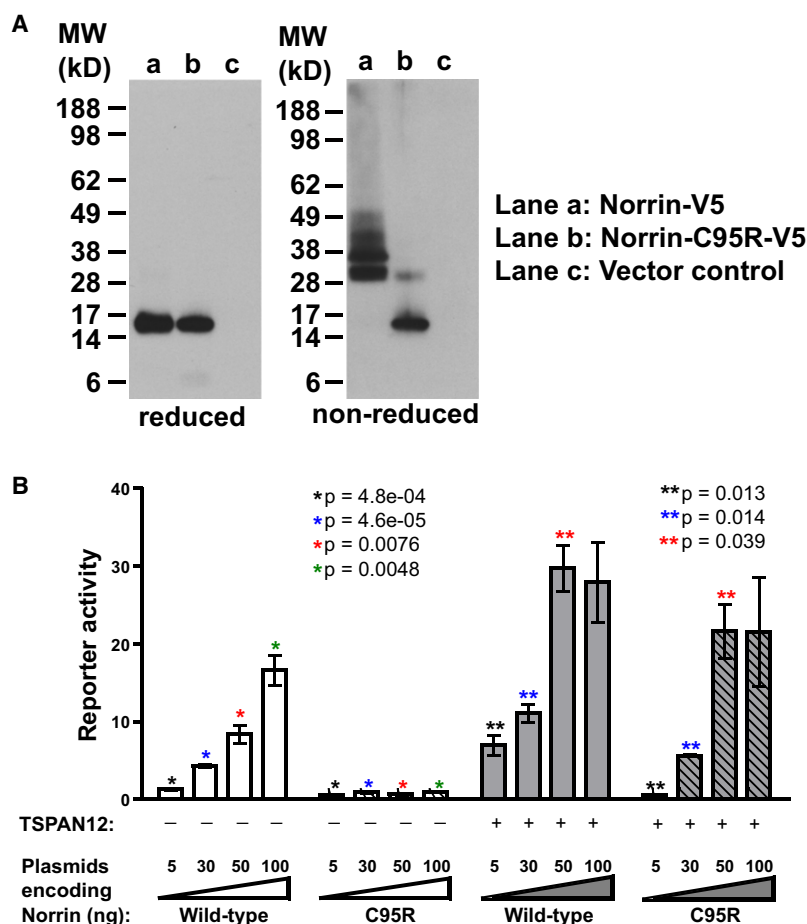
A

Figure 6. TSPAN12 Rescues the Defect of Norrin-C95R

(A) Western blots of extracellular matrix extracts from 293 cells transfected with the indicated plasmids under reducing (left) or nonreducing (right) conditions using anti-V5 antibody.

(B) Topflash assay in 293 cells transfected with plasmids encoding FZD4 and LRP5 and increasing amounts of plasmids encoding wild-type or C95R mutant Norrin, together with either TSPAN12 plasmid or vector control. Bars represent the mean of triplicate samples; error bars represent SD.

Lane a: Norrin-V5
Lane b: Norrin-C95R-V5
Lane c: Vector control

B

has been reported in a human patient with retinopathy (Isashiki et al., 1995). Wild-type Norrin or Norrin-C95R tagged with the V5 epitope were expressed in 293 cells and isolated from the extracellular matrix (ECM). SDS-PAGE under reducing conditions revealed that wild-type Norrin and Norrin-C95R are both efficiently expressed. Consistent with previous reports (Perez-Vilar and Hill, 1997), analysis under nonreducing conditions demonstrated that wild-type Norrin formed dimers and multimers. In contrast, Norrin-C95R was predominantly monomeric with a small fraction of dimers that might have formed by alternative disulfide bonding but formed no larger assemblies (Figure 6A). We transfected 293 cells with increasing amounts of wild-type or mutant Norrin cDNAs together with the receptors to induce Topflash activity in the presence or absence of TSPAN12. Expression of wild-type Norrin efficiently induced Norrin/β-catenin signaling in a dose-dependent manner, and the presence of TSPAN12 strongly enhanced this activity at all ligand concentrations (Figure 6B). Norrin-C95R, on the other hand, was inactive in cells without TSPAN12, even at the highest dose of 100 ng Norrin plasmid, although this mutant form of Norrin can still efficiently bind FZD4 (Figure S11). Intriguingly, the presence of TSPAN12 partially restored the activity of Norrin-C95R, and at the highest ligand concentration, signaling strengths of the wild-type and mutant Norrins were comparable (Figure 6B). The rescue activity of TSPAN12 in this experiment did not result from modulating Norrin-C95R binding to FZD4 (Figure S11), suggesting that it may function by compensating for the inability of monomeric Norrin-C95R to promote receptor multimerization.

Since we were unable to express Norrin-C95R at high enough concentration to carry out a biochemical analysis of receptor clustering, we utilized the previously described mutation FZD4-M157V, which strongly impairs Norrin/β-catenin signaling but maintains the ability to bind Norrin (Xu et al., 2004). Aided by structural information (Dann et al., 2001), the M157V mutation has been proposed to affect Norrin-induced FZD4 dimerization and consequently multimerization (Dann et al., 2001; Toomes et al., 2004; Xu et al., 2004). Consistent with previous reports (Xu et al., 2004), we found that signaling mediated by FZD4-M157V was severely impaired. Interestingly, TSPAN12
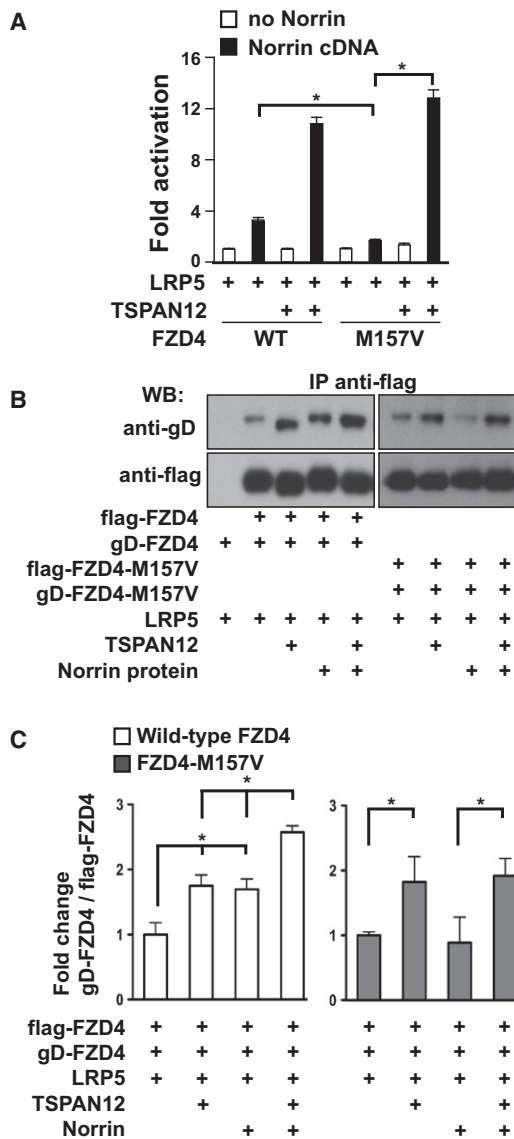
flag-AP-Norrin to cells was similar in the presence or absence of TSPAN12 at all Norrin concentrations tested, suggesting that coexpression of TSPAN12 does not alter the ability of FZD4 to bind Norrin.

Finally, surface FZD4 levels from HeLa cells overexpressing flag-FZD4/LRP5 in the presence or absence of TSPAN12 were evaluated with an HRP-coupled anti-flag antibody and found to be unaltered (Figure 5H).

TSPAN12 Enhances Receptor Clustering

Because our results do not support a role for TSPAN12 in altering ligand-receptor binding or receptor expression, and in light of reports that oligomerization of Frizzleds and LRP5/6 is important for their function (Cong et al., 2004), we went on to investigate whether TSPAN12 regulates the organization of the Norrin-receptor complex. Toward this aim, we took advantage of the unique biochemical properties of Norrin. Norrin belongs to the subgroup of cysteine knot proteins that form dimers via intermolecular disulfide bonds (Vitt et al., 2001). In addition, it has been reported that Norrin dimers can further assemble into higher-molecular-weight multimers, and through reduction of the intermolecular disulfide bonds or mutation of the cysteine in position 95, Norrin multimers can be disrupted (Perez-Vilar and Hill, 1997).

To investigate if multimer formation plays a role in Norrin function, we mutated cysteine 95 to arginine as the C95R mutation

A

B

C

Figure 7. TSPAN12 Rescues FZD4-M157V Multimerization and Signaling Defects

(A) Topflash assay in 293 cells transfected with plasmids indicated below the graphs and stimulated with Norrin or no ligand.

(B) 293 cells transfected with the indicated plasmids were incubated on ice with or without recombinant Norrin, lysed, and IPed with anti-flag antibody. Membranes were probed consecutively with anti-gD and anti-flag antibodies.

(C) Ratios of gD-FZD4 and flag-FZD4 band densities were calculated and plotted from triplicate experiments similar to the example shown in (B). Data are normalized to the value represented by the first column in each graph. Bars in all panels represent the mean of triplicate samples; error bars represent SD. Asterisks indicate a significant difference between a pair of samples with a p value < 0.05.

coexpression fully rescued the signaling defect of FZD4-M157V (Figure 7A).

We then utilized FZD4-M157V to directly investigate the role of TSPAN12 in FZD4 multimerization. 293 cells were transfected with TSPAN12, control vector, or CD9 and cotransfected with

flag-FZD4 and gD-FZD4 or flag-FZD5 and gD-FZD5 or with flag-FZD4-M157V and gD-FZD4-M157V. Cells were incubated on ice with medium containing Norrin or no ligand. Cell lysates were IPed with anti-flag antibody and probed for coimmunopre-cipitation of gD-FZD4. To enable quantification of protein-protein interactions, no crosslinking reagent was used in this experiment.

Similar baseline levels of association between gD-FZD4 and flag-FZD4 or gD-FZD4-M157V and flag-FZD4-M157V were detected (Figure 7B and data not shown). TSPAN12 increased the amount of gD-FZD4 pulled down by flag-FZD4 compared to samples without a tetraspanin (Figures 7B and 7C) or with tet-raspanin CD9 (Figures S12A and S12B) but did not enhance the multimerization of FZD5 (Figures S12C and S12D). Norrin alone also increased the amount of gD-FZD4 pulled down by flag-FZD4, and the combination of Norrin and TSPAN12 further increased FZD4 clustering (Figure 7B, left panels and Figure 7C, open bars). Importantly, the M157V mutation severely impaired the ability of Norrin to cluster gD-FZD4 with flag-FZD4, whereas coexpression of TSPAN12 compensated this defect (Figure 7B, right panels and Figure 7C, filled bars). Together, these data indicate that TSPAN12 and Norrin both promote FZD4 multimerziation and suggest that initiation of Nor-rin/β-catenin signaling requires (1) factors that promote FZD4 multimerization and (2) activation of FZD4 by ligand binding.

DISCUSSION

TSPAN12 Is Required for FZD4/β-Catenin Signaling Induced by Norrin but Not Wnts

The central conclusion from the present study is that the tetra-spanin TSPAN12 is required for retinal vascular development and Norrin/β-catenin but not Wnt/β-catenin signaling, thus uncovering a mechanistic distinction between these two signaling systems. Consistent with a specific role for TSPAN12, $Tspan12^{-/-}$ mice lack the embryonic phenotypes (Figure S9) that were found in mutants of several Wnts (Daneman et al., 2009; Stenman et al., 2008; van Amerongen and Berns, 2006). In addition, $Tspan12^{-/-}$ mice do not phenocopy $Fzd4$ mutant phenotypes that are independent of Norrin, e.g., malnutrition and impaired growth due to the lack of esophageal skeletal muscle (Wang et al., 2001). Furthermore, $Lrp5$ mutant mice exhibit Norrin-independent low bone mass phenotypes, whereas bone mass in $Tspan12^{-/-}$ mice is normal (data not shown). Finally, although hyaloid vessel regression is delayed in $Tspan12^{-/-}$ and $Norrin^{-/-}$ mice, the degrees of delay appear to be less severe than what was observed in $Fzd4$ and $Lrp5$ mutant mice, likely due to the activity of Wnt7b mediated by FZD4 (Lobov et al., 2005). Thus, the spectrum of phenotypes seen in the $Tspan12^{-/-}$ mice is consistent with our in vitro results and indicates a specific role of TSPAN12 in Norrin/β-catenin signaling but not Wnt/β-catenin signaling.

TSPAN12 and Norrin Share Similar Profiles of Evolutionary Conservation

The human genome harbors 33 tetraspanins, and numerous tet-raspanins are found in multicellular eukaryotes (Garcia-Espana et al., 2008). TSPAN12 is highly conserved in evolution, and

a high degree of conservation extends to birds (94% identity between *Homo sapiens* and *Gallus gallus*) and fish (70% identity between *Homo sapiens* and *Danio rerio*). However, no TSPAN12 ortholog could be identified in flies or nematodes. Interestingly, Norrin orthologs are also found only in birds (87% identity between *Homo sapiens* and *Gallus gallus*) and fish (68.6% identity between *Homo sapiens* and *Danio rerio*) but not in flies and nematodes (using NCBI Homologene). Although *Tspan12* and *Norrin* reside in different chromosomes, it appears possible that these two molecules coevolved and became functionally linked.

Regulation of FZD4 Clustering by Norrin and TSPAN12

Receptor oligomerization has been reported to play an important role in Wnt/β-catenin signaling (Bilic et al., 2007; Carron et al., 2003; Cong et al., 2004; Kaykas et al., 2004). Our study suggests that FZD4 clustering is also required for Norrin/β-catenin signaling, but the mechanisms to regulate receptor oligomerization appear to be different between Norrin and Wnts. Data in this report suggest that pre-existing FZD4 oligomers are insufficient to transduce Norrin/β-catenin signaling, and enhancement of FZD4 clustering via binding to Norrin multimers and association with TSPAN12 is required to fully activate the system. Given that TSPAN12 alone can enhance FZD4 multimerization, it is unclear why TSPAN12 does not affect signaling induced by Wnts. It is possible that Wnts can induce strong signaling on small receptor clusters or organize receptor multimers via a distinct mechanism, thus circumventing a requirement for TSPAN12. More detailed structural and biochemical characterization of the receptor complexes induced by Norrin or Wnts will be required to explain why TSPAN12-mediated FZD4 multimerization regulates Norrin but not Wnt activities.

TSPAN12 Is a Candidate Gene for FEVR

FEVR (familial exudative vitreoretinopathy) is a progressive retinal vascular disease with autosomal dominant, autosomal recessive, and X-linked recessive inheritance patterns. Several disease-associated loci have been identified, corresponding to the *Fzd4*, *Lrp5*, and *Norrin* genes (Warden et al., 2007). Interestingly, a family with FEVR patients was recently identified in which linkage to any known EVR locus could be excluded (Toomes et al., 2005). This report also suggested that only about 40% of FEVR patients carry mutations in either *Fzd4* or *Lrp5*. In light of the phenotypic similarities of *Tspan12*, *Fzd4*, *Lrp5*, and *Norrin* mutant mice, it seems likely that FEVR or related vascular diseases of the retina could be caused by mutations in *Tspan12*.

EXPERIMENTAL PROCEDURES

Histology

Tissues were fixed with 4% PFA and embedded in OCT. LacZ, DAB, and IB4 staining was performed according to standard procedures. Antigen retrieval using DAKO Target Retrieval Solution was done before staining with IB4 (Sigma) and MECA-32 (BD), which were detected with a secondary antibody conjugated to Alexa-594 or Alexa-488 (Molecular Probes), respectively.

Hyaloid Vessel Preparation

Neonatal eyes were fixed in 4% PFA overnight. Cornea, lens, and iris were removed. 1.5% low melting agarose was injected into the vitreous. The solid-ified agarose was extricated and heat melted on a glass slide, washed with warm water, air-dried, and imaged without mounting.

Plasmids

Expression vectors encoding multiple TSPANs, Frizzleds, Norrin, Wnts, and LRPs were in the pCMV6XL vectors. cDNAs encoding epitope-tagged proteins were generated by PCR and subcloned into pEGFPN1, pCMV, or pRK5. Topflash and Fopflash plasmids and pRL-CMV (Renilla luciferase) were from Promega. Norrin-C95R and FZD-M157V were generated by site-directed mutagenesis.

Luciferase Assays

Firefly and Renilla luciferase activity was measured using the Dual Stop and Glo system (Promega). In 24-well plates, 160K 293 cells/well were transfected with 400 ng DNA and 1.5 μl Fugene6 (Roche). The DNA mix contained 100 ng each of FZD and LRP5 plasmids, 50 ng of TSPAN12 plasmid or vector control, and 150 ng of reporter mix (105 ng Topflash or Fopflash, 30 ng pRL-CMV, and 15 ng pCAN-myc-Lef-1). Cells were stimulated 24 hr (hr) after transfection for 16–18 hr with 125 ng/ml Norrin or 200 ng/ml Wnt3a, 5a, 7a (R&D Systems) or by coexpressing the indicated amount of Norrin or Wnt plasmids. Reporter activity was calculated as firefly/renilla activity in each well. Within each plot all data were normalized to the datapoint represented by the first bar.

Knockdown of TSPAN12 in HRMVEC

HRMVECs (ACBRI) were transfected with 20 nM of pooled or single siRNA (Dharmacon, for target sequences see Supplemental Data) with RNAimax. After 24 hr, cells were split at 50 K into 6-well plates. Twelve hours later cells were stimulated with 1.25 μg/ml Norrin or 0.3 μg/ml hWnt3a (R&D Systems) for 48 hr. Total RNA was extracted using Trizol (Invitrogen) and digested with DNase (Stratagene) and used for Axin2 and Meca-32 RT-qPCR.

β-Catenin Stabilization Assay

293 cells were transfected in 24-well plates with 100 ng each of FZD4, LRP5 plasmids, and TSPAN12 plasmid or vector control. After 24 hr cells were stimulated with 500 ng/ml Norrin or 500 ng/ml Wnt3a (R&D Systems) for 12 hr and lysed in 300 μl lysis buffer (150 mM NaCl, 50 mM Tris/HCl [pH 8.0], 1 mM EGTA, 1× protease inhibitor cocktail [EDTA free, Roche], 1% NP-40, 0.1% N-dodecyl-beta-D-maltoside [Calbiochem], 3 mM MgCl$_2$ and 30 U/ml DNaseI) on ice for 1 hr. Lysates were cleared by centrifugation and 200 μl supernatant were incubated for 1 hr at 4°C with Convacalin-A beads to adsorb junctional β-catenin. Supernatants were cleared by centrifugation and processed for western blot using anti-β-catenin (Transduction labs) and anti-actin (Novus) antibodies.

Immunofluorescence Staining and Protein Colocalization Analysis

HeLa cells grown on 4-well chamber sides (Labtek) were transfected for 36–40 hr, cooled on ice, and stained with rabbit anti-flag Ab (Sigma, 1:500) on ice for 1 hr. After extensive washing, cells were briefly fixed with 4% PFA, blocked and permeabilized with 10% goat serum, 0.1% Triton X-100 in PBS, and stained with anti-HA or anti-myc antibody. Images were taken with a 60× oil lens using a deconvolution microscope.

Immunoprecipitation

Semiconfluent 293 cells in 175 cm^2 dishes were transfected with 15 μg of Frizzled, 6.3 μg of LRP5-HA, and 18 μg HA-TSPAN12 or 16.65 μg vector control plus 1.35 μg HA-CD9 (to achieve similar expression levels of TSPAN12 and CD9) using 135 ul Fugene6 for 36–40 hr. Cells were washed (subsequent steps were at 0°C) with culture medium and incubated for 1 hr with 40 ml CM containing flag-AP-Norrin. After several washes with PBS, cells were subjected to mild surface crosslinking with 40 ml of 0.02 mM DTSSP (Pierce) in PBS for 1 hr (stopped with Tris [pH 7.5], 20 mM final). Cells were pelleted and washed with TBS before lysis in 20 ml lysis buffer (see β-Catenin Stabilization Assay) for 1 hr. Subsequent steps were at 4°C. Lysates were spun at 180K g for 1 hr and preadsorbed with agarose beads. Subsequently, flag-AP-Norrin was bound to anti-flag-beads (Sigma) overnight. After extensive washing with lysis buffer, beads were eluted with 150 μl 1× LDS sample buffer (Invitrogen). Eluates were analyzed by western blotting with HRP-conjugated primary antibodies to detect flag and HA, and anti-rho antibody (ABR, clone 1D4) was

detected using mouse Trueblot reagent (eBioscience). For immunoprecipitation with anti-HA-affinity matrix (Roche), cells were transfected, lysed, and processed as described above.

For coimmunoprecipitation of gD-FZD with flag-FZD, semiconfluent 293 cells in 25 cm^2 dishes were transfected with 390 ng of each FZD plasmid, 910 ng of LRP5, and 3380 ng of HA-TSPAN12 or vector control for 36 hr. When Norrin was added, cells were placed on ice and incubated with 3 ml of ice-cold medium containing 600 ng/ml Norrin or no ligand for 45 min. Cells were lysed with the lysis buffer (see above) containing 600 ng/ml Norrin or no ligand. After centrifugation at 280K g for 10 min, supernatants were incubated for 60 min with anti-flag beads while rocking on ice. After three brief washes with ice-cold wash buffer (100 mM salt, 50 mM Tris [pH8] and plus 10% v/v lysisbuffer), proteins were eluted with 110 μl of 1× LDS sample buffer and processed for western blotting using an anti-gD antibody and mouse Trueblot reagent or HRP-conjugated anti-flag antibody.

Quantification of Plasma Membrane FZD4
HeLa cells overexpressing FZD4 were incubated on ice with HRP-anti-flag Ab (Sigma) at dilutions from 1:1.000 to 1:10.000 in culture medium, washed, lysed in TBS 1% Triton X-100, and mixed with an equal volume of TMB ELISA substrate (Rockland). After several minutes the reaction was stopped with an equal volume of 1M H$_3$PO$_4$ and the reaction product was quantified at 450 nm.

Extracellular Matrix Extracts of Wild-Type and Mutant Norrin
293 cells were transfected with plasmids encoding V5-tagged wild-type Norrin or Norrin-C95R. After 4 days all cells were removed using an enzyme-free cell dissociation reagent (Sigma). ECM was extracted with 6M Guanidinium hydrochloride/0.1 M NaAcetate (pH 5.5) (60 μl per cm^2) for 30 min at room temperature on a rocking platform. Four hundred microliters of ECM extract were ethanol precipitated and washed. Pellets were dissolved in 100 μl LDS sample buffer with and without DTT and analyzed by western blotting with anti-V5 Ab (Serotec).

Statistics
If not mentioned otherwise, averages were calculated from triplicate experiments. Groups were compared using a two-tailed, unpaired Student's t test. p values < 0.05 were considered significant.

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