The Role of *Vldlr* in Intraretinal Angiogenesis in Mice

Chun-hong Xia,¹ Eric Lu,¹ Haiquan Liu,¹ Xin Du,² Bruce Beutler,² and Xiaohua Gong^{1,3}

PURPOSE. To identify and characterize the *r26* mouse line, which displays depigmented patches in the retina, and to determine the causative gene mutation and study the underlying mechanism.

METHODS. Fundus examination, fluorescein angiography, histology, and immunostaining were used to determine the retinal phenotypes. Genome-wide linkage analysis, DNA sequencing, and an allelic test were used to identify the causative gene mutation. Wild-type and mutant gene products were examined by Western blot and transient transfection.

RESULTS. Homozygous *r26/r26* mice displayed depigmented patches in the fundus that overlapped the hyperfluorescent spots in the angiogram. Histology showed overgrown retinal vessels in the subretinal space. Immunostaining verified the presence of endothelial cells in the photoreceptor layer. Chromosome mapping and DNA sequencing revealed a point mutation, c.2239C>T, in the very-low-density lipoprotein receptor (*Vldlr*) gene. An allelic test in *Vldlr* knockout ($-/-$) mice confirmed that *r26/r26* mice display a phenotype similar to that of *r26/r26* mice. The *Vldlr* protein was predominantly localized at the plasma membrane of transfected cells, whereas the truncated *Vldlr* was diffusely expressed in the cell cytosol. The *r26* truncated *Vldlr* was undetectable in mutant retinas by Western blot.

CONCLUSIONS. The *r26* is a recessive mutant caused by a missense mutation in the *Vldlr* gene. This results in a truncated *Vldlr* protein that lacks the C-terminal 127 amino acid residues including the single transmembrane domain and fails to localize at the plasma membrane. Thus, the *r26* is a loss-of-function *Vldlr* mutation. *Vldlr* on the cell surface probably mediates an antiangiogenic signal to prevent retinal endothelial cells from migrating into the photoreceptor cell layer. (*Invest Ophthalmol Vis Sci.* 2011;52:6572–6579) DOI:10.1167/iovs.10-7082

Retinal vasculature is essential for normal visual function of the eye. Vascular abnormalities, including ischemia, neovascularization, and vessel leakage occur in common eye diseases such as diabetic retinopathy, age-related macular degeneration (AMD), and macular edema, which are some of the leading causes of blindness in adults.^{1–3} Retinal angiomatous proliferation (RAP), characterized by subretinal neovascularization originating from retinal vessels, is a subtype of AMD and accounts for 10% to 15% of neovascular AMD.^{4–8} Recent stud-

ies have reported that very-low-density lipoprotein receptor (*Vldlr*) knockout mice replicate some characteristic phenotypes of human RAP, suggesting that *Vldlr* mutant mice can be used as animal models for studying the underlying mechanism of the development of RAP.^{9–13}

Vldlr knockout ($-/-$) mice develop retinal angiomatous proliferation with subretinal neovascularization arising from retinal vessels.^{10–13} In *Vldlr* $^{-/-}$ mice, retinal vessels grow into the subretinal space, which results in retinal-choroidal anastomosis, subretinal fibrosis, retinal pigment epithelium (RPE) hyperplasia, and photoreceptor degeneration. The loss of *Vldlr* facilitates the proliferation, migration, and capillary-like formation of retinal vascular endothelial cells and enhances the angiogenic properties of endothelial cells in vitro and in vivo.¹⁴ Thus, *Vldlr* negatively regulates retinal angiogenesis during development. Moreover, recent findings have indicated *Vldlr* as one of the functional candidate genes in human AMD.¹⁵ However, the underlying mechanism for how the *Vldlr* mutation promotes retinal angiogenesis remains unclear; it is also unclear whether this retinal phenotype is associated with the function of *Vldlr* as a receptor for the uptake of very-low-density lipoprotein (VLDL) or as a mediator for other intracellular signaling.

We report the identification and characterization of a new recessive *Vldlr* mutation, *r26*, from *N*-ethyl-*N*-nitrosourea (ENU)-mutagenized mice. The *r26* mutant mice contain a missense point mutation of the *Vldlr* gene, leading to the expression of a truncated *Vldlr* protein with a premature termination at amino acid residue 747. Similar to the *Vldlr* $^{-/-}$ mice, the *r26* homozygous mice display uncontrolled intraretinal angiogenesis. This work further demonstrates the essential role of the C terminus of *Vldlr* in the regulation of intraretinal angiogenesis. This truncated *Vldlr* mouse line is a useful model for the study of the underlying mechanism for *Vldlr* signaling and human RAP.

METHODS

Animals, Fundus Photography, and Fluorescence Angiography

Animals were cared for in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and all studies were conducted in accordance with a protocol approved by the Animal Care and Use Committee (ACUC) at the University of California, Berkeley. ENU-mutagenesis and mouse breeding were performed as previously described.^{16,17} Allelic testing was conducted by crossing the homozygous *r26* mutant mice with the homozygous *Vldlr* $^{-/-}$ mice.

Fundus photography and fluorescence angiography were performed as described previously.¹⁷

Immunofluorescent Staining, Vessel Painting, and Retinal Histology Analysis

Immunofluorescent staining and vessel-painting experiments were performed as described previously.¹⁷ For immunostaining, a rat anti-CD31 antibody (BD Pharmingen, San Diego, CA) was used to stain retinal vessels in frozen sections, and a goat anti-*Vldlr* antibody (R&D Systems,

From the ¹School of Optometry and Vision Science Program, and the ³Helen Wills Neuroscience Institute, University of California, Berkeley, Berkeley, California; and the ²Department of Genetics, The Scripps Research Institute, La Jolla, California.

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Corresponding author: Chun-hong Xia, 695 Minor Hall, University of California, Berkeley, CA 94720-2020; chxia@berkeley.edu.

Minneapolis) was used to stain transfected cells. For vessel-painting experiments, a solution of 0.1 mg/mL of lipophilic dye DiI (Sigma-Aldrich, St. Louis, MO) was perfused transcardially into the animals, and flat-mounted retinas were imaged using a fluorescent microscope (Zeiss Axiovert 200 equipped with Apotome; Carl Zeiss, Jena, Germany).

Retinal histology analysis was performed as described previously.¹⁷

Chromosome Mapping and Sequencing Analysis

For genome-wide linkage analysis, homozygous *r26* mutant mice in C57BL/6J strain background were mated to wild-type C3H/HeN mice to generate heterozygous hybrid mice. The backcrossed mice were generated by crossing the heterozygous hybrid mice with homozygous *r26* mutant mice. Backcrossed mice were phenotyped, and tail DNAs were genotyped. A total of 59 microsatellite markers were used.

For sequencing analysis, total RNA was isolated from the retinas of homozygous *r26* mutant mice (TRIzol reagent; Invitrogen Life Technologies, Carlsbad, CA), and cDNAs were synthesized as described previously.¹⁷ The *Vldlr* coding region was then amplified (Platinum pfx DNA polymerase; Invitrogen Life Technologies). PCR fragments with overlapping regions were sequenced with various primers spanning the whole coding sequence of *Vldlr*.

Western Blot Analysis

Four retinas were pooled from two mice with the same genotype and were homogenized in the lysis buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100) containing protease inhibitors (Roche Diagnostic Corporation, Indianapolis, IN). The protein lysates were sonicated, and protein concentration was determined (Coomassie Assay Reagent kit; Pierce, Rockford, IL). The samples were then mixed with an equal volume of 2× loading buffer (120 mM Tris-PO₄ [pH 6.8], 4% SDS, 20% glycerol, 10% β-mercaptoethanol, and 0.002% bromophenol blue). Equal amounts (150 μg protein) of the samples were loaded onto 4% to 12% Bis-Tris gel (NuPAGE; Invitrogen, Carlsbad, CA) for separation, and the gel was transferred to PVDF membrane (Bio-Rad Laboratories, Hercules, CA). A goat anti-*Vldlr* an-

tibody (R&D Systems) and a mouse anti-β-actin antibody (Sigma-Aldrich) were used to detect the specific proteins, and chemiluminescence (SuperSignal West Pico Chemiluminescent substrate kit; Thermo Scientific, Rockford, IL) was used to develop the blots.

Construction of *Vldlr* Expression Plasmids and Transient Transfection In Vitro

Two plasmids expressing full-length *Vldlr* or truncated *r26* mutant *Vldlr* were constructed. RNA isolated from the retinas of C57BL/6J wild-type mice was used to synthesize cDNA, and the cDNA was used as the template for PCR amplification of the full-length *Vldlr* and truncated *r26* *Vldlr* fragments. The primer pair of 5'-*EcoRI*-GCATGGGCACGTCCGCGC-3' and 5'-*SacII*-AGCCAGATCATCATCTGTGC-3' generated a DNA fragment that spans the ATG and the last codon (before the stop codon) of the *Vldlr*, and this fragment was then subcloned into the *EcoRI* and *SacII* sites of the mEos2-N1 vector; the primer set of 5'-*EcoRI*-GCATGGGCACGTCCGCGC-3' and 5'-*SacII*-TCATTCTTCGAGATTG-3' amplified a truncated DNA fragment including the ATG and the codon before the stop codon caused by the *r26* mutation, and the fragment was also subcloned into the *EcoRI* and *SacII* sites of the mEos2-N1 vector. In both constructs, the mEos2 protein is in frame with the N-terminal *Vldlr* protein.

Plasmid DNAs of *Vldlr*/mEos2-N1 and *r26-Vldlr*/mEos2-N1 were transiently transfected into HEK293 cells in vitro (Superfect transfection reagent; Qiagen, Valencia, CA). Briefly, HEK293 cells (1.4×10^5) were seeded in 35-mm glass dishes in MEM complete medium, and transfection was performed 24 hours after cell plating. The expression of full-length or *r26* mutant *Vldlr* was examined by confocal microscope (Carl Zeiss) 24 or 48 hours after transfection.

RESULTS

Depigmented Patches in the *r26/r26* Mutant Fundus

Using an indirect ophthalmoscope, we observed that a male mouse from the G3 generation of ENU-mutagenized mice dis-

FIGURE 1. Fundus photographs show depigmented patches in the *r26* homozygous mutant mice. In comparison to the fundus image from a 3-week-old heterozygous *r26/+* control mouse (A), the homozygous *r26/r26* mutant mouse at the same age displayed depigmented patches (B). A homozygous *r26/r26* mutant mouse at 4 weeks of age showed depigmented patches (arrows) in the fundus photograph (C) that match the hyperfluorescent spots (arrows) in the angiogram (D).

played depigmented patches in the retina. This G3 male founder was mated with wild-type C57BL/6J female mice to generate the *r26* mutant mouse line. We confirmed by fundus examination that homozygous *r26/r26* mutant mice developed depigmented patches in the retina (Figs. 1B, 1C), whereas heterozygous *r26/+* littermates had normal retinas (Fig. 1A). Moreover, fluorescein angiography revealed that depigmented patches overlapped with hyperfluorescent spots in the *r26/r26* mutant (Figs. 1C, 1D).

Retinal Histology

Histology analysis was performed to examine the morphologic changes in *r26* mutant retinas. At the age of 4 weeks, com-

pared to the wild-type retina (Fig. 2A), the *r26/r26* mutant displayed all retinal layers without any obvious loss of photoreceptor cells (Fig. 2B). However, some areas of the retinal outer nuclear layer (ONL), the subretinal space, and the retinal pigment epithelium (RPE) were distorted, with the presence of vascular-like cells (Figs. 2B, 2C, arrows). Changes and degeneration of photoreceptor cells became apparent as *r26/r26* mutant mice grew older. At the age of 2 months, some photoreceptor cells completely lost their outer- and inner-segments, and the ONL was adjacent to the RPE in the *r26/r26* retina (Fig. 2D). At the age of 14 months, severe photoreceptor cell degeneration was observed in *r26/r26* mutant mice; moreover, vessellike cellular struc-

FIGURE 2. Histology of *r26* mutant retinas. Compared with the 4-week-old wild-type *+/+* control (A), the homozygous *r26/r26* mutant at the same age showed a relatively normal number of photoreceptor nuclear layers (B). However, some areas of the ONL were distorted, and abnormal retinal vessels were growing from the inner retinal layers into the subretinal space (B, arrows). (C) Enlarged view of a region in (B) shows retinal vessels in the subretinal space, growing from the OPL toward the disorganized RPE layer (arrows). The photoreceptor cells were distorted along the overgrown vessels. (D) A 2-month-old *r26/r26* retinal section displays a region of retinal ONL that grew to the RPE. (E, F) Fourteen-month-old *r26/r26* retinal sections show degenerated photoreceptor layers with four to seven remaining rows of ONL. The subretinal space and RPE layer were disorganized, although the Bruch's membrane seemed to be intact (E). Arrows: retinal vessel in the ONL (F). Scale bars: (A, B) 50 μ m; (C-F) 20 μ m.

tures appeared in the disorganized subretinal region near the RPE layer (Figs. 2E, 2F, arrows).

Abnormal Intraretinal Vascular Growth beyond the Outer Plexiform Layer

Immunostaining with the endothelial cell-specific CD31 antibody and vascular perfusion with a fluorescent dye (vessel painting) were used to examine the retinal vasculature in *r26/r26* homozygous mutant mice by comparing it to that of the wild-type or *r26/+* heterozygous control. In 3-week-old wild-type control mice, CD31 fluorescence signals showed that retinal vascular endothelial cells were restrictively present in the outer plexiform layer (OPL), inner plexiform layer (IPL), and ganglion nerve fiber layer (GL), but not in the photoreceptor ONL and beyond. However, in *r26/r26* mutant retinas, CD31-positive vascular endothelial cells were detected, not only in the OPL, IPL, and GL, but also in the ONL (Fig. 3A, arrowheads) and the subretinal space (data not shown). Thus, immunostaining revealed uncontrolled growth of retinal vessels in the photoreceptor cell layer and subretinal space of *r26/r26* mutant retinas.

Three-dimensional (3D) reconstructions of retinal vasculature were obtained from z-stack fluorescent images of whole-mounted retinas isolated from the control *r26/+* and mutant *r26/r26* mice, which had been transcardially perfused with a lipophilic fluorescent dye.¹⁷ The front views of 3D images of retinal vasculature showed no obvious difference between heterozygous *r26/+* control and homozygous *r26/r26* mutant mice (Fig. 3B, top). However, a cross-sectional 3D view (90° rotation of the front view) revealed that a normal three-layer retinal vasculature, located in the GL, IPL, and OPL, was present in the *r26/+* control (Fig. 3B, bottom left), whereas the *r26/r26* mutant displayed abnormal vessels that extended beyond the OPL toward the subretinal space (Fig. 3B, bottom right, arrow). Both CD31 immunostaining data of frozen retinal

sections and the 3D images of fluorescent dye-perfused retinal vasculature clearly demonstrated that the overgrowth of retinal vasculature is the cause of abnormal vessels in the photoreceptor cell layer and the subretinal space of the *r26/r26* mutant mice.

A *Vldlr* Missense Mutation in *r26/r26* Mice

To identify the causative gene mutation in the *r26* mutant mouse line, we performed genomewide linkage analysis. Fifty-nine microsatellite markers were tested among 30 meioses, and the *r26* locus was confined to chromosome 19 with a peak LOD score of 4.8 (Fig. 4A). A previous study reported similar retinal phenotypes in the *Vldlr*^{-/-} mice,¹⁰ and the *Vldlr* gene resides on chromosome 19.¹⁸ Thus, we performed sequence analysis of the *Vldlr* gene in *r26* mutant mice. Total RNA was isolated from *r26/r26* mutant retinas and subsequently used to synthesize cDNA, and the coding region of *Vldlr* was amplified and sequenced. A change of C to T at nucleotide 2239 of the *Vldlr* coding sequence was identified. This single nucleotide change resulted in a premature stop codon at amino acid residue 747 of the *Vldlr* protein. The missense mutation was predicted to express a truncated *Vldlr* protein with the first 746 amino acids but without the C-terminal 127 amino acid residues that include a single transmembrane domain.

To verify the functional change of this truncated *Vldlr* mutant, we performed an allelic test by crossing homozygous *Vldlr*^{-/-} and *r26/r26* mice. Interestingly, the *r26/r26* mice developed a retinal vascular phenotype similar to *r26/r26* and *Vldlr*^{-/-} mice, examined by indirect ophthalmoscope and fluorescein angiography (data not shown). Retinal histology of a 6-week-old *r26/r26* mouse showed abnormal vascular growth from the OPL toward the subretinal space and RPE layer (Fig. 4B); this defect was identical with that in the *r26/r26* mutant retina. Thus, the *r26* mouse is a loss-of-function mutation of *Vldlr*.

FIGURE 3. Abnormal retinal vessel growth in the *r26* mutant retina. **(A)** Immunostaining of 3-week-old wild-type and *r26* mutant retinas. In the wild-type control retina, vascular endothelial cells stained by an anti-CD31 antibody were observed in the OPL, IPL, and GL. However, in the homozygous *r26* mutant retina, CD31-positive signals were expressed not only in the OPL, IPL and GL, but also in the photoreceptor ONL. **(B)** 3D images of retinal vasculature labeled with a lipophilic fluorescent dye in 8-week-old *r26* littermates. *Top*: 3D front views of retinal vasculature; *bottom*: the same images rotated 90°. The heterozygous *r26* control (*r26/+*) shows a normal three-layer vessel network in the GL, IPL, and OPL. No apparent difference is visible between the front views of the homozygous *r26* mutant (*r26/r26*) retinal vasculature and the *r26/+* control; *bottom*: overgrown vessels that extend beyond the OPL toward the subretinal space in the *r26/r26* retina (arrow).

FIGURE 4. (A) Genome-wide linkage analysis. The *r26* locus is confined to chromosome 19 with a peak LOD score of 4.8 on 30 meioses. Fifty-nine microsatellite markers were used. (B) An allelic test between *Vldlr*^{-/-} and *r26* mutant mice. As shown by retinal histology sections, the *r26*^{-/-} mouse generated by mating homozygous *Vldlr*^{-/-} and homozygous *r26*/*r26* mice displayed a retinal vascular phenotype identical with that of the *r26*/*r26* mouse. Scale bar, 20 μ m. (C) Sequence analysis of the *Vldlr* gene in the *r26* mutant. A single-nucleotide change (C to T) was identified. This point mutation leads to a premature stop codon at amino acid residue 747 of the Vldlr protein. The resulting truncated Vldlr protein (amino acids 1-746) lacks the 127 C-terminal amino acids, which include the single transmembrane domain.

Western blot analysis was performed to examine the expression of the wild-type Vldlr and the *r26* truncated Vldlr proteins in retinal homogenates (Fig. 5A). The full-length Vldlr protein was detected in the retinas of the wild-type mice (w1 and w2 in Fig. 5A). As expected, Vldlr protein was not detected in the retinas from *Vldlr*^{-/-} mice (k1 and k2). Moreover, the truncated Vldlr protein (r1 and r2) was undetectable in the *r26*/*r26* retinal homogenates.

To further characterize the *r26* *Vldlr* mutation, we constructed two expression plasmids that express either the full-length Vldlr tagged with mEos2 fluorescent protein or the *r26* truncated Vldlr tagged with mEos2 fluorescent protein. Both plasmids were transiently transfected into HEK293 cells in vitro. As shown in Figure 5B, 24 hours after transfection, the full-length Vldlr protein was predominantly localized in the plasma membrane (as visualized by mEos2 fluorescent protein in green), whereas the *r26* truncated Vldlr protein was mainly localized in intracellular regions. Forty-eight hours after transfection, the plasma membrane enriched localization of the full-length Vldlr was revealed by both the tagged fluorescent protein and immunostaining signals using a Vldlr antibody (Fig. 5C, top), whereas a diffuse cytosolic distribution of the truncated *r26*-Vldlr was observed in transfected cells (Fig. 5C, bottom). Thus, unlike the full-length Vldlr protein, the *r26*

truncated Vldlr protein fails to localize at the plasma membrane.

DISCUSSION

We have identified an ENU-induced *r26* mouse mutant, which is a missense mutation of the *Vldlr* gene, a c.2239C>T for a p.R747X substitution. This mutation results in a truncated Vldlr protein lacking the transmembrane domain. The *r26* mutant mice develop subretinal neovascularization similar to that of *Vldlr*^{-/-} mice. Therefore, the *r26* mouse line is a loss-of-function mutation of Vldlr. However, unlike the *Vldlr*^{-/-} mice that lack the expression of Vldlr protein in vivo, the *r26* mutant mice probably express a soluble form of Vldlr. Thus, the *r26* mutant line may be a useful and complementary model for investigating the role of Vldlr signaling in the regulation of retinal angiogenesis in vivo. A recent study has reported an increased proliferation and migration of *Vldlr*^{-/-} retinal endothelial cells.¹⁴ However, the molecular and cellular mechanisms for how Vldlr controls intraretinal angiogenesis during development remain largely unknown.

Vldlr belongs to the evolutionarily conserved LDL receptor family, and it contains typical structural/functional domains

FIGURE 5. (A) Western blot to examine Vldlr expression in wild-type (w), *r26/r26* (r), and *Vldlr*^{-/-} (k) retinas. Retinas from two wild-type mice (w1 and w2) expressed the full-length Vldlr protein. The *Vldlr*^{-/-} mice (k1 and k2) had no expression of any Vldlr proteins, as predicted, and the *r26/r26* (r1 and r2) retinas had no detectable truncated Vldlr protein by Western blot. (B, C) Plasmids expressing full-length Vldlr/mEos2 and *r26*-Vldlr/mEos2 fusion proteins were transiently transfected into HEK293 cells. (B) Twenty-four hours after transfection, the Vldlr fusion proteins were imaged by mEos2 under a confocal microscope. Together with differential interference contrasts (DIC) images, the confocal images make it apparent that the full-length Vldlr protein was predominantly localized at the cell membrane, whereas the *r26* truncated Vldlr protein was diffusely expressed mainly in the cell bodies. (C) Forty-eight hours after transfection, the cells were fixed and stained with a goat anti-Vldlr antibody. The antibody staining also confirmed the membrane-enriched expression of full-length Vldlr and cytosolic expression of *r26*-Vldlr. Scale bar, 20 μ m.

like many other members of the LDL receptor family. Vldlr can be structurally divided into three regions: the extracellular region that includes ligand-binding repeats domain, the EGF-like repeat domain, the YWTD β -propeller domain, and the O-linked glycosylation domain; a single transmembrane domain region; and a short cytosolic tail region that contains the NPXY motif (Fig. 6).^{19,20} Vldlr was initially thought to be an endocytic receptor that mediates the binding and uptake of apoE-containing lipoproteins, such as VLDL and β -VLDL.²¹ Early studies reported that homozygous *Vldlr*^{-/-} mice had normal lipoprotein profiles without any significant phenotypes, except that the animals were somewhat smaller and leaner at a young age.⁹ Interestingly, recent findings reveal that retinal vasculature defects are the main phenotype observed in the *Vldlr*^{-/-} mice.^{10–13} Vldlr may function in endothelial cells as an endocytic receptor to mediate the endocytosis of cell surface-bound ligands that may act as proangiogenic factors in the retina.

However, Vldlr has been demonstrated to play a signaling function by its interaction with Reelin to modulate neuronal

migration, neurodevelopment, and other physiological processes in the central nervous system.²² This has been well documented by the Reelin-Vldlr/ApoER2 signaling pathway. Reelin is a large secreted protein that is primarily expressed in nerve tissues. Vldlr and ApoER2 are functional Reelin receptors. Defective Reelin signaling leads to disordered laminated cortical structure.²³ Indistinguishable phenotypes were uncovered among the ataxic *reeler* mutant strain,²⁴ Disabled-1 (*dab1*) gene mutant mice,^{25,26} and mice lack both Vldlr and ApoER2,²⁷ suggesting that Reelin, Dab1, Vldlr, and ApoER2 function in the same signaling pathway. Reelin signaling is initiated by the binding of Reelin to both Vldlr and ApoER2, and the ligand/receptor binding triggers the tyrosine phosphorylation of Dab1, thus regulating neuronal migration.²⁸ Dab1 binds to the NPXY motif in the cytosolic domain of ApoER2 and Vldlr.²⁹ The NPXY motif is essential for Reelin signaling, since it is the critical structural domain for Dab1 binding to both receptors.³⁰ It has been reported recently that Vldlr-mediated Reelin signal prevents neurons from entering the marginal zone, suggesting that Vldlr provides a stop signal

FIGURE 6. Structural domains of the Vldlr protein. In the wild-type protein, the ligand-binding repeats domain, the EGF-like repeats domain, the YWTD β -propeller domain, and the O-linked glycosylation domain are located in the extracellular space; a single transmembrane domain follows the extracellular domains and anchors the protein to the cell membrane; a short C-terminal region with an NPXY motif resides in the intracellular cytosol. The *r26* mutation leads to the mutant Vldlr-R747X protein. The mutant Vldlr protein has all extracellular domains but lacks the transmembrane domain and the C-terminal intracellular portion.

for migrating cortical neurons.³¹ However, Dab1 expression is limited to neurons, and it is unclear whether Dab1 or Dab2 is expressed in retinal endothelial cells.³² Therefore, the downstream targets of Vldlr that mediate endothelial cell migration in angiogenesis need to be explored. It is also possible that other cell types expressing Vldlr in the retina may be the key in the regulation of retinal angiogenesis.

The *r26* mutation leads to a premature stop codon at amino acid residue 747 of the Vldlr protein. The truncated Vldlr-R747X protein contains only the first 746 amino acid residues and lacks the C-terminal 127 amino acids that include the single transmembrane domain. Unlike the plasma membrane localization of the full-length Vldlr protein, the *r26* truncated Vldlr protein displays an intracellular distribution in transfected cells *in vitro*. As shown in Figure 6, the *r26* truncated Vldlr-R747X protein lacks the transmembrane domain. It is also possible that the truncated proteins will be secreted into the extracellular space *in vivo*. Regardless of the intracellular or extracellular distribution of the *r26* truncated Vldlr protein *in vivo*, the expression level of this truncated Vldlr must be extremely low *in vivo*, since its protein amount in the retinal homogenates is undetectable by Western blot. Thus, the *r26* mutant line is a valuable model for a loss-of-function mutation of Vldlr.

Previous reports of *Vldlr*^{-/-} mice and our present study have demonstrated that Vldlr loss-of-function mutations lead to an uncontrolled overgrowth of intraretinal vessels.^{10–13} Vldlr provides an essential cue to prevent retinal vascular endothelial cells from migrating into the photoreceptor cell layer. It is unknown what triggers the migration of endothelial cells into photoreceptor/subretinal space in the Vldlr mutants and what the Vldlr ligand is in retinal endothelial cells.

We hypothesize that Vldlr binds an anti-angiogenic factor to prevent the endothelial cells from migrating into the photoreceptor layer. Further investigation is needed to understand whether Vldlr has a co-receptor like ApoER2 in a Reelin-like signaling pathway in the retina and which intracellular downstream molecules, such as Dab1-like molecules, are involved in this signaling pathway. It is also necessary to determine whether Vldlr is expressed in other retinal cells besides endothelial cells to inhibit the vessel growth in photoreceptor cells or subretinal space. A better understanding of the Vldlr signaling pathway will be very helpful for developing new therapeutic

methods for treating certain types of AMD, such as RAP, with abnormal intraretinal angiogenesis.

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