EPHB4 kinase–inactivating mutations cause autosomal dominant lymphatic-related hydrops fetalis

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Hydrops fetalis describes fluid accumulation in at least 2 fetal compartments, including abdominal cavities, pleura, and pericardium, or in body tissue. The majority of hydrops fetalis cases are nonimmune conditions that present with generalized edema of the fetus, and approximately 15% of these nonimmune cases result from a lymphatic abnormality. Here, we have identified an autosomal dominant, inherited form of lymphatic-related (nonimmune) hydrops fetalis (LRHF). Independent exome sequencing projects on 2 families with a history of in utero and neonatal deaths associated with nonimmune hydrops fetalis uncovered 2 heterozygous missense variants in the gene encoding Eph receptor B4 (EPHB4). Biochemical analysis determined that the mutant EPHB4 proteins are devoid of tyrosine kinase activity, indicating that loss of EPHB4 signaling contributes to LRHF pathogenesis. Further, inactivation of Ephb4 in lymphatic endothelial cells of developing mouse embryos led to defective lymphovenous valve formation and consequent subcutaneous edema. Together, these findings identify EPHB4 as a critical regulator of early lymphatic vascular development and demonstrate that mutations in the gene can cause an autosomal dominant form of LRHF that is associated with a high mortality rate.

Introduction
Hydrops fetalis is defined as excessive fluid accumulation or edema in at least 2 fetal compartments. Nonimmune hydrops fetalis is the cause in more than 85% of cases, of which 15% have been reported to have a lymphatic-related abnormality (1). In 20% of nonimmune hydrops fetalis cases, the cause is not known. Lymphatic-related (nonimmune) hydrops fetalis (LRHF) has been included in a subgroup of primary lymphedemas under the umbrella term generalized lymphatic dysplasia (GLD) by Connell et al. (2). In this classification, GLD was defined as lymphedema associated with systemic or visceral involvement (including hydrops fetalis), even if the lymphedema was not widespread. The GLD group includes patients with a widespread developmental abnormality of the lymphatic system, often presenting prenatally with hydrothoraces or nonimmune hydrops fetalis.

Hennekam syndrome (OMIM 235510) is an example of a GLD that is inherited in an autosomal recessive manner. Mutations in collagen and calcium binding EGF domains 1 (CCBE1) and FAT atypical cadherin 4 (FAT4) have been identified as causal (3–5). Another recessively inherited form of GLD with a high incidence of LRHF has recently been reported as caused by mutations in piezo-type mechanosensitive ion channel component 1 (PIEZO1) (6), adding to the genetic heterogeneity of the GLD group.

We have recently ascertained 2 families with a history of nonimmune hydrops and postnatal lymphatic dysfunction, but with a pattern suggestive of autosomal dominant inheritance and with sporadic occurrence in one of the families. We have identified heterozygous inactivating mutations in the kinase domain of Eph receptor B4 (EPHB4) as causative for this condition. This suggests not only that LRHF/GLD is genetically heterogeneous, but also that it should be considered in both dominant and recessive forms. The importance of EPHB4 for lymphatic vascular development is further supported by analysis of a genetic mouse model with lymphatic endothelial-specific deletion of Ephb4.

Results
 Genetic analysis of LRHF identifies causative mutations in EPHB4. We report 2 multigenerational families (one from Norway [GLD_NOR] and one from the UK [GLD_UK]) (Figure 1). Clinical findings in these families included antenatal nonimmune hydrops fetalis or bilater-
Filter criterion, and MIER2 was the only gene that fulfilled that (Figure 1A). However, the MIER2 variant (NM_017550.1: c.865C>T, p.Arg289Trp) has been reported as a SNP (rs148482834), with a heterozygous genotype observed at a frequency of 0.001.

Meanwhile, an independent study of a GLDNOR family was undertaken. In this family, the condition initially appeared to be sporadic in monozygotic twins (GLDNOR:II.2 and GLDNOR:II.3), who both had subcutaneous edema at birth that resolved in infancy (Table 1). GLDNOR:II.3 required ventilation and thoracentesis for bilateral chylothoraces. Both sisters had sons with nonimmune hydrops. One died at 1.5 days of age; the other was moribund in the neonatal period, but the edema eventually resolved. Both sons also had an ASD. Three genes (protein tyrosine phosphatase, non-receptor type 11 [PTPN11], forkhead box C2 [FOXC2], and VEGFR3) were ruled out in GLDNOR:II.2 by Sanger sequencing, and a high-resolution microarray comparative genomic hybridization (CGH) (Affymetrix 6.0) was normal in GLDNOR:II.3. When analyzing the WES data, the only gene to fulfill an autosomal dominant model with de novo occurrence was EPHB4. Sanger sequencing of additional family members showed all affected family members carried the variant (c.2345T>G, p.Ile782Ser) (Figure 1B). GLDNOR:II.2 and GLDNOR:II.3 were both found to be mosaic for this variant, and MIER2 was the only gene that fulfilled that (Figure 1A). However, the MIER2 variant (NM_017550.1: c.865C>T, p.Arg289Trp) has been reported as a SNP (rs148482834), with a heterozygous genotype observed at a frequency of 0.001.

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The possibility that the mutations were interfering with the phosphorylation state of the WT protein, different ratios of WT and mutant proteins were cotransfected and phosphorylation of the receptor analyzed as described above. Results showed no dominant negative effect, as phosphorylation of the total receptor decreased when increasing amounts of mutated receptor were cotransfected (Figure 3B). Furthermore, Ephrin B2–dependent EPHB4 activation was evaluated in lymphatic endothelial cells (LECs) after expression of Myc-DDK–tagged EPHB4. To distinguish phosphorylation levels of Myc-DDK–tagged exogenous expressed WT and mutant EPHB4 from endogenous expressed WT EPHB4, an anti-DDK antibody was used for the immunoprecipitation and isolation of only the overexpressed forms of Myc-DDK–tagged EPHB4. Ephrin B2 treatment increased phosphorylation levels of WT Myc-DDK–tagged EPHB4, but not mutant proteins (Figure 4), confirming the negative effect of both mutations on the receptor activity after ligand stimulation in LECs.

LRHF-associated mutations lead to inactive EPHB4 kinase. EPHB4 binds the transmembrane Ephrin B2. Binding of Ephrin B2 to EPHB4 stimulates phosphorylation and activates downstream signaling cascades (7, 8). The 2 EPHB4 mutations (p.Arg739Glu and p.Ile782Ser) occur at highly conserved residues located in the tyrosine kinase domain of the EPHB4 protein (Supplemental Figure 2 and Supplemental Figure 3A). Moreover, p.Arg739Glu is located within the catalytic loop HRD (His-Arg-Asp) motif, also highly conserved in many tyrosine kinases (Supplemental Figure 3B). To investigate the effect of the mutations identified in the patients with LRHF, corresponding expression constructs for WT and mutant proteins by site-directed mutagenesis were generated and analyzed for their phosphorylation activity after transient transfection in HEK293T cells. The tyrosine phosphorylation levels of WT, p.Arg739Glu, and p.Ile782Ser mutants were analyzed by immunoprecipitation and Western blotting with anti-EPHB4 and anti–p-tyrosine–specific antibodies. Mutant proteins showed no phosphorylation (Figure 3A), suggesting that both mutations alter EPHB4 signaling in patients with LRHF/GLD. To test the possibility that the mutations were interfering with the phosphorylation state of the WT protein, different ratios of WT and mutant proteins were cotransfected and phosphorylation of the receptor analyzed as described above. Results showed no dominant negative effect, as phosphorylation of the total receptor decreased when increasing amounts of mutated receptor were cotransfected (Figure 3B). Furthermore, Ephrin B2–dependent EPHB4 activation was evaluated in lymphatic endothelial cells (LECs) after expression of Myc-DDK–tagged EPHB4. To distinguish phosphorylation levels of Myc-DDK–tagged exogenous expressed WT and mutant EPHB4 from endogenous expressed WT EPHB4, an anti-DDK antibody was used for the immunoprecipitation and isolation of only the overexpressed forms of Myc-DDK–tagged EPHB4. Ephrin B2 treatment increased phosphorylation levels of WT Myc-DDK–tagged EPHB4, but not mutant proteins (Figure 4), confirming the negative effect of both mutations on the receptor activity after ligand stimulation in LECs.

Table 1. Clinical features of family members carrying EPHB4 mutations

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<th>Family</th>
<th>GLD&lt;sub&gt;nor&lt;/sub&gt;</th>
<th>GLD&lt;sub&gt;uk&lt;/sub&gt;</th>
<th>GLD&lt;sub&gt;uk&lt;/sub&gt;</th>
<th>GLD&lt;sub&gt;uk&lt;/sub&gt;</th>
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<td>\</td>
<td>\</td>
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<td>CT, An</td>
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<td>T, Fa</td>
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<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
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<tr>
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<td>+</td>
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<td>+</td>
<td>NA</td>
<td>+</td>
<td>NA</td>
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<td>NA</td>
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<td>-</td>
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<td>-</td>
<td>+</td>
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<td>-</td>
<td>BL at 15 yr</td>
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<td>+</td>
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<td>-</td>
<td>NA</td>
<td>-</td>
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</table>

<sup>a</sup>Monozygotic twins. As, ascites; An, anemia; BL, bilateral lower limb; CT, chylothoraces; Fa, facial edema; F, female; IUD, intrauterine death; M, male; ND, neonatal death; NT, nuchal translucency scan; PC, pericardial effusions; PE, pleural effusions; PFO, patent foramen ovale; RA, right arm edema; RD, respiratory distress; SC, subcutaneous edema; T, truncal; TC, thoracocentesis in utero; \, not recorded; -, no; +, yes.

Figure 2. Imaging of the lymphatic system in LRHF. Anterior view of lower limb lymphoscintigraphy 2 hours after injection with radionuclide. (A) GLD<sub>uk</sub>:I.2, rerouting through skin and superficial tissues in the right leg and markedly reduced transport in the left leg. (B) GLD<sub>uk</sub>:II.4, normal uptake of tracer in the lymph nodes in the groin area, but with some rerouting in the calves (seen as the dark shading; arrows). (C) Unaffected subject with symmetrical transport of radionuclide within collecting lymph vessels in the leg.
EPHB4 deficiency in mice results in subcutaneous edema and abnormal lymphatic development. Ephrin B2/EPHB4 signaling is critically required for the development of the cardiovascular system during early embryogenesis (9,10). Ephrin B2 and EPHB4 are also essential for lymphatic vessel remodeling and valve formation during late embryonic and early postnatal development (11,12), but whether they have an earlier role in lymphatic vessel morphogenesis that could explain Ephb4 loss of function–induced LRHF/GLD in humans is not known. Whole-mount immunofluorescence analysis confirmed the previously reported venous and lymphatic endothelial-specific expression of EPHB4 in embryonic skin and mesenteries (Supplemental Figure 4, A and B). To assess the potential contribution of lymphatic endothelial EPHB4 loss of function to LRHF/GLD, we deleted Ephb4 specifically in the lymphatic vasculature using tamoxifen-inducible Prox1-CreERT2 mice crossed with a conditional Ephb4fl/fl Prox1-CreERT2 mouse line (Figure 5A and Supplemental Figure 5). The mice were further crossed with the R26-mTmG double reporter to monitor Cre activity and to label gene-deleted cells with GFP (Ephb4fl/fl Prox1-CreERT2 R26-mTmG mice, referred to here as Ephb4 mutants). Ephb4 deletion was induced from the earliest stage of lymphatic development by administration of 4-hydroxytamoxifen (4-OHT) for 5 consecutive days starting at E10.5 (Figure 5A). At E15.5, a high proportion of mutant embryos showed subcutaneous edema (Figure 5B and C). In addition, a proportion of dermal lymphatic vessels contained blood in 71% of edematous mutant embryos (n = 14), but not in nonedematous mutants (n = 5) or in control embryos (n = 20) (Figure 5C and data not shown). Whole-mount immunofluorescence of the skin revealed tortuous and dilated dermal lymphatic vessels in the Ephb4 mutants (Figure 5C). Notably, abnormal vessel morphology was also observed in vessels that showed a low contribution of GFP+ (i.e., Ephb4-deficient cells) (Figure 5C), suggesting that edema and/or blood filling of lymphatic vessels secondarily caused vessel dilation. In support of a non–cell-autonomous effect of early embryonic deletion of Ephb4 on dermal lymphatic vasculature, inactivation of Ephb4 from E12.5, when dermal lymphatic vessel formation begins (ref. 13 and Figure 5A), resulted in normal vasculature despite efficient gene targeting (Figure 5D). These results suggest E10–E12 as a critical time-window for Ephb4 function during lymphatic development.

Ephb4 is required for the formation of lymphovenous and lymphatic valves. Previous studies have shown that between E10.5 and E13.5, formation of specialized lymphovenous valves (LVVs) occurs at the connection sites between the primordial thoracic duct (pTD) and the cardinal vein (refs. 14–16 and Figure 6A). It was therefore reasoned that edema in Ephb4 mutants might be due to defective LVVs leading to inefficient lymph drainage. To investigate this, we induced Cre recombination in the developing LVVs in Ephb4fl/fl Prox1-CreERT2 R26-mTmG embryos by 4-OHT treatment between E10.5 and E12.5. Analysis of immunostained transverse vibratome sections of E13.5 control embryos showed preferential and efficient targeting of the dual LVVs by the Prox1-CreERT2 transgene, while pTD endothelium exhibited mosaic labeling (Figure 6B). Control LVVs (11 out of 11) consisted of 2 well-defined leaflets extending to the lumen of the cardinal vein (Figure 6, C and D, and Supplemental Video 1). In contrast, the majority of EPHB4-deficient LVVs (9 out of 13) did not show extended leaflets, but instead consisted of abnormal clusters of GFP+ cells (Figure 6, C and D, and Supplemental Video 2).

Interestingly, studies using a function-blocking antibody and a chemical genetic approach showed that EPHB4 kinase signaling regulates lymphatic valve formation (11), while genetic
lymphatic phenotype in humans that can be classified as LRHF/GLD. However, this phenotype shows highly variable expression. Some individuals present with severe in utero swelling, which may cause perinatal demise (or fully resolve to become completely asymptomatic), others with no edema but only an ASD. This phenotype can be distinguished from the majority of Hennekam syndrome cases, in which the swelling presents in the antenatal period but persists throughout life (3–5). The large number of miscarriages in GLDNOR may well be related to this disorder. In this regard, it is of interest that EPHB4 and Ephrin B2 have been shown to be instrumental in human placental development (18). Invasive cytotrophoblasts use the EPHB4 expression on veins to ensure that migration of these cells into EPHB4-expressing uterine veins is limited and instead biased toward the arterial side of the circulation (19). Expression of EPHB4 at half the levels normally encountered may disturb the complex migration patterns seen in the process of placentation. A failure of the invasive cytotrophoblasts to take on an arterial phenotype is suspected as leading to the loss of pregnancy during the late first or early second trimester (19).

studies have demonstrated an important function for its ligand, Ephrin B2, in the formation of both lymphatic and venous valves (12, 17). Using our genetic loss-of-function model, we confirmed the essential role of EPHB4 in lymphatic valve morphogenesis. Deletion of Ephb4 during embryonic valve formation led to a complete absence of valves that form in control mesenteries by E18.5 by LECs expressing high levels of PROX1 (Supplemental Figure 4C). In addition, early postnatal deletion of Ephb4 led to a complete loss of lymphatic valves (Supplemental Figure 4D). These results demonstrate a critical role of Ephb4 in the formation and early postnatal maintenance of lymphatic valves and highlight conserved mechanisms regulating the formation of valves at different anatomical sites.

Discussion
This study identifies the EPHB4 receptor tyrosine kinase as a critical regulator of early lymphatic vessel development and a causative gene for LRHF and primary lymphedema. We have shown here that kinase-inactivating mutations in EPHB4 can produce a lymphatic phenotype in humans that can be classified as LRHF/GLD. However, this phenotype shows highly variable expression. Some individuals present with severe in utero swelling, which may cause perinatal demise (or fully resolve to become completely asymptomatic), others with no edema but only an ASD. This phenotype can be distinguished from the majority of Hennekam syndrome cases, in which the swelling presents in the antenatal period but persists throughout life (3–5). The large number of miscarriages in GLDNOR may well be related to this disorder. In this regard, it is of interest that EPHB4 and Ephrin B2 have been shown to be instrumental in human placental development (18). Invasive cytotrophoblasts use the EPHB4 expression on veins to ensure that migration of these cells into EPHB4-expressing uterine veins is limited and instead biased toward the arterial side of the circulation (19). Expression of EPHB4 at half the levels normally encountered may disturb the complex migration patterns seen in the process of placentation. A failure of the invasive cytotrophoblasts to take on an arterial phenotype is suspected as leading to the loss of pregnancy during the late first or early second trimester (19).
Perinatal deaths were also of a higher frequency in the autosomal recessive form of LRHF/GLD caused by PIEZO1 mutations but, in this condition, were probably related to the hydrops fetalis (6).

Two GLDuk family members (GLDuk:II.4 and her son, GLDuk:III.2) carry the variant, but have no clinical history of preor postnatal swelling. On lymphoscintigraphy (GLDuk:II.4), quantification showed entirely normal levels of transport of lymph within the legs, but imaging was suggestive of rerouting through skin and superficial tissues rather than a main lymphatic tract as seen in the control (Figure 2B). She had a small ASD and, interestingly, her son had large, multiple ASDs requiring surgical closure. Variable expression has been observed in other primary lymphedemas, e.g., PIEZO1-related LRHF/GLD (6).

Like other forms of GLD (4, 6), this condition presents antenatally with nonimmune hydrops or pleural effusions. The swelling may completely resolve with no residual lymphatic phenotype, which is similar to observations in the recently identified PIEZO1-related GLD (6). However, the report of 1 affected individual with bilateral lower limb edema (GLDuk:II.2) with abnormal lymph scans suggests that there may be residual, lymphatic weakness in the survivors. Further studies will be needed to investigate the specific nature and extent of the lymphatic dysfunction in these patients.

LEC-specific deletion of Ephb4 in mouse embryos led to subcutaneous edema and abnormal lymphatic vessel morphology, thus recapitulating aspects of the human LRHF phenotype. Temporal analysis of Ephb4 function demonstrated a critical requirement of Ephb4 during early stages of lymphatic development. Specifically, we found that Ephb4 regulates the formation of LVVs that are critical for efficient lymphatic function by maintaining unidirectional flow of lymph into blood (14, 20, 21). We additionally confirmed the previously reported critical role of Ephb4 in both formation and maintenance of lymphatic valves (11). Lymphatic valve defects are, however, an unlikely cause of in utero swelling due to their late embryonic development (22, 23). We postulate, therefore, that defective LVV formation, caused by the lack of Ephb4, could contribute to the LRHF seen in the GLDNor and GLDuk patients. In agreement with this, defective LVVs were recently demonstrated in mouse models of primary lymphedemas caused by loss of function of FOXC2, connexin 37, and GATA2 (16, 24).

Edema in the mouse embryos lacking Ephb4 specifically in the lymphatic endothelia appears to be milder than that observed in the patients, suggesting that defective LVVs may only partially explain human LRHF/GLD. It is well known that Ephb4 is also expressed in venous and capillary endothelium (9, 10), and therefore, the impact of the mutations on the venous system needs to be considered. In accordance with this hypothesis, some of the patients (GLDNOR:II.2 and GLDNOR:II.3; GLDuk:II.2) showed varicose veins, which would be consistent with a valve defect in the venous system and would support the contribution of vascular deficiency to the observed phenotype. Together with the varicose veins, the ASD observed in the patients could also be an important part of this phenotype. Both have been reported in lymphedema distichiasis syndrome with a frequency of approximately 7% (ASDs) (25) and 100% (varicose veins) (26).

Kinase activity is critical for Ephb4 forward signaling in lymphatic endothelia (11). Our in vitro data show that Ephb4 mutants carrying the LRHF-associated mutations p.Arg739Glu and p.Ile782Ser are kinase dead, but do not have a dominant negative effect on WT protein. In contrast, VEGFR3 mutants in Milroy disease have a slower turnover (27), which may affect the signaling capacity of the WT tyrosine kinase receptor due to accumulation of mutant receptors on the cell surface. Unlike typical
receptor tyrosine kinases that dimerize upon ligand stimulation, Eph receptors form higher order clusters, with cluster size being an important determinant of the quality and strength of cellular response (28, 29). Inclusion of a kinase-dead receptor may thus significantly weaken the signaling strength of higher order clusters and thereby alter cellular responses. EPHB2 receptor-mediated endocytosis requires the kinase activity of the receptor (30), so kinase-dead EPHB4 could also show defective endocytosis, influencing clustering dynamics and cellular responses. Further studies will aim to investigate those and other functional consequences of the LRHF/GLD-associated mutations.

In conclusion, we report on kinase-inactivating EPHB4 mutations in 11 individuals from 2 extended family pedigrees presenting with a phenotypic spectrum from severe, lethal nonimmune hydrodrops to ASD only. The inheritance pattern is typical of autosomal dominant inheritance with variable expression. Using a genetic mouse model, we have further shown that Ephb4 deficiency in lymphatic endothelium leads to defective LVVs, which may critically contribute to edema formation in LRHF/GLD patients. This is the first report, to our knowledge, of a human phenotype associated with EPHB4 mutations and also, we believe, the first report of an autosomal dominant form of LRHF.

Methods

Exome sequencing. For GLDUX, sequencing libraries were made following the protocol from Roche/Nimblegen’s SeqCap EZ Exome Library v2.0 kit. The libraries were then sequenced on HiSeq2000 (Illumina) machines. Sequence reads were aligned to the reference genome (hg19) using Novoalign (Novocraft Technologies). Duplicate reads, resulting from PCR clonality or optical duplicates, and reads mapping to multiple locations were excluded from downstream analysis. Depth and breadth of sequence coverage were calculated with custom scripts and the BedTools package (31).

All variants were annotated using a custom annotation pipeline. Single-nucleotide substitutions and small indel variants were identified and quality filtered within the SamTools software package (32) and in-house software tools (33). Variants were annotated with respect to genes and transcripts with the Annovar tool (34). Variants were filtered for novelty by comparing them with dbSNP135 and 1000 Genomes SNP calls and with variants identified in 900 control exomes (primarily of European origin), which were sequenced and analyzed by the same method. Summary statistics for the exome sequencing are given in Supplemental Tables 1 and 2. Analysis of the exome-variant profiles was performed with Lifescope v1.3 software. All variants were annotated using a custom annotation pipeline. Variants from the exome were filtered for known variants in dbSNP, intronic and UTR variants, synonymous variants, and variants in our in-house database. Variants with fewer than 5 variation reads were also omitted. Summary statistics for the exome sequencing are given in Supplemental Table 3.

Confirmation sequencing. Samples of available family members were analyzed by Sanger sequencing. Primers were designed for the coding regions and associated splice sites for exons 13 and 14 of EPHB4 and all exons of MIER2 using Primer3 software (35) or ExonPrimer (https://www.helmholtz-muenchen.de/). Primer sequences are listed in Supplemental Table 4. PCR products were sequenced using BigDye Terminator v1.1 and v3.1 chemistry (Life Technologies) and an ABI3130xl Genetic Analyzer or 3730 DNA analyzer (Life Technologies). Sequencing traces were visually inspected in Finch TV v1.4 (Genospiza Inc.) and SequScape Software v2.5 (Life Technologies).

The twins in GLDUX were also examined for mosaicism by Sanger sequencing on DNA extracted directly from blood (new sample), urine, saliva, and skin biopsy (fibroblasts).

The variants from the 2 families have been submitted to the Leiden Open Variation Database (LOVD²) (genomic variants 0000095140 [c.2216G>A; p.(Arg739Gln)] and 0000095141 [c.2345T>G; p.(Ile782Ser)]) [http://databases.lovd.nl/shared/genes/EPHB4]).

Site-directed mutagenesis of EPHB4 constructs. The human EPHB4 mammalian expression plasmids pCMV6-XL6-EPHB4 (SOCI735) and Myc-DDK-tagged pCMV6-Entry-EPHB4 (RC208559) were obtained from OriGene and used as templates for site-directed mutagenesis using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent). All primers were designed using QuikChange Primer Design (Agilent) and are listed in Supplemental Table 5. All constructs were verified by DNA sequencing.

Cell culture and transfection. HEK293T cells (provided by Tris McKay, St. George’s University of London) were maintained in DMEM supplemented with 10% FBS. Transfection of HEK293T cells was performed with GeneLucene Transfection Reagent (Merk) following the manufacturer’s protocol; 6 × 10⁵ cells/well were seeded in 6-well plates the day before transfection, and then they were transfected with 3 μl GeneJuice and 1 μg of DNA. Human dermal LECs (C-12217) were obtained from PromoCell and maintained in supplemented endothelial cell growth medium MV2 (C-22022, PromoCell) containing recombinant human VEGF-C (2179-VC-025, R&D Systems). Transfection of LECs was performed with Viromer YELLOW (VY-01LB-01, Lipocalyx) following the manufacturer’s recommendations. 2 × 10⁵ cells/well were seeded in fibronectin-coated (F1141, VY-01LB-01, Lipocalyx) following the manufacturer’s recommendations. 2 × 10⁵ cells/well were seeded in fibronectin-coated (F1141, Sigma-Aldrich) 6-well plates the day before transfection and then transfected with 3 μl GeneJuice and 1 μg of DNA. For both cell types, lysates were collected 24 hours after transfection and subjected to immunoprecipitation and Western blot analysis.

Ligand activation of EPHB4 receptor. Ephrin B2/Fc (7397-EB-050, R&D Systems) or Fc fragment alone (CSB-NP005401h, Stratech) was clustered by incubation with a goat anti-human IgG Fc antibody (40C-CHR022G-FIT, Stratech) at a 1:2 ratio for 1 hour at 4°C. Eighteen hours after transfection, LECs were serum-starved for 6 hours and then treated with 1 μg/ml clustered Ephrin B2/Fc or Fc alone for 30 minutes before cell lystate collection.

Immunoprecipitation and Western blot analysis. For immunoprecipitation of overexpressed WT and mutant EPHB4, transfected cells were harvested in 100 μl of lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 0.5% Triton X-100) supplemented with protease and phosphatase inhibitors (Sigma-Aldrich). After clarification by centrifugation, 100 μg of total HEK293T cell protein lysate was incubated with 0.8 μg of goat anti-human EPH4 antibody (AF3038, R&D Systems) overnight at 4°C. After incubation with protein A sepharose beads (Sigma-
Alrich) for 4 hours at 4°C, immune complexes were precipitated by centrifugation. 100 μl of total LEC protein lysate was incubated with 4 μg of mouse anti-DDK antibody (clone OTI4C5, TAA50011, Origene) overnight at 4°C and immune complexes precipitated with protein G sepharose beads (Sigma-Aldrich). Immunoprecipitates were separated by SDS-PAGE and transferred to PVDF membranes. Immunoblot analysis was performed with goat anti-human EphB4 (AF3038, R&D Systems) and mouse anti-phosphotyrosine (clone 4G10, 05-321, Millipore) antibodies. All uncropped Western blots are shown in Supplemental Figures 6 and 7.

Mouse lines. We generated Ephb4fl/fl Prox1-CreERT2 R26-mTmG mice as follows: R26-mTmG mice were acquired from the Jackson Laboratory (36). Prox1-CreERT2 mice were previously described (17). For the generation of the Ephb4fl line, a conditional KO strategy, flanking Ephb4 exons 2 and 3 with LoxP sites, was used to target the Ephb4 locus. The targeting vector was built using homologous recombination in bacteria (37). A C57BL/6 mouse BAC served as a template for the extraction of the homology arms of the targeting vector. The targeting vector contained a flanked neomycin phosphotransferase (Neo) selectable marker cassette. After linearization, the targeting construct was electroporated into A2Z1, a C57BL/6/OLA-Hsd-derived embryonic stem cell line. PCR screens and Southern blot analyses revealed clones that had undergone the desired homologous recombination event. Several of these clones were expanded and injected into BALB/cOlaHsd blastocysts to generate chimeric males that were then bred to C57BL/6/OlaHsd females. Black-coated offspring were genotyped on both sides of the homology arms of the targeting vector. The targeting vector contained a flanked neomycin phosphotransferase (Neo) selectable marker cassette. After linearization, the targeting construct was electroporated into A2Z1, a C57BL/6/OLA-Hsd-derived embryonic stem cell line. PCR screens and Southern blot analyses revealed clones that had undergone the desired homologous recombination event. Several of these clones were expanded and injected into BALB/cOlaHsd blastocysts to generate chimeric males that were then bred to C57BL/6/OlaHsd females. Black-coated offspring were genotyped on both sides of the homology arms of the targeting vector. The targeting vector contained a flanked neomycin phosphotransferase (Neo) selectable marker cassette. After linearization, the targeting construct was electroporated into A2Z1, a C57BL/6/OLA-Hsd-derived embryonic stem cell line. PCR screens and Southern blot analyses revealed clones that had undergone the desired homologous recombination event.


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