tmem33 is essential for VEGF-mediated endothelial calcium oscillations and angiogenesis

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Angiogenesis requires co-ordination of multiple signalling inputs to regulate the behaviour of endothelial cells (ECs) as they form vascular networks. Vascular endothelial growth factor (VEGF) is essential for angiogenesis and induces downstream signalling pathways including increased cytosolic calcium levels. Here we show that transmembrane protein 33 (tmem33), which has no known function in multicellular organisms, is essential to mediate effects of VEGF in both zebrafish and human ECs. We find that tmem33 localises to the endoplasmic reticulum in zebrafish ECs and is required for cytosolic calcium oscillations in response to Vegfa. tmem33-mediated endothelial calcium oscillations are critical for formation of endothelial tip cell filopodia and EC migration. Global or endothelial-cell-specific knockdown of tmem33 impairs multiple downstream effects of VEGF including ERK phosphorylation, Notch signalling and embryonic vascular development. These studies reveal a hitherto unsuspected role for tmem33 and calcium oscillations in the regulation of vascular development.

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he formation of a complex vascular network is an essential process during embryonic development, which is vital for growth of tissues and is frequently dysregulated during disease in the adult. Endothelial cells (ECs) line the inner lumen of blood vessels and their organisation into complex branching networks requires co-ordination of molecular outputs coupled to specific cellular behaviours via a process primarily orchestrated by signalling from vascular endothelial growth factor (VEGF)3. VEGF is a morphogen that signals via different ligands to induce motile and invasive behaviour, which drives blood vessel sprouting. VEGFA primarily controls angiogenesis from arteries via its cognate receptor VEGFR2/KDR, whereas VEGFC promotes sprouting from veins via VEGFR3/FLT42. Migrating ECs extend filopodia to sense VEGF signals via Kdr (VEGFR2), as they form a new sprouting vessel3. Leading angiogenic ECs are termed tip cells, which upregulate dll4 transcription, inducing Notch signalling in neighbouring cells, and this acts to limit excessive angiogenic sprouting3. Neighbouring Notch-expressing cells join the sprout as stalk cells, which in zebrafish tend to exhibit reduced proliferative capacity compared with tip cells4,5.

VEGA has been shown to promote proliferation of ECs in vitro via VEGFR2-mediated activation of the RAS/RAF/ERK pathway without affecting migration8. Others, however, have shown that inhibition of ERK phosphorylation in vivo inhibits EC migration but not proliferation during angiogenesis7. ERK activation is induced via PLCG1 phosphorylation in vitro9, which generates inositol 1,4,5-trisphosphate (IP3). IP3 subsequently activates inositol triphosphate receptor (IP3R) Ca2+ channels within the endoplasmic reticulum (ER) to increase cytosolic Ca2+ concentrations and activate protein kinase C to phosphorylate ERK9. ERK activation is required to promote angiogenesis and has been shown to promote expression of tip cell markers including dll47,10 and flt42. VEGF and Notch therefore balance formation of tip and stalk cells within developing blood vessels and modulate the relative migration and proliferation of ECs during angiogenesis11. Ca2+ is a universal secondary messenger, which achieves specificity using complex signalling modalities. These include encoding information to activate cellular responses within cytosolic Ca2+ oscillations12. How EC Ca2+ oscillations integrate complex molecular outputs to precisely control discrete cellular behaviours in a developing vascular network remains unknown.

ECs are generally thought to be non-excitable and as such utilise store-operated calcium entry (SOCE) as their primary means to maintain Ca2+ levels in the ER after influx13,14. The principal trigger for SOCE activation occurs when intracellular calcium stores with the ER are depleted and these are refilled via interaction of TRPC1 and ORAI1 on the plasma membrane with ER-resident STIM1, which forms the calcium-release activated channel15–18. SOCE relies on modulation of the actin cytoskeleton, which requires Ca2+-dependent proteins for ER motility19.

We have identified transmembrane protein 33 (TMEM33) as a component of the ER Ca2+ signalling machinery required for angiogenesis. TMEM33 is a three-pass transmembrane domain protein conserved throughout evolution with two paralogues in the budding yeast Saccharomyces cerevisiae, Pom33 and Per33, showing enrichment in the nuclear pore and ER, respectively20,21. In the fission yeast Schizosaccharomyces pombe, the TMEM33 orthologue Tts1p contributes to maintenance of the cortical ER network22. Human TMEM33 has been shown to localise to the nuclear envelope and ER in vitro, where it has been suggested to regulate the tubular structure of the ER by suppressing the membrane-shaping activity of reticulons23,24. However, its function within the ER in multicellular organisms remains unknown.

Here we describe the first characterisation of tmem33 in a multicellular organism and show that tmem33 is required in an EC-specific manner for Vegfa-mediated Ca2+ oscillations, to promote angiogenesis in zebrafish embryos. The requirement for tmem33 during the response to VEGF is conserved from zebrafish to humans. Furthermore, tmem33-mediated endothelial Ca2+ oscillations are critical for formation of endothelial filopodia and contribute to activation of ERK and induction of Notch signalling to co-ordinate vascular morphogenesis.

Results

tmem33 knockdown impairs vascular and pronephric development. We find tmem33 is expressed ubiquitously during zebrafish segmentation (Fig. 1a–c) and by 26 h post fertilisation (hpf) is enriched in the trunk vasculature and pronephros (Fig. 1d). TMEM33 expression has been previously identified within the nuclear envelope and ER in human cells25,26. We expressed a full-length C-terminal tmem33-EGFP fusion messenger RNA in developing zebrafish embryos and found Tmem33-EGFP fusion protein to localise to structures indicative of nuclear envelope (Fig. 1e–g, blue arrowheads) and ER (Fig. 1e–g, white arrowheads) of ECs within the caudal artery.

To knock down tmem33 expression and establish its function during embryonic development, we next used two splice blocking morpholinos targeting exon 3, (Supplementary Fig. 1a, b, Supplementary Tables 1 and 2). All experiments were conducted using co-injection of both morpholinos. tmem33 morphants exhibited reduced segmental artery (SeA) length compared with controls (Fig. 1h–k, white arrowheads, p). By 30 hpf, SeAs in control morphants had reached the dorsal roof of the neural tube and began to sprout laterally to form the dorsal longitudinal anastomotic vessel (DLAV) (Fig. 1h, i, yellow arrowhead), whereas tmem33 morphants often displayed abnormal spade-shaped tip cell morphology (Fig. 1k, white arrowhead) and were observed to stall at the level of the horizontal myoseptum. Consistent with this, tmem33 morphants displayed reduced SeA migration rate (Supplementary Fig. 2a–d, yellow arrowheads, Supplementary Fig. 2e). Expression of an arterial marker Tg(0.8flt1:RFP)25 in tmem33 morphants was indistinguishable from controls and restricted to the dorsal aorta (DA) and SeAs from day 1, suggesting the DA was correctly specified in tmem33 morphants (Fig. 1l, n). Onset of circulation and blood flow was normal in tmem33 morphants (Supplementary Fig. 3 and Supplementary Movies 1–3). Despite this early delay in sprouting angiogenesis, by 48 hpf, most SeAs in tmem33 morphants had completed their dorsal migration, but many failed to migrate laterally, resulting in a primitive and discontinuous DLAV (Fig. 1l–o, yellow arrowheads, q). In addition, tmem33 morphants displayed absent paracardial lymphangioblasts (Fig. 1l, n, red arrowheads). The thoracic duct (TD) is the first major lymphatic vessel to develop in zebrafish25 and forms by ventral migration of paracardial lymphangioblasts. In keeping with the absence of paracardial lymphangioblasts, the TD was absent in tmem33 morphants (Fig. 1r, s, asterisks).

tmem33 knockdown reduces EC Ca2+ oscillations and filopodia. VEGF signalling is essential for SeA formation in zebrafish7–9 and lymphatic development36,37,39. Although TMEM33 has never been implicated in angiogenesis or VEGF signalling, the similarity of the phenotype of embryos with loss-of-function of tmem33 or VEGF signalling led us to hypothesise that tmem33 may regulate VEGF signalling in ECs. Furthermore, as VEGF increases EC intracellular Ca2+ oscillations37, which is dependent upon the interaction of plasma membrane proteins with ER-resident proteins via SOCE40,41, we also postulated that tmem33 may regulate VEGF-induced EC Ca2+ signalling, given its indicative expression in the ER (Fig. 1e–g).
To determine the effect of *tmem33* knockdown on EC calcium signalling in vivo, we generated an endothelial Ca\(^{2+}\) reporter line *Tg(fli1a:gal4FFubs3;uas-gCaMP7a*) \(^{39}\), hereafter referred to as *fli1a:gal4FFubs3;uas-GCaMP7a*. Recently, a similar transgenic has been employed as an indirect readout of VEGF signalling activity in zebrafish ECs. \(^{39}\) To first establish whether *fli1a:gal4FFubs3;uas-GCaMP7a* embryos reliably reported fluctuations in endothelial Ca\(^{2+}\) signalling, we treated embryos with Thapsigargin to inhibit the sarcoplasmic or ER Ca-ATPase (SERCA) family of Ca\(^{2+}\) pumps \(^{40}\) and thereby raise endothelial Ca\(^{2+}\) levels. Indeed, Thapsigargin treatment significantly increased GCaMP7a fluorescence in tip cells (Supplementary Fig. 4a,b,d) and reduced cytosolic Ca\(^{2+}\) oscillations in tip cells (Supplementary Fig. 4e). Conversely, as VEGF-mediated elevations in cytosolic Ca\(^{2+}\) are dependent upon IP3R function \(^{37}\), we treated embryos with an IP3R antagonist 2-APB, to reduce cytosolic Ca\(^{2+}\) (Supplementary...
negatively regulates VEGF signalling 41, significantly reduced endothelial Ca$^{2+}$ oscillations. Notch signalling, which mediates the response to VEGF in vivo39.

Having established fli1a:GCaMP7a embryos treated with 2-APB displayed reduced EC GCaMP7a fluorescence and reduced frequency of Ca$^{2+}$ oscillations in tip cells (Supplementary Fig. 4a,c,e).

Having established fli1a:gal4FFib3;uas-GCaMP7a as a reporter of EC cytosolic Ca$^{2+}$ levels in vivo, we examined the effect of tmem33 knockdown on EC Ca$^{2+}$ oscillations. tmem33 knockdown in fli1a:gal4FFib3;uas-GCaMP7a embryos significantly reduced the frequency of Ca$^{2+}$ oscillations and GCaMP7a fluorescence intensity in EC tip cells (Fig. 2a–c and Supplementary Movies 4,5). Furthermore, treatment of fli1a:gal4FFib3;uas-GCaMP7a embryos with the VEGFR inhibitor Tivozanib/ AV951 reduced Ca$^{2+}$ oscillations in a similar manner to tmem33 knockdown (Supplementary Fig. 5a–d,i). In contrast, N-[N-(3,5-Difluorophenacetyl)-1-alkyl]-S-phenylglycine t-butyl ester (DAPT)-mediated inhibition of Notch signalling, which negatively regulates VEGF signalling41, significantly increased cytosolic Ca$^{2+}$ oscillations within EC tip cells (Supplementary Fig. 5e,f,i). These data indicate tmem33 knockdown reduced endothelial Ca$^{2+}$ activity to a similar level as VEGF inhibition and are consistent with studies that demonstrate EC calcium signalling mediates the response to VEGF in vivo39.

The delayed SeA sprouting, abnormal tip cell morphology and aberrant DLAV anastomosis following tmem33 knockdown (Fig. 1h–o) are similar to phenotypes observed following inhibition of filopodia formation in endothelial tip cells42. Knockdown of tmem33 in Tg(fli1a:lifeACT-mClover)sh467, which labels filamentous actin in ECs, substantially reduced the number and length of filopodia present on EC tip cells (Fig. 2f–i), suggesting defective filopodia formation may contribute to impaired angiogenesis in tmem33 morphants. As filopodia are known to express VEGF receptors and transduce the migratory signal upon VEGF ligand binding43, we examined whether loss of filopodia could account for the reduction in EC Ca$^{2+}$...
oscillations observed in tmem33 morphants (Fig. 2a–e, Supplementary Movies 4,5). Tf(fli1a:life-AC5-mClover) embryos treated with the actin depolymerising agent Latrunculin B did not form filopodia (Supplementary Fig. 6a–c, black arrowheads), instead forming rapidly depolymerising F-actin foci in keeping with previous reports. Treatment of fli1a:gad4Epgb1:uas-GCaMP7a embryos with Latrunculin B had no significant effect on frequency of endothelial Ca$^{2+}$ oscillations in tip cells in comparison with controls (Supplementary Fig. 6d–f, white arrowheads). This suggests loss of EC filopodia in tmem33 morphants is not responsible for reduced EC Ca$^{2+}$ oscillations.

tmem33 mutant zebrafish embryos display genetic compensation. As morpholino knockdown is transient and may induce off-target effects, we next generated zebrafish tmem33 mutants using transcription activator-like effector nuclease (TALENs). We identified a mutant allele (hereafter referred to as sh443) with a 2 bp deletion in exon 3, which induces a premature stop codon within exon 4 encoding the second conserved transmembrane helix (Supplementary Fig. 7a,b). tmem33sh443 mutants display substantially reduced expression of tmem33 mRNA (Supplementary Fig. 7c–e) likely via nonsense-mediated decay, indicating this mutation represents a severe loss-of-function or null allele. However, in contrast to tmem33 morphants, vascular (Supplementary Fig. 7l,g) and pronephric development was normal in tmem33sh443 mutants and homozygous tmem33 mutant adults were viable. Recent studies describe mechanisms by which the zebrafish genome can compensate for genetic mutations but not morpholino knockdown. Consistent with such compensation, homozygous tmem33sh443 mutants injected with tmem33 morpholinos titrated to a level, which did not significantly induce p53 expression (Supplementary Fig. 7h), displayed significantly longer SeAs compared with heterozygote or wild-type embryos (Supplementary Fig. 7i–l, arrowheads). In addition to the described effect on vascular and lymphatic development, tmem33 morphants exhibited increased glomerular size (Supplementary Fig. 7m–o, arrows) and homozygous tmem33sh443 mutants were protected against the effect of the tmem33 morpholino on glomerular size (Supplementary Fig. 7m–o, arrows). These data suggest tmem33sh443 mutants exhibit genetic compensation or transcriptional adaptation.

tmem33 crispant embryos phenocopy tmem33 morphant embryos. As tmem33 mutants appeared to display genetic compensation, and in order to confirm the morpholino-induced phenotype, we next knocked down tmem33 using Clustered Regularly Interspaced Short Palindromic Repeats interference (CRISPRi). This acts via steric inhibition of transcription and has been previously successful in zebrafish embryos. In comparison with co-injection of mRNA encoding a catalytically inactive Cas9 nuclease (dCas9) and control single-guide RNAs (sgRNAs) lacking the dCas9 binding motif (Supplementary Table 2), co-injection of sgRNAs flanking the start codon of tmem33 with dCas9 mRNA into zebrafish embryos (hereafter referred to as crispants) reduced tmem33 expression by in situ hybridisation (Supplementary Fig. 8a,b, arrowhead) and quantitative reverse-transcriptase PCR (qRT-PCR) (Supplementary Fig. 8c). tmem33 CRISPRi significantly reduced Ca$^{2+}$ oscillations in tip cells (Supplementary Fig. 8d–h) and endothelial filopodia number and length (Supplementary Fig. 8i–l, arrowheads). We were able to rescue the angiogenic defects in tmem33 morphants and crispants by co-injection of full-length tmem33 mRNA (Supplementary Fig. 9) confirming that these were due to reduced expression of tmem33. Interestingly, at minimal phenotypic doses, induction of p53 expression was substantially reduced in tmem33 crispants in comparison with tmem33 morphants injected with 4 ng morpholino (Supplementary Fig. 8m), suggesting CRISPRi may be less subject to nonspecific effects than morpholinos. Taken together, these data demonstrate that transcriptional inhibition of tmem33 via CRISPRi induces endothelial defects highly similar to those generated by morpholino-mediated translational inhibition of tmem33 (Fig. 2). These data indicate tmem33 is required for endothelial cytosolic Ca$^{2+}$ oscillations, endothelial filopodia formation and normal angiogenesis.

tmem33 promotes angiogenesis in an EC-specific manner. The morphant and crispant phenotypes described above were induced by global translational/transcriptional inhibition and tmem33 is expressed in tissue other than ECs and the developing kidney. We therefore sought to examine the effect of EC- and pronephros-specific inhibition of tmem33. As CRISPRi employs a genetically encoded catalytically inactive Cas9 nuclease (dCas9), we postulated that co-expressing dCas9 under the control of a tissue-specific promoter and gene-targeting sgRNAs may facilitate tissue-specific knockdown of tmem33. We therefore generated fli1a:dCas9, cryaa:cfp and enpep:dCas9, cryaa:cfp constructs (Supplementary Fig. 10a,b) that drive transient dCas9 expression under control of either EC or pronephros-specific promoters, respectively. These constructs were injected alongside Tol2 mRNA and gene-targeting sgRNAs to elicit tissue-specific gene knockdown.

In comparison with co-injection of dCas9 mRNA and control sgRNAs lacking the dCas9-binding motif (Supplementary Fig. 10c, arrowheads, g arrows, k, l), co-injection of sgRNAs targeting tmem33 and dCas9 mRNA into progeny of an intercross between Tg(fli1a:AC-TgRFP)sh511/+ and Tg(wt1b:egfp)/+ fish induced both abnormal vascular and pronephric development (Supplementary Fig. 10d, arrowheads h, arrows k, l) as previously demonstrated. In contrast, embryos injected with tmem33 sgRNAs and fli1a:dCas9, cryaa:cfp construct displayed significantly reduced DLAV continuity but normal kidney development (Supplementary Fig. 10e, arrowheads i, arrows k, l), whereas embryos injected with tmem33 sgRNAs and enpep:dCas9, cryaa:cfp construct, restricting CRISPRi knockdown to the kidney, developed distended glomeruli, whereas the vasculature was normal (Supplementary Fig. 10f, arrowheads j, arrows k, l). We next generated a stable transgenic line expressing dCas9 in EC Tg(fli1a:dCas9, cryaa-gerulene)52, hereafter referred to as Tg(fli1a:dCas9). To avoid the potential for reduced of knockdown efficiency by fluorescent tagging, we employed untagged dCas945. We screened for embryos expressing dCas9 in ECs by in situ hybridisation (Fig. 3a–c) and validated EC-restricted expression of dCas9 using immunohistochemistry (Fig. 3d–g). As co-injection of dCas9 mRNA and control sgRNAs were consistent with normal vascular and pronephric development (Supplementary Fig. 8, S10), to test efficacy of EC gene knockdown in stable dCas9 expressing transgenics, we first injected progeny of outcrosses from Tg(fli1a:dCas9)+ × Tg(fli1a:EGFP)+ with an sgRNA targeting dll4 (Fig. 3h–j). Injection of sgRNA therefore served as an internal control in Cerulean Fluorescent Protein (CFP)-negative embryos. CFP-positive embryos displayed significantly increased DLAV diameter in comparison with CFP-negative embryos (Fig. 3h,i, arrowheads, j), consistent with previously observed ectopic DLAV sprouting in dll4 mutants and mutants. Injection of tmem33 sgRNAs into Tg(fli1a:dCas9) embryos delayed SeA sprouting (Fig. 3k–m, compare white arrowheads with yellow arrowheads) and induced discontinuous DLAV formation (Fig. 3n–p, yellow arrowheads) only in CFP-positive embryos, similar to tmem33 morphants (Fig. 1h–o) and crispants (Supplementary Fig. 10d,e, arrowheads, k).
**Fig. 3** Injection of sgRNAs targeting *tmem33* into stable Tg(*fli1a:dCas9;cryaa:Cerulean*)<sup>j512</sup> embryos recapitulates knockdown of *tmem33*. a dCas9 is expressed within the developing vasculature (red arrowhead) including SeAs (black arrowheads) in ~50% of progeny from a Tg(*fli1a:dCas9;cryaa:Cerulean*)<sup>j512</sup>/+ outcross at 26 hpf. At this stage the dominant *cryaa:Cerulean* marker is not expressed. b dCas9 is not expressed within the developing vasculature in CFP+ Tg(*fli1a:dCas9;cryaa:Cerulean*)<sup>j512</sup> transgenic embryos at 50 hpf. Probe trapping in notochord is highlighted (blue arrowhead). c dCas9 is expressed within the dorsal aorta (red arrowhead) and SeAs (black arrowheads) in CFP+ Tg(*fli1a:dCas9;cryaa:Cerulean*)<sup>j512</sup> transgenic embryos at 50 hpf. Probe trapping in notochord is highlighted (blue arrowhead). d-g Colocalisation of dCas9 and GFP in 72 hpf Tg(*fli1a:EGFP*) transgenic embryos at 50 hpf. Probe trapping in notochord is highlighted (blue arrowhead). e-h i1a:dCas9;cryaa:Cerulean)sh512 injected with sgRNAs targeting *tmem33* display reduced SeA length (yellow arrowheads) (unpaired t-test ***<i>p </i>=<i> 0.001; t = 4.203 DF = 35; 2 repeats; n = 9 embryos per group). Scale bars 20 μm. h, i Tg(*fli1a:dCas9;cryaa:Cerulean*) embryos injected with sgRNA targeting *dll4* phenocopies ectopic vascular looping within the DLAV previously observed in *dll4* morphants and mutants at 3 dpf (white arrowheads). j Tg(*fli1a:dCas9;cryaa:Cerulean*)-positive embryos injected with sgRNAs targeting *dll4* display increased DLAV diameter (unpaired t-test ***<i>p </i>=<i> 0.001; t = 6.716 DF = 62; 3 repeats; n = 9–12 embryos per group). Scale bars 20 μm. k-m Tg(*fli1a:dCas9;cryaa:Cerulean*) embryos injected with sgRNAs targeting *tmem33* display reduced SeA length (yellow arrowheads highlight normal SeAs, white arrowheads highlight delayed SeAs) at 30 hpf (unpaired t-test ***<i>p </i>=<i> 0.001; t = 6.716 DF = 62; 3 repeats; n = 9–12 embryos per group). Scale bars 50 μm. n-p Tg(*fli1a:dCas9;cryaa:Cerulean*) embryos injected with sgRNAs targeting *tmem33* display absent parachordal lymphangioblasts (red arrowhead) and reduced DLAV continuity (yellow arrowheads) (unpaired t-test ***<i>p </i>=<i> 0.001; t = 6.399 DF = 56; 3 repeats; n = 9–11 embryos per group). Scale bars 50 μm. q-s Tg(*fli1a:dCas9;cryaa:Cerulean*) embryos injected with sgRNAs targeting *tmem33* display no significant difference in glomerular area, compared with Tg(*fli1a:dCas9;cryaa:Cerulean*)-negative siblings embryos (unpaired t-test, ns = not significant; t = 0.4048; DF = 74; 3 repeats; n = 12–13 embryos per group). Scale bars 200 μm. Source data are provided as a Source Data file.
Furthermore, Tg(fli1adCas9) embryos injected with tmem33 sgRNA displayed absent parachordal lymphangioblasts (Fig. 3n, o, red arrowheads) only in CFP-positive embryos, similar to tmem33 morphants (Fig. 1l–o, red arrowheads). By contrast, Tg(fli1adCas9) embryos injected with tmem33 sgRNA displayed no significant difference in glomerular area between CFP-positive and -negative embryos (Fig. 3q–s, arrows). Collectively, these data demonstrate tissue-specific CRISPRi-mediated uncoupling of tmem33 functions within the endothelium and pronephros, and indicate EC-specific and pronephros-specific functions of tmem33 during angiogenesis and pronephric development, respectively, in zebrafish. Importantly, all approaches to knockdown tmem33 function were compatible with normal gross embryonic morphology at phenotypic doses (Supplementary Fig. 11).

**tmem33 functions downstream of VEGF during angiogenesis.**

Elevations in cytosolic Ca\(^{2+}\) are induced when EC tip cells respond to extracellular VEGF via the Kdr receptor\(^{39}\) and EC Ca\(^{2+}\) oscillations are reduced in tip cells following tmem33 knockdown (Fig. 2a–e, Supplementary Movies 4, 5 and Supplementary Fig. 8d–h). We therefore sought to establish epistasis between VEGF signalling and tmem33. We overexpressed vegfa165 mRNA, which increases EC Ca\(^{2+}\) signalling (Supplementary Fig. 5g, h, j) and examined the effect on EC Ca\(^{2+}\) activity in the presence or absence of tmem33 CRISPRi knockdown (Fig. 4a–e and Supplementary Movies 6–9). As expected, vegfa165 overexpression increased the frequency of cytosolic Ca\(^{2+}\) oscillations in endothelial tip cells (Fig. 4a, b, arrowheads, e) but this was significantly reduced by simultaneous tmem33 knockdown by CRISPRi (Fig. 4d,e) similar to knockdown of tmem33 alone without vegfa165 overexpression (Fig. 4c–e). Thus, tmem33 is required for Vegfa-induced Ca\(^{2+}\) oscillations in endothelial tip cells.

In addition, VEGF promotes proliferation of intersegmental vessel (ISV) ECs via a process normally limited by Notch signalling\(^4\). We therefore asked whether tmem33 knockdown suppresses EC number in ISVs. Vessels are referred to as ISVs where the transgene employed did not allow distinction between SEAs and SEVs. Knockdown of tmem33 reduced EC number within ISVs (Fig. 4f–h), similar to previous studies following VEGF inhibition\(^5\). Furthermore, VEGF receptor expression was unaffected in tmem33 crispsants (Fig. 4i). Taken together, these data demonstrate that tmem33 mediates the effects of VEGF during angiogenesis and is required for VEGF-mediated cytosolic Ca\(^{2+}\) signalling within endothelial tip cells. Furthermore, these data suggest tmem33 knockdown inhibits EC proliferation in response to VEGF.

**Tmem33 is required for VEGFα-mediated angiogenesis in HUVECs.** As tmem33 mediates the effects of VEGF in zebrafish, we next sought to establish whether this was conserved in humans. We used RNA interference to knock down Tmem33 in human umbilical vein ECs (HUVECs) (Fig. 5a) and examined the effect on EC migration and tube formation in response to VEGFA (Fig. 5b). Consistent with our findings in zebrafish, Tmem33-deficient HUVECs migrated less than controls (Fig. 5b). In addition, HUVEC tubular morphogenesis induced by VEGFA was significantly reduced by Tmem33 knockdown (Fig. 5c), indicating the requirement for Tmem33 during the response to VEGFA is conserved in humans.

**Tmem33 knockdown reduces EC Notch and ERK signalling.**

VEGF signalling via VEGF receptor 2 (kdr), and VEGF receptor 4 (kdr4) in zebrafish, induces transcription of dll4 in endothelial tip cells via the MEK-ERK signalling pathway. Upregulation of Dll4 in endothelial tip cells induces Notch signalling in neighbouring stalk cells, which balance tip and stalk cell identity via regulation of VEGFR expression\(^41\). As tmem33 mediates the response to VEGF, we examined whether Dll4/Notch signalling was...
perturbed by tmem33 knockdown. Using a transgenic Notch reporter Tg(csl:venus)⁵⁹, which reliably reports endothelial Notch signalling (Supplementary Fig. 12a–f), we observed no significant differences in reporter expression at 26 hpf (Fig. 6a, b red arrowhead, c), but by 48 hpf, Notch reporter expression was significantly reduced in the DA (Fig. 6d, e, red arrowheads, f) and SeAs displayed reduced Venus fluorescence in tmem33 morphants (Fig. 6d, e, white arrowheads). In keeping with this, expression of dll4, notch1b and the Notch targets hey2/gridlock and her12 were reduced by tmem33 knockdown using global CRISPRi at 48 hpf but not 26 hpf (Fig. 6g, h and Supplementary Table 3). In situ hybridisation confirmed reduced arterial expression of all genes assayed by qPCR at 48 hpf (Fig. 6i–p, arrowheads). Phosphorylation of the serine/threonine kinase ERK preferentially occurs in angiogenic ECs⁷. In keeping with this requirement downstream of VEGF signalling during angiogenesis, tmem33 knockdown reduced phosphorylated ERK (pERK) in sprouting SeAs (Fig. 6q–t, white arrowheads, u). pERK promotes expression of dll4 in zebrafish angiogenic ECs⁷,¹⁰ and dll4 expression was reduced by tmem33 knockdown (Figs. 6h, n, arrowheads). These data indicate tmem33 is required for ERK phosphorylation and Notch signalling, which act downstream of VEGF in angiogenic ECs.

As tmem33 promotes sprouting angiogenesis cell autonomously (Fig. 3), we examined whether several other molecular and cellular consequences of tmem33 inhibition were due to tmem33 function within ECs. Similar to knockdown of tmem33 by morpholino and CRISPRi, Tg(fli1a:dCas9) embryos injected with tmem33 sgRNA displayed reduced endothelial Ca²⁺ oscillations (Supplementary Fig. 13a–c, white arrowheads), reduced filopodia number and length (Supplementary Fig. 13d–g, black arrowheads), reduced EC number in ISVs (Supplementary Fig. 13h–j) and reduced endothelial Notch signalling (Supplementary Fig. 13k–m, arrowheads) in CFP-positive embryos in comparison with CFP-negative siblings. Collectively, this indicates tmem33 is required cell autonomously to promote endothelial Ca²⁺ oscillations, filopodia formation, Notch signalling and to regulate EC number during angiogenesis.

SOCE inhibition inhibits angiogenesis. Although tmem33 knockdown impairs angiogenesis and reduces endothelial cytosolic Ca²⁺ oscillations, it was possible that these effects were due to different roles of tmem33 rather than causally linked. We therefore sought to determine whether inhibiting EC cytosolic Ca²⁺ release was sufficient to cause the observed phenotype of
We inhibited SOCE using SKF-96365, a compound that inhibits STIM1 and TRPC1 function. SKF-96365 treatment between 21 hpf and 27 hpf reduced SeA length (Fig. 7a–c arrowheads) as induced by tmem33 knockdown (Fig. 1j, k white arrowheads). Embryos treated from 21 hpf until 50 hpf displayed incomplete DLAV formation (Fig. 7d–f, arrowheads) similar to tmem33 knockdown (Fig. 1n, o). Importantly, although tmem33 expression was unaffected by SKF-96365 treatment (Fig. 7g), frequency of endothelial Ca^{2+} oscillations was significantly reduced in tip cells (Fig. 7h–j). Furthermore, Notch reporter expression (Fig. 7k–m) and endothelial tip cell filopodia number (Fig. 7n–p) was reduced in ECs following SKF-96365 treatment similar to tmem33 knockdown (Fig. 6d–f and Fig. 2f–i, respectively). SKF-96365-treated embryos also displayed abnormal spade-shaped tip cell morphology (Fig. 7b, e, o, blue arrowheads) as induced by tmem33 knockdown (Fig. 1k, white arrowhead). Given the similar angiogenic defects induced by tmem33 knockdown and SKF-96365 treatment, we examined whether inhibition of SOCE could account for reduced EC number and migration previously observed in tmem33 morphants (Fig. 4f–h, and Supplementary Fig. 2). We treated embryos from 21 hpf until 30 hpf and quantified EC proliferation.
Fig. 6 tmem33 knockdown reduces endothelial Notch and ERK signalling during angiogenesis. a–c Notch reporter expression is unaffected by injection of 0.4 ng tmem33 morpholinos at 26 hpf. Notch signalling activity in control embryos is present in neural tube (blue arrowhead) and DA (red arrowhead). (c, unpaired t-test; t = 0.5553; DF = 20; 2 repeats; n = 5 or 6 embryos per group). Scale bars 50 µm. d–f Injection of 0.4 ng tmem33 morpholinos reduces Notch reporter expression at 48 hpf. Notch expression in control Tg(cal/venus) embryos is present in the neural tube (blue arrowhead), DA (red arrowhead) and SeAs (white arrowheads) (f, unpaired t-test; p = 0.0451; t = 2.138; DF = 20; 2 repeats; n = 6 and 4 embryos per group). Scale bars 50 µm. 
g Expression of notch1b, dll4, hey2 and her12 are not significantly altered by tmem33 CRISPRi at 26 hpf (one-way ANOVA using post hoc Tukey’s comparison test. n = 3 repeats). h Expression of notch1b, dll4, hey2 and her12 are significantly reduced at 48 hpf by tmem33 CRISPRi (one-way ANOVA using post hoc Tukey’s comparison test. p < 0.05; *p < 0.01. n = 3 repeats). i–p In comparison with control morphants (i–l), embryos injected with 0.4 ng tmem33 morpholino (m–p) displayed reduced expression of notch1b within the DA (i, m, red arrowheads) and SeAs (i, m, white arrowheads), dll4 within SeAs (j, n, white arrowheads), hey2 within the DA (k, o, red arrowheads) and her12 within the DA (l, p, red arrowheads) and SeAs (l, p, white arrowheads). Scale bars 100 µm. q–t tmem33 knockdown by CRISPRi reduces endothelial ERK phosphorylation (q, r, SeAs highlighted by yellow outline and white arrowheads). Intensity plot of ERK staining is shown (s, t, SeAs highlighted by yellow outline and white arrowheads). Scale bars 50 µm. u tmem33 crispants display significantly reduced levels of pERK phosphorylation. Pixel intensity normalised to neural tube ERK fluorescence (unpaired t-test. **p = 0.0097, t = 2.993 DF = 14. n = 2 repeats, 4 embryos per group). Source data are provided as a Source Data file.

and migration in the presence and absence of SKF-96365 between 24 and 30 hpf (Supplementary Fig. 14 and Supplementary Movies 10, 11), and observed that both SeA migration (Supplementary Fig. 14a–e, arrowheads) and tip and stalk cell proliferation (Supplementary Fig. 14a–f,d–h) were significantly reduced in migrating SeAs treated with SKF-96365. Consistent with this, expression of VEGF receptors in tmem33 crispants were unaffected (Fig. 4i) and thus unlikely to account for reduced EC number (Fig. 4f–h and Supplementary Fig. 13h–j). Collectively, these data demonstrate that SOCE inhibition impairs angiogenesis in vivo by limiting EC migration, proliferation, disrupting signalling pathways downstream of VEGF and inhibiting filopodia formation similar to tmem33 knockdown. This is consistent with our hypothesis that reduced cytosolic EC Ca2+ oscillations account for the angiogenic defects observed caused by tmem33 knockdown. Thus, tmem33 is essential for VEGF-mediated endothelial Ca2+ oscillations that are required for tip cell filopodia formation, the downstream consequences of VEGF signalling and developmental angiogenesis (Fig. 8).

Discussion

No previous study has examined the function of tmem33 in a multicellular organism. Previous studies in yeast20–22 and transformed human cells23,24 had localised tmem33 to the ER, but no function has been demonstrated during development. We find tmem33 is expressed widely throughout the developing zebrafish embryo and localised to structures indicative of ER in zebrafish ECs. In keeping with its expression patterns, tmem33 knockdown demonstrated a requirement for tmem33 during normal angiogenesis and pronephric development. Furthermore, TMEM33 is also required for Vegfa-mediated angiogenesis in human ECs, indicating a conserved function between zebrafish and human.

We find that tmem33 is required for Vegfa-mediated cytosolic Ca2+ oscillations in endothelial tip cells. Although increases in cytosolic Ca2+ are a well-established response to Vegf in ECs37, it remained unclear whether Ca2+ signalling was required for its effects in vivo and, if so, how Ca2+ co-ordinated EC behaviours during angiogenesis. Recent studies have established that Vegfa/Kdr signalling is required for endothelial Ca2+ oscillations during sprouting angiogenesis in vivo30. Our findings are consistent with these, as Vegfr inhibition abrogated Ca2+ oscillations and overexpression of vegfa165, mRNA or Notch inhibition increased Ca2+ oscillations in tip cells. Reduction of Ca2+ oscillations in tip cells following either tmem33 knockdown or acute SOCE inhibition were associated with reduced formation of filopodia, abnormal tip cell migration and aberrant Anastomosis. Impaired SeA migration and DLAV anastomosis have been observed in embryos with reduced EC filopodia formation, suggesting this may contribute to the angiogenic defects induced by tmem33 knockdown42. Importantly, the frequency of cytosolic Ca2+ oscillations in tip cells was unaffected following loss of filopodia. This is in keeping with studies that demonstrated tip cells without filopodia retained their ability to respond to VegfA52 and suggests reduced filopodia formation induced by tmem33 knockdown was secondary to reduced EC Ca2+ oscillations. Ca2+ oscillations have been reported to promote F-actin reorganisation and cell migration in vitro51,52. It therefore seems likely to be that tmem33-mediated cytosolic Ca2+ oscillations induced by VEGF signalling promote tip cell migration in vivo via organisation of the actin cytoskeleton. As such, tmem33-mediated Ca2+ oscillations permit tip cells to respond quickly to the proangiogenic Vegfa stimulus.

Vegfa-mediated activation of VEGF signalling is also required for angiogenic sprouting and induction of tip cell marker expression including dll47,10,33,53. tmem33 knockdown reduced ERK phosphorylation andDll4/Notch signalling, and delayed tip cell migration in a similar way to ERK inhibition7,53 without significantly affecting vegfr expression. This indicates tmem33 function contributes to activation of these pathways in vivo, likely to be via Ca2+-dependent mechanisms downstream of Vegfa. Interestingly, induction ofDll4/Notch signalling at 26 hpf was not dependent on tmem33 function; however, its attenuation at 48 hpf, suggests tmem33 contributes to maintenance ofDll4/Notch signalling in ECs. Consistent with this, inhibition of SOCE reduced Notch reporter activity at 48 hpf, suggesting an input from VEGF-mediated calcium signalling between 1 and 2 dpf to maintainDll4/Notch signalling within developing arteries. Furthermore, recent studies indicate a requirement for Ca2+ binding during folding of Notch receptors and ligand engagement, including theDll4–Notch1 interaction54. Reduced expression ofdll4 induced by tmem33 knockdown is in keeping with observed reductions in ERK phosphorylation. ERK phosphorylation is required for development of the lymphatic system33,35,35; therefore, reduced ERK phosphorylation induced by tmem33 knockdown may contribute to defective lymphatic sprouting.

Surprisingly, tmem33 mutants exhibited normal angiogenesis, yet displayed substantial reductions in tmem33 expression. The relative ease with which one can now generate targeted lesions within the zebrafish genome and widespread adoption of these approaches by the community has generated substantial evidence that many loss-of-function mutants fail to recapitulate morpholino-induced phenotypes36. Although this was initially attributed to well-established off-target effects of morpholinos37, recent studies have indicated a greater degree of compensation within the zebrafish genome than previously considered45. The tmem33msh allele is predicted to generate a premature stop codon within coding exon 4, located 53 nucleotides downstream.
Abnormal tip cell morphology (blue arrowhead) remain in SKF-96365-treated embryos by 50 hpf. Scale bars 50 µm. e SKF-96365-treated embryos display reduced SeA length by 27 hpf (unpaired t-test; p = 0.0001; t = 11.22 DF = 14; 2 repeats, n = 4 embryos per group). d, e SKF-96365 treatment impairs DLAV formation (white asterisks) in comparison with control embryos, which form the DLAV normally by 50 hpf (white arrowheads).

Increased frequency of abnormal vascular branching (stunted SeAs or incomplete DLAV anastomosis) by 50 hpf (unpaired t-test; p = 0.0001; t = 14.28 DF = 18. n = 2 repeats, n = 5 embryos per group). g tmem33 expression is not significantly altered by SKF-96365 treatment (unpaired t-test; t = 0.911, DF = 4, n = 3 repeats). h–j SKF-96365 reduces frequency of endothelial Ca²⁺ oscillations in Tg(fli1a:gal4FFubs3;uas-gcamp7a) embryos. Intensity projection over time showing calcium oscillations in control embryos, highlighting intensity change over duration of time lapse (unpaired t-test; p = 0.005; t = 2.594, DF = 10, n = 2 repeats, 3 embryos per group). k, l Notch reporter expression is reduced in SeAs (white arrowheads) and DA (red arrowhead) of Tg(csl:venus) embryos. Scale bars 50 µm. m DA expression of Notch reporter is reduced by SKF treatment (unpaired t-test; **p = 0.0097, t = 3.982 DF = 15, 2 repeats, n = 4 embryos per group). n, o SKF-96365 treatment reduces endothelial filopodia number (black arrowheads) and abnormal spade-shaped tip cell morphology (blue arrowhead), in Tg(fli1a:lifeACT-mClover) embryos. Scale bars 50 µm. p SKF-96365 treatment reduces mean filopodia number per SeA per embryo by 26 hpf (unpaired t-test; p = 0.005; t = 2.525, DF = 10, n = 2 repeats, n = 5 embryos per group). Source data are provided as a Source Data file.

Fig. 7 SOCE inhibition limits sprouting angiogenesis and disrupts signaling pathways downstream of VEGF. a, b SKF96365 impairs angiogenesis in Tg(fli1a:EGFP) embryos (white arrowheads) and induces abnormal tip cell morphology (blue arrowhead) by 27 hpf. Scale bars 50 µm. c Calcium oscillations in control embryos, highlighting intensity change over duration of time lapse (unpaired t-test; p = 0.0001; t = 11.22 DF = 14; 2 repeats, n = 4 embryos per group). d, e SKF-96365 treatment impairs DLAV formation (white asterisks) in comparison with control embryos, which form the DLAV normally by 50 hpf (white arrowheads). Abnormal tip cell morphology (blue arrowhead) remain in SKF-96365-treated embryos by 50 hpf. Scale bars 50 µm. f SKF-96365-treated embryos display increased frequency of abnormal vascular branching (stunted SeAs or incomplete DLAV anastomosis) by 50 hpf (unpaired t-test; p = 0.0001; t = 14.28 DF = 18. n = 2 repeats, n = 5 embryos per group).

Increased frequency of abnormal vascular branching (stunted SeAs or incomplete DLAV anastomosis) by 50 hpf (unpaired t-test; p = 0.0001; t = 14.28 DF = 18. n = 2 repeats, n = 5 embryos per group). g tmem33 expression is not significantly altered by SKF-96365 treatment (unpaired t-test; t = 0.911, DF = 4, n = 3 repeats). h–j SKF-96365 reduces frequency of endothelial Ca²⁺ oscillations in Tg(fli1a:gal4FFubs3;uas-gcamp7a) embryos. Intensity projection over time showing calcium oscillations in control embryos, highlighting intensity change over duration of time lapse (unpaired t-test; p = 0.005; t = 2.594, DF = 10, n = 2 repeats, 3 embryos per group). k, l Notch reporter expression is reduced in SeAs (white arrowheads) and DA (red arrowhead) of Tg(csl: venus) embryos. Scale bars 50 µm. m DA expression of Notch reporter is reduced by SKF treatment (unpaired t-test; **p = 0.0097, t = 3.982 DF = 15, 2 repeats, n = 4 embryos per group). n, o SKF-96365 treatment reduces endothelial filopodia number (black arrowheads) and abnormal spade-shaped tip cell morphology (blue arrowhead), in Tg(fli1a:lifeACT-mClover) embryos. Scale bars 50 µm. p SKF-96365 treatment reduces mean filopodia number per SeA per embryo by 26 hpf (unpaired t-test; p = 0.005; t = 2.525, DF = 10, n = 2 repeats, n = 5 embryos per group). Source data are provided as a Source Data file.
the cytosol from intracellular stores. Our data suggest the VEGF-mediated release of Ca\(^{2+}\) into the cytosol from intracellular stores. Our data suggest the VEGF-mediated release of Ca\(^{2+}\) from ER stores during angiogenesis is dependent on Tmem33 function within the ER membrane. Furthermore, resultant Ca\(^{2+}\) oscillations generated in tip cells downstream of Vegfa contribute to phosphorylation of ERK and induction or maintenance of downstream targets including Dll4/Notch signalling, to co-ordinate cellular behaviours during vascular morphogenesis. ER, endoplasmic reticulum; P, plasma membrane.

**Fig. 8** Proposed model for tmem33 function in endothelial cells during angiogenesis. When VEGFA binds to its cognate receptor, e.g., Kdrl, the resulting phosphorylation of PLC\(\gamma\) generates inositol 1,4,5-trisphosphate (IP3) and this, in turn, binds to IP3 receptors on the ER membrane, which release Ca\(^{2+}\) into the cytosol from intracellular stores. Our data suggest the VEGF-mediated release of Ca\(^{2+}\) from ER stores during angiogenesis is dependent on Tmem33 function within the ER membrane. Furthermore, resultant Ca\(^{2+}\) oscillations generated in tip cells downstream of Vegfa contribute to phosphorylation of ERK and induction or maintenance of downstream targets including Dll4/Notch signalling, to co-ordinate cellular behaviours during vascular morphogenesis.

zebrafish endothelium and pronephros using tissue-specific CRISPRi. Using this approach we were able to uncouple tissue-specific functions of tmem33 and show that tmem33 is required cell autonomously in both tissues for their normal development. tmem33 functions within ECs to promote endothelial Ca\(^{2+}\) oscillations and filopodia formation during angiogenesis, and also to promote Notch signalling and regulate EC number. Tissue-specific CRISPRi offers a relatively straightforward method to study gene function within different tissues and represents an alternative to laborious cell transplantation procedures. Tissue-specific CRISPRi can be employed transiently to uncouple tissue-specific functions of genes, via co-injection of gene-targeting sgRNAs, dCas9 expression constructs and Tol2 mRNA. However, we observed more severe defects using this approach than when using stable transgenic lines expressing dCas9. We suspect this may be due to variable copy number integration mediated by Tol2 and thus variable dCas9 expression, in addition to inherent toxicity of DNA microinjections. At minimal phenotypic doses, CRISPRi is less prone to induction of p53 than some morpholinos and although this will vary depending upon the sgRNA, target and morpholino employed, our experience with the genes we have studied to date suggests CRISPRi may be susceptible to fewer off-target effects.

The localisation of tmem33 within the ER in zebrafish ECs suggest it may have an important function in regulation of endothelial SOCE. Consistent with this, acute antagonism of SOCE induced similar reductions in cytosolic Ca\(^{2+}\) oscillations, filopodia formation and endothelial Notch activity as tmem33 knockdown, without affecting tmem33 expression. Furthermore, SOCE inhibition reduced EC proliferation, consistent with reduced EC number induced by tmem33 knockdown. Genome-wide protein interaction studies in Drosophila embryos, have shown that the Drosophila Tmem33 orthologue, Kr-h2, physically interacts with SERCA\(^{\text{59}}\), and although this requires functional validation, the interaction appears to be conserved in humans. This suggests an ancestral function of Tmem33 in regulation of SOCE. Tmem33 may also act to augment the activity of Ca\(^{2+}\) channels such as IP3R within the ER. Alternatively, tmem33 may be involved in mediating cortical distribution of the ER, as has been described for a yeast orthologue Tts1p\(^{\text{22}}\). Interestingly, rapid changes in cytosolic Ca\(^{2+}\) are known to induce dynamic changes in actin cytoskeleton organisation in vitro\(^{\text{60}}\). The actin cytoskeleton has been linked to normal SOCE function by facilitating interaction of ER-resident proteins with their plasma membrane counterparts\(^{\text{19,61}}\). As tmem33 knockdown reduces Ca\(^{2+}\) oscillations and inhibits filopodia formation, this represents an intriguing possibility. VEGF has been shown to induce distinct modalities of calcium oscillations that correlate with different EC behaviours, including migration and proliferation\(^{\text{62}}\). We find that inhibition of SOCE impairs both migration and proliferation of ECs and knockdown of tmem33 similarly impairs EC migration and reduces EC number, suggesting an essential requirement for Ca\(^{2+}\) oscillations in both processes. The endothelial actin cytoskeleton is necessary for efficient mitosis and migration, and is notably disrupted by tmem33 knockdown or SOCE inhibition. This suggests that reduced EC proliferation and migration induced by inhibition of SOCE or reduced migration and EC number induced by tmem33 knockdown may represent a consequence of Ca\(^{2+}\)-dependent disruption of the endothelial actin cytoskeleton. Further studies will be required to address the precise function of Tmem33-mediated Ca\(^{2+}\) within the ER in ECs in response to VEGF.
Methods

Zebrafish strains, morpholinos and sgRNA. All zebrafish were maintained according to institutional and national ethical and animal welfare guidelines. All experiments were performed under UK Home Office licences 40/3708 and 70/8588. The following published zebrafish lines were employed: Tg(flh:EGFP)^{63} Tg(−0.8flh:enrHRFP)^{33,35} Tg(−201flh:EGFP)^{64} Tg(k1:EGFP-NLS)^{65,67} Tg(kdr:HRAS-mCherry-CAXA)^{98,96} and Tg(cfd:tmG)^{105,106}. For details on mutant and transgenic line generation, see Supplementary Methods. A quantity of 2–3 or 2 ng of Sp2b and Sp3 morpholinos were injected. Where these were co-injected, a total of 0.4 ng or 4 ng was injected. Four nanograms of morpholino was found to exhibit some induction of p53 (Supplementary Fig. 7b) and was titrated to 0.4 ng to reduce this; however, phenotypes were equivalent at each dose. One nanogram of each sgRNA was injected alongside 500 pg of dCas9 mRNA^35. Negative control sgRNAs contained the DNA-binding element of the active gRNA but lacked the stem-loop forming region associated with binding to the Cas9 or dCas9 protein. For morpholino, sgRNA and primer sequences, see Supplementary Methods.

Generation of tmem33^sh443 mutant allele. TALENs specific for tmem33 (ENSDARG00000041332) were designed against the following sequence 5′-cttcgctgggtgctgtcagtctgtgctgtga-3′. TALENs were assembled using the Golden Gate TALEN and TAL Effector Kit (Addgene, MA, USA)^{44} to generate the pTmm33TallR plasmids. Following linearisation of pTmM33Tal1R with NotI, capped mRNA was generated by in vitro transcription and 1500 pg TALEN mRNA was injected per embryo. Individual G0 embryos were tested by PCR and restriction fragment length polymorphism using tmem33ex3F and tmem33ex3R primers (Table S1), to identify somatic mutations that destroyed the EcoNI restriction site. The progeny of TALEN-injected G0 adults were incrossed and genotyped to confirm the presence of the sh434 alleles. All sh434 embryos were used for F3 and F4 generations.

Generation of fltladCas9 and enpepCas9 constructs. pME-dCas9 was generated by cloning the nls-dCas9-NLS coding sequence from pFRTS-dCas9, a gift from Dean Dister, into pME-MCS2, which contains the multiple cloning site of pc2S+. fltladCas9 cx regulatory and enpepCas9 cx regulatory constructs (Supplementary Fig. 1a) were subsequently generated using the Tol2 Kit via standard methods^67. Each construct was injected into Nacre embryos alongside Tol2 mRNA at 25 pg/ml.

Generation of transgenic lines. Tg(flh;Gal4UAS:UGA:GFP)^{79,82,83} was generated via injection of Tol2UAS:Gal4UAS^7 in progeny of Tg(flh;Gal4UAS)^{78} heterozygous outcrosses and tol2 mRNA according to standard protocols^67. Tg(flh:LifeAct-mCherry)^{106,68} was generated by fusing the LifeAct F-Actin binding motif to the N terminus of mCherry by PCR (Table S1) to generate pME-LifeAct-mChlor. pLifeAct-mChlor construct was generated via injection of tol2 mRNA via standard methods^67 and the following components: fltlad enhancer/promoter^29, pME-LifeAct-mChlor, pDestTol2-pA267 and p3E-SV40-pA67. Tg(flh:DsRedEx2)^{68} was generated by using the Tol2 Kit via standard methods^67 and the following components: fltlad enhancer/promoter^29, PENTRD5RedEx2^29, pDestTol2-pA267 and p3E-SV40-pA67. Tg(flh:AC-TargFP)^{63,67} was generated by amplifying the AC-TargFP coding sequence from AC-TargFP (Chromeotek) and adding attB1/attR2 sites (Table S1) to generate pME-AC-TargFP. pME-AC-TargFP construct was generated using the Tol2 Kit^67 and components listed above. Tg(flh: dCas9, cryaCasCreer)^{52,52} was co-injected into fltladCas9, cryaCasCreer plasmid with Tol2 mRNA. Embryos were injected at one-cell stage with 25 ng/ml Tol2 mRNA and corresponding plasmid DNA.

mRNA microinjections. Microinjections of both vegfa^ex3^ (300 pg), tmem33 (250 pg) and tmem33^gfp (1200 pg) mRNA were performed on single-cell-stage embryos with 1 nl injection volume.

RNA in situ hybridisation and immunohistochemistry. Alkaline phosphatase wholemount in situ hybridisation experiments were performed using standard methods as described previously^31. Detailed protocols are available upon request. EC dCas9 expression was detected using Cas9 in vivo probe^32. Immunohistochemistry to detect pERK was performed using Phospho-p44/42 Erk1/2 (Thr202, Tyr204) Rabbit mAb (#4370, cell signal, 1:250) and quantified by normalising EC signal against ERK staining within the neural tube as described^27. Immunochemistry to detect dCas9 was performed on whole-mount and sectioned embryos as described^35 using mouse anti-CRISP/Cas9 (7A9-A3A) (Novus Biologicals, NB2-36446, 1:1000), chicken anti-GFP (Abcam, ab19370, 1:500) primary antibodies, goat anti-mouse IgG (H&L) Alexa Fluor® 647 (Thermo Fisher, A21235, 1:1000), and goat anti-chicken IgY (H&L) Alexa Fluor® 488 (Thermo Fisher, A11039, 1:1000) secondary antibodies. Sectioned embryos were fixed at 3 dpf in 4% paraformaldehyde overnight, washed into 30% sucrose and sectioned at 14 μm thickness on a Jung Frigocut cryostat (Leica).
Cell migration of HUVECs and MLEC3s, treated as indicated, was assayed using the Cytoselect® 24-well Wound Healing Assay kit (Cell Biolabs). Images were captured with a Nikon DSFi1 digital camera coupled to a Nikon Eclipse TS100 microscope at ×4 magnification. Cell-free area was quantified with ImageJ software.

**Statistical analysis**
All statistical analysis employed two-tailed tests and are described in figure legends. All error bars display the mean and SD, except for qPCR error bars, which display the mean and SEM. Numbers of experimental repeats are listed in figure legends. P-values, unless exact value is listed, are as follows: *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001. F-values, t-values and degrees of freedom are listed in individual figure legends.

**Reporting Summary**
Further information on experimental design is available in the Nature Research Reporting Summary linked to this Article.

**Wound-healing EC migration assay.** Cell migration of HUVECs and MLEC3s, treated as indicated, was assayed using the Cytoselect® 24-well Wound Healing Assay kit (Cell Biolabs). Images were captured with a Nikon DSFi1 digital camera coupled to a Nikon Eclipse TS100 microscope at ×4 magnification. Cell-free area was quantified with ImageJ software.

**Matrilig EC tube-formation assay.** HUVECs transfected with the corresponding siRNA were plated onto Gelmatre Reduced Growth-Factor Matrix (Invitrogen) in Medium 200 with newborn reconstituted human umbilical venous endothelial cells and VEGF (25 ng/ml) when indicated. Tube formation was quantified after incubation at 37 °C for 16 h. Images were recorded with a Nikon DSFi1 digital camera coupled to a Nikon Eclipse TS100 microscope at ×4 magnification.

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