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Asymmetric division coordinates collective cell migration in angiogenesis

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Abstract

The asymmetric division of stem or progenitor cells generates daughters with distinct fates and regulates cell diversity during tissue morphogenesis. However, roles for asymmetric division in other more dynamic morphogenetic processes, such as cell migration, have not previously been described. Here we combine zebrafish *in vivo* experimental and computational approaches to reveal that heterogeneity introduced by asymmetric division generates multicellular polarity that drives coordinated collective cell migration in angiogenesis. We find that asymmetric positioning of the mitotic spindle during endothelial tip cell division generates daughters of distinct size with discrete “tip” or “stalk” thresholds of pro-migratory Vegfr signalling. Consequently, post-mitotic Vegfr asymmetry drives Dll4/Notch-independent self-organisation of daughters into leading tip or trailing stalk cells, and disruption of asymmetry randomises daughter tip/stalk selection. Thus, asymmetric division seamlessly integrates cell proliferation with collective migration, and as such, may facilitate growth of other collectively migrating tissues during development, regeneration and cancer invasion.

Organogenesis requires the precise integration of diverse morphogenetic processes. For example, in angiogenesis the coordinated specification, proliferation and collective migration of leading “tip” and trailing “stalk” endothelial cells drives nascent blood vessel formation^{1–3}. Activation of VEGFR-2/-3 signalling by gradients of VEGF ligand induces tip cell (TC) formation and directed migration^{3,4}. Moreover, VEGFR activation promotes expression of the Notch ligand delta-like 4 (Dll4) in TCs, which activates Notch in adjacent stalk cells (SCs) to down-regulate VEGFR function and laterally inhibit TC identity^{5–8}. Consequently, relative levels of *Vegfr* and/or *Dll4* expression directly influence TC selection and collective migration via VEGFR-Notch feedback^{9,10}.

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Cell proliferation is also essential for new vessel growth^{11,12}, but it is unclear how cell divisions, which must inherently partition VEGFR and Notch signalling components between daughters, do not disrupt hierarchical tip-stalk organisation and synchronised collective movements. For example, symmetrical partitioning of VEGFR-2 and Dll4 during TC division would prompt competition between daughters for re-assignment of TC identity. Considering that the temporal dynamics of Delta-Notch-mediated lateral inhibition are relatively slow (upwards of 5h)^{13,14}, division would severely impede angiogenesis (Supplementary Fig. 1a). Hence, it is unknown how multicellular polarity and uninterrupted directed collective migration are maintained during proliferative growth.

Results

Endothelial cell divisions generate asymmetric daughters

To investigate the post-mitotic dynamics of tip-stalk re-selection we performed a detailed analysis of tip/stalk behaviour at single cell resolution *in vivo*. Live-cell imaging of intersegmental vessel (ISV) sprouting in *Tg(kdr:nlEGFP)^{fl109}* zebrafish embryos indicated that endothelial cell movements were highly characteristic and dependent on cell positioning within new branches (Fig. 1a to e; Supplementary Fig. 1b, c). Tracking of individual nuclei revealed that ISV TCs move dorsally from the dorsal aorta (DA) to the dorsolateral anastomotic vessel (DLAV) position at quicker rates than adjacent SCs (Supplementary Fig. 1b, c; Supplementary Video 1). Further analyses of single lyn-mCherry-expressing cells revealed that TCs rapidly migrated (Fig. 1a, c; Supplementary Video 2) whereas SCs were less motile and alternatively underwent cell elongation¹⁵ (Fig. 1b, d; Supplementary Video 3). Moreover, averaging of multiple datasets indicated that cells exhibit wide variance in motility (Supplementary Fig. 1d) but revealed that the first, second and third endothelial cells to sprout from the DA (the leading TC, adjacent SC and following SC, respectively) displayed highly characteristic motilities during ISV branching (Fig. 1e). During ISV sprouting, 76% of TCs and 27% of SCs divided (Supplementary Fig. 1e), with mitosis oriented perpendicular to the vessel long axis, as previously described^{16,17}. However, continued tracking of the distal-most daughter of TC or SC divisions demonstrated that mitosis had no effect on movement, relative to non-dividing cells (Fig. 1f). Hence, characteristic behaviour was instantly re-established following division without pausing for tip/stalk re-selection. Consistent with these observations, tracking of daughter cells after completion of mitosis (Supplementary Fig. 1f) revealed that the tip-stalk hierarchy and characteristic TC/SC motilities were seamlessly re-established following division (Fig. 1g to l; Supplementary Fig. 1g, h). For example, distal TC daughters (cell 1.1) retained TC-like movement whereas proximal daughters (cell 1.2) instantly adopted the motility of SCs (Fig. 1g, i to l; Supplementary Video 4). Similarly, only distal daughters of SC divisions retained parental motility (Fig. 1h, i and Supplementary Video 5). Hence, daughters rapidly self-organise into leading and trailing cells following division, which maintains uninterrupted collective migration during vessel proliferation (Fig. 2g).

Post-mitotic tip/stalk selection is Dll4/Notch-independent

To confirm that this represents a Dll4/Notch-independent mechanism, *dll4* expression was abrogated using a well-validated morpholino oligonucleotide⁵. In the absence of *dll4*, lateral

inhibition of TC identity was lost and non-dividing cells at SC positions atypically exhibited TC-like motilities (Fig. 2a-c and Supplementary Video 6). Despite this, daughters of TC and SC divisions still displayed differential motilities, with only the distal daughter retaining parental behaviour (Fig. 2d to f and Supplementary Videos 7 and 8). Similar results were obtained upon inhibition of Notch signalling using the γ -secretase inhibitor, dibenzazepine (DBZ; Supplementary Fig. 2a, b). Hence, endothelial cell division generates daughters with differential motilities, independent of Dll4/Notch-mediated lateral inhibition. Although Dll4-Notch signalling did not determine post-mitotic behaviour, it likely reinforces differences introduced by division, as proximal daughters of *dll4*-deficient TC divisions recovered to be more motile than controls 5h after division (cell 1.2; Fig. 2f). Interestingly, slower proximal daughters of TC divisions were frequently overtaken by adjacent SCs (Fig. 2d). Likewise, the distal daughters of SC divisions were faster than and often overtook the slower proximal daughters of TC divisions (Supplementary Fig. 2c, d). Hence proximo-distal positioning within the ISV, the cell-cell junctional context and/or proximity of cells to Vegf ligand after division do not account for observed differences in daughter motility (Fig. 2g, h). Overall, these findings reveal a Dll4/Notch-independent role for mitosis in tip/stalk selection and suggest that endothelial cell divisions may be intrinsically asymmetric.

***in silico* prediction of asymmetric cell division**

Asymmetric division determines differential daughter cell fate in many systems^{18,19}, but has not been implicated in the control of cell motility. To investigate a potential role for asymmetric division in collective migration we adapted our previously validated MemAgent-Spring computational Model (MSM) of Notch/Vegf-mediated tip/stalk selection^{20,21} to accurately replicate *in vivo* TC and SC dynamics observed during ISV branching (Supplementary Fig. 3). Importantly, the MSM was extended/adapted to facilitate a simple cell division function and improved lamella extension/retraction functionality (Supplementary Fig. 4a, b; see Methods). The key assumptions that determine the behavior of the MSM can be summarised as (1) ISVs branch in response to a Vegf gradient; (2) binding of Vegf to Vegfr induces the formation of filopodia and lamellipodia; (3) the amount of filopodia and lamellipodia determines the rate of cell migration; (4) upon cell division Vegfr mRNA and protein are partitioned between daughter cells according to their present localisations; (5) a slower-acting Dll4-Notch-Vegfr-mediated mechanism then determines daughter TC and SC phenotypes. Once calibrated to match *in vivo* TC and SC dynamics in the absence of cell division (Supplementary Fig. 3b, c), further simulation of symmetric division was not sufficient to replicate biological data (Fig. 3a; Supplementary Video 9). Simulations of symmetric divisions that equally partitioned Vegfr mRNA and protein between daughter cells resulted in daughters that were less motile than the parental TC. These symmetric daughters then actively competed for the tip position and mutually repressed each other's motility via time-consuming Dll4-Notch oscillations, slowing down ISV branching (Fig. 3a). In contrast, simulation of asymmetric divisions to create daughters that differentially partitioned key components of the MSM (cell size, Vegfr protein, *vegfr* mRNA; see methods) generated daughters with distinct tip and stalk cell-like behaviours (Fig. 3b; Supplementary Video 10). The MSM indicated that asymmetric divisions rapidly generated daughters with distinct motilities by ensuring that one daughter retained higher levels of Vegfr signalling (Fig. 3b). The model therefore predicted that asymmetric division

rather than symmetric division of these key components is necessary to generate the observed post mitotic motilities.

Asymmetric division generates daughters of distinct size

To validate this *in silico* prediction *in vivo* we first quantified post-mitotic differences in cell size, a key hallmark of asymmetric division^{18,19}. Firstly, nuclear localised Eos fluorescent protein expressed in TCs of *Tg(kdrl:nlsEos)^{ncv6}* embryos was irreversibly and stably photoconverted from green (gEos) to red (rEos) using 405nm light (Fig. 3c, d). The nuclear localisation signal fused to Eos ensured dispersal of the stable pool of rEos throughout the cytoplasm upon mitotic nuclear envelope breakdown (Supplementary Fig. 5a), followed by partitioning between daughters at cytokinesis and re-distribution to daughter cell nuclei after reassembly of the nuclear envelope (Fig. 3c, e). Importantly, the sum total of nuclear rEos subsequently inherited by both daughter cells exactly corresponded to the initial levels found in parental tip cells (Fig. 3f). Hence, post-mitotic nuclear rEos levels were a direct readout of the relative partitioning of cytoplasmic volumes of TC daughters at cytokinesis. Consistent with *in silico* predictions, differential inheritance of photoconverted rEos confirmed that TC divisions were intrinsically asymmetric and generated daughters of distinct size (Fig. 3e, f). Moreover, similar analysis of sprouting mesencephalic veins revealed that asymmetric division was a conserved feature of migrating TCs (Supplementary Fig. 5b-e). Asymmetries in TC daughter size were further confirmed upon analysis of single dividing TCs, with distal daughters of TC divisions being consistently 1.8 to 1.9 times larger than proximal daughters (Fig. 4a, b). Importantly, larger TC daughters were consistently more motile than smaller cells (Fig. 4c) and the size of individual TC daughters was directly correlated with their rate of post-mitotic motility (Fig. 4d). Lastly, the difference between the sizes of both daughters from TC divisions (distal cell size – proximal cell size) was putatively correlated with resulting differences in daughter cell motility (distal cell motility – proximal cell motility), but critically, in rare cases where daughters were near symmetrically sized they displayed near identical motilities (Fig. 4e). Hence, intrinsically asymmetric divisions coordinate collective cell migration *in vivo* by generating TC daughters of differential size and motility.

Polarised positioning of the mitotic spindle drives asymmetric division

Asymmetric positioning of the mitotic spindle is known to drive asymmetric division of stem/progenitor cells in many systems^{18,19}. As such, to define the mechanistic basis of post-mitotic asymmetries in tip cell daughter size, we investigated spindle positioning during tip cell division. High temporal resolution *in vivo* live imaging of tip cells expressing α -tubulin-GFP revealed that, after initially assuming a central position, the entire mitotic spindle consistently shifted to the proximal pole of dividing tip cells during metaphase (Fig. 4f; Supplementary Video 11). Consequently, the plane of tip cell division was proximally biased during anaphase and telophase, generating daughters of unequal size. Hence, similar to the asymmetric division of stem/progenitor cells, polarised positioning of the mitotic spindle functions to generate intrinsically asymmetric daughters of tip cell division.

TC division generates daughters with distinct Vegfr activity

Further dissection of the computational model indicated that the distinct motilities of TC daughters observed in control and *dll4* knockdown embryos could largely be recapitulated

upon the cell-size dependent asymmetric partitioning of *vegfr* mRNA (Supplementary Fig. 4c-h), but not by even severe asymmetries in Vegfr protein or cell area. These observations were a consequence of longer half-life of *vegfr* mRNA versus Vegfr protein^{22–24} (see Methods). Hence, the model predicted that asymmetries in cell size might regulate cell motility by differentially partitioning Vegfr signalling components to generate daughters with differential Vegfr activity. Consistent with this model prediction, live-cell imaging of dividing TCs followed by flash-fixation on ice and immunofluorescence staining for phospho-p44/42 mitogen-activated protein kinase (pErk), a key downstream component of the Vegfr pathway^{25–29}, revealed that divisions generated daughters with differential levels of Vegfr signalling (fig. 5). Firstly, live-cell imaging coupled with immunofluorescence staining demonstrated that leading TCs contained significantly more pErk than adjacent SCs or cells residing in the DA (Fig. 5a-c). Inhibition of Vegfr abrogated pErk staining, confirming that endothelial Erk phosphorylation was Vegfr-dependent (Fig. 5d). Moreover, cells possessing higher pErk levels were frequently more motile than adjacent cells and maximal rates of motility were observed in cells containing pErk at TC levels or above (Fig. 5b, e). Hence, the differential motilities of TCs and SCs correlated with distinct levels of Vegfr activity. Importantly, movies in which TC divisions were captured shortly prior to fixation indicated that distal daughters consistently displayed higher Vegfr-dependent pErk levels relative to proximal daughters (Fig. 5f). Furthermore, capture of mitotic TCs at the point at which they first split to form distinct daughters revealed that distal daughters asymmetrically acquired higher pErk levels (Fig. 5g). Quantification demonstrated that pErk levels were low during division when only one dividing TC was distinguished, likely due to mitotic disruption of Vegfr endocytosis and/or recycling^{30–32} (Fig. 5h). However, when two daughter TCs were first distinguished, each immediately displayed differential Vegfr signalling, with distal daughters re-establishing TC-like activity and proximal daughters adopting SC-like levels (Fig. 5h). Hence, asymmetric division introduce heterogeneity that self-generates daughters with distinct levels of Vegfr activity.

Differential partitioning of filopodia does not drive asymmetric division

Consistent with these observations, the larger distal daughters of TC division possessed more Vegfr-dependent filopodial protrusions³ than smaller proximal daughters (Fig. 4a; Supplementary Fig. 6). In agreement with previous work, the disruption of filopodia formation did not impact TC guidance³³, but also did not disrupt asymmetries in TC daughter motility, which remained dependent on cell size (Supplementary Fig. 6c to j). However, the tight correlation of Vegfr-induced filopodia numbers with daughter cell motility (Supplementary Fig. 6b) further indicated that underlying asymmetries in Vegfr activity drive differential post-mitotic motility.

Kdr1 mRNA is asymmetrically partitioned during TC division

Modelling of asymmetric tip cell division suggested that differential size-dependent partitioning of Vegfr signalling components, such as *vegfr* mRNA, generates daughters with distinct Vegfr signalling levels. Consistent with this model prediction, live-cell imaging and fluorescent *in-situ* hybridisation (FISH) revealed that mRNA encoding *kinase insert domain receptor-like* (*kdr1*) mRNA, a key *VEGFR* orthologue in zebrafish^{34,35}, was asymmetrically partitioned in TC daughters (Fig. 5i, j). Interestingly, prior to division *kdr1* mRNA was

localised at distal peri-nuclear sites in migrating TCs (Supplementary Fig. 6k-p), suggesting roles for mRNA targeting in Vegfr function. However, this localisation of *kdr* mRNA was disrupted during mitosis and appeared more homogeneously localised, similar to *egfp* mRNA. Consequently, during division both *kdr* mRNA and *egfp* mRNA were asymmetrically partitioned, with distal daughters inheriting 1.7 to 1.9 times more mRNA than proximal daughters (Fig. 5j), suggesting global asymmetries in cellular components. Thus, asymmetric divisions generate daughters with differentially partitioned *kdr* mRNA, discrete tip/stalk thresholds of pro-migratory Vegfr signalling and distinct tip/stalk motilities.

Vegfr asymmetry is required for daughter tip/stalk positioning

Finally, we aimed to determine whether post-mitotic Vegfr asymmetry mechanistically drives re-organisation of daughters into leading and trailing collectively migrating cells. Treatment of embryos with the pan-specific Vegfr inhibitor SU5416 at doses of 0.6 μ M or above severely disrupted endothelial cell Vegfr signalling and pErk staining (Fig. 6a). However, a partial SU5416 dose of 0.3 μ M attenuated pErk levels to approximately 60% of controls, reducing Vegfr activity in all sprouting cells to SC-like levels (Fig. 6a, b). Subsequent incubation with 0.3 μ M SU5416 during division did not disrupt the orientation of endothelial cell division but generated TC daughters that all displayed SC-like motilities (Fig. 6c, d and Supplementary Video 12). Hence, in the absence of differential Vegfr thresholds, TC daughters assumed symmetric motile behaviours. Similar results were also observed using the more selective Vegfr-2 inhibitor, ZM323881 (Fig. 6e, f). In contrast, SCs retained the ability to generate daughters with differential motilities (Fig. 6d, f). Moreover, in the presence of 0.3 μ M SU5416 we noted that proximal daughters of TC divisions frequently and atypically assumed the TC position. In control or *dll4* knockdown embryos, distal TC daughters robustly maintained TC positioning and were only occasionally overtaken by proximal daughters (Fig. 6g, i, l and Supplementary Video 13). However, upon incubation with 0.3 μ M SU5416 during division, TC positioning was randomised with either daughter capable of taking the lead (Fig. 6i, l). In contrast, competition between TC daughters and adjacent SCs was not affected by the presence of 0.3 μ M SU5416. After TC division, proximal daughters in control embryos were occasionally overtaken by adjacent SCs (Fig. 6h, j and Supplementary Video 14), likely due to transiently elevated Vegfr activity observed in SCs residing adjacent to dividing TCs (Supplementary Fig. 6k). This shuffling behaviour was augmented by *dll4* knockdown, but was unaffected by partial Vegfr inhibition (Fig. 6j, l). Hence, SU5416-induced symmetric TC division selectively enhanced competition only between TC daughters and randomised assignment of tip-stalk positioning after division. Taken together, these data reveal that post-mitotic Vegfr asymmetry functions to instantly re-establish the tip-stalk hierarchy and maintain uninterrupted collective migration during proliferative growth.

Discussion

Our results define a previously unidentified role for asymmetric cell division in the control of collective cell migration. In particular, we show that post-mitotic Vegfr heterogeneity self-generates leader-follower cell hierarchies that drive coordinated collective movements in angiogenesis (Fig. 6m). Generation of cell type diversity by asymmetric division remarkably

mirrors the proposed role of mitosis in lymphatic progenitor emergence^{36,37}, suggesting a wider role for Vegfr asymmetry in divergent vascular fate choices. Furthermore, our observation of tip/stalk selection by asymmetric division redefines the current view that tip/stalk identity is exclusively specified by Dll4/Notch-dependent lateral inhibition. It was recently proposed that the temporal dynamics of Dll4/Notch-mediated lateral inhibition are too slow to account for many of the rapid, adaptive switches of cell identity observed during blood vessel branching¹⁴. Vegfr asymmetry may account for these observations, as self-organisation of daughters into leading/trailing cells is near instantaneous and eradicates the need to pause for re-specification of tip/stalk identity by lateral inhibition. As such, asymmetric divisions functionally integrate proliferation with seamless re-establishment of tip-stalk hierarchy to robustly maintain uninterrupted collective migration during tissue growth. Consequently, endothelial cell migration and angiogenesis are acutely temporally delayed in the absence of asymmetric divisions. Considering that maintenance of multicellular leader-follower polarity underlies all collectively migrating cell systems³⁸, asymmetric divisions may drive the integrated growth of multiple tissues during embryonic development, wound healing and cancer invasion.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Endothelial cell division generates daughters with distinct behaviours.

(a and b) Time-lapse images of individual lyn-mCherry expressing tip (a; cell 1) or stalk (b; cell 2) cells in sprouting ISVs of *Tg(kdrl:nlsEGFP)^{zf109}* embryos from around 22 hours post-fertilisation (hpf). (c and d) Quantification of the dorsal movement of cell nuclei and the top, bottom and centre of migrating tip (c; $n=8$ ISVs, 7 embryos) or stalk (d; $n=9$ ISVs, 8 embryos) cells. (e and f) Quantification of the averaged motility of all tracked endothelial cell nuclei from 22 hpf (e, $n=136$ ISVs, 34 embryos) or of tracked nuclei from ISVs in which TCs or SCs did or did not divide (f, $n=83$ ISVs, 1 no div; $n=51$ ISVs, 1 div; $n=58$ ISVs, 2 no div; $n=42$ ISVs, 2 div, 34 embryos). In f, If a TC or SC divided, only the distal-most daughter continued to be tracked. (g and h) Time-lapse images of sprouting ISVs from 0.3h prior to either TC division (g) or SC division (h) in control *Tg(kdrl:nlsEGFP)^{zf109}* embryos. Brackets indicate dividing cells. Nuclei are pseudocoloured according to their initial position. (i) Quantification of the dorsal movement of daughter cells following TC and SC division in control embryos (i, $n=43$ ISVs, 1.1/1.2; $n=41$ ISVs, 2.1/2.2, 34 embryos).

Dashed lines represent trend-lines of endothelial cell motilities from **e**. (**j**) Time-lapse images of an individual lyn-mCherry expressing TC in a sprouting ISV of a *Tg(kdrl:nlsEGFP)^{zf109}* embryo from 22 hpf. Bracket indicates the point of TC division. (**k** and **l**) Quantification of the dorsal movement of cell nuclei and the top, bottom and centre of the migrating distal daughter cell (**k**; cell 1.1) or proximal daughter cell (**l**; cell 1.2) following TC division. Brackets indicate the point of TC division. Source data for **c**, **d**, **e**, **f** and **i** are shown in Supplementary Table 2. Error bars: mean $\pm$ SEM. * $P < 0.0001$, two way ANOVA test. Scale bars, 25 μ m.

Figure 2. Post-mitotic asymmetry is independent of Dll4-Notch signalling.

(a) Time-lapse images of sprouting ISVs from 22 hpf in *Tg(kdr1:nlsEGFP)^{zf109}* embryos upon *dll4* knockdown (*dll4* KD). Nuclei are pseudocoloured according to their initial position. Arrowheads indicate a super-motile SC. (b) Quantification of the dorsal movement of tracked endothelial cell nuclei from 22 hpf upon *dll4* knockdown ($n=80$ ISVs, 23 embryos). Dashed lines represent endothelial cells motilities from Fig. 1e. In the absence of *dll4* SCs adopt TC-like motilities. (c) Quantification of the total number of cells per ISV at 32.5 hpf, number of mitotic TCs or SCs per ISV, position of TC or SC divisions and the time

of TC or SC divisions in control or *dll4* knockdown embryos following live-cell imaging from 22 to 32.5 hpf ($n=70$ ISVs, 18 embryos, control; $n=76$ ISVs, 23 embryos, *dll4* KD). More endothelial cells sprout into ISVs and more SC divisions are observed in the absence of *dll4*. (**d, e**) Time-lapse images of sprouting ISVs from 0.3h prior to either TC division (**d**) or SC division (**e**) in *dll4* knockdown *Tg(kdrl:nlsEGFP)^{zf109}* embryos. Brackets indicate dividing cells. Nuclei are pseudocoloured according to their initial position. (**f**) Quantification of the dorsal movement of daughter cells following TC and SC division upon *dll4* knockdown ($n=33$ ISVs, 17 embryos, 1.1/1.2; $n=29$ ISVs, 16 embryos, 2.1/2.2) embryos. Dashed lines represent trend-lines of endothelial cell motilities from Fig. 1e. (**g** and **h**) Daughter cell behaviour following TC or SC division in control (**g**) or *dll4* knockdown (**h**) vessels. Source data for **b** and **f** are shown in Supplementary Table 2. Error bars: mean $\pm$ SEM. * $P<0.0001$, two way ANOVA test. † $P<0.05$ control versus *dll4* knockdown. ** $P<0.05$ *dll4* KD 1.2 versus control 1.2 from Fig. 1i. Unpaired two-tailed Student's *t* test. Scale bars, 25 μ m.

Figure 3. *in silico* and *in vivo* prediction of post-mitotic cell size asymmetry.

(a and b) MemAgent-Spring model simulations of either symmetric (a) or asymmetric (b) TC divisions ($n=10$). Arrows and arrowheads indicate periods of either mutual activation or repression of TC daughters, respectively. (c) Photo-conversion and post-mitotic inheritance of rEos during TC division. (d) Images of an ISV in a *Tg(kdrl:nlsEos)^{ncv6}* embryo prior to or following irradiation with 405 nm light and quantification of the relative stability of TC rEos fluorescence versus gEos, which increases over time ($n=8$ cells). (e and f) Time-lapse images of rEos fluorescence in a dividing photo-converted TC (e) and quantification of rEos levels inherited by each TC daughter, as well as the total rEos fluorescence present before (cell 1) and after (cells 1.1 + 1.2) TC division (f, $n=15$ dividing TCs from 7 embryos). Bracket in e indicates TC division. Error bars: mean $\pm$ SEM. * $P<0.01$ 1.1 versus 1.2. Unpaired two-tailed Student's *t* test. Scale bars, 25 μ m.

Figure 4. TC division generates daughter cell size asymmetry.

(a and b) Time-lapse images of a dividing lyn-mCherry- and nlsEGFP-expressing TCs and quantification of either cell size (a; $n=14$ pre-div TCs; $n=13$ post-div TCs, from 12 embryos) or cell volume (b; $n=5$ dividing TCs from 5 embryos) both before (cell 1) and directly after (cell 1.1 and cell 1.2) TC division. Bracket and arrowheads indicate a mitotic TC and the *de novo* interface between daughter cells, respectively. (c) Quantification of the dorsal movement of TC daughters of the indicated sizes ($n=22$ cells from 9 embryos). (d) Correlation of the size of individual TC daughters with their rate of post-mitotic motility

($P < 0.001$, Pearson's correlation, $R^2 = 0.56$, $n = 24$ cells from 11 embryos). (e) Correlation of the difference in size between TC daughters (distal cell size – proximal cell size) with their differences in cell motility (distal cell motility – proximal cell motility; $P < 0.02$, Pearson's correlation, $R^2 = 0.46$, $n = 12$ dividing cells from 11 embryos). (f) Time-lapse images of an individual dividing lyn-mCherry and α -tubulin-GFP-expressing tip cell in a *Tg(kdrl:ras-mCherry)^{s896}* embryo, and quantification of spindle movement during mitosis (NEB, nuclear envelope breakdown; ana., anaphase; tel., telophase; $n = 8$ dividing cells from 8 embryos). Error bars: mean $\pm$ SEM. * $P < 0.05$ 1.1 versus 1.2. ** $P < 0.01$ versus anaphase. Unpaired two-tailed Student's *t* test. Scale bars, 25 μ m.

Figure 5. TC division asymmetrically partitions Vegfr activity.

(a, b, f, g) Time-lapse images of nlsEGFP-expressing sprouting ISVs and localisation of pErk (red). Nuclei are pseudocoloured according to their initial position. Brackets in f and g indicate dividing TCs. The highly motile TC in b exhibits much higher levels of Vegfr signaling activity adjacent SCs. The distal daughters of TC division in f and g exhibit higher levels of Vegfr signaling activity than the proximal daughter. (c) Quantification of pErk fluorescence intensity at the indicated positions. TCs and SCs display distinct levels of Vegfr-dependent pErk ($n=44$ embryos). (d) Quantification of TC pErk levels in

Tg(kdr1:nlsEGFP)^{zf109} embryos upon incubation with DMSO, 2 μ M SU5416, 5 μ M DMH4, 1 μ M Ki8751 and 5 μ M ZM323881 ($n=9$ embryos, DMSO, SU5416, DMH4, Ki8751; $n=6$ embryos, ZM323881). (e) Correlation between pErk levels and endothelial cell motility ($n=21, 36, 50, 68, 48$ and 40 cells for $<5, <10, <15, <20, <25$ and >25 , respectively, from 14 embryos). (h) Quantification of pErk levels during and after TC division. Distal daughters of TC divisions exhibit higher levels of Vegfr signaling activity than proximal daughters ($n=85$ no-div TCs; $n=9$ during div., 0h div. and 0.3h div.; $n=18 >0.3h$ div., 44 embryos). (i) Time-lapse images of a dividing nlsEGFP-expressing TC and localisation of ZO-1 protein (white), *kdr1* mRNA (red) and *egfp* mRNA (green). Bracket, white arrowheads and red arrowheads indicate the mitotic TC, the *de novo* daughter cell-cell junction and the pre-existing TC-SC junction, respectively. White lines indicate the position of ZO-1 positive cell-cell junctions. (j) Quantification of *kdr1* and *egfp* mRNA segregation in the distal-most versus the proximal-most daughters of TC division ($n=19$ TC divisions, 16 embryos, *kdr1*; $n=13$ TC divisions, 10 embryos, *egfp*). Error bars: mean $\pm$ SEM. * $P<0.05$ versus 1.2. ** $P<0.001$ versus 1. † $P<0.05$ versus 4. †† $P<0.001$ versus DMSO-treated TC. *** $P<0.05$ versus <5 pERK intensity. Unpaired two-tailed Student's *t* test. Scale bars, 25 μ m.

Figure 6. Disruption of Vegfr asymmetry randomises daughter tip/stalk positioning.

(a and b) Quantification (a) and localisation (b) of pErk levels in endothelial cells of *Tg(kdrl:nlsEGFP)^{zf109}* embryos upon incubation with DMSO or the indicated concentration of SU5416. 0.3 μ M SU5416 reduces TC pErk to SC levels ($n=129$ ISVs, 17 embryos, DMSO; $n=109$ ISVs, 17 embryos, 0.3 μ M; $n=113$ ISVs, 18 embryos, 0.6 μ M). (c, g and h) Time-lapse images of sprouting ISVs from 0.3 h prior to TC division in 0.3 μ M SU5416-treated (c) or control (g and h) embryos. Nuclei are pseudocoloured according to their initial position. Brackets indicate dividing TCs. Open arrowheads indicate the position of a normal 1.1 TC daughters following division. (d) Quantification of the dorsal movement of daughter

endothelial cells following TC and SC division in 0.3 μ M SU5416-treated embryos ($n=54$ ISVs, 1.1/1.2; $n=10$ ISVs, 2.1/2.2, 22 embryos). **(e)** Quantification of pErk levels in endothelial cells upon incubation with DMSO or the indicated concentration of ZM323881 ($n=42$ ISVs, 8 embryos). 80 nM ZM323881 reduces TC pErk to SC levels. **(f)** Quantification of the dorsal movement of daughter endothelial cells following TC and SC division in 80 nM ZM323881-treated embryos ($n=38$ ISVs, 1.1/1.2; $n=11$ ISVs, 2.1/2.2, 17 embryos). **(i)** Quantification of the number of distal 1.1 TC daughters overtaken by proximal 1.2 TC daughters following division ($n=59$ divisions, 26 embryos, control; $n=48$ divisions, 20 embryos, *dll4* MO; $n=48$ divisions, 22 embryos, SU5416). **(j)** Quantification of the number of proximal 1.2 TC daughters overtaken by adjacent SCs following TC division ($n=57$ divisions, 26 embryos, control; $n=38$ divisions, 20 embryos, *dll4* MO; $n=65$ divisions, 24 embryos, SU5416). **(k)** Quantification of pErk levels in non-dividing TCs, SCs adjacent to TCs that do not divide, SCs adjacent to a dividing TC or SCs adjacent to a TC that had previously divided. SC pErk levels rise during division of an adjacent TC ($n=71$ cells, 28 embryos, cell 1 and no div. 2; $n=11$ cells, 11 embryos, during div. 2; $n=14$ cells, 8 embryos, after div. 2). **(l)** Endothelial cell rearrangements following TC division. **(m)** Model summarising the mechanisms of asymmetric division in angiogenesis. Source data for **d** and **f** are shown in Supplementary Table 2. Error bars: mean $\pm$ SEM. * $P<0.001$ versus DMSO 1. ** $P<0.001$ versus control. † $P<0.001$ versus 1. ‡ $P<0.05$ versus 2 no-division. Unpaired two-tailed Student's *t* test. Scale bars, 25 μ m.