Endothelial cell rearrangements during vascular patterning require PI3-kinase-mediated inhibition of actomyosin contractility

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Angiogenesis is a dynamic process relying on endothelial cell rearrangements within vascular tubes, yet the underlying mechanisms and functional relevance are poorly understood. Here we show that PI3Kα regulates endothelial cell rearrangements using a combination of a PI3Kα-selective inhibitor and endothelial-specific genetic deletion to abrogate PI3Kα activity during vessel development. Quantitative phosphoproteomics together with detailed cell biology analyses in vivo and in vitro reveal that PI3K signalling prevents NUAK1-dependent phosphorylation of the myosin phosphatase targeting-1 (MYPT1) protein, thereby allowing myosin light chain phosphatase (MLCP) activity and ultimately downregulating actomyosin contractility. Decreased PI3K activity enhances actomyosin contractility and impairs junctional remodelling and stabilization. This leads to overstretched endothelial cells that fail to anastomose properly and form aberrant superimposed layers within the vasculature. Our findings define the PI3K/NUAK1/MYPT1/MLCP axis as a critical pathway to regulate actomyosin contractility in endothelial cells, supporting vascular patterning and expansion through the control of cell rearrangement.
issue growth and homeostasis require the establishment of a functional hierarchical tubular network of blood vessels. Blood vessels are mainly formed by a process known as sprouting angiogenesis in which new vascular sprouts arise from parental vessels, grow, and fuse to an adjacent sprout or a pre-existing vessel. Newly formed sprouts are highly dynamic with endothelial cells interchanging their relative position within the vascular tube. This collective cell migration across the vascular tubes relies on cell rearrangement; yet the regulation of this cell behaviour during the formation and patterning of blood vessels is poorly understood.

Endothelial cell rearrangement occurs through the reorganization of cell–cell junctional contacts thereby allowing the modification of cell–cell adhesion strengths. In endothelial cells, there are two types of vascular endothelial-cadherin (Cdhs/VE-cadherin)-based junctional patterns, namely continuous or straight, and discontinuous or serrated. Straight junctional VE-cadherin-based junctional organization is mainly found in stable and mature junctions, whereas the serrated VE-cadherin junctional pattern is considered as immature or remodeling junctions. Although these VE-cadherin junctional patterns are not visible in the endothelium during zebrafish developmental angiogenesis, computational models have proposed that spatial heterogeneity of these junctional patterns is necessary for cells to rearrange in vivo. Yet, how these junctional profiles impact on the capacity of endothelial cells to rearrange remains poorly understood. Formation, remodelling, and stabilization of cell–cell adhesions in cultured endothelial cells are mediated by actin structures. At mature junctions, linear VE-cadherin is aligned to parallel cortical actin bundles. Instead, serrated immature junctions are connected to perpendicular or radial tensile actin cables. At mature junctions, linear VE-cadherin is aligned to parallel cortical actin bundles. Instead, serrated immature junctions are connected to perpendicular or radial tensile actin cables. The switch between stable and immature junctions is mediated by actomyosin contraction-based pulling forces at the cell–cell junctions.

Among the different players of the angiogenic process, class I PI3-kinases (PI3K) have emerged as a critical node, for both the physiology of endothelial cells and the pathogenesis of venous malformations. PI3Ks are lipid kinases that signal downstream of a variety of cell surface receptors and regulate cellular functions including growth, proliferation, migration, and metabolism. Upon activation, these enzymes generate the lipid phosphatidylinositol-3,4,5-trisphosphate, a second messenger that triggers signalling pathways, such as those mediated by the serine/threonine kinase Akt and its substrates. Of the class I PI3K isoforms, PI3Ka has been shown to be the only isoform required for endothelial-mediated vascular development. Several studies have demonstrated that PI3Ka signalling primary regulates cell motility during angiogenesis in mouse and zebrafish.

Our study uncovers a PI3K downstream pathway, namely NUAK1/MYPT1/MLCP, as a critical node in the regulation of cell rearrangement during vessel growth. We have found that blockade of PI3Ka signalling impairs junctional remodelling, inhibits cell rearrangement and drives endothelial cells to grow in superimposed aberrant layers. We identify that a failure of cells to rearrange results in cell stretching and inability to remodel and stabilize new cell–cell contacts upon anastomosis. Through a combination of in vivo and in vitro approaches together with an unbiased and deep quantitative phosphoproteomic screening, we have discovered that PI3Ka mediates cell rearrangement by inhibiting actomyosin contractility through NUAK1/MYPT1/MLCP.

Results
PI3Ka mediates rearrangement of endothelial cells. Here, we investigated how endothelial cell rearrangements within the vascular sprouts contribute to vessel expansion and patterning. We and others have shown that the PI3Ka isoform regulates endothelial cell motility (Supplementary Fig. 1, Supplementary Movies 1 and 2, and refs. 15,21,22). Therefore, we predicted that cell rearrangement would be altered upon blockage of this signalling node. To validate our hypothesis, we first studied vessel growth in zebrafish embryos treated with a PI3Ka isoform-specific inhibitor (GDC-0326, ref. 23). We focused our studies between 27 and 38 h post fertilization (hpf), when intersegmental vessels (ISVs) that arise from the dorsal aorta reach the dorsal roof and form the dorsal longitudinal anastomotic vessels (DLAVs). Treatment with GDC-0326 efficiently inhibited PI3K signalling (Supplementary Fig. 2a) and this led to aberrant endothelial cell junctional patterns. Junctions were frequently disconnected in the dorsal region of the ISV axis and the junctional elongation was reduced in both ISVs and DLAV (Fig. 1a, b). We also observed the presence of ring-shaped junctions in these embryos that suggests lack of tight contact between adjacent endothelial cells (Fig. 1a). Moreover, GDC-0326-treated embryos showed a delay in the growth of ISVs (Supplementary Fig. 2b-d), without an overall delay in embryo development (Supplementary Fig. 2e). These aberrant junctional patterns are indicative of defects in cell rearrangements. Conversely, in vehicle-treated embryos, ISV outgrowth was accompanied by a normal extensive junctional remodelling leading to a pattern of highly elongated endothelial cell junctions (Fig. 1a, b).

In order to translate our observations into a mammalian model, we inactivated PI3Ka in endothelial cells using genetic approaches and we studied retinal angiogenesis. To maximize Pik3ca deletion and avoid compensations by other PI3K isoforms, we studied Pik3ca<sup>KD/lox</sup> mice, in which one Pik3ka allele is a constitutive kinase-dead (KD) and the other is a lox-P-flanked Pik3ka allele. Pik3ca<sup>KD/lox</sup> mice were crossed into the Pdgfb-iCreER transgenic mouse, which expresses a tamoxifen-inducible CRE recombinase specifically in endothelial cells (further referred to as Pik3ca<sup>KD/IEC</sup>) (Supplementary Table 1 provides details of the mouse models used). To ensure that Pik3ca<sup>lox</sup> allele is completely knocked out in our experimental settings, we assessed PI3Ka half-life in Pik3ca<sup>lox/lox</sup> mice crossed into the Pdgfb-iCreER transgenic mouse (referred to as Pik3ca<sup>IEC/IEC</sup>). Complete depletion of the PI3Ka protein was achieved at 96 h after 4-hydroxytamoxifen (4-OHT) treatment (Supplementary Fig. 3a). Thus, to reach complete PI3Ka depletion in vivo, we administered 4-OHT at postnatal day (P) 1 and P2 and investigated Pik3ca<sup>KD/IEC</sup> retinas at P7. As compared to control retinas, Pik3ca<sup>KD/IEC</sup> P7 retinas showed a decrease in phospho-S6 (pS6) (S240/4) and an enhanced nuclear FOXO1 staining confirming the inactivation of the PI3K pathway (Supplementary Fig. 3b, c).

In line with our previous findings in zebrafish embryos, we observed that endothelial cells in Pik3ca<sup>KD/IEC</sup> retinas showed an increased number of junctional gaps (Fig. 1c–e; yellow arrowheads). Furthermore, in a proportion of vascular tubes, the junction gaps resulted either in isolated rings or single-dots of VE-cadherin, indicating lack of tight contacts between endothelial cells (Fig. 1c; red asterisks). To investigate this phenotype in further detail, we analysed VE-cadherin-based cell–cell junctions in cultured endothelial cells from Pik3ca<sup>KD/IEC</sup> mice by immunostaining for β-catenin and F-actin (Fig. 1f). These experiments showed that Pik3ca<sup>KD/IEC</sup> endothelial cells failed to establish mature cell–cell junctions; instead, most of the junctions remained immature and connected to radial actin fibres (Fig. 1f–h). Together these data indicate that PI3Ka signalling is involved in cell–cell junctional remodelling in endothelial cells, a process required for cellular rearrangements within the vasculature.
Defects in cell rearrangements lead to anastomosis failure. P7 Pik3ca<sup>KD/iΔEC</sup> retinas exhibited a significant reduction in vascular radial outgrowth (Fig. 2a and Supplementary Fig. 4a) with no detectable differences in sprouting activity (Supplementary Fig. 4c, d). This phenotype was further increased at P10, with the sprouting front of Pik3ca<sup>KD/iΔEC</sup> retinas neither reaching the periphery of the retina nor invading deeper retinal layers (Fig. 2a and Supplementary Fig. 4b). However, P7 Pik3ca<sup>KD/iΔEC</sup> retinas showed an increase in vessel density with vascular tubes growing in multiple layers (Fig. 2a–c; Supplementary Fig. 4e and
Fig. 1 Defects in junctional remodelling upon inactivation of PI3Kα in endothelial cells. a Lateral views of intersomitic vessels (ISV) in vehicle and GDC-0326 (50 µM)-treated transgenic Tg(kdr:EGFP)s843 (shown in red) embryos stained for ZO-1 (green) at 33 h post fertilization (hpf). Single ZO-1 staining is shown in upper row. DA refers to dorsal aorta and DLAV refers to dorsal longitudinal anastomotic vessels. White lines indicate ZO-1 negative staining; punctuate white lines indicate elongation of junction; yellow arrowheads show ring-shape junctions. b Quantification of the length of the dorsal part of the ISVs without ZO-1 (left graph) and length of the ISVs with continuous ZO-1 staining (right graph) in vehicle and GDC-0326 treated embryos (n ≥ 54 ISVs per treatment). c Representative maximum intensity projections of anti-VE-cadherin (green) and isolectin B4 (IB4, red) immunostained control and Pik3ca<sup>KD</sup>/iEC mouse retinas at P7. Single channel images of whole mount retinas stained with IB4 from control and Pik3ca<sup>KD</sup>/iEC mice stained for β-catenin (green) and F-actin (red) after being treated with 4-OHT for 72 h and re-plated on gelatin-coated slides for 24 h. Yellow arrowheads indicate straight junctional pattern; orange arrowheads indicate serrated junctional pattern. d Quantitative number of VE-cadherin-negative vessels (junctional gaps) per unit area (n ≥ 5 retinas per genotype) (d) and length of vessel structures without VE-cadherin (length of junctional gaps) (n ≥ 6 retinas per genotype) (e). f Confocal immunofluorescence images of primary mouse endothelial cells isolated from control and Pik3ca<sup>KD</sup>/iEC mice stained for β-catenin (green) and F-actin (red) after being treated with 4-OHT for 72 h and re-plated on gelatin-coated slides for 24 h. Yellow arrowheads indicate straight junctional pattern; orange arrowheads indicate serrated junctional pattern. g Graph shows the average of β-catenin-positive area along junctional linescans (n ≥ 109 cells from six independent experiments). h Quantification of percentages of cells with serrated, straight or mixed junctions (n = 5 independent experiments). Scale bars, 30 µm (a), 20 µm (c), 10 µm (c small panel, f). Data in b, d, e, g, h represent mean ± SEM (error bars). *P < 0.05; **P < 0.01; ***P < 0.001 were considered statistically significant. Statistical analysis was performed by the two-sided Mann-Whitney test.

Supplementary Movies 3 and 4); this superposition was more pronounced at P10 (Supplementary Fig. 4b, f). Also, the vascular tubes in Pik3ca<sup>KD</sup>/iEC retinas showed smaller calibre (Fig. 2b, c). Analysis of Pik3ca<sup>KD</sup>/WT and Pik3ca<sup>ΔEC</sup>/iEC retinas (Supplementary Table 1) showed that the former appeared normal, whereas Pik3ca<sup>ΔEC</sup>/iEC retinas looked similar to Pik3ca<sup>KD</sup>/iEC retinas but with milder vascular defects (Supplementary Fig. 4a, b).

Current models of enhanced vessel density involve an increase in the number of endothelial cells<sup>16,17,27,28</sup>. However, immunostaining of endothelial cell nuclei revealed a significant reduction in the total number of endothelial cells (Fig. 3a, b) and in...
endothelial cells in S-phase in Pik3ca\textsuperscript{KD/ΔEC} retinas (Supplementary Fig. 5a, b). Endothelial cells were highly stretched in Pik3ca\textsuperscript{KD/ΔEC} retinal vessels, with the distance between adjacent endothelial cell nuclei increased by twofold (Fig. 3a, b). This suggests that these cells failed to elongate alongside each other, a process known as cell pairing\textsuperscript{29}, and as a consequence the cells overstretched. Given the lower number of endothelial cells in GDC-0326-treated embryos and in Pik3ca\textsuperscript{KD/ΔEC} retinas, a reduction in cell number might interfere with cell rearrangement during sprouting angiogenesis.
Statistical analysis was performed by the two-sided Mann–Whitney test.

Vehicle (left panel) or GDC-0326 (50 μM) (right panel). Endothelial cell membrane is visualized in red and the actin cytoskeleton is visualized by F-actin staining. Images of control and Pik3caKD/iΔEC retinas stained for collagen IV (green) and IB4 (red). Single channels are also shown. White punctuated islet in the image of a Pik3caKD/iΔEC retina shows higher magnification of selected region to the right. Red arrowheads indicate a retracting sprout. Quantification of retracting sprouts per area (n = 4 retinas per genotype). Images from a time-lapse movie (starting at 30 hpf) showing lateral views of ISV morphogenesis in transgenic Tg(αΔflox::kdrl:mCherry-CAAX)S96 embryos treated with vehicle (left panel) or GDC-0326 (50 μM) (right panel). Endothelial cell membrane is visualized in red and the actin cytoskeleton is visualized by F-actin staining. Single channels are also shown. Red arrow shows a retracting event between two endothelial cells. Schematic illustration showing the vascular defects driven by inactivation of Pik3ka (designed by Ana Angulo-Urarte). During vessel growth remodelling, stabilization, and elongation (punctuated arrow) of junctional contacts is required between neighbouring endothelial cells to rearrange. Upon inactivation of Pik3ka, endothelial cells fail to stabilize (black arrow) and elongate (crossed punctuated arrow) junctional contacts. Scale bars, 40 μm (a), 20 μm (a amplified panels, e), 10 μm (c, e amplified panels) 15 μm (g). Data in b, d, and f represent mean ± SEM (error bars). *P < 0.05, **P < 0.01, ***P < 0.001. Statistical analysis was performed by the two-sided Mann–Whitney test.

PI3Ka inhibition upregulates phospho-MYPT1. To define the molecular and signalling changes occurring upon inactivation of PI3Ka in endothelial cells, we performed untargeted mass spectrometry (MS)-based phosphoproteomics analyses in primary mouse endothelial cells from Pik3caflox/flox (control) and Pik3caKD/iΔEC mice. We analysed four biological replicates per genotype, each of which was treated with vehicle (ethanol) for 24 h or 4-OHT for 24 and 96 h (Fig. 4a). We identified a total of 6836 phosphopeptides in the 24 samples analysed, which were quantified using a previously described label-free methodology, generating 328,128 data points. This analysis identified: (i) 224 differently regulated phosphopeptides in the Pik3caKD/flox endothelial cells relative to control; (ii) 122 deregulated phosphopeptides in Pik3caKD/iΔEC endothelial cells upon 24 h treatment with 4-OHT; and (iii) 202 deregulated phosphopeptides in Pik3caKD/iΔEC endothelial cells upon 96 h treatment with 4-OHT (Fig. 4b, c).

We clustered the phosphorylation sites that were significantly deregulated at 96 h post 4-OHT treatment by gene ontology (Supplementary Fig. 3a). Of particular relevance for the cell rearrangement phenotype observed, we found a total of 23 phosphosites from 14 cytoketone-related proteins that were differently modulated between Pik3caKD/iΔEC and control endothelial cells. These included the myosin light chain phosphatase (MLCP), CDC42, RAC1, junctional proteins, focal adhesions, intermediate filaments, and microtubules (Fig. 4d).

Among the different candidates, Pik3caKD/iΔEC endothelial cells showed an upregulation on the S445 phosphosite (pS445) of the phosphatase 1 regulatory subunit 12 A (Ppp1r12A) also known as MYPT1 (Fig. 4d). This protein was of particular interest because, together with PP1β and M20, it composes the MLCP complex and regulates the dephosphorylation of the myosin light chain (MLC) 2 (refs. 33–36), which is required for the contraction of actomyosin (illustration is shown in Supplementary Fig. 8a). We validated the increase of pS445 MYPT1 in Pik3caKD/iΔEC endothelial cells by western blot (Fig. 4e; Supplementary Fig. 8b, c). Also, we confirmed the PI3Kα-mediated regulation of pS445 MYPT1 in wild-type endothelial and HEK-293 cells treated with GDC-0326 (Fig. 4e; Supplementary Fig. 8b, c).
PI3Kα is required to suppress actomyosin contractility. Zagoroska et al. found that S445 MYPT1 phosphorylation triggers the binding of MYPT1 to 14-3-3, thereby blocking the ability of the MLCP complex to dephosphorylate MLC2 in S19 and T18/S19 (ref.35). Thus, we predicted that inhibition of PI3Kα, and the resultant increase of MYPT1 S445 phosphorylation, might promote the detachment of MLCP from the actomyosin machinery, thereby increasing actomyosin contractility. Indeed, we detected that the ability of MYPT1 to bind β-actin in an overlay assay was reduced upon inhibition of PI3Kα (Fig. 5a). By knocking down MYPT1 protein expression with small interference RNA (siRNA), we found that phosphorylation of MLC2 at S20 (S19 in humans) was increased upon MYPT1 downregulation in endothelial cells (Fig. 5b–d). Enhanced phosphorylation of MLC2 on S20 was also found upon both genetic and pharmacologic inhibition of PI3Kα in mouse endothelial cells (Supplementary Fig. 9a, b). Moreover, pS20 MLC2 staining was enriched at the subcortical region of Pik3caKD/iΔECPik3caKD/iΔEC endothelial cells (Fig. 6a–c; Supplementary Fig. 9c). This was correlated with subcortical accumulation of F-actin.
Fig. 4 Phosphoproteomics elucidate downstream effectors of PI3Kα in endothelial cells. a Schematic illustration of the untargeted label-free mass spectrometry analysis. The study was conducted in Pik3ca^{fl/ox} (control) and Pdgfb-iCre;Pik3ca^{KD/ox} (Pik3caKD/iΔEC) mouse lung endothelial cells under exponential growing conditions upon preincubation with vehicle (EtOH) or 4-OHT for the indicated time points. The vehicle condition for analysis of the heterozygous inactivation of PI3Kα (Pik3ca^{KD/ox}) without induction of CRE activity) was included as a further control and four different mice were analysed in each condition (a total of 24 samples). b Volcano plots exhibiting changes in phosphopeptides across genotypes. The Y axis represent the negative log10 of P value and the X axis shows the log2 of the fold change between control and Pik3caKD/iΔEC endothelial cells treated with vehicle (EtOH) for 24 h, 4-OHT for 24 h or 4-OHT for 96 h. Red and yellow dots represent significantly regulated phosphopeptides (P < 0.01 and P < 0.05 respectively) with a fold-change higher than 0.8 or lower than −0.8. c Venn diagram showing the number and percentage of phosphopeptides which are significantly upregulated or downregulated between experimental groups. Number and percentage of overlapping phosphopeptides between groups are also shown. d Heatmap indicating fold-changes in the phosphorylation of proteins related to the cytoskeleton. Phosphopeptides identified to be down- or upregulated in Pik3ca^{KD/ΔEC} vs. Ctrl are shown in blue and red, respectively across EtOH and 4-OHT treatments. Values shown represent mean fold-change over Ctrl. e Western blot validation of pS445 MYPT1 in mouse lung endothelial cells and HEK-293 cells upon genetic and pharmacological inhibition of PI3Kα. Control and Pik3ca^{KD/ΔEC} endothelial cells were treated with 4-OHT for 72 h, re-platted for 24 h and subjected to immunoblotting. Wild-type endothelial cells and HEK-293 cells were treated with vehicle or GDC-0326 for 48 h and subjected to immunoblotting. Quantification of at least three independent experiments is shown in Supplementary Figure 8.

Fig. 5 MYPT1 promotes dephosphorylation of MLC2 in endothelial cells. a Immunoblot analysis of HEK-293 cells treated with vehicle or GDC-0326 for 48 h using the indicated antibodies. Endogenous MYPT1 was immunoprecipitated and its ability to interact with actin was assessed in an overlay assay. Bars to the right show quantification of actin bound to total MYPT1 from three independent experiments. b Western blot analysis of MYPT1, pS20 MLC2 and β-actin in lysates of wild-type mouse lung endothelial cells transfected with siControl (siCtrl) or siMYPT1. Bars to the right show quantification of pS20 MLC2 normalized to β-actin from three independent experiments. c Images of endothelial cells transfected with siCtrl or siMYPT1, seeded on gelatin-coated plates 72 h post-transfection, and immunostained for β-catenin (green), pS20 MLC2 (red) and F-actin (blue). d Quantification of total cell pS20 MLC2 immunostaining intensity (shown as integrated density) of images shown in c (n ≥ 6 independent experiments). Scale bars, 15 µm (c). Data in a, b, and d represent mean ± SEM (error bars). *P < 0.05, **P < 0.01. Statistical analysis was performed in a, and b by the two-sided Student’s t test and in d by the two-sided Mann–Whitney test.
Fig. 6 Inactivation of PI3Kα results in increased actomyosin contractility. **a** pS20 MLC2 (green), F-actin (red) and β-catenin (blue) immunostaining of endothelial cells isolated from control and Pik3caKD/iΔEC mice treated with 4-OHT for 72 h and re-plated on gelatin-coated slides for 24 h. **b** Yellow islets show higher magnification of selected regions in **a**. Vertical blue arrows indicate endothelial junction between two endothelial cells. **c** Representative fluorescence intensities of pS20 MLC2, β-catenin and F-actin immunostaining corresponding to the area depicted by the white line in **b**. **d** pS20 MLC2 (green) and IB4 (red) staining of control and Pik3caKD/iΔEC P7 retinas. **e** Quantification of the intensity of pS20 MLC2 staining per vascular area (shown as integrated density) (n ≥ 5 retinas per genotype). **f** F-actin (green) and IB4 (red) staining of control and Pik3caKD/iΔEC P7 retinas. **g** High magnification of selected regions shown in **f** illustrates the increase in F-actin intensity induced in Pik3caKD/iΔEC endothelium. **h** Quantification of F-actin staining per vascular area (shown as integrated density) (n ≥ 6 retinas per genotype). Scale bars, 15 μm (**a**), 20 μm (**d, f, g**). Data in **e** and **g** represent mean ± SEM (error bars). *P < 0.05, **P < 0.01. Statistical analysis was performed by the two-sided Mann-Whitney test.

(Fig. 6a–c; Supplementary Fig. 9d). Instead, control cells showed stress fibres evenly distributed between the cytoplasm and the subcortical area of the cell (Fig. 6a–c). Analysis of Pik3caKD/iΔEC retinal vessels confirmed an increase in the intensity of pS20 MLC2 (Fig. 6d, e) and F-actin staining (Fig. 6f, g). Together, these experiments show that PI3Kα activity is required to downregulate actomyosin contractility and may also explain why Pik3caKD/iΔEC endothelial cells fail to stabilize junctions in vivo (Fig. 1).

NUA1 inhibition restores PI3Kα-driven vascular phenotypes. Two non-related protein kinases are able to phosphorylate...
PIK3caKD/i, namely NUAK family kinase 1 (NUAK1) and large tumour suppressor kinase 1 (LATS1). Up to date, only NUAK1 has been reported to regulate phosphorylation of MLCK. Based on these findings, we tested whether restoring MLCP activity by blocking NUAK1 reduces pS20 MLCK and suppresses actomyosin contractility. Treatment of endothelial cells with a selective NUAK1 inhibitor (NUAKi) reduced phosphorylation of MLCK and F-actin staining (Fig. 7a). Inhibition of ROCK, the main kinase that phosphorylates MLCK, was not sufficient to completely abrogate actomyosin contractility in Pik3caKD/i endothelial cells (Supplementary Fig. 10a, b). In contrast, ROCK inhibitor completely abrogated pS20 MLCK and disrupted stress fibres in control cells (Supplementary Fig. 10a, b). This further supports the role of PI3Ka in the regulation of the phosphorylation of MLCK in an MYPT1/MLCP-dependent manner.

Next, we attempted to rescue the vascular phenotypes triggered by the inactivation of PI3Ka in vivo by treating newborn mice with NUAKi. While inhibition of NUAK1 did not impact on the outgrowth of the vasculature, it normalised the hyperbranched vascular plexus and the vessel width and prevented the three-dimensional growth in Pik3caKD/iEC retinas (Fig. 7c, d and Supplementary Fig. 10c). In line with the observations in cultured endothelial cells, administration of ROCK inhibitor in vivo did not rescue the Pik3caKD/iEC retinal phenotype (Supplementary Fig. 10d-f). This further supports that PI3K signalling does not regulate pMLC2 in a ROCK-dependent manner. Altogether, these data demonstrate that regulation of the NUAK1/MYPT1/MLCK axis by PI3Ka is required to inhibit actomyosin contractility in cultured endothelial cells and in growing vessels in vivo (Fig. 7c).

Discussion

In this study, we present evidence demonstrating that the PI3K downstream pathway, the NUAK1/MYPT1/MLCP axis, controls actin dynamics in endothelial cells. We uncover that failure of endothelial cells to rearrange results in vascular tubes composed of stretched cells, which grow in a superimposed fashion and fail to stabilize upon anastomosis. Our data support that a tight regulation of endothelial cell movement, beyond tip cells, is necessary for the adequate patterning of the vascular plexus. Cell rearrangement is critical for any process that implies collective cell migration such as in epithelial sheets and neural crest cells during development, and in cancer cells during collective invasion. Thus, our discoveries may be translated to the cellular and molecular dynamics orchestrating these critical pathophysiological processes.

The combination of a PI3Ka-selective inhibitor together with an endothelial-specific inducible genetic system has allowed us to fully abrogate PI3Ka activity during vessel development and unravel functions of this signalling hub. We show here that inhibition of PI3Ka hinders endothelial cell pairing and triggers defects in cell elongation. These aberrant cellular behaviours ultimately result in stretches of unicellular vascular tubes devoid of lumen. Thus, our data demonstrate that rearrangement of endothelial cells is required to form well-organized, multicellular vascular tubes. Our experimental strategies also reveal cell proliferation defects in PI3Ka-inhibited endothelial cells. Although it is possible that such defects contribute to the overall phenotype, the observation that blockade of proliferation per se does not interfere with junctional patterns argues for impaired junctional remodelling and cell motility as an independent defect in Pik3caKD/iEC retinas. Previous data showing that partial decrease in PI3K output selectively decreases endothelial cell migration with no defects in cell proliferation further support this interpretation. Pik3caKD/iEC retinas show an atypical phenotype consisting of hyperbranching but reduced numbers of endothelial cells. In contrast, canonical reduction of endothelial cell proliferation in vivo, by over-activation of Notch or depletion of VEGFR2, results in a hypobranched plexus. Our data thus highlight that increased numbers of branches is not always associated with an increase in endothelial cell proliferation as previously described. Taken together, the present data establish that PI3Ka is an important regulator of sprouting angiogenesis and confirm that angiogenic endothelial cells are exclusively regulated by this signalling hub.

Previous studies have described that PI3K signalling regulates planar cell rearrangement in epithelial cells by stimulating junctional lengthening and stability. Our data show that inactivation of PI3K signalling also impairs junctional remodelling in cultured endothelial cells and identify that this also occurs during vessel growth in vivo. Aberrant junctional patterns are indicative of defects in cell rearrangements. Therefore, this suggests that cell rearrangement defects in our PI3Ka-inhibited in vivo models are caused by impaired junctional remodelling. We also identify that the spatial heterogeneity of junctional patterns required for cells to rearrange is lost in Pik3caKD/iEC endothelial cells, with a concomitant shift towards serrated immature junctions. While current understanding of junctional patterns associates serrated junctions with highly motile cells and straight junctions with non-moving cells, our data suggest that the loss of junctional pattern heterogeneity, regardless of the type, blocks cell rearrangement. This agrees with previous results based on computational models which indicate that cells rearrange when differential adhesion strengths are found between neighbouring cells.

Mechanistically, we show that PI3Ka mediates endothelial cell rearrangement by inhibiting actomyosin contractility at the subcortical edge of the cells. This is in line with previous reports documenting that inactivation of PI3K in vitro leads to both enhanced actin contractility and aberrant actin pattern. Together, this supports the notion that actomyosin activity has to be low for cell rearrangements to occur. This is not unique of endothelial cells as it has also been observed in Drosophila epithelial tracheal tubes and in tumour cells. Upon contraction, the actomyosin machinery transmits force to cell —cell contacts and regulates junctional remodeli...
**Fig. 7** Blockade of NUAK1 restores the endothelial phenotypes imposed by PI3Kα inactivation. 

**a** Confocal images of control and Pik3caKD/ΔEC endothelial cells treated with 4-OHT for 72 h, re-plated on gelatin-coated slides for 24 h and treated with 10 μM WZ4003 (NUAK1 inhibitor; NUAKi) or DMSO as control for 10 min, and stained for pS20 MLC2, β-catenin and F-actin. 

**b** Quantification of subcortical pS20 MLC2 (upper graph) and F-actin (lower graph) immunostaining intensities (shown as integrated density) (n ≥ 14 images of three independent experiments).

**c** IB4-stained control and Pik3caKD/ΔEC retinas treated with DMSO as control or WZ4003 (NUAKi) at P6 (16:00), and isolated at P7 (1000 hours).

**d** Quantification of branch points per unit area, vessel width per unit area, and superimposed vascular tubes per unit area (n ≥ 4 retinas per genotype and treatment).

**e** Molecular mechanism by which PI3Kα suppresses actomyosin contractility. Scale bars, 15 μm (a), 100 μm (c). Data in **b** and **d** represent mean ± SEM (error bars). *P < 0.05, **P < 0.01. Statistical analysis was performed by the two-sided Mann-Whitney test.
adhesion properties and junctional growth upon new cell-cell contacts. Given the similarity between the vascular defects in Pik3caKDΔΔΔΔ retinas and GDC-0326-treated zebrafish and those observed in cdh5-mutant zebrafish embryos and mouse retinas, we propose that PI3Ka and VE-cadherin cooperate to regulate cell rearrangement and contact expansion.

We have identified a molecular pathway regulated by PI3K signalling that points towards a role of PI3K in regulating actin dynamics. Specifically, we have discovered that inactivation of PI3Ka leads to enhanced phosphorylation of MYPT1 at S445, a site which can be phosphorylated by NUAK1. Given that Akt inhibits liver kinase B1 (LKB1) through phosphorylation-dependent nuclear retention56 and LKB1 activates NUAK1, it is tempting to speculate that PI3Ka blocks the ability of LKB1 to phosphorylate NUAK1. By preventing the phosphorylation of MYPT1 on S445 by NUAK1, PI3Ka then promotes MLCP phosphatase activity (Fig. 7e). Supporting this idea, genetic endothelial depletion of LKB1 opposes Pik3caKDΔΔΔΔ vascular phenotypes, with enhanced retinal angiogenesis and increased endothelial cell proliferation and migration57. However, taking into account the complexity of the PI3K signalling cascade, other pathways may also contribute to the vascular defects seen in the Pik3caKDΔΔΔΔ retinas.

Taken together, our study discovers the PI3Ka/MYPT1/MLCP signalling axis as a crucial hub in endothelial cell rearrangement and highlights the key role of cell rearrangement in the orchestration of collective cell migration during angiogenesis. The regulation of actomyosin remodelling by PI3K signalling has been observed in a variety of primary and tumour cells; yet the molecular mechanism behind this regulation has not been fully understood. Therefore, our findings may be translated into other developmental and pathologic situations.

Methods

Reagents. All chemicals, unless otherwise stated, were from Sigma-Aldrich. Growing mediums for cultured cells were from Gibco.

Zebrafish. Maintenance of zebrafish (Danio rerio) and experimental procedures involving zebrafish embryos were carried out at the Biocentrum/Universitat Basel according to Swiss national guidelines of animal experimentation (TscVh). Zebrafish lines were maintained under licences 1014 H and 1014G1 issued by the Veterinäramt-Basel-Stadt. The fish were maintained using standard procedures and embryos obtained via natural spawning38, and embryos were staged by hours post-fertilization35 (ref. 39). All experiments were performed in accordance with federal guidelines and were approved by the Kantonales Veterinäramt Basel-Stadt. The reagents and protocol used were approved by the Ethics Committee for Animal Care (ECAC). Zebrafish were used in experiments conducted in accordance with the guidelines and laws of the Catalan Departament d’Agricultura, Ramaderia i Pesca (Catalunya, Spain) under the Project license number: MAH 8609, following protocols approved by the local Ethics Committees of IDIBELL-CEEA. All PI3Ka mutant mice and littermate controls were bred in the C57/BL6 genetic background (Supplementary Table 1). For the analysis of angiogenesis in the postnatal mouse retina, CD31-mediated recombination was induced in newborn Cre- expressing transgenic (l.p.) injections of 25 pg of 4-OHT (2.5 μl of a 10 mg ml−1 solution in absolute ethanol) at P1 and P2. Eyes were harvested at P7 and P10 for analysis. Control animals were littermate Pik3cafl/fl mice without CRE expression and injected with 4-OHT. For mosaic inactivation of PI3Ka, 2.5 μg ml−1 recombinant mouse31 was crossed with Pik3caKDΔΔΔΔ mice and 0.8 μg of 4-OHT (2.5 μl of a 0.33 mg ml−1 solution in absolute ethanol) was injected i.p. at P1, and eyes were harvested at P7. CRE-mediated recombination was assessed by the expression of membrane-bound GFP. Injected retinas from 26.3 mg ml−1 of Pik3cafl/fl mice were used as control for the analysis. The Z26·mg ml−1 allele was kept heterozygous. To block proliferation, wild-type pups were i.p. injected with 10 μg g−1 of animal of mitomycin C solution (Sigma, #M4287) as described in ref. 30 at P5. At P7, all pups were i.p. injected with Edu (Invitrogen) to assess proliferation (Edu staining protocol below). For pharmacological rescue experiment studies the following protocols were used: ROCK was inhibited in half of the pups from the same litter by ip injection of 30 mg kg−1 of animal of Y-27632 (Calbiochem, #680000) dissolved in DMSO at P6 (18:00) and P7 (10:00), and eyes were harvested at P7 (14:00). NUAK1 was inhibited in half of the pups by a single subcutaneous injection of 30 mg kg−1 of animal of WZ4003 (Selleckchem, #S7317) at P6 (16:00 hours), and eyes were harvested at P7 (10:00). Control mice were injected with DMSO only.

Cells. Mouse lungs were digested with Dispase (Life Technologies, #17105-041; 4 units ml−1) for 1 h at 37 °C, followed by positive selection with anti-mouse vascular endothelial-cadherin (Pharmingen, #555289) antibody coated with magnetic beads (Dynal Biotech, #110-35). Cells were seeded on a 12-well plate and were coated with gelatin (0.5%) in DMEM/F12 supplemented with 20% foetal calf serum and EC growth factor (PromoCell, #C30140) and 1% penicillin/streptomycin. After the first passage, the cells were re-purified with vascular endothelial-cadherin antibody-coated magnetic beads. Cells were cultured until passage 5. Human umbilical vein endothelial cells (HUVECs, Lonza, #C2519A) were cultured in EBM-2 culture medium supplemented with EGM-2 BulletKit (Lonza, #CC-3162) on 0.5% gelatin-coated coverslips and cultured up to passage 5. Human embryonic kidney cells (HEK-293, ATCC, CRL-1573) were expanded in DMEM (Lonza, #H12-733F) with 10% of inactivated FBS and 1% penicillin/streptomycin. To induce gene deletion in mouse lung endothelial cells 4-OHT (1 μM) or vehicle (ethanol) was added to the cultured medium for 24 h, followed by replacing the medium without 4-OHT or vehicle. All the experiments were performed 96 h after the addition of 4-OHT or vehicle. For pharmacological inhibition of ROCK and NUAK1 kinases, cells were cultured for 24 h followed by treatment with vehicle (DMSO), 10 μM ROCK inhibitor for 10 or 30 min, or 10 μM NUAK1 inhibitor for 10 min. For pharmacological inhibition of PI3Ka, cells were treated with vehicle (DMSO) and 1 μM GDC-0326 inhibitor (Genentech) for 48 h.

siRNA transfection. Solution A (493 μM of Opti-MEM (Gibco, #15985026) with 7.5 μg of 20 μM control (Dharmacon, #D-091206-13) or 20 μM MYPT1 (Dharmacon, #D-073772-02) SMART siRNA oligo). Embryo treatment lasted 3 h. Solution B of Opti-MEM with 7.5 μM of lipofectamine RNAi Max (Thermo Fisher Scientific, #13778075) was prepared and incubated for 5 min at room temperature (RT) in separated tubes following the manufacturer’s instructions. Subsequently, Solution B was added to solution A and incubated for 20 min at RT, followed by adding A + B to endothelial cells (750,000 cells) in suspension resuspended in 500 μl of Opti-MEM without antibiotics on coverslips in six-well plates (three coverslips per well previously coated with 0.5% gelatin). The medium was changed the day after, and cells were either fixed for immunofluorescence assays or lysed for western blotting 72 h after transfection.

Protein extraction, immunoprecipitation and immunoblotting. Zebrafish embryos were subjected to a depolymerizing protocol to avoid the interference of yolk proteins and other embryo proteins. Subsequently, whole embryo lysates were subjected to 1X SDS lysis buffer containing 10 μg ml−1 of a 1X lysis buffer filled with 1 ml depolymer buffer (55 mM NaCl, 1.8 mM KCl and 1.25 mM NaHCO3). By pipetting with a narrow tip the yolk sac was disrupted. The embryos were shaken for 5 min at 200 x g to dissolve the yolk. The cells were pelleted at 300 x g for 20 s and the supernatant discarded. Two additional washes were performed by adding 1 ml of wash buffer (110 mM NaCl, 3.5 mM KCl, 2.7 mM CaCl2, 10 mM Tris/Cl pH 8.5), shaking 2 min at 200 x g and pelleting the cells as before. For protein extraction, depolymerized zebrafish embryos, mouse lung endothelial cells and HEK-293 cells were lysed in 50 mM Tris HCl pH 7.4, 5 mM EDTA, 150 mM NaCl and 1% Triton X-100 supplemented with 2 mg ml−1 aprotinin, 1 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM phenylmethyl sulfonyl fluoride, 10 g ml−1 Na2-tosyl-yl-lysine chloromethyl ketone hydrochloride, 1 mM sodium orthovanadate, 1 mM okadaic acid and 1 mM DTT followed by
cleavage of lysates by centrifugation. Supernatants were resolved on 8, 10 or 12% SDS–PAGE gels, transferred onto nitrocellulose or PVDF membranes. Membranes were blocked in 5% non-fat dry milk in PBS for 60 min at room temperature (RT), and probed with specific primary antibodies overnight at 4 °C in 1% BSA in 0.1% Tween 20 buffer (referred to as ‘TBST’, then washed three times with TBST and incubated with peroxidase-conjugated secondary antibodies in 5% (v/v) skimmed milk in TBST at RT for 1 h. The following primary antibodies were used: p-AKT (Ser 473) (Cell Signaling Technology, #9272, diluted 1:1000), AKT (Cell Signaling Technology, #9272, diluted 1:1000), ACT (Cell Signaling Technology, #2603, diluted 1:1000), pS19/S20 MLC2 (Rockland Antibodies, #039600-401-416, diluted 1:100) and p38 (Millipore, #AB234, diluted 1:2000), PDK3a (monoclonal clone U3A4(4)), Ser445 MYPT1 (MRC Reagents, #55802C, 1 μg ml−1), MYPT1 (MRC Reagents, #S110D, ref.35,1 μg ml−1), phophoshamphophosphate variant of the antigen used to raise the antibody), pS19/S20 MLC2 (Rockland Antibodies, #039600-401-416, diluted 1:500), β-actin (Abcam, #ab94900, diluted 1:10 000) and α-tubulin (Sigma-Aldrich, #T6764, diluted 1:10 000). The following secondary antibodies were DAKO were used in a 1:5000 dilution: swine anti-rabbit (PO4399, rabbit anti-goat (PO449), rabbit anti-mouse (PO2600), and rabbit anti-sheep (PO3353)).

MYPT1 immunoprecipitation (IP) was performed using a sheep polyclonal MYPT1 antibody (MRC Reagents, #S110D) covalently coupled to protein G-Sepharose (GE Healthcare, #17-0618-01) (1 μg of antibody per 1 μl of beads) with a dimethyl pimelidimide cross-linking procedure(3). Cells treated with DMSO or GDC-0326 for 48 h were lysed with IP buffer (50 mM Tris pH 8.0, 150 mM NaCl, 0.1% SDS, 1% NP40, 0.5% sodium deoxycholate, 2 μg ml−1 aprotinin, 1 mM pepstatin A, 1 ng ml−1 leupeptin, 10 μg ml−1 TLCK, 1 mM PMSF, 1 mM NaF, 1 mM NaVO4, and 1 μM okadaic acid) and clarified by centrifugation at maximum speed for 15 min at 4 °C. Cell lysate (1 mg) was incubated with 5 μg of coupled antibody for 1 h at RT. Immunoprecipitates were washed three times with IP buffer and resuspended in 1× SDS Sample Buffer. Immunoprecipitates and cell lysates (50 μg) were subjected to electrophoresis on 8–12% SDS–PAGE and transferred to nitrocellulose membranes. For sheep antibodies, the membranes were incubated for 30 min with TBST containing 10% (w/v) skimmed milk. The membranes were then incubated for 1 h in TBST containing 0.1% (w/v) skimmed milk followed by overnight incubation at 4 °C with the MYPT1 primary antibody (1 μg ml−1) overnight at 4 °C. The incubation with phosphospecific MYPT1 sheep antibody was performed with the addition of 10 μg ml−1 of a dephosphopeptide variant of the antigen used to raise the antibody. Uncropped scans are shown in Supplementary Figures 11–13.

Live 2D wound healing assay. Mouse lung endothelial cells and HUVECs were plated on six-well plates and grown for the gelatin to grow to confluence for 24 h. Cell monolayers were scratched with a p200 pipette tip to induce cell migration. Phase-contrast images were performed every 10 min using a widefield microscope (Nikon Eclipse Ti) equipped with a ×10air objective and an Andor Zyla 4.2 plus sCMOS camera. An Okolab cage incubator and humidified CO2 gas chamber set to 37 °C and 5% CO2 were used during the imaging process.

Immunofluorescence analysis in zebrafish, retinas and cells. For immunofluorescence and imaging of zebrafish embryos, dechorionated zebrafish embryos were fixed in 2% paraformaldehyde and 0.1% Tween 20 in PBS overnight at 4 °C. After fixation, embryos were washed four times with 0.1% Tween 20 in PBS (hereafter referred to as ‘IPST’), permeabilized with 0.3% Triton-X 100 in PBS at room temperature (RT) for 15–30 min, and blocked with 1% BSA, 5% goat serum, 0.2% Triton-X 100 in PBS at RT for 30 min. Embryos were then incubated with the primary antibody overnight at 4 °C followed by continuous shaking. Thereafter, embryos were incubated with mouse anti-human ZO-1 (Zymed, #33-9911, diluted 1:200) in 500 μl of blocking buffer at 4 °C overnight, washed in PBST at least six times for over 3 h, and incubated with Alexa-633 goat anti-mouse IgG (Invitrogen, #A21236, diluted 1:200) and Alexa-Fluor 633-conjugated phalloidin (Invitrogen, #A12380, diluted 1:400) for 1 h at RT. Colour washes with PBS were performed, adding 1 μg ml−1 of 4′,6-diamidino-2-phenylindole (DAPI; Molecular Probes, #D1306) in the last one.

Images were taken at ×40 (NA = 1.1) water immersion objective, maximum intensity projections were used for quantification and five ISVs were quantified per embryo. Measurements of ISVs length were made straight from the edge of the aorta to the leading edge of the sprout and the number of endothelial nuclei in that length was quantified. The number of endothelial nuclei in the DLAV and DA was quantified per segment within five ISVs. Junctional length and junctional gaps length were measured in embryos stained for ZO-1. Only flat-mounted embryos were selected for quantification avoiding crooked ones. ZO-1 intensity, vascular parameter were quantified in at least four images of comparable vascular areas per retina and of at least three mice of each genotype or experimental conditions. All images shown in the figures are maximum intensity projections unless otherwise specified. For the quantification of retinal vessel progression overview, widefield images of IB4-stained retinal vasculature were used. The width of the x10 objective of the Nikon Eclipse 80i microscope of vessel growth from the centre of the optic nerve to the edge of the angiogenic front was measured per retina leaftlet. The mean of all leaftlet measurements was obtained per retina and compared between control and mutant groups. For all other quantifications high-resolution confocal images at ×40 oil immersion objective were used. The number of filopodia and sprouts were quantified at the angiogenic front. The total number of filopodia and sprouts were normalized to a vessel length of 100 μm at the angiogenic front. Endothelial branch points, vessel width, superimposed vascular tubes, number of junctional gaps, length of junctional gaps, distance between nuclei, endothelial cell numbers, collagen IV empty sleeves and lumen disconnections were quantified behind the angiogenic front in fields sized 100 μm × 100 μm. For quantification of cell shape, single GFP-positive cells located behind the sprouting front were considered. Distance width, distance between neighbouring cells, length of junctional gaps and area of GFP-positive cells was determined using ImageJ software with the proper scale set (4). Description of cell shape, single GFP-positive cells located behind the sprouting front was considered as a region of interest (ROI). Integrated density of S20 MLC2 or F-actin was measured in the IB4-positive area for each image. Then, to calculate the corrected total fluorescence (CTF), the following formula was used: CTF = Integrated Density − (Area selected × Mean fluorescence of background readings). The background readings were taken from three areas close to the vasculature but negative for IB4. 3D reconstructions were generated by using the Leica LAS-X.

In phase-contrast wound healing assays, quantification of cell migration was made by measuring the percentage of cell-free area. Cells from the first, second and third fields of view were manually identified using the blind counting tool in plugin and cell velocity, directionality and travelled distance were calculated with the chemotaxis tool plugin.
In cultured endothelial cells, maximum intensity projections of confocal images with the x63 oil immersion objective were acquired. Signal intensity of pS20 MLC2 and F-actin immunostaining/cell was quantified using the junctional staining of β-catenin to select individual endothelial cells (ROI A1). Using the enlarge command two concentric areas were drawn in each cell (distance of ~2.5 μm (ROI A2) and ~7.5 μm (ROI A3) from the junctional β-catenin positive staining). Integrated density was measured. For measuring total intensity levels, area from ROIs A1 was used as templates to measure total integrate intensity of pS20 MLC2 and F-actin staining in individual endothelial cells. For measuring subcortical levels: Integrate density from ROIs A2 to ROI A3 was calculated in individual endothelial cells. The mean of the integrate density of four cells per image, and at least four images per genotype/treatment were used for the quantification of β-catenin positive area. Junctional staining of β-catenin was used to measure the perimeter of individual cells. At least four cells per image and four images per genotype were used for the quantification. To measure the type of junctional coverage, percentage of junctional pattern was considered. ≥60% saturated pattern/cell = saturated, ≥ 60% of straight junctional pattern/cell = straight, ≤50% of each pattern/cell = mixed. Five cells per image and at least five images per genotype were used for the quantification.

**Peptide identification and quantification.** Mascot Daemon 2.5.0 was used to automate peptide identification from MS data. Peak list files (MGF files) from RAW data were generated with Mascot Distiller v2.5.1.0 and searched into the Mascot search engine (v2.5) in order to match MS/MS data to theoretical peptide fragmentation data26. The searches were performed against the SwissProt Database (uniprot_sprot_2014_08.fasta) with an FDR of 1% (specific FDR for each phosphopeptide identification is included in Supplementary Data 1). A maximum of two trypsin allowed cleavages and a mass tolerance of ±10 ppm for the MS scans and ± 25 ppm for the MS/MS scans were allowed. Carbamidomethyl Cys as fixed modification, and phosphorylation at Ser, Thr, and Tyr, PyroGlu on N-terminal Gln and oxidation of Met as variable modifications were considered. The accuracy of phosphosite location within the identified peptides was assessed using delta score values as described by Savitski et al.27. Delta scores for each phosphopeptide are reported in Supplementary Data 1. In-house developed software (Pascal) was used for label-free peptide quantification. Pescal constructs extracted ion chromatograms (XIC) for each identified peptide and measures the area of the XICs for all the peptides identified across all samples. Thresholds for XIC generation was set to ≥7 ppm and 20 min ms/retention time windows, respectively and undetectable peptides were given an intensity value of 0. Values of two technical replicates per sample were averaged and intensity values for each peptide were normalized to total sample intensity. Normalized quantitative data were used to calculate fold changes between groups and statistical significance (assessed by Student’s t test) when necessary. The construction of volcano plots and heatmaps was automated with software generated in R software using the ggplot package. Venn diagrams were constructed using the software Venny (v2.1, http://bioinfogp.cnb.csic.es/tools/venny/).

### Statistics

Data were analysed using GraphPad Prism software and were presented as mean ± SEM (error bars). Sample size and experimental replicates were indicated in the figure legends. Statistical analyses were performed using the non-parametric Mann–Whitney’s test when necessary. The α value was set to 0.05. Significant differences were indicated by P values. Data were regarded as significantly different at P ≤ 0.001.

**Code availability**

The mass spectrometry data is deposited in the PRIDE repository (www.ebi.ac.uk/pride/archive) with the dataset identifier PXD007060. The code used for analysing the mass spectrometry data are annotated in Supplementary Table 2.

### Reporting Summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

The mass spectrometry data that support the findings of this study have been deposited and are publicly available at the ProteomeXchange Consortium via the PRIDE repository (www.ebi.ac.uk/pride/archive) with the dataset identifier PXD007060. The rest of the data and materials generated within this study are available from the corresponding author upon request. A reporting summary for this article is available as a Supplementary Information file.

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Author contributions
A.A.-U., P.R.C. and M.G. conceived the project; A.A.-U., P. Casado, S.D.C., P.K., M.P.K., A.M.F., P. Castel, V.R., M.M.-G., C.W., H.S., and L.M. performed experiments and analyzed data with the supervision of J.M., O.C., F.V., M.A., H.G., S.H., H.-G.B., P.R.C. and M.G. A.A.-U., S.D.C. and M.G. wrote the manuscript. J.M., O.C., F.V., M.A., H.G., S.H., H.-G. B., P.R.C. and M.G. provided funding.

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