YAP/TAZ-CDC42 Signaling Regulates Vascular Tip Cell Migration

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Angiogenesis and vascular remodeling are essential for the establishment of vascular networks during organogenesis. Here we show that the Hippo signaling pathway effectors YAP and TAZ are required, in a gene dosage-dependent manner, for the proliferation and migration of vascular endothelial cells (VECs) during retinal angiogenesis. Intriguingly, nuclear translocation of YAP and TAZ induced by *Lats1/2*-deletion blocked endothelial migration and phenocopied *Yap/Taz*-deficient mutants. Furthermore, overexpression of a cytoplasmic form of YAP (YAPS127D) partially rescued the migration defects caused by loss of YAP and TAZ function. Finally, we found that cytoplasmic YAP positively regulated the activity of the small GTPase CDC42, deletion of which caused severe defects in endothelial migration. These findings uncover a previously unrecognized role of cytoplasmic YAP/TAZ in promoting cell migration by activating CDC42 and provide new insight into how Hippo signaling in endothelial cells regulates angiogenesis.

Hippo signaling | angiogenesis | cell migration | CDC42

Introduction

Angiogenesis is a process of growth and remodeling in vascular networks that is essential for normal development. In adulthood, angiogenesis is activated as a reparative process, for example, during wound healing (1, 2). Aberrantly regulated angiogenesis can also be a component of disease (3) and can play a key role in tumor growth and metastasis (4), inflammatory diseases (5), diabetic retinopathy, and retinopathy of prematurity (6).

Retinal angiogenesis in mice begins at postnatal day 0 (P0). The retinal vasculature initiates its expansion from the optic nerve head and migrates outwards along a preexisting network of astrocytes (7, 8). This results in the formation of the superficial vascular plexus within the retinal ganglion cell layer during the first 8 days (9, 10). Endothelial cells then migrate along nerve fibers to establish deep and intermediate vascular layers (9, 11). Cell proliferation and migration are essential for angiogenesis and these cell responses are regulated by many different signaling pathways, including the VEGF, Notch, Wnt, FGF, BMP, and integrin signaling responses (9, 12-16). VEGFA and CDC42 are known to regulate extension of the angiogenic front and filopodia formation in angiogenic tip cells (2, 17, 18).

The Hippo signaling pathway is an evolutionarily conserved, pivotal regulator of cell proliferation and organogenesis. YAP and TAZ are key components of the Hippo signaling pathway and function as transcription cofactors that regulate downstream gene expression via association with DNA binding proteins such as TEAD1-4 (19, 20). YAP and TAZ can drive the expression of genes that regulate cell proliferation and survival (*diap1*, *bantam*, *cyclin E,* and *E2F1*), the Hippo pathway (*Kibra*, *Crb*, and *Fj*), and cell-cell interaction (*E-Cadherin*, *Serrate*, *Wingless*, and *Vein*)(20). The activity of YAP and TAZ is regulated by the LATS1 and LATS2 kinases. These kinases phosphorylate YAP and TAZ, thus preventing their nuclear translocation and regulating transcriptional activity. Although the function of YAP and TAZ in the nucleus has been subject to extensive studies (20, 21), the role of these proteins in the cytoplasm is not fully understood.

In the present study, we used the mouse postnatal retina as a model for investigating the function of YAP and TAZ during angiogenesis. We show that YAP and TAZ are required for vascular network formation by regulating endothelial cell proliferation and migration and that the influence of YAP and TAZ on angiogenesis is gene dosage-dependent. Importantly, we show that cytoplasmic YAP, but not the nuclear form, is crucial for modulating endothelial cell migration by regulating the Rho family GTPase CDC42 activity. These findings identify a previously unrecognized role of cytoplasmic YAP in regulating angiogenesis via CDC42.

Results

YAP and TAZ are required for vascular development in the retina

We examined the expression of YAP in retinal endothelial cells. YAP was detected mainly in the cytoplasm in most retinal VECs (Fig. S1A-D) and in both the nucleus and cytoplasm in some VECs (Fig. S1C and D). Whole mount retina staining also showed that YAP was mainly localized in the cytoplasm in both

Significance

New blood vessel formation is a physiological process seen in development, as well as in wound healing and tumorigenesis. Although the process of blood vasculature formation has been well documented, little is known about the molecular mechanisms that regulate endothelial migration during vascular network formation. In this study, we identified a critical role for Hippo effectors YAP and TAZ in the regulation of vascular network remodeling through controlling endothelial cell proliferation, filopodia formation, and cell migration. We found a striking cytoplasmic function of YAP in the regulation of endothelial cell migration through controlling the Rho family GTPase CDC42 activity. These findings identify a previously unrecognized YAP/TAZ function involved in the vascular network remodeling during angiogenesis.

Reserved for Publication Footnotes

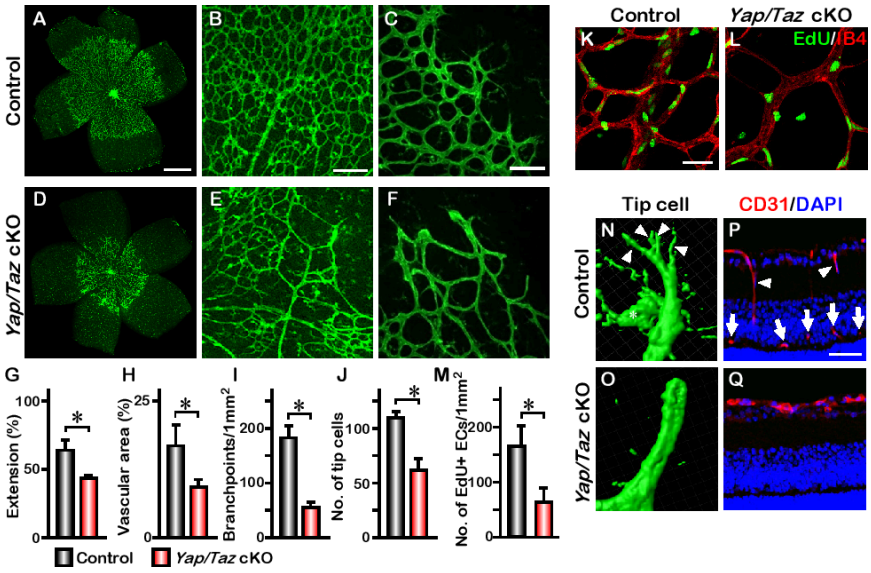

Fig. 1. YAP/TAZ regulate vascular endothelial migration in the developing retina. (A-F) IB4 labeling of P5 retina vasculature from littermate *Yap*$^{flox/flox}$; *Taz*$^{flox/flox}$ mice with *Pdgfb-iCreERT2* (*Yap/Taz* cKO) (D) or without Cre (Control) (A). Higher-magnification images of the vascular plexus and front are shown in (B, C, E, and F). (G-J) Quantification of the vascular extension (n=6), vascular area (n=3), number of branchpoints (n=6), and tip cells (n=4); mean ± SD, *P<0.01. (K-M) Whole-mount EdU staining of P5 Control and *Yap/Taz* cKO retina. Statistical analysis of the number of EdU-positive cells is shown in (M) (n=4); mean ± SD, *P<0.01. (N and O) Imaris image analysis of P5 retina tip cells. Arrowheads and asterisk in (N) indicate filopodia and a macrophage respectively. (P and Q) Immunohistochemistry of retinal sections of P11 eyes. Control retina shows some migrating endothelial cells (arrowheads) and the deep vascular plexus (arrows). Scale bars represent 500 μm (A), 200 μm (B), 100μm (C), and 50 μm (K and P).

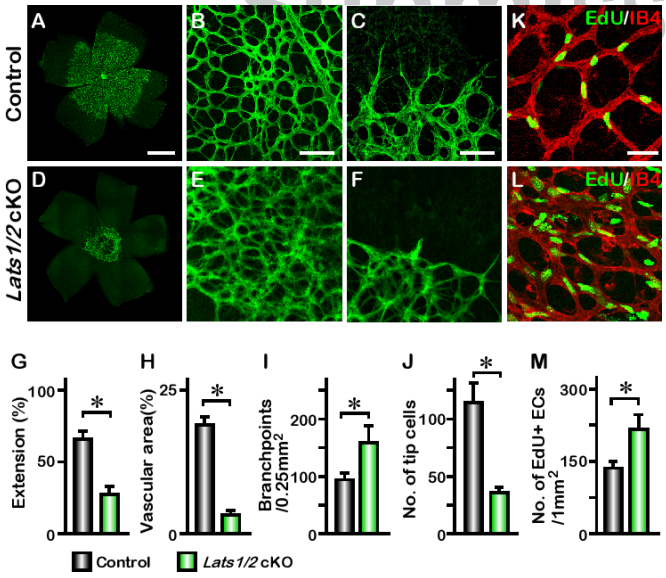

Fig. 2. Deletion of *Lats1/2* disrupts retinal vascular extension and filopodia formation. (A-F) IB4 labeling of P5 retina vasculature from Control and *Lats1*$^{flox/flox}$; *Lats2*$^{flox/flox}$;*Pdgfb-iCreERT2* (*Lats1/2* cKO) neonates. Higher-magnification images of the vascular plexus and front are shown in (B, C, E, and F). (G-J) Quantification of vascular extension (n=6) , vascular area (n=4), number of branchpoints (n=4), and tip cells (n=4); mean ± SD, *P<0.01. (K-M) Whole-mount EdU staining of P5 Control and *Lats1/2* cKO retina. Statistical analysis of the number of EdU-positive cells is shown in (M) (n=4); mean ± SD, *P<0.01. Scale bars represent 500 μm (A), 200 μm (B), 100 μm (C), and 50 μm (K).

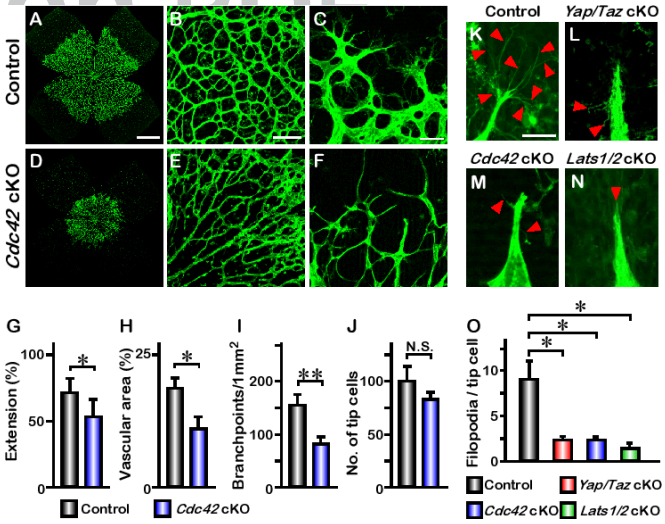

Fig. 3. Phenocopy of *Cdc42* cKO filopodial loss in *Yap/Taz* and *Lats1/2* cKO. (A-F) Whole mount IB4 staining of P5 retina from Control (A) and *Cdc42*$^{flox/flox}$; *Pdgfb-iCreERT2* (*Cdc42* cKO) neonates (D). Higher-magnification images of the vascular plexus and front are shown in (B, C, E, and F). (G-J) Quantification of vascular extension (n=4), vascular area (n=3), number of branchpoints (n=6), and tip cells (n=3); mean ± SD, *P<0.05, **P<0.01, N.S.; not significant. (K-N) Comparison of filopodia formation of each genotype. Tip cells are labeled by IB4. Red arrowheads indicate filopodia. (O) Quantification of the number of filopodia per tip cell (n=4); mean ± SD, *P<0.01. Scale bars represent 500μm (A), 100 μm (B), 50 μm (C), and 10 μm (K).

the migrating tip cells and the central region of retinal vessels (Fig. S1E). To determine the function of YAP in retinal VECs, we bred the conditional *Yap*$^{flox/flox}$ allele with the *Pdgfb-iCreERT2* mouse line to delete *Yap* in endothelial cells in a temporally regulated manner. The expression of *Pdgfb-iCreERT2* in the developing retinal VECs was confirmed by breeding with *Rosa26-Loxp-STOP-Loxp-tdTomato* reporter mice (Fig. S2) (22). Upon tamoxifen treatment from P1 to P3, *Yap*$^{flox/flox}$; *Pdgfb-iCreERT2* (referred to as *Yap* cKO) mice did not show overt abnormalities when examined at P5 (Fig. S3). To investigate whether the lack of phenotype in *Yap* cKO is due to a redundant function with TAZ (homolog of YAP in mammals), we generated endothelial-specific *Taz* knockout mice, *Taz*$^{flox/flox}$; *Pdgfb-iCreERT2* (referred to as *Taz* cKO). Similar to the *Yap* cKO mice, the *Taz* cKO mice appeared normal without an obvious vascular phenotype (Fig. S3). However, the deletion of both *Yap* alleles and one allele of *Taz*, *Yap*$^{flox/flox}$; *Taz*$^{wt/flox}$; *Pdgfb-iCreERT2,* (referred to as *Yap* cKO; *Taz* cHet) led to reduced vascular density (Fig. S3) and decreased extension of the retinal vascular field (vascular extension) (Fig. S3). Furthermore, deletion of both alleles of *Yap* and *Taz* in endothelial cells, *Yap*$^{flox/flox}$; *Taz*$^{flox/flox}$; *Pdgfb-iCreERT2* (referred to as *Yap*/*Taz* cKO), caused a severe vascular phenotype with prominently impaired retinal vessel sprouting, vascular area, and

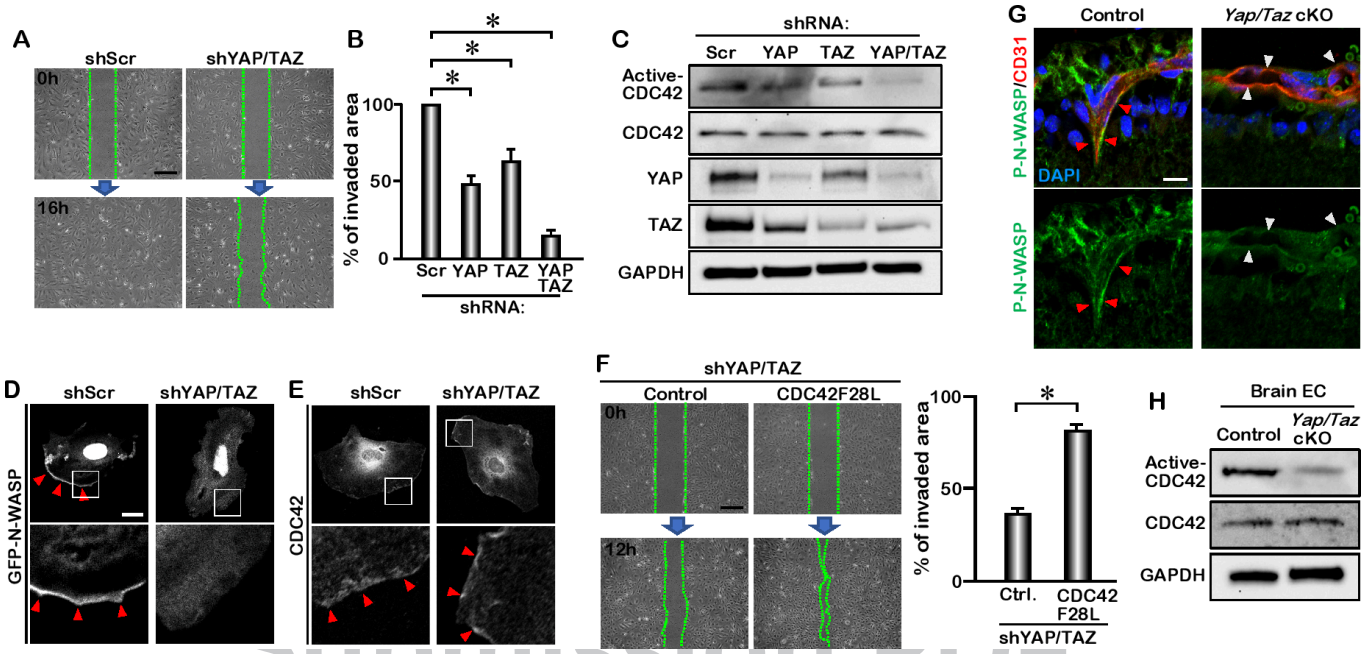

Fig. 4. Downregulation of CDC42 activity in *YAP/TAZ* deficient endothelial cells. (A) Scratch assay of Scrambled-shRNA (shScr) and YAP/TAZ-shRNA (shYAP/TAZ) infected HUVECs. Images are taken at 0 and 16 hours after cell scratch. (B) Quantification of invaded area within the scratched region of *YAP/TAZ* knockdown HUVEC (n=3); mean ± SD, *P<0.01. (C) Active-CDC42 pull-down assay and western blot analysis of *YAP/TAZ* knockdown HUVECs. (D-E) GFP-N-WASP expression (D) and endogenous CDC42 expression (E) in *YAP/TAZ* knockdown HUVECs. The boxed areas are enlarged on the lower panels. Arrowheads indicate GFP-N-WASP and endogenous CDC42 expression at the edge of the cell. (F) Scratch assay of *YAP/TAZ* knockdown HUVECs expressing CDC42F28L. Images are taken at 0 and 12 hours after the scratch. Quantification of invaded area within the scratched region (n=3); mean ± SD, *P<0.01. (G) Phosphorylated N-WASP expression in the retinal VECs of P11 eyes. Arrows indicate the VECs. (H) Active-CDC42 pull-down assay of P5 brain VECs. Scale bars represent 50μm (A and F) and 10 μm (D).

reduced number of vascular branches (Fig. 1A-I and Fig. S3). This severe vascular phenotype persisted until later developmental stages (Fig. S4), indicating that *Yap* and *Taz* are required for vessel morphogenesis in a gene dose-dependent manner. Quantitative PCR (Q-PCR) on RNA isolated from the brain VECs of *Yap*/*Taz* cKO mice confirmed a significantly lower level of each transcript as well as the expression of YAP target genes, *Ctgf* and *Cyr61* (Fig. S5). Severe reduction of vascular density in *Yap*/*Taz* cKO mutants led us to investigate the possibility that endothelial cell proliferation was affected. 5-ethynyl-2'-deoxyuridine (EdU) was delivered to P4 pups via intraperitoneal injection 16 hours before the analysis. We found that the number of proliferating endothelial cells was greatly reduced in the *Yap*/*Taz* cKO retinas compared with the littermate controls (Fig. 1K-M), suggesting that YAP and TAZ are required for endothelial cell proliferation during angiogenesis.

Angiogenic sprouting is promoted by active filopodial protrusions and tip cell migration (23). To determine whether the vascular defect in *Yap*/*Taz* cKO mice involves tip cell migration, we examined the abundance and morphology of tip cells. The number of tip cells was significantly reduced in *Yap*/*Taz* cKO mice (Fig. 1C, F, J and S3E). Furthermore, tip cells in the double mutant mice exhibited only a few filopodia extending from vessel termini (Fig. 1N and O). The reduced vascular extension and the morphology of the tip cells in *Yap*/*Taz* cKO mice led us to investigate whether YAP and TAZ are necessary for VEC migration. During retinal angiogenesis, vasculature expands from the optic stalk at P1 and reaches the periphery by about P8 (24). VECs then migrate downwards into the regions where neurons reside to form the deep and intermediate vascular plexus by 3 weeks of age. P11 retina sections showed that there were some migrating endothelial cells and an intermediate vascular plexus in the control, but not in the *Yap*/*Taz* cKO retinas (Fig. 1P and Q). Whole mount CD31 staining at P13 also indicated that endothelial specific deletion of *Yap* and *Taz* prevented the migration that forms the deep and intermediate vascular layers (Supplemental Movies). These data suggest that YAP and TAZ are required for endothelial cell proliferation and migration during vascular development.

Deletion of the upstream *Lats1*/2 results in cell migration defect

To investigate whether other components of the Hippo signaling pathway are involved in regulating cell proliferation and migration, we deleted the upstream kinases *Lats1*/2 by breeding *Lats1*$^{flox/flox}$; *Lats2*$^{flox/flox}$ mice with *Pdgfb-iCreERT2* to generate *Lats1*$^{flox/flox}$; *Lats2*$^{flox/flox}$; *Pdgfb-iCreERT2* (referred to as *Lats1*/2 cKO). This eliminates LATS-dependent phosphorylation of YAP and TAZ in endothelial cells and prevents their phosphorylation-dependent sequestration in the cytoplasm (25, 26). The *Lats1*/2 cKO retinas exhibited a migration defect with reduced extension distance compared with the control mice (Fig. 2A, D, and G). The angiogenic network in *Lats1*/2 cKO mice also displayed hyperplasia with increased vascular complexity evident by a 60% increase in branchpoints and reduced vascular area (Fig. 2B, E, H, and I). The proliferation rate of VECs was significantly increased whereas vascular area was reduced in *Lats1*/2 cKO retina (Fig. 2K-M) and expression of YAP target genes was significantly increased in *Lats1*/2 cKO endothelial cells (Fig. S5). These results contrasted with the proliferation phenotype and gene expression in the *Yap*/*Taz cKO* retina (Fig. 1K-M and S5). Although other effectors might be affected in the *Lats1*/2 cKO mice, these data suggest that nuclear YAP/TAZ might be mainly required for VECs proliferation, but not for cell migration.

Loss of CDC42 caused abnormal vessel morphology and migration defect

The Rho GTPase CDC42 has been shown to be required for blood vessel formation during vasculogenesis by promoting filopodia formation in endothelial tip cells (17, 18, 27, 28). To

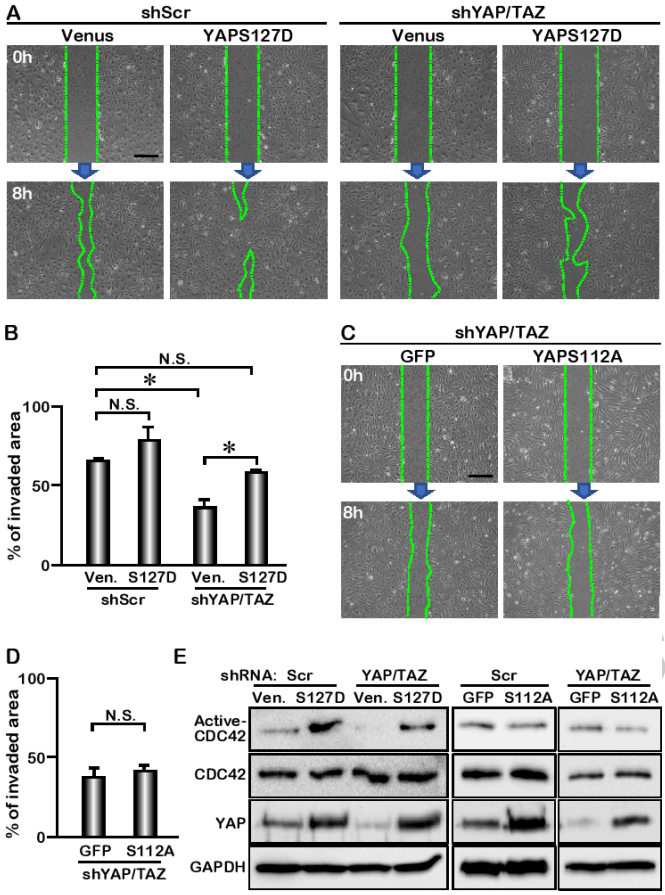

Fig. 5. Cytoplasmic YAP regulates CDC42 activity in HUVEC. (A) Scratch assay of *YAP/TAZ* knockdown HUVECs expressing Venus or YAPS127D. (B) Quantification of invaded area within the scratched region (n=3); mean ± SD, *P<0.01, N.S.; not significant, Ven,; Venous, S127D; YAPS127D. (C) Scratch assay of *YAP/TAZ* knockdown HUVECs expressing YAPS112A. (D) Quantification of invaded area within the scratched region (n=3); mean ± SD, N.S.; not significant, S112A; YAPS112A. (E) Active-CDC42 pull-down assay of YAPS127D-lentivirus-infected (S127D) and YAPS112A-adenovirus-infected (S112A) HUVECs. CDC42, YAP, and GAPDH expression levels are detected by western blot analysis. Scale bars represent 50 μm (A and C).

confirm the activity of CDC42 in the formation of filopodia, we combined the *Cdc42*$^{flox/flox}$ allele (29) with *Pdgfb-iCreERT2* mice to generate an endothelial specific deletion of *Cdc42*, *Cdc42*$^{flox/flox}$; *Pdgfb-iCreERT2* (referred to as *Cdc42* cKO). *Cdc42* cKO retinas exhibited reduced radical extension of vasculature at P5 (Fig 3A, D, and G). The vascular density was also reduced in *Cdc42* cKO mice (Fig. 3B, E, and I). The number of tip cells at the sprouting front did not show a significant difference between *Cdc42* cKO and the littermate controls (Fig. 3C, F and J).

Cdc42 cKO retina tip cells had few filopodia (Fig. 3M), and lacking of filopodia in tip cells was also observed in *Yap*/*Taz* cKO and *Lats1*/*2* cKO retinas (Fig. 3L, N). Quantitative analysis showed a significant decrease in filopodia density in these mice (Fig. 3O). The converging phenotype of endothelial specific deletion of *Yap*/*Taz*, *Cdc42*, or *Lats1*/*2* in the filopodia-mediated vascular sprouting and branching in the retina suggests that these molecules might operate in a common pathway in angiogenic tip cell development.

YAP and TAZ regulate CDC42 activity in migrating endothelial cells

The similar tip cell phenotype in *Yap*/*Taz* cKO and *Cdc42* cKO mice lead us to examine how YAP/TAZ regulates endothelial cell migration and whether YAP and TAZ regulate CDC42 activity and its cellular localization in endothelial cells. We depleted YAP and TAZ *in vitro* in human umbilical vein endothelial cells (HUVECs) using lentiviruses expressing short hairpin RNAs targeting human *YAP* and *TAZ*, and assessed HUVECs migration by a wound healing scratch assay. To distinguish the effect of proliferation from cell migration, HUVECs were treated with hydroxyurea for 4 hours before the migration assay. Quantification analysis of the invaded area 16 hours after the scratch demonstrated that knocking down *YAP* and *TAZ* in HUVECs inhibited cell migration (Fig. 4A and B). The cell migration defect was exacerbated when both *YAP* and *TAZ* were knocked down, consistent with the mouse *in vivo* data showing that YAP and TAZ are required for cell migration and that this requirement is gene dosage-dependent.

We found that, while the total CDC42 level did not change, the level of active CDC42 was greatly reduced upon knockdown of *YAP* and *TAZ* (Fig. 4C). For better visualization of active CDC42 in a single cell, we transfected HUVECs with a GFP-tagged CDC42/RAC interactive binding domain of neural Wiskott Aldrich syndrome protein (GFP-N-WASP)(30), which binds to endogenous active CDC42. The active CDC42 was located at the lamellipodial edge of the control HUVECs (Fig. 4D). In *YAP*/*TAZ* knockdown HUVECs, only active CDC42 was diminished in the protruding edge (Fig. 4D), while CDC42 localization was not disrupted (Fig. 4E), suggesting that YAP and TAZ regulate CDC42 activation rather than its cellular localization in HUVECs. The migration defect in *YAP*/*TAZ* knockdown HUVECs can be rescued by a constitutively active form of CDC42 (CDC42F28L) (Fig. 4F), which is capable of spontaneously exchanging GDP for GTP(31, 32), suggesting that YAP/TAZ regulation of the HUVEC migration at least in part channels through CDC42 activity. To confirm the effect of YAP/TAZ *in vivo*, we examined the expression of phosphorylated-N-WASP, an effector of CDC42 (33), in the developing mouse retinal vasculature. Phosphorylated-N-WASP was detected in the migrating endothelial cells in control retinas at P11; however, the level of phosphorylated-N-WASP was greatly reduced in the *Yap*/*Taz* cKO retinal VECs (Fig. 4G). These data suggest that YAP/TAZ regulate cell migration through activating CDC42 mediated N-WASP pathway *in vivo.* Moreover, CDC42 activity was down regulated in the brain endothelial cells from *Yap*/*Taz* cKO mice. (Fig. 4H). Collectively, these observations indicate that the endothelial migration defect in the *Yap*/*Taz* cKO retinas is at least partially due to the downregulation of CDC42 activity.

Cytoplasmic YAP promotes endothelial cell migration

YAP is a mechanical sensor whose cellular localization changes in response to various environmental stimuli including cell-cell interaction and alterations of cytoskeletal dynamics (34, 35). We assessed whether YAP cellular localization affected CDC42 activity. When HUVECs were at low density, YAP was localized in the nucleus and translocated to the cytoplasm when cells reached confluency (Fig. S6A). Consequently, the level of phosphorylated YAP was greatly upregulated in the over-confluent cells (Fig. S6B). The active CDC42 level also increased dramatically in the over-confluent cells compared with the cells at low density (Fig. S6B). In the wound scratch assay on over-confluent HUVECs, YAP remained in the cytoplasm while the cells migrated (Fig. S6C), suggesting a pro-migratory role of the cytoplasmic YAP. The decrease of CDC42 activity in *LATS1*/*2* knockdown HUVEC and *Lats1*/*2* cKO brain endothelial cells (Fig. S6D and E) further supports the hypothesis that the cytoplasmic YAP regulates the migration of endothelial cells.

To further investigate whether cytoplasmic YAP promotes cell migration and activates CDC42, we transduced HUVECs with a lentivirus expressing YAPS127D. Substitution of Ser127 with Asp (S127D) generates a YAP protein that is sequestered in the cytoplasm mimicking phospho-YAP (Fig. S7). HUVECs

treated with lenti-YAPS127D migrated with a trend faster than cells treated with the control lentivirus (Venus), although the difference in migration did not reach significance (Fig. 5A and B). YAPS127D did partially rescue the migration defects caused by shRNA-mediated *YAP/TAZ* knockdown, suggesting that phospho-YAP promotes endothelial cell migration (Fig. 5A and B). We further examined the effect of nuclear YAP, using a constitutively active form of nuclear YAP (YAPS112A, in which Serine 112 is mutated to Alanine) in retinal angiogenesis, by breeding *Pdgfb-iCreERT2* with transgenic mice under the control of *CAG-LoxP-CAT-Stop-Loxp* cassette. No substantial effect on retinal angiogenesis was detected in Tg-YAPS112A(Fig. S8), suggesting that nuclear YAPS112A overexpression does not alter neovasculature formation in the retina. Unlike the *Lats1/2* cKO phenotype, VEC proliferation was not upregulated by YAPS112A although YAP targets genes (*Ctgf* and *Cyr61*) were upregulated in VECs (Fig. S8). Notably, YAPS112A did not rescue the migration defect in *YAP/TAZ* knockdown HUVECs (Fig. 5C and D). To examine whether this pro-migration function of YAP is through activation of CDC42, we overexpressed YAPS127D in HUVECs and found that the level of active CDC42 was greatly increased (Fig. 5E). The reduction of active CDC42 with shRNA-mediated *YAP/TAZ* knockdown was also rescued by YAPS127D, but not by YAPS112A expression (Fig. 5E). These results indicate an important role of cytoplasmic YAP in promoting cell migration by activating CDC42 (Fig. S9). The partial rescue of the cell migration and CDC42 activity with YAPS127D could be due to the fact that only the phospho-YAP mimic is overexpressed in the HUVECs in which both YAP and TAZ are knocked down. Although YAP and TAZ play redundant roles in regulating retinal angiogenesis, they may have distinct functions in interacting with different proteins in the cytoplasm to regulate cell migration.

Discussion

Angiogenesis is a highly regulated process. This reflects the potentially detrimental consequences of a deficiency or an excess of blood vessels. The Hippo signaling pathway has been implicated in vascular development (36-38) but the underlying mechanisms have not been fully described. In this study, we found that the cytoplasmically localized phospho-YAP, which is not involved in transcription, plays an important role in promoting cell migration via activating CDC42.

Cell autonomous function of YAP/TAZ vascular development *in vivo*. A migration defect in epithelial-mesenchymal transition (EMT) during cardiac cushion formation causes early embryonic lethality instigated by deletion of a floxed *Yap* allele using *Tie2-Cre*. This made it difficult to study YAP function in the developing vasculature (37). Global knockdown of *Yap* via siRNA injection in mice revealed that YAP is important for mediating the stability of endothelial cell junction and vascular remodeling (36), however, the cell autonomous function of YAP could not be addressed due to the systemic distribution of the siRNA. We report here that deletion of *Yap* using endothelial cell-expressing *Pdgfb-iCreERT2* allows for assessment of postnatal retinal vascular development. Combined endothelial deletion of *Yap* and *Taz* in mice revealed gene dosage-dependent effects on retinal vascular sprouting, endothelial cell proliferation, and migration.

We found that YAP target genes such as *Ctgf* and *Cyr61* are down regulated in *Yap/Taz* cKO brain endothelial cells, while they are up-regulated in *Lats1/2* cKO endothelial cells, suggesting that transcriptional activity of YAP/TAZ might contribute to the regulation of proliferation in endothelial cells. In contrast to *Yap/Taz* cKO and *Lats1/2* cKO, YAPS112A overexpression alone is insufficient for retinal VEC proliferation, and the level or strength of YAP activity or additional factors such as TAZ may control EC proliferation. Furthermore, nuclear YAPS112A overexpression in transgenic mice does not alter angiogenesis in the retina as opposed to the vascular defects in *Lats1/2* cKO mice, suggesting that LATS1/2 could regulate other effectors, in addition to the subcellular localization of YAP/TAZ for the vascular morphogenesis.

The majority of studies of YAP and TAZ report their transcriptional activity in the nucleus via association with TEAD transcription factors and that phosphorylation of YAP and TAZ by the upstream kinases induces their cytoplasmic retention and degradation (20). Studies have revealed that phosphorylated YAP and TAZ are associated with 14-3-3 to bind to proteins in the cytoplasmic and tight junctions (39, 40). In kidney cells, it has been reported that cytoplasmic YAP and TAZ interact with angiomotin (AMOT) to facilitate the localization of YAP and TAZ to tight junctions and to promote phosphorylation by the upstream kinases in the Hippo pathway (41). In addition, cytoplasmically localized phospho-YAP and -TAZ have been shown to interact with DVL to inhibit Wnt/β-catenin and SMAD signaling (42, 43). Expression of YAPS112A in YAP/TAZ knockdown HUVECs cannot rescue the migration defect while the cytoplasmic mutant YAPS127D can, suggesting that cytoplasmic YAP but not nuclear YAP is required for cell migration. Hence, our data reveal a previously unrecognized function of cytoplasmic YAP/TAZ in the regulation of endothelial cell migration.

The crosstalk between Hippo signaling and CDC42. The small Rho family GTPase CDC42 is required for lumen formation during vasculogenesis and filopodia formation in endothelial cells (17, 27, 44). When *Cdc42* was deleted in endothelial cells using *Cdh5(PAC)-CreERT2* (17), vascular extension was not significantly changed between the *Cdc42* mutant and littermate controls. In our study, we observed a reduced vascular extension phenotype using *Pdgfb-iCreERT2* to delete the floxed *Cdc42* allele. The difference in the phenotype between these two studies could be due to the distinct Cre line used and the timing of tamoxifen administration. A previous study reported that deletion of *Cdc42* in kidney progenitor cells resulted in reduced YAP nuclear localization and target gene expression, suggesting that CDC42 acts upstream of YAP in mouse kidney development (45). Our study demonstrated that cytoplasmic YAP promoted CDC42 activation, providing a complementary mechanism of crosstalk between the Hippo pathway and CDC42. How cytoplasmic YAP regulates CDC42 activity in endothelial cell migration remains to be defined. A recent study indicates that YAP regulates RhoA activity through the controlling the expression of ARHGAP29 (Rho GTPase activating protein) (46). While CDC42 is not able to be directly activated by YAPS127D, there is a possibility that cytoplasmic YAP regulates CDC42-GEF or CDC42-GAP activity in migrating endothelial cells. The result of the rescue experiment using CDC42F28L, which can bind to GTP in the absence of GEF, supports this hypothesis.

YAP and TAZ join a collection of cellular factors and signaling molecules with the known ability to promote vascular sprouting and angiogenesis. Although our findings clearly demonstrate that YAP can activate CDC42 activity to promote endothelial cell migration, multiple mechanisms likely contribute. More evidence continues to demonstrate crosstalk between different signaling pathways to control vascular development. The Notch, VEGF, and BMP signaling pathways have been shown to play important roles in regulating vascular sprouting and tip cell formation during angiogenesis (9, 12-14). One report showed that BMP9 crosstalks with the Hippo pathway by repressing YAP target genes in endothelial cells (47). It seems possible that, in turn, YAP and TAZ could regulate BMP, Notch, and other pathways to control vascular development. Future investigations would need to identify the cellular mechanism underlying how cytoplasmic YAP activates CDC42 and to test the potential synergistic activities between YAP and regulators in other signaling pathways. The new findings of cytoplasmic YAP activity may help to develop

pharmacologic and genetic strategies to further enhance the pro-angiogenic potential for treating patients suffering from ischemic diseases.

Materials and Methods

Animals

All animal experiments were performed with the approval of the institutional animal care and use committee of Cincinnati Children's Hospital Medical Center. Please see SI Materials and Methods for origins of knockout and transgenic mice.

Cell culture

HUVECs were maintained in EGM-2 medium (Lonza) and were infected with an adenovirus, retrovirus, and lentiviruses. Plasmid transfection was performed using PolyJet DNA In Vitro Transfection Reagent (Signagen Laboratories). Additional details can be found in SI Materials and Methods.

Immunostaining and EdU labeling.

Eyes were fixed with 4% paraformaldehyde (PFA) for 1 hour, and then retinas were incubated with IB4-FITC (Molecular Probes) overnight. For EdU studies, P4 neonates were administered an intraperitoneal (IP) injection of 5-ethynyl-2-deoxyuridine (EdU, 5μg/g of mouse body weight). EdU incorporation was assessed using Click-IT EdU system (Invitrogen). Detailed information is described in SI Materials and Methods.

Active CDC42 assay

CDC42 activity was performed as previously described (48). Additional details can be found in SI Materials and Methods.

Statistics

All datasets were taken from n ≥ 3 biological replicates. Data are presented as mean ± SD. We calculated p values with unpaired Student's t test or Tukey-Kramer test with Excel (Microsoft Office); $P < 0.05$ was considered significant.

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