VPS28 regulates brain vasculature by controlling neuronal VEGF trafficking through extracellular vesicle secretion

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Highlights

- Vps28 is highly expressed in neurons and involved in the secretion of neuronal EVs
- Vps28, as a subunit of ESCRT-1 complexes, participates in the formation of MVB
- Vps28 plays an important role in VEGFA transport and promotes neurovascular communication
VPS28 regulates brain vasculature by controlling neuronal VEGF trafficking through extracellular vesicle secretion

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SUMMARY
Extracellular vesicles (EVs) participate in intercellular communication and contribute to the angiogenesis. However, the understanding of the mechanisms underlying EVs secretion by neurons and their action on the vascular system of the central nervous system (CNS) remain rudimentary. Here, we show that vascular protein sorting 28 (Vps28) is essential for the sprouting of brain central arteries (CtAs) and for the integrity of blood-brain barrier (BBB) in zebrafish. Disruption of neuron-enriched Vps28 significantly decreased EVs secretion by regulating the formation of intracellular multivesicular bodies (MVBs). EVs derived from zebrafish embryos or mouse cortical neurons partially rescued the brain vasculature defect and brain leakage. Further investigations revealed that neuronal EVs containing vascular endothelial growth factor A (VEGF-A) are key regulators in neurovascular communication. Our results indicate that Vps28 acts as an intercellular endosomal regulator mediating the secretion of neuronal EVs, which in turn communicate with endothelial cells to mediate angiogenesis through VEGF-A trafficking.

INTRODUCTION
The formation and connection of a functional vasculature is essential for embryogenesis and plays a crucial role in tissue and organ homeostasis. The development of cranial vasculature in zebrafish follows a stereotypical growth pattern with regard to vasculogenesis and angiogenesis (Isogai et al., 2001). Cerebral vascularization is critical because the interaction of the vasculature with the CNS forms the BBB, which is essential in maintaining a self-balancing environment for proper functioning of the brain (Daneman and Prat, 2015; Langen et al., 2019; Obermeier et al., 2013). The basilar artery (BA), primordial hindbrain channels (PHBCs), and the central arteries (CtAs) constitute the main vascular network of the CNS during the embryogenesis in zebrafish (Isogai et al., 2001; Ulrich et al., 2011). CtAs constitute an important network of vessels penetrating through the hindbrain and are necessary for the correct connection between the PHBCs and the BA (Gore et al., 2012; Ulrich et al., 2011).

CNS vascularization is controlled and organized by multiple neural-derived classical angiogenic factors, such as vascular endothelial growth factor (VEGF) and Wnt7a/b, through neurovascular communication (Paredes et al., 2018). Neural tube-derived VEGF-A is essential for the formation of the perineural vascular plexus and induction of ectopic ingression of blood vessels into the CNS parenchyma (Himmels et al., 2017; James et al., 2009). In radial glia-ablated zebrafish with reduced Vegfab expression within the spinal cord, the vertebral arteries were reported to be completely absent (Matsuoka et al., 2017). VEGF is evolutionarily conserved among vertebrate species. VEGF and sFlt1 expressed by motor neurons are required for blood vessel patterning around the motor neuron columns of the developing spinal cord in mouse and chick embryos (Himmels et al., 2017). Astrocyte-derived VEGF guides the retinal angiogenesis in the early stages of life in newborn mice (Gerhardt et al., 2003). Recently, microRNAs have been described as an additional factor essential for development of endothelial cells (ECs) during CNS angiogenesis and establishment of BBB integrity (Madeleine et al., 2017; Xu et al., 2017). Although numerous research studies have reported that neural-derived signals control CNS angiogenesis, the mechanism underlying the communication for transport of these neural signals and support for survival and sprouting of ECs is not fully understood.

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Figure 1. vps28 knockout displayed the abnormal angiogenesis of brain vascular CtAs in zebrafish larval

(A) Schematic showed that the vps28 mutant generated by the CRISPR/Cas9 system was with 22 bp deletion in exon 6, and the predicted Vps28 mutant protein was shortened with only 118 amino acids.
(B) The phenotype of vps28 mutant with intracranial hemorrhage (black arrows) in 54 hpf zebrafish larval. Scale bar: 100 μm.
(C) Proportion of the intracranial hemorrhage occurred in vps28 mutants at 54 hpf.
investigate whether Vps28 participates in the development of brain vasculature

Vps28 diminishes the endosomal trafficking of Awd in adipocytes of Drosophila larval (Mezzofanti et al., 2011). MVBI biogenesis is mediated by the endosomal sorting complex required for transport (ESCRT) complexes (Henne et al., 2011). The ESCRT system consists of four major subcomplexes: ESCRT-0, ESCRT-I, ESCRT-II, and ESCRT-III (Henne et al., 2011). Vacular protein sorting 28 (Vps28), which was first identified in yeast and is conserved in mammals and plants, is a component of the ESCRT-1 complex that regulates the MVB-dependent cargo sorting (Henne et al., 2011; Katzmann et al., 2001). Functional loss of Vps28 diminishes the endosomal trafficking of Awd in adipocytes of Drosophila larval (Mezzofanti et al., 2019). Vps28 is also expressed in neurons and is involved in the stabilization of dendrites in hippocampal neurons by regulating the activity of rapamycin complex 1 (Firkowska et al., 2019). Vps28 directly interacts with Gβγ and regulates organization of mitotic spindles (Dionisio-Vicuna et al., 2018). Furthermore, VPS28A and VPS28B double mutants displayed obvious developmental defects in regulating vacuole formation and endosomal sorting of proteins in Arabidopsis (Liu et al., 2020). However, the involvement of Vps28 in the development of brain vascular remains undetermined.

In the present study, we demonstrate that Vps28 expressed by neurons regulates vascularization of the CNS by controlling the transfer of VEGF-A through the secretion of EVs. In Vps28-deleted zebrafish, the angiogenesis of CtAs was significantly disrupted and the BBB integrity was compromised, thereby causing severe intracranial hemorrhage. Interestingly, whole mount in situ hybridization of vps28, vps28 transgenic line, and fluorescence-activated cell sorting (FACS) experiments indicated that vps28 is mainly expressed in CNS neurons, suggesting that the CNS vascularization and communication is disturbed in Vps28 mutants. Vps28 loss-of-function dramatically decreased the secretion of EVs by influencing MVBI formation, both in vitro and in vivo. We further confirmed that EVs secreted by neurons contain VEGF-A and participated in the generation of CtAs. In summary, our study demonstrates that Vps28 plays a critical role in the vascularization of CNS by regulating the formation of MVBI and affecting the secretion of VEGF-A-containing EVs.

RESULTS

Vps28 is required for the formation of cranial vasculature during angiogenesis

VPS28 is enriched in neurons and plays crucial roles in neural development (Firkowska et al., 2019). To investigate whether Vps28 participates in the development of brain vasculature in vivo, we generated vps28 mutants of zebrafish using the CRISPR/Cas9 technology, guided by a specific gRNA, targeting the vps28 exon6 in the zebrafish genome. One vps28 mutant allele with a 22 bp deletion in vps28 was obtained. The vps28 mutants encoded a truncated protein, terminated at the amino acid position 118 (Figure 1A), resulting in the lack of the conserved domain (40–220 aa) of the Vps28 superfamily; the relevant protein sequence of the wild type and the mutant Vps28 are presented in Figure S1. The vps28 mutants appeared to be morphologically normal for up to 36 h postfertilization (hpf) (data not shown). However, they displayed decreased pigment granules and cranial hemorrhages at 54 hpf and the larvae became necrotic and died at 3 days postfertilization (dpf) (Figure 1B). Approximately 44.2% of the vps28 mutants exhibited an obvious intracranial hemorrhage phenotype, whereas only 1.8% of the vps28 siblings (comprising vps28+/− and vps28+/-) displayed spontaneous hemorrhage (Figure 1C).

To demonstrate whether vps28 is essential for the brain vascular angiogenesis, vps28 heterozygotes were crossed into the background of double transgenic zebrafish Tg (Kdrl: eGFP, Gata1: DsRed) background. In contrast to vps28 siblings, DsRed-expressing blood cells were found to be accumulated in the brain of vps28 mutants. In addition, vps28-deficient zebrafish embryos showed severe impairment of CtAs development (Figure 1D), whereas development of the other brain vascular and intersegmental vessels (ISVs) was normal (data not shown).
To further investigate the processes leading to these differences within the hindbrain vasculature, confocal time-lapse imaging analysis was performed in Tg (Kdrl: eGFP) zebrafish embryos. It was found that the growth of CtAs, sprouting from the PHBC and BA, was impaired, leading to failure in the formation of an elaborate network of CtAs in vps28 mutants (Figure 1E). In addition, to determine whether the blood circulation is required for the CtAs formation in vps28 mutants, a tntt2a morpholino was used to generate the “silent heart” and “no blood flow” embryos (Sehnert et al., 2002). In tntt2a mutants, in which red blood cells were accumulated in the trunk region, the formation of CtAs appeared normal in tntt2a morphants compared with that in control and the deficit of angiogenesis in vps28 mutants also occurred in the absence of circulation (Figure S2). These results suggested that the lack of CtAs development is primarily because of the defect in Vps28 and not because of the deficiency of blood circulation.

Next, we investigated whether the intracranial hemorrhage in vps28 mutant embryos was caused by the impairment of BBB function. The leakage tracer, 4',6-diamidino-2-phenylindole (DAPI), was microinjected into the circulatory system through the common cardinal vein (CCV) in Tg (Kdrl: eGFP) embryos at 54 hpf and the CNS vasculature in the embryos was then examined via confocal live imaging. The number of DAPI-positive parenchymal nuclei was used for quantification of the degree of blood leakage in the zebrafish brain (Tam et al., 2012; Xu et al., 2017). We found a significantly higher accumulation of DAPI-positive parenchymal cell nuclei in vps28 mutant embryos compared with that in vps28 siblings (Figures 1F and 1H). Based on the position of DAPI-positive parenchymal cell nuclei, we observed that the leakage across the BBB in vps28 mutants was mainly concentrated in the PHBC, middle cerebral vein, and only a small amount of it occurred in the tip cells of deficient CtAs (Figure 1G). Overall, these results indicate that Vps28 is essential for the angiogenesis of CtAs and for the integrity of the brain vasculature.

Vps28 is enriched in neurons

To determine the function of Vps28 in the development of CtAs, we first assessed the expression pattern of Vps28 in zebrafish using whole mount in situ hybridization. Vps28 was extensively expressed at 30% epiboly and was widely expressed at the somite stage. However, it was abundantly and specifically expressed in the CNS at 24 hpf and 48 hpf (Figure 2A). To better understand the function of Vps28 in CNS angiogenesis, we constructed a Tg (Vps28: eGFP) transgenic zebrafish. GFP was expressed under the control of the Vps28 promoter. We found that GFP was widely expressed at 30% epiboly and 5-somite stage, and was specifically expressed in the brain at 24 and 36 hpf, strictly mimicking the endogenous expression pattern of vps28 (Figure 2B).

To ascertain the cellular localization of Vps28 in the brain, Tg (Vps28: eGFP) zebrafish were crossed into the Tg (Kdrl: mCherry) background to verify whether Vps28 was expressed in the ECs. We found that Vps28 was strongly expressed throughout the brain in the Tg (Vps28: eGFP) embryos at 48 hpf (Figure 2D). Consistent with the Vps28 expression pattern at 48 hpf, vps28 was extensively expressed in the CNS at 72 hpf but not in the dorsal longitudinal vessel (DLV) and mesencephalic vein (MsV) (Figure 2E). Flow cytometry and real-time PCR analyses were performed to detect the enrichment of vps28. It was observed that vps28 was more enriched in the neurons than in the ECs (Figure 2C). Furthermore, we observed robust vps28 expression in primary cultures of G0 mouse cortical neurons (Tubb3 * ) (Figure 2F). To determine whether neuronal-derived vps28 is involved in the CtAs angiogenesis, we directly microinjected vps28, under the control of an HuC promoter, into vps28 mutants. The neuronal-derived vps28 could partially rescue the defects in CtAs development (Figures 2G and 2H). Taken together, these results suggest that neuron-enriched Vps28 might play an important role in CtAs angiogenesis in the brain vasculature.

VPS28 knockdown inhibits EVs secretion in vitro

VPS28 belongs to the ESCRT family and is one of the subunits of the ESCRT-I complex that participates in the formation of MVBs and secretion of EVs (Henne et al., 2011). We sought to identify whether the abnormal angiogenesis of CtAs in vps28 mutants was because of altered MVB formation and EVs secretion. We knocked down VPS28 in human embryonic kidneys (HEK) 293T cell line by transfecting an siRNA targeting VPS28. The efficiency of VPS28 siRNA knockdown was confirmed via western blot analysis (Figure 3A). Subsequently, we detected MVB formation in VPS28 knockdown HEK 293T cells using immunofluorescence. VPS28 knockdown resulted in a clustered localization of HGS, an MVB marker, as evidenced by
Figure 2. Vps28 was enriched in CNS and cultured mouse cortical neurons

(A) The expression pattern of vps28 at 30% epiboly, 5-somite (5s), 24 hpf, and 36 hpf stage in zebrafish.

(B) Relative expression pattern of vps28 in Tg (Vps28: GFP).

(C) Relative expression level of vps28 in zebrafish ECs and neurons, which were sorted by flow cytometry from Tg (Kdrl: eGFP) and Tg (Huc: eGFP) embryos at 2 dpf, respectively.

(D and E) Expression of vps28 in Tg (vps28: eGFP; Kdrl: mCherry) at 48 hpf and 72 hpf. vps28 was mainly expressed in the zebrafish CNS than DLV (arrowhead) and MsV (arrow) at 72 hpf; DLV, dorsal longitudinal vein; MsV, Mesencephalic vein (E).

(F) Confocal images of primary mouse cortical neurons immunolabeled with Vps28 at 5 days in culture, a majority of the Tubb3 expressing neurons express detectable levels of Vps28 in primary mouse cortical neurons.

(G) Effects of vps28 driven by the HuC promoter (huc: vps28) on CTA defects of vps28 mutants.

(H) Graphical representations of the CTA numbers in (G). Data are represented as mean +/- SD. **p < 0.01.
the increased size of HGS+ particles in cells (Figure 3B). Transmission electron microscopy (TEM) analyses revealed significant reduction in the number and density of MVBs in VPS28 knockdown cells (Figure 3C).

To determine whether the decrease in the number of MVBs in VPS28 knockdown cells was influenced by the first step in MVB biogenesis, we transfected HEK 293T cells with the GTPase-defective mutant, Rab5(Q79L), which forms enlarged early and late endocytic endosomes (Stenmark et al., 1994; Villarroya-Beltri et al., 2016; Wegner et al., 2010). VPS28 knockdown dramatically affected the morphology of the MVBs and significantly decreased the number of endosomes compared with that in the negative control (Figure 3D).

Next, we attempted to ascertain whether the loss of function of VPS28 in HEK 293T cells would affect EVs secretion. The levels of EVs markers, CD63 and TSG101, were analyzed in whole cell lysates (WCL), 2K pellet, and purified EVs via western blot analysis. Knockdown of VPS28 in 293T cells led to a significant decrease in TSG101 levels in ultracentrifuged pellets, but not in the WCL and 2K pellet. However, a drastic reduction in CD63 levels was observed in the secreted EVs and 2K pellet, as well as in WCL. In addition, calnexin was abundant in the WCL and 2K pellet but was barely detected in pure EVs, indicating that the extracted EVs were relatively pure and were free of contamination from other cell compartments (Figure 3E). Taken together, these results suggest that, in vitro, VPS28 regulates the secretion of EVs by influencing the formation of MVBs.
Vps28 knockout decreases EVs secretion in vivo

Next, we determined whether in vivo EVs secretion was disrupted in Vps28 knockout mutants, in line with the results of the in vitro experiments. The neuron-specific distribution of CD63-enriched endosomes was detected in zebrafish embryos by transient expression of CD63-GFP driven by the HuC promoter (HuC: CD63-GFP), which was used to visualize the fluorescent reporter CD63-GFP in EVs in vivo and the fusion of MVBs with the plasma membrane in vitro (Verweij et al., 2018; Xu et al., 2017). In vivo confocal imaging in zebrafish Vps28 mutants transiently expressing HuC:CD63-GFP showed significantly decreased number of GFP-positive neuronal endosomes in the mutants compared with that in Vps28 siblings (Figures 4A and 4B). The results suggest that Vps28 may regulate CD63-filled endosomes. Next, we characterized the total number of EVs by dissociating 2.5 dpf-old Vps28 mutants and control embryos, subjecting them to collagenase D treatment, and isolating EVs from the supernatant via ultracentrifugation (Verweij et al., 2019). Nanoparticle tracking analysis (NTA) of the ultracentrifuged particles revealed that the number of pellets secreted by Vps28 knockout embryos was reduced compared with that in the control group (Figure 4C). Consistent with the NTA results, western blot analysis of the EVs marker CD63 revealed that...
knockout of Vps28 in zebrafish led to a significant decrease in CD63 levels in the ultracentrifuged pellets but not in the WCL (Figure 4D), suggesting that Vps28 significantly regulates the secretion of EVs in vivo by affecting the number of endosomes.

To further explore whether Vps28 influences EV secretion by affecting the formation of MVBs in vivo, we performed a TEM analysis and determined the number and morphology of MVBs in zebrafish CNS. MVBs and intraluminal vesicles (ILVs) were relatively easy to observe in wild type embryos, but were almost undetectable in Vps28-depleted zebrafish embryos (Figure 4E). Moreover, the number of MVBs per cell decreased in Vps28 mutant embryos compared with that in the control embryos (Figure 4F). Overall, these results indicate that, in vivo, Vps28 regulates the secretion of EVs by influencing the formation of MVBs.

**EVs are involved in the formation of CtAs in the brain**

EVs play a significant role in many aspects of cellular functions and pathological states, including angiogenesis, inflammation, immune responses, tissue homeostasis, neurodegenerative diseases, and cancer (Gurunathan et al., 2019; Howitt and Hill, 2016). In view of the impairment of the EVs secretion pathway in Vps28 mutants, we hypothesized that neuronal EVs may participate in regulating vascular angiogenesis in the brain by delivering specific substances to ECs. To test this hypothesis, we first isolated EVs from the zebrafish whole embryos using gradient ultracentrifugation. TEM and NTA analysis of the purified pellets revealed saucer-type small membrane vesicles with an average diameter of 113 ± 77 nm (mean ± SD, Figures 5A and 5B), suggesting that they were EVs. Next, we transiently microinjected the isolated EVs into the CCV of Tg (Kdrl: eGFP) vps28 mutants at 30 hpf to evaluate whether EVs played a significant role in brain vascularization. Injection of zebrafish EVs into the CCV partially promoted sprouting of CtAs from the PHBCs in the zebrafish brain at 54 hpf (Figures 5C and 5D). To further confirm whether neuronal EVs are involved in CtAs development, we extracted EVs from the conditioned medium of cultured primary mouse cortical neurons. Using TEM and NTA analyses of the purified pellets, EVs with an average diameter of 127 ± 80 nm (mean ± SD) were identified (Figures 5E and 5F). We microinjected the extracted neuronal EVs into the CCV of double transgenic zebrafish, Tg (Kdrl: eGFP; Gata1: DsRed) at 30 hpf and compared
the formation of parts of normal CtAs in vps28 mutants with that in non-injected morphants (Figure 5G). Moreover, co-injection of DAPI into the circulation system of Vps28 mutants at 54 hpf followed by counting of DAPI-positive nuclei of brain parenchymal cells showed that a reduced count of DAPI-positive nuclei was observed in the neuronal EVs rescued group compared with that in the vps28 mutant group (Figure 5H). These results indicated that EVs play an important role in CtAs development and in alleviating the hemorrhagic phenotype in vps28 mutants.

**EVs containing VEGF-A participate in regulating brain vascular angiogenesis**

VEGF signaling plays an important role in the development of the vascular system by regulating the vascular specification and differentiation (Jin et al., 2017). The decrease in CtAs sprouts in Vegfa-deficient zebrafish embryos during cerebrovascular development was akin to the deficiency in CtAs formation in the brains of Vps28 mutants. Moreover, it has been reported that astrocyte-derived VEGF-A is involved in the homeostatic maintenance of the BBB homeostasis (Argaw et al., 2012). Therefore, we hypothesized that EVs may transfer VEGF-A from neurons to ECs, thereby controlling the vascularization of CNS. To test this hypothesis, we first detected the expression of VEGF-A in cultured primary cortical neurons. Immunofluorescence analysis showed robust VEGF-A expression, indicating that VEGF-A is abundant in the nervous system (Figure 6A). Furthermore, the presence of VEGF-A was also observed in EVs isolated from the cell supernatant of cultured primary cortical neurons (Figure 6B). These results indicated that neuronal EVs can transfer VEGF-A in the CNS.

To demonstrate whether VEGF-A-containing EVs in the circulatory system are involved in brain angiogenesis, we transiently microinjected human VEGF-A-121 into the CCV of Tg (Kdrl: eGFP) zebrafish embryos at the onset of the CtAs sprouting stage. Confocal imaging revealed that VEGF-A-121 partially rescued the abnormal CtAs formation (Figure 6C). Moreover, overexpression of VEGF-A-121 dramatically decreased the intracranial hemorrhage, as evidenced by reduced DAPI leakage (Figures 6C and 6D). Likewise, overexpression in the EVs extracted from zebrafish embryos also alleviated the CtAs deficiency (Figures 6E and 6F). More importantly, the VEGF-A mRNA and protein levels in Vps28-depleted embryos were similar to those in Vps28 siblings (Figures 6G and 6H), but the levels were significantly diminished in the EVs of Vps28 (Figure 6I). In addition, we microinjected the EVs extracted from wild type zebrafish into the Tg (Kdrl: eGFP; Gata1: DsRed) zebrafish embryos at 1-cell stage and observed that the formation of CtAs in vps28 mutants was almost normal compared with that in uninjected mutants (Figures 6J and 6K). However, when VEGF signaling was blocked with vegfaa MO, the formation of CtAs did not differ between vps28 mutants and microinjected with vegfaa morphants-extracted EVs (Figures 6J and 6K). Collectively, these results indicate that the expression of VEGF in EVs is important for hindbrain CtAs angiogenesis and mediates the neurovascular crosstalk in the CNS.

**DISCUSSION**

As opposed to the classical VEGF signaling pathway that regulates angiogenesis in the CNS, our study demonstrates that the ESCRT-1 complex subunit, Vps28, which is abundantly expressed in the nervous system, affects angiogenesis of CtAs through the secretion of VEGF-A-containing EVs by influencing the generation of MVBs (Figure 6L). It highlights the function of the neuron–VEGF–EVs–EC–angiogenesis axis in neurovascular communication.

Vps28, which belongs to class E VPS proteins and participates in the ESCRT-I complex, is localized in ubiquitin-rich endosomes and is required for receptor trafficking (Bishop et al., 2002). In addition, Vps28 plays an important role in embryogenesis by regulating cell-division patterns in plant development (Liu et al., 2020). In the present study, Vps28 knockout via the CRISP/Cas9 system in zebrafish resulted in significant intracranial hemorrhage at 48 hpf, probably because of the disruption of cerebrovascular integrity, leading to larval mortality at approximately 80 hpf. Interestingly, vps28 mutants displayed normal angiogenesis of ISV from the DA. This phenotype demonstrated that different mechanisms govern brain vascular angiogenesis and ISV angiogenesis in Vps28 null-allele embryos.

An extensive body of evidences suggests that angiogenesis is temporally coordinated with the barrier genesis (Engelhardt and Liebner, 2014; Umans et al., 2017). Prnd is involved in blood vessel development and endothelial barrier integrity in the CNS (Chen et al., 2020), and CTGF participates in both retinal angiogenesis and BBB (Moon et al., 2020). Neurons and glia also play important roles in coupled angiogenesis and
barriergenesis (Biswas et al., 2020). We found that depletion of vps28 caused DAPI-leakage in CNS and defects in the development of CtAs; however, whether vps28 regulates vascular barriergenic differentiation needs to be further investigated.

In vertebrates, CNS vessel growth and maturation is largely mediated by neural and vascular communication (Biswas et al., 2020; Paredes et al., 2018). An impairment of neural Tgfbr2 secretion inhibits EC migration and reduces branching in angiogenesis (Hellbach et al., 2014). Here, we demonstrated that the expression of Vps28 is higher in the CNS than in the ECs, suggesting that neuron-derived Vps28 has...
a non-cell-autonomous function in controlling the sprouting of the CtAs. However, it is important to determine whether the lower expression of Vps28 in the ECs can also regulate brain vascular development in the brain.

EVs, with diameter ranging from 50 to 200 nm, have a cup-shaped structure and originate intracellularly from endosomes. EVs are generated from MVBs containing cargo-laden ILVs. MVBs fuse with the plasma membrane of various cell types, following which the ILVs are secreted as EVs (van Niel et al., 2018). The ESCRT consists of five distinct protein complexes, ESCRT-0, ESCRT-I, ESCRT-II, ESCRT-III, and AAA ATPase Vps4 complex, which play important roles in MVB morphogenesis and synthesis of EVs (Henne et al., 2011, 2013). Depletion of the TSG101 and Hrs (ESCRT-0 protein), STAM1 (ESCRT-I protein), or CHMP6, ALIX (ESCRT-III) decreased the secretion of EVs (Gurunathan et al., 2019; Larios et al., 2020). Vps28 belongs to the ESCRT-I family that is involved in the intracellular trafficking of proteins and MVB formation (Firkowska et al., 2019; Mezzofanti et al., 2019). Nevertheless, the function of Vps28 in the secretion of EVs has not yet been elucidated. We found that VPS28 regulates the secretion of CD63-enriched EVs by mediating the formation of MVBs in an ESCRT-dependent manner both in vitro and in vivo. Our research uncovers the functions of the ESCRT-I protein, Vps28, in addition, we show that it is involved in the generation of EVs and plays an essential role in neural regulation during the formation of the brain vasculature.

During development of the brain vasculature, a few neural tissues secreting EVs were reported to promote CNS vascular formation. By delivering their cargo comprising miR-132, neuron-derived EVs contribute to the regulation of the BBB integrity in the vascular development in the zebrafish brain (Xu et al., 2017). This prompted us to determine whether defective CtA formation in vps28 mutants was caused by the interruption of EV delivery. We harvested EVs from zebrafish whole embryos and from cultured mouse cortical neurons, microinjected them EVs into the 1-cell stage embryos or into the blood circulation system of 30 hpf zebrafish embryos through the CCV, and found that parts of CtAs could be rescued in vps28 mutants. Interestingly, the mouse neuron-derived EVs were more effective in promoting brain angiogenesis and preventing blood leakage.

Neural-derived VEGF-A plays a critical role in brain angiogenesis. VEGF-A secreted from astrocytes is required for retinal vasculature angiogenesis and BBB formation (Argaw et al., 2012; Bozoyan et al., 2012; Scott et al., 2010). In the present study, we detected that the VEGF-A mRNA and protein levels in vps28 mutants were not significantly different from those in vps28 siblings. However, the VEGF-A protein levels in vps28 mutant-derived EVs were dramatically lower than that in EVs from vps28 siblings. In addition, direct injection of the VEGF121 protein into the CCV was able to partially rescue the CtAs angiogenesis and prevented vascular leakage in the brain. Furthermore, EVs derived from vegfaa morphants could not promote the angiogenesis of CNS. In summary, we demonstrated that Vps28 is enriched in the CNS and executes a non-cell-autonomous function in controlling the angiogenesis of the CtAs via transfer of VEGF-A-containing EVs to ECs.

Limitations of the study
In our study, we found that vps28 participated in the angiogenesis of CtAs and the integrity of BBB in zebrafish. We show that the vps28 is involved in the development of CtAs by regulating the EVs secretion of VEGF. However, the mechanism through which vps28 regulates the integrity of BBB needs to be further studied.

STAR+METHODS
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• QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.104042.

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AUTHOR CONTRIBUTIONS
X.D., Y.Z., G.Z., and X.L. designed the project and edited the manuscript. Q.Z. supported the vps28 mutants and provided the idea of the subject. X.D., D.J., and L.W. performed the experiment and wrote the manuscript. Y.H., X.W., and Y.Z. performed the experiment. L.Y. and J.Z. helped to identify the vps28 mutants.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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| REAGENT or RESOURCE                 | SOURCE         | IDENTIFIER                          |
|------------------------------------|----------------|-------------------------------------|
| Antibodies                         |                |                                     |
| Antibodies                         |                |                                     |
| Rabbit polyclonal anti-VPS28       | Proteintech    | Cat#15478-1-AP; RRID: AB_2878144     |
| Rabbit polyclonal anti-Calnexin    | Proteintech    | Cat#10427-2-AP; RRID: AB_2069033     |
| Rabbit polyclonal anti-TSG101      | Proteintech    | Cat#28283-1-AP; RRID: AB_2881104     |
| Rabbit polyclonal anti-CD63        | Proteintech    | Cat#25682-1-AP; RRID: AB_2783831     |
| Mouse Monoclonal anti-TUBB3        | Proteintech    | Cat#66375-1-Ig; RRID: AB_2814998     |
| Rabbit polyclonal anti-HGS         | Proteintech    | Cat#10390-1-AP; RRID: AB_2118914     |
| Mouse Monoclonal anti-Beta Actin   | Proteintech    | Cat#66009-1-Ig; RRID: AB_2687938     |
| Mouse Monoclonal anti-VEGF         | Santa cruz     | Cat#sc-7269; RRID: AB_628430         |
| Rabbit Monoclonal anti-VEGF        | Abcam          | Cat#ab52917, RRID:AB_883427          |
| Coralite488-conjugated Affinipure Goat | Proteintech    | Cat#SA00013-2, AB_2797132            |
| Anti-Rabbit IgG(H+L) secondary antibodies | Proteintech    | Cat# SA00013-3, RRID:AB_2797133     |
| HRP-conjugated Affinipure Goat Anti-Rabbit IgG(H+L) secondary antibodies | Proteintech    | Cat# SA00001-1, RRID:AB_2722565     |
| HRP-conjugated Affinipure Goat Anti-Rabbit IgG(H+L) secondary antibodies | Proteintech    | Cat# SA00001-2, RRID:AB_2722564     |
| Chemicals, peptides, and recombinant proteins |                |                                     |
| **VEGF121**                        | GenScript      | Accession # P15692-9                 |
| Critical commercial assays         |                |                                     |
| mMESSAGE mMACHINE T7 Kit          | Invitrogen     | Cat# AM1344                          |
| MAXIscript In Vitro Transcription Kit | Invitrogen    | Cat# AM1314                          |
| Direct-ZoI7M RNA MiniPrep          | Zymo Research  | Cat#R2052                            |
| HiScript II Q Select RT SuperMix   | Vazyme         | Cat#R233-01                          |
| PowerUp SYBR Green Master Mix      | Thermo Fisher  | Cat# A25742                          |
| ABclonal MultiF Seamless Assembly Mix | ABclonal Technology | Cat# RK21020                     |
| ZK MultiF Seamless Assembly Mix    | ABclonal Technology | Cat# RK21020                     |
| DAPI                              | Beyotime       | Cat# C1002                           |
| Protease                          | SIGMA          | Cat#PS147                            |
| Trypsin-EDTA (0.05%)               | Gibco          | Cat#25300054                         |
| Fetal Bovine Serum                | Gibco          | Cat# 10099141                        |
| Poly-D-Lysine                     | Gibco          | Cat#A3890401                         |
| Ham’s F-12K                       | Gibco          | Cat# 21127030                        |
| DMEM                              | Gibco          | Cat# 11965084                        |
| Antibiotic-Antimycotic            | Gibco          | Cat# 15240062                        |
| Lipofectamine 3000                | Invitrogen     | Cat#L3000015                         |
| Opti-MEM                          | Gibco          | Cat#31985088                         |
| Fetal Bovine Serum                | SBI            | Cat#EXO-FBS-50A-1                    |
| Collagenase D                     | Roche          | Cat#11088859001                      |
| RIPA                              | Beyotime       | Cat# P0013B                          |
| Experimental models: Cell lines   |                |                                     |
| Human: 293T cell                  | ATCC           | CRL-3216                             |
| Mouse: primary neuron cell        | This paper     | N/A                                  |

(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Xiaohua Dong (njfishxiaohua@163.com).

Materials availability
All plasmids or zebrafish lines generated in this study are available from the Lead Contact with a completed Materials Transfer Agreement.

Data and code availability
- All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.

REAGENT or RESOURCE SOURCE IDENTIFIER

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Experimental models. Organisms/strains** | | |
| Tg (kdrl: EGFP) | China Zebrafish Resource Center (CZRC) | ZFIN ID: ZDB-ALT-050916-14 |
| Tg (Gata1: DsRed) | China Zebrafish Resource Center (CZRC) | ZFIN ID: ZDB-ALT-051223-6 |
| Tg (vps28: egFP) vps28+/− | This paper | N/A |
| Tg (huc: eGFP) | China Zebrafish Resource Center (CZRC) | ZFIN ID: ZDB-ALT-060301-2 |
| Tg (kdrl: mCherry) | China Zebrafish Resource Center (CZRC) | ZFIN ID: ZDB-ALT-111104-1 |

| Oligonucleotides | | |
|-----------------|--------|------------|
| siRNA targeting sequence: VPS28 #1 | Wagenaar et al., 2015 | N/A |
| GAAGUGAAGUGUUGAAAGATT | | |
| siRNA targeting sequence: VPS28 #2 | Wagenaar et al., 2015 | N/A |
| AAUCACGCUAUGUGACGAATT | | |
| Morpholino: MO-vegfaa | Gene Tools | ZFIN ID: ZDB-MRPHLNO-050513-12 |
| GTATCAAATACCAACAAGTTCAT | | |
| Morpholino: MO-trn12a | Gene Tools | ZFIN ID: ZDB-MRPHLNO-060317-4 |
| CATGTTTGCTCTGATCTGACAGCA | | |
| SgRNA targeting sequence: vps28#1 | This paper | N/A |
| GGACGACCGATCACATTGAAGG | | |
| Primers for vps28, see Table S1 | This paper | N/A |
| Primers for this study, see Table S1 | This paper | N/A |

| Recombinant DNA | | |
|-----------------|--------|------------|
| pYSY-gRNA | YSY Biotech | N/A |
| Tol2-vps28-GFP | This paper | N/A |
| Hu:C:CD63-mCherry | This paper | N/A |
| huc: vps28 | This paper | N/A |
| Hu:C:CD63-GFP | Xu et al., 2017 | N/A |
| Rab5CA (Q79L)-GFP | Bohdanowicz et al., 2012 | Addgene-35140 |

| Software and algorithms | | |
|--------------------------|--------|------------|
| Graphpad Prism | Graphpad Software | https://www.graphpad.com |
Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

**Zebrafish**

Zebrafish were raised under the standard conditions at 28°C in the zebrafish facility of the Model Animal Research Center, Nanjing University, and Tongren Hospital, Shanghai Jiao Tong University School of Medicine. The breeding and experimental protocols involved in using zebrafish were approved by the IACUC of the Model Animal Research Center, Nanjing University. The developmental stage of zebrafish was determined according to the previously reported (Kimmel et al., 1995). The following transgenic fish: Tg (Kdrl: eGFP), Tg (Huc: eGFP), Tg (Gata1: DsRed), provided by the China zebrafish resource center were used in this study. The vps28+/− mutant fish line and Tg (vps28: eGFP) transgenic line were generated in this study. 1-cell stage embryos or 30hpf stage larval were used for microinjection. Embryos used in this study were less than 3 dpf, which sex cannot be determined and unlikely to affect the results. The animal used in this study was approved by the Animal Ethics Committee of Tongren Hospital in China.

**Mouse primary cortical neuron culture and 293 T cell culture**

Mouse primary cortical neurons were separated from postnatal 0 day C57BL/6 mouse. In brief, cerebral cortices were dissected and digested with 1 mL 0.25% trypsin at 37°C for 15 min and then replace trypsin with 10% F12/10% FBS in DMEM for stop digestion for two times. Dissociated cells then plated onto petri dishes coated with 0.01 mg/mL Poly-D-Lysine (Gibco) with 10% F12/10% FBS in DMEM for 4 h. After that, neurons were cultured in serum-free medium with 2% B27/1% Glutamax in Neurobasal medium in cell incubator at 37°C with 5% CO2 and half medium were replaced every 3 days. And the replaced medium was collected for future EVs extracted. The sex of mouse used for primary cortical neuron culture was random.

HEK293T cell line was purchased from ATCC, and cultured in DMEM medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS, Gibco) and 1% penicillin-streptomycin (Gibco) in cell incubator at 37°C with 5% CO2. The sex of HEK293T cell came from is uncertain, but it is unlikely to affect the results.

**METHODS DETAILS**

**Mutation and transgenic lines**

CRISPR/Cas9 technology was used for generation of vps28 mutants (Dong et al., 2014). Capped Cas9 mRNA were synthesized in vitro by using the mMESSAGE mMACHINE T7 Kit (Ambion) (Dong et al., 2017). SgRNA were prepared to recognize the coding sequence of the exon 6 of vps28, and the target site of sgRNA was shown in Table S1. The templates of sgRNA were amplified by PCR with pYSY-grRNA vector (YSY, China) (Dong et al., 2017). sgRNA were synthesized using the MAXIscript In Vitro Transcription Kit (Ambion, USA). Cas9 mRNA and sgRNA were mixed and microinjected to 1-cell stage zebrafish embryos with a final concentration of 300 ng/μL:100 ng/μL. Founder (F0) were raised into adulthood and then crossed with wildtype to obtain next generation (F1). Then, when F1 was grown up, the genotype of F1 was identified by tail fin genomic PCR and sequencing to screening vps28+/− zebrafish (F1). vps28−/− were in-crossed to generate vps28−/− mutant embryos (F2) and used for the experimental analyses. Genotyping was used to identified between vps28+/+, vps28+/− and vps28−/−. Primers used for genotyping vps28 mutants was shown in Table S1. Fragment sizes are 159 bp for vps28+/+ and 137 bp for the vps28−/−, which could be identified by 2.5% agarose gel electrophoresis under 100 voltages for 30 min.

The Tg (Vps28: GFP) line was generated using the Tol2 transposon system. Zebrafish Vps28 promoter fragment (1672 bp upstream of vps28 translation start site) and GFP fragment were subcloned into the Tol2 vector. Primers used for generating the construct are given the Table S1. The recombined Tol2 plasmid (100 ng/μL) and transposase mRNA (300 ng/μL) were microinjected into 1-cell stage embryos to generation the founder (F1) of Tg (Vps28: GFP) line. The transgenic line was isolated by the expression of GFP in the next generation. Tg (Vps28: GFP; Kdrl: mCherry) fish were created by crossing individual transgenic line with the corresponding transgenic complement.
Flow cytometry, RNA extracted, real-time PCR and whole mount in situ hybridizations

Tg (Kdrl: eGFP) and Tg (Huc: eGFP) embryos were used for EC and neuron sorting, respectively. Firstly, zebrafish embryos at 48 hpf were dechorionated with 0.5 mg/mL pronase (Roche, Switzerland) and washed with 1× PBS for three times. Then the embryos were transferred into 15 mL tube containing 10 mL 0.25% trypsin (Gibco, California, USA) and were aspirated with the tips every 20 min for 2 h. Digested cells were collected by centrifugation with 200 g for 10 min at 4°C, suspended with 2% FBS/PBS solution, and then filtered with 40 μm cell strainer (BD Falcon, 352340). Finally, the cell suspension was used to sort out ECs or neurons by BD FACS Fortessa with 488 and 560 nm lasers.

Total RNAs of single zebrafish embryos, flow cytometry-sorted zebrafish ECs and neurons were extracted by using Direct-Zol™ RNA MiniPrep (Zymo Research, R2052, USA). Complementary DNA (cDNA) was synthesized with the HiScript II Q Select RT SuperMix for qPCR (Vazyme, R233-01, China). qPCR was performed by using PowerUp SYBR Green MasterMix (Thermo Fisher, A25742, USA) with ABI StepOne Plus. Whole mount in situ hybridization were performed as standard procedure (Dong et al., 2017). The primer sequences of specific genes are listed in Table S1.

Transfection with siRNAs and plasmids in 293T cell

For knock down of VPS28 in 293T cell, two reported VPS28 siRNA were used in this study (Wagenaar et al., 2015). The sequence of siRNA were listed in key resources table. siRNA1 and siRNA2 were co-transfected into 293T cell with a final concentration of 10 nM.

Transfection were performed in a 6-well cell culture plate using Lipofectamine 3000 (Invitrogen, life technologies). Briefly, 5 μL of lipofectamine 3000 was mixed with 245 μL OptiMEM (Gibco), 1.5 μL of siRNA1 (20 μM) and 1.5 μL of siRNA2 (20 μM) or 3 μL negative control (20 μM) were mixed with 247 μL OptiMEM (Gibco). siRNA mixture added into lipofectamine 3000 mixture and incubated for 5 min, the entire 500 μL mixture was added to a single well of 6-well cell culture plate. As for plasmids transfections, 2500 ng Rab5CA (Q79L)-GFP (Bohdanowicz et al., 2012) (Addgene plasmid 35140) or 2500 ng pCMV-CD63-mCherry plus 5 μL P3000 (Invitrogen, life technologies) and siRNAs were mixed with OptiMEM (Gibco), plasmids/P3000/siRNA/lipofectamine 3000 mixture incubated for 5 min and then added to a single well of 6-well cell culture plate. At 24 h post-transfection, the cells were changed with fresh media and prepared for immunofluorescence or imaging after 48 h post-transfection.

EVs collection from in vitro cultured 293T cell, primary cortical neuron and zebrafish embryos

293T cell was cultured in DMEM supplemented with 1% penicillin-streptomycin and 10% EVs-depleted FBS (EXO-FBS-50A-1, SBI, USA). Cell culture supernatant were collected at 24, 48 and 72 h after transfected with VPS28 siRNA. The supernatant of primary cortical neuron was collected every 3 days during the cell culture. The supernatant of zebrafish embryos was obtained as follow step, about 150 zebrafish embryos of 54 hpf were treated with Collagenase D (Roche), used at a concentration of 2 mg/mL in a total volume of 1.5 mL. Embryos were incubated at 37°C/4°C for 30 min, while the sample was intermittently crushed through 1 mL syringes to make the large clumps disappeared.

Then, the EVs from 293T cell, primary cortical neuron and zebrafish embryos were collected by serial centrifugation as follows, 320 g for 10 min, 2000 g for 15 min at 4°C (precipitates were collected as 2k pellet), 10,000 g for 30 min, and ultracentrifugation at 100,000 g for 70 min at 4°C. EVs pellet were washed in cooled PBS and obtained by ultracentrifugation at 100,000 g for 70 min at 4°C (Optima XPN-100 Ultracentrifuge, Beckman Coulter).

Microinjection of plasmid, morpholino, EVs, VEGF-A and DAPI into zebrafish embryos

HuC:CD63-GFP plasmid kindly provide by Prof. Juilin Du lab (Xu et al., 2017). HuC:CD63-GFP (75 ng/μL) plasmid is microinjected into 1-cell stage embryos to detected the expression of GFP in vps28 mutants. For rescue assay, HuC: vps28 (50 ng/μL) plasmid is microinjected into 1-cell stage embryos. vegfaa and tnt2a antisense oligonucleotides are designed as previously described and obtained from Gene Tools (Jin et al., 2017; Nasevicius et al., 2000). The sequence of vegfaa MO and tnt2a MO are listed in key resources table. 8 ng of vegfaa MO and 4 ng of tnt2a MO is microinjected into 1-cell stage of zebrafish embryos to generate the specific morphants. EVs (10⁷ particles/μL) derived from wildtype embryos and vegfaa morphants at 54hpf is microinjected into the 1-cell stage embryos.
VEGF-A protein, primary neuron cell derived-EVs and wildtype embryos derived-EVs microinjected into the blood circulation system through the common cardinal vein (CCV) at 30hpf, respectively. 10 mg/mL DAPI (Beyotime, China) was injected into the blood circulation system at 54 hpf and taken confocal images immediately after injection.

**Plasmid construction**

pCMV5 were digested with EcoRI and XbaI, CD63 and mCherry were cloned into pCMV5 with primers listed in Table S1 by ABclonal MultiF Seamless Assembly Mix (RK21020, ABclonal Technology, Wuhan, China). For huc:vps28 construct, the HuC promoter sequences was subcloned from HuC:CD63-GFP with primer listed in Table S1, the vps28 CDS fragment was obtained with the primer listed in Table S1, and then the huc containing fragment, vps28 containing fragment and pGEM-T easy vector fragment were mixed with 2X MultiF Seamless Assembly Mix followed their operation manual (RK21020, ABclonal Technology, Wuhan, China).

**Immunofluorescence**

For immunofluorescence assays, cells were plated onto slides (YA0352, Solarbio, Beijing, China), cultured for 12 h, fixed with 4% paraformaldehyde for 20 min at 4°C. Cells were incubated for HGS Rabbit Polyclonal antibody (Proteintech, 10390-1-AP) (1:100), Mouse Monoclonal anti-TUBB3 (Proteintech, 66375-1-Ig), VPS28 antibody (Proteintech, 15478-1-AP) and Anti-VEGFA antibody [EP1176Y]-C-terminal (Abcam, Cat#ab52917), followed by CoraLite488-conjugated Affinipure Goat Anti-Rabbit IgG(H+L) secondary antibodies (Proteintech, SA0003-2) (1:250) or CoraLite594 – conjugated Goat Anti-Mouse IgG(H+L) (Proteintech, SA00013-3), respectively. Cells were transfected with Rab5CA (Q79L)-GFP and pCMV-CD63-mCherry, fixed with 4% paraformaldehyde. Samples were examined with ZEISS LSM880 confocal microscope (Zeiss) and images were processed using ZEN software.

**Western blot**

Cells, cell pellet and EVs pellet were lysed with RIPA (Radio Immunoprecipitation Assay) lysis buffer (P0013B, Beyotime, Shanghai, China) containing 1 mM PMSF (Phenylmethanesulfonyl fluoride) (ST506, Beyotime, China). Proteins were separated by 8%–20% acrylamide/bisacrylamide gels and transferred to PVDF membranes (Millipore, Billerica, USA). The proteins were blocked in 5% blocking regent (1×TBST containing 5% defatted milk powder) for 1 h at room temperature. Membranes were incubated with primary antibodies (1:1000–1:3000) in antibody dilution buffer (G2025, Servicebio, Wuhan, China) for overnight at 4°C. After that, membranes were washed with 1×TBST for three time and then incubated with HRP (horseradish peroxidase)-conjugated second antibodies (1:1000–1:5000) in antibody dilution buffer for 1 h at room temperature. The proteins were visualized with Tanon 6200 (Tanon, Shanghai, China). The following antibodies were used, rabbit anti-human VPS28 (Proteintech, 15478-1-AP), rabbit anti-human Calnexin (Proteintech, 10427-2-AP), rabbit anti-human TSG101 (Proteintech, 28283-1-AP), rabbit anti-human CD63 (Proteintech, 25682-1-AP), anti-human β-Actin (Proteintech, 66009-1-Ig) and anti-mouse VEGF (Santa cruz, sc-7269).

**NTA measurement**

EVs particle size and concentration were measured by NTA at Viva Cell Biosceinces with ZetaView PMX 110 (Particle Metrix, Meerbusch, Germany) and corresponding software ZetaView 8.04.02. Isolated total EVs samples were diluted to 100 μL 1X PBS buffer. Concentrated samples were pre-diluted 300x, and were measured at 11 different positions with stage temperature control set at 27°C.

**Confocal imaging**

Larvae were embedded in 1% low-melting agarose (BBI Life Sciences, Shanghai, China) at 54 hpf at room temperature. Time-lapse images were taken with a 10-min interval, Z-stake of imaging ranged from 10 to 15 μm with the interval 2 μm. Imaging was captured with Zeiss LSM880 confocal microscopy (Zeiss, Germany).

**Transmission electron microscopy**

A total 10 μL isolated EVs were put on a formvar/carbon-coated copper grid for 10 min and were fixed in 1% glutaraldehyde for 10 min, washed in water, and contrasted in a uranylacetate/methylcellulose mix for
10 min at room temperature to negatively stain the exosomal fractions. Then the samples were examined immediately at 80 kV with a JEM-1011 transmission electron microscope.

Larvae at 2 dpf were fixed in 2.5% glutaraldehyde overnight at 4°C. Preparations were then washed with 0.1 M phosphate buffer (pH = 7.4) for three times with 15 min interval. Dehydrated through a graded with acetone series, and embedded in Epon812 (PELCO, 18010). Ultrathin sections (70 nm thickness) were prepared, stained with uranyl acetate (PELCO, 19481) and lead citrate (TEDPELLA, 19312), and examined by electron microscopy (Tecnai G2 Spirit Biotwin).

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Data were analyzed by Graphpad Prism (Version 9.0.0) using t tests between two groups. Differences were considered significant with \( p < 0.05 \). Error bars indicate SD.