Reck enables cerebrovascular development by promoting canonical Wnt signaling


ABSTRACT

The cerebral vasculature provides the massive blood supply that the brain needs to grow and survive. By acquiring distinctive cellular and molecular characteristics it becomes the Blood Brain Barrier (BBB), a selectively permeable and protective interface between the brain and the peripheral circulation that maintains the extra-cellular milieu permissive for neuronal activity. Accordingly, there is great interest in uncovering the mechanisms that modulate the formation and differentiation of the brain vasculature. By performing a forward genetic screen in zebrafish we isolated no food for thought (nft<sup>y72</sup>), a recessive late-lethal mutant that lacks most of the intra-cerebral Central Arteries (CtAs), but not other brain blood vessels. We found that the cerebral vascularization deficit of nft<sup>y72</sup> is caused by an inactivating lesion in reck (reversion-inducing-cysteine-rich protein with Kazal motifs or ST15; Suppressor of Tumorigenicity 15 protein), which encodes a membrane-anchored tumor suppressor glycoprotein. Our findings highlight Reck as a novel and pivotal modulator of the canonical Wnt signaling pathway that acts in endothelial cells to enable intra-cerebral vascularization and proper expression of molecular markers associated with BBB formation. Additional studies with cultured endothelial cells suggest that, in other contexts, Reck impacts vascular biology via the Vascular Endothelial Growth Factor (VEGF) cascade. Together, our findings have broad implications for both vascular and cancer biology.
INTRODUCTION

The cerebral vasculature is essential for brain development, activity and homeostasis (Vallon et al., 2014). It supplies the metabolic needs of this organ, which consumes a fifth of the oxygen and a quarter of the glucose used by the body (Mergenthaler et al., 2013; Rolfe and Brown, 1997). Assembly of the cerebral vasculature involves endothelial cells initially found within vasculogenic perineural vessels. Some of these cells form angiogenic sprouts that invade the brain, yielding intracerebral vessels that branch and interconnect with the perineural vasculature (Ruhrberg and Bautch, 2013). Cerebral vasculature morphogenesis is accompanied by barrierogenesis, the process of endothelial differentiation yielding the blood-brain barrier (BBB). Key BBB hallmarks include contiguous intercellular tight junctions, lack of fenestrations and selective/enriched expression of particular tight junction components and nutrient/efflux transporters (Hagan and Ben-Zvi, 2014; Obermeier et al., 2013; Siegenthaler et al., 2013). The BBB regulates the brain’s extra-cellular ion balance, facilitates nutrient transport into the parenchyma, prevents the entrance of harmful molecules and metastatic cells and enables neuroepithelial immune surveillance (Carson et al., 2006; Hawkins and Davis, 2005; Mergenthaler et al., 2013; Ousman and Kubes, 2012; Rolfe and Brown, 1997). Cerebral angiogenic growth and barrierogenesis are modulated by neuroepithelial cues that activate endothelial signaling cascades (Hagan and Ben-Zvi, 2014). These include canonical Wnt or Wnt/β-catenin signaling, which promotes both aspects of vascular development specifically in the Central Nervous System (CNS; the brain and the spinal cord), and global regulators of angiogenic growth with positive (VEGF and SDF1) or negative (Notch and TGF-β) roles (Arnold et al., 2014; Bussmann et al., 2011; Fujita et al., 2011; Gridley, 2010; Larrivee et al., 2012; Mackenzie and Ruhrberg, 2012; Masckauchan and Kitajewski, 2006; Reis and Liebner, 2013). Reck (Reversion-inducing cysteine-rich protein with Kazal motifs) is a dimeric multi-domain glycosylphosphatidylinositol (GPI)-anchored protein isolated as a tumor suppressor whose over-expression normalized the aberrant morphology of transformed fibroblasts. Reck is an inhibitor for metalloproteinases (MP) of the MMP (Matrix MetalloProteinase) and ADAM (A Disintegrin And Metalloproteinase) families (Chang et al., 2008; Hong et al., 2014; Nagini, 2012; Omura et al., 2009). MPs promote cell migration by weakening the mechanical barrier properties of the extra-cellular matrix (ECM) and reducing cell-cell adhesion via intercellular junction destruction (Page-McCaw et al., 2007; Seals and Courtneidge, 2003). For example, fly Reck limits basement membrane degradation (Srivastava et al., 2007) and glioma migration (Silveira Correa et al., 2010). MPs also modulate signaling pathways by cleaving ligands, receptors, ECM components (Page-McCaw et al., 2007; Seals and Courtneidge, 2003) and adherens junctions (Rims and McGuire, 2014). They can also act non-proteolytically (Mantuano et al., 2008; Mori et al., 2013). Accordingly, Reck also modulates cell signaling with MP-dependency (Miki et al., 2010; Muraguchi et al., 2007). Finally, consistent with its multi-domain structure, Reck associates with the ERBB2 receptor to block its activity in an MP-independent fashion (Hong et al., 2011). Reck
modulates the development of forelimbs, dorsal root ganglia neurons (DRG), brain (Muraguchi et al., 2007; Park et al., 2013; Prendergast et al., 2012; Yamamoto et al., 2012) and vasculature. *Reck* knockout mice show perineural vascular plexus disorganization and reduced intra-cerebral vascularization (Chandana et al., 2010; Miki et al., 2010; Oh et al., 2001). In this study we provide key mechanistic insights into how Reck modulates cerebrovascular development at both the cellular and molecular levels.
RESULTS

no food for thought mutants (nft<sup>y72</sup>) lack intra-cerebral blood vessels and DRG

In a zebrafish genetic screen (Shaw et al., 2006) we isolated the recessive-lethal mutant nft<sup>y72</sup> for its brain-specific vascularization deficit. While nft<sup>y72</sup> lacks intra-cerebral Central Arteries or CtAs (Fig. 1A-B), its other cephalic blood vessels form and carry circulation normally (Fig. 1A-B and Movies S1A-S1D). Importantly, in the mutants gross cerebral organization is undisturbed (Fig. S1). Cardiac contractility appears normal; see movies S1K-L. In the trunk, blood vessels form and function properly (Fig. 1C-D and Movies S1E-H) and the lymphatic thoracic duct is patterned correctly (Fig. 1F,H). However, the neural crest-derived DRG are missing (Fig. 1E,G). The shape, patterning and size of the head and body are unaffected (Fig. 1I-L), except for minor jaw defects; see (Prendergast et al., 2012).

nft<sup>y72</sup> is a genetically-null mutant allele of reck

nft<sup>y72</sup> maps to a genetic interval spanning Df(Chr24:reck)<sup>w15</sup> (chromosome 24 deficiency removing reck and other genes), which was isolated as a sensory deprived (sdp; now reck) allele in a screen for DRG-deficient mutants. All four sdp alleles are recessive lethal and genetic nulls (Prendergast et al., 2012). Given the positional and/or phenotypic similarities between nft<sup>y72</sup>, sdp (Prendergast et al., 2012) and Reck knockout (Reck<sup>−</sup>) mice (Chandana et al., 2010) we tested nft<sup>y72</sup> and sdp for complementation. We found that nft<sup>y72</sup>/sdp trans-heterozygotes and both nft<sup>y72</sup> and sdp homozygotes have prominent CtA and DRG deficits (Fig. S2A-H; Table S1). To compare nft<sup>y72</sup> and Df(Chr24:reck)<sup>w15</sup> with respect to additional cardiovascular phenotypes see Figs. S2E-I, S3 and movies S1G-N.

DNA sequencing from nft<sup>y72</sup> revealed a G-to-A transition at position 761 of the 2,868 nt open reading frame of reck (Prendergast et al., 2012) yielding a missense, non-conservative substitution of the evolutionarily-conserved Cys<sup>254</sup> residue to Tyr at the fourth Cysteine Knot 4 (CK4; Fig. 2A). A similar Cys substitution occurs in sdp<sup>w13</sup> at CK1 (Prendergast et al., 2012) (Fig. S3). To confirm this reck transition is the causative mutation in nft<sup>y72</sup> we provided exogenous wild type (WT) reck mRNA to one-cell stage embryos from nft<sup>y72</sup>/+ in-crosses; see (Prendergast et al., 2012). This treatment rescues the CtA and DRG deficits of nft<sup>y72</sup> (henceforth called reck<sup>y72</sup>) without yielding a surplus of these structures (Fig. 2B-F), indicating that reck plays permissive roles in CtA and DRG formation. Together with the results of experiments using tissue-specific gene expression to rescue CtA formation in reck<sup>y72</sup> (Figs. 5, S6 and Graph S1), the identical intra-cerebral vascularization deficits of reck<sup>y72</sup> and Df(Chr24:reck)<sup>w15</sup> (Fig. 5J) and the differential subcellular localization of the wild type Reck and the mutant Reck<sup>y72</sup> proteins (Fig. 2G-J), our observations imply that reck<sup>y72</sup> is an amorphic allele of reck.
The mutant Reck\(^{72}\) protein is inactive because it fails to reach the outer cell surface

Since secretion of disulfide-bridged proteins is often impaired by Cys substitutions (Bodin et al., 2007; Boute et al., 2004; Claffey et al., 1995; Halliday et al., 1999; Mason, 1994; Schrijver et al., 1999) we hypothesized that the inactivity of Reck\(^{72}\) is due to failure to reach the outer cell surface; see (Simizu et al., 2005). We thus assayed the cell surface localization of epitope-tagged (3xFLAG and 2xHA) WT (Reck) and mutant (Reck\(^{72}\)) zebrafish proteins in non-permeabilized immunofluorescently stained cells; see (Imhof et al., 2008). In 293T cells epitope-tagged Reck is detected at the cell surface (Fig. 2G-H; these proteins are active: Figs. 5, S6). In contrast, Reck\(^{72}\) is un-detectable at the cell surface (Fig. 2I,J). Quantification of COS7 cell lysates shows the localization disparity is not due to differential abundance (Fig. 2K,L). We also co-expressed differentially tagged versions of Reck and Reck\(^{72}\) and found that the WT form still localizes correctly. Our findings thus provide a simple molecular explanation for the recessive amorphic nature of the \(reck^{72}\) allele: Reck\(^{72}\) fails to reach the outer cell surface without disrupting the targeting of its WT counterpart.

The intra-cerebral vascularization deficit of \(reck^{72}\) is due to decreased CtA-forming cell emigration

To elucidate the endothelial cellular bases of the intra-cerebral vascularization deficit of \(reck\) mutants (\(reck^{-}\)) we exploited the advantages of the hindbrain (Hb) vasculature as a model for cerebrovascular development, as we did in (Ulrich et al., 2011). The WT Hb harbors both extra-cerebral (peri-neural) and intra-cerebral vessels. These lie, respectively, ventral to the Hb or inside it (Fig. 3A). The extra-cerebral vasculature comprises the two lateral Primordial Hindbrain Channels (PHBCs) and, at the midline, the Posterior Communicating Segments (PCS) and Basilar Artery (BA). The PHBCs communicate with the midline vessels via arteriovenous connections (avc). The intracerebral vessels or CtAs sprout dorsally from the PHBCs into each rhombomere center. The 24-72 hpf assembly of the Hb vasculature primarily involves endothelial cell migration and follows a reproducible sequence. The PHBCs and PCS form first (Fig. 3B). Then PHBCs launch ventral sprouts towards the midline, which coalesce into the BA (Fig. 3C-D). While the BA forms the CtAs emerge from the PHBCs, penetrate the Hb and connect to the PCS and BA (Fig. 3D-E). Most BA-forming sprout remnants disappear by 48 hpf, those that persist become avc (Fig. 3D-E); see (Bussmann et al., 2011; Corti et al., 2011; Fujita et al., 2011; Fukuhara et al., 2014; Isogai et al., 2001; Ulrich et al., 2011).

At the cellular level the intra-cerebral vascularization deficit of \(reck^{-}\) could be due to defects in the abundance and/or distribution of endothelial cells (Fig. 3F-K). Quantification of these parameters reveals that endothelial abundance is slightly reduced at 36 hpf but not at 50 hpf (Fig. 3J), consistent with a minor transient delay in the mutant’s vascular development and eliminating the possibility that reduced endothelial cell abundance (due to impaired cell specification, proliferation and survival) causes the lack of CtAs in \(reck^{-}\). In contrast, endothelial cell distribution is abnormal in the mutant:
the PHBCs (but not the BA) are hyperplastic (Fig. 3J). We also found that \textit{reck}^{y22} has overabundant avc (Fig. 3I,K,M), reminiscent of the murine perineural vascular plexus disorganization of \textit{Reck}− (Chandana et al., 2010). Notably, the mutant’s avc harbor only few cells (Fig. 3J). Together with the results of our time-lapse imaging showing that the PHBCs in \textit{reck}^{y22} fail to form CtA sprouts (Fig. S4 and Movies S2A, S2B), our observations indicate that the primary cellular defect leading to the intracerebral vascularization deficit of \textit{reck}− is a dramatic reduction in CtA-forming endothelial cell emigration from the PHBCs, which in turn induces the hyperplasia of the latter (Fig. 3L-M).

\textit{reck} limits avc abundance even without circulatory flow

Blood vessel perdurance and circulatory flow can be linked (Bussmann et al., 2011; Chen et al., 2012; Fish et al., 2008; Kochhan et al., 2013; Nicoli et al., 2010; Watson et al., 2013). For instance, drug-induced heartbeat inhibition reduces avc abundance, which suggests that in WT embryos the few avc that persist do so because they were carrying robust flow (Corti et al., 2011; Helisch and Schaper, 2003). Hence we hypothesized that in \textit{reck}− the CtA deficit increases circulatory pressure through extra-cerebral vessels (which have robust flow: Movies S1A-B, Table S1), secondarily enhancing avc maintenance and/or formation (Fig. S4, Movies S2A, S2B). We thus asked if the avc overabundance of \textit{reck}^{y22} is suppressed by lack of circulatory flow. We abrogated cardiac contractility with \textit{silent heart} (\textit{sih}^{hitb}), a recessive \textit{cardiac troponin-t2a} inactivating mutation (Sehnert et al., 2002). Unlike drug-based circulatory flow inhibition, genetic abrogation of circulation increases avc abundance, even in \textit{reck}^{y22}, and has little impact on CtA abundance (Table S2 and Fig. S5) (Bussmann et al., 2011; Corti et al., 2011; Fujita et al., 2011; Fukuhara et al., 2014; Isogai et al., 2001; Ulrich et al., 2011). Thus, \textit{reck} limits avc abundance even without flow, strongly suggesting that the avc overabundance of \textit{reck}^{y22} is unrelated to circulatory pressure gains.

\textit{reck} is expressed in the cerebral endothelium where it is required non-cell autonomously for intra-cerebral vascularization

To visualize the cephalic expression of \textit{reck} during cerebrovascular development we performed RNA \textit{in situ} Hybridization Chain Reaction or HCR (Choi et al., 2014) using 36 and 48 hpf WT embryos. Consistent with prior reports (Chandana et al., 2010; Miki et al., 2010; Prendergast et al., 2012), we found that in WT embryos \textit{reck} is expressed in cerebral vessels and neural crest derivatives. For example, at 48 hpf \textit{reck} expression highlights the MtA, PHBCs and the CtAs, as well as the Branchial Arches (BAx); see Fig. 4A-C. Based on these observations we hypothesized that CtA development requires \textit{reck} activity in the cerebral endothelium. To test this hypothesis we performed cell transplants between WT donors and \textit{reck}^{y22} hosts, making chimeras with mosaicism in the Hb and/or its vessels (Carmany-Rampey and Moens, 2006). Although cell transplants target the Hb’s endothelium rarely despite yielding frequent mosaicism in both the Hb and the trunk’s vasculature, we found that CtAs, like other vessels (Zygmunt et al., 2011), are of mixed clonal origin (Fig. 4D-F).
Moreover, WT endothelial cells form chimeric CtAs in *reck*<sup>y72</sup> hosts (Fig. 4G; n=3 chimeras), consistent with the notion that intra-cerebral vascularization requires endothelial *reck* activity in a non-cell autonomous manner. Accordingly, mosaic endothelial expression of WT Reck (but not *reck*<sup>y72</sup> or EGFP) is sufficient to rescue the CtA deficit of *reck*<sup>y72</sup> (Figs. 5, S6 and Graph S1).

**reck** is required for canonical Wnt signaling in the cerebral endothelium

The intra-cerebral vascular deficit of *reck*<sup>−</sup> best fits the model that Reck is a positive modulator of pathways that foster cerebral vascular development (Wnt, VEGF, SDF1) and/or an inhibitor of cascades that antagonize it (Notch, TGF-β). Since our experiments eliminated the latter possibility we focused on testing the hypothesis that Reck promotes Wnt, VEGF and/or SDF1 signaling. We visualized the expression of molecular markers in WT and *reck*<sup>y72</sup>, including components and/or targets of these pathways. We found no obvious differences between genotypes in the neuroepithelial expression of genes encoding Wnt (*wnt1*), VEGF-A (*vegfaa* and *vegfab*) and SDF (*sdf1b*) ligands and Reck-targeted MPs (*mmp2* and *mmp14a*). Analysis of the expression of pan-endothelial genes (*fli1a*, *tie1* and *ve-cdh/cdh5*) and markers of specific cerebral vessels (*dab2*, *dll4* and *cxcr4a*) failed to reveal any obvious expression abnormalities, beyond the anticipated CtA labeling deficit in *reck*<sup>y72</sup> (Amoyel et al., 2005; Bai et al., 2005; Brown et al., 2000; Bussmann et al., 2011; Fujita et al., 2011; Janssens et al., 2013; Larson et al., 2004; Lyons et al., 1998).

However, consistent with the cerebrovascular role and expression of *reck* (Figs. 1A-B, 4, 5, S6), we found that *reck*<sup>y72</sup> displays abnormalities in the expression of both artificial and endogenous targets of the canonical Wnt signaling pathway in the cerebral endothelium (Figs. 6, 7). For example, in WT embryos the fluorescent reporter of canonical Wnt signaling *Tg(7xTCF-Xla.Siam:GFP)*<sup>ia4</sup> (Moro et al., 2013) is expressed both in brain vessels (PHBCs and nascent CtAs at 36 hpf; CtAs at 48 hpf) and non-vascular cephalic tissues. In contrast, *reck*<sup>y72</sup> displays a selective loss of *Tg(7xTCF-Xla.Siam:GFP)*<sup>ia4</sup> cerebrovascular expression (Fig. 6A-N”).

In addition, *reck*<sup>y72</sup> shows altered expression of two genes that are endogenous targets of canonical Wnt signaling in the mammalian cerebral endothelium and serve as markers of BBB differentiation; namely GLUT1 (Glucose Transporter 1 or SLC2A1/solute carrier family 2 (facilitated glucose transporter), member 1) and *plvap* (plasmalemma vesicle associated protein or vsg1/vessel-specific gene 1); see Fig. 7. Glut1 is a BBB component with Wnt-activated expression. *plvap* encodes a Wnt-repressed marker of fenestrated endothelium that highlights the immature BBB; see (Daneman et al., 2009; Hallmann et al., 1995; Liebner et al., 2008; Posokhova et al., 2015; Qian et al., 2005; Tam et al., 2012; Zhou and Nathans, 2014; Zhou et al., 2014). We found that at 72 hpf Glut1-immunostaining labels the MtA, PHBCs and CtAs of WT embryos (Fig. 7A-D”), but in *reck*<sup>y72</sup> mutants Glut1 is undetectable in the PHBCs despite the fact that it remains in the MtA (Fig. 7E-G”). Conversely, *plvap* mRNA highlights the dorsal aspect of the CtAs but not the extra-cerebral PHBCs in WTs at 48 hpf (Fig. 7H). However, in *reck*<sup>y72</sup> the PHBCs display ectopic *plvap* expression (Fig. 7I).
We next asked if the intra-cerebral vascularization deficits and aberrant expression of BBB-related markers found in \textit{reck}^{72} can be phenocopied via the inactivation of canonical Wnt signaling in otherwise WT embryos. To do this we used transgenes that enable heat-induced global inhibition of canonical Wnt signaling via distinct mechanisms. \textit{Tg(hsp70l:Xla.TCFAC-EGFP)} drives expression of a dominant negative form of the transcription factor TCF3 (T-cell factor 3 or lymphoid enhancer factor 3/LEF3) that lacks its DNA-binding HMG domain and is fused to EGFP. These truncated forms of TCF3 (TCFAC) act downstream of the destruction complex in Wnt-receiving cells by binding to β-catenin, preventing its interaction with endogenous TCF3 (Martin and Kimelman, 2012). \textit{Tg(hsp70l:Mmu.Axin1-YFP)}^{135} expresses a fusion of Axin1 (a pivotal component of the β-catenin destruction complex) and fluorescent YFP to promote β-catenin degradation (Kagermeier-Schenk et al., 2011; MacDonald et al., 2009; Stamos and Weis, 2013). We found that blocking canonical Wnt signaling with these tools yields defects in intra-cerebral angiogenesis and endothelial expression similar to those displayed by \textit{reck}^{72} (Fig. S7). For example, forced expression of TCFAC greatly inhibits CtA angiogenesis and abrogates Glut1 expression (Fig. S7E-H") without disrupting the trunk’s vascular patterning (Fig. S8). Similarly, Axin1-YFP reduces CtA abundance by ~50% (Fig. S9E-F) and induces ectopic \textit{plvap} expression in the PHBCs (Fig. S7J).

We also attempted to rescue CtA angiogenesis in \textit{reck}^{72} mutants via forced activation of canonical Wnt signaling. Briefly, we over-expressed constitutive active (ca) forms of β-catenin (caβ-catenin), either ubiquitously with the heat-inducible \textit{Tg(hsp70l:caβ–catenin-2A-TFP)}^{130} transgenic line (Veldman et al., 2013) or with endothelial-specificity via \textit{flt1}-driven mosaic expression; see (Wada et al., 2013; Wu et al., 2012; Yost et al., 1996). Forced expression of caβ-catenin inhibited CtA angiogenesis in WT animals and, unsurprisingly, failed to rescue CtA angiogenesis in \textit{reck}^{72} mutants. It is likely that in these experiments the over-expression of caβ-catenin ended up inhibiting canonical Wnt signaling via transcriptional squelching (titration of endogenous transcription factors), as previously demonstrated (Prieve and Waterman, 1999). In addition, \textit{flt1}-driven mosaic endothelial expression of a dominant negative form of GSK3β (DN-GSK3-GFP) (Taelman et al., 2010) had no effect on CtA angiogenesis in WT animals and failed to promote CtA angiogenesis in \textit{reck}^{72} mutants, consistent with the fact that chemical activation of Wnt signaling using the GSK3β inhibitors CHIR99021 and LiCl likewise failed to rescue both the DRG and CtA deficits of \textit{reck}^{72} mutants (Fig. S10); see (Klein and Melton, 1996; Ring et al., 2003; Vanhollebeke et al., 2015; Veldman et al., 2013). Overall, it is likely that the inability to achieve physiological levels of canonical Wnt signaling with proper spatio-temporal dynamics via forced expression of modified components of this pathway or with drugs explains why these treatments failed to rescue the defects of \textit{reck}^{72} mutants.

Nonetheless, our observations demonstrate that the role of canonical Wnt signaling in promoting intra-cerebral angiogenesis and proper expression of markers of barriergenic differentiation is evolutionarily conserved between zebrafish and mammals; see (Tam et al., 2012; Umans and Taylor,
In addition, our other findings indicate that the cerebrovascular activity of canonical Wnt signaling is Reck-dependent, thus uncovering this tumor suppressor as a novel and key modulator of this pathway.

Reck promotes VEGF signaling in cultured endothelial cells

VEGF signaling, critical for intra-cerebral vascularization, is mediated primarily by receptors encoded by the zebrafish kdrl (kinase insert domain receptor-like) and mammalian KDR/VEGFR2 “ohnologs” (Bussmann et al., 2008; Habeck et al., 2002; Mackenzie and Ruhrberg, 2012; Sivaraj et al., 2013; Sohet and Daneman, 2013; Wittko-Schneider et al., 2013). We noted that the kdrl transcriptional reporters Tg(kdrl:RFP)$^{s896}$ and/or Tg(kdrl:GFP)$^{la116}$ (Chi et al., 2008; Choi et al., 2007) are weakly expressed in the PHBCs of reck$^{y72}$ and Df(Chr24:reck)$^{w15}$ homozygotes and reck$^{y72}$/Df(Chr24:reck)$^{w15}$ trans-heterozygotes (Fig. S9A-B), but a similar reduction is not apparent in trunk vessels. In contrast, endothelial fluorescence from the fli1a transcriptional reporter Tg(fli1a:EGFP)$^{y1}$ (Lawson and Weinstein, 2002) appears unaffected in reck$^{-}$. We verified these qualitative observations by performing quantitative confocal imaging to determine the Normalized Fluorescent Intensity (NFI) of Tg(kdrl:RFP)$^{s896}$ using the signal of Tg(fli1a:EGFP)$^{y1}$ as reference; as in (Venkiteswaran et al., 2013). These measurements confirmed that in reck$^{y72}$ mutants the fluorescence of Tg(kdrl:RFP)$^{s896}$ is decreased in the PHBCs, but not in other cephalic vessels like the MtA (Fig. S9D). Consistent with the existence of distinct genetic circuits governing the activity of VEGF signaling in arterial and venous vessels (Covassin et al., 2006) we found that in CtA-deficient kdrl$^{y17}$ mutants (Covassin et al., 2006) Tg(kdrl:RFP)$^{s896}$ fluorescence is unaffected in the PHBCs but reduced in the MtA (Fig. S9C-D). These findings suggest that the expression of kdrl in the PHBCs, as in the trunk vessels (Wythe et al., 2013), is insensitive to VEGF signaling; but see also (Lawson et al., 2003; Lawson et al., 2002; Liang et al., 2001). We next over-expressed Axin1-YFP to ask if inhibition of canonical Wnt signaling reduces Tg(kdrl:RFP)$^{s896}$ expression; see (Veldman et al., 2013; Wang and Nakayama, 2009). Indeed, this treatment dramatically reduces Tg(kdrl:RFP)$^{s896}$ expression (Fig. S9E-F). These observations suggest that the cerebrovascular inactivation of canonical Wnt signaling in reck$^{y72}$ reduces kdrl expression thereby secondarily impairing VEGF activity. Yet, additional observations are inconsistent with this possibility. First, the cerebrovascular phenotypes of reck$^{-}$ and kdrl$^{-}$ are not identical. Both mutants lack CtAs, but in kdrl mutants the BA is missing and cerebral endothelial abundance is reduced (Bussmann et al., 2011). Second, plvap is ectopically expressed in the PHBCs of reck$^{y72}$ (Fig. 7I) and embryos in which canonical Wnt signaling is silenced via Axin1-YFP over-expression (Fig. S7J). In contrast, in kdrl$^{um19}$ mutants plvap is not ectopically expressed (Fig. S9G-H). This finding is consistent with the emerging notion that intra-cerebral angiogenesis and barriergenic differentiation are genetically uncoupled (Hagan and Ben-Zvi, 2014) and suggests that VEGF activity is dispensable for cerebrovascular canonical Wnt signaling. Third, we found no obvious changes in the expression of VEGF-responsive markers like dll4. Finally, RNA in situ HCR
failed to reveal any obvious reduction in the abundance of kdrl transcripts in the cerebral vasculature of recky72, but these were nearly undetectable in kdrlmu19 mutants likely due to nonsense-mediated mRNA decay; see (Brogna and Wen, 2009).

To investigate whether Reck can impact VEGF signaling in other contexts we used HUVECs (Human Umbilical Vein Endothelial Cells), as they are VEGF-responsive and express RECK (Miki et al., 2010). In these experiments we manipulated VEGF availability, RECK abundance and KDR kinase activity with the SU5416 inhibitor (Sakao and Tatsumi, 2011), quantified VEGF signaling readouts (KDR, AKT and ERK1/2 phosphorylation levels) and measured RECK and KDR abundance (Fig. S9I-J’); see (Koch et al., 2011; Lanahan et al., 2013; Zachary, 2003). We found that RECK-knockdown impairs VEGF signaling by reducing pKDR and pAKT levels without affecting pERK abundance (Fig. S9I, J, I’), which is consistent with the different thresholds and kinetics of AKT and ERK phosphorylation (Olszewska-Pazdrak et al., 2009). Finally, RECK-knockdown, but not chemical abrogation of VEGF signaling, reduces KDR abundance (Fig. S9I-J’). Together, the results of our zebrafish and HUVEC studies suggest that Reck promotes VEGF signaling in contexts other than embryonic cerebrovascular development.
DISCUSSION

Our findings highlight Reck as a pivotal player in vascular development required for the branching morphogenesis and barriergenic differentiation of cerebral blood vessels (Fig. 8). At a cellular level reck acts in the first process by ensuring the migration-dependent formation of intra-cerebral vessels and the remodeling of the perineural network. The results of our cell transplantation and tissue-specific reck<sup>y72</sup> rescue experiments indicate that, consistent with its cerebrovascular expression pattern, reck is required in the brain’s endothelium in a non-cell autonomous manner to promote CtA angiogenesis; see (Chandana et al., 2010; Miki et al., 2010; Prendergast et al., 2012; Vanhollebeke et al., 2015).

Molecularly, reck is required for proper expression of Glut1 and plvap, two barriergenic differentiation markers regulated by canonical Wnt signaling. Accordingly, the cerebrovascular expression of a transgenic reporter of canonical Wnt signaling is lost in reck<sup>y72</sup>. Our study thus reveals that canonical Wnt signaling promotes intra-cerebral angiogenesis and barriergenic gene expression with evolutionarily conservation from fish to mammals; see (Daneman et al., 2009; Liebner et al., 2008; Posokhova et al., 2015; Tam et al., 2012; Zhou and Nathans, 2014; Zhou et al., 2014) and, that Reck plays a pivotal role in enabling cerebrovascular canonical Wnt signaling; see also (Vanhollebeke et al., 2015).

These novel mechanistic insights about reck might illuminate the etiology and treatment of CNS vascular diseases caused by aberrant Wnt signaling (FEVR/Familial Exudative Vitreoretinopathy; OPPG/Osteoperosis-pseudogioma Syndrome and Norrie disease) and suggest strategies for manipulating vascular barriers to enable drug delivery into the CNS (Daneman et al., 2009; Dejana, 2010; Hagan and Ben-Zvi, 2014; Hawkins and Davis, 2005; Liebner et al., 2008; Obermeier et al., 2013; Siegenthaler et al., 2013; Vallon et al., 2014; Zhou et al., 2014). Accordingly, future studies will determine whether reck, like canonical Wnt signaling, regulates vascular development and/or maintenance throughout the CNS; see (Daneman et al., 2009; Liebner et al., 2008; Stenman et al., 2008).

How reck promotes canonical Wnt signaling remains undefined at the biochemical level. Reck associates with Gpr124 (G-protein coupled receptor 124), another novel member of the canonical Wnt signaling pathway acting downstream of Wnt7 ligands that plays roles in cerebrovascular and DRG development similar to those of Reck (Posokhova et al., 2015; Vanhollebeke et al., 2015; Wang et al., 2014; Zhou and Nathans, 2014). Thus, Reck-Gpr124 heteromers might modulate Wnt7 binding, the assembly of the Wnt signaling complex, its internalization and/or MP-mediated processing events that gate its activity. However, it is worth highlighting that the effects of inactivating reck and gpr124 are not fully equivalent. For example, gpr124 mutants begin to recover intra-cerebral angiogenesis at 5 dpf and 50% of them survive to become adults without any apparent deficits in cerebral vascularization (Vanhollebeke et al., 2015). In contrast, in mutants of the different reck alleles CtA
angiogenesis does not recover after 5 dpf and lethality is fully penetrant by 10 dpf. Similarly, the CtA and DRG deficits of gpr124 morphants (Vanhollebeke et al., 2015), but not of reck/y2 mutants, can be rescued via chemical activation of Wnt signaling.

Finally, our zebrafish and endothelial cell culture studies suggest that Reck promotes VEGF signaling in contexts other than cerebrovascular development, consistent with the involvement of Gpr124 in VEGF-mediated tumor angiogenesis (Wang et al., 2014). RECK is expressed in tumor vessels (Clark et al., 2011; Miki et al., 2010; Oh et al., 2001; Rahmah et al., 2012) and epigenetically inactivated in human cancers. Reduced RECK abundance is a poor prognosis tumor signature linked to high metastasis and short survival in patients with cerebral glioma, neuroblastoma and other tumors (Nagini, 2012; Noda and Takahashi, 2007). Given the involvement of both canonical Wnt and VEGF signaling in both tumor vascularization and metastasis (Carmeliet, 2005; Goel and Mercurio, 2013; Hu et al., 2009; Klaus and Birchmeier, 2008) our findings might also prove relevant in these pathological settings.
MATERIALS AND METHODS

Zebrafish husbandry
Zebrafish (Danio rerio) handled under NYU’s IACUC/IBC-approved protocols.

Zebrafish lines

Transgenic fluorescent reporters: Endothelial: Tg(kdrl:RFP)896 (Chi et al., 2008), Tg(kdrl:EGFP)la116 (Anderson et al., 2008) Tg(kdrl:eGFP-NLS)2909 (Blum et al., 2008), Tg(fli1a:eGFP)yi1 (Lawson and Weinstein, 2002); erythrocytes: Tg(gata1a:DsRed)3d2 (Traver et al., 2003) DRG (and other neuronal populations): Tg(neurog1:eGFP)61 (McGraw et al., 2008); canonical Wnt signaling activity: Tg(7xTCF-Xla.Siam:GFP)64 (Moro et al., 2013). Transgenes for inhibiting canonical Wnt signaling: Tg(hsp70l:Mmu.Axin1-YFP)35 (Kagermeier-Schenk et al., 2011) and Tg(hsp70l:Xla.TCF4C-EGFP); see (Martin and Kimelman, 2012). Transgene for activating canonical Wnt signaling: Tg(hsp70l:caβ–catenin-2A-TFP)50 (Veldman et al., 2013). Mutants: kdrl17 and kdrlm19 (Covassin et al., 2009; Meng et al., 2008), reck72 (this study), reck14 and Df(Chr24:reck)5 (Prendergast et al., 2012), sih109 (Sehnert et al., 2002). Genotyping protocols in Supplemental Material and Methods.

Zebrafish heat-shock (HS) treatments for inducible inhibition or activation of canonical Wnt signaling

Tg(hsp70l:Xla.TCF4C-EGFP), Tg(hsp70l:caβ–catenin-2A-TFP)50 and their HS-controls: 40°C for 30 minutes at 30 hpf; fixed at 48 hpf. Tg(hsp70l:Mmu.Axin1-YFP)35 and its HS-controls: Heat-shock: 39°C for 1 hour at 24 hpf, fixed and/or imaged live at 48 hpf. Tg(hsp70l:Xla.TCF4C-EGFP) and Tg(hsp70l:Mmu.Axin1-YFP)35 transgenes provided paternally.

Antibodies

Primaries (zebrafish experiments): rabbit anti-RFP (1:500; Clonetech’s Living colors #632496), rabbit anti-GFP (1:2000; Life Technologies #A1122), mouse anti-phospho-FAK y397 (1:100; Millipore #MAB1144), rabbit Glut1 (1:200; Novus Biologicals #NB300666), mouse zrf1 (1:10; ZIRC), mouse anti-3A10 (1:100; DSHB) and mouse anti-HuC (1:100; Sigma-Aldrich #), mouse anti-HA (1:500).

Primaries (cell culture experiments): pVEGFR-2 (1:1,000; #2478), VEGFR-2 (1:5,000; #2479), pAKT (Ser473; 1:1,000; #4058), AKT (1:10,000; #9272), pERK1/2 (Ser473; 1:15,000; #4370), ERK1/2 (1:15,000; #4695), Reck (1:5,000; #3433), GAPDH (1:10,000; #4058) and HA (1:10,000; #2367) from Cell Signaling. GFP (1:5,000; Life Technologies; A11121) and FLAG (1:20,000; SIGMA #F1804). Secondaries (zebrafish and cell culture experiments): Life Technologies’ AlexaFluor-labeled anti-mouse or anti-rabbit antibodies from donkey (1:1000; catalog #A31571, A10040, A21206 and A100036).
Phalloidin staining
Fixed embryos incubated in a Phalloidin-488 (Sigma-Aldrich) solution for 2 hr at room temperature; as in (Snow et al., 2008).

Whole mount RNA in situ hybridizations (WISH)
Non-fluorescent chromogenic WISH; as in (Zygmunt et al., 2011). Fluorescent Hybridization Chain Reaction (HCR-WISH): As in (Choi et al., 2014). See Supplemental Material and Methods.

Cell culture experiments
HUVEC (Lifeline Cell Technology; FC-0003): cells were infected with anti-RECK shRNA lentiviral particles, puromycin-selected, starved overnight and stimulated with or without VEGFA. SU5416 (S8442 SIGMA) used for VEGFA signaling inhibition. HEK 293T (ATCC; #CRL-11268), COS-7 (ATCC; #CRL-1651): Lipofectamine-2000 (Life Technologies) was used to transfect constructs for expressing epitope-tagged Reck and Reck<sup>y72</sup>. Cells were tested for contamination by the commercial provider. Cells were used for western blot or immunofluorescence 48 hours post-transfection. See Supplemental Material and Methods.

Cell transplantations
As in (Carmany-Rampey and Moens, 2006; Zygmunt et al., 2011).

Vectors for expressing Reck, Reck<sup>y72</sup> (both epitope-tagged), EGFP, caβ-catenin and DN-GSK3-GFP in zebrafish and/or cultured cells
*Tol2* transgenesis and *Tol2*/Gateway-based vectors were used for *flt1*-driven mosaic endothelial expression in zebrafish. Gateway-based vectors were used for CMV-driven expression in cultured cells. See (Bussmann et al., 2010; Hogan et al., 2009; Kwan et al., 2007; Villefranc et al., 2007).

*reck<sup>y72</sup>* rescue via microinjection of WT *reck* mRNA
*reck* mRNA (100 pg) injected into 1-cell stage embryos.

Chemical activation of canonical Wnt signaling using GSK3β inhibitors
Dechorionated embryos were incubated with DMSO vehicle (0.7% in egg water; negative control) or GSK3β inhibitors starting at the 16-somite stage (16 hpf) until 72 hpf. The following GSK3β inhibitors were used: CHIR99021 (10 μM; diluted in DMSO) and LiCl (100 mM; diluted in egg water); as in (Vanhollebeke et al., 2015; Veldman et al., 2013).
Confocal microscopy and image processing

**Image acquisition:** Leica SP5 confocal microscope with 40x dipping/water immersion objectives (NA=0.8 or 1.1), bi-directional scans at 200 lines/s in 1024x1024 pixel windows; z-stacks at 1 µm z intervals. **Image processing:** Images are Maximum Intensity Projections. Whole-head images assembled from combining separately-collected anterior and posterior regions via ImageJ and Photoshop.

**Live quantification of** Tg(kdrl:RFP)s896 fluorescence: Tg(kdrl:RFP)s896/+; Tg(fli1a:eGFP)y1/+ embryos (WT and reca72) were imaged and the RFP signals were normalized to those of GFP at every z-level with a custom ImageJ macro (Venkiteswaran et al., 2013) and the resulting values averaged over all z-levels. **Embryo mounting:** Fixed (1% agarose/PBS), live/single time points (1% agarose/fish medium with Tricaine and PTU), live/time-lapse (0.1% agarose under 1% agarose); see (Kaufmann et al., 2012; Lawson and Weinstein, 2002).
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Competing interests

None.

Author contributions


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Fig. 1. *nft*<sup>y72</sup> lacks intra-cerebral blood vessels and DRG but shows normal body morphology. (A-H) Confocal and bright field (I-L) lateral images. Anterior, left; dorsal, up. hpf: A,B,E,G (72); C,D (48); F,H (96); I-L (60). (A,B) Central Arteries (CaA) are found in WT (A) (white arrowheads) but are missing in *nft*<sup>y72</sup> (B); the other head vessels are present in *nft*<sup>y72</sup>. Blood vessels (*Tg(kdrl:RFP)*<sup>y896</sup>), red. (C-D) WT and (C) *nft*<sup>y72</sup> (D) show identical trunk vascular patterns. Endothelium (*Tg(fli1a:eGFP)*<sup>y1</sup>), green; somite boundaries, blue; see (Zygmuniet al., 2011). (E,G) DRG (yellow circles, red HuC immunofluorescence) are present in WT (E) but absent in *nft*<sup>y72</sup> (G). (F,H) Blood vessels, green (*Tg(fli1a:eGFP)*<sup>y1</sup>) and red (*Tg(kdrl:RFP)*<sup>y896</sup>); lymphatics (asterisks), green only (*Tg(fli1a:eGFP)*<sup>y1</sup>). (J,L) Head close-ups of (I,K). Key: MtA, Metencephalic Artery; PHBC, Primordial Hindbrain Channel; DLAV, Dorsal Longitudinal Anastomotic Vessel; Se, Intersegmental vessel; PAC, Parachordal Chain; Fb, Forebrain; Mb, Mindbrain; Hb, Hindbrain. Scale Bars: (A,B) 100 µm. (C,D,E,G) 50 µm. (F,H) 25 µm. (I-L) 200 µm. See also Fig. S1, movies S1A-N and Table S1.
**Fig 2.** *nft*<sup>y72</sup> is a genetically null allele of *reck*. (A) WT zebrafish *Reck* (top) is 955 aa long and features the same domains and motifs found in mammalian RECK; see (Takahashi et al., 1998). N-terminal signal peptide (SP; brown), Cysteine Knot motifs (CK1-5; blue), Epidermal Growth Factor-like repeats (EGF1-2; black), Fibronectin-like type I module (Fnl1; green), Kazal motifs (K1-3; red), C-terminal GPI transferase cleavage site (G; pink). Mutant *Reck*<sup>y72</sup> (bottom) harbors a missense amino-acid change in an evolutionarily conserved Cys residue within CK4. (B-F) Providing WT *reck* mRNA into *reck*<sup>y72</sup> restores formation of both CtAs (B-D; scale bar in B-C: 50 µm) and DRG (E-F; scale bar: 100 µm). Embryos with unilateral CtA rescue (B-C; endothelium, green (*Tg(fli1a:eGFP)y1*)) or, bilateral DRG rescue (F; yellow circles, red HuC immunofluorescence; only one side shown). Quantification of CtA rescue expressed as the percentage of embryos (from incrossing heterozygous mutant carriers) with *reck*<sup>y72</sup>-like CtA deficits (D). (G-L) Exogenous co-expression of epitope-tagged zebrafish *Reck* (WT *Reck* or mutant *Reck*<sup>y72</sup>) with cytosolic EGFP in cultured mammalian cells. (G-J) Immunofluorescence-based detection of surface 3xFLAG-*Reck* (red) and EGFP-fluorescence (green) in non-permeabilized 293T cells. 3xFLAG-*Reck*<sup>y72</sup> fails to reach the cell surface (J). (K-L) WT and mutant *Reck*<sup>y72</sup> expressed in COS7 cells show similar abundance. (K) Western Blot of total cell lysates from cells expressing WT or mutant 3xFLAG-*Reck*. (L) Densitometry-based quantification total cell lysates from cells expressing WT Reck or mutant *Reck*<sup>y72</sup> zebrafish proteins tagged with 3xFLAG or 2xHA. Protein levels normalized with EGFP and GAPDH. Scale bar: 10 µm. Error bars: S. E. M. See also Figs. S2, S3, movies S1A-N and Table S1.
Figure 3. **reck**^{72}'s CtA deficit is due to impaired endothelial cell migration from the perineural PHBCs. (A-E) WT Hb vasculature anatomy (A; anterior half detail) and development (B-E; cross-sections “cut” by plane in (A). Dorsal, up. PHBCs, red; BA, dark blue; PCS, light blue; avc (PCS-connected, yellow; BA-connected, orange), CtAs; green. (F-K) Abundance and distribution of Hb endothelial cells and avc at 36 and 50 hpf in WT and **reck**^{72}. (F-I) Confocal images (50 hpf). Endothelium, red (Tg(kdrl:RFP)\textsuperscript{s896}); endothelial nuclei (Tg(kdrl:eGFP-NLS)\textsuperscript{zf109}), green. Anterior, left. Scale bars: 100 μm. (F, G) Lateral views; dorsal, up. (H, I) Dorsal views (ventral-level) of extracerebral vessels; left side, bottom. Arrowheads, avc (PCS-connected, yellow; BA-connected, orange). (J, K) Quantifications. (J) Endothelial cell abundance and distribution. (K) avc abundance. Asterisks and bars color-matched. Asterisks, significant differences (p < 0.001) between age-matched genotypes (Student's t-test). n=10 embryos per genotype and stage. Vertical lines, SD. (L, M) Diagrams of the Hb vasculature phenotypes (anterior half detail) in WT (L) and **reck**^{72} (M). The mutant shows a dramatic CtA deficit, hyperplastic PHBCs and too many avc. See also Figs. S4, S5, movies S2A-B and Table S2.
Figure 4. reck is expressed in the cerebral endothelium where it is required non-cell autonomously for intra-cerebral vascularization. (A-C) Confocal lateral views of a 48 hpf WT Hb. Anterior, left; dorsal, up. (A) Endothelium, green (Tg(fli1a:eGFP)y1). (B) Fluorescent intensity of reck transcripts detected via RNA in situ HCR. Warmer and cooler colors represent, respectively, signals of higher and lower fluorescent intensity. reck is expressed in cerebral vessels (MtA, CtAs and PHBC) and BAx. (D) Workflow of cell transplantation experiments. Chimeras analyzed at ~72 hpf. (E-G) Confocal lateral views of the Hb of two chimeras. Anterior, left. Dorsal, up. Donor’s endothelium, green (Tg(kdrl:EGFP)y116); host endothelium, red (Tg(kdrl:RFP)s896); lineage-tracer (Rhodamine 647 Dextran), blue. (E-F) Chimera made using WT embryos. Note mosaicism in both the Hb environment (blue) and the CtAs (green and red). (G) Chimera made using a WT donor and a recky72 host. Note mosaic CtAs (asterisks) containing both WT (green) and mutant (red) endothelial cells. Scale bars: 100 µm.
Figure 5. Mosaic endothelial expression of WT Reck is sufficient to rescue the CtA deficit of reck<sup>y72</sup>. (A-I) Dorsal views (dorsal-level to show CtAs) of the 72 hpf Hb vasculature (red,
*Tg(kdr1:RFP)*) of *reck* injected with constructs driving endothelial expression of exogenous Reck, Reck (both HA-tagged, see Fig. 2L) or EGFP proteins (green). Anterior, left. Right side, up. (C,F,I) White asterisks mark CtAs with exogenous expression of listed proteins. Scale bars, 100 µm. (J) Quantification of CtA abundance in the Hb of *reck* and *Df(Chr24:reck)* with or without (“Uninj”) exogenous endothelial expression of listed proteins. Asterisks, significant differences (p < 0.001); n.s., not significant. Student’s *t*-test. *reck* mutants scored: Reck (n=28), Reck (n=14), EGFP (n=19), Uninj (n=20). *Df(Chr24:reck)* mutants scored: Uninj (n=19). See also Fig. 4, S6 and Graph S1.
Figure 6. The cerebrovascular expression of the transgenic reporter of canonical Wnt signaling Tg(7xTCF-Xla.Siam:GFP)iad is specifically lost in reck^72. Confocal images (anterior, left) of 36 and 48 hpf Hbs from WT andreck^72. Views: Lateral (A-H’; dorsal, up); dorsal (I-N”; right side, up). Endothelium, red (Tg(kdrl:RFP)s896); Wnt reporter, green (Tg(7xTCF-Xla.Siam:GFP)iad). In WT the Wnt reporter expression highlights the PHBCs (A-D”), CtAs (A-D”, I-K”) and additional non-vascular tissues. However, in reck^72 expression of the Wnt reporter is undetectable in PHBCs (E-H”, L-N”) and occasional CtAs (N-N”), but is present elsewhere. Detail zooms: Regions in white dashed boxes (A,E,I,L) shown in C-C”, G-G”, K-K” and N-N”, respectively; regions in yellow dashed boxes (C, G) shown in D-D” and H-H”, respectively. See also Fig. 8.
Figure 7. *reck*<sup>72</sup> shows aberrant cerebrovascular expression of the Wnt-responsive markers of barriergenic differentiation Glut1 and *plvap*. (A-G”) Confocal lateral images of the 72 hpf Hb vasculatures of WT (A-D”) and *reck*<sup>72</sup> (E-G”). Anterior, left. Dorsal, up. Endothelium, red (*Tg(kdrl:RFP)*<sup>s896</sup>); Glut1 immunofluorescence, green. Colored dashed boxes (A, E) demarcate a region of the following vessels: white, MtA (zooms: B-B”, F-F”); yellow, CtAs (zooms: C-C”); blue, PHBCs (zooms: D-D”, G-G”). Merged images of zooms: B”, C”, D”, F”, G”. Glut1 decorates the MtA, CtAs and PHBCs of the WT (n=7 embryos). In contrast, Glut1 decorates the MtA, but not the PHBCs, of *reck*<sup>72</sup> (n=10 embryos). (H-I) Transmitted light images of the 48 hpf heads of embryos subjected to whole mount RNA *in situ* hybridization with *plvap* riboprobes. (H) WT. *plvap* is expressed in the dorsal aspect of CtAs but not in the PHBCs (n=57 embryos). (I) *reck*<sup>72</sup>. *plvap* is ectopically expressed in the PHBCs (n=8 embryos). Scale bar: 100 µm. Merged images of detail zooms: C”, D”, G”, H”, K”, N”. Scale bar: 100 µm. See also Fig. S7-S9.
Figure 8. Model. Reck enables cerebrovascular canonical Wnt signaling to promote intra-cerebral vascularization and proper expression of barrierogenesis markers. Diagrams. Hb vasculature (cross-sections) in WT and $reck^−$. Intra-cerebral CtAs, green. Extra-cerebral (peri-neural) vessels: PHBCs, red; BA, dark blue; ave, orange. In WT animals (left panel) Reck ensures proper endothelial cell responsiveness to the canonical Wnt signals that promote CtA angiogenesis, induce cerebrovascular Glut1 expression (light blue) and repress plvap expression in the PHBCs. In $reck^−$ (right panel) cerebrovascular canonical Wnt signaling is inactive, which dramatically impairs intra-cerebral vascularization, prevents Glut1 expression and induces ectopic expression of $plvap$ in the PHBCs. $reck^−$ also show a disorganized perineural vessel network.
REFERENCES


