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 Structural basis of the junctional anchorage of the cerebral cavernous malformations complex

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Introduction

Cerebral cavernous malformation (CCM) is a common cerebrovascular anomaly, affecting ~1 in 200 individuals and predisposing patients to a lifetime risk of seizures, hemorrhagic stroke, and other neurological deficits (Leblanc et al., 2009; Faurobert and Albiges-Rizo, 2010). Heterozygous loss of KRIT1 (also known as CCM1) causes a familial form of CCM (Laberge-le Couteulx et al., 1999; Sahoo et al., 1999), characterized by the development of multiple vascular dysplasias within the brain but also in the spinal cord, retina, and skin (Leblanc et al., 2009; Faurobert and Albiges-Rizo, 2010). A similar disease is associated with heterozygosity for loss of function of CCM2 or CCM3, and these three proteins interact with each other (Leblanc et al., 2009; Faurobert and Albiges-Rizo, 2010). CCM lesions typically arise in adulthood and consist of beds of dilated capillary vessels with little intervening brain tissue. There is a reduction in endothelial cell (EC)–cell adhesion within lesions but not in the surrounding normal brain tissue (Leblanc et al., 2009; Faurobert and Albiges-Rizo, 2010). Loss of KRIT1 leads to defects in EC junctional integrity (Glading et al., 2007) associated with increased RhoA activity (Stockton et al., 2010) and apicobasal polarity (Lampugnani et al., 2008), and loss of CCM1 similarly disrupts EC–cell junctions (Glading and Ginsberg, 2010). Inhibiting the Rho effector, Rho kinase, reverses the effect of KRIT1 silencing on EC junctions (Stockton et al., 2010) and slows the development and progression of CCMs in a murine model of the human disease (McDonald et al., 2012).

In mice, deletion of Krit1 leads to early embryonic lethality (approximately embryonic day 10 [E10]) caused by gross defects in multiple vascular beds (Whitehead et al., 2004). Endothelial-specific deletion of Krit1 early in life produces hemorrhagic vascular lesions in the cerebellum and retina that resemble CCMs (Boulday et al., 2011). Combined with the more global effect of the constitutive deletion, these data suggest that KRIT1 functions in multiple endothelial beds but at only specific developmental times. Constitutive global or endothelial-specific deletion of CCM2 produces virtually identical phenotypes to those of KRIT1 (Kleaveland et al., 2009; Whitehead et al., 2009). Similarly, in zebrafish, loss of krit1, ccm2, or heg (heart of glass) function leads to aberrant vascular morphogenesis (Hogan et al., 2008) and...
cardiac dilatation (Mably et al., 2003, 2006), and the vascular phenotypes in HEG1- and KRIT1-deficient mice are similar (Kleaveland et al., 2009). Therefore, KRIT1, CCM2, and HEG1 interact physically and genetically and play major roles in regulating vascular development and integrity.

KRIT1 contains a C-terminal band 4.1, ezrin, radixin, and moesin (FERM) domain and a series of N-terminal ankyrin repeats (Sahoo et al., 2001). FERM domains are comprised of F1, F2, and F3 subdomains (Fehon et al., 2010). KRIT1 FERM domain localizes KRIT1 to EC–cell junctions (Glading et al., 2007), and KRIT1 is required for the junctional localization of its binding partner, CCM2 (Stockton et al., 2010). KRIT1 F1 subdomain binds Rap1 in a GTP-dependent manner, and KRIT1–Rap1 interaction is important for KRIT1 localization in EC junctions and KRIT1 function (Liu et al., 2011). KRIT1 FERM F3 subdomain is predicted to comprise a phosphotyrosine-binding (PTB) domain. Many proteins bind to membrane proteins via their FERM domains. In cases in which structural data are available, the membrane protein cytoplasmic domain interacts with the F3 subdomain (Calderwood, 2004; Fehon et al., 2010). Here, we use protein crystallography to determine the structural basis of the KRIT1–HEG1 interaction. Surprisingly, the interaction differed from well-characterized FERM domain–membrane protein interactions (Calderwood, 2004; Anthis et al., 2009; Fehon et al., 2010) in which the F3 subdomain alone provides a binding site. Instead, the HEG1 binding interface was localized in a hydrophobic groove between the F1 and F3 subdomains of KRIT1. The biochemical relevance of this structure was validated by structure-based mutants of HEG1 or KRIT1 that disrupted their interaction; the biological relevance of the interaction was established by showing that KRIT1 mutants with reduced binding to HEG1, failed to localize to EC–cell junctions, and did not support cardiovascular development in zebrafish. These data reveal the structural basis of anchoring of KRIT1, the central scaffold of the CCM complex, to cell–cell junctions via HEG1, and establish the biological importance of this new form of FERM domain–membrane protein interaction.

Results and discussion

Defining the KRIT1–HEG1 interaction

The HEG1 cytoplasmic tail interacts with KRIT1 protein in the complex milieu of mammalian cell lysates (Kleaveland et al., 2009). To test whether HEG1–KRIT1 is a direct protein–protein interaction, we purified recombinant GST-fused KRIT1 FERM domain and HEG1 cytoplasmic tail (residues 1,274–1,381) proteins and found that HEG1 tail bound directly to the KRIT1 F123 protein (Fig. 1A). Coomassie blue-stained SDS-PAGE gels are representative of three experiments. All lanes were from the same gel. (B) HEG1, HEG1 (N1367A/F1370A), HEG1 C26, and HEG1 C26 (N1367A/F1370A) tail model proteins bind to recombinant GFP-KRIT1 F123 from HEK293 cell lysates. HEG1 a1,363 truncated tail model protein binds to KRIT1 F123 to a much lesser extent. Top section shows the sequence of HEG1 cytoplasmic tail and indicates the mutated residues. All lanes were from the same membrane. Bold letters refer to the mutated sequence. (C) Calorimetric titration of 600 µM HEG1 peptide, out of the syringe, into 40 µM KRIT1 FERM domain in the sample cell (Kd = 1.2 µM). The top line defines the background. The bottom line defines the data fitting.
of HEG1 (HEG1 C26) were sufficient for KRIT1 binding, the NPxY/F motif was dispensable. Mutating the N$^{1.367}$PSF$^{1.370}$ sequence to A$^{1.367}$PSA$^{1.370}$ (HEG1 NAFA and HEG1 C26 NAFA mutants) had no effect on KRIT1 binding (Fig. 1B). In contrast, removal of 19 residues 1,363–1,381 at the C terminus of HEG1 (HEG1 Δ1,363) drastically reduced KRIT1 binding, suggesting that the key determinant is the C-terminal 19 residues of HEG.

Isothermal titration calorimetry (ITC; Fig. 1C and Table 1) revealed that the KRIT1 FERM domain bound to HEG1 C26 with $K_d = 1.2 \pm 0.14 \mu M$. Thus, the HEG1 C-terminal 19 residues are the principal binding site for the KRIT1 FERM domain, and the N$^{1.367}$PSF$^{1.370}$ motif is dispensable for this interaction.

### Table 1. Thermodynamic parameters for binding of KRIT1 FERM domain to HEG1 cytoplasmic tail

| Clone       | $n$ | $K_d$ (µM) | $\Delta H^\circ$ (Kcal/mol) | $\Delta G^\circ$ (Kcal/mol) | $T \Delta S^\circ$ (Kcal/mol) |
|-------------|-----|------------|-----------------------------|-----------------------------|-----------------------------|
| Wild-type   | 1.01 | 1.2 ± 0.14 | −10.0                      | −8.1                        | −1.9                        |

$K_d = 1 / K_a$, $\Delta G^\circ = -RT \ln K_a$, $\Delta H = \Delta H^\circ + T \Delta S$. $K_a$, association constant; $\Delta H$, enthalpy changes; $\Delta G$, Gibbs energy changes; $R$, gas constant; $T$, absolute temperature; $\Delta S$, entropy changes.

### Structure of the KRIT1-HEG1 complex

The human KRIT1 FERM domain, KRIT1(417–736), was purified and crystallized with a recombinant human HEG1 peptide containing the 26 C-terminal residues. The structure (Protein Data Bank accession no. 3U7D) was solved and refined to a 2.5-Å resolution with a $R_{work}$ of 23.4% and $R_{free}$ of 30.9% (Table 2). The current KRIT1 model excludes residues 647–652 in the β1C–β2C loop, which are poorly defined in the electron density map (Fig. 2, A and B). Each asymmetric unit contained two KRIT1–HEG1 complexes that are almost identical, root-mean-square deviation = 0.6 Å, with distinct electron density visible for the C-terminal seven residues of the HEG1 tail. The last three residues (YF$^{1.379}$YF$^{1.381}$) showed the best electron density (Fig. 3A), whereas the first 19 residues were not observed, suggesting that they remained unstructured.

The KRIT1 FERM domain is similar to that found in ERM proteins and contains three subdomains arranged as a cloverleaf (Fig. 2B): F1 (residues 420–510; Fig. 2B, green and blue), F2 (residues 516–630; Fig. 2B, red), and F3 (residues 638–730; Fig. 2B, orange). The F1 domain has an ubiquitin-like fold (DALI server Z score = 7.1, root-mean-square deviation = 2.1 Å, Protein Data Bank no. 3NOB), although it has a novel helix α2A inserted in the β4A–β5A loop (residues 480–494; Fig. 2, A and B, blue helix). This helix, which is not observed in other ubiquitin-like folds, is kinked by $\sim 70^\circ$ in the middle because of the presence of Pro$^{388}$ breaking the hydrogen bond network (Fig. 3B). It is, however, kept into position by extensive hydrophobic contacts between Trp$^{487}$ and residues from the β3A to β5A strands. The F2 domain contains a core four-helix equivalent to that found in acyl-CoA–binding protein, and the F3 domain shares a fold of an adaptable ligand module seen for PTB, pleckstrin homology, and EHV1 (Enabled/ Vasodilator-stimulated phosphoprotein homology) domains.

### Table 2. Data collection and refinement statistics for the KRIT1 FERM domain in complex with the HEG1 cytoplasmic tail

| Crystallographic statistic | Complex (3U7D) |
|----------------------------|----------------|
| Data collection            |                |
| Space group                | P2$_1$         |
| Cell dimensions:           |                |
| a, b, c (Å)                | 73.1, 76.8, 79.2|
| α, β, γ (°)                | 90.0, 113.6, 90.0|
| Resolution (Å)             | 2.62–2.49      |
| $R_{merge}$                | 5.8 (31.2)     |
| $I/\sigma I$               | 5.3 (2.3)      |
| Completeness (%)           | 98.7 (97.9)    |
| Redundancy                 | 2.2 (2.2)      |
| Refinement                 |                |
| Resolution (Å)             | 2.49           |
| No. of reflections         | 26,420         |
| $R_{work}/R_{free}$        | 23.4/30.9      |
| No. of atoms               | 5,136          |
| Protein                    | 5,126          |
| Water                      | 10             |
| B factors                  | 50.3           |
| Protein                    | 50.3           |
| Water                      | 35.0           |
| R.m.s. deviations:         |                |
| Bond lengths (Å)           | 0.017          |
| Bond angles (°)            | 1.746          |

R.m.s., root-mean-square. Highest resolution shell is shown in parentheses.
pocket (Fig. 2 B). The KRIT1 β4A–α2A loop mainly interacts by hydrogen bonding with the HEG1 sequence (Fig. 3 A, left section), in which the backbone carbonyl group of KRIT1 Gln473 bonds to the backbone amide of HEG1 Phe1381, and KRIT1 Lys475 side chain hydrogen bond to the backbone carbonyl of HEG1 Asp1379. KRIT1 helix α2A forms a hydrophobic pocket (Fig. 2 B). The KRIT1 β4A–α2A loop mainly interacts by hydrogen bonding with the HEG1 sequence (Fig. 3 A, left section), in which the backbone carbonyl group of KRIT1 Gln473 bonds to the backbone amide of HEG1 Phe1381, and KRIT1 Lys475 side chain hydrogen bond to the backbone carbonyl of HEG1 Asp1379. KRIT1 helix α2A forms a hydrophobic pocket

Figure 2. Crystal structure of the KRIT1 FERM domain bound to the HEG1 cytoplasmic tail peptide reveals a new mode of binding. (A) Sequence of human KRIT1 [UniProt O00522] FERM domain with secondary structure elements shown below the sequence. (B) View of the KRIT1 FERM domain bound to the HEG1 peptide. The HEG1 peptide is shown in yellow. The KRIT1 FERM domain consists of three subdomains: F1 (green and blue), F2, and F3. The linkers F1–F2 (residues 511–515) and F2–F3 (residues 631–637) are colored in gray, and the new features of the F1 domain that are not present in ubiquitin and radixin are shown in blue, i.e., helix α2A (residues 480–494). (C) The HEG1 binding pocket at the KRIT1 F1 and F3 interface. The interaction is mainly formed by hydrogen bonds from residues in loop β4A–α2A and hydrophobic residues from helix α2A and α1C. (D) Surface electrostatic potentials of the KRIT1 FERM domain with the HEG1 peptide shown as a stick model. The HEG1 residues buried in the hydrophobic pocket are shown in yellow, whereas the charged residues exiting the pocket are shown in white.
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**Structure of the CCM membrane anchor**

(Fig. 3 B), composed of Ile<sup>490</sup> and Leu<sup>494</sup>, with Leu<sup>472</sup> from the βA strand to accommodate the aromatic ring of HEG1 Tyr<sup>1380</sup>. The HEG1 Tyr<sup>1380</sup> hydroxyl group is stabilized by hydrogen bonding with one of the indole nitrogens of KRIT1 His<sup>485</sup> (Fig. 3 A). The F3 domain contributes to the interaction mainly from KRIT1 residues Leu<sup>717</sup> and Leu<sup>721</sup> of helix αC completing the hydrophobic pocket surrounding the HEG1 C-terminal Tyr<sup>1380</sup> and Phe<sup>1381</sup> residues (Fig. 3 A). This structure represents a new mode of interaction of a FERM domain with a transmembrane protein and reveals the basis of the high affinity interaction of HEG1 with KRIT1. Furthermore, it differs from the classical PTB domain–peptide interaction (Smith et al., 2006) and explains the lack of dependence of the interaction on the HEG1 NPSF motif.

To test whether the HEG1 C-terminal aromatic dipeptide is required for binding to KRIT1, we formed an affinity matrix with an immobilized synthetic HEG1 C-terminal 19-residue peptide (HEG1-C19) or a truncated HEG1 peptide lacking the two C-terminal residues (HEG1-C19delYF). HEG1-C19 bound to KRIT1 FERM domain whereas, HEG1-C19delYF exhibited a >20-fold reduction in binding (Fig. 4 A). Thus, the C-terminal Tyr-Phe dipeptide is critical for HEG1–KRIT1 interaction.

**The biological role of the KRIT1–HEG1 interaction**

The structure described in this study created the opportunity to examine the functional importance of the KRIT1–HEG1 interaction. Specifically, KRIT1 Leu<sup>717</sup> and Leu<sup>721</sup> help establish the hydrophobic pocket that buries the C terminus of HEG1. In consequence, a KRIT1(L717,721A) mutant exhibited markedly reduced binding to the HEG1-C19 peptide as judged by affinity chromatography (Fig. 4 B). Furthermore, injection of 2.2 mM HEG1 peptide into a solution of 40 µM KRIT1(L717,721A) FERM protein did not generate significant heat in an ITC experiment, indicating a >100-fold reduction in affinity (unpublished data). KRIT1(L717,721A) FERM protein was well folded as judged by its sharp melting temperature of 49.5°C in differential scanning calorimetry (Fig. S1 A). Furthermore, we made the corresponding mutant in the full-length human and zebrafish KRIT1 (kr1(L714,718A)), and both proteins were well-expressed in HEK293 cells (Fig. S1, B and C). KRIT1 has other important binding partners, including CCM2 (Zawistowski et al., 2005) and Rap1 (Serebriiskii et al., 1997). KRIT1(L717,721A) mutant interacted with both CCM2 (Fig. 4 C) and Rap 1 (Fig. 4 D) to the same extent as the wild-type (WT) protein. Thus, the KRIT1(L717,721A) is well folded and expressed, exhibits markedly reduced affinity for HEG1, but maintains interactions with other known binding partners of KRIT1.

HEG1 is a transmembrane receptor that localizes at EC–cell junctions (Kleaveland et al., 2009) and may therefore interact with KRIT1 to localize KRIT1 to junctions. In human umbilical vein ECs (HUVECs), KRIT1 localized at EC–cell junctions (Fig. 4 E, top), whereas KRIT1(L717,721A) did not (Fig. 4 E, bottom). KRIT1(L717,721A) junctional localization was not restored by stabilization of junctions by inhibition of Rho kinase (Fig. S2). Both WT and mutant KRIT1 also exhibited nuclear and cytoplasmic localizations as previously reported (Glading et al., 2007).

**Figure 3. Structural details of the HEG1 binding pocket in KRIT1.** (A) Electron density of the HEG1 peptide in complex with the KRIT1 FERM domain. Electron density (2Fo-Fc map contoured at 1.2σ) of the HEG1 peptide. The HEG1 peptide is colored as in Fig. 2 C, and the KRIT1 FERM domain is as in Fig. 2 B. Some of the key KRIT1 residues are highlighted. (B) Close view of the novel helix α2A in the KRIT1 F1 domain. The helix is kinked by ~70° in the middle because of the presence of Pro<sup>488</sup>. The helix position is stabilized by hydrophobic contacts with residues from the β sheet. (C) The surface charge map of the KRIT1 FERM domain shows a basic surface at the F1–F2–F3 subdomain interface. The position of Arg<sup>452</sup> is highlighted as mutation of this residue to Glu reduces KRIT1 binding to Rap1.
Figure 4. Reduced binding of KRIT1 [L717,721A] to HEG1. [A] HEG1 C19 cytoplasmic tail model protein binds to recombinant GFP-KRIT1 FERM from HEK293 cell lysates. HEG1 C19delYF, which lacks the last two residues, does not bind to GFP-KRIT1 FERM. (bottom) The equal loading of tail proteins as judged by SDS-PAGE and Coomassie blue staining. Blots are representative of three experiments. [B] HEG1 C19 tail model protein binds to recombinant GFP-KRIT1 FERM from HEK293 cell lysates but not GFP-KRIT1 FERM[L717,L721A]. Both WT and mutant proteins are expressed at similar levels. All lanes were from the same gel. [C] Both GFP-fused KRIT1 WT and GFP-fused KRIT1[L717,721A] are associated with CCM2 at equivalent levels as assessed by coimmunoprecipitation and immunoblotting. All lanes were from the same gel. [D] Both GST-KRIT1 F123 and GST-KRIT1 F123[R452E] mutant serves as a negative control as it does not bind to Rap1. [E] HA-tagged KRIT1 WT colocalizes with VE-cadherin at HUVEC cell–cell junctions. In contrast, HA-KRIT1[L717,L721A] does not. HA-KRIT1, green. VE-cadherin, red. IP, immunoprecipitation; LALA, KRIT1[L717A,L721A]. Bar, 50 µm.
The KRIT1-HEG1 interaction is important for cardiovascular development and Rho signaling in ECs

Zebrafish embryos lacking heg or krit1 (also known as santa [San]) exhibit a similar dilated heart phenotype (Mably et al., 2003, 2006). To test whether the HEG1–KRIT1 interaction is important in zebrafish heart development, we coinjected krit1 WT or krit1(L714,718A) (the orthologue of human KRIT1 [L717,721A]) cRNAs and San morpholino (MO) into zebrafish embryos at the one-cell stage and assessed cardiac dilation at 48 h postfertilization (hpf) as previously described (Liu et al., 2011). Injecting San MO alone resulted in heart dilation in 95% of zebrafish embryos. Authentic dilation was verified by indentifying the endocardium in the fluorescence images, which is indicated by the dotted lines. All microscopic images were taken at 48 hpf. Bars, 500 µm. (B) Bar graphs showing effects of Krit1 [L714,718A] mutant on zebrafish cardiovascular development. Data are expressed as number of embryos without dilated heart phenotype divided by total number of embryos used per experiment × 100; means ± SD. *, P < 0.05 compared with San MO + HA-WT cRNA group. Data are from three independent experiments. Total number of animals used: 94 in San MO + HA-WT group and 71 in San MO + HA-Krit1[L714,718A] group. (C) Both HA-tagged zebrafish Krit1 WT and Krit1[L714,718A] proteins were expressed at similar levels in injected embryos as revealed by immunoprecipitation with a rabbit anti-HA antibody from zebrafish embryo lysates and immunoblotting with a mouse anti-HA antibody. (D) Effect of disruption of the HEG1–KRIT1 interaction on RhoA/Rho kinase signaling in EC. Silencing of KRIT1 caused a marked increase in myosin light chain phosphorylation that was not reversed by expression of KRIT1 [L717,721A]. (E) Extent of KRIT1 depletion and reconstitution in the experiment depicted in D. IP, immunoprecipitation; LALA, KRIT1[L717A,L721A]; MLC myosin light chain.
20% reversal of the dilated heart phenotype. Both WT and krit1(L714,718A) zebrafish krit1 cDNAs contained an N-terminal HA tag, and similar protein expression levels were confirmed by immunoprecipitation blot from zebrafish embryo lysates (Fig. 5 C). Silencing KRIT1 in HUVECs increased RhoA/Rho kinase signaling in HUVECs as judged by increased myosin light chain phosphorylation, and coexpressing an siRNA-resistant KRIT1 reversed this effect. In contrast, expressing siRNA-resistant KRIT1(L717,721A) did not reduce myosin light chain phosphorylation (Fig. 5, D and E; and Fig. S3). KRIT1(L717,721A) expression in the absence of KRIT1 silencing did not impair zebrafish heart development (not depicted) or increase RhoA/Rho kinase signaling in HUVECs (Fig. S4). Thus, the direct interaction between HEG1 and KRIT1 is important for zebrafish cardiovascular development and suppression of RhoA signaling in EC.

Additional implications of the KRIT1-HEG1 mode of interaction

Our data show that KRIT1 interacts with the HEG1 cytoplasmic tail in an unusual manner and establish a structural mechanism for HEG1 to recruit KRIT1, and potentially CCM2, to cell–cell junctions. The HEG1 cytoplasmic tail is 111 amino acids long and has little predicted secondary structure, and we show here that KRIT1 binds strongly at the C terminus. If fully extended, the HEG1 tail could reach distances of ~30 nm, which could permit it to recruit KRIT1 from afar after KRIT1 release from intracellular sites such as microtubules by Rap1 (Béraud-Dufour et al., 2007). A common structural feature shared by PTB domains, such as KRIT1 F3, is the presence of a peptide binding pocket at the B5-α1 interface, which often engages peptides containing NPxY/F motifs, and we note that when HEG1 is bound, this F3 peptide binding pocket remains accessible (Fig. 2 B). Because KRIT1 is autoinhibited by the binding of KRIT1 N terminus NPxy/F motifs to F3 via this binding pocket (Béraud-Dufour et al., 2007; Francalanci et al., 2009), our results explain how HEG1 can efficiently bind to full-length, presumably autoinhibited, KRIT1 (Liu et al., 2011). Alternatively, it is possible that the F3 subdomain of HEG1-bound KRIT1 can simultaneously engage other membrane proteins to create membrane microdomains at cell–cell junctions.

Materials and methods

Cell culture and transfection

HeK293 cells were maintained in Dulbecco’s modified Eagle’s medium (Cellgro) supplemented with 10% FBS (Sigma-Aldrich), 1% nonessential amino acids, 1% l-glutamine, and 1% penicillin and streptomycin (all obtained from Invitrogen). HEK293 cells were transfected using lipofectamine Plus (Invitrogen) according to the manufacturer’s protocol. HUVECs (ScienCell) were maintained in EC medium with the manufacturer’s supplements (ScienCell). HUVECs were transfected using Nucleofector II and HUVEC Nucleofector kit (both obtained from Lonza) according to the manufacturer’s protocol.

Antibodies, cDNAs, siRNA, and reagents

Monoclonal anti-HA and monoclonal anti-FLAG M2 antibodies (both obtained from Sigma-Aldrich) were used for immunoblotting at 1:4,000. Rabbit polyclonal anti-HA antibody (Takara Bio Inc.) was used for immunoprecipitation. Mouse monoclonal anti-KRIT1 (15B2) and rabbit polyclonal anti-KRIT1 (6832) antibodies were developed using recombinant KRIT1 FERM domain as the antigen as previously described (Liu et al., 2011). Anti-KRIT1 15B2 antibody was used for immunoprecipitation. Anti-KRIT1 6832 antibody was used for immunoblotting at 1:1,000. Polyclonal anti-GFP antibody (Takara Bio Inc.) was used for immunoprecipitation. Monoclonal anti-GFP antibody (Takara Bio Inc.) was used for immunoblotting at 1:5,000.

Human KRIT1 cDNAs encoding HA-, GFP-, and GST-tagged full-length KRIT1 and KRIT1 FERM domain were previously described (Liu et al., 2011). In brief, HA, GFP, and GST-tagged constructs were cloned in pCDNA3.1(−) (Invitrogen), pEGFP-C1 (Takara Bio Inc.), and pGEX2TK vectors, respectively. Human KRIT1(L717,721A) mutant was generated by site-directed mutagenesis using the site-directed mutagenesis kit (QuikChange; Agilent Technologies). The mutant was then subcloned into pGEX2TK (OE Healthcare), pCDNA3.1 (Invitrogen), and pEGFP-C1 (Takara Bio Inc.) vectors for expressing GST, HA-, and GFP-tagged proteins, respectively. Zebrafish kr1t1 cDNA was a gift from B. Weinstein (National Institutes of Health, Bethesda, MD) and subcloned into pcDNA3.1 vector with an N-terminal HA tag as previously described (Liu et al., 2011). Zebrafish kr1t1 (L714,718A) mutant was generated using the QuikChange site-directed mutagenesis kit. pk3c-CMM2 encoding FLAG-tagged human CCM2 has been previously described and was the gift of D. Marchuk (Duke University, Durham, NC; Zawistowski et al., 2005). pMT2-HA-Rap1 A(G12V) was a gift from J. Bos (University Medical Center Utrecht, Utrecht, Netherlands) as previously described (Liu et al., 2011). The recombinant human HEG1 cytoplasmic tail model protein affinity matrix was previously described (Liu et al., 2011). HEG1 tail truncation mutants, C-26 and C-19, were generated by PCR from the HEG1 full-length tail construct and subcloned into the same pHisAvi vector. HEG1 tail (N1367A, F1370A) mutant was generated using the QuikChange site-directed mutagenesis kit.

In vitro protein interaction assay

Bacterial expression plasmids encoding GST-KRIT1 FERM or GST vector were expressed in BL21 (DE3) (EMD Millipore), and recombinant proteins were purified using glutathione–Sepharose beads according to manufacturer’s instructions (GE Healthcare). HEG1 and vHb intracellular tail model proteins were prepared as previously described (Pfaff et al., 1998). In brief, His6-tagged HEG1 and vHb intracellular tails containing an in vivo biotinylation peptide tag at the N terminus were cloned into pET15b. Tail proteins were expressed and purified from Escherichia coli. Pull-down assay using cell lysates were previously described (Liu et al., 2011). In brief, HEK293 cells were transfected with the indicated cDNAs. 24 h after transfection, cells were lysed in cold lysis buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 0.5% NP-40, and 5 mM MgCl2) plus protease inhibitor cocktail (Roche). A total of 10 µg of immobilized bead-bound proteins was added to 350 µg of clarified cell lysates. Reactions were kept at 4°C overnight. After washing the beads with lysis buffer, samples were fractioned on 4–20% SDS-PAGE gel (Invitrogen). Bound proteins were analyzed by immunoblotting or Coomassie blue staining.

Immunoprecipitation

HEK293 cells were transfected with indicated cDNAs and incubated for 24 h. Cells were scraped on ice in lysis buffer plus protease inhibitor cocktails (Roche). A total of 2 µg monoclonal anti-GFP antibody was added to 350 µg cell lysates and incubated at 4°C overnight. Protein G–Sepharose (Invitrogen) was added to the reaction mixture and further incubated for 4 h at 4°C. After three washes with lysis buffer, beads were mixed with sample buffer and subjected to SDS-PAGE. Immunoprecipitated proteins were detected by immunoblotting.

ITC

Proteins were dialyzed into the ITC buffer (20 mM sodium phosphate, pH 6.5, and 150 mM NaCl). The thermodynamic parameters are determined using an isothermal titration calorimeter (ITC 200; MicroCal) at 25°C in 20 mM sodium phosphate, pH 6.5, and 150 mM NaCl. For binding, 0.6 or 2.2 mM recombinant human HEG1 C26 peptide (residues 1,356–1,381) was titrated from the syringe into the sample cell containing 40 µM KRIT1 FERM domain WT or KRIT1 FERM (L717,721A) mutant protein, respectively. Titration was performed by injecting volumes of 2.5 µl into the sample cell, during which the time between injections was 2 min. Further data evaluation was performed using the Origin program (MicroCal).

Protein crystallization

Human KRIT1 residues 417–736 were expressed and purified as described previously (Liu et al., 2011). In brief, KRIT1 was cloned into the expression vector pLEICS-07 (Protex) and expressed in E. coli BL21 Star...
Zebrafish experiments

Tg(krt1:EGFP) Danio rerio (zebrafish) embryos were raised at 28.5°C.

Online supplemental material

Fig. S1 shows that krt1(L177,212A) mutant protein is well folded and its expression in HEK293 cells is similar to WT. Fig. S2 shows that krt1(L177,212A) junctional localization was not restored by stabilization of junctions by inhibition of Rho kinase. Fig. S3 quantifies the phospho–myosin light chain increase caused by krt1 deletion in HUVECs. Fig. S4 shows that krt1(L177,212A) did not increase RhoA/Rho kinase signaling in HUVECs. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201205109/DC1.

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