The mechanism of activation of the actin binding protein EHBP1 by Rab8 family members

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EHBP1 is an adaptor protein that regulates vesicular trafficking by recruiting Rab8 family members and Eps15-homology domain-containing proteins 1/2 (EHD1/2). It also links endosomes to the actin cytoskeleton. However, the underlying molecular mechanism of activation of EHBP1 actin-binding activity is unclear. Here, we show that both termini of EHBP1 have membrane targeting potential. EHBP1 associates with PI(3)P, PI(5)P, and phosphatidylserine via its N-terminal C2 domain. We show that in the absence of Rab8 family members, the C-terminal bivalent Mical/EHBP Rab binding (bMERB) domain forms an intramolecular complex with its central calponin homology (CH) domain and auto-inhibits actin binding. Rab8 binding to the bMERB domain relieves this inhibition. We have analyzed the CH:bMERB auto-inhibited complex and the active bMERB:Rab8 complex biochemically and structurally. Together with structure-based mutational studies, this explains how binding of Rab8 frees the CH domain and allows it to interact with the actin cytoskeleton, leading to membrane tubulation.

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**EHB1** was originally identified as an Eps15-homology domain-containing protein 1/2 (EHD1/2) interacting partner that plays a central role in GLUT4 transport and couples endocytic vesicles to the actin cytoskeleton\(^1,2\). EHB1 co-localizes with the actin cytoskeleton and overexpression of either EHB1 or EHD2 leads to extensive actin reorganization\(^2\). Disruption of EHB1/EHDs by siRNA leads to inhibition of transferrin endocytosis and GLUT4 transportation\(^3\). The Ras superfamily GTPase Rab10 has also been shown to regulate the translocation of GLUT4 in adipocytes\(^4\). In our previous work, we showed that EHB1 is an effector molecule for Rab8 family members, including Rab10, and forms complexes with 1:1 stoichiometry\(^4,5\). Recent work has also shown that a Rab10-EHB1-EHD2 trimeric complex plays a crucial role in lipid droplet engulfment during lipophagy in hepatocytes\(^6\). Moreover, *Caenorhabditis elegans* EHB1 promotes endosomal tubulation by linking the membrane lipid PI(4,5)P\(_2\) to the actin cytoskeleton and this interaction is enhanced upon Rab10 binding\(^7\).

Apart from having roles in vesicular trafficking and autophagy, EHB1 is implicated in early development and cancer. In *C. elegans*, EHB1 depletion leads to an endocytic recycling defect in the intestine and in non-polarized germline cells and the phenotype was recapitulated upon Rab8/10 deletion\(^8\). *Drosophila* EHB1 has been shown to play an essential role in eye development by regulating the exocytosis of Scabrous, a positive regulator of Notch signaling\(^9\). Notch signaling has been implicated in metastatic prostate cancer, and a genome-wide SNP association study shows that EHB1 is associated with aggressive disease\(^10\)-\(^13\). EHB1 controls the invasiveness of PTEN-positive prostate cancer cells and is essential for the anti-invasive effect of the drug atorvastatin\(^14\).

Despite having information on EHB1 at the functional level, convincing biochemical data on EHB1 activation are missing. In this work, we have identified and characterized an autoinhibited state of human EHB1, which is mediated by an intramolecular association between the CH domain and the bMERB domain. We have elucidated the structure of the CH domain in complex with the bMERB domain, providing an explanation for the specificity of EHB1\(_{bMERB}\) toward its CH domain. Transient kinetic experiments show that the binding of Rab8 family members to the bMERB domain releases the CH domain. Furthermore, we have solved the crystal structure of the bMERB domain in complex with human Rab8a. Structural analyses supported by mutagenesis and biochemical experiments identify key residues for the interaction and explain why the generation of a stable CH-bMERB-Rab8 ternary complex is not possible. Our biochemical and structural data suggest that the interaction between the C-terminal bMERB domain and the central CH domain has a regulatory role in the function of EHB1 and binding of Rab8 family members to the bMERB domain releases the CH domain, which can then interact with the actin cytoskeleton.

**Results**

**Domain architecture and localization of EHB1.** EHB1 consists of an N-terminal C2 domain, a central CH, a C-terminal bMERB domain, and a CaaX box at the C-terminus that is a substrate for FTerase\(^4\). The UniPort database has reported three EHB1 isoforms and for biochemical studies, the domain boundaries are taken from isoform 1 (Q8NDI1-1), whereas for cellular localization experiments, fluorescent constructs are based on isoform 3 (Q8NDI1-3) (Fig. 1a). Previously, we have reported that the bMERB domain, together with the CaaX box, is sufficient to target an EGFP-fusion protein to the endosome and co-localize with active Rab8/10\(^4\). Here, we show that the full-length protein, as well as the construct lacking the NT-C2 domain, is targeted to structures that appear to be endosomes. Surprisingly, the EGFP-EHB1\(_{bMERB}\) construct is cytosolic, suggesting that even in the full-length background, the CaaX box is dispensable for endosomal localization. However, this could be due to the presence of the N-terminal EGFP tag, which could hinder membrane association via the NT-C2 domain. To rule out this possibility, we expressed an isolated NT-C2 EGFP (C-terminal) fusion construct and showed that this construct is cytosolic as well as having the potential to associate with membranous structures (Fig. 1b). Thus, our localization data suggest that both termini of EHB1 have membrane targeting potential. Further, we could show that the full-length EHB1 co-localizes with Rab8/10 active constructs (Fig. 1c, d).

**C-terminal high affinity Rab-binding site of bMERB domain.** Previously, we reported that the EHB1 bMERB domain preferentially binds to Rab8 family members\(^4\). In this work, we have used a smaller version of the bMERB\(_{1060-1212}\) domain containing all three predicted \(\alpha\)-helices\(^4\), which, consistent with our prior work, forms a stable complex with Rab8a with a similar affinity to the longer bMERB\(_{1047-1220}\) construct. Earlier, we reported that some bMERB family members have two Rab-binding sites, a high-affinity C-terminal binding site, and a lower or similar affinity N-terminal binding site\(^4,5\). However, in the case of the EHB1 bMERB domain, we observed only a single-binding site\(^4\). To localize the exact binding site, we made two deletion constructs in which either the N or the C-terminal helix was deleted (Fig. 2a, insets). Both the full-length as well as the N-terminally truncated constructs form stable complexes with Rab8a as observed by analytical size exclusion chromatography (aSEC) experiments. Further, isothermal titration calorimetry (ITC) measurements show that both bMERB constructs bind to Rab8a: GppNHp with a \(K_D\) value of 0.3 \(\mu\)M (Fig. 2b). In contrast, we could not detect any interaction with the (potential) N-terminal low-affinity binding site.

**Next, we independently measured the association (\(k_{on}\)) and dissociation rate (\(k_{off}\)) constants for \(K_D\) calculation (\(K_D = k_{off}/k_{on}\)). For \(k_{on}\) measurements, association kinetics of Rab8a loaded with fluorescent 2′,3′-O-(N-methyl-anthraniloyl) mantGppNHp were monitored with increasing concentrations of the bMERB\(_{1060-1212}\) domain using a stopped-flow apparatus. \(k_{on}\) for Rab8a\(_{mantGppNHp}\) was 2.79 \(\times\) 10\(^7\) M\(^{-1}\) s\(^{-1}\) and the intercept on the \(y\)-axis yielded the \(k_{off}\) (13.4 s\(^{-1}\)) (Fig. 2c). Direct \(k_{off}\) was determined by the displacement of the Rab8a\(_{mantGppNHp}\) from the bMERB\(_{1060-1212}\) domain by an excess of Rab8a\(_{mantGppNHp}\) \(k_{off}\) of 13.8 s\(^{-1}\) was observed (Fig. 2d) and \(K_D\) value of 0.48 \(\mu\)M was calculated, in reasonable agreement with the value of 0.31 \(\mu\)M obtained in ITC experiments.
Phosphomimetic mutation of Rab8/10 switch II threonine. LRRK2, a serine/threonine kinase phosphorylates threonine of switch II of Rab8a/10. Phosphorylated Rabs are GDI resistant, thus increasing their lifetime on the membrane. Further, phosphorylation also increases Rab8a/10 binding to the effector proteins RILPL1/L2. Structure-based sequence alignment shows that the switch II threonine of Rab8a/10 does not directly interact with Mical1bMERB and Mical clbMERB (Fig. 2c). However, it is still unknown whether phosphorylation of Rab8a/10 has any effect on the EHBP1bMERB interaction. Therefore, we prepared the phosphomimetic mutants Rab8aT72E/10T73E by site-directed mutagenesis and checked for complex formation.
with the bMERB domain by aSEC/ITC experiments (Fig. 2f and Supplementary Fig. 1). No effect of phosphomimetic mutations of Rab8a/10 on the EHBPI\textsubscript{bMERB} interaction was observed.

CH and bMERB domain association and release by Rab8. It has been suggested that some bMERB family members exist in an auto-inhibited state in the absence of Rab and that the bMERB domains have to be released or exposed for activation to occur\textsuperscript{19-24}. Since it was difficult to investigate a possible intramolecular interaction between the CH and bMERB domains of EHBPI using constructs containing both domains, we examined the interaction between the separately purified bMERB and CH domains of EHBPI, initially employing aSEC experiments. Clear complex formation with the CH domain was observed for the construct lacking the N-terminal helix of the bMERB domain (Fig. 3a). However, in the case of the full-length construct, only a partial shift in the CH domain peak was observed, indicating a weaker equilibrium between free and bMERB-bound CH domains. No complex formation was observed for the construct lacking the N-terminal helix (Fig. 3a). Using ITC measurements, binding was observed between full-length bMERB domain and CH domains and similar to Rab8a, CH domain binding to bMERB domain is an enthalpy driven process with a $\Delta H$ value of 1.2 $\mu$M and stoichiometry of 1:1. The C-terminally truncated construct showed a slightly higher affinity ($K_D$ = 0.78 $\mu$M), whereas no binding was observed for the construct lacking the N-terminal helix, clearly indicating that helices 1 and 2 constitute the CH-binding site (Fig. 3b).

Using transient kinetic measurements, we determined the $k_{on}$ and $k_{off}$ rate constants for the CH and full-length bMERB domain interaction. The observed pseudo first order association rates of Cy3-CH were plotted against increasing concentrations of the bMERB domain and a $k_{on}$ of 2.76 $\times$ 10$^7$ M$^{-1}$ s$^{-1}$, while $k_{off}$ was 103.7 s$^{-1}$ as obtained from the y-axis intercept (Fig. 3c). $k_{off}$ was also measured directly by displacing Cy3-CH from the bMERB domain by mixing with an excess of unlabeled CH domain, leading to a value of 117 s$^{-1}$ (Fig. 3d). These values led to a calculated $K_D$ value of 3.75$\pm$2.43 $\mu$M. This is higher than the value obtained by ITC, suggesting some interference with bMERB binding by the Cy3 label. We note that the association rate constant for CH and Rab8a binding to bMERB is nearly identical, whereas the $k_{off}$ for CH is ca. ten times higher than for Rab8a (Fig. 2c, d).

To test whether binding of Rab8a to the bMERB domain can release the CH domain, we generated the Cy3-CH:bMERB complex and mixed it rapidly with an excess of Rab8a. This led to an increase in fluorescence intensity, indicating that Rab8a can indeed displace the CH domain and $k_{off}$ was 162 s$^{-1}$ (Fig. 3e), which is significantly larger than that for spontaneous dissociation (Fig. 3d), suggesting an active displacement mechanism via a ternary complex between the three proteins.

In similar experiments using different bMERB domains and their respective CH, LIM, or CH-LIM domains, no complex formation was detected using aSEC/ITC experiments (Fig. 3f and Supplementary Fig. 2).

The CH domain interacts with actin filaments. To serve as an actin-binding domain (ABD), a tandem repeat of CH1 and CH2 is usually required\textsuperscript{25}. The CH1 domain directly interacts with F-actin, while CH2 plays a supporting role\textsuperscript{26}. The human EHBPI CH domain is quite similar to the CH2 domain of alpha-actinin4 (36% identity), which usually has a lower actin-binding affinity; however, the C. elegans CH\textsubscript{EHBP1} domain was shown to interact with actin filaments\textsuperscript{7}. Using actin co-sedimentation assays, we could demonstrate an interaction with the human EHBPI CH domain, with a $K_D$ value of 9.34$\pm$1.86 $\mu$M (Fig. 4a, b). The affinity is relatively high for a single CH2 domain; for alpha-actinin and utrophin isolated CH2 domains a $K_D$ of $>1000$ $\mu$M was reported\textsuperscript{27,28}.

A similar interaction of the CH domain from Micals/Mical-like family members could not be detected (Fig. 4c).

The overall structure of the CH:bMERB complex. Although crystals of the EHBPI CH:bMERB diffracting to 4.0 Å were obtained, these were twinned and the crystal quality could not be improved. Since we had noted earlier that helices 1 and 2 of the bMERB domain are sufficient to form a complex with the CH domain, we then used the bMERB\textsubscript{H1-2}:CH complex and obtained crystals (SG: P 2$\text{\_}1$) diffracting to 2.2 Å. The structure was solved as described in material and methods (Data and refinement statistics are shown in Supplementary Table 1).

The asymmetric unit contained two copies of the CH: bMERB\textsubscript{H1-2} complex, sharing the same overall architecture (Supplementary Fig. 3a, b). According to a DALI search\textsuperscript{29} against the protein data bank, the EHBPI CH domain is most similar to the CH2 domains of alpha-actinin\textsuperscript{430} and beta-spectrin\textsuperscript{31} (Supplementary Fig. 3c). As expected, the bMERB\textsubscript{H1-2} is composed of two helices and the CH domain adopts a similar fold to the corresponding free EHBPI CH domain (PDB 2D89\textsuperscript{32} Supplementary Fig. 3c). The interface of the bMERB\textsubscript{H1-2}:CH complex shows both hydrophobic and hydrophilic interactions with a buried surface area of 593 $\AA^2$ (Fig. 4d, e). Most of the interactions with the CH domain lie on $\alpha$-helix 2, with some additional interacting residues provided by $\alpha$-helix 1 (Fig. 4d). An array of hydrophobic residues including L1099, M1103 of $\alpha$-helix 1 and M1116, W1119, F1120 and V1123 from $\alpha$-helix 2 forms a contiguous hydrophobic patch on the bMERB surface with extensive contacts to L534, M537, and Y541 of the C-terminal helix of the CH domain (Fig. 4d, e). Besides these hydrophobic interactions, several polar interactions were observed at the CH:bMERB binding interface, including D532CH \textsubscript{R1100}, T535CH \textsubscript{R1100}, T538CH \textsubscript{W1119},
Y541CH-N1124, and Q542CH-N1127. The Y541CH side chain also forms a hydrogen bond with the backbone carbonyl of F1120, and the R1131 side chain forms another hydrogen bond with the carbonyl of G549CH (Fig. 4d inset). Some of the interface residues are conserved in different bMERB family members (Fig. 4f).

Key elements of the CH:bMERB interface. To identify the crucial key residues required for complex formation, we purified a series of CH as well as bMERB domain mutants and checked their interaction using ITC measurements. Beginning with the C-terminal interacting residues of the CH domain, we could show that mutation of the conserved D532 to alanine leads to a more...
Fig. 2 Rab8 preferentially binds to the C-terminal Rab-binding site of the bMERB domain. a The bMERB domain (green), GppNHp Rab8a1-176 (gray), and a mixture of both (blue) were loaded onto a Superdex 75 10/300 GL column and monitored for complex formation. Complex formation was observed in the case of the full-length domain (indicated as green helices in the inset) as well as a construct lacking the N-terminal helix, indicating that helices 2-3 are crucial for Rab8aGppNHp interaction, clearly showing that only the high-affinity C-terminal Rab8 binding site is present in the EHBPI bMERB domain. b Binding affinities were measured by titrating GppNHp Rab8a1-176 (500 μM) to the bMERB domain (50 μM). Integrated heat peaks were fitted to a one-site-binding model yielding the binding stoichiometry (N), the enthalpy (ΔH), the entropy (ΔS), and the dissociation constant (Kd). The data are representative of at least three repetitions. N.D. denotes not detected. c Observed pseudo first order association rate constants between 0.5 μM mntGppNHp Rab8a1-176 and different concentrations of the bMERB domain (1.5-8 μM). Association was monitored by the change in fluorescence intensity using a stopped-flow apparatus at 25 °C. Association of the bMERB domain with mntGppNHp Rab8a1-176 leads to an increase in intensity. As an example of the data obtained, the association between 0.5 μM mntGppNHp Rab8a1-176 and 4 μM of the bMERB domain is shown in the inset. d Dissociation of Rab8a from the bMERB domain was monitored using the decrease of fluorescence after mixing a complex of mntGppNHp Rab8a1-176 with the bMERB domain (2 μM) with a 20-fold excess of unlabeled GppNHp Rab8a1-176. e Sequence alignment of Rab8a/10, using Clustal Omega. Switch I/II regions are indicated in red and light blue colors, respectively. The residues involved in binding with the Mical cL bMERB domain are denoted by gray (Rab8a) and magenta (Rab10), and T72/73 phosphorylated by LRRK2 is shown in the orange box. f Phosphomimetic mutation of switch II threonine does not affect bMERB binding of both Rab8 and Rab10. Binding affinities were measured by ITC experiments. The data are representative of at least three repetitions.

than 40-fold reduction in binding affinity (Fig. 5d), while mutation of L534 and T538 completely abolishes the interaction with the bMERB domain (Fig. 5e, f). However, mutation of M537, Y541, and Q542 to alanine only led to minor decreases in affinity (Fig. 5g–i). The side chain of L534CH inserts itself into the pocket created by L1099, R1100, M1103, and M1116 of the bMERB domain. D532, T535, and T538 of the CH domain stabilize the hydrophobic surface of the bMERB domain, which seems to be crucial for the interaction (Fig. 4d, e and Fig. 5a, b). Sequence alignments of the CH domain from EHBPI and Mical family members show that only D532 and L534 are conserved, whereas the essential T538 is not conserved (Supplementary Fig. 4f).

Conversely, we mapped the effect of the continuous hydrophobic surface (Fig. 5b) of the full-length bMERB domain by mutating M1103, M1116, W1119, F1120, and L1099/R1100 (LR motif)4 to alanine. Each mutation led to a significant decrease in binding affinity with wild type CH domain (Fig. 5j–n), indicating that indeed the continuous patch of hydrophobic residues is crucial for the interaction (Fig. 5j–n and Fig. 4d, e). Interestingly, sequence alignment of the bMERB domains from EHBPI and Mical family members shows that the LR motif and total hydrophobicity of the binding interface is quite conserved in EHBPI and Micals. However, it appears that small changes in the amino acid composition may determine the specificity, as seen for the crucial non-conserved M1103 (Fig. 4e and Supplementary Fig. 3e).

Subsequently, we investigated whether a mutation at the CH-binding site of the bMERB domain has any effect on Rab8 binding and could show that bMERB hydrophobic mutants still form stable complexes with Rab8a with similar affinity (Supplementary Fig. 4). Since we were not able to obtain wild type bMERB:Rab8a crystals, we attempted to crystallize these mutants: Rab8a complexes, finally succeeding with the mutants of the full-length bMERB:Rab8a and bMERB:F1120A:Rab8a.

Structure of the bMERB:Rab8a complex. In order to investigate how the binding of Rab8a releases the CH domain from the bMERB domain, we aimed to determine the structure of a full-length bMERB domain in complex with Rab8a. We were able to solve the structures of the bMERB:M1116A:Rab8a and bMERB:F1120A:Rab8a complexes to resolutions of 1.914 Å and 2.0 Å, respectively, as described in materials and methods. The complex structures are quite similar, and we describe the bMERB:M1116A:Rab8a structure in detail. Two copies with an overall similar architecture of bMERB:M1116A:Rab8a complex per asymmetric unit were observed (Supplementary Fig. 5a, b). Consistent with previously reported bMERB structures, the EHBPI bMERB domain displays the same three helical–fold organization4,24 and helices 1–2 of the bMERB:M1116A domain have a slightly different conformation in both copies, indicating that this part is somewhat flexible (Supplementary Fig. 5b); however, the Rab8a binding site adopts the same conformation in both copies. The EHBPI bMERB domain shows an RMSD of 2.3 Å for 122 residues to MicalM1 (PDB 5SZI), RMSD of 2.5 Å for 120 residues to MicalM2 (PDB 5SZG) and RMSD of 3.6 Å for 119 residues to MicalM3 (PDB 5LPN), whereas Rab8a is quite similar to Rab8a/10 of MicalM1bMERB:Rab8a /MicalM2bMERB:Rab10 complex (RMSD 0.6 Å) (Supplementary Fig. 5c–e).

Similar to previously reported bMERB:Rab complex structures4, the major interactions between Rab8a and bMERB4,24 involve α-helix 3, and some additional interaction surface is provided by residues from α-helix 2. Hydrophobic side chains of Rab8a switch I (I141 and I143) and switch II (F70, I73, and Y77) are buried in a hydrophobic core of the interface formed by α-helices 2–3 (Y1149, L1156, L1160, L1179, L1182, and V1183) (Fig. 6c, d). A conserved triad of aromatic amino acids (F45, W62, and Y77) also forms hydrophobic interactions with V1186 and L8, F45, and I47 of Rab8a interact with V1193 of the bMERB domain (Fig. 6b and Supplementary Fig. 5b). Besides these interactions, the inter-switch region (F45, I47, Q60, and W62) and Rab subfamily motif1 (RabSF1, Y5, and L8)33 of Rab8 also interact with the bMERB:EHBPI domain. Several polar interactions were observed between Rab8a and the bMERB domain, including side chains of T4Rab8a-E1200, Y5Rab8a-D1197, D44Rab8a-R1189, D44Rab8a-Y1149, Q60Rab8a-D1190, R69Rab8a-Q1176, Y77Rab8a-N1187, and the F45Rab8a backbone carbonyl forms a hydrogen bond with R1189 side chain (Fig. 6a–d). T72Rab8a is not involved in any direct interaction with the bMERB domain, explaining why mutant T72E has no effect on bMERB:EHBPI binding (Supplementary Fig. 5b).

bMERB Ct-hydrophobic patch is crucial for Rab8 interaction. Previously, we have shown that the N-terminus of Rab8 family members provides specificity with respect to interaction with bMERB4. Here, we sought to determine the contribution of essential bMERB residues that are necessary for Rab8 interaction. Similar to the CH-binding site, a robust cluster of hydrophobic residues was observed in the bMERB:Rab-binding interface (Fig. 6a–d) and to test the importance of this hydrophobic patch, we mutated the leucine residues of bMERB to serine and valine to alanine (Fig. 6c, d). The L1156S mutation leads to a 47-fold reduction in affinity (Fig. 6f); L1156 is part of the LR motif that is conserved in bMERB family members (Supplementary Fig. 3e). L1156 has only one close hydrophobic contact, with I141 of Rab8a. It nevertheless appears to be essential for maintaining the
integrity of the hydrophobic patch formed by helices 2 and 3 of the bMERB domain. The effect of L1160S on Rab binding was less pronounced, leading to a 2–3-fold reduction in binding affinity (Fig. 6g). However, mutations L1179S and L1182S led to a tenfold decrease in binding affinity (Fig. 6i–j). We could not detect binding in the case of the V1183A mutant that is involved in hydrophobic interactions with I43, I73, and Y77 (Fig. 6k). Altogether, our data suggest that the continuous C-terminal hydrophobic patch on bMERB is essential for the formation and stability of the Rab-binding site. Besides the hydrophobic patch residues, we also mutated several polar residues of the bMERB domain to alanine. Mutation of Q1176A does not have any effect (Fig. 6h). V1186A displays a more than 30-fold reduction in binding affinity (Fig. 6l) and this residue forms hydrophobic interactions with a conserved triad of aromatic amino acids (F45, W62, and Y77). Mutation of N1187A and R1189A led to a 5 and
Fig. 3 Interaction between the CH and the bMERB domain of EHBP1 and disruption by Rab8. a The bMERB domain (green), CH domain (orange), and a mixture of both (blue) were loaded onto a Superdex 75 10/300 GL column to monitor for complex formation. Clear complex formation was observed for the construct lacking the C-terminal helix (indicated as green helices in the insets). However, in the case of the full-length construct, only a shift in the CH domain peak was observed, indicating distribution between free and EHBP1 bMERB bound CH domain at the concentrations used. No complex formation or shift in the CH peak was observed for the construct lacking the N-terminal helix. b Binding affinities were measured by titrating the CH domain (800 µM) to either the full length or N/C-terminally truncated bMERB domain (60 µM). Integrated heat peaks were fitted to a one-site-binding model yielding the binding stoichiometry (N), the enthalpy (ΔH), and the entropy (ΔS), and the dissociation constant (Kₐ). The data are representative of at least three repetitions. c Observed association first-order rate constants between 0.5 µM Cy3 labeled CH domain with different concentrations of full-length bMERB domain (2–16 µM). Kinetics were registered as a change in fluorescence using a stopped-flow apparatus at 25 °C. Association of bMERB to the Cy3-CH leads to a decrease in the fluorescence. As an example, the kinetics of association between 0.5 µM Cy3 labeled CH domain and 5 µM of bMERB domain is shown in the inset. d, e Dissociation of the CH domain was measured by monitoring the increase of fluorescence after mixing a complex of Cy3 labeled CH with full-length bMERB domain (2 µM) with a 20-fold excess of either unlabeled CH domain or GppNHp Rab8a. f Results of systematic analysis of interactions between the bMERB domains of different family members with their respective CH/LIM/CH-LIM domains (from Supplementary Fig. 2). Interactions were measured by ITC. N.D. denotes not detected.

Structural basis of CH domain release upon Rab8 binding. To unravel the structural basis for the release of the CH domain from the bMERB domain upon Rab8 binding, we superimposed the bMERBH1-2:CH complex structure with that of bMERBM1116A:Rab8a (Fig. 7a). The first two helices of the bMERB domain adopt the same conformation in both structures, while the third helix of the bMERB domain in the presence of Rab8 adopts a defined conformation which would infringe spatially on the CH domain binding site, suggesting that the CH-bMERB:Rab8a triple complex could not be formed because of a steric clash (Fig. 7a and Supplementary Fig. 8a). This finding complements our transient kinetic data showing that the binding of Rab8a to the bMERB domain releases the CH domain. The structures also suggest that in the absence of Rab8a, helix 3 of bMERB domain must adopt a different conformation, or be flexible, so that the full-length bMERB domain can form a stable complex with the CH domain. To compare the Rab-and CH-binding sites on the bMERB domain, we have cut the second helix in the middle and aligned the resulting two hairpins (Fig. 7b). This alignment shows that the two halves of the bMERB domain are quite similar, a characteristic feature of bMERB family members indicating that bMERB domains are evolved by gene duplication of this helical hairpin.

Further, to understand the biological significance of the CH domain release and to identify the probable F-actin binding site in the CH domain, we have utilized the filament A EBD_E254K: F-actin (PDB 6D8C, 3.6 Å) cryo-EM structure. A superposition of the CH₁FLNa and CHÉHBP1 domains indicates a similar structure (RMSD of 2.5 Å, 99 aligned Cas) (Fig. 7d and Supplementary Fig. 8b-d). To understand the binding mode, the CHÉHBP1 domain was superimposed onto the CH₁FLNa domain. To optimize the contacts to the actin filament and to obtain the best fit to the CH-binding pocket, the CHÉHBP1 model was manually adjusted to relieve minor clashes, mainly by shifting the short helix 522–529 (residues PSMDVLLA) and the following loop, and then energy minimized with Macromodel (Schrodinger suite) and PHENIX. It is clear that the main contact site of the CHÉHBP1 domain that corresponds to ABS2 is occupied by bMERB in the CH:bMERB complex structure, indicating that the bMERB domain has to dissociate to facilitate F-actin binding of the CH domain. The optimized CHÉHBP1:F-actin model has a smaller buried interaction interface area (4640 Å²) between the CH domain and F-actin compared with the CH₁FLNa:F-actin (6000 Å²) (Fig. 7e). This is mainly due to missing ABS-N and a smaller ABS2 in the CHÉHBP1 domain (Fig. 7d and Supplementary Fig. 7c). Since the CHÉHBP1 domain has lower affinity to F-actin compared with filamin A, the smaller buried interface could indeed correspond to the physiological situation. However, we cannot rule out the possibility of different or more extensive F-actin induced conformational changes in the CHÉHBP1 domain upon binding. Further structural studies are required to understand the F-actin:CHÉHBP1 interaction. However, our model suggests that for F-actin association, the CHÉHBP1 domain has to be free, i.e., not bound to the bMERB domain.

Discussion
EHBP1 has a central role in vesicular trafficking and lipophagy, yet its regulation is not understood. This work offers mechanistic insight into the EHBP1 activation mechanism by Rab8 family members. We have shown that both N- and C-termini of EHBP1 have the potential to interact with the membrane; the NT-C2 domain binds to PI(3)P, PI(5)P, and phosphatidylserine, lipid molecules present in the early endosomes and plasma membrane respectively. Previously, we reported that the EHBP1 CaaX box can be farnesylated. The presence of two membrane-associating moieties can fine-tune the EHBP1 function by regulating its localization.

We have found that unlike Mical1, EHBP1 has only a single highly conserved C-terminal high affinity Rab-binding site and the so-called low-affinity binding site can be occupied by the CH domain. In the absence of Rab8, EHBP1 exists in a closed form with the C-terminal bMERB domain interacting with the central CH domain so that its binding with actin filaments is perturbed. Several previous studies indicated that an intramolecular interaction occurs between the bMERB domain with the various other domains (CH/LIM/CH-LIM), and were suggested to be inhibitory. Fremont et al., proposed a model for Mical1 activation in which an intramolecular association occurs between the C-terminal bMERB domain and the N-terminal mono-oxidase together with the CH and LIM domains (MO-CH-LIM), forming an enzymatically dead complex. Binding of Rab35 can release the auto-inhibition, resulting in increased F-actin depolymerization. Mical-like family members also engage in intramolecular interaction between their N-terminal CH domain (Mical-L1) or LIM domain (Mical-L2) with their C-terminal bMERB domain. The present work is the first to perform
biochemical characterization of the auto-inhibited bMERB complexes, but, we could not detect any interaction between the bMERB domain of Mical family members with their respective CH/LIM/CH-LIM domain (Supplementary Fig. 2), although this could be due to a weaker affinity, which is high enough to lead to an intramolecular interaction, but not enough to be detected in the intermolecular situation. Further experiments with longer constructs are required.

Here, we report the CH:bMERB and bMERB:Rab8 complex structures and demonstrate that the integrity of both N- and C-terminal hydrophobic patches is crucial for the CH and Rab interactions. We show that the CH and Rab-binding sites of the

**Fig. 4 CH domain interaction with F-actin and structure of the EHBP1 CH:bMERBH1-2 complex.**

a The EHBP1 CH domain co-sediments with F-actin filaments in vitro. SDS-PAGE of pellets and supernatants from high-speed centrifugation performed at a fixed concentration of F-actin (10 µM) and with varying concentrations of the EHBP1 CH domain (0–60 µM) is shown. b The normalized fraction of F-actin bound EHBP1 CH domain as a function of total EHBP1 CH domain concentration. Values were calculated from densitometry of SDS-PAGE. The data from two technical repeats are shown as means ± s.d. (n = 2). The error bars are included in the plot but are too small to be displayed on several of the points. c Results of systematic analysis of interactions between the CH domains of different bMERB family members with F-actin via co-sedimentation experiments. Only the EHBP1 CH domain interacts with F-actin. Experiments were repeated at least two times independently with similar results. Source data are provided as a Source Data file. d Cartoon representation of the CH:bMERBH1-2 complex structure. The bMERB domain is colored green and the CH domain in orange. The inset shows the zoom-in overview of the CH:bMERB interaction interface. Hydrogen bonds and polar interactions are shown in gray dashed lines. e Surface electrostatic potential of bMERBH1-2 calculated in PyMOL using the APBS-PDB2PQR plugin and visualized in red to blue (−5 kT/e to +5 kT/e). The C-terminal helix of the CH domain is shown. f Sequence alignment of the interacting regions of the CH and the bMERB domain of different bMERB family members. Residues directly involved in the CH:bMERB interactions are shown over the top of sequence alignment and the conserved LR motif is shown in the black box.
bMERB domain have significant similarities (e.g., the conserved LR motif of helix 1<sub>CH bs</sub> and helix<sub>2Rab bs</sub> is essential for CH/Rab interaction and similarly CH and Rab aligned interacting residues M1116/L1179, W1119/L1182, and F1120/V1183 are for the CH/Rab interaction). Small changes in the amino acid sequence of the two halves determine the specificity toward CH or Rab binding.

Structural alignment of the EHBPI<sub>bMERB</sub>-Rab8 complex with other published structures shows that the C-terminal high affinity Rab-binding site is quite conserved in all structures (Supplementary Fig. 5c–e). However, the conformation of helix 1 and helix 2 differs at the CH<sub>2ndRab</sub>-binding site. Further, superposition of the Mical1<sub>bMERB</sub>-Rab10 (1:2) complex with EHBPI<sub>bMERB</sub>M1116A-Rab8a shows that the conformation of the Mical1<sub>bMERB</sub>-Rab10 (1:2) complex with EHBPI<sub>bMERB</sub>M1116A-Rab8a shows that the conformation of

**Fig. 5** The N-terminal hydrophobic patch of bMERB domain is essential for the CH domain interaction. a Schematic illustration of the interactions between the bMERB<sub>1-2</sub> domain and the CH domain C-terminal helix. Hydrophobic interactions are indicated by light orange dashed lines, ionic interactions, and H-bonds are indicated by gray dashed lines. b Hydrophobic residues at the CH-binding site of the EHBPI<sub>bMERB</sub> domain. c-n Mutational alanine screening of the CH:bMERB complex binding interface via ITC measurements. Mutation of the CH domain residues that are involved in stabilization of the hydrophobic patch results in a significant defect in binding and the integrity of the hydrophobic patch is crucial for the CH interaction. The data are representative of at least three repetitions. N.D. denotes not detected.
Rab8aGppNHp complex. Rab8aGppNHp (gray, chain B) binds to EHBP1 bMERB (blue, chain D) via its N-terminal regions, the switch regions as well as the inter-switch region. Switch I and switch II are shown in red and blue, respectively. GppNHp and Mg^2+ are depicted as sticks and a green sphere, respectively.

**Fig. 6** The C-terminal hydrophobic patch of the bMERB domain is crucial for Rab8a interaction. 

(a) Cartoon depiction of the EHBP1 bMERB_R1189A:Rab8aGppNHp complex. Rab8aGppNHp (gray, chain B) binds to EHBP1 bMERB (blue, chain D) via its N-terminal regions, the switch regions as well as the inter-switch region. Switch I and switch II are shown in red and blue, respectively. GppNHp and Mg^2+ are depicted as sticks and a green sphere, respectively.

(b) Schematic illustration of the interactions between the bMERB domain and Rab8aGppNHp. Hydrogen bonds and ionic interactions are shown in gray dashed lines and light orange dashed lines indicate hydrophobic interactions. RabSF1, RabF1, RabF2, RabF3, and RabF4 motifs are shown in orange, green, pink, purple, and brown respectively.

(c) Electrostatic potential of the bMERB domain calculated in PyMOL using the APBS-PDB2PQR plugin and visualized in red to blue (−5 kT/e to +5 kT/e). The dashed line highlights the region that interacts with Rab8a.

(d) The C-terminal hydrophobic patch of the EHBP1 bMERB domain. 

(e-n) Mutational characterization of the bMERB-Rab8a complex interface. Binding of GppNHp Rab8a1-176 with different EHBP1 bMERB mutants was systematically tested and affinities were measured by ITC experiments. The data are representative of at least three repetitions. N.D. denotes not detected.
EHBP1 helix 1 and helix 2 clashes with the 2nd Rab10 molecule. For effectors displaying a 1:2 stoichiometry with Rab8 (like Mical 1), we have previously proposed a model where the two Rab-binding sites have separate functions, with the first Rab binding leading to membrane recruitment, and subsequently to the release of auto-inhibition of the CH/LIM domains by binding of the second Rab. In contrast, EHBP1 appears to have the intrinsic ability to be targeted to the membrane, and Rab8 only binds to the highly conserved C-terminal Rab-binding site of the bMERB domain, changing helix 3 conformation, and leading to release of auto-inhibition.

In agreement with recent work on Mical1, we show that phosphomimetic mutation of the switch II threonine of Rab8/10 does not affect its interaction with the EHBP1bMERB domain and bMERB / bMERB (1060-1138) form a sterical clash. 

### References

1. Mical1

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**Image Descriptions**

- **Figure a**: Diagram showing the interaction between EHBP1 helix 1 and helix 2 with Rab10 molecule.
- **Figure b**: Model of Rab8 binding to the CH/LIM domains, showing helix 3 conformation change.
- **Figure c**: Alignment of bMERB1060-1138 and bMERB1139-1212 showing amino acid differences.
- **Figure d**: Crystal structure of EHBP1_CH / bMERB_BS with Filamin A_CH1 / ABS-N / ABS 2' / ABS2.
- **Figure e**: Actin (n-2) and Actin (n) interactions with EHBP1_CH / bMERB_BS.
is not part of the bMERB binding interface (Supplementary Fig. 5b). In contrast, a study of the RILPL2:Rab8 interaction has shown that phosphorylation of switch II T72 is essential for the interaction and also stabilizes the RILPL2:Rab8 complex (PDB 6RIR)38, suggesting that the phosphorylation of switch II threonine has a selective effect on different effector molecule interactions.

In the last part of the work, we have shown that unlike a conventional F-actin binding domain, which requires an open conformation CH1-CH2 structure, the CHEHBP1 (CH2-type) domain binds to F-actin25,26. Previously, it was shown that the C. elegans CHEHBP1 domain directly interacts with F-actin, and the authors suggested that Rab10 binding to the bMERB domain enhances the CH domain:F-actin interaction7. However, no difference was detected in the F-actin binding of the free CH domain versus a CH-bMERB fusion construct1. The authors did not consider an auto-inhibition model; instead, they proposed a model where Rab10 binding leads to multimerization of EHBP1, leading to a side by side placement of CH domains from the dimeric EHBP1, but no experimental proof is provided. Recently, Miyake et al. have reported a closed conformation mouse Mical-L1 (LIM:bMERB) and binding of Rab13 opens up the Mical-L1, allowing its interaction with F-actin23. By homology modeling, the authors built the LIM:bMERB:Rab13 tripartite complex and binding of Rab13 to bMERB releases the CH domain and further, our CH:F-actin model suggests that for F-actin interaction, the CH domain has to be free, since the bMERB-binding site is also part of the F-actin binding site (Fig. 8).

**Methods**

**Plasmid cloning.** Prokaryotic and eukaryotic expression constructs were generated by standard cloning techniques, using Phusion polymerase, restriction digestion, and ligation by T4 DNA ligase. Point mutants were generated by quick-change site-directed mutagenesis, using Phusion polymerase. A detailed overview of all expression vectors employed in this study is presented in Supplementary Table 2. Primers used for plasmid cloning are listed in Supplementary Table 3. All plasmids were verified by DNA sequencing.

**Fluorescence microscopy.** The full-length human mCherry-tagged Rab constructs (Rab8a-GST, Rab8b-GST) used in this paper were described previously6. Full-length human EHBP1 constructs, NT-C2-EHBP1construct2 and EHBP1CaaX constructs were cloned into the pEGFP (C1) vector between XhoI and SmaI sites by conventional PCR using Human EHBP1 cDNA (Isoform 3, Dharmacon). The NT-C2 domain was cloned into the pEGFP (N1) vector between XhoI and SmaI sites and all constructs were verified by DNA sequencing. Cos7 cells (ATCC: CRL-1651) were maintained in DMEM medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and penicillin/streptomycin at 37 °C in the presence of 5% CO2. Cells were grown on a coverslip in 6-well plates until they reached 60–70% confluency and transiently transfected using linear polyethylenimine, MW 25000 (PEI, Polysciences Inc, 3:1 PEI:DNA (12:4 µg). Expression was checked 16–24 h post transfection. Cells were fixed with 3.7% paraformaldehyde in PBS for 15 min at room temperature. After washing with PBS, coverslips were mounted on glass slides with SlowFade Gold antifade reagent (Invitrogen). For EHBP1 constructs, single-plane images were taken by an EVOS FL fluorescence microscope equipped with 60x/1.42 Plan-Apochromat oil immersion objective. EHBP1:Rab co-localization images were taken with a Zeiss LSM800 confocal microscope equipped with a Plan-Apochromat 63x/1.4 Oil DIC M27 oil immersion objective. 3D stacks of 0.37 µm steps were acquired and images from all focal...
planes were rendered as a single maximum-intensity projection image using ImageJ software and later assembled with Adobe Illustrator.

**Lipid overlay assay.** To assess the lipid-binding properties of the EHBP1 NT-C2 domain, we performed a protein-lipid overlay assay with recombinant His6-MBP (MBP: maltose-binding protein) NT-C2 domain fusion protein and His6-MBP was used as a control. 1 ng of His6-MBP-NT-C2 domain or His6-MBP as also blotted (MBP: maltose binding protein)-NT-C2 domain fusion protein and His6-MBP was dialyzed overnight in their respective buffer. Samples were centrifuged at 15,700 g for 30 min at 4 °C and protein concentration was determined by Bradford assay (Bio-Rad). 500 µM of GppNHF Rab8a1-176 was titrated into the cell containing 50 µM bMERB domain and for bMERB/CH interactions 600–800 µM of CH domain was titrated into the cell containing 80–60 µM bMERB domain. For the control experiments, the buffer was titrated into the cell containing the bMERB domain and in the second control experiment, the CH domain was titrated against buffer. The binding isotherms were integrated and the data were fitted to a one-site-binding model using Origin 7.0 (MicroCal). The reported ITC result is the representative one of at least three independent measurements.

**Analytical size exclusion chromatography.** Recombinant protein expression and purification. Human Rab G-domains (Rab8a1-176 and Rab101-175) and phosphomimetic Rab (Rab8a1-176_T72E and Rab101-175_T72A) were expressed and purified as described previously. Rab were preparatively loaded with GppNHF (Guanosine-5′-[β,y-imido]-triphosphate) or mant GppNHF (2′/3′-O-Methyl-anti-ribofuranosyl)-guanosine-5′-[β,y-imido](tri-phosphate) and the reaction was performed as described previously. Nucleotide exchange efficiency was quantified by C18 reversed-phase column (Prontosil C18, Bischhoff Chromatography) with HPLC in 50 mM potassium phosphate buffer pH 6.6, 10 mM tetraethylammonium bromide, and 12% acetonitrile (v/v) and for the mant GppNHF exchange run the buffer contains 25% acetonitrile (v/v). Protein samples were heat precipitated at 95 °C for 5 min and centrifuged at 15,700 g for 10 min and loaded (25 µM, 20 µl) on the column. Peaks were integrated and to determine the nucleotide retention times, a nucleotide standard run was performed. All other proteins were recombinantly expressed in E. coli BL21 DE3 RIL (Agilent) cells in LB media supplemented with proper antibiotics and cells were grown at 37 °C to OD600 nm = 0.8–1.0 and stored at 4 °C for 30 min. Further expression was induced by the addition of 0.5 mM IPTG, and cells were allowed to grow at 20 °C for 14–16 h. Cells were pelleted and stored at –80 °C until ready for purification. Cells were mechanically lysed by passing through a fluidizer (Microfluidic) in a buffer (50 mM Hepes pH 8.0, 800 mM NaCl or LiCl, 2 mM βME (2-Mercaptoethanol) having 1% CHAPS and lysates were cleared by centrifugation at 75,600 g for 30 min. Subsequently, the proteins were purified by Ni^2+ affinity chromatography (HiTrap, GE Healthcare). For the His6-MBP-NT-C2 domain purification, the protein was concentrated after first Ni^2+ affinity chromatography and subjected to gel filtration (Supersite 75 26/60, GE Healthcare) in the final buffer (20 mM Hepes 7.5, 200 mM NaCl and 2 mM DTE). For the human EHBP1 CH, bMERB domain and other protein purifications, cells were lysed in a buffer (50 mM Hepes pH 8.0, 500 mM NaCl or LiCl, 2 mM βME (2-Mercaptoethanol) having 1% CHAPS and lysates were cleared by centrifugation at 25,000 rpm for 30 min. Subsequently, the proteins were purified by Ni^2+ affinity chromatography (HiTrap, GE Healthcare). The His6-tag was cleaved by Tobacco Etch Virus (TEV)-protease, and a second Ni^2+ affinity purification was performed to remove the TEV protease and His6-tag. The final purification step was achieved by gel filtration (Superdex 75 26/60, GE Healthcare) (final buffer: 20 mM Hepes 7.5 or 8.0, 100 mM NaCl and 2 mM DTE). The purified protein was collected and concentrated; flash-frozen in liquid N2, and stored at –80 °C.

**Isothermal titration calorimetry.** Protein–protein interaction measurements were conducted by ITC using an ITC200 microcalorimeter (MicroCal). bMERB/Rab interaction measurements were performed in buffer containing 20 mM Hepes 7.5, 50 mM NaCl, 1 mM MgCl2, and 1 mM Tris (2-carboxymethyl) phosphine (TCEP) whereas bMERB/CH interactions were performed in buffer containing 20 mM Hepes 7.5, 150 mM NaCl and 1 mM TCEP at 25 °C. Wild types and mutant proteins were dialyzed overnight in their respective buffer. Samples were centrifuged at 15,700 g for 30 min at 4 °C and protein concentration was determined by Bradford assay (Bio-Rad). 500 µM of GppNHF Rab8a1-176 was titrated into the cell containing 50 µM bMERB domain and for bMERB/CH interactions 600–800 µM of CH domain was titrated into the cell containing 80–60 µM bMERB domain. For the control experiments, the buffer was titrated into the cell containing the bMERB domain and in the second control experiment, the CH domain was titrated against buffer. The binding isotherms were integrated and the data were fitted to a one-site-binding model using Origin 7.0 (MicroCal). The reported ITC result is the representative one of at least three independent measurements.

**Cy3 labeling of the CH domain.** 100 µM of the CH domain is incubated with 300 µM Cy3-thioester (Jena biosience) in the buffer containing 50 mM Hepes pH 7.5, 130 mM NaCl and 50 mM MPAA (4-Mercaptophenylacetic acid, Merck) at RT for 1 h. Free dye was removed by passing through a PD10 column (GE Healthcare) in buffer containing 50 mM Hepes pH 7.5, 150 mM NaCl, and 2 mM DTE. To check the extent of labeling, samples were analyzed by mass spectrometry (LC/ESI-MS) (Supplementary Fig. 2k).

**Crystallization and structure determination.** Initial crystallization condition screens for all protein complexes described in the paper were performed with the JCSG PEG, PACT, and Proteum Complex Suite (Qiagen). The sitting-drop vapor diffusion method was used, with a reservoir volume of 70 µl and a drop volume of 0.1 µl protein (300–400 µM complexes, 1:1 Rabeffector) and 0.1 µl reservoir solution at 20 °C. The best conditions were then optimized using the sitting-drop vapor diffusion method varying drop sizes in order to obtain well diffracting crystals. Complex of bMERB/Rab8a1-176 complex was crystallized in 0.18 M Tri-ammonium citrate and 20% (v/v) PEG 3350. The complex of bMERBF1120A:Rab8a1-176_GppNHp (400 µM of 1:1 complex) was crystallized in 0.1 Mes pH 6.0, 5% (v/v) PEG 3000, and 30% (v/v) PEG 200. The complex of bMERBF1120A:Rab8a1-176_GppNHp was crystallized in 0.1 Mes pH 6.5, 10% (v/v) PEG 3000, 50% 1-Propanol and 12% (v/v) acetonitrile (v/v) and for the crystallization drop and flash-cooled in liquid nitrogen. Diffraction data were collected at 100 K on beamline X10SA at the Swiss Light Source (Paul Scherrer Institute, Villigen, Switzerland). For the bMERBF1120A:CH complex, a native data set was collected at a wavelength of 1.000010 Å whereas two data sets from a single crystal were taken for the bMERBF1120A:CH complex at a wavelength of 0.919532 Å. A native data set was collected for bMERBF1120A:Rab8a1-176_GppNHp complex at a wavelength of 0.919550 Å. Data were integrated and scaled with XDS. The Crystal of bMERBF1120A:CH complex diffraction to a resolution of 2.2 Å (space group P2_1, a = 54.06 Å, b = 48.19 Å, c = 100.3 Å) and two copies of the complex is present in the asymmetric unit of the crystal. The initial model for bMERBF1120A:CH complex was obtained by molecular replacement using PHASER with the NMR structure of the EHBP1 CH domain (PDB 2DK9) as a search model. The partial model was completed with PHENIX AutoBuild and manual building in Coot. For the bMERBF1120A:Rab8a1-176_GppNHp complex, the crystal diffraction to a resolution of 1.914 Å (space group C2 with a = 116.36 Å, b = 35.38 Å, c = 165.67 Å) and two copies of the complex constitute the asymmetric unit of the crystal. The initial model was obtained by MR using PHASER and the Rab8a (PDB 3Z52) was used as a search model. The initial model was completed with PHENIX AutoBuild and by manual building in Coot. The crystal was refined to convergence with phenix.refine or Refmac5 of the CCP4 package. The final model of the bMERBF1120A:Rab8a1-176_GppNHp complex was refined with phenix.refine using refined Translation/Libration/Screw tensors, which further lowered the Rfree by 9%. For the bMERBF1120A:Rab8a1-176_GppNHp Complex, the crystal was refined to a resolution of 2.0 Å (space group C2 with a = 117.34 Å, b = 35.66 Å, c = 168.56 Å) and bMERBF1120A:Rab8a1-176_GppNHp complex was used as a model for molecular replacement.
Table 1. Structural Actin co-sedimentation assay

Concentrations of the EHBP1 CH domain (5 μM) were purchased from Cytoskeleton and polymerized into F-actin according to the manufacturer’s protocol. F-actin (10 μM) was incubated for 1 h at RT with different concentrations of the EHBP1 CH domain (5–80 μM) in the buffer containing 5 mM Tris-HCL pH 7.5, 0.18 mM CaCl₂, 15 mM KCl, 1 mM DTT, and 1.8 mM NaN₃. Samples were centrifuged at 100,000 g for 1 h at 4 °C. The supernatant and pellet were subjected to 18% SDS-PAGE, followed by Coomassie Brilliant Blue staining. The quantitative analyses were performed using the Bio-Rad image analysis software in the ChemiDoc system (Bio-Rad).

For comparative actin co-sedimentation assays, 10 μM F-actin was incubated with 40 μM CH domain from EHBP1, Mical1, Mical3, and Mical-L1, and co-sedimentation was performed as described above.

Bioinformatics. Multiple sequence alignments were generated using Clustal Omega. The protein interaction interfaces from the asymmetric unit were examined in detail using the PDBePISA server (Proteins, Interfaces, Structures and Assemblies). DALI server was used for structural comparison.

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

Data availability. Data supporting the findings of this paper are available from the corresponding authors upon reasonable request. A reporting summary for this paper is available as a Supplementary Information file.

Protein coordinates and structure factors have been submitted to the Protein Data Bank under accession numbers PDB 6ZSH (bMERBH1-2:CH), PDB 6ZST (bMERBH1116A:Rab8a), and PDB 6ZSY (bMERBH1116A:Rab8b).

Source data are provided with this paper.

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Author contributions

A.R. and R.S.G. conceived and designed the study. A.R. and N.B. carried out protein expression/purification. A.R. carried out all biochemical studies, in vivo localization experiments, and crystallization work. N.B. performed lipid overlay assay and actin-co-sedimentation assay. A.R. and I.V. determined the X-ray-structures. I.V. performed molecular modeling. A.R., I.V., and R.S.G. analyzed and interpreted the data. A.R., I.V., and R.S.G. wrote the paper.

Competing interests

The authors declare no competing interests.

Additional information

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