Structural mechanism for amino acid-dependent Rag GTPase nucleotide state switching by SLC38A9

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The Rag GTPases (Rags) recruit mTORC1 to the lysosomal membrane in response to nutrients, where it is then activated in response to energy and growth factor availability. The lysosomal folliculin (FLCN) complex (LFC) consists of the inactive Rag dimer, the pentameric scaffold Ragulator, and the FLCN:FNIP2 (FLCN-interacting protein 2) GTPase activating protein (GAP) complex, and prevents Rag dimer activation during amino acid starvation. How the LFC is disassembled upon amino acid refeeding is an outstanding question. Here we show that the cytoplasmic tail of the human lysosomal solute carrier family 38 member 9 (SLC38A9) destabilizes the LFC and thereby triggers GAP activity of FLCN:FNIP2 toward RagC. We present the cryo-EM structures of Rags in complex with their lysosomal anchor complex Ragulator and the cytoplasmic tail of SLC38A9 in the pre- and post-GTP hydrolysis state of RagC, which explain how SLC38A9 destabilizes the LFC and so promotes Rag dimer activation.

The mechanistic target of rapamycin complex 1 (mTORC1) couples cell growth to nutrient, energy and growth factor availability 1–4. mTORC1 is recruited to the lysosomal membrane when amino acids are replete via the Rag guanosine triphosphatases (GTPases) (known as Rags) 1–4,5. Unlike other small GTPases, Rags exist as obligate heterodimers of functionally redundant RagA or B in complex with functionally redundant RagC or D. They exist in two stable states—inactive (RagA or B GDP:RagC or D GTP) and active (RagA or B GTP:RagC or D GDP) states—during low and high cellular amino acid levels, respectively 1–4. Rags interact with the pentameric scaffold Ragulator, which serves as a lysosomal anchor through the lipitation of one of its subunits 1,6. Whereas inactive Rags fail to recruit mTORC1, active Rags directly bind to the mTORC1 subunit Raptor and so recruit mTORC1 to the lysosomal membrane 7,8.

The lysosomal membrane protein SLC38A9 is both an amino acid transporter and an Arg-dependent Rag activator 11–15. The N-terminal cytoplasmic tail of SLC38A9 (residues 1–119, hereafter, SLC38A9 NT) is not required for amino acid transport itself, but is sufficient to interact with inactive Rags and stimulate mTORC1 activity 11,12,13. The N-terminal tail is sequestered in the absence of bound Arg, but is liberated and available to bind to Rags in its presence 14,15, suggesting a mechanism for amino acid-dependent regulation of Rag GTPases. It has been reported that the SLC38A9 N-terminal tail regulates the Rags directly by serving as a guanine nucleotide exchange factor (GEF) for RagA 16. However, we previously found that RagA spontaneously exchanges guanosine diphosphate (GDP) to guanosine triphosphate (GTP), raising the question as to whether a GEF is required in this pathway. Spontaneous nucleotide exchange by RagA is blocked, however, when the Rags are bound in the lysosomal folliculin complex (LFC) 19.

Folliculin GTPase activating protein (FLCN GAP) activity 19, which is blocked in the LFC 20,21, is essential for the inactivating phosphorylation of TFE3, a member of the MiT/TFE family of transcription factors controlling lysosomal biogenesis and autophagy 12,22–28. Taken together, the LFC both blocks FLCN GAP activity and prevents spontaneous nucleotide exchange by RagA. These observations suggested the hypothesis that disassembly of the LFC is a prerequisite for Rag activation by amino acids, particularly with respect to the mTORC1-dependent downregulation of the MiT/TFE family of transcription factors 20,21. Here we show that the cytoplasmic tail of SLC38A9 triggers FLCN GAP activity toward RagC by destabilization of the LFC. We solved the cryo-EM structures of Rags in complex with Ragulator and SLC38A9 NT in the pre- and post-GTP hydrolysis state of RagC and discuss their implications on the current model of Rag activation.

Results

SLC38A9 NT triggers FLCN GAP activity. We reconstituted a complex comprising Ragulator, inactive Rags and SLC38A9 NT from purified components and tested the effect of SLC38A9 NT on FLCN:FNIP2 GAP activity toward RagC (Fig. 1a–c). To individually control the nucleotides bound to RagA and RagC, xanthosine-specific RagC was used throughout this study (RagC D181N). FLCN:FNIP2 GAP activity toward RagC was enhanced in the presence of SLC38A9 NT, consistent with a positive role in Rag activation. In the presence of SLC38A9 NT, RagC-bound xanthosine triphosphate (XTP) was undetectable (Fig. 1d). We termed the complex of inactive Rag-Ragulator with SLC38A9 NT the ‘pre-GAP complex’. To assess if SLC38A9 NT can actively disassemble the LFC to form the pre-GAP complex, we added SLC38A9 NT to a pre-formed LFC and analyzed the resulting complexes after overnight incubation via size exclusion chromatography (SEC; Fig. 1d). After addition of SLC38A9 NT, the peak at 9.7 ml corresponding to the LFC (Fig. 1d, green triangle) is virtually gone (Fig. 1d, yellow trace). Instead, two peaks at 10.5 ml and 11.2 ml are present, which correspond, respectively, to the isolated FLCN:FNIP2 complex (10.5 ml) and the pre-GAP Rag-Ragulator complex comprising Ragulator, inactive Rags and SLC38A9 NT from purification (11.2 ml). Taken together, these data show that SLC38A9 NT can disassemble the LFC, ultimately triggering FLCN GAP activity toward RagC.

Structure of the pre-GAP complex. To unravel the molecular mechanism of FLCN:FNIP2 GAP activation by SLC38A9 NT, we...
determined the structure of the pre-GAP complex (Ragulator-RagAGDP-RagCXTP-SLC38A9NT) (Figs. 1a,b and 2a) by single-particle cryo-EM. The structure of this ~170-kDa complex was resolved at an overall resolution of 3.2 Å (Fig. 2b, Table 1 and Extended Data Fig. 1). Atomic models of the Ragulator and Rag subunits were readily placed into the density on the basis of previously solved structures\(^{19,29,30}\). The remaining unassigned density was located in the cleft between the two Rag G domains and could be assigned to SLC38A9NT in isolation; purple (circle), FLCN:FNIP2 in isolation. o/n, overnight. Note that, in the SDS-PAGE analysis, FLCN:FNIP2 can be detected in the same fraction as the pre-GAP complex because of peak overlap due to the very similar molecular weight of FLCN:FNIP2 and the pre-GAP complex (~187 kDa versus ~170 kDa) (yellow cross). The presence of SLC38A9NT in this fraction can only be explained by the fact that it is bound to Ragulator-Rag, as it elutes at 16.1 ml in isolation (red square). Uncropped gel images for b and d are shown in the source data. Data for graphs in a, c and d are available as source data.

The ordered core and sole secondary structure element of SLC38A9NT is an α-helical stretch deeply buried in the G domain cleft directly contacting both Rag G and roadblock domains. SLC38A9NT then folds back on top of the N-core making direct contact with the GDP nucleotide bound to RagA. The most N-terminal resolved residue (Pro39) is located directly adjacent to the RagC switch I helix (α2) (Fig. 2c), whose conformation depends on the nucleotide binding state of RagC. Thus, the interaction of SLC38A9NT with the Rags is intimately connected to their nucleotide binding state. SLC38A9NT contains within it the so-called N-plug, which is inserted into the transmembrane domain of SLC38A9 in the absence of arginine\(^{17}\).

The global architecture of the Ragulator-Rag subcomplex is unchanged compared to the one observed in the LFC, but binding of SLC38A9NT has two effects. First, it leads to a slight inward rotation of both Rag G domains. Second, it occupies the cleft between the Rag G domains (Fig. 2d). Together, these effects prevent binding of FLCN:FNIP2 in the GAP-incompetent conformation observed in the LFC as multiple loops and α-helix 1 of the FLCN longin domain (α-1) would clash with SLC38A9NT and the RagA G domain, respectively (Fig. 2e). This explains how SLC38A9NT binding to Rags breaks up the LFC and drives FLCN:FNIP2 into the GAP-active conformation.

To gain further insight into the dynamics of the SLC38A9NT interaction with inactive Rags, we measured its hydrogen-deuterium exchange (HDX), in isolation and bound to inactive Rags. In isolation, SLC38A9NT shows high exchange rates (>50%) throughout its entire sequence at the earliest time point tested, leading us to conclude that the entire region is intrinsically disordered (Extended Data Fig. 3a). Consistent with the assignment from the cryo-EM structure, residues 37–97 of SLC38A9NT became partially protected from HDX when bound to inactive Rags (Fig. 3a). Only residues 50–55 located in a solvent-exposed loop were not significantly protected at any of the exchange times (Fig. 3b). The α-helical N-core is strongly protected, with HDX differences >30% even after 600 s (Fig. 3a,b and Extended Data Fig. 3b,c). Thus, the HDX results corroborate the cryo-EM structure and show that the order observed for SLC38A9NT is induced by formation of the complex.

The SLC38A9NT core harbors multiple residues shown to be crucial for the interaction with and activation of Rags in vivo\(^{11,12}\). Alanine mutations of residues Ile68, Tyr71 and Leu74, all located in the N-core, abolished the interaction with Rags\(^{3}\) (Fig. 3c). Mutation of residue His60, located at the solvent-exposed start of the N-core, had no influence on the interaction with Rags. Mutations of stretches located in the N-plug and the part interacting with RagC α2 also abolished the Rag interaction in vivo\(^{11}\) (Fig. 3c). The high

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**Fig. 1 | SLC38A9NT triggers FLCN GAP activity and disassembles the LFC.** a, SEC profile of the reconstituted SLC38A9NT-RagA\(^{\text{GDP}}\)-RagC\(^{\text{XTP\_\_\_\_}}\)-Ragulator complex. b, SDS-PAGE analysis of the peak fraction indicated in a. c, HPLC-based RagC XTPase assay to measure FLCN GAP activity. Data are plotted as the mean ± s.d. and individual data points of two independent experiments with technical triplicates (n = 6). U. XTP signal was undetectable; norm., normalized; XTP, xanthosine triphosphate serving as a GTP analog.

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consistency of the cryo-EM and HDX-MS results with the known biology of SLC38A9 mutants validates that the observed structure and dynamics correspond to the state relevant for Rag regulation in cells.

Structure of the post-GAP complex. Having shown that SLC38A9 is capable of disassembling the LFC and activating FLCN to generate the RagAGDP:RagCGDP intermediate, we considered how this complex might progress to the active RagAGTP:RagCGDP dimer. We found that SLC38A9NT, RagAGDP:RagCXDP and Ragulator form a complex that is stable on SEC (Fig. 4a,b); we refer to this as the post-GAP complex. We used cryo-EM to solve its structure at an overall resolution of 3.9 Å (Fig. 4c, Table 1 and Extended Data Figs. 4 and 5).

Aside from the difference in the nucleotide bound to RagC, clearly visible in density, the post-GAP complex structure is nearly identical to the pre-GAP complex structure (Cα r.m.s.d.)=0.8 Å (Fig. 4d,e). The RagC switch I region of the pre- and post-GAP complexes is in the same conformation (Fig. 4f). By comparison, in the structure of the mTORC1 subunit Raptor in complex with active Rags9, containing RagC bound to GDP as in the post-GAP complex, the RagC switch I region was disordered and could not be resolved (Fig. 4f). In fact, all other known Rag structures bound to GDP have at least partially disordered switch I regions compared to the respective GTP-bound structure (Extended Data Fig. 6). This unusual switch I conformation is consistent with intrinsic tryptophan (Trp) fluorescence data. Trp fluorescence reports on the conformational change of RagC switch I when transitioning from the GTP to the GDP-bound state19,31. Usually, this is triggered by GTP hydrolysis, but we could not detect a FLCN:FNIP2-induced change in RagC Trp fluorescence in the presence of SLC38A9NT, even though GTP hydrolysis was confirmed by the HPLC-based GTPase assay (Fig. 1c and Extended Data Fig. 7a). This observation can be explained by the unaltered RagC switch I conformation between the pre- and post-GAP complex cryo-EM structures (Fig. 4d,f). Thus, the Trp fluorescence RagC GTPase assay corroborates the unexpected finding that the double GDP-bound Rag dimer is trapped in the inactive conformation by SLC38A9NT. We conclude that, in the post-GAP complex, RagCGDP is uniquely trapped in the GTP conformation through its interaction with SLC38A9. We propose that the free energy stored by this abnormal strained conformation promotes SLC38A9 dissociation. This could occur upon some appropriate trigger, or might be spontaneous at cellular
Given that RagA
SLC38A9 blocks RagA nucleotide exchange. Given that RagA spontaneously exchanges its nucleotide, that this reaction is inhibited by the LFCNN and that SLC38A9NT directly interacts with the

concentrations of these molecules. Furthermore, it cannot be excluded that the transmembrane part of SLC38A9 influences the interaction of SLC38A9NT with Rags in the context of the full-length protein.

SLC38A9 blocks RagA nucleotide exchange. Given that RagA spontaneously exchanges its nucleotide, that this reaction is inhibited by the LFCNN and that SLC38A9NT directly interacts with the GDP nucleotide bound to RagA, suggesting a stabilization rather than destabilization of the nucleotide state, we revisited the report of RagA GEF activity by SLC38A9NT (ref. 18). We employed an HPLC-based nucleotide exchange assay to directly measure GTP and GDP bound to RagA. Irrespective of the RagC nucleotide state, spontaneous GDP to GTP exchange in RagA is reduced when SLC38A9NT is present compared to the respective Ragulator-Rag complex in the absence of SLC38A9NT (Fig. 5a). We confirmed this finding with a (2’ or 3’)-O-(N-methylanthraniloyl) (mant) GDP fluorescence-based nucleotide exchange assay where the detected mantGDP fluorescence signal decreases upon its release from Rags into the solution12 (Fig. 5b). These measurements show that SLC38A9NT is not a GEF for RagA, but rather antagonizes nucleotide exchange.

Whereas the HPLC and fluorescence-based assays presented here directly detect the nucleotide bound to RagA in bulk, the cross-linking assay previously interpreted as evidence for SLC38A9NT GEF activity10 only detects a subpopulation of radioactively labeled nucleotides after UV crosslinking. With the structures now available, the previous crosslinking data can be reinterpreted in a new light. In the pre-GAP complex, SLC38A9NT directly interacts with the RagC switch I region as well as the GDP nucleotide bound to RagA, which would very probably impact the crosslinking efficiency of GDP to RagA (Extended Data Fig. 7b). Given the structures and the new biochemical data presented here, the crosslinking data are best explained by changes in the accessibility of the nucleotide, rather than exchange.

Discussion

The cleft between the G domains of the Rag GTPase heterodimer emerges as a regulatory platform with a total of three different protein (complexes) shown to bind directly in this region, namely FLCN:FNIP2, SLC38A9 and mTORC1 via the Raptor subunit9,10,19,21. Owing to the overlapping binding sites, these interactions cannot occur simultaneously on a Rag heterodimer. The main factor that determines the availability of Rags for interaction with the respective binding partner is their nucleotide binding state, which is indirectly regulated by amino acids. We propose a model in which FLCN:FNIP2 binds to inactive Rag GTPases forming the LFC when amino acid levels are low. During this phase, the N-plug of the cytoplasmic tail of SLC38A9 occludes the arginine binding site of the SLC38A9 transmembrane domain, making it unavailable for interaction with inactive Rag GTPases17. With increasing amino acid levels, arginine outcompetes N-plug binding in the transmembrane domain, leading to LFC disassembly and resulting in activation of FLCN:FNIP2 GAP activity (Fig. 5c). Dissociation of SLC38A9 is probably facilitated by the release of conformational strain in RagC. Our in vitro data raise the possibility that an additional trigger could be involved in SLC38A9 dissociation, although it might also occur spontaneously. SLC38A9 dissociation would then enable GDP to GTP exchange on RagA, generating the active Rag dimer. Activation of mTORC1 with respect to the MiT/TFE transcription factors would then lead to the inactivation of MiT/TFE-dependent transcription16,22,23,26, completing the chain of events.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41594-020-0490-9.

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Table 1 | Cryo-EM data collection, refinement and validation statistics

|                        | Pre-GAP complex (EMD-21686, PDB 6JW2) | Post-GAP complex (EMD-21687, PDB 6WJ3) |
|------------------------|--------------------------------------|----------------------------------------|
| Data collection and processing | Magnification 29,000 36,000 | Voltage (kV) 300 200 |
|                         | Electron exposure (e−/Å2) 59 60.5 | Defocus range (µm) −0.8 to −2.3 −1.0 to −2.5 |
|                         | Pixel size (Å) 0.8522 1.137 | Symmetry imposed CI CI |
| Initial particle images (no.) | 1,419,155 2,309,565 | Final particle images (no.) 129,553 106,659 |
| Map resolution (Å) | 3.2 3.9 | FSC threshold 0.143 0.143 |
| Map resolution range (Å) | 2.6–11 3.4–11 | Model composition Nonhydrogen atoms 8,909 8,912 |
|                         | Protein residues 1,167 1,168 | Ligands 1 GDP; 1 XTP (U3J) 1 GDP; 1 XDP (U3J) |
| B factors (Å2, min/max/avg) | Protein 38.89/226.58/87.85 54.13/204.34/103.71 | Ligand 49.91/65.12/57.44 96.98/113.93/105.46 |
|                         | R.m.s. deviations Bond lengths (Å) 0.002 0.002 | Bond angles (°) 0.454 0.484 |
| Validation MolProbity score | 1.91 1.76 | Clashscore 3.83 7.82 |
|                         | Poor rotamers (%) 2.81 0.00 | Ramachandran plot Favored (%) 94.05 95.20 |
|                         | Allowed (%) 5.95 4.71 | Disallowed (%) 0.00 0.09 |

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Fig. 3 | HDX-M5 difference of SLC38A9NT reveals pre-GAP complex dynamics. a, HDX difference plot of SLC38A9NT in complex with inactive Rags and in isolation at 6-s exchange time. Data are plotted as the mean ± s.d. of technical replicates (n = 4). b, Uptake difference mapped onto SLC38A9NT in the pre-GAP complex structure. Boundaries of the N-core and the solvent-exposed loop are indicated. c, Residues shown to be crucial for SLC38A9 function in vivo (red) mapped onto the pre-GAP complex structure. Data for the graph in a are available as source data online.

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Fig. 4 | Rag GTases are trapped in the inactive conformation in the post-GAP complex. a. SEC profile of the reconstituted SLC38A9NT–RagAGDP–Ragulator complex. XDP, xanthosine diphosphate serving as a GDP analog. b. SDS-PAGE analysis of the peak fraction indicated in a. c. Cryo-EM density of the post-GAP complex. d. Overlay of the post-GAP (dark gray, colored) and pre-GAP (light gray) complex structures represented as pipes and planks. The boxed (red) region is show in e and f. e. Overlay of the pre- (blue/pink) and post-GAP (light red/red) complex models focused on the RagC nucleotide pocket. Cryo-EM densities of the pre- (left) and post-GAP (right) complexes are compared to highlight the clear distinction between the bound XTP and XDP, respectively. f. RagC nucleotide-binding region of the pre-GAP (left, blue), post-GAP (middle, red) and Raptor-Rag-Ragulator (right, green, PDB 6U62, EMD-206600) complex structures and respective cryo-EM density (top). Bottom: canonical small GTPase elements involved in nucleotide binding are highlighted in the models. The uncropped gel image for b is shown as source data. Data for the graph in a are available as source data.

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Methods

Protein expression and purification. RagA/RagC(GTP) GTPases (MBP tag on RagC) and Ragulator (GST tag on Lamtor 1, His tag on Lamtor 2) were expressed in Sf9 insect cells via baculovirus infection and purified as described in ref. 1. Rags were expressed and purified as described in ref. 2, 3. In brief, Sf9 and HEK293-GNTI cells were resuspended and lysed in wash buffer (25 mM HEPES, 130 mM NaCl, 2.5 mM MgCl2, 2 mM EGTA, 0.5 mM tris-(2-carboxyethyl)phosphine (TCEP), pH 7.4) supplemented with 1% Triton X-100 and protease inhibitor (Roche, one tablet per 100 ml lysis buffer) by gentle rocking at 4 °C for 20 min. Lysate was cleared by centrifugation at 30,000g and 4°C for 45 min. For RagA/RagC(GDP), the supernatant (SN) was applied to amylose resin (NEB), washed with wash buffer (±200 mM NaCl) and eluted by overnight on-column tobacco etch virus (TEV) protease (home-made) cleavage. For Ragulator, the SN was applied to Ni-nitrilotriacetic acid (NTA) resin (Thermo Scientific), washed with wash buffer (±200 mM NaCl) and eluted with wash buffer (± 250 mM imidazole). The eluate was applied to glutathione resin (GE Healthcare), washed with wash buffer (±200 mM NaCl) and eluted by overnight on-column TEV protease cleavage. For pre-GAP complex assembly, the assembled complex was injected onto a Superdex 200 column (GE Healthcare) and protease inhibitor as described above. After clarification of the lysate by centrifugation (see above), the SN was applied to glutathione resin and incubated for 2h at 4 °C. The resin was washed consecutively with wash buffer supplemented with 1% Triton X-100, 1% Triton X-100 and 200 mM NaCl and 0.1% CHAPS, respectively. Immobilized GST-SLC38A9NT was eluted from the resin by overnight TEV protease cleavage. The eluate was concentrated using spin filters (Millipore Sigma) and purified to homogeneity via SEC equilibrated with wash buffer.

Codon-optimized DNA coding for SLC38A9NT was cloned into a pCAG-GST vector for expression in HEK293-GNTI cells. HEK293-GNTI cells were transfected with the DNA and INN1 (aldimine-modification) by lipofection (Aldrich) of cells at a density of 1.3–1.8 × 106 cells per ml. Cells were collected after 48–72 h, resuspended and lysed in wash buffer supplemented with 1% Triton X-100 and protease inhibitor as described above. After clarification of the lysate by centrifugation (see above), the SN was applied to glutathione resin and incubated for 2h at 4°C. The resin was washed consecutively with wash buffer supplemented with 1% Triton X-100, 1% Triton X-100 and 200 mM NaCl and 0.1% CHAPS, respectively. Immobilized GST-SLC38A9NT was eluted from the resin by overnight TEV protease cleavage. The eluate was concentrated using spin filters and purified to homogeneity via SEC using a Superdex 75 column (GE Healthcare) equilibrated with wash buffer.

All stated resolutions are according to the 0.143 cutoff of the respective gold-standard FSC3. Density maps used for atomic coordinate refinement and illustration in all figures along with the respective half maps and masks used during refinement and FSC calculation have been deposited in the Electron Microscopy Data Bank (EMDB) with accession codes EMD-21860 (pre-GAP complex) and EMD-21687 (post-GAP complex).

Atomic model building and refinement. For the pre-GAP complex coordinate model, the coordinates of all five Ragulator subunits as well as RagA and RagC are based on the LFC structure (PDB 6NZD) and were rigid body fitted separately during refinement and FSC calculation have been deposited in the Electron Microscopy Data Bank (EMDB) with accession codes EMD-21860 (pre-GAP complex) and EMD-21687 (post-GAP complex).
0.5 Å and re-refined against half map 1 of the final 3D reconstruction using Phenix real-space refinement with the same parameters used in the coordinate refinement described above. Model-map FSCs of the coordinates re-refined against half map 1 were calculated using the same half map 2 (FSC test). No overfitting could be detected (Extended Data Fig. 2b). The final model was validated using Phenix comprehensive validation including MolProbity™ and EMRinger™ analysis.

The post-GAP complex coordinate model was based on the previously refined pre-GAP coordinate model. After rigid body fitting using UCSF Chimera, coordinates were refined following the protocol described above for the pre-GAP complex, with the map weight set to 0.5 during automated real-space refinement to reflect the lower resolution of the density. Model assessment and validation were performed as described for the pre-GAP complex (Extended Data Fig. 5). All model and validation parameters of the pre and post-GAP coordinate models are summarized in Table 1. Coordinated in the PDB with accession codes 6WJ2 (pre-GAP complex) and 6WJ3 (post-GAP complex), respectively.

Hydrogen-deuterium exchange mass spectrometry. Amide hydrogen exchange was carried out at 37 °C for 6, 60, 600 and 60,000 s, respectively by diluting 5 μl of 5 μM SLC38A9 × RagC to 1 μM with 1.2 × molar excess of SLC38A9NT was added. After signal equilibration, the assay was started by the addition of 20 μl of FLCN:FNIP2 to a final concentration of 35 nM and the fluorescence signal was recorded in 1-s intervals for 1,000 s. The signal before FLCN:FNIP2 addition was used for baseline subtraction and subsequently normalized to the signal immediately after FLCN:FNIP2 addition. This also served as the t = 0 time point. Data are plotted as the mean and s.d. of each time point.

RagA mantGDP nucleotide exchange assay. The assay was carried out with the same instrument and cuvette as the tryptophan fluorescence RagC GTase assay, in quadruplicate (see above). Mant fluorescence was collected using 360-nm excitation (10-nm slit) and 440-nm emission (10-nm slit). Experiments were performed in wash buffer at RT with stirring. The final concentration of Rags was 100 nM. A 500-μl volume of wash buffer was added to the cuvette and, after baseline equilibration, 20 μl of a protein mixture containing Rags, 1.2 × molar excess of Regulator and with or without 1.2 × molar excess of SLC38A9NT was added. After signal equilibration, the assay was started by addition of 20 μl of GTP to a final concentration of 10 μM (100 μM molar excess over Rags) and fluorescence was measured in 1-s intervals for 1,000 s. The signal before protein mixture addition was used for baseline subtraction and subsequently normalized to the signal immediately after GTP addition. This also served as the t = 0 time point. Data are plotted as the mean and s.d. of each time point.

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

Tryptophan fluorescence RagC GTase assay. Fluorimetry experiments were performed in quadruplicate using a FluoroMax-4 instrument (Horiba) and a quartz cuvette compatible with magnetic stirring (Starna Cells) and a 10-mm path length. The tryptophan fluorescence signal was collected using 297-nm excitation (1.5-nm slit) and 340-nm emission (20-nm slit). Experiments were performed in wash buffer at RT with stirring. The final concentration of Rags was 350 nM. A 500-μl volume of wash buffer was added to the cuvette and, after baseline equilibration, 20 μl of a protein mixture containing Rags, 1.2 × molar excess of Regulator and with or without 1.2 × molar excess of SLC38A9NT was added. After signal equilibration, the assay was started by the addition of 20 μl of FLCN:FNIP2 to a final concentration of 35 nM and the fluorescence signal was recorded in 1-s intervals for 1,000 s. The signal before FLCN:FNIP2 addition was used for baseline subtraction and subsequently normalized to the signal immediately after FLCN:FNIP2 addition. This also served as the t = 0 time point. Data are plotted as the mean and s.d. of each time point.

Data availability
EM density maps have been deposited in the EMDB with accession codes EMD-21686 (pre-GAP complex) and EMD-21687 (post-GAP complex). Atomic coordinates have been deposited in the PDB with accession codes 6WJ2 (pre-GAP complex) and 6WJ3 (post-GAP complex). Source data are provided with this paper.

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Author contributions
S.A.F designed and carried out all experiments and carried out all data analysis. R.E.L performed initial GEF and LFC disassembly experiments. S.A.F and J.H.H. conceptualized the project and wrote the first manuscript draft. All authors contributed to editing the manuscript.

Competing interests
J.H.H. is a scientific founder and receives research funding from Casma Therapeutics.

Additional information
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Correspondence and requests for materials should be addressed to J.H.H.

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Extended Data Fig. 1 | Pre–GAP complex cryo-EM structure determination. a, Exemplary raw cryo-EM micrograph at −2.2 μm defocus. Scale bar 50 nm. b, Power spectrum of micrograph shown in a with CTF estimation. c, Exemplary 2D class averages. Scale bar 150 Å. d, Cryo-EM data processing workflow. Used software is indicated with italic font. red asterisk indicates the map used for model building to subsequently generate simulated maps w/o SLC38A9. e, Particle orientation distribution of the final particle set. f, Fourier shell correlation (FSC) of the final 3D reconstruction. g–h, Overlay of the final density map with the masks used during refinement (g, blue, transparent), FSC calculation (h, pink, transparent). i, Both masks from g and h overlaid with the final density map.
Extended Data Fig. 2 | Pre-GAP complex atomic coordinate building and refinement. a, Overlay of half-map (green) and map-model (purple) FSC to assess map to model agreement. b, Overlay of FSC work (blue) and FSC test (yellow) of the cross-validation test to assess overfitting. The refinement target resolution is indicated by a vertical dashed line. c, Final model composition and chain assignment. Parts not resolved by the cryo-EM density are represented by thin black lines. Red lines indicate regions where side chains are truncated to alanine. d, Model fit in the cryo-EM density (mesh) of selected regions. The threshold level used to display the density in UCSF Chimera is given in parentheses.
Extended Data Fig. 3 | HDX-MS analysis of SLC38A9NT in isolation and bound to Rags. a, Deuterium uptake and peptide coverage (grey lines) of SLC38A9NT in complex with inactive Rags (left) or in isolation (right) at 6, 60, 600 and 60,000 s exchange time. b, HDX difference plots of SLC38A9NT in complex with inactive Rags and in isolation at 60 (left), 600 (middle) and 60,000 s (right) exchange time. Plotted are the mean±SD of technical replicates (n = 3). c, Individual SLC38A9NT MS peptide spectra of selected peptides in isolation (black) and in complex with inactive Rags (red). Undeuterated reference spectra are shown at the top. Data for graphs in b are available as source data online.
Extended Data Fig. 4 | Post-GAP complex cryo-EM structure determination. **a**, Exemplary raw cryo-EM micrograph at −2.2 μm defocus. Scale bar 50 nm. **b**, Power spectrum of micrograph shown in a with CTF estimation. **c**, Exemplary 2D class averages. Scale bar 150 Å. **d**, Cryo-EM data processing workflow. Used software is indicated with italic font. **e**, Particle orientation distribution of the final particle set. **f**, Fourier shell correlation (FSC) of the final 3D reconstruction.
Extended Data Fig. 5 | Post-GAP complex atomic coordinate building and refinement. 

**a**, Overlay of half-map (green) and map-model (purple) FSC to assess map to model agreement.

**b**, Overlay of FSC work (blue) and FSC test (yellow) of the cross-validation test to assess overfitting. The refinement target resolution is indicated by a vertical dashed line.

**c**, Model fit in the cryo-EM density (mesh) of selected regions. The threshold level used to display the density in UCSF Chimera is given in parentheses.
Extended Data Fig. 6 | Overview of Rag GTPase structures in different states. a–g. Top (middle) and side view (right) of published Rag GTPase structures in surface representation (cyan, RagA G domain; blue, RagC G domain; pink, GTP or GTP analogue; red, GDP or GDP analogue; yellow, RagA or RagC switch I region; grey, RagA or RagC C-terminal roadblock domain). The dashed line connects the Cα atoms of RagA Trp165 and RagC Tyr221 representing the width of the G domain cleft. The two vertical solid lines represent the RagA Trp165 and RagC Tyr221 Cα position in a. PDB codes, experimental method, de facto nucleotide state and Rag binding partners (if any) are summarized on the left.
Extended Data Fig. 7 | Tryptophan fluorescence-based RagC XTPase assay. a, Intrinsic tryptophan fluorescence based RagC XTPase assay of Ragulator-RagA<sup>GDP</sup>:RagC<sup>CTP</sup> in the absence (blue) and presence (orange) of SLC38A9NT. Tryptophan fluorescence of a Ragulator-RagA<sup>GTP</sup>:RagC<sup>CTP</sup> substrate used in the same assay serves as a positive control (green). Plotted is the mean ± SD mantGDP fluorescence at each time point of one experiment performed in quadruplicates (n = 4). The experiment has been performed twice with similar results. norm., normalized. b, Top view of the pre-GAP complex structure illustrating the SLC38A9<sup>NT</sup>-RagC switch I and GDP (RagA, red) interaction. SLC38A9 (yellow) and RagC switch I (blue) are displayed in surface representation. Data for graph in a is available as source data online.
Reporting Summary

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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection

- Cryo-EM: SerialEM3 7.1; MS: Xcalibur 2.1; HPLC: Agilent OpenLab CDS; SEC: GE Unicorn 5.01

Data analysis

- Cryo-EM reconstruction: MotionCor2, CTFFIND4.1.13, gautomatch-v0.53, cryOLLO, Relion3.0.6, Relion3.1-beta, cryoSPARC v2, ccpem-1.4.1, Phenix dev-3736, UCSF pyem, miscEM (https://github.com/simonfromm/miscEM)
- Atomic coordinate building and refinement: UCSF Chimera 1.13, Coot 0.8.9.1, Phenix dev-3736
- HDX-MS: Proteome Discoverer 2.1, HDXaminer
- Data display: gnuplot 5.0, UCSF Chimera 1.13, Adobe Illustrator 22.1

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Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

EM density maps have been deposited in the EMDB with accession numbers EMD-21686 (pre-GAP complex) and EMD-21687 (post-GAP complex). Atomic coordinates have been deposited in the PDB with accession numbers 6WJ2 (pre-GAP complex) and 6WJ3 (post-GAP complex).
Field-specific reporting

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size | Cryo-EM: The data for the pre-GAP and post-GAP complex data set was collected in one session. The sample size was not predetermined and followed common standards practiced in the field.

Data exclusions | Cryo-EM: After motion correction and CTF estimation, cryo-EM micrographs which were empty, experienced a lot of motion, had non-vitreous ice, extensive contaminations or poor CTF estimation statistics were excluded from further processing. HDX-MS: all peptide spectra were manually inspected and those with poor quality were excluded from further data analysis.

Replication | HDX experiment was performed triplicate or quadruplicate for 6, 60, 600 and 60,000 sec timepoints. GTPase and nucleotide exchange assays were carried out at least in triplicates.

Randomization | Randomization was not relevant to this study and not performed.

Blinding | Researchers were not blinded during this study.

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Materials & experimental systems

| n/a | Involved in the study |
|----|-----------------------|
| ☒ | Antibodies |
| ☒ | Eukaryotic cell lines |
| ☒ | Palaeontology and archaeology |
| ☒ | Animals and other organisms |
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| ☒ | Clinical data |
| ☒ | Dual use research of concern |

Methods

| n/a | Involved in the study |
|----|-----------------------|
| ☒ | ChIP-seq |
| ☒ | Flow cytometry |
| ☒ | MRI-based neuroimaging |

Eukaryotic cell lines

Policy information about [cell lines](#)

| Cell line source(s) | HEK 293 Gn-Ti cells: UC Berkeley cell culture facilities; SF9: UC Berkeley cell culture facilities |
|---------------------|------------------------------------------------------------------------------------------------|
| Authentication      | None of the cell lines used were authenticated. |
| Mycoplasma contamination | Cell lines were not tested for mycoplasma contamination. |
| Commonly misidentified lines (See [ICLAC](#) register) | n.a. |