Ragulator and SLC38A9 activate the Rag GTPases through noncanonical GEF mechanisms

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The mechanistic target of rapamycin complex 1 (mTORC1) growth pathway detects nutrients through a variety of sensors and regulators that converge on the Rag GTPases, which form heterodimers consisting of RagA or RagB tightly bound to RagC or RagD and control the subcellular localization of mTORC1. The Rag heterodimer uses a unique "locking" mechanism to stabilize its active (^{GTP}RagA–RagC^{GDP}) or inactive (^{GDP}RagA–RagC^{GTP}) nucleotide states. The Ragulator complex tethers the Rag heterodimer to the lysosomal surface, and the SLC38A9 transmembrane protein is a lysosomal arginine sensor that upon activation stimulates mTORC1 activity through the Rag GTPases. How Ragulator and SLC38A9 control the nucleotide loading state of the Rag GTPases remains incompletely understood. Here we find that Ragulator and SLC38A9 are each unique guanine exchange factors (GEFs) that collectively push the Rag GTPases toward the active state. Ragulator triggers GTP release from RagC, thus resolving the locked inactivated state of the Rag GTPases. Upon arginine binding, SLC38A9 converts RagA from the GDP- to the GTP-loaded state, and therefore activates the Rag GTPase heterodimer. Altogether, Ragulator and SLC38A9 act on the Rag GTPases to activate the mTORC1 pathway in response to nutrient sufficiency.

mTORC1 | Ragulator | Rag GTPases | SLC38A9 | guanine exchange factor

The mechanistic target of rapamycin complex 1 (mTORC1) is a master regulator of cell growth and proliferation. Nutrients, including amino acids and glucose, as well as growth factors, regulate the kinase activity of mTORC1 (1–4). In nutrient-rich conditions, mTORC1 stimulates anabolic processes and inhibits catabolic ones (5). Dysregulation of mTORC1 promotes aberrant growth and contributes to numerous human diseases (6). In mammalian cells, mTORC1 activation requires two steps: translocation of mTORC1 to the lysosomal surface and stimulation of its kinase activity, each of which is controlled by a different small GTPase(s). In the presence of nutrients, the Rag GTPases contact the Raptor subunit of mTORC1 and recruit mTORC1 to the lysosomal surface (7) where, if the growth factors are present, the Rheb GTPase turns on its kinase activity (8–10). This AND gate ensures that mTORC1 is only activated in the presence of both nutrients and growth factors.

The heterodimeric Rag GTPases occupy a key position in the mTORC1 pathway, as they integrate multiple inputs from upstream sensors. Distinct from canonical small signaling GTPases, the Rag GTPases form an obligatory heterodimer consisting of RagA or RagB bound to RagC or RagD (11, 12), and employ a unique locking mechanism that stabilizes either the on (^{GTP}RagA–RagC^{GDP}) or the off (^{GDP}RagA–RagC^{GTP}) state (13). The binding of a single GTP to either of the two subunits induces intra- and intersubunit conformational changes ("cross-talk") that have three consequences: (*i*) preventing the prebound GTP from dissociating, (*ii*) preventing a second GTP from associating with the other subunit, and (*iii*) in the case of accidental binding of the second GTP, triggering its hydrolysis (13). These properties are desirable for generating a steady signaling output under constant conditions, but its stability also poses a problem for upstream regulators when nutrient level changes.

There are two branches to the nutrient-sensing pathway upstream of the Rag GTPases. Cytosolic leucine, arginine, and *S*-adenosylmethionine are sensed by Sestrin2 (14, 15), CASTOR1 (16), and SAMTOR (17), respectively, which signal through the GATOR1 and GATOR2 proteins (18–20). Lysosomal arginine is sensed by SLC38A9 (21–23), which directly contacts the Rag–Ragulator complex on the lysosomal surface. Ragulator is a pentameric complex that consists of p18, p14, MP1, c7orf59, and HBXIP (24, 25). It directly binds the C-terminal roadblock domains (CRDs) of the Rag GTPases and tethers the Rag heterodimer on the lysosomal membrane (26, 27). SLC38A9 is a multipass transmembrane protein that also localizes to the lysosome and is an arginine sensor that upon activation stimulates mTORC1 (21–23). SLC38A9 shares homology with amino acid transporters but, unlike most such transporters, it has a large N-terminal domain on the cytosolic side of the lysosomal membrane (28). This N-terminal domain is necessary and sufficient to bind to the Rag–Ragulator complex, and its overexpression in cells activates mTORC1 even in the absence of amino acids (21, 23). Currently, the biochemical mechanisms through which Ragulator and SLC38A9 act on the Rag GTPases remain elusive, although a previous study from our laboratory indicated that Ragulator has guanine exchange factor (GEF) activity for RagA (25). How Ragulator and SLC38A9 act is likely difficult to understand because of the communication between the Rag subunits.

Recently, we established a suite of quantitative methods to measure the affinity and kinetics of nucleotide binding by the Rag GTPases (13). To study the roles of Ragulator and

Significance

Amino acids are basic building blocks for all organisms and are essential for cell growth and proliferation. Cells use a set of protein machines to sense the availability of amino acids to carry out basic responses. In particular, the mTORC1 pathway is a central sensor of amino acids in mammalian cells. Residing on the lysosomal surface upon activation, mTORC1 processes signals from the upstream Rag GTPases and stimulates downstream effectors through phosphorylation cascades. Ragulator and SLC38A9 are key components of the lysosomal branch of the amino acid-sensing machinery upstream of the Rag GTPases, but how they regulate the Rag GTPases at the molecular level remains poorly understood. Here we used kinetic analyses to define their functions.

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SLC38A9, we purified both and included them in the kinetic assays. Surprisingly, we found that Ragulator and SLC38A9 both function as unique GEFs for Rag subunits through distinct molecular mechanisms. Ragulator triggers GTP release from RagC and thus resolves the inactivated locked state of the Rag GTPases. SLC38A9 stimulates GDP release from RagA upon its activation by arginine. Together, these two components push the Rag GTPase heterodimer toward its active state.

Results

Ragulator Modulates the Nucleotide Binding Preference of the Rag GTPase Heterodimer. To generate the Ragulator complex for biochemical characterization, we coexpressed its five subunits in bacteria. All of the subunits coeluted in the same fraction after gel-filtration separation, suggesting its integrity (*SI Appendix*, Fig. S1*A*). We then assembled the Rag–Ragulator complex by incubating the purified Rag GTPase heterodimer with the Ragulator complex at a 1:1 stoichiometric ratio (*SI Appendix*, Fig. S1*A*). Coomassie staining confirmed that all seven subunits were assembled together (*SI Appendix*, Fig. S1*B*). We used this heptamer to biochemically characterize the Rag GTPases upon Ragulator binding.

Using a previously established cross-linking assay to examine the nucleotide occupancy of both Rag GTPases at the same time (13), we first asked if Ragulator might modulate the nucleotide binding affinities of the Rag GTPases. An increasing amount of the Rag–Ragulator complex was incubated with ^{32}P-labeled guanine nucleotides. We then induced photo-cross-linking and resolved the bound nucleotide to each Rag subunit by SDS/PAGE analyses (Fig. 1 *A* and *B*). Quantification of the radioactive signal gave binding affinities (K_d) of 10 and 36 nM for RagA and RagC to GTP, respectively, and of 63 and 64 nM for RagA and RagC to GDP, respectively (Fig. 1*C*). In comparison with the data obtained with the Rag GTPase heterodimer alone, the affinity of RagA and RagC for GDP remains similar while the affinity of RagA for GTP increased by 4.7-fold by Ragulator and that of RagC decreased by 3.3-fold. Considering the locking mechanism within the Rag GTPase heterodimer, in which only

one subunit can occupy the GTP-bound state (13), there is a 16-fold thermodynamic shift of the GTP binding preference toward RagA. This result is consistent with Ragulator being an activator of the mTORC1 pathway. Because in our previous study (25) we could not differentiate the binding of nucleotides to individual Rag subunits, we did not detect the modulation of nucleotide binding preference found here.

Ragulator Increases the Off-Rate of GTP from RagC. The Rag GTPase heterodimer functions through a distinct locking mechanism, in which GTP binding induces intra- and inter-subunit conformational changes (13). We first checked how Ragulator affects the intrasubunit conformational changes of the Rag GTPases. Upon GTP binding to a Rag GTPase subunit, Switch I swings to the top of the nucleotide-binding pocket, forming a lid (20, 29, 30). This conformational change prevents the release of the bound GTP, leading to a 1,300- and 46-fold reduction of the off-rate of GTP from RagA and RagC, respectively (13). Using a similar assay, we measured the on- and off-rates of GTP to the Rag–Ragulator complex. While the on-rates remain similar (*SI Appendix*, Fig. S2), we found Ragulator specifically accelerates the off-rate of GTP from RagC by 16-fold (Fig. 2 *A* and *C*). In contrast, the off-rate for GTP of RagA is largely unaffected by the presence of Ragulator (Fig. 2*A*). We observed a slightly slower off-rate of GDP from RagA, likely caused by intersubunit locking (Fig. 2*B*). This result sharply contrasts with canonical signaling GTPases and their corresponding GEFs, in which nucleotide (GTP or GDP) release is indistinguishably accelerated (31–34), suggesting Ragulator functions on RagC through a unique mechanism.

To further validate this result, we performed a dose-dependence assay by incorporating increasing amounts of Ragulator into the GTP off-rate measurements. Not until we included a stoichiometric amount of Ragulator did we observe a full stimulation of GTP release from RagC (*SI Appendix*, Fig. S3), which suggests Ragulator does not act on the Rag GTPases catalytically (no turnover is required). Therefore, we conclude that Ragulator modulates the nucleotide binding preference for both RagA and RagC subunits by binding and possibly an internal conformational change, thus resolving the locked inactivated state by accelerating the GTP off-rate from RagC (Fig. 2*D*).

Using a differential binding assay (*SI Appendix*, Fig. S4), we reexamined the reaction condition that our laboratory used to examine the activity of Ragulator in a previous study (25). We found that a stoichiometric amount of the xanthine nucleotide was not sufficient to block the misloading of the guanine nucleotide into a putatively xanthine-specific Rag subunit [RagA–RagC(D181N)]. This misloading could lead to misinterpretation of the data therein (*Discussion*).

Ragulator Resolves the RagC^{GTP}-Locked State by Opening Up the Nucleotide-Binding Pocket of RagC. Based on the results above, we considered two possible molecular mechanisms for how Ragulator accelerates GTP release from RagC. First, Ragulator could stimulate GTP hydrolysis of RagC and convert the bound GTP to GDP, thus accelerating its release because the GDP off-rate is much faster than the GTP off-rate (Fig. 2*C*). Second, Ragulator could open up the nucleotide-binding pocket of RagC, or interfere with the intersubunit cross-talk ("locking"), and thus release the bound GTP. To differentiate between these two possibilities, we first checked the GTP hydrolysis rate of the Rag GTPases in the presence of Ragulator by including increasing amounts of Ragulator in single- or multiple-turnover GTP hydrolysis assays. We found no difference in hydrolysis rates under either condition (*SI Appendix*, Fig. S5), suggesting that Ragulator does not stimulate GTP hydrolysis of either subunit.

To probe the conformation of the nucleotide-binding pocket of RagC, we performed a half-site on-rate measurement. In this
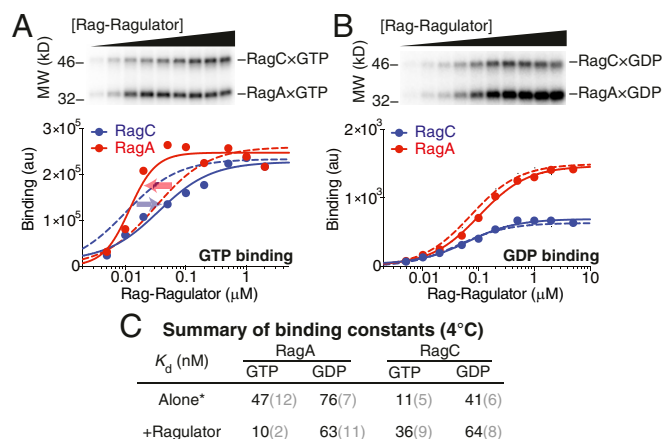

Fig. 1. Ragulator modulates the GTP binding affinity of the Rag GTPases. (*A* and *B*) Equilibrium binding assay to determine GTP (*A*) or GDP (*B*) binding affinity of the Rag–Ragulator heptamer. Increasing amounts of the Rag–Ragulator complex were incubated with trace amounts of radioactively labeled nucleotide. The dissociation constant (K_d) was extracted by fitting the band intensity of the same cross-linked species against the protein concentration using a quadratic equation. Dashed lines represent normalized data obtained with the Rag GTPases alone. Arrows point to the change of binding curves upon addition of Ragulator. (*C*) Summary of nucleotide binding affinity of the Rag–Ragulator heptamer. The asterisk indicates Rag GTPase alone data were taken from ref. 13 for comparison. Gray numbers in parentheses denote the SDs of the reported values calculated from two independent experiments.
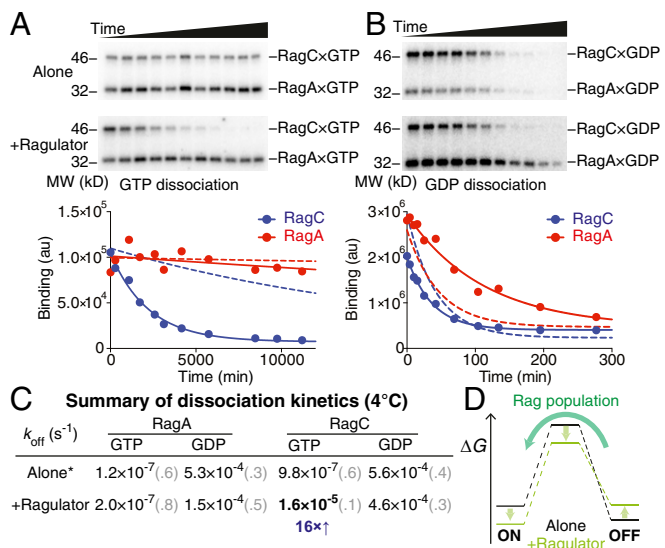
C Summary of binding constants (4°C)

K_d (nM)	RagA		RagC	
	GTP	GDP	GTP	GDP
Alone*	47(12)	76(7)	11(5)	41(6)
+Ragulator	10(2)	63(11)	36(9)	64(8)

Fig. 2. Ragulator accelerates the release of GTP from RagC. (*A* and *B*) Pulse-chase experiment to determine the GTP (*A*) and GDP (*B*) off-rate from the Rag–Ragulator heptamer. An excess amount of unlabeled nucleotide was used to chase away the radioactively labeled nucleotide prebound to Rag–Ragulator. The off-rate was extracted by fitting the band intensity against time to a single exponential decrease. Dashed lines represent normalized data with the Rag GTPases alone. (*C*) Summary of off-rates from the Rag–Ragulator heptamer. The asterisk indicates Rag GTPase alone data were taken from ref. 13 for comparison. Gray numbers in parentheses denote the SDs of the reported values calculated from two or three independent experiments. (*D*) Free-energy diagram to show the thermodynamic and kinetic effect of Ragulator on the Rag GTPases. Ragulator stabilizes the on-state and lowers the barrier toward it.

experiment, half of the nucleotide-binding pockets of the Rag GTPase heterodimer are statistically occupied, so we can specifically determine the on-rate of GTP to the remaining site. Here, a first-order reaction (concentration independence) suggests the nucleotide-binding pocket resides in a closed conformation, while a second-order reaction (concentration dependence) suggests a relatively open conformation (cf. supplementary figure 2 J and K in ref. 13). In the absence of Ragulator, both subunits were closed, as reflected by their first-order rate constants (dashed lines, *SI Appendix*, Fig. S6). Interestingly, while the half-site on-rate for RagA remains first-order in the presence of Ragulator, that of RagC becomes second-order (*SI Appendix*, Fig. S6), suggesting RagC adopts a relatively open conformation even when RagA binds GTP.

Intersubunit cross-talk within the Rag GTPase heterodimer is important for maintaining its normal function and responding appropriately to amino acid signals (13). To test whether Ragulator affects cross-talk, we harnessed a Rag GTPase mutant, RagA–RagC(L91P), which is partially defective in intersubunit communication (13). We measured the off-rate of GTP from RagA–RagC(L91P) alone or in the presence of Ragulator (Fig. 3*A*). Consistent with a previous study, the GTP off-rate from RagA–RagC(L91P) is 10-fold faster than that from wild-type Rag GTPases (Fig. 3*B*, magenta). Interestingly, Ragulator further accelerates the off-rate of GTP from the RagC(L91P) subunit by 4.6-fold but not from RagA (Fig. 3*B*, orange), suggesting Ragulator functions in parallel with intersubunit locking and the effect is specific to RagC. Combining the results above, we conclude that Ragulator opens up the nucleotide-binding pocket of RagC, and thus resolves the ^{GDP}RagA–RagC^{GTP}– locked state and converts it to ^{GDP}RagA–RagC^{GDP} (Fig. 3*C*). In this state the Rag GTPase heterodimer is unlocked, making the nucleotide-binding pocket of RagA accessible to GTP. Thus,

Ragulator has a similar effect on the nucleotide loading state of RagC as its GTPase activating protein (GAP), the FLCN–FNIP complex (35, 36).

SLC38A9 Directly Binds the Rag GTPase Heterodimer Independent of Ragulator. To investigate the molecular mechanism through which SLC38A9 acts, we first probed its binding capacity to the Rag GTPase heterodimer and/or Ragulator. After incubating with Ragulator in vitro, the N-terminal domain of SLC38A9 (SLC38A9N, amino acids 1 to 119) failed to form a complex with it, as we observed discrete peaks in the gel-filtration profiles, whose identities we confirmed by SDS/PAGE analyses (*SI Appendix*, Fig. S7 *A* and *B*). Strikingly, a substantial amount of SLC38A9N coeluted with the Rag GTPases in vitro after gel-filtration separation (Fig. 4 *A* and *B*), suggesting a direct interaction with the Rag heterodimer. This interaction is independent of Ragulator (*SI Appendix*, Fig. S7 *C* and *D*) but depends on the nucleotide loading state of the Rag GTPases (Fig. 4*C*): RagA(T21N)–RagC (approximating the ^{GDP}RagA– RagC^{GTP} state) coimmunoprecipitated a much higher amount of SLC38A9N than wild-type RagA–RagC or the RagA(Q66L)– RagC mutant (the ^{GTP}RagA–RagC^{GDP} state), suggesting that SLC38A9N preferentially binds the heterodimer when RagA is loaded with GDP. Furthermore, in cells lacking p18 (LAMTOR1), a critical component of the Ragulator pentamer, we observed a similar amount of the Rag GTPases associating with SLC38A9N (Fig. 4*C*), further confirming our in vitro binding assay. These results corroborate that a direct interaction occurs between the Rag GTPases and SLC38A9N. Moreover, the nucleotide binding states of the Rag GTPases in the heterodimer strongly regulate the interaction, consistent with previous results in cells (cf. figure 4C in ref. 23).

SLC38A9 Is a GEF for RagA. Because SLC38A9N binds the Rag GTPase heterodimer directly, we hypothesized that it might modulate their nucleotide binding capacity and/or affinity or kinetics. To test these hypotheses, we performed the cross-linking assay with an excess amount of SLC38A9N that was preincubated with nucleotide-free Rag GTPases before the mixture was loaded with trace amounts of radioactively labeled nucleotides. Surprisingly, we failed to detect any nucleotide, neither GTP nor GDP, cross-linked to RagA in the presence of SLC38A9N (Fig. 5*A*), sharply contrasting with the unaltered nucleotide binding capacity of RagC. Moreover, this effect was independent of Ragulator (Fig. 5*A*). To further confirm this

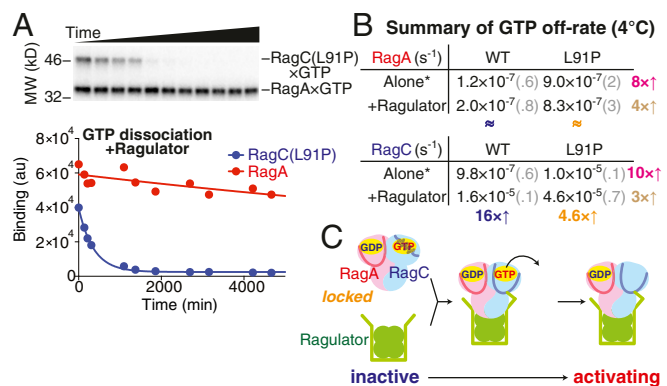**Fig. 3.** Ragulator opens up the nucleotide-binding pocket of RagC. (*A*) Pulse-chase experiment to determine the GTP off-rate from RagA–RagC (L91P) in the presence of Ragulator. (*B*) Summary of GTP off-rates. Gray numbers in parentheses denote the SDs of the reported values calculated from two independent experiments. (*C*) Ragulator opens up the nucleotide-binding pocket of RagC, allowing GTP to be released.
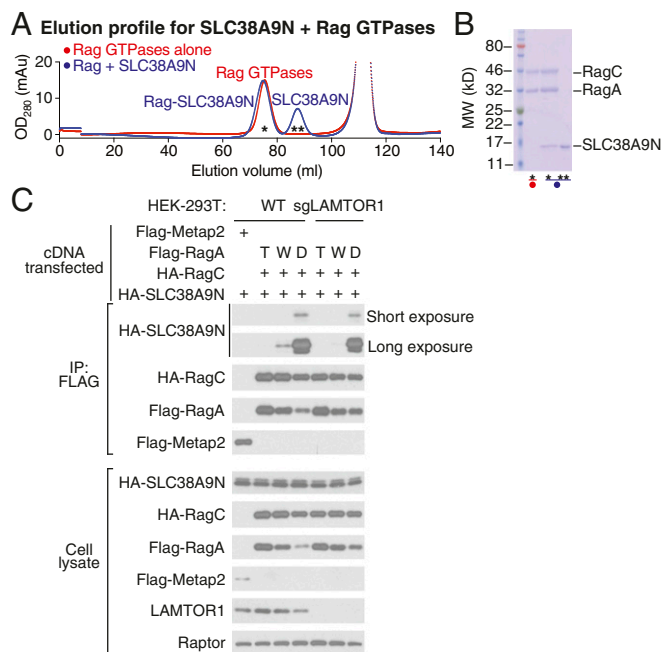
Fig. 4. SLC38A9N directly interacts with the Rag GTPase heterodimer. (*A* and *B*) Gel-filtration profile (*A*) and Coomassie-stained gel (*B*) of the Rag GTPases (red) and a mixture of Rag GTPases and SLC38A9N (blue). For Rag+SLC38A9N, the Rag GTPases were incubated with SLC38A9N before separation on a gel-filtration column. The peak representing the Rag GTPases shifts upon addition of SLC38A9N. Coomassie-stained gel analysis confirmed the coelution. (*C*) Coimmunoprecipitation (IP) assay to detect the effects of the nucleotide binding states of the Rag GTPases on the binding to SLC38A9N. SLC38A9N strongly binds the Rag GTPase heterodimer when RagA is in the GDP-loaded state. Similar behavior was observed in p18-knockout cells that lack a functional Ragulator. D, RagA(T21N) mutant; T, RagA(Q66L) mutant; W, wild-type RagA.

result, we performed a dose-dependent assay in which increasing amounts of SLC38A9N were incubated with the Rag GTPases (*SI Appendix*, Fig. S8*A*) before inducing cross-linking. A stoichiometric amount of SLC38A9N was sufficient to block nucleotide binding to RagA (*SI Appendix*, Fig. S8*B*).

Based on the results above, we suspected that SLC38A9N might serve as a GEF by actively displacing nucleotides from RagA. To this end, we included saturating amounts of SLC38A9N in a nucleotide-release assay where unlabeled nucleotides were used to chase away bound, radioactively labeled nucleotides (Fig. 5*B*). The off-rate of GTP from RagA is moderately stimulated by SLC38A9N, while that of RagC remains unchanged as an internal control (*SI Appendix*, Fig. S9). These results are consistent with the notion that SLC38A9N acts on RagA, and that RagA in its GTP-bound form binds only very poorly to SLC38A9N (Fig. 4*C*). In contrast, we observed a dramatic effect of SLC38A9N on the GDP off-rate (Fig. 5 *C* and *D*): The moment it was added to the reaction mixture, the GDP bound to RagA was readily chased away almost completely. Quantification of the time points revealed an 18-fold stimulation of the GDP off-rate from RagA with the Rag GTPases alone, and a 60-fold stimulation with the Rag–Ragulator heptamer (Fig. 5*E* and *SI Appendix*, Fig. S10). In accordance with the locking mechanism proposed previously, the GDP off-rate of RagC is reduced, although to a lesser extent. Considering that the fast nucleotide-release rate approaches the limit of the time resolution of our cross-linking analysis (each time point takes ~1.5 min), we speculate that the actual rate enhancement on RagA may be even higher. The stimulatory effect of SLC38A9N is catalytic, as a substoichiometric amount of SLC38A9N robustly triggered GDP release from RagA (*SI Appendix*, Fig. S11). Combining the results above, we show that SLC38A9N reduces the half-life of the

inactivated Rag GTPases to shorter than 0.5 min, which is then consistent with the temporal requirement for the activation of mTORC1 by amino acids (half-life of ~10 min).

Nucleotide Loading State of the Rag GTPase Heterodimer Feeds Back on SLC38A9 Binding. Based on the results above, we propose the following unified model (Fig. 5*F*): When lysosomal arginine binds to SLC38A9, its N-terminal domain becomes active and binds to the inactivated form of the Rag GTPase heterodimer (RagA-GDP form; Fig. 4*C*). In this state, SLC38A9N executes its GEF activity and kicks out the bound GDP from RagA (Fig. 5 *C* and *D*). Because cellular GTP concentrations are much higher than those of GDP, a GTP molecule is likely to bind to the then-empty nucleotide-binding pocket, which converts RagA into its GTP-bound form and thus becomes activated. As RagA-GTP binds poorly to SLC38A9, the Rag heterodimer likely dissociates from SLC38A9 (Fig. 4*C*), turning it over to activate other Rag GTPases (*SI Appendix*, Fig. S11).

Two key features remain to be tested in this model: First, arginine has been shown to regulate the interaction between SLC38A9 and the Rag–Ragulator complex (37). What, then, is the molecular mechanism? Does arginine activate the N-terminal domain to promote this interaction? Second, will the binding of GTP to RagA weaken the interaction between SLC38A9N and the Rag GTPase heterodimer, thus facilitating the turnover of SLC38A9N? To directly observe these two processes, we designed a dynamic binding assay (Fig. 6*A*). We first incubated the Rag GTPase heterodimer with full-length SLC38A9 in the absence or presence of arginine and examined the amount that coimmunoprecipitated with it. Consistent with our prediction, a higher amount of the Rag GTPase heterodimer bound to SLC38A9 in the presence of arginine, suggesting that arginine likely triggers conformational changes within SLC38A9 that promote the binding of its N-terminal domain to the Rag GTPase heterodimer (Fig. 6*B*, "Bound" lanes). A similar binding preference has also been observed in cells (*SI Appendix*, Fig. S12). To test whether GTP binding to RagA will trigger its release from SLC38A9, we added nucleotides to the +arginine sample and probed the amount of Rag GTPases that remained bound. Satisfactorily, the Rag GTPase heterodimer dissociated from SLC38A9 when we added GTP to the mixture, but not GDP (Fig. 6*B*, "RB" lanes). This directly implies that GTP loading to RagA weakens its affinity for SLC38A9, which dissociates and can perform another round of catalysis on inactive Rag GTPase heterodimers.

To test whether the dissociation of the Rag GTPases from SLC38A9 is necessary for mTORC1 activation, we immunoprecipitated stably expressed SLC38A9 or RagC and probed for endogenous mTORC1 components Raptor and mTOR (Fig. 6*C*). While RagC coimmunoprecipitated a considerable amount of Raptor and mTOR in an amino acid-regulated fashion, we failed to detect any Raptor or mTOR coimmunoprecipitating with SLC38A9 (Fig. 6*C*), suggesting that SLC38A9 cannot interact with the Rag GTPases when they are bound to mTORC1.

Discussion

Ragulator and SLC38A9 are two critical regulators of the lysosomal branch of the nutrient-sensing pathway upstream of mTORC1. Using kinetic analyses, we find that Ragulator triggers GTP, but not GDP, release from RagC and thus resolves the inactivated locked state of the Rag GTPase heterodimer. SLC38A9 stimulates GDP release from RagA upon its activation by arginine, which leads to its dissociation from the activated Rag heterodimer. Altogether, these two components push the Rag GTPases toward the activated state, which then recruits mTORC1 to the lysosomal surface in response to nutrient availability.

Both Ragulator and SLC38A9 modulate the nucleotide loading state of the Rag GTPases. In a broad sense, they function as guanine exchange factors. However, the biochemical
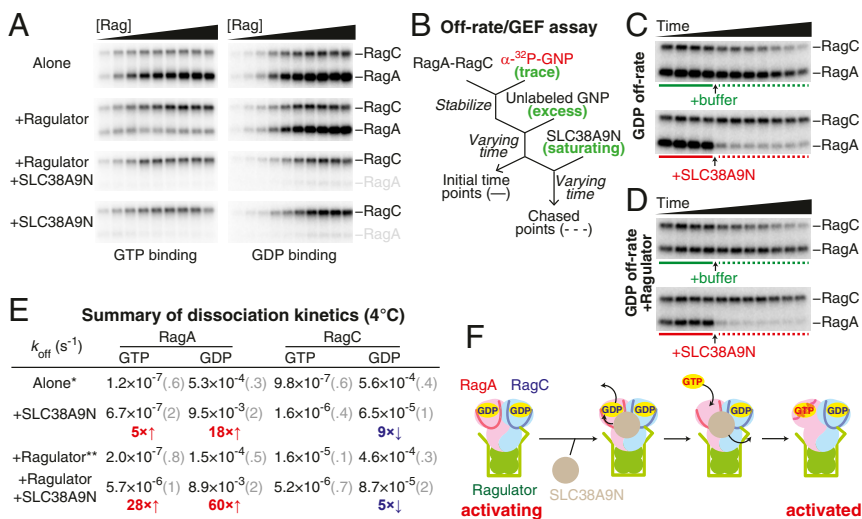
A

	[Rag]	[Rag]	
Alone			–RagC / –RagA
+Ragulator			–RagC / –RagA
+Ragulator +SLC38A9N			–RagC / –RagA
+SLC38A9N			–RagC / –RagA
	GTP binding	GDP binding	

B Off-rate/GEF assay

RagA·RagC → α-³²P-GNP (trace)
Stabilize → Unlabeled GNP (excess)
Varying time → SLC38A9N (saturating)
Initial time points (—) → Varying time
→ Chased points (- - -)

C Time

GDP off-rate — –RagC / –RagA (+buffer)
–RagC / –RagA (+SLC38A9N)

D Time

GDP off-rate +Ragulator — –RagC / –RagA (+buffer)
–RagC / –RagA (+SLC38A9N)

E Summary of dissociation kinetics (4°C)

k_{off} (s⁻¹)	RagA GTP	RagA GDP	RagC GTP	RagC GDP
Alone*	1.2×10^{-7}(.6)	5.3×10^{-4}(.3)	9.8×10^{-7}(.6)	5.6×10^{-4}(.4)
+SLC38A9N	6.7×10^{-7}(2) 5×↑	9.5×10^{-3}(2) 18×↑	1.6×10^{-6}(.4)	6.5×10^{-5}(1) 9×↓
+Ragulator**	2.0×10^{-7}(.8)	1.5×10^{-4}(.5)	1.6×10^{-5}(.1)	4.6×10^{-4}(.3)
+Ragulator +SLC38A9N	5.7×10^{-6}(1) 28×↑	8.9×10^{-3}(2) 60×↑	5.2×10^{-6}(.7)	8.7×10^{-5}(2) 5×↓

F

RagA RagC / GDP GDP → GDP GDP → GDP → GTP GDP
Ragulator SLC38A9N
activating → activated

Fig. 5. SLC38A9N is a GEF for RagA. (*A*) Equilibrium binding assay to monitor nucleotide binding to the Rag GTPases alone, in complex with Ragulator, and/or in the presence of an excess amount of SLC38A9N. SLC38A9N abolishes nucleotide binding to RagA. (*B*) GEF assay to determine the off-rate of nucleotide from the Rag GTPases. A saturating amount of SLC38A9N was added during the time course of the reaction to probe its effect. (*C* and *D*) GDP off-rates of the Rag GTPases (*C*) and Rag–Ragulator heptamer (*D*). SLC38A9N strongly accelerates GDP release from RagA. See *SI Appendix*, Fig. S10 for quantification. (*E*) Summary of nucleotide off-rates. The asterisk indicates Rag GTPase alone data were taken from ref. 13 for comparison. Double asterisks indicate +Ragulator data were taken from Fig. 2. Gray numbers in parentheses denote the SDs of the reported values calculated from two independent experiments. (*F*) Model for the function of the N-terminal domain of SLC38A9 (SLC38A9N).

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details obviously differ from other GEFs. Canonical GEFs, such as CDC25 for Ras (31), RCC1 for Ran (32), and TRAPP for Ypt1p (33, 34), trigger nucleotide exchange from their GTPase targets regardless of the identity of the bound nucleotide. The higher cellular concentration of GTP ensures that once the bound nucleotide is released, a new GTP molecule will likely bind to the GTPase and thus activate it. In sharp contrast to these GEFs, Ragulator specifically accelerates GTP, but not GDP, release from RagC, suggesting that it acts through a unique molecular mechanism. Although the structure of Ragulator with full-length Rag GTPases is still missing so the molecular details cannot yet be visualized, we speculate that this behavior evolved along with the locking mechanism: Upon GTP binding, Switch I of the Rag GTPases likely goes through a dramatic conformational rearrangement, which swings to the top of the nucleotide-binding pocket. This conformational change leads to a closed binding pocket and is likely responsible for the extremely slow off-rate of GTP. On the other hand, GDP binding is incapable of triggering such a movement, and therefore it releases at a much faster rate. Ragulator partially opens up the closed nucleotide-binding pocket

when RagC is loaded with GTP, and thus resolves the locked, inactivated Rag heterodimer. Based on the steric restriction, we suspect the functional region of Ragulator is the N-terminal part of p18, because it extends toward the nucleotide-binding domain of RagC, as observed in the crystal structure containing Ragulator–RagA(CRD)–RagC(CRD) (26, 27).

In a previous study, Bar-Peled et al. (25) suggested that Ragulator serves as a GEF for RagA. We now recognize that an imperfect experimental setup may have led to this misinterpretation of the results. First, the assay used previously could not definitively distinguish nucleotide binding to each Rag subunit. Second, the reaction condition was not optimized to fully prevent misloading of the guanine nucleotide to the putatively xanthine-specific Rag GTPase mutants. Third, the results were likely complicated by a small amount of SLC38A9 that copurified with Rag–Ragulator in the mammalian protein expression system used. As a result, we conclude that while Ragulator does impact the affinity of RagA for GTP, it has the most pronounced effects on RagC, a function that we failed to detect in the Bar-Peled et al. study.
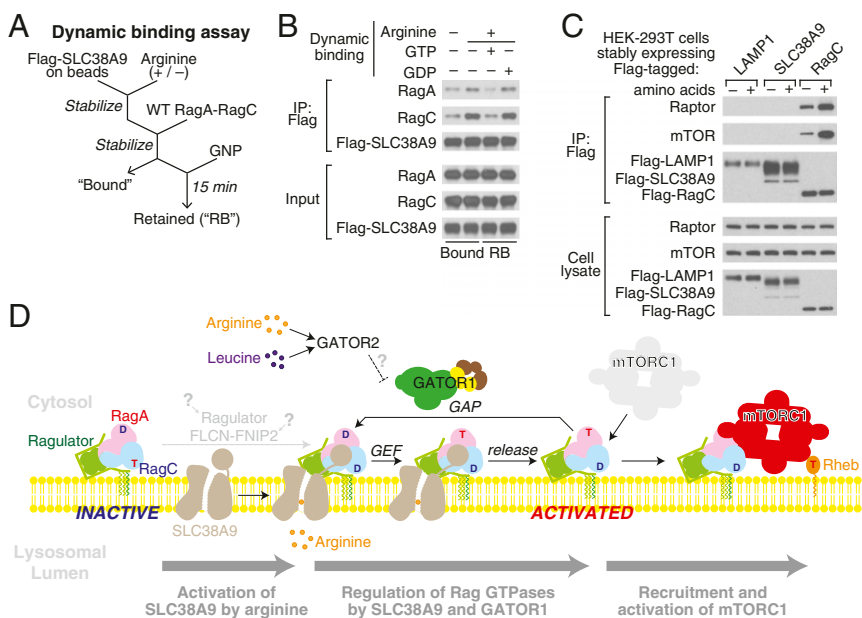

A Dynamic binding assay

Flag-SLC38A9 on beads + Arginine (+ / −)
Stabilize → WT RagA·RagC
Stabilize → GNP
"Bound" → 15 min
→ Retained ("RB")

B

Dynamic binding	Arginine	−	+		
	GTP	−	−	+	−
	GDP	−	−	−	+
IP: Flag	RagA				
	RagC				
	Flag-SLC38A9				
Input	RagA				
	RagC				
	Flag-SLC38A9				
		Bound		RB	

C HEK-293T cells stably expressing Flag-tagged: LAMP1 / SLC38A9 / RagC

amino acids: − + / − + / − +

IP: Flag:
Raptor
mTOR
Flag-LAMP1 / Flag-SLC38A9 / Flag-RagC

Cell lysate:
Raptor
mTOR
Flag-LAMP1 / Flag-SLC38A9 / Flag-RagC

D

Cytosol — Arginine, Leucine → GATOR2 ⊣ GATOR1 (GAP) → mTORC1
RagA(D) Ragulator / FLCN-FNIP2? / Ragulator ᵀRagC
INACTIVE → SLC38A9 → GEF → release → ACTIVATED → mTORC1 / Rheb
Lysosomal Lumen — Arginine
Activation of SLC38A9 by arginine → Regulation of Rag GTPases by SLC38A9 and GATOR1 → Recruitment and activation of mTORC1

Fig. 6. Arginine regulates the enzymatic function of SLC38A9. (*A* and *B*) Dynamic binding assay to probe the effect of arginine on SLC38A9 and the effect of GTP binding on the SLC38A9–Rag interaction (*A*). Arginine enhances SLC38A9 binding to the Rag GTPases, while GTP binding to the Rag GTPases weakens it (*B*). A 50% decrease in Rag GTPase binding is expected upon GTP addition because of the equal chance of GTP association with either subunit. RB, Rag GTPases that remain bound to SLC38A9 upon the nucleotide chase. (*C*) Coimmunoprecipitation assay to probe potential interaction between mTORC1 and SLC38A9 or RagC. An amino acid-regulated interaction was observed between mTORC1 and RagC, but not between mTORC1 and SLC38A9. (*D*) Model for mTORC1 activation upon amino acid sufficiency. The Rag GTPases cycle between SLC38A9 and mTORC1, which physically separates the activation of the Rag GTPases from the recruitment of mTORC1. D, GDP; T, GTP.

The binding of canonical GEFs to their substrate GTPases is usually independent of the nucleotide loading state (32). This concept is consistent with the nonspecific stimulation of nucleotide release. In contrast, the binding of SLC38A9N to the Rag GTPase heterodimer strongly depends on the nucleotide loading state of RagA, which suggests an elegant functional cycle (Fig. 6D). When RagA is loaded with GDP and arginine is present, the N-terminal domain of SLC38A9 binds to it and catalyzes the release of GDP. As GTP associates with RagA, it likely triggers conformational changes that actively reject the bound SLC38A9N and detach it from the activated Rag GTPases. SLC38A9N is then available for another round of activation. This cycle has several biological implications. First, it allows SLC38A9 to activate multiple Rag GTPases in the presence of lysosomal arginine, defining SLC38A9 as an enzyme instead of a scaffolding protein. Second, it physically separates the activation of the Rag GTPases (by the activated SLC38A9) from the recruitment of mTORC1 (by the activated Rag GTPases), because RagA in its GTP-bound form binds weakly with SLC38A9. Third, it suggests that a signaling supercomplex between SLC38A9, Ragulator–Rag, and mTORC1 is unlikely to be possible, as the binding of the Rag GTPases to SLC38A9 and mTORC1 is strictly anticorrelated (21, 23). Instead, SLC38A9 only transiently interacts with the Rag GTPase heterodimer and "charges" it into the active nucleotide loading state (^{GTP}RagA–RagC^{GDP}) to recruit mTORC1. Therefore, detachment of the activated Rag GTPases from SLC38A9 is likely an essential step during mTORC1 activation (Fig. 6D).

In addition to the lysosomal amino acid-sensing branch, cytosolic sensors such as Sestrin2 and CASTOR1 signal to the Rag GTPases through the GATOR1 and GATOR2 branch of the pathway (Fig. 6D). In the absence of cytosolic amino acids, GATOR1 will remain active and convert RagA to its GDP-bound

form, so that even if SLC38A9 GEFs RagA in response to lysosomal arginine, the bound GTP will be hydrolyzed to prevent pathway activation. This "futile cycle" reveals a coincident detector mechanism that ensures that mTORC1 becomes active only in the presence of both cytosolic and lysosomal amino acids (Fig. 6D). Lastly, the finding that SLC38A9 is a GEF for RagA likely explains why overexpression of just its N-terminal domain can render the mTORC1 pathway insensitive to amino acid starvation (23). At high expression levels, SLC38A9N should counter the stimulation of the GATOR1 GAP activity that presumably occurs when cytosolic leucine or arginine is low.

Materials and Methods

Materials. Reagents used in this study can be found in *SI Appendix*.

Protein Purification. Details of the protein purification procedure are described in *SI Appendix*.

Kinetic and Equilibrium Binding Assays. All of the kinetic and equilibrium analyses were performed using established protocols (13), in which Ragulator and/or SLC38A9N was included as indicated.

Coimmunoprecipitation Experiments. Details of the coimmunoprecipitation experiments are described in *SI Appendix*.

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