SLC38A9 is a component of the lysosomal amino acid sensing machinery that controls mTORC1

Manuele Rebsamen1, Lorena Pochini2, Taras Stasyk3, Mariana E. G. de Araújo3, Michele Galluccio2, Richard K. Kandasamy1, Berend Snijder1, Astrid Fauster1, Elena L. Rudashevskaya1, Michele Bruckner1, Stefania Scorzon1, Przemyslaw A. Filipek3, Kilian V. M. Huber1, Johannes W. Bigenzahn1, Leonhard X. Heinz1, Claudine Kraft4, Keiryn L. Bennett1, Cesare Indiveri2, Lukas A. Huber5 & Giulio Superti–Furga1

Cell growth and proliferation are tightly linked to nutrient availability. The mechanistic target of rapamycin complex 1 (mTORC1) integrates the presence of growth factors, energy levels, glucose and amino acids to modulate metabolic status and cellular responses6–9. mTORC1 is activated at the surface of lysosomes by the RAG GTPases and the Ragulator complex through a not fully understood mechanism monitoring amino acid availability in the lysosomal lumen and involving the vacuolar H+–ATPase10–12. Here we describe the uncharacterized human member 9 of the solute carrier family 38 (SLC38A9) as a lysosomal membrane-resident protein competent in amino acid transport. Extensive functional proteomic analysis established SLC38A9 as an integral part of the Ragulator–RAG GTPases machinery. Gain of SLC38A9 function rendered cells resistant to amino-acid-induced mTORC1 activation. Thus SLC38A9 is a physical and functional component of the amino acid sensing machinery that controls the activation of mTOR.

Amino acids are essential for mTORC1 activity, as growth factors cannot efficiently activate mTOR in their absence13. Notwithstanding the growing number of proteins involved in the activation of mTOR at the lysosomal surface, the molecular nature of the amino acid sensing mechanisms have remained elusive14–16. Several members of the solute carrier (SLC) group belonging to families capable of transporting amino acids at the plasma membrane have been shown to regulate mTOR activity17,18, raising the possibility that SLCs may also be involved in the lysosomal sensing. We hypothesized the existence of an ubiquitously expressed SLC belonging to a family competent for amino acid transport19 with a subcellular localization compatible with lysosomal amino acid sensing. Among the list of SLCs robustly expressed in two different cell lines, we focused on member 9 of the SLC38 family as it was completely uncharacterized, showed vesicular staining16 and had been associated to lysosomes by proteomic analysis17 (Extended Data Fig. 1a). The SLC38 family contains eleven members, and is part of a phylogenetic cluster of amino acid transporters comprising the SLC32 and SLC36 families20 (Extended Data Fig. 1b). SLC38A9 is predicted to encompass eleven transmembrane helices and a 120-residue cytoplasmic amino-terminal region. Treatment with peptide-N-glycosidase (PNGase) F showed that SLC38A9 is highly glycosylated and enabled detection of the endogenous protein (Extended Data Fig. 2a, b). Supporting a possible role in growth regulatory pathways, silencing of SLC38A9 by short hairpin RNA (shRNA) in HEK293T cells resulted in a reduction of cell size and cell proliferation (Extended Data Fig. 2c, d).

To test whether SLC38A9 would associate with the complex regulating mTORC1, we engineered HEK293 cells to express tagged SLC38A9 in an inducible fashion and verified the localization of the protein to lysosomes (Extended Data Fig. 3a–c). We purified endogenously assembled protein complexes using tandem affinity purification (TAP) coupled to one-dimensional gel-free liquid chromatography tandem mass spectrometry (LC–MS/MS). The gel-free approach was critical as upon boiling SLC38A9 formed insoluble aggregates that failed to enter sodium dodecyl sulfate–polyacrylamide gels (SDS–PAGE; Extended Data Fig. 2e, f). The analysis identified all the five members of the Ragulator/LAMTOR complex and the four RAG GTPases (known as RAGA–D or RRAGA–D) as specific interactors of SLC38A9 (Fig. 1a, Extended Data Fig. 3d). Such collective high sequence coverage of all components of the Ragulator–RAG GTPases complex strongly indicated that SLC38A9 was an additional uncharacterized member. When co-expressed in HEK293T cells, SLC38A9 co-immunoprecipitated with LAMTOR1 and overexpressed LAMTOR1 bound endogenous SLC38A9 (Fig. 1b, c). We validated complex membership entirely at the endogenous level in different cell lines. Immunoprecipitation of SLC38A9 resulted in the specific recruitment of endogenous RAGA and LAMTOR1 and, conversely, immunoprecipitated RAGA bound SLC38A9 (Fig. 1d). This association was not observed when SLC38A9 was silenced, confirming specificity. Association of endogenous SLC38A9 and RAGA was demonstrated in HeLa and K562 cells (Fig. 1e, f) and in murine NIH/3T3 fibroblasts and RAW 264.7 macrophages (Extended Data Fig. 2g, h). To further challenge specificity, we applied the identical proteomic strategy to the two highest expressed members of the SLC38 family, SLC38A1 and SLC38A2, and SLC36A1 (also known as PAT1), which has been previously associated with the Ragulator–RAG GTPase complex21. Despite very high bait recovery, none of the Ragulator–RAG GTPase complex members was identified among the interactors, highlighting that the association of SLC38A9 with this complex is a unique property of this family member (Extended Data Fig. 3d). Moreover, when we immunoprecipitated SLC38A9, SLC38A1, SLC38A2, SLC36A1 as well as a lysosomal member of the SLC38 family, SLC38A7 (ref. 20) and a second member of the SLC36 family, SLC36A4 (also known as PAT4), only SLC38A9 co-immunoprecipitated endogenous LAMTOR1, LAMTOR3, RAGA and RAGC, with both low and high expression levels (Fig. 1g).

Immunostaining of tagged SLC38A9 in HeLa cells revealed extensive colocalization with the late endosome/lysosome markers LAMP1, CD63 and the late endosome/multivesicular bodies lipid LBPA, but not with early endosome (EEA1) or Golgi (giantin) markers (Fig. 1h–j, Extended Data Fig. 4a, b). This supports SLC38A9 being a lysosomal component of the Ragulator–RAG GTPase complex.

Full membership to this multiprotein complex would entail physical association with any of the several detected members in reciprocal purifications. We performed affinity purification coupled to mass spectrometry analysis with LAMTOR1, 3, 4 and 5, as well as RAGA and RAGC GTPases. At the core of the interacting network obtained by combining the six independent purifications we found all the expected members of the Ragulator–RAG GTPases complex, RAPTOR as well as SLC38A9 (Fig. 2a, Extended Data Fig. 5a). The overall low sequence

©2015 Macmillan Publishers Limited. All rights reserved
coverage of SLC38A9 could be ascribed to inefficient proteolytic cleavage of the inaccessible transmembrane portions of the protein as it mirrored the coverage obtained when SLC38A9 was used as bait (Extended Data Fig. 5b, c). The interaction of endogenous SLC38A9 with all baits was confirmed by immunoprecipitation (Fig. 2b, c). The quality of the proteomic survey was also indicated by detection of the subunit was confirmed by immunoprecipitation (Fig. 2b, c). The quality of the proteomic survey was also indicated by detection of the subunit

Interestingly, we did not detect any other SLC member of the amino acid transporter families in any of the purifications with the members of Ragulator/GTPases complex, indicating that SLC38A9 is, at least in this cellular system, the only prominently interacting SLC.

Deletion studies indicated that the N-terminal cytoplasmic tail of SLC38A9 (amino acids 1–112), devoid of any transmembrane region, was sufficient and required to bind the Ragulator–RAG GTPases complex,

Figure 1 | SLC38A9 is a lysosomal component of the amino acid sensing machinery controlling mTORC1. a, Interactors of SLC38A9 identified by TAP–LC–MS/MS. Data shown are based on two independent experiments (n = 2), each analysed in two technical replicates. b–g, Lysates from HEK293T cells transfected with the indicated tagged constructs or empty vector (−) (b, c, g), control (empty vector or shGFP) or shSLC38A9-transduced HEK293T (d) and HeLa (e), or K562 (f) cells were subjected to immunoprecipitation. PNGase-treated immunoprecipitates (IP) and protein extracts (XT) were analysed by immunoblot with the indicated antibodies. Results are representative of two independent experiments (n = 2). <, ST-HA-SLC38A9; *, non-specific band. HA, haemagglutinin; ST, streptavidin; V5, V5 epitope tag. h–j, Confocal microscopy images of HeLa cells transfected with tagged SLC38A9 construct and immunostained with anti-HA and LAMP1 (h), EEA1 (i) or giantin (j) antibodies. Representative cells are shown. Scale bar, 10 μm.

Figure 2 | SLC38A9 is an integral component of the Ragulator–RAG GTPases machinery. a, Interactors of LAMTOR1, LAMTOR3, LAMTOR4, LAMTOR5, RAGA and RAGC were identified by TAP–LC–MS/MS. Proteins that interacted with all the bait proteins are shown, with the addition of RAGD that was not detected in RAGC pull-down. Previously published interactors of the Ragulator–RAG GTPases complex detected are indicated. Data shown are based on two independent experiments for each condition (n = 2), each analysed in two technical replicates. b–e, HEK293T cells were transfected with the indicated tagged constructs or empty vector (−). Immunoprecipitates and cell extracts were treated with PNGase and analysed by immunoblot. SLC38A9 mutant constructs are labelled with the number of the encoded amino acids (d) or with the amino acid motif substituted to alanine (e). Results are representative of two independent experiments (n = 2). Long exp., long exposure. WT, wild type.
whereas this interaction was completely lost when the remaining eleven transmembrane-containing region (113–561), which retains lysosomal localization, was used (Fig. 2d, Extended Data Fig. 4c, d). We further mapped the minimal interacting region to amino acids 31–112 and identified four conserved motifs in this portion (Extended Data Fig. 6a, b). Mutation of any of the first three motifs completely abolished binding, while disruption of the fourth had no effect (Fig. 2e, Extended Data Fig. 6c). Importantly, none of the described mutations affected the orientation observed in lysosomes (Extended Data Fig. 7b). Addition of SLC38A9 or with control empty vector. Values represent means of per

SLC38A9 transports amino acids and interacts with the RAG GTPases in a nucleotide-loading and amino-acid-sensitive manner. a, Time course of [3H]glutamine uptake in proteoliposomes reconstituted with purified recombinant SLC38A9 or with control empty vector. Values represent means ± s.d. from eight different experiments (n = 8). b, Inhibition by amino acids of [3H]glutamine uptake in proteoliposomes. Values represent means of residual activity with respect to control (without added competitor) ± s.d. from three independent experiments (n = 3). c, Uptake of the indicated [3H]-labelled amino acids by SLC38A9 in proteoliposomes. Values represent means of per cent in respect to glutamine transport measured in the same experiment ± s.d. from three independent experiments (n = 3). d, Time course of glutamine efflux from proteoliposomes reconstituted with SLC38A9. Values represent means of specific transport ± s.d. from three independent experiments (n = 3). e, HEK293T cells were transfected with the indicated combination of tagged RAG GTPases mutant constructs or empty vector (–). PNGase-treated immunoprecipitates and cell extracts were analysed by immunoblot. W, wild type; 66, Q66L; 21, T21N; 75, S75N; 120, Q120L. f, g, HEK293T cells stably expressing the indicated constructs were starved for amino acids and serum for 50 min (AA starv +) and stimulated with amino acids for 20 min (AA stim +). Immunoprecipitates and cell extracts were analysed by immunoblot. In g results of two biological replicates are shown. *, IgG light chain. Results are representative of two (e, f, n = 2) or three (g, n = 3) independent experiments. In a–c significance was estimated by Student’s t-test (**P < 0.01 or ***P < 0.001).
mutational state, even more than what was observed for the Ragulator complex (Fig. 3c, Extended Data Fig. 8). The low-affinity nucleotide-binding mutants RAGA(T21N) and RAGB(T54N) showed a strong increase in SLC38A9 recruitment, contrasting with the behaviour of RAGC(S75N) that abolished the binding of SLC38A9 to the heterodimer. GTP-bound RAGA(Q66L)/RAGB(Q99L) mutants showed also reduced SLC38A9 binding (Fig. 3c, Extended Data Fig. 8). These results indicate that the interaction of SLC38A9 with the critical GTases moieties of the complex is highly conformation-specific. In cells stably expressing tagged SLC38A9, amino acid starvation strengthened the interaction between SLC38A9 and endogenous RAGC and, to a minor extent, RAGA, without significantly affecting LAMTOR1 and LAMTOR3 recruitment (Fig. 3f). Similarly, amino acid stimulation reduced the amount of recruited RAGC and RAGA. Altogether, the amino-acid-sensitive character of these binding properties are evocative of the ones exerted by Ragulator and Folliculin and point to a possible function of SLC38A9 in modulating the nucleotide status of the RAG GTases. Amino acid sensitivity required the transmembrane region, as the recruitment of RAGC by the N-terminal region alone was not affected by amino acid availability (Fig. 3g). This is consistent with the notion that the eleven transmembrane helices-encompassing region is the moiety physically engaging amino acids and required to convey sensitivity.

Withdrawal of amino acids results in rapid inactivation of mTORC1. Cells stably expressing SLC38A9 showed sustained mTORC1 activation upon amino acid starvation, as monitored by the phosphorylation of the substrates S6 kinase and ULK1 (Fig. 4a, Extended Data Fig. 9a). This resulted in a delayed and reduced induction of autophagy upon amino acid starvation, as shown by quantification of LC3B relocalization (Fig. 4b, Extended Data Fig. 9b), as well as sustained phosphorylation and delayed nuclear translocation of the transcription factor TFEB (Extended Data Fig. 9c). Sustained mTOR activity triggered by SLC38A9 expression during starvation was inhibited by Torin 1 (Extended Data Fig. 9e). In contrast, the v-ATPase inhibitor concanamycin A had no effect in this setting, whereas it efficiently blocked mTORC1 activation induced by cycloheximide, which mimics amino acid stimulation by blocking protein synthesis and thus inducing accumulation of intracellular amino acids (Fig. 4e). This further suggests that SLC38A9 participates in mTORC1 activation at the lysosome rather than contributing to the import of extracellular amino acids at the plasma membrane. Moreover, SLC38A9 levels did not appear to be induced upon amino acid starvation, in contrast to several SLCs responsible for importing amino acids at the plasma membrane (Extended Data Fig. 10b, c).

Altogether, the work presented here identifies SLC38A9 as a novel integral component of the lysosomal machinery that controls mTORC1 activity in response to amino acids (Fig. 4g). SLC38A9 is the first member of the entire machinery shown to be competent for binding and transporting amino acids. As other solute carrier proteins, it should be eminently druggable. We failed to observe a strong dependence on SLC38A9 in amino acid stimulation-induced mTOR lysosomal recruitment, which could be due to technical reasons or, more intriguingly, to separate, partly independent mechanisms controlling localization and activation of mTOR. Together with the adaptation observed upon prolonged SLC38A9 silencing, this suggests that additional sensing components are likely to operate in this pathway. Considering the low transport capacity and the physical association with the Ragulator–RAG complex.
SLC38A9, a transceptor-type of SLC24,28–30, reminiscent of yeast amino acid sensors GAP1 and ssy1p, in which amino acid engagement is used for allosteric signal transduction rather than mere transport.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

Received 25 October 2013; accepted 20 November 2014. Published online 7 January 2015.

1. Dibble, C. C. & Manning, B. D. Signal integration by mTORC1 coordinates nutrient input with biosynthetic output. Nature Cell Biol. 15, 555–564 (2013).
2. Laplante, M. & Sabatini, D. M. mTOR signaling in growth control and disease. Cell 149, 274–293 (2012).
3. Conru, M., Albert, V. & Hall, M. N. mTOR in aging, metabolism, and cancer. Curr. Opin. Genet. Dev. 23, 53–62 (2013).
4. Kim, E., Goraksha-Hicks, P., Li, L., Neufeld, T. P. & Guan, K. L. Regulation of TORC1 transduction rather than mere transport. Nature Cell Biol. 10, 935–945 (2008).
5. Sancak, Y. et al. The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1. Science 320, 1496–1501 (2008).
6. Sancak, Y. et al. Ragulator-Rag complex targets mTORC1 to the lysosomal surface and is necessary for its activation by amino acids. Cell 141, 290–303 (2010).
7. Zoncu, R. et al. mTORC1 senses lysosomal amino acids through an inside-out mechanism that requires the vacuolar H⁺-ATPase. Science 334, 678–683 (2011).
8. Bar-Peled, L., Schwetzter, L. D., Zoncu, R. & Sabatini, D. M. Ragulator is a GEF for the rag GTPases that signal amino acid levels to mTORC1. Cell 150, 1196–1208 (2012).
9. Jewell, J. L., Russell, R. C. & Guan, K. L. Amino acid signaling upstream of mTOR. Nature Rev. Mol. Cell Biol. 14, 133–139 (2013).
10. Bar-Peled, L. et al. A tumor suppressor complex with GAP activity for the Rag GTPases that signal amino acid sufficiency to mTORC1. Science 340, 1100–1106 (2013).
11. Tsun, Z. Y. et al. The folliculin tumor suppressor is a GAP for the RagC/D GTPases and regulates the lysosome via mTOR and TFEB. Nature Rev. Drug Discov. 9, 215–236 (2010).
12. Wu, B. et al. Competitive intra- and extracellular nutrient sensing by the transporter homologue Sys1p. J. Cell Biol. 173, 327–331 (2006).
13. Kriel, J., Haesendonckx, S., Rubio-Texeira, M., Van Zeebroeck, G. & Thevelein, J. M. From transporter to transceptor: signaling from transporters provokes re-evaluation of complex trafficking and regulatory controls. BioEssays 35, 870–879 (2011).
14. Taylor, P. M. Amino acid transporters: eminences grises of nutrient signaling mechanisms? Biochem. Soc. Trans. 37, 237–241 (2009).

Acknowledgements We thank D. M. Sabatini, S. Wang and Z. Tsun for discussing results before publication and generously providing Flag-SLC38A9 and Flag-METAP2 stably expressing cells, all members of the Superti-Furga laboratory for discussions, the Bennett laboratory for the proteomics analysis, F. Paulier and the Barlow laboratory for the RNA seq-analysis and M. Gstaiger for providing expression vectors. This work was supported by the Austrian Academy of Sciences, ERC grant to G.S.-F. (I-Five 290179), EMBO long-term and Marie Curie fellowships to M.R. (ALTF 1346-2011, IEF 301663), EMBO long-term fellowship to R.K.K. (ALTF 314-2012), Swiss NSF fellowship (P300P3_147897) to B.S., Vienna Science and Technology Foundation (WWTF VRG10-001) and the Austrian Science Fund (FWF P 23522-B20) to C.K., the Italian Ministry of Instruction University and Research, PON-research and competitiveness 2007-2013 (no. PON01_00937) to C.L., the Austrian Federal Ministry for Science and Research (GenAu projects, APP-III and BIN-III) to L.A.H., K.L.B. and G.S.-F., the Austrian Science Fund (FWF) projects, APP-III and BIN-III) to L.A.H., K.L.B. and G.S.-F., the Austrian Science Fund (MCBO/SFB021 to L.A.H.

Author Contributions M.R. and G.S.-F. conceived the study. L.P., M.G. and C.J. designed and performed transport assays. M.R., T.S., M.E.G.d.A., E.L.R., M.B., K.L.B., L.A.H. and G.S.-F. designed the other experiments. R.K.K. and B.S. performed bioinformatic data and image analysis. K.V.M.H., J.W.B., L.X.H., C.K. generated reagents and provided scientific insight. M.R., L.A.H. and G.S.-F. performed TAP-mass spectrometry experiments. M.R., T.S., M.E.G.d.A., E.L.R., M.B., K.L.B., L.A.H. and G.S.-F. performed transport and transport assays. M.R., T.S., M.E.G.d.A., E.L.R., M.B., K.L.B., L.A.H. and G.S.-F. designed and performed the other experiments. R.K.K. and B.S. performed bioinformatic data and image analysis. K.V.M.H., J.W.B., L.X.H., C.K. generated reagents and provided scientific insight. M.R. and G.S.-F. wrote the manuscript. All authors contributed to the discussion of results and participated in manuscript preparation.

Author Information The protein-protein interactions have been submitted to the IMEx (http://www.imexconsortium.org) consortium through IntAct (http://www.ebi.ac.uk/intact/) and assigned the identifier IM-23283. The SLC network has the IntAct accession number EBI-9975668 and the RAGA-RAG-Camtornor network is EBI-9975664. RNA-Seq data is available in ArrayExpress (http://www.ebi.ac.uk/arrayexpress) under the accession number E-MTAB-3102. Reprints and permissions information is available at www.nature.com/reprints. The authors declare competing financial interests: details are available in the online version of the paper. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to G.S.-F. (goupert@cemm.oeaw.ac.at).
LETTER

METHODS

Antibodies. Antibodies were used against SLC38A9 (HPA043785 Sigma), LAMTORI (8975 Cell Signaling), LAMTOR3 (8169 Cell Signaling), RAGA (4357 Cell Signaling), RAGC (5466 Cell Signaling), sheep polyclonal to human SLC38A9 (ab7291 Abcam), RAGC (sc-55559 Santa Cruz), HA (HEK-36 Sigma), SLA-1 (ABclonal), Flag-tagged SLC38A9 isoform 1 (GenScript) were generated using a modified pLKO.1 vector.

Generation of stably expressing cells. Cell samples were treated with PNGase (NEB, 250 U for 30 min) to remove the glycosylation of immunoglobulin heavy chains. In case of detection of endogenous SLC38A9, proteins were analyzed by LC–MS/MS. Proteomics. Flp-in HEK293 T-Rex cell lines inducibly expressing ST-HA-tagged SLC38A9, SLC38A1, SLC38A2, SLC36A1, RAGA, RAGC, GFP or LAMTOR complex subunits were generated as described10. Tandem affinity STREP-HA purifications were performed as previously described11. In brief, cells were stimulated with doxycycline/tetrascline for 24 h to induce expression of ST-HA-tagged bait proteins. LAMTOR3 pull-downs were done using murine protein version and performed after 9 h starvation in serum-free medium. Protein complexes were isolated by TAP using streptavidin agarose followed by elution with biotin, and a second purification step using HA-agarose beads. Proteins were eluted with 100 mM formic acid, neutralized with triethylammonium bicarbonate (TEAB) and digested with trypsin and the peptides were analysed by LC–MS/MS.

MS data analysis and interaction data filtering. Peak list data were extracted from RAW files using ProteoWizard (release 3.0.3201, http://proteowizard.sourceforge.net) and searched against human SwissProt database version v2013_01_20130110 (37,261 sequences and common contaminants). The search engines MASCOT (v2.3.02, MatrixScience, London, UK) and Phenyx (v2.5.14, GeneBio, Geneva, Switzerland)12 were used. The searches were submitted to MASCOT using in-house specific precursor and fragment ion mass tolerances ± 0.1 and ± 0.6 Da, respectively. Using the high-confidence identifications from this search, precursor and fragment ion masses were recalibrated for a second-pass search on MASCOT and Phenyx with precursor and fragment ion mass tolerances ± 0.05 and ± 0.3 Da, respectively. One tryptic missed-cleavage was permitted. Carbamidomethyl cysteine and oxidized methionine were set as fixed and variable modifications, respectively. A false discovery rate of <0.25% and <0.1% were used for proteins and peptides, respectively, as described13. SAINT AP-MS filtering software14 was used to filter the interactions using GFP TAP as negative control. All prey proteins with a SAINT AvgP of >0.95 were identified as high-confidence interactors. In addition, proteins with a spectral count of 1 or a CRAPome37 frequency of >0.1 were excluded. For the LAMTOR-RAG network, we retained only those proteins that interacted with all the bait proteins (RAGD was not detected in RAGC pull-down).

Immunofluorescence. HEK293T cells were plated on fibronectin-coated glass coverslips and, after 16 h, induced with doxycycline. After 24 h, cells were washed with PBS, fixed (PBS, 4% formaldehyde) and permeabilized (PBS, 0.3% saponin, 10% FBS). Nuclei were stained with DAPI (10 μg/mL in PBS). Cells were washed with PBS, fixed (2% PFA, 30 min), permeabilized (0.5% saponin, 5% FBS, 1 h) and then blocked with 10% FBS in PBS. Cells were incubated with primary antibodies diluted in blocking buffer for 1 h at room temperature. Cells were washed with PBS, fixed (PBS, 4% formaldehyde) and permeabilized (PBS, 0.3% saponin, 10% FBS). Cells were washed in PBS, fixed (2% PFA, 30 min), permeabilized (0.5% saponin, 5% FBS, 1 h) and then blocked with 10% FBS in PBS. Cells were incubated with primary antibodies diluted in blocking buffer for 1 h at room temperature. Cells were then washed with PBS, fixed (2% PFA, 30 min), permeabilized (0.5% saponin, 5% FBS, 1 h) and then blocked with 10% FBS in PBS. Cells were incubated with primary antibodies diluted in blocking buffer for 1 h at room temperature. Upon washing 6 times in cytoskeleton buffer supplemented with 50 mM NH4Cl, the coverslips were mounted using Vectashield hardening medium (Vectorlabs).

©2015 Macmillan Publishers Limited. All rights reserved.
were deconvoluted using Huygens professional Deconvolution and Analysis Software (Scientific Volume Imaging). The Z-stack ids files were then visualized in ImageJ (open source version), converted into a colour stack image and a representative Z plane was selected. The single plane images were finally converted to Adobe Photoshop CS6 format. Representative cells are shown in all figures at the same exposure and magnification.

Cell size and autophagosome measurements. HEK293T cells transduced with shRNA against SLC38A9 or GFP cells were seeded 24 h before fixation (PBS, 4% formaldehyde), permeabilized (PBS, 0.3% Saponin, 10% PBS) and stained with DAPI. Images were taken by automated microscopy using the PerkinElmer Operetta with ×20 magnification in confocal mode. Images were analysed using CellProfiler (http://www.cellprofiler.org), CellClassifier (http://www.pelkmanslab.org/?page_id=63), Population Context measurement code (https://www.pelkmanslab.org/?page_id=1150) and custom Matlab code written specifically for this study. CellProfiler was used to detect individual nuclei on each image, and iterative machine learning using CellClassifier was applied to detect properly segmented interphase nuclei. Population context measurement code was used to measure the local cell density of each individual cell, and cell size measurements were restricted to sparse cells to avoid local crowding from confounding the measurements. We used the typical nucleus diameter (that is, the diameter of a circle with the same area as that measured for each nucleus) as a robust proxy for cell size. We confirmed that the cell size reduction induced by SLC38A9 shRNA treatment were present for a broad range of different cell densities. EGFP-LC3B and SLC38A9 or METAP2 expressing cells were seeded in 96-well plates for imaging. After 24 h cells were washed with PBS and starved for amino acids and serum for the indicated time. Three by three images were acquired per well with the Operetta at ×20 magnification on living cells to minimize disruption of EGFP-LC3B-positive autophagosomes. After imaging cells were fixed, DAPI stained and reimagined. Autophagosomes were quantified from the GFP channel using custom Matlab analysis, based on a thresholding of the integrated Laplacian of Gaussian transformation for diameters between 8 and 30 pixels. Candidate spots with a local GFP-signal enrichment of less than 42% were discarded, and remaining spots were considered autophagosomes, and normalized to the cell number and area for each condition. Each condition was measured in three replicate wells accounting for over 85,000 cells. Adjusted hill curves were fit and data were normalized to the maximum fitted value in the METAP2 control cell line.

Cell proliferation measurements. HEK293T cells transduced with shRNA against SLC38A9 or GFP were seeded and counted every 24 h using Easy (Roche). Amino acids starvation and stimulation. EGFP-LC3B and SLC38A9 or METAP2 expressing cells were seeded in 96-well plates for imaging. After 24 h cells were washed with PBS and starvation was performed by incubating the cells for 50 min in amino-acid-free RPMI without serum. Cell were then stimulated for 10 or 20 min by the addition of RPMI containing a two-time concentrated solution of amino acids in amino-acid-free RPMI medium adjusted to pH 7.4 and filtered. RPMI containing a two time concentrated solution of amino acids was obtained by complementing amino-acid-free RPMI medium powder (R8999-04A, US biological) with 20 mM HEPES/Tris pH 6.5. 600 µl of proteoliposomes were passed through a Sephadex G-75 column (0.7 cm diameter × 15 cm height) preequilibrated with 200 mM HEPES/Tris pH 6.5. 600 µl of proteoliposomes were passed through a Sephadex G-75 column (0.7 cm diameter × 15 cm height) preequilibrated with 200 mM HEPES/Tris pH 6.5, for removing the residual external radioactivity. The time course of [3H]glutamine efflux was then measured stopping the efflux reaction at each time interval by applying proteoliposome samples on a Sephadex G-75 column (0.6 x 8 cm) to separate the external from the internal radioactivity. In competition experiments, the indicated amino acids (1 mM) were added together with [3H]glutamine (10 µM) and transport was measured at 60 min. For efflux measurements, aliquots of the same pool of proteoliposomes passed through a Sephadex G-75 column (0.7 cm diameter × 15 cm height) preequilibrated with 200 mM HEPES/Tris pH 6.5 were incubated with external 10 µM [3H]glutamine. After 120 min of loading, proteoliposomes were washed and reimagined through a Sephadex G-75 column (0.7 cm diameter × 15 cm height) preequilibrated with 200 mM HEPES/Tris pH 6.5 for removing the residual external radioactivity.

Reconstitution of SLC38A9 in proteoliposomes and transport measurements. After purification, His-SLC38A9 was incubated overnight at 37 °C in absence or in presence of 1 U thrombin (GE healthcare) and then assayed by immunoblotting using anti-His or anti-SLC38A9 antibody. To assess the orientation of SLC38A9, reconstituted proteoliposomes were centrifuged at 108,000 × g for 90 min, resuspended in 20 mM HEPES/Tris pH 6.5, incubated overnight at 37 °C with 1 U thrombin in the same conditions of the purified protein. After incubation proteoliposomes were dissolved by 2.5% SDS and 0.2 M Tris/HCl pH 6.8. and immunoblotting analysis was performed as described before. Statistical analysis. A normal distribution of data was assumed and appropriate tests were applied.

31. Varjosalo, M. et al. Interlaboratory reproducibility of large-scale human protein-complex analysis by standardized AP-MS. Nature Methods 10, 307–314 (2013).
32. Giambruno, R. et al. Affinity purification strategies for proteomic analysis of transcription factor complexes. J. Proteome Res. 12, 4018–4027 (2013).
33. Pichichero, A. et al. Viral immune modulators perturb the human molecular network by common and unique strategies. Nature 487, 486–490 (2012).
34. Colinge, J., Masselot, A., Giron, M., Dessingy, T. & Magnin, J. OLAV: towards high-throughput tandem mass spectrometry data identification. *Proteomics* **3**, 1454–1463 (2003).

35. Bennett, K. L. et al. Proteomic analysis of human cataract aqueous humour: Comparison of one-dimensional gel LCMS with two-dimensional LCMS of unlabelled and iTRAQ(R)-labelled specimens. *J. Proteomics* **74**, 151–166 (2011).

36. Choi, H. et al. SAINT: probabilistic scoring of affinity purification-mass spectrometry data. *Nature Methods* **8**, 70–73 (2011).

37. Mellacheruvu, D. et al. The CRAPome: a contaminant repository for affinity purification-mass spectrometry data. *Nature Methods* **10**, 730–736 (2013).

38. Snijder, B. et al. Population context determines cell-to-cell variability in endocytosis and virus infection. *Nature* **461**, 520–523 (2009).

39. Galluccio, M. et al. Over-expression in *E. coli* and purification of the human OCTN1 transport protein. *Protein Expr. Purif.* **68**, 215–220 (2009).

40. Pochini, L., Scalise, M., Galluccio, M., Amelio, L. & Indiveri, C. Reconstitution in liposomes of the functionally active human OCTN1 (SLC22A4) transporter overexpressed in *Escherichia coli*. *Biochem. J.* **439**, 227–233 (2011).
Extended Data Figure 1 | Expression of SLC members of amino acid transporter families. a, Table of SLCs belonging to amino acid transporter families robustly expressed in HEK293 and K562 cells as monitored by RNA-seq. SLC members of amino acid transporter-containing families (SLC1, 6, 7, 16, 17, 18, 32, 36, 38 and 43 families) expressed (FPKM > 0.5) in both cell lines were ranked according to their expression level, top ten are shown. The number of PubMed entries was obtained by querying the GeneSymbol (24 October 2013). b, Expression of members of the SLC32, SLC36 and SLC38 families in HEK293 and K562 cells.

**a**

| RefSeqNT     | GeneSymbol | EntrezGeneID | HEK293_FPKM | K562_FPKM | Average_FPKM | PubMed_entry |
|--------------|------------|--------------|-------------|------------|---------------|--------------|
| NM_001145144 | SLC1A5     | 6510         | 47.08       | 56.21      | 51.65         | 92           |
| NM_001013251 | SLC3A2     | 6520         | 22.28       | 54.08      | 38.18         | 62           |
| NM_018976    | SLC38A2    | 54407        | 17.46       | 31.18      | 24.32         | 80           |
| NM_001077484 | SLC38A1    | 81539        | 24.41       | 9.34       | 10.87         | 60           |
| NM_014331    | SLC7A11    | 23657        | 9.35        | 7.12       | 8.24          | 148          |
| NM_173514    | SLC38A9    | 153129       | 5.81        | 8.83       | 7.32          | 1            |
| NM_003045    | SLC7A1     | 6541         | 9.89        | 2.87       | 6.38          | 81           |
| NM_052831    | SLC18B1    | 116843       | 6.10        | 3.68       | 4.89          | 1            |
| NM_003038    | SLC1A4     | 6509         | 1.40        | 7.93       | 4.66          | 36           |
| NM_080546    | SLC4A1     | 23446        | 5.85        | 3.10       | 4.48          | 19           |

| RefSeqNT     | GeneSymbol | EntrezGeneID | HEK293_FPKM | K562_FPKM |
|--------------|------------|--------------|-------------|------------|
| NM_001077484 | SLC38A1    | 81539        | 24.41       | 9.34       |
| NM_018976    | SLC38A2    | 54407        | 17.46       | 31.18      |
| NM_006841    | SLC38A3    | 10991        | 0.05        | 0.00       |
| NM_018018    | SLC38A4    | 55089        | 0.01        | 0.00       |
| NM_033518    | SLC38A5    | 92745        | 0.03        | 3.22       |
| NM_153811    | SLC38A6    | 145389       | 0.99        | 1.90       |
| NM_018231    | SLC38A7    | 55238        | 0.92        | 1.49       |
| NM_001080442 | SLC38A8    | 146167       | 0.00        | 0.00       |
| NM_173514    | SLC38A9    | 153129       | 5.81        | 8.83       |
| NM_138570    | SLC38A10   | 124565       | 0.72        | 1.95       |
| NM_173512    | SLC38A11   | 151258       | 0.00        | 0.00       |
| NM_078483    | SLC38A1    | 206358       | 0.77        | 0.49       |
| NM_181776    | SLC38A2    | 153201       | 0.00        | 0.00       |
| NM_181774    | SLC38A3    | 285641       | 0.00        | 0.00       |
| NM_152313    | SLC38A4    | 120103       | 4.19        | 0.11       |
| NM_080552    | SLC32A1    | 140679       | 0.00        | 0.00       |
Extended Data Figure 2 | Biochemical and functional characterization of SLC38A9. a, b, Where indicated, HEK293T cells were transfected with tagged SLC38A9 constructs (+) or empty vector (−). Cell lysates were left untreated (Untr.) or incubated 1 h at 37 °C in presence or absence of PNGase and analysed by immunoblot. Results are representative of two independent experiments (n = 2). c, Cell size measurements of HEK293T cells after short hairpin (shRNA)-mediated knockdown against GFP (control, dashed black line) or SLC38A9 (grey line), measured by automated microscopy and image analysis. Sparse and interphase cells were selected using image analysis and machine learning, and nucleus diameter was used as robust proxy for cell size. Smoothed distributions of 2,400 and 4,165 cells, respectively, are shown. d, Cell proliferation measurement of HEK293T cells transduced with lentivirus-encoded shRNA against SLC38A9 or GFP. 10⁵ cells were seeded and counted every 24 h. Mean values ± s.d. from triplicates. Results are representative of two independent experiments (n = 2). e, f, Where indicated, HEK293T cells were transfected with tagged SLC38A9. Cell lysates were prepared and left untreated (Untr.) or incubated 1 h at 37 °C with PNGase and analysed by immunoblot. Where indicated, cell lysates were boiled for 5 min at 95 °C after PNGase treatment. g, h, Lysates from murine NIH/3T3 (g) or Raw 264.7 (h) cells were subjected to immunoprecipitation with the indicated antibodies, treated with PNGase and analysed by immunoblot. Results are representative of two independent experiments (n = 2). <, SLC38A9; *, non-specific band.
Extended Data Figure 3 | SLC38A9 proteomic analysis: bait localization and results.  

a, Single-channel and merged confocal microscopy images of DAPI stained nuclei and indirect immunofluorescence against HA-tagged SLC38A9 and endogenous lysosomal markers LAMP1 (top panel) and LAMP2 (middle panel) and the non-induced and secondary antibody only control (bottom panel) in HEK293 Flp-In TReX cells. Scale bar, 10 μm. Intensity profiles for SLC38A9 (green) and LAMP1, LAMP2 or secondary antibody control (red) along the cross-section lines indicated in the respective merged channel images are shown.

b, Quantification of HA-SLC38A9 signal above background (dashed lines in a) that colocalizes with LAMP1, LAMP2 or secondary antibody only positive areas. Average and s.d. of at least two images is shown, analysing colocalization in 22, 34 and 27 cells respectively.

c, HEK293 Flp-In TReX cells inducibly expressing SLC38A9 were treated or not with doxycycline (Dox) for 24 h. Where indicated, cell lysates were treated with PNGase and analysed by immunoblot.

d, Tabular view summarizing the proteomic analysis of SLC38A9, SLC38A1, SLC38A2 and SLC36A1. Comparison of the SLC38A9 interactors identified by TAP–LC–MS/MS to the same analysis performed with the other transporters. Spectral counts (Sp. c., average of biological replicates) and sequence coverage (Sq. c., percentage, average of biological replicates) are indicated. Data shown are based on two independent experiments for each condition (n = 2), each analysed in two technical replicates.
Extended Data Figure 4  | SLC38A9 localizes to the late endosome/lysosome compartment. a–h, HeLa cells were transfected with the indicated ST-HA tagged SLC38A9 construct. Merged and single-channel confocal microscopy images of indirect immunofluorescence of HA-tagged SLC38A9 (red) and endogenous lysosomal marker LAMP1 (green) are shown. Representative cells are shown. Scale bar, 10 μm.
Extended Data Figure 5 | SLC38A9 is an integral component of the Ragulator–RAG GTPase complex. a, Tabular view of spectral counts (Spec. count, average of biological replicates) and sequence coverage (Seq. cov., percentage, average of biological replicates) of the core Ragulator–RAG GTPase network and published interactors detected. Data shown are based on two independent experiments for each condition (n = 2), and analysed in two technical replicates. b, c, SLC38A9 peptides detected in LAMTOR1, 3, 4 and 5 (b) or in SLC38A9 (c) TAP–LC–MS/MS analysis are mapped on SLC38A9 sequence and highlighted in bold. Transmembrane helices are highlighted in light brown. Potential tryptic cleavage sites are in red.
Extended Data Figure 6 | The cytoplasmic N-terminal region of SLC38A9 binds the Ragulator–RAG GTPase complex through evolutionary conserved motifs. a, Sequence alignment of the N-terminal cytoplasmic region (amino acids 1–112) of human, mouse, rat, Xenopus and zebrafish SLC38A9. Amino acids selected for deletion and motifs substituted to alanine are highlighted. Black and grey shading indicates >60% amino acid sequence identity and similarity, respectively. b, c, HEK293T cells were transfected with the indicated tagged SLC38A9 constructs. Immunoprecipitates and cell extracts were analysed by immunoblot. SLC38A9 mutant constructs are labelled with the number of the encoded amino acids (b) or with the amino acid motif substituted to alanine (c). Results are representative of two independent experiments (n = 2).
Extended Data Figure 7 | Characterization of SLC38A9-mediated amino acid transport in proteoliposomes. a, Purification of SLC38A9. Lanes represent empty vector control and SLC38A9 expressed in E. coli and purified by Ni-chelating chromatography. Immunoblot of the same fractions using anti-His or anti-SLC38A9 antibody are shown. b, Orientation of SLC38A9 in proteoliposomes. Purified His-SLC38A9 protein or proteoliposomes reconstituted with SLC38A9 were incubated overnight at 37 °C in presence or in absence of 1 U thrombin. Proteoliposomes were then solubilized with SDS and analysed by immunoblot. Results are representative of two independent experiments (n = 2). c, Time course of glutamine uptake by SLC38A9 in proteoliposomes reconstituted with the purified protein fraction. Transport was calculated by subtracting the radioactivity associated to proteoliposomes reconstituted with the empty vector fraction. Values represent means of specific transport ± s.d. from three independent experiments (n = 3). d, Time course of glutamine uptake in proteoliposomes reconstituted with purified SLC38A9 wild-type or N128A mutant protein. Values represent means of specific transport ± s.d. from 3 independent experiments (n = 3). Significance was estimated by Student’s t-test (*P < 0.01). Immunoblot analysis of purified protein reconstituted in the proteoliposomes. e, Effect of pH on the reconstituted SLC38A9. Reconstitution and transport assay were performed at the indicated pH. Results are means of specific transport rate ± s.d. from three different experiments (n = 3). f, Inhibition of the [3H]glutamine uptake in proteoliposomes. 1 mM MeAIB ([α-(methylamino)isobutyric acid) was added together with 10 μM [3H]glutamine. Transport was measured at 60 min. Values represent means of percent residual activity with respect to control (without added inhibitor) ± s.d. from three independent experiments (n = 3).
Extended Data Figure 8 | Nucleotide-loading/conformation dependent interaction of RAGB/RAGC heterodimers with SLC38A9. HEK293T cells were transfected with the indicated combination of tagged RAG GTPases mutant constructs or empty vector (–). Anti-HA immunoprecipitates and cell extracts were treated with PNGase and analysed by immunoblot. W, wild type; 75, S75N; 120, Q120L; 99, Q99L; 54, T54N. Results are representative of two independent experiments (n = 2).
Extended Data Figure 9 | Stable expression of SLC38A9 mediates sustained mTORC1 activation upon amino acid starvation.  

a, SLC38A9 or METAP2 stably expressing HEK293T cells were starved for the indicated time in medium without amino acids and serum. Cell lysates were analysed by immunoblot. Results are representative of two independent experiments (n = 2). 

b, Representative images in the GFP channels of HEK293T cells stably expressing EGFP–LC3B and SLC38A9 or METAP2 starved for 120 min (related to Fig. 4b). Scale bar, 40 μm. 

c, HEK293T cells stably expressing TFEB–STHA and SLC38A9 or METAP2 were starved for the indicated time. Cytoplasmic and nuclear fraction were analysed by immunoblot. Results are representative of two independent experiments (n = 2). 

d, Immunoblot analysis of HEK293T cells stably expressing the indicated SLC38A9 constructs. 

e, SLC38A9 (S) or METAP2 (M) stably expressing HEK293T were starved for 50 min and then stimulated with amino acids for 20 min. Where indicated, cells were treated with concanamycin A (5 μM) or DMSO during both incubation times. Cell lysates were analysed by immunoblot with the indicated antibodies. Results are representative of two independent experiments (n = 2). 

f, SLC38A9 (S) or METAP2 (M) stably expressing HEK293T were treated for 30 min with DMSO (D), concanamycin A (C, 5 μM) or Torin 1 (T, 250 nM), and then starved for the indicated times in presence of the inhibitors. Cell lysates were analysed by immunoblot. Results are representative of two independent experiments (n = 2).
Extended Data Figure 10 | Expression of SLC38A9 is required for amino acid-induced mTORC1 activation and is not affected by starvation. a, HeLa cells were transfected with siRNA targeting SLC38A9 (SLC), LAMTOR1 (LT1) or non-targeting control (Cntr). After 72h, cells were starved for 50 min in medium without amino acids and serum and then stimulated with amino acids in presence of insulin (1 μM). Cell lysates were analysed by immunoblot. Results are representative of three independent experiments (n = 3).

b, c, HEK293T cells were starved for the indicated times. SLC38A9 expression was analysed by quantitative PCR (b) and immunoblot (c). In b, mean values ± s.d. from technical triplicates are shown. Results are representative of two independent experiments (n = 2). <, SLC38A9; *, non-specific band.