Dynamics of mTORC1 activation in response to amino acids

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Abstract Amino acids are essential activators of mTORC1 via a complex containing RAG GTPases, RAGULATOR and the vacuolar ATPase. Sensing of amino acids causes translocation of mTORC1 to lysosomes, an obligate step for activation. To examine the spatial and temporal dynamics of this translocation, we used live imaging of the mTORC1 component RAPTOR and a cell permeant fluorescent analogue of di-leucine methyl ester. Translocation to lysosomes is a transient event, occurring within 2 min of aa addition and peaking within 5 min. It is temporally coupled with fluorescent leucine appearance in lysosomes and is sustained in comparison to aa stimulation. Sestrin2 and the vacuolar ATPase are negative and positive regulators of mTORC1 activity in our experimental system. Of note, phosphorylation of canonical mTORC1 targets is delayed compared to lysosomal translocation suggesting a dynamic and transient passage of mTORC1 from the lysosomal surface before targetting its substrates elsewhere.

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exchange factor *Bar Peled et al* J) and by an additional complex known as the GATOR acting as a GTPase activating protein (*Bar Peled et al* J) although it is also possible to activate mTORC1 downstream of amino acids in a way that is independent of the RAGs but still sensitive to the vacuolar ATPase (*Jewell et al* J). In addition to the fundamental role of amino acids acting via the RAG/RAGULATOR axis, a small GTPase termed RHEB is also essential for mTORC1 activation

(*Dibble and Manning* *J*

significantly from the endogenous complex (unpublished results). We have tried to overcome this problem in two different cell lines and using two different strategies. In HEK-293 cells, we have down-regulated endogenous RAPTOR while simultaneously expressing exogenous tagged constructs; in the near haploid HAP-1 cells we have tagged the endogenous RAPTOR gene with GFP. We describe here the dynamics of activation of the mTORC1 complex in these cells, with special emphasis in the HAP-1 cells. To examine whether mTORC1 responds directly to lysosomal amino acids, we have also generated a cell permeant fluorescent analogue of leucine in order to image RAPTOR translocation in response to this compound.

Results

Amino acid-dependent mTOR localization in fixed cells

We compared the timing of the translocation of the endogenous mTOR to a punctate compartment in response to amino acids with the phosphorylation of S6K, one of mTORC1's main substrates (*Figure A C*). As previously reported (*Sancak et al JJ*), in fed cells mTOR was partially in LAMP-1-positive punctate structures but became cytosolic during starvation from growth factors and amino acids (B). Upon re-stimulation with just amino acids, mTOR was found in a punctate distribution with a peak at 5 min (B, C). In a parallel experiment, mTOR phosphorylated S6K with a peak at 10 min of amino acid re-stimulation and phosphorylation continued to be strong even at 20 min (A). The discrepancy in the timing of mTOR translocation versus activity could be a natural delay until the complex becomes fully competent for phosphorylation. We noted however that in time points where the intensity of mTOR staining on lysosomes was back to low levels, phosphorylation of S6K was still strong (20 min), indicating that the pool of mTOR undertaking phosphorylation may not have been fully on the lysosomes. We also examined the localization of the direct mTORC1 target 4EBP1 as well as the S6K target the S6 protein under these conditions using antibodies that stain the endogenous phosphorylated proteins. The phosphorylated form of 4EBP1 did not localize on LAMP-1-positive lysosomes upon amino acid stimulation where activated mTOR would be expected to reside (*Figure* ; *Figure figure supplement A C*). Similarly, the phosphorylated form of S6 was also not localised to LAMP-1-positive lysosomes at any time during recovery (*Figure* ; *Figure figure supplement D E*).

An important protein in mTOR signalling is RHEB, a small GTPase absolutely required for mTOR activity (*Dibble and Manning J*). Current models suggest that RHEB and the mTOR complex interact on the lysosomes for subsequent mTOR activation. In our hands, overexpressed RHEB capable of activating mTOR (fu6j.222-128(capa5imil29(stl)n229(stl)n8.04o5(inte84(the)-.66T1293-406(2013)]TJ0stl)n

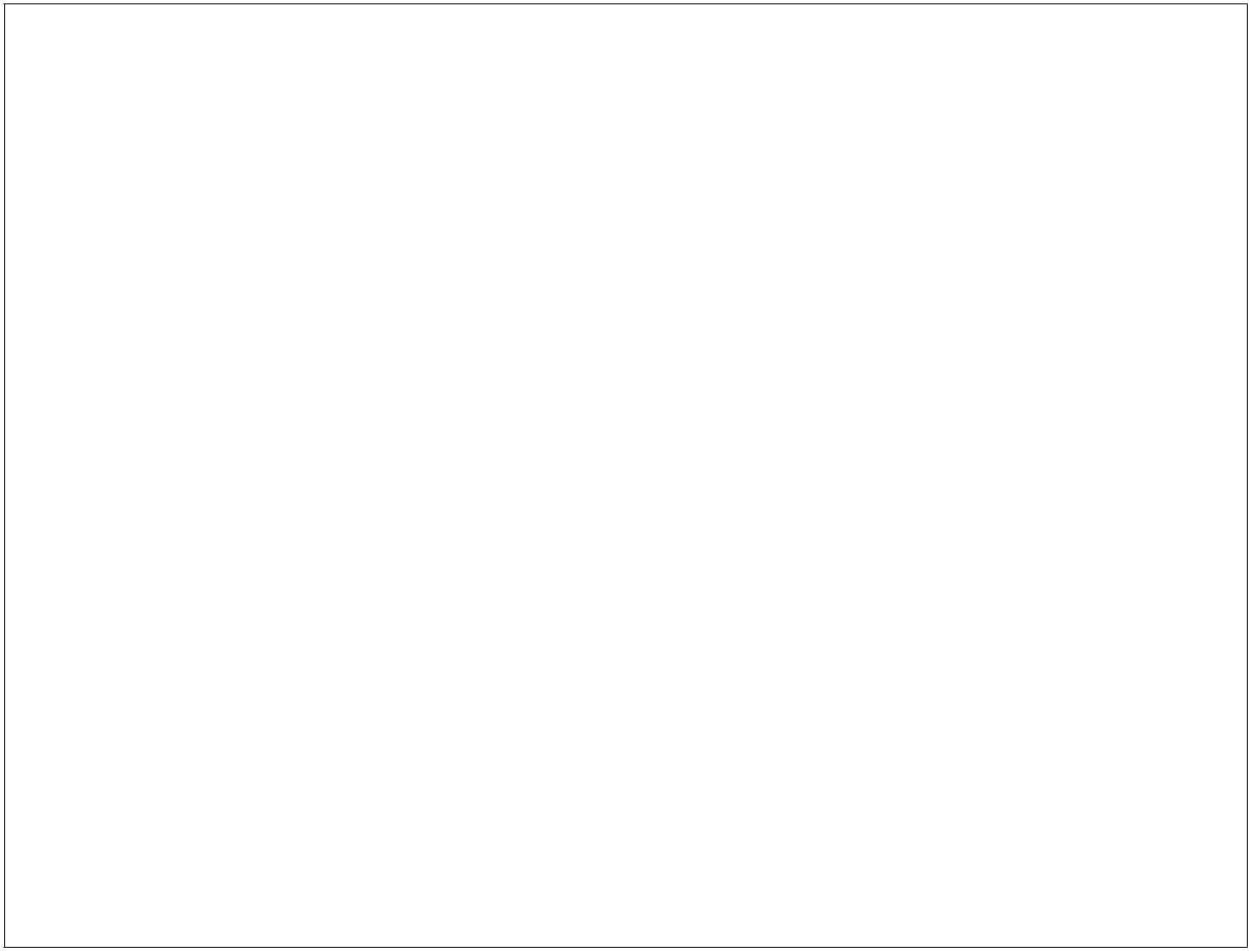

Figure 1. Activation of mTOR components in response to amino acids. HEK-293 cells were starved of amino acids and growth factors, and re-stimulated for the indicated times with MEM amino acids. (A) The phosphorylation of S6K at T389 was established as a surrogate for mTORC1 activity. Band marked with an asterisk serves as loading control. (B) Under the same conditions the localization of mTOR and the LAMP-1 lysosomal protein were examined by indirect immunofluorescence using antibodies that recognise the endogenous protein. (C) The total intensity of mTOR- or LAMP-1-

Dynamics of RAPTOR-GFP in HEK-293 cells

To examine the dynamics of mTOR in response to amino acids we tried to express subunits of mTORC1 tagged with GFP in order to follow localization in live cells. Tagging either mTOR or RAPTOR with GFP at either N' or C' terminus and generating either transient or stable cell lines of varying expression levels did not provide us with model systems that recapitulated the dynamics of endogenous mTOR (data not shown). One approach that was partially successful was to transfect cells with siRNAs targeting the 3' and 5' untranslated regions of endogenous RAPTOR while simultaneously expressing transiently in the same cells RAPTOR-GFP (*Figure D*). In these cells, endogenous RAPTOR expression was greatly reduced and exogenous expression (resistant to the siRNAs) could be titrated to be similar to the endogenous. In live imaging experiments using these cells after a round of starvation (removing amino acids and growth factors) and re-stimulation (adding back amino acids) we observed a rapid translocation of RAPTOR-GFP to small and fine punctate structures within 1 to 2 min of amino acid addition (*Figure E*, panels labelled "aa add"). In contrast, re-stimulation of these cells with starvation medium alone did not reveal a translocation of RAPTOR-GFP (*Figure E*, panel labelled "starved"). Although this was a reproducible effect, it was evident in a minority of the cells in the population, primarily in those expressing very low levels of exogenous RAPTOR-GFP. This cell variability made it very difficult to prove by biochemical methods that mTOR was being activated and we did not pursue this line of experimentation further.

Generation of HAP-1 cells expressing RAPTOR-GFP from the endogenous locus

An alternative approach was to introduce to the endogenous RAPTOR a GFP tag. For this we used HAP-1 cells which are derived from the near haploid cell line KBM-7 via an unsuccessful attempt at reprogramming (*Carette et al J* ; *Burckstummer et al J*). Using a CRISPR/Cas9 approach we derived a clone that expressed RAPTOR-GFP from the endogenous locus and at equal levels to the parental cells (*Figure A*). The fluorescence signal in these cells was sufficiently bright to be discerned by wide field microscopy (*Figure B*) although not strong enough for long illumination in confocal microscopy (data not shown). The levels of endogenous mTOR in these cells were equal to the wild type cells (*Figure A C*) and the RAPTOR-mTOR complex could be immunoprecipitated with antibodies to endogenous mTOR (*Figure C*) using the lysis conditions in 0.3% CHAPS previ-

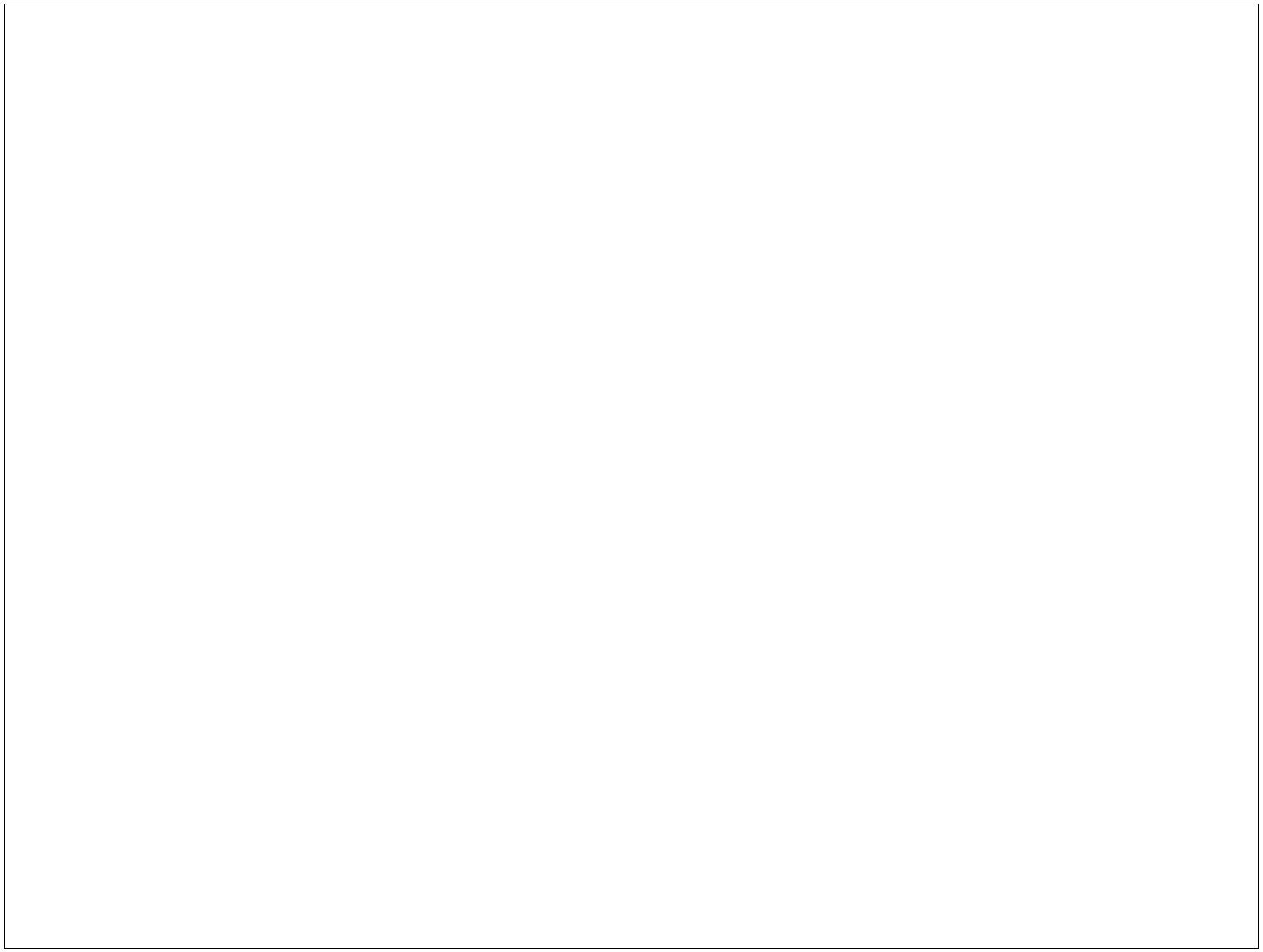

Figure 2. Characterization of HAP-1 cells expressing RAPTOR-GFP in place of endogenous RAPTOR. (A) Immunoblots of parental HAP-1 cells or HAP-1 cells expressing RAPTOR-GFP, in triplicate. (B) Fluorescence microscopy of cells as in A; both photos were taken at the same setting asamKghtsa inds-TJ-1i98Td42

compared to the fed condition (*Figure F*, blot labelled 1% BSA). We also examined the response of the two HAP-1 cells and the HEK-293 cells to serum starvation on its own or in combination with a subsequent amino acid starvation. The activity of mTORC1 in both HAP-1 cells was remarkably resistant to overnight serum withdrawal whereas in HEK-293 cells this treatment completely inhibited

conditions that enhance the phosphorylation response as shown in *Figure F*. In these experiments we

lysosomal surface or that de-phosphorylation of S6K continuously antagonises the phosphorylation step in a way that does not allow the phosphorylated protein to accumulate at the early time points. To address the second possibility directly we examined the rate of dephosphorylation of S6K after a round of stimulation for different time points. First cells were starved and then re-stimulated with amino acids and growth factors for either 20 min or 50 min, at which point a mTOR inhibitor was

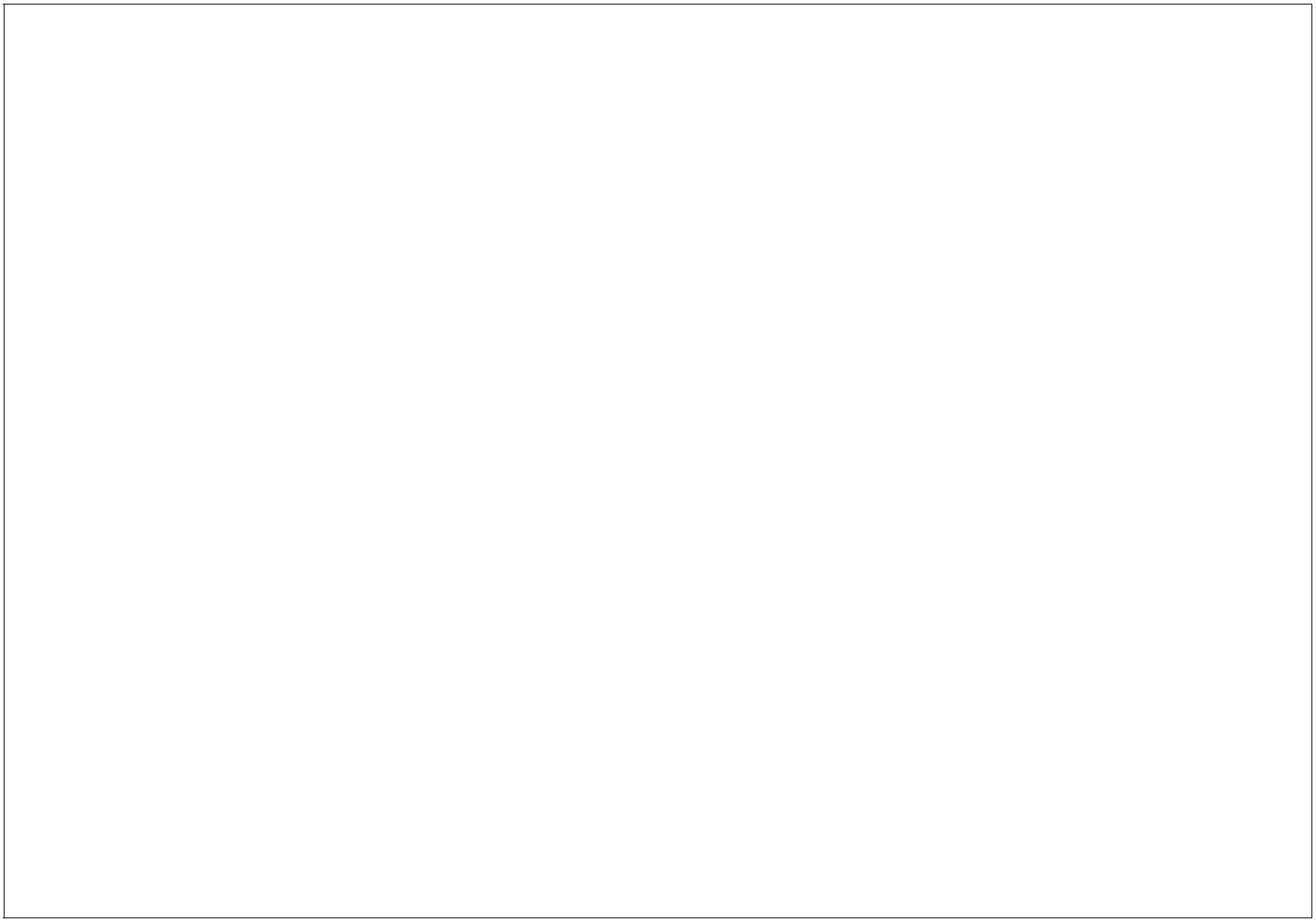

Figure 4. Translocation of RAPTOR-GFP to lysosomes and its dynamics in response to amino acid stimulation. (A) HAP-1 cells expressing RAPTOR-GFP were co-transfected with plasmids expressing mRFP-LAMP2 for 24 hr. The cells were then starved of amino acids and growth factors for 60' and assembled on an imaging chamber still in starvation medium. Live imaging was started in a starvation medium for 2' followed by replacement with medium containing a mixture of MEM and NE amino acids for an additional 40'. Images were captured every 10''. Shown here are frames from such a movie in the two channels (GFP and red) the merged images and the co-localizing areas for the indicated time points. The extent of co-localization between RAPTOR-GFP and mRFP-LAMP2 as determined by the Pearson's coefficient is plotted on the graph. Images were background-subtracted using the rolling ball method in FIJI (*Schindelin et al*) with a diameter of 10 pixels. Co-localization analysis was performed using Imaris software (Bitplane/Oxford Instruments) with thresholds for co-localization set using the auto function to avoid user bias. See also *Video* . (B) HAP-1 cells expressing RAPTOR-GFP were starved of amino acids and growth factors, and re-stimulated for 20'. The cells were stained for GFP (RAPTOR) and endogenous LAMP-1, and the extent of co-localization was determined by the Pearson's co-efficient (PCC) shown. (C) HAP-1 cells expressing RAPTOR-GFP were starved of amino acids and growth factors for 60' and set up for live imaging as above. Re-stimulation after 5' of imaging was in the starvation medium (starved), in the starvation medium containing a mixture of MEM and NE amino acids (aa's), or in medium containing amino acids and 2 mM concanamycin A (aa's + conc) for an additional 40'. Images were captured every 10''. Selected frames are shown. Note that in the concanamycin A-treated samples we found it necessary to pre-treat the cells for the last 10' of starvation with the compound before adding it again during re-stimulation. The bar in all panels represents 10 mm.

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but we also saw examples where several lysosomes containing analogue became RAPTOR-positive simultaneously. In addition, we frequently saw that RAPTOR translocated to large vacuolar-like organelles containing the fluorescent analogue (example in *Figure*). Amino acid methyl esters are known to continuously accumulate within lysosomes wherein the methyl ester moiety is hydrolysed and amino acids are trapped (*Reeves*); this provides a condition of continuous amino acid supply to provide sustained mTOR activation. To examine if this would also affect translocation, we quantitated translocation dynamics of RAPTOR-GFP in response to the fluorescent analogue

(*Figure F*). It was clear that translocation under these conditions was sustained (albeit starting more slowly than the response with amino acids) and the RAPTOR-GFP protein did not come off the lysosomes throughout our live imaging time interval (30 min). This result provides additional evidence that translocation of the complex to the lysosomal surface is in direct response to amino acid levels in the lysosomal lumen.

Activation of mTORC1 by the amino acid analogue depends on Sestrin2 and on intact lysosomes

To our knowledge, the leucine analogue is the first fluorescent reagent capable of activating mTORC1 and at the same time visible by microscopy. In order to understand its mechanism of action further we set up parallel experiments in HEK-293 cells (where the analogue can activate mTORC1 completely on its own) and in the edited HAP-1 cells expressing RAPTOR-GFP (where activation is

stimulation of mTORC1 via the fluorescent analogue was insensitive to EIPA (*Figure 1*, *Figure 1 figure supplement C*).

These data suggest that the fluorescent analogue requires intact and functional lysosomes to signal to mTORC1 and that its mechanism of being sensed depends on Sestrin2 although it does not require delivery via macropinocytosis.

Discussion

We examined the spatiotemporal dynamics of mTOR activation and its kinetic dependence on amino acid presence. Pioneering work from several laboratories had already provided strong evidence that mTOR itself translocates to lysosomes during amino acid re-stimulation (*Sancak et al JJ , J J*; *Zoncu et al J*), but this work had relied exclusively on a single antibody that can stain the endogenous protein and it did not provide any information on the dynamics of the process. The RAPTOR-GFP edited cells that we used here provide a unique tool to address this question in a near-physiological setting. Although they are slightly less respons(kinetiDe]TJ174.4es)-1]TJ95Tm 10.4eless

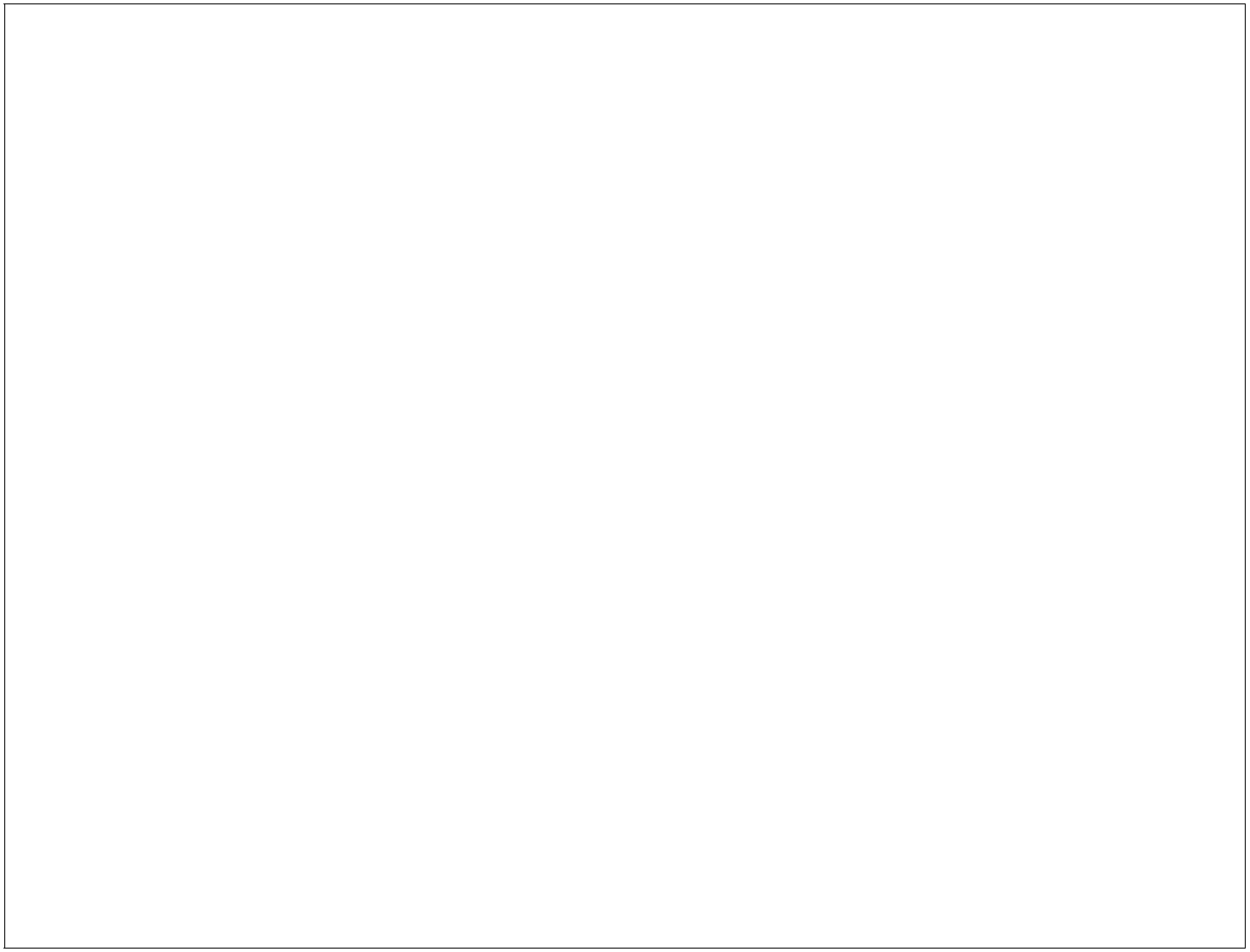

Figure 5. Dynamics of RAPTOR-GFP translocation to lysosomes in comparison to mTOR activity. HAP-1 cells expressing RAPTOR-GFP were set up for parallel experiments: live imaging to reveal RAPTOR dynamics (B, D, E, F

thereafter. We have also demonstrated for the first time that the presence of an amino acid analogue in the lysosomal lumen is sufficient to induce the translocation of RAPTOR-GFP there. This analogue, a methyl ester of di-leucine with a fluorescent moiety attached, produces a sustained translocation of the complex presumably owing to its property to continuously accumulate within the lysosomal lumen. All of these data provide for the first time a dynamic view of mTORC1 activation in response to amino acids.

Despite tremendous progress, the exact mechanism by which mTORC1 senses amino acids appears very complex and incomplete at present (reviewed and commented on in: *Goberdham et al* ; *Lee et al* ; *Shimobayashi and Hall*). Clearly, several different sensors are involved (sometimes for the same amino acid, e.g. for arginine), and the topology of the phenomenon involves the cytosol as well as the lysosomal membrane and the lysosomal lumen. Our own data in two cell types argue that, in addition to intact lysosomes and the lysosomal ATPase, the cytosolic sensor Sestrin2 is required as well, even when the activating moiety is a lysosomally targeted amino acid methyl ester derivative. One possible explanation may be that amino acids are first imported into the lysosomes and then exported in a regulated manner (requiring intact lysosomes and lysosomal function) in order to be sensed by Sestrin2 and other such cytosolic sensors. The import step may depend on macropinocytosis (*Palm et al* ; *Yoshida et al*) but the timing of the RAPTOR translocation step places a restraint on possible trafficking steps (see below). Our data cannot exclude the possibility that glutaminolysis in synergy with leucine also activate mTORC1 in the HAP-1 cells (*Durán et al*) but we consider it less likely to operate in HEK-293 cells

Figure *figure supplement* **). Even when the Golgi localization of RHEB was disrupted with BFA (a treatment that had a very modest effect on mTORC1 activity), very little of the protein co-localized with RAGC, a major mTORC1 regulator residing on lysosomes (***Figure* **;**

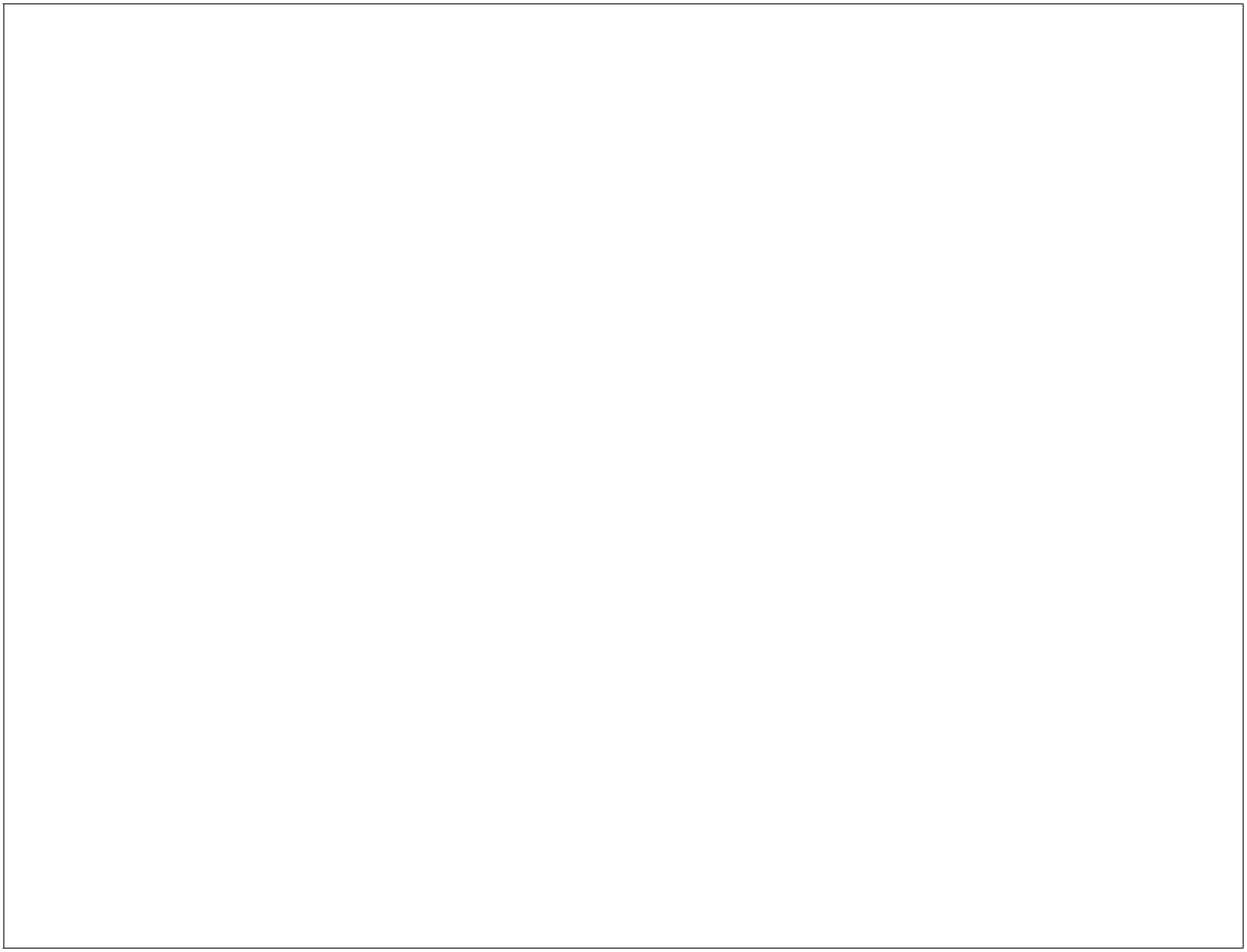

Figure 7. Activation of mTORC1 in HAP-1 RAPTOR-GFP cells and in HEK-293 cells depends on Sestrin2 and intact lysosomes. (A) HAP-1 cells expressing RAPTOR-GFP were treated with siRNA against Sestrin2 or with a non-targetting control (NT) as indicated. After 72 hr, cells were kept in normal medium (Iscove's modified Dulbecco's medium -IMDM plus 10% FBS) or starved of amino acids and growth factors for 60' in medium containing salts and 1% dialysed FBS. The starved cells were then re-stimulated for 10', 20' or 30' with a mixture of amino acids and growth factors (aa) or with fluorescent analogue (fa) added to the starvation medium as indicated. After lysis and electrophoresis the samples were immunoblotted for the indicated proteins. The intensity of the S6K phospho T389 band is plotted in the graphs. This experiment is representative of two. (B) The same experiment as in A was done with HEK-293 cells. This experiment is representative of two. (C) HAP-1 cells expressing RAPTOR-GFP were kept in normal medium or starved and re-stimulated for 20' and 30' with amino acids and growth factors (aa) or with fluorescent analogue (FA) in the presence or absence of GPN as indicated. After lysis and electrophoresis the samples were immunoblotted for the indicated proteins. (D) Cells as in C were loaded with lysotracker or with fluorescent analogue (FA) for 30' during aa re-stimulation in the presence or absence of GPN as indicated. After fixation the cells were examined by fluorescence microscopy. (E) HEK-293 cells were kept in normal medium or starved and re-stimulated for 20' with MEM amino acids (aa) or with fluorescent analogue (FA) in the presence or absence of GPN as indicated. After lysis and electrophoresis the samples were immunoblotted for the indicated proteins. (F) Cells as in E were loaded with lysotracker or with fluorescent analogue (FA) for 20' during aa re-stimulation in the presence or absence of GPN as indicated. After fixation the cells were examined by fluoresceAftt-290(GP1(examincopy)12(.)-288(()TJ87(same)bari

adenovirus-price-list/) and were tested for mycoplasma contamination every 2 years in our lab using Mycoguard (Genecopoeia, http://www.genecopoeia.com/product/mycoplasma-detection-kit/). HAP-1 cells and the RAPTOR-GFP edited cells were obtained from Haplogen (now part of Horizon Genomics https://www.horizondiscovery.com/) and were tested for mycoplasma contamination every 2 years. The following antibodies were used. Mouse anti-b-COP (a kind gift from the late Thomas Kreis), mouse anti-LAMP2 (Developmental Studies Hybridoma Bank, PRID: AB_528129), rabbit anti-phosphoS6K1 (Cell Signaling, PRID: AB_330944), rabbit anti-phosphoS6 (Cell Signaling, RRID: AB_916156), rabbit anti-mTOR (Cell Signaling, RRID:AB_2105622), rabbit anti-RAGC (Cell Signaling, PRID:AB_2180068), rabbit anti-RAPTOR (Cell Signaling, PRID:AB_10694695), mouse ant-RHEB (Abnova, PRID:AB_1112097), rabbit anti-phospho4EBP1 (Cell Signaling, PRID: AB_560835), rabbit anti-Giantin (Covance, PRID:AB_291560), rabbit anti-phosphoULK1 (Cell Signaling PRID: AB_10829226), rabbit Sestrin2 (Proteintech Group, PRID:AB_2185480). The following Plasmids were used. pRK5 HA GST Rheb1 from D Sabatini (Addgene 14951), RAGB from D Sabatini (Addgene 19301), myc-RAPTOR from D Sabatini (Addgene 1859). RAPTOR-EGFP was generated from myc-RAPTOR by excising the RAPTOR open reading frame, tailing it with restriction sites for Xho1 and EcoR1 and cloning it into the pEGFP-C1 vector. To target endogenous RAPTOR we used the following oligos:

si-1 targetting 3' untranslated region with sequence A.G.A.G.A.G.A.G.G.A.A.G.A.A.G.G.A.G.A. U.U

si-2 targeting 5' untranslated region with sequence G.G.G.C.U.G.A.U.G.A.G.A.U.G.A.G.U.U.U.U. U.

Generation of RAPTOR-GFP cell line

The RAPTOR-GFP cell line was created using a strategy based on that of Auer, et al (*Auer et al J*). HAP-1 cells were transfected with several plasmids: (1) a plasmid expressing Cas9, (2) a donor plasmid containing the GFP coding sequence flanked by zebra fish-specific guide RNAs and the zebra fish guide RNA sequence under the U6 promoter, (3) a plasmid expressing a guide RNA sequence (TGGAGAAGCGTGTCAGATAG) targeting the 3' end of the RAPTOR gene. When the donor plasmid is cleaved in the transfected cells, it will likely be integrated in the site targeted by the RAPTOR guide RNA. GFP positive transfected cells were sorted using FACS and subjected to single cell dilution to obtain clonal cell lines. These clonal cell lines were screened to search for cells which contained the GFP cassette integrated at 3' end of the RAPTOR gene. These cells are commercially available from Horizon Genomics.

Generation of fluorescent amino acid analogue

The synthetic route outlined in the scheme shown in Supplementary *Figure B* was followed using standard methods. All reagents are commercially available. The final product was freeze dried from water plus 5% acetic acid and re-suspended at 0.5 M in DMSO as a stock solution kept at –20˚C.

Starvation of cells and re-stimulation with aa's

For starvation (both HEK-293 and HAP-1), cells were washed twice with pre-warmed starvation medium (140 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM Glucose, 20 mM Hepes, 5 mM KCl, pH 7.4) containing either 1% BSA or 1% dialysed FBS prior to incubation with this medium for 60 min. At the end of 60 min, the medium for HEK-293 cells was replaced with new starvation medium containing 2X solution of MEM amino acid solution (50X stock, containing the following amino acids in one letter code R, C, H, I, L, K, M, F, T, W, Y, V, and sold by LifeTechnologies), and incubated in this solution for the indicated times. For HAP-1 cells, the starvation medium was replaced with new starvation medium containing 1 mM glutamine, MEM amino acids as above, 2X solution of NE amino acids (100X stock, containing the following amino acids in one letter code G, A, N, D, E, P, S and sold by LifeTechnologies), insulin (1:500 dilution of liquid supplement at 9–11 mg/ml sold by Sigma-Aldrich) and EGF (20 ng/ml) as indicated in the Figure legends. In some experiments using the fluorescent analogue, we also added 0.4 mM arginine to the above solution.

Immunofluorescence and live imaging

Cells for immunofluorescence were grown on glass coverslips and fixed in 3.7% formaldehyde in 200 mM Hepes pH 7.2. Staining for immunofluorescence and digital photography were done as described before (*Karanasios et al* J). Live-cell imaging was performed as previously described (*Karanasios et al* J

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