

DAPK2 is a novel regulator of mTORC1 activity and autophagy

Y Ber¹, R Shiloh¹, Y Gilad¹, N Degani¹, S Bialik¹ and A Kimchi^{*1}

Autophagy is a tightly regulated catabolic process, which is upregulated in cells in response to many different stress signals. Inhibition of mammalian target of rapamycin complex 1 (mTORC1) is a crucial step in induction of autophagy, yet the mechanisms regulating the fine tuning of its activity are not fully understood. Here we show that death-associated protein kinase 2 (DAPK2), a Ca²⁺-regulated serine/threonine kinase, directly interacts with and phosphorylates mTORC1, and has a part in suppressing mTOR activity to promote autophagy induction. DAPK2 knockdown reduced autophagy triggered either by amino acid deprivation or by increases in intracellular Ca²⁺ levels. At the molecular level, DAPK2 depletion interfered with mTORC1 inhibition caused by these two stresses, as reflected by the phosphorylation status of mTORC1 substrates, ULK1 (unc-51-like kinase 1), p70 ribosomal S6 kinase and eukaryotic initiation factor 4E-binding protein 1. An increase in mTORC1 kinase activity was also apparent in unstressed cells that were depleted of DAPK2. Immunoprecipitated mTORC1 from DAPK2-depleted cells showed increased kinase activity *in vitro*, an indication that DAPK2 regulation of mTORC1 is inherent to the complex itself. Indeed, we found that DAPK2 associates with components of mTORC1, as demonstrated by co-immunoprecipitation with mTOR and its complex partners, raptor (regulatory-associated protein of mTOR) and ULK1. DAPK2 was also able to interact directly with raptor, as shown by recombinant protein-binding assay. Finally, DAPK2 was shown to phosphorylate raptor *in vitro*. This phosphorylation was mapped to Ser721, a site located within a highly phosphorylated region of raptor that has previously been shown to regulate mTORC1 activity. Thus, DAPK2 is a novel kinase of mTORC1 and is a potential new member of this multiprotein complex, modulating mTORC1 activity and autophagy levels under stress and steady-state conditions.

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Macroautophagy (hereafter referred to as autophagy) is a highly regulated intracellular bulk degradation process found ubiquitously in eukaryotes. During autophagy a double-membrane vesicle, termed an autophagosome, engulfs cytoplasmic materials, including whole organelles. The autophagosome is later fused with the lysosome and its content degraded by hydrolases.¹ Basal levels of autophagy are maintained within the cell during steady state, and are involved in cell homeostasis activities such as turnover of long-lived proteins, preventing accumulation of protein aggregates, and removal of damaged cellular structures.² Beyond this homeostatic function, autophagy is stimulated during various stress conditions, such as nutrient deprivation, intracellular Ca²⁺ increase, hypoxia, ER stress and oxidative stress, to ensure continuous cell survival under stress.³

A critical step in the induction of autophagy comprises the inactivation of a key negative regulator of the process, the mammalian target of rapamycin (mTOR).⁴ mTOR is a conserved serine/threonine protein kinase that acts as a master regulator in the cell. mTOR forms a rapamycin-sensitive complex named mTORC1 with its binding partner raptor (regulatory-associated protein of mTOR), which mediates mTOR's substrate presentation.⁵ mTORC1 senses nutrient availability, growth factors and energy levels, and, in

response, regulates cell growth, metabolism and protein synthesis, mainly by phosphorylation of substrates involved in protein translation: the p70 ribosomal S6 kinase (p70S6K) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1). Under nutrient-rich conditions, mTORC1 suppresses autophagy to basal levels by phosphorylating and inhibiting the autophagy proteins ULK1 (unc-51-like kinase 1) and Atg13. Upon autophagic stimulus, mTORC1 activity is inhibited and the ULK1 complex is activated, leading to autophagy induction.⁶ The activity levels of mTORC1 are regulated by several mechanisms, such as interacting proteins, cellular localization and phosphorylation events. Raptor phosphorylation has been suggested as a mechanism by which upstream kinases such as AMPK,⁷ RSK⁸ and ULK1⁹ can regulate mTORC1 activity.

Death-associated protein kinase 2 (DAPK2; also named DRP-1) is a 42-kDa Ca²⁺/calmodulin (CaM)-regulated serine/threonine kinase,¹⁰ and a closely related homolog of DAPK, a gene originally discovered in an attempt to find positive regulators of cell death.¹¹ DAPK2 was identified based on homology to the catalytic domain of DAPK. DAPK2 is a soluble cytoplasmic protein, which triggers massive membrane blebbing and appearance of double-membrane autophagic vesicles upon its overexpression (for a review see Shiloh *et al.*¹²). DAPK2's substrates and interacting proteins

¹Department of Molecular Genetics, Weizmann Institute of Science, Rehovot 76100, Israel

*Corresponding author: A Kimchi, Department of Molecular Genetics, Weizmann Institute of Science, Herzl Street, Rehovot 76100, Israel. Tel: +972 8 9342428; Fax: +972 8 9315938; E-mail: adi.kimchi@weizmann.ac.il

Abbreviations: 4E-BP1, eukaryotic initiation factor 4E-binding protein 1; DAPK, death-associated protein kinase; mTOR, mammalian target of rapamycin; mTORC, mTOR complex; p70S6K, p70 ribosomal S6 kinase; raptor, regulatory-associated protein of mTOR; ULK1, unc-51-like kinase 1; WT, wild type

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are mostly unknown, with the exception of the myosin II regulatory light chain, which has been shown to be an *in vitro* and *in vivo* substrate.¹³ Although many publications have studied DAPK, its substrates and its role in cell death and autophagy,^{14,15} very little is known about DAPK2 substrates, cellular functions or the molecular pathways that it regulates.

In this work, we studied the involvement of DAPK2 in the autophagic module. We identified DAPK2 as a novel interacting protein of mTORC1, and as a negative regulator of the complex both during steady-state growth conditions and in response to different stress autophagic signals. We identified mTOR's binding partner, raptor, as a substrate of DAPK2, and found Ser721 as its phosphorylation site.

Results

Depletion of DAPK2 reduces thapsigargin-induced autophagy by relieving mTORC1 inhibition. Previous work carried out in our lab showed that DAPK2 over-expression induced the appearance of double-membrane autophagic vesicles in HEK293 and MCF-7 cells.¹⁶ To map

functionally the position of DAPK2 along the different stages of the autophagic pathway, we studied the outcome of DAPK2 knockdown on the levels of autophagy induced by different autophagic stimuli. As DAPK2 is a Ca²⁺-regulated protein, we first decided to test the involvement of DAPK2 in autophagy induced by elevation of intracellular Ca²⁺ levels. It was previously reported that an increase in intracellular Ca²⁺ levels, such as those achieved by thapsigargin (TG) treatment, induce autophagy in an AMPK-dependent pathway.¹⁷ TG is a potent inhibitor of ATP-dependent Ca²⁺ pumps in the ER, resulting in increased intracellular Ca²⁺ levels.¹⁸ As high levels of TG (i.e. μM range) can also block autophagy flux,¹⁹ HEK293T cells were treated with 100 nM TG, which was shown to induce autophagy accompanied by an increase in the degradation rate of long-lived proteins.¹⁷ Incubation of cells with TG caused an increase in the number of autophagosomes in cells transfected with control siRNA (ctrl siRNA), as reflected by the appearance of GFP-LC3 punctate staining. We found that depletion of DAPK2 significantly reduced GFP-LC3 puncta (Figures 1a and b). Consistent with this, there was a reduction in the LC3II/actin ratio in DAPK2-depleted cells after TG treatment compared with

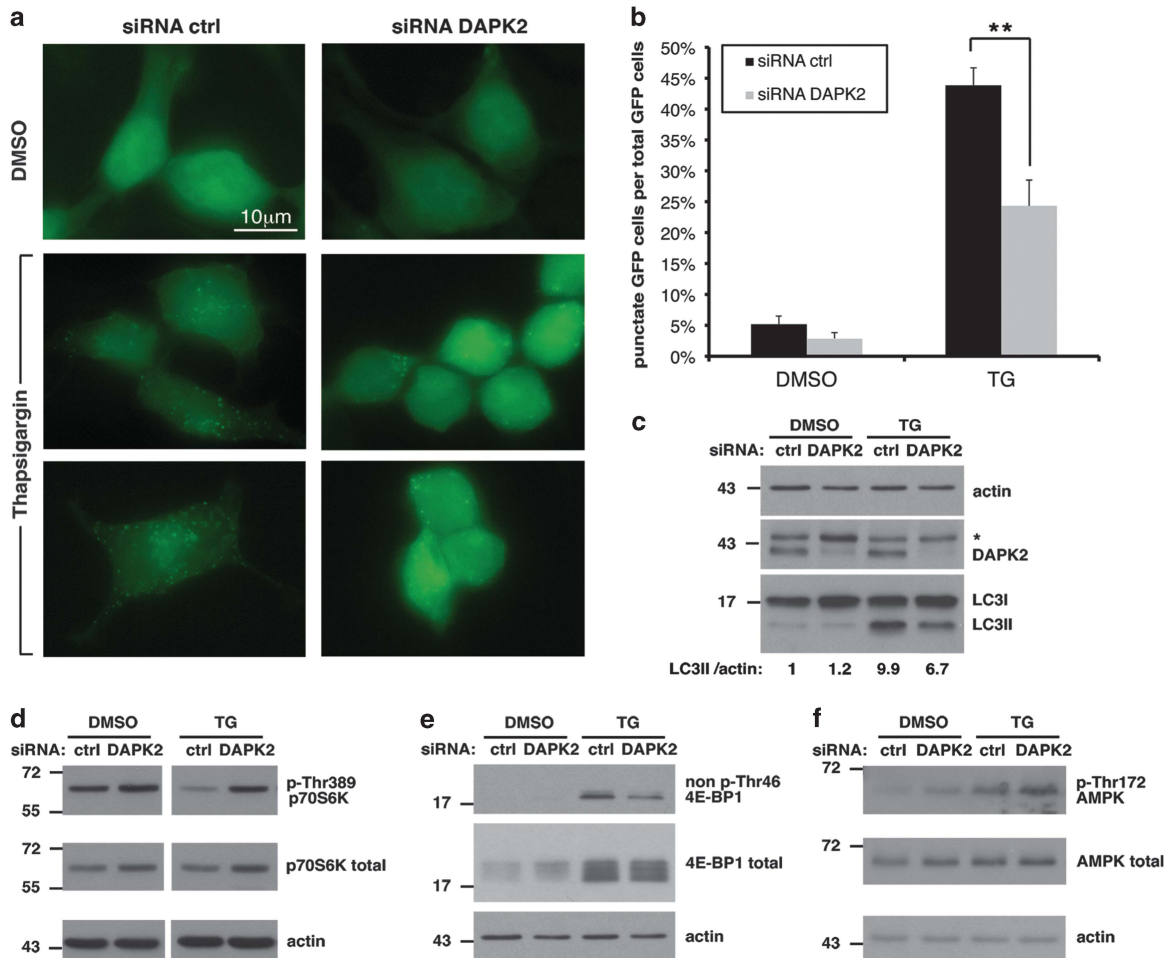


Figure 1 Depletion of DAPK2 reduces TG-induced autophagy by relieving mTOR inhibition. HEK293T cells were transiently co-transfected with GFP-LC3 and ctrl siRNA or DAPK2 siRNA. After 48 h, the cells were replated and treated with 100 nM TG for an additional 24 h. (a) Fluorescent micrographs of representative cells showing diffuse or punctate GFP-LC3. (b) Quantitation of the number of cells with GFP-LC3 puncta. S.D. is calculated from the mean of three independent experiments (***P* < 0.01). (c–f) Samples of cellular extracts were analyzed by western blot using the indicated Abs (*nonspecific band)

control cells (Figure 1c and Supplementary Figure S1A). To ensure that the changes in LC3II are due to genuine changes in the autophagic flux, we used bafilomycin A1. Indeed, treatment with TG increased the autophagic flux (Supplementary Figure S1B, compare lanes 1, 3 and 7), whereas knockdown of DAPK2 reduced this elevation (Supplementary Figure S1B, compare lanes 7 and 8).

Next, we examined the relationship between DAPK2 and mTORC1 to further determine if DAPK2 integrates into the signaling pathway(s) operating upstream of mTORC1, or whether it directly affects the autophagic core machinery. TG-mediated reductions in the phosphorylation levels of the mTORC1 substrate, p70S6K, were alleviated upon knockdown of DAPK2, indicating that mTOR inhibition by TG was interrupted (Figure 1d and Supplementary Figure S1C). Accordingly, knockdown of DAPK2 also alleviated the TG-induced increase in levels of the non-phosphorylated form of a second mTORC1 substrate, 4E-BP1, detected by specific antibodies (Abs) recognizing only the non-phosphorylated form of the protein, as well as by the appearance of faster migrating forms of 4E-BP1, corresponding to the

non-phosphorylated 4E-BP1 (Figure 1e and Supplementary Figure S1D). Interestingly, DAPK2 regulation of mTORC1 activity during treatment with TG did not depend on AMPK signaling, as DAPK2 depletion did not affect AMPK activation, as reflected by its phosphorylation levels on Thr172 (Figure 1f).

Depletion of DAPK2 reduces amino acid starvation-induced autophagy by relieving mTORC1 inhibition.

Next, we examined whether DAPK2 regulates additional autophagic stimuli that use different types of signaling pathways, such as deprivation of amino acids. Amino acid starvation upregulates autophagy by inhibiting mTORC1 through the RAG GTPase protein family.^{20,21} To this end, DAPK2 was knocked down in cells cultured with media lacking amino acids (Earle's balanced salt solution (EBSS)). Depletion of DAPK2 reduced the appearance of GFP-LC3 puncta in EBSS-treated cells (Figures 2a and b). Consistent with this, DAPK2 knockdown caused a reduction in the LC3II/actin ratio in starved cells (Figure 2c and Supplementary Figure S2A).

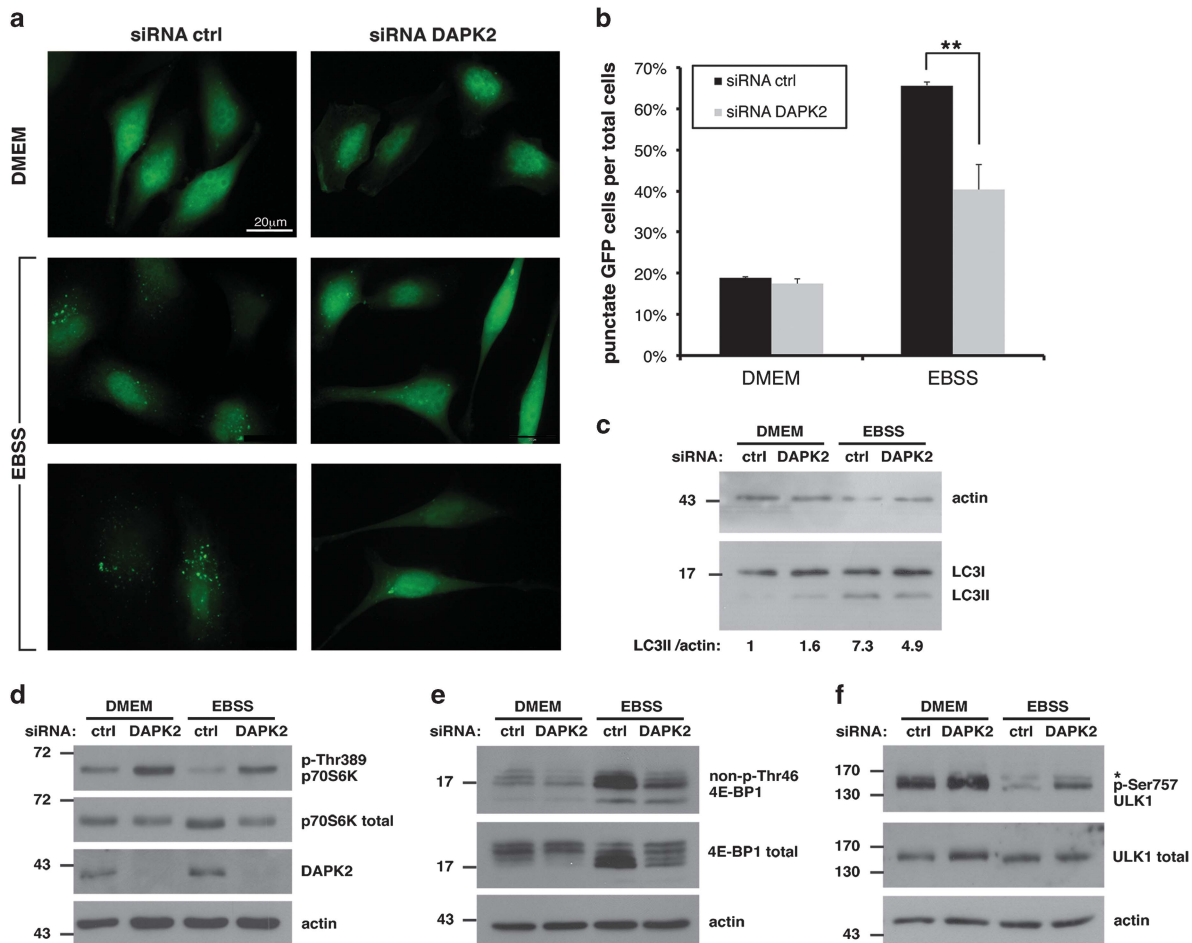


Figure 2 Depletion of DAPK2 reduces amino acid starvation-induced autophagy by relieving mTOR inhibition. HeLa cells stably expressing GFP-LC3 were transfected with ctrl siRNA or DAPK2 siRNA. After 48 h, the cells were replated and treated with EBSS for 2 h. (a) Fluorescent micrographs of representative cells showing diffuse or punctate GFP-LC3. (b) Quantitation of the number of cells with GFP-LC3 puncta. S.D. is calculated from the mean of three independent experiments (** $P < 0.01$). (c-f) Samples of cellular extracts were analyzed by western blot using the indicated Abs (*nonspecific band)

Examination of mTORC1 activity in cells revealed that similar to TG-induced autophagy, knocking down DAPK2 reduced the levels of amino acid starvation-induced autophagy through the relief of mTORC1 inhibition. We found that the decline in the levels of phosphorylated p70S6K in EBSS-treated cells was partially compromised upon DAPK2 knock-down (Figure 2d and Supplementary Figure S2B). Consistent with this, the increased levels of the non-phosphorylated 4E-BP1 observed in response to starvation were attenuated, as was the appearance of the faster migrating non-phosphorylated forms of total 4E-BP1 proteins (Figure 2e and Supplementary Figure S2C). We also examined the levels of phosphorylated ULK1 on Ser757, a specific mTORC1 phosphosite that inhibits ULK1 activity.²² As expected, the levels of phospho-Ser757 ULK1 were significantly reduced upon amino acid starvation. Notably, this decline was partially prevented by DAPK2 depletion (Figure 2f and Supplementary Figure S2D). Similar results were obtained when HEK293T cells were depleted of DAPK2 and treated with EBSS (Supplementary Figures S3A and D).

Depletion of DAPK2 reduces the inhibitory effect of rapamycin on mTORC1-mediated 4E-BP1 phosphorylation.

In addition to TG and amino acid starvation-induced autophagy, we also examined the effect of DAPK2 knock-down on a direct mTORC1 inhibitor, rapamycin. Rapamycin forms a complex with FKBP12, which binds mTOR^{23,24} and inhibits its activity by causing disassembly of the mTORC1

complex.⁵ Although rapamycin rapidly inhibits phosphorylation of p70S6K by mTOR, 4E-BP1 phosphorylation slowly decreases within 2 h. Structural analysis of the complex suggested that the binding of rapamycin immediately prevents the binding of large substrates such as p70S6K or ULK1 to the complex, but not the binding of smaller substrates such as 4E-BP1.²⁵ Thus, as long as the binding between mTOR and raptor still exists, 4E-BP1 is still subjected to phosphorylation by mTOR, for a certain time frame. Indeed, DAPK2 depletion could not block rapamycin-induced autophagy, which is ULK1 dependent (Figure 3a), or the complete inhibition of p70S6K phosphorylation (Figure 3b). However, 4E-BP1 phosphorylation by mTOR was affected by DAPK2 depletion, as the reduction of 4E-BP1 phosphorylation was attenuated (Figure 3c and Supplementary Figure S4). This result suggests that DAPK2 might regulate mTORC1 directly, rather than regulating the upstream signaling pathway, consistent with the finding that two autophagic inducers, TG and amino acid starvation, which use different upstream signaling pathways, were susceptible to DAPK2 depletion.

Depletion of DAPK2 increases mTORC1 activity during basal growth conditions, both in cells and *in vitro*.

Interestingly, knocking down DAPK2 by siRNA in growing HEK293T and HeLa cells led to an increase in the basal phosphorylation levels of p70S6K, indicating increased activity of mTORC1 (see Figures 1d and 2d, compare ctrl

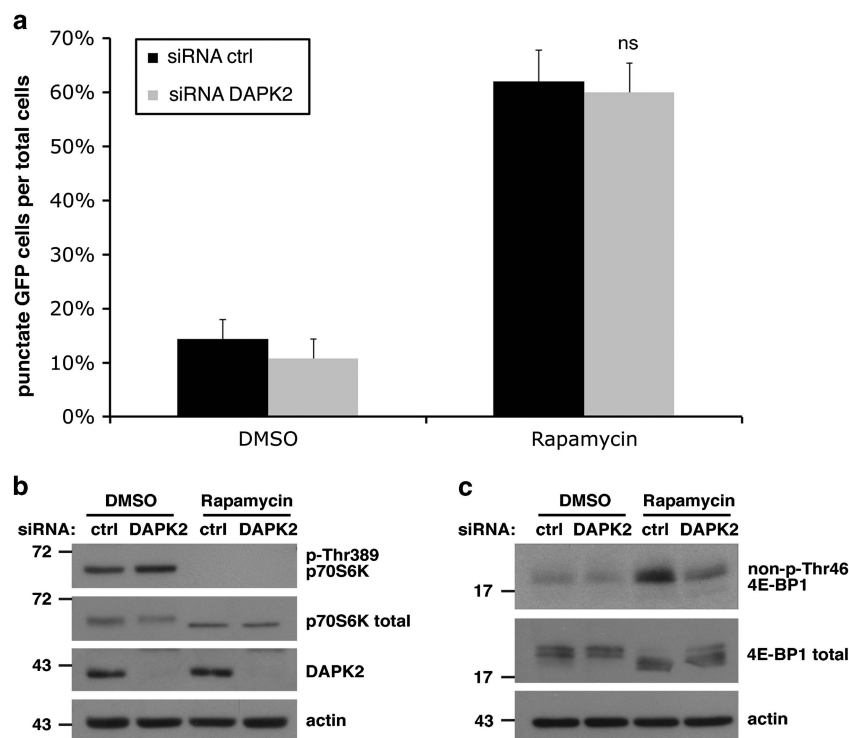


Figure 3 Depletion of DAPK2 reduces the inhibitory effect of rapamycin on mTOR. HeLa cells stably expressing GFP-LC3 were transiently transfected with ctrl siRNA or DAPK2 siRNA. After 48 h, the cells were replated and treated with 100 nM rapamycin for 2 h. (a) Fluorescent micrographs of representative cells showing diffuse or punctate GFP-LC3. S.D. is calculated from the mean of two independent experiments. NS, not statistically significant. (b and c) Samples of cellular extracts were analyzed by western blot using the indicated Abs

siRNA and DAPK2 siRNA in non-stressed cells). Basal autophagy, however, was not affected by DAPK2 knockdown (Figures 1b and 2b). This phenomenon was also observed in another human cell line, A549 (Supplementary Figure S5A). To rule out off-target effects, this result was also confirmed in cells transfected with shRNA against DAPK2, targeted against a different sequence of DAPK2 from the ones targeted by the siRNA pool (Supplementary Figure S5B). Significantly, DAPK2 knockdown also affects mTORC1 complex activity in *in vitro* kinase assays. Endogenous mTOR that was immunoprecipitated from DAPK2-depleted cells displayed increased activity towards the mTORC1-specific substrate, GST-4E-BP1, compared with mTOR prepared from control siRNA transfectants (Figure 4). Interestingly, depletion of DAPK2 did not affect the binding affinity between mTOR and raptor (Supplementary Figure S5C), or between raptor and 4E-BP1 (Supplementary Figure S5D).

Altogether, the results obtained by knocking down DAPK2 indicate that DAPK2 is a negative regulator of mTORC1 under steady-state conditions, as well as under various autophagic stimuli. DAPK2 depletion increased mTORC1 activity during different stress stimuli, during basal growth conditions and even in the presence of short-term rapamycin treatment. In addition, immunoprecipitated mTOR from DAPK2-depleted cells showed increased catalytic activity *in vitro*.

DAPK2 interacts with components of mTORC1. The above results imply that DAPK2 regulates autophagy and mTORC1 activity by directly regulating components of the mTORC1 complex. To investigate this possibility, we first checked if DAPK2 physically interacts with components of the complex. To this end, co-immunoprecipitation experiments were performed under conditions that retain the interactions among the well-known components of the whole mTORC1 complex (i.e. CHAPS-based lysis buffer).⁵ First, FLAG-tagged DAPK2 was coexpressed with HA-tagged raptor in HEK293T cells. Immunoprecipitation assays performed in both directions showed that DAPK2 and raptor specifically interact with each other (Figures 5a and b). In addition, DAPK2 interacted with two other members of mTORC1: myc-mTOR and FLAG-ULK1 (Figures 5c and d, respectively).

However, these interactions were detected only when myc-mTOR or FLAG-ULK1 were immunoprecipitated and not in the reciprocal direction (data not shown). Interestingly, DAPK2 showed enhanced association with raptor during amino acid starvation (Figure 5e), similar to the effect amino acid starvation has on the association between mTOR and raptor.⁵

DAPK2 phosphorylates raptor *in vitro* on Ser721. Next, we tested whether the status of the catalytic activity of DAPK2 influences its ability to bind with raptor. To this end, the binding properties between raptor and DAPK2 wild-type (WT) or its kinase-dead mutant, DAPK2 K42A, were compared.¹⁰ Interestingly, DAPK2 K42A bound significantly stronger to raptor compared with DAPK2 WT (Figure 6a). Often, mutation of residues that block ATP binding to the catalytic site, such as DAPK2's K42A, can block release and turnover of the bound substrate, as it cannot be phosphorylated, rendering the mutant kinase a 'substrate trap'. Because raptor showed greater binding to the kinase-dead mutant, we hypothesized that raptor could be a substrate of DAPK2. To test this, HA-tagged raptor was immunopurified from HEK293T cells, and subjected it to an *in vitro* kinase assay with purified DAPK2. Recombinant MLC, a known DAPK2 substrate, was used as a positive control (Figure 6b, right panel). The immunoprecipitated raptor displayed background phosphorylation, most likely due to the copurification of endogenous kinase(s), despite the use of strong detergents in the lysis/immunoprecipitation (Figure 6b, left panel, lane 3). Still, an increase in phosphorylation of raptor was detected above this background when active DAPK2 was introduced (Figure 6b, left panel, lane 2). DAPK2 failed to phosphorylate its other binding partners, mTOR and ULK1 (Supplementary Figures S6A and B).

To map the phosphorylation site(s) on raptor, a bacterially produced recombinant raptor protein was generated. Raptor is a highly conserved protein, containing several protein-to-protein interaction domains: raptor N-terminal conserved domain, followed by three HEAT repeats, which are then followed by seven WD repeats in the C terminus of the protein.⁵ Interestingly, most of the known regulatory

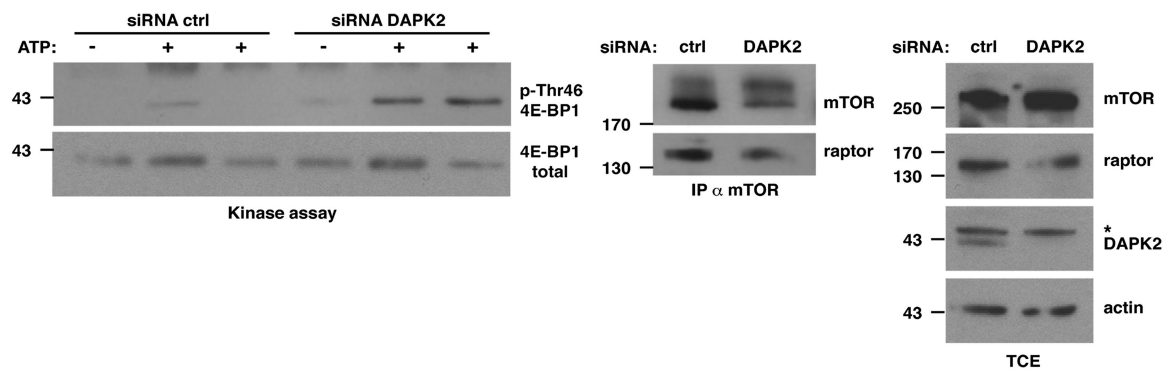


Figure 4 Depletion of DAPK2 increases *in vitro* kinase activity of mTORC1. HEK293T cells were transfected with ctrl siRNA or DAPK2 siRNA for 72 h. The *in vitro* kinase assay was performed using recombinant GST-4E-BP1 as a substrate, and anti-phospho Abs were used to assess the extent of phosphorylation on western blots. A fraction of the immunoprecipitated mTOR (IP) that was used in the assay was also subjected to western blot (middle panel) to assess the levels of pulled down mTOR and raptor. *Nonspecific band

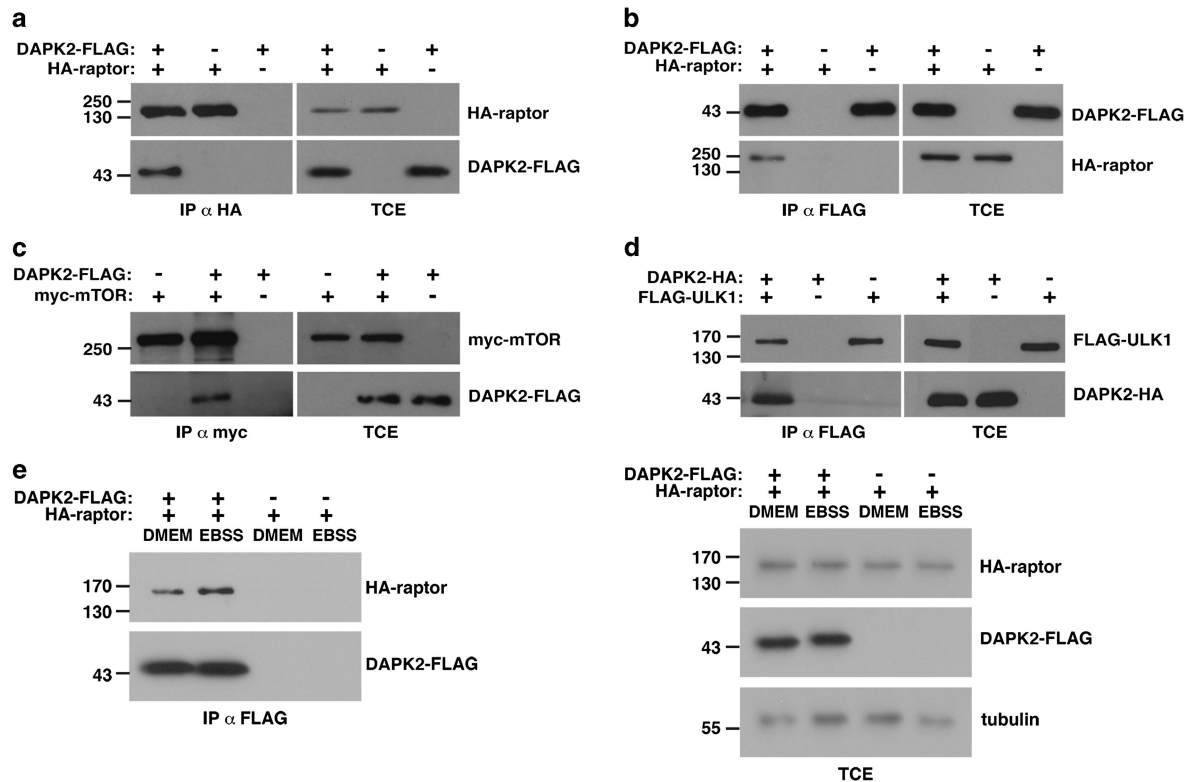


Figure 5 DAPK2 interacts with components of mTORC1. (a and b) HEK293T cells were co-transfected with DAPK2-FLAG and HA-raptor. Lysates were subjected to immunoprecipitation using anti-HA (a) or anti-FLAG (b) Abs, and protein levels in the immunoprecipitate (IP) or total cell extract (TCE) were detected by western blot. (c) HEK293T cells were co-transfected with DAPK2-FLAG and myc-mTOR. Lysates were subjected to immunoprecipitation using anti-myc Ab. (d) HEK293T cells were transfected with DAPK2-HA together with FLAG-ULK1. Lysates were subjected to immunoprecipitation using anti-FLAG Ab. (e) HEK293T cells were transfected with DAPK2-FLAG, together with HA-raptor. After 24 h, the cells were incubated in EBSS for 2 h. Lysates were subjected to immunoprecipitation using anti-FLAG Ab

phosphorylation sites of raptor are clustered in a region extending between the HEAT repeats and the WD repeats. A fragment that includes this cluster, amino acids 691–930, was therefore produced in bacteria (Supplementary Figure S6C). Use of the recombinant fragment provided the additional advantage of eliminating any bound kinases that affect background phosphorylation of full-length cellular raptor. Significantly, DAPK2 was able to phosphorylate the raptor fragment (Figure 6c), indicating that at least one of its phosphorylation targets is located within this segment of the raptor protein. Importantly, the recombinant raptor fragment strongly bound a bacterially produced recombinant full-length DAPK2 *in vitro* (Figure 6d). This result indicated that indeed DAPK2 directly binds raptor.

To identify raptor phosphorylation site(s), a non-radioactive *in vitro* kinase assay using purified DAPK2 and the raptor fragment was performed and analyzed by liquid chromatography-mass spectrometry/mass spectrometry. A sample without DAPK2 was used as control to ensure that the phosphorylation(s) identified were DAPK2 dependent. The analysis identified a single phosphorylation site at the equivalent of amino acid Ser721 of the full-length raptor protein (Figure 7a). Interestingly, this serine resides within a putative DAPK family consensus phosphorylation motif (Figure 7b). To verify experimentally that DAPK2 phosphorylates raptor on this site, an additional recombinant fragment

of raptor with a Ser721 to Ala mutation was produced. Indeed, while DAPK2 phosphorylated the WT raptor fragment, it was not able to phosphorylate the mutated raptor fragment (Figure 7c).

The mapping of the phosphorylation site was further validated using a specific Ab against Ser721. Ser721 is located within a stretch of amino acids RXRXXS/T to which anti-phospho Abs are available. Indeed, the phospho-RXRXXpS/T Ab detected raptor when it was phosphorylated by DAPK2 WT, but failed to do so when raptor was reacted with DAPK2 K42A in an *in vitro* kinase assay (Figure 7d). The phospho-RXRXXpS/T Ab failed to detect the mutated S721A raptor fragment upon incubation with DAPK2 WT (Figure 7e), suggesting that DAPK2 indeed phosphorylates raptor on Ser721.

Thus, we proved that the physical interaction between DAPK2 and raptor involves phosphorylation at Ser721, a site located within a highly phosphorylated region of raptor that has been shown to regulate mTORC1 activity.

Discussion

Here, we characterized DAPK2 as a novel negative regulator of mTORC1, both under autophagic stress and during basal growth conditions. We integrated DAPK2 into a specific point in the autophagic regulatory network, and identified raptor as a novel substrate of DAPK.

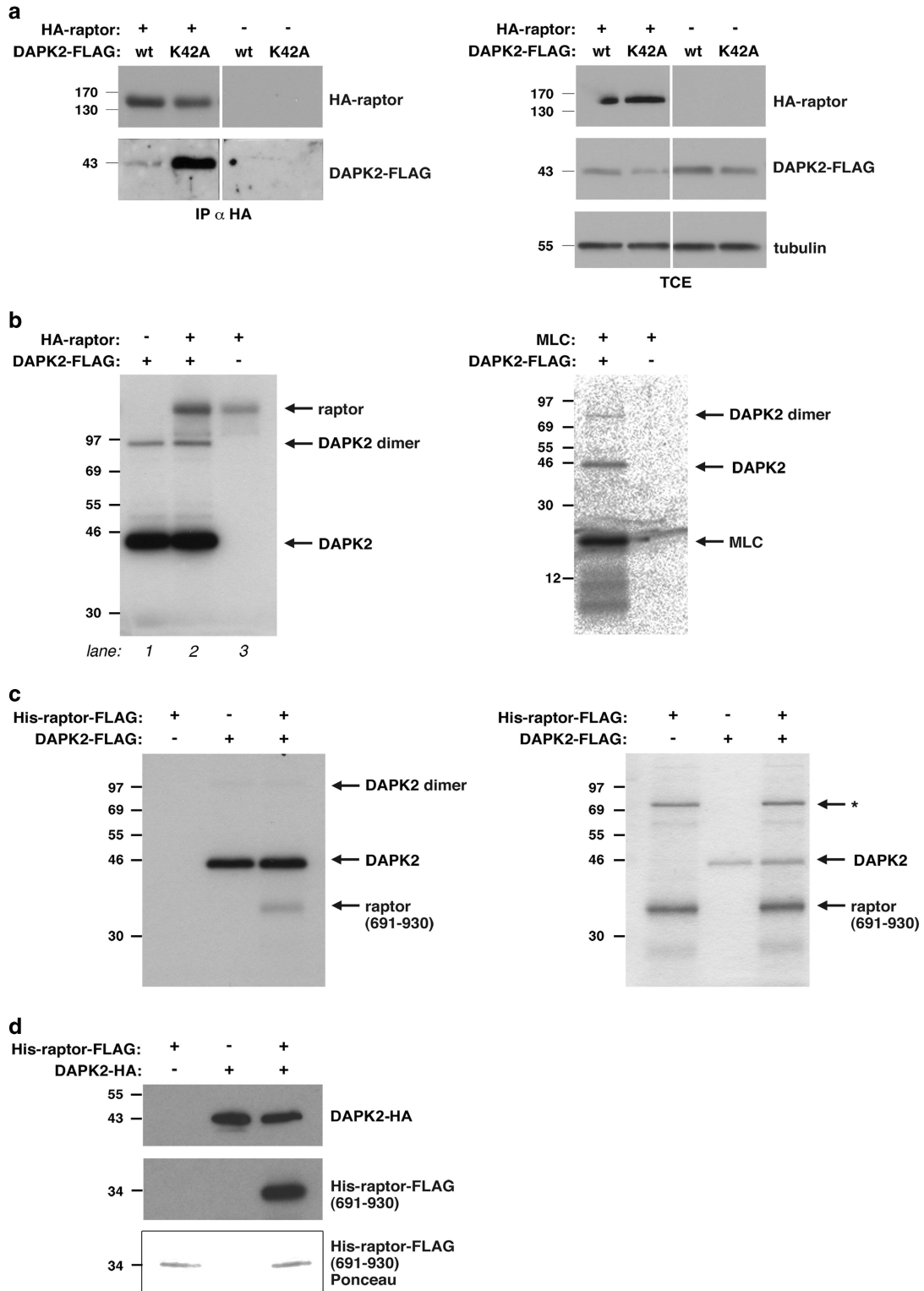


Figure 6 DAPK2 phosphorylates raptor *in vitro*. (a) HEK293T cells were transfected with DAPK2-FLAG WT or DAPK2-FLAG K42A together with HA-raptor. Lysates were subjected to immunoprecipitation using anti-HA Ab, and protein levels in the immunoprecipitate (IP) or total cell extract (TCE) were detected by western blot. (b) HA-raptor was immunoprecipitated from HEK293T cells and was incubated with immunopurified DAPK2-FLAG in a kinase reaction mixture containing ^{33}P -labeled ATP. MLC was used as a positive control for DAPK2 activity. Reactions were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and exposed to X-ray film. (c) Recombinant raptor fragment was incubated with immunopurified DAPK2-FLAG in a kinase reaction mixture containing ^{32}P -labeled ATP and reactions were resolved by SDS-PAGE (autoradiogram, left). Gel code-stained gel shows equal amount of substrate loading (right). (d) Recombinant raptor fragment and recombinant DAPK2 were subjected to protein-binding assay. Bound proteins were subjected to western blot with the indicated Abs. Fractions of the input were also resolved by SDS-PAGE and blot stained with Ponceau S to assess equal loading of raptor (bottom blot)

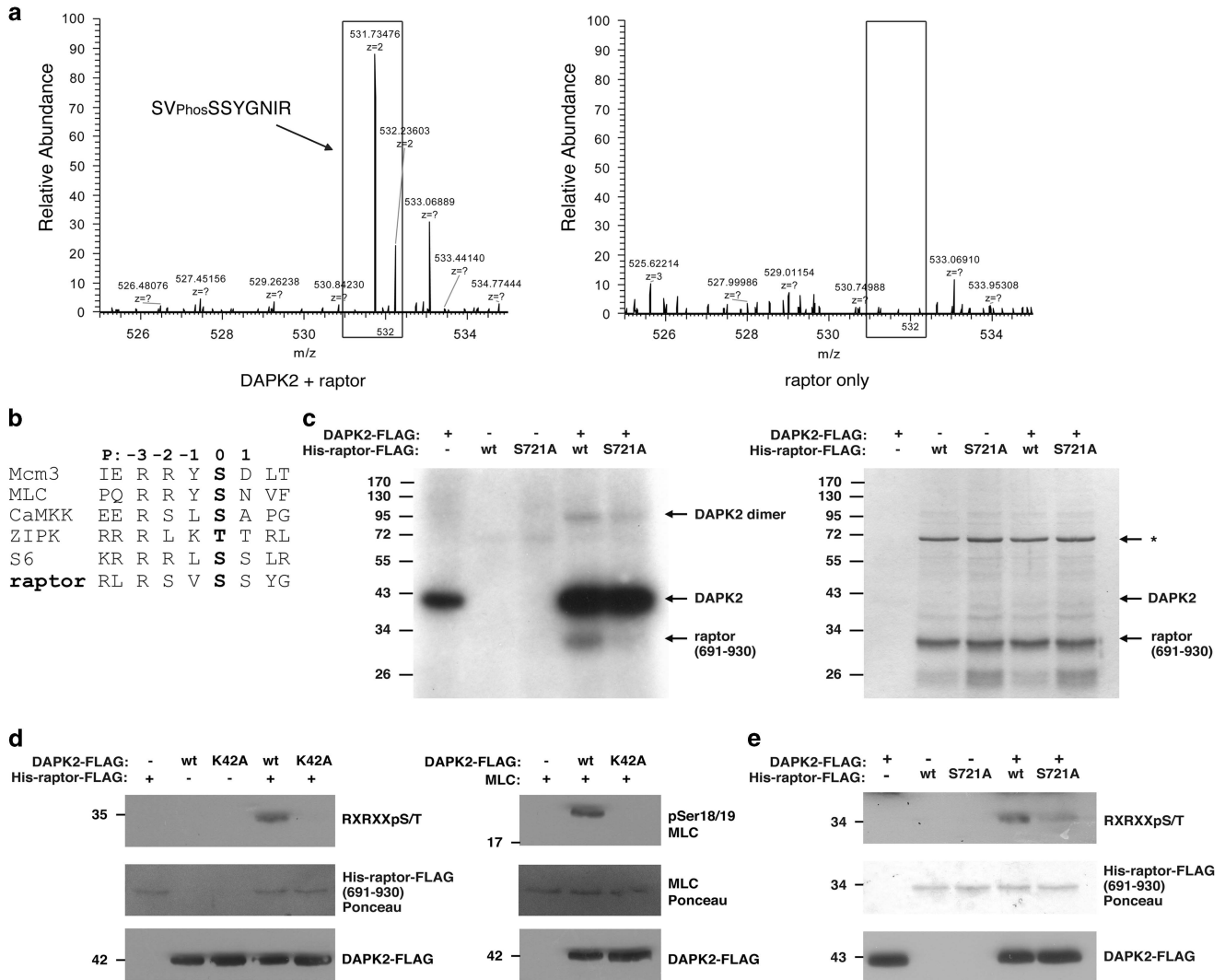


Figure 7 DAPK2 phosphorylates raptor fragment *in vitro* on Ser721. (a) Raptor fragment was subjected to an *in vitro* kinase assay with immunoprecipitated DAPK2-FLAG, and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). A sample without DAPK2 was used as control. The gel was stained with Gel code and the raptor bands in each sample were excised and analyzed by liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) as shown in the graphs. Each peak represents a peptide; the graph height represents the relevant abundance of the peptide in the fraction. (b) Alignment of raptor amino acids 716–724 with the phosphorylation site of several known substrates of DAPK. (c) Immunoprecipitated DAPK2-FLAG was reacted in an *in vitro* kinase assay with raptor fragment WT or mutated at the equivalent site of S721. The kinase reaction mixture contained 32 P-labeled ATP (left). The samples were resolved on SDS-PAGE and the gel stained with Gel code to assess equal amount of substrate loading. *Nonspecific band. (d) Immunoprecipitated DAPK2-FLAG WT or DAPK2-FLAG K42A were reacted in an *in vitro* kinase assay with raptor fragment and subjected to western blot. MLC was used as a positive control for the kinase activity of the DAPK2 proteins. (e) Immunoprecipitated DAPK2-FLAG was reacted in an *in vitro* kinase assay with raptor fragment WT or mutated in the equivalent site of S721 and subjected to western blot

Previous studies carried out in our lab showed that DAPK2 overexpression induces the appearance of double-membrane vesicles.¹⁶ Here we show, for the first time, two different physiological autophagic settings where knockdown of DAPK2 interferes with the execution of the pathway: increases in intracellular Ca^{2+} levels and deprivation of amino acids. In both settings, depletion of DAPK2 reduced the percentage of autophagic cells in the stressed cell population. This result suggests that DAPK2 has a part in the autophagy regulatory process, acting in parallel to other signaling pathways that control autophagy induction. The knockdown of DAPK2 was previously shown to be involved in other cellular pathways, such as normal myeloid differentiation²⁶ and β -catenin-induced anoikis resistance of malignant epithelial cells.²⁷ However,

none of these works identified DAPK2's interacting partners or substrates, and therefore did not integrate DAPK2 into a specific signaling pathway. Interestingly, the two autophagic stresses, increase in Ca^{2+} levels and amino acid starvation, induce autophagy by different signaling pathways: CaMKK β and AMPK mediate Ca^{2+} -induced autophagy¹⁷ and the RAG protein family mediate amino acid-induced autophagy.^{20,21} These two pathways converge at the level of mTORC1. Indeed, in both stresses, DAPK2-depleted cells showed increased activity levels of mTORC1, as reflected by the phosphorylation of its downstream substrates p70S6K, 4E-BP1 and ULK1. Furthermore, mTORC1 that was pulled down from DAPK2-depleted cells showed increased ability to phosphorylate 4E-BP1 *in vitro*. Taken together, these results suggest that DAPK2

function is inherent to the complex itself, proving that we functionally mapped DAPK2 to the level of mTORC1.

mTORC1 is a central signaling complex within the cell. It mediates the cellular response to a wide range of cellular stresses by balancing between anabolic processes, such as protein synthesis, and catabolic processes, such as autophagy and consumption of energy storages.²⁸ mTORC1 includes several proteins that can mediate mTOR's kinase activity by substrate recognition (such as raptor) or regulate it under specific conditions (such as PRAS40 and DEPTOR^{29,30}). The upstream regulation of mTORC1 is very complex, and includes signaling events from multiple pathways. We have identified DAPK2 as a new binding partner of mTORC1. Under conditions that maintain the integrity of the whole complex, DAPK2 co-immunoprecipitated with several members of the mTOR complex (i.e. mTOR, raptor and ULK1). Interaction with several complex members was also demonstrated for other mTORC1-interacting proteins such as PRAS40³¹ and p62.³² An *in vitro* recombinant protein-binding assay revealed that DAPK2 binds raptor, suggesting that raptor is the direct binding partner of DAPK2 within mTORC1.

In attempts to identify a possible regulatory mechanism by which DAPK2 inhibits mTORC1, we examined different proteins from mTORC1 as candidate substrates of DAPK2. DAPK2 was able to phosphorylate raptor *in vitro*, whereas it failed to phosphorylate mTOR or ULK1. Using a recombinant fragment of raptor, we identified by mass spectrometry a specific phospho-site on Ser721 that is phosphorylated by DAPK2. This site was later confirmed using a mutated fragment. Raptor phosphorylation and its effect on mTORC1 activity has been the subject of many research papers. It was shown to both promote mTORC1 activity^{8,33,34} and to repress it.^{7,9} In many cases, the phosphorylation event includes more than one phosphorylation site. For example, AMPK phosphorylates raptor on both Ser722 and Ser792,⁷ and mTOR phosphorylates raptor on Ser859 and Ser863.³³ Using only a fragment of raptor, we cannot rule out the possibility that DAPK2 phosphorylates additional sites on raptor.

DAPK2 is part of the DAPK family, a family of kinases that regulate cell death, cytoskeletal dynamics and immune functions. Recently, DAPK has also been shown to be a critical regulator of autophagy (reviewed in Levin-Salomon *et al.*¹⁴). Interestingly, even though DAPK and DAPK2 are both regulated by binding to Ca²⁺/CaM, and they share highly conserved kinase domains, they participate differently in autophagy. First, based on our current knowledge, they integrate into different points within the autophagic pathway. While we show that DAPK2 regulates the induction step of autophagy by negatively regulating mTORC1, DAPK was shown to positively contribute to the autophagosome nucleation step by regulating the Vps34 complex.^{35,36} Second, they are involved in different autophagic stresses. DAPK seems to be involved mostly in autophagy during cell death scenarios (e.g. oxidative stress, ER stress), but not in prosurvival autophagy such as starvation-induced autophagy (G Oberkovitz and A Kimchi, unpublished observations). It would be interesting to understand the differences in the activation and/or interacting partners that direct DAPK and DAPK2 to the autophagic signaling pathway during different autophagic stimuli.

Finally, the tumor-suppressive function of these two family members is an intriguing subject to be followed. Since its original identification, DAPK was shown to be a *bona fide* tumor suppressor gene. DAPK expression is lost in many cancer cell lines and fresh tumor specimens, mostly due to hypermethylation of the DAPK promoter region. In contrast, only a few reports connecting DAPK2 to cancer have so far been documented. DAPK2 expression is downregulated in Hodgkin lymphoma cells owing to promoter methylation, and its reintroduction into these cells induces apoptosis and suppresses tumor growth.³⁷ However, the mechanism by which DAPK2 may function as a tumor suppressor has not been discovered. In this work, we identified a possible link between DAPK2 and cancer. We show that DAPK2 is a novel interacting protein and a negative regulator of mTORC1. Several of mTORC1's negative regulators have been previously shown to be tumor suppressor genes, such as *TSC2* and *LKB1*. mTORC1 is also activated by known oncogenes such as AKT and PI3K.³⁸ Some of the previous findings depicting DAPK2 as a tumor suppressor might be explained by the regulation it exerts on mTORC1. For example, it is possible that the reduced proliferation observed in Hodgkin lymphoma cells upon the reconstitution of DAPK2 was due to reduced activity of mTORC1. Thus, DAPK2 might function as a tumor suppressor, downregulating mTORC1's oncogenic activation, which leads to induction of several processes required for cancer cell growth, survival and proliferation.

Materials and Methods

Protein analysis. Cells were lysed in PLB buffer (100 mM NaCl, 0.1 M sodium phosphate, pH 7.5, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate). The buffer was supplemented with 1% protease inhibitor (Sigma-Aldrich, St. Louis, MO, USA) and 1 mM PMSF. In experiments assaying levels of phosphorylated proteins, phosphatase inhibitors (1 mM NaF, 1 mM sodium vanadate, 40 mM β GPO₄) were also added. Proteins were separated by SDS-PAGE and blotted onto nitrocellulose membranes, which were incubated with Abs to FLAG, LC3B, α -tubulin and actin (Sigma-Aldrich), phospho-p70S6K (Thr389), p70S6K, phospho-4E-BP1 (Thr37/46), non-phospho-4E-BP1 (Thr46), 4E-BP1, phospho-AMPK α (Thr172), AMPK α , phospho-ULK1 (Ser757), phospho-RXRXXpS/T, phospho-MLC (Ser19), mTOR and raptor (Cell Signaling, Danvers, MA, USA), ULK1 (Santa Cruz, Santa Cruz, CA, USA), HA (Biotest, Boca Rato, FL, USA), DAPK2 (Abcam, Cambridge, MA, USA; ProSci). Myc Ab was a gift from M Oren (Weizmann Institute of Science, Rehovot, Israel). Detection was carried out with either HRP-conjugated goat anti-mouse or anti-rabbit secondary Abs (Jackson ImmunoResearch, Suffolk, UK), followed by enhanced chemiluminescence (SuperSignal; Pierce, Thermo Scientific, Rockford, IL, USA). Protein densitometric analysis was performed using EZQuant-Gel software (EZQuant, Tel-Aviv, Israel) on scanned blots.

DNA constructs and transfection procedures. FLAG- or HA-tagged DAPK2 and FLAG-tagged DAPK2 K42A¹⁰ were expressed from pcDNA3 expression vectors. myc-mTOR, myc-mTOR D2357E/V2364I, myc-Raptor and HA-raptor (all in pRK5 vector) were purchased from Addgene (Cambridge, MA, USA). FLAG-ULK1 was generated by removing the GFP from mULK1-GFP (kindly provided by N Mizushima) and inserting the FLAG-tag at the N terminus. The GFP-LC3 plasmid, pEGFP1-LC3, was a kind gift from N Mizushima and T Yoshimori. Control plasmid consisted of empty pcDNA3 plasmid. Ser to Ala mutation was generated by PCR-mediated site-directed mutagenesis. All cell lines were transfected by standard calcium phosphate technique.

RNA interference. DAPK2 expression was transiently knocked down in cells using siGENOME SMARTpool siRNA reagent (Dharmacon, Lafayette, CO, USA) against human DAPK2 (NM_014326). For control siRNA, siCONTROL non-targeting siRNA was used (Dharmacon). shRNA targeting DAPK2 (nucleotides 563–581, NM_014326) and HcRed (nucleotides 99–117, AF363776) were designed

in our lab. The 19-mer oligos were annealed and ligated into the *Bgl*II and *Hind*III of pSUPER-based shRNA vector.

Cell culture and induction of cell stress. HeLa, HEK293T and A549 cells were grown as previously described.¹⁶ To generate stable cell lines expressing GFP-LC3, HeLa cells were transfected with the GFP-LC3 plasmid and grown in the presence of G418 (1 mg/ml) (Calbiochem, Merck, Darmstadt, Germany). Selected clones were individually isolated to create monoclonal cell lines. The cells were treated with the following reagents: DMSO (Sigma-Aldrich), TG (Sigma-Aldrich), EBSS (Biological Industries, Beit-Haememk, Israel) and rapamycin (Sigma-Aldrich).

GFP-LC3 punctate staining assay. HeLa cells stably expressing GFP-LC3 or HEK293T cells transiently expressing GFP-LC3 were plated on 13 mm glass coverslips. The treated cells were fixed with 3.7% formaldehyde and viewed by fluorescent microscopy. The percentage of cells displaying more than five GFP-LC3 puncta per total GFP-positive cells was determined.

Far western (raptor overlay assay). The far western assay was carried out as described by Dunlop *et al.*⁹ Briefly, recombinant GST-4E-BP1 protein was resolved by SDS-PAGE and transferred to PVDF membrane. Raptor-containing lysates were incubated with the membrane overnight. The membrane was then washed and probed with anti-Myc Ab, followed by processing and visualization using the final steps of the standard western blotting protocol.

Immunoprecipitation. Cells were washed with PBS and extracted in ice-cold lysis buffer as described above. Protein extracts were precleared with protein G-agarose beads (Santa Cruz) and then incubated with anti-FLAG M2 beads or anti-HA beads (Sigma-Aldrich), or with the beads prebound to the relevant Abs for 2 h at 4 °C. Immunoprecipitates were washed three times with the lysis buffer and the protein was eluted from the beads with an excess of FLAG-HA peptides (Sigma-Aldrich). Co-immunoprecipitation of the mTORC1 protein members (mTOR, raptor and ULK1) and DAPK2 were carried out using CHAPS-based lysis buffer (40 mM HEPES (pH 7.4), 2 mM EDTA, 10 mM pyrophosphate, 10 mM glycerophosphate, 0.3% CHAPS). For immunoprecipitation of potential DAPK2 substrates used in *in vitro* kinase assays, cells overexpressing the protein of interest were lysed in Triton-based lysis buffer (40 mM HEPES (pH 7.4), 2 mM EDTA, 10 mM pyrophosphate, 10 mM glycerophosphate, 0.3% Triton). Immunoprecipitation of endogenous mTOR was carried out as previously described³¹ using anti-mTOR Ab (Santa Cruz).

***In vitro* kinase assay for mTOR activity.** For kinase assays, endogenous mTOR immunoprecipitates were washed three times in low salt wash buffer, followed by two additional washes in 25 mM HEPES (pH 7.4) and 20 mM KCl. Kinase assays were performed for 20 min at 30 °C in a final volume of 30 μ l consisting of mTOR kinase buffer (25 mM HEPES (pH 7.4), 50 mM KCl, 10 mM MgCl₂, 250 μ M ATP) and 1 μ g GST-4E-BP1 as the substrate. Reactions were stopped by the addition of sample buffer and boiling for 5 min, and analyzed by SDS-PAGE and immunoblotting.

***In vitro* DAPK2 kinase assay.** Hundred nanograms of DAPK2 was incubated with 750 ng–1.5 μ g substrate in a kinase assay buffer (50 mM HEPES, pH 7.5, 20 mM MgCl₂, 50 μ M ATP, 5 μ Ci [γ -³³P]ATP or [γ -³²P]ATP (Perkin-Elmer, Waltham, MA, USA), protease and phosphatase inhibitors, 500 mM EGTA or 0.5 mM CaCl₂, 1 μ M CaM) at 30 °C for 30 min. Reactions were stopped by the addition of sample buffer and resolved by SDS-PAGE. Radioactive signals were detected with Amersham Hyperfilm using ³³P or ³²P intensifying screen (Kodak, Rochester, NY, USA). For reactions in which radioactive ATP was not used, the concentration of ATP in the assay was increased to 100 μ M, and the samples were transferred to nitrocellulose membrane and subjected to western blot using phospho-specific Abs.

Bacterial plasmid construction and expression. For bacterial expression, WT and S721A mutant of raptor (amino acids 691–930) and full-length DAPK2 were cloned into the pET28-TevH expression vector.³⁹ Cloning of both plasmids was performed using Transfer-PCR.⁴⁰ Raptor and DAPK2 plasmids were expressed in *Escherichia coli* BL21(DE3) cells. Proteins were purified using excess peptide. GST-4E-BP1 construct was a gift from N Sonenberg. GST-4E-BP1 was expressed in BL21(DE3) (Novagen, Merck), and purification was carried out using the standard GST method (GE Healthcare, Little Chalfont, UK).

***In vitro* binding assay.** Recombinant raptor fragment was incubated with recombinant DAPK2 bound to anti-HA beads for 2 h at 4 °C. Immunoprecipitates were washed three times with CHAPS-based lysis buffer and the protein was eluted from the beads with an excess of HA peptides (Sigma-Aldrich).

In-gel proteolysis and mass spectrometry analysis. The proteins in the gel were reduced with 2.8 mM DTT (60 °C for 30 min), modified with 8.8 mM iodoacetamide in 100 mM ammonium bicarbonate (in the dark, room temperature for 30 min) and digested in 10% acetonitrile and 10 mM ammonium bicarbonate with modified trypsin or chymotrypsin (Promega, Madison, WI, USA; 1 : 10) overnight at 37 °C.

The resulting peptides were resolved by reverse-phase chromatography on 0.075 \times 200 mm² fused silica capillaries (J&W, Santa Clara, CA, USA) packed with Repronil reversed-phase material (Dr Maisch GmbH, Ammerbuch, Germany). The peptides were eluted with linear 65 min gradients of 5–45% and 15 min at 95% acetonitrile with 0.1% formic acid in water at flow rates of 0.25 μ l/min. Mass spectrometry was performed by an ion-trap mass spectrometer (OrbitrapXP; Thermo-Scientific, Rockford, IL, USA) in a positive mode using repetitively full MS scan followed by collision induces dissociation, with multistage activation, of the seven most dominant ion selected from the first MS scan. The mass spectrometry data was analyzed using the Protein Discoverer 1.4 (Thermo-Scientific) using both Sequest search engines, searching against the specific sequence of the protein. Identifications were filtered according to mass accuracy and 1% false discovery rate. The position of the phosphorylation in the peptide was validated with the phospho-RS tool. The relative quantities of the phosphopeptides were determined using the peak area calculation.

Statistical analysis. The statistical significance of differences between means was assessed by two-tailed Student's *T*-test. Values of *P* < 0.05 were considered significant.

Conflict of Interest

The authors declare no conflict of interest.

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