

Cytoplasmic and nuclear distribution of the protein complexes mTORC1 and mTORC2: rapamycin triggers dephosphorylation and delocalization of the mTORC2 components rictor and sin1

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The mammalian target of rapamycin (mTOR) is part of two distinct complexes, mTORC1, containing raptor and mLST8, and mTORC2, containing rictor, mLST8 and sin1. Although great endeavors have already been made to elucidate the function and regulation of mTOR, the cytoplasmic nuclear distribution of the mTOR complexes is unknown. Upon establishment of the proper experimental conditions, we found mTOR, mLST8, rictor and sin1 to be less abundant in the nucleus than in the cytoplasm of non-transformed, non-immortalized, diploid human primary fibroblasts. Although raptor is also high abundant in the nucleus, the mTOR/raptor complex is predominantly cytoplasmic, whereas the mTOR/rictor complex is abundant in both compartments. Rapamycin negatively regulates the formation of both mTOR complexes, but the molecular mechanism of its effects on mTORC2 remained elusive. We describe that in primary cells short-term treatment with rapamycin triggers dephosphorylation of rictor and sin1 exclusively in the cytoplasm, but does not affect mTORC2 assembly. Prolonged drug treatment leads to complete dephosphorylation and cytoplasmic translocation of nuclear rictor and sin1 accompanied by inhibition of mTORC2 assembly. The distinct cytoplasmic and nuclear upstream and downstream effectors of mTOR are involved in many cancers and human genetic diseases, such as tuberous sclerosis, Peutz–Jeghers syndrome, von Hippel–Lindau disease, neurofibromatosis type 1, polycystic kidney disease, Alzheimer's disease, cardiac hypertrophy, obesity and diabetes. Accordingly, analogs of rapamycin are currently tested in many different clinical trials. Our data allow new insights into the molecular consequences of mTOR dysregulation under pathophysiological conditions and should help to optimize rapamycin treatment of human diseases.

INTRODUCTION

In mammalian cells, two mammalian target of rapamycin (mTOR)-containing complexes have been identified. mTORC1 is composed of mTOR, raptor (regulatory associated protein of mTOR) and mLST8 (also known as GβL). Whereas the function of mLST8 is not really clarified, raptor functions as a scaffold for recruiting mTORC1 substrates, such as the p70S6K (ribosomal p70S6 kinase) and 4EBP1 (eukaryotic initiation factor 4E binding protein-1), both regulators of protein translation. mTORC2 contains mTOR, rictor (rapamycin-insensitive

companion of mTOR), mLST8 and sin1 (stress-activated protein kinase-interacting protein). Rictor and sin1 appear to stabilize each other through binding, building the structural foundation for mTORC2. The role of the mTORC2 component protor (protein observed with rictor), which lacks any obvious functional domains (if any), still remains elusive. mTORC2 phosphorylates the kinase Akt, which in conjunction with PDK1 (phosphoinositide-dependent kinase-1)-mediated phosphorylation drives full activation of Akt (1–6).

Upstream of mTOR activated receptor tyrosine kinases activate the phosphatidylinositol-3-kinase (PI3K). Phosphorylation of

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the membrane lipid phosphatidylinositol-4,5-bisphosphate by PI3K produces the second messenger phosphatidylinositol-3,4,5-trisphosphate (PIP₃) in a reaction that can be reversed by the phosphatase PTEN (phosphatase and tensin homolog). PDK1 and Akt bind to PIP₃, and PDK1 phosphorylates Akt. Akt-mediated phosphorylation downregulates tuberin's GTPase-activating potential toward Rheb (Ras homolog enriched in brain), which regulates mTOR through FKBP38, a member of the FK506-binding protein (FKBP) family (1–5,7,8). Tuberin is part of the hamartin/tuberin (TSC1/TSC2) complex and mutations in either the *TSC1* or *TSC2* gene cause tuberous sclerosis (9). Rheb has been reported to have a negative effect on mTORC2, probably indirectly through a p70S6K-dependent negative feedback loop (10). Recently, it was reported that the TSC1/TSC2 complex can physically associate with mTORC2 and positively regulates mTORC2 in a manner independent of its activity toward Rheb (11). Besides Akt, other enzymes have been identified to regulate tuberin's functions. The *LKB1* Peutz–Jeghers tumor suppressor gene product phosphorylates and activates AMPK (5'AMP-activated protein kinase), a positive regulator of tuberin (12,13). Phosphorylation of tuberin by GSK3 β (glycogen synthase kinase 3 β) depends on AMPK-priming phosphorylation of tuberin and triggers its activation to inhibit mTOR. Wnt inhibits the GSK3 β -mediated activation of tuberin to block mTOR (14). Loss of functional tuberin triggers accumulation of the hypoxia-inducible transcription factor (HIF) and upregulation of the expression of HIF-responsive genes including VEGF. The von Hippel–Lindau (VHL) tumor suppressor protein is part of a multiprotein complex involved in ubiquitination and degradation of HIF. mTOR is a key upstream regulator of HIF and the interactive roles of mTOR and VHL for the control of HIF activity are currently under investigation (15–18). Ras is aberrantly activated in tumors deficient for the neurofibromatosis type 1 gene, *NF1*. Ras has many functions in the cell, one of which is to activate the PI3K-TSC1/TSC2-mTOR cascade (19,20). A synergistic role of tuberin and PC1 (encoded by the polycystic kidney disease gene, *PKD1*) was suggested because the cytoplasmic tail of PC1 interacts with tuberin and mTOR, tuberin is required for the proper localization of PC1 and patients with mutations in both, *TSC2* and *PKD1*, have earlier onset and more severe polycystic kidney disease than patients harboring only *PKD1* mutations (21,22). In addition, the tumor necrosis factor- α (TNF- α) activates mTOR through phosphorylation and inactivation of hamartin by the kinase IKK β (23).

These signaling components upstream and downstream of mTOR are frequently altered in a wide variety of human diseases, which has provoked intense interest in mTOR from virtually all major therapeutic areas. mTOR plays an important role in Alzheimer's disease (24–29), cardiac hypertrophy (16,30–32), obesity and type 2 diabetes (4,16,32,33). Ras, PI3K, Akt, Rheb, TNF- α and Wnt are well known to play critical roles in many human cancers (1–5,7,16). Mutations in *TSC1*, *TSC2*, *LKB1*, *PTEN*, *VHL*, *NF1* and *PKD1* trigger the development of the syndromes tuberous sclerosis, Peutz–Jeghers syndrome, Cowden syndrome, Bannayan–Riley–Ruvalcaba syndrome, Lhermitte–Duclos disease, proteus syndrome, VHL disease, Neurofibromatosis type 1 and polycystic kidney disease, respectively (9,12–15,17–23). In addition, the

tuberous sclerosis proteins have been implicated in the development of several sporadic tumors (34–38) and in the control of the cyclin-dependent kinase inhibitor p27, known to be of relevance for several cancers (39–42).

Accordingly, mTOR inhibitors are of great therapeutic interest for many human diseases. Analogs of the mTOR inhibitor rapamycin are approved drugs to prevent the rejection of transplanted organs and to block restenosis after angioplasty. Very recent data suggest that inhibition of mTOR results in clinical benefit in renal cell carcinoma patients and in the treatment of renal tumors and lung cysts found in tuberous sclerosis (1–3,9,43). Rapamycin binds to the protein FKBP12 generating a drug–receptor complex that binds and inhibits mTORC1. Since FKBP12-rapamycin does not bind to mTORC2, rapamycin was thought to only inhibit mTORC1. However, recently it was shown that rapamycin also suppresses the assembly and function of mTORC2 and that this property is important for the effects of this drug (44). Clinical data obtained so far suggest that rapamycin treatment shows promise only against some tumors, which very likely reflects the fact that the mechanism of action of rapamycin against mTORC2 is not yet understood. It is of highest importance to learn more about this mechanism with the aim to allow predictions what kind of tumors could respond to this drug (1–3).

Besides regulation of translation, the mTOR pathway has also been implicated in the control of many other cellular processes, such as e.g. ribosome biogenesis, macro-autophagy or transcription (1–5,7). Accordingly, it is not surprising that several proteins involved in this signaling pathway, including PI3K (45), PDK1 (46), Akt (47–52), PTEN (53,54), tuberin (55–58) or p70S6K and its target S6 (59–61), have been localized to both the cytoplasm and the nucleus. Over 100 different putative Akt substrates have been reported, of which many are known to be exclusively cytoplasmic, whereas others are exclusively nuclear (6). In addition, mTOR has been found to be localized to both the cytoplasm and the nucleus, although the results are somewhat controversial, which very likely is due to the fact that these analyses have been performed in different transformed immortalized cell lines (62–65). Neither the distribution of the other mTORC1 or mTORC2 components, nor the assembly of these complexes has been studied yet with regard to their cytoplasmic and nuclear localization.

In the study presented here, we have established the experimental conditions allowing the analysis of the expression and of the assembly of the mTORC1 and mTORC2 protein components in the cytoplasm and in the nucleus of non-transformed, non-immortalized, diploid human primary fibroblasts. For the first time, we report a cytoplasmic and nuclear study of the mTOR complexes. Using primary human cells, we found different cytoplasmic and nuclear distributions of mTORC1 and mTORC2, which has been confirmed using the immortalized murine fibroblast cell line NIH3T3. Although rapamycin is known to trigger dissociation of both mTOR complexes, the molecular mechanism of its effects on mTORC2 remained elusive. Here we report that long-term, but not short-term, treatment with rapamycin triggers dephosphorylation and cytoplasmic translocation of nuclear rictor and sin1 accompanied by inhibition of mTORC2 assembly.

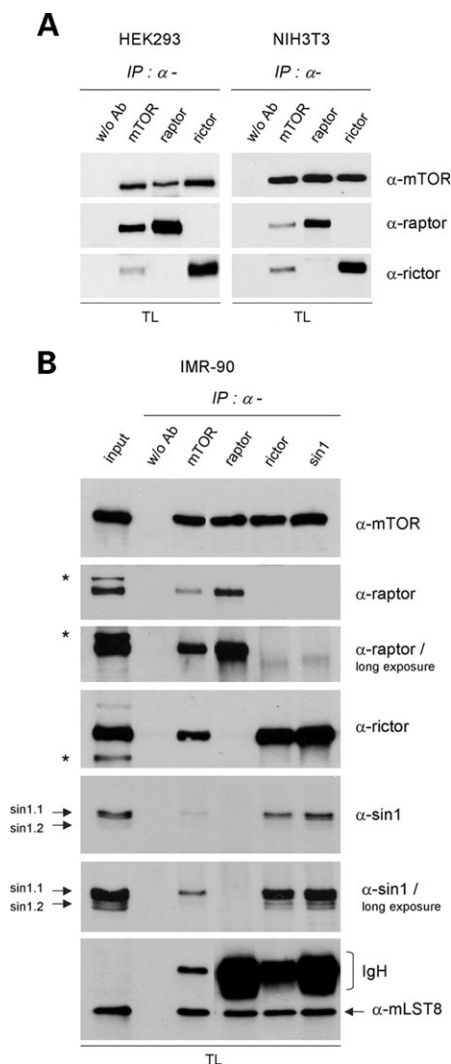


Figure 1. Detection of mTORC1 and mTORC2 complexes in non-transformed non-immortalized primary IMR-90 fibroblasts. (A) Total lysates of HEK293 and NIH3T3 cells were prepared in buffer containing 0.3% CHAPS. Endogenous mTOR, raptor and rictor were immunoprecipitated as indicated. Precipitates were analyzed for protein levels of mTOR, raptor and rictor by immunoblotting. (B) Experiments as described above were performed in non-immortalized human IMR-90 fibroblasts harboring a normal diploid 46,XX karyotype. mTOR, rictor, raptor and sin1 immunoprecipitates were analyzed for mTOR, raptor, rictor, sin1 and mLST8 protein levels as indicated. Non-specific bands are indicated with an asterisk.

RESULTS

Identification of a non-transformed non-immortalized diploid primary human cell system to study mTORC1 and mTORC2

A wide variety of upstream regulators of mTOR are deregulated in many different types of diseases and tumors (see Introduction). Accordingly, it is not surprising that the regulation of mTOR has been reported to vary depending on the cancer cell type or transformed cell line used in the different studies (44,62–65). Another problem of many mTOR studies is that they have been performed with ectopically over-expressed components of the mTOR pathway or using *in vitro*

assay systems. Cells, which have already been shown to allow the analysis of endogenous mTORC1 and mTORC2 complexes, are either transformed or immortalized, such as, e.g. HEK293 cells (adenovirus transformed human embryonic kidney) or NIH3T3 cells (immortalized mouse embryonic fibroblasts) (Fig. 1A). It was the aim of this study to first identify a non-transformed, non-immortalized human cell system, which can be used to investigate endogenous proteins involved in the regulation of mTOR. In addition, it should preferentially be a system that is commercially available to all colleagues in the field and which can easily be cultivated with reasonable proliferation rates. In an extensive search, we identified IMR-90 cells to fulfill all these criteria. IMR-90 cells are non-transformed, non-immortalized human diploid fibroblasts (HDFs), which can be obtained from the American Type Culture Collection (ATCC #CCL-186). They are derived from normal fetal lung tissue and harbor finite lifetime being capable of attaining 58 population doublings before the onset of senescence. We obtained IMR-90 cells at passage number 10 (population doubling 26). In the course of this study, we cultivated them for eight or less additional passages (≤ 47 total population doublings). IMR-90 cells have a normal diploid karyotype 46,XX and we regularly proved for genome stability by standard karyotyping in our laboratory (data not shown). Most importantly, we could demonstrate that using IMR-90 cells endogenous mTOR, raptor, rictor and sin1 can be immunoprecipitated to reasonable levels (Fig. 1B; compare also with the levels obtained with HEK293 and NIH3T3 cells, Fig. 1A). Using this approach, we could confirm interaction of endogenous mTOR with raptor, rictor, sin1 and mLST8. In addition, even on long exposures we neither detected raptor co-immunoprecipitated with rictor or sin1, nor rictor or sin1 on raptor precipitates demonstrating the specificity of the immunoprecipitations (Fig. 1B). Here it is important to note that all presented data on co-immunoprecipitated proteins have been generated from one single immunoprecipitation of the indicated protein. For example, analyses of mTOR, raptor, rictor, sin1 and mLST8 have been performed using one anti-mTOR precipitate (Fig. 1B, lane 3). In summary, these data demonstrate that IMR-90 fibroblasts are non-transformed non-immortalized diploid primary human cells well suitable to investigate the regulation of the mTOR pathway studying endogenous proteins.

Establishment of a protocol allowing analysis of cytoplasmic and nuclear mTOR complexes

mTOR has been found to be localized to both the cytoplasm and the nucleus. However, the results are somewhat controversial very likely because these analyses have been performed in different transformed or immortalized cell lines (62–65). Neither the distribution of raptor, rictor, sin1 or mLST8, nor the assembly of the mTOR complexes has been studied yet with regard to their cytoplasmic and nuclear localization. In many of the studies investigating the cytoplasmic/nuclear localization of other components of the insulating signaling pathway, such as PI3K, PDK1, Akt, PTEN, tuberlin or p70S6K and its target S6, a commonly performed fractionation protocol has been used (45–65). In this protocol, the outer cell membrane is disrupted in a buffer containing NP-40 to obtain the cytoplasmic fraction. Afterwards nuclear

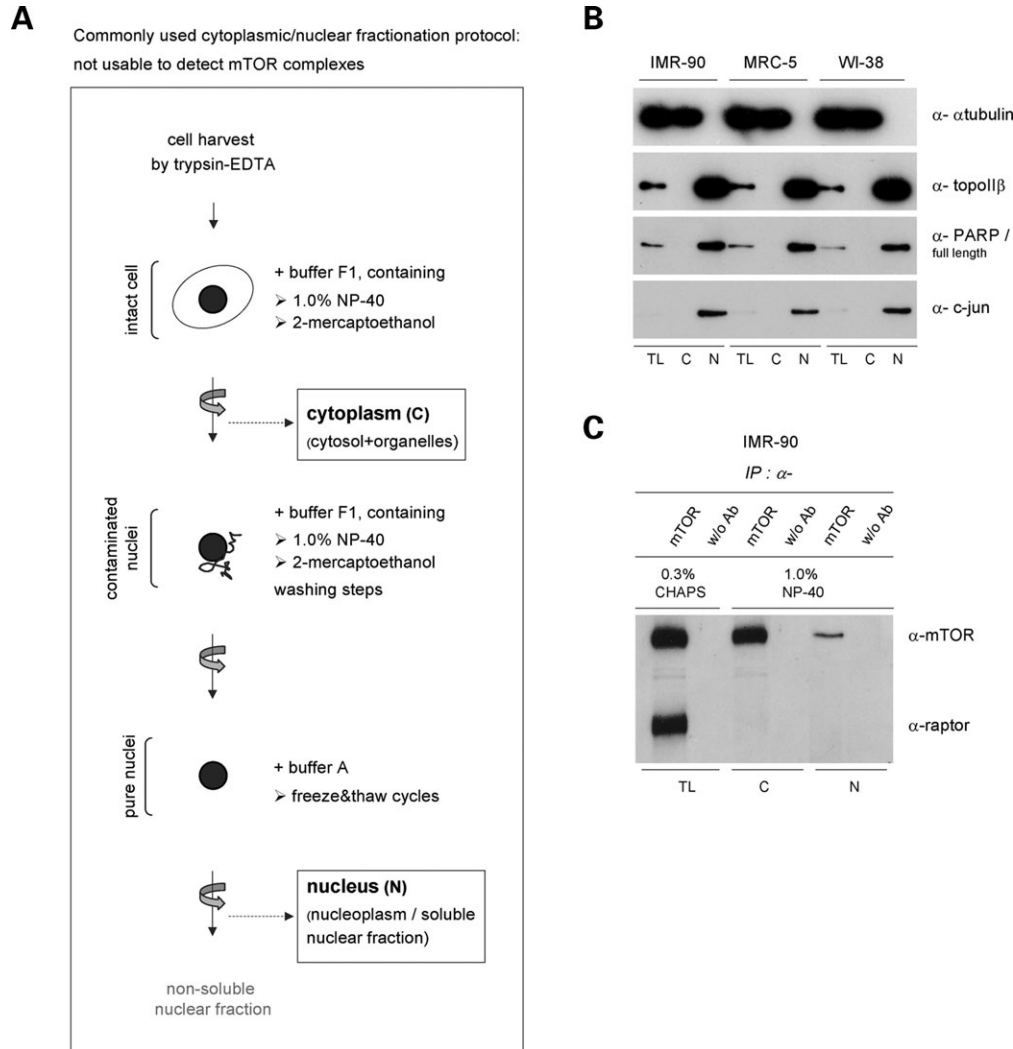


Figure 2. The commonly used fractionation protocol is not usable to detect cytoplasmic and nuclear mTORC1 complexes (**A**) Schematic presentation of the stepwise isolation of cytoplasmic and nuclear proteins involving the commonly used, non-ionic detergent NP-40. Briefly, extracts are prepared by first disrupting the outer cell membrane to obtain the cytoplasmic fraction (C) including cytosol and organelles and then extracting proteins from the purified nuclei containing the soluble nuclear fraction (N). NP-40 is used at a final concentration of 1% (v/v) and solubilizes proteins of the outer cell membrane to release cytoplasmic proteins without affecting the integrity of crude nuclei which are then extracted by physical disruption of the nuclear membrane using freeze&thaw cycles. For a detailed description of this protocol, compare protocol #1a (p #1a) in Materials and Methods. (**B**) Non-transformed, non-immortalized human fibroblasts of different origin were separated into cytoplasmic and nuclear fractions as described in (A). Purity of fractions was proven by analyzing cytoplasmic and nuclear marker proteins including α -tubulin (cytoplasmic) and topoisomerase II β , PARP and c-jun (nuclear). (**C**) Cytoplasmic and nuclear fractions of IMR-90 cells were separated as described in (A and B). So obtained lysates were used for the immunoprecipitation of endogenous mTOR. mTOR and co-immunoprecipitated raptor were analyzed via immunoblotting of precipitates.

proteins are extracted upon disruption of the nuclear membrane via freeze&thaw cycles (Fig. 2A). Studying the distribution of cytoplasmic (α -tubulin) and nuclear (topoisomerase II β , PARP and c-jun) marker proteins revealed that this protocol enables separation of cytoplasmic and nuclear extracts to high purity also in primary human cells (Fig. 2B). However, immunoprecipitation approaches demonstrated that this approach cannot be used to study endogenous mTOR complex assembly. For example, although the interaction of endogenous mTOR with raptor can be visualized with high efficiency in total lysates of IMR-90 cells (Figs 1B and 2C), this interaction can be detected neither in the cytoplasmic nor in the nuclear fractions generated using this commonly used approach

(Fig. 2C). Since fractionation protocols using NP-40 are widely used, we found it of highest importance to inform the colleagues in the field that they do not allow the investigation of cytoplasmic and nuclear mTOR complex formation.

It was a major aim of this project to analyze cytoplasmic and nuclear mTOR regulations. Accordingly, we next established a protocol allowing the detection of mTOR complex assembly in the cytoplasm and in the nucleus. First, we compared different protocols involving either the detergent NP-40 (p #1a, described in Fig. 2) or hypo/hypertonic buffers (p #2) or a combination of both (p #3) (for details of the different protocols, see Materials and Methods). Analyzing cytoplasmic and nuclear marker proteins revealed that the level of purity

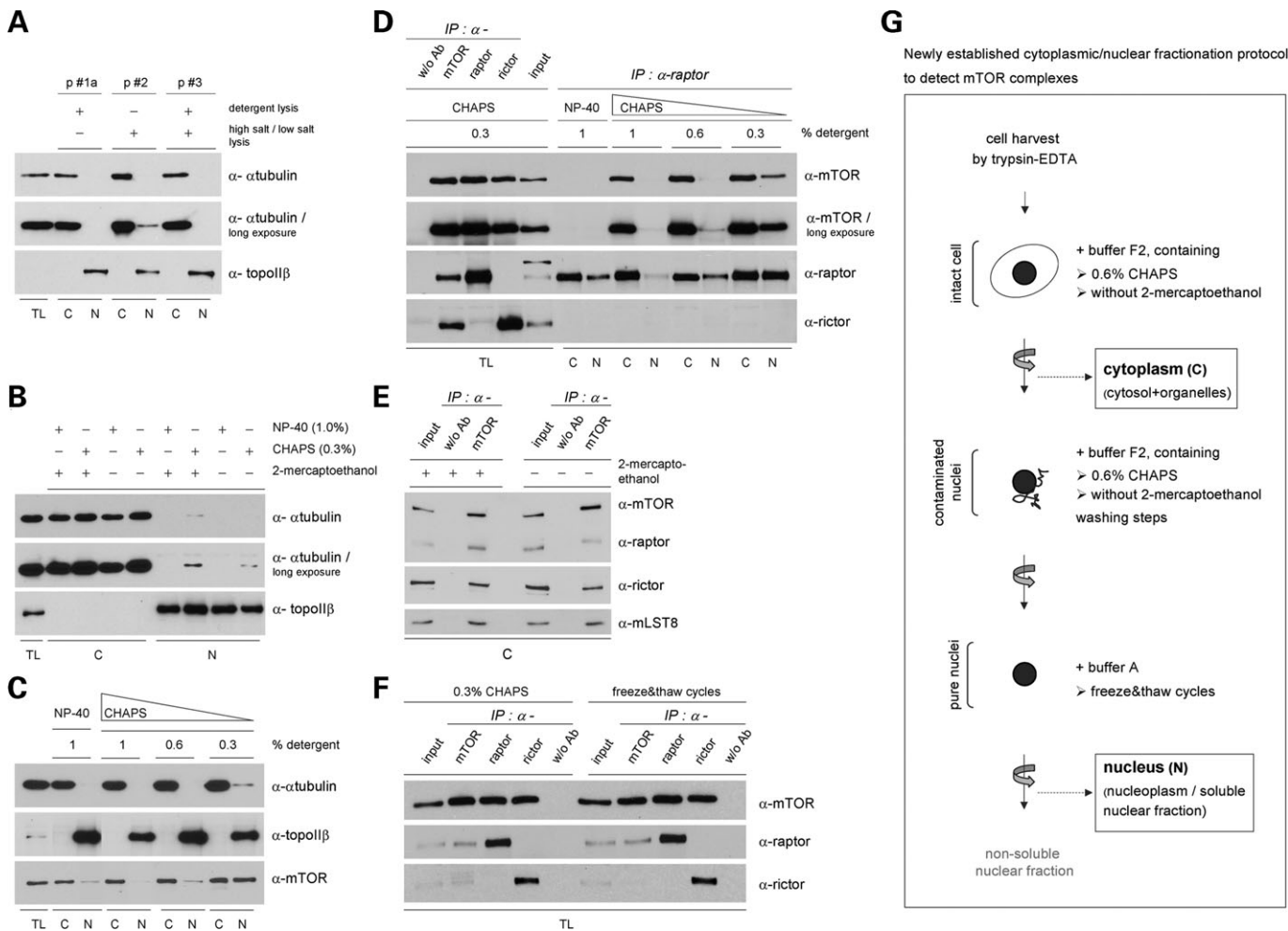


Figure 3. Establishment and verification of a protocol allowing the detection of cytoplasmic and nuclear assembly of mTOR complexes. (A) Cytoplasmic and nuclear fractions of logarithmically growing IMR-90 cells were separated according to three different protocols involving either the use of a detergent (p #1a, also compare Fig. 2) or the complementary use of hypo/hypertonic buffers (p #2) or a combination of both (p #3). Extracts were analyzed for cytoplasmic and nuclear cross-contamination with antibodies against indicated proteins via immunoblotting. Total lysates of the same pool of cells were co-analyzed as a control. For a detailed description of the used experimental procedures, compare Materials and Methods and references therein. (B) Cells grown as in (A) were basically fractionated according to protocol #1a (p #1a) except that NP-40 was replaced by CHAPS in the presence or absence of 2-mercaptoethanol as indicated. Purity of fractions was assessed by analyzing α -tubulin and topoII β via western blotting. Again total lysates were co-analyzed as a control. (C) IMR-90 fibroblasts were separated into cytoplasmic and nuclear fractions as described in (B) using varying amounts of detergents as indicated. To assess the purity of so obtained fractions extracts were analyzed for indicated proteins. mTOR protein levels were co-analyzed as a control. (D) Experiments were performed as in (C) with subsequent immunoprecipitation of endogenous mTOR, raptor and rictor from total lysates or of endogenous raptor from cytoplasmic and nuclear fractions. Precipitates were analyzed for co-immunoprecipitated mTOR, raptor and rictor. (E) Cells as described above were fractionated using CHAPS at a final concentration of 0.6% in the presence or absence of 2-mercaptoethanol. Cytoplasmic fractions were subjected to immunoprecipitations of endogenous mTOR and precipitates were analyzed for the level of indicated proteins. (F) Total lysates from logarithmically growing IMR-90 cells were extracted as indicated and immunoprecipitation of endogenous mTOR, raptor and rictor were performed. Precipitates were analyzed for the levels of co-immunoprecipitated proteins as indicated. (G) Schematic presentation of the newly established fractionation protocol allowing the detection of the mTOR complexes in the cytoplasm and nucleus. For a detailed description, compare Materials and Methods.

significantly decreased when high-salt/low-salt lysis is used instead of detergent, primarily detected via α -tubulin protein analysis demonstrating cytoplasmic contamination of the nuclear fraction (Fig. 3A). Ionic detergents are strong solubilizing agents tending to denature proteins, thereby destroying protein activity, function and interactions. Non-ionic and zwitterionic detergents are known to be good for gentle extraction. This is especially true for CHAPS because of its fair solubilization and weak denaturation potential. Accordingly, 0.3% CHAPS is often used as the detergent to generate total protein extracts to analyze mTOR complexes (although in another

buffer and together with high speed centrifugation). We next tested whether in the commonly used fractionation protocol p #1a (compare Fig. 2A), the 1.0% NP-40 can be replaced by 0.3% CHAPS. Studying α -tubulin protein levels demonstrated that this replacement is also accompanied by cytoplasmic contamination of the nuclear fractions (Fig. 3B). In a next step, we modified the percentage of CHAPS for the disruption of the outer cell membrane to identify a concentration allowing pure cytoplasmic/nuclear fractionation and the detection of mTOR complex assembly in the cytoplasm and in the nucleus. Studying α -tubulin and topoisomerase II β protein

levels as cytoplasmic and nuclear markers and immunoprecipitation experiments demonstrated that 0.6% CHAPS is the optimal concentration allowing to study the assembly of mTOR complexes in the cytoplasm and in the nucleus of human primary IMR-90 cells (Fig. 3C, D). 2-mercaptoethanol is a strong reductant reducing disulfide bonds holding the proteins structure together. In the course of the optimization of the extraction protocol, we tested whether omitting 2-mercaptoethanol has any effect on the purity of the cytoplasmic and nuclear extracts or on the efficiency of the detection of mTOR complex assembly. Since we could not detect any effect, neither a positive nor a negative one on the complex formation and we found 2-mercaptoethanol to increase cytoplasmic contamination in the nuclear fraction (Fig. 3B and E), we decided to omit it with the hope to avoid any probably forthcoming negative effects of this reductant in protein interaction studies. In a last step, we wanted to ensure that the buffer A together with the freeze&thaw cycles, which we use in our fractionation protocol to disrupt nuclear membranes, has no negative effects on the mTOR complex assembly measured via immunoprecipitation. Total lysates extracted with 0.3% CHAPS have been compared with total lysates obtained via freeze&thaw cycles in buffer A (without prior cytoplasmic and nuclear fractionation) with regard to mTORC1/2 assembly. Immunoprecipitations demonstrated that the results with 0.3% CHAPS and with buffer A are identical (Fig. 3F). In summary, to our knowledge, we have established the first protocol to analyze cytoplasmic and nuclear mTOR complex regulations in primary human cells (Fig. 3G).

Cytoplasmic and nuclear distribution of mTORC1 and mTORC2

Using this newly established fractionation protocol, we then started to analyze the cytoplasmic and nuclear distribution of mTOR components in non-transformed, non-immortalized primary human IMR-90 fibroblasts. Western blot analyses and immunoprecipitation approaches revealed that all components of mTORC1 and mTORC2 can be detected in both the cytoplasm and the nucleus. The protein levels of mTOR, mLST8, rictor and sin1 were significantly higher in the cytoplasm compared with the nucleus. Only the mTORC1-specific protein raptor is high abundant in both compartments (Figs 4A–C). Although the levels of all mTORC2 proteins (mTOR, mLST8, rictor and sin1) were clearly lower in the nucleus, the interaction of mTORC2 proteins was abundantly detectable in both compartments using different approaches to immunoprecipitate mTOR, rictor or sin1 (Figs 4A–C). On the other hand, despite of high levels of nuclear raptor, only very low amounts of mTOR are detectable on nuclear raptor precipitates. The fact that raptor can efficiently be immunoprecipitated in both compartments suggests a lower affinity of raptor to mTOR in the nucleus. This concept of a putative lower affinity is further supported by the detection of only very low amounts of raptor on mTOR precipitates of nuclear IMR-90 extracts. Still, long exposures of these nuclear precipitation experiments demonstrate that mTORC1 assembly also exists in the nucleus, although at very low levels (Figs 3D and 4A–C). All these data on the distribution and on the assembly of mTOR complex

proteins in the cytoplasm and in the nucleus of primary human cells were confirmed analyzing the immortalized murine cell line NIH3T3 (Fig. 4D–F). To our knowledge, this is the first analysis of the cytoplasmic/nuclear distribution of mTOR complex components. In summary, we provide evidence that mTOR, mLST8, rictor and sin1 are less abundant in the nucleus than in the cytoplasm, whereas raptor is also more abundant in the nucleus. Whereas the mTOR/rictor complex is abundant in both compartments, our finding that mTORC1 assembly occurs predominantly in the cytoplasm, suggests a lower affinity between mTOR and raptor in the nucleus.

Comparison of short-term and long-term rapamycin treatment on the assembly of mTOR complexes and on the cell cycle

Rapamycin bound to the protein FKBP12 generates a drug-receptor complex that binds and inhibits mTORC1 (1–7). Based on the knowledge that deregulation of mTOR is a hallmark of many human cancers and genetic diseases, rapamycin and its analogs became approved drugs and are currently in many different clinical trials (see Introduction). Since FKBP12-rapamycin does not bind to preformed mTORC2, rapamycin was originally thought to only inhibit mTORC1. However, recently it was shown that it also suppresses the assembly and function of mTORC2 through a yet unknown mechanism and that this property is important for the effects of this drug. It has been discussed that binding of FKBP12-rapamycin to free mTOR could prevent the subsequent binding of rictor, what could be an explanation for the observation that whereas the negative effects of rapamycin on mTORC1 are already visible after short-term rapamycin treatment, mTORC2 assembly is only affected upon prolonged drug treatment. Importantly, in addition, analysing many different cancer cell lines and other immortalized cells the effects of rapamycin on mTORC2 have been shown to depend on the studied cell system (44). Accordingly, we wanted to investigate the effects of rapamycin on mTORC1 and mTORC2 assembly in non-transformed non-immortalized primary human cells. The levels of raptor on immunoprecipitated mTOR decreased already after 1 h drug treatment, whereas the mTOR-associated rictor levels remained unaffected (Fig. 5A). To prove full functionality of rapamycin against mTORC1 already after 1 h incubation, we confirmed the well-known inhibition of the mTORC1 target p70S6K (represented by its phosphorylation on T389) (Figs 5A and 6A). In agreement with the report described above, we found prolonged, but not short-term, incubation with rapamycin to also decrease mTORC2 assembly in primary human cells (Figs 5A and 6B). Besides its many different functions and regulations, mTOR is also known to be involved in cell cycle control (1–7). To get more insights into the difference between short and long-term rapamycin effects, we performed cytofluorometric cell cycle analysis. Logarithmically growing primary IMR-90 cells harbor a cell cycle distribution of over 60% G0/G1 cells and over 30% S phase. Neither the DNA distribution analysis nor the forward scatter cell size study revealed any effect of 1 h rapamycin treatment on the cell cycle (Fig. 5B).

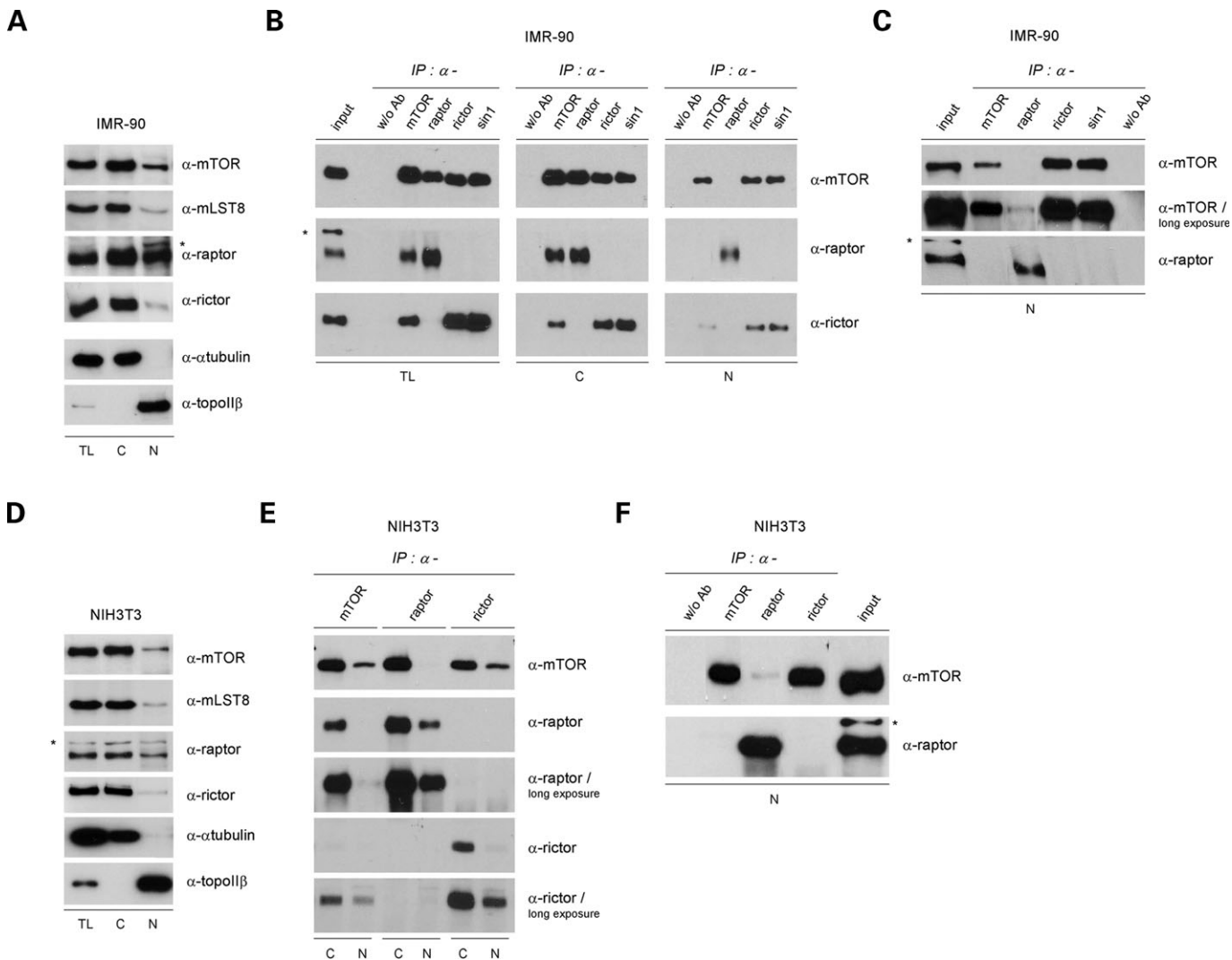


Figure 4. Cytoplasmic and nuclear distribution of mTORC1 and mTORC2 complexes in non-transformed non-immortalized primary IMR-90 fibroblasts. (A) Logarithmically growing IMR-90 fibroblasts were separated into cytoplasmic and nuclear fractions and analyzed for the distribution of indicated proteins via immunoblotting. In addition, total lysates were prepared and co-analyzed as a control. (B) Subcellular fractionation of cells from the same pool was performed in CHAPS-containing fractionation buffer F2 (compare protocol #1b) and equal amounts of so obtained extracts were used for the immunoprecipitation of endogenous mTOR, raptor, rictor and sin1. Precipitates were analyzed for levels of indicated proteins via immunoblotting. Again, total lysate of the same pool of cells was co-analyzed as a control. (C) To allow a better visualization of the nuclear mTORC1 complex, twice the amount of nuclear extracts used in (B) was re-subjected to immunoprecipitation with indicated antibodies. Raptor and co-immunoprecipitated mTOR were analyzed via immunoblotting. (D–F) Experiments were performed as described in (A–C) except that NIH3T3 mouse embryonic fibroblasts were used.

In contrast, prolonged incubation triggered a significant increase of G0/G1 cells up to over 80% accompanied by a decrease of S phase cells under 14%. This rapamycin-induced cell cycle arrest was confirmed by the detection of an increase in the amount of the smaller G0/G1 cells detected via forward scatter analysis (Fig. 5B). Since, in this experiment, the effects of mTORC2 assembly were only detectable upon 24 h rapamycin treatment accompanied by a cell cycle arrest, it was interesting to study whether another cell cycle blocking approach also affects mTORC2. Serum deprivation of IMR-90 cells triggers a cell cycle arrest in G0/G1. Although this cell cycle arrest was also accompanied by a slight downregulation of mTORC2 assembly, these effects were not as pronounced as in 24 h rapamycin-treated cells (Fig. 7).

In summary, we found that in primary human cells short-term treatment with rapamycin already affects the assembly of mTORC1 independently of rapamycin's potential to arrest the cell cycle, which can only be visualized upon prolonged drug treatment. mTORC2 assembly remains unaffected upon 1 h drug treatment and decreases upon long-term incubation.

Different effects of rapamycin on cytoplasmic and nuclear rictor and sin1

In the experiments described above, we found rapamycin to negatively affect the assembly of both mTORC1 and mTORC2, although with different kinetics. The molecular mechanism how rapamycin inhibits mTORC1 is fully

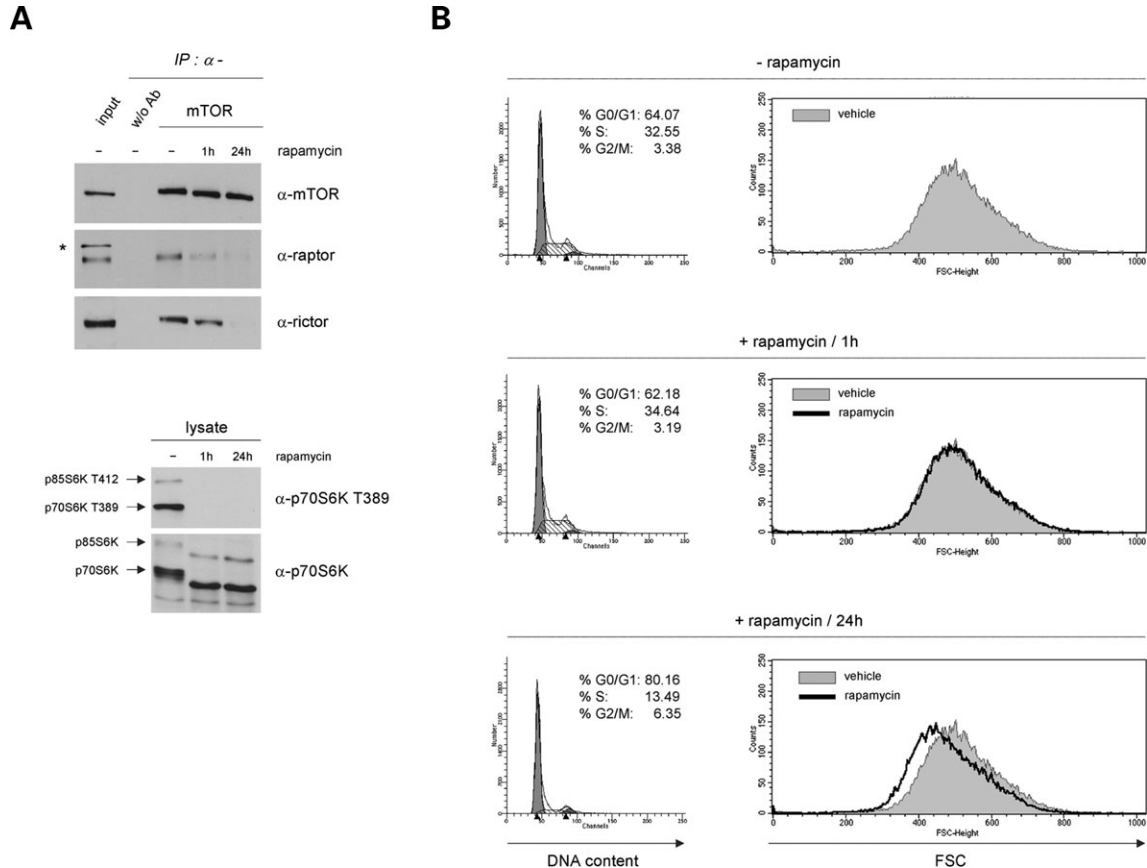


Figure 5. Effects of rapamycin on mTORC1 and mTORC2 complexes in non-transformed non-immortalized primary IMR-90 fibroblasts. (A) Logarithmically growing IMR-90 cells were treated with rapamycin at a final concentration of 100 nM for the indicated times and total lysates were prepared in buffer containing 0.3% CHAPS. The assembly of mTOR into mTORC1 and mTORC2 was studied by immunoprecipitating endogenous mTOR which was then analyzed for co-immunoprecipitated raptor and rictor via immunoblotting. The same extracts were used to analyze the phosphorylation status of p70S6K at T389 as well as the protein level of p70S6K via western blotting. (B) IMR-90 fibroblasts derived from the pool of cells used in (A) were stained with propidium iodide to compare the DNA distribution of untreated versus rapamycin treated cells. In addition, cell size was investigated via forward scatter analyses.

characterized and well known (1–7,44). On the other hand, the mechanism of its effects on mTORC2 assembly remained elusive. mTORC2 consists of mTOR, mLST8, rictor and sin1, of which the latter two are specific for mTORC2. mLST8 has been demonstrated to bind mTOR constitutively. Originally, it was suggested that mLST8 regulates mTOR kinase activity. However, more recent data provide evidence that mLST8 is dispensable for mTOR function (2,66). In humans, sin1 has five different protein isoforms, generated by alternative splicing. Three isoforms, sin1.1, sin1.2 and sin1.5, assemble into mTORC2 to generate distinct complexes. However, only sin1.1 and sin1.2 are expressed ubiquitously and only mTORC2/sin1.1 and mTORC2/sin1.2 are regulated by insulin. Both, sin1 and rictor are phosphorylated proteins and the phosphorylated and unphosphorylated versions can be distinguished via their different electrophoretic mobilities. Phosphatase treatment approaches demonstrated that the slower migrating forms represent the hyperphosphorylated proteins. Furthermore, it has been demonstrated that hypophosphorylated rictor and sin1 have lower affinity to mTOR than the phosphorylated proteins (67–69). We have confirmed these data in IMR-90 cells by showing that on mTOR precipitates we only detect the slower migrating sin1, whereas a

sin1-specific immunoprecipitation using the same cell extract allows detection of slower and faster migrating sin1 proteins (Fig. 1B). Interestingly, we also found hyper- and hypophosphorylated sin1 on rictor precipitates demonstrating that rictor is also bound to unphosphorylated sin1 (Fig. 1B). Since we observed that rapamycin affects the assembly of rictor and sin1 with mTOR in primary human IMR-90 cells (Figs 5A, 6B and 7A) and rapamycin was already suggested to influence rictor phosphorylation (70), it was of interest to investigate the effects of this drug on the phosphorylation status of rictor and sin1 in IMR-90 cells. Whereas the electrophoretic mobility of raptor remained unchanged, we found a pronounced shift to the faster migrating unphosphorylated forms of rictor and sin1.1 upon 24 h rapamycin incubation (Figs 6A and B, 7B and 8A). Although short-term rapamycin treatment neither affects mTORC2 assembly nor the cell cycle (Fig. 5A and B), we observed that, although to a very low level, faster migrating forms of rictor and sin1.1 already appeared after 1 h drug incubation (Figs 6A and B and 8A). These observations warrant further investigation of the question whether phosphorylation of rictor and sin1 must be negatively affected to allow rapamycin to trigger downregulation of mTORC2 assembly.

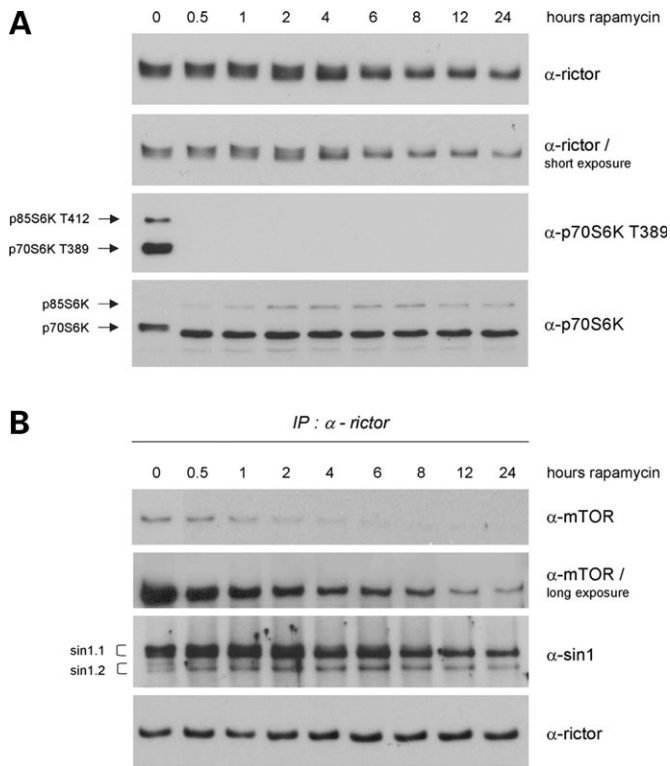


Figure 6. Time course analysis of rapamycin effects on mTORC2. (A) Logarithmically growing IMR-90 cells were treated with rapamycin at a final concentration of 100 nM for the indicated times and total lysates were prepared in buffer containing 0.3% CHAPS. Rictor and the phosphorylation status of p70S6K at T389 as well as the protein level of p70S6K were studied via western blotting. (B) mTORC2 assembly was studied by immunoprecipitation of rictor in IMR-90 fibroblasts derived from the pool of cells used in (A).

Since we have observed mTORC2 assembly to be abundant in both the cytoplasm and the nucleus (Fig. 4), we next investigated the effects of rapamycin on cytoplasmic and nuclear proteins. In agreement with the data obtained with total lysates, the electrophoretic mobility of raptor (and also of mLST8) remained unaffected by rapamycin in the cytoplasm and in the nucleus. Surprisingly, we found rapamycin to differently affect the phosphorylation status of cytoplasmic and nuclear rictor and sin1.1. In the cytoplasm, both proteins start to become unphosphorylated already after 1 h rapamycin treatment, whereas the electrophoretic mobility of the nuclear proteins remains unaffected. After prolonged rapamycin incubation, nuclear rictor and sin1.1 also exhibited altered phosphorylation status (Fig. 8B). Upon 24 h drug treatment, we also made another unexpected observation: nuclear rictor and sin 1.1 protein levels decreased accompanied by a pronounced increase of their protein levels in the cytoplasm. This translocation from the nucleus to the cytoplasm is specific for rictor and sin1.1 and could not be detected for mTOR, raptor, sin1.2 or mLST8 (Fig. 8B).

The next step was to compare the effects of rapamycin on the mTORC2 proteins with its influence on mTORC2 assembly. In agreement with the data obtained with rictor detection on mTOR-specific immunoprecipitates (Fig. 5A), we found a pronounced decrease of mTOR on rictor precipitates upon

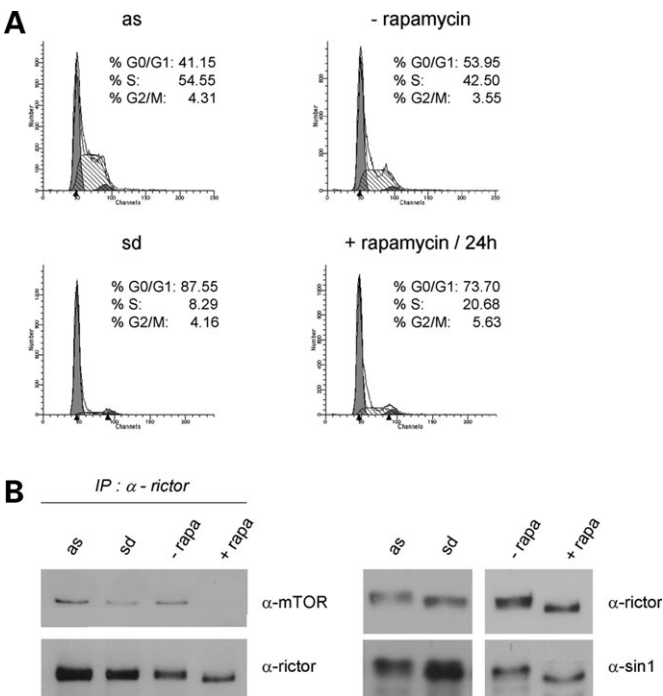


Figure 7. Effects of serum deprivation on mTORC2 assembly. (A) Logarithmically growing IMR-90 cells (as, asynchron) were serum deprived for 48 h in medium containing 0.2% serum (sd, serum deprived) or treated with rapamycin (+ rapamycin). Cell cycle analyses were performed on a flow cytometer. For the rapamycin experiment, the control cells were treated with DMSO alone for 24 h (-rapamycin). (B) Cells treated as described in (A) were analyzed for mTORC2 assembly via anti-rictor immunoprecipitations and for the gel migration properties of rictor and sin1 via western blotting.

24 h rapamycin treatment. However, the amount of sin1 bound to rictor remained unchanged (Figs 6B and 9A). These findings provide evidence that mTORC2 assembly is negatively regulated by rapamycin, although the interaction of rictor and sin1 is not affected. Accordingly, in rapamycin-treated cells, a rictor/sin1 complex exists, which is not bound to mTOR. Fractionation experiments further revealed that this 'free' rictor/sin1 complex almost exclusively exists in the cytoplasm of long-term drug-treated primary human IMR-90 cells (even to a slightly higher amount as in untreated cells). In the cytoplasm of drug-treated cells, this 'free' complex consists of the unphosphorylated faster migrating rictor and sin1 proteins (Fig. 9B).

In summary, we show that rapamycin differently regulates cytoplasmic and nuclear mTORC2 in non-transformed non-immortalized primary human fibroblasts. Short-term drug treatment does neither affect mTORC2 assembly nor the cell cycle control and only a slight dephosphorylation of rictor and sin 1.1, which specifically occurs in the cytoplasm, can be observed. Upon prolonged incubation rapamycin causes cell cycle arrest, accompanied by dephosphorylation of rictor and sin1.1 and by translocation of these two proteins from the nucleus to the cytoplasm. We provided evidence that long-term rapamycin treatment downregulates mTORC2 assembly via inhibition of the interaction between a cytoplasmic complex of unphosphorylated rictor and sin1 with mTOR.

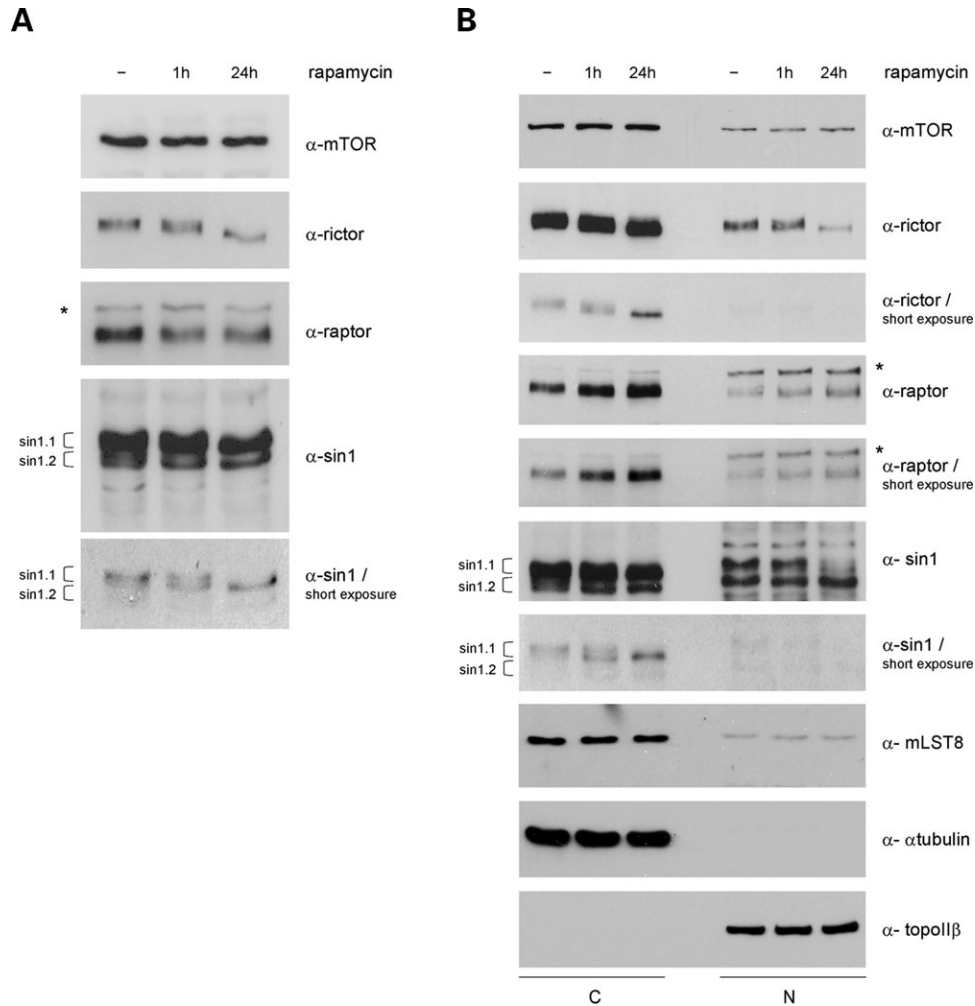


Figure 8. Rapamycin triggers delocalization and dephosphorylation of rictor and sin1. (A) IMR-90 fibroblasts were treated with 100 nM rapamycin for the indicated time points. Total extracts were prepared and subjected to SDS-PAGE. mTOR, rictor, raptor, sin1 and mLST8 were analyzed via western blotting. (B) Cytoplasmic and nuclear extracts from the same pool of cells used in (A) were prepared in CHAPS-containing lysis buffer. Equal amounts of so obtained fractions were separated by SDS-PAGE and immunodetection with indicated antibodies was performed.

DISCUSSION

This study provides two blocks of new data on the regulation of TOR in mammalian cells. For the first time, we describe the cytoplasmic and nuclear distribution of endogenous mTORC1 and mTORC2. In addition, new important insights into the effects of rapamycin on mTORC2 regulation are presented. For both aspects as well as for all forthcoming studies on mTOR, we felt it to be essential to identify an optimal biological cell system and to establish a biochemical protocol allowing the investigation of cytoplasmic and nuclear mTOR complexes. Varying results on mTOR regulation have been reported in many different immortalized or transformed cell lines, which is mainly due to the fact that a wide variety of upstream regulators of mTOR are deregulated in many different types of diseases and tumors or that the results were obtained from ectopic overexpression experiments or using *in vitro* kinase assays (9,12–15,17–42,44,62–65). In the course of this study, we identified IMR-90 cells to represent an optimal biological system for mTOR studies fulfilling the

following criterias. (1) They are of human origin. (2) They are diploid harboring a stable normal 46,XX karyotype. (3) They are primary cells which are not transformed. (4) They are not immortalized but capable of attaining 58 population doublings before the onset of senescence. (5) They can easily be cultivated with reasonable proliferation rates. Logarithmically growing IMR-90 cells harbor a cell cycle distribution of over 30% S phase cells. (6) They are commercially available to all colleagues from the ATCC (#CCL-186). (7) They abundantly express all mTOR complex components, including mTOR, mLST8, raptor, rictor and sin1. Endogenous proteins and endogenous mTOR complex assembly can reliably be analyzed using commonly used antibodies via western blotting and immunoprecipitation in total lysates, as well as in cytoplasmic and nuclear fractions.

In the study presented here, we established a protocol which allows cytoplasmic/nuclear fractionation to high purity and enables the analysis of endogenous mTOR complexes in the cytoplasm and in the nucleus of IMR-90 cells, but also, e.g. in NIH3T3 cells (for a schematic presentation of this protocol,

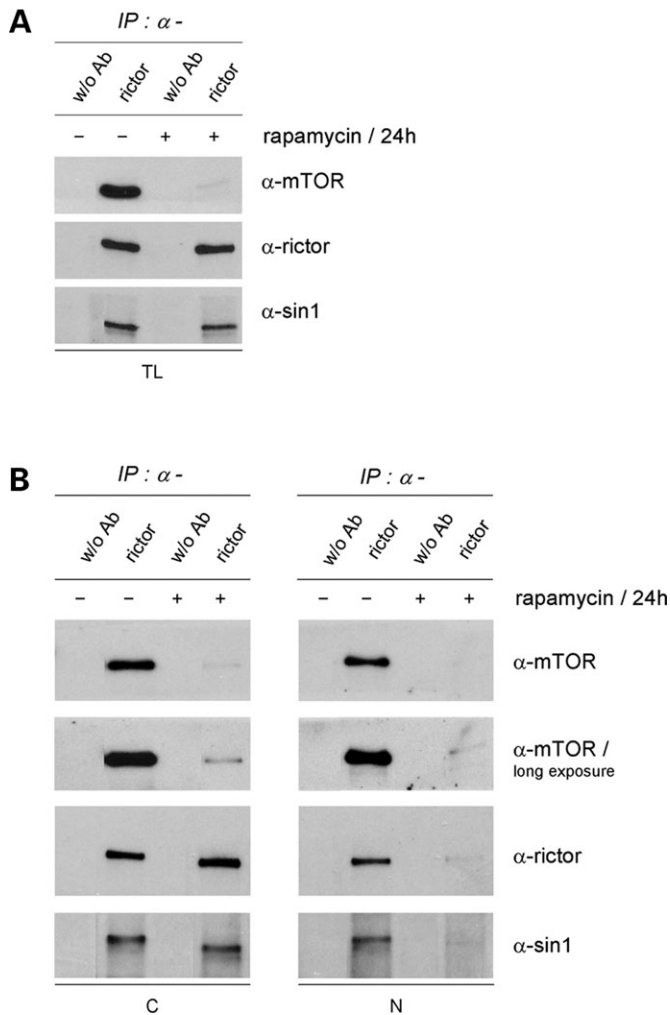


Figure 9. Rapamycin triggers dissociation of mTORC2 complexes in the cytoplasm and nucleus. (A) Logarithmically growing IMR-90 cells were treated with 100 nM rapamycin for 24 h and total lysates were prepared. Immunoprecipitations with anti-riCTOR antibody were performed and analyzed for co-immunoprecipitated mTOR and sin1 via immunoblotting. Protein levels of immunoprecipitated rictor were co-analyzed as controls. (B) Experiments were performed as described in (A) except that cytoplasmic and nuclear fractions were prepared using buffer F2 in the newly established protocol p #1b and analyzed for cytoplasmic and nuclear mTORC1 and mTORC2 assembly.

see Fig. 3G). To our knowledge, we here report the first analysis of the cytoplasmic and nuclear distribution of mTORC1/2. We found mTOR, mLST8, rictor and sin1 to be less abundant in the nucleus than in the cytoplasm. However, different approaches to immunoprecipitate cytoplasmic and nuclear mTOR, rictor and sin1 followed by immunodetection of co-precipitated mTORC2 components revealed that mTORC2 assembly is abundant in both cell compartments. On the other hand, despite high protein levels of raptor in both the cytoplasm and the nucleus, mTORC1 complex assembly predominantly occurs in the cytoplasm. Taken together, our data presented here provide evidence that nuclear raptor has lower affinity to mTOR than cytoplasmic raptor. For the first time, our results make it obvious that upstream of mTORC1 the cytoplasmic and the nuclear mTOR pathway regulation represent significant differences. This newly discovered

difference in the regulation of cytoplasmic and nuclear mTORC1 is of high importance for two reasons. (1) Many upstream regulators of mTOR have already been demonstrated to be expressed in both compartments. IRS1, PI3K, PDK1 and Akt function at the plasma membrane. In agreement with its role in the regulation of translation, all the other components of the mTOR pathway including, e.g. tuberlin, Rheb, mTOR and p70S6K, have also been localized to the cytoplasm. Besides regulation of translation, mTOR has also been implicated in the regulation of ribosome biogenesis, macroautophagy or transcription (1–7). Accordingly, it is not surprising that proteins involved in this pathway are also localized within the nucleus. PI3K has been shown to be nuclear (45), PDK1 is a nucleo-cytoplasmic shuttling protein (46) and Akt also translocates to the nucleus (47–52). Several recent reports have brought conclusive evidence that the tumor suppressor PTEN, once considered as a strictly cytoplasmic protein, also shuttles to the nuclear compartment, where it joins the components PI3K, PDK1 and Akt (53,54). Tuberlin has also been reported to be cytoplasmic and nuclear (55–58). In the future, a detailed comparison of mTORC1 upstream regulations in the cytoplasm and in the nucleus will reveal important new insights into the mTOR pathway. (2) One major substrate of mTORC1 is p70S6K, which has also been found to be localized to both the cytoplasm and the nucleus. The p70S6K target S6 is dispersed throughout the cytoplasm. Within the nucleus, S6 protein is concentrated to the nucleoli and almost absent from the nucleoplasm, which is a consequence of the fact that eukaryotic ribosomes are assembled in the nucleolus before export to the cytoplasm (59–61). p70S6K also blocks IRS1, which activates PDK1. Via this feedback loop, Akt is also under downstream regulation of mTORC1. Over 100 different Akt substrates have already been reported, of which many are cytoplasmic, whereas others are exclusively nuclear (1–6). In the future, especially for a better understanding of the regulation of mTORC1 downstream targets, considering our data that mTORC1 is differently regulated in the cytoplasm and in the nucleus will be of high relevance.

Deregulation of mTOR is a hallmark of many human cancers and genetic diseases (see Introduction). Owing to their immunosuppressive and antiproliferative effects, rapamycin and related drugs (analogues) are being currently evaluated as part of many transplant immunosuppression regimens, as well as in clinical trials for diabetes or cancers of the breast, prostate, lung, liver or kidney (1–3,9,43). For example, first reports on clinical trials using sirolimus (rapamycin) justify the need for larger trials to clarify whether sirolimus should become the standard of care for patients with tuberous sclerosis or lymphangioleiomyomatosis (71–73). From all these first trials, it became evident that not all patients and tumors respond to rapamycin. Understanding the molecular consequences of rapamycin-induced mTOR inhibition and the regulation of its tissue and tumor specificity is critical for rapamycin to be most effectively used in the clinic (1–7). We believe that our first description of different effects of rapamycin on cytoplasmic and nuclear mTORC2 presented here could provide important contributions to the understanding of the different tumor sensitivities to this drug. Obviously, the success of rapamycin treatment might also depend on the

question whether cytoplasmic or nuclear upstream regulators or downstream targets of mTOR contribute to the development of the tumor one wants to attack. We found short-term rapamycin treatment to not affect mTORC2 assembly in primary IMR-90 cells, although dephosphorylation of rictor and sin1 already appeared. However, this dephosphorylation occurred at only low levels and was exclusively detectable on cytoplasmic rictor and sin1. Prolonged drug treatment triggered complete dephosphorylation of rictor and sin1.1 accompanied by translocation of these two proteins from the nucleus to the cytoplasm. This translocation is specific for rictor and sin1.1 and could not be detected for mTOR, raptor, sin1.2 or mLST8. In summary, we here provide evidence that long-term rapamycin treatment downregulates mTORC2 assembly via inhibition of the interaction between a cytoplasmic complex of unphosphorylated rictor and sin1 with mTOR. Rapamycin bound to the protein FKBP12 generates a drug–receptor complex that binds and inhibits mTORC1, but rapamycin does not bind to preformed mTORC2 (1–7). Still rapamycin was already shown to also suppress the assembly and function of mTORC2 (44). Here we have confirmed this potential, which is important for the effects of this drug, studying endogenous proteins in IMR-90 cells. It has been discussed that binding of FKBP12-rapamycin to free mTOR could prevent the subsequent binding of rictor (44). The accumulation of a ‘free’ rictor/sin1 complex (not bound to mTOR) upon long-term rapamycin treatment described here could be in agreement with this model of function.

Not only the mechanism but also the kinetics of rapamycin’s effects on mTORC1 and mTORC2 are different. Although 1 h drug treatment already significantly inhibits mTORC1 assembly, the assembly of mTORC2 remains unaffected and is only inhibited upon prolonged rapamycin incubation. We also observed that in short-term experiments rapamycin does not mediate any effect on cell cycle regulation, whereas long-term treatment causes a cell cycle arrest of primary human IMR-90 cells. On the one hand, these data demonstrate that rapamycin’s effects on mTORC1 are clearly independent of its potential to influence cell cycle control. On the other hand, the question arises whether the rapamycin-mediated effects on cell cycle control are a consequence of its potential to inhibit mTORC2 assembly or whether the translocation and complete dephosphorylation of nuclear rictor and sin1.1 and the accompanied decrease of mTORC2 assembly is a consequence of the cell cycle arrest. These data clearly warrant further and detailed analyses of the cell cycle regulation of mTORC1 and mTORC2. This is of special interest since it has recently been reported that rapamycin triggers different effects on mTORC2 regulation in different tumor cells (44) and one could speculate that this represents the different cell cycle responses of these cells to rapamycin.

MATERIALS AND METHODS

Cells, cell culture and flow cytometry

HEK293 cells (adenovirus transformed human embryonic kidney) and NIH3T3 cells (immortalized mouse embryonic fibroblasts) were grown as previously described in Rosner

et al. (42). IMR-90 (ATCC #CCL-186), MRC-5 (ATCC #CCL-171) and WI-38 (ATCC #CCL-75) are non-transformed, non-immortalized human diploid fibroblasts (HDFs) with finite lifetime and are derived from normal fetal lung tissue. HDFs were obtained from the American Type Culture Collection at passage number 10 (population doubling 26), 14 (population doubling 22) and 14 (population doubling 23), respectively, and were cultured in Dulbecco’s modified Eagle’s medium at 4.5 g/l glucose supplemented with 10% calf serum. In the course of working with these cells, IMR-90, reported to be capable of attaining 58 population doublings before the onset of senescence, and MRC-5 and WI-38 were grown for eight or less additional passages (≤ 47 total population doublings) and regularly analyzed by standard karyotyping to confirm a normal diploid karyotype.

For cytofluorometric DNA analyses, cells were fixed by rapid submersion in ice-cold 85% ethanol. After overnight fixation at -20°C , DNA was stained with 0.25 mg/ml propidium iodide, 0.05 mg/ml RNase, 0.1% Triton X-100 in citrate buffer, pH 7.8 and DNA distribution of a total of 2×10^4 – 4×10^4 cells per sample was analyzed on a Beckton Dickinson FACScan.

Experiments including the mTOR inhibitor rapamycin (Calbiochem) were performed in the absence (DMSO vehicle control) or presence of the drug at a final concentration of 100 nM for the indicated times.

Total cell lysis, cytoplasmic and nuclear fractionation

Total cell lysates (TL) containing both soluble cytoplasmic and nuclear proteins were prepared by two different methods.

Unless otherwise indicated, extracts of cellular total protein were prepared by physical disruption of cell membranes by repeated freeze–thaw cycles. Briefly, cells were washed with PBS, harvested by trypsin-EDTA and washed twice with PBS to remove traces of trypsin and growth medium. Pellets were lysed in buffer A containing 20 mM HEPES, pH 7.9, 0.4 M NaCl, 2.5% glycerol, 1 mM EDTA, 1 mM PMSF, 0.5 mM NaF, 0.5 mM Na_3VO_4 supplemented with 2 $\mu\text{g}/\text{ml}$ aprotinin, 2 $\mu\text{g}/\text{ml}$ leupeptin, 0.3 $\mu\text{g}/\text{ml}$ benzamidinchlorid, 10 $\mu\text{g}/\text{ml}$ trypsininhibitor by freezing and thawing. Supernatants were collected by centrifugation and stored at -80°C . For immunoprecipitation of mTOR complexes, total cellular protein was extracted as described in Sarbassov and Sabatini (74) to achieve maximum mTOR complex recovery. Therefore, cells were harvested by trypsin-EDTA, rinsed twice with cold PBS and lysed on ice for 20 min in buffer containing 40 mM HEPES pH 7.5, 120 mM NaCl, 1 mM EDTA, 10 mM 2–glycerophosphate, 50 mM NaF, 0.5 mM Na_3VO_4 and 0.3% CHAPS supplemented with 2 $\mu\text{g}/\text{ml}$ aprotinin, 2 $\mu\text{g}/\text{ml}$ leupeptin, 0.3 $\mu\text{g}/\text{ml}$ benzamidinchlorid, 10 $\mu\text{g}/\text{ml}$ trypsin inhibitor. Soluble fractions of lysates were isolated by centrifugation at 20 000 g for 20 min at 4°C .

Subcellular fractionations were performed with the aim of establishing proper conditions to maintain the integrity of mTOR complexes at minimal cross-contamination of cytoplasmic and nuclear fractions. Therefore, four different fractionation protocols (designated p #1a, #1b, #2 and #3) were used. Again, cells were harvested by trypsin-EDTA, collected by centrifugation and washed two times in ice-cold PBS.

According to protocol #1a (p #1a), remaining cell pellets were resuspended in five packed cell volume buffer F1 containing 20 mM Tris, pH 7.6, 50 mM 2-mercaptoethanol, 0.1 mM EDTA, 2 mM MgCl₂, 1 mM PMSF supplemented with protease inhibitors (2 mg/ml aprotinin, 2 mg/ml leupeptin, 0.3 mg/ml benzamidinchlorid, 10 mg/ml trypsininhibitor) and incubated for 2 min at room temperature and for another 10 min on ice. Thereafter, NP-40 was added at a final concentration of 1% (v/v) and lysates were homogenized by passing through a 20 G needle for three times. Nuclei were pelleted by centrifugation at 600 g for 5 min at 4°C and supernatant containing cytoplasmic proteins (C) was collected and stored at -80°C. Remaining nuclei were washed three times in buffer F1 containing 1% NP-40. During the last wash nuclei were stained with Trypan blue and microscopically examined for number, purity and integrity. The nucleic pellets were lysed in buffer A containing 20 mM HEPES, pH 7.9, 0.4 M NaCl, 2.5% glycerol, 1 mM EDTA, 1 mM PMSF, 0.5 mM NaF, 0.5 mM Na₃VO₄, 0.5 mM DTT, supplemented with protease inhibitors by repeated freezing and thawing. Supernatants containing soluble nucleic proteins (N) were collected by centrifugation at 20 000 g for 20 min and stored at -80°C. Cells fractionated according to protocol #1b (p #1b) were processed as described in p #1a except that non-ionic detergent NP-40 was replaced by zwitterionic detergent CHAPS. CHAPS was used at a final concentration of 0.6%. In addition, reducing agent 2-mercaptoethanol was omitted from buffer F1. Buffer F2 represents buffer F1, with 0.6% CHAPS instead of 1.0% NP-40 and without 2-mercaptoethanol. Protocols #2 and #3 (p #2 and p #3) involve the use of hypo/hypertonic buffers or are based on a combination of hypotonic buffers and non-ionic detergent NP-40. Cytoplasmic/nuclear fractionation according to these protocols was done as previously described (51,62,75).

Unless otherwise indicated, equal amounts of cytoplasmic and nuclear fractions were analyzed to allow the comparison of a protein's cytoplasmic versus nuclear distribution within the cell.

Immunoprecipitation

For immunoprecipitation of endogenous mTORC1 and mTORC2 complexes, total lysates were prepared in CHAPS-containing lysis buffer as described above. Cytoplasmic and nuclear fractions were separated according to protocol #1b (p #1b). Basically, immunoprecipitations were carried out as described in Rosner *et al.* (42). Briefly, crude cell extracts (200–500 µg of total lysates or cytoplasmic and nuclear fractions) were diluted in CHAPS containing total cell lysis buffer and pre-cleared with 30 µl protein A/G-Sepharose beads (Pierce) for 30–60 min at 4°C. Indicated primary antibodies were added to the cleared lysates and incubated with constant rotation for 12–16 h (overnight) at 4°C. About 30 µl of a 50% slurry of protein A/G sepharose were then added and the incubation continued for another 90 min at 4°C. So captured immunoprecipitates were washed four times with lysis buffer containing 40 mM HEPES pH 7.5, 120 mM NaCl, 1 mM EDTA, 10 mM 2-glycerophosphate, 50 mM NaF, 0.5 mM Na₃VO₄ and 0.3% CHAPS supplemented with 2 µg/ml aprotinin, 2 µg/ml leupeptin, 0.3 µg/ml benzamidinchlorid, 10 µg/ml trypsin inhibi-

tor. The final washing step was carried out in wash buffer containing 50 mM HEPES pH 7.5, 40 mM NaCl and 2 mM EDTA. Immunoprecipitated proteins were then denatured and separated from the Sepharose beads by adding SDS sample buffer and boiling for 5 min. For immunoprecipitation, antibodies specific for the following proteins were used: mTOR (Cell Signaling, #2972), raptor (Bethyl Laboratories, #A300-553A), rictor (Bethyl Laboratories, #A300-458A) and sin1 (Bethyl Laboratories, #A300-910A).

Immunoblotting

Denatured samples prepared from total lysates and immunoprecipitated proteins were resolved by 5–10% SDS-PAGE and transferred to nitrocellulose. Blots were stained with Ponceau-S to visualize the amount of loaded protein. For immunodetection, antibodies specific for the following proteins were used: mTOR (Cell Signaling, #2972), raptor (Bethyl Laboratories, #A300-506A), rictor (Bethyl Laboratories, #A300-459A), sin1 (Bethyl Laboratories, #A300-910A), mLST8 (Gibco, 86B8, Cell Signaling, #3274), α-tubulin (DM1A, Calbiochem, #CP06), topoisomerase IIβ (clone 40, Transduction Laboratories, #611492), PARP (Cell Signaling, #9542), jun (clone 3, Transduction Laboratories, #610326). Rabbit polyclonal and monoclonal antibodies were detected using anti-rabbit IgG, an HRP-linked heavy and light chain antibody from goat (A120-101P, Bethyl Laboratories); mouse monoclonal antibodies were detected using anti-mouse IgG, an HRP-linked heavy and light chain antibody from goat (A90-116P, Bethyl Laboratories). Signals were detected with the enhanced chemiluminescence method (Pierce).

Conflict of Interest statement. None declared.

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