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mTORC2 can associate with ribosomes to promote cotranslational phosphorylation and stability of nascent Akt polypeptide

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The mechanisms that couple translation and protein processing are poorly understood in higher eukaryotes. Although mammalian target of rapamycin (mTOR) complex 1 (mTORC1) controls translation initiation, the function of mTORC2 in protein synthesis remains to be defined. In this study, we find that mTORC2 can colocalize with actively translating ribosomes and can stably interact with rpL23a, a large ribosomal subunit protein present at the tunnel exit. Exclusively during translation of Akt, mTORC2 mediates phosphorylation of the nascent polypeptide at the turn motif (TM) site, Thr450, to avoid cotranslational Akt ubiquitination. Constitutive TM phosphorylation occurs because the TM site is accessible, whereas the hydrophobic motif (Ser473) site is concealed in the ribosomal tunnel. Thus, mTORC2 can function cotranslationally by phosphorylating residues in nascent chains that are critical to attain proper conformation. Our findings reveal that mTOR links protein production with quality control.

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Introduction

Coordinated control of mRNA translation and processing of nascent polypeptides is critical for normal cell function, but the mechanisms that couple these events remain unclear. Deregulated protein synthesis and processing underlie a number of pathological conditions (Macario and Conway de Macario, 2005; Sonenberg and Hinnebusch, 2009). Insights on how translation and protein processing are connected have been gained on studies of the unfolded protein response, involving secretory and transmembrane proteins, but little is known on cytosolic proteins such as protein kinases (Frydman, 2001; Young *et al*, 2004).

The mammalian target of rapamycin (mTOR) is an atypical protein kinase that forms two structurally distinct complexes, mTORC1 and mTORC2 (Polak and Hall, 2009). The rapamycin-sensitive mTORC1 regulates translation initiation and ribosome biogenesis (Proud, 2007; Ma and Blenis, 2009; Sonenberg and Hinnebusch, 2009). mTORC1, consisting of the evolutionarily conserved mTOR, raptor, and mLST8, interacts with the translation initiation complex. In the presence of growth signals, such as nutrients and growth factors, mTORC1 phosphorylates the initiation regulators, S6K and 4E-BP, resulting in the modulation of a number of initiation factors and the assembly of the 48S initiation complex (Holz *et al*, 2005; Dann *et al*, 2007). The cellular function of mTORC2, consisting of mTOR, rictor, SIN1, and mLST8, is less clear but so far includes actin cytoskeleton reorganization and cell survival (Jacinto *et al*, 2004, 2006; Sarbassov *et al*, 2004). The mTORC2-mediated phosphorylation of the antiapoptotic proteins Akt/PKB and/or SGK could promote cell survival (Sarbassov *et al*, 2005; Jacinto *et al*, 2006; Alessi *et al*, 2009). mTORC2 phosphorylates the hydrophobic motif (HM) and turn motif (TM) sites of several members of the AGC (protein kinase A, PKG, PKC) kinase family, such as Akt, PKC, and SGK (Sarbassov *et al*, 2005; Facchinetti *et al*, 2008; Garcia-Martinez and Alessi, 2008; Ikenoue *et al*, 2008; Jacinto and Lorberg, 2008; Lee *et al*, 2010). Many members of this family, including the mTORC1-controlled S6K, become phosphorylated at these conserved motifs by poorly defined mechanisms (Newton, 2003; Hauge *et al*, 2007). The TM and HM are part of the carboxyl-terminal tail (C-tail; Figure 1A), a segment that characterizes AGC kinases and interacts with the N- and C-lobes of the kinase domain (Yang *et al*, 2002; Kannan *et al*, 2007). HM phosphorylation of Akt is induced by growth factors, necessary for full Akt activation, and often upregulated in cancer cells (Alessi *et al*, 1996; Sarbassov *et al*, 2005; Jacinto *et al*, 2006). In contrast, TM phosphorylation is constitutive (Alessi *et al*, 1996; Bellacosa *et al*, 1998). In the absence of TM phosphorylation, the stability of Akt and conventional PKC (cPKC) is dependent on the folding chaperone Hsp90 to prevent ubiquitination and degradation (Facchinetti *et al*, 2008; Ikenoue *et al*, 2008). Thus, TM phosphorylation is critical for the proper C-tail folding and stability of Akt and cPKC.

In this study, we demonstrate that constitutive phosphorylation of the TM site of Akt occurs during translation and that mTORC2 associates with actively translating ribosomes (polysomes) to fulfill this function. These findings unravel that mTORC2, like mTORC1, functions in translation and has a role in cotranslational folding of nascent cytosolic polypeptides such as Akt.

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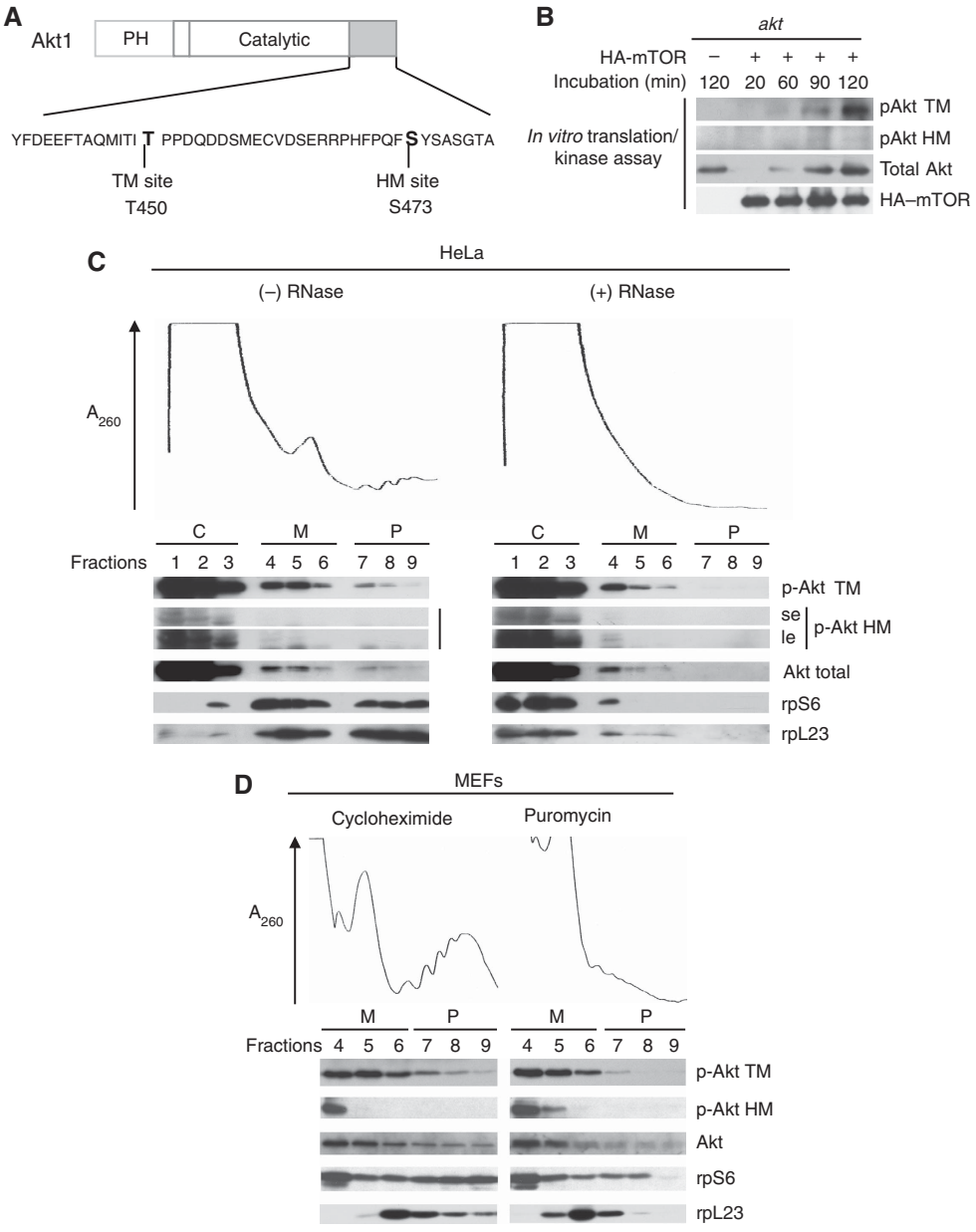

Figure 1 The turn motif site of Akt is phosphorylated during translation *in vitro* and *in vivo*. (**A**) Sequence of the C-tail of murine Akt1. Akt has a pleckstrin homology (PH) domain at the N terminus. A C-tail (grey box) that is conserved among AGC kinases follows the catalytic domain and contains the conserved turn (TM) and hydrophobic motifs (HM), which get phosphorylated at Thr450 and Ser473, respectively. (**B**) Wild-type *akt* was used as template in a coupled *in vitro* translation (bacterial components) and kinase assay by incubating at the indicated times with mock (−) or HA–mTOR (+) immunoprecipitates from HEK293 cells. Aliquots of reaction were fractionated by SDS–PAGE and immunoblotted with the indicated antibodies. (**C**) HeLa cells were starved and restimulated with serum, then treated with cycloheximide (CHX). Cell extracts were untreated (−) or treated (+) with RNase then subjected to sucrose gradient fractionation. Absorbance (A_{260}) (*y* axis) versus increasing density (*x* axis) of fractions was monitored (upper panels), and aliquots of each fraction were subjected to SDS–PAGE and western blotting using specific antibodies. Cytosolic (C), monosome (M; 40s-, 60s/80s containing), and polysome-containing (P) fractions are labelled. Both short (se) and long (le) exposures for phosphorylated Akt at the HM site are shown. (**D**) Wild-type MEFs were starved and restimulated with serum. Cells were then treated with CHX or puromycin and A_{260} profile of fractions was obtained. Fractions were processed as in 1C. Monosome (M)- and polysome (P)-containing fractions were run in SDS–PAGE and immunoblotted as in 1C.

Results

The phosphorylation of Akt at the TM site, but not the HM site, occurs during translation

We sought to determine why phosphorylation of the TM and HM sites of Akt are regulated differently, yet are both mTORC2-dependent events. As we have previously shown that mTOR mediates phosphorylation of the Akt TM site *in vivo* and *in vitro* (Facchinetti *et al*, 2008), we hypothesized that it phosphorylates these two sites under different cellular contexts. Although HM phosphorylation occurs post-translationally on Akt membrane localization, the constitutive nature of TM phosphorylation hints that it happens during or shortly after translation. To test if TM phosphorylation occurs during translation, we developed a coupled *in vitro* translation/kinase assay using translation components from bacteria, wherein no TORC orthologues exist. In the absence of added HA–mTOR as the kinase source, *in vitro*-translated

Akt lacked TM phosphorylation (Figure 1B). Upon inclusion of HA–mTOR, an increase in TM phosphorylation (Thr450) was observed simultaneously with the appearance of total Akt at 60 min. Furthermore, TM phosphorylation and total Akt synthesis increased concurrently with prolonged *in vitro* translation reaction (Figure 1B). The absence of lag time between synthesis and TM phosphorylation suggests that this phosphorylation event likely occurs during translation. In contrast, HM phosphorylation was hardly observed under these conditions (Figure 1B) but was detectable at longer incubation (Supplementary Figure S1), suggesting post-translational phosphorylation.

Next, we analysed if TM phosphorylation during translation can also be detected *in vivo*. We purified polysomes from serum-restimulated HeLa cells that were treated with cycloheximide (CHX) before harvest in order to preserve intact polysomes. As expected, most total and phosphorylated Akt are present in the cytosolic (C) fractions (Figure 1C). Upon overexposure of immunoblots, we observed the presence of Akt (Akt total) in the 40 s and 60/80 s fractions (collectively labelled as monosomes M), as well as small but detectable amounts in the polysome-containing (P) fractions. TM phosphorylation in the different fractions displayed similar patterns as total Akt and is detected in the monosome (fractions 4–6) and high-density, polysome-containing fractions (fractions 7–9) (Figure 1C). Strikingly, Akt HM phosphorylation is practically undetectable in the monosome and polysome fractions, but was highly abundant in the cytosolic fractions (fractions 1–3). As these cells were serum restimulated and that HM phosphorylation of Akt is induced under this condition, these results indicate that a minuscule fraction of Akt, most likely associated with translating ribosomes, becomes phosphorylated at the TM but not at the HM site. To confirm that the phospho-TM-containing fractions (7–9) originate from polysome complexes and not other protein complexes comigrating at high-density fractions, we treated the cell extracts with RNase to disrupt the ribosomes. The small ribosomal protein S6 and the large ribosomal protein L23 that are both abundant in polysomes in non-RNase-treated cells shifted to the cytosolic and monosome fractions upon RNase treatment, confirming the disruption of ribosomes (Figure 1C). In RNase-treated extracts, TM phosphorylation diminished significantly in the high-density fractions (5–9) but accumulated in the low-density fractions (1–4) (Figure 1C). HM phosphorylation remained in the cytosol. We further verified if TM phosphorylation occurs during translation using another cell line, murine embryonic fibrolasts (MEFs), and another drug, puromycin, to specifically disassemble polysomes *in vivo*. Likewise, TM, but not HM, phosphorylation is present in polysomes in these cells (Figure 1D). In puromycin-treated MEFs, TM phosphorylation was essentially absent in high-density fractions. Taken together, these results indicate that TM, but not HM, phosphorylation of Akt occurs during translation.

TM phosphorylation by mTORC2 is cotranslational because the Akt TM site is accessible during synthesis of the nascent polypeptide

We considered how the Akt TM site, but not the HM site, could be phosphorylated during translation. Structural studies of ribosomes from lower organisms predict that the ribosomal tunnel where nascent polypeptides traverse before exiting the ribosome can accommodate a linear polypeptide of about 30 amino acids (aa) (Nissen *et al*, 2000; Bhushan *et al*, 2010). It is believed that these last C-terminal residues are shielded in the tunnel before the newly synthesized protein is extruded from the ribosome. The TM phosphorylation site is 30 aa residues away, whereas the HM site (Ser473) is only 7 aa away from the terminal residue (Figure 1A). Hence, the TM site may be accessible for phosphorylation, whereas the HM site is only post-translationally regulated due to its inaccessibility. Therefore, we lengthened the C terminus of Akt by ligating either 15 or 30 additional aa residues, making the HM site either 22 aa (Akt–His) or 37 aa (Akt–HA–His2X) away from the final residue (Supplementary Figure S2). Using both long-tail Akt template and N-terminally tagged His–Akt, we found equivalent phosphorylation of the TM site by HA–mTOR (Figure 2A). However, HM phosphorylation became more efficient using both long-tail Akt but not N-terminally tagged His–Akt. Because the HM site of Akt–His is only 22 aa away from the terminal residue and predictably should be protected by the tunnel, we questioned whether this phosphorylation is co- or post-translational in comparison with the TM site. Using the Akt–His template, we performed *in vitro* translation but added HA–mTOR only after terminating the reaction by treatment with neomycin and RNase. Under this condition, TM site phosphorylation of Akt–His was undetectable (Figure 2B, lane 3), whereas HM phosphorylation diminished only slightly. These findings clearly illustrate that the TM is not post-translationally phosphorylated, whereas the HM site of Akt–His can undergo post-translational phosphorylation by mTOR. Without neomycin/RNase treatment and when the *in vitro* translation reaction was allowed to continue for another hour after addition of HA–mTOR (Figure 2B, lane 4), Akt continued to be translated as indicated by an increase in total Akt–His. During this additional hour of incubation in the presence of HA–mTOR, the level of TM phosphorylation did not correspond to total Akt–His levels. In fact, it was equivalent to the amount observed in lane 2, wherein HA–mTOR was included throughout the 1-h reaction. This suggests that only a fraction of total Akt (in lane 4), most likely the newly synthesized polypeptides coming from the second hour reaction, became phosphorylated at this site. In contrast, HM phosphorylation is equivalent to total Akt–His expression. This demonstrates that both the released polypeptides from the first hour of reaction and the newly synthesized polypeptides from the second hour reaction became phosphorylated at the HM upon delayed addition of mTOR. These results reveal that mTORC2 phosphorylates the TM site exclusively during translation and that it cannot phosphorylate this site after the Akt polypeptide is released from the ribosome.

Next, we truncated the C-tail of Akt such that the TM site is only 14 aa from the terminal residue. Using this construct, we noted that our commercial Akt (total) antibody does not recognize this truncated Akt (Figure 2C). However, when we used a T7 antibody to detect an epitope that is present on the N terminus of our His–Akt constructs (Supplementary Figure S2), the truncated His–Akt along with the full-length and intermediate forms of the other His–Akt constructs were apparent, confirming the presence of *in vitro*-translated products (Figure 2C and Supplementary Figure S3) (Note: We did not use anti-His antibody, as the bacterial translation components are His-tagged). In contrast to the wild-type and

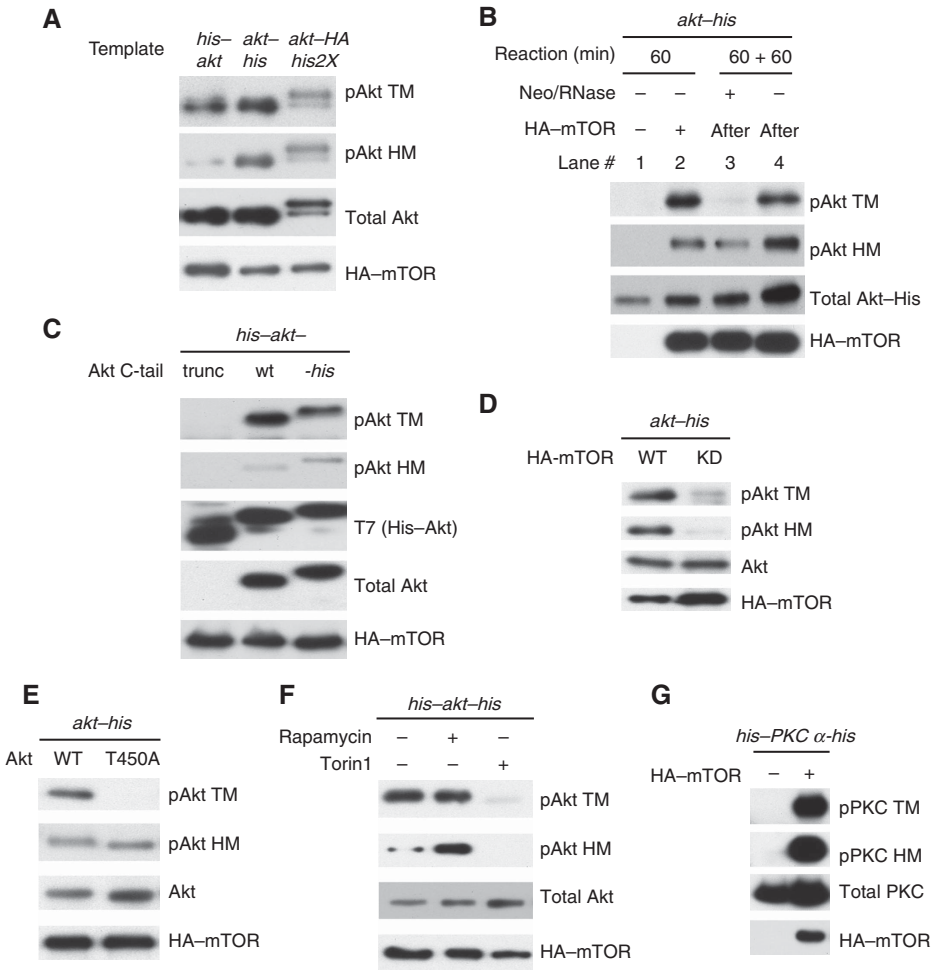

Figure 2 mTOR phosphorylates the TM site only during translation, but it can phosphorylate the HM site either co- or post-translationally if the carboxyl-tail of Akt is lengthened. (**A**) N terminally tagged *his–akt* or long-tail Akt (*akt–his* and *akt–HA–his2X*) were used as templates for coupled *in vitro* translation (bacterial components) and kinase assay for 1 h in the presence of HA–mTOR immunoprecipitates purified from HEK293 cells. Amount of phosphorylated or total Akt was detected by immunoblotting. (**B**) *akt–his* was used for *in vitro* translation and kinase assay. Mock-transfected (lane 1) or HA–mTOR (lane 2) immunoprecipitates from HEK293 cells were added during (+) the entire 60 min of translation reaction. In lanes 3 and 4, HA–mTOR was only added after the first 60 min *in vitro* translation reaction in the presence (lane 3) or absence (lane 4) of 15 μg each of neomycin and RNase. *In vitro* translation/kinase assay reaction was allowed to continue for an additional 60 min after the addition of HA–mTOR in lanes 3 and 4. Phosphorylated and total proteins were detected by immunoblotting. (**C**) *his–akt* templates that are either truncated at the C terminus (trunc), full length (wt; His–Akt), or lengthened at the C terminus (-His; His–Akt–His) were subjected to *in vitro* translation and kinase assay as in 2A for 2 h. (**D**, **E**) *In vitro* translation and kinase assay using (**D**) wild-type HA–mTOR (WT) or kinase-dead (KD) HA–mTOR as kinase and Akt–His as substrate, (**E**) HA–mTOR as kinase, and either Akt–His wild-type (WT) or mutant Akt (T450A) as substrate was performed as in 2A. (**F**) His–Akt–His was subjected to *in vitro* translation/kinase assay using HA–mTOR purified from vehicle- or rapamycin-treated (1 μM, 60 min) cells. Where indicated, Torin1 (10 μM) was added during the translation reaction. (**G**) His–PKCα–His was used as the substrate for *in vitro* translation/kinase assay.

long-tail (His–Akt–His) Akt, we did not observe TM phosphorylation of truncated His–Akt even at prolonged incubation (Figure 2C). These results demonstrate that mTOR cannot phosphorylate the TM site when rendered inaccessible, but more importantly, the TM, unlike the HM, cannot be phosphorylated post-translationally. Alternatively, truncation of the C-tail of Akt does not allow TM phosphorylation due to conformational constraints.

We then further analysed the role of mTORC2 in phosphorylating the TM site during translation. Both the TM and HM phosphorylation required the kinase activity of mTOR, as little to no phosphorylation was observed using the kinase-dead HA–mTOR (Figure 2D). The phosphorylation observed for the TM site is indeed specific to this Thr residue, as mutation to an Ala abolished phosphorylation in the presence of mTOR (Figure 2E). As we obtained inefficient phosphorylation of Akt during translation using rictor immunoprecipitates (data not shown), we used pharmacological inhibition of mTOR complexes to distinguish the kinase activity of mTORC1 versus mTORC2. Before harvest and purification of HA–mTOR from HEK293 cells, we inhibited mTORC1 by treatment with rapamycin. During the translation reaction, we added Torin1, the mTOR active site inhibitor that blocks both mTORC1 and mTORC2 (Thoreen *et al*, 2009). Inhibition by rapamycin did not block phosphorylation of Akt, whereas Torin1 abolished phosphorylation at both TM and HM sites (Figure 2F). Because previous studies demonstrate that mTORC2 also controls phosphorylation of cPKC at the homologous TM and HM sites (Sarbassov *et al*, 2004; Guertin *et al*, 2006; Facchinetti *et al*, 2008; Ikenoue *et al*, 2008), we

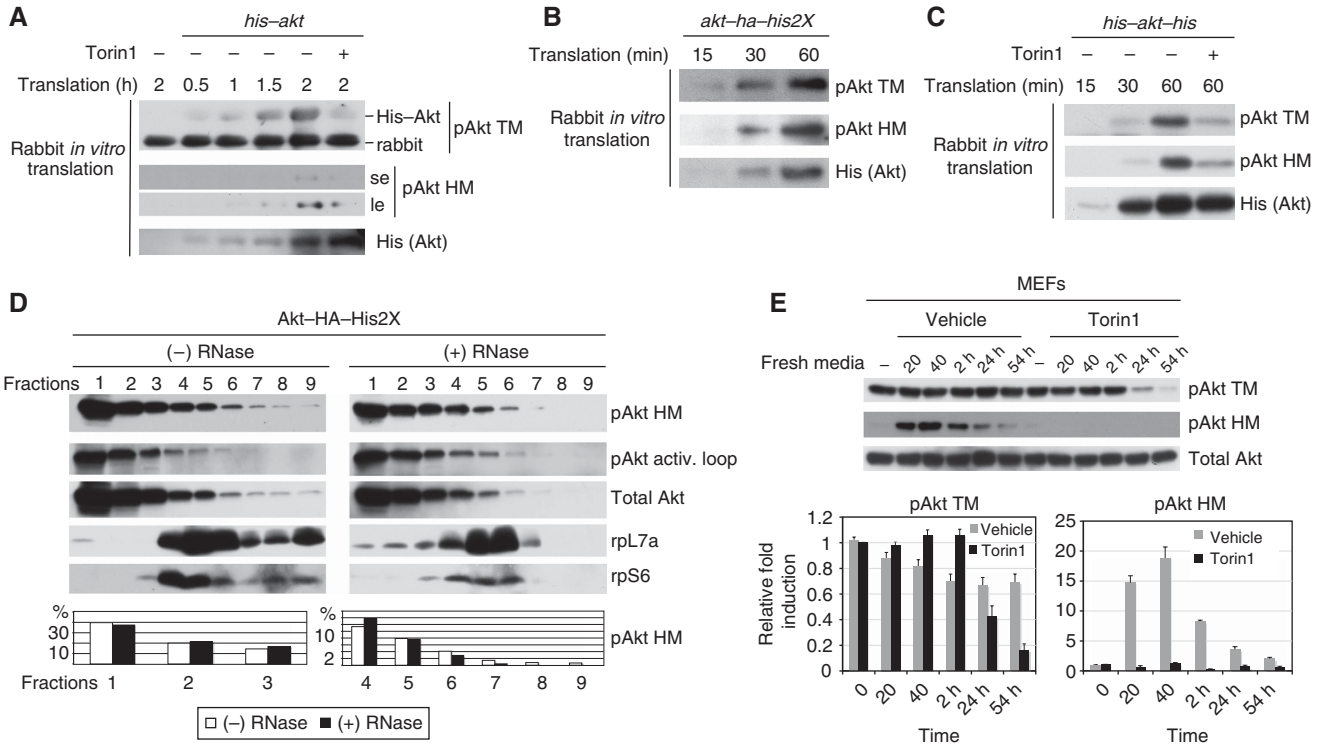

Figure 3 TM site phosphorylation can be reconstituted *in vitro* using eukaryotic translation system and is inhibited by Torin1 *in vivo* and *in vitro*. (**A–C**) *In vitro* translation of (**A**) *his–akt*, (**B**) *akt–ha–his2X*, or (**C**) *his–akt–his* template using rabbit reticulocyte lysate components was performed at the indicated times, in the absence (−) or presence of Torin1 (100 nM). (**D**) HeLa cells transiently transfected with Akt–HA–His2X expression construct was processed as in Figure 1C. The relative amount of each band to total bands (fractions 1–9) of the phosphorylated HM site from each group was quantitated and plotted (see bottom panel). (**E**) Growing MEFs were either harvested (−) or replenished and incubated with fresh media containing serum and either DMSO (vehicle) or Torin1 (250 nM) at the indicated times (minutes or hours). Total lysates were subjected to SDS–PAGE and immunoblotting. Results from four independent experiments were normalized to total Akt, averaged and fold induction relative to untreated (−) cells were plotted (lower panel). Error bars represent s.e.m.

examined if we can reconstitute the mTORC2-dependent phosphorylation of PKC using coupled *in vitro* translation/kinase assay. Using a PKCα construct with an extended tail, we observed efficient phosphorylation at both the TM and HM sites in the presence of HA–mTOR (Figure 2G). Thus, our results collectively demonstrate that mTOR, as part of an intact mTORC2, phosphorylates its target sites in Akt and PKC when these sites are accessible during translation.

Akt is cotranslationally phosphorylated using in vitro and in vivo eukaryotic systems

To verify if we can reconstitute cotranslational TM phosphorylation in a eukaryotic system, we used rabbit reticulocyte lysates for the *in vitro* translation reaction. Because there was abundant Akt in this system (Figure 3A), we used lengthened *akt* templates (Supplementary Figure S2) whose products migrate at higher MW in SDS–PAGE, enabling us to detect *in vitro*-translated Akt. mTORC2 components were also present in the reticulocyte lysates (data not shown). *In vitro*-translated His–Akt increased over time corresponding to enhanced TM site phosphorylation (Figure 3A). His–Akt phosphorylation, but not phosphorylation of the rabbit Akt, was blocked when Torin1 was included during the reaction, indicating that mTOR mediated the phosphorylation of *in vitro*-translated Akt. On the other hand, HM phosphorylation was inefficient but detectable after 2 h translation reaction (Figure 3A). When we used the long-tail template *akt–ha–his2X* or *his–akt–his*, we observed efficient phosphorylation of both the TM and HM sites, which can be diminished by Torin1 (Figure 3B and C). These results demonstrate that the mTOR-mediated cotranslational phosphorylation of Akt can be reconstituted *in vitro* using rabbit reticulocyte lysates.

We next tested how the long-tail Akt can be phosphorylated *in vivo*. We expressed HA–Akt or long-tail Akt in MEFs. Unlike the TM site, wherein starvation did not abolish phosphorylation, the HM site of long-tail Akt became dephosphorylated (Supplementary Figure S4). This further confirms that the HM, but not the TM, can be post-translationally regulated and that the TM phosphorylated Akt is resistant to starvation-induced dephosphorylation. Hence, to ascertain if the long-tail Akt can undergo cotranslational phosphorylation *in vivo*, we purified polysomes from cells expressing Akt–HA–His2X. Unlike endogenous wild-type Akt, HM phosphorylation was observed in high-density fractions (7–9) and this phosphorylation shifted to lower density fractions upon RNase treatment (Figure 3D). In contrast, there was little to no phosphorylation of the activation loop in high-density fractions. Thus, the HM site of long-tail Akt can be phosphorylated cotranslationally *in vivo*, but is still subject to post-translational regulation in response to growth conditions.

It was reported that mTOR active site inhibitor blocked the HM, but not TM phosphorylation of Akt (Feldman *et al*, 2009). Our findings above suggest that as TM phosphorylation occurs during translation and is resistant to starvation-induced dephosphorylation, pre-existing Akt would remain

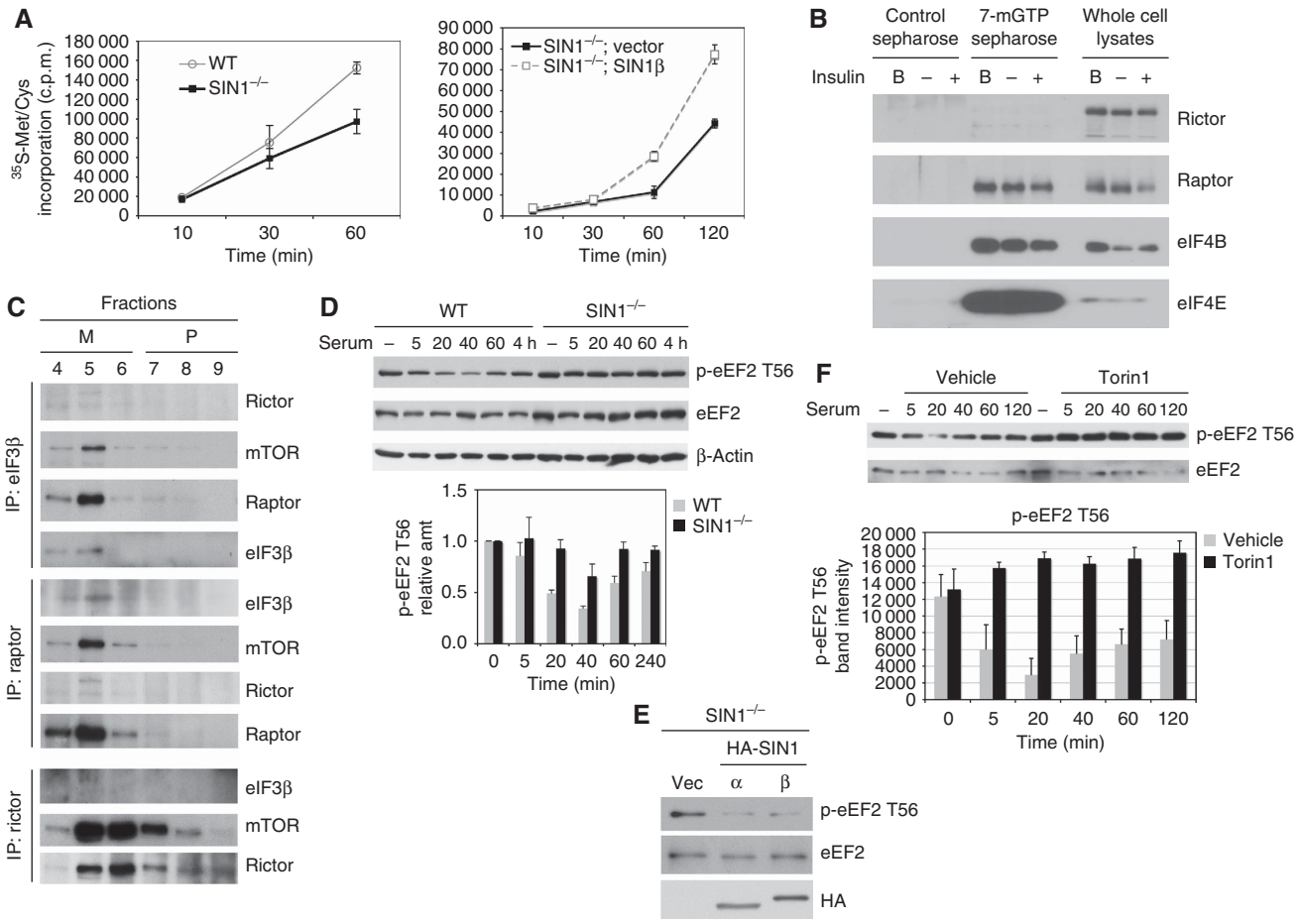

Figure 4 mTORC2 is not part of the initiation complex, but is required for efficient translation. (**A**) Wild-type or SIN1$^{-/-}$ MEFs (left panel) or SIN1$^{-/-}$ MEFs transfected with either empty or SIN1β (SIN1β) expression vector (right panel) were starved of serum and amino acids, then metabolically labelled with ^{35}S-Met/Cys in media containing serum for the indicated time (min). Data represent mean ± s.d. from three independent experiments. (**B**) HeLa cells were normally grown (basal conditions [B]), or serum-starved (−) and restimulated with insulin (+). Cell lysates were incubated with 7-mGTP sepharose or control beads. Bound and total (whole cell lysates) proteins were analysed by immunoblotting. (**C**) Serum-restimulated wild-type MEFs were fractionated as in Figure 1C. Monosome (4–6) and polysome (7–9) fractions were immunoprecipitated with the indicated antibodies, and co-immunoprecipitated proteins were detected by immunoblotting. Rictor blots were overexposed relative to mTOR and raptor blots. (**D**) Wild-type or SIN1$^{-/-}$ MEFs were starved then restimulated with serum at different time points (min) and total extracts were analysed. A representative blot is shown (upper panel). Phosphorylated eEF2 levels were normalized to actin (loading), and mean ± s.d. from three independent experiments was plotted (lower panel). (**E**) SIN1$^{-/-}$ MEFs were transfected with either empty vector (vec), HA-tagged SIN1α or SIN1β. Cells were harvested 2 days after transfection and total extracts were analysed. (**F**) Wild-type MEFs were starved then restimulated with serum containing either DMSO (vehicle) or 250 nM Torin1 at the indicated times (minutes). Total extracts were analysed as in D. Band signals of p-eEF2 T56 were quantitated by densitometry (arbitrary units) and expressed as mean ± s.d. from three independent experiments.

phosphorylated. Therefore, only the pool of nascent Akt would not undergo TM phosphorylation upon mTOR inhibition. Because Akt has a half-life of about 36 h (Basso *et al*, 2002), we would therefore expect the appearance of non-phosphorylated Akt only after prolonged mTORC2 inhibition. Indeed, upon incubation of MEF cells with Torin1, acute treatment did not abolish TM phosphorylation (Figure 3E). Attenuation of TM phosphorylation was only evident after 24-h incubation. On the other hand, HM phosphorylation was abolished at all time points. These results suggest that on mTOR inhibition, pre-existing Akt does not become dephosphorylated at the TM but newly synthesized Akt cannot undergo TM and HM phosphorylation. Collectively, the above results confirm that the TM site undergoes cotranslational phosphorylation, whereas HM phosphorylation happens post-translationally because the HM site is not accessible during translation.

mTORC2 is required for efficient translation, but is not part of the translation initiation complex

As mTORC2 can phosphorylate Akt during translation and given that mTORC1 function involves translation initiation, we questioned whether mTORC2 also has a role in translation. By metabolic labelling of newly synthesized proteins, we found that although there was no difference at early time points as previously reported (Thoreen *et al*, 2009), a marked decrease in translation occurred by 60 min in serum-restimulated SIN1$^{-/-}$ cells in comparison with wild type (Figure 4A). Re-expression of SIN1β in SIN1$^{-/-}$ cells restored translation levels, confirming that the defect is due to the absence of SIN1. Treatment of cells using Torin1 further diminished translation at longer time point (60 min) in both wild-type and SIN1$^{-/-}$ MEFs, suggesting that both mTORC1 and mTORC2 can function in translation (Supplementary Figure S5). The previous findings that phosphorylation of

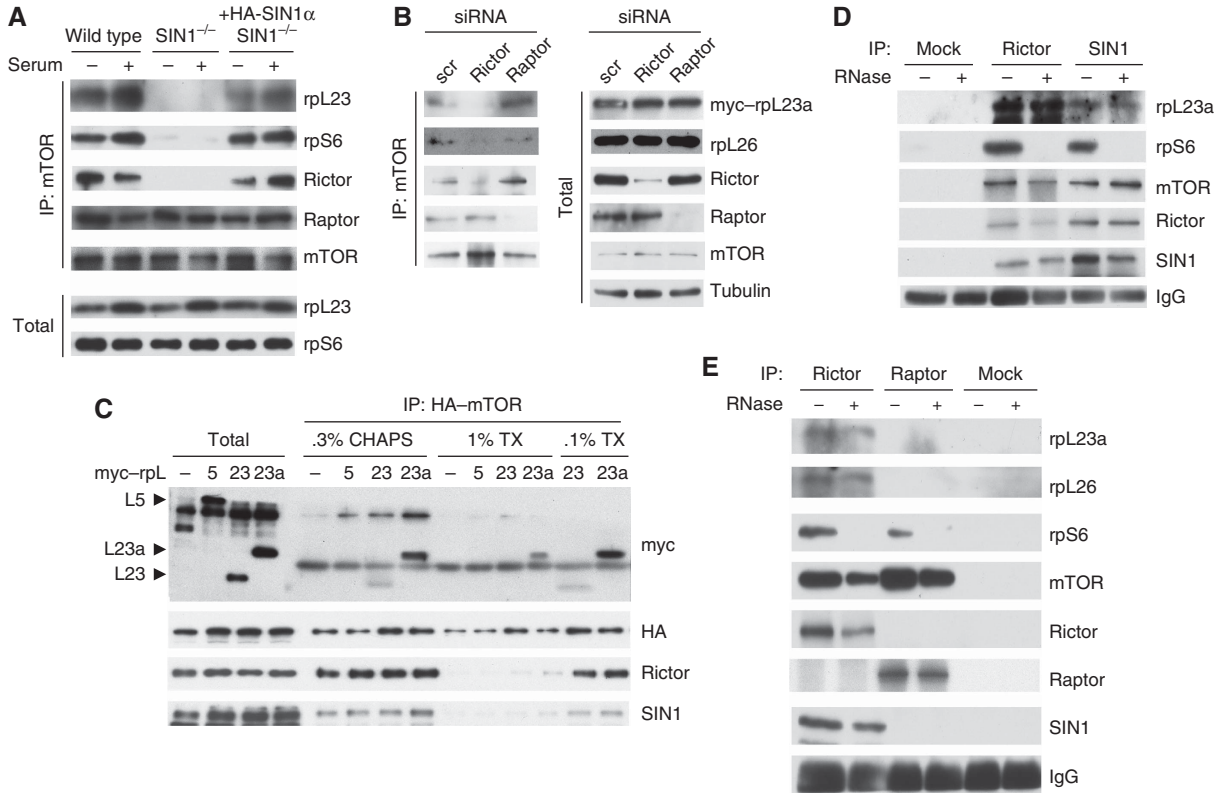

Figure 5 An intact mTORC2 associates with ribosomal proteins and form stable interactions with rpL23a. (**A**) Wild-type, SIN1$^{-/-}$ MEFs, or HA–SIN1α-reconstituted SIN1$^{-/-}$ MEFs were starved (−) or starved then restimulated with serum (+). mTOR co-immunoprecipitated proteins and total lysate proteins were detected by immunoblotting. (**B**) HEK293 cells were transfected with either scrambled control, rictor, or raptor siRNA. mTOR was immunoprecipitated from total lysates and co-immunoprecipitated and total proteins were detected by immunoblotting. (**C**) HEK293 cells were co-transfected with HA–mTOR and each of the myc-tagged ribosomal protein constructs (rpL5, rpL23, or rpL23a). HA–mTOR immunoprecipitates were washed with buffer containing either 0.3% CHAPS, 1 or 0.1% Triton X-100. Co-immunoprecipitated myc-rpL proteins were detected by immunoblotting. Migration of ribosomal proteins in SDS–PAGE is indicated by arrowheads. (**D**, **E**) HEK293 cells were starved, restimulated, and treated with CHX (30 min) and protein crosslinker DSP (75 μg/ml) before harvest. Extracts were either untreated (−) or treated (+) with RNase (5 U, 30 min), followed by immunoprecipitation of either mock, rictor, SIN (**D**) or raptor (**E**) and detection of co-immunoprecipitated proteins.

the translation initiation regulators S6K and 4E-BP1 in mTORC2-disrupted cells is not attenuated suggested that mTORC2 may not control early initiation events (Guertin *et al*, 2006; Jacinto *et al*, 2006; Shiota *et al*, 2006). To verify this, we compared the binding of raptor versus rictor to the initiation complex containing translation initiation factors. Indeed, raptor, but not rictor, associates robustly with the 7-methylguanosine cap complex and also with eIF3β in 40S-containing fractions (Figures 4B and C). Hence, mTORC2 is probably not involved in early initiation signalling events. Next, when we examined eEF2 dephosphorylation, an event that enhances translation elongation (Proud, 2007), eEF2 had decreased phosphorylation at Thr56, most evident from 5 to 40 min after serum restimulation in wild-type cells (Figure 4D). In SIN1$^{-/-}$ MEFs, this dephosphorylation was not discernible (Figure 4D) but became evident upon SIN1 re-expression (Figure 4E). Furthermore, dephosphorylation of eEF2 in Torin1-treated wild-type cells was absent despite serum-repletion (Figure 4F). Taken together, these results support a role for mTORC2 in translation.

mTORC2 associates with ribosomal proteins and stably interacts with rpL23a, which is present at the tunnel exit

To characterize how mTORC2 is involved in translation, we examined the binding of mTORC2 components with ribosomal proteins. In wild-type MEFs, mTOR efficiently binds the ribosomal proteins L23 and S6 in both starved- and serum-restimulated conditions (Figure 5A). As mTORC2 components dissociate in the absence of SIN1 (Jacinto *et al*, 2006), we assessed whether mTOR or rictor can still bind to the ribosomes upon mTORC2 disruption. In SIN1$^{-/-}$ cells, the association of mTOR with the large ribosomal protein rpL23 was severely disrupted (Figure 5A). There was also diminished interaction of mTOR with rpS6 in SIN1$^{-/-}$ cells. Binding of rictor with rpL23 and rpS6 was likewise attenuated in these cells (Supplementary Figure S6). The association of mTOR with the ribosomal proteins was restored upon re-expression of HA-SIN1α in SIN1$^{-/-}$ MEFS (Figure 5A). Moreover, knockdown of rictor, but not raptor, led to diminished association of mTOR with rpL23a, rpL26, rpS6, and rpL23 (Figure 5B and Supplementary Figure 7A). Taken together, these results indicate that an intact mTORC2 associates with ribosomal proteins.

Next, we asked how mTORC2 could associate with ribosomes. If mTORC2 phosphorylates the Akt TM site cotranslationally, mTORC2 may bind close to the ribosomal tunnel exit. At the exit, the tunnel widens up and the rim of this exit point is surrounded by a ring of highly conserved ribosomal proteins (Kramer *et al*, 2009). Among these ribosomal proteins, rpL23a orthologues (yeast rpL25, bacterial rpL23) have

been demonstrated to be involved in the interactions of the ribosome with a variety of ribosome-associated factors that have a role in nascent chain processing (Kramer *et al*, 2009). Another ribosomal protein, L5, has been reported to bind other cellular signalling proteins (Horn and Vousden, 2008), but neither rpL5 nor rpL23 (orthologue of bacterial rpL14) are present in the tunnel exit. Thus, we investigated how mTOR may interact with these large ribosomal proteins. We co-expressed mTOR with rpL5, rpL23 or rpL23a. By co-immunoprecipitation, we observed a strong interaction of mTOR with rpL23a in the presence of either CHAPS or 0.1% Triton X-100 (Figure 5C). Using stronger lysis conditions, such as 1% Triton X-100, the impaired interaction of mTOR with rpL23a correlated with disassembly of mTORC2 complex (Figure 5C). Endogenous levels of rictor and SIN1 can also bind to rpL23a, rpS6, and another exit tunnel protein rpL26 (Figure 5D and E). To obtain clues whether this interaction occurs in intact ribosomes, we treated cell extracts with RNase before immunoprecipitation in order to disrupt assembled ribosomes. RNase treatment did not affect the binding of rictor or SIN1 to rpL23a, whereas their association with rpS6 was abolished (Figure 5D). In contrast, raptor does not bind to rpL23a and rpL26 (Figure 5E). We also performed a pull-down assay to examine the binding of recombinant large ribosomal proteins with the mTORC2 components from wild-type and SIN1$^{-/-}$ MEFs. All large ribosomal proteins pulled down mTOR, rictor, and SIN1, but not raptor from wild-type cell extracts (Supplementary Figure S7B and S7C). In SIN1$^{-/-}$ cells, the binding of mTOR with all ribosomal proteins was abolished and association of rictor with L23a was greatly diminished. Collectively, these results further support that mTORC2 binds to the large ribosomal subunit and likely interacts with proteins at the rim of the exit tunnel.

An intact mTORC2 has enhanced association with polysomes

As mTORC2 phosphorylates nascent Akt during translation and binds to large ribosomal subunit proteins, we predicted that mTORC2 components should associate with translating ribosomes. Indeed, mTOR, rictor, and SIN1 are relatively abundant in the polysome fractions (fractions 7–9) (Figure 6A). Importantly, mTOR that is phosphorylated at Ser2481, a phosphosite reported to be a marker for intact mTORC2 and active mTORC (Copp *et al*, 2009; Soliman *et al*, 2010), is present in polysomes as well. This demonstrates that an active mTOR complex and not just nascent mTORC2 component polypeptides cofractionate with polysomes (Figure 6A). Upon RNase treatment, mTOR is not found in the high-density fractions, suggesting that mTOR colocalizes with actively translating ribosomes. Amounts of rictor and SIN1 were also attenuated in the high-density fractions (fractions 7–9) upon RNase treatment. In contrast, the mTORC1 component, raptor, was enriched in the C and M fractions and hardly detected in the high-density fractions, indicating that it is likely not a part of actively translating ribosomes (Figure 6A, see quantitation on bottom panels). Similar results were obtained using MEF cells and puromycin to disrupt polysomes (Supplementary Figure S8). We also analysed the association of mTOR with ribosomal proteins in fractionated cell lysates. By immunoprecipitation, mTOR associates with the ribosomal proteins L26, L7a, and S6, as well as with rictor, in the monosome and polysome fractions (Supplementary Figure S9). Together, these results demonstrate that mTORC2 colocalizes with translating ribosomes.

To distinguish whether intact mTORC2 or individual mTORC2 components bind to translating ribosomes, we examined colocalization of mTOR and rictor with polysomes in SIN1-deficient cells. Polysome profile of wild-type versus SIN1$^{-/-}$ MEFs revealed decreased polysome levels in mTORC2-disrupted cells, consistent with previous observations using mTOR inhibitors (Figure 6B; Yu *et al*, 2009; Dowling *et al*, 2010). In order to compare amounts of mTORC2 components colocalizing with polysomes between the two cell lines, we loaded more extracts from SIN1$^{-/-}$ cells to normalize levels of polysomes. This is evident by increased ribosomal proteins particularly in fractions 7–9 (polysomes) in SIN1$^{-/-}$ cells (Figure 6C). Phosphorylated mTOR at Ser2481 was found in wild-type MEFs, including high-density fractions, and was absent in all fractions from SIN1$^{-/-}$ MEFs, compatible with a disrupted mTORC2 (Figure 6C). Both mTOR and rictor protein levels were pronouncedly decreased in the polysome fractions (Figure 6C; see also bottom panels for quantitation). Intriguingly, although mTOR was still present in the high-density fractions from SIN1$^{-/-}$ MEFs, it was present as faster migrating forms compared with wild type. On the other hand, rictor became abundant in the monosome fractions of SIN1$^{-/-}$ MEFs but had retarded migration in comparison with wild-type MEFs. These results suggest that the mTOR and rictor that colocalize with ribosomes in SIN1-deficient cells are modified differently or are qualitatively distinct from assembled mTORC2 in the polysomes. Nevertheless, taken together, our results reveal that an intact mTORC2 is required for efficient binding of mTOR, and rictor to translating ribosomes.

Cotranslational phosphorylation of Akt by mTORC2 prevents premature ubiquitination of Akt

The above results together with our recent findings that the mTORC2-dependent TM phosphorylation is important for Akt C-terminal folding (Facchinetti *et al*, 2008) raise the possibility that mTORC2 may function in regulating cotranslational folding. Previous studies documented that ubiquitination of misfolded proteins can occur cotranslationally (Schubert *et al*, 2000; Turner and Varshavsky, 2000). As the specific lack of Thr450 phosphorylation enhanced Akt ubiquitination (Facchinetti *et al*, 2008), we asked whether Akt becomes ubiquitinated during translation in mTORC2-disrupted cells. Using fractionated extracts from wild-type versus SIN1-deficient cells, a pronounced increase in Akt ubiquitination was observed in SIN1$^{-/-}$ MEFs, particularly in the monosome and polysome fractions (Figure 7A). As another AGC kinase, cPKCα, is unstable in mTORC2-disrupted cells (Facchinetti *et al*, 2008; Ikenoue *et al*, 2008), we also evaluated whether it is ubiquitinated during translation in SIN1$^{-/-}$ MEFs. Similar to Akt, enhanced cPKCα ubiquitination occurs in monosome and polysome fractions from SIN1$^{-/-}$ compared with wild-type cells (Figure 7A). To verify whether the enhanced ubiquitination of Akt in monosome and polysome fractions is specifically due to lack of TM site phosphorylation, we immunoprecipitated transfected wild type or Akt T450A mutant from fractionated cells. Expression of T450A mutant, but not the wild-type form, led to increased ubiquitination in the monosome and polysome fractions, similar to the results using SIN1$^{-/-}$ cells (Figure 7B). These results indicate that

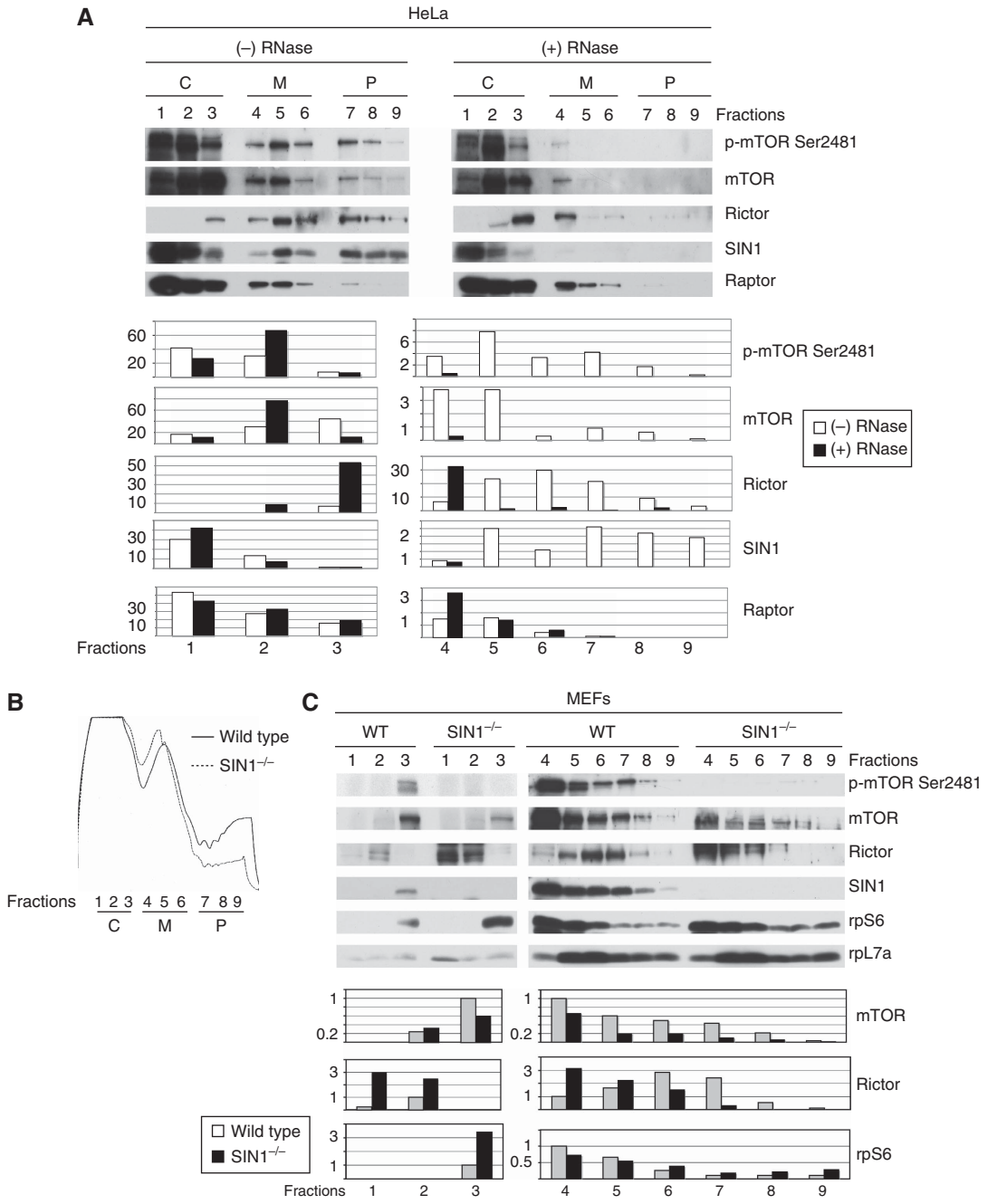

Figure 6 An intact mTORC2 colocalizes with polysomes. (**A**) HeLa cell lysate fractions from Figure 1C were run on SDS–PAGE and immunoblotted with the indicated antibodies. The amount of each band relative to the combined total amount from fractions 1–9 was calculated and plotted (bottom panel). (**B**) Polysome profile of fractionated extracts from wild-type or SIN1$^{-/-}$ MEFs was obtained. *Y* axis represents A_{260}. (**C**) Fractions from B were subjected to SDS–PAGE and analysed for the indicated proteins. More extracts from SIN1$^{-/-}$ MEFs were loaded to obtain comparable polyribosomal protein amounts between the two cell lines. Quantitation of the amount in cytosolic fractions (1–3) was expressed relative to the strongest band in wild-type fraction (assigned a value of 1.0), whereas amount in high-density fractions (4–9) was expressed relative to fraction 4 of wild type (assigned a value of 1.0).

premature Akt or cPKC ubiquitination due to misfolding occurs during translation in the absence of mTORC2-mediated TM site phosphorylation.

Discussion

Translation and processing of nascent polypeptides are highly coupled events that result in the production of mature and functional proteins. We now show that mTOR has a role in connecting these two processes. The rapamycin-sensitive mTORC1 forms a complex with the translation initiation machinery, leading to phosphorylation events that promote initiation of translation (Holz *et al*, 2005). Our studies reveal that mTORC2 also functions during translation, but in contrast to mTORC1, mTORC2 can interact with actively translating ribosomes and is not associated with the initiation complex. Our findings support a role for mTORC2 in the cotranslational phosphorylation of Akt that is required for

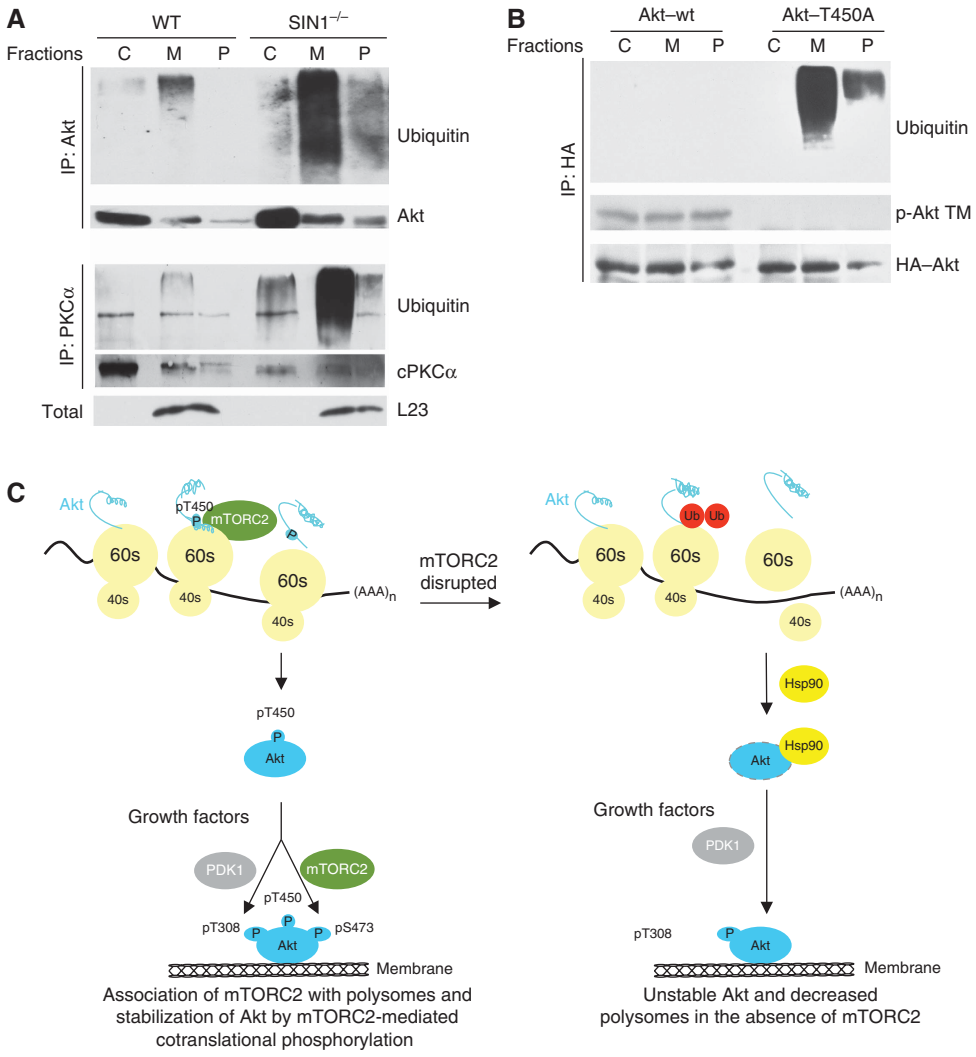

Figure 7 Akt and PKCα become ubiquitinated during translation in the absence of mTORC2 or turn motif phosphorylation. (**A**) MEF cells were starved, treated with MG132, serum-restimulated and CHX-treated before harvest. Fractions obtained as in Figure 6C were immunoprecipitated using either Akt or PKCα antibodies, followed by SDS–PAGE and immunoblotting (C (fractions 1–3), M (4–6), P (7–9)). (**B**) HEK293 cells transiently transfected with either HA-Akt or HA-Akt–T450A were treated as in A, then fractionated, immunoprecipitated with HA antibody, followed by SDS–PAGE and immunoblotting. (**C**) Model for mTORC2 function during translation. mTORC2 phosphorylates the TM site (Thr450) of Akt during translation by association of mTORC2 with translating ribosomes at the vicinity of the tunnel exit. Newly released Akt polypeptide becomes fully mature and active upon phosphorylation by PDK1 at the activation loop (Thr308) and by mTORC2 at the HM (Ser473). In the absence of an intact mTORC2, total translation is defective, polysome recovery is decreased, and Akt tends to be ubiquitinated during translation.

proper Akt folding or maturation. Cotranslational folding mechanisms are especially critical during synthesis of nascent polypeptides due to high local concentrations of non-native chains that have increased chances of premature aggregation (Young *et al*, 2004). By coupling translation with folding, mTOR keeps the rate of protein production and quality assurance in check.

As a protein kinase, mTOR cotranslationally processes nascent polypeptides by phosphorylation of critical and accessible residues that are required for the maturation and stability of newly synthesized kinases such as Akt (Figure 7C). Previously, the autophosphorylation at a tyrosine residue in the activation loop of the Ser/Thr protein kinase, DYRK, was also demonstrated to occur cotranslationally (Lochhead *et al*, 2005). This tyrosine autophosphorylation was described to be a 'one-off' (now-or-never) event, as the nascent kinase transitions from an intermediate to a mature form. In this study, we have shown that the TM phosphorylation of Akt also occurs during translation before disengagement of the nascent polypeptide from the ribosomes. Unlike DYRK, Akt cotranslational phosphorylation requires another protein kinase mTOR, but the TM phosphorylation by mTORC2 is also a one-off event to stabilize nascent Akt. Failure to phosphorylate the TM site predisposes Akt and cPKC to binding with folding chaperones (Gao and Newton, 2002; Facchinetti *et al*, 2008; Ikenoue *et al*, 2008). The colocalization of Akt/cPKC ubiquitination with monosomes and polysomes is consistent with previous observations that ubiquitination can ensue at the translation level as well (Figure 7A; Schubert *et al*, 2000; Turner and Varshavsky, 2000; Bengtson and Joazeiro, 2010). Intriguingly, a recent study identified rictor as a functional E3 ligase that associates with Cullin-1 and that this complex promotes SGK1 ubiquitination (Gao *et al*, 2010). Thus, rictor, depending on its

partner, can function in both polypeptide stabilization and degradation. Whether it can link these two processes cotranslationally remains to be investigated.

Several lines of evidence support that the mTORC2-controlled TM phosphorylation is inextricably linked to translation. First, our *in vivo* analysis demonstrates that TM, but not HM, phosphorylation colocalizes with polysomes. Although an argument can be made that the tiny fraction of Akt that is TM, but not HM, phosphorylated in the polysomes represents existing Akt (Figure 1C), we are not aware of any studies that reported the presence of a compartmentalized pool of Akt that is devoid of HM phosphorylation under growth-stimulating conditions. Second, abrogation of TM phosphorylation in wild-type cells only became obvious upon prolonged treatment with Torin1 (Figure 3E), consistent with the interpretation that TM phosphorylation of pre-existing Akt is resistant, whereas newly synthesized Akt is sensitive to mTOR inhibition. Our coupled *in vitro* translation and kinase assays provide the strongest evidence that TM phosphorylation is exclusively cotranslational. Under these coupled conditions, phosphorylation of the TM site appears concurrently with newly synthesized Akt in an mTOR-dependent manner (Figures 1B and 3A) and is lacking when mTOR is only added upon translation termination (Figure 2B). In contrast, phosphorylation of the HM site is inefficient and delayed. Truncation of Akt at the C-tail prevented TM site phosphorylation due to inaccessibility by mTOR during translation. Thus, the TM site is phosphorylated by mTOR during translation and while the polypeptide is attached to the ribosome.

As the Akt TM site is only 30 aa away from the terminal residue and thus may just emerge from the tunnel before release of the full-length Akt polypeptide, our findings suggest that mTOR must closely contact the ribosomal tunnel exit to allow Akt phosphorylation before peptide release. The inaccessible HM site that is buried inside the tunnel is only post-translationally phosphorylated when Akt has been released and upon its membrane localization in the presence of sufficient growth signals (Yang *et al*, 2002). When the Akt C-tail is lengthened so that the HM site is more than 30 aa away from the terminal residue, both the HM and TM sites became phosphorylated efficiently. Our findings are consistent with structural predictions that about 30 aa of a linear nascent polypeptide can occupy the ribosomal tunnel (Nissen *et al*, 2000), hence unavailable for cotranslational modification until emergence from the exit site. Despite phosphorylation during translation, the HM site of long-tail Akt expressed *in vivo* remained sensitive to withdrawal of growth signals, again underscoring the presence of post-translational regulatory mechanisms for the HM.

How mTOR becomes recruited to the translating ribosome is unclear at the moment. As there is less mTORC2 than active ribosomes in the cell, mTORC2 likely associates with a distinct pool or is recruited in the presence of specific signals that have yet to be identified. The nascent polypeptide itself may transduce a signal by its interaction with the ribosomal exit tunnel and induce conformational changes that permit binding to nascent chain regulatory proteins (Rospert, 2004; Baram and Yonath, 2005). We have shown that an intact mTORC2 is necessary for enhanced association with polysomes. The stable interaction of mTORC2 components with the tunnel exit protein rpL23a, but not with other ribosomal proteins, such as L23, L5, and S6, suggest that mTORC2 may bind in proximity to the nascent chain exit site (Kramer *et al*, 2009). Such an interaction would enable it to phosphorylate the TM site of Akt, as it emerges from the exit.

Could mTORC2 perform other functions in the translation complex that ultimately allows TM site phosphorylation? Like mTORC1, which regulates a number of translation-related events, including S6K phosphorylation (Proud, 2007; Ma and Blenis, 2009), mTORC2 may perform other functions during translation that could promote Akt–TM phosphorylation either directly or indirectly. Some reports proposed that a Pro-directed kinase (e.g., ERK, JNK, CDK) is the likely relevant direct TM site kinase (Shao *et al*, 2006; Alessi *et al*, 2009). So far, prolonged pharmacological treatment to inhibit activities of these kinases did not abolish TM site phosphorylation (data not shown), unlike the effects seen upon mTORC2 inhibition (Figure 3E; Facchinetti *et al*, 2008; Ikenoue *et al*, 2008). Whether other Pro-directed kinases may be mTORC2-dependent remains to be investigated.

Another possibility is that mTORC2 is involved in translation itself and that disruption of this function abrogates TM phosphorylation. Previous studies using mTOR active site inhibitors implicate mTORC2 in translation, as these inhibitors pronouncedly affect translation in comparison with the mTORC1 inhibitor, rapamycin (Feldman *et al*, 2009; Yu *et al*, 2009). Other studies proposed that Torin1-induced translation defects, based on 4E-BP phosphorylation, are instead due to rapamycin-independent mTORC1 function (Thoreen *et al*, 2009). In this study, we demonstrate that mTORC2-disrupted cells had aberrant translation readouts. Total translation examined at prolonged time points revealed both mTORC2- and mTORC1-dependence. The dephosphorylation of eEF2 was defective in mTORC2-deficient cells, indicating that mTORC2 may regulate either the kinase and/or phosphatase that control eEF2 phosphorylation. In SIN1$^{-/-}$ or mTORC2-inhibited cells, polysome recovery was also diminished (Figure 6B; Yu *et al*, 2009; Dowling *et al*, 2010). mTORC2 may also regulate other AGC protein kinases that could be involved in translation events. For example, cPKC can modulate translation by phosphorylation of eIF6 (Ceci *et al*, 2003), a protein that regulates the assembly of 80S ribosomes. Part of the defect in translation in SIN1$^{-/-}$ cells may speculatively be due to defective cPKC signalling. In summary, we have demonstrated here that Akt cotranslational TM phosphorylation is dependent on mTORC2 and that mTORC2 could have multiple roles during translation. Further studies are needed to determine other cotranslational substrates of mTORC2 and precisely how the two mTOR complexes orchestrate translation initiation and protein processing. Our findings imply that the cotranslational functions of mTORC2 could be tapped for therapeutics against cancer and particularly diseases caused by protein misprocessing, such as neurodegenerative and aging-related disorders.

Materials and methods

Plasmids, reagents

The open reading frame (ORF) of human Akt1 was subcloned into pET28a at the *Bam*HI and *Sal*I sites to generate His–Akt–His. The N-terminal His tag of His–Akt–His is also followed by additional 11 amino acids of T7 tag (Supplementary Figure S2). Details of the sequence of the different tagged Akt and PKCα templates used are summarized in Supplementary Figure S2. Myc-rpL5 (BC026934), -rpL23 (BC025918), and -rpL23a (BC125640) were generated by

subcloning the PCR product of murine ORF of each of these genes (clones obtained from Open Biosystems; Huntsville, AL) into pCI vector at the *Eco*RI and *Xba*I sites. HA–SIN1α in pMIGW vector and most antibodies used in these studies were described previously (Jacinto *et al*, 2006; Facchinetti *et al*, 2008). rpL23 antibody was generously provided by Dr Yanping Zhang (UNC, Chapel Hill); Torin1 was from Drs D Sabatini (Whitehead Inst.) and N Gray (Harvard). mTOR pSer2481, rpL7a, rpL26, rpS6, eEF2, eIF4B, and eIF4E antibodies were obtained from Cell Signalling (Danvers, MA); T7 antibody from Novus (Littleton, CO); eIF3β and rpL23a antibodies from Santa Cruz Biotechnology (Santa Cruz, CA); ubiquitin antibody from Invitrogen (Carlsbad, CA). CHX, RNAse, and puromycin were obtained from Sigma (St Louis, MO). DSP and MG132 were purchased from Pierce (Rockford, IL) and Tocris (Ellisville, MO), respectively. siRNA constructs for raptor and rictor were obtained from Dharmacon (Lafayette, CO).

Cell culture, fractionation, and polysome analysis

MEFs, HeLa, or HEK293 cells were cultured, transfected, stimulated, and harvested as described previously (Jacinto *et al*, 2006). To generate stable HA–SIN1α-reconstituted MEFs, the pMIGW retroviral vector containing HA–SIN1α was transfected into Phoenix cells by calcium phosphate method. Supernatants containing the retrovirus were collected 48 and 72 h later. SIN1$^{-/-}$ MEFs were incubated with virus containing medium in the presence of polybrene (10 μg/ml, Sigma). Two days later, the cells were selected under puromycin (1 μg/ml, Sigma). Cells used for fractionation were seeded and grown for 48 h in DMEM supplemented with 10% serum (normal or basal conditions). Normally, growing cells were starved of serum overnight and resuspended in PBS for 30 min before restimulation with serum for 60 min, and thereafter incubated with either CHX (100 mg/ml; 30 min or 2 h), then lysed in hypotonic buffer (20 mM potassium acetate, 12 mM magnesium acetate, 20 mM Tris–HCl, pH 7.4) by Dounce homogenization (35 strokes). Cell debris and nuclei were removed by centrifugation at 14 000 *g* (2 × 5 min). Where indicated, cell extracts were incubated with RNase (50 ng; 20 min, 37°C). To analyse wild-type versus SIN1$^{-/-}$ MEF polysomes, cells were treated with DSP (70 μg/ml) and incubated for 20 min at room temperature. Cells were harvested as above, except cell debris was further treated with 0.5 ml hypotonic buffer with 0.3% CHAPS and homogenized using 35 strokes. Supernatants were combined and the A_{260} of lysates was measured. 10–30 O.D. units of lysates were layered on a 10 ml 17–47% (wt/vol) sucrose gradient (10 mM sodium chloride, 12 mM magnesium chloride, 20 mM Tris–HCl, pH 7.4) and centrifuged for 4 h at 23 000 r.p.m. in a AH-629 Sorvall rotor. The A_{260} was monitored and recorded using density gradient fractionator (Brandel, Gaithersburg, MD). Fractions were concentrated to equal volume by Vivaspin concentrator (Sartorius, Elk Grove, IL). For serum repletion, cells grown to confluency were replaced with fresh media containing 10% serum with the addition of either DMSO or Torin1.

Metabolic labelling

MEFs (3×10^6) were seeded into 12-well dishes and cultured for 24 h. Cells were starved as indicated followed by incubation with [^{35}S]methionine/cysteine mixture in DMEM and serum (Perkin-Elmer, Waltham, MA) at different times. For SIN1$^{-/-}$ cells expressing empty vector or HA–SIN1β, cells were starved 24 h after transfection. The cells were lysed in 70 μl of suspension buffer (0.5% NP40 and 0.1 M NaCl in PBS). The supernatant and the pellet were separated after centrifugation for 10 min at 4°C. Supernatant was subjected to trichloroacetic acid (TCA) precipitation. 30 μg of supernatant was mixed with 20% TCA, then incubated on ice for 1 h. Precipitated proteins were recovered by centrifugation at 13 000 r.p.m. for 15 min and washed once with 100% acetone to remove unincorporated radioactivity. Protein pellets were resolubilized in 30 μl of PBS containing 2% SDS. Twenty μl of the protein solution was mixed with 5 ml scintillation fluid and counted in 1209 RackBeta liquid scintillation counter for 1 min. Experiments were carried out in triplicates.

Immunoprecipitation, pull-down assay, and immunoblotting

Unless otherwise indicated, for immunoprecipitation or pull-down assay of total cell lysates, cells were harvested using lysis buffer (40 mM HEPES, pH 7.9, 120 mM NaCl, 1 mM EDTA) containing 0.3% CHAPS. Lysates (300–500 μg) were incubated for 90 min with indicated antibodies, and immune complexes were collected by further incubation with protein A/G-Sepharose beads for 1 h. After incubation, the beads were washed three times with lysis buffer. For pull-down assays, HEK293 cells were transfected with HA–mTOR. HEK293 or MEF cell lysates were incubated with GST fusion proteins bound to glutathione-sepharose, then washed twice with binding buffer (0.2% NP40, 1 mM DTT, 20% glycerol, 1 × protease inhibitor, in PBS). For co-immunoprecipitation of RNase-treated extracts, before addition of antibody, extracts were centrifuged at 14 000 *g* for 5 min and supernatants were recovered. 1% Triton X-100 was added to supernatants followed by the addition of immunoprecipitating antibody and Protein A/G agarose slurry. Immunoblots were performed using antibodies as described above.

Cap-binding assay

HeLa cells were serum-starved overnight followed by restimulation with insulin (100 nM). Cells were lysed in low-salt lysis buffer (10 mM Hepes pH 7.2, 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EGTA, 20 mM NaF, 0.1 mM Na_3VO_4) containing 0.5% NP40 and 0.1% Brij35. Beads were washed three times in lysis buffer.

Coupled in vitro translation and kinase assay

In vitro transcription/translation was performed using PURExpress system II kit (NEB, Ipswich, MA), according to the manufacturer's instruction in a 25 μl reaction volume containing purified bacterial translation factors with 200 ng Akt template for 1 h unless noted otherwise. *In vitro* translation using rabbit reticulocyte lysates was performed using TNT T7-coupled transcription/translation system (Promega, Madison, WI), according to the manufacturer's directions. Because of slight variations in efficiency of the commercial kits, the maximal amount of translated Akt varies with incubation time from one kit to another. However, amount of translation product is typically maximal after 2 h. Immunoprecipitated HA–mTOR (purified from 2 mg lysate) expressed in HEK293 cells was included in each bacterial translation reaction as indicated. After 1 h incubation at 37°C, the reaction was stopped by addition of SDS–sample buffer.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Conflict of interest

The authors declare that they have no conflict of interest.

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