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Activation of mTORC2 by Association with the Ribosome

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SUMMARY

The target of rapamycin (TOR) is a highly conserved protein kinase and a central controller of growth. Mammalian TOR complex 2 (mTORC2) regulates AGC kinase family members and is implicated in various disorders, including cancer and diabetes. Here, we investigated the upstream regulation of mTORC2. A genetic screen in yeast and subsequent studies in mammalian cells revealed that ribosomes, but not protein synthesis, are required for mTORC2 signaling. Active mTORC2 was physically associated with the ribosome, and insulin-stimulated PI3K signaling promoted mTORC2-ribosome binding, suggesting that ribosomes activate mTORC2 directly. Findings with melanoma and colon cancer cells suggest that mTORC2-ribosome association is important in oncogenic PI3K signaling. Thus, TORC2-ribosome interaction is a likely conserved mechanism of TORC2 activation that is physiologically relevant in both normal and cancer cells. As ribosome content determines growth capacity of a cell, this mechanism of TORC2 regulation ensures that TORC2 is active only in growing cells.

INTRODUCTION

Target of rapamycin (TOR) is a central controller of cell growth and metabolism in response to nutrients, growth factors, and energy status. TOR is found in two structurally and functionally distinct multiprotein complexes termed TOR complex 1 (TORC1) and TORC2 (Wullschleger et al., 2006). The TOR complexes, originally described in yeast (Loewith et al., 2002), are conserved across all eukaryotes and regulate a wide spectrum of cellular processes that mediate cell growth (Laplante and Sabatini, 2009; Soulard et al., 2009; Wullschleger et al., 2006; Yang and Guan, 2007). In mammalian cells, mammalian TORC1 (mTORC1) contains mTOR, raptor, and mLST8 and is sensitive to the immunosuppressant and anticancer drug rapamycin (Hara et al., 2002; Kim et al., 2002; Loewith et al., 2002). mTORC1 controls transcription, ribosome biogenesis, protein synthesis, lipid synthesis, nutrient transport, autophagy, and other growth-related processes. The best-characterized substrates of mTORC1 are S6K and 4E-BP via which mTORC1 controls translation (Sonenberg and Hinnebusch, 2009). mTORC2 consists of mTOR, rictor, mSIN1, mLST8, and PRR5/PRR5L (also known as protor1 and 2) and is insensitive to rapamycin, although long-term rapamycin treatment can indirectly inhibit mTORC2 in some cell types (Cybulski and Hall, 2009; Jacinto et al., 2004; Sarbassov et al., 2004; Sarbassov et al., 2006; Sparks and Guertin, 2010). mTORC2 directly phosphorylates and activates the AGC kinases Akt (also known as PKB), SGK1, and likely PKC (Facchinetti et al., 2008; García-Martínez and Alessi, 2008; Hresko and Mueckler, 2005; Ikenoue et al., 2008; Jacinto and Lorberg, 2008; Sarbassov et al., 2004; Sarbassov et al., 2005). mTORC2 promotes cell survival via Akt and mediates organization of the actin cytoskeleton (Cybulski and Hall, 2009; Sparks and Guertin, 2010).

Both mTORC1 and mTORC2 are activated by growth factors, including insulin, IGF-1, and others. Growth factors activate mTORC1 via phosphatidylinositol 3-kinase (PI3K), PDK1, Akt, the TSC1-TSC2 complex, and Rheb, a small guanosine triphosphate (GTP)-binding protein that binds and activates mTORC1 directly (Avruch et al., 2009; Manning and Cantley, 2003). In contrast, the mechanism via which growth factors activate mTORC2 has been elusive. Growth factors activate mTORC2 via PI3K (Frias et al., 2006; García-Martínez and Alessi, 2008; Yang et al., 2006), but signaling steps beyond PI3K are distinct from those upstream of mTORC1 and unknown (Cybulski and Hall, 2009; Sparks and Guertin, 2010). The nature of the upstream regulators of TORC2 in unicellular model organisms such as yeasts, which lack a growth factor signaling pathway, is completely unknown (Soulard et al., 2009).

Here, we describe a genetic screen in yeast and subsequent studies in mammalian cells that identify the ribosome as an activator of TORC2. We demonstrate that the ribosome, independent of protein synthesis, is required for mTORC2 signaling in vivo and mTORC2 kinase activity in vitro. Active mTORC2 is associated with the ribosome. Insulin stimulates the association of mTORC2 with the ribosome via PI3K signaling. Findings with cancer cells suggest that ribosome-dependent mTORC2 activation is physiologically relevant in tumors with hyperactive PI3K signaling.

RESULTS

A Genetic Screen Reveals that NIP7 Is Required for TORC2 Signaling in Yeast

To identify upstream activators of TORC2, we performed a genetic screen in the budding yeast *S. cerevisiae*. Furthermore,

we assumed that whatever activates TORC2 in yeast would also be upstream of mTORC2 in mammals. In other words, we assumed that growth factor signaling was grafted onto a heretofore unknown ancestral input controlling TORC2 in unicellular yeast. This reasoning was supported by the fact that growth factor signaling was grafted onto the ancestral nutrient input in the case of TORC1.

mTORC2 phosphorylates the hydrophobic motif in the AGC kinase SGK1 and thereby activates SGK1. In yeast, TORC2 similarly phosphorylates and activates the SGK1 ortholog YPK2. Our genetic screen was based on the observation that overexpression of constitutively active YPK2 ($YPK2^{D239A}$, hereafter referred to as YPK2*) suppresses the lethality of a TORC2 defect (Aronova et al., 2008; Kamada et al., 2005). Assuming that YPK2* would also suppress lethality caused by a defect in an upstream activator of TORC2, we performed a so-called reverse suppressor screen to isolate yeast mutants that depend on YPK2* for viability (Figure 1A and Experimental Procedures). This is referred to as a reverse suppressor screen because it starts with a suppressor mutation (YPK2*) to identify unknown "suppressee" mutations—the reverse order of a normal (forward) suppressor screen. The screen was predicted to identify loss-of-function mutations in an essential upstream activator of TORC2 or in the essential components of TORC2, including TOR2, AVO1 (mSIN1 ortholog), and AVO3 (rictor ortholog). The screen yielded a total of 44 independent mutants defective in TORC2, thereby validating the screen. The 44 mutants consisted of 25, 13, and 6 *tor2*, *avo1*, and *avo3* mutants, respectively. In addition, we obtained a temperature-sensitive *nip7* mutant that we hereafter refer to as *nip7-1* (Figure 1A and Figures S1A and S1B). Sequence analysis of the *nip7-1* allele identified a point mutation that converts glycine 71 to aspartic acid. Western blot analysis of extracts from *nip7-1* cells showed that the point mutation mildly and strongly decreased NIP7 protein levels at permissive temperature (25°C) and nonpermissive temperature (37°C), respectively (Figure S1A). YPK2*, but not wild-type YPK2, suppressed the growth defect of the *nip7-1* mutant at semipermissive temperature (30°C and 34°C) (Figure S1B). NIP7 is an essential protein required for maturation of rRNA of the 60S ribosomal subunit (Zanchin et al., 1997). Confirming this role of NIP7 in ribosome biogenesis, we observed a reduction in the amounts of the 60S subunit, the 80S ribosome, and polysomes, with a concomitant appearance of halfmer polysomes, in extracts of the *nip7-1* mutant grown at semipermissive temperature (30°C) (Figure S1C).

To examine further whether NIP7 is required for TORC2 signaling, we investigated whether the *nip7-1* mutant phenocopied TORC2 mutants. The *nip7-1* mutant indeed exhibited several defects similar to those observed in temperature-sensitive TORC2 mutants (*avo3-1* and *tor2-21*) (Aronova et al., 2008; Beeler et al., 1998; Facchinetti et al., 2008; Helliwell et al., 1998; Kamada et al., 2005; Schmidt et al., 1997). First, the *nip7-1* mutant exhibited reduced signaling through the cell wall integrity pathway, as evidenced by decreased MPK1 phosphorylation and PKC1 protein levels, depolarization of the actin cytoskeleton, and restoration of growth in the presence of the osmotic stabilizer sorbitol (Figure 1B and Figure S1D). Second, the *nip7-1* mutant showed impaired sphingolipid biosynthesis, including hypersensitivity to myriocin, an inhibitor of the first step in the sphingolipid biosynthetic pathway, and restoration of growth in the presence of Ca^{2+} in a *csg2Δ* background (Figure S1E). Third, the *nip7-1* mutant showed decreased YPK2 kinase activity, as measured by an in vitro kinase assay with immunopurified YPK2 and, as a control, kinase-dead $YPK2^{K373A}$ (Figure 1C and data not shown for kinase dead). Finally and most importantly, TORC2 kinase activity was reduced in the *nip7-1* mutant, as measured by an in vitro kinase assay with TORC2 immunopurified from wild-type cells and *nip7-1* mutant cells grown at 30°C (Figure 1D). The above results strongly suggest that NIP7 is required, directly or indirectly, for TORC2 kinase activity and signaling.

mNIP7 Is Required for mTORC2 Activity and Ribosome Maturation in Mammals

Yeast NIP7 shares 75% identity with an uncharacterized mammalian protein also termed NIP7 (Figure S2A). We examined whether mammalian NIP7 (mNIP7) is required for mTORC2 signaling. mTORC2 directly phosphorylates Ser473 in the hydrophobic motif of Akt and Ser422 in the hydrophobic motif of SGK1 and thereby activates Akt and SGK1 toward substrates such as FoxO3a (Thr32) and NDRG1 (Thr346), respectively (García-Martínez and Alessi, 2008; Sarbassov et al., 2005). mTORC2 also autophosphorylates sites in rictor (Sarbassov et al., 2004; Jacinto et al., 2004) and is required for phosphorylation of Thr450 in the turn motif of Akt (Facchinetti et al., 2008; Ikenoue et al., 2008). Knockdown of mNIP7 in HeLa and HEK293 cells strongly decreased basal and insulin-stimulated phosphorylation of Ser473 and Thr450 in Akt, Thr32 in FoxO3a, Thr346 in NDRG1, and rictor in mTORC2 (Figure 2A, Figure S2B, and data not shown). mNIP7 knockdown had no effect on Erk phosphorylation or mTORC1-dependent S6K phosphorylation (Figure 2A). These findings suggest that mNIP7 is specifically required for mTORC2 signaling. Finally, we observed that mNIP7 knockdown reduced (by 58%) mTORC2 kinase activity in vitro, with no effect on mTORC2 amount or integrity (Figure 2B). These findings indicate that mNIP7 is required for mTORC2 activity and signaling, and not for mTORC2 synthesis, assembly, or stability. Furthermore, NIP7 is required for TORC2 signaling in both yeast and mammalian cells, suggesting a conserved mechanism of TORC2 activation.

Is mNIP7 a 60S maturation factor like NIP7 in yeast? Knockdown of mNIP7 reduced the amounts of the 60S ribosomal subunit and the 80S ribosome (80S), with no effect on the amount of the 40S subunit (Figure S2C). Knockdown of mTORC2 component mSIN1 had no effect on the ribosome profile (Figure S2C), indicating that the effect of mNIP7 on ribosome maturation was not due to a defect in signaling downstream of mTORC2. Thus, NIP7 is conserved from yeast to human as a 60S ribosome maturation factor and a TORC2 activator.

Ribosomes, but Not Protein Synthesis, Are Required for mTORC2 Signaling

Does mNIP7 control mTORC2 via its role in ribosome maturation? To address this question, we examined whether ribosome content affects mTORC2 signaling. Knockdown of ribosomal protein Rpl7 (60S subunit) reduced the amounts of the 60S

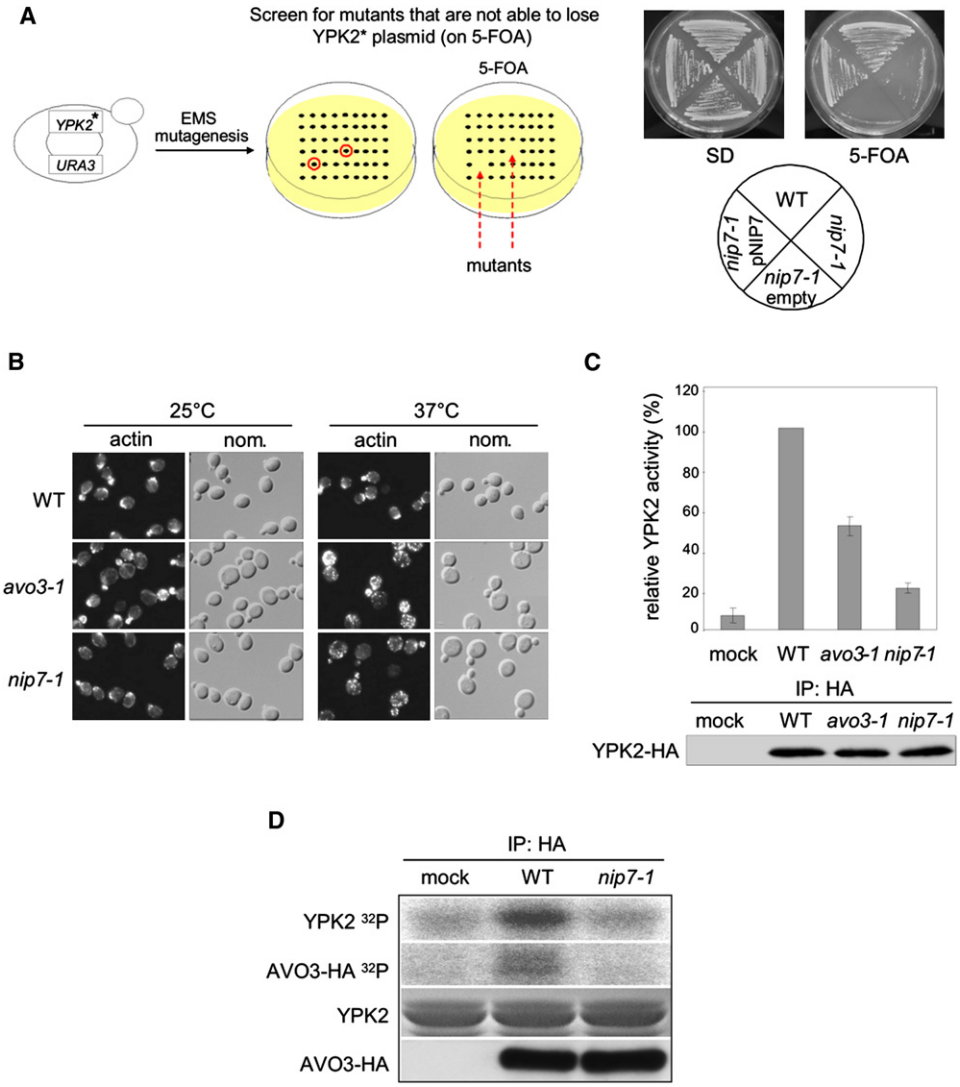

Figure 1. NIP7, Identified by a Reverse Suppressor Screen, Is Required for TORC2 Activity In Vivo and In Vitro

(A) Schematic representation of the reverse suppressor screen. A wild-type strain (JK9-3d**a**) overexpressing constitutively active YPK2* ($YPK2^{D239A}$) on an *URA3*-based plasmid was mutagenized with ethyl-methanesulfonate (EMS). Mutants that could not grow on plates containing 5-FOA were chosen for further analysis. A plasmid-borne *NIP7* gene (pNIP7) was isolated by complementation of the *nip7-1* mutation.

(B) *nip7-1* mutant exhibits depolarization of actin cytoskeleton. Wild-type (JK9-3d**a**), *avo3-1* (BAS65-2a), and *nip7-1* (DS1) cells grown in YPD at 25°C were shifted to 37°C for 6 hr. The actin cytoskeleton was stained with rhodamine-coupled phalloidin. A representative figure of three independent experiments for each strain is shown.

(C) *nip7-1* mutant shows decreased YPK2 activity. Wild-type cells (JK9-3d**a**) (mock) and wild-type, *avo3-1*, or *nip7-1* cells expressing plasmid-borne YPK2-HA (pYPK2-HA) were grown in YPD at 30°C. YPK2-HA was immunoprecipitated and subjected to in vitro kinase assay using cross-tide as a substrate. Substrate phosphorylation was quantified, and the average ± standard deviation from the mean based on three independent experiments is shown. Immunoprecipitated YPK2-HA was detected by western blotting (bottom).

(D) *nip7-1* mutant shows decreased TORC2 kinase activity in vitro. Wild-type cells (JK9-3d**a**; mock) and wild-type (DS2; WT) or *nip7-1* (DS3; *nip7-1*) cells expressing AVO3-HA were grown in YPD at 30°C. AVO3-HA was immunoprecipitated and subjected to a TORC2 kinase assay in vitro using recombinant YPK2 protein as a substrate. Also shown are Coomassie blue-stained total YPK2 protein and immunoblot of immunoprecipitated AVO3-HA.

See also Figure S1.

subunit and assembled ribosomes (80S monosomes and polysomes), whereas knockdown of ribosomal protein Rps16 (40S subunit) reduced the amounts of the 40S subunit and assembled ribosomes (Figure S2C). We also observed that knockdown of Rpl7 or Rps16 reduced the amounts of other proteins in the corresponding ribosomal subunit (Figure 3A and Figure S3A), consistent with the published finding that ribosomal subunit assembly determines the level of ribosomal proteins (Idol et al., 2007). Like knockdown of mNIP7, knockdown of either Rpl7 or Rps16 decreased basal and insulin-stimulated

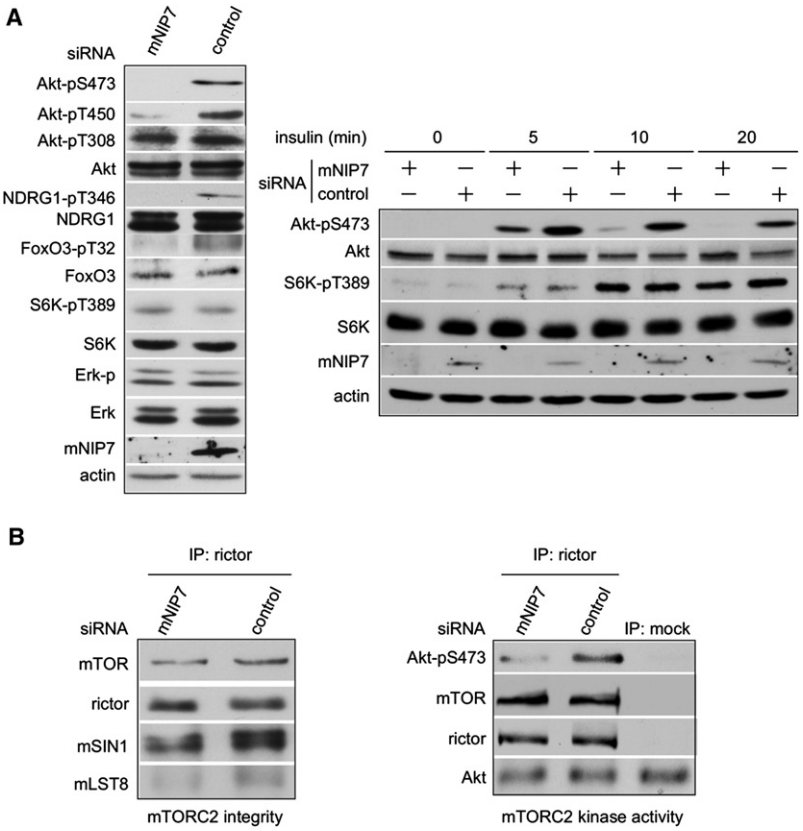

Figure 2. mNIP7 Is Required for mTORC2 Activity In Vivo and In Vitro

(A) siRNA-mediated knockdown of mNIP7 inhibits mTORC2 signaling. HeLa cells, 48 hr after transfection with the indicated siRNA, were harvested (left) or serum starved for 3 hr and then restimulated with insulin for the indicated times before harvesting (right). Phosphorylation and protein levels were determined by immunoblotting with the appropriate antibodies, as indicated.

(B) Knockdown of mNIP7 inhibits mTORC2 kinase activity toward Akt with no effect on mTORC2 integrity. rictor immunoprecipitates were immunoblotted with the indicated antibodies to determine mTORC2 integrity. mTORC2 in vitro kinase assay was performed using immunopurified mTORC2 (rictor) and recombinant, kinase-dead Akt as a substrate.

See also Figure S2.

phosphorylation of Akt (Ser473 and Thr450), FoxO3a (Thr32), NDRG1 (Thr346), and rictor, with no effect on Erk or S6K phosphorylation (Figure 3A, Figure S2B, and Figure S3A). Finally, we observed that Rpl7 knockdown reduced mTORC2 kinase activity, as determined by an in vitro kinase assay with immunopurified mTORC2 and recombinant, kinase-dead Akt as a substrate. Rpl7 knockdown had no effect on mTORC2 amount or integrity, as determined by unaffected coimmunoprecipitation of mTOR, mSIN1, or mLST8 with rictor (Figure 3B). Thus, assembled ribosomes (not 60S or 40S ribosomal subunits alone) are required for mTORC2 kinase activity and signaling. Furthermore, the above findings suggest that mNIP7 controls mTORC2 via its role in ribosome maturation.

mTORC2 promotes cell survival via phosphorylation and activation of Akt, which in turn phosphorylates and inhibits proapoptotic Bad (Brazil et al., 2004; Datta et al., 1997; Jacinto et al., 2006; Yang et al., 2006). To investigate further the physiological relevance of ribosome-mediated mTORC2 regulation, we examined the effect of mNIP7, Rpl7, or Rps16 knockdown on induction of apoptosis by etoposide or hydrogen peroxide (H_2O_2). Both treatments induce cell death in an Akt-sensitive manner (He et al., 2010; Kim et al., 2001; Wang et al., 2000). Knockdown of mNIP7, Rpl7, or Rps16 enhanced the induction of apoptosis by etoposide or hydrogen peroxide, as indicated by an increase in caspase 3 and PARP cleavage and a decrease in cell viability (Figure 4A and Figure S4). Knockdown of mSIN1 (mTORC2) similarly enhanced apoptosis. Knockdown of Bad blocked the proapoptotic effect of mSIN1, mNIP7, Rpl7, or Rps16 knockdown (Figure 4B). Thus, ribosomes appear to regulate mTORC2 signaling in a physiologically relevant manner.

Although ribosomal knockdown reduces mTORC2 kinase activity and signaling without affecting mTORC2 synthesis, is the process of protein synthesis per se required for mTORC2 activation? To address this question, we examined whether the protein synthesis inhibitors salubrinal, cycloheximide, anisomycin, or puromycin acutely inhibit mTORC2 signaling. Salubrinal blocks translation initiation by selective inhibition of eIF2α dephosphorylation (Boyce et al., 2005; Cnop et al., 2007) (Figure S3B). Cycloheximide, anisomycin, and puromycin inhibit translation elongation. Unlike knockdown of mNIP7, Rpl7, or Rps16, salubrinal, cycloheximide, anisomycin, and puromycin had no effect on basal or insulin-stimulated Akt Ser473 phosphorylation (Figures S3B–S3D and Figure S5C). All drugs were used under conditions known to affect protein synthesis in the expected manner, as determined by polysome gradient analysis (Figures S3B–S3D and data not shown). Thus, ribosomes mediate mTORC2 signaling independently of protein synthesis.

Active mTORC2 Is Associated with the Ribosome

To investigate the molecular mechanism by which ribosomes activate mTORC2 signaling, we examined by four unrelated methods whether ribosomes and mTORC2 physically interact. First, we determined whether mTORC2 coimmunoprecipitates with ribosomal protein Rpl26. Endogenous Rpl26 coimmunoprecipitated with endogenous mTOR, rictor, mSIN1, and ribosomal protein Rpl7 (Figure 5A), but not with raptor. This suggests that mTORC2, but not mTORC1, associates with the ribosome. Second, we determined whether mTORC2 copurifies with total ribosomes isolated by sedimentation through a sucrose cushion (see Experimental Procedures). mTOR and rictor, but not raptor, cosedimented with Rpl26, Rpl7, and Rps16 (Figure 5B). Third, although ribosomes activate mTORC2 independently of protein synthesis, we also determined whether mTORC2 cosediments

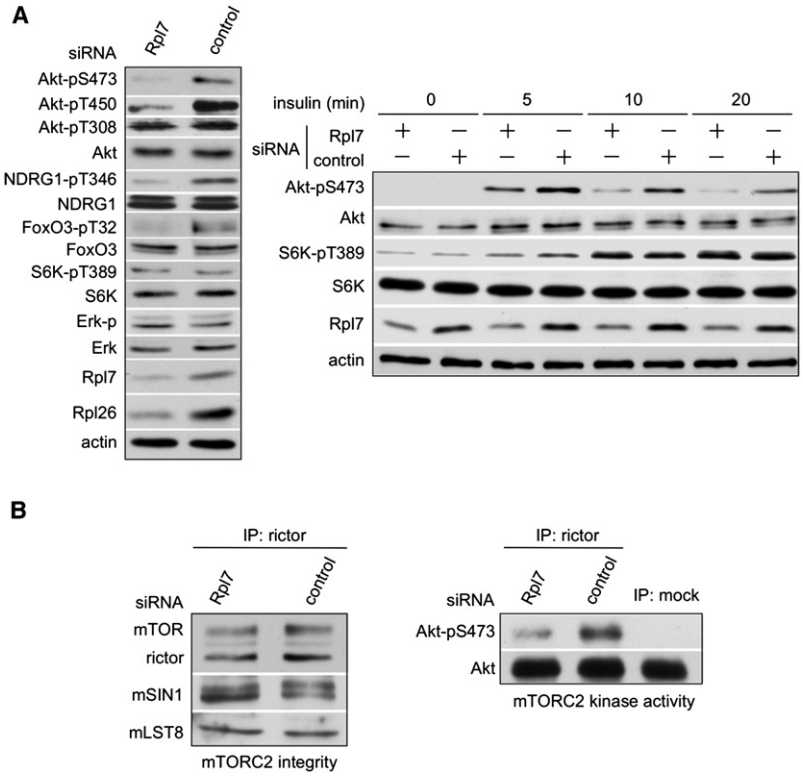

Figure 3. Ribosomes Are Required for mTORC2 Activity and Signaling

(A) siRNA-mediated knockdown of Rpl7 inhibits mTORC2 signaling. HeLa cells, 24 hr after transfection with the indicated siRNA, were harvested (left, basal activity) or serum starved for 3 hr and then restimulated with insulin for the indicated times before harvesting (right, insulin-stimulated activity). Phosphorylation and protein levels were determined by immunoblotting with the appropriate antibodies, as indicated.

(B) Knockdown of Rpl7 inhibits mTORC2 kinase activity toward Akt with no effect on mTORC2 integrity. rictor immunoprecipitates were immunoblotted with the indicated antibodies. mTORC2 in vitro kinase assay was performed using immunopurified mTORC2 (rictor) and recombinant, kinase-dead Akt as a substrate.

See also Figure S3.

with polysomes in a sucrose gradient. Lysates were fractionated in a sucrose gradient to separate polysomes from 80S, 60S, and 40S ribosomes. mTOR, rictor, and mSIN1 were found in both the polysomal and ribosomal fractions, to the same extent as Rpl7, Rpl26, and Rps16 (Figure S5A). Finally, we determined whether mTORC2 copurifies with mRNA-bound ribosomes. mRNA-bound ribosomes were purified by pull-down of poly(A) mRNA with oligo(dT) cellulose (Figure S5B) (Ceci et al., 2003). In this experiment, mTOR and rictor copurified with Rpl26, Rpl7, and the 40S ribosomal protein RACK1. RNase A treatment of a lysate before pull-down prevented isolation of any of the above proteins (Figure S5B), confirming interaction of mTORC2 with mRNA-bound ribosomes. The above data taken together suggest that mTORC2 physically interacts with translating (mRNA-bound) and nontranslating 80S ribosomes. An interaction between mTORC2 and the 80S ribosome is also supported by published mass spectrometry studies that identified several large and small subunit ribosomal proteins in mTORC2 immunoprecipitates (Pearce et al., 2007; Thedieck et al., 2007). We also note that nonionic detergent was required during any of our four above ribosome purifications to detect copurification of mTORC2 and that protein synthesis inhibitors had no effect on the interaction between mTORC2 and Rpl26 (Figure S5C).

To obtain more insight into the mTORC2-ribosome interaction, we performed coimmunoprecipitation experiments after knockdown of mTORC2 subunits or ribosomal proteins. First, the amount of mTOR in Rpl26 immunoprecipitates from rictor or mSIN1 knockdown cells was significantly reduced compared to control cells (Figure S6A), suggesting that mTORC2 interacts with the ribosome via rictor and/or mSIN1. We could not distinguish a requirement specifically for rictor or mSIN1 because knockdown of either rictor or mSIN1 alone also results in loss of the other. Second, knockdown of Rpl7 (60S subunit) abolished the interaction between mTORC2 and Rpl26, whereas knockdown of Rps16 (40S subunit) only moderately decreased the interaction between mTORC2 and Rpl26 (60S subunit) (Figure S6B). These results suggest that mTORC2 associates with the ribosome via rictor and/or mSIN1 binding to the 60S subunit.

The above findings suggest that ribosomes bind and activate mTORC2. To test this model further, we investigated whether ribosome-bound mTORC2 is indeed active. We performed an mTORC2 kinase assay with ribosomes purified either by Rpl26 immunoprecipitation or by sedimentation through a sucrose cushion, as described above. In both cases, a ribosome-associated kinase phosphorylated Ser473 in recombinant, kinase-dead Akt (Figure 5C). The mTOR inhibitor PP242 (Feldman et al., 2009) abolished the in vitro phosphorylation of Ser473 (Figure 5C), confirming that the ribosome-associated Ser473 kinase was mTORC2. Thus, ribosome-associated mTORC2 is active. Furthermore, we analyzed whether mTORC2 kinase activity is required for mTORC2-ribosome interaction. Treatment of HeLa cells with PP242 abolished Ser473 phosphorylation, as expected, but had no effect on mTORC2-Rpl26 interaction (Figure S6D), suggesting that mTORC2 activity is not required for mTORC2-ribosome association.

Insulin-PI3K Signaling Stimulates mTORC2-Ribosome Association

We next investigated whether mTORC2 association with the ribosome is regulated by insulin. Insulin treatment of serum-starved cells significantly increased the amount of mTOR and rictor that coimmunoprecipitated with Rpl26 (Figure 6A). Importantly, this stimulation of the interaction between mTORC2 and Rpl26 correlated with phosphorylation of the mTORC2 target site Ser473 in endogenous Akt. Thus, insulin stimulates mTORC2-ribosome association.

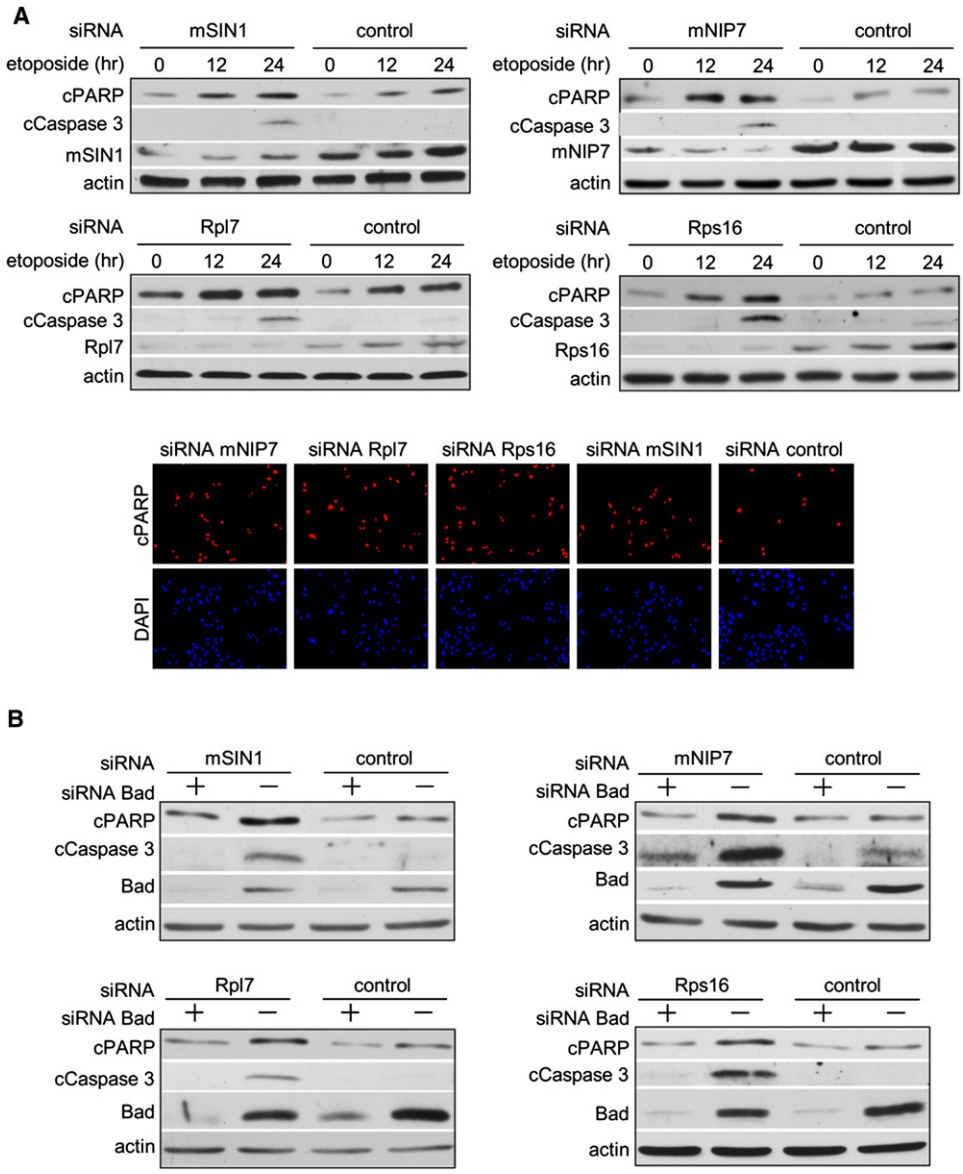

Figure 4. Ribosome or mTORC2 Knockdown Enhances Stress-Induced, Bad-Dependent Apoptosis

(A) siRNA-mediated knockdown of mSIN1, mNIP7, Rpl7, or Rps16 increases etoposide-induced apoptosis. HeLa cells were transfected with the various siRNAs and then treated with 25 μM etoposide for the indicated times (top) or for 24 hr (bottom). Extracts were analyzed by immunoblotting to assess efficiency of siRNA knockdown and induction of apoptosis with the indicated antibodies (top). Apoptosis was assessed by blotting for cleaved PARP (cPARP) and cleaved caspase 3 (cCaspase 3). Cells were fixed and stained with DAPI and cleaved PARP antibody to detect apoptotic cells (bottom).

(B) Bad is required for enhanced apoptosis in cells with knockdown of mSIN1, mNIP7, Rpl7, or Rps16. HeLa cells were transfected with the indicated siRNA and then treated with 25 μM etoposide for 24 hr. Extracts were analyzed by immunoblotting to assess the induction of apoptosis with cleaved PARP antibody. The efficiency of Bad knockdown is shown.

See also Figure S4.

Insulin activates mTORC2 via PI3K (Frias et al., 2006; García-Martínez and Alessi, 2008; Yang et al., 2006). To investigate whether PI3K signaling regulates mTORC2 association with the ribosome, we examined whether the mTORC2-Rpl26 interaction was affected upon inhibition or hyperactivation of PI3K signaling. Inhibition of PI3K strongly decreased mTORC2-Rpl26 interaction, as determined by a reduction in the amount of mTOR and rictor that coimmunoprecipitated with Rpl26 in lysates from cells treated with the PI3K inhibitor LY294002 (Figure 6B and Figure S6D). In contrast, hyperactivation of PI3K signaling by knockdown of PTEN, a negative regulator of PI3K signaling, increased the amount of mTOR and rictor in Rpl26 immunoprecipitates, compared to control cells (Figure 6C). Again, the change in mTORC2-Rpl26 interaction upon PI3K inhibition or hyperactivation was paralleled by a corresponding change in phosphorylation of the mTORC2 target site Ser473

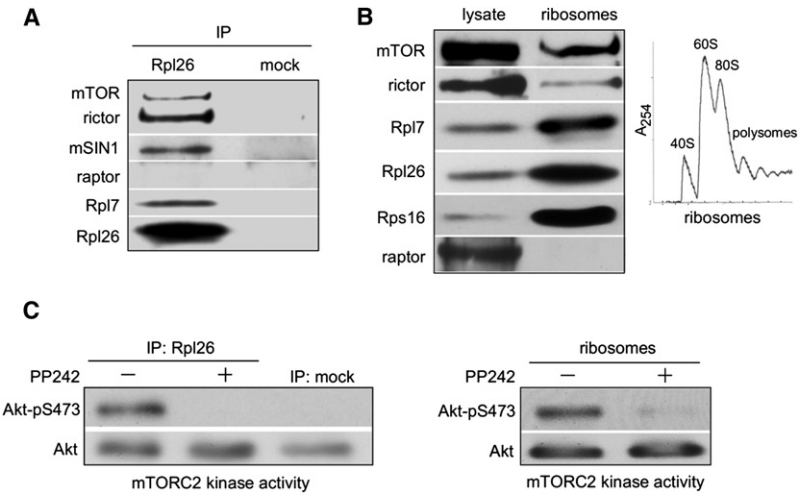

Figure 5. Active mTORC2 Is Associated with the Ribosome

(A) Endogenous Rpl26 coimmunoprecipitates with endogenous mTOR, rictor, and mSIN1. Rpl26 and mock immunoprecipitations were performed with HeLa cell extracts and analyzed by immunoblotting with the indicated antibodies.

(B) mTORC2 associates with ribosomes. Ribosomes were purified from HeLa cells by sedimentation through a sucrose cushion. Ribosomes were probed with the indicated antibodies (left) or were resedimented through a sucrose gradient to monitor 40S, 60S, and 80S ribosomes and polysomes. The absorbance profile of the sucrose gradient was determined at 254nm (right).

(C) Ribosome-associated mTORC2 is active. mTORC2 kinase assays were performed with Rpl26 or mock immunoprecipitates using recombinant, kinase-dead Akt as a substrate and in the presence or absence of the mTOR inhibitor PP242 (left). mTORC2 kinase assay was performed with ribosomes purified as described in (B), with recombinant, kinase-dead Akt as a substrate and in the presence or absence of PP242 (right).

See also Figure S5.

in endogenous Akt (Figures 6B and 6C). Taken together, the above data suggest that insulin stimulates mTORC2-ribosome association in a physiologically relevant manner via PI3K.

mTORC2-Ribosome Interaction Promotes Akt Signaling in Cancer Cells

PI3K-dependent association of mTORC2 with the ribosome, ribosome-mediated mTORC2 activation, and the physiological relevance of mTORC2-ribosome interaction in promoting cell survival prompted us to analyze mTORC2-ribosome association in cancer cells with hyperactive PI3K signaling. First, we examined whether PTEN-deficient metastatic melanoma cells have elevated mTORC2 activity. Approximately 60% of metastatic melanomas have reduced PTEN expression and elevated Akt phosphorylation (Robertson, 2005; Stahl et al., 2004). We analyzed PTEN expression and Akt Ser473 phosphorylation in

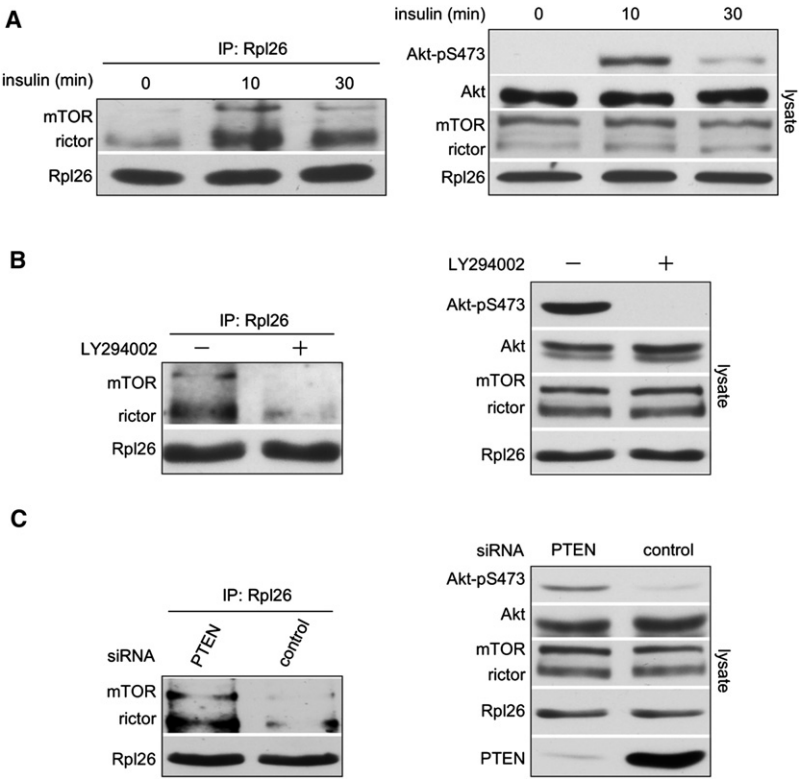

Figure 6. Insulin-PI3K Signaling Stimulates mTORC2-Ribosome Association

(A) Insulin stimulates mTORC2-ribosome association. HeLa cells were serum starved and then restimulated with insulin for the indicated time. Rpl26 immunoprecipitates (IP: Rpl26) and cell extracts (lysate) were immunoblotted with the indicated antibodies.

(B) PI3K inhibition decreases mTORC2-ribosome association. HeLa cells were treated with LY294002 (50 μM final concentration) for 30 min before harvesting. Rpl26 immunoprecipitates and cells extracts (lysate) were immunoblotted with the indicated antibodies.

(C) siRNA-mediated knockdown of PTEN increases mTORC2-ribosome association. HeLa cells were transfected with the indicated siRNA and then harvested after 48 hr. Rpl26 immunoprecipitates and cell extracts (lysate) were analyzed by immunoblotting with the indicated antibodies.

See also Figure S6.

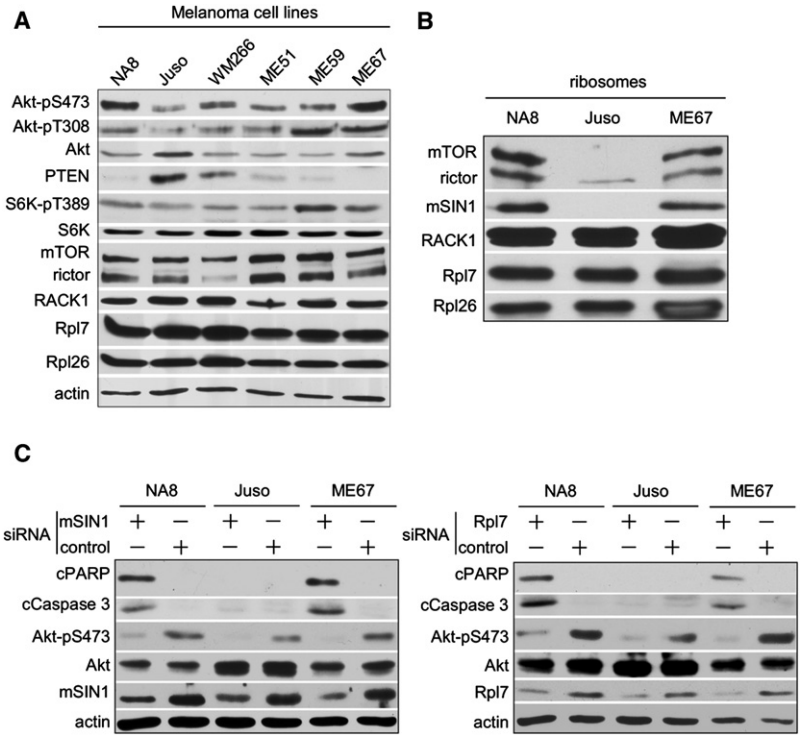

Figure 7. mTORC2-Ribosome Interaction Promotes Akt Signaling in Cancer Cells

(A) Akt Ser473 phosphorylation inversely correlates with PTEN expression in human melanoma cells. Cell extracts from six human melanoma cell lines were analyzed by immunoblotting with indicated antibodies.

(B) PTEN-deficient cell lines exhibit increased mTORC2-ribosome association. Ribosomes were purified from PTEN-deficient (NA8 and ME67) and PTEN-positive melanoma cell lines (Juso) by sedimentation through a sucrose cushion. Ribosomes were probed with the indicated antibodies.

(C) Knockdown of mSIN1 or Rpl7 induces apoptosis in PTEN-deficient (NA8 and ME67) melanoma cell lines. Cells were transfected with the indicated siRNA and harvested after 72 hr. Extracts were analyzed by immunoblotting to check the efficiency of the knockdown and the induction of apoptosis (PARP and caspase 3 cleavage) with the indicated antibodies.

See also Figure S7.

six human metastatic melanoma cell lines generated from different patients (Certa et al., 2001; Deschodt-Lanckman et al., 1990; Gervois et al., 1996). PTEN expression inversely correlated with Akt Ser473 phosphorylation (Figure 7A). For further study, we chose the two cell lines NA8 and ME67 with low PTEN expression and high Akt Ser473 phosphorylation (mTORC2 activity) and, as a control, the cell line Juso with the opposite signaling profile. To examine mTORC2-ribosome association, ribosomes were isolated from the three cell lines and probed for mTOR, rictor, and mSIN1. In all cases, the total yield of ribosomes obtained by sedimentation through a sucrose cushion was similar. However, the PTEN-deficient NA8 and ME67 cells exhibited significantly increased levels of mTORC2 associated with the ribosomal fraction as compared to the PTEN-positive Juso cells (Figure 7B), consistent with our PTEN knockdown studies in HeLa cells described above (Figure 6C). We also examined mTORC2-ribosome interaction in colon cancer cells harboring an activating mutation in the PI3K gene *PIK3CA*. The *PIK3CA* mutant cell lines HT29 and HCT116 exhibited both Akt Ser473 hyperphosphorylation (mTORC2 activity) and increased mTORC2-ribosome association as compared to SW60 colon cells harboring an unaltered *PIK3CA* gene (Figure S7A). Thus, mTORC2-ribosome interaction correlates with mTORC2 activity in both melanoma and colon cancer cells.

To investigate further the physiological relevance of mTORC2 activation via ribosome association, we examined the effect of mSIN1 and Rpl7 knockdown on mTORC2 signaling and cell survival in NA8, ME67, and Juso cells. Knockdown of either mSIN1 or Rpl7 decreased Akt Ser473 phosphorylation (Figure 7C), supporting a role for ribosome-dependent activation of mTORC2 in cancer. Furthermore, knockdown of either mSIN1 or Rpl7 enhanced apoptosis both in the presence and absence of etoposide. Interestingly, the increase in apoptosis was more pronounced in the PTEN-deficient NA8 and ME67 cells than in Juso cells, suggesting that NA8 and ME67 cells are more "addicted" to mTORC2 (Figure 7C and Figure S7B). The above results taken together suggest that mTORC2-ribosome association, mediating PI3K-mTORC2-Akt signaling and cell survival, is functionally important in cancer cells.

DISCUSSION

We investigated the upstream regulation of TORC2. A genetic screen in yeast and subsequent studies in mammalian cells revealed that ribosomes are upstream of TORC2. In particular, we found the following. First, the genetic screen in yeast revealed that knocking down ribosome biogenesis inhibits TORC2 kinase activity in vitro (Figure 1D) and TORC2 signaling in vivo (Figures 1B and 1C and Figure S1). Second, the ribosome is required for mTORC2 activity in vitro (Figure 2B and Figure 3B) and mTORC2 signaling in vivo in mammalian cells (Figure 2A, Figure 3A, and Figure S3A). Knockdown of ribosome maturation factor mNIP7 or ribosomal proteins (Rpl7 or Rps16) in mammalian cells decreased mTORC2 kinase activity and mTORC2 signaling. Third, the ribosome (translating or nontranslating) interacts directly with mTORC2 (Figures 5 and Figure S5). mTORC2 copurified with ribosomes isolated by four independent methods. Furthermore, mTORC2 copurified with ribosomes isolated from growing cells or cells treated with protein synthesis inhibitors. Fourth, ribosome-bound mTORC2 is active (Figure 5C), and ribosome-free mTORC2 is inactive in vitro (Figure 2B, Figure 3B, and Figure S6B). Fifth, PI3K-dependent insulin signaling stimulates binding of the ribosome to mTORC2 (Figure 6 and Figure S6D) with the same kinetics that it stimulates mTORC2

activation (Figure 6A). Finally, the mTORC2-ribosome interaction correlates with mTORC2 activity in both melanoma and colon cancer cells (Figure 7 and Figure S7). Melanoma and colon cancer cells with high PI3K activity (due to loss of PTEN or an activating mutation in the PI3K gene) exhibited both enhanced mTORC2-ribosome interaction and increased mTORC2 activity. Our findings suggest that the translating or nontranslating 80S ribosome binds and activates mTORC2 in response to growth factor-stimulated PI3K signaling (Figure S7C). TORC2-ribosome association is a mechanism of TORC2 activation that is likely conserved from unicellular yeast to human. The ribosome is presumably a primordial activator of TORC2 onto which growth factor signaling was grafted during the evolution of multicellularity.

In a parallel and complementary study, Oh et al. also showed that mTORC2 associates with the ribosome (Oh et al., 2010). Furthermore, they showed that mTORC2 phosphorylates the Akt turn motif cotranslationally and the Akt hydrophobic motif posttranslationally. Thus, Oh et al. investigated the role of the mTORC2-ribosome interaction in downstream signaling by mTORC2. Our study addresses the separate issue of upstream regulation of mTORC2. We show that an mTORC2-ribosome interaction activates mTORC2, and this activation is independent of translation. In other words, an mTORC2-ribosome interaction activates mTORC2 regardless of whether mTORC2 is phosphorylating a substrate co- or posttranslationally. We note that Oh et al. did not examine a requirement for the ribosome in posttranslational phosphorylation.

A connection between ribosomes and TOR signaling is well established. TORC1 activates ribosome biogenesis and protein synthesis and inhibits autophagy as key readouts in the control of cell growth. Why should ribosomes control TORC2? Ribosome content determines growth capacity of the cell and TORC2 regulates growth-related processes. Thus, regulation of TORC2 by ribosomes ensures that TORC2 is not inappropriately activated in cells that are unable to grow. The above also implies that TORC1, via activation of ribosome biogenesis and inhibition of autophagy-mediated ribosome turnover, indirectly controls TORC2. Indeed, Sarbassov et al. (Sarbassov et al., 2006) have shown that inhibition of mTORC1 by long-term rapamycin treatment indirectly inhibits mTORC2. Our findings suggest that the effect of rapamycin on mTORC2 is due, at least in part, to a reduction in ribosome content. Interestingly, the literature also indicates that ribosomal defects induce apoptotic cell death, although the underlying mechanism is not understood (Warner and McIntosh, 2009). We find that ribosomal defects inhibit mTORC2 and its downstream effector Akt, which in turn leads to Bad-dependent apoptosis. Thus, our findings also provide a mechanism for the induction of apoptosis by a ribosomal defect.

Our findings suggest that ribosomes bind and activate mTORC2 directly. The fraction of total mTORC2 that associates with ribosomes varies depending on the cell type and the growth conditions. For example, under normal growth conditions, ~20% of total rictor (mTORC2) was associated with ribosomes in HeLa cells, whereas ~30%–40% of rictor was associated with ribosomes in PTEN-deficient cells such as melanoma and PTEN knockdown cells. Thus, ribosome association appears to be a major if not the sole mechanism of TORC2 activation. Consideration of the fraction of total ribosomes that associate with mTORC2 is also potentially informative. Given that ribosomes are 100- to 1000-fold more abundant than signaling kinases such as mTORC2, only a small fraction of total ribosomes bind mTORC2. This excess of ribosomes would require strong regulation of mTORC2-ribosome binding by upstream PI3K signaling to achieve physiologically relevant regulation of mTORC2. Alternatively, TORC2 could be regulated by a specific subpopulation of ribosomes. Previous studies have demonstrated that TORC2 is associated with membranes, including the endoplasmic reticulum (ER) and Golgi apparatus, and that mTORC2 isolated from ER microsomes phosphorylates Akt Ser473 in vitro (Drenan et al., 2004; Hresko and Mueckler, 2005; Liu and Zheng, 2007; Schroder et al., 2007; Sturgill et al., 2008). These findings suggest that TORC2 might associate specifically with membrane-bound ribosomes. In support of this notion, we observed that mTORC2 copurifies with ribosomes only when the ribosomes are isolated in the presence of detergent. Membrane-bound ribosomes constitute ~10% of total ribosomes and include both translating and nontranslating ribosomes (Seiser and Nicchitta, 2000). It is also interesting to note that Komili et al. (Komili et al., 2007) have proposed a ribosome code in which there is a specialization of ribosomes for specific cellular process.

Our findings are consistent with other studies proposing the ribosome as a kinase platform. The two kinases PKCβII and Pim-1 are associated with the ribosome via RACK1 and Rps19, respectively (Ceci et al., 2003; Chiocchetti et al., 2005; Grosso et al., 2008b). Overall, the ribosome appears to be a signaling platform for mTORC2 and other kinases. Furthermore, ribosomal proteins have been shown to modulate the activity of NF-κB, p53, and c-Myc (Lindström, 2009).

PI3K-Akt signaling is upregulated and contributes to tumorigenesis in ~60% of advanced-stage melanomas (Stahl et al., 2003, 2004). PTEN expression or Akt inhibition increases sensitivity of melanoma cells to apoptosis-inducing agents and prevents tumor development (Madhunapantula and Robertson, 2009; Stahl et al., 2004). Our data, with melanoma, cancer colon, and HeLa cells, suggest that PI3K signaling promotes Akt phosphorylation via stimulation of mTORC2-ribosome binding. Furthermore, disruption of the mTORC2-ribosome supercomplex selectively induces apoptosis in PTEN-deficient melanoma cells (Figure 7C). The extent to which cells of other cancers require ribosome-dependent mTORC2 activation is unclear, although we expect that other cancers driven by mutations promoting PI3K signaling may also depend on mTORC2-ribosome association. Disrupting the mTORC2-ribosome interaction may be a useful strategy in the treatment of melanomas, colon carcinomas, and possibly other cancers.

Several findings suggest that upregulation of the protein synthesis machinery contributes to the development of cancer and other diseases (Ruggero and Pandolfi, 2003). Consistent with our findings, a ribosomal protein deficiency inhibits Akt-driven tumorigenesis (Hsieh et al., 2010). Furthermore, the *myc* oncogene enhances ribosome biogenesis, and *myc* oncogenicity in mice can be blocked by mutations in ribosomal protein genes (Barna et al., 2008; Ruggero, 2009). Our findings and the observation that mTORC2 is required for tumor progression in

at least some cancers (Guertin et al., 2009; Gulhati et al., 2009; Masri et al., 2007) suggest that myc and increased ribosomal content may promote tumorigenicity via stimulation of mTORC2 and its downstream effector Akt.

Curiously, our genetic screen in yeast yielded 44 TORC2 mutants but only a single mutant (*nip7-1*) that was defective in ribosome biogenesis. Why did we not obtain more mutants that were defective in ribosomal maturation factors or ribosomal proteins? First, ribosomal genes are duplicated in yeast, thereby precluding identification of recessive, loss-of-function mutations in these genes. Second, YPK2 is downstream of the ribosome in activation of TORC2 but is not downstream of the ribosome in mediating protein synthesis, precluding full suppression of a ribosome biogenesis defect by YPK2*. YPK2* only partly suppresses the *nip7-1* mutation and only at semipermissive temperature.

EXPERIMENTAL PROCEDURES

Detailed protocols for apoptosis assays and statistical analyses can be found in the Extended Experimental Procedures.

Yeast Strains, Media, Kinase Assays, Actin Staining, and Antibodies

Yeast strains used in this study are listed in the Table S1. All strains are isogenic derivatives of JK9-3d**a**. Plasmids used in this study are described in Table S2. Standard techniques and media were used for yeast manipulation (Kamada et al., 2005; Loewith et al., 2002). Unless indicated otherwise, cells were grown in rich YPD medium. YPK2 and TORC2 kinase assays were performed as described previously (Casamayor et al., 1999; Kamada et al., 2005; Loewith et al., 2002; Wullschleger et al., 2005). Rhodamine phalloidin staining of polymerized actin was performed as described (Loewith et al., 2002; Mulet et al., 2006). Immunoprecipitations were performed as described previously (Loewith et al., 2002; Wullschleger et al., 2005).

Reverse Suppressor Screen

A wild-type strain (JK9-3d**a**) was transformed with an *URA3*-based plasmid overexpressing YPK2* (pYPK2*) (Figure 1A). Cells were randomly mutagenized with 100 mM ethyl-methanesulfonate (EMS) (Sigma) for 15 min in SD-Ura medium, washed, and then allowed to recover in SD-Ura medium without mutagen for 2–4 hr. Cells were plated on solid SD-Ura or SD supplemented with 5-FOA and incubated at 30°C. The SD medium supplemented with 5-FOA counterselected against *URA3* such that only those cells that had spontaneously lost the *URA3*-based pYPK2* plasmid were able to form a colony. Mutants that were unable to grow on SD 5-FOA (and hence in the absence of YPK2*) were isolated from the master SD-Ura plate. ~45,000 colonies from mutagenized cells were screened, from which 45 mutants were isolated and the corresponding mutations were identified. Mutated genes were isolated by complementation with a *LEU2* centromeric plasmid-based yeast genomic library. Complementing members of the genomic library were selected by growth on SD-Leu medium containing 5-FOA. Genomic inserts of library-derived plasmids were identified by sequencing. Complementation with subclones of isolated inserts identified the complementing ORF within a given insert. Sequencing of the genomic copy of *NIP7* and meiotic segregation studies confirmed that *NIP7* was indeed the relevant mutant gene. *nip7-1* was found to be temperature sensitive upon subsequent characterization. For experiments at semipermissive temperature (30°C), *nip7-1* cells were grown in YPD at permissive temperature (25°C), diluted to OD_{600} = 0.1, and grown at 30°C to approximately OD_{600} = 0.6–0.8. For experiments at the nonpermissive temperature (37°C), *nip7-1* mutant was grown in YPD at 25°C and then shifted to 37°C for 6 hr.

Cell Culture, Immunoprecipitations, Immunoblotting, and mTORC2 Kinase Assay

HeLa, melanoma cells, and colon cancer cells were cultured, transfected, stimulated, and harvested as described previously (Jacinto et al., 2004; Thedieck et al., 2007). In brief, cells were seeded and grown for 48 hr in DMEM supplemented with 10% serum (basal conditions). Cells were starved of serum for 3 hr before restimulation with 100 nM insulin (Sigma).

For mSIN1, mNIP7, Rpl7, Rps16, PTEN, or Bad knockdown, a pool of four different synthetic siRNA or of the appropriate control siRNA (Dharmacon) was used as described (Thedieck et al., 2007). All transfections were done according to the manufacturer's instructions (Lipofectamine, Invitrogen transfection).

Protein extracts were prepared as previously described (Jacinto et al., 2004; Thedieck et al., 2007), resolved on SDS-PAGE, and transferred to nitrocellulose membranes (Protran, Whatman). Immunoprecipitation, immunoblotting, and mTORC2 kinase assays were performed as previously described (Jacinto et al., 2004; Thedieck et al., 2007).

Polysome Profiles, Ribosome Purification, and Poly(A) mRNA Pull-Down

Polysome analysis using sucrose gradients was performed as described previously (Grosso et al., 2008b; Idol et al., 2007). For ribosome purification by sedimentation through a sucrose cushion, HeLa or melanoma cells were washed in PBS, trypsinized, and lysed in buffer A (50 mM Tris-HCl [pH 7.4], 100 mM NaCl, 30 mM $MgCl_2$, 0.3% CHAPS, 100 ug/ml cycloheximide, 40 U/ml RNase inhibitor, protease inhibitor cocktail, and 100 ug/ml cycloheximide). Whole-cell extracts were clarified at 4°C, 10 min at 15,000 × g. Extracts were loaded on a 30% sucrose cushion in 50 mM Tris-acetate (pH 7.5), 50 mM NH_4Cl, 12 mM $MgCl_2$, and 1 mM DTT and ultracentrifuged for 17 hr in a SW41Ti Beckman rotor at 39,000 rpm. For the mTORC2 kinase assay, the ribosomal pellet was resuspended in mTORC2 kinase buffer (Jacinto et al., 2004; Thedieck et al., 2007). Ribosome-sucrose gradient fractionation was performed as previously described (Grosso et al., 2008a). For poly(A) pull-down, HeLa cells were washed with PBS, trypsinized, and lysed in buffer A. Whole-cell extracts were clarified at 4°C, 10 min at 8000 × g. Lysates corresponding to 5 × 10^7 cells were incubated with oligo(dT) cellulose (Invitrogen) for 1 hr at room temperature. After incubation, the oligo(dT) cellulose was pelleted and washed five times with buffer A. The bound fraction was eluted with elution buffer (100 mM Tris [pH 7.4], 500 mM NaCl, 10 mM EDTA, 1% sodium dodecyl sulfate (SDS), and 5 mM DTT). Purified ribosome fractions and the bound and unbound fractions after poly(A) pull-down were concentrated with Vivaspin 500 (Sartorius Stedim) and analyzed by immunoblotting with the indicated antibodies.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, seven figures, and two tables and can be found with this article online at doi:10.1016/j.cell.2011.02.014.

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