

## Article

# Oxygen sufficiency controls TOP mRNA translation via the TSC-Rheb-mTOR pathway in a 4E-BP-independent manner

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**Cells encountering hypoxic stress conserve resources and energy by downregulating the protein synthesis. Here we demonstrate that one mechanism in this response is the translational repression of TOP mRNAs that encode components of the translational apparatus. This mode of regulation involves TSC and Rheb, as knockout of *TSC1* or *TSC2* or overexpression of Rheb rescued TOP mRNA translation in oxygen-deprived cells. Stress-induced translational repression of these mRNAs closely correlates with the hypophosphorylated state of 4E-BP, a translational repressor. However, a series of 4E-BP loss- and gain-of-function experiments disprove a cause-and-effect relationship between the phosphorylation status of 4E-BP and the translational repression of TOP mRNAs under oxygen or growth factor deprivation. Furthermore, the repressive effect of anoxia is similar to that attained by the very efficient inhibition of mTOR activity by Torin 1, but much more pronounced than *raptor* or *ric1* knockout. Likewise, deficiency of *raptor* or *ric1*, even though it mildly downregulated basal translation efficiency of TOP mRNAs, failed to suppress the oxygen-mediated translational activation of TOP mRNAs. Finally, co-knockdown of TIA-1 and TIAR, two RNA-binding proteins previously implicated in translational repression of TOP mRNAs in amino acid-starved cells, failed to relieve TOP mRNA translation under other stress conditions. Thus, the nature of the proximal translational regulator of TOP mRNAs remains elusive.**

**Keywords:** hypoxia, mTOR, TOP mRNAs, translational control, 4E-BP

### Introduction

TOP mRNAs, characterized by the presence of an oligopyrimidine tract at their 5' terminus (5' TOP motif) encode more than 90 proteins of the translational apparatus (Meyuhas and Drazzen, 2009). The translation of these mRNAs is selectively regulated by mitogenic (serum or insulin) and growth signals via the phosphatidylinositol 3-kinase (PI3K)-Akt-mammalian target of rapamycin (mTOR) pathway (Stolovich et al., 2002; Patursky-Polischuk et al., 2009). Activated Akt phosphorylates tuberous sclerosis complex 2 (TSC2) at multiple sites within the TSC1-TSC2 tumor suppressor heterodimer. This phosphorylation blocks TSC2 ability to act as a GTPase-activating

protein (GAP) for Ras-homolog enriched in brain (Rheb), thereby allowing Rheb-GTP to operate as an activator of mammalian target of rapamycin (mTOR) complex 1 (mTORC1) (Duran and Hall, 2012). Accordingly, deficiency of either TSC1 or TSC2 renders mTORC1 activity completely refractory to mitotic arrest (Sancak et al., 2008).

mTORC1 consists of mTOR, regulatory-associated protein of mTOR (raptor) and mammalian lethal with SEC13 protein 8 (mLST8) (Guertin et al., 2006; Ikenoue et al., 2008; Jewell et al., 2013). mTORC1 activates protein synthesis by direct phosphorylation of: (i) ribosomal protein S6 kinase (S6K) at T389 (Weng et al., 1998), which becomes fully active and affects the protein synthesis machinery (Meyuhas and Drazzen, 2009); and (ii) eukaryotic translation initiation factor (eIF) 4E-binding proteins (4E-BPs) at multiple sites, which consequently dissociates from and derepresses eIF4E (Gingras et al., 2001). Previous reports have demonstrated

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that translational control of TOP mRNAs by mitogens relies on TSC1, TSC2, Rheb, and mTOR (Bilanges et al., 2007; Patursky-Polischuk et al., 2009). However, while the translation of TOP mRNAs is controlled in an S6K-independent manner (Stolovich et al., 2002; Pende et al., 2004), it has recently been suggested that translational repression of these mRNA upon acute mTOR inhibition is mediated by active 4E-BPs (Thoreen et al., 2012).

mTOR operates also within a second multiprotein complex, mTORC2, which is composed of mTOR, rapamycin-insensitive companion of mTOR (rictor), mLST8, stress-activated protein kinase (SAPK)-interacting protein (Sin1) and protein observed with rictor (protor) (reviewed by Oh and Jacinto, 2011). This complex has been implicated in phosphorylation of AGC kinases (Akt, SGK1, and PKC) and IMP (Oh and Jacinto, 2011; Dai et al., 2013). Although only mTORC1 is acutely sensitive to the allosteric inhibitor rapamycin, newly developed competitive inhibitors that target the active site of mTOR have been shown to potently and directly inhibit both complexes (Benjamin et al., 2011).

TOP mRNAs are translationally regulated also by amino acid sufficiency (Tang et al., 2001; Stolovich et al., 2005) and this mode of regulation involves two stress granule (SG)-associated RNA-binding proteins, T-cell intracellular antigen-1 (TIA-1) and TIA-1-related (TIAR). Thus, binding of TIA-1 and TIAR to the first 39 nucleotides of TOP mRNAs in amino acid-starved cells is associated with selective accumulation and translational repression of these mRNAs in SG (Damgaard and Lykke-Andersen, 2011).

In addition to being regulated by mitogenic and nutritional stimuli, mTORC1 is downregulated by oxygen stress. Thus, hypoxia-induced upregulation of the transcription factor hypoxia-inducible factor 1 (HIF-1) (Semenza, 2009) is followed by transcriptional activation of the regulated in development and damage responses 1 (REDD1) gene (Shoshani et al., 2002). Consequently, the latter binds 14-3-3 and thereby alleviates the 14-3-3-mediated inhibition of TSC1-TSC2 complex (DeYoung et al., 2008). However, hypoxia can also inhibit mTORC1 independently of REDD1 via the induction of energy stress, possibly due to reduced oxidative phosphorylation. AMPK (AMP-dependent kinase) acts as a sensor of cellular energy status and is activated by an increase in the cellular AMP:ATP ratio. AMPK activation in response to increased AMP levels relies on its phosphorylation by the tumor suppressor LKB1 (Towler and Hardie, 2007) and leads to phosphorylation of TSC2 and raptor, and thereby it enhances inhibition of Rheb/mTORC1 (Inoki et al., 2003b; Gwinn et al., 2008).

Here, we set out to establish the pathway that transduces hypoxia signals that downregulate the translation efficiency of TOP mRNAs. Our results show that the pathway involves TSC, Rheb, and mTOR but does not rely on 4E-BP, mTORC1, or mTORC2 as well as TIA-1 and TIAR.

## Results

### *TOP mRNAs are translationally controlled by oxygen sufficiency*

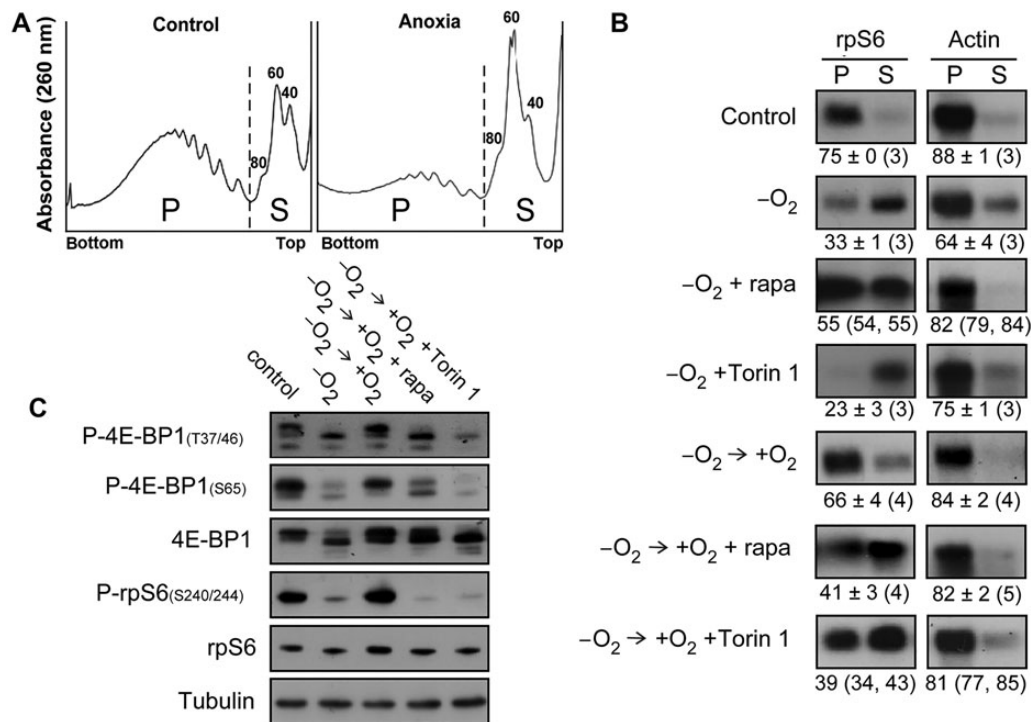
The biogenesis of the translational machinery is a highly energy-consuming process that relies on the availability of growth factors and nutrients (Tang et al., 2001; Stolovich et al., 2002). We assumed, therefore, that it should also reflect the cellular energy balance, and examined the translational behavior of a TOP mRNA in oxygen-deprived (anoxia) HEK293 cells. This treatment led to

repression of global protein synthesis, as can be judged from the shift of translationally engaged ribosomes (residing in polysomes) in untreated cells to the subpolysomal fraction under anoxic condition (Figure 1A). However, analysis of the impact of anoxia on an individual mRNA demonstrates that rpS6 mRNA, a typical TOP mRNA, was translationally repressed to a much greater extent than a typical non-TOP mRNA encoding  $\beta$ -actin, as demonstrated by its shift from polysomal to subpolysomal fractions. The translation of rpS6 mRNA was restored by 2 h of oxygen replenishment. Restoration of rpS6 mRNA translation was mTOR-dependent, as it was similarly blocked by rapamycin and Torin 1, selective mTOR allosteric and ATP-competitive inhibitors, respectively (Thoreen et al., 2009) (Figure 1B). Notably, this translational behavior parallels that of mTORC1 activity, as monitored by the phosphorylation of rpS6 and 4E-BP1, even though the latter was much less affected by rapamycin (Figure 1C), as previously noted (Choo et al., 2008). Apparently, the addition of rapamycin to oxygen-starved cells led to partial protection of rpS6 mRNA translation from the anoxic stress, yet the underlying mechanism is presently unclear. Contrarily, when Torin 1 was added to oxygen-deprived cells, it imposed further translational repression on this mRNA (Figure 1B).

### *The translation of TOP mRNAs is resistant to oxygen starvation in cells deficient for TSC2 or TSC1 or cells overexpressing Rheb*

Oxygen deficiency signals to TSC1-TSC2 via the HIF-1/REDD1 (DeYoung et al., 2008) and the LKB1/AMPK (Liu et al., 2006) pathways. We hypothesized, therefore, that deficiency of any of these four signaling proteins would derepress TOP mRNAs. However, results presented in Supplementary Figures S1 and S2 show that TOP mRNA translation in MEFs lacking Hif1 $\alpha$  (Ryan et al., 1998), REDD1 (Sofer et al., 2005), AMPK $\alpha$  (Laderoute et al., 2006) or LKB1 (Bardeesy et al., 2002) is repressed by anoxia, just as observed in wild-type MEFs. One plausible explanation is that an anoxic signal is transduced to TOP mRNAs in a TSC1-TSC2 complex-independent fashion, and therefore none of the aforementioned pathways is involved in this mode of regulation. To examine this possibility, we monitored the translation efficiency of TOP mRNAs in oxygen-deprived TSC1<sup>-/-</sup> or TSC2<sup>-/-</sup> MEFs. As in the case of serum starvation (Patursky-Polischuk et al., 2009), deficiency of TSC2 or TSC1 conferred nearly complete or partial resistance to anoxia, respectively, on TOP mRNA translation (Figure 2A) and on mTORC1 activity (Figure 2B). Furthermore, the anoxic stress seems to be transduced to TOP mRNA translation via a pathway(s) that does not exclusively rely on either HIF-1/REDD1 or LKB1/AMPK.

Previous reports have shown that TOP mRNAs can be derepressed in serum-starved cells by overexpression of wild-type or active Rheb (RhebL64), respectively (Patursky-Polischuk et al., 2009; Damgaard and Lykke-Andersen, 2011). To examine the involvement of Rheb in oxygen-mediated translational control of TOP mRNAs, HEK293 cells were co-transfected with a plasmid encoding human growth hormone mRNA that starts with the 5' TOP motif of mouse rpS16 mRNA (pS16-GH; Levy et al., 1991) and vectors expressing active RhebL64 or Rheb defective in its effector domain (Rheb-5A), and therefore, in the ability to stimulate mTORC1 (Inoki et al., 2003a). Indeed RhebL64, but not Rheb-5A,



**Figure 1** TOP mRNAs are translationally repressed by oxygen deprivation in an mTOR-dependent fashion. **(A)** Typical polysomal profiles and its portioning to polysomal and subpolysomal fractions. HEK293 cells were either untreated (control) or 16 h starved for oxygen (anoxia). The cytoplasmic extract was size fractionated by centrifugation through a sucrose gradient. The tube content was collected from the bottom and the absorbance at 260 nm was recorded. The vertical dashed line separates between the polysomal (P) fraction (left) and the subpolysomal (S) fraction (right). 80, 60, and 40 represent 80S monosomes, 60S, and 40S ribosomal subunits, respectively. **(B)** HEK293 cells were untreated (control); oxygen-deprived for 6 h ( $-O_2$ ) in the absence or presence of 20 nM rapamycin or 50 nM Torin 1; and oxygen replenished for 2 h ( $-O_2 \rightarrow +O_2$ ) in the absence or presence of 20 nM rapamycin or 50 nM Torin 1. Subsequently, cells were harvested and cytoplasmic extracts were prepared and subjected to polysomal analysis using cDNAs corresponding to rpS6 (a TOP mRNA) and actin (a non-TOP mRNA). **(C)** HEK293 cells were treated as in **B**, and their cytoplasmic proteins were subjected to western blot analysis using the indicated antibodies.

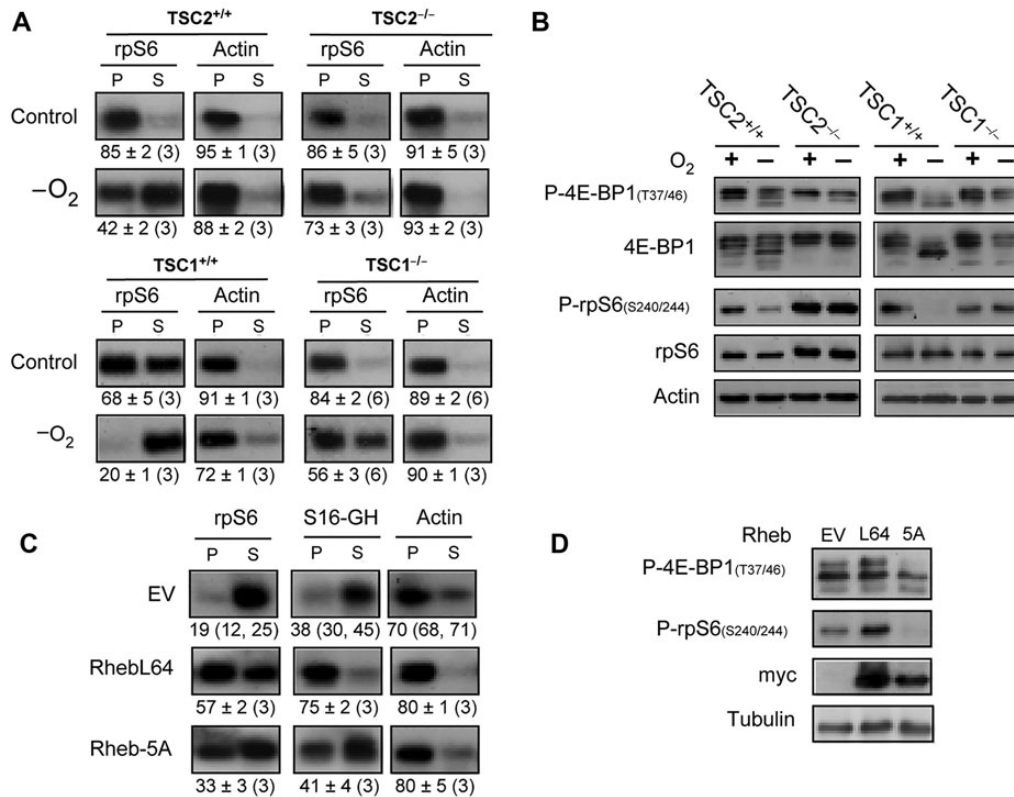
alleviated very efficiently the translational repression of chimeric (S16-GH) and endogenous (rpS6) TOP mRNAs, as well as mTORC1 activity in oxygen-starved cells (Figure 2C and D). This protective ability was not confined to RhebL64, as also wild-type Rheb and Rheb<sup>S130A</sup>, another active form of Rheb (Zheng et al., 2011), could alleviate the translational repression of rpS6 and mTORC1 activity under anoxic conditions (Supplementary Figure S3A and B).

#### 4E-BPs do not play a role in translational control of TOP mRNAs under physiologically relevant stresses

The apparent inhibition of oxygen-induced translational activation of TOP mRNAs by rapamycin (Figure 1B) and the relief by TSC deficiency or Rheb overexpression of anoxia-induced translational repression suggest signaling to TOP mRNAs through mTORC1. Furthermore, this notion is further supported by the tight correlation between the translation efficiency of these mRNAs and the phosphorylation status of rpS6 and 4E-BP (Figures 1 and 2). It has previously been shown that translation efficiency of TOP mRNAs relies neither on S6K1 nor on rpS6 phosphorylation, two downstream effectors of mTORC1 (Pende et al., 2004; Ruvinsky et al., 2005). Instead, it has recently been shown that Torin 1-induced translational repression of these mRNAs is selectively alleviated in 4E-BP deficient cells, thus implicating the latter as a major translational regulator of TOP mRNAs (Supplementary

Figure S4; Thoreen et al., 2012). To examine whether 4E-BP similarly mediates the translational repression of TOP mRNAs under oxygen starvation, we used 4E-BP DKO MEFs that lack all three 4E-BPs, as they do not express 4E-BP3 (Dowling et al., 2010). However, the 4E-BP deficiency in these cells failed to protect TOP mRNAs from oxygen deprivation (Figure 3A and C), or serum starvation for 14 h (Supplementary Figures S5 and S6) or 48 h (Figure 3B and C). Likewise, this deficiency failed to protect rpl32 mRNA from translational repression, even when Torin 1 was added for just 2 h, concomitantly with serum refeeding following 14 h starvation (Figure 4B). Contrarily, cyclin D3 mRNA, a non-TOP mRNA, whose translation relies on the availability of active eIF4E (Dowling et al., 2010), was protected from repression, whether induced by serum starvation or Torin 1 treatment, in 4E-BP deficient cells (Figure 4C). Finally,  $\beta$ -actin mRNA was refractory to serum starvation or Torin 1 treatment irrespective of the presence or absence of 4E-BP (Figure 4D).

In a complementary experiment we replenished 4E-BP DKO MEFs with a constitutively active variant of 4E-BP (4E-BP<sup>4Ala</sup>), whose mTORC1-sensitive phosphorylation sites were mutated to alanines, and therefore, it constitutively binds to and represses eIF4E (Rong et al., 2008). If, indeed, this protein functions as a selective repressor of TOP mRNAs, it would have constitutively inhibited the translation of these mRNAs, even in unstressed



**Figure 2** The deficiency of TSC2 or TSC1 or overexpression of Rheb can rescue TOP mRNAs from translational repression in oxygen-deprived cells. **(A)** TSC2<sup>+/+</sup> and TSC2<sup>-/-</sup> MEFs were either untreated (control) or oxygen-starved for 16 h (-O<sub>2</sub>), harvested and their cytoplasmic extracts were subjected to polysomal analysis. **(B)** Cytoplasmic proteins from MEFs treated as in **A** were subjected to western blot analysis. **(C)** HEK293T cells were transiently cotransfected with expression vectors encoding rpS16-GH or an empty vector (EV) pRK7, Myc-RhebL64 or Myc-Rheb-5A. After 32 h, cells were starved for oxygen (16 h), harvested, and subjected to polysomal analysis with probes directed toward endogenous (rpS6) and exogenous (S16-GH) TOP mRNAs, as well as actin mRNA. **(D)** Cytoplasmic proteins from cells treated as in **C** were subjected to western blot analysis.

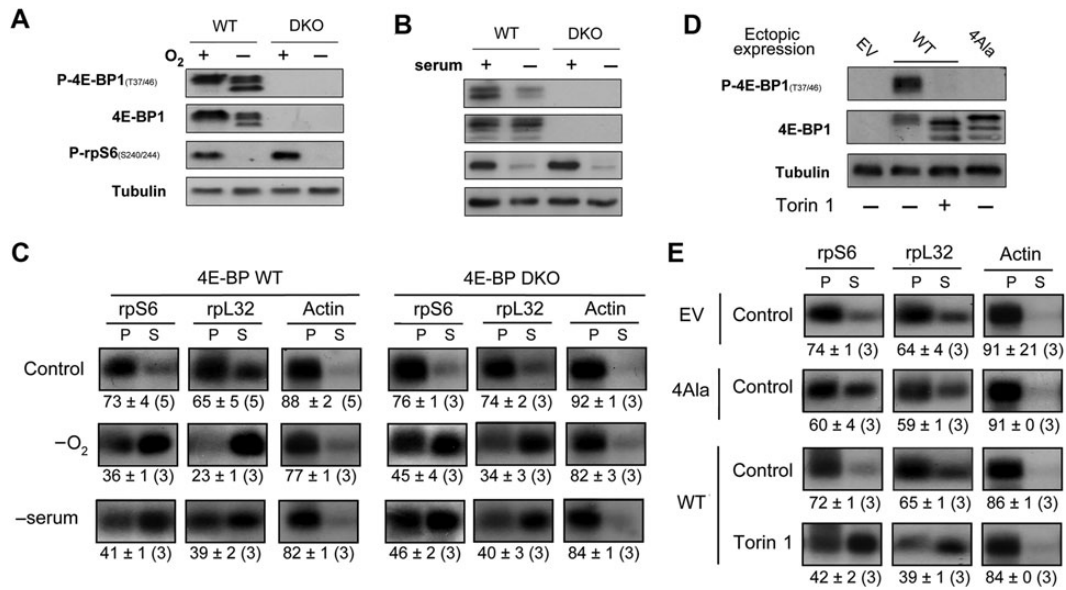
cells. However, instead, the effect of this mutant on the translation efficiency of TOP mRNAs is indistinguishable from that of wild-type 4E-BP1 (4E-BP<sup>WT</sup>), expressed in 4E-BP DKO MEFs. Evidently, the ability of exogenous 4E-BP<sup>WT</sup> to restore the sensitivity of TOP mRNAs to Torin 1 and the translational sensitivity of cyclin D3 mRNA to serum starvation of these cells attests to its proper expression (Figure 3D and E and Supplementary Figure S6).

Next, we applied a reciprocal approach and ask whether constitutive hyperphosphorylation of 4E-BP can protect TOP mRNA from anoxia-induced translational repression. To this end, we used Dicer deficient hemangiosarcoma cells (Dicer<sup>-/-</sup>). These cells show constitutive upregulation of insulin-like growth factor 1 receptor (IGF-1R) and its downstream targets, as exemplified by protection of phosphorylated Akt (Thr308) or 4E-BP1 (Thr37/46) under anoxic conditions (Figure 5A). Nonetheless, the translation of TOP mRNAs was repressed in these cells upon oxygen deprivation, just as observed for Torin 1-treated Dicer<sup>-/-</sup> cells, which completely eliminated the phosphorylation of 4E-BP1 (Figure 5B and C). Similar results were obtained with proliferating EBV-transformed human lymphoblastoid cell line L2, which displays constitutive repressed translation of rpS6 mRNA, despite apparent phosphorylated 4E-BP1 (yet Torin 1 sensitive, Figure 5D and E). Collectively, these 4E-BP loss- and gain-of-function experiments indicate that

4E-BP has no role in repression of TOP mRNA translation in cells deprived of oxygen or serum.

#### Translational activation of TOP mRNAs by oxygen is largely in a raptor- or rictor-independent fashion

The apparent rapamycin sensitivity of oxygen-mediated translational activation of TOP mRNAs (Figure 1B) coincides with a model that relates translational efficiency of these mRNAs with mTORC1 activity. However, data that disprove the involvement of the two well-characterized mTORC1 effectors 4E-BP and S6K (Figures 3–5) (Pende et al., 2004) prompted us to verify the role of mTORC1 in oxygen-mediated translational activation of TOP mRNAs. To this end, we used mouse MEFs, whose raptor gene could be conditionally knocked out by 4-hydroxytamoxifen (4HT)-inducible Cre recombinase fused to a 4HT responsive estrogen receptor (iRapKO). This gene knockout led to a pronounced decrease in raptor level and mTORC1 activity, as exemplified by the hypophosphorylation of 4E-BP1 and rpS6 (Figure 6A). Notably, this diminution might reflect the loss of *raptor* gene in 86% of cells if, indeed, homologous recombination occurred in both alleles. Nevertheless, *Raptor* knockout resulted in just 25% and 13% reduction in the basal translation efficiency of mRNAs encoding rpS6 and rpl32, respectively, in untreated cells (see control in



**Figure 3** 4E-BP deficiency failed to alleviate the translational repression of TOP mRNAs in oxygen-deprived cells. **(A and B)** 4E-BP WT and 4E-BP DKO MEFs were either untreated (+) or oxygen starved (–) for 12 h **(A)**, or either untreated (+) or serum starved (–) for 48 h **(B)**. Cytoplasmic proteins from these cells were subjected to western blot analysis with the indicated antibodies. **(C)** Cells treated as described in **A** and **B** were harvested and subjected to polysomal analysis with the indicated probes. **(D)** 4E-BP DKO MEFs were infected with an empty retroviral vector (EV) pBabe-puro or retroviral vector encoding either wild-type 4E-BP (WT) or 4E-BP<sup>4Ala</sup> (4Ala). After selection with puromycin, cells were either untreated or treated with 250 nM Torin 1 for 2 h and their cytoplasmic proteins were subjected to western blot analysis with the indicated antibodies. **(E)** Cells derived as described in **D** were harvested and subjected to polysomal analysis with the indicated probes.

Figure 6B and Supplementary Figure S7). Notably, the moderate inhibitory effect that could have been induced by 100% raptor knockout would have been augmented only marginally, but still comparable with that observed for rpS6 mRNA (16% reduction) upon *riCTOR* knockout (Figure 6C). Moreover, the mildness of the effect of *riCTOR* knockout is underscored by the fact that oxygen deprivation or very efficient mTOR inhibition by Torin 1 had a much greater effect on the polysomal association of rpS6 mRNA (from 72% to 31% or 37%, respectively) (Figure 6B).

Evidently, iRapKO MEFs untreated or treated with 4HT displayed a similar translational repression of rpS6 mRNA by oxygen deprivation that was not further affected by co-treatment with rapamycin (Figure 6B). In contrast, Torin 1 augmented the repressed translation efficiency of rpS6 in oxygen-starved MEFs, yet in a nonspecific fashion, as it also had a similar effect on the translation of non-TOP mRNA encoding actin. Remarkably, however, the loss of mTORC1 activity had no adverse effect on translational activation of rpS6 mRNA by oxygen to the basal translation efficiency that characterizes 4HT-treated iRapKO MEFs (Figure 6B).

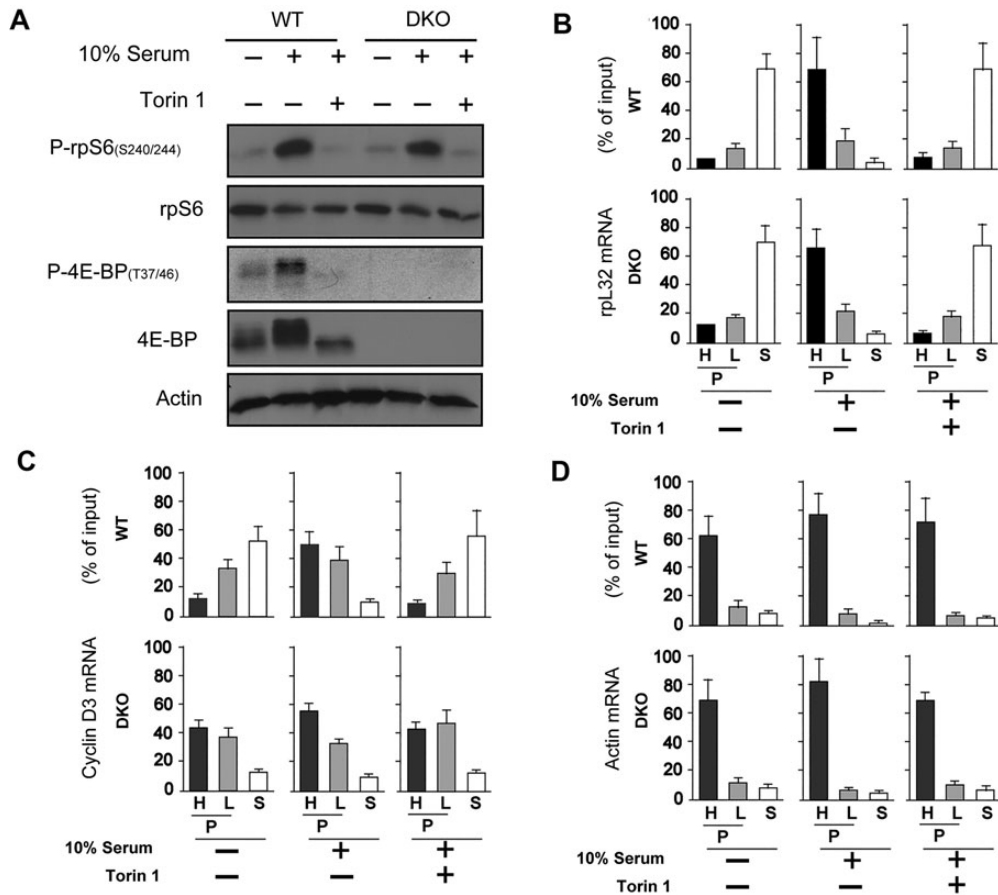
The rapamycin-sensitive translational activation of TOP mRNAs in oxygen-replenished cells (Figure 1B) seems *a priori* inconsistent with a role of mTORC2 in this mode of regulation. However, the higher sensitivity of rpS6 mRNA translation to Torin 1 than to rapamycin in uninduced iRapKO MEFs (Figure 6B) prompted us to examine the role of mTORC2 in this mode of regulation. The results presented in Figure 6A and C clearly show that oxygen-induced translational activation of rpS6 mRNA was completely refractory to the loss of rictor, and consequently to that of mTORC2 activity (as can be judged by the hypophosphorylation of Ser473 in Akt) in inducible *riCTOR* gene knockout MEFs (iRiCKO). Taken together, these results support the

notion that mTORC1 or mTORC2 are dispensable for resumption of the translational activation of TOP mRNAs to its basal level in raptor or rictor knockout MEFs, respectively.

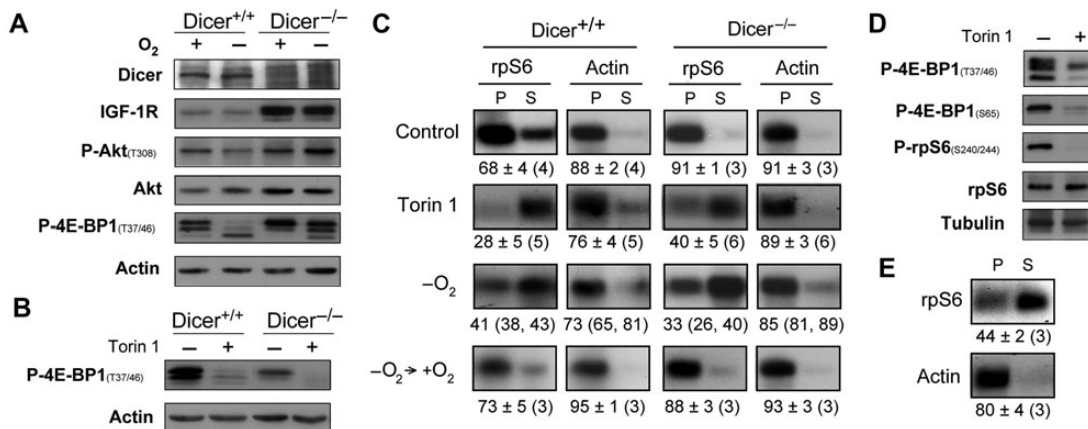
#### *TIA-1 and TIAR co-deficiency can relieve the translational repression of TOP mRNAs in amino acid-starved cells, but not in oxygen-deprived cells*

The coimmunoprecipitation of TOP mRNAs with TIA-1 and TIAR and the critical role of these proteins in translational repression of TOP mRNAs by amino acid starvation (Damgaard and Lykke-Andersen, 2011) suggest that TIA-1 and TIAR play a major role in this mode of regulation. To examine whether these proteins are involved also in anoxia-induced repression of TOP mRNA translation, we co-knocked down TIA-1 and TIAR with a lentivirus encoding shRNA directed against a common sequence within the corresponding mRNAs (Figure 7A). Indeed, TIA-1 and TIAR deficiency was able to rescue TOP mRNA translation upon amino acid withdrawal, but failed to do so if cells were starved for oxygen or both amino acids and serum (Figure 7B). These results suggest that the repressive role, played by TIA-1 and TIAR toward TOP mRNA translation, is confined to amino acid insufficiency, and is not part of a general repressive complex.

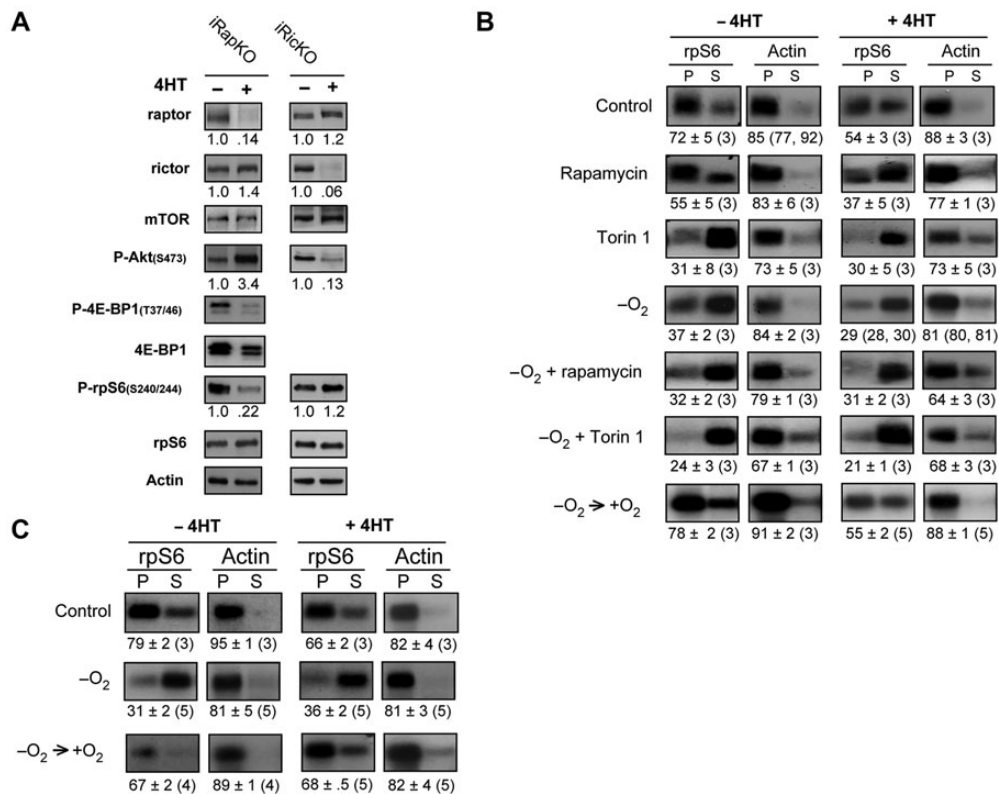
It has been shown previously that GCN2, a kinase that phosphorylates eukaryotic initiation factor 2 $\alpha$  (eIF2 $\alpha$ ) at Ser51, is essential for amino acid-induced translational repression of TOP mRNAs (Damgaard and Lykke-Andersen, 2011). Indeed, phosphorylation of eIF2 $\alpha$  was upregulated upon depriving WT MEFs (eIF2 $\alpha$ <sup>S/S</sup>) of oxygen (8 h), amino acids (16 h), or serum (48 h) (Figure 7C) concomitantly with translational repression of rpS6 mRNA (Figure 7D). Nonetheless, the fact that a similar repression was



**Figure 4** Translation of cyclin D3 mRNA, but not those encoding rpL32 and actin, is 4E-BP dependent. (A) Wild-type (WT) and 4E-BP DKO (DKO) MEFs were maintained in 0.5% FBS for 14 h and stimulated with 10% FBS in the absence or presence of 250 nM Torin 1 for 2 h. Levels and the phosphorylation status of the indicated proteins were determined by western blotting. (B–D) WT and DKO MEFs were treated as in A and the cytoplasmic extract was size fractionated by sucrose gradient centrifugation (Supplementary Figure S5 for polysomal profiles). Distribution of rpL32 (B), cyclin D3 (C), and  $\beta$ -actin (D) mRNAs among heavy (H,  $\geq 4$  ribosomes) and light (L, 2 to 3 ribosomes) polysomal fractions as well as subpolysomal fraction (S) was monitored by reverse transcriptase-quantitative PCR (RT-qPCR). Values are expressed as a percentage of total mRNA loaded onto the sucrose gradient (input). Data are presented as mean  $\pm$  SD (two independent biological replicates). Each biological replicate was carried out in three technical replicates.



**Figure 5** Hyperphosphorylation of 4E-BP failed to derepress TOP mRNA translation. (A and B) *Dicer*<sup>+/+</sup> and *Dicer*<sup>-/-</sup> hemangiosarcoma cells were either untreated (+), oxygen starved (-) for 12 h (A) or treated with 50 nM Torin 1 (+) for 3 h (B) and cytoplasmic proteins were subjected to western blot analysis with the indicated antibodies. (C) Cells treated as described in A and B as well as cells deprived of oxygen for 12 h and then resupplied with oxygen for 3 h were harvested and subjected to polysomal analysis. (D and E) Untreated L2 lymphoblastoids were subjected to western blot (D) and polysomal (E) analyses.



**Figure 6** Raptor and rictor are dispensable for translational activation of TOP mRNAs by oxygen. **(A)** iRapKO or iRicoKO cells were either untreated (–) or treated (+) with 4HT for 4 days and cytoplasmic proteins were subjected to western blot analysis with the indicated antibodies. The relative abundance of raptor, rictor, and phospho-Akt(Ser473) was normalized to that of actin, whereas phospho-rpS6(Ser240/244) was normalized to rpS6. The results are numerically presented relative to those obtained without 4HT and were arbitrarily set at 1. **(B)** iRapKO cells were either treated (for 4 days) or untreated with 4HT, and then kept under normoxia (control) in the absence or presence of 20 nM rapamycin or 50 nM Torin 1 for 3 h; deprived of oxygen for 10 h (–O<sub>2</sub>) in the absence or presence of 20 nM rapamycin or 50 nM Torin 1 for 3 h; or deprived of oxygen for 10 h and then subjected to oxygen supply for 3 h (–O<sub>2</sub> → +O<sub>2</sub>). **(C)** iRicoKO cells were either treated (for 4 days) or untreated with 4HT, and then kept under normoxia (control) deprived of oxygen for 10 h (–O<sub>2</sub>), or deprived of oxygen for 10 h and then subjected to oxygen supply for 3 h (–O<sub>2</sub> → +O<sub>2</sub>). Cells treated as in **B** and **C** were harvested and subjected to polysomal analysis.

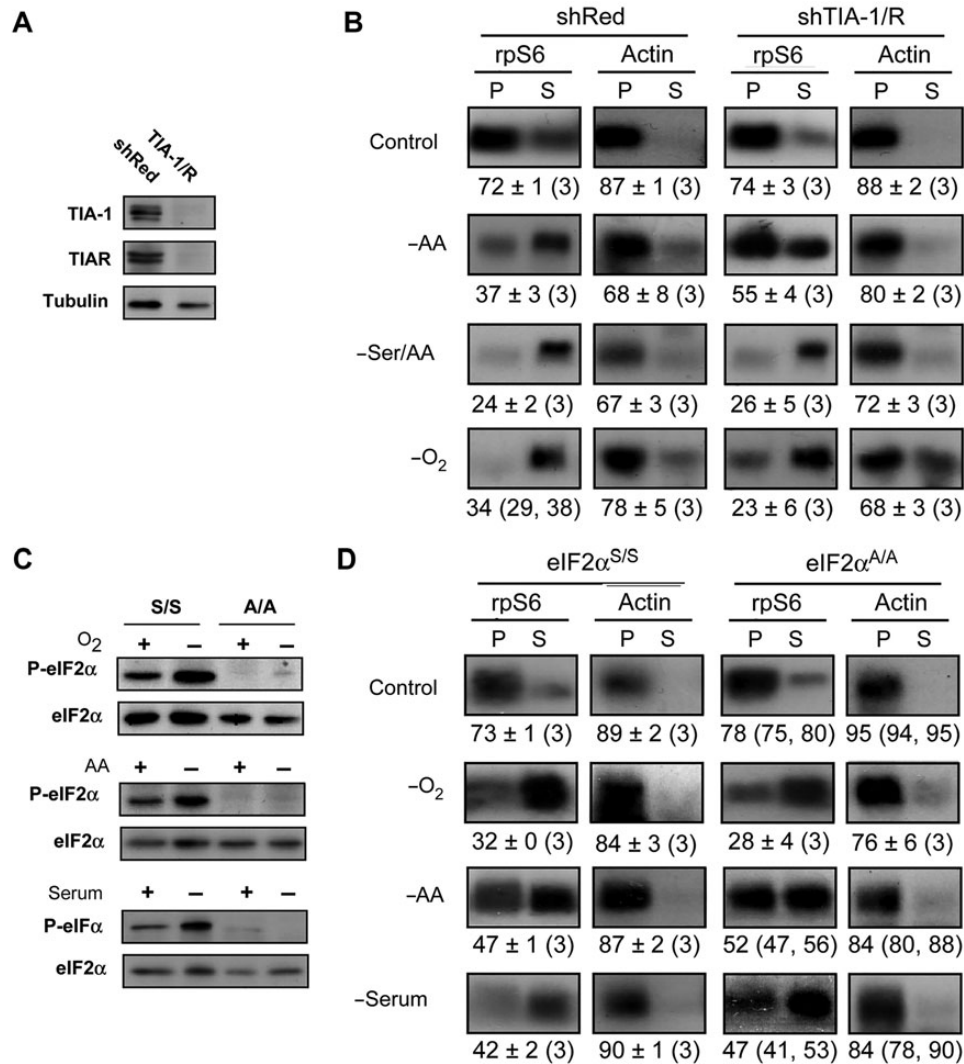
observed in eIF2 $\alpha^{A/A}$  MEFs (Scheuner et al., 2001), whose Ser51 was substituted by alanine, implies that eIF2 $\alpha$  phosphorylation is not involved in translational control of TOP mRNAs under any of the examined condition. Moreover, these results suggest that if, indeed, GCN2 plays a regulatory role in TOP mRNA translation, it does so in an eIF2 $\alpha$ -independent manner.

## Discussion

Biogenesis of the protein synthesis machinery, and particularly of ribosomes, is a highly resource-consuming process (Granneman and Tollervey, 2007). Thus, cells that encounter unfavorable conditions attenuate the production of components of the translational machinery and cease to grow (Pardee, 1989). Indeed, the present report demonstrates that mTOR-sensitive translational repression of TOP mRNAs is one mechanism that is exploited by cells to selectively downregulate wasteful biogenesis of the protein synthesis machinery under nutritional and oxygen stresses. This mechanism is highly relevant to tumor cells that exploit adaptive means to survive in hypoxic microenvironments. Indeed, chronic hypoxia exerts selective pressure in neoplastic cells that limits the growth of early lesions until the tumor starts to secrete pro-angiogenic factors, and thereby turns on the ‘angiogenic switch’ (Jubb et al., 2010).

The apparent protection of TOP mRNA translation from starvation for oxygen or serum in cells lacking either TSC1 or TSC2 (Figure 2; Patursky-Polischuk et al., 2009) suggests that these repressive signals converge at the TSC1-TSC2 complex. Nevertheless, we cannot exclude the possibility that the deficiency of the TSC1-TSC2 complex simply promotes an upregulation of a compensatory activity (Rheb?), and thereby relieves the translational repression under these stress conditions. Indeed, over expression of Rheb can similarly alleviate the translational repression of TOP mRNAs (Figure 2; Patursky-Polischuk et al., 2009).

The ability of AMPK1 and REDD1 to inhibit mTORC1 by their positive effect on the activity of the TSC1-TSC2 complex is well documented (Sengupta et al., 2010). However, data presented here show that the deficiency of AMPK or REDD1 and their modulators, LKB1 or HIF-1 $\alpha$ , respectively, failed to relieve the translational repression of TOP mRNAs under anoxia (Supplementary Figures S1 and S2). These seemingly opposing observations can be explained by the redundancy of the roles of AMPK and REDD1 deficiency in transducing the oxygen deficiency signal to translational repression of TOP mRNAs, and/or by the involvement of additional effectors that operate in parallel. Indeed, it has previously been shown that a hypoxia-inducible Bcl-2 homology 3 domain-containing



**Figure 7** Translational repression of TOP mRNAs by anoxia does not rely on TIA-1, TIAR, or phospho-eIF2 $\alpha$ . **(A)** HEK293 cells were infected with viruses expressing HcRed (red) shRNA or an shRNA that can co-target both TIA-1 and TIAR (TIA-1/R). After 48 h, cells were subjected to selection by puromycin and harvested at 48 h later. The abundance of TIA-1 and TIAR was monitored by western blot analysis of cytoplasmic proteins with the indicated antibodies. **(B)** HEK293 cells were infected and selected as described in **A** and then either kept untreated (control), amino acid starved for 8 h (-AA), amino acid starved during the last 3 h of 24 h serum starvation (-Ser/AA), or deprived of oxygen (-O<sub>2</sub>) for 16 h. Cells were harvested and their cytoplasmic extract were subjected to polysomal analysis. **(C)** eIF2 $\alpha$ <sup>S/S</sup> and eIF2 $\alpha$ <sup>A/A</sup> MEFs were untreated (+), Oxygen (O<sub>2</sub>) deprived for 16 h (-), amino acid (AA) starved for 16 h (-), or serum starved for 48 h (-). Cytoplasmic proteins were subjected to western blot analysis. **(D)** Cytoplasmic extracts from eIF2 $\alpha$ <sup>S/S</sup> and eIF2 $\alpha$ <sup>A/A</sup> MEFs, treated as described in **C**, were subjected to polysomal analysis.

protein (Bnip3) and a p38-regulated/activated kinase (PRAK) directly downregulate Rheb and inhibit mTORC1 activity under hypoxia or energy depletion (Li et al., 2007; Zheng et al., 2011). Hence, the exact nature of the pathways that transduce stress signals emerging from oxygen deficiency to activation of TSC1-TSC2 complex in the context of TOP mRNA translational repression is yet to be unveiled.

The percentage of mammalian transcripts with a C residue at the cap site is quite low (Schibler et al., 1977), yet this site is invariably occupied by a C residue in TOP mRNAs, and has been proved to be critical to their translational control (Levy et al., 1991; Avni et al., 1994). Indeed, it was hypothesized that the 5' TOP motif rendered TOP mRNAs poor competitors for eIF4E, the cap-binding proteins, and thereby selectively more sensitive to repressed translation

by 4E-BP (Shama et al., 1995; Thoreen et al., 2012). However, a series of observations strongly contradict this notion. First, *in vivo* transcribed TOP mRNAs are translationally inhibited *in vitro* by the cap analog m<sup>7</sup>G(5')pppG(5'), with the same kinetics as non-TOP mRNAs (Shama et al., 1995), suggesting that the affinity of both classes of mRNAs for eIF4E is similar. Second, eIF4E overexpression failed to relieve the translational repression of TOP mRNAs in mitotically arrested cells (Shama et al., 1995) or upon mTOR inhibition (Huo et al., 2012). Third, 4E-BP deficiency failed to alleviate the translational repression induced by oxygen or serum starvation (Figures 3 and 4). Fourth, repression of TOP mRNA translation by amino acid starvation was quantitatively indistinguishable in 4E-BP<sup>WT</sup> and 4E-BP DKO MEFs (Thoreen et al., 2012). Fifth, substitution of endogenous 4E-BPs by a constitutively



active mutant, 4E-BP<sup>4Ala</sup>, failed to confer persistent translational repression on TOP mRNA (Figure 3E). Sixth, Torin 1 inhibited TOP mRNA translation in 4E-BP<sup>WT</sup> rescued 4E-BP DKO MEFs to a much greater extent than that observed in 4E-BP DKO MEFs infected with 4E-BP<sup>4Ala</sup> (Figure 3E). This observation further supports the notion that the effects of Torin 1 cannot be mediated by dephosphorylation of 4E-BPs, since a similar extent of 4E-BP dephosphorylation was attained by Torin 1 and by the 4Ala mutation (Figure 4D). Seventh, oxygen-induced translational activation of TOP mRNAs was downregulated by rapamycin and Torin 1 to the same extent (Figure 1B), even though only the latter can fully inhibit 4E-BP phosphorylation (Thoreen et al., 2009). Finally, constitutively hyperphosphorylated 4E-BP in Dicer<sup>-/-</sup> cells or EBV-transformed lymphoblastoids was unable to derepress TOP mRNA translation (Figure 5). It appears, therefore, that translation repression of TOP mRNAs by physiologically relevant stresses, like deprivation of oxygen, amino acids, or serum, does not rely on 4E-BP activity.

The apparent ability of 4E-BP deficiency to protect TOP mRNA translation from acute (2 h) pharmacological inhibition of mTOR activity by Torin 1 (Thoreen et al., 2012) (Figure 3E and Supplementary Figure S4) is the only exception for the uncoupling between 4E-BP and TOP mRNAs. Apparently, this observation led Thoreen and colleagues to conclude that mTORC1 translationally regulates TOP mRNAs primarily through 4E-BP phosphorylation (Thoreen et al., 2012). It can be argued that the difference in results obtained by anoxia for 12 h or serum starvation for 14 h versus those obtained by 2 h Torin 1 treatment simply reflects late vs. early mechanisms. However, it is quite hard to conceive that cells in a multicellular organism, unlike manipulated cultured cells, ever experience an acute nutritional, anoxic, or mitogenic stress, and if so, it is not clear why cells switch later on to a non-overlapping mechanism (from 4E-BP-dependent to 4E-BP-independent). An alternative, and more likely, explanation is that the relative resistance of TOP mRNAs in 2 h mTOR-inhibited 4E-BP DKO MEFs does not reflect 4E-BP deficiency, but rather another, as yet unknown, modification these cells underwent during their establishment. Notably, 4E-BP-independence of TOP mRNA translation is underscored by the fact that the translation repression of cyclin D3 mRNA by serum starvation or Torin 1 treatment was relieved in 4E-BP DKO MEFs (Figure 4 and Supplementary Figure S6). These results suggest that mRNAs, like those encoding cyclins D3, E1 or E2 and ornithine decarboxylase, that harbor extensive secondary structure elements in their 5' UTRs, rather than the 5' TOP motif, are the primary targets of 4E-BP-mediated translational repression (Koromilas et al., 1992; Larsson et al., 2012).

A basic assumption in the model that implicated 4E-BP as the major translational regulator of TOP mRNAs was that 4E-BP mediates the role of mTORC1 in this mode of regulation. However, it is not only the role of 4E-BP in this model that has been questioned here, but also the role of mTORC1. Thus, Torin 1 treatment and anoxia led to a similar translational repression of rpS6 (about 33% in polysomes) in HEK293 cells (Figure 1), and in iRapKO MEFs untreated or treated with 4HT (Figure 6). Evidently, the lack of a specific additive repressive effect of the combined treatments (anoxia + Torin 1) implies that anoxia, like Torin 1, exerts its inhibitory effect by downregulating mTOR activity. Nevertheless, the

minor or marginal decrease in the translation efficiency of rpS6 or rpL32 mRNAs, respectively, in iRapKO MEFs expressing just 14% of their basal raptor level (Figure 6B and Supplementary Figure S7) suggests that mTORC1 plays only a partial role in the translational control of TOP mRNAs. Consistently, we have previously shown that knockdown of raptor by 87% in HEK 293 cells had no repressive effect on the translation of rpL32 mRNA (Patursky-Polischuk et al., 2009).

It can be argued, however, that the apparent minor effect of raptor knockout simply reflects the residual raptor expression. Indeed, acute rapamycin treatment, which partially inhibits mTORC1 activity, repressed the translation efficiency of rpS6 mRNA to the same extent as 4HT treatment (Figure 6B). However, these two treatments seem to operate through distinct mechanisms, as illustrated by the additive effect of the combined treatments. Thus, when 4HT-induced iRapKO MEFs were rapamycin treated, the polysomal association of rpS6 mRNAs decreased to 37% in polysomes, vs. 55%, when individually treated by either 4HT or rapamycin (Figure 6B). Collectively, these results suggest that anoxia downregulates the translation efficiency of TOP mRNAs, at least partially, through an mTORC1-independent mechanism.

Interestingly, despite the apparent involvement of mTOR in this mode of regulation, as evidenced by its equal sensitivity to rapamycin and Torin 1, it is not carried out by mTORC2 either, as rictor appears dispensable for translational activation of TOP mRNAs (Figure 6). These results extend the spectrum of signals that are transduced through mTOR to translational activation of TOP mRNAs largely in an mTORC1- or mTORC2-independent manner, as we have previously shown for insulin-induced translational activation of these mRNAs (Patursky-Polischuk et al., 2009). Phosphorylation of IMP2 by mTOR is another example of a rapamycin-sensitive, yet raptor-independent, effect of this kinase, as mTOR knockdown strongly inhibited IMP2 phosphorylation, whereas depletion of raptor had no effect on this modification, both *in vitro* and *in vivo* (Dai et al., 2011). Based on the apparent reduced polysomal association of TOP mRNA in untreated raptor or rictor knockout cells, we cannot exclude the possibility that mTORC1 and mTORC2 are involved in basal translation efficiency of TOP mRNAs. Yet, our results demonstrate that neither raptor nor rictor is absolutely required for mTOR activity toward these mRNAs. Hence, we propose that mTOR regulates induced TOP mRNAs translation, and possibly the activity of a subset of other targets, also through an as-yet-unidentified third complex, or in a complex-independent fashion.

The discrete translational behavior of TOP mRNAs suggests that the 5' TOP motif is recognized by a specific translational *trans*-acting factor. The search for proteins that interact with the 5' TOP motif has yielded throughout the years a number of candidates (Kaspar et al., 1992; Pellizzoni et al., 1996, 1997). However, none of these has been validated in an *in vitro* or *in vivo* functional assay as bona fide translational regulator of TOP mRNAs (Caldarola et al., 2009). The only two proteins that have been shown to be coimmunoprecipitated with TOP mRNAs and involved in their translational repression in amino acid-starved cells are the two RNA-binding proteins TIA-1 and TIAR. Furthermore, simultaneous knockdown of both proteins derepressed TOP mRNA translation in amino acid-deprived cells (Figure 7B; Damgaard and

Lykke-Andersen, 2011), implying their critical role under this stress condition. Nonetheless, they do not appear to be the long-sought general translational repressor of TOP mRNAs, as their co-knockdown failed to alleviate the repression exerted by anoxia or starvation for both amino acids and serum (Figure 7B). A promising non-protein modulator was identified through an unbiased screen for microRNA 10a (miR-10a)-associated mRNAs, which disclosed a large proportion of TOP mRNAs (Orom et al., 2008). miR-10a binds in a seed sequence-independent fashion a CG-rich sequence immediately downstream of the 5' TOP motif. Furthermore, it has been claimed that overexpression of this miR can derepress the translation of TOP mRNAs under amino acid starvation, suggesting its positive regulatory role. Yet, its role in oxygen-regulated TOP mRNA translation is still unclear, as complete loss of microRNAs in *Dicer*<sup>-/-</sup> cells failed to compromise translational activation of rpS6 mRNA upon oxygen replenishment (Figure 5). It appears, therefore, that despite more than two decades since the identification of the 5' TOP motif, the nature of its *trans*-acting factor and its mode of action (positive or negative) is still elusive.

## Materials and methods

More details of materials and experimental procedures are listed in Supplementary material.

### Cell culture

Human embryonic kidney (HEK) 293, HEK293T cells, as well as MEFs from *TSC2*<sup>+/+</sup>; *p53*<sup>-/-</sup>, *TSC2*<sup>-/-</sup>; *p53*<sup>-/-</sup> (Zhang et al., 2003), *TSC1*<sup>+/+</sup>, *TSC1*<sup>-/-</sup> (Kwiatkowski et al., 2002), *AMPK*<sup>+/+</sup>, *AMPK*<sup>α</sup> null (*AMPK*<sup>-/-</sup>) (Laderoute et al., 2006), *HIF-1α*<sup>+/+</sup>, *HIF-1α*<sup>-/-</sup> (Ryan et al., 1998), *REDD1*<sup>+/+</sup>, *REDD1*<sup>-/-</sup> (Sofer et al., 2005), 4E-BP WT and 4E-BP1; 4E-BP2 double knockout (4E-BP DKO) MEFs (Le Bacquer et al., 2007) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. *eIF2α*<sup>S/S</sup> and *eIF2α*<sup>A/A</sup> MEFs were grown in DMEM containing 10% FBS, 2 mM glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 50 μM β-mercaptoethanol (Scheuner et al., 2001). MEFs from *LKB1*<sup>+/+</sup> and *LKB1*<sup>-/-</sup> embryos (Bardeesy et al., 2002) were grown as *eIF2α*<sup>S/S</sup> MEFs, except for the inclusion of 5 mM β-mercaptoethanol (Bardeesy et al., 2002). L2 is a human lymphoblastoid cell lines derived by transformation with Epstein-Barr virus, and represents a normal female (provided by National Laboratory for Genetics of Israeli Populations) and a female with deletion at the tip of chromosome 17 (provided by the Genetic Laboratory of Hadassah Hospital), respectively. Cells were grown in suspension (generation time: 26 h) as described (Avni et al., 1994) and harvested for polysomal or western blot analyses at mid-log phase ( $5 \times 10^5$  to  $7 \times 10^5$  cells/ml). *iRiCKO* and *iRapKO* are inducible rictor and raptor, respectively, knockout MEF cell lines that contain a floxed rictor or raptor allele and tamoxifen-inducible Cre recombinase (Cybulski et al., 2012). Induction of *rictor* or *raptor* knockout was accomplished by treating cells with 1 mM 4HT for 4 days. Anoxia was accomplished by incubating cells in GasPak 100 Anaerobic System in the presence of GasPak EZ Anaerobe Container System Sachet (Becton, Dickenson and Company). Mitotic arrest was achieved by

incubation in serum-free medium for 48 h. Amino acid starvation was carried out as previously described (Tang et al., 2001). Starvation of cells for both serum and amino acid was attained by keeping the cells in DMEM (without serum) for 32 h (*TSC1* and *TSC2*) or 18–21 h (HEK 293) and then for additional 16 h (*TSC1* and *TSC2*) or 3–6 h (HEK 293) in Earle's salt solution, MEM-Eagle vitamin solution, 0.37% NaHCO<sub>3</sub>, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. HEK293 and HEK293T cells were transfected using polyethylenimine (PEI) procedure. Briefly, 25 μl of 2 mg/ml PEI (Sigma-Aldrich, average molecular weight 25000) were added to 750 μl of serum-free medium containing 12.5 μg of DNA. The solution was mixed and kept for 5 min at room temperature prior to its addition to 60%–70% confluent cell culture in a 100 mm plate containing 10 ml complete medium. The medium was changed the next morning and cells were harvested about 48 h posttransfection.

### Generation of *Dicer* and *p53* doubly knockout hemangiosarcoma cell lines

Mice homozygous for the *Dicer*-conditional allele (*Dicer*<sup>c/c</sup>) were crossed with mouse homozygous for *p53* null allele to produce *Dicer*<sup>c/c</sup>; *p53*<sup>-/-</sup> mice (Mudhasani et al., 2008). Due to the absence of functional *p53* protein, these mice develop and succumb to tumors at very young age. One such tumor was dissected to generate *Dicer*<sup>c/c</sup>; *p53*<sup>-/-</sup> cell line. A part of the tumor was histochemically analyzed by hematoxylin and eosin staining and determined as hemangiosarcoma. A cell line from the tumor was established after harvesting cells by trypsin treatment and culturing in DMEM containing 10% FBS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. To delete *Dicer* gene, cells were transduced retrovirally with Cre-Puro-IRE-GFP expression vector PMIG-Cre (Chen et al., 2005) and selected in media supplemented with puromycin (3 μg/ml) for a week. Subsequently, single cell-derived individual clones from the infected culture were isolated and genotyped by PCR using two forward primers (CCAAGATGCA GTGATCATTCC and CCATTGGTGCCAAGACAATG) and a reverse primer (CAGGCTCCACTCCCTAAC) for the establishment of *Dicer*<sup>+/+</sup>; *p53*<sup>-/-</sup> and *Dicer*<sup>-/-</sup>; *p53*<sup>-/-</sup> cell lines.

## Supplementary material

Supplementary material is available at *Journal of Molecular Cell Biology* online.

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