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S6K1 Alternative Splicing Modulates Its Oncogenic Activity and Regulates mTORC1

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SUMMARY

Ribosomal S6 kinase 1 (S6K1) is a major mTOR downstream signaling molecule that regulates cell size and translation efficiency. Here, we report that short isoforms of S6K1 are overproduced in breast cancer cell lines and tumors. Overexpression of S6K1 short isoforms induces transformation of human breast epithelial cells. The long S6K1 variant (Iso-1) induced opposite effects. It inhibits Ras-induced transformation and tumor formation, while its knockdown or knockout induces transformation, suggesting that Iso-1 has a tumor-suppressor activity. Furthermore, we found that S6K1 short isoforms bind and activate mTORC1, elevating 4E-BP1 phosphorylation, cap-dependent translation, and Mcl-1 protein levels. Both a phosphorylation-defective 4E-BP1 mutant and the mTORC1 inhibitor rapamycin partially blocked the oncogenic effects of S6K1 short isoforms, suggesting that these are mediated by mTORC1 and 4E-BP1. Thus, alternative splicing of S6K1 acts as a molecular switch in breast cancer cells, elevating oncogenic isoforms that activate mTORC1.

INTRODUCTION

The PI3K/Akt/mTOR pathway is one of the major signaling pathways hyperactivated in many cancers and leads to uncontrolled proliferation, increased survival, motility, and invasiveness of cancer cells (Mamane et al., 2006; Manning and Cantley, 2007; Yuan and Cantley, 2008). mTOR resides in two distinct complexes: mTOR complex-1 (mTORC1) and complex-2 (mTORC2) (Zoncu et al., 2011). mTORC1 core contains mTOR, Raptor, and G-β-L and is considered to be sensitive to rapamycin. mTORC2 contains Rictor, as the mTOR partner instead of Raptor, and, depending on the cell type, is less sensitive to rapamycin (Sarbassov et al., 2004, 2006). The best-characterized substrates of mTORC1 are S6 Kinase 1(S6K1) and eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1), while Akt is a substrate of mTORC2 (Sarbassov et al., 2006; Shiota et al., 2006). Several components of the mTOR signaling cascade have been identified as oncogenes or tumor suppressors that activate or repress this pathway, respectively (Zoncu et al., 2011; Shaw and Cantley, 2006). Among the two well-characterized mTORC1 substrates, S6K1 and 4E-BP1, the latter has been shown to be important for efficient protein translation, proliferation, and oncogenic transformation (Hsieh et al., 2010; Ohanna et al., 2005; She et al., 2010). S6K1 has been implicated in the regulation of cell size (Ohanna et al., 2005), and its role in transformation is controversial; in some reports, it seems dispensable for transformation (Hsieh et al., 2010), while in others it appears to be required (Alliouachene et al., 2008). A link between S6K1 function and cancer was suggested by the finding that *RPS6KB1*, the gene encoding for S6K1, resides in the chromosomal region 17q22-17q23, which is often amplified in breast and lung cancers (Bepler and Koehler, 1995; Monni et al., 2001). However, direct evidence that S6K1 expression or activity is sufficient to lead to cellular transformation is lacking. We have previously reported that the splicing factor oncoprotein SRSF1 is upregulated and in some cases amplified in several types of tumors, including breast and lung (Karni et al., 2007). SRSF1 modulates the splicing of *RPS6KB1*, which encodes S6K1 in both mouse and human (Karni et al., 2007). Furthermore, SRSF1 activates the mTORC1 pathway, which phosphorylates S6K1 downstream of Akt (Karni et al., 2008). The spliced variants of S6K1 yield a long active kinase p85/p70 S6K1 (will be referred to as Iso-1) and shorter splicing variants (will be referred to as Iso-2 in mouse and h6A and h6C in human). We have shown previously that SRSF1 increases the expression of the shorter S6K1 isoform, and that this isoform possesses oncogenic activity and can transform immortal mouse fibroblasts (Karni et al., 2007).

In this study, we examine the oncogenic and signaling activities of S6K1 splicing isoforms and their expression in cancer. Our findings suggest that while Iso-1 is tumor suppressive in vitro and in vivo and can block Ras-induced transformation, the short kinase inactive S6K1 splicing isoforms possess oncogenic properties. We show that the short isoforms of S6K1 bind mTOR and activate mTORC1, leading to increased 4E-BP1 phosphorylation, cap-dependent translation, and upregulation

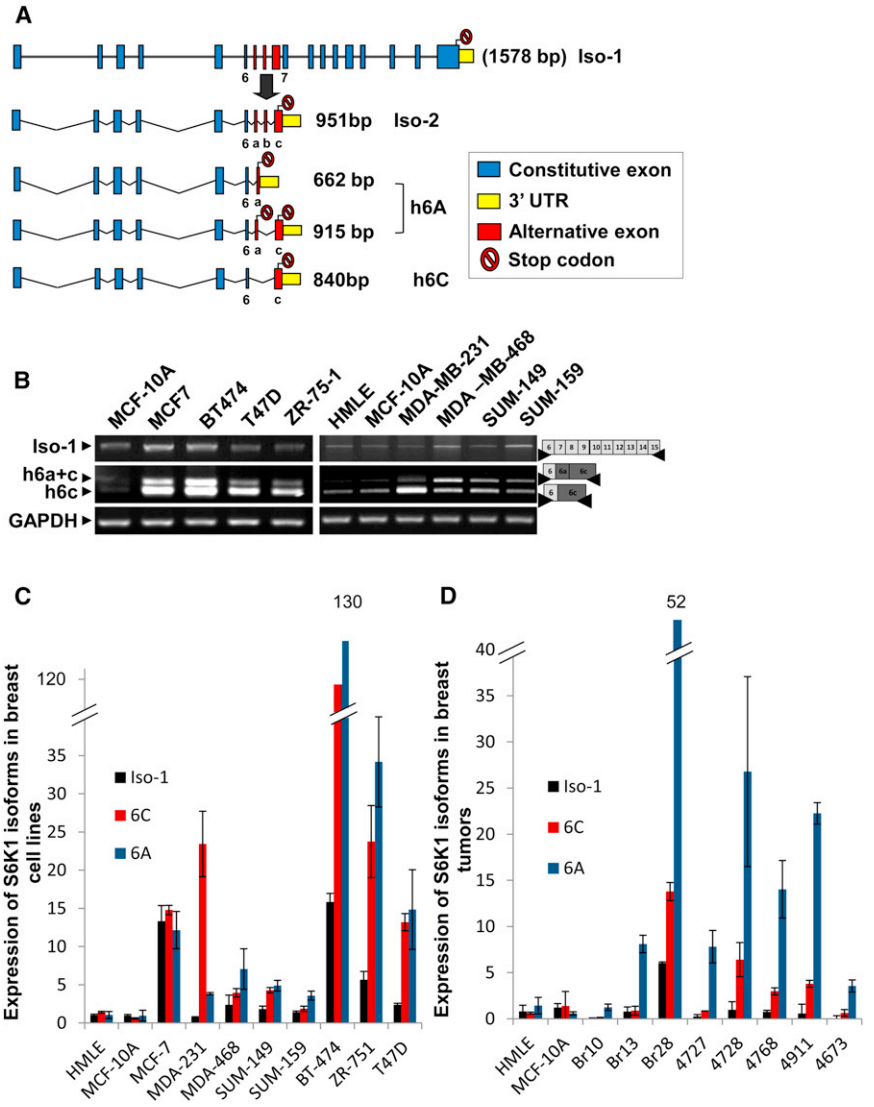

Figure 1. Increased Expression of Human S6K1 Short Variants 6A and 6C in Breast Cancer Cell Lines and Tumors

(A) Schematic representation of *RPS6KB1* pre-mRNA and its splicing isoforms. Isoform-1(encoding for p70/p85 S6K1 protein) is composed of 15 exons (blue boxes). S6K1 mouse spliced variant-2 (Iso-2) contains three alternative exons between exon 6 and 7: a, b, c (red boxes), a different polyadenylation site and 3′ UTR region (yellow area), and a stop codon in exon 6C. Human short S6K1 isoforms, h6C and h6A, lack the alternative 6b exon and contain a combination of two alternative exons: exon a followed by 3′ UTR or followed by exon c. In both cases, a stop codon in exon a terminates translation at the same amino acid and both transcripts give rise to one protein, h6A. The other isoform includes only exon 6C followed by a poly(A) tail and encodes a protein with a different C terminus. All S6K1 short variants are identical up to the sixth exon and differ from Iso-1 and from each other in their C terminus.

(B) RNA from the indicated immortalized breast cell lines (HMLE,MCF-10A) or breast cancer cell lines (MCF-7, BT474, T47D, ZR-75-1, MDA-MB-231, MDA-MB-468, SUM149, SUM159) was extracted and the levels of S6K1 h6A and h6C short isoforms and Iso-1 were detected by RT-PCR with primers specific for each isoform. The splice variants are indicated by boxes at the right side of each transcription variant.

(C and D) qRT-PCR quantitation of S6K1 isoforms: Iso-1, h6A, and h6C. Quantitation was done on total RNA extracted from breast cell lines (C) or tumors (D). All samples were normalized to β-actin mRNA levels and to the average expression of the immortal breast cell lines (HMLE and MCF-10A). Error bars represent SD of three repeats.

See also Figure S1.

of the antiapoptotic protein Mcl-1. Furthermore, mTORC1 activation is critical for the oncogenic activity of S6K1 short isoforms as the mTORC1 inhibitor rapamycin or expression of a phosphorylation-defective mutant of 4E-BP1 (Hsieh et al., 2010; She et al., 2010) partially inhibit the oncogenic properties of these isoforms. Taken together, our results suggest that S6K1 alternative splicing acts as a switch between a tumor suppressor protein and an oncoprotein, which is deregulated in breast cancer and modulates mTORC1 activity.

RESULTS

S6K1 Short Isoforms Are Upregulated in Breast Cancer Cell Lines and Tumors

The gene *RPS6KB1* encoding for p85/p70 S6K1 can be alternatively spliced to form a number of truncated isoforms. In mouse cells, the splicing factor SRSF1 induces the inclusion of three additional exons (a-b-c) located between exon 6 and 7 (Figure 1A). By PCR, cloning, and sequencing, we have discovered that in human there are two alternative exons in this region: a and c, which can be included together or individually generating two protein isoforms that we have termed h6A and h6C (Figures 1A; Figures S1D and S1E; Table S1). All of these isoforms in mouse or human that include combinations of exons 6 (a–c) are termed S6K1 short isoforms. Inclusion of the alternative exons mentioned above results in exposure of alternative polyadenylation sites and alterations in the reading frame that, in turn, generate a stop codon in exon 6c in mouse and exons 6a or 6c in humans. The presence of these stop codons creates transcripts containing approximately half of the original S6K1 coding sequence (Iso-1), and lacking more than half of the conserved kinase domain (Figure 2A).

In all of these alternative splicing events, the presence of a polyadenylation sequence, and in the case of h6A also a premature stop codon (PTC) located less then 55 bp from the next exon junction complex, prevents degradation of the generated transcripts by the Nonsense Mediated Decay (NMD) mechanism (Figures 1A and S1E) (Schoenberg and Maquat, 2012). We found

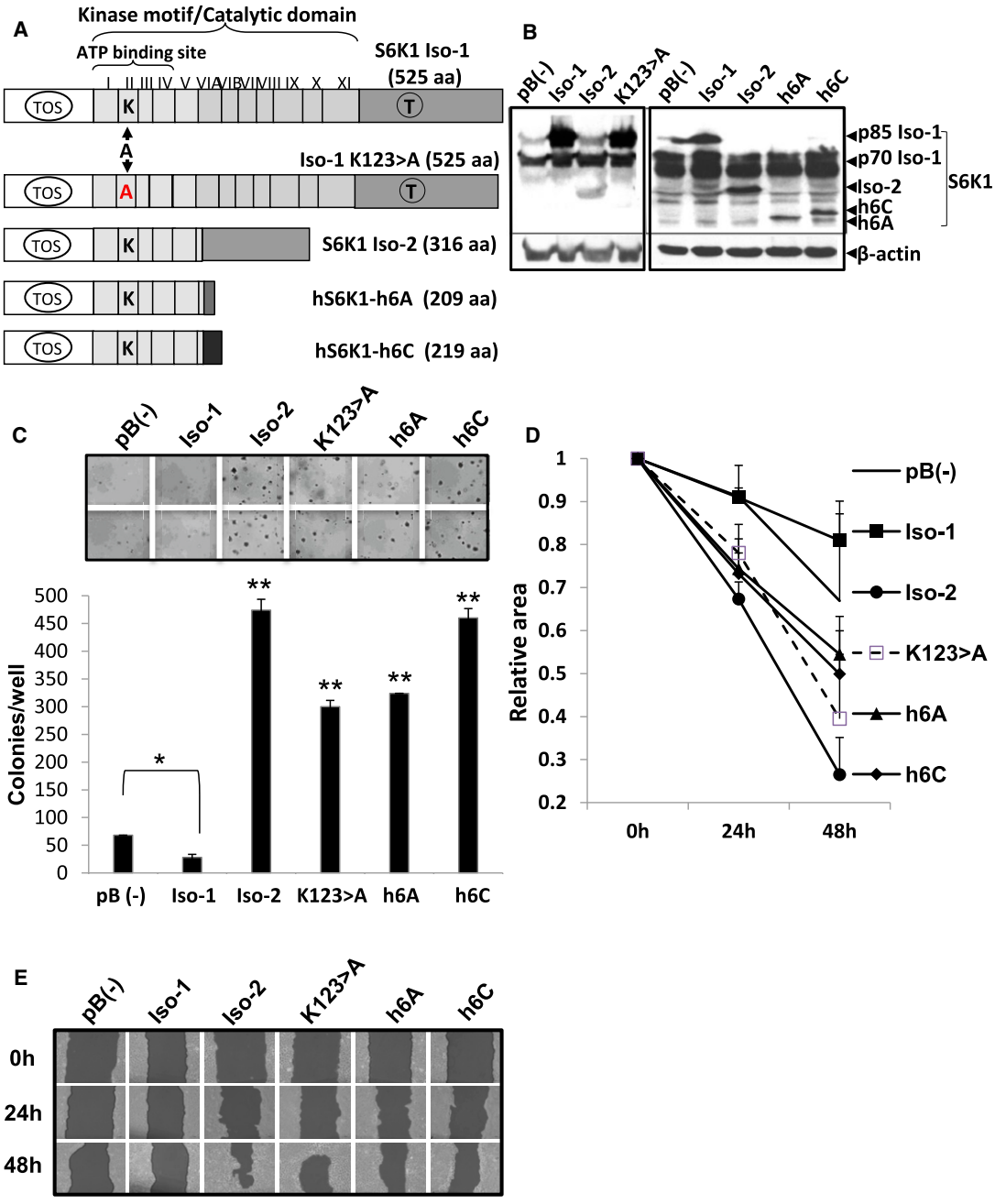

Figure 2. S6K1 Short Isoforms Enhance Transformation of Breast Epithelial Cells

(A) The predicted protein structure of mouse and human *RPS6KB1* splicing isoforms. All isoforms contain Raptor binding motif mTOR-signaling (TOS) at the N terminus (white boxes) and a lysine residue (K123) at the ATP binding site in the catalytic domain that is essential for its protein kinase activity. S6K1 isoform-1 kinase dead version (Iso-1 K123 > A) contains a lysine to alanine (K123A) substitution. S6K1 short isoforms lack 6 out of the 12 conserved kinase helical domains as well as the C-terminal autoinhibitory domain, which harbors the mTOR activatory phosphorylation site at threonine 389.

(B) MCF-10A cells were transduced with retroviruses encoding for the indicated S6K1 isoforms and protein extracts subjected to western blotting. Membranes were probed with a monoclonal antibody against the N′ terminus of S6K1 to detect the endogenous and exogenous isoforms. β-actin was analyzed as a loading control.

(C) Pools of MCF-10A cells transduced with the indicated retroviruses as in (B) were seeded into soft agar in duplicates and colonies were allowed to grow for 14 days. Data represent the average number ± SD of colonies per well. n = 2. *p = 0.0049 **p ≤ 0.005 relative to empty vector. The results shown are a representative experiment out of three individual experiments.

(D) MCF-10A pools of cells transduced with the indicated retroviruses as in (B) were stimulated to migrate by physical wounding of cells seeded in monolayer. n = 3.

(E) Representative images of the wound area of cells described in (D).

See also Figure S2.

that while in immortal breast cells (MCF-10A, HMLE) the expression of S6K1 short isoforms is relatively low, in breast cancer cell lines inclusion of exons 6a and 6c is significantly increased, especially in metastatic breast carcinoma cell lines (Figures 1B and S1A). Indeed, while in both primary and immortal breast cells S6K1 short protein isoforms were hardly detected at the protein level, in breast cancer cell lines elevated protein levels of S6K1 short isoforms were detected (Figure S1C) (Karni et al., 2007; Rosner and Hengstschläger, 2011). In human breast tumor samples, we found elevated expression of S6K1, h6A, and h6C isoforms compared to the immortal breast cell lines (Figures 1D, S1F, and S1G). Interestingly, whereas most analyzed breast cancer cell lines and tumor samples presented high expression of S6K1 short isoforms, we did not find elevated expression of the full-length isoform Iso-1 in most tumors (Figures 1B–1D). Two of the cell lines that showed elevated Iso-1 expression (MCF-7, BT474) possess amplification of the *RPS6KB1* gene, and, except for MCF-7, all tumors and cell lines showed an increase in short isoforms/Iso-1 ratio (Figures S1F and S1G), indicating that an alternative splicing switch in S6K1 occurs in breast cancer.

All S6K1 protein isoforms are identical in their N terminus but share only partial homology in their kinase domain and differ from each other in their C terminus. Iso-1, Iso-2, h6A, and h6C contain distinct sequences in their C terminus consisting of 330, 121, 12, or 24 amino acids, respectively (Figure 2A).

S6K1 Short Isoforms Enhance Motility and Anchorage-Independent Growth

We sought to examine the oncogenic activity of S6K1 isoforms in human breast epithelial cells. We found that Iso-2, h6A, and h6C were able to transform human immortal breast MCF-10A cells and mouse NIH 3T3 cells enabling them to form colonies in soft agar despite the relatively low expression of the short isoforms in comparison to Iso-1 (Figures 2B, 2C, and S2A–S2C). The kinase dead version of Iso-1 (Iso-1 K123 > A) enhanced transformation and increased the ability of MCF-10A and NIH 3T3 cells to form colonies in soft agar as well (Figures 2C and S2C). An in vitro kinase assay using S6 as a substrate shows that the short isoform Iso-2 and the kinase dead K123 > A version of Iso-1 have no kinase activity (Figure S2G). The oncogenic effects of S6K1 short isoforms did not require a functional kinase activity since all short S6K1 isoforms share truncated kinase domains and cannot phosphorylate ribosomal protein S6, or mTOR, both known S6K1 substrates (Figures S2E–S2G and 5A) (Chiang and Abraham, 2005; Holz and Blenis, 2005). In contrast to the expression of S6K1 short isoforms, overexpression of Iso-1, the long active kinase, did not enhance colony formation in soft agar and even reduced the basal level of colony formation (Figure 2C).

One characteristic of cellular transformation is enhancement of cell motility (Hanahan and Weinberg, 2011). MCF-10A cells expressing the shorter isoforms or the kinase dead version of Iso-1 (Iso-1 K123 > A) showed accelerated migration rate compared to cells transduced with S6K1 Iso-1 or the empty vector in a tissue culture wound healing assay (Figures 2D and 2E). In order to exclude the possibility that these effects are the result of changes in proliferation, we measured the proliferation rate of the cells. Cells overexpressing the shorter variants, empty vector, or Iso-1 K123>A did not show enhanced proliferation rate in comparison to Iso-1 (Figure S2D). Altogether, these results suggest that S6K1 short kinase inactive isoforms promote transformation of MCF-10A cells.

Expression of S6K1 Short Isoforms Enables Growth-Factor-Independent Three-Dimensional Acini Formation and Elevates 4E-BP1 Phosphorylation

The immortal breast epithelial cells MCF-10A possess the ability to grow into spheroid structures (acini) when grown on matrigel. To study the effects of S6K1 isoforms on growth of three-dimensional (3D) reconstituted basement membrane cultures, we seeded MCF-10A cells transduced with S6K1 isoforms in the presence or absence of growth factors necessary for acini formation in MCF-10A cells (Arias-Romero et al., 2010). Cells overexpressing S6K1 truncated isoforms, as well as the kinase-dead version of Iso-1, formed large, hyperproliferative 3D structures (Figures 3A–3C) with slight morphological disruption (Figure S3A), even when grown in the presence of only one of the growth factors. This suggests that S6K1 isoforms can replace the strong proliferative signal provided by either epidermal growth factor (EGF) or insulin/insulin growth factor (IGF)-1. This observation is further underscored by the fact that the kinase active isoform of S6K1 (Iso-1) failed to support growth-factor-independent acinus formation and may even inhibit basal proliferation in matrigel (Figures 3A, 3B, and S3A).

Insulin/IGF-1 and EGF activate their corresponding receptors leading to activation of several mitogenic signaling cascades, among them the Ras-Raf-MAPK-ERK and the PI3K-Akt-mTOR pathways (Shaw and Cantley, 2006; Yuan and Cantley, 2008; Mendoza et al., 2011). We hypothesized that S6K1 short isoforms affect acinus proliferation by activating the insulin/IGF-1 and EGF signaling downstream of the receptors, bypassing the need for growth factor activation (Figures 3A and 3B). Thus, we sought to investigate the activities of these signaling pathways. We measured the phosphorylation state of known downstream effectors of these pathways: Akt, ERK, and 4E-BP1 (Shaw and Cantley, 2006; Yuan and Cantley, 2008; Mendoza et al., 2011) in MCF-10A cells overexpressing S6K1 isoforms with and without growth factor activation. We found that overexpression of S6K1 kinase inactive isoforms stimulated the phosphorylation of 4E-BP1, even under serum starvation conditions (Figures 3D, 3E, and S3D). Under these conditions, we observed only a slight and inconsistent activation of Akt or ERK (Figures S3B–S3D). Notably, while overexpression of S6K1 Iso-1 failed to increase 4E-BP1 phosphorylation under serum starvation conditions compared to cells transduced with empty vector (Figures 3D, 3E, and S3D), knockdown of this isoform in MCF-10A cells increased 4E-BP1 phosphorylation (Figures 3F and 3G), supporting the notion that Iso-1 plays an opposite role than S6K1 kinase inactive isoforms. Thus, the increased level of 4E-BP1 phosphorylation we observed, even in serum- and growth-factor-deprived cells, may contribute to the transforming phenotype of cells harboring S6K1 short isoforms (Figures 2C–2E, 3A, 3B, and S2C). Moreover, 4E-BP1 phosphorylation is higher in most breast cancer cells compared to immortal nontransformed cells (Avdulov et al., 2004) in correlation with elevated levels of S6K1 short isoforms (Figures 1B, 1C, S1A, and S3E).

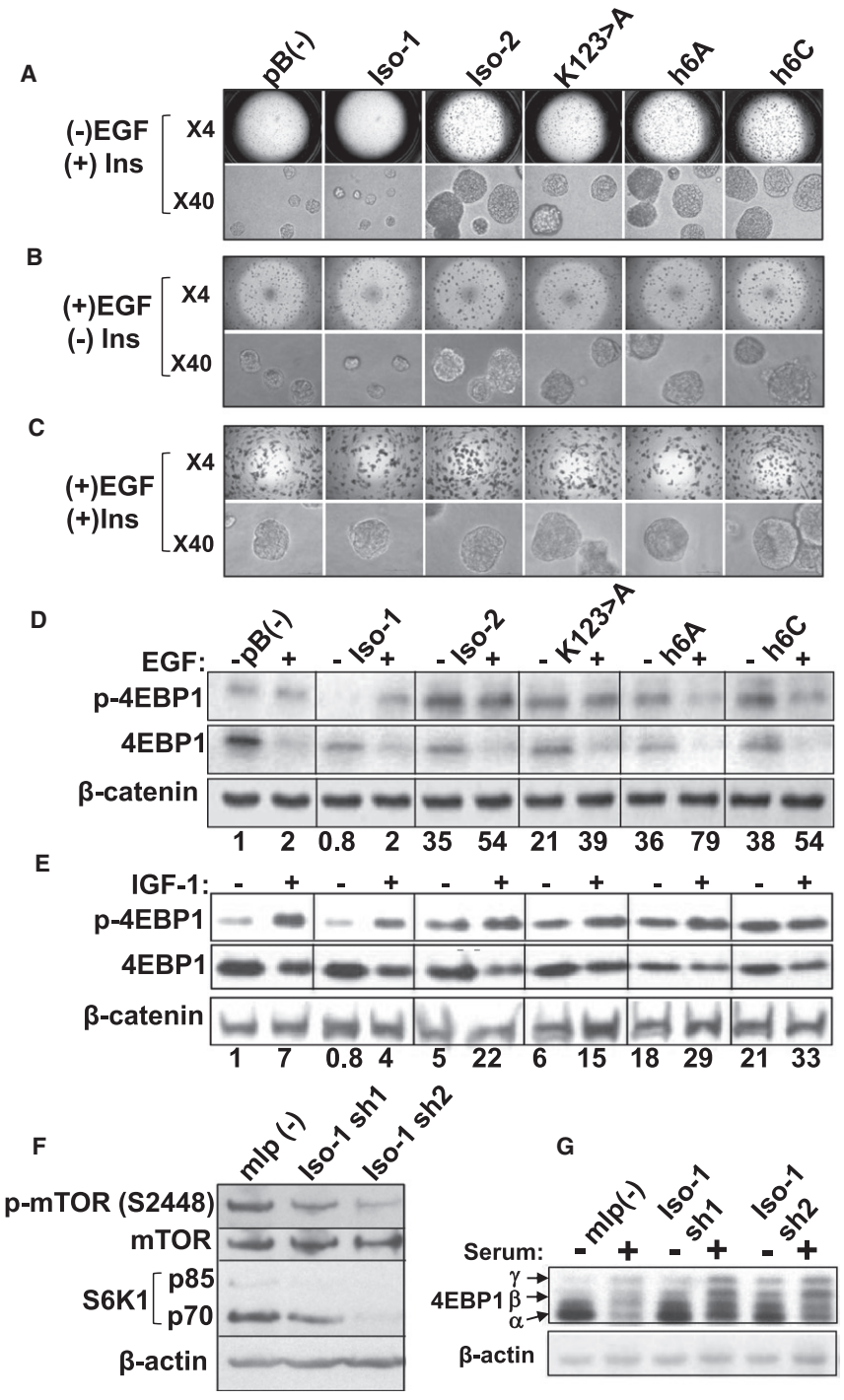

Figure 3. S6K1 Inactive Isoforms Enable Growth-Factor-Independent 3D Growth of MCF-10A Cells and Enhance 4E-BP1 Phosphorylation

(A–C) Phase images of MCF-10A cells transduced with the indicated retroviruses seeded in matrigel as described in Experimental Procedures, in the absence of EGF (A) or insulin (B) or in the presence of both (C). Cells were allowed to grow for 2 weeks to form acini structures. These results were obtained in at least three individual experiments.

(D–G) Total protein was isolated from cells and subjected to western blotting using the indicated antibodies.

(D and E) MCF-10A-transduced cells described above were seeded in six-well plates (3×10^5 cells/well). Cells were starved for 24 hr and then induced with EGF (D) or IGF-1 (E) for 4 hr. Fold increase of 4E-BP1 was normalized (phosphorylated/total protein levels) to that of untreated (starved) pB(−) (empty vector), which was arbitrarily set at 1 (quantitation values are shown under each panel).

(F) MCF-10A cells were transduced with the indicated retroviruses expressing empty vector (mlp[−]) or the indicated S6K1 Iso-1-specific shRNAs (Karni et al., 2007).

(G) MCF-10A cells transduced with the indicated retroviruses as described in (F) were grown for 24 hr in the presence of 0.5% or 5% serum. Arrows show the phosphorylation states of 4E-BP1, where gamma is the fully phosphorylated form. β-actin was analyzed as a loading control.

See also Figure S3.

S6K1 Iso-1 Inhibits Ras-Induced Transformation In Vitro and In Vivo

Our results, unexpectedly, indicated that the full-length S6K1 Iso-1 did not support growth-factor-independent acinus formation, as opposed to the short isoforms (Figures 3A–3C). To further study the possibility that S6K1 Iso-1 acts as a tumor suppressor, we silenced its expression in the immortal MCF-10A cell line using Iso-1-specific small hairpin RNA (shRNAs) (Karni et al., 2007) (Figure 3F). We measured reduced phosphorylation of mTOR S2448, a known substrate of p70 S6K1 (Holz and Blenis, 2005; Chiang and Abraham, 2005) in these cells (Figure 3F). Knockdown of S6K1 Iso-1 in MCF-10A cells increased both colony formation in soft agar and acinus number and size in matrigel (Figures 4A and S4C). Similar results were obtained using NCI-H460 lung carcinoma cells (Figures S4A and S4B). Even though overexpression of Iso-1 did not significantly decrease cell motility (Figures 2D and 2E), its knockdown in MCF-10A cells was sufficient to increase motility (Figure S4D).

To examine if Iso-1 can suppress the oncogenic potential of transformed cells, we coexpressed S6K1 Iso-1 with an active RAS mutant (H-Ras^{V12}) in MCF-10A cells (Figure S4E). Cells coexpressing the kinase active S6K1 isoform (Iso-1) and oncogenic RAS formed fewer and smaller colonies in soft agar compared to cells coexpressing Ras and empty vector, suggesting that S6K Iso-1 possesses tumor-suppressive activity (Figure 4B). Moreover, cells coexpressing Iso-1/Ras did not gain the spindle/

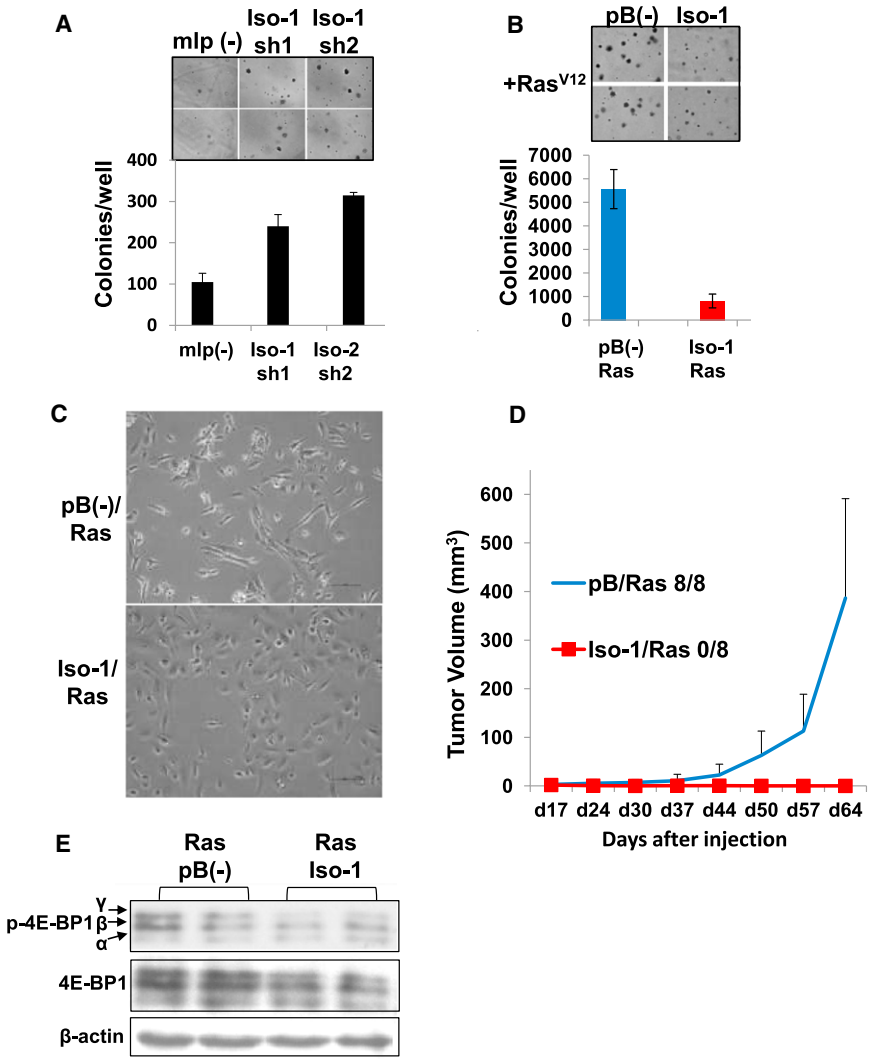

Figure 4. S6K1 Iso-1 Knockdown Increases Transformation and Its Overexpression Blocks RAS-Induced Transformation In Vitro and In Vivo

(A) MCF-10A pools of cells were transduced as in (Figure 3F) and seeded into soft agar in duplicates, and colonies were allowed to grow for 14 days. Data represent the average number ± SD of colonies per well. n = 2.

(B) MCF-10A cells were transduced with retroviruses encoding pB(−) empty vector or S6K1 Iso-1 followed by transduction with an active Ras mutant (H-Ras^{V12}). Cells were seeded into soft agar in duplicates, and colonies were allowed to grow for 14 days.

(C) MCF-10A pools of cells transduced with the indicated retroviruses as in (B) were photographed 24 hr after seeding (×100 magnification).

(D) RAS-transformed MCF-10A cells expressing empty vector (pB[−]) or S6K1 Iso-1 were injected into NOD-SCID mice (2 × 10^6 cells/injection). Tumor volume was measured weekly and tumor growth curve was calculated as described in Experimental Procedures; error bars indicate SD of eight tumors. n/n = number of tumors per number of injections.

(E) Ras-transformed MCF-10A cells expressing the indicated retroviruses as described in (B). Total protein from stable pools was extracted and subjected to western blotting. The membranes were probed with the indicated antibodies. β-actin was analyzed as a loading control.

See also Figure S4.

fibroblastic shape that is characteristic of cells transformed with an oncogene such as Ras (Janda et al., 2002) (Figure 4C). The same cell pools were injected subcutaneously into NOD-SCID mice. Supporting the results in vitro, we found that RAS-transformed cells coexpressing Iso-1 did not form tumors in vivo (zero tumors formed of eight injections) as opposed to the empty vector (eight of eight tumors formed) (Figures 4D and S4F). 4E-BP1 phosphorylation levels were lower in cells coexpressing Iso-1 and H-Ras^{V12} than in cells expressing H-Ras^{V12} alone (compare the γ bands in Figure 4E). Moreover, cells coexpressing Iso-1 and Ras^{V12} showed about 20% decrease in Cap-dependent translation in correlation with the decreased phosphorylation of 4E-BP1 (Figure S4G). Taken together, these data suggest that S6K1 Iso-1 is a putative tumor suppressor.

S6K1 Short Isoforms Bind mTORC1 and Enhance Cap-Dependent Translation and Mcl-1 Expression

We hypothesized that S6K1 short isoforms might directly bind and activate mTORC1. To test this possibility, we cotransfected myc-tagged mTOR and T7 tagged Iso-1 or Iso-2 (as a representative of the short isoforms). Immunoprecipitation of transfected S6K1 with anti-T7 antibody revealed that only S6K1 Iso-2 interacts with mTOR (Figures 5A and 5B). The fact that we did not see an interaction between S6K1 Iso-1 and mTOR might be explained by T389 phosphorylation of S6K1 (Figure 5B). This phosphorylation activates S6K1 Iso-1 (Kim et al., 2002), leading to its release from mTORC1 (Holz et al., 2005).

Cells overexpressing S6K1 short inactive isoforms showed elevated levels of cap-dependent translation under serum starvation conditions as measured by a reporter gene measuring Cap- versus IRES-mediated translation from the same transcript (Figures 5C and 5D). Indeed, Iso-1 overexpressing MCF-10A cells exhibited only a slight decrease in 4E-BP1 phosphorylation that was consistent with a slight decrease of cap-dependent translation, compared to cells expressing empty vector (Figures 3D, 3E, and 5D). Moreover, on the background of H-Ras^{V12} transformation, Iso-1 decreased both 4E-BP1 phosphorylation and cap-dependent translation (Figures 4E and S4G), suggesting that Iso-1 tumor-suppressive effect can be clearly detected on the background of a strong oncogene such as mutant Ras but not in a nontransformed cell system such as MCF-10A cells.

We next examined if S6K1 short isoforms alter the expression of proteins known to be controlled by mTORC1-4E-BP1

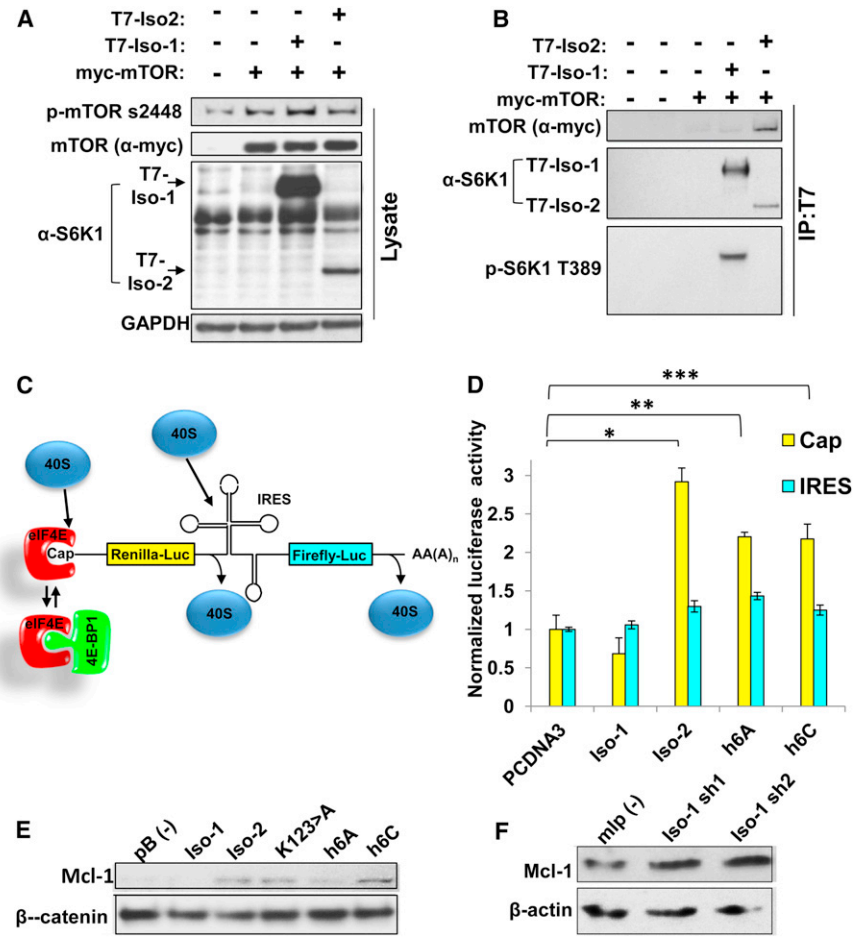

Figure 5. S6K1 Kinase Short Isoforms Interact with mTOR, Enhance Cap-Dependent Translation, and Increase Mcl-1 Expression

(A and B) HEK293 cells were cotransfected with myc-tagged mTOR and the indicated T7-tagged S6K1 isoforms. Whole-cell lysates were examined for construct expression (A) and for immunoprecipitation of T7-tagged S6K1 isoforms (B). Myc-tagged bound mTOR and T7-tagged S6K1 isoforms were detected by immunoblotting using anti-myc or monoclonal antibody against the N′ terminus of S6K1, respectively. The first two left lanes represent anti-T7 anibody alone and pull down from untransfected HEK293 cells, respectively.

(C) Schematic representation of pLPL Cap-Renilla-IRES-Luciferase bicistronic dual reporter vector (Gerlitz et al., 2002).

(D) MCF-10A cells were cotransfected with dual reporter vector (C) and with the indicated S6K1 isoforms and starved for serum and growth factors for 24 hr posttransfection. Cap-dependent translation (Renilla luciferase activity) and IRES-mediated translation (Firefly luciferase activity) were measured (n = 4 experiments,*p = 0.019, **p = 0.008, ***p = 0.033).

(E) MCF-10A cells were transduced with retroviruses encoding for the indicated S6K1 isoforms. Transductants (3×10^5) were seeded in six-well plates and starved for 24 hr for serum and growth factors. After western blotting, the membranes were probed with the indicated antibodies. β-catenin was analyzed as a loading control.

(F) MCF-10A cells described in (Figure 3F) were seeded (3×10^5 cells/well) in a six-well plate. Twenty-four hours later, cells were lysed and total protein was extracted and separated by SDS-PAGE. The membranes were probed with the indicated antibodies. β-actin was analyzed as a loading control.

See also Figure S5.

translational regulation and can contribute to cell transformation. One such protein is Mcl-1 (Hsieh et al., 2010), a key antiapoptotic protein that was shown to be translationaly controlled by mTORC1 (Mills et al., 2008). We found that Mcl-1 protein levels are elevated in MCF-10A cells overexpressing S6K1 kinase inactive isoforms, but not Iso-1 or empty vector (Figure 5E). Interestingly, Mcl-1 was also elevated by Iso-1 knockdown (Figure 5F). S6K family consists of two kinases, S6K1 and S6K2 (*RPS6KB1* and *RPS6KB2*, respectively) (Lee-Fruman et al., 1999), that are known to have redundant activities (Nardella et al., 2011; Pende et al., 2004). We did not detect any changes in S6K2 protein levels in either Iso-1 knockdown or S6K1 kinase inactive isoforms overexpression (Figures S5A and S5B), indicating that the biological effects we observe are due to up or downregulation of S6K1 Iso-1 or the short S6K1 isoforms.

Loss of S6K1/2 Enhances Cap-Dependent Translation, Mcl-1 Expression, and Transformation

In order to establish definitive proof that S6K1 Iso-1 is a tumor suppressor and to rule out any possible compensation or interference by S6K2, we analyzed S6K1 and S6K2 double-knockout (DKO) mouse embryonic fibroblasts (MEFs) (Figure 6A) (Dowling et al., 2010; Pende et al., 2004). Consistent with our previous results, serum-starved S6K DKO MEFs showed elevated phosphorylation levels of 4E-BP1 (Figure 6A). In addition, S6K DKO MEFs formed significantly higher number of colonies in soft agar and also presented 5-fold increase in cap-dependent translation, indicating that these cells are transformed (Figures 6B and 6C). Furthermore, western blot analysis of S6K DKO MEFs revealed high levels of expression of Mcl-1 (Figure 6D) in agreement with what was observed in Iso-1 knockdown in MCF-10A cells (Figure 5F). The fact that the DKO cells show minor/negligible mTOR phosphorylation (Figure 6A) and no S6 phosphorylation (Figure S2F), but were transformed, supports the notion that mTOR S2448 phosphorylation is not essential for its ability to phosphorylate 4E-BP1.

Expression of Iso-1 in S6K DKO MEFs (Figure S2E) partially reduced cap-dependent translation, colony survival, and growth of colonies in soft agar showing that Iso-1 harbors tumor-suppressive ability (Figures 6E–6G). The transformed phenotype of S6K DKO MEFs was not completely reversed by the ectopic expression of S6K1 Iso-1, probably due to the absence of

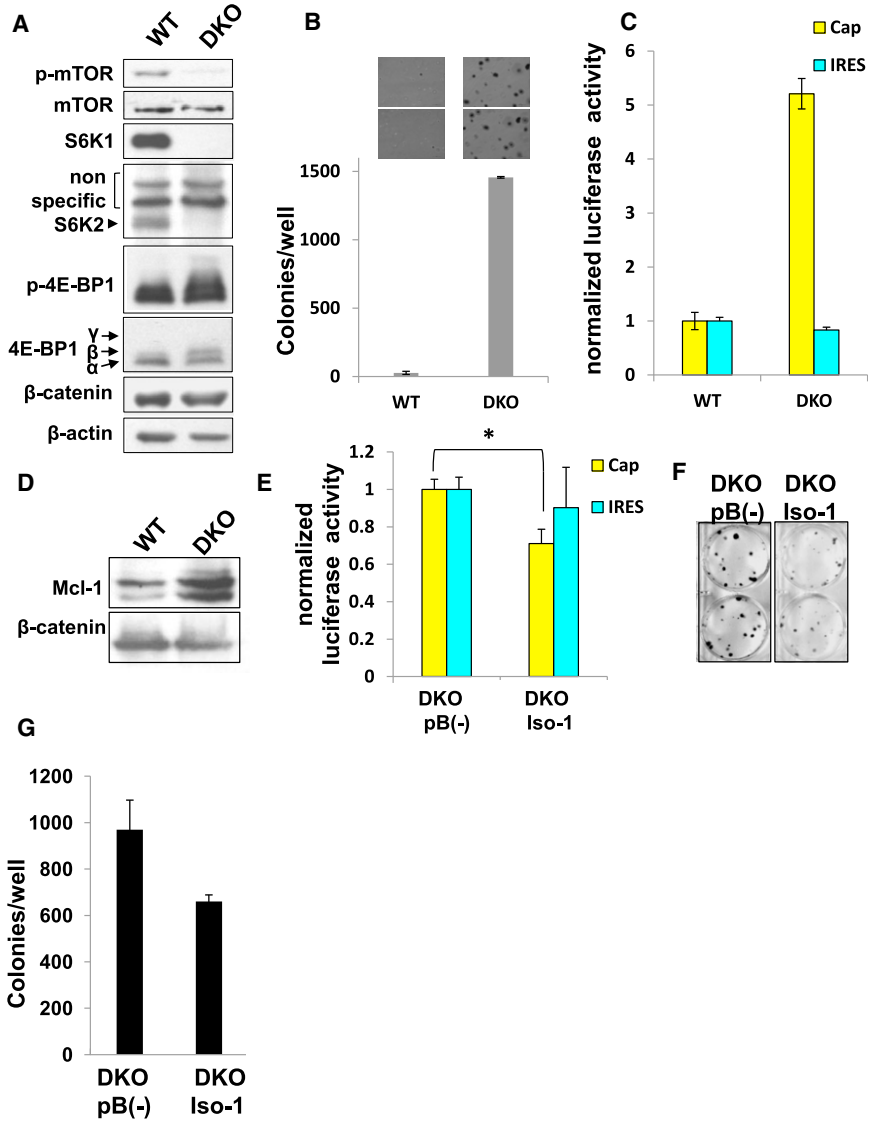

Figure 6. Loss of S6K1/2 Enhances Cap-Dependent Translation, Mcl-1 Expression, and Transformation

(A) Wild-type (WT) or S6K1 and S6K2 DKO MEFs were seeded at 80% confluency and serum starved for 5 hr. Total protein was extracted and subjected to western blotting. The membranes were probed with the indicated antibodies.

(B) WT and DKO MEFs were seeded into soft agar in triplicates and colonies were allowed to grow for 14 days. Data represent the average number ± SD of colonies per well. n = 3. The results shown are a representative experiment out of three individual experiments.

(C) WT and DKO MEFs were transfected with dual reporter vector (Cap-Renilla-IRES-Luciferase described in Figure 5C). Twenty-four hours post-transfection cells were serum starved for another 24 hr. Cap-dependent translation (Renilla luciferase activity) and IRES-mediated translation (Firefly luciferase activity) were measured (data represent the average number ± SD of n = 6 experiments).

(D) Total protein from WT and DKO MEFs described in (A) was separated by SDS-PAGE. After western blotting, the membranes were probed with the indicated antibodies.

(E) S6K1 and S6K2 DKO MEFs cells transduced with retroviruses encoding for empty vector (pB[−]) or S6K1 Isoform-1(Iso-1) were transfected with dual reporter vector (Cap-Renilla-IRES-Luciferase) 24 hr posttransfection cells were serum starved for another 24 hr. Cap-dependent translation (Renilla luciferase activity) and IRES-mediated translation (Firefly luciferase activity) were measured (n = 3 experiments,*$p < 0.004$).

(F) MEF cells described in (E) were seeded (200 cells/well) in six-well plates and grown for 14 days. Colonies were fixed and stained with methylene blue.

(G) MEF cells described in (E) were seeded into soft agar in triplicates and colonies were allowed to grow for 14 days. Data represent the average number ± SD of colonies per well. n = 3. The results shown are a representative experiment out of three individual experiments.

S6K2 in this cell system that might contribute to the tumor-suppressive phenotype of S6K1.

The Oncogenic Activities of S6K1 Short Isoforms Are Mediated by mTORC1 Inactivation of 4E-BP1

S6K1 short isoforms activated mTORC1 as measured by 4E-BP1 phosphorylation (Figures 3D, 3E, and S3D). We next examined, using two complementary strategies, if this activation is required for their oncogenic activities: (1) inhibition of mTORC1 by the pharmacological inhibitor rapamycin, and (2) expression of a phosphorylation-defective 4E-BP1, which is mutated in its phosphorylation sites and cannot dissociate from eIF4E upon mTORC1 activation (Avdulov et al., 2004; She et al., 2010). We transduced MCF-10A cells expressing the mouse S6K1 short isoform (Iso-2) with phosphorylation-defective 4E-BP1 (4E-BP1^{5A} or 4E-BP1^{4A}), where each of the five (or four) insulin and rapamycin-responsive 4E-BP1 phosphorylation sites have been mutated to alanine (Hsieh et al., 2010).

Coexpression of both Iso-2 and mutant 4E-BP1 decreased colony formation in soft agar 2-fold relative to cells expressing Iso-2 alone (Figures 7A, 7B, S6A, and S6B). Similarly, rapamycin reduced the number of colonies formed in soft agar in cells overexpressing Iso-2 to the background level of cells expressing empty vector (Figure 7B). These results suggest that mTORC1 activation and 4E-BP1 phosphorylation play a major role in the oncogenic capabilities of S6K1 short isoforms.

Expression of mutant 4E-BP1 together with Iso-2 also partially decreased cell motility compared to cells expressing Iso-2 alone (Figures 7C and 7D).

Next, we investigated whether 4E-BP1 phosphorylation is important for Iso-2-mediated growth-factor-independent 3D proliferation in matrigel. Expression of 4E-BP1^{5A} strongly inhibited Iso-2's ability to induce growth-factor-independent acinus formation in matrigel (Figure 7E). Taken together, our results suggest that the mechanism of action of S6K1 short isoforms is mediated mostly by 4E-BP1 phosphorylation through mTORC1 activation.

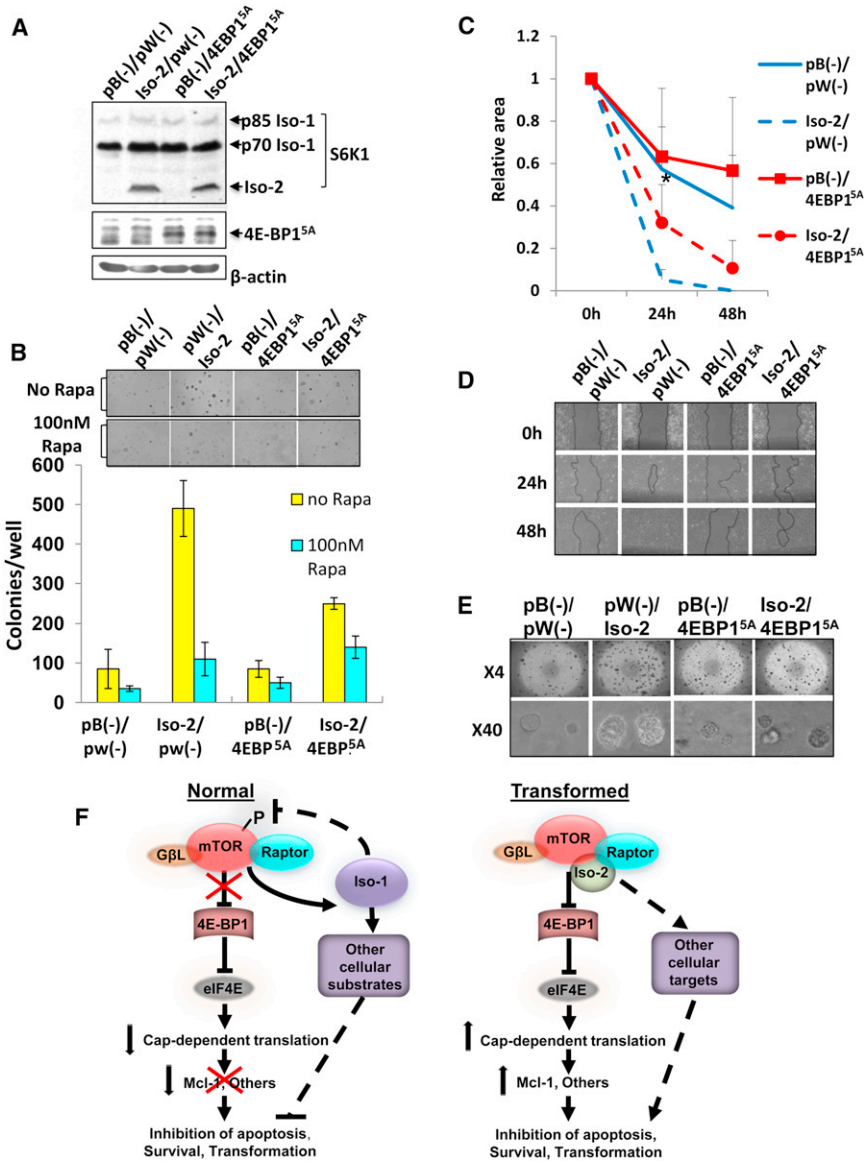

Figure 7. 4E-BP1 Inactivation and mTORC1 Activity Is Required for the Oncogenic Activities of S6K1 Short Isoforms

(A) MCF-10A cells were cotransduced with retroviruses encoding for empty vector pWZI-Hygro (pW) or pW-4E-BP1 phosphorylation defective mutant in which all five phosphorylation sites were mutated to alanine (4E-BP1^{5A}) (Hsieh et al., 2010) and empty vector pBABE (pB[−]) or pB- Iso-2. Total protein from stable pools was extracted and separated by SDS-PAGE. Membranes were probed with monoclonal antibodies against 4E-BP1 or S6K1 to detect the endogenous and exogenous isoforms. β-actin was analyzed as a loading control.

(B) MCF-10A pools of cells transduced with the indicated retroviruses as in (A) were seeded into soft agar in duplicates, with or without 100 nM rapamycin, and colonies were allowed to grow for 14 days. Colonies from ten fields of each well were counted, and representative fields of colonies were photographed in phase image (×100 magnification). Data represent the average number ± SD of colonies per well. n = 2.

(C) MCF-10A pools of cells transduced with the indicated retroviruses as in (A) were stimulated to migrate by physical wounding of cells seeded in monolayer. Data represent the average number of quantified wound area and SD from three individual experiments.

(D) Representative images of the wound area of cells described in (C).

(E) Phase images of MCF-10A cells transduced with the indicated retroviruses seeded in matrigel. Cells were allowed to grow for 2 weeks to form acini structures.

(F) A proposed model of mTORC1 regulation by S6K1 isoforms. In nontransformed cells upon mitogen stimulation, S6K1 Iso-1 is activated by mTORC1 and generates a feedback signal loop resulting in phosphorylation of mTOR at S2448 in the repressor domain. This might attenuate mTOR's ability to phosphorylate and repress 4E-BP1 leading to decreased cap-dependent translation. Other cellular substrates might contribute to the tumor-suppressive activity of Iso-1 independently of cap-dependent translation (left panel). In transformed cells S6K1 short isoforms are upregulated, bind mTORC1 and increase its activity. mTORC1 activation leads to enhanced 4E-BP1 phosphorylation, cap-dependent translation elevation of the antiapoptotic protein Mcl-1 and other proliferating or anti apoptotic proteins and increased cell survival and transformation (right panel).

See also Figure S6.

DISCUSSION

We have previously shown that mouse S6K1 is alternatively spliced to form a short isoform (Iso-2), which is essential for the oncogenic activity of the splicing factor oncoprotein SRSF1(Karni et al., 2007). Here, we report that in human cells S6K1 has two alternatively spliced short isoforms that are overproduced in breast cancer cell lines and tumors. Furthermore, all of S6K1 short splicing variants lack an autoinhibitory C terminus domain (Ali and Sabatini, 2005) and half of the kinase domain and do not exhibit kinase activity, at least on the known S6K1 substrate rpS6 (Figures 2A and S2E–S2G). Overexpression of mouse or human S6K1 short isoforms enhanced transformation, anchorage-independent growth, cell motility, and growth-factor-independent 3D acinus formation of human breast epithelial cells (Figures 2, 3A–3C, S2C, and S3A). Surprisingly, the long, kinase active S6K1 isoform (Iso-1) inhibited 3D acinus formation and reduced 4E-BP1 phosphorylation, cap-dependent translation, and transformation in vitro and in vivo, demonstrating many properties that characterize a tumor suppressor. Our results suggest that only S6K1 short isoforms, but not Iso-1, interact with and activate mTORC1 leading to elevated 4E-BP1 phosphorylation, enhanced cap-dependent translation, and upregulation of the antiapoptotic protein Mcl-1. Inhibition of mTORC1

or 4E-BP1 phosphorylation can partially reverse the oncogenic activity of S6K1 short isoforms, suggesting that their oncogenic properties are at least in part mediated by this pathway.

A Switch in *RPS6KB1* Alternative Splicing Upregulates Oncogenic Isoforms in Breast Cancer

We found that many breast cancer cell lines and tumors switch the splicing of *RPS6KB1* to elevate the human short isoforms of S6K1 h6A and h6C (Figures 1B–1D, S1F, and S1G). There are several documented examples of an alternative splicing switch that induces a prooncogenic isoform at the expense of a tumor-suppressive isoform. In many cancers, tumor-suppressive isoforms are downregulated, while prooncogenic ones are upregulated (David and Manley, 2010). We found that while all the short S6K1 isoforms, as well as the kinase-dead form of Iso-1, induced anchorage-independent growth, enhanced motility, and growth-factor-independent 3D acinus formation in matrigel, the active S6K1 isoform (Iso-1) did not (Figures 2, 3, and S2C). Moreover, in most of these assays Iso-1 showed an opposite effect, indicating that these splicing isoforms possess antagonistic activities. Several lines of evidence suggest that Iso-1 acts as a functional tumor suppressor. (1) When coexpressed with an active Ras^{V12} mutant, Iso-1 inhibited Ras-induced transformation in vitro and in vivo (Figure 4), suggesting it possesses an antitumorigenic activity. (2) Iso-1 knockdown in MCF-10A cells induced transformation, colony formation in soft agar, acinus formation, increased motility, and elevated Mcl-1 levels (Figures 4A, 5F, S4C, and S4D). (3) Loss of S6K1 and S6K2 had dramatic effect on cellular transformation, as immortalized MEFs from S6K1/2 DKO mice were transformed, formed large numbers of colonies in soft agar, and showed increased levels of the antiapoptotic protein Mcl-1 (Figure 6), which is translationally regulated by mTORC1(Hsieh et al., 2010). (4) Reintroduction of S6K1 Iso-1 partially inhibited the transformed phenotypes of S6K1/2 DKO cells indicating that Iso-1 possesses tumor-suppressive activities, but might also require the presence of S6K2 in these cells to restore its full capability as an antitumorigenic protein (Figure 6). It has been shown previously that S6K1 is required for insulinoma development in a mouse model where an active Akt mutant (*Myr-Akt*) was overexpressed in pancreatic beta cells under the insulin promoter (Alliouachene et al., 2008). Similarly, S6K1 deletion on the background of $PTEN^{+/-}$ and low expression of S6K2 in pheochromocytoma cells impaired adrenal tumorigenesis (Nardella et al., 2011). In both of these reports, the cancer was induced by activation of the same pathway (PI3K-Akt) and in a specific tissue. It is conceivable that under other experimental settings the outcome might be different, as already demonstrated for S6K1 deletion in other tissues (Nardella et al., 2011). The lack of an effect on tumor development in the latter cases is possibly due to redundancy between S6K1 and S6K2. Moreover, to the best of our knowledge, no study has directly shown that overexpressed or mutated S6K1 or S6K2 acts as an oncogene. Hence, the present report provides direct evidence for the pro- and antitumorigenic effects of stable knockdown or overexpression of S6K1 long isoform (Iso-1), respectively. It should be noted that the pro-oncogenic effect of Iso-1 knockdown does not involve S6K2, as the latter remained unchanged (Figure S5). Notably, the catalytic activity of S6K1 is essential for its tumor-suppressive activity, as the kinase-dead point mutant completely abrogated this activity. In addition, the fact that Iso-1 inhibited Ras transformation, but only partially inhibited cap-dependent translation raises the possibility that Iso-1 phosphorylates additional substrates other than those in the mTOR pathway, that contribute to its tumor suppressor activity independently of cap-dependent translation. Importantly, S6K1 short isoforms are catalytically inactive and did not induce phosphorylation of the known S6K1 substrates, the ribosomal protein S6 or mTOR itself in an in vitro kinase assay and upon transfection into cells (Figures 5A, 5E, S2E–S2G, and S3C). Altogether, these results suggest that Isoform-1 of S6K1 has tumor-suppressive properties, while the short isoforms are pro-oncogenic. Our results suggest that the gain of h6A and h6C observed in breast cancer cells and tumors is a mechanism to switch off a tumor-suppressive isoform and to turn on an oncogenic one.

RPS6KB1 Splicing Isoforms Modulate the Activity of mTORC1

Surprisingly, we found that most S6K1 short isoforms activated mainly 4E-BP1 phosphorylation without a significant activation of Akt or ERK and in a growth-factor-independent manner (Figures 3 and S3). In accordance with increased 4E-BP1 phosphorylation, cells expressing S6K1 short isoforms showed elevated cap-dependent translation and upregulation of Mcl-1, while Iso-1-expressing cells showed low or basal cap-dependent translation, similar to the control cells expressing empty vector or reduced cap-dependent translation in Ras-transformed cells (Figures 5 and S4G). Moreover, loss of S6K1 and S6K2 enhanced cap-dependent translation in MEF cells that was partially inhibited by S6K1 Iso-1 reintroduction (Figure 6), indicating that S6K1 and S6K2 activities suppress cap-dependent translation and transformation (Figure 6). Although surprising, previous studies support this result: Cells from a knockin mouse of a mutant S6 gene ($rpS6^{P-/-}$), where all five phosphorylation sites were replaced to alanines, showed a 2-fold increase in global translation as measured by methionine incorporation and a 2-fold increase in proliferation rate as compared to MEFs from WT littermates (Ruvinsky et al., 2009). Combining these two findings, both inactivation of the "kinase" (S6K1/2 DKO) and inhibition of phosphorylation of the substrate ($rpS6^{P-/-}$) gave similar results of increased translation. This suggests that S6 phosphorylation might inhibit rather than increase translation. Other studies did not find reduced translation upon S6K1/2 loss, even though they did not observe increased translation (Mieulet et al., 2007). These results suggest that the growth-factor-independent growth observed in matrigel (Figure 3) could be explained by the ability of S6K1 short isoforms to activate mTORC1 that, in turn, inactivates 4E-BP1 even under low nutrient conditions. Enhanced cap-dependent translation contributes to cancer development by elevating the translation of several oncogenes and antiapoptotic genes such as *Mcl-1*, *Bcl-X*, *MDM2*, *HIF-1*α, *β-catenin*, *c-myc*, *cyclin D1*, and others, which are translationally regulated by cap-dependent translation and the mTORC1 pathway (Hsieh et al., 2010; Karni et al., 2002; Karni et al., 2005; Mamane et al., 2004). We found that, at least in the case of the antiapoptotic protein

Mcl-1, overexpression of S6K1 short isoforms, Iso-1 knockdown or S6K1/2 knockout enhanced its expression (Figures 5E, 5F, and 6D). To examine if S6K1 short isoforms can interact with mTOR, we coexpressed mTOR with S6K1 Iso-1 or Iso-2 and found that only the short isoform could pull down mTOR, whereas Iso-1 did not, even though its expression was much higher (Figures 5A and 5B).

The Oncogenic Activities of S6K1 Short Isoforms Are Partly Mediated through 4E-BP1 and mTORC1 Activation

We examined the contribution of mTORC1-4E-BP1 axis to S6K1 short-isoform-mediated oncogenesis and found that inhibition of eIF4E/4E-BP1 dissociation greatly inhibited motility, anchorage-independent growth, and growth-factor-independent acinus formation in matrigel (Figures 7 and S6). These results suggest that most of the oncogenic effects of S6K1 short isoforms are mediated by mTORC1 activation. However, we cannot rule out the possibility that S6K1 short isoforms affect other oncogenic signaling pathways as suggested by the fact that expression of a dominant-negative 4E-BP1 did not fully suppress colony formation in soft agar and motility induced by S6K1 short isoforms. These results also suggest that S6K1 is not only a substrate of mTOR, but that it may also modulate the activity of mTOR increasing 4E-BP1 phosphorylation when S6K1 short isoforms are elevated (Figures 3 and S3D). In addition, S6K1 Iso-1 can phosphorylate mTOR at serine 2448 (Figures 3F, 5A, and 6A), although the consequences of this phosphorylation on mTOR activity are not fully understood (Chiang and Abraham, 2005; Holz and Blenis, 2005). S6K1 short isoforms cannot be phosphorylated by mTOR at threonine 389 and by phosphoinositide-dependent kinase 1 (PDK1) at threonine 229, as they lack both phosphorylation sites. However, both Iso-1 and the short isoforms should be able to bind mTORC1, since all of them contain the Raptor binding motif called mTOR-signaling (TOS motif) (Schalm and Blenis, 2002). Thus, while Iso-1 activity responds to mitogenic and nutritional stimuli, as well as energy status, the short isoforms are refractory to any of these signals, yet they can still affect mTORC1. Indeed, our data suggest that these short isoforms transmit through mTORC1 activation, a constitutive mitogenic/metabolic signal, even in the absence of growth factors (Figures 3 and S3). In addition, S6K1 short isoforms cannot induce mTOR S2448 phosphorylation, as they lack a functional kinase domain and are catalytically inactive (Figures 5A and S2E–S2G). Thus, our current hypothesis is that Iso-1 phosphorylates mTOR and inhibits cap-dependent translation and transformation. Iso-1 also phosphorylates other cellular substrates that might lead to suppression of transformation. In contrast, the short isoforms bind to the same or other cellular targets and thereby induce opposite effects. S6K1 short isoforms can bind directly to mTORC1 and enhance its activity possibly by competing with Iso-1 for mTOR binding (Figure 7F). Nevertheless, the mechanism by which S6K1 short isoforms activate mTOR is yet to be determined.

We have discovered alternatively spliced variants of the gene encoding S6K1. Our findings provide additional insight into the oncogenic switch that can be caused by alternative splicing. Furthermore, another mode of mTORC1 activation is identified in this study, which may open a new direction in manipulating mTORC1 activity for cancer therapy. In addition, we show that, the balance between S6K1 short isoforms and Iso-1 is tipped toward those short isoforms in most tumors examined, suggesting that this splicing event can be a useful marker for breast cancer development. Moreover, since S6K1 short isoforms activate mTORC1 and this activation is important for tumorigenesis, we expect that the newly approved active site mTOR inhibitors will act more efficiently on tumors where the S6K1 alternative splicing switch has occurred.

EXPERIMENTAL PROCEDURES

Cells

NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) calf serum (BS), penicillin, and streptomycin. Human breast cells, MCF-10A, were grown in DMEM/F12 supplemented with 5% (v/v) horse serum, 50 ng/ml EGF, 10 μg/ml insulin, 0.5 μg/ml hydrocortisone, 100 ng/ml cholera toxin, penicillin, and streptomycin (Arias-Romero et al., 2010). MCF-7, MDA-MB-231, and HEK293 cells were grown in DMEM, HMLE cells were grown in MEBM/DMEM/F12, and MDA-MB-468 cells were grown in Leibovitz-F12, supplemented with 10% (v/v) fetal bovine serum, penicillin, and streptomycin. SUM159 cells were grown in Ham's F12 with 5% (v/v) calf serum, 5 μg/ml insulin, and 1 μg/ml hydrocortisone. S6K1/2 double-knockout MEFs were generated as described in Pende et al. (2004) and were immortalized by the 3T3 protocol as described in Dowling et al. (2010).

Stable Cell Lines

NIH 3T3 and MCF-10A cells were infected with pBABE-puro retroviral vectors encoding T7-tagged S6K1 isoforms human and mouse cDNAs as described (McCurrach and Lowe, 2001). Infected cells were selected with puromycin (2 μg/ml) or hygromycin (200 μg/ml) for 72–96 hr. In the case of double infection with pWZL-hygro-Ras (McCurrach and Lowe, 2001), cells were treated with hygromycin for 72 hr after selection with puromycin. In the case of infection with MLP-puro-shRNAs vectors, MCF-10A cell transductants were selected with puromycin (2 μg/ml) for 96 hr.

Immunoblotting

Cells were lysed in Laemmli buffer and analyzed for total protein concentration as described (Golan-Gerstl et al., 2011). Primary antibodies were as follows: Sigma: β-catenin (1:2,000) phospho-ERK (1:10,000 T202/Y204); Santa Cruz: β-actin (1:200), total Akt (1:200 sc1619), GAPDH (1:1,000 sc25778), Myc (1:1,000 sc40); Novagen: T7 tag (1:5,000); BD Trunsduction Laboratories: total S6K1-anti-p70; Cell Signaling Technologies: total ERK1/2 (1:1,000); phospho-4E-BP1 Thr70 (1:1,000 #2855), 4E-BP1 (1:1,000 #9452), phospho-S6K1 Thr389 (1:1,000 #9205), p-S6 ser240/244 (1:1,000 #2215), total S6 (1:1,000 #2217), phospho-Akt ser473 (1:1,000 #9271), RAS (1:1,000 #3339), p-S2448-mTOR (1:1,000 #2972), secondary antibodies: HRP-conjugated goat anti-mouse, anti-rabbit, or anti-goat IgG (H^+L) (1:10,000 Jackson Laboratories).

Immunoprecipitation

HEK293 cells were transfected with myc-mTOR and pCDNA3-T7-Iso-2/h6A/h6C/pCDNA3 or pCDNA-T7-Iso-1 S6K1 with empty pCDNA. Forty-eight hours after transfection, cells were lysed in CHAPS buffer as described (Sarbassov et al., 2004). After protein quantitation, 1 μg of anti-T7 antibody bound to 40 μl of 50% protein G-Sepharose was incubated with 800 μg of total protein lysate overnight. After washing four times with CHAPS buffer and beads were incubated with 50 μl of 2 × Laemmli buffer and separated by SDS-PAGE.

Colony-Survival Assay

S6K1/2 DKO MEF cell populations were transduced with retroviruses encoding for empty pB(–) vector or T7-Iso-1 S6K1. After selection, cells were seeded sparsely (200 cells/well of six-well plates) and were grown for 14 days. Colonies were fixed and stained with methylene blue as described (Karni et al., 2007).

Dual Luciferase Reporter Assay

MCF-10A cells were cotransfected using Fugene 6 (Roche) with the dual reporter vector pLPL (Cap-Renilla-IRES-Luciferase) (Gerlitz et al., 2002) and the indicated S6K1 isoforms. Cap-dependent translation (Renilla luciferase activity) and IRES-mediated translation (Firefly luciferase activity) were measured with the Promega Stop and Glow assay kit according to the manufacturer's instructions.

EGF and IGF-1 Activation

MCF-10A cells (3×10^5) were seeded in six-well plates. Twenty-four hours later, cells where washed with PBS, and the medium was replaced with a serum and growth factor-free media for 24 hr. Cells were stimulated with 50 ng/ml IGF-1 or 50 ng/ml EGF for 4 hr. After 4 hr, cells were lysed in Laemmli buffer for western blot analysis.

Anchorage-Independent Growth

Colony formation in soft agar was assayed as described (Karni et al., 2007; McCurrach and Lowe, 2001). After 14–21 days, colonies from ten different fields in each of two wells were counted for each transductant pool, and the average number of colonies per well was calculated. The colonies were stained as described (Karni et al., 2007; McCurrach and Lowe, 2001).

Growth Curves

MCF-10A cells were infected with the indicated retroviruses. Following selection, 2,000 or 3,500 cells per well were seeded in 96-well plates and grown in DMEM/F12 media with 5% or 0.2% horse serum. Cells were fixed and stained with methylene blue as described (Karni et al., 1999), and the absorbance at λ = 650 nm of the acid-extracted stain was measured on a plate reader (Bio-Rad).

3D Morphogenesis of Mammary Epithelial Cell Assay

MCF-10A cells were infected with the indicated retroviruses. Following selection, 1,500 cells per well were seeded in 96-well plates in growth-factor- reduced matrigel (BD Bioscience, Cat# 25300), as described in Zhan et al. (2008). The method is also described in Debnath et al. (2003). In some cases, 2.5 or 5 ng/ml of EGF or 10 μg/ml of insulin was added to assay medium of seeded cells.

Wound-Healing Assay

MCF-10A cells were seeded in a six-well plate until formation of a confluent monolayer, and a "wound" was created by scratching the monolayer with a p200 pipette tip as described in Liang et al. (2007). Cells were washed with PBS and growth medium was replaced to DMEM/F12 media with 5% horse serum without EGF and insulin. The wound was photographed (×100 magnification) after matching the reference point in a phase contrast microscope and wound area was measured using Photoshop record measurement analysis tools.

Xenograft Tumor Formation in Mice

Pools of Ras-MCF-10A cells expressing the indicated S6K1 isoforms were injected subcutaneously into the rear flanks of NOD-SCID mice (2×10^6 cells per site in 100 μl of serum-free media containing 0.25 v/v matrigel (BD Bioscience) using a 26-gauge needle. Tumor growth was monitored as described (Karni et al., 2007). Veterinary care was provided to all animals by the Hebrew University animal care facility staff in accordance with AAALAC standard procedures and as approved by the Hebrew University Ethics committee (no. MD-08-11517-4).

Statistical Analysis

All data presented as histograms refer to a mean value ± SEM of the total number of independent experiments. An unpaired, two-tailed t test was used to determine p values for Figures 2C and 5B.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, six figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2012.11.020.

LICENSING INFORMATION

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