

S6K1 and E2FB are in mutually antagonistic regulatory links controlling cell growth and proliferation in *Arabidopsis*

Rossana Henriques,^{1,†,*} Zoltán Magyar^{1,2} and László Bögre^{1,*}

¹Royal Holloway; University of London; School of Biological Sciences; Egham, UK; ²Institute of Plant Biology; Biological Research Centre; Szeged, Hungary

[†]Current Affiliation: Center for Research in Agricultural Genomics (CRAG); Consortium CSIC-IRTA-UAB-UB; Parc de Recerca UAB; Edifici CRAG; Barcelona, Spain

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Plant development is dependent on the coordination between growth and cell proliferation. The nutrient sensing TOR kinase and its downstream target, the 40S ribosomal S6 Kinase, are central controllers of cell growth that were also shown to determine cell size by inhibiting the onset of mitosis in yeast and animal cells. We have shown that the *Arabidopsis* S6 Kinase1 inhibits cell proliferation through the RBR-E2FB complex. S6K1 interacts with RBR via its N-terminal RBR binding motif, promotes its nuclear localization and consequent RBR-dependent repression of cell cycle genes through E2FB. Here we show that S6K1 and E2FB are in a mutually antagonistic relationship both in their protein abundance and in their activity. We propose that this double inhibitory regulatory connection between S6K1 and E2FB forms a regulatory switch that might be important to determine whether cells divide or grow.

Growth and cell proliferation are intimately interlinked processes. In yeast, growth precedes and is required for cell proliferation, while in animal cells it is thought to be either upstream, or in parallel pathways that respond to specific stimuli such as growth or mitogenic factors.^{1,2} Nutrients and growth factors regulate the entry into cell proliferation by stimulating the accumulation of G1-phase specific cyclins in yeast, plant and animal cells. In addition, growth conditions can also affect the size of cells; yeast cells grown at favorable conditions are larger than when grown in poor media.²⁻⁴ The TOR signaling pathway is an evolutionary conserved nutrient sensor that sets the growth capacity of cells by regulating protein synthesis.⁵⁻⁷ It was shown that nutrient limiting conditions and inhibition of TOR activity, specifically the TORC1 complex, lead to the onset of mitosis and thus to reduced cell size both in yeast and in animal cells.^{4,8} Starvation activates the AMP-activated kinase (AMPK1) that was also shown to regulate mitosis directly or through the inhibition of TOR.⁹⁻¹¹ Importantly, both upstream regulators and downstream effectors of the TORC1 complex, such as the 40S ribosomal protein S6 kinase were shown to be involved in the cell size control.¹²⁻¹⁴

In plant meristems, cell size homeostasis is also strictly controlled during cell proliferation. Similarly to animals, the TOR signaling pathway was shown to regulate growth, protein synthesis and metabolism in *Arabidopsis*.¹⁵⁻²¹ A conserved downstream TOR target is the *Arabidopsis* S6 Kinase (S6K) that phosphorylates the ribosomal protein S6.²¹⁻²³ We have shown that S6K1

is a repressor of cell proliferation.²⁴ Interestingly, this function requires the RETINOBLASTOMA RELATED PROTEIN (RBR) and its target E2FB. S6K1 forms a complex with RBR and E2FB, promotes the nuclear localization of RBR and potentiates its repressor activity on E2FB targets, such as on the plant specific mitotic cyclin dependent kinase *CDKB1;1* gene and on the S-phase specific ribonucleotide reductase *RNR2* gene.²⁴ In this report we show that S6K1 and E2FB have an antagonistic relationship both at the protein abundance and activity levels. This double inhibition wiring in regulatory networks is an important feature of molecular switches that regulate cellular decisions.²⁵

Silencing of *S6K1* promotes E2FB transcriptional activity. We have shown before that overexpression of the *E2FB/DPA* heterodimer induces the expression of *CDKB1;1*.²⁶ In agreement, transient overexpression of the *E2FB/DPA* heterodimer in protoplasts resulted in higher activity of the *CDKB1;1* promoter, an effect abolished when the E2F-specific binding site was mutated (Fig. 1A). We also found that depletion of *S6K* expression by RNA interference, resulted in an increased activity of the *CDKB1;1* promoter²⁴ (Fig. 1B). To demonstrate that S6K silencing affects *CDKB1;1* promoter activity through E2FB, we co-silenced *E2FB* and *S6K* and found that the *CDKB1;1* promoter activity was reduced.

***Arabidopsis* S6K1 and E2FB oppositely affect each other at the post-translational level.** We showed that S6K1, E2FB and RBR co-immunoprecipitated in vivo and thus are in a protein

*Correspondence to: Rossana Henriques and László Bögre; Email: rossana.henriques@cragenomics.es and l.bogre@rhul.ac.uk

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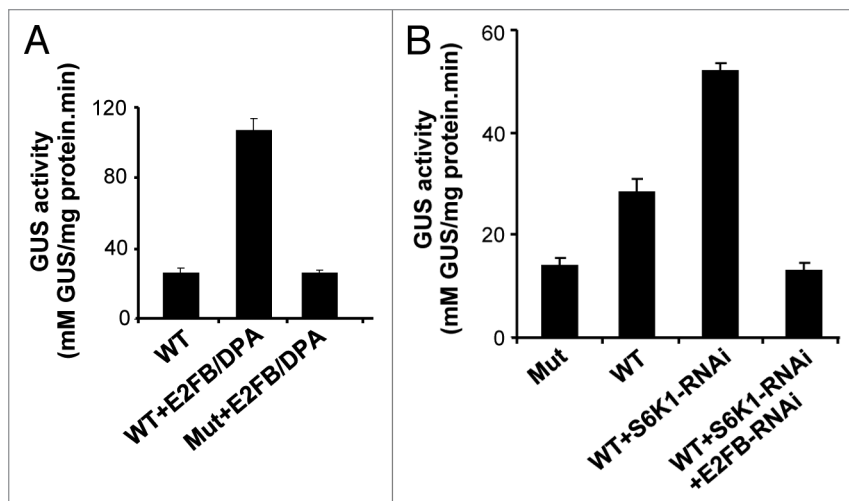


Figure 1. S6K1 regulates the *CDKB1;1* promoter activity through E2FB. (A) The *CDKB1;1* WT promoter (WT) and *CDKB1;1* mutant (Mut) promoter, where the consensus E2F binding site was mutated³⁵ were fused to the *GUS* reporter gene and GUS activity measured in cells co-transformed with *E2FB/DPA* constructs. (B) Determination of activity of the *CDKB1;1* WT and Mut promoter fused to the *GUS* reporter gene in cells transformed with a *S6K1*-RNAi construct alone or together with *E2FB*-RNAi.

complex.²⁴ We investigated whether changes in the level of any of these proteins could affect the abundance of the complex components. Surprisingly, we found that silencing of *S6K* led to an increase of E2FB protein level, as detected through the Myc-epitope tagged construct of E2FB, expressed under the control of CaMV35S promoter in transformed protoplasts (Fig. 2A). To demonstrate that *S6K1* kinase activity is required for the regulation of E2FB protein level, we overexpressed a mutant, kinase-dead version of *S6K1* that also resulted in increased E2FB levels (Fig. 2A). In contrast to E2FB, *S6K* silencing had no effect on RBR protein level (Fig. 2B).

Next we investigated how E2FB and RBR levels affected *S6K* abundance by co-transformation of *S6K1*-HA with either *E2FB*-RNAi or *RBR*-RNAi constructs. When *E2FB* was silenced the *S6K1*-HA level increased. In contrast, *RBR* silencing led to a decrease in *S6K1*-HA (Fig. 2C).

We also determined whether the E2FB and *S6K1* levels are regulated through a change in protein stability. To do this, we compared Myc-E2FB protein levels in control and *S6K* silenced cells in conditions when the de novo protein synthesis was inhibited by cycloheximide (CHX). The E2FB level was elevated in *S6K*-silenced cells already at time 0, and this did not diminish in the presence of CHX, while in control cells the level of E2FB decreased (Fig. 2D, upper panel). In a corresponding experiment, we found that silencing of *E2FB* led to an increased *S6K1*-HA level, which diminished slowly compared with the control cells (from less than 1h to around 6h) (Fig. 2D, lower panel). This data suggested that the opposite function of E2FB and *S6K* on cell proliferation could be reinforced by their reciprocal effect on each others' stability.

S6K1 phosphorylates RBR specifically when E2FB is silenced. We have shown that *S6K1* regulates RBR activity to repress the mitotic *CDKB1;1* gene. We therefore investigated

whether *S6K* could directly phosphorylate RBR and if this activity was regulated by the E2FB levels. To do this, we expressed in *Arabidopsis* cells a wild type *S6K1*-HA construct and a kinase-dead (*S6K1*-KD-HA) mutant version of it. We purified *S6K1* through the HA-tag from *Arabidopsis* cells with and without *E2FB* silencing and assayed the in vitro phosphorylation of a GST-RBR-pocket substrate protein, a region that is responsible for binding to the E2F transcription factors. *S6K1*-HA, immunoprecipitated from control cells had undetectable phosphorylation activity on the RBR pocket region. In contrast, immunoprecipitation of *S6K1*-HA from cells co-transformed with an *E2FB*-RNAi construct resulted in an active *S6K1*-HA that phosphorylated the GST-RBR-pocket substrate (Fig. 2E and F). To demonstrate that *S6K1* kinase activity, and not another co-purified kinase, is responsible for RBR phosphorylation, we also transformed a kinase-dead mutant form of *S6K1* (*S6K1*-KD-HA) which did not phosphorylate the GST-RBR pocket fragment, even when E2FB was silenced (Fig. 2E and F). Interestingly, the kinase-dead form of *S6K1* was much less abundant than the active *S6K1* (Fig. 2D). These results indicate that *S6K1* regulates RBR by direct phosphorylation specifically when E2FB levels are low.

In our previous work we showed that *S6K1* negatively regulates cell proliferation by promoting the cell cycle inhibitory function of the plant RB ortholog, RBR.²⁴ A similar effect was recently reported when *S6K1* was expressed under the control of the auxin responsive element DR5.²⁷ We have shown that *S6K1* interacts with RBR and E2FB leading to E2FB repression. In agreement, *S6K* silencing increases the activity of E2FB target genes, including the plant specific mitotic *CDKB1;1*.²⁶ Recently, we have shown that sucrose levels control the amount and activity of RBR/E2Fs complexes, leading to different outcomes in cell growth and proliferation.²⁸ In the presence of sucrose RBR is phosphorylated, dependent on *CYCD3;1* and *KRP2* levels (Fig. 3). This inactivates RBR, leading to the release of E2FB that will stimulate cell proliferation. In agreement, overexpression of E2FB accelerates cell cycle progression and cells divide with a smaller cell size.^{26,29} The dramatic effect of E2FB overexpression on total cell mass also indicated an inhibition of cell growth but the underlying molecular mechanisms were not known.²⁶ Here we show that *S6K1* and E2FB are in a mutually antagonistic relationship both in their protein abundance and in their activity. Silencing of *E2FB* activates *S6K1* that phosphorylates RBR, while silencing of *S6K1* leads to more E2FB that can trans-activate the mitotic regulator, *CDKB1;1*. The mutual inhibition of E2FB and *S6K1* is regulated by protein stability, as we showed that *S6K1* protein was stabilized in *E2FB* silenced cells, and E2FB became stabilized when *S6K1* was silenced (Fig. 2A–D). These observations seem to depend on E2FB and *S6K1* activities. Overexpression of a dominant negative kinase-inactive *S6K1* mutant construct

also resulted in an increased E2FB protein level; while silencing of *RBR*, which releases active E2FB, destabilized the S6K1 protein (Fig. 3). The mechanism how E2FB and S6K regulate each other's protein stability is not yet known. There are several candidate F-box protein genes carrying E2F-binding elements in their promoters³⁰ that could be induced by elevated E2FB levels and regulate protein stability. RBR, E2FA and E2FB are known to be unstable especially under sucrose-depleted conditions.^{31,32} S6K might regulate E2FB turnover by phosphorylation either directly or indirectly. This post-translational modification could then be a mark for proteasomal degradation similarly to E2FC and DPB.^{33,34}

Collectively, these findings are consistent with a model that S6K1 represses cell proliferation by enhancing RBR function through several routes: by the regulation of RBR localization, by enhancing RBR binding to E2FB and by repressing E2FB accumulation. On the other hand accumulation of active E2FB represses cell growth through destabilization of S6K1. These opposing functions could provide the molecular link between cell growth (S6K) and cell proliferation (E2FB) (Fig. 3).

Materials and Methods

Constructs. The constructs used in this work have been described elsewhere, *E2FB*-RNAi,²⁸ *S6K1*-RNAi, *S6K1*-HA,²⁴ *E2FB*/DPA,²⁶ *pRT100::S6K1-KD-HA* (kinase-dead S6K1 where Lys163, responsible for phosphate coordination in the active center, was replaced by Arg) was kindly provided by Dr. M. Teige, and confirmed by sequencing. *E2FB* was cloned in frame with the N-terminal double Myc epitope,³⁶ and the Myc tagged *E2FB* was further cloned into the pK7WG2D Gateway vector (Invitrogen) following the manufacturer's instructions.

In vitro RBR kinase assay and immunoblotting. S6K1 activity toward the RBR-pocket region (RBR-PR) was measured in protoplasts transformed with *S6K1*-HA, *S6K1*-HA+*E2FB*-RNAi, *S6K1*-kinase dead (KD)-HA and *S6K1*-KD-HA+*E2FB*-RNAi constructs. HA antibody was used to immunoprecipitate S6K1 and kinase assays were performed as described.³⁷ The pGEX::RBR-PR polypeptide was purified accordingly to the manufacturer's instructions (Promega) and 1 μ g was used as substrate.

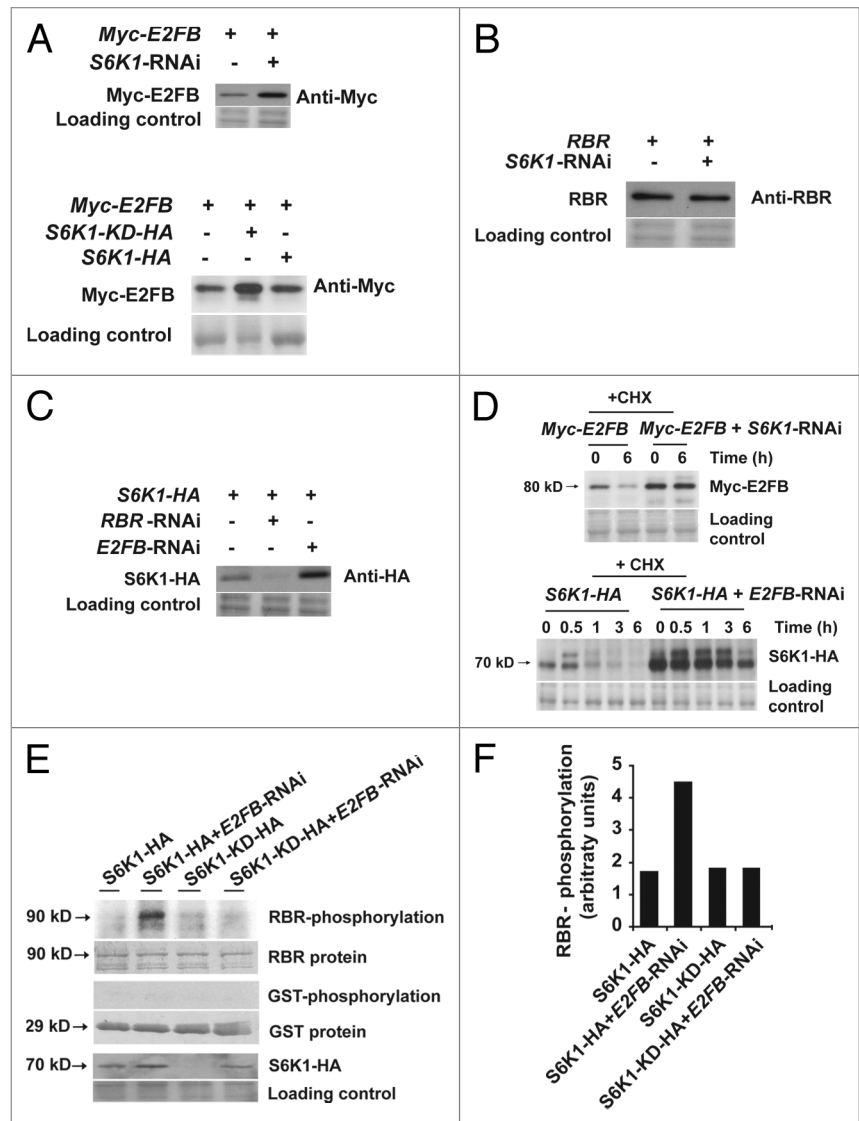


Figure 2. Mutual inhibition of S6K1 and E2FB is regulated at the stability and activity levels. (A) S6K regulates E2FB accumulation. Detection of Myc-E2FB expressed from a construct transformed to control cells (first lane, upper panel) or to cells co-transformed with the *S6K1*-RNAi (second lane, upper panel). Myc-E2FB in control cells (first lane, lower panel), in cells co-transformed with *S6K1*-KD-HA, a kinase-dead form with the active center mutated (second lane, lower panel) and in cells co-transformed with *S6K1*-HA (third lane, lower panel). (B) RBR expressed from a construct transformed to control cells (first lane) or in cells co-transformed with the *S6K1*-RNAi construct (second lane). (C) RBR-E2FB regulate S6K1 accumulation. *S6K1*-HA expressed from a construct transformed to control cells (first lane), to cells co-transformed with the *RBR*-RNAi (second lane) or to cells co-transformed with *E2FB*-RNAi (third lane). (D) Regulation of E2FB and S6K stability. Upper panel: detection of Myc-E2FB expressed from a construct transformed to control cells or to cells co-transformed with the *S6K1*-RNAi construct in the presence of 100 μ M of cycloheximide (CHX) at 0 and 6 h. Lower panel: detection of *S6K1*-HA expressed from a construct transformed to control cells or to cells co-transformed with the *E2FB*-RNAi construct in the presence of 100 μ M of CHX from 0 to 6 h. (E) Phosphorylation of RBR-pocket-GST fusion protein (first row) and only GST (third row) with immunopurified *S6K1*-HA and *S6K1*-KD-HA expressed in control cells or in cells where *E2FB* was silenced by RNAi (*E2FB*-RNAi). Fifth row: detection of *S6K1*-HA in these samples. Note the elevated expression level of *S6K1*-HA when *E2FB* was silenced, a result that corresponds to (A). (F) Quantification of the phosphorylation signals shown in (D).

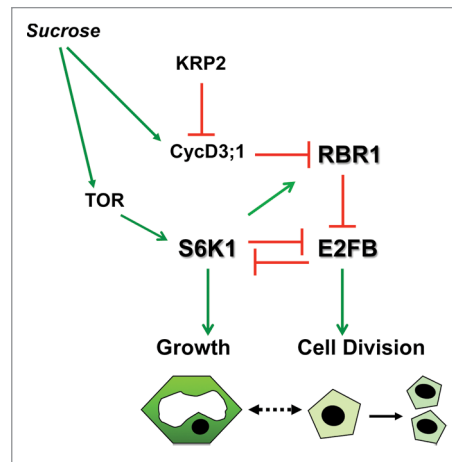


Figure 3. Model explaining the antagonistic functions of E2FB and S6K1. Sucrose availability increases the CycD3;1 amount and presumably the TOR kinase activity, and decreases KRP2. CycD3;1 in complex with CDKA phosphorylates RBR, which releases E2FB and that drives cells into the mitotic cell cycle. On one hand E2FB destabilizes S6K1 and negatively affects cell growth. On the other hand active S6K1 destabilizes E2FB and potentiates RBR to inhibit cell proliferation. This antagonistic relationship of S6K1 and E2FB could play an important role to keep cell proliferation as is the case of small meristematic cells, or allow cells to grow and increase the cell size.

Reactions were resolved in a 10% SDS-PAGE gel and the phosphorylation signal was detected on a Typhoon 9410 phosphorimager and quantified by the ImageQuant software (GE Healthcare). Immunoblotting was performed as described.²⁴

Protein stability assays. Protoplast cells were transformed with HA-S6K1 or Myc-E2FB constructs alone or in combination with *E2FB*-RNAi or *S6K1*-RNAi constructs, respectively

and treated further with 100 μ M of cycloheximide (CHX) for up to six hours and protein levels were followed by western blot analyses by using specific antibodies against the epitope tag as described in reference 24.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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