

## Control of contact-inhibition by 4E-BP1 upregulation

Rania Azar,<sup>1,†</sup> Christiane Susini,<sup>1</sup> Corinne Bousquet<sup>1</sup> and Stéphane Pyronnet<sup>1,2,\*</sup>

<sup>1</sup>INSERM U858; Institut de Médecine Moléculaire de Rangueil (I2MR); Département Cancer; and <sup>2</sup>Pôle Digestif; CHU de Toulouse; Toulouse, France

<sup>†</sup>Current address: Department of Clinical Pharmacy; Faculty of Pharmacy; Lebanese University; Hadath, Lebanon

**A**lthough contact inhibition is a fundamental process for multicellular organisms, how proliferation is inhibited at high cellular densities remains poorly characterized. Here we show that 4E-BP1, one major repressor of cap-dependent translation, plays a critical role in density-mediated cell cycle arrest. 4E-BP1 promoter is activated and 4E-BP1 protein amount increases as cells reach confluence. Conversely, a much less marked density-dependent inhibition of cell proliferation is observed upon 4E-BP1 silencing. We further show that at high density, progression through the G<sub>1</sub> phase of the cell cycle is faster and Cyclin D1 protein is induced in different cell types where 4E-BP1 has been either downregulated (stable shRNA expression or transient siRNA transfection) or removed (knock-out). Thus 4E-BP1 appears as an important mediator of contact inhibition.

### Introduction

In eukaryotic cells, the ribosome is recruited to the mRNA 5' end via the cap-binding complex eIF4F (eukaryotic translation initiation factor 4F).<sup>1</sup> This protein complex is composed of three translation initiation factors named eIF4E (the cap-interacting protein), eIF4A (an RNA-helicase) and eIF4G (a scaffolding protein which binds directly to eIF4E and eIF4A). eIF4G also interacts with the mRNA<sup>2</sup> and with eIF3,<sup>3</sup> another factor associated with the 40S small ribosomal subunit. Thus, via its simultaneous association with eIF4E and eIF3, one biochemical function of eIF4G is to dock the small ribosomal subunit at the mRNA 5' cap and therefore to ensure efficient protein synthesis.<sup>4</sup>

The rate of protein synthesis is tightly controlled through the cell cycle.<sup>5,6</sup> We and others have shown for instance that protein synthesis augments as cells enter G<sub>1</sub> and progress through S.<sup>7,8</sup> These changes can be attributed in part to the control exerted by 4E-BP1 (eIF4E-binding protein 1). In resting cells, the hypophosphorylated forms of 4E-BP1 indeed compete with eIF4G for binding to eIF4E, preclude eIF4F assembly, and therefore maintain protein synthesis repressed. As cells are stimulated to proliferate, the enhancement of 4E-BP1 phosphorylation permits the release of eIF4E which can then assemble with eIF4G to form a cap-binding complex which in turn relieves translation silencing. Once cycling cells have attained confluence they are contact-inhibited and the rate of protein synthesis is in contrast diminished. It has been shown that 4E-BP1 is likely involved as cell-density-mediated inhibition of protein synthesis correlates with activation (hypophosphorylation) of 4E-BP1.<sup>9,10</sup> However, when cell reach confluence the causal link between 4E-BP1 activation (and consequent inhibition of cap-binding complex assembly) and exit from the cell cycle has never been demonstrated.

Here we show that 4E-BP1 gene transcription increases in a cell density-dependent manner, and that 4E-BP1 protein accumulation is involved in cell cycle arrest at contact inhibition likely through downregulation of cyclin D1 expression.

### Results and Discussion

To explore the role of 4E-BP1 in contact inhibition we have monitored the expression of 4E-BP1 protein, and its capability

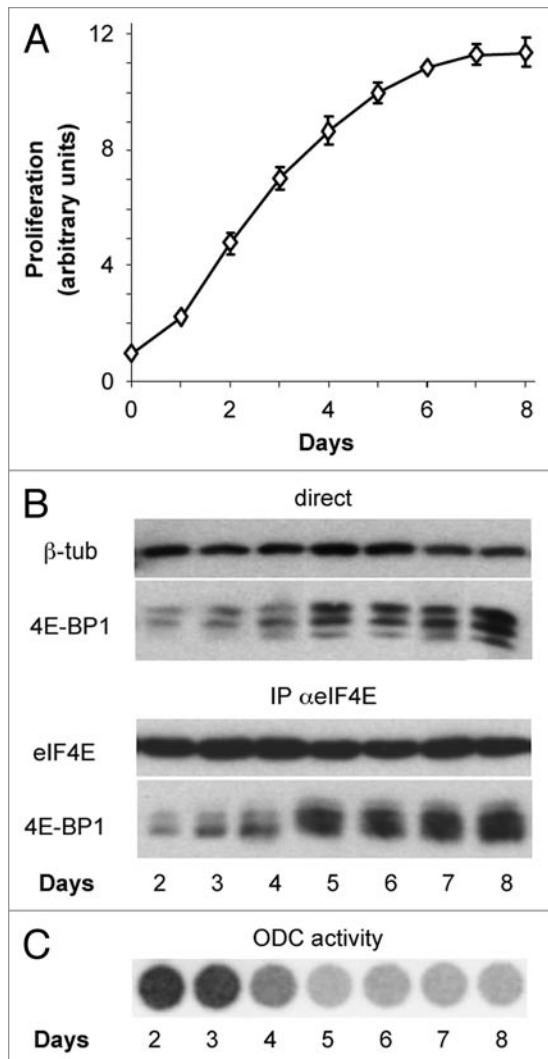
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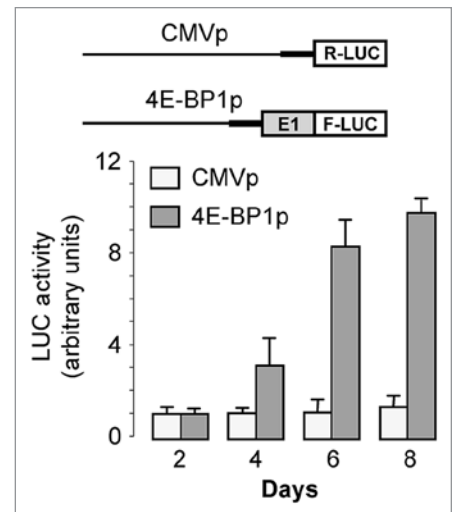
\*Correspondence to: Stéphane Pyronnet;  
Email: [stephane.pyronnet@inserm.fr](mailto:stephane.pyronnet@inserm.fr)



**Figure 1.** Confluence-dependent activation of 4E-BP1. (A) Proliferation assay with Panc-1 cells. Data are the means (+/- SE) of three independent experiments performed in triplicates and are expressed as a function of the value obtained the day of seeding. (B) Equal amounts of proteins obtained from cells at different days of proliferation were examined by SDS-PAGE immunoblotting either directly (top) or following eIF4E immunoprecipitation (bottom) using antibodies against  $\beta$ -tubulin (internal control), 4E-BP1 and eIF4E as indicated. (C) Protein extracts obtained as above were processed for ODC activity.

to interact with the cap-binding protein eIF4E, as a function of cell density in the established pancreatic cancer cell line Panc-1. A proliferation assay first showed that Panc-1 cells grow exponentially at low density (Fig. 1A, days 0–4). Proliferation was then slackened at higher density until contact inhibition occurred (Fig. 1A, days 5–8). A concomitant western-blot analysis revealed that 4E-BP1 accumulation became apparent as the rate of proliferation slowed and was maximal in contact inhibited cells (Fig. 1B, top, days 5–8). This suggested that density-dependent inhibition of protein synthesis generally

observed when cells attain confluence might be due in part to sequestration of the cap-dependent translation initiation factor eIF4E by 4E-BP1. Therefore, to verify this hypothesis we performed a kinetic analysis of 4E-BP1 binding to eIF4E by co-immunoprecipitation. The data revealed that the amount of 4E-BP1 bound to immunoprecipitated eIF4E was in direct proportion to the level of 4E-BP1 (Fig. 1B, bottom). Then, to control whether eIF4E sequestration by 4E-BP1 was actually correlated to inhibition of translation initiation, we analysed the expression ornithine decarboxylase



**Figure 2.** Confluence-dependent activation of 4E-BP1 promoter activity. LUC activities were assayed 36 hours following Panc-1 cells transfection as described in Material and Methods. Data are the means (+/- SE) of three independent experiments performed in triplicates and are expressed as a function of the values obtained for day 2.

(ODC), a protein known to be regulated at the level of translation initiation.<sup>11,12</sup> The enzymatic activity of ODC, which reflects ODC protein amount, paralleled confluence-dependent 4E-BP1 induction and consequent eIF4E sequestration (Fig. 1C). These data indicated that on mRNAs known to be sensitive to eIF4E/4E-BP1-dependent translational control can be downregulated due to 4E-BP1 accumulation as cell density increases.

We have previously shown that cell density-dependent accumulation of 4E-BP1 can be attenuated by silencing of the Smad4 transcription factor.<sup>13</sup> This suggested the existence of a transcriptional mechanism. To address this question, we have tested whether 4E-BP1 promoter activity could be stimulated by cell density in a reporter gene system. We used the 4E-BP1 genomic sequence spanning from nucleotides -628 to +219 and fused to a firefly LUC reporter open reading frame (ORF) because we have shown that this construct permits efficient transcription following transient transfection of Panc-1 cells.<sup>13</sup> As an internal control, activity from a co-transfected Renilla luciferase coding sequence driven by the CMV promoter (depicted in Fig. 2A) was also followed.

The measures of both LUC reporter genes revealed that while CMV promoter activity did not change significantly, a strong increase (up to ten-fold) in 4E-BP1 promoter activity was monitored (Fig. 2B), thus indicating that 4E-BP1 promoter is the target of a positive cell-density-dependent regulation.

Next, we searched for the existence of a causal link between transcriptional induction of 4E-BP1 (and consequent sequestration of eIF4E) and contact inhibition. To this end, a proliferation assay was performed using a stable panc-1 cell line (named MirBP1)<sup>13</sup> where 4E-BP1 can be silenced on demand, via doxycycline-inducible expression of 4E-BP1-targeted shRNAs. In this model, apparition of the green fluorescent protein (GFP) serves as a control of doxycycline induction, as described in Materials and Methods (see below). Silencing of 4E-BP1 became apparent 48 hours following treatment with doxycycline (Fig. 3A, MirBP1, day 3 after seeding) and was maintained to the end of the proliferation assay (day 7). In the doxycycline-treated mock-transfected inducible cell line (named MirNeg),<sup>13</sup> a cell-density-dependent accumulation of 4E-BP1 was in contrast clearly observed (Fig. 3A, MirNeg), as shown in the non-transfected parental Panc-1 cell line (Fig. 1B). Interestingly, the rate of proliferation and the density of cells at the end of the assay were both higher in MirBP1 cells (where 4E-BP1 was silenced) as compared to MirNeg cells (Fig. 3B). To better understand how 4E-BP1 silencing and consequent release of eIF4E accelerated cell proliferation and mediated contact inhibition we then explored the impact of 4E-BP1 silencing on cell cycle phases by flow cytometry. The data revealed that cells where 4E-BP1 level was down-regulated (Fig. 3C, MirBP1 cells) passed through the G<sub>1</sub> phase of the cell cycle faster than cells where the amount of 4E-BP1 was left unchanged (MirNeg cells). This was observed when flow cytometry was performed from both sparse (day 3) and dense (day 6) cells.

One critical regulator of the cell cycle whose expression has been shown to be sensitive to eIF4E/4E-BP1-dependent translational control is cyclin D1.<sup>14-16</sup> Furthermore, cyclin D1 interacts with

CDK4 or CDK6, two cyclin-dependent kinases which have been implicated in the progression through G<sub>1</sub>,<sup>17</sup> and down-regulation of cyclin D1 has been shown to be involved in confluence-dependent exit from the cell cycle.<sup>18</sup> It was therefore highly probable that the faster progression through G<sub>1</sub> we observed upon 4E-BP1 silencing in Panc-1 cells (Fig. 3C) was due, at least in part, to upregulation of cyclin D1 protein. As expected, a western-blot analysis showed that cyclin D1 protein amount was higher once 4E-BP1 was silenced in Panc-1 cells (Fig. 4, left, compared MirBP1 to MirNeg). Finally, we wanted to extend our observation that 4E-BP1 can control cyclin D1 expression outside the pancreatic cancer situation. To this purpose, we used two additional models of cultured cells: human immortalized keratinocytes (HaCat cells) and mouse embryonic fibroblasts (MEFs). Interestingly, cyclin D1 protein amount was slightly increased upon 4E-BP1 silencing by siRNA transfection in HaCat cells (Fig. 4, middle), and frankly higher in MEFs isolated from 4E-BP1<sup>-/-</sup> mice as compared to MEFs isolated from 4E-BP1<sup>+/+</sup> mice (Fig. 4, right).

From these data we concluded that 4E-BP1 is a critical regulator of cell cycle exit when cells are let grown to confluence. This may have important impacts in cancer where anarchic proliferation substitutes to contact inhibition and participates in tumor growth. Indeed, the possibility that translational control of gene expression may be targeted for cancer therapy has drawn much attention the past-decade.<sup>16,19</sup> In this context, our observations further argue in favour of a potent anti-cancer benefit of compounds that stimulate or mimic 4E-BP1 activity.

## Material and Methods

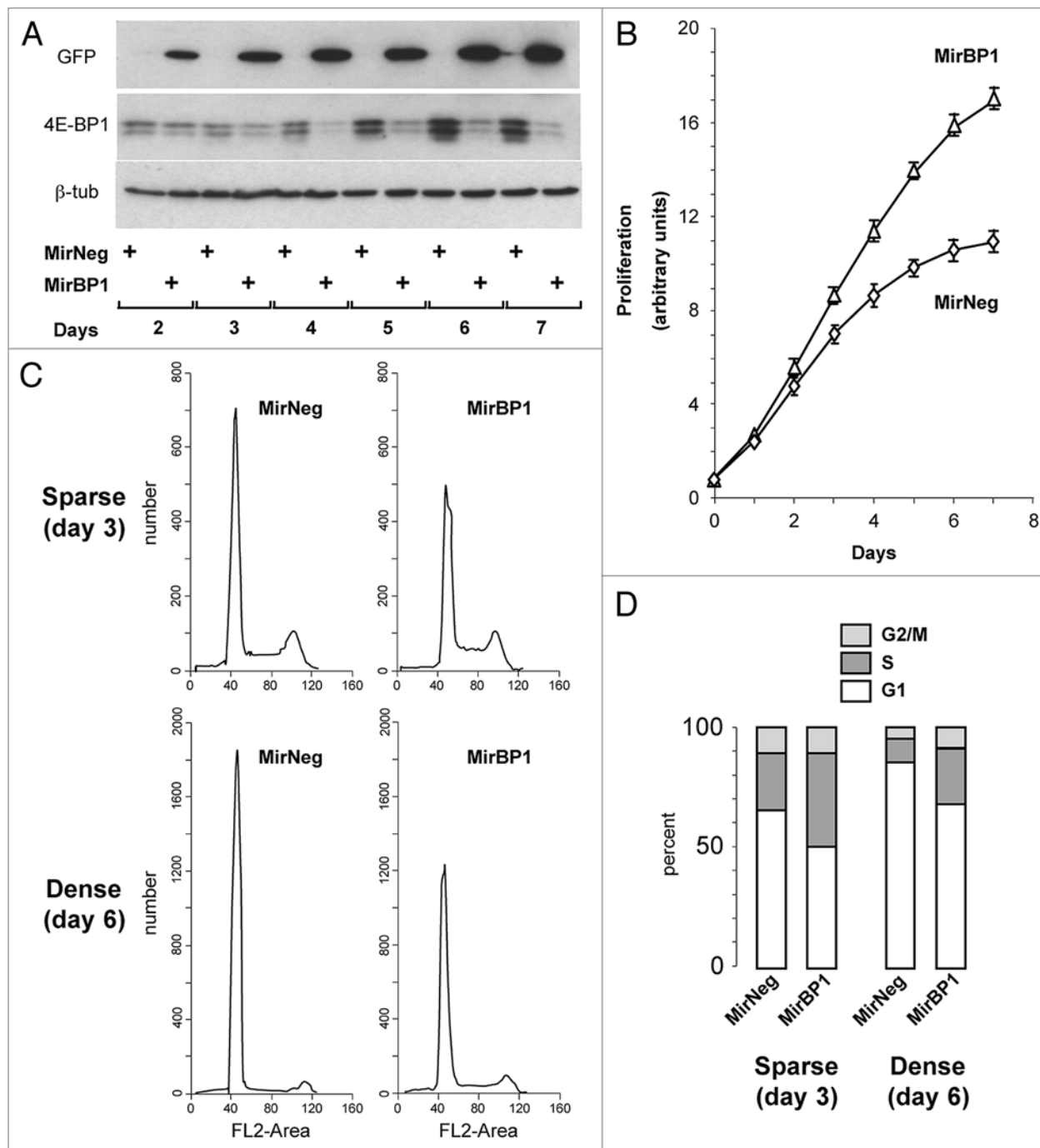
**Cell culture and drug treatment.** Panc-1 WT, Panc-1 pTet-on advanced vector (Clontech) and pTRE-Tight-miRNeg-transfected (MirNeg) or pTRE-Tight-miRBP1-transfected (MirBP1) were obtained previously.<sup>13</sup> MirNeg or MirBP1 Panc-1 clones permit doxycycline-inducible expression of GFP together with either non-targeted or 4E-BP1-targeted shRNAs, respectively. Upon induction by

doxycycline, expression of GFP (encoded by pTRE-Tight vectors) serves as an internal control, while expression of endogenous 4E-BP1 is downregulated in the MirBP1 clone, but not in MirNeg cells. These two clones and the other cell lines including HaCaT and immortalized MEFs isolated from 4E-BP1/4E-BP2 double-knockout mice (4E-BP1<sup>-/-</sup>) or from their normal littermates (4E-BP1<sup>+/+</sup>) were cultured as described previously.<sup>13</sup> Induction of MirNeg or MirBP1 was achieved by treatment of cells with 100 ng/ml doxycycline (Sigma) for indicated times.

**ODC activity.** ODC activity was measured as previously described.<sup>20</sup> Briefly, cell lysates were cleared, protein content was quantified and equal amounts of total protein (100 µg) were incubated with 2.5 µCi of <sup>14</sup>C-labelled ornithine (Amersham) and 50 µM pyridoxal 5-phosphate for 1 h at 37°C. Incubations were performed in 96-well microtiter plates. Liberated <sup>14</sup>CO<sub>2</sub> was trapped in a covering 3 MM paper saturated with a solution of barium hydroxide. The 3 MM paper was rinsed with acetone, dried and exposed to an X-ray film.

**Flow cytometry and proliferation assay.** For cell cycle analysis, cells were harvested, stained with propidium iodide (Pharmingen) as described previously,<sup>21</sup> and analyzed on a FACS Caliber Flow Cytometer using Cell Quest (BD) software. For proliferation assays, cells were plated in six-well dishes and counted with a Coulter counter (Coulter Electronics).

**Immunoprecipitation and western analysis.** Cells were harvested, lysed, protein concentration of extracts was measured using Protein Assay reagent (Bio-Rad), and equal amounts of proteins were either immunoprecipitated with mouse monoclonal antibody to eIF4E (Santa-Cruz Biotechnology) or subjected directly to SDS-PAGE and electroblotted onto Immobilon-P membranes (Millipore) as described previously.<sup>22</sup> Membranes were incubated with mouse monoclonal antibodies to β-tubulin (Sigma), or with rabbit polyclonal antibodies to 4E-BP1 (Cell Signaling), eIF4E (Cell Signaling), cyclin D1 (Cell Signaling) or GFP (Ab-cam). Bound antibodies were detected with peroxidase-coupled goat antibodies to mouse



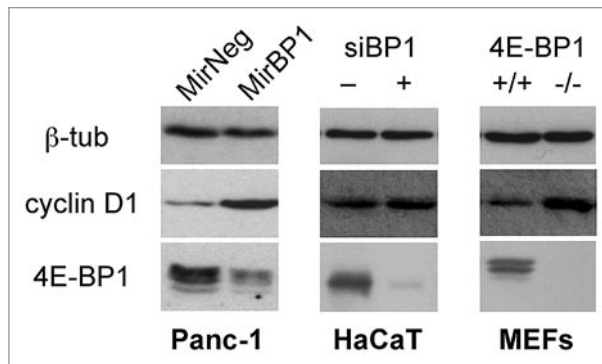
**Figure 3.** Accelerated G<sub>1</sub> phase upon 4E-BP1 silencing. (A) Equal amounts of protein extracts from miRNeg or miRBP1 Panc-1 cells incubated in the presence of 100 ng/ml of doxycycline were examined by SDS-PAGE immunoblotting using antibodies against GFP, 4E-BP1 or  $\beta$ -tubulin (internal control) as indicated. (B) Proliferation assays with miRNeg or miRBP1 Panc-1 cells incubated in the presence of 100 ng/ml of doxycycline. Data are the means ( $\pm$  SE) of three independent experiments performed in triplicates and are expressed as a function of the value obtained the day of seeding. (C) Flow cytometry analysis of miRNeg or miRBP1 Panc-1 cells incubated in the presence of 100 ng/ml of doxycycline. (D) The data obtained in (C) were quantified. Histograms show the relative amount (%) of cells that distribute in the different phases of the cell cycle.

or rabbit IgG (Pierce) using chemoluminescence detection reagent (Pierce).

**Luciferase assay.** To determine 4E-BP1 and CMV promoter activities, the dual-luciferase reporter assay system (Promega)

that permit the measure of firefly and Renilla luciferases simultaneously was used. In brief, cells ( $10^5$  cells/well) were plated in six-well plates, transiently co-transfected with a plasmid vector carrying

human 4E-BP1 promoter (extended to exon1 sequence; p4E-BP1)<sup>13</sup> fused to firefly Luciferase and a plasmid vector carrying CMV promoter (pCMV) fused to Renilla Luciferase (pRL-CMV; Promega)



**Figure 4.** Cyclin D1 expression is dependent on 4E-BP1 level in different cell types. Equal amounts of protein extracts from Panc-1 cells stably expressing non-targeted or 4E-BP1-targeted shRNAs (left), HaCat cells transiently transfected with non-targeted or 4E-BP1-targeted siRNAs (middle) and 4E-BP1<sup>+/+</sup> or 4E-BP1<sup>-/-</sup> MEFs (right) were examined by SDS-PAGE immunoblotting using antibodies against  $\beta$ -tubulin (internal control), cyclin D1 or 4E-BP1 as indicated.

as depicted in **Figure 2**. Cells were transfected with Exgen 500 (Euromedex) and let grown for 36 hours in normal culture medium before harvesting in Passive Lysis Buffer (Promega) at the indicated times after seeding. Extracts were assayed for firefly and Renilla luciferase activities and detected with Centro LB 960 (Berthold Technologies) as described previously.<sup>22</sup>

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