4E-BP1 is a target of Smad4 essential for TGF β -mediated inhibition of cell proliferation

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Assembly of the multi-subunit eukaryotic translation initiation factor-4F (eIF4F) is critical for protein synthesis and cell growth and proliferation. eIF4F formation is regulated by the translation-inhibitory protein 4E-BP1. While proliferation factors and intracellular pathways that impinge upon 4E-BP1 phosphorylation have been extensively studied, how they control 4E-BP1 expression remains unknown. Here, we show that Smad4, a transcription factor normally required for TGF β -mediated inhibition of normal cell proliferation, enhances 4E-BP1 gene-promoter activity through binding to a conserved element. 4E-BP1 expression is specifically modulated by treatment with TGF β and by manipulations of the natural Smad4 regulators (co-Smads) in cells isolated from Smad4^{+/+} human tumours, whereas no response is observed in cells isolated from Smad4^{-/-} human tumours or in cells where Smad4 has been knocked down by specific siRNAs. In addition, cells where 4E-BP1 has been knocked down (inducible shRNAs in human pancreatic cancer cells or siRNAs in non-malignant human keratinocytes) or has been knocked out (mouse embryonic fibroblasts isolated from 4E-BP1^{-/-} mice) proliferate faster and are resistant to the antiproliferative effect of TGF β . Thus, 4E-BP1 gene appears critical for TGF β /Smad4-mediated inhibition of cell proliferation.

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Introduction

In eukaryotic cells, most mRNAs are translated through the cap-binding protein eukaryotic translation initiation factor-4E (eIF4E), which interacts with the ribosome-associated protein eIF4G, thus docking the ribosome at the mRNA 5' end (Gingras *et al*, 1999; Gebauer and Hentze, 2004). eIF4E's function is controlled by the translation-inhibitory protein 4E-BP1 (Richter and Sonenberg, 2005), which competes with eIF4G for a common binding site on eIF4E. Upon various

stimuli, 4E-BP1 is hyper-phosphorylated and frees eIF4E, which can in turn stimulate protein synthesis and consequent cell growth and proliferation (Bjornsti and Houghton, 2004; Sonenberg and Pause, 2006). While the signalling pathways (mainly the PI3K/AKT/mTOR cascade) that control 4E-BP1 phosphorylation have been extensively studied (Armengol *et al*, 2007), little is known about the regulation of its expression. Yet, PI3K also impinges upon 4E-BP1 gene transcription (Azar *et al*, 2008), and regulation of 4E-BP1 gene transcription appears critical for resistance to stress (Tettweiler *et al*, 2005; Yamaguchi *et al*, 2008). Consistently, in addition to inactivation by phosphorylation, alterations in 4E-BP1 levels have been described under different pathological conditions. For instance, eIF4E gain of function consequent to downregulation of the amount of 4E-BP1 has been shown to participate in the genesis and maintenance of the cancer phenotype (Avdulov *et al*, 2004). Similarly, increased levels of eIF4E stimulates protein synthesis, induces cell growth and proliferation (De Benedetti and Graff, 2004; Mamane *et al*, 2004), and promotes tumour formation in animal models (Ruggero *et al*, 2005; Wendel *et al*, 2007). To gain insight into the molecular mechanisms that control 4E-BP1 expression, we first performed *in silico* analysis (Genomatix) of its genomic sequence. Among the transcription factors identified (Supplementary Figure S1), the p53 and Smad4 tumour suppressors appeared as highly probable candidates. The use of cells with or without p53 expression indicated that differences in the activity of p53 have no effect on 4E-BP1 promoter activity (A Elia, MJ Clemens, R Azar and S Pyronnet, data not shown). Many arguments, however (see below), identified Smad4 as a stronger candidate.

Smad4 is a transcription factor also termed DPC4 (Deleted in Pancreatic Cancer locus 4) because it was first identified as a candidate tumour-suppressor gene frequently altered in human pancreatic adenocarcinomas (Hahn *et al*, 1996). Smad4 acts as a tumour suppressor likely through its function in TGF β signalling (Siegel and Massagué, 2003; ten Dijke and Hill, 2004). For instance, upon pancreatic damage, production of TGF β exerts a protective effect through a Smad4-dependent transcriptional programme, which blocks epithelial-cell proliferation and maintains a polarized phenotype. Following Smad4 loss, epithelial cells are no longer protected and are susceptible to transformation. Thus, given that (i) 4E-BP1 gene sequence (exon-1) contains a putative Smad4-binding element (Supplementary Figure S1), (ii) Smad4 is a pancreatic tumour suppressor and (iii) pancreas is the organ that expresses the most 4E-BP1 mRNA (Tsukiyama-Kohara *et al*, 1996), we hypothesized that 4E-BP1 could be a transcriptional target of Smad4 important for TGF β 's antiproliferative action, at least in pancreatic cells.

Results and discussion

To test the possibility that 4E-BP1 acts downstream of TGF β in a Smad4-dependent manner, we first analysed the

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expression levels of 4E-BP1 and Smad4 in four different cell lines (Panc-1, MiaPaca-2, BxPC-3 and Capan-1) that exhibit various Smad4 and/or TGF β -receptor status (Grau *et al*, 1997; Giehl *et al*, 2000). Whereas the TGF β -Smad4 signalling cascade is fully active in Panc-1 cells, it is altered in the three other cell lines as TGF β -RII receptor (MiaPaca-2 cell line) or Smad4 (BxPC-3 and Capan-1 cell lines) is absent, as described in the Supplementary Table S1. The data we obtained showed that the amounts of 4E-BP1 protein and mRNA were elevated in Smad4^{+/+} cell lines (Panc-1 and MiaPaca-2), but low in Smad4^{-/-} cell lines (BxPC-3 and Capan-1; Figure 1A and B). To explore the molecular mechanism that could link Smad4 to 4E-BP1 transcription, we have first delineated the 4E-BP1 genomic sequence that permits efficient transcription following transient transfection of Smad4^{+/+} pancreatic cells. A series of deletion mutants spanning from nucleotide -960 (relative to the putative +1 transcription start site) to the end of exon-1 (+219) has been generated and fused to a firefly LUC reporter open reading frame (ORF; Figure 1C). In Smad4^{+/+} Panc-1 cells, the higher transcriptional activity was obtained when a promoter fragment of 4E-BP1 gene was extended to the end of exon-1

(Figure 1C, -628/+219). Similar data were obtained with the other Smad4^{+/+} cell line MiaPaca-2 (Figure 1D). Furthermore, the activity of such extended promoter fragment reflected cell-line-dependent variability of endogenous 4E-BP1 and Smad4 levels (compare Figure 1D to A and B), whereas other fragments lacking exon-1 did not (not shown), suggesting that Smad4 responsiveness resided in 4E-BP1 exon-1. This hypothesis was further supported by chromosome immunoprecipitation assays (ChIP). We first showed that endogenous Smad4 binding to endogenous 4E-BP1 promoter was detected in Smad4^{+/+} MiaPaca-2 (Figure 1E) or Panc-1 (not shown) cells solely when primers encompassing exon-1 were used. Consistently, no PCR fragment was obtained following anti-Smad4-ChIP assay of DNA extracted from Smad4^{-/-} cells, and the amount of Smad4 bound to 4E-BP1 promoter was in direct proportion to the endogenous level of Smad4 protein in the different cell lines (Figure 1F).

Interestingly, sequence alignments of the 4E-BP1 nucleotide sequences from different species indicated that the putative Smad4-binding element (SBE: CAGCCAGA) in exon-1 is conserved among mammals (Supplementary Figure S1). To confirm that this putative sequence is a true

Figure 1 4E-BP1 and Smad4 in pancreatic epithelial cell lines. (A) Immunoblotting of equal amounts of proteins from different cell lines using antibodies against Smad4 and β -tubulin (internal control) (top), eIF4E (middle) and 4E-BP1 (bottom). (B) Semi-quantitative RT-PCR of total RNAs from different cell lines using primers specific to 4E-BP1 and GAPDH (internal control). (C) Transcriptional activity of 4E-BP1 promoter deletion mutants inserted upstream of firefly LUC. LUC activity was assayed 36 h following Panc-1 cell transfection. Transfection efficiency was normalized to the concurrent transfection of a CMV-*Renilla* LUC reporter plasmid. LUC activities are representative of three independent experiments performed in triplicates, and are expressed as percents of the value obtained for the -628/+219 promoter fragment after normalization to the residual firefly LUC activity of pGL2-Basic. (D) Cell-line-dependent transcriptional activity of the 4E-BP1 -628/+219 promoter fragment. Firefly LUC activity normalized to the concurrent transfection of a CMV-*Renilla* LUC reporter plasmid are the means ($\pm$ s.e.) of three separate experiments performed in triplicates. (E) ChIP assay of MiaPaca-2 cell extracts using a specific antibody against Smad4 and a set of primers that covers the -278/+219 4E-BP1 promoter fragment. (F) ChIP assay of DNA of different cell lines using a specific antibody against Smad4 and the E1 primers described in panel E.

Smad4-binding element, the human sequence was mutated (Figure 2A, hMut) and tested in different cell lines where Smad4 status was manipulated. The data first revealed that mutations in the putative SBE rendered 4E-BP1 promoter much less active in Smad4^{+/+} cells (Figure 2B, left, mock), but had no effect in Smad4^{-/-} cells (Figure 2B, right, mock). Then, inactivation of Smad4 by transfection with a dominant-negative truncated form (DN) in Smad4^{+/+} cells clearly inhibited wild-type 4E-BP1 promoter activity but had no effect on the mutated promoter (Figure 2B, left). Conversely, re-expression of the transcription factor in Smad4^{-/-} cells stimulated wild-type 4E-BP1 promoter activity, but had no effect on the mutated sequence (right). In

addition, activity of the mutated 4E-BP1 promoter in Smad4^{+/+} cells became equivalent to that of the wild-type promoter in Smad4^{-/-} cells (Figure 2C). Finally, the use of specific siRNAs showed that silencing of Smad4 substantially diminished endogenous 4E-BP1 expression (Figure 2D). These data indicated that binding of Smad4 to a responsive element located in 4E-BP1 exon-1 enhanced 4E-BP1 promoter activity.

Smad4 belongs to a large family of proteins (Xu, 2006), which also includes Smad1, 2, 3, 5, 6, 7 and 8 (also termed co-Smads). Smad1, 2, 3, 5 and 8 are phosphorylated by TGF β -receptor kinases and are, therefore, referred to as R-Smads (receptor-activated Smads). Upon phosphorylation, R-Smads

Figure 2 4E-BP1 exon-1 contains a functional Smad4-binding element (SBE). (A) Alignment of 4E-BP1 exon-1 sequences from rat, mouse and human, which contain a conserved computer-predicted SBE (box). Mutated nucleotides that leave unchanged the human amino-acid sequence (hMut) but destroy the putative SBE are underlined. (B) SBE destruction renders 4E-BP1 promoter insensitive to Smad4. Left: Firefly LUC activity normalized as in Figure 1D was assayed in Smad4^{+/+} cells (Panc-1 and MiaPaca-2) 36 h following co-transfection with either wild-type (hWT) or mutated (hMut) –628/+219 4E-BP1 promoter fragment and vector alone (mock) or a dominant negative form of Smad4 (DN). Right: Firefly LUC activity normalized as in Figure 1E was assayed in Smad4^{-/-} cells (BxPC-3 and Capan-1) 36 h following co-transfection with either wild-type (hWT) or mutated (hMut) –628/+219 4E-BP1 promoter fragment and vector alone (mock) or Smad4. Values are the means ($\pm$ s.e.) of three separate experiments performed in triplicates. (C) SBE destruction or absence of Smad4 has a similar effect on 4E-BP1 promoter activity. Firefly LUC activity normalized as in Figure 1E was assayed in Smad4^{+/+} (Panc-1 and MiaPaca-2) or Smad4^{-/-} (BxPC-3 and Capan-1) cells 36 h following transfection with either wild-type (hWT) or mutated (hMut) –628/+219 4E-BP1 promoter fragment. Values are the means ($\pm$ s.e.) of three separate experiments performed in triplicates. (D) Smad4 silencing alters 4E-BP1 expression. MiaPaca-2 cells were transfected with either a nonspecific siRNA (NS) or a siRNA targeting Smad4 mRNA for 48, 72 or 96 h, and equal amounts of proteins were examined by SDS-PAGE immunoblotting as shown.

interact with Smad4, and the neo-formed heterodimeric complex translocates to the nucleus and targets genes that contain Smad4-binding elements. Smad1, 5 and 8 are activated by BMPs (bone morphogenetic proteins), whereas Smad2 and 3 (which were used in this study) act specifically downstream of TGF β . Smad6 and 7 belong to the I-Smads (inhibitory Smads) subgroup. They bind to Smad4 and preclude its association with R-Smads, and hence inhibit TGF β signalling.

To explore the possibility that 4E-BP1 transcription could be specifically modulated by Smad4 downstream of TGF β , we first focused on the Smad4 activators Smad2 and 3, and on the Smad4 inhibitor Smad7. Cell lines were transfected with different combinations of Smads and 4E-BP1 promoter activity was monitored (Figure 3). Expression of Smad2 or Smad3 alone had no (or poor) effect on 4E-BP1 promoter activity in either cell line. Expression of Smad4 alone slightly (160%) induced 4E-BP1 promoter activity in Panc-1 cells (which already contain Smad4), but was not significant in MiaPaca-2 cells (which contain the highest level of Smad4 and which carries a defective TGF β -RII receptor). Induction of 4E-BP1 promoter activity following transfection with Smad4 was more pronounced in the Smad4^{-/-} cell lines BxPC-3 (180%) and Capan-1 (200%). In addition, Smad4-dependent induction of 4E-BP1 promoter activity was not affected by coexpression of Smad2, but was substantially enhanced by coexpression of Smad3 in every cell line. Conversely, expression of Smad7 inhibited 4E-BP1 promoter activity in Smad4^{+/+} cells (Panc-1 and MiaPaca-2), had no effect in Smad4^{-/-} cells (BxPC-3 and Capan-1) and precluded Smad4-dependent stimulation of 4E-BP1 promoter activity observed following transfection of Smad4 in every cell line. These data indicated that 4E-BP1 promoter activity was sensitive to the co-Smads known to act specifically downstream of TGF β .

We have then tested more directly whether 4E-BP1 expression and/or activity can be regulated by TGF β in a Smad4-dependent manner. As suspected, TGF β had no effect on 4E-BP1 in the Smad4^{-/-} cell line Capan-1 (Figure 4A). However, treatment of Smad4^{+/+} Panc-1 cells with TGF β provoked accumulation of 4E-BP1 and a corresponding enhancement in 4E-BP1 activity as attested by increased binding of 4E-BP1 to eIF4E (Figure 4A). Strikingly, a reporter assay showed that TGF β actually induced 4E-BP1 promoter activity in Panc-1 cells, whereas no induction was detected in the three other cell lines lacking either TGF β -RII (MiaPaca-2) or Smad4 (BxPC-3 and Capan-1) (Figure 4B). Such stimulation in Panc-1 cells was specific to TGF β as it was precluded by co-incubation with anti-TGF β blocking antibodies (anti-TGF β ; Figure 4B). Similarly, the enhanced stimulation of 4E-BP1 promoter activity observed upon TGF β treatment in Panc-1 cells transfected with Smad4 was precluded with co-treatment with anti-TGF β blocking antibodies (Supplementary Figure S2). TGF β -stimulated 4E-BP1 promoter activity was also precluded either by mutations that destroy the SBE (hMut) or by coexpression of the dominant negative truncated form (DN) of Smad4 (Figure 4C). Conversely, restoration of Smad4 in Smad4^{-/-} BxPC-3 and Capan-1 cells rescued 4E-BP1 promoter responsiveness to TGF β (Figure 4D). These data indicated that TGF β activated 4E-BP1 promoter activity through Smad4.

To test for the implication of 4E-BP1 in the inhibition of epithelial cell proliferation by TGF β , we have created a

Figure 3 Regulation of 4E-BP1 promoter activity by co-Smads. Firefly LUC activity normalized as in Figure 1E was assayed in different cell lines co-transfected with the -628/+219 4E-BP1 promoter fragment and various combinations of Smad2, 3, 4 or 7 as described. Values are the means ($\pm$ s.e.) of three separate experiments performed in triplicates.

pancreatic cell line where the level of endogenous 4E-BP1 protein can be manipulated on demand, by using a doxycyclin-inducible vector and RNA interference properties. First, an siRNA complementary to the 4E-BP1 3'UTR (siBP1) was validated for its ability to actually silence 4E-BP1 expression

Figure 4 TGF β controls 4E-BP1 activity through Smad4-dependent induction of 4E-BP1 promoter. **(A)** Regulation of 4E-BP1 expression and binding to eIF4E by TGF β . Capan-1 or Panc-1 cells were treated with TGF β and equal amounts of proteins were subjected to immunoprecipitation and/or examined by SDS-PAGE immunoblotting as shown. **(B)** Regulation of 4E-BP1 promoter activity by TGF β in different cell lines. Firefly LUC activity normalized as described in Figure 1E was assayed in different cell lines following transfection with –628/+219 4E-BP1 promoter fragment for 36 h and treatment with TGF β alone or together with blocking TGF β antibodies (α TGF β) for 24 h. Values are the means ($\pm$ s.e.) of three separate experiments performed in triplicates. **(C)** Requirement of Smad4 in TGF β -induced 4E-BP1 promoter activity. Firefly LUC activity normalized as described in Figure 1E was assayed in Panc-1 cells following co-transfection with wild-type (hWT) or mutated (hMUT) –628/+219 4E-BP1 promoter fragment and vector alone (mock) or a dominant-negative (DN) form of Smad4 for 36 h and treatment with TGF β for 24 h. Values are the means ($\pm$ s.e.) of three separate experiments performed in triplicates. **(D)** Overexpression of Smad4 enhances 4E-BP1 promoter induction by TGF β . Firefly LUC activity normalized as described in Figure 1E was assayed in BxPC-3 or Capan-1 cells following co-transfection with the –628/+219 4E-BP1 promoter fragment and vector alone (mock) or a Smad4 for 36 h and treatment with TGF β for 24 h. Values are the means ($\pm$ s.e.) of three separate experiments performed in triplicates.

at least for 72 h following transient transfection of Panc-1 cells, as compared with a nonspecific siRNA directed against LUC (siNS; Figure 5A). Then, to avoid inconveniences due to transient transfection of siRNAs, we have generated a cell line (derived from Panc-1 cells) stably transfected with a doxycycline-inducible vector driving simultaneous expression of GFP (used as a marker protein) and either a nonspecific microRNA (directed against LacZ; miRNeg) or a microRNA directed against 4E-BP1 (derived from siBP1; miRBP1; Supplementary Figure S3). Panc-1 cells were chosen because they possess both Smad4 and functional TGF β receptors (Supplementary Table S1), and because their proliferation has been shown to be inhibited by TGF β in a Smad4-dependent manner (Grau *et al*, 1997). Dose-response (Figure 5B, right, top) and time-course (right, bottom) assays showed that efficient extinction of 4E-BP1 was achieved following treatment with 100 ng/ml doxycycline for 72 h. Extinction was specific as doxycycline treatment of miRNeg-stably

transfected cells had no effect on 4E-BP1 amount (Figure 5B, left). A proliferation assay then revealed that 4E-BP1 protein silencing increased proliferation rate (Figure 5C), thus showing that 4E-BP1 attenuates pancreatic epithelial-cell proliferation. Finally, the importance of 4E-BP1 in the antiproliferative effect of TGF β was evaluated. As expected, TGF β was capable of inhibiting Panc-1-cell proliferation (Figure 5D, compare miRNeg with miRNeg + TGF β). Interestingly, silencing 4E-BP1 protein in contrast accelerated proliferation (compare miRNeg with miRBP1) and prevented the inhibitory effect of TGF β (compare miRNeg + TGF β with miRBP1 + TGF β). Taken together, these data indicate that 4E-BP1 is a new target of Smad4 that is important for the antiproliferative effect of TGF β in pancreatic cancer cells.

Finally the implication of 4E-BP1 in TGF β -mediated inhibition of cell proliferation was tested outside the tumour situation. To this end, we used two distinct models of

Figure 5 Involvement of 4E-BP1 in TGF β -mediated inhibition of pancreatic cancer-cell proliferation. **(A)** Validation of 4E-BP1 siRNAs. Panc-1 cells were transiently transfected with either a nonspecific (siNS) or a 4E-BP1 (siBP1) siRNA and equal amounts of protein was examined by SDS-PAGE immunoblotting as shown. **(B)** Validation of Panc-1 inducible cell lines. Panc-1 cells stably transfected with inducible miRNeg (left) or miRBP1 (right) were treated for 72 h with increasing concentrations of doxycycline (top), or for different times with 100 ng/ml of doxycycline. Equal amounts of proteins were examined by SDS-PAGE immunoblotting as shown. **(C)** 4E-BP1 knockdown accelerates MiaPaca-2-cell proliferation. MiaPaca-2 cells stably transfected with inducible miRNeg or miRBP1 were incubated in the presence of 100 ng/ml doxycycline or vehicle alone and cell proliferation was monitored. Values are the means ($\pm$ s.e.) of three separate experiments performed in triplicates. **(D)** 4E-BP1 is involved in TGF β -mediated proliferation inhibition of Panc-1 cells. Panc-1 cells stably transfected with inducible miRNeg or miRBP1 were all incubated in the presence of 100 ng/ml doxycycline, and untreated or treated with TGF β . Cell proliferation was monitored as described under Materials and methods. Values are the means ($\pm$ s.e.) of three separate experiments performed in triplicates.

cultured cells, which were not isolated from tumours and whose proliferation has been shown to be inhibited by TGF β : human immortalized keratinocytes (HaCaT cells; Kortlever *et al*, 2008) and mouse embryonic fibroblasts (MEFs; Datto *et al*, 1999). TGF β -mediated inhibition of HaCat cells' proliferation was almost completely prevented upon silencing of 4E-BP1 by specific siRNAs (Figure 6A), and 4E-BP1-knockout (4E-BP1 $^{-/-}$) MEFs are partially resistant to the antiproliferative effect of TGF β as compared with wild-type (4E-BP1 $^{+/+}$) MEFs (Figure 6B). This is consistent with our observations that 4E-BP1 knockdown accelerates progression through the G1 cell-cycle phase (data not shown), and that TGF β , in contrast, slows cell-cycle progression through downregulation of the G1-phase cyclin-D1 (Ko *et al*, 1995; Halder *et al*, 2005; Kolb *et al*, 2007, and data not shown).

These data, taken together, establish a role of 4E-BP1 in mediating the antiproliferative effects of TGF β . It is likely that through 4E-BP1 induction, the TGF β /Smad4 signalling pathway controls translational expression of critical regulators of cell growth and proliferation (such as cyclin-D1). In this regard, the inducible 4E-BP1-shRNAs pancreatic cancer cell line we have created and the 4E-BP1-null MEF model provide useful systems to identify novel routes of TGF β -mediated inhibition of cell proliferation.

Materials and methods

Cell lines, culture, treatment and proliferation assay

Pancreatic cancer cell lines (Panc-1, MiaPaca-2, BxPC-3 and Capan-1) were cultured as described (Puente *et al*, 2001). To obtain Panc-1 and

Figure 6 Role of 4E-BP1 in TGF β -mediated inhibition of keratinocyte- and fibroblast-cell proliferation. (A) 4E-BP1 is involved in TGF β -mediated inhibition of HaCaT-cell proliferation. HaCaT cells transfected with nonspecific siRNAs (siNS) or with siRNAs specific to 4E-BP1 (siBP1) were untreated or treated with TGF β and cell proliferation was monitored as described under Materials and methods. Values are the means ($\pm$ s.e.) of three separate experiments performed in triplicates (top). TGF β upregulates 4E-BP1 in HaCaT cells. HaCaT cells transfected as above were untreated or treated with TGF β for 48 h. Equal amounts of proteins were then subjected to SDS-PAGE immunoblotting as shown (bottom). (B) 4E-BP1 is involved in TGF β -mediated inhibition of MEF proliferation. 4E-BP1^{+/+} and 4E-BP1^{-/-} MEFs were untreated or treated with TGF β and cell proliferation was monitored as described under Materials and methods. Values are the means ($\pm$ s.e.) of three separate experiments performed in triplicates (top). TGF β upregulates 4E-BP1 in MEFs. 4E-BP1^{+/+} and 4E-BP1^{-/-} MEFs were untreated or treated with TGF β for 48 h. Equal amounts of proteins were then subjected to SDS-PAGE immunoblotting as shown (bottom).

MiaPaca-2 cells stably expressing inducible miRNeg or miRBP1, cells were first transfected with the pTet-on advanced vector (Clontech) using Exgen 500 (Euromedex) and cultured in the presence of genetecin (Invivogen). Resistant clones were isolated, transiently transfected with pTRE-Tight-LUC (Clontech) and assayed for LUC activity following treatment with doxycycline (Sigma). Clones that gave satisfactory induction of LUC upon doxycycline treatment were then amplified and co-transfected with linear hygromycin selection marker (Clontech) and with either pTRE-Tight-miRNeg or pTRE-Tight-miRBP1, and cultured in the presence of hygromycin. Resistant clones were then isolated and clones that gave satisfactory induction of GFP and concomitant knockdown of endogenous 4E-BP1 upon treatment with doxycycline were selected and amplified.

HaCaT and immortalized MEFs isolated from 4E-BP1/4E-BP2 double-knockout mice (4E-BP1^{-/-} cells) or from their normal littermates (4E-BP1^{+/+}; Le Bacquer *et al*, 2007) were grown in Dulbecco's Modified Eagle's Medium (Gibco BRL) with 10% heat-inactivated fetal bovine serum (Gibco BRL) and penicillin-streptomycin (Gibco BRL).

Proliferation assays with Panc-1 cells were performed in the presence of 100 ng/ml doxycycline as follows: cells were plated and allowed to grow in six-well dishes for 24 h, serum-starved for 24 h and incubated with 1% serum in the absence or presence of 10 ng/ml TGF β (Euromedex). HaCaT cells were plated, allowed to grow in six-well dishes for 24 h and transfected with siRNAs targeting 4E-BP1 (Applied Biosystems, forward 5'-CAAGAACGAA CCCUCCUU-3' and reverse), using the siPort NeoFx transfection reagent (Applied Biosystems), according to the manufacturer's instructions. Twenty-four hours following transfection, cells were serum-starved for 24 h and incubated with 1% serum in the absence or presence of 10 ng/ml TGF β (Euromedex). 4E-BP1^{+/+} and 4E-BP1^{-/-} MEFs were plated and allowed to grow in six-well dishes for

24 h, serum-starved for 24 h and incubated with 0.5% serum in the absence or presence of 10 ng/ml TGF β (Euromedex). At the end of each experiment, cells were trypsinized and counted with a Coulter counter (Coulter Electronics) at the indicated times.

Immunoprecipitation and western blot analysis

Cells were harvested, lysed, the 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) as described previously (Cormier *et al*, 2001) or subjected directly to SDS-PAGE and electroblotted onto Immobilon-P membranes (Millipore) as described previously (Pyronnet *et al*, 2000, 2001). Membranes were incubated with mouse monoclonal antibodies to β -tubulin (Sigma) or Smad4 (Santa Cruz Biotechnology), or with rabbit polyclonal antibodies to 4E-BP1 (Cell Signaling), eIF4E (Cell Signaling) or GFP (Ab-cam). Bound antibodies were detected with peroxidase-coupled goat antibodies to mouse or rabbit IgG (Pierce) using chemiluminescence detection reagent (Pierce). Figures represent contiguous lanes.

Semi-quantitative RT-PCR and ChIP

For RT-PCR, RNA was extracted with RNeasy (Qiagen) and equal amounts of RNAs were subjected to RT-PCR using the Superscript One Step RT-PCR kit (Invitrogen) according to the manufacturers' instructions. Primers used were as follows: 4E-BP1—forward primer: 5'-ATGTCCGGGGCAGCAGCTGCAGCCAG-3', reverse primer: 5'-TTAAATGTCCATCTGAACTGTGACTC-3' and GAPDH—forward primer: 5'-GTCGGAGTCAACGGATTGG-3', reverse primer: 5'-AAAAGCAGCCCTGGTGACC-3'. For ChIP assay, genomic DNA was extracted, fragmented and subjected to immunoprecipitation using antibodies to Smad4, and immunoprecipitates were

PCR-amplified using a ChIP kit (Upstate) according to the manufacturer's instructions.

Constructs, mutagenesis, transfections and LUC activities

Fragments of the human 4E-BP1 promoter were PCR-amplified using primers extended by *KpnI* (forward primers) or by *XhoI* or *HindIII* (reverse primers) restriction sites, digested by *KpnI/XhoI* or *KpnI/HindIII* restriction endonucleases and inserted into the *KpnI/XhoI* or *KpnI/HindIII*-linearized pGL2-Basic (Promega) luciferase (LUC) reporter vector (see Figure 2D for details). The 4E-BP1 exon-1 has been cloned in frame with the LUC ORF. Mutations were generated by PCR using a Quick Change mutagenesis kit (Stratagene) according to the manufacturer's instructions. Smad2, 3, 4 and 7 vectors were as described previously (Jonckheere *et al*, 2004). pRL-CMV (Promega) containing the *Renilla* LUC coding sequence driven by the CMV promoter was used as a transfection efficiency internal control. Following co-transfection of pRL-CMV with any pGL2 or Smad construct using Exgen 500 (Euromedex), cells were harvested in Passive Lysis Buffer (Promega) and extracts were assayed for firefly and *Renilla* LUC activities using the Dual Luciferase Reporter Assay System (Promega), and detected with a Luminoscan Ascent Microplate Luminometer (Thermo Labsystems), as described previously (Lahlou *et al*, 2005). Cells were transfected with siRNAs targeting Smad4 (Applied Biosystems, forward 5'-CAUCCUAGUAAUGUGUUA-3' and reverse) or 4E-BP1

(see above), using siPort NeoFx transfection reagent (Applied Biosystems), according to the manufacturer's instructions.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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