Contribution of HIF-1α in 4E-BP1 gene expression

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Abstract

The eukaryotic translation initiation factor 4E (eIF4E) is necessary for the translation of capped mRNAs into proteins. Cap-dependent mRNA translation can be however inhibited by the eIF4E-binding protein 1 (4E-BP1). The hypophosphorylated forms of 4E-BP1 indeed sequester eIF4E and thus block translation initiation and consequent protein synthesis. Different reports indicate that, in addition to hypophosphorylation, 4E-BP1 function can be also regulated at the level of protein expression. This is the case in contact inhibited cells or in cells exposed to hypoxia. The molecular mechanisms responsible for 4E-BP1 protein accumulation in these conditions remain however unknown. In the present study, we found that 4E-BP1 gene promoter contains a hypoxia-responsive element (HRE) that mediates 4E-BP1 gene up-regulation via the hypoxia-inducible factor 1 alpha (HIF-1α) transcription factor. Gene reporter assays then revealed that the presence of such HRE in the promoter of 4E-BP1 gene is involved in 4E-BP1 accumulation in contact inhibited cells and in cells exposed to hypoxia. We also reveal that the TGF-β-dependent transcription factor SMAD4 cooperates with HIF-1α to fully activate 4E-BP1 gene transcription under hypoxia. These data therefore suggest that HIF-1α contributes to 4E-BP1 gene expression under different conditions.
**Introduction**

One molecular mechanism whereby cells can counteract undesired rises in proliferation rates is the inhibition of protein synthesis. In eukaryotic cells, most mRNAs are translated into proteins *via* the recruitment of ribosomes at the mRNA 5’ cap structure. This process necessitates the cap-binding protein eIF4E (eukaryotic translation initiation factor 4E), which facilitates ribosome loading on the mRNA 5’ end through its interaction with eIF4G, a large scaffolding protein. eIF4G is indeed bound to other translation initiation factors including eIF4A and eIF3, whose functions are to aid in melting mRNA 5’ end secondary structures (eIF4A) or to serve as a docking site for the ribosome (eIF3). Assembly of this translation initiation complex is the target of tight controls that limit protein synthesis especially when there is a need to block cell proliferation. Among these mechanisms of control, sequestration of eIF4E by the eIF4E-binding protein 1 (4E-BP1) plays a prominent role. The binding of 4E-BP1 to eIF4E prevents eIF4G association with eIF4E and consequently precludes ribosome recruitment at the mRNA 5’ cap structure. 4E-BP1 activity (i.e. sequestration of eIF4E) has been shown to be enhanced in various organisms exposed to different conditions of stress or in contact inhibited cells (see below).

The regulation of 4E-BP1 activity was initially described as being dependent mainly on phosphorylation events. To sequester eIF4E, 4E-BP1 must be actually maintained in a hypophosphorylated state. mTOR being the major kinase of 4E-BP1, hypophosphorylation of 4E-BP1 is observed when mTOR activity is inhibited. This is the case in many conditions of stress, including nutrient deprivation, or exposure to oxidative or genotoxic compounds. More recently however, different conditions have been shown to trigger not only 4E-BP1 hypophosphorylation but also 4E-BP1 gene expression. For instance in Drosophila, 4E-BP1 induction prolongs the survival of flies upon nutrient deprivation. Similarly, the survival of endocrine cells in the pancreas of mice exposed to a reticulum stress is dependent on the activation of 4E-BP1 expression. In these examples, stress-induced 4E-BP1 expression was shown to be mediated by transcription factors (FOXO in Drosophila and ATF4 in mouse endocrine pancreas) that bind to and activate the transcription of 4E-BP1 gene. Based on these data, stress-dependent 4E-BP1 activation, which can result from hypophosphorylation and/or enhanced expression, is believed to exert a protective effect by limiting excessive synthesis of proteins and consequently limiting cell proliferation that could otherwise have deleterious effects.
Contact inhibition and hypoxia are two other situations where 4E-BP1 protein expression is induced. We and others have actually observed in different cell types of various organisms that continuous cell culture and contact inhibition\(^8,9\) or exposure to a low concentration of oxygen \((O_2)^{10-12}\) provoke an accumulation of 4E-BP1 protein. However, how induction of 4E-BP1 protein amount occurs under these conditions remains unknown. We show here that in contact inhibited cells or in cells exposed to 1\% O\(_2\), 4E-BP1 protein accumulation is dependent at least partially on a hypoxia-responsive element in the 4E-BP1 gene promoter that binds the transcription factor HIF-1\(\alpha\). Furthermore, we show that HIF-1\(\alpha\) cooperates with the transcription factor Smad4 to fully induce 4E-BP1 gene transcription when pancreatic cells are exposed to hypoxia.

**Materials and Methods**

**Cell culture, treatment and proliferation assay.** Pancreatic cancer cell lines: wild-type (WT), mock-transfected (BxC) or sst2-stably-transfected (Bx2) BxPC-3 cells and MiaPaca-2 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 1 g/l glucose (LONZA) and supplemented with 10% FCS, 2 mM L-glutamine (GIBCO), 2.5 \(\mu\)g/ml Fungizone (GIBCO), 5 U/ml streptomycin/penicillin (GIBCO), and 0.01% plasmocin (InvivoGen). The medium of stable BxC and Bx2 cells contained also 400 \(\mu\)g/ml geneticin (InvivoGen). For extinction of gene expression, cells were plated in six-well dishes, allowed to grow for 24 h and transfected with 10 nM of siRNAs targeting either 4E-BP1 (Applied Biosystems, forward 50-CAAGAACGAACCCUUCCUU-30 and reverse) or targeting HIF-1\(\alpha\) (Dharmacon, siGENOME® SMARTpool® HIF-1\(\alpha\) siRNA and siGENOME® nontargeting siRNA) using the siPort NeoFx transfection reagent (Applied Biosystems), according to the manufacturer’s instructions. Protein or mRNA stability assay was performed using 10 \(\mu\)g/ml cycloheximide (Sigma) or 5 \(\mu\)g/ml actinomycin-D (Sigma), respectively. WT- and DN-HIF-1\(\alpha\) expression plasmids were kindly provided by Pr H. Prats. For chemical hypoxia, cells were incubated with 0.5 mM CoCl\(_2\) (Sigma). For hypoxic conditions, cells were incubated at 37°C in a 5% CO\(_2\), 94% N\(_2\), and 1% O\(_2\) atmosphere (hypoxic incubator, Binder GmbH, Tuttlingen, Germany). For proliferation assays, cells were plated in six-well dishes and counted with a Coulter counter (Coulter Electronics).
Analysis of 4E-BP1-eIF4E interaction and Western-blotting. Cells were harvested in lysis buffer and cell lysates were subjected to immunoprecipitation and/or Western-blotting as previously described. Membranes were incubated with: mouse monoclonal antibodies to β-tubulin (Sigma), p27Kip1 (BD Transduction Laboratories), HIF-1α (Zymed laboratories), eIF4E (Cell Signaling), total or phospho-specific 4E-BP1 (Cell Signaling). Rabbit polyclonal antibodies against eIF4GI were kindly provided by Pr N. Sonenberg (Department of Biochemistry, McGill University). Membranes were then subjected to immunoblotting using goat horseradish peroxidase-conjugated secondary antibodies to mouse or rabbit IgG (Pierce). Peroxidase activity was revealed using the enhanced chemiluminescence (ECL) system (Pierce). Quantitative analyses were carried out by using Phoretix 1D software (Samba technologies).

RNA isolation and Northern-blotting. Total RNA was isolated using RNeasy Kit (Qiagen) according to the manufacturer’s instructions. 10 μg of total RNA was denatured in RNA sample buffer (39 mM MOPS pH=7, 58.5% deionized formamide, 10.8% formaldehyde, 3% ethidium bromide) for 15 min at 65°C, separated by electrophoresis on formaldehyde agarose gels, and transferred onto a nylon membrane (Hybond™-N+; Amersham Biosciences) by capillary transfer in a 10X SSC buffer (Invitrogen). After UV-cross linking (Cross-linker; Stratagene) and prehybridization for 2 hours at 68°C with QuikHyb® (Stratagene), filters were hybridized for 3 hours with [32P]-labeled probes made from the agarose gel-purified RT-PCR products of each gene using the RadPrime DNA Labeling System (Invitrogen) and 10 μg salmon sperm DNA (Stratagene). After washing, hybridized membranes were exposed to a PhosphorImager (Molecular Dynamics). Equal loading of RNA was confirmed by staining of the ribosomal RNA with ethidium bromide. Signals were quantified using the ImageQuant software (Amersham).

Luciferase Reporter Gene Analysis. To determine 4E-BP1 promoter activity, the dual-luciferase reporter assay system (Promega) was used as prescribed previously. Fragments of 4E-BP1 promoter were PCR-amplified using primers extended by KpnI (forward primers: 5’ ggggtaccattaatttaggcagctaatcag 3’) and by XhoI (reverse primers: 5’ tccgctcgaggtctcctgtgcgctgcac 3’) restriction sites, digested by KpnI/XhoI restriction endonucleases and inserted into KpnI/XhoI-linearized pGL2B. The pCMV-Renilla Luciferase plasmid was used to evaluate transfection efficiency. Cells transfected in 6-well dishes were incubated in culture medium for 36 h, and harvested in Passive Lysis Buffer (Promega). Luciferase activities were detected
with Centro LB 960 (Berthold Technologies). The relative luciferase activity was calculated by normalizing the activity of firefly luciferase to that of renilla luciferase.

**Chromatin Immunoprecipitation analysis.** Experiments were performed using EZ ChIP™ assay Kit (Upstate Biotechnology) according to the manufacturer’s instructions using mouse monoclonal antibody against HIF-1α and primers for -278/+64 as described previously.\(^\text{15}\)

**Statistical analysis.** Statistical analysis was performed by using the unpaired t test. * or # indicate a P value < 0.05.

**Results and discussion**

**Characterization of the model of cell-density-dependent accumulation of 4E-BP1 protein**

To explore the molecular mechanism involved in the accumulation of 4E-BP1 protein triggered by contact inhibition, we used a pancreatic cancer cell line (BxPC-3) which expresses (Bx2) or not (BxC) the somatostatin receptor 2 (sst2). This model has been chosen because in most human pancreatic tumors and derived cell lines, sst2 expression is dramatically decreased as compared to normal pancreas.\(^\text{16}\) As sst2 normally triggers anti-proliferative signals,\(^\text{17}\) its absence leads to uncontrolled cell proliferation. We have however shown that sst2 re-expression conversely limits the proliferation of pancreatic cancer cells, likely via the induction of 4E-BP1 protein and the restoration of contact inhibition.\(^\text{18}\) Here, we first wished to confirm that sst2-triggered contact inhibition is dependent on 4E-BP1 protein accumulation. A proliferation assay actually showed that contact inhibition in sst2-expressing cells (Fig. 1A) is abolished (Fig. 1B) when cell-density-dependent accumulation of 4E-BP1 protein is prevented by the use of specific siRNAs (Fig. 1C).

To verify whether cell-density-dependent accumulation of 4E-BP1 has a functional consequence on eIF4F complex assembly, we have then monitored 4E-BP1 expression and phosphorylation, and eIF4E binding to 4E-BP1 or eIF4GI in the course of BxC versus Bx2 cell proliferation. As expected, 4E-BP1 accumulation with cell density was more pronounced in sst2 expressing cells (Fig. 2A). 4E-BP1 activity (i.e. binding to eIF4E and consequent disruption of eIF4F complex) is dependent on its phosphorylation status. 4E-BP1 is phosphorylated at multiple sites including T37/T46, S65 and T70.\(^\text{2}\) However, phosphorylation at S65 has been shown to play a prominent role in 4E-BP1 binding to eIF4E.\(^\text{2}\) In both BxC
and Bx2 cells, the level of phosphorylation at S36/T47 or T70 (Fig. 2B) was in direct proportion to the level of 4E-BP1 protein (Fig. 1A), suggesting that phosphorylation at these residues is regulated neither by cell density nor by sst2. In contrast, phosphorylation at S65 did not follow the density-dependent increase in 4E-BP1 amount seen in mock transfected cells (Fig. 2B, BxC cells), and S65 was not phosphorylated at all in sst2-expressing cells (Fig. 2B, Bx2 cells), even at high cell density when 4E-BP1 accumulates. Consistent with such increase in 4E-BP1 amount and impaired phosphorylation at S65, a cap-affinity purification then revealed that density-dependent disruption of the eIF4F complex (here visualized by the concurrent binding of 4E-BP1 or eIF4GI to eIF4E) occurred predominantly in sst2-expressing and contact-inhibited cells (Fig. 2C). These data suggest that 4E-BP1 phosphorylation at S65 is inversely correlated to cell density, and is severely impaired by sst2 signaling. This latter observation is consistent with our previous reports describing how sst2 inhibits the PI3K/mTOR axis,19,20 the major signaling pathway that impinges upon 4E-BP1 phosphorylation.2

**Cell-density-dependent accumulation of 4E-BP1 is transcriptional**

We have then searched for the molecular mechanism that could explain how 4E-BP1 gene expression is up-regulated by the combination of sst2 expression and cell density. We first ruled out the possibility that 4E-BP1 accumulation was due to increased stability in 4E-BP1 protein or mRNA. The stability of 4E-BP1 protein or mRNA was monitored in mock-versus sst2-transfected cells which were cultured to high density and incubated with either cycloheximide (to monitor protein stability) or actinomycin D (to monitor mRNA stability). The data revealed that 4E-BP1 protein stability was not affected by sst2 expression or cell density, and that the protein is relatively stable over a period of 48 hours, in contrast to p27 protein which was degraded faster during the same period (Fig 3A, left). The amount of 4E-BP1 mRNA followed a slightly different kinetic over the same 48-hours period. In the first 16 hours after Actinomycin D treatment, the amount of 4E-BP1 mRNA increased to about 150% of the initial level, specifically in Bx2 cells (Fig. 3A, right). However following 48 hours of treatment with actinomycin D, the stability of 4E-BP1 mRNA was equally high in BxC and Bx2 cells (as compared to actin mRNA which was degraded faster; Fig. 3A, right), suggesting a minor contribution of mRNA stability in the induction of 4E-BP1 protein expression in contact-inhibited cells.

To more directly test for the involvement of transcription, we then used a luciferase reporter system. We have previously described the relative activity of various 4E-BP1
promoter fragments in BxC versus Bx2 cells, and showed that a promoter fragment encompassing nucleotides -278 to +64 was sufficient to confer sst2-dependent upregulation of luciferase activity.\textsuperscript{13} The use of this plasmid construction in the course of BxC and Bx2 cell proliferation confirmed that 4E-BP1 promoter activity is enhanced in sst2-expressing cells and, more importantly, revealed that 4E-BP1 promoter activity is augmented as cell density increases (Fig. 3B). As previously described, this fragment possesses most of the GC-boxes known to bind Egr-1,\textsuperscript{21} a transcription factor that limits 4E-BP1 gene transcription. Upon activation of sst2 signaling however, Egr-1 dissociates from this segment and 4E-BP1 gene transcription is released.\textsuperscript{13} This fragment is also expected to contain the features required for induction of 4E-BP1 by cell density as it is sufficient to promote a mild but significant cell-density-dependent 4E-BP1 promoter activation in cells that do not express sst2 (Fig. 3B, BxC).

\textbf{Sst2- and cell-density-triggered accumulation of 4E-BP1 is dependent on HIF-1\textalpha{}}

One possible candidate transcription factor that could account for cell-density-dependent induction of 4E-BP1 promoter activity was HIF-1\textalpha{}. Cell density has actually been shown to induce HIF-1\textalpha{},\textsuperscript{22} and a computer analysis of human 4E-BP1 gene sequence revealed the presence of a putative HRE located in the -278/+64 4E-BP1 promoter segment (Fig. 4A) that mediates cell-density-dependent activation of 4E-BP1 promoter. To test for the involvement of HIF-1\textalpha{,} we first confirmed that HIF-1\textalpha{ was up-regulated by cell density in our cell lines (Fig. 4B), and that HIF-1\textalpha{ bound the 4E-BP1 promoter in a cell-density-dependent manner by chromatin immunoprecipitation (ChIP) of the 4E-BP1 promoter segment that contains the putative HRE using anti-HIF-1\textalpha-specific antibodies (Fig. 4C). Interestingly, cell-density-dependent induction of HIF-1\textalpha expression and binding to the 4E-BP1 promoter were higher in sst2-expressing cells, suggesting that the combination of sst2-signaling and cell density produced an additive effect on HIF-1\textalpha-mediated induction of 4E-BP1 promoter activity. The use of a 4E-BP1 promoter carrying a mutated HRE (Hmut) then indicated that the putative HRE detected by computer analysis was functional (Fig. 4D, left), and silencing HIF-1\textalpha using specific siRNAs demonstrated that HIF-1\textalpha is involved in cell-density-dependent induction of 4E-BP1 promoter activity (Fig. 4D, right). Finally, to confirm that sst2 signaling cooperates with cell density to fully activate the 4E-BP1 promoter, we manipulated HIF-1\textalpha function by transfecting BxC or Bx2 cells with expression vectors encoding either wild type (WT) or a dominant negative form (DN) of HIF-1\textalpha. The data we
obtained revealed that artificial overexpression of WT-HIF-1α in dense BxC cells enhances 4E-BP1 promoter activity, while in contrast overexpression of DN-HIF-1α in dense Bx2 cells represses 4E-BP1 promoter activity (Fig. 4E). Together, these data show that HIF-1α mediates cell-density-dependent induction of 4E-BP1 promoter activity and that such induction is enhanced in sst2-expressing cells. One probable explanation for the additive effect of sst2 signaling and cell density on 4E-BP1 promoter activity is suggested by the observation that the HRE is located in the vicinity of the Egr-1-binding GC-boxes. As described earlier,13 sst2-induced 4E-BP1 promoter activity is indeed due to the release of Egr-1 from 4E-BP1 promoter. It is therefore conceivable that Egr-1 dissociation facilitates HIF-1α binding to the 4E-BP1 promoter.

Hypoxia-triggered induction of 4E-BP1 is dependent on HIF-1α and SMAD4

Since we have identified a cell-density-activated functional HRE in the 4E-BP1 promoter, we thought that 4E-BP1 gene transcription could be directly regulated by HIF-1α under hypoxia. To explore this hypothesis, we first monitored the expression of 4E-BP1 and HIF-1α in BxPC-3 parental cells incubated in 1% oxygen (O2) for different times. Surprisingly, only minor changes in 4E-BP1 amount were observed despite induction of HIF-1α over a 24 hours period under hypoxia (Fig. 5A, left). This apparent discrepancy with earlier reports could be due to cell-type specificity as we here used pancreatic cancer cells while earlier reports described the effect of hypoxia on 4E-BP1 expression in sea urchin embryos,10 human cervix cancer HeLa cells11 and human nerve growth factor-differentiated PC12 cells.12 One important feature in pancreatic cancer cells in the status of the transcription factor Smad4, a tumor suppressor deleted in 50% of pancreatic tumors (Smad4 is also called deleted in pancreatic cancer locus 4 or DPC4).23 In conjunction with other members of the SMAD family (such as Smad3), Smad4 acts downstream of TGFβ receptors to induce the transcription of SMAD-binding-elements (SBE)-containing genes. We have shown that SMAD3/4 normally controls 4E-BP1 promoter activity via interaction with a SBE15 which is also located in the vicinity of the HRE (Fig. 4A). Another intriguing observation is that SMAD3/4 and HIF-1α have been shown to assemble into a same protein complex which is responsible for transcriptional activation of different genes under hypoxia.24,25 Thus, deletion of Smad4 in BxPC-3 pancreatic cancer cells could explain the moderate induction of 4E-BP1 protein expression under hypoxia. Consistently, when we used the MiaPaca-2 pancreatic cancer cell line which carries two normal Smad4 alleles, we observed that 4E-BP1 was in
contrast induced when cells were incubated under hypoxia (Fig. 5A, right), and that such induction was significantly prevented by pre-incubation of cells with specific HIF-1α siRNAs (Fig 5A, right). A reporter test also showed that 4E-BP1 promoter (containing both the HRE and the SBE) was activated in MiaPaca-2 cells incubated in the presence of cobalt chloride (CoCl₂), a chemical inducer of hypoxia (Fig. 5B). Finally, while only a non significant induction was detected in Smad4⁻/⁻ BxPC-3 cells, 4E-BP1 promoter activity was much more activated in MiaPaca-2 cells under hypoxia (Fig. 5C, left). The use of modified 4E-BP1 promoters in Smad4⁺/+ MiaPaca-2 cells then demonstrated that mutation of either HRE (Hmut) or SBE (Smut) did not fully prevented hypoxia-induced promoter activity, while mutation of both elements (HSmut) did (Fig 5C, right).

In summary, we show that the 4E-BP1 gene contains a functional hypoxia-responsive element which binds HIF-1α in two conditions of 4E-BP1 protein induction: high cell density and hypoxia. These data thus reveal that HIF-1α may contribute to 4E-BP1 gene expression under different conditions. Furthermore, HIF-1α appears to cooperate with SMAD4 to fully induce 4E-BP1 gene transcription under hypoxia. This suggests that TGF-β signaling might cooperate with hypoxia to control 4E-BP1 function and that such regulation might be impaired in pancreatic tumors where Smad4 is deleted.

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Legends

Figure 1. 4E-BP1 is necessary for cell-density mediated inhibition of cell proliferation.

A, contact inhibition in sst2-expressing cells. The proliferation of mock- (BxC) versus sst2-transfected (Bx2) BxPC-3 cells was monitored over an 8-days period. Data represent the mean +/- SE of triplicates repeated 3 times. B, sst2-dependent inhibition of cell proliferation is prevented by 4E-BP1 siRNAs. Proliferation was monitored as in A except that cells were transfected with either control (siCTRL) or 4E-BP1-targeting (si4E-BP1) siRNAs 24 hours after seeding. Data represent the means +/- SE of triplicates repeated 3 times. C, 4E-BP1 silencing by specific siRNAs. The amounts of β-tubulin and 4E-BP1 proteins were visualized by Western-blotting 2 and 6 days after siRNA transfection (3 and 7 days after seeding). Numbers are densitometric quantifications of 4E-BP1 and are normalized to the values obtained for cells collected 3 days after seeding and transfected with siCTRL. They are representative of three separate Western-blots.
Figure 2. Cell-density-dependent function of 4E-BP1 is enhanced in sst2-expressing cells.

A, cell-density-dependent accumulation of 4E-BP1 is enhanced in sst2-expressing cells. The amounts of β-tubulin, eIF4GI, eIF4E and 4E-BP1 were visualized in BxC versus Bx2 cells by Western-blotting over an 8-days period after seeding. Data are representative of three separate Western-blots (top), and those obtained for 4E-BP1 were quantified and expressed as a function of the values obtained for BxC and Bx2 at day 2 (bottom). B, 4E-BP1 phosphorylation at S65 is inhibited in sst2-expressing cells. The phosphorylation of 4E-BP1 at different sites was monitored in BxC versus Bx2 cells by Western-blotting using phospho-specific antibodies over an 8-days period. Data are representative of three separate Western-blots. C, cell-density-dependent sequestration of eIF4E by 4E-BP1 is enhanced in sst2-expressing cells. The relative binding of eIF4GI or 4E-BP1 to eIF4E was visualized by Western-blotting following immunoprecipitation (IP) with anti-eIF4E specific antibodies over an 8-days period. Numbers are densitometric quantifications of eIF4GI (top) or 4E-BP1 (bottom) and are normalized to the values obtained for BxC or Bx2 cells 2 days after seeding. They are representative of three separate IPs.

Figure 3. Cell-density-dependent accumulation of 4E-BP1 is transcriptional.

A, 4E-BP1 protein or mRNA stability is not affected in sst2-expressing cells. The stabilities of 4E-BP1, p27 and β-tubulin proteins (Western-blot) or 4E-BP1 and actin mRNAs and 28S rRNA (Northern-blot) were monitored in the presence of cycloheximide (proteins) or actinomycin (RNAs) for a 48-hours period (top). Data are representative of three separate experiments. The data obtained for 4E-BP1 were quantified and expressed as percents +/- SE of the values obtained at time 0 (bottom). B, cell-density-dependent induction of 4E-BP1 promoter is enhanced in sst2-expressing cells. A segment (nucleotides -278 to +64) of the 4E-BP1 promoter which encompasses 5 of the 6 GC-rich Egr-1 binding sites (ovals numbered 1-6) was inserted in a firefly luciferase reporter (thick lines: 4E-BP1 transcribed sequence). BxC or Bx2 cells were transfected with the -278/+64 reporter vector 24 hours after seeding and firefly luciferase activity was measured 1, 3 and 5 days following transfection. Data represent the means +/- SE of triplicates repeated 3 times and which were normalized to the activity measured for a co-transfected CMV-promoter-based Renilla luciferase reporter.

Figure 4. Contribution of HIF-1α in cell-density-dependent induction of 4E-BP1 promoter.
A, 4E-BP1 promoter contains computer-predicted HRE and SBE (filled boxes: wild type elements; open boxes: mutated elements). B, cell-density-dependent induction of HIF-1α is enhanced in sst2-expressing cells. The amounts of HIF-1α and β-tubulin were monitored in BxC versus Bx2 cells by Western-blotting over an 8-days period after seeding. Numbers are densitometric quantifications of HIF-1α and are normalized to the value obtained for BxC cells 2 days after seeding. They are representative of three separate Western-bLOTS. C, HIF-1α binds to 4E-BP1 promoter. ChIP assays using anti-HIF-1α-specific antibodies or non-specific IgGs (IP) were performed on DNA extracted from BxC or Bx2 cells which were cultivated at low (LD) or high (HD) density. Primers were directly tested on purified DNA (input). Numbers are densitometric quantifications of PCR amplifications and are normalized to the value obtained for the LD input of Bx2 cells. They are representative of three separate ChIP assays. D, HIF-1α and the HRE are necessary for cell-density-dependent induction of 4E-BP1. Bx2 cells where transfected with wild-type or Hmut -278/+64 reporter vector 1 day (LD) or 5 days (HD) after seeding and firefly luciferase activity was measured 36 hours following transfection. Data represent the means +/- SE of triplicates repeated 3 times and which were normalized as in Figure 3B (left). Bx2 cells where transfected with either control (siCTRL) or HIF-1α-targeting (siHIF-1α) siRNAs 24 hours after seeding and HIF-1α, β-tubulin and 4E-BP1 were visualized by Western-blotting 1 day (LD) or 5 days (HD) following transfection. Numbers are densitometric quantifications of 4E-BP1 and are normalized to the value obtained for LD-cultured and siCTRL-transfected cells. They are representative of three separate Western-bLOTS (right). E, sst2 signaling cooperates with cell density to fully activate the 4E-BP1 promoter. BxC or Bx2 cells where co-transfected with the -278/+64 reporter and either mock-, HIF-1α- or DN-HIF-1α-expression vector 5 days after seeding and firefly luciferase activity was measured 36 hours following transfection. Data represent the means +/- SE of triplicates repeated 3 times and which were normalized as in Figure 3B.

**Figure 5. Contribution of HIF-1α and Smad4 in 4E-BP1 promoter induction under hypoxia.**

A, 4E-BP1 induction under hypoxia is dependent on Smad4 status and on HIF-1α. The amounts of HIF-1α, β-tubulin and 4E-BP1 were monitored in Smad4<sup>−/−</sup> BxPC-3 and Smad4<sup>+/+</sup> MiaPaca-2 cells by Western-blotting 3 days after seeding and over a 12-hours period under hypoxia (1% oxygen). MiaPaca-2 cells were pre-treated or not with HIF-1α specific siRNAs.
Numbers are densitometric quantifications of 4E-BP1 and are normalized to the values obtained for cells in normoxia. They are representative of three separate Western-blot. B, 4E-BP1 promoter induction in cells treated with CoCL2. MiaPaca-2 cells where transfected with the -278/+64 reporter vector 1 day after seeding, let grown for 2 days and firefly luciferase activity was measured over a 24-hours period in the presence of 0.5mM of CoCL2. Data represent the means +/- SE of triplicates repeated 3 times and which were normalized as in Figure 3B. C, 4E-BP1 promoter induction under hypoxia necessitates both HIF-1α and Smad4 functions. Smad4−/− Bx-PC-3 or Smad4+/+ MiaPaca-2 cells where transfected with the -278/+64 reporter vector 1 day after seeding, let grown for 2 days and firefly luciferase activity was measured over a 24-hours period under 1% oxygen (left). MiaPaca-2 cells where transfected with wild-type, Hmut, Smut or HSmut -278/+64 reporter vector 1 day after seeding, let grown for 2 days and firefly luciferase activity was measured before and after a 24-hours period under 1% oxygen (right). Data represent the means +/- SE of triplicates repeated 3 times and which were normalized as in Figure 3B.
Azar et al., Figure 1

A

B

C

Proliferation (arbitrary units)

BxC

Bx2

siCTRL

si4E

siCTRL

si4E

siCTRL

si4E

β-tub

4E-BP1

siRNA

siRNA

siRNA

BxC

Bx2

β-tub

4E-BP1

37

days

8

0

20

12

4

0

2

4

6

8

days

0

2

4

6

8

0

2

4

6

8

days

0

2

4

6

8

0

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4

6

8

days

0

2

4

6

8

days

siRNA

siRNA

siRNA

β-tub

4E-BP1

1.0 0.1 1.2 0.1

1.0 0.2 2.3 0.2

siCTRL

si4E-BP1

+ +

+ +
Azar et al., Figure 2

A

B

C

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Azar et al., Figure 3
Azar et al., Figure 4

A

-278/+64

HRE

SBE

Luc

Hmut

Smut

H5mut

ccCACGTGga

ccCAGCCAGAc

B

HRE

SBE

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Azar et al., Figure 5

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BxPC-3
(Smad4−/−)

MiaPaca-2
(Smad4+/+)

0 2 4 8 12
1% oxygen (hours)

HIF-1α
β-tub
4E-BP1

0 2 4 8 12
1% oxygen (hours)

siRNA HIF-1α
HIF-1α
β-tub
4E-BP1

B

-278/+64
Hmut

MiaPaca-2

0.5 mM CoCl2 (hours)

Relative LUC activity (fold)

0 4 8 12 24

MiaPaca-2

C

-278/+64
Hmut
Smut
HSmut

MiaPaca-2

0 1% oxygen (hours)

Relative LUC activity (fold)

0 4 12 24

MiaPaca-2
Molecular Cancer Research

Contribution of HIF-1α in 4E-BP1 gene expression

Rania Azar, Charline Lasfargues, Corinne Bousquet, et al.

Mol Cancer Res Published OnlineFirst November 21, 2012.

Updated version Access the most recent version of this article at: doi:10.1158/1541-7786.MCR-12-0095

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