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 upregulation 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. Mol Cancer Res; 11(1); 54–61. ©2012 AACR.

Introduction

One molecular mechanism whereby cells can counteract undesired increases 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 eukaryotic translation initiation factor 4E (eIF4E), 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; ref. 1). 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 (1). 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 later).

The regulation of 4E-BP1 activity was initially described as being dependent mainly on phosphorylation events (2). 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 (3), or exposure to oxidative (4) or genotoxic (5) compounds. More recently, however, different conditions have been shown to trigger not only 4E-BP1 hypophosphorylation, but also 4E-BP1 gene expression (2). For instance in Drospihila, 4E-BP1 induction prolongs the survival of flies upon nutrient deprivation (6). 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 (7). In these examples, stress-induced 4E-BP1 expression was shown to be mediated by transcription factors (FOXO in Drospihila and ATF4 in mouse endocrine pancreas) that bind to and activate the transcription of 4E-BP1 gene. On the basis of 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 2 other situations in which 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 (O₂; refs. 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% O₂, 4E-BP1 protein accumulation is dependent at least partially on a hypoxia-responsive element (HRE) in the 4E-BP1 gene promoter that binds the transcription factor HIF-1α. Furthermore, we show that HIF-1α 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 (BxO or stt2-stably transfected (Bx2) BxPC-3 cells, and MiaPaCa-2 cells were maintained in Dulbecco’s modified Eagle’s medium containing 1 g/L glucose (LONZA) and supplemented with 10% fetal calf serum (FCS), 2 mmol/L L-glutamine (GIBCO), 2.5 μg/mL Eugenine (GIBCO), 5 U/mL streptomycin/penicillin (GIBCO), and 0.01% plasmocin (InvivoGen). The medium of stable BxC (GIBCO), 5 U/mL streptomycin/penicillin (GIBCO), and supplemented with 10% fetal calf serum (FCS), 2 mmol/L L-glutamine (GIBCO), 2.5 μg/mL Eugenine (GIBCO), and 0.01% plasmocin (InvivoGen). The medium of stable BxC and Bx2 cells contained also 400 μg/mL Geneticin (Invivo-Gen). For extinction of gene expression, cells were plated in 6-well dishes, allowed to grow for 24 hours and transfected with 10 nmol/L of siRNAs targeting either 4E-BP1 (Applied Biosystems), forward 5'-CAAGAACGAACCCUUCCUU-3' and reverse) or targeting HIF-1α (Dharmacon, siGENOME SMARTpool HIF-1α 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 conducted using 10 μg/mL cycloheximide (Sigma) or 5 μg/mL actinomycin-D (Sigma), respectively. WT- and DN-HIF-1α expression plasmids were kindly provided by Prof. H. Prats (INSERM UMR-1037, Toulouse, France). For chemical hypoxia, cells were incubated with 0.5 mmol/L Prof. H. Prats (INSERM UMR-1037, Toulouse, France).

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 (13, 14). Membranes were incubated with mouse monoclonal antibodies to β-tubulin (Sigma), p27Kip1 (BD Transduction Laboratories), HIF-1α (Zymed laboratories), eIF4E (Cell Signaling), and total or phosphospecific 4E-BP1 (Cell Signaling). Rabbit polyclonal antibodies against eIF4G1 were kindly provided by Prof. N. Sonenberg (Department of Biochemistry, McGill University, Sainte-Anne-de-Bellevue, QC, Canada). Membranes were then subjected to immunoblotting using goat horse-radish peroxidase–conjugated secondary antibodies to mouse or rabbit immunoglobulin G (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. Ten microgram of total RNA was denaturated in RNA sample buffer (39 mmol/L MOPS pH 7, 58.5% deionized formamide, 10.8% formaldehyde, and 3% ethidium bromide) for 15 minutes 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 10× saline sodium citrate (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 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 (13). Fragments of 4E-BP1 promoter were PCR-amplified using primers extended by KpnI (forward primers: 5'-ggggtcacatttaggccagcat-3') and by XhoI (reverse primers: 5'-tcgcctgagcttcggtgcagc-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 hours, 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 conducted 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 (15).

Statistical analysis
Statistical analysis was conducted by using the unpaired t test. "*" or "#" indicate a P value less than 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 with normal pancreas (16). As sst2 normally triggers antiproliferative signals (17), its absence leads to uncontrolled cell proliferation. We have however shown that sst2 reexpression conversely limits the proliferation of pancreatic cancer cells, likely via the induction of 4E-BP1 protein and the restoration of contact inhibition (18). Here, we first wish 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 (2). However, phosphorylation at S65 has been shown to play a prominent role in 4E-BP1 binding to eIF4E (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, Bx2 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 upregulated 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

![Figure 1](image)

**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-day 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 mean ± 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 3 separate Western blot analyses.
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-hour 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 with 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 (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 (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 (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).

**Sst2- and cell density–triggered accumulation of 4E-BP1 is dependent on HIF-1α**

One possible candidate transcription factor that could account for cell density–dependent induction of 4E-BP1 promoter activity was HIF-1α. Cell density has actually been shown to induce HIF-1α (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-1α, we first confirmed that HIF-1α was upregulated by cell density in our cell lines (Fig. 4B), and that HIF-1α 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-1α-specific antibodies (Fig. 4C). Interestingly, cell density–dependent induction of HIF-1α 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-1α–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-1α using specific siRNAs showed that HIF-1α 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-1α function by transfecting BxC or Bx2 cells with expression vectors encoding either WT or a dominant negative form (DN) of HIF-1α. The data we obtained revealed that artificial overexpression of WT-HIF-1α in dense BxC cells enhances 4E-BP1 promoter activity, whereas 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**

Because 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% O2 for different times. Surprisingly, only minor changes in 4E-BP1 amount were observed despite induction of HIF-1α over a 24-hour 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, whereas earlier reports described the effect of hypoxia on 4E-BP1 expression in sea urchin embryos (10), human cervix cancer HeLa cells (11), 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;
ref. 23). In conjunction with other members of the SMAD family (such as Smad3), Smad4 acts downstream of TGF-β 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 SBE (15), 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 2 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 preincubation of cells with specific HIF-1α siRNAs (Fig 5A, right). A reporter test also showed that 4E-BP1 promoter

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, WT 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-day 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 3 separate Western blot analyses (right). E, sst2 signaling cooperates with cell density–dependent induction of 4E-BP1. Bx2 cells were transfected with WT or Hmut –278/+64 reporter vector 1 day (low density) or 5 days (high density) after seeding, and firefly luciferase activity was measured 36 hours following transfection. Data represent the mean ± SE of triplicates repeated 3 times and which were normalized as in Fig. 3B, left. Bx2 cells were 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 (low density) or 5 days (high density) following transfection. Numbers are densitometric quantifications of 4E-BP1 and are normalized to the value obtained for low density–cultured and siCTRL-transfected cells. They are representative of 3 separate Western blot analyses (right). E, sst2 signaling cooperates with cell density to fully activate the 4E-BP1 promoter. BxC or Bx2 cells were cotransfected 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 mean ± SE of triplicates repeated 3 times and which were normalized as in Fig. 3B.

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and on HIF-1α. The amounts of HIF-1α, β-tubulin, and 4E-BP1 were monitored in Smad4−/− BxPC-3 and Smad4+/+ MiaPaca-2 cells by Western blotting 3 days after seeding and over a 12-hour period under hypoxia (1% O2). MiaPaca-2 cells were pretreated 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 3 separate Western blot analyses. B, 4E-BP1 promoter induction in cells treated with CoCl2. MiaPaca-2 cells were 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-hour period in the presence of 0.5 mmol/L of CoCl2. Data represent the mean ± SE of triplicates repeated 3 times and which were normalized as in Fig. 3B. C, 4E-BP1 promoter induction under hypoxia necessitates both HIF-1α and Smad4 functions. Smad4−/− Bx-PC-3 or Smad4+/− MiaPaca-2 cells were 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-hour period under 1% O2 (left). MiaPaca-2 cells were transfected with WT, 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-hour period under 1% O2 (right). Data represent the mean ± SE of triplicates repeated 3 times and which were normalized as in Fig. 3B.

In summary, we show that the 4E-BP1 gene contains a functional HRE, which binds HIF-1α in 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 seems 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 in which Smad4 is deleted.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: R. Azar, S. Pyronnet Development of methodology: R. Azar, C. Lasfargues, C. Bousquet Acquisition of data (provided animals, collected samples, provided facilities, etc.): R. Azar, C. Bousquet, S. Pyronnet Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R. Azar, C. Lasfargues, S. Pyronnet Writing, review, and/or revision of the manuscript: R. Azar, C. Lasfargues, C. Bousquet, S. Pyronnet Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): R. Azar, S. Pyronnet Study supervision: S. Pyronnet

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References


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