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Far Upstream Element Binding Protein 1 Activates Translation of p27^{Kip1} mRNA Through Its Internal Ribosomal Entry Site

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Abstract

The cyclin dependent kinase inhibitor p27 plays an important role in controlling the eukaryotic cell cycle by regulating progression through G1 and entry into S phase. It is often elevated during differentiation and under conditions of cellular stress. In contrast, it is commonly downregulated in cancer cells and its levels are generally inversely correlated with favorable prognosis. The cellular levels of p27 are regulated, in part, by translational control mechanisms. The 5'-untranslated region (5'-UTR) of the p27 mRNA harbors an internal ribosome entry site (IRES) which may facilitate synthesis of p27 in certain conditions. In this study, Far Upstream Element (FUSE) Binding Protein 1 (FBP1) was shown to directly bind to the human p27 5'-UTR and to promote IRES activity. An eight-nucleotide element downstream of a U-rich region within the 5'-UTR was important for FBP1 binding and p27 IRES activity. Overexpression of FBP1 enhanced endogenous p27 levels and stimulated translation initiation. In contrast, repression of FBP1 by siRNA transfection downregulated endogenous p27 protein levels. Using rabbit reticulocyte lysates, FBP1 stimulated p27 mRNA translation *in vitro*. The central domain of FBP1, containing four K homology motifs, was required for p27 5'-UTR RNA binding and the N terminal domain was important for translational activation. These findings indicate that FBP1 is a novel activator of p27 translation upon binding to the 5'-UTR.

Keywords

p27^{Kip1}; cell cycle; IRES; FBP; 5'-untranslated region

Introduction

P27^{Kip1} (p27) is a Cip/Kip family cyclin-CDK inhibitor and plays a critical role in eukaryotic cell cycle regulation (Sgambato et al., 2000). Cellular levels of p27 are controlled by multiple signaling mechanisms that regulate transcription, translation, and protein

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Authors' contributions. Y.Z. and K.M. initiated the work, designed the experiments and wrote the paper. Y.Z. performed the experiments.

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stability. In many types of human cancer, p27 is aberrantly regulated and reduced p27 expression in tumors is usually associated with enhanced malignancy and poor prognosis (Chu et al., 2008).

The cellular level of p27 can be regulated at the translational level. Translational control of p27 mRNA is not completely understood but involves the 5' untranslated region (5'-UTR). The 5'-UTR of the p27 message harbors an internal ribosome entry site (IRES) that mediates cap-independent translation initiation (Cho et al., 2005, Kullmann et al., 2002, Jiang et al., 2007, Miskimins et al., 2001). Ribosome entry appears to occur within an U-rich region located just upstream of the AUG start codon (Coleman and Miskimins, 2009). Many factors are involved in p27 IRES regulation. P27 IRES activity can be stimulated by polypyrimidine tract binding protein (PTB) and inhibited by HuR (Cho et al., 2005, Kullmann et al., 2002). In addition, pseudouridine synthase, encoded by the DKC1 gene, is important for translation through the p27 IRES. Mutation of the DKC1 gene impairs translational pre-initiation complex formation on the p27 mRNA (Yoon et al., 2006, Ruggero et al., 2003, Bellodi et al.).

At present there is an incomplete understanding of the factors involved in regulating translation of p27 mRNA through its 5'-UTR, or how such factors function together to regulate expression of the protein. In this study Far Upstream Element (FUSE) Binding Protein 1 (FBP1) was shown to bind with specificity to the p27 5'-UTR. FBP1 was first identified as a DNA binding protein that promotes expression of the *c-myc* through transcriptional activation (Avigan et al., 1990). Data presented here suggest that FBP1 is a novel p27 IRES regulatory factor that enhances p27 expression by promoting translational initiation.

Materials and Methods

Plasmids construction

PCR primers and template information is summarized in Table 1. Human FBP1 cDNA was a gift from David L. Levens (NCI). Internal deletion mutants were synthesized by two-step PCR. For expression in eukaryotic cells, wild-type FBP1 or its mutants were subcloned into pCDNA3.1-HisB at sites KpnI and XhoI. For expression in prokaryotic cells, the same cDNAs were subcloned into pRSETB at sites XhoI and HindIII. The dual luciferase bicistronic plasmid pTKLL472 was previously described (Jiang et al., 2007).

Cell culture, transfection, and reporter gene assays

Human breast cancer cell lines MCF7 and human embryonic kidney cell line 293T were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin at 37 °C in a humidified atmosphere containing 5% CO₂. Transient transfection of DNAs was performed using DreamFect (Boca Scientific) following the manufacturer's guidelines. For stable transfection, MCF7 cells cultured in 35 mm dishes were transfected with pCDNA3.1-HisB-FBP1. One day later, cells were trypsinized and plated in 150 mm dishes. G418 was added to a final concentration of 1 mg/ml and continuously maintained in selection medium. Colonies of cells were isolated, expanded, and expression of Xpress-tagged FBP1 was tested by western blotting. FBP1 siRNA was purchased from IDT, and control siRNA was purchased from Ambion. siRNAs were transfected using HyperFect reagent (Qiagen) according to manufacturer's protocol. Dual luciferase assays were performed using the Dual-Glo Luciferase Assay system (Promega) as previously described (Jiang et al., 2007).

Recombinant protein expression and purification

Competent Rosetta *E. coli* cells (Novagen) were transformed with pRSETB constructs carrying wild-type or mutant FBP1 following the manufacturer's guidelines. Recombinant proteins were expressed and purified as previously described (Wei et al., 2007). All purified recombinant proteins were dialyzed and then verified by Coomassie Blue staining of SDS-polyacrylamide gels and by western blotting (data not shown).

Preparation of cell lysates

Cells were harvested and resuspended in five volumes of cell lysis buffer (10 mM Tris pH 8.0, 140 mM NaCl, 1.5 mM MgCl₂, 0.1% NP40). The cell lysates were briefly sonicated on ice and clarified by centrifugation at 10,000 g and then stored at -80 °C until used.

Western blotting

Western blotting was performed as previously described (Miskimins et al., 2001). FBP1 antibody was purchased from Transduction Laboratory; Xpress antibody was purchased from invitrogen.

Preparation of biotinylated RNA probe and biotin-labeled RNA pulldown assay

The full-length human p27 5'-UTR was amplified by PCR using primers described in Table 1. The PCR product was used as a template for synthesis of biotin-labeled RNAs using the AmpliScribe T7-Flash Transcription Kit (Epicentre Biotechnologies) according to the manufacturer's guidelines. Biotin-labeled RNA pulldown assays were performed using Dynabeads M-280 Streptavidin (Invitrogen). For each sample, 20 µl of resuspended beads were used. Beads were washed twice with solution A [0.1 M NaOH, 0.05 M NaCl prepared in diethylpyrocarbonate (DEPC) treated water], once in solution B (0.1 M NaCl in DEPC treated water) and once in B&W buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 2.0 M NaCl). The beads were resuspended in 60 µl B&W buffer and then 1 µl SUPERase-In (Ambion) and 2 µg biotin-labeled RNA were added. The samples were incubated at room temperature for 20 min with occasional mixing. Free biotin-labeled RNAs were removed by collecting the beads using a magnetic stand and then washing the beads twice with B&W buffer. The RNA loaded beads were resuspended with protein samples for a total volume of 200 µl. The samples were incubated at 15 °C for 30 min with mixing every 5 min. The beads were then washed five times with cell lysis buffer to remove unbound proteins. After the last washing step, beads were resuspended in 20 µl SDS-PAGE sample buffer and the eluted proteins analyzed by Western blotting.

Preparation of radio-labeled RNA probes, RNA electrophoretic mobility shift assays (REMSA), and UV-crosslinking assays

³²P-labeled RNA was synthesized using a MAXIscript Kit (Ambion) following the manufacturer's protocol. REMSA was performed according to a previous publication (Thomson et al., 1999). Specifically, radiolabeled RNA probe (30,000 cpm) was incubated with 10 µl recombinant protein (2–20 µg) in the presence of 1 µl RNase inhibitor SUPERase-In (Ambion) at room temperature for 15 min. A 4% native polyacrylamide gel was pre-run at 200 V for 30 min in 0.5X TBE buffer (0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA) before sample loading. The sample was then loaded and the gel run at 4 °C, 200 V for 4 hours. The gel was dried and subjected to autoradiography.

The UV crosslinking assay was performed as described in a previous publication (Thomson et al., 1999). For each sample, 30,000 cpm radiolabeled RNA probe was incubated with 10 µl recombinant protein (~20 µg protein) for 15 min at room temperature. Then the mixture was UV irradiated (3 X10⁵ J for 5 min) on ice using a UV crosslinker. After crosslinking, 1

μl RNase1/T1 was added and the samples were incubated at 37 °C for 1 hour to degrade free RNA. Then the samples were separated by 10% SDS-PAGE. The gel was dried using a vacuum gel drier and then the bands were detected by autoradiography.

Preparation of Capped RNAs and *in vitro* translation

The reporter constructs were amplified using primers described in Table 1. The PCR products were used as templates for capped RNA synthesis using the mMESSAGE mMACHINE T7 kit (Ambion) following the product instructions.

Rabbit Reticulocyte Lysate (RRL) was purchased from Promega. The *in vitro* translation reaction was performed in two steps. The first step was performed by mixing 0.25 μl amino acid mixture lacking leucine, 0.25 μl amino acid mixture lacking methionine, 0.5 μl SUPERase-In, 2 μg FBP1 mRNA or revFBP mRNA, 17.5 μl RRL, and water to a final volume of 25 μl. The samples were then incubated at 30 °C for 90 min. Synthesis of FBP1 was confirmed by western blotting. For the second step, the translation products from the first step were combined with fresh *in vitro* translation reaction mixture and reporter mRNA. The resulting products were analyzed by luciferase assays.

Sucrose gradient fractionation and RNase protection assays (RPA)

Sucrose gradients were generated using a Gradient Station (BIOCOMP Model 153, with Gradient forming software Version 2.17D and Fractionation software Version 5.50). The gradient was from 15% to 60% sucrose in gradient buffer (20 mM HEPES pH 7.6, 150 mM potassium acetate, 5 mM MgCl₂, 1 mM DTT). The program setting was SW50, long, Suc 15%–60%, 2 step, 1:50 min, angle 55 and speed 25. Cell lysates were generated by resuspending cell pellets from one 150 mm dish in 1 ml gradient buffer. The cells were sonicated three times on ice, 10 second each. The lysate was centrifuged at 1300 g at 4 °C for 15 min. The supernatant was then loaded to the top of the sucrose gradient. The gradient was centrifuged in an SW 50.1 rotor at 49,600 rpm for 90 min. Fractionation was performed using the same Gradient Station machine with the following parameters: DIST 30mm, speed 6.5 mm, rinse ACD5. The fractionation speed was 1 mm, DIST 4 mm. The number of fractions from each gradient was 15–20, with a volume of 400 μl each. Collected fractions were used for RNA isolation using Tri-Reagent. p27 mRNA in each sucrose gradient fractions was quantified by RPA as described previously (Jiang et al., 2007).

Results

FBP1 binds with specificity to the p27 5'-UTR

The human p27 mRNA has a long 5'-UTR with a high GC content (Coleman et al., 2001). It also contains a U-rich region located ~40 nucleotides upstream of the AUG start codon. This U-rich region appears to exist as a large single-stranded loop that is important for ribosome entry during cap-independent translation (Coleman and Miskimins, 2009). Previous studies have shown that the U-rich region specifically interacts with several RNA-binding proteins, including PTB and HuR (Cho et al., 2005, Kullmann et al., 2002). To further study proteins that may influence p27 translation, biotinylated RNA pulldown experiments were used to isolated p27 5'-UTR-binding proteins from MCF7 cell extracts. Bound proteins were separated by SDS-PAGE and then identified by mass spectrometry (data not shown). The results confirmed binding of PTB, hnRNP1/2, and HuR (Cho et al., 2005, Millard et al., 2000, Kullmann et al., 2002). In addition, these experiments identified FBP1 as a putative p27 5'-UTR-binding protein. To confirm this possibility and to map the region bound by FBP1, a series of RNA binding experiments was performed (Fig. 1A). Biotinylated RNA pulldown assays (Fig. 1B) using MCF7 cytosolic extracts show that FBP1 associates with the p27 5'-UTR but not with a control RNA of similar length. UV-crosslinking assays using

recombinant FBP and radiolabeled p27 5'-UTR further indicate that FBP1 binds directly to the p27 5'-UTR (Fig. 1C). REMSA confirms direct binding of FBP1 to the p27 5'-UTR. This assay also demonstrates the specificity of the interaction because unlabeled p27 5'-UTR RNA effectively competes with the radiolabeled probe for FBP1 binding, while a non-specific control RNA has only a limited effect (Fig. 1D).

To map the region of the p27 5'-UTR necessary for binding FBP1, a series of deletion mutations was introduced into the sequence (Fig. 1A). REMSA indicates that FBP1 binds to the 3' half of the 5'-UTR (nucleotides 226–472) but not to the 5' half (nucleotides 1–219). Deletion of the U-rich element near the 3' end of the 5'-UTR, which has been shown to be important for binding of other factors, did not affect FBP1 binding (Fig. 1E). RNA toe printing assays (data not shown) suggested that regions just outside of the U-rich element might be important for FBP binding. Based on this, mutations were generated in which short sequences adjacent to either end of the U-rich region were deleted (constructs Δ S1 and Δ S2 in Fig. 1A). Biotinylated RNA pulldown assays show that the sequence deleted in Δ S1 is not important for FBP1 binding. However, no FBP1 binding was detected when the Δ S2 probe was used in the assay. This deletion spans nucleotides 431–439 (5'-GCGAAGAG-3') just downstream of the U-rich element and the results indicate that this region is critical for FBP1 binding to the p27 5'-UTR.

FBP1 stimulates p27 IRES activity

A bicistronic reporter plasmid, pTKLL472, was used to examine p27 IRES activity. This reporter construct was transiently cotransfected with an expression vector encoding Xpress-tagged FBP1 or with empty vector into MCF7 breast cancer cells. Expression of Xpress-tagged FBP1 was confirmed by western blotting (Fig. 2A, left panel). Overexpression of FBP1 significantly enhanced p27 IRES activity as indicated by the ratio of firefly and Renilla luciferase activities (Fig. 2A, right panel). Since the S2 element is important for FBP1 binding to the p27 5'-UTR, IRES activity of a bicistronic construct in which only this 8 nucleotide region is deleted, pTKLL Δ S2, was compared to the full-length construct (Fig. 2B). Deletion of the S2 element significantly impaired p27 IRES activity, suggesting that FBP1 enhances p27 translation through this element.

To confirm the effects of FBP1 on p27 IRES activity and to examine its effects on expression of endogenous p27, a stably transfected MCF7 cell line that constitutively overexpresses FBP1 was generated. This line displays a higher level of endogenous p27 than the parental MCF7 cell line (Fig. 2C, left panel). Transient transfection of this line with the p27 IRES reporter construct shows that p27 IRES activity is also upregulated by stable expression of FBP1 (Fig. 2C, right panel). To further demonstrate the role of FBP1 in p27 expression, an siRNA targeted to FBP1 was used to knock down endogenous FBP1 in MCF7 cells. Two days after siRNA transfection, cells transfected with FBP1 siRNA had decreased FBP expression and lower p27 levels relative to control cells (Fig. 2D), suggesting that FBP1 plays an important role in regulating p27 expression in this cell line.

FBP promotes p27 translation *in vitro*

To further test whether FBP1 directly regulates p27 at the translational level, a p27 5'-UTR luciferase reporter mRNA was used for *in vitro* translation assays (Fig. 3A). First, FBP1 was synthesized *in vitro* using in RRL. Expression of FBP1 was confirmed by western blotting (Fig. 3B). As a control, a similar reaction was performed using a template derived from a construct in which the FBP1 cDNA was inserted in the reverse orientation. Next, a second *in vitro* translation reaction was performed using fresh RRL, combined with either the FBP1 reaction mixture or the control reaction mixture, to translate the p27 5'-UTR luciferase reporter mRNA. p27 5'-UTR-driven luciferase expression was enhanced approximately 6

fold in the reactions containing newly synthesized FBP1 (Fig. 3C). This suggests that FBP1 directly promotes translational initiation through the p27 5'-UTR.

In addition, the efficiency of p27 mRNA translation was examined in parental MCF7 cells, or MCF7 cells stably expressing FBP, using sucrose gradient assays (Fig. 3D). The amount of p27 mRNA in each gradient fraction was measured by RNase protection assays. A greater proportion of p27 mRNA was associated with polysomal fractions from cells that overexpress FBP1, suggesting a higher level of translation initiation.

The N-terminal and central domains of FBP1 are required for promoting p27 IRES activity

Previous work has shown that FBP1 has three major functional regions, the N-terminal, central, and C-terminal domains (Duncan et al., 1996). For *c-myc* gene regulation, the FBP1 central domain recognizes and binds to FUSE. This region of the protein has four mRNPK homology (KH) motifs that function in nucleic acid interactions. The N terminal domain is involved in protein-protein interactions and may function in transcriptional repression (Chung et al., 2006), while the C terminal domain functions in transcriptional activation by interacting with TFIID (Liu et al., 2001, Braddock et al., 2002, Duncan et al., 1996). To investigate the role of each domain in p27 IRES regulation, FBP1 deletion mutations were generated (Fig. 4A) and the effects on RNA binding and p27 IRES activity was tested. REMSA binding assays show that the FBP1 mutant lacking the central domain (del-CEN) does not form an RNA-protein complex with the p27 5'-UTR (Fig. 4B). Thus, the FBP1 KH domains are most likely required for recognizing the p27 message. Deleting the C-terminal domain (del-Cterm) had no effect on binding to the p27 5'-UTR. FBP1 with an N-terminal deletion (del-Nterm) appears to bind to the p27 5'-UTR but the complex formed migrates more rapidly than that formed by the wild-type protein or the del-Cterm mutant. To examine the importance of these domains in activating p27 IRES activity, the constructs encoding the deletion mutants were co-transfected with the pTKL472 reporter construct into 293T cells. Expression of the proteins was confirmed by western blotting (Fig. 4C). The C terminal deletion mutant activated IRES activity to levels nearly equivalent to wild-type FBP1. However, FBP1 deletions lacking either the N terminal or central domains were incapable of stimulating IRES activity (Fig. 4D).

Discussion

In this study evidence is provided that FBP1 binds to the 5'-UTR of human p27 mRNA and activates p27 expression. Overexpression of FBP is associated with higher levels of endogenous p27 and also enhanced p27 IRES activity. In contrast, knockdown of FBP1 using siRNAs leads to reduced expression of p27. These findings suggest that binding of FBP1 to the p27 5'-UTR activates IRES-dependent initiation of translation.

Previous studies have shown that a U-rich region located ~40 nucleotides upstream of the start codon of p27 mRNA is important for p27 IRES activity (Fig 5) (Coleman and Miskimins, 2009, Millard et al., 2000). The region of the p27 5'-UTR that includes the U-rich region exists as a large single-stranded loop that functions in ribosome recruitment during initiation of translation. The ribosome entry window for IRES-mediated translation is between 36 and 50 nucleotides upstream of the p27 start codon (Coleman and Miskimins, 2009). The U-rich region is also a regulatory site for p27 IRES activity. Several IRES trans-activating factors (ITAFs), including PTB, hnRNP and HuR are recruited to the mRNA through the U-rich region (Cho et al., 2005, Millard et al., 2000, Kullmann et al., 2002, Zheng and Miskimins). However, the U-rich region is not essential for FBP1 binding, which requires an 8 nucleotide element just downstream. This element, referred to as S2, is located 32 to 40 nucleotides upstream of the p27 start codon. Deletion of the S2 element interferes with FBP binding but has little effect on binding of HuR or PTB. Functional tests showed

that the S2 element is also important for p27 IRES activity. Since the S2 element is close to the ribosome entry site of the 5'-UTR, it is possible that FBP1 modulates either ribosome recruitment or ribosome scanning toward the translation start codon.

From the current data alone, it cannot be concluded that the S2 element is the actual contact site for FBP binding. It is possible that this region is involved in forming structural elements that influence FBP binding and that FBP contact sites reside elsewhere. A previous publication characterized the complex between FBP1 and single-stranded DNA (ssDNA) for KH3 and KH4 (Braddock et al., 2002). In this model the central portion of the recognition sequence for KH3 was the tetrad TTTT and for KH4 it was ATTC. A later publication indicates that the core of the ideal binding site for all of the FBP1 KH motifs is TGT (Benjamin et al., 2008). However, there is evidence that ssDNA and RNA bound to KH motifs have very different conformations (Braddock et al., 2002, Lewis et al., 2000). Therefore, sequence motifs for ssDNA cannot be directly extrapolated to RNA binding elements. The narrow groove of the nucleic acid binding region of the KH motifs favors pyrimidines over purines (Braddock et al., 2002). The S2 element (5'-GCGAAGAG-3') contains a single pyrimidine, suggesting that it could not directly contact FBP1. Rather, it is more likely to be a structural element or a spacer between two KH motif contact sites. Further analysis of the interactions between FBP1 and the p27 5'-UTR will be required to resolve this question.

FBP1 was first identified as a DNA binding protein that activates c-myc gene transcription (Duncan et al., 1996). Upregulation of c-myc affects hundreds of downstream factors and thus regulates cell differentiation, proliferation and apoptosis (Chung and Levens, 2005, Pelengaris and Khan, 2003). Generally, a more active c-myc pathway is associated with increased cell proliferation and decreased cell apoptosis. The role of FBP1 in c-myc pathway has been well established (He et al., 2000, Wang et al., 1998). However, our findings suggest a novel function for FBP in stimulating expression of the cell cycle repressor p27. This function of FBP1 is different in several aspects. FBP1 regulates p27 mRNA translation in the cytosol, rather than gene transcription in nucleus. The N-terminal domain of FBP1 is important for p27 translational activation but this region has a repressive function in transcriptional control (Duncan et al., 1996). The C terminal region of FBP1 harbors an activation domain that is essential for enhancing transcription but does not appear to have any role in promoting p27 translation. In general, in terms of cell cycle progression and tumorigenesis, up-regulation of p27 and up-regulation of c-myc have opposite consequences for the cell. At present it is not clear how FBP1 mediates these opposing functions. It is possible that nuclear FBP1 enhances cell proliferation by promoting c-myc gene expression, while cytoplasmic FBP1 promotes cell cycle arrest by controlling translation of key target proteins. In this regard, a recent publication indicates that FBP1 acts as a suppressor of proliferation through a translational control mechanism (Olanich et al.). In this case FBP1 binds to the 3'-UTR of the oncoprotein nucleophosmin mRNA to repress its translation. It will be of interest to determine how the balance between FBP1 transcriptional and translational functions is regulated.

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References

- Avigan MI, Strober B, Levens D. A far upstream element stimulates c-myc expression in undifferentiated leukemia cells. *J Biol Chem.* 1990; 265:18538–18545. [PubMed: 2211718]

- Bellodi C, Krasnykh O, Haynes N, Theodoropoulou M, Peng G, Montanaro L, Ruggero D. Loss of function of the tumor suppressor DKC1 perturbs p27 translation control and contributes to pituitary tumorigenesis. *Cancer Res.* 70:6026–6035. [PubMed: 20587522]
- Benjamin LR, Chung HJ, Sanford S, Kouzine F, Liu J, Levens D. Hierarchical mechanisms build the DNA-binding specificity of FUSE binding protein. *Proc Natl Acad Sci U S A.* 2008; 105:18296–18301. [PubMed: 19015535]
- Braddock DT, Louis JM, Baber JL, Levens D, Clore GM. Structure and dynamics of KH domains from FBP bound to single-stranded DNA. *Nature.* 2002; 415:1051–1056. [PubMed: 11875576]
- Cho S, Kim JH, Back SH, Jang SK. Polypyrimidine tract-binding protein enhances the internal ribosomal entry site-dependent translation of p27Kip1 mRNA and modulates transition from G1 to S phase. *Mol Cell Biol.* 2005; 25:1283–1297. [PubMed: 15684381]
- Chu IM, Hengst L, Slingerland JM. The Cdk inhibitor p27 in human cancer: prognostic potential and relevance to anticancer therapy. *Nat Rev Cancer.* 2008; 8:253–267. [PubMed: 18354415]
- Chung HJ, Levens D. c-myc expression: keep the noise down! *Mol Cells.* 2005; 20:157–166. [PubMed: 16267388]
- Chung HJ, Liu J, Dundr M, Nie Z, Sanford S, Levens D. FBPs are calibrated molecular tools to adjust gene expression. *Mol Cell Biol.* 2006; 26:6584–6597. [PubMed: 16914741]
- Coleman J, Hawkinson M, Miskimins R, Miskimins WK. The major transcription initiation site of the p27Kip1 gene is conserved in human and mouse and produces a long 5'-UTR. *BMC Mol Biol.* 2001; 2:12. [PubMed: 11696240]
- Coleman J, Miskimins WK. Structure and activity of the internal ribosome entry site within the human p27 Kip1 5'-untranslated region. *RNA Biol.* 2009; 6:84–89. [PubMed: 19106631]
- Duncan R, Collins I, Tomonaga T, Zhang T, Levens D. A unique transactivation sequence motif is found in the carboxyl-terminal domain of the single-strand-binding protein FBP. *Mol Cell Biol.* 1996; 16:2274–2282. [PubMed: 8628294]
- He L, Liu J, Collins I, Sanford S, O'Connell B, Benham CJ, Levens D. Loss of FBP function arrests cellular proliferation and extinguishes c-myc expression. *EMBO J.* 2000; 19:1034–1044. [PubMed: 10698944]
- Jiang H, Coleman J, Miskimins R, Srinivasan R, Miskimins WK. Cap-independent translation through the p27 5'-UTR. *Nucleic Acids Res.* 2007; 35:4767–4778. [PubMed: 17617641]
- Kullmann M, Gopfert U, Siewe B, Hengst L. ELAV/Hu proteins inhibit p27 translation via an IRES element in the p27 5'UTR. *Genes Dev.* 2002; 16:3087–3099. [PubMed: 12464637]
- Lewis HA, Musunuru K, Jensen KB, Edo C, Chen H, Darnell RB, Burley SK. Sequence-specific RNA binding by a Nova KH domain: implications for paraneoplastic disease and the fragile X syndrome. *Cell.* 2000; 100:323–332. [PubMed: 10676814]
- Liu J, Akoulitchev S, Weber A, Ge H, Chuikov S, Libutti D, Wang XW, Conaway JW, Harris CC, Conaway RC, Reinberg D, Levens D. Defective interplay of activators and repressors with TFIH in xeroderma pigmentosum. *Cell.* 2001; 104:353–363. [PubMed: 11239393]
- Millard SS, Vidal A, Markus M, Koff A. A U-rich element in the 5' untranslated region is necessary for the translation of p27 mRNA. *Mol Cell Biol.* 2000; 20:5947–5959. [PubMed: 10913178]
- Miskimins WK, Wang G, Hawkinson M, Miskimins R. Control of cyclin-dependent kinase inhibitor p27 expression by cap-independent translation. *Mol Cell Biol.* 2001; 21:4960–4967. [PubMed: 11438653]
- Olanich ME, Moss BL, Piwnicka-Worms D, Townsend RR, Weber JD. Identification of FUSE-binding protein 1 as a regulatory mRNA-binding protein that represses nucleophosmin translation. *Oncogene.* 30:77–86. [PubMed: 20802533]
- Pelengaris S, Khan M. The many faces of c-MYC. *Arch Biochem Biophys.* 2003; 416:129–136. [PubMed: 12893289]
- Ruggero D, Grisendi S, Piazza F, Rego E, Mari F, Rao PH, Cordon-Cardo C, Pandolfi PP. Dyskeratosis congenita and cancer in mice deficient in ribosomal RNA modification. *Science.* 2003; 299:259–262. [PubMed: 12522253]
- Sgambato A, Cittadini A, Faraglia B, Weinstein IB. Multiple functions of p27(Kip1) and its alterations in tumor cells: a review. *J Cell Physiol.* 2000; 183:18–27. [PubMed: 10699962]

- Thomson AM, Rogers JT, Walker CE, Staton JM, Leedman PJ. Optimized RNA gel-shift and UV cross-linking assays for characterization of cytoplasmic RNA-protein interactions. *Biotechniques*. 1999; 27:1032, 1039–1042. [PubMed: 10572651]
- Wang X, Avigan M, Norgren RB Jr. FUSE-binding protein is developmentally regulated and is highly expressed in mouse and chicken embryonic brain. *Neurosci Lett*. 1998; 252:191–194. [PubMed: 9739993]
- Wei Q, Eviatar-Ribak T, Miskimins WK, Miskimins R. Galectin-4 is involved in p27-mediated activation of the myelin basic protein promoter. *J Neurochem*. 2007; 101:1214–1223. [PubMed: 17403142]
- Yoon A, Peng G, Brandenburger Y, Zollo O, Xu W, Rego E, Ruggero D. Impaired control of IRES-mediated translation in X-linked dyskeratosis congenita. *Science*. 2006; 312:902–906. [PubMed: 16690864]
- Zheng Y, Miskimins WK. CUG-binding protein represses translation of p27Kip1 mRNA through its internal ribosomal entry site. *RNA Biol*. :8.

Figure 1.

FBP1 binds with specificity to p27 5'-UTR. **A.** Diagram of the human p27 5'-UTR with the U-rich region shown in black. Truncations and deletions used for RNA-binding assays are shown. **B.** Biotinylated-RNA pulldown assays showing binding of FBP1 to the p27 5'-UTR. Protein extracts were prepared from MCF7 cells. Biotin-labeled probes included a control RNA derived from a plasmid vector and the full-length p27 5'-UTR as shown in A. RNA-bound FBP1 was eluted and assayed by western blotting. For the input lane total MCF7 cell extract was used. **C.** UV-crosslinking assay showing the binding of purified recombinant FBP1 to 32 P labeled p27 5'-UTR. In the lane labeled (-) no recombinant FBP1 was added to the reaction. **D.** REMSA with cold p27 5'-UTR or control RNA as competitor. Increasing amounts of each competitor were added to the assays as indicated by the wedges. **E.** REMSA showing the binding of FBP1 to full-length p27 5'-UTR (wt) and to deletion mutants. The probes correspond to the diagram in A. **F.** Binding of FBP1 to the full-length p27 5'-UTR or the S1 and S2 deletions. Extracts prepared from MCF7 were used for biotin-labeled RNA pulldown assays. Biotin-labeled probes are shown in A.

Figure 2.

FBP regulates p27 expression and IRES activity. **A.** MCF7 cells were cotransfected with the bicistronic reporter construct pTKLL472 and either empty vector (pCDNA3.1-HisB) or pCDNA3.1-HisB-FBP encoding Xpress-tagged FBP1. One day after transfection, the expression of FBP1 was tested by western blotting using anti-Xpress antibodies (left panel). Cell lysates were assayed for firefly and Renilla luciferase activity as an indicator of IRES activity (right panel). The mean of two independent experiments is shown and error bars represent standard error. **B.** MCF7 cells were transfected with the bicistronic reporter construct pTKLL472 or pTKLL472ΔS2. One day after transfection cell lysates were assayed for luciferase activity. The ratio of firefly to Renilla luciferase indicates p27 IRES activity. The mean of two independent experiments is shown and error bars represent standard error. **C.** An MCF7 cell line constitutively overexpressing FBP1 was generated as described in Methods. The levels of FBP1, p27 and β-actin in the parental MCF7 cell line and the FBP1 overexpressing cell line were compared by western blotting (left panel). The bicistronic reporter construct pTKLL472 was transiently transfected into MCF7 cells or MCF7 cells stably expressing Xpress-tagged FBP1. Firefly and Renilla luciferase assays were performed as described in A. **D.** MCF7 cells were transfected with control (non-targeting) or FBP1-specific siRNAs. FBP1 siRNA transfected cells were harvest 48 hours after transfection. The levels of FBP1, p27, and β-actin were examined by western blotting.

Figure 3.

FBP stimulates p27 mRNA translation *in vitro*. **A.** Diagram of reporter mRNA p27 5'-UTR-Luc and FBP mRNA or Rev-FBP mRNA used in the following experiments. **B.** FBP was synthesized in RRL as described in methods. The endogenous FBP1 and newly synthesized FBP1 in RRL were detected by western blotting. **C.** Reporter mRNA was translated in the second step of an *in vitro* translation reaction using freshly added RRL as described in the Methods section. The translation efficiency of each sample was measured by luciferase assays. **D.** The MCF7 cell line constitutively overexpressing FBP was generated as described above. Cell lysates from MCF7 or the MCF7-FBP1 stable cell line were fractionated in sucrose gradients. RNAs from each fraction were isolated and used for RPA analysis to detect p27 mRNA. The resulting p27 bands were quantified and normalized to the total amount of p27 mRNA in all fractions. Thus, the value of each fraction represents the percentage of p27 mRNA in the total lysate. Lower number fractions represent the top of the gradient. In FBP1 stable cells, a higher ratio of p27 mRNA is detected in polyribosomal fractions, which suggests that overexpression of FBP1 stimulates p27 translation initiation.

Figure 4. FBP1 domains involved in binding p27 5'-UTR and stimulating p27 IRES activity. **A.** Diagram of the human FBP1. Truncations and deletions used for the experiments are shown. **B.** Purified recombinant FBP1 and FBP1 mutants described in A were used for REMSA assays using radiolabeled human p27 5'-UTR probe. **C.** FBP1 and FBP1 mutants subcloned in pCDNA3.1-HisB were co-transfected with reporter construct pTKLL472 into 293T cells. Expression of the recombinant proteins was tested by western blotting using anti-Xpress antibody. **D.** One day after transfection cell lysates were assayed for luciferase activity. The value of firefly to Renilla luciferase ratio is shown. The mean of two independent experiments is shown and error bars represent standard error.

Figure 5.
p27 IRES regulation model

Table 1

Primer sequences

Primer	Sequence	Template	Comments
5'FBP-PC 3'FBP-PC	5'-atcggtagctatggcagactattcaa-3' 5'-gatctcgagttattggccctgaggtg-3'	FBP plasmid	primers for subcloning hFBP cDNA into pCDNA3.1-HisB
5'FBPdN 3'FBPdN	5'-atcggtagctgtccagatggaatgg-3' 5'-gatctcgagttattggccctgaggtg-3'	pCDNA3.1-HisB-FBP	primers for subcloning hFBP del N mutant cDNA into pCDNA3.1-HisB
5'FBPdC 3'FBPdC	5'-atcggtagctatggcagactattcaa-3' 5'-gatctcgagttaaatctttctctat-3'	pCDNA3.1-HisB-FBP	primers for subcloning hFBP del C mutant cDNA into pCDNA3.1-HisB
5'FBP-PR 3'FBP-PR	5'-cagctcgagcatggcagactattcaa-3' 5'-gctaagcttattggccctgaggtgct-3'	pCDNA3.1-HisB-FBP	primers for subcloning FBP cDNA into pRSETB
5'FBPdN-PR 3'FBPdN-PR	5'-cagctcgagcgtccagatggaatgg-3' 5'-gctaagcttattggccctgaggtgct-3'	pCDNA3.1-HisB-FBP	primers for subcloning hFBP del N mutant cDNA into pRSETB
5'FBPdC-PR 3'FBPdC-PR	5'-cagctcgagcatggcagactattcaa-3' 5'-atcggtagctatggcagactattcaa-3'	pCDNA3.1-HisB-FBP	primers for subcloning hFBP del C mutant cDNA into pRSETB
5'FBPdM-PR 3'FBPdM-PR	5'-atcggtagctatggcagactattcaa-3' 5'-gatctcgagttattggccctgaggtg-3'	pCDNA3.1-HisB-FBP-dM	primers for subcloning hFBP del M mutant cDNA into pRSETB
5'p27UTR 3'p27UTR	5'-ggatcctaatacgaactactataggtcttctcgtcagcctccctt-3' 5'-ctttctccggcggtggtcgtcgtcggg-3'	pTKLL472	primers for p27 5'UTR DNA amplification
5'p27UTR226 3'p27UTR	5'-ggatcctaatacgaactactataggtcttctcgtcagcctccctt-3' 5'-gaccaggcaaggcgagaggggtg-3'	pTKLL472	primers for p27 5'UTR226-472 truncation DNA amplification
5'p27UTR 3'p27UTR219	5'-ggatcctaatacgaactactataggtcttctcgtcagcctccctt-3' 5'-ctttctccggcggtggtcgtcgtcggg-3'	pTKLL472	primers for p27 5'UTR 1-219 truncation DNA amplification
5'p27UTR 3'p27UTR	5'-ggatcctaatacgaactactataggtcttctcgtcagcctccctt-3' 5'-ctttctccggcggtggtcgtcgtcggg-3'	pTKLL472-ΔU	primers for p27 5'UTR U-rich region deletion DNA amplification
5'-472-Luc-PolA 3'-472-Luc-PolA	5'-ggatcctaatacgaactactataggtcttctcgtcagcctccctt-3' 5'-ttttttttttttttttttttttttagtaagaatttcgtcatcg-3'	pTKLL472	primers for p27 5'UTR-Luciferase-poly A DNA amplification
1st PCR	5'-atcggtagctatggcagactattcaa-3' 5'-gctttttatggtttcaaccattccatctgg-3' 5'-ccagatggaatggttgaaccataaaaagc-3' 5'-gatctcgagttattggccctgaggtg-3'	pCDNA3.1-HisB-FBP pCDNA3.1-HisB-FBP	primers for internal deletion of middle domain of FBP
2nd PCR	5'-atcggtagctatggcagactattcaa-3' 5'-gatctcgagttattggccctgaggtg-3'	1st PCR product	
1st PCR	5'-cccaagcttcttcgtcagcctccctccac-3' 5'-cgggtctgcacgaccgccactctcaaaaaacaaa-3' 5'-ttttttttttgagagtggtcggtcgtgcagaccg-3' 5'-ctttctccggcggtggtcgtcgtcggg-3'	pTKLL472 pTKLL472	primers for internal deletion of S2 in p27 5'-UTR
2nd PCR	5'-cccaagcttcttcgtcagcctccctccac-3' 5'-ctttctccggcggtggtcgtcgtcggg-3'	1st PCR product	
1st PCR	5'-cccaagcttcttcgtcagcctccctccac-3' 5'-accgaacaaaacaaagcgcagcccggaaccctctcg-3' 5'-cgagagggttcgggctgcgttttttttgcgtg-3' 5'-ctttctccggcggtggtcgtcgtcggg-3'	pTKLL472 pTKLL472	primers for internal deletion of S1 in p27 5'-UTR
2nd PCR	5'-cccaagcttcttcgtcagcctccctccac-3' 5'-ctttctccggcggtggtcgtcgtcggg-3'	1st PCR product	