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p53 Acts as a Safeguard of Translational Control by Regulating Fibrillarin and rRNA Methylation in Cancer

Virginie Marcel,^{1,2,9} Sandra E. Ghayad,^{1,2,9} Stéphane Belin,^{1,2,9} Gabriel Therizols,^{1,2} Anne-Pierre Morel,^{1,2} Eduardo Solano-González,³ Julie A. Vendrell,^{1,2,4,5} Sabine Hacot,^{1,2} Hichem C. Mertani,^{1,2} Marie Alexandra Albaret,^{1,2} Jean-Christophe Bourdon,⁵ Lee Jordan,⁶ Alastair Thompson,⁵ Yasmine Tafer,^{1,2} Rong Cong,⁷ Philippe Bouvet,⁷ Jean-Christophe Saurin,^{1,2,8} Frédéric Catez,^{1,2} Anne-Catherine Prats,³ Alain Puisieux,^{1,2} and Jean-Jacques Diaz^{1,2,*}

¹Centre de Recherche en Cancérologie de Lyon UMR Inserm 1052 CNRS 5286, Centre Léon Bérard, F-69373, Lyon, France

²Université de Lyon, Université Lyon 1, ISPB, Lyon F-69622, France

³Université de Toulouse, UPS, TRADGENE, EA4554, Institut des Maladies Métaboliques et Cardiovasculaires, 1 Avenue Jean Poulhès, BP 84225, F-31432 Toulouse, France

⁴ISPB, Faculté de Pharmacie, Université Lyon 1, Lyon, France

⁵Dundee Cancer Centre, Clinical Research Centre, University of Dundee, Dundee DD1 9SY, UK

⁶Department of Pathology, Ninewells Hospital and Medical School, Dundee DD1 9SY, UK

⁷Laboratoire Joliot-Curie, Ecole Normale Supérieure de Lyon, Université de Lyon, CNRS USR 3010, SFR BioSciences UMS3444, Lyon 69364, France

⁸Gastroenterology Unit, Édouard Herriot Hospital, Hospices Civils de Lyon, 69002 Lyon, France

⁹These authors contributed equally to this work

*Correspondence: jean-jacques.diaz@lyon.unicancer.fr

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SUMMARY

Ribosomes are specialized entities that participate in regulation of gene expression through their rRNAs carrying ribozyme activity. Ribosome biogenesis is overactivated in p53-inactivated cancer cells, although involvement of p53 on ribosome quality is unknown. Here, we show that p53 represses expression of the rRNA methyl-transferase fibrillarin (*FBL*) by binding directly to *FBL*. High levels of *FBL* are accompanied by modifications of the rRNA methylation pattern, impairment of translational fidelity, and an increase of internal ribosome entry site (IRES)-dependent translation initiation of key cancer genes. *FBL* overexpression contributes to tumorigenesis and is associated with poor survival in patients with breast cancer. Thus, p53 acts as a safeguard of protein synthesis by regulating *FBL* and the subsequent quality and intrinsic activity of ribosomes.

INTRODUCTION

Recent findings support the emerging notion that the dysregulation of ribosome biogenesis in cancer cells is not just a consequence of oncogenesis, but represents a key step in this complex process (Barna et al., 2008; Bywater et al., 2012; Yoon et al., 2006). Ribosome biogenesis is a multistage process that involves transcriptional and posttranscriptional regulation and a stringent quality control to produce functional ribosomes

(Bashan and Yonath, 2008; Henras et al., 2008). Ribosome biogenesis is overactivated in cancer cells, notably by a loss of function of RNA polymerase I (Pol I) repressors such as p53 (Bywater et al., 2012; Zhai and Comai, 2000).

The posttranscriptional steps of ribosomal RNA (rRNA) processing determine the structure and function of the mature molecule (King et al., 2003; Baxter-Roshek et al., 2007; Puglisi, 2009). Indeed, rRNAs are ribozymes that support the decoding and proofreading steps and catalyze the formation of the peptide

Significance

Ribosome production is increased in cancer cells. This enhanced production of ribosomes plays a crucial role in tumor progression. Our results show that p53 regulates the pattern of ribosomal RNA posttranscriptional modification that impairs translational fidelity and increases the initiation of internal ribosome entry site (IRES)-dependent translation. By demonstrating that p53 regulates the biogenesis and intrinsic activity of ribosomes, our study demonstrates that p53 deficiency participates in the “translational reprogramming” in cancer cells and contributes to the uncontrolled expression of key oncogenic genes.

bond during translation (Cech, 2000; Demeshkina et al., 2012). rRNAs are subjected to intense and highly specific chemical modifications (methylations and pseudouridylations). The exact role of these modifications has not yet been fully elucidated, although it appears that they help optimize the complex ribosomal architecture required to produce an efficient ribosome (Baxter-Roshek et al., 2007).

Changes in the posttranscriptional modifications of rRNAs influence translational fidelity (i.e., nonsense suppression or amino acid misincorporation) and the mode of translation initiation (i.e., CAP versus internal ribosome entry site [IRES]) of key cancer genes (Ruggero, 2013; Basu et al., 2011; Baxter-Roshek et al., 2007). Moreover, a defect in rRNA methylation or pseudouridylation may cause specific clinical syndromes and is correlated with an increased incidence of cancer (Belin et al., 2009; Montanaro et al., 2008). This study aims to determine whether p53 controls rRNA methylation and subsequently affects translational regulation.

RESULTS

FBL Expression Is Inversely Associated with p53 Activity in Cell Lines and Human Breast Cancer Samples

Fibrillarin (FBL) is an indispensable, highly conserved protein essential in the processing of pre-rRNAs (Newton et al., 2003; Tollervey et al., 1993). In eukaryotes, it is the only known methyltransferase that performs the specific 2'-O-ribose-methylation directed by a large family of small *trans*-acting guide RNA (box C/D antisense snoRNAs). Because abnormal rRNA methylation could influence translational control and because p53 regulates rRNA transcription, we explored whether *FBL* expression was associated with p53 activity. We measured *FBL* mRNA and protein levels in different cell lines in which p53 expression and/or activity was modulated by different strategies. In immortalized human mammary epithelial cells (HME), p53 activity was impaired either by reducing its expression using an shRNA approach (HME-shp53) or by inactivation using an SV40 T/t antigen trapping strategy (HMLE; Elenbaas et al., 2001). In response to p53 knockdown or inactivation, there was a significant increase in *FBL* expression: 1.5-fold for the mRNA and 2-fold for the protein (Figures 1A–1D; Figures S1A and S1B available online). As expected, we observed a decrease in the expression of *CDKN1A*, which encodes p21, and *MDM2*, two p53 target genes, which validated the reduction in p53 activity in these cellular models (Figures 1B, 1D, S1A, and S1B). The inverse correlation between *FBL* and p53 expression was confirmed in a second series of immortalized HMEC lines (Figures S1C and S1D) and in an isogenic human HCT-116 colorectal cellular model: HCT-116-p53^{+/+} cells that express wild-type p53 protein and HCT-116-p53^{-/-} cells that lack p53 protein expression (Bunz et al., 1998). *FBL* mRNA and protein levels were increased in HCT-116-p53^{-/-} cells compared to those of HCT-116-p53^{+/+} cells (Figures 1E, 1F, and S1E), demonstrating that the increase in expression of *FBL* in response to p53 inactivation is not restricted to mammary cell lines.

To assess more directly the impact of p53 on *FBL* expression, HME cells were treated with a p53-specific siRNA instead of an shRNA to induce a transient knockdown of p53 expression (Figures 2A, 2B, S2A, and S2B). In this condition, increased *FBL*

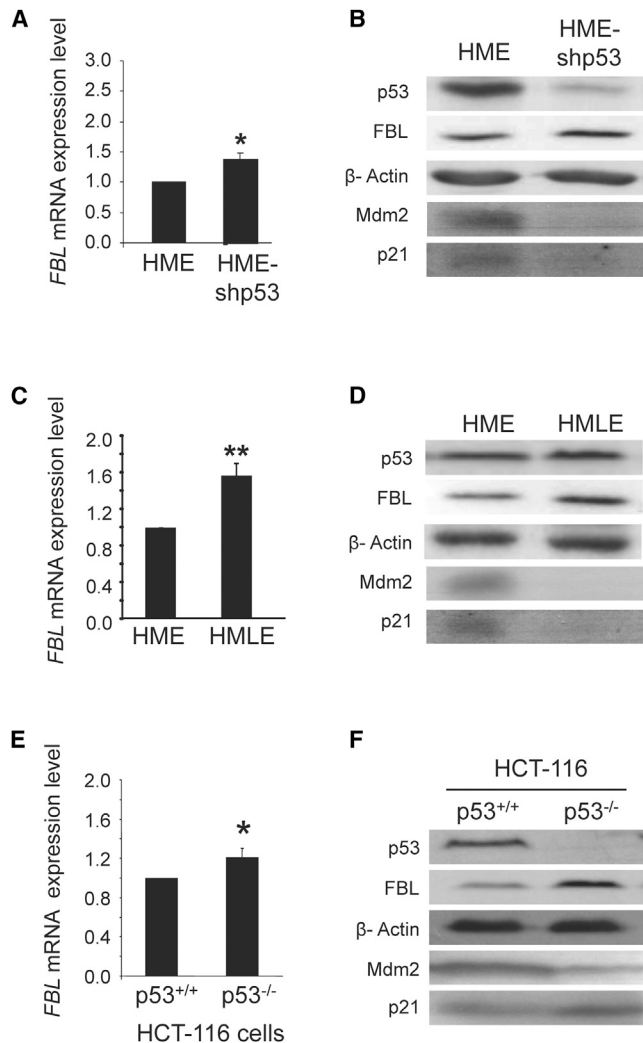


Figure 1. p53 Regulates *FBL* Expression at both the mRNA and Protein Levels

The expression of endogenous *FBL* in the indicated cell lines was analyzed at the mRNA level by RT-qPCR (A, C, and E) and at the protein level by western blot (B, D, and F). All graphs represent mean and SD of at least three experiments. * $p < 0.05$ and ** $p < 0.01$ according to Student's *t* test.

See also Figure S1.

expression at the mRNA and protein levels correlated with the inhibition of p53 expression (Figures 2A and 2B). Taken together, these results exclude the possibility that the changes in *FBL* expression levels resulted from an off-target effect and firmly link *FBL* expression to p53 expression. Additionally, to investigate the impact of p53 activation on *FBL* expression, we treated HME cells with the topoisomerase inhibitors doxorubicin and camptothecin, which are prominent activators of p53. The results showed that the expected p53 induction in response to treatment was accompanied by a decrease in *FBL* mRNA and protein levels (Figures 2C and 2D). Quantification of the western blots demonstrated a significant association between the increase of p53 protein levels and the decrease of *FBL* protein levels (Figures S2C–S2E).

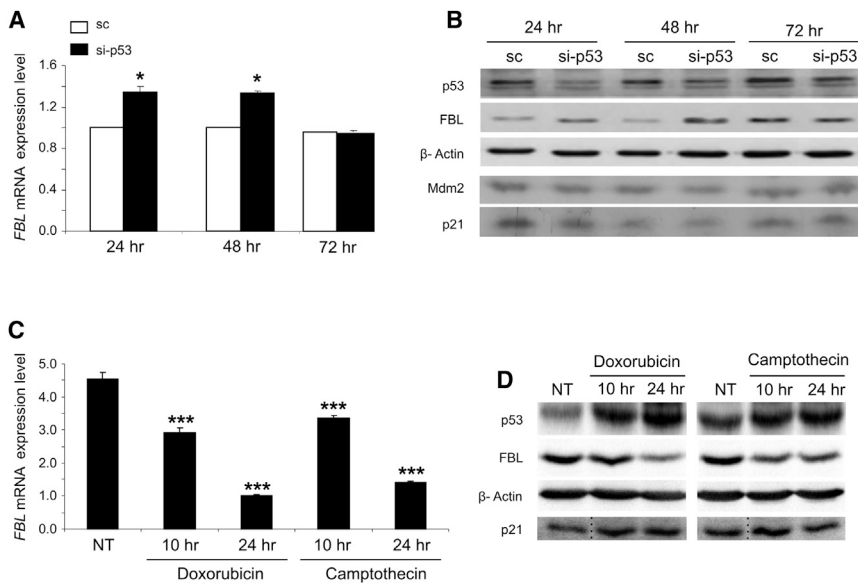


Figure 2. Modulation of p53 Expression Alters *FBL* Expression

Endogenous *FBL* expression was analyzed in HME cells at the mRNA level by RT-qPCR (A and C) and at the protein level by western blot (B and D). The p53 expression is modulated by using an siRNA (A and B) or by treating or not (NT) with 2 μ g/ml doxorubicin or 1 nM camptothecin (C and D). The p21 lanes in (D) were spliced together from discontinuous lanes of the same blot as indicated by dotted lines. All graphs represent mean and SD of at least three experiments. * $p < 0.05$ and *** $p < 0.001$ according to Student's t test. See also Figure S2.

detectable protein levels (Figures 4B and S4B). The coexpression of wild-type p53 significantly decreased the luciferase activity by 80%, while no significant variation in luciferase activity was observed after co-expression of any p53 mutant. Similar results were observed in HME-

shp53, the coexpression of wild-type, but not mutant, p53 protein reducing the luciferase activity (Figure 4C). These results suggest that p53 represses promoter activity through intron 1 of *FBL* in both breast and colon cellular models.

To determine whether p53 directly binds to *FBL* gene DNA, chromatin immunoprecipitation (ChIP) assays were performed in HME-derived cells (Figures 4D, 4E, S4C, and S4D). Compared to nontreated HME cells, camptothecin treatment increased p53 binding to both the *CDKN1A* promoter and the intron 1 of *FBL* at p53RE-1 and p53RE-2. In contrast, decrease in p53 expression in HME-shp53 cells was associated with a drastic reduction in p53 binding to both the *CDKN1A* promoter and intron 1 of *FBL* compared with nontreated HME cells (Figures 4D, 4E, S4C, and S4D). These data show that p53 binds the *FBL* intron 1 both in the basal condition and when p53 is activated. Altogether, these results demonstrate that *FBL* is a p53 target gene and that *FBL* expression is directly repressed by p53.

p53-Mediated Alteration of the rRNA Methylation Pattern

Because p53 directly represses *FBL* expression, we determined whether p53 inactivation alters rRNA methylation by using a previously described, site-specific semiquantitative RT-qPCR-based method (Belin et al., 2009; Maden, 1988). We analyzed the change of rRNA methylation at 18 sites distributed along the 5.8S, 18S, and 28S rRNAs that are known to be methylated. These sites include those localized within key functional domains of rRNAs, i.e., the decoding center (DC) in the 18S rRNA, and the peptidyl transferase center (PTC) and the helix 69 (H69) of 28S rRNA. In general, most of the sites were significantly more frequently methylated in HME-shp53 than in HME cells (Figures 5A and S5A). This overall increase in the site-specific rRNA methylation pattern is consistent with our finding that *FBL* expression level is higher in HME-shp53 than in HME cells, and suggests that methylation could be regulated in a site-by-site manner.

In human cancers, the *TP53* tumor suppressor gene is frequently inactivated, mainly by mutations (Olivier et al., 2006). To determine whether *FBL* expression is associated with p53 mutation status independently of other genetic variations in cell lines, we analyzed the *FBL* expression levels in a panel of three wild-type p53 breast cell lines and five mutant p53 breast cancer cell lines. High levels of *FBL* mRNA and protein were significantly associated with the expression of mutant p53 (Figures 3A, 3B, and S3). This result prompted us to investigate the clinical correlation between the p53 mutation status and *FBL* expression. We analyzed the *FBL* mRNA expression levels by RT-qPCR in relation to the p53 mutation status in a cohort of 80 randomly selected primary breast tumors (Table S1). Consistent with the results obtained in cell lines, *FBL* mRNA levels were significantly higher in mutant p53 tumors compared to wild-type p53 tumors (Figure 3C). We also performed a retrospective statistical analysis of the gene expression array data described by Miller and colleagues (Miller et al., 2005). *FBL* mRNA levels were significantly higher in mutant p53 tumors ($n = 58$) than in wild-type p53 tumors ($n = 193$; $p < 10^{-4}$, t test). Altogether, these results show a significant and reproducible inverse association between the p53 level and/or activity and the expression of *FBL* at both the mRNA and protein levels, suggesting that p53 can repress *FBL* expression in cellular models of breast and colon cancer as well as in human breast tumors.

p53 Represses *FBL* Expression by Directly Binding to DNA

Using the MatInspector software and the p53FamTag database, two putative p53 responsive elements (p53RE-1 and p53RE-2) were identified within the *FBL* intron 1, suggesting a direct transcriptional regulation of *FBL* expression by p53 (Cartharius et al., 2005; Sbisà et al., 2007; Figures 4A and S4A). Based on these predictions, we developed a luciferase reporter (p*FBL*-Luc) assay to assess whether p53 regulates *FBL* promoter activity. HCT-116-p53^{-/-} cells were cotransfected with p*FBL*-Luc and a plasmid expressing either wild-type or mutant p53 protein at

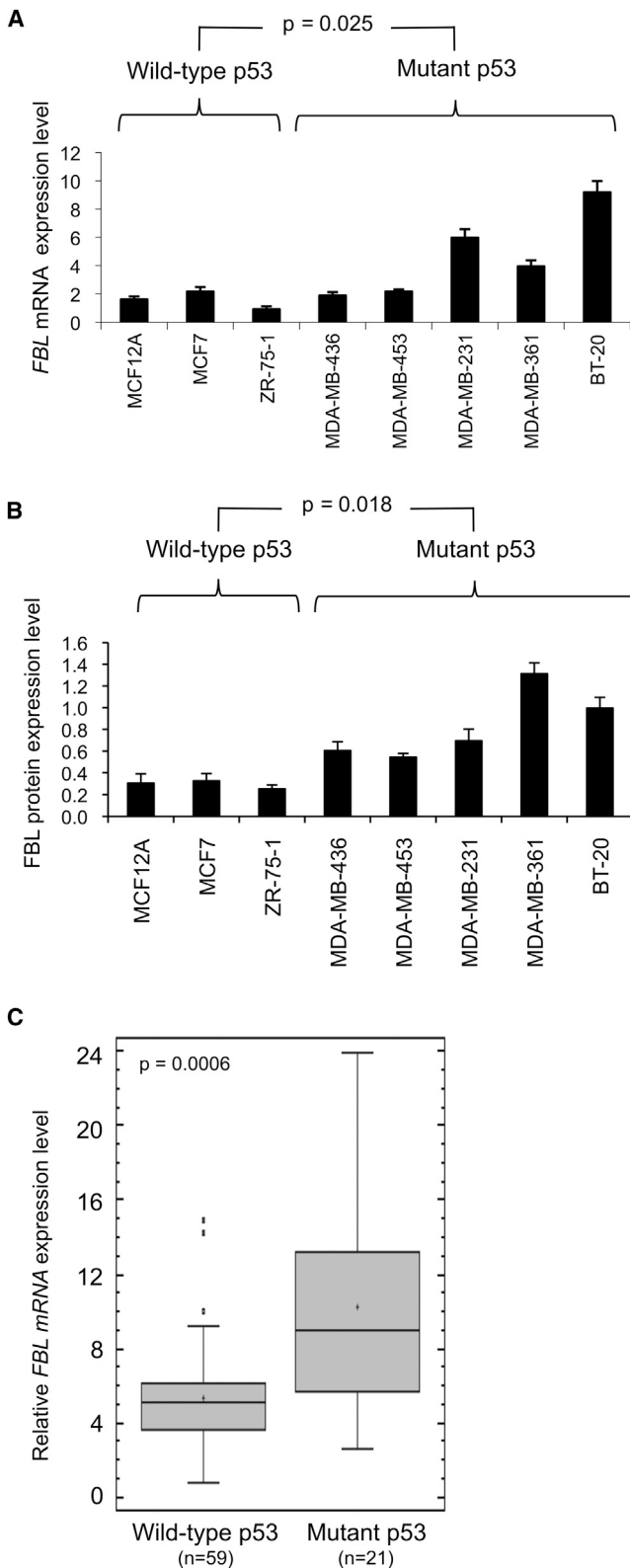


Figure 3. p53 Regulates *FBL* Expression in Human Breast Cell Lines and Tumors

(A) Quantification of *FBL* mRNA expression analyzed by RT-qPCR and normalized to *RNA18S*.

As expected, metabolic labeling with [5,6-³H]-uridine and L-[methyl-³H]-methionine showed that p53 inactivation led to a significant increase in rRNA synthesis (Figure S5B; Zhai and Comai, 2000), with a faint increase in the global rRNA methylation rate (Figure S5C). This showed that the amount and/or activity of the rRNA methylation machinery was sufficient to sustain the global rRNA methylation rate following the increase in rRNA synthesis after p53 inactivation. In addition, because selection of site methylation is ensured by guide C/D-box snoRNAs that complex with FBL, we verified whether snoRNA expression levels were altered in p53-inactivated cells. Northern blot analyses revealed a modification of snoRNA levels according to p53 levels, suggesting that p53 could also be involved in the regulation of some C/D-box snoRNA as is the case for some H/ACA snoRNP (Figures S5D–S5F; Krastev et al., 2011). However, no correlation was found between the amount of snoRNA and the level of the corresponding rRNA methylation sites (Figures 5A and S5A). These results show that inactivation of p53 resulted in a site-specific modification of the rRNA methylation pattern that correlates with *FBL* expression.

p53 Alters the Translational Fidelity by Modulating *FBL* Expression

The chemical modifications of rRNA that have been conserved throughout evolution in all species are involved, at least in eukaryotes, in the control of translational fidelity and in the control of translation initiation modalities (i.e., CAP-dependent versus IRES-dependent; Ruggero, 2013; Baxter-Roshek et al., 2007; Chaudhuri et al., 2007; Ruggero et al., 2003). We first analyzed two different examples of translational fidelity, nonsense suppression and amino acid misincorporation (Belin et al., 2009). The bypass of a premature stop-codon (Figures 5B–5D) and the misincorporation of amino acids (Figures 5E–5G) were both significantly increased after p53 inhibition or inactivation in different cell lines. This suggested that the translational alteration could be due to p53-mediated increase of *FBL* expression level. To investigate this possibility, we analyzed the misincorporation of amino acids in response to knockdown of *FBL* expression. As shown in Figure 5G, reduction of *FBL* expression in HCT-116-p53^{-/-} prevented the increase in amino acid misincorporation, demonstrating that the decrease in translational quality control in response to p53 inhibition is dependent on *FBL* overexpression. In contrast, the ability of ribosomes to induce a –1 frameshift from a severe acute respiratory syndrome-coronavirus (SARS-CoV)-1 programmed ribosome frameshift signal was similar in the three cell lines (Figures 5H–5J). These results suggest that p53 inactivation could decrease translational fidelity in an *FBL*-dependent manner.

(B) Quantification of *FBL* protein expression analyzed by western blot. All graphs represent mean and SD of at least three experiments.

(C) Box-and-whisker plots of *FBL* mRNA expression quantification in wild-type p53 (n = 59) versus mutant p53 (n = 21) primary breast tumor samples. The bottom and the top of the boxes represent the 25th and 75th percentiles, respectively. The median values are visible as a line, the mean as a cross in the box and SD as error bars. The p value has been determined by a Mann-Whitney W test.

See also Figure S3 and Table S1.

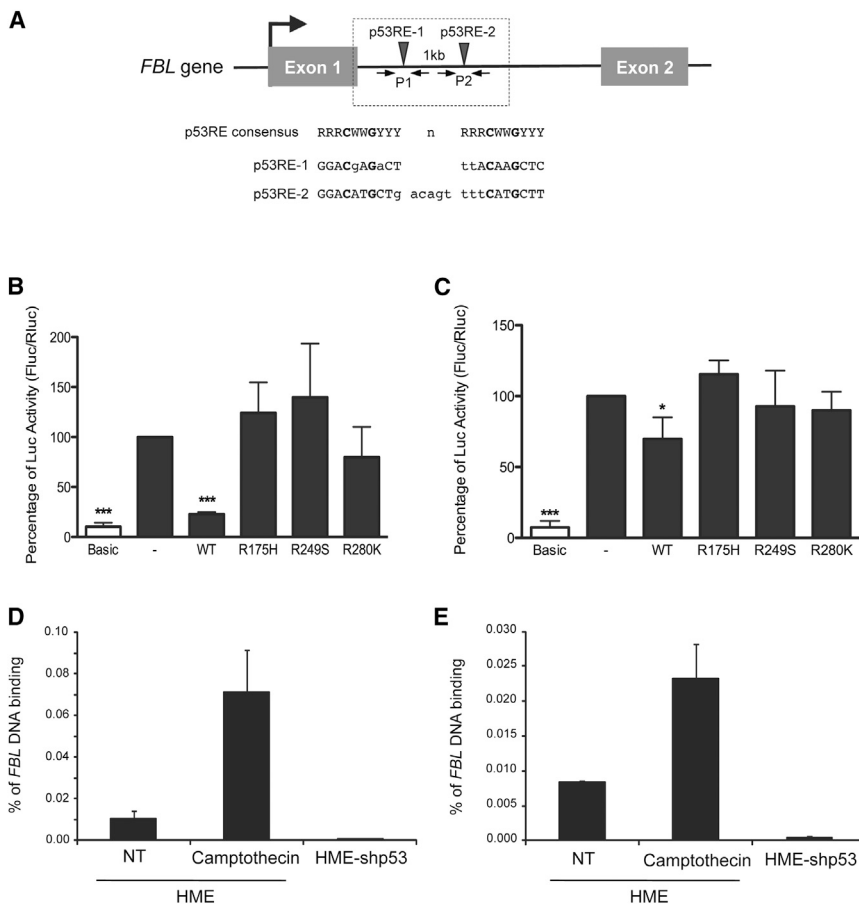


Figure 4. p53 Represses FBL Promoter Activity by Directly Binding to DNA

(A) Alignment of the two putative p53 response elements (p53RE-1 and p53RE-2, black arrows) located within the intron 1 of *FBL* with the p53RE consensus (R, G/A; W, A/T; Y, C/T). n, spacer within p53RE consensus; dotted box, nucleotide region of the *FBL* gene cloned in the p*FBL*-Luc reporter vector; P1 and P2, primers pairs used in ChIP assays.

(B and C) Luciferase reporter assays were performed in the absence of p53 (–) and in the presence of the wild-type (WT) or the indicated mutant p53 protein in HCT-116-p53^{-/-} (B) and in HME-shp53 cells (C). *Firefly* luciferase activity is normalized to the *renilla* luciferase activity. Basic, luciferase reporter vector with no *FBL* sequence. (D and E) ChIP using an anti-p53 antibody and primer pairs P1 (D) or P2 (E) were performed in nontreated (NT) or 1 nM camptothecin-treated HME cells, or in HME-shp53 cells. All graphs represent mean and SD of at least three experiments. *p < 0.05 and ***p < 0.001 according to Student's t test.

See also Figure S4.

p53 Increases IRES-Mediated Initiation of Translation by Modulating FBL Expression

To determine whether the CAP- or IRES-dependent mode of translation was modified, we used a bi-cistronic vector strategy that has been extensively used to identify a vast number of IRES-containing sequences (Belin et al., 2009; Komar and Hatzoglou, 2011). At this stage of the study, we focused our analysis on *IGF1R* due to its role in tumorigenesis and because *IGF1R* possesses the longest GC-rich 5'UTR that contains a well-identified IRES among all human transcripts yet characterized (Giraud et al., 2001; Pollak et al., 2004). Using a bi-cistronic luciferase assay, we observed a significant increase in the global luciferase activity in the p53-inactivated HME-shp53 and HCT-116-p53^{-/-} cells compared with cells expressing wild-type p53 (Figure 6A). This increase was due to a preference to initiate IRES-dependent translation. Indeed, the translation of the *firefly* luciferase driven by the IRES of *IGF1R* was significantly increased, while no significant variation was detected for the *renilla* luciferase driven by a CAP-dependent mechanism (Figures S6A and S6B). Similar results were obtained in other HME-derived cell lines (Figures 6B, white bars; Figures S6C and S6D, sc bars). Moreover, HME and HMLE cells expressed similar levels of *firefly* luciferase mRNA and *renilla* luciferase mRNA independently of variations in p53 protein levels, which supports the hypothesis of IRES-mediated translational regulation rather than transcriptional regulation of *IGF1R* expression (Figure S6E).

To evaluate whether a similar regulation exists in human cell lines, we explored the *IGF1R* IRES activity in breast cell lines that express wild-type or mutant p53 protein (Figure 6C). The activity level of the *IGF1R* IRES was significantly higher in the mutant p53 cells that expressed high levels of *FBL* compared with the wild-type p53 cells that expressed low levels of *FBL*. Thus, translation initiation mediated by the *IGF1R* IRES was higher in breast cell lines that had inactivated p53 instead of wild-type p53.

To confirm that the IRES activity was modulated by rRNA methylation, we analyzed the *IGF1R* IRES activity after using an *FBL*-siRNA knockdown approach that reduced the *FBL* protein level (Figures S6C, S6D, and S6F). As shown in Figure 6B, reduction of *FBL* expression significantly decreases the IRES-dependent translation initiation of *IGF1R* in HME, HME-shp53 and HMLE cells. This result demonstrates that the efficiency of IRES-dependent translational initiation is modulated by rRNA methylation through modulation of *FBL* expression.

To determine whether the p53-mediated translational control of the *IGF1R* mRNA observed with recombinant expression vectors also occurs with endogenous *IGF1R* mRNA, we compared the distribution of the *IGF1R* mRNA within polysomal fractions of HME and HME-shp53 cells. The polysomal fraction corresponds to mRNA bound to more than one ribosome and therefore contains actively translated mRNA. As shown in Figure 6D, a significant 12-fold polysomal enrichment in *IGF1R* mRNA was observed in HME-shp53 compared with HME cells. In addition, a significant increase in the IGF1R protein level was observed in p53-inactivated HMLE cells compared to HME cells independently of any variation in *IGF1R* mRNA levels (Figures 6E and

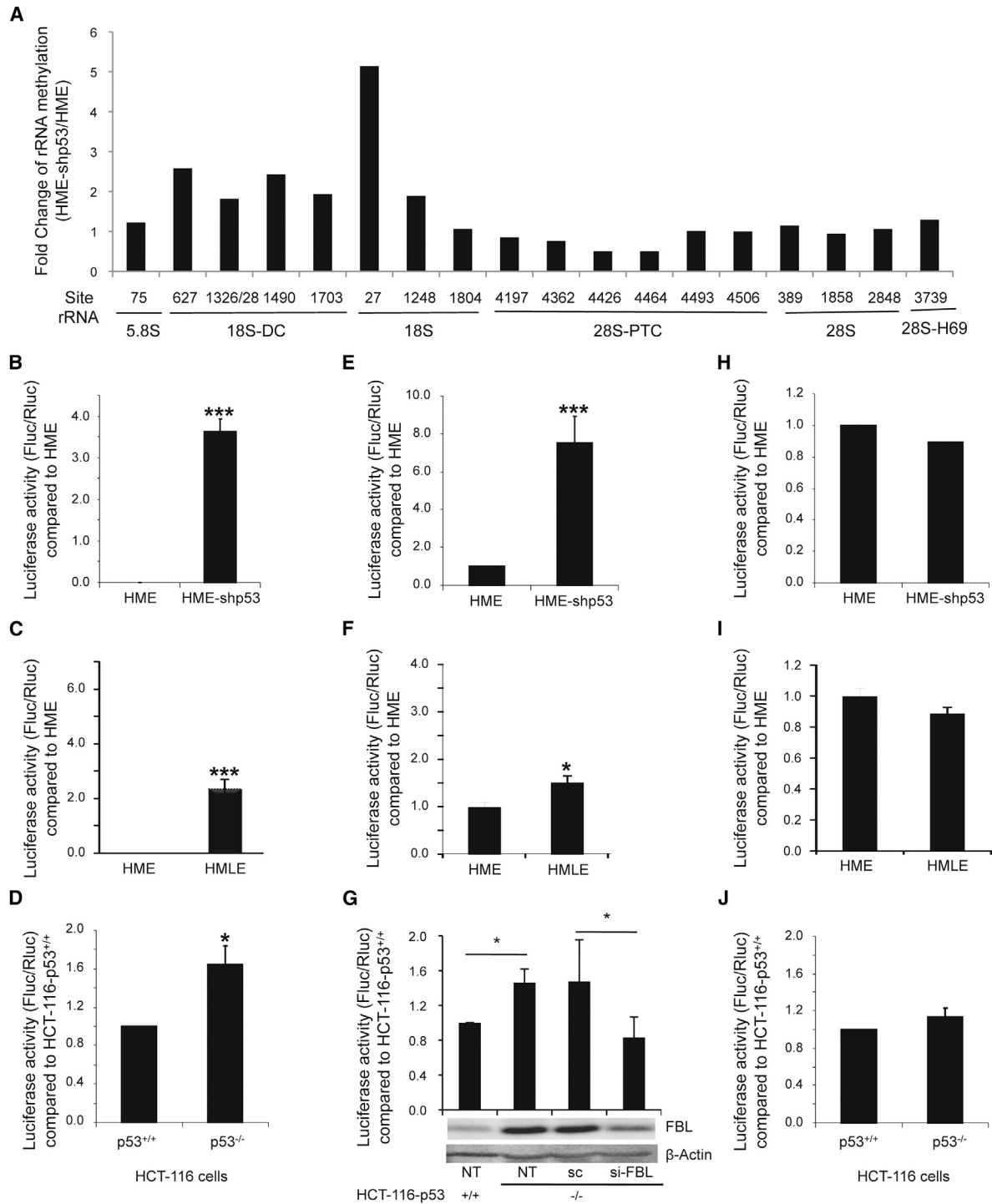
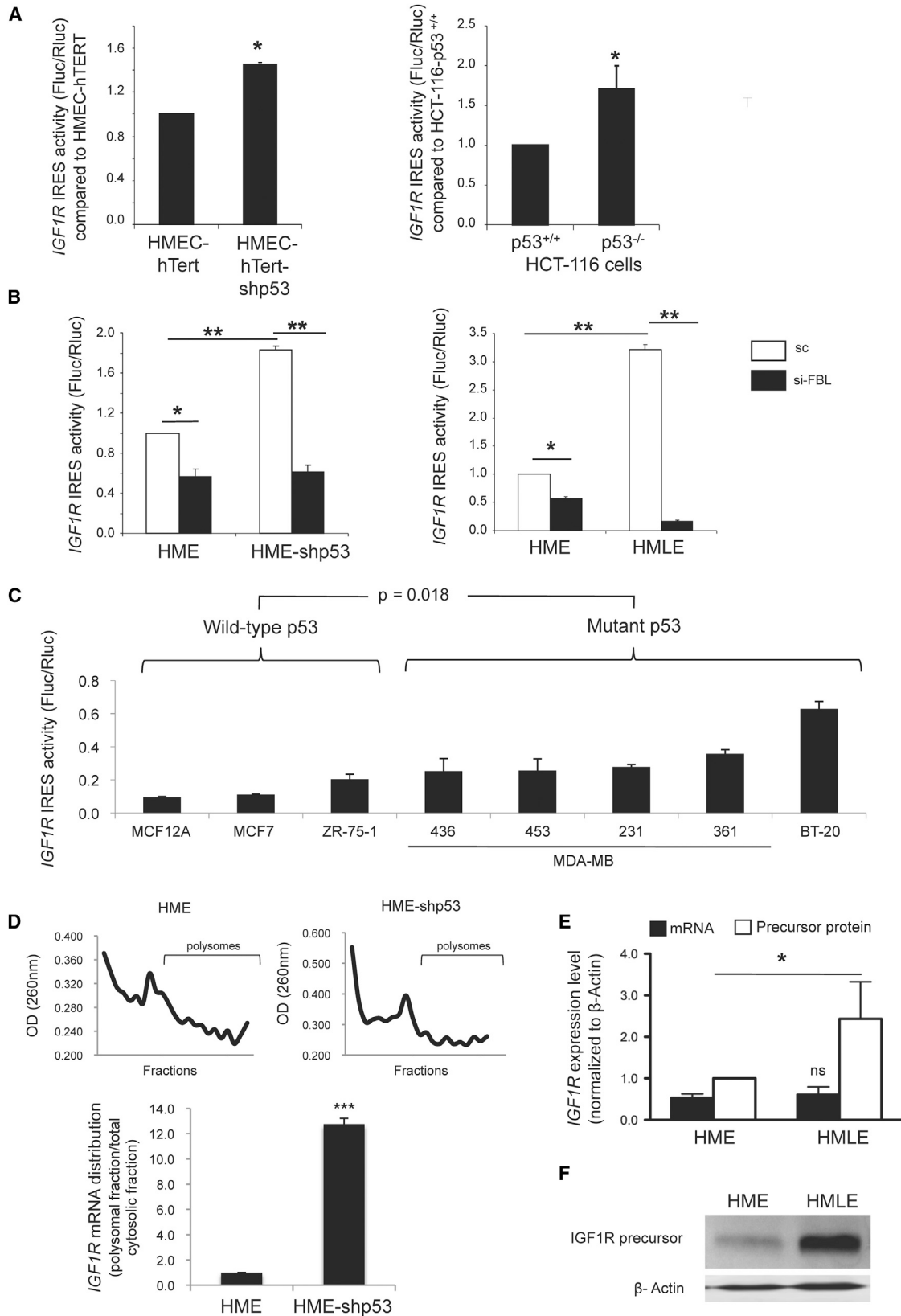


Figure 5. p53 Regulates the rRNA Methylation Pattern and the Translational Fidelity of Ribosomes

(A) The fold difference in rRNA methylation at 18 sites distributed throughout the 5.8S, 18S, and 28S rRNAs between HME-shp53 and HME cells were analyzed by RT-qPCR.

(B–J) Translational fidelity was analyzed by transfecting cells with the pGL3mut1 vector (premature stop mutant, B–D), the pGL3mut2 vector (amino acid substitution mutant, E–G), or the SARS-CoV-1 programmed ribosome frameshift vector (H–J) in the indicated cells. (G) Translational fidelity was analyzed in nontransfected cells (NT) and after transfection of siRNA control (sc) or siRNA targeting *FBL* (si-FBL). *FBL* expression levels were verified by western blot (G, lower panel). All graphs represent mean and SD of at least three experiments. * $p < 0.05$ and *** $p < 0.001$ according to Student's t test.

See also Figure S5.



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6F). These results demonstrate that p53 inactivation increases the translation of *IGF1R* mRNA.

To investigate whether this p53-mediated translational control is restricted to *IGF1R* mRNA, we analyzed translational control of several other known cellular and viral mRNAs containing IRESs, including *MYC*, *FGF1*, *FGF2*, and *VEGFA* that play key roles in oncogenesis (Dang, 2012; Turner and Grose, 2010; Carmeliet and Jain, 2011) and encephalomyocarditis virus. Bi-cistronic luciferase assays and mRNA polysomal profiling assays showed an increase of translational efficiency of these mRNA in p53 inactivated cells compared to p53 wild-type cells (Figures S6G–S6I). Altogether these results show that p53 inactivation impacts the translational control of several genes involved in tumorigenesis through deregulation of *FBL* expression.

FBL Overexpression Contributes to Tumorigenesis

Six independent stable clones derived from MCF7 cells overexpressing either a FBL-GFP protein or a GFP protein were developed (Figure S7A) to determine the effect of *FBL* overexpression on several characteristics of cancer cells. Cell proliferation of the stable clones was first monitored for 72 hr and the index of cell proliferation was calculated from the slope of the growth curve (Figure 7A). Cells overexpressing FBL-GFP exhibit a significant 2-fold increase in proliferation rate compared with GFP control cells. To determine whether the FBL-induced cell proliferation is mediated by *IGF1R*, whose translation is increased in *FBL*-overexpressing cells, proliferation of two clones was monitored in response to Osi-906, an IGF1R tyrosine kinase activity inhibitor (Figure 7B; Mulvihill et al., 2009). Inhibition of IGF1R pathway activity abolished the difference in proliferation rate between GFP-G3 and FBL-GFP-F2 clones, suggesting that IGF1R pathway activity is required in these cells, for FBL-induced cell proliferation.

Anchorage-independent cell proliferation was then investigated using soft agar assays. Compared to GFP control clones, those overexpressing FBL-GFP formed significantly more colonies (Figures 7C and 7D). Finally, the effect of *FBL* overexpression on cell growth in response to doxorubicin treatment was investigated (Figure 7E). A significantly higher concentration of doxorubicin is required to reach 50% of inhibitory effect in FBL-GFP cells compared to GFP control cells (mean half-maximal inhibitory concentration [IC₅₀]: 24.6 μ M versus 53.1 μ M, respectively). Altogether, these data show that *FBL* overexpression promotes cell proliferation in both an anchorage-dependent and -independent manner and protects the MCF7 breast cancer cells from doxorubicin. Moreover, the FBL-induced cell proliferation required IGF1R pathway, supporting the notion that *FBL* overexpression can directly contribute to tumorigenesis by altering translational control of key cancer genes.

High Levels of FBL Are an Independent Marker of a Poor Outcome in Breast Cancer

To investigate whether the level of *FBL* mRNA in tumors is associated with prognosis, we analyzed the relapse-free survival and the breast cancer-specific survival of patients with breast cancer in regard to *FBL* expression. High expression of *FBL* mRNA was significantly associated with a poor relapse-free survival rate and poor breast cancer-specific survival rate (Figures 7F and 7G). We also performed retrospective statistical analyses of published gene expression array data (Györfy et al., 2010; Sabatier et al., 2011; Weigelt et al., 2005). This investigation confirmed that the high levels of *FBL* mRNA are associated with poor breast cancer-specific survival and relapse-free survival rates (Figures S7B–S7F).

However, because the high levels of *FBL* expression are associated with p53 mutations and because p53 mutations are known to be associated with poor disease-free and overall survival rates (Olivier et al., 2006), a multivariate analysis was conducted to adjust for possible confounding variables. The analysis included the number of invaded lymph nodes, histological grade, estrogen and progesterone receptors status, ErbB2 status, p53 mutation status, and *FBL* mRNA levels. The best model associated with poor survival contained two independent markers: progesterone receptor-negative status and high *FBL* expression (Table S2). These analyses showed that *FBL* expression is associated with poor survival independent of other commonly used clinical markers.

DISCUSSION

It is now clearly established that ribosome synthesis is increased in cancers due to the overexpression of oncogenes or the inactivation of tumor suppressor genes leading to a sustained increase in RNA Pol I activity (Bywater et al., 2012). Moreover, studies performed in different animal and cellular models of various eukaryotic organisms have shown that heterogeneity in ribosome composition, due to regulated posttranscriptional modifications of ribosomal proteins and rRNA, is likely to be the more common mechanism (Xue and Barna, 2012). Xue and Barna have made detailed contributions to extend the concept of “specialized ribosomes” to eukaryotes and highlighted the adaptive capabilities of the ribosomes in the control of cell fate through selective protein synthesis. Moreover, it is now well demonstrated that within the ribosomes, the rRNAs catalyze and control protein synthesis through their ribozyme activity that could be finely optimized by their rRNAs methylations and pseudouridylations (Baxter-Roshek et al., 2007; Belin et al., 2009). In this study, we show a p53-mediated alteration of ribosome biogenesis and translational control of cancer cells that

Figure 6. p53 Regulates the IGF1R IRES-Dependent Translation

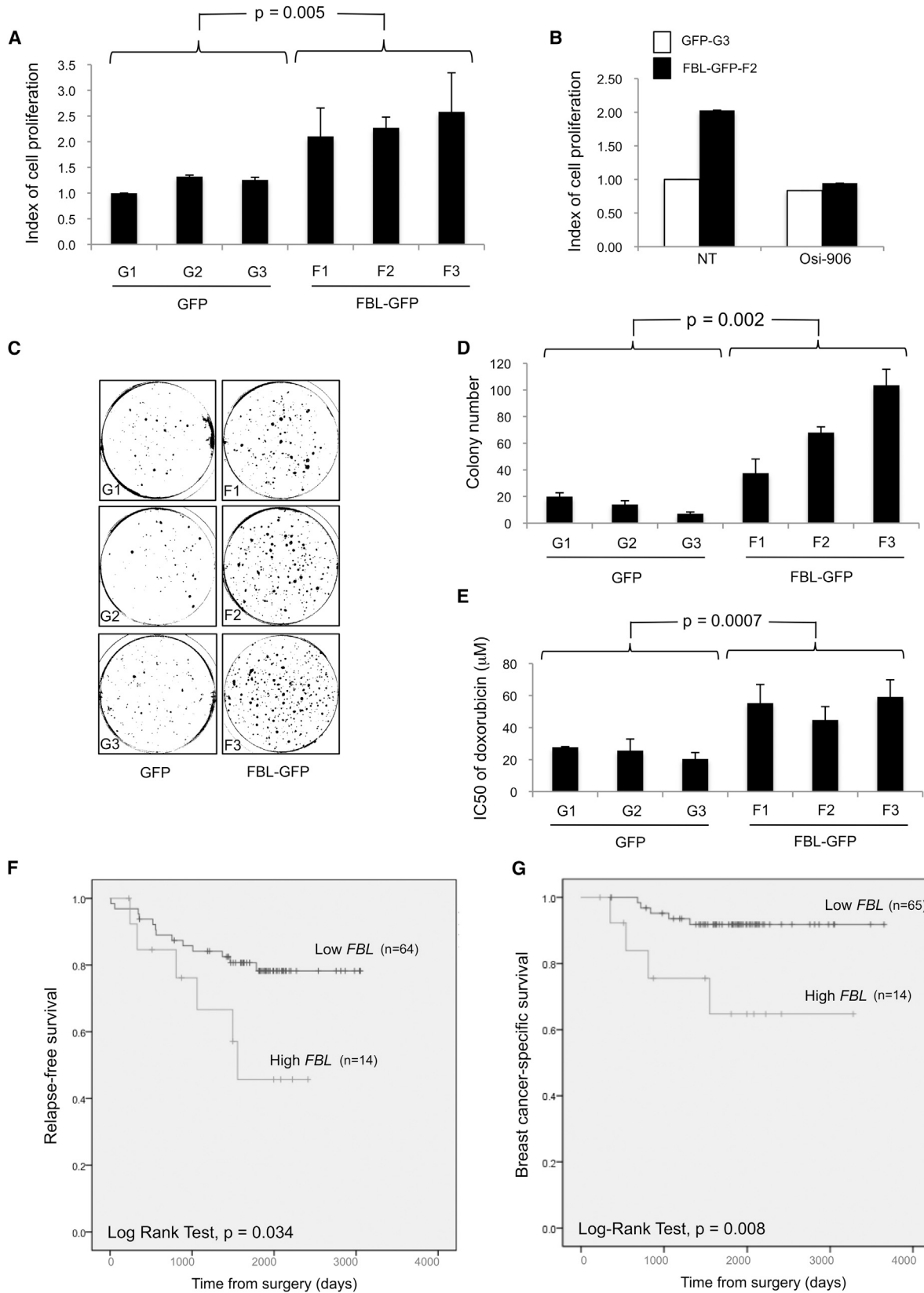
(A and B) The *IGF1R* IRES-dependent translation initiation was determined by using luciferase bi-cistronic vectors in the indicated cells (A) and in cells after the downregulation of *FBL* by siRNA approach (B).

(C) Analysis of the *IGF1R* IRES-dependent translation initiation in a panel of breast cell lines expressing either wild-type or mutant p53 proteins.

(D) Typical polysomal profiles after fractionation of postmitochondrial supernatants from HME and HME-shp53 cells in a 10%–40% sucrose gradient are shown (upper). The distribution of the *IGF1R* mRNA within polysomes was determined by RT-qPCR (lower).

(E and F) Endogenous *IGF1R* expression at mRNA (E, black bars) and protein levels (E, white bars; and F) was analyzed in HME and HMLE cells. All graphs represent mean and SD of at least three experiments. *p < 0.05, **p < 0.01 and ***p < 0.001 according to Student's t test.

See also Figure S6.



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could contribute in gene expression dysregulation and cancer development (Ruggero, 2013).

We demonstrate a role of p53 in the control of rRNA methylation patterning by directly regulating *FBL* expression levels that leads to the synthesis of modified “cancer ribosomes.” The notion that *FBL* is a p53-target gene is supported by genome-wide analyses, such as ChIP-seq assays showing that p53 binds the *FBL* gene (ENCODE database, Nikulenkov et al., 2012) and by transcriptomic analysis showing an inverse expression of *FBL* in response to p53 inactivation by siRNA or activation by doxorubicin in several cell lines (Troester et al., 2006). p53 response elements have also been identified in the first intron of approximately 25% of p53 target genes, some of them being associated with gene repression, including genes involved in ribosome biogenesis such as *NOLC1*, a snoRNP chaperone gene (Menendez et al., 2009; Krastev et al., 2011).

We found that the methylation pattern of rRNAs varies between sites, which is consistent with published data (Baxter-Roshek et al., 2007; Belin et al., 2009; Basu et al., 2011) and suggests that the rRNA modification pattern is modulated in a site-by-site manner. Our data showed that p53 inactivation is sufficient to alter rRNA methylation patterning. In addition to regulating *FBL* expression, the role of p53 in optimizing the rRNA functional quality is reinforced by our observations that the level of some C/D-box snoRNAs is modulated according to the p53 status and that p53 is involved in the assembly process of the other major family of snoRNP (H/ACA box; Krastev et al., 2011). Deciphering the mechanisms by which p53 inactivation alters the site-specific rRNA methylation pattern through *FBL* induction will require biochemical and structural studies dedicated to the understanding of formation, dynamics, and activities of the rRNA methylation complex. However, we can hypothesize that the improper induction of *FBL* expression observed in cancer cells leads to an alteration of the coordination between pre-rRNA production and the rRNA methylation enzymatic machineries.

Today, several pieces of data, including ours, indicate that modulation of methylation at only some rRNA sites is sufficient to affect the translational regulation process and that it could alter cellular behavior without inducing a lethal phenotype (King et al., 2003; Baudin-Baillieu et al., 2009; Higa-Nakamine et al., 2012). Indeed, the depletion of methylation of several rRNA methylation sites in yeast and human cells has been associated with a decrease in translation fidelity (such as an increase in nonsense suppression, frameshifts, and amino acid misincorporation; Baxter-Roshek et al., 2007; Baudin-Baillieu et al., 2009). Moreover, modulating rRNA methylation by RPL13a

depletion in HeLa cells modified the control of the translation initiation by IRES (Basu et al., 2011). Modification in the rRNA methylation pattern in breast cancer cells exhibiting an induced aggressive phenotype was also associated with the alteration of IRES activity of key factors such as vascular endothelial growth factor (VEGF) and p53 (Belin et al., 2009). In the present study, we demonstrate that the repression of *FBL* expression by p53 is accompanied by an increase of IRES-dependent translation initiation, affecting cellular as well as a viral IRES-containing mRNAs. These data are consistent with previous reports showing that the *FGF2* mRNA translation is inhibited by p53, whereas the *FGF2* mRNA IRES is aberrantly activated in transformed cells when p53 is inactivated (Galy et al., 2001). It remains to systematically explore the effect of methylation sites, individually and as a pattern, in the intrinsic activity of the ribosome.

Modulation of intrinsic activity of the ribosome by altering rRNA methylation may involve structural changes of ribosomes. The inhibition of rRNA methylation altered the IRES translation initiation by impairing the association of the 40S and 60S subunits (Basu et al., 2011). Moreover, structural and biochemical studies showed that ribose methylation modifies the conformational state of the RNA backbone, stabilizes the RNA loops, and influences the overall structure of the modified RNA regions. Ribose methylation helps maintain the tertiary structure of rRNAs and potentially the rRNA-mRNA, rRNA-tRNA or rRNA-protein interactions (Blanchard and Puglisi, 2001; Liang et al., 2009). Consistently, several 18S rRNA regions promote structural modifications when a viral IRES is bound to the 40S subunit (Spahn et al., 2004) and the efficient translation of *IGF1R* mRNA results from its IRES directly contacting an 18S rRNA domain through a Shine-Dalgarno-like interaction (Meng et al., 2010). These data support the notion that rRNA methylation could participate in translational control by regulating IRES translation initiation.

Our clinical analysis shows that a high level of *FBL* in primary breast tumors is associated with poor survival independent of other biological markers. Elevated expression levels of *FBL* were previously reported in primary and metastatic prostate cancers compared with normal prostate epithelium and in squamous cell cervical carcinoma compared with normal cervix samples (Choi et al., 2007; Koh et al., 2011; Su et al., 2013). Furthermore, we have shown the direct contribution of *FBL* overexpression in tumorigenesis. The maintenance and progression of cancer phenotype induced by *FBL*-mediated enhanced translation may involve several key cancer proteins whose synthesis is dependent upon IRES-containing mRNA. As shown here,

Figure 7. Contribution of *FBL* Overexpression to Cancer Phenotype

(A) Cell proliferation of three each independent stable MCF7 clones expressing *FBL*-GFP (F1, F2, and F3) or GFP (G1, G2, and G3).

(B) Proliferation of the indicated cell clones not treated (NT) or treated with 1 μ M Osi-906 for 72 hr.

(C and D) Anchorage-independent growth of MCF7 clones using soft agar assay. Representative images are shown in (C) and the numbers of colonies determined in three experiments are shown in (D).

(E) Impact of *FBL* overexpression on drug response was investigated by determining the IC_{50} of doxorubicin using MTS assays. Error bars represent SD. The p values have been determined by a Mann-Whitney W test.

(F and G) Kaplan-Meier analysis of relapse-free survival rates (event = relapse) (F) and of breast cancer-specific survival rates (event = death related to breast cancer disease) (G) according to *FBL* mRNA level in primary breast tumor samples. The data are dichotomized at the upper quartile value into high and low expression groups.

See also Figure S7 and Table S2.

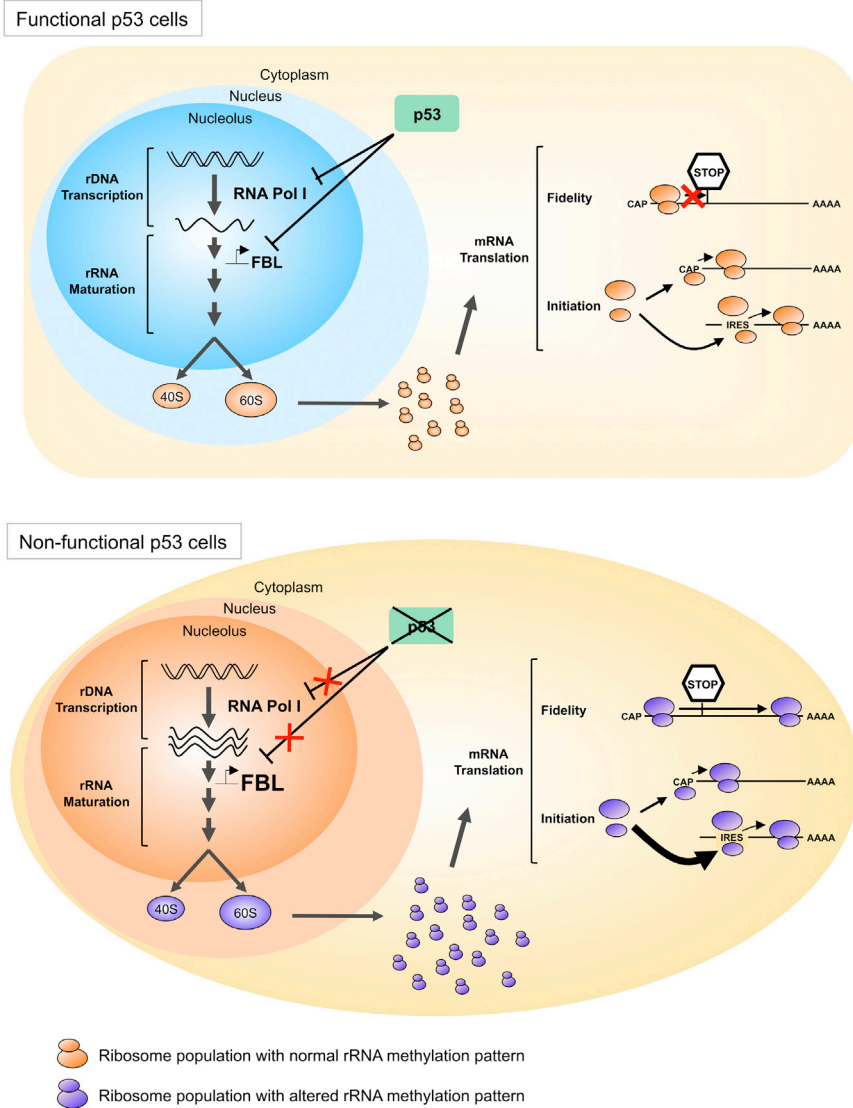


Figure 8. Model of the Implication of p53 in the Control of Ribosomes Quantity and Ribosomes Quality, and Their Consequences on Translation

In cells expressing functional p53 (top), p53 negatively regulates RNA Pol I activity to control ribosome quantity and *FBL* expression levels to control ribosome quality. This regulation would aim to coordinate the methylation of ribosomes and the rate of ribosome production according to the cell needs. These quality-controlled ribosomes allow a high translational fidelity together with a correct control of the balance between CAP- and IRES-dependent initiation of translation. In cells expressing a mutant or nonfunctional p53 (bottom), loss of the repression of RNA Pol I activity leads to an increase in rRNA synthesis. In parallel, p53 inactivation leads to an increase in *FBL* expression levels, resulting in a modification of the rRNA methylation patterns. Ribosomes with modified rRNA methylated translate mRNA with a lower fidelity (bypass of stop codon, amino acid misincorporation) and are more likely to initiate translation through IRES of mRNA coding for pro-oncogenic, anti-apoptotic, and survival proteins.

growing class of translationally regulated cancer-promoting genes (Ruggero, 2013).

Finally, the detailed description of rRNA chemical modification patterning in cancer cells, occurring in part through the p53-mediated regulation of ribosome biogenesis enzymatic machineries, and the increasing knowledge of the ribosome structure at the atomic level (Anger et al., 2013), opens up the possibility to target these “cancer ribosomes” to develop anticancer molecules using strategies similar to those used for

these proteins may include IGF1R, which is involved in tumor progression, cell survival, and response to chemotherapy (Pollak et al., 2004), c-Myc, which exhibits pleiotropic pro-oncogenic functions (Dang, 2012), FGF1/2, which are involved in epithelial-mesenchymal transition (Sakuma et al., 2012), and VEGFA, which is involved in tumoral angiogenesis (Carmeliet and Jain, 2011). Thus, high levels of *FBL* observed in human samples could have a role in tumor progression and could affect the clinical outcome of patients through alteration of translational regulation.

Our results allow us to propose a model in which p53 regulates not only the ribosome production rate, but also their structure, function, and intrinsic activity (Figure 8). In this model, p53 alteration in pathological cells results in the production of ribosomes with decreased translational fidelity and increased translation of the IRES-containing mRNAs selectively. Thus, the p53-mediated ribosome alterations could be in part responsible for the “translational instability” of cancer cells and contribute to the expression of the continuously

development of antibiotics specifically targeting prokaryotic ribosomes (Yonath, 2009).

EXPERIMENTAL PROCEDURES

Cell Culture, Transfection, and Luciferase Assay

Cells were maintained in culture following ATCC recommendations. siRNA and plasmids (Belin et al., 2009) were transfected using lipofectamine 2000 (Invitrogen). Cells were treated with 2 μ g/ml doxorubicin or 1 nM camptothecin (Sigma). Luciferase activity was measured using the Dual Luciferase Reporter Assay kit (Promega). Anchorage-dependent cell proliferation was analyzed using a real-time monitoring cell proliferation assay based on variation of electric impedance using the xCELLigence RTCA system (ACEA Biosciences) for 72 hr in nontreated cells or in presence of 1 μ M Osi-906 (Selleckchen). Anchorage-independent cell proliferation was analyzed by soft agar assays. The IC₅₀ values for doxorubicin were determined by MTS assays (Promega).

Western Blot and Chromatin Immunoprecipitation

Protein extraction and western blot were performed as described (Belin et al., 2010) using the following antibodies: anti-FBL (38F3, Abcam); anti-p53 (DO-1, Santa Cruz); anti- β -actin (AC-15, Sigma); anti-Mdm2 (4B2, Bethyl

Laboratories); anti-p21 (F-5, Santa Cruz); and anti-IGF1R α (N20, Santa Cruz). For ChIP assays, chromatin was prepared from 1% formaldehyde fixed cells by sonication. Immunoprecipitation was performed on 200–1000 bp DNA fragments using the DO-1 anti-p53 antibody and immunoprecipitated DNA was quantified by qPCR using Sybr Green technology.

Total mRNA, Polysomal mRNA, and rRNA Methylation Quantification

Total RNA and RNA issued from cytosolic and polysomal fractions was extracted and purified using either Trizol reagent (Invitrogen) or TriPure Isolation reagent (Roche). Cytosolic ribosomes were obtained from postmitochondrial fractions, and polysomal ribosomes by separation of postmitochondrial fractions on a 10%–40% sucrose gradient by ultracentrifugation. Total, cytosolic, and polysomal mRNA levels were quantified by RT-qPCR using M-MLV and Sybr Green technologies as described (Ghayad et al., 2009). Site-specific rRNA methylation was quantified using a RT-qPCR based method, which relies on the inhibition of reverse transcription reaction by ribose methylation at low dNTP concentration and on the detection of total rRNA as an internal reference, by reverse transcription at high dNTP concentration (Belin et al., 2009). RT products were then quantified by qPCR.

Breast Tumor Samples

FBL mRNA expression was analyzed in a cohort of 80 primary breast tumors collected at Ninewells Hospital from white women who received no neoadjuvant treatment prior surgery (Tayside Tissue Bank, Dundee; Table S1). Informed consent was obtained from all patients and ethical approval was received from the Tayside Tissue Bank (REC Reference 07/S1402/90) under delegated authority from the Local Research Ethics Committee. Relapse-free survival was calculated among breast cancer patients from the date of diagnosis to the date of relapse (event = relapse). Breast cancer-specific survival was calculated from the date of diagnosis to the date of breast cancer specific death (event = death related to breast cancer disease). The characterization of the classical molecular markers (histological grade, invaded lymph node, p53 mutation as well as estrogen, progesterone, and *errB2* status) was previously determined and reported (Bourdon et al., 2011).

Statistical Analyses

Statistical analyses were performed using the Statgraphics 3 plus software (Statgraphics Centurion). The log-rank test (univariate analysis), Kaplan-Meier plots, and Cox proportional hazards model (multivariate analysis) were performed using SPSS Software. A *p* value < 0.05 was considered to be statistically significant. All graphs present the mean and standard variations of at least three independent experiments and Student's *t* test has been performed for experimental data.

Experimental procedures are detailed in the [Supplemental Experimental Procedures](#).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and two tables and can be found with this article online at <http://dx.doi.org/10.1016/j.ccr.2013.08.013>.

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