

The Proto-Oncogene PBF Binds p53 and Is Associated With Prognostic Features in Colorectal Cancer

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The PTTG1-binding factor (PBF) is a transforming gene capable of eliciting tumor formation in xenograft models. However, the precise role of PBF in tumorigenesis and its prognostic value as a cancer biomarker remain largely uncharacterised, particularly in malignancies outside the thyroid. Here, we provide the first evidence that PBF represents a promising prognostic marker in colorectal cancer. Examination of a total of 39 patients demonstrated higher PBF expression at both the mRNA ($P = 0.009$) and protein ($P < 0.0001$) level in colorectal tumors compared to matched normal tissue. Critically, PBF was most abundant in colorectal tumors associated with Extramural Vascular Invasion (EMVI), increased genetic instability (GI) and somatic *TP53* mutations, all features linked with recurrence and poorer patient survival. We further demonstrate by glutathione-S-transferase (GST) pull-down and coimmunoprecipitation that PBF binds to the tumor suppressor protein p53, as well as to p53 mutants ($\Delta 126-132$, M133K, V197E, G245D, I255F and R273C) identified in the colorectal tumors. Importantly, overexpression of PBF in colorectal HCT116 cells interfered with the transcriptional activity of p53-responsive genes such as *mdm2*, *p21* and *sfn*. Diminished p53 stability ($> 90\%$; $P < 0.01$) was also evident with a concurrent increase in ubiquitinated p53. Human colorectal tumors with wild-type *TP53* and high PBF expression also had low p53 protein levels ($P < 0.05$), further emphasizing a putative interaction between these genes in vivo. Overall, these results demonstrate an emerging role for PBF in colorectal tumorigenesis through regulating p53 activity, with implications for PBF as a prognostic indicator for invasive tumors.

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INTRODUCTION

Colorectal cancer is the second leading cause of cancer-related deaths in developed countries [1]. Ten-year survival rates for patients with colorectal cancer have increased to ~50% in the past 30 yr. However, a better understanding of the underlying molecular mechanisms will be needed to identify suitable therapeutic targets to improve long-term survival. Genetic alterations that initiate and promote colorectal tumorigenesis have been well-defined and include mutations in the adenomatous polyposis coli (*APC*) gene, the β -catenin gene *CTNNB1* and *BRAF* [2,3]. The precise role of proteins that have been proposed to affect later stages of colorectal tumorigenesis such as p53, Smad4, DCC and K-Ras [4] still need further investigation to facilitate their usefulness as potential therapeutic targets.

Among these, inactivation of the tumor suppressor protein p53 has been identified as a critical event in the pathogenesis of most cancers, with well-established roles in cell-cycle regulation, apoptosis and senescence [5]. New roles for p53 in intestinal

tumorigenesis have recently been identified in murine models, including those involved in invasiveness control [6,7], creation of an inflammatory microenvironment [8] and the induction of epithelial-mesenchymal transition (EMT) [8]. In addition, it

Abbreviations: EMT, epithelial-mesenchymal transition; PTTG1, pituitary tumor-transforming gene 1; PBF, PTTG1-binding factor; siRNA, small interfering RNA; HA, hemagglutinin; GST, glutathione-S-transferase; GI, genetic instability; EMVI, extramural vascular invasion.

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has emerged that p53-regulated transcription of miRNAs such as the miR-34 family may be involved in controlling migration [9], invasion [9] and EMT induction [10,11] of colorectal cancer cells. These studies emphasize the importance of identifying how p53 activity is modulated in colorectal cancer to help define the pathways integral to tumorigenesis.

Typically p53 inactivation is considered a late event involved in the transition of benign tumors to invasive colorectal cancer [4,12]. It has been well-characterised that inactivating mutations of the *TP53* gene can occur in up to 50% of colorectal tumors, and their frequency increases with advancing tumor stage [13]. In contrast, far less is known about how p53 activity is regulated by oncogenes that are commonly overexpressed in colorectal cancer. A well-described example is the human securin pituitary tumor-transforming gene 1 (PTTG1) which is a marker for invasive colorectal carcinoma [14] and was identified as a key signature gene associated with tumor metastasis [15]. The functional interaction between PTTG1 and p53 has been demonstrated in transformed cells [16,17]. However, it is unclear whether proteins that physically interact with PTTG1, such as the PTTG1-binding factor (PBF) [18], Ku70 [19], ribosomal protein S10 [20] and DNAJA1 [20], can modulate the effects of PTTG1 on p53 activity.

Also known as PTTG1-interacting protein (PTTG1IP), PBF was first identified through its ability to bind and facilitate nuclear translocation of PTTG1 [18]. In subsequent studies we have described PBF overexpression in thyroid [21], pituitary [22] and breast cancers [23]. Functional studies highlighted that PBF was a transforming gene in vitro and induced subcutaneous high-grade malignant tumor formation in athymic nude mice [21]. PBF overexpression in breast MCF-7 cells led to increased cell invasion, which could be abrogated both by small interfering RNA (siRNA) treatment and specific mutation [23]. We further demonstrated that thyroid-targeted expression of PBF in transgenic mice resulted in the induction of hyperplastic growth and macrofollicular thyroid lesions [24]. Collectively, these observations indicated that PBF has particular relevance to endocrine and breast tumorigenesis, but its precise role in neoplasia was not established.

In this study we show for the first time that PBF is overexpressed in the non-endocrine setting of human colorectal tumors, and particularly in invasive tumors. Furthermore, we present evidence suggesting that PBF binds and acts as a novel regulator of p53 in colorectal cancers, an action that appears to be independent of PTTG1. This study therefore identifies a new pathway of p53 inactivation in tumorigenesis, and thus provides insights into understanding the pathogenesis of colorectal cancer.

MATERIALS AND METHODS

Human Tissue Samples

Matched tumor and normal tissue specimens were obtained from 39 patients undergoing surgery for colorectal cancer at the University Hospital Birmingham NHS Trust, UK. Upon gross clinical inspection normal samples of non-tumorous appearance were taken with a fresh scalpel blade at least 10 cm from the tumor (proximal or distal depending on the resection). Tumor tissue was then sampled using a fresh scalpel blade to limit contamination of colorectal samples. All specimens harvested at the time of resection were collected with appropriate local ethical committee approval and informed patient consent.

Cell Culture

Human colorectal cancer HCT116 cells were obtained from the Health Protection Agency Culture Collections, UK and routinely cultured in McCoy's 5 A (Life Technologies) supplemented with 10% fetal bovine serum (Invitrogen), penicillin (105 U/L) and streptomycin (100 mg/L) [Invitrogen]. H1299 and MCF-7 cells were routinely cultured in RPMI 1640 (Life Technologies) supplemented with 10% fetal bovine serum (Invitrogen), penicillin (105 U/L) and streptomycin (100 mg/L). All cell lines were kept at low passage number. HCT116 [25] and MCF-7 cells express WT p53, whereas H1299 cells are p53-null.

Nucleic Acids and Transfections

The QuikChange II XL site-directed mutagenesis kit (Agilent Technologies) was used to delete amino acids 149–180 (M1) and 94–149 (M2) of PBF, as well as to generate six p53 mutants [Δ 126–132 (M1), M133 K (M2), V197E (M3), G245D (M4), I255F (M5) and R273C (M6)]. WT p53 and mutants were cloned into pcDNA3 for mammalian cell expression experiments. WT PBF, M1 and M2 were cloned into pGEX4T-1 for bacterial expression. The PBF and mutant vectors for expression in mammalian cells [26], the p53 reporter plasmid phdm2-Luc [27] and the haemagglutinin (HA)-tagged ubiquitin (Ubq-HA) construct [28] have been described previously.

Small interfering RNA (siRNA) and plasmid DNA transfections were performed with Lipofectamine-2000 (Invitrogen), Fugene (Roche) and Transit LT1 (Mirus Bio LLC) according to manufacturer's protocol. Cells were transfected using pooled PBF-specific siRNA (catalog nos. 4399 and 147350) or negative control siRNA (AM4635) at a final concentration of 100 nM (Ambion). In transient reporter assays H1299 cells were transfected with PBF expression vectors (400 ng), pcDNA3-p53 (5 ng), pRL (20 ng) and phdm2-Luc (150 ng). After 24 h cells were harvested in Passive Lysis Buffer and luciferase activity measured using the Dual Luciferase Reporter Assay System (Promega).

Western Blotting

Western blot analyses were performed as described previously [23,26]. Blots were probed with specific antibodies against PBF [23,26], 1:500; HA (Covance Research Products), 1:2000 and p53(D0-1) (Santa Cruz Biotechnology), 1:1000. Antigen-antibody complexes were detected using the ECL Plus chemiluminescent detection system (Amersham Biosciences). Actin expression was determined using mouse monoclonal anti- β actin antibody clone AC-15 (Sigma-Aldrich) at 1:10,000. Protein quantification was performed on cell lysates using the Bradford assay. To quantify detected bands by densitometry, blots were scanned into Photoshop (Adobe Systems) keeping all scanning parameters the same and analysed using ImageJ software [29].

Real-Time RT-PCR and Sequencing

Total RNA was extracted using the RNeasy Micro Kit (Qiagen) and reverse transcribed using the Reverse Transcription System (Promega). Expression of specific mRNAs was determined using 7500 Real-time PCR system (Applied Biosystems) as previously described [24]. Colorectal tumor cDNA was sequenced to determine p53 mutational status as described with minor modifications [30]. Details of sequencing primers are given in Supplementary Figure S1A.

p53 Binding and Stability Assays

L- α -[³⁵S]-methionine-labelled p53 was expressed in vitro using a TNT T7 Coupled Reticulocyte Lysate System according to the manufacturer's guidelines (Promega). In vitro glutathione-S-transferase (GST) pull-down assays using [³⁵S]-p53 and GST-PBF proteins were performed using established protocols [31]. Coimmunoprecipitation (co-IP) assays were performed as described previously [26]. In p53 half-life experiments cells were incubated in 100 μ M anisomycin for 2 h prior to cell lysate extraction using standard protocols. In p53 ubiquitination experiments cells were incubated in 20 μ M MG132 for 5 h prior to cell lysate extraction.

Biotinylated Oligonucleotide Pull-down Assay

Oligonucleotide pull-downs were performed essentially as described previously [32]. An oligonucleotide (p21, Supplementary Figure S2A) containing a consensus p53 binding sequence was 5' end labelled with biotin and incubated with 25 ng recombinant human p53 protein (Active Motif, #31318). The DNA binding mixture contained 2.5 nM biotinylated DNA, 200 mM NaCl, 3 mM DTT, 25 mM HEPES-OH (pH 7.5), 0.2 mM EDTA, 0.1% Tween-20, 5% glycerol and 1 μ g poly(dA-dT). In competition reactions non-biotinylated oligonucleotide, recombinant PBF protein or BSA was also added to the binding reaction as indicated. DNA/protein complexes were captured with 5 μ g of magnetic streptavidin beads (Promega).

Subsequently, bound proteins were probed with an anti-p53 antibody (Santa Cruz Biotechnology). Prior to use magnetic beads were incubated in 7 mg/ml acetylated BSA for 30 min to prevent non-specific binding of p53. Oligonucleotide sequences are given in Supplementary Figure S2A.

Proximity Ligation Assay

The Duolink in situ proximity ligation assay (PLA) was performed according to the manufacturer's instructions (Olink Bioscience). In our experiments MCF-7 cells were seeded onto coverslips and transfected with expression vectors for p53 (pcDNA3-p53) and HA-tagged PBF (pcDNA3-PBF-HA) for 24 h prior to performing the PLA assay.

Soft Agar Assays, Irradiation and Cell Viability

Stable transfections in NIH3T3 cells and subsequent soft agar assays were performed as described previously [21]. DNA damage was induced by Caesium 137 irradiation using an irradiator IBL 437 C type H unit (CIS Bio international, Gif Sur Yvette). The cellular viability of HCT116 cultures transfected with either VO or PBF was determined using the MTT assay as described previously [33].

Immunohistochemistry and Genetic Instability

Formalin-fixed paraffin embedded sections of colorectal tissue were immunostained using an avidin-biotin peroxidase technique (Vectastain Elite, Vector laboratories, Burlingame, CA). Immunostaining was performed with specific antibodies against PBF [23,26], 1:200 as described previously [21]. FISSR-PCR to assess genetic instability (GI) of colorectal tumors was performed using primer (CA)₈RG as described previously [34].

Cell Migration

HCT116 cell migration experiments were performed using the ORIS™ Cell Migration Assay (Platypus Technologies) according to the manufacturer's protocols. Briefly, the cell stopper was removed after seeding 50,000 HCT116 cells per 96 well for 16 h. Cells were then washed gently with 100 μ l sterile PBS, fed with 100 μ l culture media and incubated for 24 h at 37 °C with 5% CO₂ prior to analysis of cell migration. At least eight wells were analysed per condition which involved imaging cells with an inverted light microscope and digital camera (Leica Microsystems). ImageJ software was used to process the image and quantify the proportion of migrating cells.

Statistical Analysis

Data are displayed as mean \pm SE. Normally distributed data were analysed using a two-tailed Student's t-test, unless otherwise indicated. A *P*-value < 0.05 was considered to be statistically significant.

RESULTS

Overexpression of PBF in Human Colorectal Tumors

Based on our previous findings that PBF expression was upregulated in thyroid cancer [21], we investigated whether PBF was also overexpressed in colorectal cancer and might therefore represent a prognostic indicator. Tumors showed 2.4-fold higher PBF mRNA expression than matched normals (Figure 1A; $n = 24$; $P = 0.009$). Immunohistochemistry revealed significant positive granular staining for PBF in the cytoplasm of adenocarcinomas, with weak or absent PBF staining in normal tissue specimens (Figure 1B). Protein expression was quantified through Western blotting in a further 15 matched normal [N] and cancer [C] colorectal specimens. 14/15 (93.3%) of tumors demonstrated significant PBF upregulation (Figure 1C), with a mean ~ 6 -fold induction of protein compared to matched normal tissue (Figure 1D; $P < 0.0001$). Altogether these results demonstrate that PBF expression is elevated in colorectal tumors and thus represents a potential prognostic marker.

PBF Promotes Colony Formation Independent of PTTG1-Binding Domain

To investigate whether the interaction between PBF and the human securin PTTG1 might have a role in tumorigenesis [18], we evaluated the transformation ability of PBF and a mutant of PBF (M1) lacking the entire PTTG1-binding domain (PTTG1 BD). Consistent with previous data [21], stable overexpression of PBF in NIH-3T3 cells resulted in the formation of highly significant numbers of colonies in soft agar assays compared to VO cells (Supplementary

Figure S3; VO 9.1 ± 1.4 colonies; PBF 201 ± 33.2 ; $P < 0.001$). However, mutant PBF (M1) was still capable of transforming NIH-3T3 cells with a significant number of colonies (Supplementary Figure S3; M1 175 ± 23.5 ; $P < 0.001$), thus implying that the transforming ability of PBF is not dependent on PTTG1.

PBF Binds to p53

We next examined whether PBF interacts with p53, a protein critical in suppressing human cancers. Initial GST pull-down assays demonstrated that L- α - 35 S]-methionine-labeled p53 binds to the full-length PBF protein but not with the GST control (WT 1–180; Figure 2A). Deletion mutants of GST-PBF altered the stringency of p53 binding. For instance, a mutant of PBF lacking amino acids 149–180 (M1) retained p53 binding, whereas the p53 interaction was lost with a mutant lacking amino acids 94–149 (M2). We next investigated whether the two proteins were able to bind in colorectal cancer HCT116 cells. Following irradiation of cells PBF was precipitated with an anti-PBF antibody. Coimmunoprecipitation of p53 with PBF was observed by probing with an anti-p53 antibody following Western blotting (Figure 2B) with a two-fold greater level of intensity than in non-irradiated HCT116 cells (Figure 2C; $P < 0.01$; $n = 3$). Analysis of total protein cell lysate confirmed increased p53 expression in irradiated cells whereas PBF protein level was unaltered compared to controls (Figure 2B).

The reciprocal co-immunoprecipitation was performed using the anti-p53 antibody for precipitation of p53 and immunoblotting for PBF, which resulted in

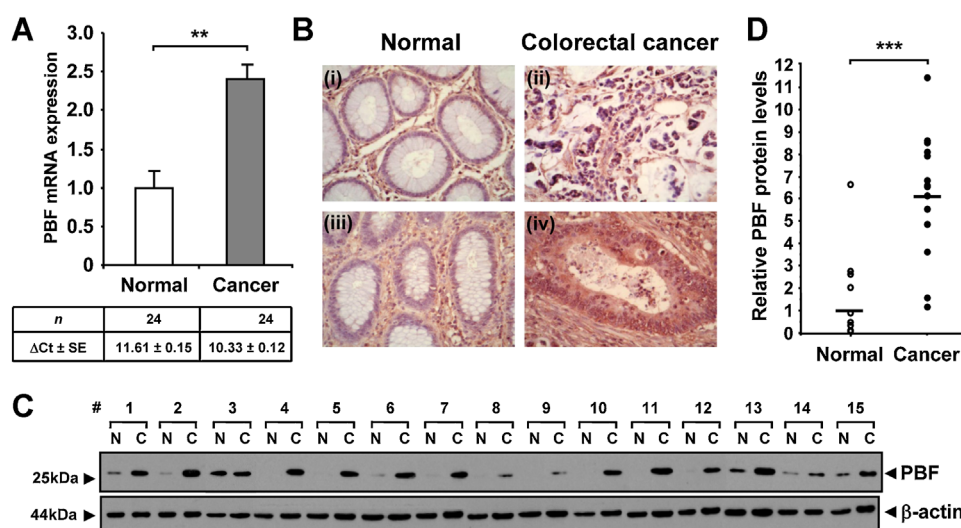


Figure 1. Elevated PBF expression in colorectal tumors. (A) PBF mRNA levels in matched normal and tumor colorectal pairs ($n = 24$). Data presented as mean \pm SE. (B) Representative images of PBF immunostaining in matched normal (i, iii) and colorectal tumor specimens (ii, iv). (C) Western blot analysis of PBF expression in matched normal [N] and cancer [C] colorectal pairs along with a β -actin loading control ($n = 15$). (D) Quantification of PBF protein expression in colorectal tumors relative to normal tissue ($n = 15$). Data presented as a scatterplot and analysed using Wilcoxon signed-rank test. $**P < 0.01$; $***P < 0.001$.

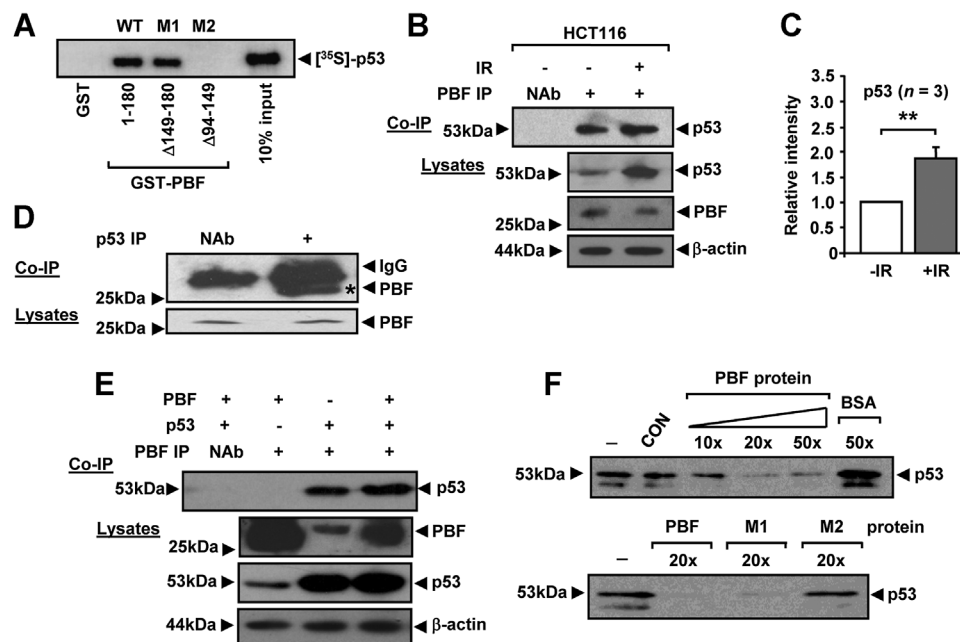


Figure 2. PBF binds p53 in vitro. (A) Binding of [³⁵S]-p53 to GST-PBF (1–180) and GST-PBF deletion mutants M1 and M2 as indicated versus a GST-only control. (B) Coimmunoprecipitation (co-IP) assay in untreated (–) or irradiated (+) HCT116 cells showing interaction between PBF and p53. Cell lysates were immunoprecipitated with an anti-PBF antibody. Purified proteins were analysed by Western blotting using an anti-p53 antibody. (lower) Analysis of total protein lysate demonstrating the presence of p53 and PBF along with a β-actin control. (C) Quantification of mean p53 protein levels ±SE from 3 independent co-IP assays described in (B). **, $P < 0.01$. (D) Reciprocal co-IP assay in untreated HCT116 cells showing interaction between PBF and p53. Asterisk indicates

position of ~30kDa band corresponding to PBF. (lower) Immunoprecipitation of PBF in total protein lysates. (E) Co-IP assays in HCT116 cells transfected with PBF p53, or PBF and p53. Co-IP assays were performed as described in (B). (lower) Western blotting analysis to confirm exogenous expression of p53 and PBF along with β-actin control. (F) Oligonucleotide pull-down analysis of p53 binding to a consensus p21 promoter in the presence of increasing amounts of recombinant PBF compared to BSA (upper), a control (CON) oligonucleotide (upper), or recombinant PBF mutant proteins M1 and M2 as indicated (lower). Values indicate fold (x) molar excess relative to p53. NAb = no antibody control.

successful co-immunoprecipitation (Figure 2D). In subsequent experiments we examined the binding interaction after exogenous expression of p53 and PBF (Figure 2E). Following the transient transfection of PBF and p53 into HCT116 cells PBF was precipitated with an anti-PBF antibody. The greatest amount of p53 co-immunoprecipitated with PBF was evident in HCT116 cells transfected with both p53 and PBF but was not present in the no-antibody control (Figure 2E).

The ability of PBF to interact with p53 was further examined using an oligonucleotide pull-down assay based on a p21 consensus binding site [32]. Binding of recombinant p53 was successively diminished by incubation with increasing amounts of recombinant PBF, but was unaltered by competition with excess BSA (Figure 2F, top panel). Furthermore, competition hinged upon the ability of PBF to bind p53, as PBF mutant M1 retained the ability to compete for p53 binding, whereas there was no significant reduction in p53 binding with PBF mutant M2 protein (Figure 2F, lower panel). In control experiments the specificity of p53 binding was demonstrated by a lack of competition with oligonucleotides containing either a mutated p53 sequence (MUT) or a non-related sequence (CON) (Supplementary Figure S2B).

In parallel experiments proximity ligation assays also demonstrated the presence of red spots of specific p53 and PBF interaction in MCF7 cells after transient (Supplementary Figure S2C). Altogether these results demonstrate that PBF protein binds to p53 both in vitro and in the cellular environment in colorectal cells.

PBF Increases Turnover and Ubiquitination of p53

p53 is an intrinsically unstable protein which is subject to degradation both in the nucleus and cytoplasm. We next examined whether the interaction of PBF with p53 resulted in altered protein stability. Half-life studies using anisomycin to block de novo protein synthesis showed that overexpression of PBF resulted in significantly increased turnover of p53 protein in HCT116 cells, with a ~90% decrease in p53 levels compared to VO controls after 120 min (Figure 3A and B; $P < 0.01$; $n = 4$). Further evidence of diminished p53 stability in PBF-transfected HCT116 cells was shown by an increased level of high mwt p53 conjugates in the presence of the proteasome inhibitor MG132 (Figure 3C), and in cells cotransfected with HA-tagged ubiquitin (Ubq-HA) and p53 (Figure 3D), which are consistent with the accumulation of ubiquitinated p53. Analysis of p53

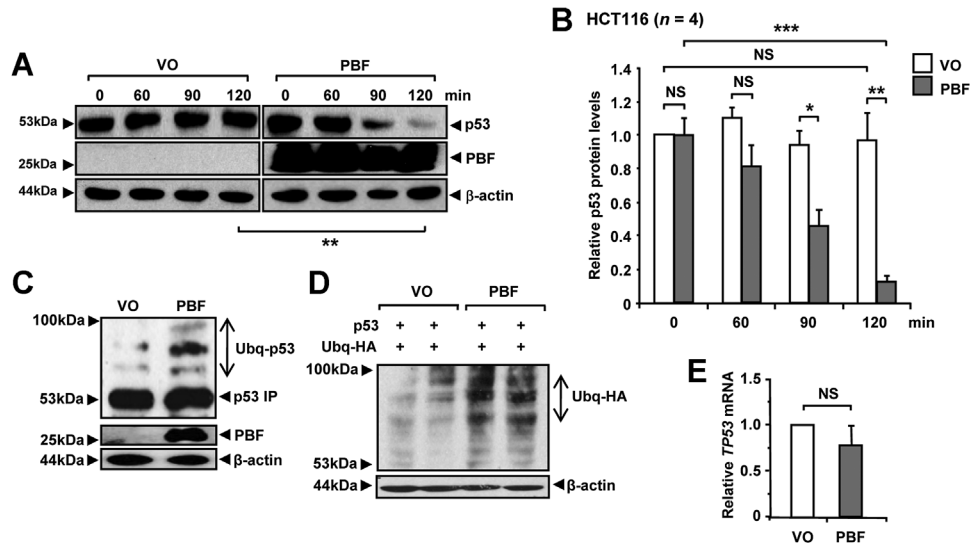


Figure 3. PBF regulates p53 stability. (A) Western Blot analysis of p53 and PBF in HCT116 cells transfected with either VO or PBF and then lysed at indicated times post-treatment with 100 μ M anisomycin. Exogenous PBF was detected with an anti-HA antibody. (B) Quantification of mean p53 protein levels relative to β -actin from 4 independent experiments as described in (A). (C) Detection of high molecular weight p53 conjugates by Western blot analysis in HCT116 cells transfected with either VO or PBF and then treated with 10 μ M

MG132. (D) Detection of high molecular weight HA-tagged ubiquitin (Ubq-HA) conjugates by Western blot analysis in HCT116 cells transfected with either VO or PBF, as well as p53 and Ubq-HA as indicated, and then treated with MG132. (E) Relative p53 mRNA levels in HCT116 cells transfected with either VO or PBF. Data presented as mean \pm SE. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. NS - not significant.

mRNA levels also revealed no change in PBF-transfected HCT116 cells (Figure 3E), which indicated that the effects of PBF on p53 were primarily at the level of protein stability and not transcriptional.

PBF Modulates p53 Activity

Given that PBF binds p53, we next analyzed the effect of PBF on the transactivation activity of p53 in transient reporter assays using p53-null H1299 cells. When coexpressed with p53, PBF significantly repressed p53-mediated *hdm2* promoter activity by up to 50% (Figure 4A; $P < 0.0001$) in H1299 cells. PBF mutant M1, which retained p53 binding in the GST pull-down assay, also repressed p53-mediated *hdm2* promoter activity (Figure 4B; $P < 0.0001$), but the PBF mutant M2, which did not bind p53, was unable to repress p53 transcriptional activity ($P = \text{NS}$).

In normal cells p53 is maintained at low levels but stabilized by irradiation-induced DNA damage. We therefore first determined the optimal p53 response in irradiated HCT116 cells (Figure 4C) and then examined the influence of manipulating PBF expression on cell survival in response to irradiation. In the absence of PBF, HCT116 cell survival was reduced by $\sim 30\%$ following exposure to irradiation (Figure 4D). In contrast, cells transfected with PBF demonstrated no decrease in cell survival, and indeed showed significantly greater cell number than irradiated VO-transfected cells ($P < 0.01$). Examination of p53 regulated genes indicated that PBF overexpression had ablated the ability of irradiation to induce mRNA levels for

genes such as p21 (Figure 4E; $P < 0.05$), *mdm2* (Figure 4E; $P < 0.05$), *bax* (Supplementary Figure S4; $P < 0.05$) and *sfn* (Supplementary Figure S4; $P < 0.05$) compared to irradiated VO-transfected cells. To determine whether the reverse relationship held true, we depleted PBF (Figure 4F) and observed significant increase in p53 activity in HCT116 cells with elevated p21 (Figure 4F; $P < 0.001$) and *sfn* (Supplementary Figure S5; $P < 0.05$) mRNA levels. Together these results provide compelling evidence for the functional ability of PBF to modulate p53 activity in colorectal cells.

PBF Expression Correlates with p53 Status

Colorectal cancers are known to contain a high frequency of *TP53* mutations that alter stability of the p53 protein as well as cellular function [35,36]. To gain further insight into the association between p53 and PBF in vivo we next compared the mutational status and relative expression of p53 in a cohort of 15 matched human normal (N) and cancer (C) colorectal specimens against their PBF expression levels as described in Figure 1C. Screening of our 15 matched colorectal specimens identified 8 tumors that contained either a nonfunctional somatic *TP53* mutation or deletion (Figure 5A and Supplementary Figure S1B). Highly stabilized p53 protein was also evident in these mutant p53 colorectal tumors ($n = 8$) with a mean 20.6 ± 3.8 -fold higher relative level of p53 expression than in WT p53 tumors ($P < 0.001$; $n = 7$; Figure 5B). Further details of the *TP53* mutations identified in these

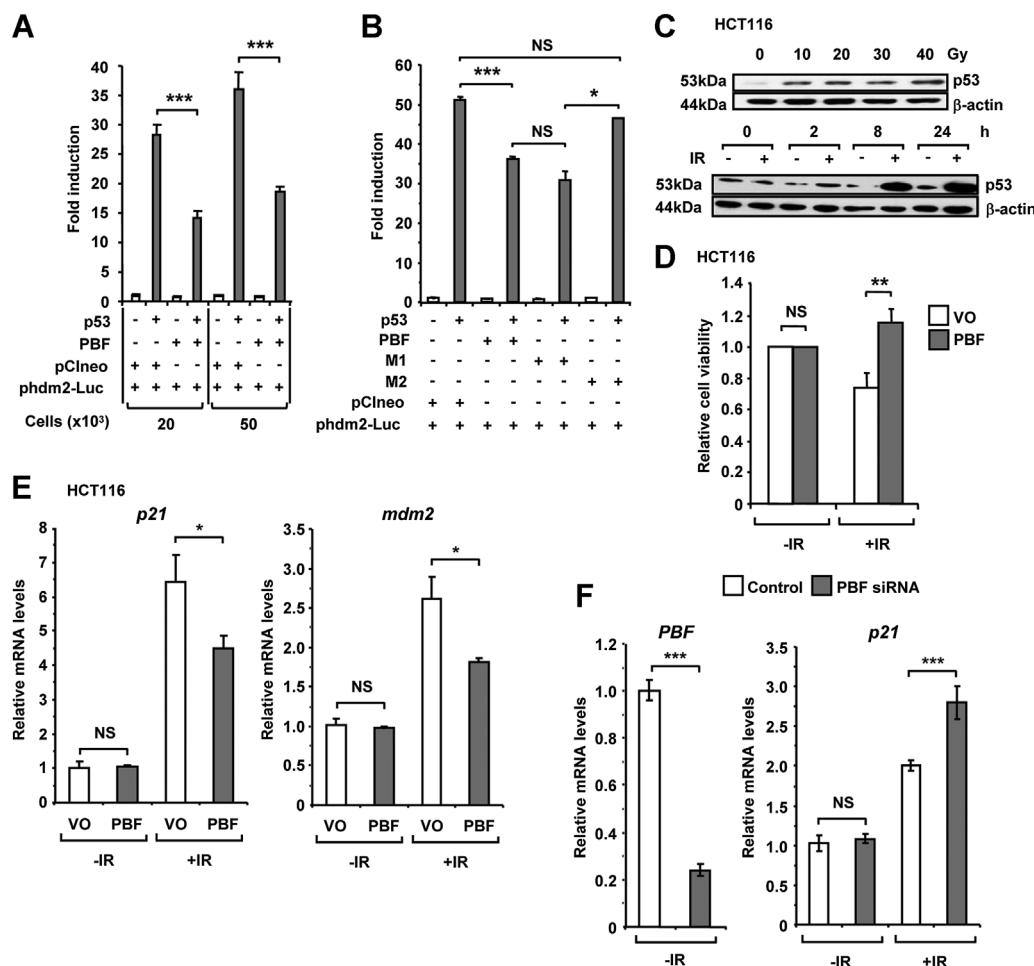


Figure 4. Altered expression of p53-regulated genes by PBF. (A) Fold induction of luciferase reporter activity in H1299 cells seeded at either 20 or 50 $\times 10^3$ per well and transfected with p53 and PBF expression vectors as indicated. (B) Fold induction of luciferase reporter activity in H1299 cells transfected with p53, PBF and PBF mutant (M1 or M2) expression vectors as indicated. (C) Western blot analysis of p53 in HCT116 cells irradiated with 0- to 40-Gy dose as indicated for 8 h (upper), or irradiated with 15-Gy dose and p53 protein levels monitored at 0, 2, 8, or 24 h after treatment compared with untreated (-) controls (lower). (D) Cell viability of HCT116 cells transfected with

either VO or PBF for 24 h and irradiated with a 40 Gy dose (+IR) compared to untreated controls (-IR). (E) Relative levels of p21 and Mdm2 mRNA in HCT116 cells transfected with either VO or PBF for 24 h and then irradiated with a 20 Gy dose (+IR) or untreated (-IR) prior to harvesting after 8 h. (F) Relative levels of PBF and p21 mRNA in HCT116 cells transfected with either PBF-specific or control siRNA for 48 h and then irradiated with a 20 Gy dose (+IR) or untreated (-IR) prior to harvesting after 8 h. Data presented as mean \pm SE. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. NS - not significant.

colorectal tumors and associated clinical data are given in Supplementary Tables S1 and S2.

To improve detection of the lower expression of p53 in WT *TP53* tumors we quantified immunoblots at higher exposure as shown in Supplementary Figure S6. An inverse relationship was apparent between p53 and PBF such that colorectal tumors with WT *TP53* and high PBF expression (> 2-fold induction) had reduced p53 protein levels compared to those with low PBF expression (Figure 5C; $P < 0.05$). In addition, all tumors with mutated *TP53* were associated with higher PBF expression compared to those with WT *TP53* (Figure 5D; $P < 0.05$). Having observed strong evidence for PBF binding to WT p53, we next examined whether PBF was also able to bind to mutant p53. Following

transient transfection of PBF and six of the p53 mutants (M1-M6) identified in the human colorectal tumors (Figure 5B), PBF was precipitated with an anti-PBF antibody. Coprecipitation for all 6 p53 mutants studied with PBF was observed following Western blotting using an anti-p53 antibody at up to 5.7-fold greater intensity than WT p53 (Figure 5E). In contrast, p53 was not detected in controls in which PBF and empty vector (-) were transfected. Analysis of total protein cell lysate demonstrated the presence of exogenous PBF and mutant p53 protein (Figure 5E).

These results provide evidence for a significant correlation between PBF and p53 expression levels in vivo, as well as the impact of mutant p53 status on these associations.

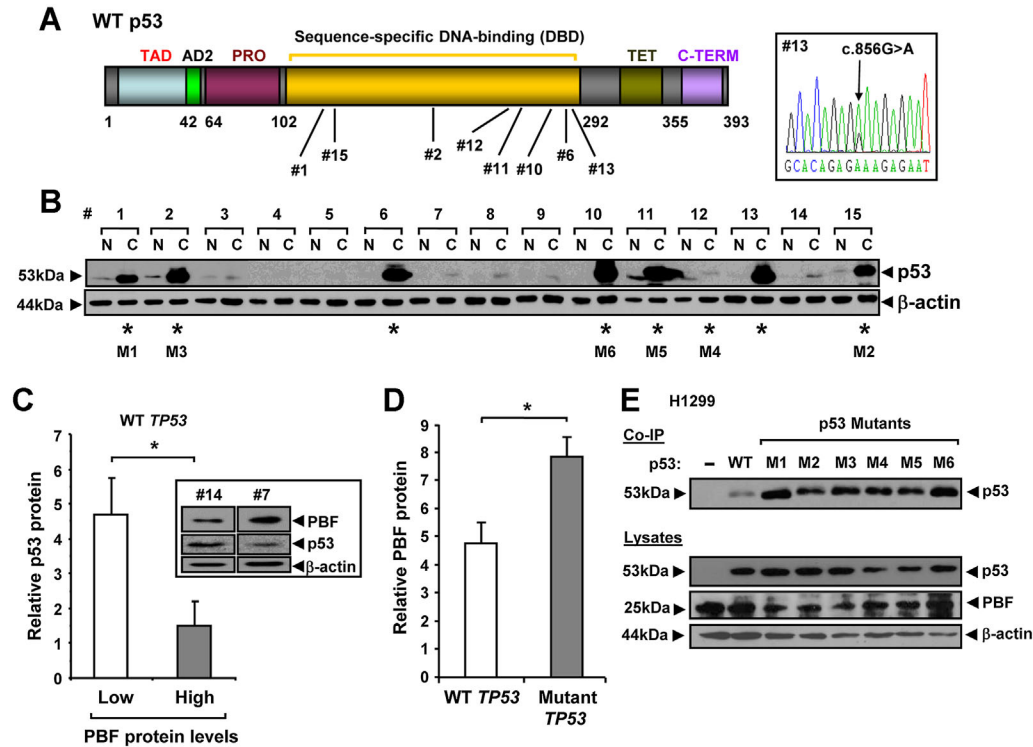


Figure 5. PBF correlates with mutational status and expression of p53 in colorectal tumors. (A) Schematic of the protein domain structure of p53 showing the relative position of p53 mutations identified in human colorectal tumors. (right) A representative electropherogram trace of the somatic *TP53* mutation (c.856G>A) in colorectal tumor from patient #13. (B) Western blot analysis of p53 expression in matched normal [N] and cancer [C] colorectal pairs ($n=15$). Cancers marked with an asterisk contain a somatic *TP53* mutation or deletion. Six mutations (M1-M6 as indicated) were used to generate p53 mutant expression constructs as described in (E). (C) Relative p53 protein levels in WT *TP53* colorectal tumors with either low ($n=3$) or high ($n=4$; >2-fold induction) PBF expression.

Magnified image of representative colorectal tumor with either low (#14) or high (#7) PBF expression along with p53 and β -actin levels. (D) Relative PBF expression in colorectal tumors with either WT or mutant *TP53*. All protein levels were normalized to β -actin. Data presented as mean \pm SE. *, $P < 0.05$. (E) Co-IP assays in H1299 cells showing interaction between PBF and p53 mutants. Lysates from cells transfected with PBF and WT p53 (WT) or the indicated p53 mutant (M1-M6) were immunoprecipitated with an anti-PBF antibody. Purified proteins were analysed by Western blotting using an anti-p53 antibody and are shown relative to control cells transfected with PBF alone (-). (below) Analysis of total protein lysate demonstrating the presence of p53, PBF and β -actin as loading control.

PBF Expression Correlates with Genetic Instability and Invasiveness

Dysregulation of p53 function has been linked to GI and invasiveness of colorectal tumorigenesis. We therefore examined whether PBF expression also correlated with levels of GI in colorectal tumors. Those tumors with greater than two-fold induction of PBF mRNA compared to their matched normals had significantly higher GI than those tumors with lower than two-fold induction (Figure 6A; $P < 0.05$), indicating an association between PBF expression and GI in colorectal tumors.

We observed that PBF expression was also significantly higher (~2-fold; $P < 0.05$) in WT *TP53* colorectal tumors with evidence of Extramural Vascular Invasion (EMVI) (Figure 6B), an independent predictor of recurrence and poorer overall survival [37,38], than those that failed to invade. In keeping with this, subsequent in vitro experiments demonstrated ~60% greater migration of PBF-transfected HCT116 cells at 24 h (Figure 6C; $P < 0.001$)

compared to VO-transfected controls, which further suggests a role for PBF in promoting cell movement in colorectal tumorigenesis.

Taken together these results demonstrate the ability of PBF to bind and regulate p53 activity, as well as correlating with clinical parameters of colorectal cancer. We therefore propose that PBF has a role in colorectal tumorigenesis through inhibition of p53 activity, which may involve the induction of cancer cell migration and invasion.

DISCUSSION

PBF is a relatively well-described protein [21,23,24,26,39-41] comprising 180 amino acids which is ubiquitously expressed [18], but shares no significant homology with other human proteins. It is highly conserved across animal species, suggesting both unique function and significant evolutionary importance. We previously reported overexpression of PBF in thyroid [21] and breast cancers [23], and demonstrated it to be a transforming gene in vitro

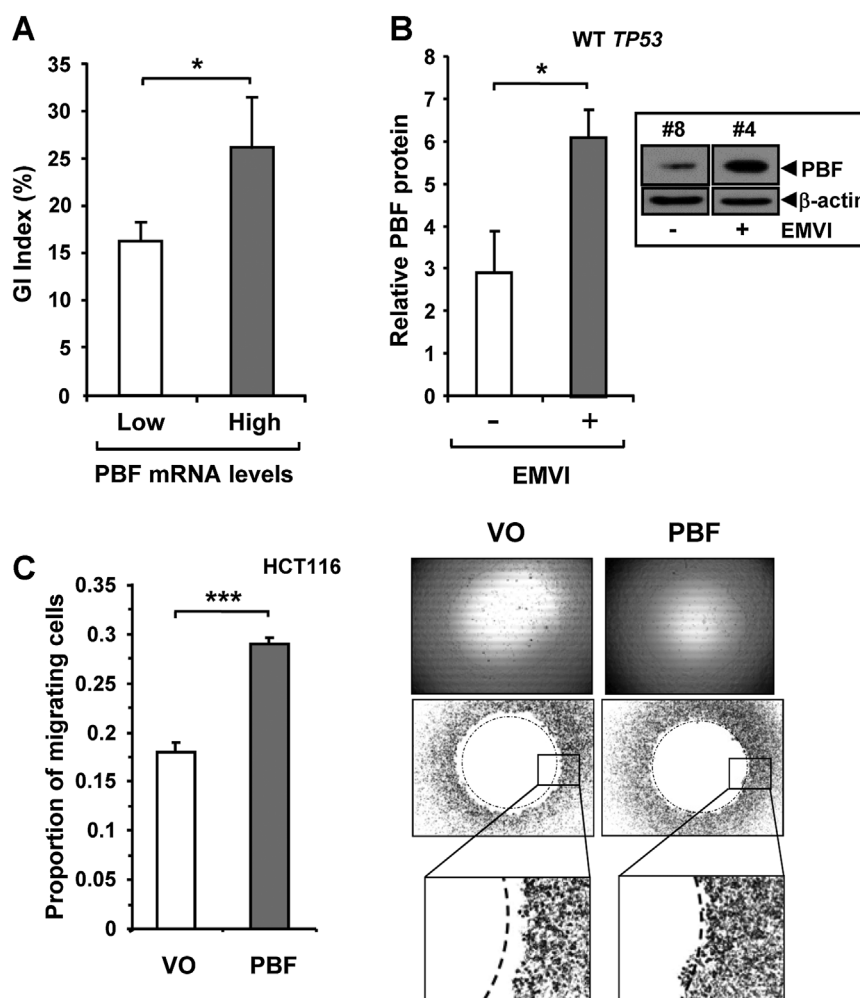


Figure 6. Increased genetic instability and invasiveness of cells with elevated PBF. (A) Relative GI indexes of colorectal tumors with either low ($n = 12$) or high PBF mRNA levels ($n = 6$; > 2-fold induction). (B) Relative PBF expression in WT *TP53* human colorectal tumors either without (-) or with (+) EMVI. Protein levels were normalized to β -actin. Representative Western blot of PBF expression in colorectal tumors either without (patient #8) or with EMVI (patient #4), along with β -actin, is also shown. (C) Relative proportion of migrating

HCT116 cells transfected with either VO or PBF after 24 h ($n = 8$). Representative images are shown of transfected cells taken with an inverted light microscope (upper panels) and then digitally processed (middle panels) for data analysis using ImageJ software. Magnified regions are shown in lower panels. Circles (dashed) of identical diameter are superimposed on images of both VO and PBF-transfected cells to highlight greater migration of PBF-transfected cells. *, $P < 0.05$; ***, $P < 0.001$.

and tumorigenic in vivo [21]. However, the precise role of PBF in tumorigenesis is unknown. Our current study provides the first evidence that PBF should be considered a new aetiological factor in colorectal cancer, as well as a potential prognostic biomarker.

Here, we characterized PBF in human colorectal tumors in order to gain possible insights into the role of PBF in tumorigenesis. Strikingly, PBF was overexpressed in the majority of colorectal tumors examined at both the mRNA (~2.5-fold) and protein (~6-fold) level, which was comparable to that seen in papillary thyroid tumors [21]. Previous studies have indicated that the expression of PBF appears to be greater in more aggressive cancers [21,42]. High PBF expression, for instance, was significantly correlated with locoregional recurrence and distant metastases in 153 patients with papillary thyroid cancer [42]. We also

observed an 8-fold increase in PBF mRNA in recurrent papillary thyroid cancer compared to 2.7-fold in tumors without recurrence [21]. Similarly in this study, PBF was most abundant in colorectal tumors with reported EMVI, raised GI and somatic *TP53* mutations, all features linked with recurrence and poorer patient survival [13,37,38,43]. Together these findings indicate that PBF represents a promising prognostic indicator for aggressive colorectal tumors.

The mechanisms governing colorectal tumorigenesis are yet to be fully defined, despite recent progress in identifying new roles for p53 in tumor progression [6,8,9,11]. The control of p53 activity itself is known to be highly complex involving a myriad of different pathways, and interactions with both positive and negative regulatory factors [5,44]. Our data clearly show that PBF interacts specifically with p53 as

evidenced by GST pull-down, coimmunoprecipitation and oligonucleotide pull-down assays. The relative level of p53:PBF coimmunoprecipitates in colorectal cells was enhanced by gamma-irradiation, which likely reflects the greater abundance of stabilized protein as p53 protein levels were, as expected, raised in irradiated HCT116 cells, in contrast to negligible changes in PBF protein. Interestingly, pull-down assays using deletion mutants of PBF showed that p53 did not bind to the C-terminal region (residues 149–180), which is known to bind PTTG1 [18]. Instead, a largely undefined region in PBF from residues 94 to 149 was implicated as deleting this region abolished the PBF: p53 interaction. In future studies we plan to map the precise interaction between PBF and p53 by further extensive mutational analysis. In particular, it will be important to investigate whether a short stretch of acidic amino acids located between residues 133–149 in PBF can mimic similar sequences identified in the E3 ligase Mdm2 that have been described to promote interaction with p53 [45,46].

In addition to interfering with binding of p53 to a consensus DNA site, we also demonstrated that PBF has a role in diminishing p53 stability as PBF overexpression significantly increased p53 turnover in colorectal cells. Further evidence was provided by increased ubiquitination of p53 in PBF-transfected cells as shown by the accumulation of high mwt p53 conjugates. Our results are therefore consistent with other proteins such as Mdm2 that can influence p53 activity in multiple ways. For example, it is known that interactions between the acidic domain of Mdm2 at the DNA-binding core of p53 are involved in ubiquitination and degradation of p53 [45,47], as well as for inhibiting DNA-binding [48]. It is unlikely however that PBF is functioning as an E3 ligase such as Mdm2 to promote p53 ubiquitination as it does not contain a well-defined domain with either ubiquitin-conjugating (UBC) [49] or E3 ligase [50] activity. Instead, we envisage that PBF may have a role in promoting the interaction of p53 with proteins known to alter its ubiquitination or acetylation status, such as Mdm2 [51], HDAC1 [52], PCAF [53] or Tip50 [54]. Importantly, we were also able to show that binding of PBF to p53 had functional consequences, with reduced transactivation in p53 reporter assays and increased survival of irradiated HCT116 cells, as well as altered mRNA expression for p53-regulated genes in both PBF-transfected and PBF-depleted cells.

In colorectal cancer overexpression of PTTG1 has been well-described in invasive disease [14], with particular significance as a marker for patient survival [15]. A role for PBF in regulating PTTG1 activity was indicated in one study where nuclear translocation of PTTG1 was enhanced by PBF in COS-7 cells [18]. We therefore expected that PBF might facilitate the role of PTTG1 as a human securin in colorectal tumorigene-

sis to inhibit mitosis and generate intrachromosomal breaks, as well as increasing the interaction between PTTG1 and p53 [16,55]. Our current data, however, suggest that the interaction of PBF with p53 might represent a new pathway in colorectal tumorigenesis that is independent of PTTG1 action. In particular, we found that a mutant of PBF (M1) lacking the entire PTTG1-binding domain (PTTG1 BD), as identified by Chien and colleagues [18], was still capable of binding p53 and abrogating function in a transactivation p53 reporter assay, as well as efficiently transforming NIH-3T3 cells in soft agar assays. In addition, preliminary co-IP experiments showed that the PBF:p53 interaction was not significantly altered in PTTG1-depleted cells (data not shown). These findings are therefore supportive of an independence of action for PBF.

A high frequency of *TP53* mutations have been identified in colorectal tumors [35]. Our data are in keeping with those in the literature with about 50% of tumors demonstrating either a somatic or deletion mutation in *TP53*, with two of the amino acids identified, that is, R273 and R282, known to be “hotspot” residues [12]. Importantly, we were able to show significant associations between PBF with the mutational status and expression of p53 in colorectal tumors. Of particular significance was that WT *TP53* tumors with high PBF expression had reduced p53 levels compared to those with low PBF expression, which further emphasizes a putative interaction between these two genes in vivo. However, greater PBF expression was also evident in colorectal tumors with somatic *TP53* mutations, a scenario in which patients have a shorter survival time than those with a WT *TP53* gene [13]. We are now planning to expand this study and investigate the wider clinical associations of these findings in a larger cohort of colorectal tumors.

It will also be important to determine the precise biological consequences of the interaction between PBF and mutant p53. Our coimmunoprecipitation experiments in p53-null H1299 cells showed a strong interaction between PBF and six different p53 mutants. It is unclear why elevated levels of PBF present in colorectal tumors were unable to bind and degrade stabilized mutant p53. It has been proposed however that Mdm2-mediated turnover of mutant p53 is ineffective in tumors due to binding of heat-shock proteins [56–58] and the inability of p53 mutant protein to transactivate *Mdm2* [59]. Recently, we showed that the action of PBF on p53 in thyroid cells appeared to be Mdm2-dependent as the inhibitor nutlin-3, which blocks binding of Mdm2 to p53, abrogated the ability of PBF to diminish p53 stability [51]. Based on these observations our current model is that elevated PBF levels in colorectal tumors are unable to promote destabilization of mutant p53 due to diminished regulation by the E3 ligase Mdm2. We are also currently investigating whether PBF might represent a novel mutant p53-interacting

partner such as Sp1 [36] that can enhance the oncogenic activity of mutant p53.

Cancer cell migration and invasion are important initial steps in tumor metastasis. Of relevance in this study was the observation that PBF expression was higher in colorectal tumors with reported EMVI, a poor prognostic feature linked with recurrence and poorer overall survival, in which malignant cells have invaded endothelial cell-lined blood vessels [37,38]. Previously, we have shown that high PBF expression was associated with cancer cell invasion in breast MCF-7 cells [23]. Similarly in this study, we demonstrated that PBF overexpression induced the migration of colorectal HCT116 cells. The mechanism for the ability of PBF to promote cell movement still needs to be clarified. However, it was recently suggested that p53 controls a specific gene signature to suppress intestinal tumor progression, and that loss of p53 in the intestinal epithelium of *Csnk1a1^{Δgut}p53^{Δgut}* mice was responsible for rapid invasiveness [6]. Therefore, based on these observations and other recent studies on p53 [7–11], we propose that the ability of PBF to bind and regulate p53 function might have a direct role in promoting colorectal cancer cell migration and invasion.

In summary, we present evidence for PBF as a novel interacting partner of p53, which modulates p53 transactivation capabilities by disrupting promoter binding and altering p53 stability. This is the first study to demonstrate overexpression of PBF in colorectal cancer, particularly in invasive WT p53 and mutant p53 tumors, thus implying that this gene should be investigated in future studies as a novel aetiological marker in colorectal tumorigenesis. We recently showed that thyroid-related inhibitory properties of PBF can be abrogated with the Src inhibitor PP1 to promote radioiodine uptake [41]. Thus, the ability of PBF activity to be regulated by inhibitors suggests that targeting PBF might also represent a promising therapeutic strategy in colorectal cancer.

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