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**Selective inhibition of  $\beta$ -F1-ATPase mRNA translation in human tumors.**

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**Running Title:** Control of  $\beta$ -F1-ATPase expression in cancer.

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**Footnotes:**

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**Abbreviations:**  $\beta$ -F1-ATPase, subunit  $\beta$  of the mitochondrial  $H^+$ -ATP synthase; p $\beta$ F1, precursor of  $\beta$ -subunit;  $\beta$ -mRNA,  $\beta$ -F1-ATPase mRNA; 3'UTR, 3' untranslated region.

### Synopsis

Down-regulation of the catalytic subunit of the mitochondrial H<sup>+</sup>-ATP synthase ( $\beta$ -F1-ATPase) is a hallmark of many human tumors. The expression level of  $\beta$ -F1-ATPase provides a marker of the prognosis of cancer patients as well as of the tumor response to chemotherapy. However, the mechanisms that participate in down-regulating its expression in human tumors remain unknown. Herein, we have studied the expression of  $\beta$ -F1-ATPase mRNA ( $\beta$ -mRNA) in breast, colon and lung adenocarcinomas and squamous carcinomas of the lung. Despite the down-regulation of the protein, tumor  $\beta$ -mRNA levels remained either unchanged (breast and lung adenocarcinomas) or significantly increased (colon and squamous lung carcinomas) when compared to paired normal tissues, suggesting a specific translation masking event for  $\beta$ -mRNA in human cancer. Consistently, we show using cell-free translation assays that a large fraction (~ 70%) of protein extracts derived from breast and lung adenocarcinomas specifically repress the translation of  $\beta$ -mRNA. We show that the 3'UTR of human  $\beta$ -mRNA is a relevant *cis*-acting element required for efficient translation of the transcript. However, an RNA chimera bearing the 3'UTR of human  $\beta$ -mRNA does not recapitulate the inhibitory effect of tumor extracts on  $\beta$ -mRNA translation. Overall, our findings support that the down-regulation of the bioenergetic activity of mitochondria in human tumors is exerted by translation silencing of  $\beta$ -mRNA.

## Introduction

Mitochondria play a central role in the physiology of eukaryotic cells. The biogenesis of mitochondria is a complex genetic program that requires the concerted transcriptional response of nuclear and mitochondrial genes [1]. However, mechanisms that control the localization, stability and translation of mRNAs also contribute to define the mitochondrial phenotype of the cell [2]. In oxidative phosphorylation, ATP is synthesized by the H<sup>+</sup>-ATP synthase, a rotatory engine of the inner mitochondrial membrane that utilizes as driving force the proton electrochemical gradient generated by the respiratory chain. The  $\beta$ -subunit of the H<sup>+</sup>-ATP synthase ( $\beta$ -F1-ATPase) is the catalytic subunit of the complex and rate-limiting component for the production of ATP [3]. The expression of  $\beta$ -F1-ATPase in the liver [4-6], in brown adipose tissue [7] and during progression through the cell cycle [8], indicates that the mitochondrial localization and translation of  $\beta$ -F1-ATPase mRNA ( $\beta$ -mRNA) are primary sites for regulating the spatial and temporal expression of the protein. Similar findings have been obtained in yeast, where the sorting of  $\beta$ -mRNA to the vicinity of mitochondria is mediated by its 3'UTR and deletion of this element leads to deficient protein import, reduced ATP synthesis, mtDNA depletion and respiratory dysfunction [9, 10]. In rat liver, the 3'UTR of  $\beta$ -mRNA has the functional activity of an internal ribosome entry sequence [11, 12] which controls the synthesis of the protein at the G2/M phase of the cell cycle [8]. The binding of developmentally regulated RNA binding proteins to the 3'UTR of  $\beta$ -mRNA has been shown to inhibit its translation during fetal stages of liver development [4]. The re-installment of the same mechanism of translational repression seems to control the expression of  $\beta$ -F1-ATPase in rat hepatomas [13].

Mitochondrial malfunctioning is implicated in the pathogenesis of various human disorders including cancer [14, 15]. Cancer cells are characterized by a predominant glycolytic metabolism even under aerobic conditions [16, 17]. The distinctive glycolytic shift of cancer cells is accompanied by silencing of the bioenergetic activity of mitochondria [3]. In this regard, we and others have reported that the expression level of  $\beta$ -F1-ATPase is consistently down-regulated in different human tumors when compared to normal tissue [3, 14, 18-24]. Moreover, the relative cellular expression level of the protein ( $\beta$ -F1-ATPase/GAPDH ratio) provides a bioenergetic signature of the tumor with potential clinical utility [16]. In fact, a diminished bioenergetic signature of the tumor is associated with poor prognosis as assessed in large cohorts of colon [14, 25], lung [3, 19] and breast [20] cancer patients. Interestingly, the bioenergetic signature of cancer cells and tumors also affords a promising predictive marker of the response to therapy [25-28]. Even though the expression of  $\beta$ -F1-ATPase is compromised in cancer and its down-regulation involved in progression of the disease, our knowledge of the mechanisms that control  $\beta$ -F1-ATPase expression in human tumors is practically missing. In this investigation we show that post-transcriptional expression of  $\beta$ -mRNA plays a key role in defining the bioenergetic phenotype of colon, lung and breast carcinomas. Consistently, we illustrate in cell-free translation assays that a large fraction of breast and lung carcinoma extracts trigger the specific repression of  $\beta$ -mRNA translation when compared to extracts derived from normal tissue of the same patients. In agreement with previous findings in the rat [4, 12], we document that the human 3'UTR of  $\beta$ -mRNA is a *cis*-acting element required for efficient translation. However, in contrast with findings in fetal rat liver [4] and rat hepatocarcinomas [13], human tumor extracts did not recapitulate the translation inhibitory effect on a reporter bearing the 3'UTR of human  $\beta$ -mRNA, indicating that control of  $\beta$ -mRNA translation in human cells requires additional elements.

## Experimental.

**Patient specimens and protein extraction.** Frozen tissue non-embedded in OCT obtained from surgical specimens of untreated cancer patients with primary ductal infiltrating carcinomas of the breast, adenocarcinomas of the colon and lung and squamous cell lung carcinomas were obtained from the Banco de Tejidos y Tumores, IDIBAPS (Instituto de Investigaciones Biomédicas Pi y Suñer), Hospital Clinic, Barcelona, Spain. Routine histopathological study of all the cases had been previously performed by an experienced pathologist and the histological type, grade and size of the tumor as well as regional lymph-node involvement recorded (Table 1). All tissue samples were anonymized and received in a coded form to protect patient confidentiality. The Institutional Review Board approved the project. Tissue sections of the tumor and normal tissue of each patient were analyzed and the regions of the tumors that did not contain significant areas of fibrosis, inflammation or necrosis were chosen [18]. For protein extraction, the samples were homogenized with a glass potter in a buffer containing 20 mM HEPES pH 7.9, 100 mM KCl, 3 mM MgCl<sub>2</sub>, 1 mM DTT, 0.5 mM PMSF and protease inhibitors (Roche) in a 1/5 (w/v) ratio and further freeze-thawed three times in liquid nitrogen. After protein extraction, the samples were centrifuged (15,000xg) at 4°C for 25 min. For *in vitro* translation assays, 200 µg of protein extracts were concentrated using centricon-10 filters (Amicon- Millipore) and the buffer exchanged to 20 mM HEPES pH 7.9, 20 mM KCl, 1 mM DTT, 0.5 mM PMSF and protease inhibitors. The protein concentration was determined with the Bradford reagent (Bio-Rad) using BSA as standard. Aliquots were stored at -80°C until use.

**Western blots.** Protein extracts were fractionated by SDS-PAGE and transferred onto PVDF membranes. The antibodies used for Western blotting were anti-β-F1-ATPase (1:20,000) and anti-GAPDH (1:20,000) [14]. Peroxidase conjugated anti-rabbit or anti-mouse IgGs (1:5000) (Nordic immunology) were used as secondary antibodies for detection by chemiluminescence (ECL, Amersham Bioscience). Membranes were exposed to X-ray films and the quantification of the immunoreactive bands (arbitrary units) was accomplished using a Kodak DC120 Zoom digital camera and the Kodak 1D Image Analysis Software for Windows.

**Quantification of mRNA expression.** Human β-mRNA levels in normal and tumor tissues were carried out by qPCR using TissueScan Tissue qPCR Arrays from OriGene Technologies, Inc (Rockville, MD). The breast cancer panel (BCRT101) contains cDNA from 7 normal breast and 41 ductal breast adenocarcinoma biopsies. The lung cancer panel (HLRT104) includes cDNA from paired normal and tumor tissue derived from 10 adenocarcinomas, 8 squamous and 6 other lung carcinoma patients. The colon cancer panel (HCRT103) includes cDNA from paired normal and tumor tissue of 24 adenocarcinoma patients. Only samples from clinic stages I, II and III patients were considered in the analysis. For detailed pathological information of these patients see Table 1. Real time qPCR was performed using an ABI PRISM 7900 SDS thermocycler and the Power SYBR Green Master Mix (Applied Biosystems) following the instructions provided by the suppliers. The following forward (F) and reverse (R) primers were used to amplify human β-F1-ATPase and β-actin cDNAs: F: 5'-cagcagatttggcaggtg-3', R: 5'-cttcaatgggtcccaccata-3' and F: 5'-cagccatgtacgttgctatccagg-3', R: 5'-aggtccagacgcaggatggcatg-3', respectively. Relative expression level of β-mRNA was determined using the comparative ΔΔCt method [29] using β-actin as control and relative to the ΔCt mean value of the normal samples.



**Cloning strategies.** To obtain the plasmid pBS-h $\beta$ F1-fl, encoding the full length human  $\beta$ -F1-ATPase cDNA, we followed the same bipartite strategy as for the rat cDNA [4] using human RNA (30  $\mu$ g) as template of the RT (AMV; Boehringer, Ingelheim, Germany). The primers and restriction enzymes used were the following: For the 5'-end fragment, h $\beta$ cDNA-ss (gcgcggtcgcagagctctccaccggactacgcca) and h $\beta$ cDNA-as (tggtctccttcaggggtaccagctt), digested with XhoI and PstI and placed in the pBluescript-SK<sup>+</sup> vector; for the 3'-end fragment, h $\beta$ 500-ss (gcgcgggggcccggatcccaacattgttgccag) and h $\beta$ -3' $\beta$ -as (gcgcggggaattctttttttttt), digested with PstI. The plasmid encoding ARF fused to the 3'UTR of human  $\beta$ -mRNA was obtained by subcloning the *Apal-EcoRI* cDNA fragment from pBS-h $\beta$ F1-fl into pARF [4]. Plasmids expressing a hybrid GFP that contains the mitochondrial targeting sequence of  $\beta$ -F1-ATPase (p $\beta$ ) with or without (p $\beta$ GFP-3' $\Delta$ ) the 3'UTR of human  $\beta$ -mRNA (p $\beta$ GFP-3' $\beta$ ) or  $\alpha$ -F1-ATPase mRNA (p $\beta$ GFP-3' $\alpha$ ) were developed in pcDNA3.1 vector (Invitrogen) using the p $\beta$ -GFP- $\beta$ -3'UTR [8] and human cDNA as template. The following primers were used: F-p $\beta$ -GFP-KpnI: gcgggtaccggaatccagctctc, R-GFP-EcoRI: gcggaattctcactgtacagctcgtccatg; F-3' $\beta$ -h-EcoRI: gcggaattcggggtctttgtcctctgta, R-3' $\beta$ -h-XhoI: gcgctcgcagtttttttttttgaggggtgta, F-3' $\alpha$ -h-EcoRI: gcggaattcactcctgtggattcacatcaa and R-3' $\alpha$ -h-XhoI: gcgctcgcagtttttttttaactatgcattatg. PCR amplifications were performed with the Expand High Fidelity PCR System (Roche, Mannheim, Germany).

**Transcription reactions and UV cross linking experiments.** *In vitro* transcriptions were carried out for 2h at 37°C using the T7 MEGAscript kit (Ambion/Applied Biosystems), the corresponding linearized plasmid DNA (1  $\mu$ g) and 6 mM m7G(5')ppp(5')G cap analog (Ambion/ Applied Biosystems) [12]. The restriction enzymes used to generate the following RNAs are indicated in brackets: h $\beta$ F1 (*EcoRI*), h $\beta$ -3'UTR (*EcoRI*) and h $\beta$ F1-3' $\Delta$  (*HindIII*), ARF (*Apal*), ARF-3' $\beta$  (*EcoRI*), p $\beta$ GFP-3' $\beta$  and p $\beta$ GFP-3' $\alpha$  (*XhoI*) and p $\beta$ GFP-3' $\Delta$  (*EcoRI*). RNAs were purified and its quality assessed by electrophoresis. UV cross linking experiments were performed as previously described [4]. In brief, 10  $\mu$ g of breast or lung tissue extracts were incubated with the radiolabeled 3'UTR of human  $\beta$ -mRNA [ $5 \times 10^5$  c.p.m.] at 30° C for 30 min. The reaction mixtures were exposed to UV light (254 nm) (Stratalinker 1800; Stratagene) for 6 min on ice, before the addition of 20 units of RNase T1 (Boehringer). For competition experiments an excess of unlabelled 3'UTR of  $\beta$ -mRNA was added 10 min before the addition of the labeled probe. The RNA-protein complexes were resolved by SDS-PAGE.

**Translation assays.** *In vitro* translations of capped RNAs were carried out using nuclease-treated rabbit reticulocyte lysates (GE-Healthcare) [4]. The reactions were performed in the presence of 1.2  $\mu$ Ci/ $\mu$ l of [<sup>35</sup>S]-Met labeling mix (Redivue Promix, GE-Healthcare), 100 mM K<sup>+</sup> and 1.5 mM Mg<sup>2+</sup> ions for 30 min at 30°C in the presence or absence of human extracts (0-10  $\mu$ g protein) derived from normal or tumor tissues. Tissue extracts were diluted in translation buffer (20 mM HEPES, 20 mM KCl, 1 mM DTT supplemented with protease inhibitors). The translation products were further analyzed by SDS-PAGE and fluorography [4]. The input RNA was recovered after translation and the content of the remaining  $\beta$ -mRNA determined by RT-qPCR [30].

**Statistical analysis.** Statistical analysis was performed using Student's t test for paired samples. One-way ANOVA was used to detect differences in the bioenergetic signature within the normal and tumor biopsies. Standard F test was used to assess significance. Statistical tests were two-sided at the 5% level of significance.

## Results

**Evidence of post-transcriptional regulation of  $\beta$ -F1-ATPase expression in human cancer.** The expression of mitochondrial  $\beta$ -F1-ATPase is down-regulated in a wide variety of carcinomas concurrently with the up-regulation of the glycolytic GAPDH (Fig. 1). In fact, a significant inverse correlation between these two protein markers of metabolism have been reported in the analysis of large cohorts of breast, colon and lung cancer patients [3, 14, 20]. Consistently, the results in Fig. 1 illustrate the significant down-regulation of the bioenergetic signature ( $\beta$ -F1-ATPase/GAPDH ratio) of breast, colon and lung adenocarcinomas as well as in squamous carcinomas of the lung when compared to the bioenergetic signature in paired normal tissues from the same patients. These findings raised the question of the mechanism(s) that could control  $\beta$ -F1-ATPase expression in human tumors. Therefore, we determined the expression level of  $\beta$ -mRNA in normal and tumor tissue of breast, colon and lung cancer patients (Fig. 1). The expression of  $\beta$ -mRNA in cancer varied significantly depending upon the tissue and histological type being considered (Fig. 1). Expression of  $\beta$ -mRNA in breast (Fig. 1A) and lung (Fig. 1C) adenocarcinomas was not significantly different from normal tissues. In contrast, its expression was significantly augmented in adenocarcinomas of the colon (Fig. 1B) and squamous carcinomas of the lung (Fig. 1C) when compared to paired normal samples. These findings indicate that the decreased expression of  $\beta$ -F1-ATPase in human tumors (Fig. 1) could not be ascribed to a limitation in the availability of  $\beta$ -mRNA due to an impaired transcription and/or increased degradation of the transcript. Otherwise, the results suggest that the lower content of  $\beta$ -F1-ATPase could originate from translational silencing of  $\beta$ -mRNA as it has been previously documented in rat liver during development [4], in brown adipose tissue [7] and in rat hepatocarcinomas [13].

**The 3'UTR of human  $\beta$ -mRNA is a relevant *cis*-acting element involved in the control of its translation.** Previous studies have demonstrated a relevant role for the 3'UTR of rat liver  $\beta$ -mRNA in the control of its translation [4, 11, 12]. Therefore, a first question was to verify whether the human 3'UTR is also necessary for efficient translation of the transcript. Translation *in vitro* of the human  $\beta$ -mRNA (h $\beta$ F1) and of a truncated version of the mRNA that lacks the 3'UTR (h $\beta$ F1-3' $\Delta$ ) revealed a *two-fold* decrease in the amount of synthesized p $\beta$ -F1-ATPase when the 3'UTR is missing (Fig. 2A). To further confirm the need of the human 3'UTR in translation we fused the human 3'UTR of  $\beta$ -mRNA to ARF and GFP mRNAs and tested the translational efficiency of the chimeric reporters (Fig. 2B). The ARF transcript was selected as a reporter to maintain uniformity with previous findings where the translational activity of the 3'UTR of rat  $\beta$ -mRNA has been described [4, 13]. The results revealed an autonomous translation enhancing activity of the human 3'UTR because the amount of ARF derived from the ARF-3' $\beta$  chimera was  $\sim$ 10-fold higher when compared to that of the ARF-mRNA (ARF) (Fig. 2B). Likewise, the amount of GFP derived from the GFP-3' $\beta$  reporter was  $\sim$ 2-fold higher than that derived from GFP chimeras that lack any 3'UTR (GFP-3' $\Delta$ ) or contained the 3'UTR of  $\alpha$ -F1-ATPase (GFP-3' $\alpha$ ), a partner subunit of  $\beta$ -F1-ATPase in the H<sup>+</sup>-ATP synthase complex. The rather large differences in the translational activity of the 3'UTR as assessed with the ARF-3' $\beta$  and GFP-3' $\beta$  chimeras could result from intrinsic translational differences of the two open reading frames. In any case, these results demonstrate that the human 3'UTR of  $\beta$ -mRNA is also necessary for efficient translation of the transcript and that it might act as a relevant element in the control of  $\beta$ -mRNA translation [4, 13].

**Breast and lung tumor extracts specifically repress  $\beta$ -mRNA translation.** The lower content of  $\beta$ -F1-ATPase in tumors, despite an unchanged or increased

availability of  $\beta$ -mRNA, suggested that its translation could be hampered in human cancer. Therefore, we next studied whether protein extracts from paired normal (N) and tumor (T) tissue derived from the same patient have a different effect in the synthesis of p $\beta$ -F1-ATPase (p $\beta$ F1) using cell-free translation assays [4, 13]. It was observed that in seven out of the eleven breast cancer patients studied (64 %), the addition of carcinoma extracts promoted a dose dependent reduction in the amount of p $\beta$ F1 synthesized when compared to assays in which normal breast extract was added (Fig. 3A, B). The higher inhibitory activity of breast tumor extracts on the synthesis of p $\beta$ F1 compared to paired normal extracts is shown in the histogram (Fig. 3B). Similar findings were obtained in six out of the eight (75 %) lung cancer patients analyzed when using extracts of lung adenocarcinomas and of its paired normal tissue (Fig. 4A,B). The higher inhibitory activity of lung tumor extracts on the synthesis of p $\beta$ F1 is shown in the histogram (Fig. 4B). It should be noted that differences in p $\beta$ F1 synthesized are not the result of an increased degradation of  $\beta$ -mRNA in the presence of tumor extracts, because the recovery of the transcript at the end of translation assays was the same irrespective of the extract added (see histograms in Figs. 3A and 4A).

To verify the specificity of the inhibitory effect of tumor extracts on human  $\beta$ -mRNA translation, we studied the effect of the extracts in the translation of an unrelated mRNA such as ARF, that was previously used as a reporter to document similar findings with the rat transcript [4, 13]. The results showed no relevant effect of breast (Fig. 3B) and lung (Fig. 4B) tumor extracts on the synthesis of ARF when compared to paired normal extracts (compare open and closed bars in Fig. 3B and 4B, respectively). These results illustrate the specific inhibition of  $\beta$ -mRNA translation exerted by tumor extracts.

Contrary to our expectations, provided the relevant functional role that the 3'UTR of human  $\beta$ -mRNA has in translation (Fig. 2) and with the findings obtained with the rat transcript [4, 13], we observed that human breast (Fig. 3B) and lung (Fig. 4B) tumor extracts were unable to recapitulate the translation inhibitory effect on RNA chimeras that contained the 3'UTR of human  $\beta$ -mRNA (compare open and closed bars in Fig. 3B and 4B, respectively). These results indicate the existence of mechanistic differences among mammals for controlling the expression of  $\beta$ -mRNA and suggest that in human tissues, in addition to the 3'UTR, other elements of the transcript are required for its appropriate translation masking in cancer. Taken together, the results indicate that in a large fraction of human breast and lung adenocarcinomas the decreased expression of  $\beta$ -F1-ATPase could originate from a specific translation repression event of  $\beta$ -mRNA.

## Discussion

A characteristic of normal proliferating cells is the reprogramming of its energetic metabolism, switching from a prevalent oxidative phosphorylation in G1 to glycolysis in the rest of cell cycle phases [16, 31, 32]. On top of these changes, a large number of studies support the malfunctioning of the bioenergetic activity of mitochondria in cancer cells and tumors (for review see [16, 33]), a condition that might be superimposed to the acquisition of the metabolic phenotype of proliferation. Several mechanisms have been proposed to explain the Warburg effect observed in tumors (for review see [16, 17]). However, irrespective of the decisive molecular alteration that could explain the Warburg phenotype, there are large biochemical similarities between



tumors and embryonic tissues, especially with regard to the expression of isoforms of glycolytic enzymes and with the control of mitochondrial biogenesis [2]. Indeed, conserved mechanisms for regulating the expression of  $\beta$ -F1-ATPase have been described between fetal rat liver and rat hepatomas [2, 13]. In following with these observations, we have shown that the expression of  $\beta$ -F1-ATPase is decreased in various human tumors, and its altered expression linked to the glycolytic switch experienced by the tumors [3, 14, 20]. However, to date the molecular mechanisms underlying the down-regulation of  $\beta$ -F1-ATPase expression in human cancers have remained largely unexplored. In this investigation we have unveiled that the control of  $\beta$ -mRNA translation is a molecular mechanism that could participate in defining the bioenergetic phenotype of human tumors, supporting a role for the misregulation of the translation of a specific transcript in the etiology of the disease [34].

Consistent with the complexity of cancer as a disease [35] and with the cell-type specific mechanisms that regulate the biogenesis of mitochondria [36], we observed, with regard to the expression of  $\beta$ -mRNA, that different tissues and histological types respond differently in oncogenesis to the drop in its bioenergetic signature. Whereas in breast and lung adenocarcinomas there are no relevant changes in  $\beta$ -mRNA expression, colon adenocarcinomas and squamous carcinomas of the lung revealed a significant increase in  $\beta$ -mRNA expression. Changes in the cellular availability of  $\beta$ -mRNA in these tissues could result from an increase in the transcription rate of the gene and/or in the stability of the transcript, since both mechanisms have been shown to control the expression of the mammalian *ATP5B* gene in order to compensate for the energetic imbalance [5, 13].

In agreement with previous findings with rat  $\beta$ -mRNA [4], we have shown that the 3'UTR of human  $\beta$ -mRNA is required for efficient translation of the transcript, acting also as a translational enhancer of reporter constructs. In following with these similarities, we have demonstrated that human  $\beta$ -mRNA translation is specifically inhibited in the presence of lung and breast adenocarcinoma extracts, as was previously shown for the translation of rat  $\beta$ -mRNA in the presence of fetal rat liver [4] and hepatocarcinoma [13] extracts, two other conditions where translation masking of  $\beta$ -mRNA has been described. However, in total contrast to these situations [4, 13], a reporter with the 3'UTR of human  $\beta$ -mRNA is unable to recapitulate the translation inhibitory activity of tumor extracts (Figs 3B and 4B). It is assumed that the binding of proteins to the 3'UTR sterically hinder the initiation of translation by preventing 43S ribosomal recruitment onto the "closed loop" pseudo-circular mRNA that is competent in translation [37]. At variance with the rat 3'UTR, where silencing of  $\beta$ -mRNA translation and of 3'UTR containing chimeras is due to the specific binding of regulatory proteins to the 3'UTR of the transcript [4, 13], the 3'UTR of human  $\beta$ -mRNA does not interact specifically with breast and lung tumor proteins (see Supplemental Figure S1). In fact, and in contrast with rat data, the RNA-protein complexes that could be visualized in UV cross linking experiments are not competed by an excess of the unlabelled human 3'UTR (see Supplemental Figure S1). This result is consistent with the absence of a specific effect of these extracts on the translation of the ARF-3' $\beta$  chimera and suggest that although the 3'UTR is a sequence element required for efficient translation of the mRNA (Fig. 2), in the human transcript additional *cis*-acting elements are required to recapitulate translational inhibition. Thus, indicating that the control of  $\beta$ -mRNA translation in human tissues is more complex than anticipated.

Translation masking of  $\beta$ -mRNA has been observed in fetal rat liver [4], brown adipose tissue [7], rat hepatomas [13, 38] and in the human tumors analyzed in this

study. Translation silencing is usually mediated by 3'UTR-mediated sequestration of the mRNA into RNPs [39, 40] and/or by miRNA-mediated inhibition of translation [41, 42]. Recently, the AU-rich element-binding protein HuR has been shown to interact with the human 3'UTR of  $\beta$ -mRNA [30]. However, HuR seems to play an ancillary role in  $\beta$ -F1-ATPase expression in human cells [30], consistent with the finding that it provides a relevant independent marker of breast cancer prognosis [30]. Since the control of  $\beta$ -F1-ATPase expression is mostly exerted at the level of translation [4, 5, 13] and we have observed no specific interactions of the human 3'UTR with protein extracts derived from breast and lung cancer biopsies the possibility exists that miRNAs [41, 42] could play a role in post-transcriptional regulation of  $\beta$ -mRNA expression in cancer. Taken together, we have unveiled the existence of a specific translation repression mechanism for human  $\beta$ -mRNA that might explain the diminished bioenergetic signature of the tumors. Deciphering the complete mechanistic picture that accompanies translational repression of  $\beta$ -mRNA will certainly contribute to transform cancer into a chronic disease.

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**Table 1: Summary of clinicopathologic characteristics of the cohorts of patients studied.**

<b><u>Breast cancer</u></b>		<b><u>Lung cancer</u></b>		<b><u>Colon cancer</u></b>	
<b>Histology</b>	<b>N°</b>	<b>Histology</b>	<b>N°</b>	<b>Histology</b>	<b>N°</b>
Normal breast	<b>14</b>	Normal lung	<b>35</b>	Normal colon	<b>27</b>
DIC	<b>50</b>	AC	<b>19</b>	AC	<b>27</b>
		SCC	<b>16</b>		
<b>Stage</b>		<b>Stage</b>		<b>Stage</b>	
I	<b>11</b>	IA	<b>4</b>	I	<b>5</b>
IIA	<b>20</b>	IB	<b>8</b>	IIA	<b>10</b>
IIB	<b>10</b>	IIA	<b>9</b>	IIB	<b>5</b>
III	<b>11</b>	IIB	<b>8</b>	IIIA	<b>2</b>
		IIIA	<b>3</b>	IIIB	<b>3</b>
		IIIB	<b>3</b>	IIIC	<b>1</b>
				N/R	<b>1</b>
<b>Grade</b>		<b>Grade</b>		<b>Grade</b>	
1	<b>4</b>	1	<b>3</b>	1	<b>4</b>
2	<b>18</b>	2	<b>17</b>	2	<b>17</b>
3	<b>30</b>	3	<b>12</b>	3	<b>4</b>
		N/R	<b>3</b>	N/R	<b>2</b>
<b>Lymph node metastases</b>		<b>Lymph node metastases</b>		<b>Lymph node metastases</b>	
NO	<b>24</b>	NO	<b>13</b>	NO	<b>17</b>
YES	<b>24</b>	YES	<b>22</b>	YES	<b>9</b>
N/R	<b>4</b>	N/R	<b>3</b>	N/R	<b>1</b>

DIC: Ductal infiltrating carcinoma;  
AC: Adenocarcinoma;  
SCC: Squamous cell carcinoma;  
N/R: non registered

**Figure Legends:**

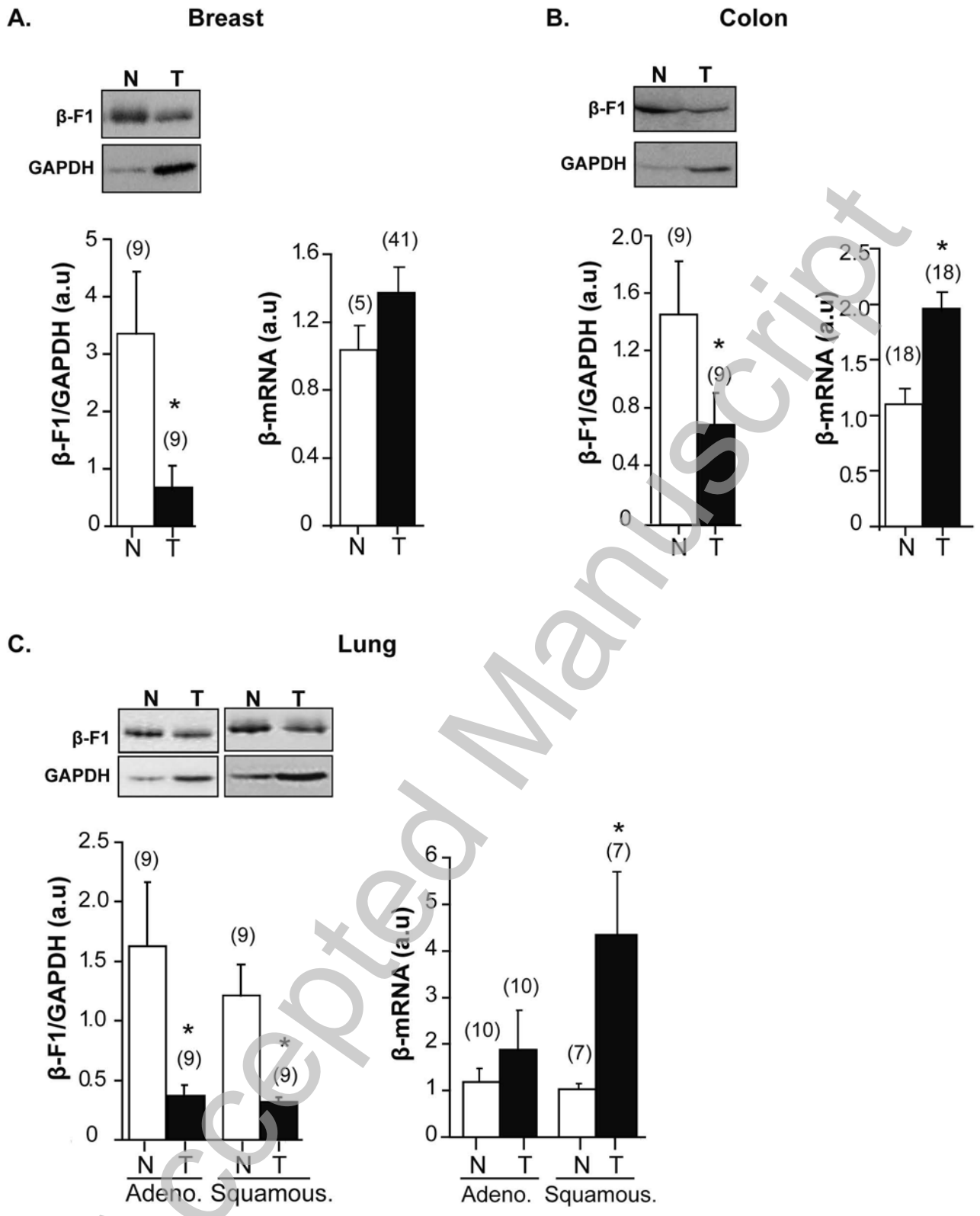
**Figure 1. Expression of  $\beta$ -mRNA in human tumors.** The bioenergetic signature ( $\beta$ -F1-ATPase/GAPDH ratio) was determined by western blotting in human breast (A), colon (B) and lung adenocarcinomas and squamous carcinomas (C) (T, closed bars) and in paired normal (N, open bars) tissue of the same patients. For illustration purposes, the result of a patient is shown for each type of carcinoma. The expression of  $\beta$ -mRNA was assessed by qPCR in normal and tumor tissue of breast (A), colon (B) and lung (C). The number of patients analyzed is shown in brackets. The results shown are means  $\pm$  SEM. \*,  $p < 0.05$  when compared to normal by Student's t test.

**Figure 2. The 3'UTR of human  $\beta$ -mRNA is required for efficient translation.** (A) The human *in vitro* synthesized and capped  $\beta$ -mRNA containing (h $\beta$ F1) or non-containing (h $\beta$ F1-3 $\Delta$ ) the 3'UTR of the transcript were translated in a cell free system. The fluorograms show the protein product (p $\beta$ F1) synthesized. (B) The *in vitro* synthesized and capped ARF- and GFP-mRNA containing (ARF3' $\beta$ , p $\beta$ GFP-3' $\beta$ ) or non-containing (ARF, p $\beta$ GFP-3' $\Delta$ ) the 3'UTR of  $\beta$ -mRNA or containing the 3'UTR of  $\alpha$ -F1-ATPase mRNA (p $\beta$ GFP-3' $\alpha$ ), were translated in a cell free system. The fluorograms of the synthesized protein products are shown. The histograms illustrate the relative amount of synthesized protein. The results shown are means  $\pm$  SEM. \*,  $p < 0.05$  when compared to h $\beta$ F1, ARF and p $\beta$ GFP-3' $\Delta$ , respectively, by Student's t test.

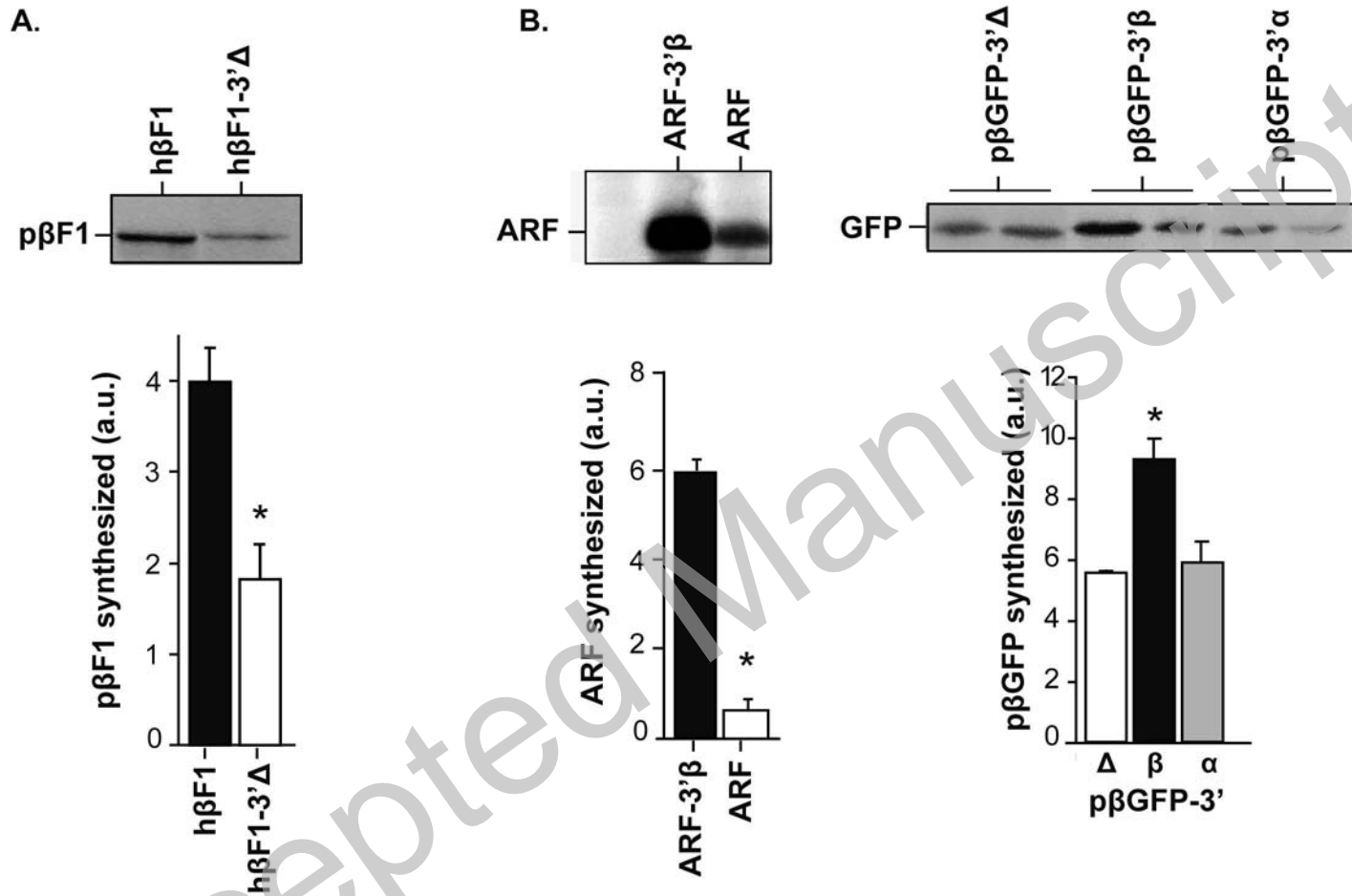
**Figure 3. Human breast tumor extracts specifically repress  $\beta$ -mRNA translation.** (A) The human *in vitro* synthesized and capped  $\beta$ -mRNA (h $\beta$ F1) was translated in a cell free system in the presence of different amounts of human breast tissue extracts derived from normal (N, open circles and continuous line) and tumor (T, closed circles and discontinuous line) biopsies. The fluorogram and plot show the synthesized precursor of  $\beta$ -F1-ATPase (p $\beta$ F1) in the presence of different protein inputs ( $\mu$ g of protein) of the extracts in a representative experiment derived from one cancer patient. The quantification of the inhibitory activity of normal (open bars) and tumor (closed bars) extracts for h $\beta$ F1 translation in seven patients is summarized under panel B. The histogram in A shows the quantity of  $\beta$ -mRNA recovered after translation assays with normal (N) and tumor (T) extracts. (B), The *in vitro* synthesized and capped ARF and ARF-3' $\beta$  RNAs were translated in a cell free system in the presence of different amounts of human breast tissue extracts derived from normal and tumor biopsies. A representative experiment (same patient as in panel A) illustrates the synthesized ARF protein in the presence of different protein inputs ( $\mu$ g of protein) of the extracts. The histograms show the translation inhibitory activity of tumor (closed bars) extracts on the different RNAs tested when compared to the activity of normal extracts (open bars). The results shown are means  $\pm$  SEM of seven different patients. \*,  $p < 0.05$  when compared to normal breast extracts by Student's t test.

**Figure 4. Human lung adenocarcinoma extracts specifically repress  $\beta$ -mRNA translation.** (A) The human *in vitro* synthesized and capped  $\beta$ -mRNA (h $\beta$ F1) was translated in a cell free system in the presence of different amounts of human lung tissue extracts derived from normal (N, open circles and continuous line) and tumor (T, closed circles and discontinuous line) biopsies. The fluorogram and plot show the synthesized

precursor of  $\beta$ -F1-ATPase (p $\beta$ F1) in the presence of different protein inputs ( $\mu$ g of protein) of the extracts in a representative experiment derived from one cancer patient. The quantification of the inhibitory activity of normal (open bars) and tumor (closed bars) extracts for h $\beta$ F1 translation in six patients is summarized under panel B. The histogram in **A** shows the quantity of  $\beta$ -mRNA recovered after translation assays with normal (N) and tumor (T) extracts. **(B)**, The *in vitro* synthesized and capped ARF and ARF-3'UTR RNAs were translated in a cell free system in the presence of different amounts of human lung tissue extracts derived from normal and tumor biopsies. A representative experiment (same patient as in panel A) illustrates the synthesized ARF protein in the presence of different protein inputs ( $\mu$ g of protein) of the extracts. The histograms show the translation inhibitory activity of tumor (closed bars) extracts on the different RNAs tested when compared to the activity of normal extracts (open bars). The results shown are means  $\pm$  SEM of six different patients. \*,  $p < 0.05$  when compared to normal lung extracts by Student's t test.

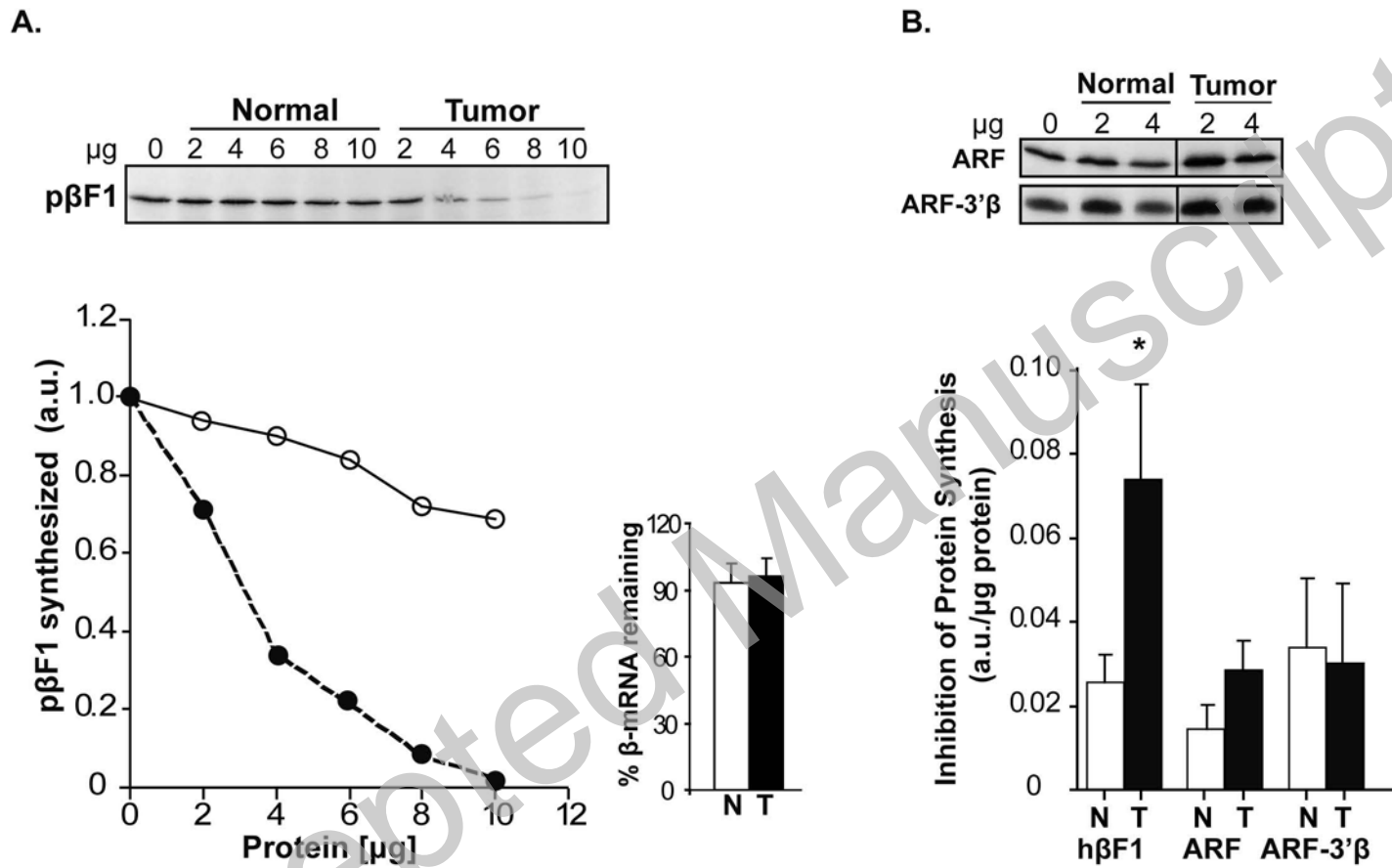


**Figure 1**

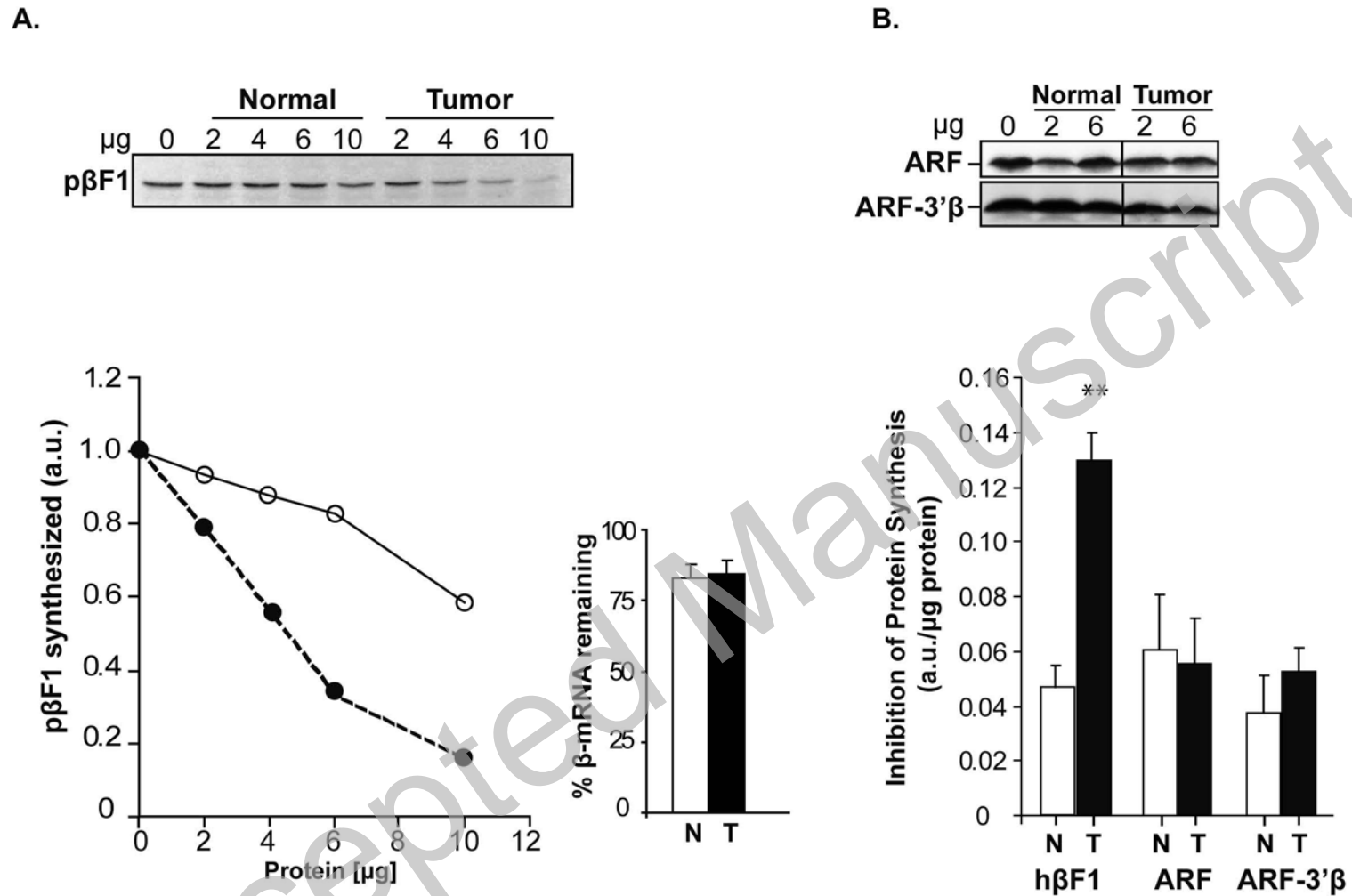


**Figure 2**





**Figure 3**



**Figure 4**