

## EBP1 Inhibits Translation of Androgen Receptor mRNA in Castration Resistant Prostate Cancer Cells

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**Abstract.** *Background: Therapies that inhibit androgen receptor (AR) are needed for treatment of castration-resistant prostate cancer (CRPC). The ErbB3 binding protein 1 (EBP1) reduces protein expression of both AR and its target genes in CRPC. Although EBP1 regulates AR in hormone-sensitive prostate cancer cells, by both destabilizing AR mRNA and inhibiting protein translation, the mechanism of EBP1 down regulation of AR in CRPC is unknown. Materials and Methods: Western blot and quantitative PCR analysis of cell lysates and polysomes were used to assess AR mRNA, protein expression and translation. Results: In contrast to hormone-dependent cells, EBP1 did not change steady state levels of AR mRNA or AR mRNA stability in hormone refractory cells. EBP1 did slow protein translation of AR mRNA. The ErbB3/4 ligand heregulin further diminished AR translation in EBP1-transfected cells, but not in control cells. Conclusion: These studies suggest that one pathway of EBP1 down-regulation of AR levels may be lost in CRPC.*

Prostate cancer begins as an androgen-dependent tumor that regresses in response to therapies that reduce testosterone concentration. Despite this treatment, the disease progresses to a uniformly fatal hormone-refractory or castration-resistant prostate cancer (CRPC). The androgen receptor (AR) regulates malignant progression in CRPC (1). Thus, new approaches to reduce AR protein expression and silence AR signaling are important for the therapy of CRPC.

ERBB family members and their ligands have been implicated in the development of castration resistance due to

their stimulatory effects on AR function (2). Androgen-independent sublines of human prostate cancer xenografts express higher levels of ErbB2 than androgen-dependent sublines and ectopic expression of *ErbB2* sensitizes cells to extremely low levels of androgen (3). ERBB2/3 kinase activity stabilizes AR protein levels *via* inhibition of ubiquitin-mediated degradation and increased recruitment of AR to AR-responsive promoters (2). ERBB3 has also recently emerged as an important factor in prostate cancer progression. Overexpression of ERBB3 is linked to a less favorable prognosis in prostate cancer (4). A high frequency of nuclear ERBB3 expression is associated with hormone-refractory prostate cancer (5) and metastasis (6).

Our laboratory has demonstrated that EBP1, an ERBB3 binding protein, is an AR co-repressor (7-9). EBP1 expression is significantly reduced in CRPC (10). Ectopic expression of EBP1 in the hormone-refractory LNCaP variant C81 cell line leads to reversion to an androgen-dependent phenotype and to lower expression of AR protein (10, 11). EBP1 affects both transcription and post-transcriptional regulation of the *AR* gene *via* its ability to bind DNA (12), RNA (13) and protein (9). The ability of EBP1 to bind RNA led us to explore its role in post-transcriptional AR regulation. We previously demonstrated that the C-terminal domain of EBP1 bound to both a (UC)-rich region in the 3' UTR of *AR* mRNA that affects mRNA stability (14) and a 5' CAG rich sequence in the coding sequence, predicted to affect protein translation (15). *EBP1* overexpression in hormone-dependent prostate cancer cells resulted in reduced *AR* mRNA steady state levels, mRNA stability and translation (13). However, EBP1 post-transcriptional regulation of AR was not examined in CRPC.

The purpose of this study was to examine whether ectopic expression of *EBP1* in hormone refractory prostate cancer cells would lower AR protein expression *via* destabilizing AR mRNA and slowing protein translation as is found in hormone sensitive cells. In addition, we wished to determine if the ERBB3/4 ligand heregulin (HRG) could destabilize AR mRNA and slow protein translation in hormone refractory cells.

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## Materials and Methods

**Cell culture and reagents.** The generation of C81 cells stably transfected with *EBP1* cDNA was previously described (10). HRGB1 and EGF were purchased from R&D Systems (Minneapolis, MN, USA) and Cell Signaling (Danvers, MA, USA), respectively.

**Linear sucrose gradient fractionation.** Logarithmically growing C81 cells and C81-*EBP1* transfected cells were harvested and cytoplasmic extracts obtained in Triton-X 100 based polysome extraction buffer (13). Sucrose gradient fractionation was performed as described previously (13). Each gradient was fractionated into 1-ml aliquots using a gradient fractionator (Brandel, Gaithersburg, MD, USA) and monitored by optical density measurement ( $A_{254}$ ). Each fraction was diluted with an equal volume of water and RNA was isolated using Trizol (Invitrogen, Carlsbad, CA, USA). AR mRNA levels in individual fractions were measured using quantitative real-time PCR (RT-qPCR) as described below. For analysis of *EBP1*, *EIF2 $\alpha$* , phospho-*EIF2 $\alpha$*  and actin protein expression, 15  $\mu$ l of each fraction was denatured in 15  $\mu$ l of 1 $\times$ Laemmli buffer and analyzed by Western blotting as described below. Where indicated, both C81 vector control and *EBP1*-transfected cells were serum-starved for 24 h and then treated with HRG (20 ng/ml) for 24 h prior to analysis. Results shown represent one of three independent experiments.

**RNA isolation and PCR analysis.** RNA was prepared from C81 vector control or *EBP1* transfected cells untreated or treated with HRG using Trizol reagent (10) or from sucrose gradient fractions as described above. RNA was DNase-treated and converted into cDNA using the AMV reverse-transcription system (Promega, Madison, WI, USA) in the presence of random hexamers (Invitrogen). The cDNA was used for conventional PCR or RT-qPCR for detection of AR mRNA with specific gene primers as previously described (13). A MYIQ real-time PCR detection system and SYBR green PCR mix (Bio-Rad, Richmond, CA, USA) were used to carry out the real-time PCR. The relative quantitation of targeted genes was determined by the comparative  $\Delta\Delta C_t$  (threshold) method using actin as an internal control (13). All data were analyzed from three independent experiments and statistical significance was validated by Student's *t*-test.

**Western blotting analysis.** Western blot analysis of C81 vector control and *EBP1*-transfected cells treated with or without HRG (20 ng/ml) was performed as described previously (16). The *EBP1* antibody was from Millipore (Billerica, MA, USA), the monoclonal anti- $\beta$ -actin and anti-GADPH antibodies were from Sigma (St. Louis, MO, USA), and the monoclonal antibody against AR was from Santa Cruz (Santa Cruz, CA, USA). Polyclonal antibodies against *EIF2 $\alpha$* , phospho-*EIF2 $\alpha$*  (Ser51), p44/42 MAPK, phospho-p44/42 MAPK (Thr202/Tyr204), SAPK/JNK and phospho-SAPK/JNK (Thr183/Tyr185) were from Cell Signaling.

**Statistical analysis.** RT-qPCR assays were performed in triplicate and repeated at least three times and Western blotting assays were repeated three times. All data presented represent one individual experiment. Where appropriate, means comparison were made using a two-tailed Student's *t*-test with alpha equal to 0.05.

## Results

***EBP1* reduces AR protein levels without changing AR mRNA levels in CRPC.** Previous work from our laboratory showed that ectopic expression of *EBP1* reduced the levels of AR protein in C81 hormone refractory cells (10). However, microarray data indicated that the steady-state level of AR mRNA in *EBP1* transfectants was not changed (10). In contrast, we previously found that *EBP1* reduced the steady state level of AR mRNA in LNCaP hormone-responsive cells and that HRG enhanced this decrease (13). We therefore tested the effects of *EBP1* overexpression and HRG treatment on steady state-levels of AR mRNA and protein in hormone refractory C81 vector and *EBP1* transfectants. We found, as previously shown (10), that AR protein levels decreased in *EBP1* transfectants as compared with vector controls (Figure 1A and B). HRG increased the levels of AR protein in C81 vector control cells and slightly decreased AR expression levels in *EBP1* transfectants (Figure 1A and B). In keeping with previous data (10), we found that mRNA levels of AR were approximately equal in C81 vector control and *EBP1*-transfected cells. Neither EGF nor HRG affected AR mRNA levels in either control or *EBP1*-transfected cells (Figure 1C). Neither ectopic expression of *EBP1* nor HRG treatment affected AR mRNA decay (data not shown), in keeping with data on AR mRNA expression levels.

***EBP1* affects AR mRNA association with polysomes.** As ectopic expression of *EBP1* reduced AR protein but not mRNA levels, we examined the effects of *EBP1* overexpression and HRG treatment on AR protein translation.

Equal amounts of cytoplasmic extracts of logarithmically growing C81 vector and C81 *EBP1*-transfected cells were fractionated by sucrose gradient centrifugation. Absorbance readings at  $A_{254}$  indicated the distribution of monosomes and polysomes. The pattern was comparable in C81 vector control (Figure 2A, left panel) and C81 *EBP1* transfectants (Figure 2A, right panel), indicating that overexpression of *EBP1* did not trigger significant changes in general protein translation. Western blot analysis of the different fractions of the gradient demonstrated the association of *EBP1* with 40S, 60S and 80S ribosomes. *EBP1* co-purified with elongation-initiation factor 2 $\alpha$  (*EIF2 $\alpha$* ), a component of the translation initiation complex, as has been previously reported in HeLa cells (17). *EIF2 $\alpha$*  was not phosphorylated in either C81 vector or *EBP1*-transfected cells (Figure 2B).

The relative abundance of AR mRNA in each polysome fraction was next used to measure the degree of engagement of AR mRNA with the translational apparatus. The percentage of AR mRNA in actively translating polysomes declined markedly following stable transfection of exogenous *EBP1* cDNA (Figure 2C, left panel), in keeping with the observation that AR protein levels are reduced in *EBP1*-transfected cells

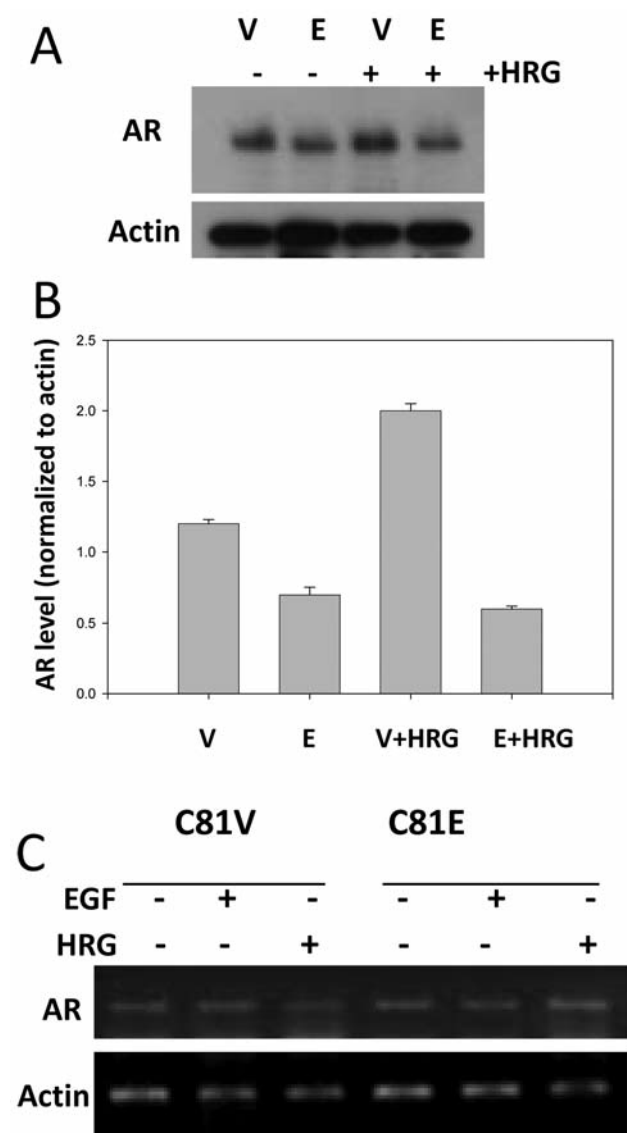


Figure 1. Differential effects of HRG on AR expression in C81 vector and EBP1 transfectants. A: C81 vector (V) and EBP1-transfected (E) cells were serum-starved overnight and treated with HRG $\beta$ 1 (20 ng/ml) for 24 h. Cell lysates were analyzed by Western blotting for expression of AR and actin as indicated. A representative Western blot of three independent experiments is shown. B: Densitometric analysis of the blot in A. C: Total RNA was extracted from vector control and EBP1 transfected C81 cells treated with HRG (20 ng/ml, 24 h) or EGF (50 ng/ml, 24 h) to detect steady-state levels of AR and actin mRNA by RT-PCR. A representative gel of three independent experiments is shown.

as compared to controls. The polysome profile of a control house-keeping transcript (actin mRNA) was not significantly altered in C81E cells as compared to C81V cells (Figure 2C, right panel). This finding suggests that ectopic expression of EBP1 reduces AR protein translation.

HRG attenuates AR mRNA translation only in EBP1 transfected cells. To investigate if HRG can regulate the association of EBP1 with polysomes, C81 vector and EBP1-transfected cell lines were treated with HRG and processed as described above. HRG treatment did not change the polysomal distribution of EBP1, EIF2 $\alpha$  or actin in either cell line as compared to untreated cells. HRG treatment induced the phosphorylation of EIF2 $\alpha$  in both C81 vector control and EBP1-transfected cells. However, the phosphorylation of EIF2 $\alpha$  was more marked in the EBP1 transfectants than in vector controls (Figure 3B).

AR mRNA continued to be associated with actively translated polysomes in C81 vector control cells in the presence of HRG (Figure 3C). In contrast, the percentage of AR mRNA associated with the non-translated fractions was about 50% greater than in untreated EBP1 transfected cells as determined using area under the curve software (PRISM, Irvine, CA, USA) (Figures 2C and 3C, left panels). The association of actin mRNA with polysomes was not significantly reduced by HRG treatment in either vector or EBP1 transfected cells (Figure 3C, right panel).

Effects of ectopic expression of EBP1 on EIF2 $\alpha$ , mitogen-activated protein kinase (MAPK) and Jun-N-terminal kinase (JNK) phosphorylation after HRG treatment. As EIF2 $\alpha$  phosphorylation levels were higher in polysome fractions of EBP1 transfectants after HRG stimulation, we examined the activation status of HRG-regulated kinases that may affect EIF2 $\alpha$  phosphorylation. A recent study demonstrated that inhibition of MAPK phosphorylation is associated with decreased stability of mitogen-activated kinase phosphatase 7 (MKP-7), a JNK phosphatase. The decreased activity of this phosphatase results in increased activation of JNK. The activation of JNK results in phosphorylation and subsequent inhibition of protein-phosphatase 1 (PP1), an EIF2 $\alpha$ -directed phosphatase. The inhibition of PP1 activity leads finally to increased phosphorylation of EIF2 $\alpha$  (18). We therefore examined the ability of HRG to induce phosphorylation of MAPK and JNK in C81 vector control and EBP1 transfectants. We showed, using total cell lysates, that HRG stimulation resulted in greater EIF2 $\alpha$  phosphorylation in EBP1 transfectants as compared to vector controls. Ectopic expression of EBP1 resulted in inhibition of HRG-stimulated phosphorylation of MAPK. In contrast, JNK phosphorylation was increased in response to HRG in EBP1-overexpressing cells as compared to vector controls (Figure 4).

## Discussion

EBP1 is an AR mRNA binding protein that reduces AR mRNA levels in hormone-dependent prostate cancer cells by promoting AR mRNA decay and inhibiting protein translation (13). In this study, we examined if ectopic expression of EBP1 could also down-regulate AR in

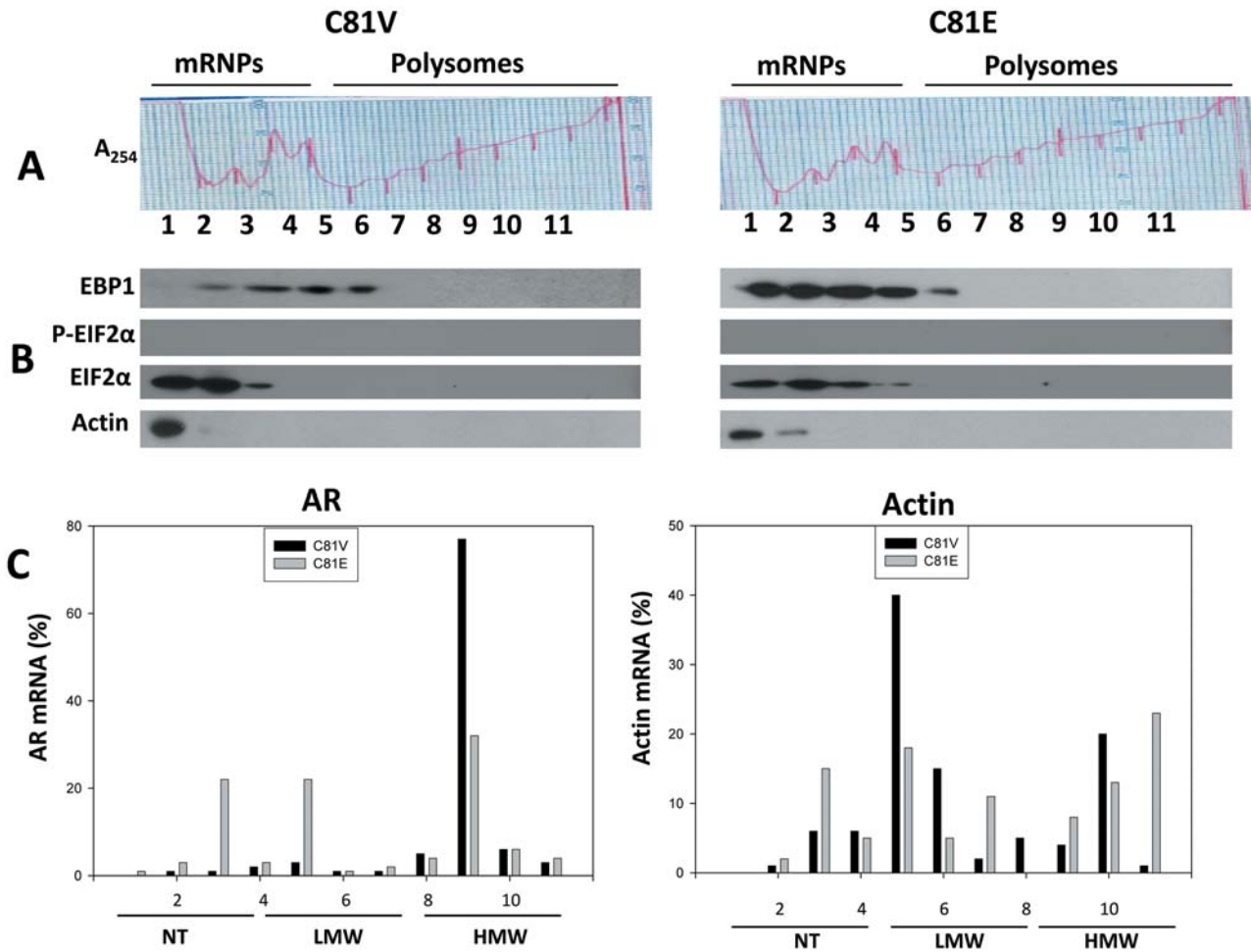


Figure 2. Overexpression of EBP1 reduces AR mRNA translation. A: Cytoplasmic extracts of C81 vector (left) and C81 EBP1 transfected cells (right) growing in logarithmic culture conditions were fractionated by centrifugation on 10-50 w/v sucrose gradients. Eleven fractions were collected and absorbance at 254-nm was recorded. B: Western blot analysis was used to measure the levels of EBP1, p-EIF2α, EIF2α, and actin in each fraction from C81 vector control (left panel) and C81 EBP1-transfected (right panel) cells. C: The levels of AR and actin mRNAs in each gradient fraction were measured by RT-qPCR and plotted as a percentage of the total AR (left panel) or actin mRNA levels (right panel) in that sample. The translational activity associated with each fraction is indicated as NT (not translated), LMW (low molecular weight polysomes, moderately translated) and HMW (high-molecular-weight polysomes, actively translated) as previously described (13). Data represent one of three independent experiments showing similar results.

hormone-refractory cells. We found, in contrast to what is observed in hormone-dependent cells, that EBP1 inhibited AR translation but had no effect on steady-state levels of AR mRNA or mRNA stability in hormone-refractory cells. We suggest that the inability of EBP1 to destabilize AR mRNA may play a role in the development of CRPC.

The reasons for the failure of EBP1 to destabilize AR mRNA and reduce steady-state mRNA levels in hormone-refractory cells are unknown. EBP1 is likely to act as part of a complex to destabilize AR mRNA and key co-regulatory RNA-binding proteins such as HuR and poly-C binding proteins 1 and 2 (CP1 and CP2) (14) may be missing in

hormone refractory cells. It is also possible that the 3'UTR regulatory element in AR mRNA, important to its stability, is mutated in CRPC.

However, as in hormone-dependent LNCaP cells, overexpression of EBP1 in hormone-refractory C81 cells resulted in a shift of AR mRNA towards translationally inactive ribosomes. This finding suggests that EBP1 might inhibit translation initiation of AR mRNA. EBP1 cosediments with 40S, 60S and 80S ribosomes in CRPC cells, consistent with previous reports that EBP1 is part of ribonucleoprotein protein translation complexes in HeLa cells (17, 19). Furthermore, our previous finding that EBP1

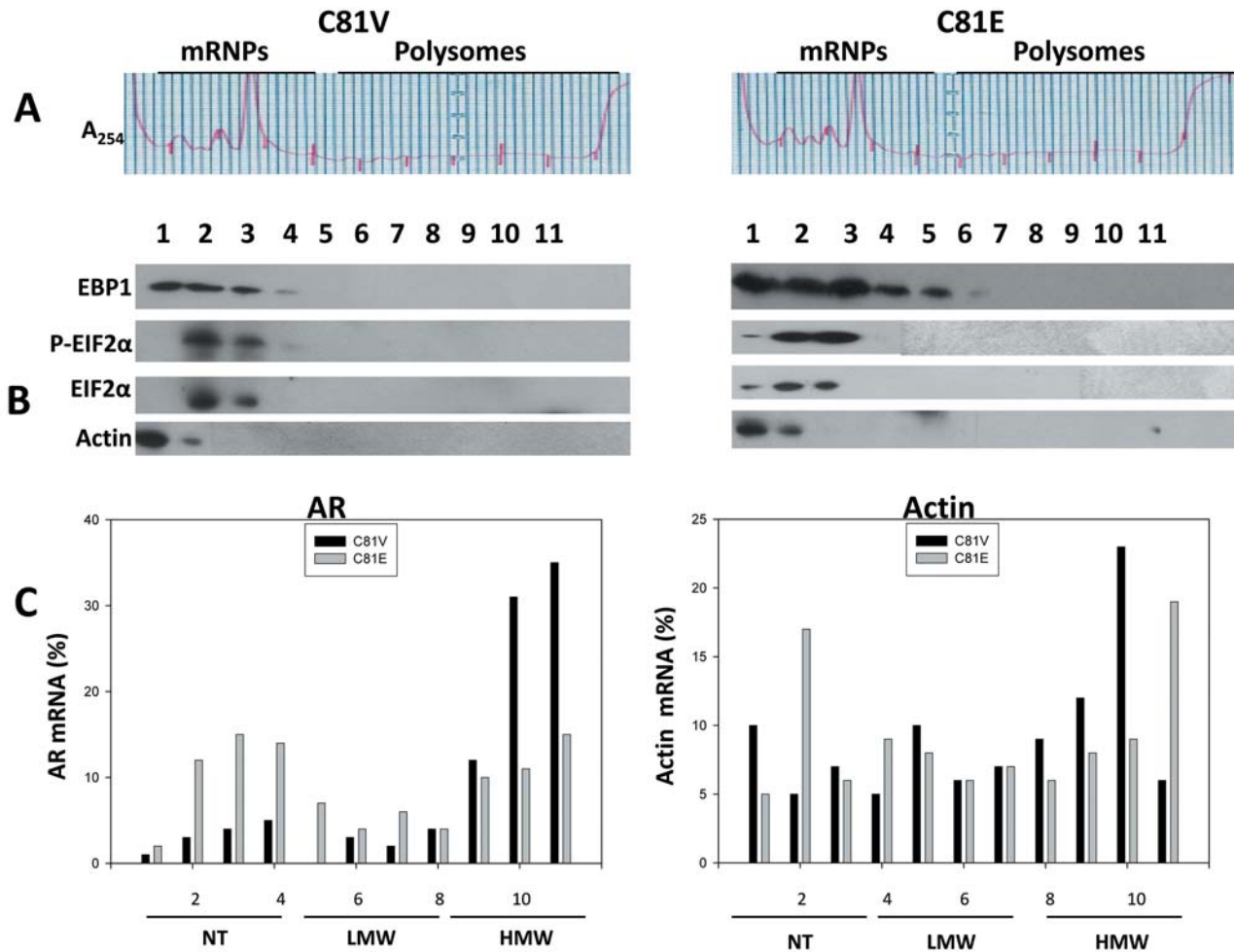


Figure 3. *HRG* attenuates *AR* mRNA translation only in *EBP1* transfected cells. *C81* vector and *EBP1*-transfected cells were serum-starved overnight and then treated with *HRG* (20 ng/ml) for 24 h. Cell lysates were processed as described in the Materials and Methods. **A:** Absorbance profiles at 254-nm. **B:** Western blot analysis of polysome fractions. **C:** The levels of *AR* and actin mRNAs in each gradient fraction were measured by RT-qPCR and plotted as a percentage of the total *AR* (left panel) or actin mRNA levels (right panel) in that sample. Data represent one of three independent experiments showing similar results.

binds a CAG trinucleotide repeat at the 5'-end of *AR* exon 1, predicted to form a stable stem-loop structure and regulate *AR* translation, supports this view (13). The fact that *EBP1* binds viral internal ribosome entry sites supports its potential role in protein translation (20, 21).

We also found that in contrast to hormone-dependent cells, *HRG* increased *AR* protein levels in control *C81* cells, but slightly reduced *AR* protein in *EBP1* transfectants. Similarly, Cai *et al.* (22) found that *HRG* failed to affect *AR* levels in *CWR-R1* hormone-refractory cells. These findings suggest that the inability of *HRG* to reduce *AR* protein levels may be a hallmark of the hormone-refractory phenotype. *HRG* was able to attenuate *AR* mRNA translation in *EBP1*-overexpressing cells, but

not in vector controls that express *EBP1* at extremely low levels (10). Similarly, we observed that knock-out of *EBP1* in hormone-dependent LNCaP cells results in the inability of *HRG* to attenuate *AR* mRNA translation (13). Our findings suggest that *HRG* affects *AR* translation *via* an *EBP1*-mediated pathway. Another example of ERBB ligand regulation of *AR* mRNA translation is the HB-EGF-induced decrease of *AR* mRNA translation (23). The HB-EGF effect is mediated by the RNA-binding protein, hnRNP-K which, as in our study, binds sequences within the *AR* coding region that leads to reduced translation rates (24).

Consistent with the reduced translation rate, we found that phosphorylation and thus inactivation of *EIF2α* in response to *HRG* was enhanced in *EBP1*-transfected *versus*

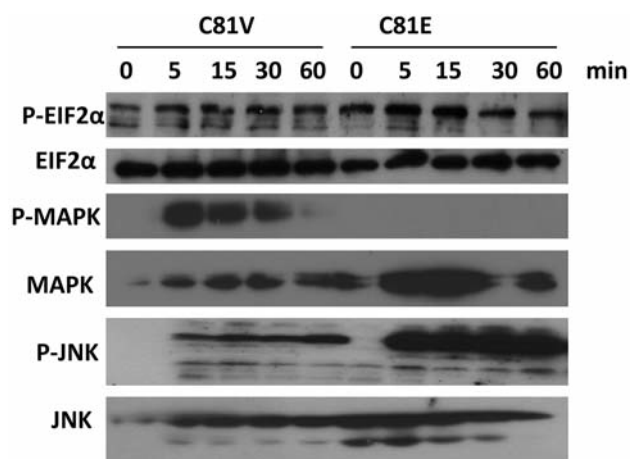


Figure 4. Effects of ectopic expression of EBP1 on EIF2 $\alpha$ , MAPK and JNK phosphorylation after HRG treatment. C81 vector control and EBP1-transfected cells were serum-starved overnight and then incubated with or without heregulin (20 ng/ml) for the times indicated. Whole-cell extracts were prepared and resolved as described in the Materials and Methods. Levels of phosphorylated and total EIF2 $\alpha$ , MAPK, and JNK were determined by Western blotting.

control cells. The mechanism of the HRG enhancement of EIF2 $\alpha$  phosphorylation is not known. However, our results indicated that EBP1 overexpression inhibited HRG-induced MAPK phosphorylation and increased activation of JNK. It is known that JNK phosphorylates and inactivates the EIF2 $\alpha$  phosphatase PP1, resulting in enhanced phosphorylation of EIF2 $\alpha$  (25). Thus, the increased activation of JNK in EBP1 transfectants after HRG stimulation may have led to enhanced EIF2 $\alpha$  phosphorylation.

In summary, our study suggests that EBP1 can inhibit AR translation in a model of CRPC, although its ability to destabilize AR mRNA is lost. The loss of a major mode of AR regulation in CRPC may contribute to malignant progression.

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