

**TRIPTOLIDE SUPPRESSES C-MYC EXPRESSION THROUGH REGULATION OF ITS ASSOCIATED  
TRANSCRIPTIONAL FACTORS AND COACTIVATORS**

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## Abstract

Pancreatic cancer is the fourth leading cause of death among all cancers. Lack of effective screening and treatment modalities leads to poor prognosis and high mortality. For these reasons new therapeutic regimens are desperately needed for the treatment of pancreatic cancer. Minnelide, which is a prodrug of triptolide, is currently being evaluated as a potential drug therapy for pancreatic cancer. Early preclinical test has shown that triptolide is highly effective of reducing tumor progression and metastases. Despite showing clinical efficacies the mechanism of triptolide is not fully understood.

One of the targets of triptolide reported is c-Myc. c-Myc has been associated with various aggressive tumors including pancreatic cancer. We hypothesize that triptolide maybe suppressing the expression of c-Myc by inhibiting the binding of its transcription factors or coactivators to the promoter and/or enhancer regions of the c-Myc gene. The Far UpStream Element binding protein 1 (FBP1) is a potential regulatory element that may be affected by triptolide.

In this study, we investigated whether triptolide regulates c-Myc expression by inhibiting FBP-1 protein expression at the transcription and/or protein level. We performed an RNA sequence to determine global regulation of transcriptome in response to triptolide treatment. In addition, we determined the effects of triptolide on c-Myc, FBP1 and FIR protein expression. We found that 100nM of triptolide inhibited gene expression of c-Myc, FBP1, FIR and XBP by 2.4, 1.3, 3.5, and 1.4 fold respectively ( $p < 0.05$ ,  $n = 2$ ). This finding is consistent with a decrease in protein expression of c-Myc (53%) and FIR (83%) only. There was no effect of FBP-1 protein expression with triptolide treatment. This data suggests that inhibition of c-Myc expression is independent of FBP-1 expression however this does not rule out the possibility of triptolide ability to alter the activity of FBP-1 preventing it from binding to TFIIH at the active XPB subunit. An attempt to create a XPB CRISPR knockout clone resulted in a potentially lethal mutation in PC cells. Additional studies are further needed to elucidate the mechanisms of triptolide but, these results suggest that XPB may serve as an effective target for future therapy.

## Table of Contents

Introduction .....	1
Methods .....	3
Results .....	5
Discussion.....	10
References .....	12

## List of Tables and Figures

Table 1: RNA sequencing of MiaPaCa1 treated with 100nM triptolide for 24 hours compared to untreated cells.....	7
Figure 1: MiaPaca2 treated with triptolide [0 – 100nm] for 6 hours and accessed for protein expression .....	8
Figure 2: Functional validation of HEK293T transfection with PsP-Cas9 sgRNA clone using surveyor assay to detect single base mismatch .....	9

## Introduction

Each year approximately 43,000 new cases of pancreatic cancer (PC) are reported in the United States, which accounts for about 2.8% of all cancers<sup>1</sup>. PC has one of the highest mortality rates among all malignancies. The National Cancer Institute estimates that the mean survivability for PC patients to be approximately 3 - 6 months and the 5-year survivability rate to be 6.7%<sup>1</sup>. Lack of effective screening and treatment modalities leads to poor prognosis and high mortality.

Surgical resection is the ideal course of action for PC. However, surgical candidacy highly depends on the location of the tumor (i.e. pancreatic head, body, tail or ampulla), presence of metastases and the degree of vascular invasion<sup>2,3</sup>. Less than 20% of diagnosed pancreatic tumors are resectable<sup>4</sup>. Chemotherapy with Folfirinox or combination treatment with gemcitabine and nab paclitaxel is the current recommended chemotherapeutic treatment for PC. Clinical studies have shown that current treatments utilized for PC provide poor objective response rates and does not significantly improve survival<sup>5</sup>. Thus, new clinical studies are desperately needed to define better treatment strategies for this lethal disease.

Triptolide is a natural compound that is extracted from a Chinese medicinal plant, *Tripterygium wilfordii*. Triptolide has been shown to induce apoptosis in a number of cancer cells including pancreatic cancer<sup>6-8</sup>. Early preclinical studies for minnelide, a prodrug for triptolide has shown promising results for treating pancreatic cancer. Data have reported as much as 60% reduction in tumor size in animal studies<sup>9,10</sup>. Triptolide has been implemented in down regulation of proliferation by inhibiting pro-mitotic growth factor VEGF and COX-2<sup>11</sup>. It also induces cellular apoptosis by down regulation of heat shock protein 70 by disrupting the activity of Sp1 and increase expression of miR142-3p<sup>12,13</sup>. Triptolide is currently under phase I clinical trials. However, despite showing clinical efficacies with strong specificity to tumor cells the mechanism by which triptolide induces cell death of pancreatic cancer needs to be further explored in order to refine optimal treatment strategies for PC.

The transcriptional factor, c-Myc has been associated with various aggressive malignant tumors including pancreatic cancer<sup>14</sup>. High level expression of c-Myc has been shown to cause increase binding of pro-mitogenic transcriptional factor to their respective promoters and enhancer targets thereby causing gene amplification in tumor cells<sup>15</sup> to promote cell growth and metabolism<sup>16</sup>. Normal binding of FBP1 to the FUSE sequence upstream of the c-Myc promoter brings FBP1 into the proximity of the transcription machinery and interacts with transcription factor 2 helicase (TFIIH) and optimizes transcription causing an upsurge in c-Myc expression<sup>17</sup> during mitosis. The subsequent rise in FBP1 binding in turn activates a negative feedback loop and recruit the FBP1 interacting repressor (FIR) which binds XPB; a helicase subunit of TFIIH thereby hindering TFIIH from activation by FBP1 and inhibit the expression of c-Myc to restore baseline. Small changes in FBP-FIR affinity can lead to drastic unregulated expression of c-myc thus leading to rapid cell proliferation and pathogenesis. In this study we investigated whether triptolide regulates c-Myc expression by inhibiting FBP1 protein expression at the transcription and/or protein level.

## **Methods**

### ***Cell Culture***

Experiments were conducted using commercially available MIA-PaCa2 cell lines purchased from American Type Culture Collection grown in DMEM supplemented with 10% FBS and 0.1% antibiotics. Cells were passaged every 3 days at a ratio 1:3. All experiments were conducted with cells passage less than 12.

### ***RNA Sequencing***

To determine the global gene regulation by triptolide on the super-enhancer region of c-Myc, we performed RNA sequencing on MIA-PaCa2 cell lines for both untreated and treated with triptolide. PC cells were seeded at 500,00 cells per well for 24 hours on a 6 well plate and were then treated with 100nM of triptolide for 6 hours. RNA isolation and cDNA library were obtained using the Illumina protocol: TrueSeq RNA sample preparation v2 LS. Samples were then sequenced using Novogene RNA-sequencing services. Sample size consisted of n=2 and data were analyzed using DEseq analysis software.

### ***c-Myc Expression***

We are particularly interested in whether any potential anti-mitogenic effect of triptolide is caused by the down regulation of c-Myc. To measure the expression level of c-Myc we determined both the mRNA expression and protein level of c-Myc in untreated and triptolide treated cultures via RT-PCR and Western blotting, respectively. We used c-Myc primers obtained from Qiagen (Quantitect primer assay) gene bank NM\_002467 (Forward Primer = AAACACAACTTGAACAGCTAC) for PCR quantification. Anti-c-Myc (Y69) rabbit monoclonal from Abcam product# ab32072 was used for total protein expression of c-Myc.

### ***Expression of FBP and FIR***

The binding of FUBP to the FUSE sequence upstream of c-Myc promoter optimizes and enhances its transcription. To determine if the level of FUBP is affected by triptolide we evaluated this regulation at a transcription and translation level. Triptolide treated samples



were assessed for RNA and protein expression levels using RT-PCR and Western blotting for FBP and FIR and XBP (ERCC3). Primers were obtained from Qiagen (Quantitect primer assay) and anti-FBP1 antibody was from Pierce-antibodies (# PA5-30291).

### ***XPB KO Mia-PaCa2 cells using CRISPR***

Methods used were adapted from Ran et al. published in Nature Protocol<sup>18</sup>. Plasmid were obtained from Addgene (plasmid ID: 48138); pSpCas9(BB)-2A-GFP. Oligos sgRNA sequence targeting XPB at exon 9 Fwd: 5'-CACCGGCTCCATATTCATCCACTT-3'; Rev: 5'-AAACAAGTGGATGAATATGGAGC-3' (D54H). Cas9-D54H constructs were sequenced using a U6 forward primer to verify clone sequence containing guide RNA. Functional validation was performed by transfecting HEK293FT cells and detecting indel mutations by surveyor nuclease assay. Mia-PaCa-2 cells were then transfected to establish a XPB KO line and treated with triptolide and assessed for resistance.

## Results

### ***Triptolide suppresses c-Myc expression at the mRNA level***

We determined the effects of triptolide on global gene regulation by performing RNA sequencing on MIA-PaCa2 cells treated with 100nM for 24 hours. We found that triptolide decreased mRNA expression by 2.4-fold when compared to control (Table 1:  $p < 0.001$ ,  $n = 2$ ). There was also a significant decrease in the mRNA expression of ERCC3 (XPB), PUF60 and FBP1 by 1.4, 3.5 and 1.3-fold respectively.

### ***Triptolide downregulates the expression of c-Myc, FUBP and FIR at the protein level***

We next investigated whether the effects of triptolide on gene activity is correlated with regulation of translation into mRNA and protein expression. We found that 100nM of triptolide decreased c-Myc protein expression by 53% (Figure 1A:  $p < 0.05$ ,  $n = 3$ ) when compared to control. Doses less than 10nM had no effect on protein expression. However, at doses above 25nM we observed a negative dose response (Figure 1 panel D).

FBP-1 is a known enhancer of c-Myc which binds the far upstream element in the promoter region approximately 1.5kb upstream from the start codon. No significant differences were seen in FBP-1 protein expression with any of triptolide doses used (Figure 1B). There was a decreasing trend in FIR protein expression with an 83% reduction seen with 100nM triptolide treatment (Figure 1C:  $p < 0.05$   $n = 3$ ).

### ***Knock out of XPB in Mia-PaCa2 pancreatic cancer cells is potentially lethal***

Next, we wanted to determine whether triptolide could inhibit the ability of FBP-1 to bind to XPB, the active site of TFIIH. We used CRISPR to create a knockout mutant which would potentially create a resistant strain of pancreatic cancer cells to triptolide. Transfected HEK293T cells treated with endonuclease resulted in a mismatch sequence as indicated by a tertiary band at 350kb and 250kb in lane PsP-Cas9 sgRNA + endonuclease compared to no nuclease. (Figure 2A black arrow). Transfection of HEK293T control cells resulted in a low transfection rate of approximately 10% (Figure 3D). However, when attempting to transfected MiaPaca2

cells we observed poor cell morphology with high level of detached cells transfected with Cas9 clone (Figure 2C black arrow). We were unable to detect fluorescence marker post transfection of MiaPaCa2 (Figure 2E).

Table 1: RNA sequencing of MiaPaCa1 treated with 100nM triptolide for 24 hours compared to untreated cells.

Gene	Control Log CPM	Triptolide Log CMP	Fold change	P adj
C-Myc	4422.2	1796.8	-2.4	3.5 e-80
ERCC3 (XPB)	9792.5	7172.9	-1.4	3.0 e-20
PUF60 (FIR)	13822.1	4088.4	-3.5	2.8 e-185
FBP1	2874.2	2125.7	-1.3	2.5 e-09

Abbreviations: C-Myc ; ERCC3 sequence for protein XPB an essential subunit for transcription II helicase . FBP1 = FUSE binding protein, PUF60 = poly U binding splicing factor 60 which codes for FBP interacting repressor. RNA sequence data was statistically analyzed using DESeq2 with an N=2. CPM = count per million.

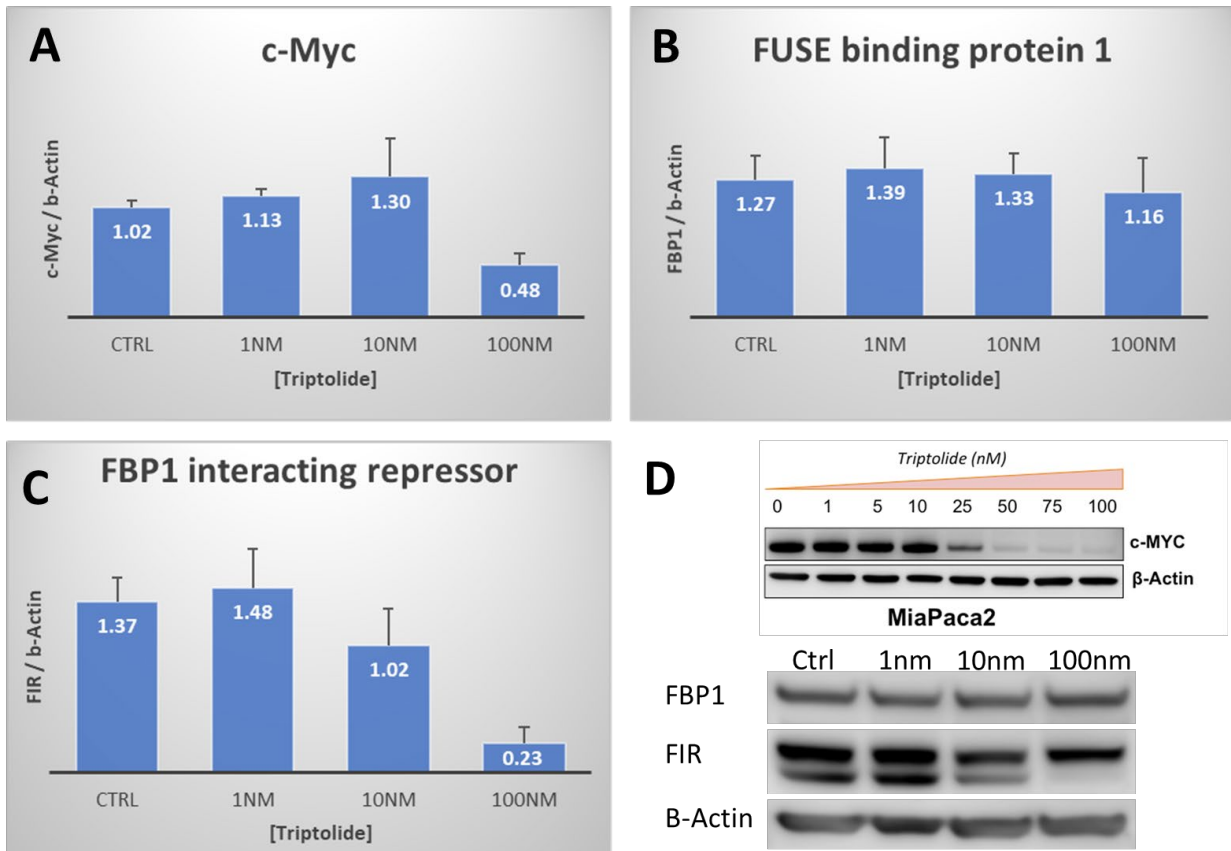


Figure 1: MiaPaca2 treated with triptolide [0 – 100nm] for 6 hours and accessed for protein expression. Presented as mean $\pm$ SEM of protein / b-Actin ratio A.) C-Myc, B.) FBP-interacting repressor (FIR) and C.) far upstream element binding protein (FBP-1) in response to treatment with 1nm, 10nm and 100nm of triptolide. \*  $p < 0.001$  vs. Control. Panel D shows representative Western bands for FBP1, FIR and control B-actin treated with 0-100nM of triptolide (D).

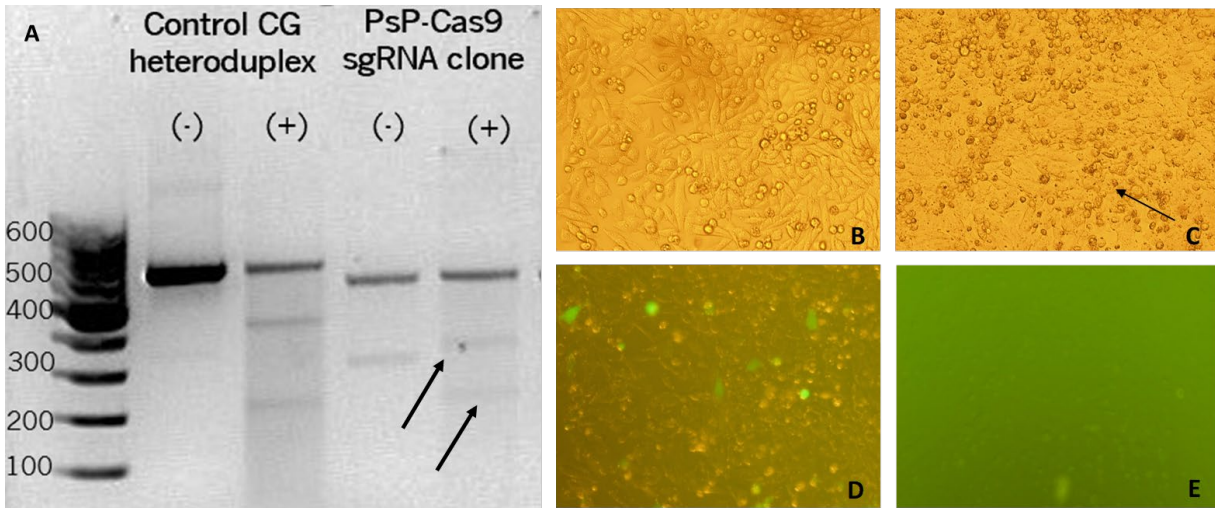


Figure 2: Functional validation of HEK293T transfection with PsP-Cas9 sgRNA clone using surveyor assay to detect single base mismatch (Figure 3A lanes 4 and 5). (+) signifies the addition of endonuclease (Panel A lanes 3 and 5) and absence (-) of endonuclease (Figure 3A lanes 2 and 4). Transfection control: HEK293T (Figure 3B, 3D) and MiaPaCa1 (Figure 3C, 3E) with PsP-Cas9 clone of image by light microscopy and fluorescence overlay. Arrow in Figure 3C shows round cells detached from the culture plate with poor morphology.

## Discussion

Triptolide has been shown to have various anticancer effects in a number of malignancies including pancreatic adenocarcinoma (PAC). Studies have shown that triptolide antimitogenic effects are associated with dysregulation of heat-shock protein, caspases, NF- $\kappa$ B, c-Myc and also enhances the synergistic effects with other chemotherapeutics. However, despite these findings the exact mechanism still remains elusive. To our knowledge this study is the first to investigate super-enhancers as a potential mechanism of triptolide regulating c-Myc in PAC.

Dysregulation and enhanced expression of c-Myc has been well characterized in pancreatic adenocarcinoma<sup>19</sup>. In this study investigated the far upstream binding protein 1 (FBP-1) as a potential target of triptolide. Normally binding of FBP-1 to the Far upstream element (FUSE) upstream of the c-Myc promoter brings FBP-1 into the proximity of the transcription machinery and interacts with TFIIH and optimizes transcription. This subsequent rise in FBP-1 binding in turns activates a negative feedback loop and recruit the FBP interacting repressor which binds XPB; a helicase subunit of TFIIH thereby hindering TFIIH from activation by FBP.

We observed that treating PC with 100nM of triptolide for 6 hours was effective in reducing c-Myc protein expression by 2.4 fold ( $p < 0.05$ ,  $n = 3$ ) when compared to no treatment. This finding was consistent with decreasing protein expression of c-Myc by 53%. We found that although triptolide decreased the mRNA level of FBP-1 this did not result in decreased protein expression. This suggest that triptolide regulation of c-Myc may not be dependent on the expression of FBP-1. Treatment with 100nM triptolide significantly suppressed FIR expression at the transcription level. This suggests that although triptolide may not significantly inhibit FBP-1 expression, it may be affecting its ability to interact with TFIIH at a sufficient level to potentiate the negative feedback loop of FIR. XPB maybe a potential target of triptolide where we also saw a decrease in mRNA expression.

To investigate whether triptolide has a direct effect on XBP and or FBP-1 ability to interact with XPB we attempted to create an XPB knock clone using CRISPR. We were successful in ligating a single guide RNA into a pSp-Cas9 plasmid containing a GFP marker. Using this plasmid clone we were able to transfect 293T cells and introduce the mutation; however, attempts to transfect MiaPaCa2 cell lines have been unsuccessful (Figure 2). It is possible that the mutation introduced into MiaPaCa2 creating an XPB KO may prove to be lethal mutation however, it is important to note that XPB function is necessary but not essential for cell replication. This study supports that triptolide may be acting on the super enhancer FBP-1 in regulating c-Myc expression and may serve as a potential target as a potential therapeutic modality for PAC treatment. Despite these finding additional studies are further needed to elucidate the mechanism behind the anticancer effects of triptolide.



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