

Post-transcriptional regulation of ABC transporters by the RNA binding protein IGF2BP1 in HCC.

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Abstract

The overexpression of ABCB1 and ABCC3 is a major factor contributing to multidrug resistance (MDR) in tumours, including hepatocellular carcinoma (HCC). IGF2BP1, the most strongly upregulated RNA-binding protein in human HCC compared to normal liver, is also overexpressed in HCC. Establishing a connection between chemoresistance development associated with ABC transporters and IGF2BP1 would significantly contribute to understanding MDR. Bioinformatics analysis revealed a correlation between the overexpression of IGF2BP1, ABCB1, and ABCC3 and poor prognosis in HCC. This study aimed to elucidate if IGF2BP1 regulates the expression of ABC transporters in HCC and the molecular mechanisms involved under constitutive conditions or after treatment with sorafenib (SFB). Transient silencing of IGF2BP1 in HepG2 cells resulted in reduced protein and mRNA levels of ABCB1 and ABCC3. IGF2BP1 depletion also decreased total ABCB1 and ABCC3 mRNA levels, and in the presence of ActD, a general inhibitor of transcription, the effect persisted, suggesting post-transcriptional regulation. The RPISeq algorithm predicted interactions between IGF2BP1 and the mRNA sequence of ABC transporters, particularly with the 3' UTR region of ABCB1. RBPmap predicted a potential binding motif for IGF2BP1 in the ABCB1 nucleotide sequence. SFB treatment induced simultaneous upregulation of IGF2BP1, ABCB1, and ABCC3, and IGF2BP1 silencing led to decreased mRNA levels of ABCB1 and ABCC3. Results suggest that IGF2BP1 plays a key role in the posttranscriptional regulation of ABC transporters in HCC cells, both constitutively and in response to SFB treatment, positioning it as a potential therapeutic target to minimize MDR.

Keywords: IGF2BP1, ABCB1, ABCC3, SORAFENIB, MULTIDRUG RESISTANCE, HEPATOCARCINOMA.

Abbreviations:

ABC: ATP-binding cassette ABC; ActD: Actinomycin; DMEM: Dulbecco's modified Eagle's medium; EX: EX-527; FBS: foetal bovine serum; HCC: Hepatocarcinoma; IGF2BP1: Insulin-like growth factor 2 mRNA-binding protein 1; MDR: multidrug resistance; MRP: Multidrug resistance-associated protein; mTOR: mammalian Target of Rapamycin; MTT: 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; P-gP: P-glycoprotein; SFB: sorafenib; SIRT: Sirtuin.

1. Introduction

Hepatocarcinoma (HCC) is a type of malignant tumour that derives from the parenchymal cells of the liver, the hepatocytes [1]. To date, a pattern of genetic alterations linked to the development of HCC has not yet been established, requiring a multidisciplinary approach to therapy for favourable outcomes [2,3]. Despite advancements in diagnostic and treatment technologies, the incidence and mortality of HCC continue increasing [3]. The majority of patients are diagnosed in advanced to terminal stages and die within 3 to 6 months after diagnosis [3].

After its approval in 2007, sorafenib (SFB) became the pioneering systemic targeted agent for first-line treatment of advanced HCC. However, it presents low efficacy due to multidrug resistance (MDR) that leads to poor patient survival. Although alternative therapies have been approved recently, SFB remains being the most prescribed drugs for HCC treatment [4,5]. One of the most critical and well-characterised mechanisms of MDR involves the overexpression of ATP-binding cassette (ABC) efflux transporters in cancer cells, such as ABCB1 (also known as P-glycoprotein; P-gP) and ABCC3 (also known as multidrug resistance-associated protein 3; MRP3). These transporters pump chemotherapeutic drugs out of the cell, thus reducing their intracellular concentrations [6,7]. Sirtuins (SIRTs), a family of enzymes histone deacetylases are also implicated in MDR, with SIRT1 and SIRT2 being overexpressed in HCC cell lines and in a subset of human HCC tissues compared to normal hepatocytes and non-tumour tissues [8]. It has been described that SIRT1 overexpression in HCC cells induces the upregulation of ABCB1 and that SIRT2 regulates another ATP binding cassette transporter expression in other cell line: ABCC1 (also known as multidrug resistance-associated protein 1; MRP1) [9–11].

Insulin-like growth factor 2 mRNA-binding protein 1 (IGF2BP1) is an RNA-binding protein involved in physiological and pathological processes, including embryogenesis and carcinogenesis, controlling the expression of multiple genes [12]. IGF2BP1 displays an oncofoetal expression pattern typically absent in healthy adult tissues, with a few exceptions [30]. Aberrant expression of IGF2BP1 is implicated in the development and progression of several cancers, often

associated with a poor prognosis [13]. However, its role in chemoresistance remains unknown.

While there is currently no direct evidence linking IGF2BP1 to chemoresistance in HCC, various reports established an association between IGF2BP1 and MDR in other tumour types. For instance, a positive correlation between IGF2BP1 and ABCB1 levels was found in ovarian cancer biopsies [14]. Additionally, microRNA-dependent overexpression of IGF2BP1 has been shown to increase MDR by promoting ABCB1 expression in ovarian cancer cells [14]. Dr. Vore et al. demonstrated that IGF2BP1 overexpression in HepG2 cells increases MRP2 translation [15]. These findings suggest IGF2BP1 as a potential therapeutic target to overcome chemoresistance. Currently, it is not known whether the modulation of IGF2BP1 can be used as therapeutic strategy to counteract ABC transporter overexpression in the context of cancer multidrug resistance, and, in particular, in HCC.

The problem of MDR in tumours is complex and multifactorial. In turn, the origin of the development of multidrug resistance is even more uncertain. Establishing a potential link between the development of chemoresistance associated with ABC transporters and IGF2BP1 would represent a significant contribution to understanding MDR, with a potential therapeutic application. Therefore, the present study is conducted for evaluating whether IGF2BP1 is involved in ABC transporters modulation in HCC and uncovered the putative intracellular pathway involved.

2. Material and methods

2.1. Bioinformatic analysis

2.1.1. Kaplan–Meier analyses

Gene-level RNA-seq reads were obtained from the TCGA-LIHC dataset, The Cancer Genome Atlas - Liver Hepatocellular Carcinoma (<https://www.cancer.gov/tcga>), which includes 368 samples with the following data: normalised expression values (RSEM) for the genes of interest, patient disease-specific survival (DSS) indicators (1 for no event, 0 for an event), and DSS time measured in days. To investigate the impact of combined expression

variations of IGF2BP1, ABCC3, and ABCB1 on the survival probability of a cohort of Liver Hepatocellular Carcinoma (LIHC) patients we employed the survcutpoint function from the R package Survminer to categorise the patients into combined high and low expression groups for each gene. Subsequently, we applied the Kaplan-Meier survival estimator to construct survival curves and assessed significant differences in survival between these groups using a Logrank test ($p < 0.05$).

2.1.2. Prediction of IGF2BP1-ABC transporters interaction

To globally assess the interaction probability between IGF2BP1 and the ABC transporters of interest, ABCB1 and ABCC3, we used different bioinformatics tools. We first evaluated the RNA-binding potential of IGF2BP1 to ABCB1 and ABCC3 mRNA using the RPI-Seq software from Iowa State University (<http://pridb.gdcb.iastate.edu/RPISeq>) [16,17]. The software requires two query fields in plain-text format, the amino acid sequence of the protein and a nucleic acid sequence of the target mRNA. The software uses existing interaction databases to predict whether domains within the primary amino acid sequence can bind to the target nucleic acid string. The input of the amino acid sequence of IGF2BP1 was selected from the National Library of Medicine's NCBI website and was inserted into the protein sequence field of the RPI-Seq website. RNA transcripts were also sourced from the NLM NCBI database. The mRNA regions (5'UTR; CDS and 3'UTR) were identified using the USCS Genome Browser program (<http://genome.ucsc.edu/index.html>) and also entered in plain text format into the RNA query field.

For the identification of RNA-protein binding regions we generate an interaction profile and interaction matrix used CatRAPID fragments module, an algorithm based on individual interaction propensities of polypeptide and nucleotide sequence fragments, especially used to evaluate interactions when protein (or RNA) is larger than 750 aa (1200 nt) [18]. This algorithm calculates and assigns an interaction score to each region of the input RNA sequence. Additionally, we identified a possible IGF2BP1 binding motif in the ABCB1 and ABCC3 mRNA sequences using the web-based RBP-binding prediction tool RBPmap (<http://rbpmap.technion.ac.il>) [17,19], that compares the input sequences to

known consensus binding sites of human and mouse proteins and classifies/ranks hits based on their fit to the consensus motif. This tool assigns a z-score and a p-value to each region in which a consensus binding motif was identified.

2.2. Chemicals

SFB was purchased from Cayman Chemical (Ann Arbor, MI, USA). EX-527 (EX), Actinomycin D (ActD), phenyl-methylsulfonyl fluoride (PMSF), pepstatin A, leupeptin and 3-(4,5-dimethyl-2-thiazolyl)-2,5- diphenyl-2H-tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St.Louis, MO, USA). DMSO was purchased from Merck (Darmstadt, Germany). Pierce™ ECL Western Blotting Substrate was obtained from Thermo Fisher Scientific (Waltham, MA, USA). All other chemicals were of analytical grade purity and used as supplied.

2.3. Cell lines and treatments

The human HCC cell lines HepG2/C3A and Huh7 were obtained from the ATCC (American Type Culture Collection; Manassas, VA, USA) and JCRB (Japanese Collection of Research Bioresources) Cell Bank (Tokyo, Japan), respectively. The HepG2/C3A cell line, also called C3A, is clonally derived from the HepG2 cell line. For both cell lines the passage number used was between 20 and 30. The lines were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Life Technologies, Grand Island, NY, USA) with L-glutamine (Gibco) supplemented with 10% foetal bovine serum (FBS; Internegocios S.A., Buenos Aires, ARG), 100 U/mL penicillin (Gibco) and 100 µg/mL streptomycin (Gibco) at 37°C in a humidified atmosphere of 95% O₂ and 5% CO₂.

For treatments with SFB, cells were plated in 6-well plates at a density of 5×10⁵ HepG2/C3A cells/well and 2×10⁵ Huh7 cells/well. After 24 h of attachment, cells were treated for 48 and 72 h with 2 µM SFB for protein expression analysis and for 24 h, 48 h and 72 h for RNA expression analysis. Total RNA was isolated for qRT-PCR or cells were harvested and used in western blot studies.

The SIRTs 1 and 2 deacetylase activity inhibitor EX was used to evaluate the participation of SIRTs 1 and 2 in the induction of IGF2BP1 produced by SFB.

HepG2/C3A cells were seeded in 6-well plates at a density of 5×10^5 cells/well. After 24 hours, the culture medium was replaced with fresh medium containing the following treatments: 1) Control (DMSO), 2) SFB 2 μ M, 3) EX 1 μ M or 40 μ M, and 4) SFB 2 μ M and EX 1 μ M or 40 μ M as applicable (combined treatment), maintained for 72 hours. Subsequently, cells were harvested and utilised in Western blot studies to evaluate the impact of the treatments.

The final concentration of DMSO in the culture media was below 0.1%.

2.4. Cytotoxicity assay

To perform the assay, HepG2/C3A and Huh7 cells were seeded in 96-well plates at a density of 10,000 and 4,000 cells/well, respectively. The next day, cells were treated with different concentrations of SFB (0-32 μ M) for 72 h. After the treatment, the MTT assay was performed.

The MTT reagent was added to the culture medium at a final concentration of 0.5 mg/mL and incubated at 37°C. After 2 h, cells were lysed and formazan crystals were solubilized by the addition of 200 μ L of DMSO and the absorbance of the metabolite produced by viable cells was quantified at 540 nm (with reference filter at 650 nm) on a microplate reader (DTX 880 multimode detector; BeckmanCoulter Inc., Fullerton, CA, USA). From the absorbance data obtained, firstly, SFB concentration-response curves were performed in order to obtain the IC₅₀ (concentration inhibiting 50 % of cell growth) of this compound, using the GraphPad program (GraphPad Software, La Jolla, CA, USA) and, secondly, viability percentages were obtained as a function of SFB (100 % viability was considered for untreated cells).

2.5. siRNA transfection

Three different and specific IGF2BP1 siRNAs and scramble siRNA were synthesised using the Silencer siRNA construction kit (Ambion, Life Technology, Carlsbad, CA, USA). The sequences of template oligonucleotides (DNA) used were designed following the guidelines described by Elbashir *et al.* [20]. Template oligonucleotides were 29 nucleotides in length. The eight nucleotides at the 3' end of each oligonucleotide was 5'-CCTGTCTC-3'. Template sequences for

IGF2BP1 were siRNA 1, sense: 5'-AAATTTGACCAAGAACTGGCC-3', antisense: 5'-AAGGCCAGTTCTGGTCAAAT-3'; siRNA 2, sense: 5'-AATTGCCTGGTCTGCTCCGG-3', antisense: 5'-AACCGGGAGCAGACCAGGCAA-3'; siRNA 3: sense: 5' -AATATATTAGGAGGAGAGGCC-3', antisense: 5' -AAGGCCTCTCCTCCTAATATA-3'; control siRNA, sense: 5'-AATTAGGTCACGGTGGCATAG-3', antisense: 5'-AACTATGCCACCGTGACCTAA-3'. Synthesis conditions were as suggested by the manufacturer.

For siRNA transient transfection, HepG2/C3A cells were seeded at a density of 3×10^5 cells/well in a 6-well plate in antibiotic free DMEM. After 24 h of attachment, cells were exposed to a transfection mixture of 100 nM of the corresponding siRNA with Dharmafect 4 siRNA Transfection Reagent (Dharmacon, Lafayette, CO, USA), both dissolved in OPTIMEM culture medium for 48 h according to manufacturer's instructions.

For treatments with SFB, fresh medium containing SFB or vehicle was added 24 h after transfection was initiated and the cells were incubated for another 48 h.

At the end of the treatment, total RNA was isolated for RT-PCR or cells were harvested and used in western blot studies.

2.6. Transcription inhibition with Actinomycin D

We blocked the transcription with ActD to study the mechanism involved in the regulation of IGF2BP1 and ABC transporters by SFB. HepG2/C3A cells were pre-treated with 5 μ g/mL ActD or its vehicle (DMSO) for 30 min. Then, SFB or its vehicle was added, and cells were further incubated for 48 h. Total RNA was isolated at the end of treatment.

In other sets of experiments, ActD treatment assay was performed for detecting ABC transporters mRNA stability. HepG2/C3A cells were transfected with IGF2BP1-siRNA or C-siRNA for 24h and treated with 5 μ g/ml ActD or its vehicle (DMSO) for 6 h.

Total RNA was isolated at the end of treatment and used for RT PCR analysis.

2.7. Western Blotting

Western blotting was performed using total cell lysates. Preparations containing 20 µg of total protein were loaded onto 10% SDS–polyacrylamide gel and subjected to electrophoresis. After electro transfer onto PVDF membranes (GE Healthcare Life Sciences, Chicago, IL, USA), the blots were blocked overnight at 4 °C with PBS containing 0.3 % Tween 20 and 5 % non-fat dry milk, and then incubated overnight with primary antibodies: anti-IGF2BP1 (1:1000, ab82968, Abcam, Cambridge, UK); anti-ABCB1 (1:1000, C219, Calbiochem, Darmstadt, Germany), anti-ABCC3 (1:1000, #14182, Cell Signalling, Danvers, MA, USA or M0318, Sigma-Aldrich Corp, Sr. Louis, MO, USA), anti-β-catenin (1:10000, L87A12, Cell Signalling Technology, Danvers, MA, USA) and anti-p-β-catenin (Ser33 phosphorylated, SC-16743, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Equal loading was systematically checked by detection of GAPDH (1:1000, FL-335, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immune complexes were detected by incubation with horseradish peroxidase-linked secondary antibodies (1:5000) for 1 h. Immunoreactive bands were detected by chemiluminescence (Pierce™ ECL Western Blotting Substrate, Thermo Scientific) and analyzed using Gel-Pro Analyzer software (Media Cybernetics, Silver Spring, MD, USA) [21].

2.8. RNA isolation and RTqPCR analysis

After cell treatments, total RNA was isolated using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) cDNA was synthesized from 2 µg of total RNA with the Superscript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) using random hexamers according to manufacturer's instructions. Quantitative real time PCR (qPCR) was performed on an Applied Biosystems™ StepOne™ Real-Time PCR System using the 5 × HOT FIREPol® EvaGreen ® qPCR Mix Plus (ROX) (Solis Biodyne, Tartu, Estonia). All the primers were used at a final concentration of 1 µM. Forward and reverse primers were 5'-CCTGCTGGCTCAGTATGGT-3' and 5'-GACATTCAACCAGTGCCTCS-3' for IGF2BP1, 5'-TACTTGGTGGCACATAAAC-3' and 5'-CCAAAGACAACAGCTGAAA-3' for ABCB1, 5'-GTCCGCAGAATGGACTTGAT-3' and 5'-TCACCACTTGGGGATCATTT-3', 5'-CAACCTTCTGCAGCTCCTC-3' and 5'-TTCTGACCCATACCCACCAT-3' for GAPDH. IGF2BP1, ABCB1 and ABCC3 mRNAs were normalised to GAPDH mRNA as housekeeping.

2.9. Statistical analysis

Results were expressed as mean \pm S.E.M. Statistical analysis was performed using the Student's t-test (for two experimental groups), one-way ANOVA followed by Newman Keuls test and two-way ANOVA with repeated measures followed by Tukey's test (for more than two experimental groups). Significance was set at $p < 0.05$. Analyses were performed using GraphPad Prism 6 software (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. *IGF2BP1, ABCB1 and ABCC3 overexpression is correlated with poor prognosis*

We investigate the probability of survival within a cohort of 368 patients sourced from the TCGA-LIHC dataset. Among these patients, clinical information regarding the type of chemotherapy they underwent, was available for 57 individuals. Notably, 29 patients were administered SFB as part of their treatment regimen (Table 1). While these patients also underwent additional treatments alongside SFB, exploring the impact on survival probability when considering the combined expressions of a specific group of genes, provides valuable insights into whether any expression patterns significantly influence patient survival ($p < 0.05$). Our analysis reveals that the simultaneous variation in the expression of IGF2BP1, ABCC3, and ABCB1 appears to substantially influence the overall survival. Patients with the lowest probability of survival demonstrated high expression levels of all three genes. Notably, there is a clear tendency for patients with low expression of the three genes to be associated with a better overall prognosis (Fig. 1).

Table 1.**Clinical information from the cohort of patients in the TCGA-LIHC database.**

The Table shows the number of patients presenting information on the chemotherapy to which they were exposed.

Drug	Number of patients
[Not Available]	311
5-Fluorouracil	1
Veliparib	1
Veliparib + temozolomide	1
Doxorubicin	3
Alvesin	1
Cisplatin	3
Everolimus	1
Everolimus, gemcitabine and cisplatin	1
Gemcitabine	6
LY228820	1
Mitomycin C	1
Oxaliplatin	1
Regorafenib	1
SOM 230	1
Sorafenib	29
Sunitinib	1
Tamoxifen	1
Temozolomide	2
Tensirolimus	1

3.2. IGF2BP1 depletion leads to ABCB1 and ABCC3 downregulation

To investigate the role of IGF2BP1 in regulating ABCB1 and ABCC3 expression, we designed three siRNAs targeting different regions of human IGF2BP1 mRNA (siRNA 1, 2, and 3). We observed a significant decrease in IGF2BP1 protein expression in HepG2/C3A cells treated with all siRNAs compared to cells treated with control siRNA ($38 \pm 2\%$, $41 \pm 13\%$, and $39 \pm 6\%$ vs. control siRNA $100 \pm 12\%$, respectively; Fig. 2 A). Next, we analysed the protein expression of ABCB1 and ABCC3 to evaluate the role of constitutive levels of IGF2BP1 in modulating ABC transporters. Transient silencing of IGF2BP1 using any of the three siRNAs led to a significant decrease in ABCB1 ($40 \pm 10\%$, $41 \pm 17\%$, and $42 \pm 7\%$ vs. control siRNA $100 \pm 3\%$, respectively) and ABCC3 ($42 \pm 4\%$, $67 \pm 5\%$, and $62 \pm 4\%$ vs. control siRNA 100 ± 1 , respectively) protein levels (Fig. 2 A). Although all three siRNAs showed efficient knockdown of IGF2BP1 in HepG2/C3A cells, siRNA 1, hereafter referred to as IGF2BP1 siRNA, was chosen for subsequent experiments due to its highest inhibitory effect.

We also measured the mRNA levels of IGF2BP1, ABCB1 and ABCC3 in HepG2/C3A with IGF2BP1 knock down. We observed that IGF2BP1, ABCB1 and ABCC3 levels were significantly decreased after IGF2BP1 silencing (IGF2BP1: $58 \pm 4\%$ vs. control $100 \pm 6\%$, ABCB1: $72 \pm 3\%$ vs. control $100 \pm 8\%$ and ABCC3: $58 \pm 4\%$ vs. control $100 \pm 8\%$; Fig. 2 B).

3.3. ABCB1 and ABCC3 are modulated by IGF2BP1 in a posttranscriptional manner under constitutive expression.

Based on the results of transient silencing of IGF2BP1, where we observed a decrease in the protein and mRNA expression of ABCB1 and ABCC3 in HepG2/C3A cells, we decided to evaluate the mechanism by which these transporters were modulated by IGF2BP1. To achieve this, IGF2BP1 was silenced and transcription was subsequently inhibited with ActD in HepG2/C3A cells. Consistent with this, IGF2BP1 depletion reduced total ABCB1 and ABCC3 mRNA levels and in the presence of ActD the effect of IGF2BP1 depletion on ABC transporters persisted, indicating a post-transcriptional mechanism (Fig. 3 A).

Finally, due to the regulatory nature of IGF2BP1, that implies regulation of most of its target mRNAs through interaction with the 3' UTR, we performed an *in silico*

analysis of the interaction probability of the protein sequence of IGF2BP1 and that of ABCB1 and ABCC3 mRNAs.

First, the predicted interaction between IGF2BP1 and the mRNA from both ABC transporters was evaluated at the level of the complete ABC transporter sequence and by evaluating individual regions (5' UTR - CDS - 3' UTR). This was done using the RPSeq algorithm, a family of machine learning classifiers (Random Forest:RF or Support Vector Machine:SVM) for predicting RNA-protein interactions using sequence information only. From Table 2, it can be seen that for both transporters in their full sequence the classifier values are high than 0.5 indicating a positive interaction (RF:0.75 and SVM:0.959 for ABCB1 and RF:0.8 and SVM:0.513 for ABCC3). When the regions of the mRNA are analysed, it is observed that for ABCB1 the high interaction probability is mainly due to the interaction probability of IGF2BP1 and the 3' UTR region of ABCB1(RF:0.75 and SVM:0.97). For the case of ABCC3, although at least one of the values of both classifiers is higher than 0.5 in the different regions of the mRNA, only for the 3'UTR region (RF and SVM: 0.75) the interaction can be considered as positive. Subsequently, we also predicted, using CatRapid fragment module, the probable regions of interaction of IGF2BP1 with ABC transporters. As can be seen in the interaction plot the interaction score between IGF2BP1-ABCB1 is high at the end of CDS and at the beginning of the 3' UTR of the nucleotide sequence. More specifically the nucleotides between positions 3664-3887 in the ABCB1 sequence show a high probability of interaction with the amino acid residues at positions 51-103, 226-277 and 302-353 of IGF2BP1. For ABCC3, two potential interaction regions were detected between nucleotide positions 3843-4070 and 2261-2488, although with a lower probability of binding. Additionally, several shorter secondary interaction zones with lower interaction scores were identified at positions 156-383, 905-1132, and 1399-1626. As observed in the ABCC3-IGF2BP1 interaction profile (Fig. 3B), similar to the case of ABCB1, the IGF2BP1 amino acid residues most involved in the binding are situated between positions 51-103 and 302-353. Unlike ABCB1, an interaction region is also detected in residues at positions 226-277.

Finally, we utilized the RBPmap motifs database to map the consensus IGF2BP1 binding site in the RNA sequence of ABCB1. These sites consist of nucleotide

sequences (with their possible variations) for which there is experimental evidence of binding to a specific protein, in this case, IGF2BP1 (Fig. 3C). The p-values, consensus binding motif, and its variations are described in Table 3. Consistent with findings from previous analyses, we observed a large number of consensus binding motifs to IGF2BP1 in the ABCB1 sequence, the majority were located in the 3'-UTR region.

Table 2.

Predicted interaction between IGF2BP1 and ABC transporters mRNAs.

Shown in summary of the predicted probabilities for interaction between IGF2BP1 and target ABC transporters mRNAs, based on RNA-Protein interaction PRI-Seq. The predicted values of the classifiers RF (Random Forest) and Support Vector machine (SVM), 0.0 - 1.0, suggested > .5 to be statistically significant for potential interaction between the protein and target site.

mRNA SEQUENCE NAME	RBP PROTEIN	Region (nt position)	RF Classifier	SVM Classifier
>NM_001348945.2 Homo sapiens ATP binding cassette subfamily B member 1 (ABCB1), transcript variant 1, mRNA	IGF2BP1	Complete sequence (1...5586)	0.75	0.959
		5'-UTR (1...517)	0.6	0.35
		3'-UTR (4360...5586)	0.75	0.97
		CDS (518...4359)	0.75	0.92
>NM_003786.4 Homo sapiens ATP binding cassette subfamily C member 3 (ABCC3), transcript variant 1, mRNA		Complete sequence (1...5693)	0.8	0.513
		5'-UTR (1...56)	0.5	0.46
		3'-UTR (4641...5693)	0.75	0.75
		CDS (57...4640)	0.85	0.47

Table 3

Binding sites predictions summary.

This is a summary table of the predicted binding sites, their location on the input sequence and a measure for their reliability. The protein's ascribed motifs are listed, ordered by their sequence position.

Sorafenib induced IGF2BP1, ABCB1 and ABCC3 expression

To select the SFB concentration to incubate the HepG2/C3A cell line, we performed a cytotoxicity curve and calculated IC₅₀ value. The IC₅₀ values after 72 h of SFB incubation were 8.25 ± 0.3 µM for HepG2/C3A cell line. Considering this result, we selected a concentration lower than the IC₅₀. Thus, the following experiments were done using a SFB concentration of 2 µM.

The protein levels of IGF2BP1, ABCB1 and ABCC3 were increased by SFB after 48 h of incubation in HepG2/C3A cell line (IGF2BP1: 128 ± 4% vs. control 100 ± 3%, ABCB1: 122 ± 4% vs. control 100 ± 2%, ABCC3: 142 ± 1% vs. control 100 ± 2%; Fig. 4 A). Also, an induction of the expression of all proteins was observed after 72 h of SFB incubation (IGF2BP1: 155 ± 5% vs. control 100 ± 2%, ABCB1: 138 ± 2% vs. control 100 ± 0.13%, ABCC3: 180 ± 7% vs. control 100 ± 0.5%, Fig. 4 B).

To determine whether IGF2BP1, ABCB1 and ABCC3 up-regulation results from an increased expression of their mRNA, we measured their levels by qPCR. We observed that levels of IGF2BP1, ABCB1 and ABCC3 were significantly increased after incubation of HepG2/C3A cells with SFB 2 µM for 48 h (IGF2BP1: 160 ± 10 % vs. control 100 ± 3 %, ABCB1: 136 ± 7 % vs. control 100 ± 4 % and ABCC3: 139 ± 6 % vs. control 100 ± 8 %; Fig. 4 C) and 72 h (IGF2BP1: 183 ± 12 % vs. control 100 ± 7 %, ABCB1: 193 ± 22 % vs. control 100 ± 5 % and ABCC3: 142 ± 12 % vs. control 100 ± 9 %; Fig. 4 D). Similar results were obtained in Huh7 cells, other HCC cell line (Fig. 5 A and B).

3.4. Involvement of IGF2BP1 in ABCB1 and ABCC3 modulation by SFB

To investigate the role of IGF2BP1 in the induction of ABCB1 and ABCC3 by SFB, we knocked down IGF2BP1 expression in HepG2/C3A cells and treated them with SFB. We then analysed protein expression by western blotting. Our results showed that the depletion of IGF2BP1 in HepG2/C3A cells leads to a decrease in ABCB1 when comparing C siRNA SFB: 121.5 ± 2.3 % vs IGF2BP1 siRNA SFB: 85.0 ± 6.5%, but did not affect ABCC3 induction (Fig. 6 A).

ABCB1 and ABCC3 mRNA levels were measured to assess the molecular mechanism of ABC transporters induction by SFB mediated by IGF2BP1. We found that the depletion of IGF2BP1 leads to a decrease in ABCB1 when

comparing C siRNA SFB: $209.5 \pm 4.4\%$ vs IGF2BP1 siRNA SFB: $101.0 \pm 2.4\%$, and also in ABCC3 (C siRNA SFB: $132.4 \pm 7.6\%$ vs IGF2BP1 siRNA SFB: $76.0 \pm 3.5\%$) (Fig. 6 B).

Based on our results we studied the mechanism involved in IGF2BP1 and ABCB1 induction by SFB, by incubating HepG2/C3A cells with ActD after and during SFB treatment. The results showed that the mRNA levels of IGF2BP1 and ABCB1 remained higher after SFB incubation when transcription was inhibited by ActD, suggesting that the up-regulation of these genes by SFB occurs mainly at the post-transcriptional level (Fig. 6 C).

3.5. SIRTs participation in IGF2BP1 induction by SFB.

Inhibition of SIRTs 1 and 2 activities with EX resulted in decreased ABCB1 ($48 \pm 2\%$ vs. control $100 \pm 1\%$) and ABCC3 ($63 \pm 4\%$ vs. control $100 \pm 1\%$) protein levels in HepG2/C3A cell line. Co-treatment with EX and SFB prevented the induction of ABCB1 and ABCC3 ($58 \pm 4\%$ vs. control $100 \pm 1\%$ and $103 \pm 3\%$ vs. control $100 \pm 1\%$, respectively; Fig. 7 A). These results were in line with those of Ceballos et al 2021 for HepG2 cells [22]. Treatment with the SIRTs 1 and 2 inhibitor reduced IGF2BP1 protein levels in this cell line ($35 \pm 4\%$ vs. control $100 \pm 2\%$, Fig. 7 A). The presence of EX in the combined treatment prevented SFB ($2 \mu\text{M}$) from increasing IGF2BP1 protein levels, leading to significantly lower levels of IGF2BP1 protein than those found in the control group ($76 \pm 4\%$ vs. control $100 \pm 2\%$; Fig. 7 A). When only SIRT 1 was inhibited by using EX at $1 \mu\text{M}$, a decrease in IGF2BP1 protein levels was observed ($77 \pm 8\%$ vs. control $100 \pm 2\%$). Furthermore, the combined treatment was more effective in preventing IGF2BP1 induction by SFB ($21 \pm 5\%$ vs. control $100 \pm 2\%$). For ABCB1, similar results to EX40 were observed when incubated with EX1. For ABCC3 the combine treatment partially prevented the induction of this transporter by SFB and at the same time an induction was observed in the combined treatment, with respect to the control when only SIRT1 was inhibited (Fig. 7 B).

3.6. Effect of SFB on β -catenin and $p\text{-}\beta$ -catenin protein expression.

To continue studying the pathway of ABC transporters regulation by IGF2BP1 we assessed the expression of β -catenin. SFB induced β -catenin in HepG2/C3A cells after 72 h of treatment, showing a similar expression pattern as IGF2BP1.

The ratio between total β -catenin and inactive form of β -catenin: p- β -catenin was significantly increased by SFB (Fig. 8).

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4. Discussion

Among all the mechanisms involved in chemoresistance development in HCC, one of the most important is the overexpression of drug efflux pumps such as ABCB1 and ABCC3 [5]. In a microarray analysis of 60 human HCC liver samples, among the 116 RNA binding proteins analysed, IGF2BP1 represented the most strongly up-regulated [23]. IGF2BP1 is an oncogene that was also found to be associated with chemoresistance in HCC although so far there were no studies demonstrating its association with ABC transporters. Through bioinformatic studies we were able to detect that patients with HCC undergoing chemotherapy and expressing high levels of ABCB1, ABCC3 and IGF2BP1 had a worse survival than those expressing low levels of these 3 genes. It is not known whether this effect is a consequence of ABC-associated chemoresistance and whether IGF2BP1 regulates the expression of these ABCs transporters. RNA-binding proteins (RBPs) play a pivotal role in the regulation of gene expression by directly interacting with specific sequence motifs or structural elements within the CDS, 5'-UTR, or 3'-UTR of their target RNA [24]. These proteins can control RNA localization, stability, or translation. IGF2BPs function by recruiting their target transcripts to cytoplasmic messenger ribonucleoprotein particles (mRNPs), which subsequently condense into RNA granules, such as processing bodies and stress granules [13]. This process, in turn, prevents mRNA decay and regulates events associated with translation [13]. Recently, IGF2BPs were identified as a family of reader proteins that control RNA fate in an m6A-dependent manner [25]. By silencing IGF2BP1 we observed a reduction not only in ABCB1 and ABCC3 protein expression, but also a reduction in mRNA levels of both ABC transporters. Act D experiments suggest that IGF2BP1 is modulating ABCB1 and ABCC3 by a post-transcriptional mechanism in HepG2/C3A cells. In line with our findings, Sparanese and Lee demonstrated that, in ovarian cancer cells, IGF2BP1 binds to the mRNA encoding ABCB1 protecting it from endoribonuclease cleavage [26]. In our study, the probable interaction between IGF2BP1 and the mRNAs of ABCB1 and ABCC3 were explored through a comprehensive bioinformatic analysis. Employing the RPSeq algorithm, interactions between IGF2BP1 and the complete sequence of this mRNA and the individual regions (i.e., 5' UTR,

CDS or 3' UTR) of these ABC transporters were predicted. As expected, the results indicated a positive interaction, with high classifier values, especially in the 3' UTR region of ABCB1. Additionally, RBPmap predicted a potential binding motif in the nucleotide sequence of ABCB1 for IGF2BP1. Altogether, these results strongly suggest a post-transcriptional regulation of ABCB1 by IGF2BP1 in HepG2/C3A cells.

Instead, in the case of ABCC3, both RBPmap and RPSeq methods detected binding sites only in the 3'UTR region, but with low classifier values. Furthermore, the relatively low interaction scores and imprecise IGF2BP1-ABCC3 aminoacidic residues and nucleotide interaction regions distributionidentified from CatRapid results raise uncertainties about the direct binding between IGF2BP1 and ABCC3 mRNA. Post-transcriptional regulation of ABCC3 is a quite unexplored field. Among the few miRNA reported to regulate ABCC3, the miRNA-143 was found to modulate ABCC3 [27,28]. An et al demonstrated that the lncRNA FOXD2AS1 induced ABCC3 through downregulation of miRNA-143 [28]. Since it was described that the silencing of IGF2BP1 in HCC derived cells decreases FOXD2AS1 levels [29], this could be a possible mechanism that explains the post-transcriptional regulation of ABCC3 by IGF2BP1. More experiments are needed to test this hypothesis.

mTOR (mammalian Target of Rapamycin) and Wnt/β-catenin are two well studied pathways that are activated by SFB in HCC cells [30]. Recently, it was also demonstrated that mTOR and Wnt/β-catenin are involved in IGF2BP1 up-regulation [13,31]. Whether these pathways are involved in IGF2BP1 induction by SFB is still unknown. With the intention of elucidating whether some of these signalling pathways are involved in the induction of IGF2BP1 by SFB, we assessed the expression of β-catenin. The results showed an induction of β-catenin after treatment with SFB. When analysing the relationship between the total and inactive forms of β-catenin (p-β-catenin), we observed a significant increase at the concentration of 2 μM of SFB. It is highly probable that the Wnt/β-catenin pathway is acting upstream of IGF2BP1. These findings indicate a likely involvement of β-catenin in the induction of IGF2BP1 by SFB. However, additional studies are necessary to confirm these observations and if the mTOR pathway is also involved.

As we observed an increase in IGF2BP1 after SFB treatment and also an increase in ABCB1 and ABCC3 protein expression at same concentration and incubation time, we performed experiments in similar conditions but silencing IGF2BP1, to test if IGF2BP1 is involved in the induction of ABCB1 and ABCC3 by SFB. We observed that in the presence of SFB, its induction effect was prevented for the three of the genes studied when mRNAs were measured, and for IGF2BP1 and ABCB1 but not for ABCC3 when protein levels were quantified. The observed differential modulation of ABCB1 and ABCC3 by IGF2BP1 in response to SFB raises intriguing questions about the specific regulatory mechanisms involved. Several factors may contribute to this discrepancy. Firstly, post-transcriptional regulation by IGF2BP1 might exert a more prominent influence on ABCB1 mRNA stability or translation efficiency compared to ABCC3. In fact, the probability of IGFP2B1 binding to ABCC3 mRNA was lower than its binding probability to ABCB1 mRNA. Additionally, the temporal dynamics of the regulatory processes could be a contributing factor. The observed prevention of SFB-induced effects at the ABCB1 and ABCC3 mRNA levels, but not protein levels, may suggest a post-transcriptional or translational regulatory mechanism that influences the protein synthesis efficiency or degradation rates differently for ABCB1 and ABCC3 in the presence of SFB. Further investigations, including detailed mechanistic studies and exploration of the specific molecular pathways involved, will be crucial to unravel the intricacies of this regulatory relationship.

A well described phenomenon in HCC is the up regulation of SIRT 1 and SIRT 2 [8]. It is also known that SIRT1 has shown bifunctional roles depending on the tissue context and the cancer type. Most of the evidence in HCC attributes an oncogenic role to SIRT1 [8]. SIRT1 mediates overexpression and chemoresistance associated with ABCB1 in breast tumour cells, through regulating β -catenin signalling and NF- κ B-specific transcriptional activity [32]. In HepG2 cells, SIRT1 overexpression resulted in the upregulation of ABCB1 [10]. Wen *et al.* (2020) showed that EX, a SIRT1 and 2 inhibitor, decreased the expression of ABCC3 [33]. The transfection of HepG2 cells with shRNAs against SIRT1 or SIRT2 led to a decrease in ABCB1 and ABCC3 protein levels [22]. Here, when we inhibited both SIRT 1 or SIRT 1 and 2 by incubating HepG2 cells with EX 1 μ M or 40 μ M, respectively, we found that in the presence of SFB, its

induction effects were prevented for the three of the proteins. These results suggest that SIRT1 could be involved in the SFB-dependant induction of IGF2BP1. We cannot rule out SIRT2 involvement in this process. At the moment, the association between the expression of IGF2BP1 and SIRTs is poorly studied. Xiong *et al.* demonstrated that lncRNA HULC (long non coding RNA Highly upregulated in Liver Cancer), whose expression is regulated by IGF2BP1 early in HCC development, regulates expression of SIRT1 [34,35]. Thus, this is the first study to show a connection between SIRT1 and the induction of IGF2BP1 by SFB.

Our findings suggest that IGF2BP1 is a key player in the post-transcriptional regulation of ABCB1 and ABCC3 in HCC cells. Moreover, we demonstrated the involvement of IGF2BP1 in the upregulation of ABCB1 induced by SFB treatment, positions it as a potential therapeutic target to minimise resistance to chemotherapeutic agents. The intricate network involving SIRTs, β -catenin, and mTOR provides potential avenues for therapeutic intervention to enhance the efficacy of chemotherapeutic agents in HCC. However, further investigations are essential to unravel the detailed molecular mechanisms and to validate these findings. In summary, our study sheds light on the multifaceted role of IGF2BP1 in HCC chemoresistance and provides a foundation for the development of targeted therapeutic strategies.

CRediT author statement:

Bucci-Muñoz, María: Investigation, Formal analysis, Methodology, Writing - original draft.

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Livore, Verónica: Methodology.

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Fig. 1. Relationship of IGF2BP1 and ABC transporters in HCC patients. Kaplan–Meier curves of the combined expression of high and low IGF2BP1 and ABC transporters expressing groups. $p = 0.0023$.

Fig.2. Effect of IGF2BP1 depletion in ABC transporters expression. (A) Protein levels of IGF2BP1, ABCB1 and ABCC3 after 48 h of transfection with control siRNA (C siRNA) and three different IGF2BP1-specific siRNAs (1, 2 and 3). (B) corresponding mRNA levels after transfection with control siRNA (C siRNA) and IGF2BP1-specific siRNA, siRNA1 (IGF2BP1 siRNA). a different from transfection control (C siRNA), $p < 0.05$ $n = 3-6$. GAPDH bands corresponding to IGF2BP1 and ABCB1 are duplicated due to IGF2BP1 and ABCB1 were detected from stripping of the same membrane.

Fig. 3. Mechanism of ABC transporters regulation by IGF2BP1. (A) mRNA levels of ABCB1 (left) and ABCC3 (right) in HepG2/C3A cell line after siRNA transfection for 24 h and 6 h of incubation with 5 μg/ml actinomycin D (ActD). a

different from transfection control (C siRNA); b different from IGF2BP1-specific siRNA (IGF2BP1-siRNA), c different from transfection control+ActD (C siRNA+ActD). p < 0.05, n = 3. (B) Predicted interaction sites between IGF2BP1 and ABCB1 (left) and ABCC3 (right) mRNAs. The interaction profile (top) represents the interaction score of the protein (y-axis) along the RNA sequence (x-axis), giving information about the transcript regions that are most likely to be bound by the protein. The interaction matrix (bottom) shows, as a heat-map, the protein (y-axis) and RNA (x-axis) regions predicted to interact. The shades of red of the heat-map indicate the interaction score of the individual amino acid and nucleotide pairs. (C) Probable mRNA binding region predicted by RBPmap tool.

Fig. 4. Effect of SFB on IGF2BP1, ABCB1 and ABCC3 expression in HepG2/C3A cells. (A, B) Protein levels of IGF2BP1, ABCB1, and ABCC3 after 48 h and 72 h of incubation with SFB (2 μ M). Selected lanes were cropped from different parts of the same gel and they are shown after cropping, aligning and separating them by a white space. (C, D) Corresponding mRNA levels after 48 h and 72 h of SFB (2 μ M) treatment. a different from control (C), p < 0.05, n = 3-6.

Fig. 5. Effect of SFB on IGF2BP1 and ABCB1 protein expression in Huh7. (A, B) Protein levels of IGF2BP1, ABCB1, and ABCC3 after 48 h and 72 h of incubation with SFB (2 μ M). Selected lanes were cropped from different parts of the same gel and they are shown after cropping, aligning and separating them by a white space. a different from control (C), p < 0.05, n = 3.

Fig. 6. Implication of IGF2BP1 in SFB induction of ABC transporters. (A) Protein levels of IGF2BP1, ABCB1 and ABCC3 in HepG2/C3A cell line after siRNA transfection for 24 h and 48 h of incubation with SFB (2 μ M). Selected lanes were cropped from different parts of the same gel and they are shown after cropping, aligning and separating them by a white space. (B) Corresponding mRNA levels after siRNA transfection and SFB (2 μ M) treatment. a different from transfection control (C siRNA); b different from treated control (C siRNA + SFB), c different from IGF2BP1-specific siRNA (IGF2BP1 siRNA) p < 0.05, n = 3. (C) IGF2BP1 (left) and ABCB1 (right) mRNA levels in HepG2 cell line incubated with SFB (2 μ M) for 48 h, post pre-treatment with 5 μ g/ml actinomycin D (ActD) or its vehicle. a different from control (C); b different from SFB (2 μ M), c different from control+ActD (C+ActD). p < 0.05, n = 3.

Fig. 7. Participation of SIRT1/2 in the modulation of IGF2BP1 by SFB. (A) Protein levels of IGF2BP1, ABCB1 and ABCC3 after 72 h of treatment with SFB (2 μ M), EX (40 μ M), Sfb+EX or DMSO (control). a different from control (C), b different from SFB (2 μ M), c different from combined treatment (SFB 2 μ M + EX 40 μ M). p < 0.05, n = 3. (B) Protein levels of IGF2BP1, ABCB1 and ABCC3 after 72

h of treatment with SFB (2 μ M), EX (1 μ M), Sfb+EX or DMSO (control). a different from control (C), b different from SFB (2 μ M), c different from combined treatment (SFB 2 μ M + EX 1 μ M). p<0,05, n=4. Selected lanes were cropped from different parts of the same gel and they are shown after cropping, aligning and separating them by a white space.

Fig. 8. Effect of SFB on β -catenin and p- β -catenin protein expression in HepG2/C3A cells. Protein levels of β -catenin (A) and p- β -catenin (B) after 72 h of treatment with SFB (2 μ M) or DMSO (Control, C). a different from control (C). Selected lanes were cropped from different parts of the same gel and they are shown after cropping, aligning and separating them by a white space. a different from control (C), p<0,05, n=3. Ratio of β -catenin and p- β -catenin was calculated a measured of β -catenin activation (C).

Table 1.**Clinical information from the cohort of patients in the TCGA-LIHC database.**

The Table shows the number of patients presenting information on the chemotherapy to which they were exposed.

Drug	Number of patients
[Not Available]	311
5-Fluorouracil	1
Veliparib	1
Veliparib + temozolomide	1
Doxorubicin	2
Alvesin	1
Cisplatin	3
Everolimus	1
Everolimus, gemcitabine and cisplatin	1
Gemcitabine	6
LY228820	1
Mitomycin C	1
Oxaliplatin	1
Regorafenib	1
SOM 230	1
Sorafenib	29
Sunitinib	1
Tamoxifen	1
Temozolomide	2
Tensirolimus	1

Table 2.**Predicted interaction between IGF2BP1 and ABC transporters mRNAs.**

Shown in summary of the predicted probabilities for interaction between IGF2BP1 and target ABC transporters mRNAs, based on RNA-Protein interaction PRI-Seq.

The predicted values of the classifiers RF (Random Forest) and Support Vector machine (SVM), 0.0 - 1.0, suggested > .5 to be statistically significant for potential interaction between the protein and target site.

mRNA SEQUENCE NAME	RBP PROTEIN	Region (nt position)	RF Classifier	SVM Classifier
>NM_001348945.2 Homo sapiens ATP binding cassette subfamily B member 1 (ABCB1), transcript variant 1, mRNA	IGF2BP1	Complete sequence (1...5586)	0.75	0.959
		5'-UTR (1...517)	0.6	0.35
		3'-UTR (4360...5586)	0.75	0.97
		CDS (518...4359)	0.75	0.92
		Complete sequence (1...5693)	0.8	0.513
		5'-UTR (1...56)	0.5	0.46
>NM_003786.4 Homo sapiens ATP binding cassette subfamily C member 3 (ABCC3), transcript variant 1, mRNA		3'-UTR (4641...5693)	0.75	0.75
		CDS (57...4640)	0.85	0.47

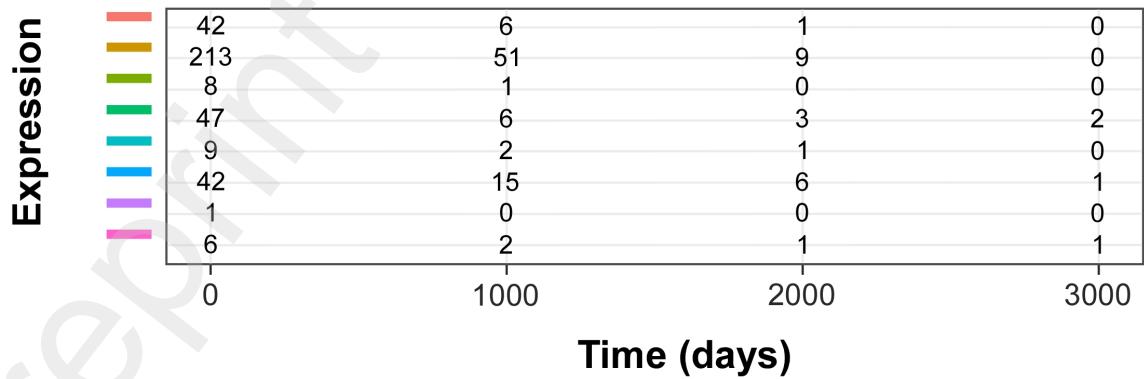
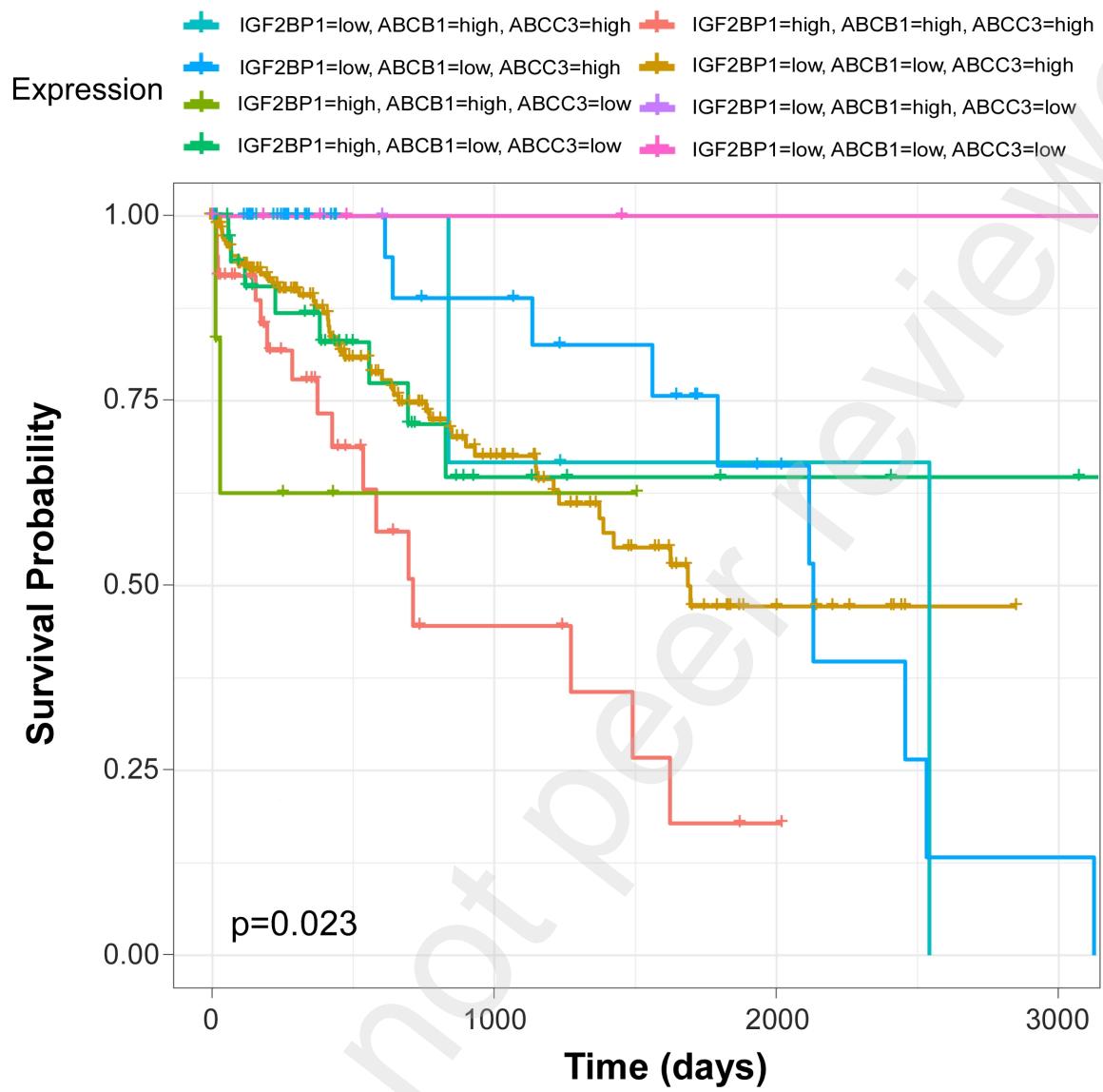
Table 3

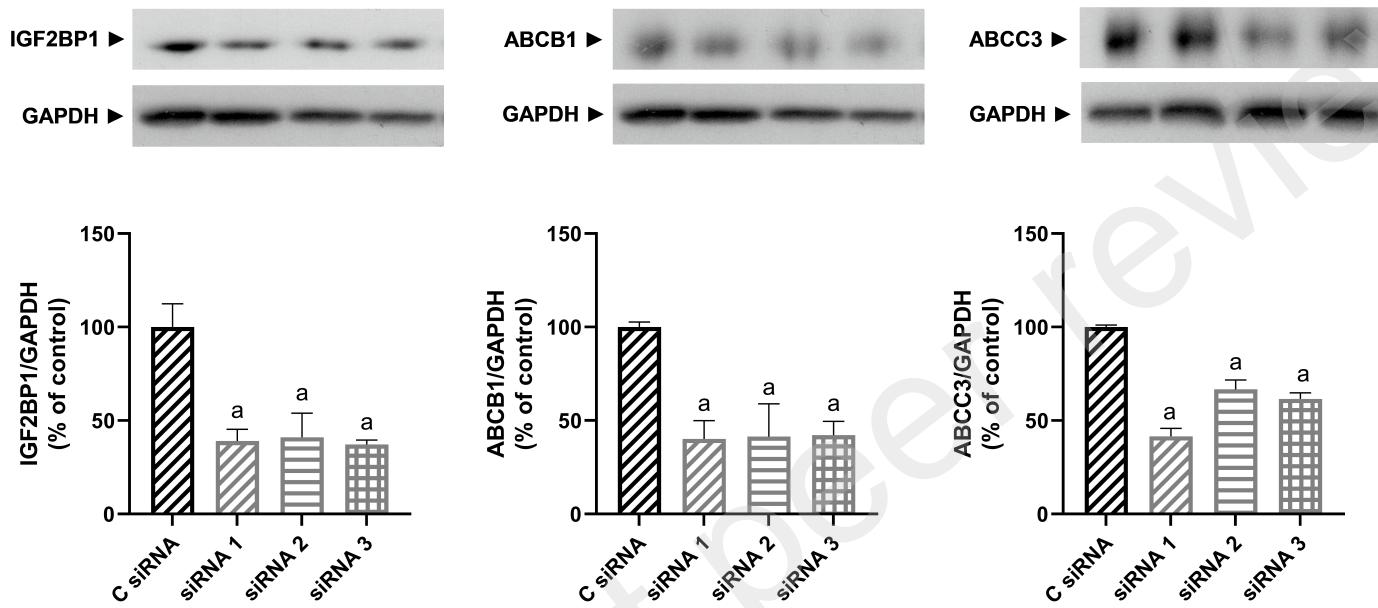
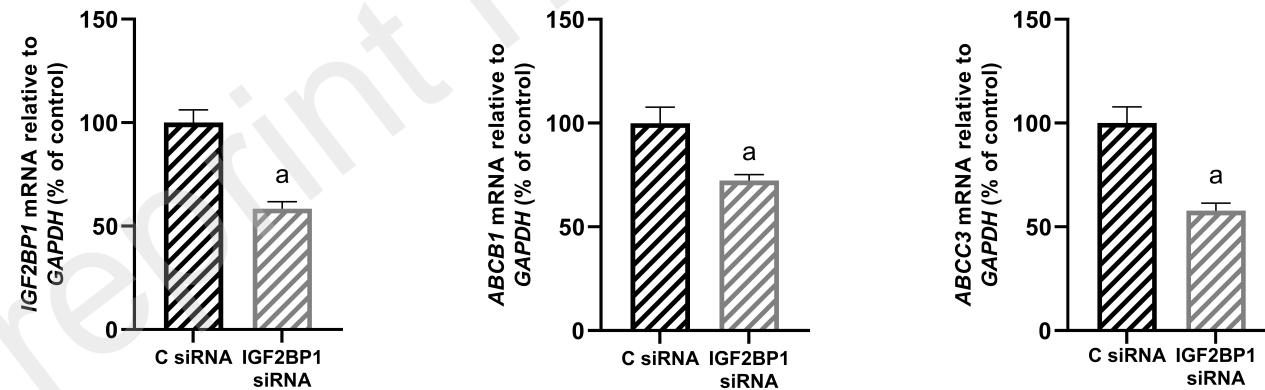
Binding sites predictions summary.

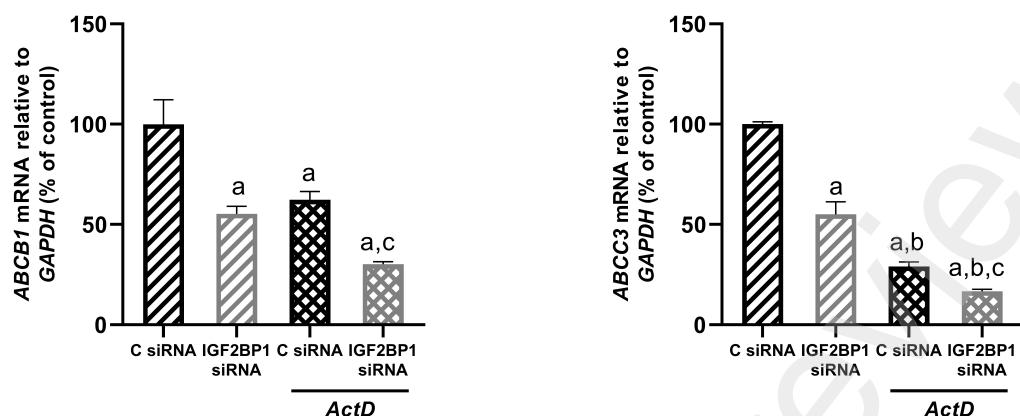
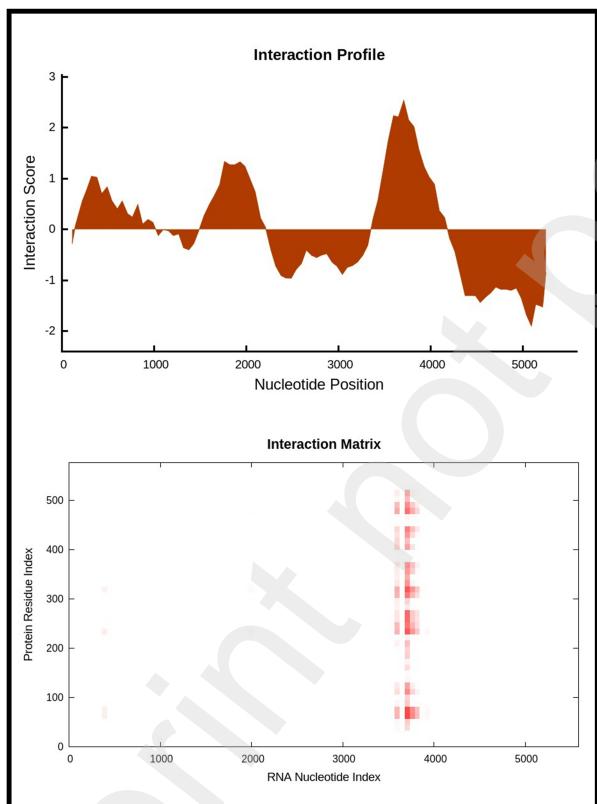
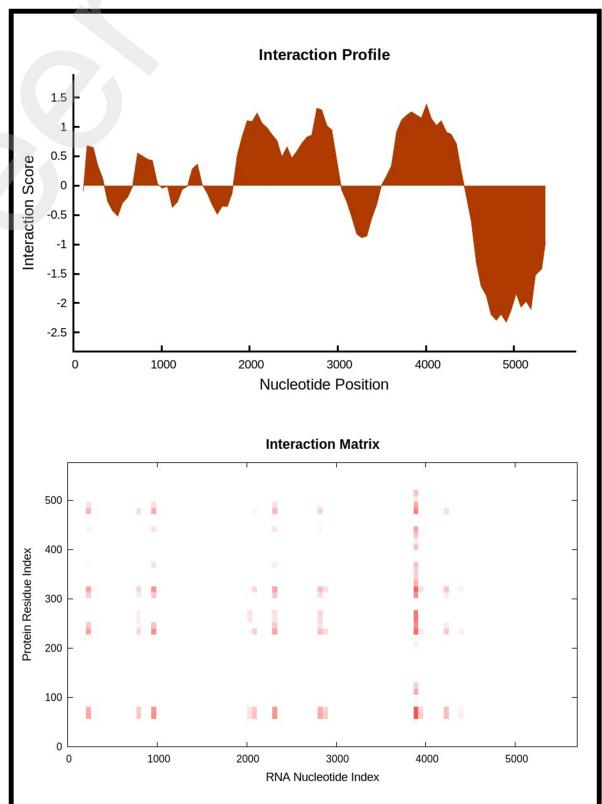
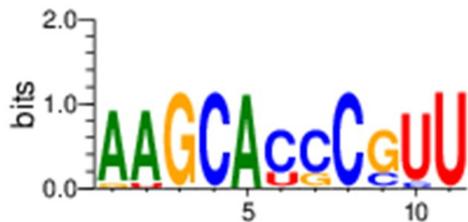
This is a summary table of the predicted binding sites, their location on the input sequence and a measure for their reliability. The protein's ascribed motifs are listed, ordered by their sequence position.

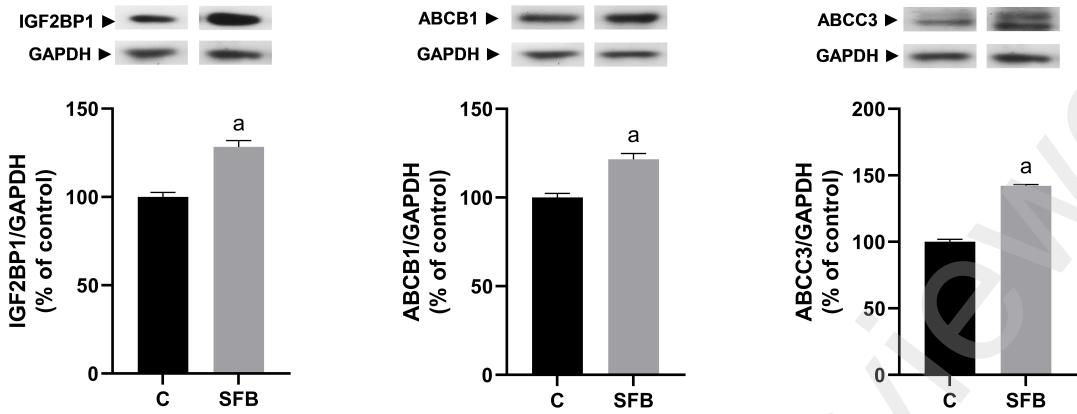
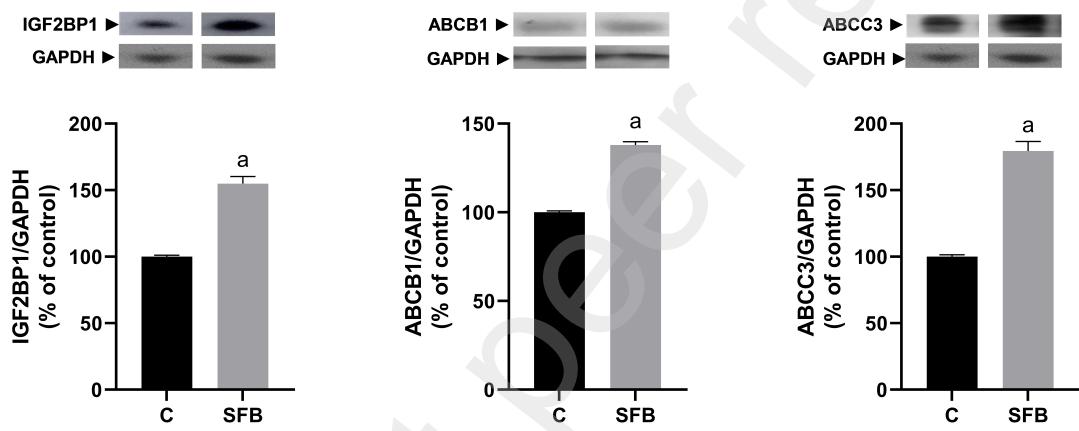
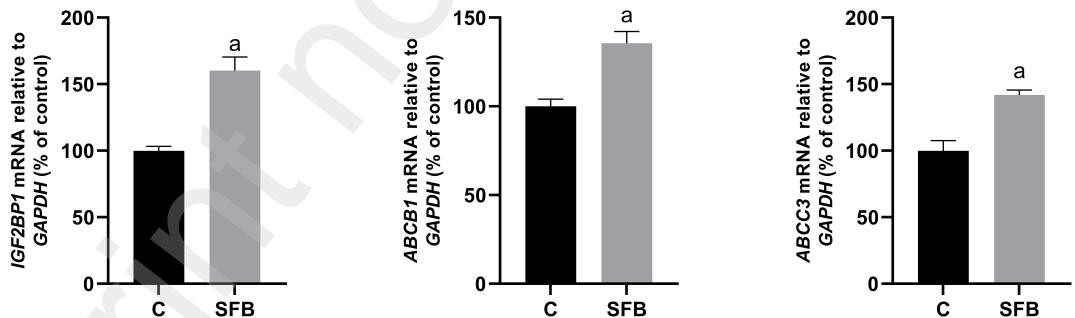
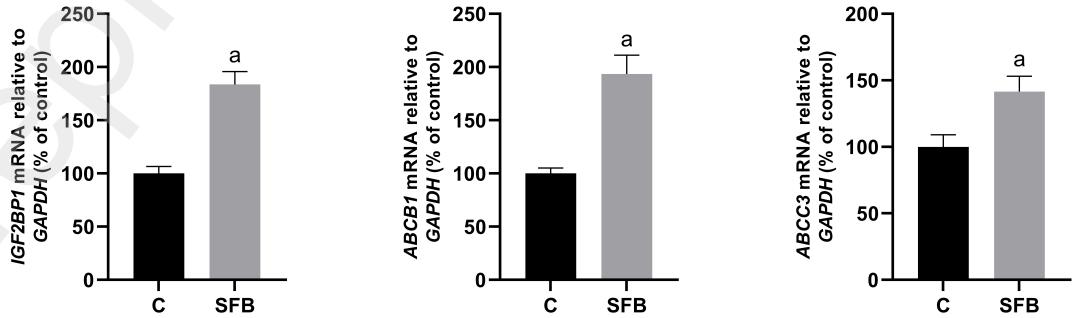
5523	5498	ucaacaaucaaaauaaugcaaauua	aaaca aaaaucauuuuuuugccuuguaau	5552	1.761	3,91E-02	3'UTR
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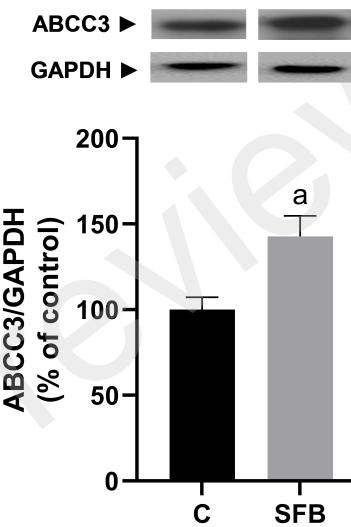
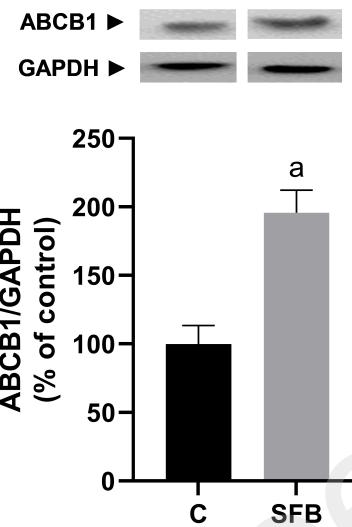
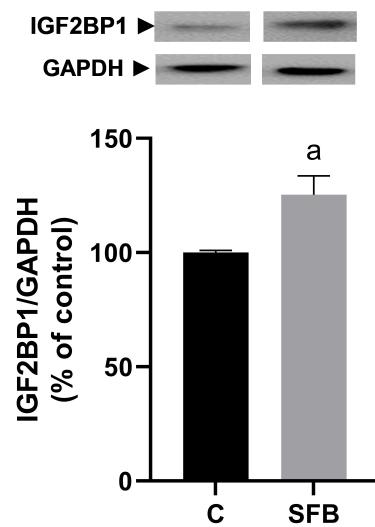
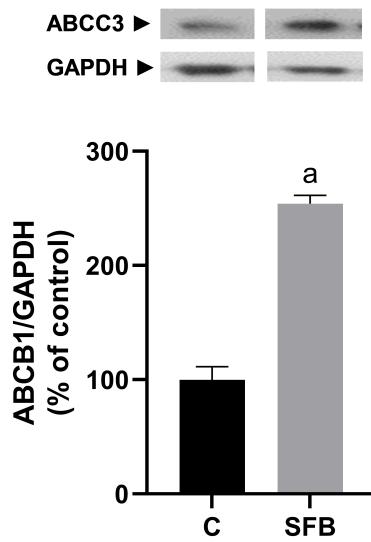
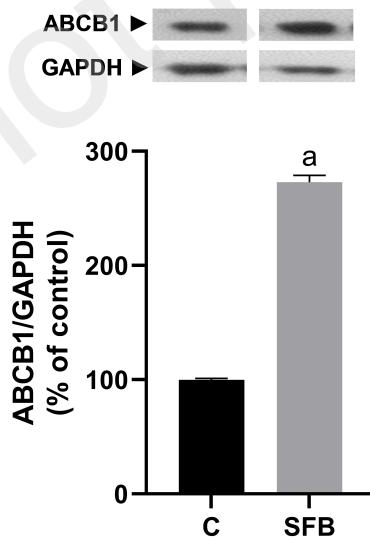
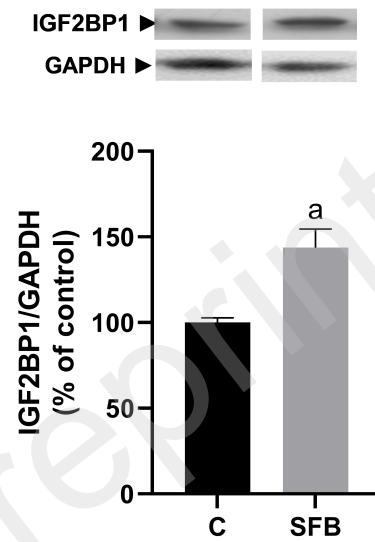
Preprint not peer reviewed

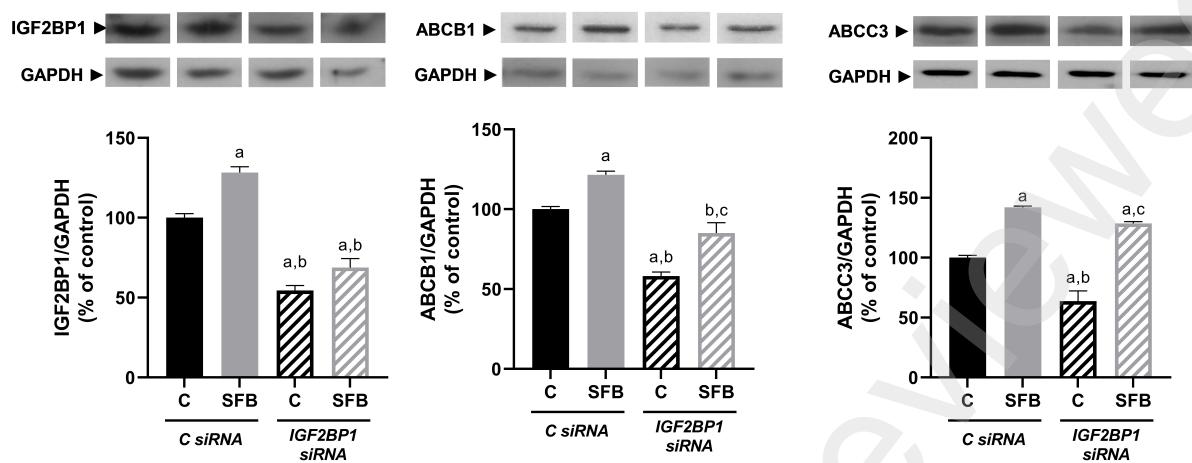
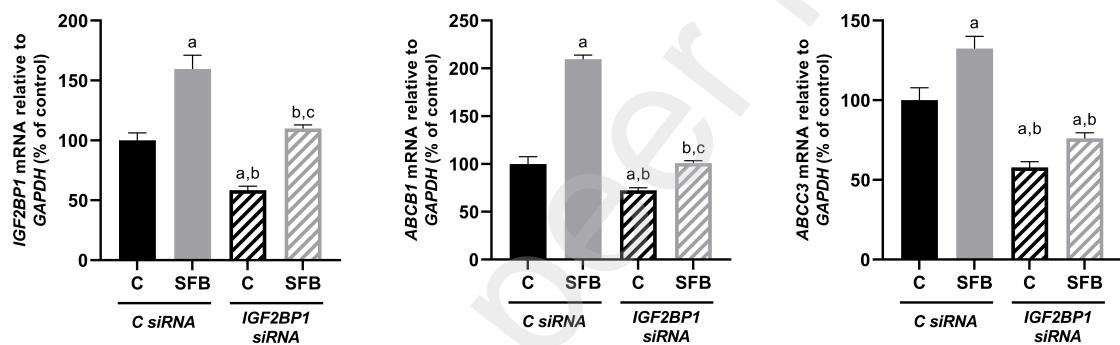
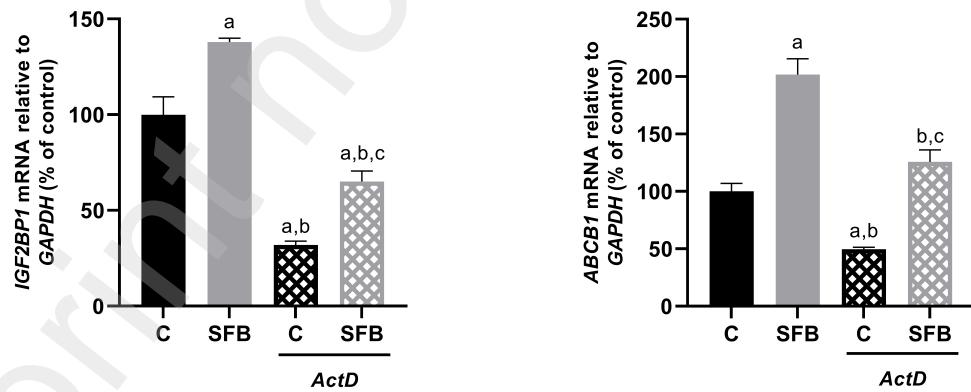


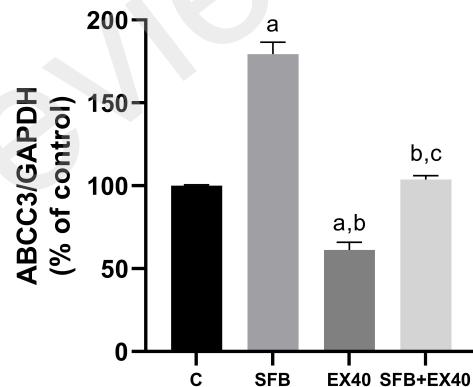
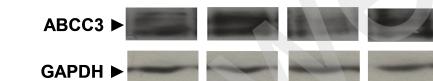
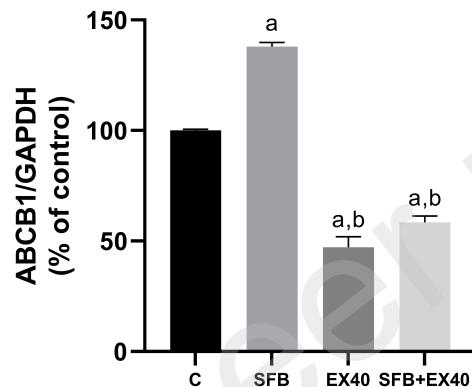
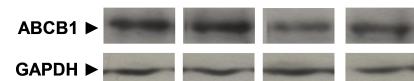
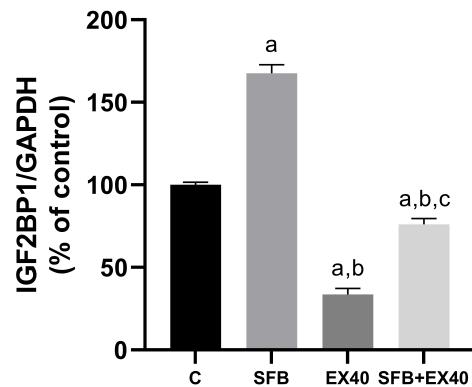
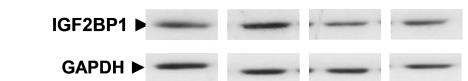
A**B**

A**B****ABCB1****ABCC3****C**

A**B****C****D**

A**B**

A**B****C**

A**B**