

RESEARCH ARTICLE

Expression of activating transcription factor 5 (ATF5) is mediated by microRNA-520b-3p under diverse cellular stress in cancer cells

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Abstract

Cellular stress response mechanisms normally function to enhance survival and allow for cells to return to homeostasis following an adverse event. Cancer cells often co-opt these same mechanisms as a means to evade apoptosis and mitigate a state of constant cellular stress. Activating transcription factor 5 (ATF5) is upregulated under diverse stress conditions and is overexpressed in a variety of cancers. It was demonstrated ATF5 is a survival factor in transformed, but not normal cells. However, the regulation of ATF5 is not fully understood. The purpose of the present study was to investigate miRNA regulation at the 3' untranslated region (UTR) of ATF5, with the goal of demonstrating a reversal of the upregulation of ATF5 induced under diverse cellular stress in cancer cells. A multifactorial approach using *in silico* analysis was employed to identify miRNAs 433-3p, 520b-3p, and 129-5p as potential regulators of ATF5, based on their predicted binding sites over the span of the ATF5 3' UTR. Luciferase reporter assay data validated all three miRNA candidates by demonstrating direct binding to the target ATF5 3'. However, functional studies revealed miR-520b-3p as the sole candidate able to reverse the upregulation of ATF5 protein under diverse cellular stress. Additionally, miR-520b-3p levels were inversely related to ATF5 mRNA under endoplasmic reticulum stress and amino acid deprivation. This is the first evidence that regulation at the 3' UTR is involved in modulating ATF5 levels under cellular stress and suggests an important role for miRNA-520b-3p in the regulation of ATF5.

Introduction

Conserved defense mechanisms such as the heat shock response (HSR) and the adaptive integrated stress response (ISR) protect eukaryotic cells from environmental or physiological challenges to homeostasis [1, 2]. Cancer cells face a hypoxic, nutrient poor environment, often with high levels of reactive oxygen species. Cancer cells are also characterized by unchecked growth, continuous proliferation, and increased DNA and protein synthesis, which leads to greater energy and nutrient requirements and a higher burden on the DNA repair systems and the protein assembly machinery. The HSR is the first line of defense triggered by proteotoxic stress

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stimulated by conditions in the tumor microenvironment and increased load on the protein folding machinery [3]. The HSR is frequently activated in cancer and high expression of chaperone heat shock proteins HSP70 and HSP27 is associated with poor prognosis in a variety of cancer types [3, 4]. An increase in protein production can additionally lead to endoplasmic reticulum (ER) stress since a portion of the proteins may require assembly in the ER, and cancer cells demonstrate an increased secretory nature, which further leads to a heavy ER load [5]. Moreover, ER stress signaling is altered in many cancer types and aids in tumor growth [5–7]. In an environment lacking in amino acids due to poor vascularization, and where continuous proliferation requires high amounts of protein production, amino acid limitation is a constant challenge. Cancer cells have adapted by shifting to the glycolytic pathway, which allows for the use of glycolysis intermediates for biosynthesis, and scavenging the microenvironment for nutrients; however, the metabolic needs of cancer cells are often unmet [8, 9].

This stress phenotype encompasses a variety of conditions, including hypoxia, oxidative stress, amino acid limitation, and endoplasmic reticulum (ER) stress [1, 10]. Cellular stress responses function to temporarily enhance cell survival and restore proper cellular function, or in times of prolonged or extreme cell stress, trigger cell death mechanisms. However, the ability of cells to co-opt the cell stress response to ensure survival can lead to an advantage in tumorigenesis. For example, cancer cells can become resistant to stress-induced cell death by developing a dependence on anti-apoptotic factors [11]. Additionally, enhanced cell survival can lead to epithelial to mesenchymal transition, and thus invasion and metastasis [12]. Apoptotic resistance can also contribute to treatment resistance towards many chemotherapeutic agents [10, 11].

In response to diverse stress conditions of the ISR (including ER stress and amino acid deprivation), stress activated protein kinases phosphorylate EIF2 α , leading to a global reduction of protein translation. However, selective translation is initiated for some stress responsive proteins with multiple upstream open reading frames in the 5' untranslated region (UTR). ATF5 is one such stress response protein. ATF5 is a transcription factor in the ATF/cAMP response-element binding protein (CREB) family and shares high homology to ATF4, but is less well characterized [13, 14]. The *Atf5* gene produces two distinct mRNA transcripts, ATF5 α and ATF5 β , differing only in the 5' UTR and resulting in the same protein [15]. Both transcripts share high homology in humans and mice, and while ATF5 α was found widely expressed in adult mice, the ATF5 β was only detected during early development [15]. Like ATF4, ATF5 α has two upstream open reading frames (ORFs), and under normal conditions, a re-initiation of translation occurs at a second upstream ORF, which overlaps the coding sequence ORF, inhibiting protein translation. However, under stress, the second upstream ORF is bypassed and post-transcriptional suppression is alleviated, a phenomenon observed to occur only with the ATF5 α 5' UTR but not the ATF5 β 5' UTR [16–18].

ATF5 is widely expressed and acts to regulate cell survival, cell cycle, homeostasis, and differentiation [14, 15, 19–23]. ATF5 protein expression is regulated at both the translational and post-translational level [16–18, 24, 25]. Under steady state conditions, ATF5 has a short half-life and is rapidly degraded due to proteasome and caspase dependent mechanisms [26]. However, ATF5 is elevated in response to cellular stress conditions such as amino-acid limitation, heat stress, ER stress, and oxidative stress and typically has a pro-survival effect via upregulation of its downstream targets, anti-apoptotic factors Mcl-1 and Bcl-2 [18, 27, 28]. Further, ATF5 is upregulated in a variety of carcinomas and is a survival factor in several cancer cell lines [20, 29]. When ATF5 function is blocked or silenced *in vitro*, apoptosis occurs in a number of cancer cells such as C6 glioma, SKOV-3 ovarian cancer, MCF-7, and other breast cancer cell lines, but not in non-transformed cells [20, 22, 29, 30]. Interference with ATF5 function *in vivo* causes cell death and tumor shrinkage of C6 glioma [29, 31]. It follows that ATF5 is a

potential target for cancer therapy, and a better understanding of its regulation could lead to enhanced or novel therapeutics for cancer treatment.

It is well established that under stress conditions ATF5 is regulated at the post-transcriptional level via the 5' UTR. Questions remain as to whether ATF5 is also regulated concurrently at the 3' UTR by microRNA (miRNA). miRNAs are evolutionarily conserved small non-coding RNAs ~22 nucleotides in length that regulate genetic expression at the translational level [32]. Briefly, a primary miRNA transcript is processed to the precursor miRNA (pre-miRNA) hairpin structure, which is then exported out of the nucleus and cleaved into a small double stranded RNA duplex, containing the mature single stranded miRNA [32]. The mature miRNA is loaded into the miRNA-induced silencing complex (miRISC) and recruits miRISC to the mRNA target site, resulting in repression of translation and/or mRNA degradation [32, 33].

Canonical miRNA seed sequences (nucleotides 2–7 at the 5' end of the miRNA) bind via Watson Crick pairing to target sites in microRNA response elements (MRE) of mRNA, primarily located in the 3' UTR [32, 34]. A single miRNA may target multiple mRNAs, while conversely multiple miRNAs may target a single mRNA [35, 36]. This lends to the inherent ability of miRNAs to dynamically fine tune genetic expression. There is evidence that multiple miRNAs can work in an additive or synergistic manner to regulate mRNA levels [34, 37]. Such cooperative miRNA modules are reported to have preferred binding regions with a distance between them of greater than 130 but less than 360 nucleotides apart [37]. Furthermore, miRNAs are more effective at repressing translation when bound at either end of the 3' UTR, and shortening of the 3' UTR results in enhanced suppression of protein synthesis by positioning microRNA closer to the stop codon [34, 38].

Genetic regulation by miRNA is highly context dependent, and is cell-type and condition specific [39]. Although the biogenesis and function of miRNAs are tightly regulated at multiple steps, dysregulation of miRNAs often occurs under conditions of cellular stress. Both miRNA activity and expression can be decreased by a variety of mechanisms, including altered levels of proteins involved in biogenesis or function, sequestration into stress granules, interference by RNA binding proteins, or transcriptional regulation [33, 40]. Genetic expression is thereby modified in a variety of cellular stress related diseases [33].

The regulation of specific miRNAs via the cellular stress response has been shown to modulate stress response proteins and impact cell fate. ATF4 mediated repression of the miR-106b-25 cluster under ER stress resulted in upregulation of the pro-apoptotic factor BIM, while an increase in miR-30c-2-3p led to a repression of the pro-survival transcription factor XBP1 [40, 41]. Similarly, a protein kinase R-like endoplasmic reticulum kinase (PERK) dependent decrease in miR-424 during ER stress was shown to upregulate ATF6 via a release of translational repression [42]. Lu et al. (2017) noted an inverse relationship of miR-214 and ATF4 under oxidative stress and demonstrated suppression of ATF4 by miR-214 and a resulting increase in cell survival [43].

The goal of the present study was to investigate the potential translational suppression of ATF5 expression by multiple predicted miRNA regulators under various stress conditions. As ATF5 is a stress responsive transcription factor, this could have ramifications in the treatment of a number of stress-related diseases, particularly cancer and cancer therapy resistance. Evidence is presented demonstrating that miR-520b-3p may play an important role in the regulation of ATF5 under conditions of cellular stress.

Materials and methods

Reagents

The pGL3-control and pRL-TK Renilla plasmids were purchased from Promega (Madison, WI). DNA oligos were obtained from Integrated DNA Technologies (Coralville, IA). The

lentiviral vector pSIREN-RetroQ-ZsGreen and retroviral vector pleGFP-C1 were purchased from Clontech Laboratories, Inc (Mountain View, CA), and pGIPZ-ATF5 shRNA lentiviral plasmids targeting the ATF5 3' UTR or coding sequence were purchased from Dharmacon, Inc. (Lafayette, CO). Restriction enzymes *NheI*, *XbaI*, *BamHI*, and *EcoRI* were purchased from New England Biolabs, Inc. (Ipswich, MA). Heat-inactivated fetal bovine serum (FBS) was purchased from Atlanta Biologicals, while dialyzed heat-inactivated FBS was from GE Healthcare. Roswell Park Memorial Institute (RPMI) 1640 medium with L-glutamine; Dulbecco's modified Eagle's medium (DMEM) 4.5g/L glucose, L-glutamine, and pyruvate; and DMEM with 4.5 g/L glucose and sodium pyruvate and without glutamine, methionine, and cystine were purchased from Corning (Corning, NY). Polyethylenimine (PEI) was purchased from Polysciences, Inc (Warrington, PA) and thapsigargin was purchased from EMD Millipore (Burlington, MA). Rabbit polyclonal anti-ATF5 (#SAB4500895) and mouse monoclonal anti- β -tubulin (#T0198, clone D66) were purchased from Sigma Aldrich (St. Louis, MO), and rabbit polyclonal anti-GRP78 (#ab21685) from Abcam (Cambridge, MA). Goat anti-rabbit (#1706515) and goat anti-mouse (#1706516) secondary antibodies conjugated to horseradish peroxidase were purchased from Bio-Rad Laboratories (Hercules, CA).

Cell lines, culture conditions and stress treatments

Human cervical adenocarcinoma (HeLa, ATCC CCL-2) and human breast adenocarcinoma MCF-7 (ATCC HTB-22) cell lines were purchased from the American Type Culture Collection (ATCC Manassas, VA). HeLa cells were cultured in DMEM medium and MCF-7 cells in RPMI medium, each supplemented with 10% FBS and hereafter referred to as complete medium. Cells were grown at 37°C in 5% CO₂ under a humidified atmosphere up to ~60–70% confluence for all experiments. All the cells tested negative for mycoplasma when tested with Hoechst staining using epifluorescence microscopy at 100x magnification. DNA transfection was performed using 1 μ g DNA, unless otherwise specified, at a ratio of 1:3 DNA to PEI for 48 h. Heat stress was induced by incubating cells in a 42°C humidified incubator for 2 h. A stock solution of thapsigargin (ThG) in DMSO was diluted in complete medium and added to cells to induce ER stress. For amino acid deprivation experiments, cells were washed twice with phosphate buffered saline and treated with DMEM lacking glutamine, methionine, and cystine, and supplemented with 10% dialyzed fetal bovine serum (dFBS). Treatment details were established via a time course trial for each stress type, and treatments are specified in the Results section.

miRNA binding site predictions

In silico analysis was performed to identify putative miRNA targets in the human ATF5 3' UTR. TargetScan version 7.1 was used to predict miRNA-mRNA binding partners [36] and miRanda algorithms were used to identify the alignment between the miRNA and the corresponding MRE within the ATF5 3' UTR [44]. Candidates were selected based on their predicted ability to undergo canonical binding of at least 7 nucleotides (nt) perfect complementary base pairing of the seed sequence to targets in the 3' UTR of ATF5. Only conserved miRNA were included, with the position of putative binding along the 3' UTR as well as predicted free energy upon binding taken into consideration [34, 36, 37]. Additionally, candidates were evaluated only where mature miRNA resulting from a single arm (5' or 3') of the precursor miRNA targeted the 3' UTR. To include miRNA with binding sites at each end of the 3' UTR and an analysis of potential concerted target suppression by miRNA with a preferred binding distance of 130–360 base pairs between binding sites, potential miRNA candidates were segregated based on target site locations in three segments: 3' UTR nucleotides 1–281, 282–562, and 563–844 [37]. Potential miRNA candidates were cross-referenced with

miRbase, the central repository for curated miRNA, to include only high confidence miRNA annotations in our analysis [45]. Candidates in each 3' UTR segment that met the preferred binding distance listed above were then ranked by context++ score percentile and total context++ score, excluding any target sites with conserved branch length of zero.

Plasmid constructs for luciferase and functional assays

To produce the human ATF5 3' UTR-containing pGL3 plasmid, the pGL3 control vector was digested with *XbaI*, gel purified, and treated with calf intestinal phosphatase to prevent recircularization. The ATF5 3' UTR was amplified from HeLa cell genomic DNA using primers that included *NheI* and *XbaI* restriction sites, with 5' -CGTAGCGCTAGCAAGGGCAGGGGTGTG GCTTCT-3' as the sense primer (located at +1 - +21 relative to the translation stop codon), and 5' -TGCTTCTCTAGAAGACAAGATGCACAAGCCAGAGGAA-3' as the antisense primer (located at +835 - +860 relative to the translation stop codon). The amplified segment was then digested with *NheI* and *XbaI* and the ATF5 3' UTR product was ligated into the pGL3 control vector in the multiple cloning region downstream of the firefly luciferase reporter gene using its *XbaI* site, which is compatible with *NheI*. Orientation was confirmed by restriction fragment digestion and direct sequencing. Mutation of the seed sites for miRNA candidates was performed using the Quickchange XL Site Directed Mutagenesis kit (Agilent Technologies, Inc, Santa Clara, CA) following the manufacturer's protocol; the primers used for mutagenesis are listed in Table 1.

Precursor miRNA (pre-miRNA) stem loop sequences were obtained from miRbase (miRbase.org) for each miRNA candidate and flanked with 100 bp of native sequence at each end. Primers were designed with *BamHI* and *EcoRI* restriction sites for cloning into the pSI-REN RetroQ vector under the hU6 promoter. The primers used were as follows (sense and antisense, respectively): 5' -TTCGGGATCCTGCATCTTTCTTTTCGAGTCCA-3' and 5' -TACGGAATTCTCCAGCCTGGGCAATAGAC-3' for pre-miR-520b, 5' -TTCGGGATCCTCTGGAAGGCTCTCCTC-3' and 5' -TACGGAATTCCCGCAACATCTCCCCTATC-3'

Table 1. List of primers used.

Mutagenesis Primers	
ATF5 3' UTR MRE1 S ^a	5' -GCTTATGCTTGTAATCCCAGGTGGTGGGAGGCCAAGGCAGGAG-3'
ATF5 3' UTR MRE1 AS	5' -CTCCTGCCTTGGCCTCCCAACCACCTGGGATTACAAGCATAAGC-3'
ATF5 3' UTR MRE2 S	5' -CCTTCCCTCCTTTCTCGTCCAAAGGTGGAATGTTTGGCCTTAGTCAATG-3'
ATF5 3' UTR MRE2 AS	5' -CATTGACTAAGGCCAAACATTTCCACCTTGGACGAGAAAGGAGGGAAGG-3'
ATF5 3' UTR MRE3 S	5' -GGGACCCATATCCTACAGGCTTTTAGCAGGCTAGGTGACCTTGG-3'
ATF5 3' UTR MRE3 AS	5' -CCAAGGTCACCTAGCCTGCTAAAAGCCTGTAGGATATGGGTCCC-3'
ATF5 3' UTR MRE4 S	5' -GTACTGATTTTTTGGGAGGTTATGAGGTTAAATAAAACGAAACATTTCTCTGGCT-3'
ATF5 3' UTR MRE4 AS	5' -AGCCAGAGGAAATGTTTCGTTTTATTTAACCTCATAACCTCCCAAAAAATCAGTAC-3'
qPCR Primers	
ATF5 S	5' -GGGTGCAGTGGCTTATGC-3'
ATF5 AS	5' -GCCCAGGCTGGTATTGAC-3'
RPLP0 S	5' -GTGGAAGTGACATCGTCTTTA-3'
RPLP0 AS	5' -ATGGTGTTCTTGCCCATC-3'
miR-520b-3p S	5' -AAAGTGCTTCCTTTTAGAGGGAAAAA-3'
SNORD44 AS	5' -GCAATGCTGACTGAACATGAA-3'
Oligo dT adapter	5' -GCATAGACCTGAATGGCGTAAGGGTGTGGTAGCGAGACATTTTTTTTTTTTTTTTTTTT-3'
miR Universal Reverse	5' -GCATAGACCTGAATGGCGTA-3'

^a S, sense, AS, antisense.

for pre-miR-433, and 5'-TTCGGGATCCACTCCCCTCCCTCCCCCTAG-3' and 5'-TACGGAATTCAAAGGAGAGCCAGGAGACCC-3' for pre-miR-129. The amplified fragments were digested with BamHI and EcoRI and ligated into the pSIREN RetroQ vector using standard protocols.

Dual luciferase reporter assay

Luciferase activity was measured using the Dual-Luciferase Reporter Assay System from Promega (Madison, WI, USA), following the protocol for manual luminometers. HeLa cells were seeded into 6-well plates and co-transfected 24 h later with 750 ng of pGL3 control vector containing the ATF5 3' UTR, 15 ng of Renilla plasmid, and 750 ng of either scrambled control, pSIREN RetroQ vectors expressing precursor miRNA, or a positive control, as described below. A pGIPZ lentiviral vector containing shRNA targeting the ATF5 coding sequence served as scrambled control, and a pSIREN RetroQ vector containing shRNA for the firefly luciferase gene served as a positive control. Transfections were carried out in HeLa cells using PEI as described above. Cells were harvested at 48 h and luciferase activity was measured using the FB12/Sirius single tube luminometer from Berthold Detection Systems, performed in quadruplicate with Renilla luciferase activity as an internal standard.

Preparation of cell lysates and immunoblotting

Cell extracts were prepared in radio-immunoprecipitation assay (RIPA) lysis buffer with 1X protease inhibitor cocktail (Sigma Aldrich). Lysates were centrifuged at 5000g for 15 min at 4°C to remove cell debris. Protein concentration was determined using a Bradford protein assay kit (Bio-Rad). Proteins were separated by SDS-polyacrylamide gel electrophoresis (10%) and transferred to a PVDF membrane (ThermoFisher Scientific, Waltham, MA). The membranes were probed with primary antibodies for ATF5 (1:1000) and/or GRP78 (1:500). Equal loading was verified with a β -tubulin antibody (1:2000). GRP78 expression levels served as stress markers for ER stress and amino acid deprivation. Blots were visualized via the Bio-Rad Chemidoc XRS system using horseradish peroxidase-coupled species-specific secondary antibodies and ECL Western blotting detection reagent (Bio-Rad) according to manufacturer's instructions. Densitometry analysis was performed using Bio-Rad ImageLab software.

Quantitative real-time PCR

Total RNA was extracted from cells using TRIzol (Invitrogen, Carlsbad, CA) and cDNA for mRNA analysis was synthesized using SuperScript III first Strand synthesis kit (Invitrogen), each according to the manufacturer's instructions. miRNA cDNA was synthesized using qScript microRNA cDNA Synthesis Kit (Quanta Biosciences, Beverly, MA), which includes a polyadenylation step prior to reverse transcription and the use of an oligo-dT adapter primer during first-strand cDNA synthesis. Primers specific for ATF5 and ribosomal protein lateral stalk subunit P0 (RPLP0) were used for mRNA analysis; since the ATF5 primers were specific for the ATF5 3' UTR, both the α and β ATF5 transcripts are detected in this analysis. Forward primers specific to miR-520b-3p and small nucleolar RNA, C/D Box 44 (SNORD44), and a universal reverse primer specific to a unique sequence at the 5' end of the adapter primer were used for miRNA analysis. Primer sequences are listed in Table 1. Quantitative gene expression analysis was performed using BlazeTaq™ SYBR Green qPCR Mix with ROX (GeneCopoeia, Rockville, MD). Quantitative PCR reactions were performed in 10 μ l reactions using the StepOnePlus real-time PCR (Applied Biosystems, Foster City, CA) in 96-well plates. Gene expression was compared with an endogenous, internal control (RPLP0 for mRNA or SNORD44 for miRNA) using the $\Delta\Delta$ Ct method [46].

Statistical analysis

Statistical analysis was performed using GraphPad Prism 7.00 (GraphPad Software, La Jolla, CA). Samples were analyzed using the student t-test with Welch's correction for unequal variance, or one-way analysis of variance (ANOVA) as appropriate. One-way ANOVA was followed by Dunnett's method of comparison post hoc test. The ANOVA trend test was also performed for qPCR analysis of mRNA after stress treatment, as appropriate. A p-value of ≤ 0.05 was considered significant. All experiments were performed at least three times in triplicate.

Results

In silico analysis predictions for miRNA candidates targeting the ATF5 3' UTR

ATF5 is encoded by the gene *Atf5* on chromosome 19 and spans 5,235 bases (Entrez Gene ID: 22809). The ATF5 α transcript is preferentially upregulated under cellular stress conditions and has a coding sequence of 849 nt (NCBI reference sequence NM_001193646) [16–18]. The 844 nt long 3' UTR of ATF5 was analyzed by TargetScan to identify miRNA candidates that could potentially target sites within the ATF5 3' UTR, with ATF5 3' UTR divided into three segments for analysis. TargetScan predictions identified miR-520b-3p, miR-433-3p, and miR-129-5p as top miRNA candidates (Fig 1). In the segment closest to the coding sequence (nt 1–281), miR-520b was predicted to have a putative binding site within an MRE located at nt 106–125. miR-433-3p was predicted to have a binding site in the middle region (nt 282–562) within an MRE at nt 323–346. miR-129-5p had two predicted binding sites toward the 3' end of the 3' UTR within two MREs at nt 562–580 and 804–822.

Predicted microRNA candidates target the ATF5 3' UTR

Luciferase assays were used to confirm the predicted binding of miR-520b-3p, miR-433-3p, and miR-129-5p to the ATF5 3' UTR. When the wild-type ATF5 3' UTR-containing luciferase vector is co-transfected with either pre-miR-520b, pre-miR-433, or pre-miR-129, a significant ($P < 0.001$) reduction in luciferase activity was observed (60%, 50%, and 70%, respectively), when compared to wild-type ATF5 3' UTR co-transfected with a scrambled control (Fig 2). Co-transfection of the wild-type ATF5 3' UTR with a combination of all three precursor miRNA candidates results in a significant ($P < 0.001$) decrease of 60% in luciferase activity, similar to that observed for each of the three miRNA alone.

A 3–4 nt sequence in each MRE seed sequence (shown in Fig 1) was deleted within the ATF5 3' UTR pGL3 plasmid and co-transfection was performed individually for each of these mutant plasmids and the corresponding precursor miRNA plasmid. Overexpression of MRE1, MRE2 and MRE4 mutants were able to restore luciferase activity to the approximate levels observed in the wild-type ATF5 3' UTR-containing plasmid. However, luciferase activity remained significantly reduced when the MRE3 mutant was co-transfected with the pre-miR-129 plasmid.

Experimental validation *in vitro* of miRNA-ATF5 3' UTR binding predictions reveals activity of miR-520b-3p

Functional assays were performed to determine whether the miRNA candidates were able to regulate ATF5 expression at the protein level under diverse stress conditions. ATF5 protein is significantly upregulated in HeLa cells (45% increase, $P < 0.05$) exposed to heat stress conditions of 42°C for 2 h (Fig 3A). miR-520b-3p was the only candidate able to significantly

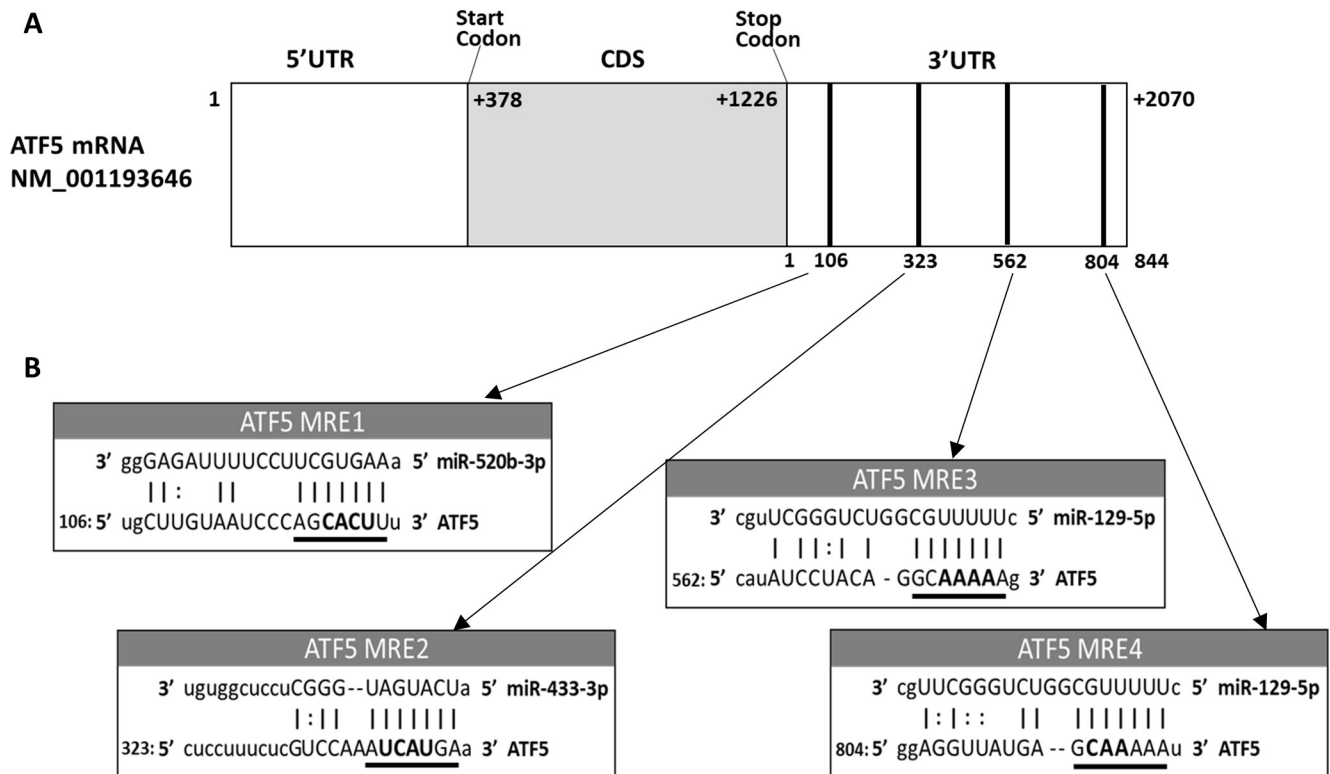


Fig 1. *In silico* predictions of potential miRNA candidates and their putative binding to the ATF5 3' UTR. (A) Schematic of ATF5 mRNA. Nucleotides in the 3' UTR are numbered from 1–844, with nt 1 indicating the 5' end closest to the translation stop codon and nt 844 indicating the 3' end closest to the polyA tail. Black bars in 3' UTR depict binding sites of miRNA candidates along the 3' UTR. (B) miRNA/ATF5 3' UTR alignments. The ATF5 MREs of the selected miRNA candidates are referred to in order of distance from the ATF5 coding sequence. The miR-520b-3p MRE (ATF5 MRE1) is located at +106–126 nt and the miR-433-3p MRE (MRE2) is located at +323–346 nt. miR-129-5p has two MREs in the ATF5 3' UTR, MRE3 and MRE4, located at +562–581 and +804–522 nt, respectively. Bolded nucleotides in the underlined MRE seed sequences were mutated by site-directed mutagenesis to confirm specific binding of miRNA to the ATF5 3' UTR in luciferase assays.

<https://doi.org/10.1371/journal.pone.0225044.g001>

downregulate ATF5 protein (37% decrease, $P < 0.05$) in HeLa cells under the same heat stress conditions (Fig 3B). Co-transfection of all three pre-miRNA vectors also resulted in a significant decrease in ATF5 protein expression (42% decrease, $P < 0.01$), but did not appear to have an additive effect.

We investigated the effect of miR-129-5p, miR-433-3p, and miR-520b-3p on ATF5 protein expression under the induction of two arms of the integrated stress response, ER stress and amino acid deprivation. Experimental conditions for ER stress and amino acid deprivation induction of ATF5 protein expression in HeLa cells were assessed via a time-course treatment of 0.5 μ M ThG or media lacking glutamine, methionine, and cystine supplemented with dialyzed FBS, respectively. An upregulation of GRP78 protein expression indicates that 0.5 μ M ThG is sufficient to induce ER stress after 6, 12, and 24 h of treatment, while ATF5 upregulation was pronounced at 3, 6, and 12 h (Fig 4A). Thus, using conditions of 0.5 μ M ThG treatment for 6 h, we assessed the effect of each miRNA on ATF5 protein expression under ER stress via Western blot analysis. Compared to negative control, miRNA-520b-3p was the only candidate miRNA that significantly ($P < 0.01$) downregulated ATF5 protein by 48% in HeLa cells under ER stress (Fig 4B). Co-transfection of all three pre-miRNA vectors resulted in a similar and significant ($P < 0.01$) decrease of 48% in ATF5 protein expression. The observed decrease in ATF5 protein expression is comparable to that of the positive control (~54% decrease, $P < 0.01$) (Fig 4B).

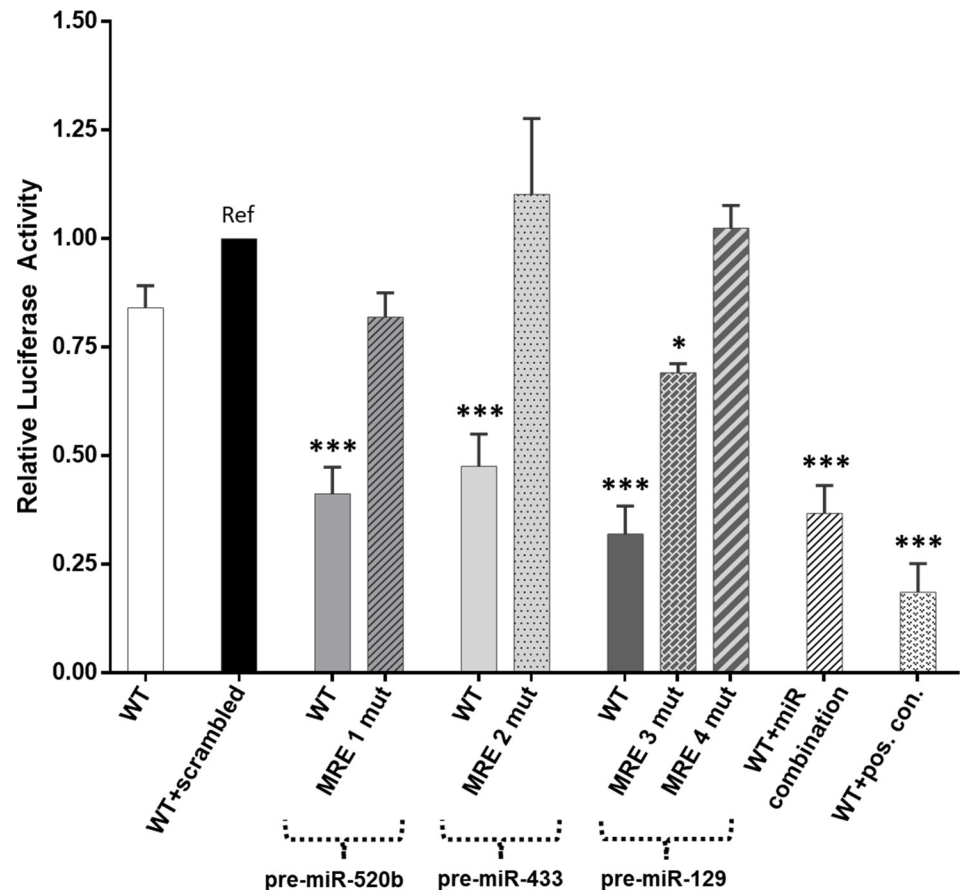


Fig 2. ATF5 3' UTR luciferase activity in the presence of miRNA candidates. Luciferase activity for the wild-type ATF5 3' UTR pGL3 vector alone or co-transfected with pre-miR-520b, pre-miR-433-3p, pre-miR-129-5p, a combination of all three, or a positive control (shRNA against luciferase). ATF5 3' UTR MRE1-4 mutant pGL3 vectors (with deletions in the seed sequences) were co-transfected with pre-miRNA corresponding to the appropriate mature miRNA. All comparisons were made with wild-type ATF5 3' UTR co-transfected with scrambled control. Columns represent the mean \pm S.E. of at least three independent experiments performed in quadruplicate and normalized to the precursor miRNA scrambled control. * $P < 0.05$, ** $P < 0.001$. WT, wild-type.

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After treatment with glutamine-, methionine-, and cystine-free media supplemented with dFBS, GRP78 expression was upregulated as early as 30 min, but was most pronounced at 6, 12, and 24 h post-treatment, indicating amino acid deprivation-induced cellular stress in HeLa cells. Similarly, ATF5 protein levels were elevated as early as 30 min, but were markedly upregulated at 12 and 24 h post-treatment (Fig 4C). We assessed the effect of the candidate miRNA on ATF5 protein expression after 24 h of amino acid deprivation treatment via Western blot analysis. Compared to negative control, miRNA-520b-3p significantly ($P < 0.05$) downregulated ATF5 protein by 41%. Co-transfection of all three pre-miRNA vectors also led to significantly ($P < 0.05$) decreased ATF5 protein expression (38%). A comparable reduction of 50% in ATF5 protein expression was observed with the positive control-transfected cells (Fig 4D).

Endogenous miR-520b-3p and ATF5 mRNA levels are altered in HeLa cells under cellular stress

To further assess a functional effect of miR-520b-3p on ATF5 expression, we tested the impact of cellular stress on endogenous levels of miR-520b-3p and corresponding ATF5 mRNA

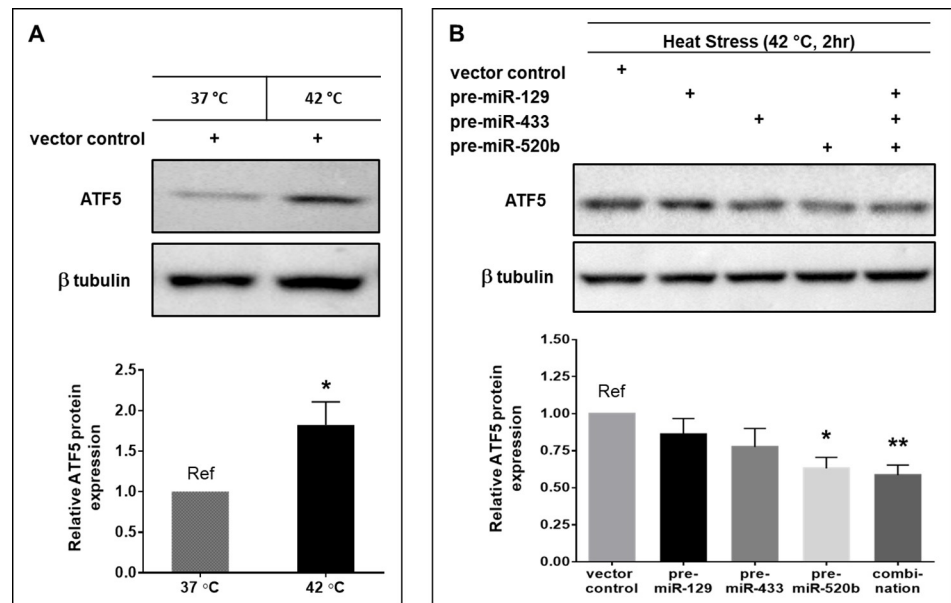


Fig 3. miR-520b-3p suppresses ATF5 protein expression under heat stress in HeLa cells. (A) HeLa cells were transfected with a vector control (pleGFP, 1 μ g) and incubated at 37 or 42°C for 2 h. (B) HeLa cells were transfected with 1 μ g of either vector control (pleGFP), pre-miR-129, pre-miR-433, or pre-miR-520b, or 0.5 μ g each of all three precursor miRNA vectors (combination), and then subjected to 42°C for 2 h. Blots are representative images of 5 independent experiments. Columns represent the mean \pm S.E. of ATF5 protein expression normalized to β -tubulin and compared to the reference (Ref). * $P < 0.05$ and ** $P < 0.01$.

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expression. Cells underwent a time-course treatment of 0.5 μ M ThG for ER stress or glutamine-, methionine-, and cystine-free media supplemented with dialyzed FBS for amino acid deprivation. Total RNA was extracted from cells at various time points and miR-520b-3p and ATF5 mRNA were measured via qPCR in matched samples. Under ER stress conditions, miR-520b-3p was significantly ($P < 0.01$) downregulated as early as 0.5 h post-treatment (72% decrease compared to non-treated control), a trend that continued for up to 3h of treatment (Fig 5A). ATF5 mRNA was significantly ($P < 0.05$) upregulated 1.8 fold at 6 h post-treatment (Fig 5B). A similar pattern was observed under conditions of amino acid deprivation, with miR-520b-3p expression significantly downregulated at both 0.5 h (84%) and 1 h (74%) post-treatment ($P < 0.05$ and $P < 0.001$, respectively; Fig 5C), while ATF5 mRNA was upregulated by 2.5-fold ($P = 0.054$) at 24 h post-treatment (Fig 5D).

520b-3p activity toward ATF5 3' UTR is confirmed in the MCF-7 cell line

We further investigated the ability of miR-520b-3p to regulate ATF5 expression at the protein level in MCF-7 cells under similar experimental conditions. Using conditions of 1 μ M ThG treatment for 6 h, we assessed the effect of miR-520b-3p on ATF5 protein expression in MCF-7 cells under ER stress. There was a 1.7-fold increase in ATF5 protein expression ($P < 0.01$) in treated vs. non-treated MCF-7 cells transfected with the negative control luciferase shRNA (Fig 6A). In contrast, pre-miR-520b transfection in MCF-7 cells subsequently treated with ER stress resulted in a significant ($P < 0.01$) 44% downregulation of ATF5 protein compared to negative control, a decrease similar to that observed for cells transfected with the ATF5 shRNA positive control (~44% decrease, $P < 0.01$). We also evaluated the effect of miR-520b-3p on ATF5 protein expression in MCF-7 cells under amino acid deprivation conditions. Similar to

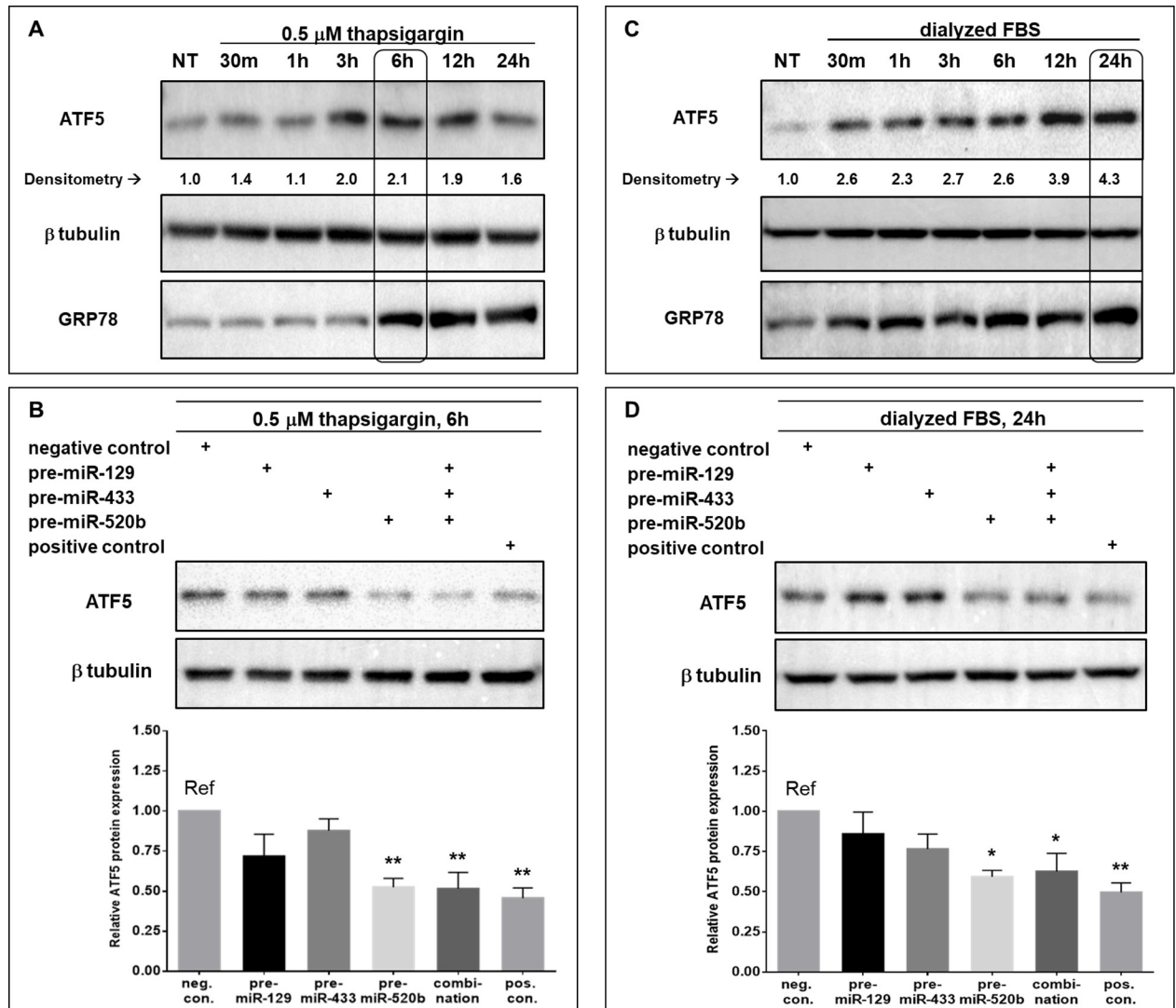


Fig 4. miR-520b-3p reverses ATF5 upregulation under ER Stress and amino acid deprivation in HeLa cells. (A) HeLa cells were transfected with 1 μg of sham vector (pleGFP) and treated with 0.5 μM ThG for the indicated times, with the exception of a non-treated control (NT). Membranes were probed for ATF5 protein expression, as well as GRP78 as an indicator of ER stress, normalized to β-tubulin and compared to NT. (B) HeLa cells were transfected with either 1 μg of negative control (shRNA against luciferase), pre-miR-129, pre-miR-433, pre-miR-520b, or positive control (shRNA against the ATF5 3' UTR), or a combination of all three miRNAs (0.5 μg of each), and then treated with 0.5 μM ThG for 6 h prior to collection of cells. (C) HeLa cells were transfected as in panel A, and treated with DMEM lacking glutamine, methionine, and cystine supplemented with dFBS for the indicated times, with the exception of NT. Membranes were probed for ATF5 protein expression, as well as GRP78 as an indicator of amino acid deprivation-induced cellular stress, normalized to β-tubulin and compared to NT. (D) HeLa cells were transfected as in panel B and then treated with amino acid deprivation as in panel C for 24 h prior to collection of cells. Blots are representative images of 4 independent experiments. Columns represent the mean ± S.E. of ATF5 protein expression normalized to β-tubulin and compared to the reference (Ref). * P<0.05 and ** P<0.01.

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that observed for ER stress conditions, ATF5 protein expression was significantly increased (1.8-fold, P<0.05) in treated cells as compared to non-treated cells, when each was transfected with the luciferase shRNA negative control (Fig 6B). In cells transfected with pre-miR-520b, ATF5 protein was again significantly downregulated compared to negative control cells (~47% decrease, P<0.05), a level of downregulation similar to that observed in the ATF5 shRNA positive control transfected cells (~51% decrease, P<0.01).

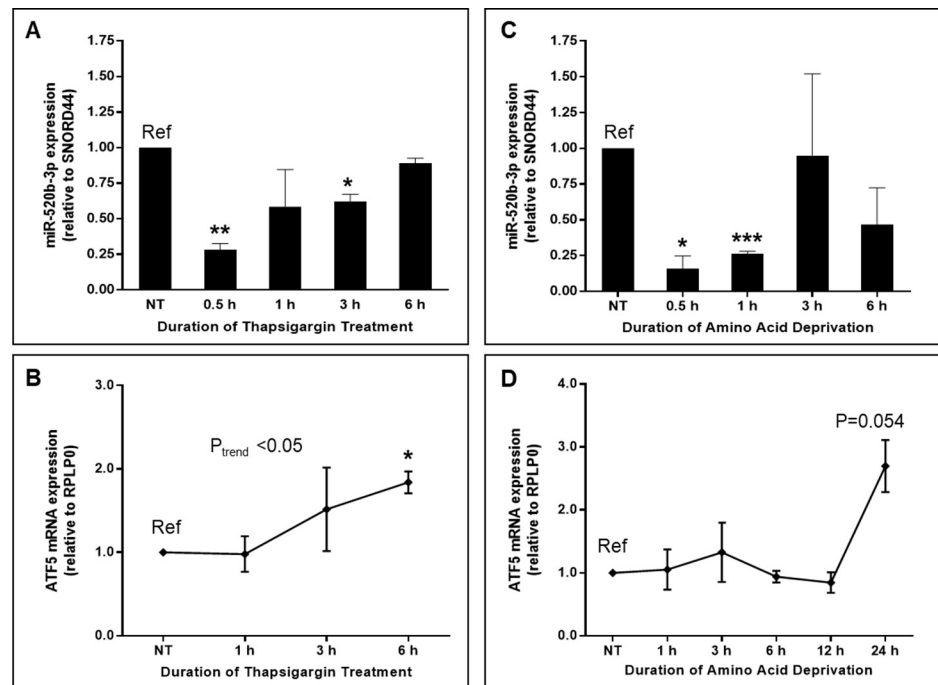


Fig 5. Diverse stress conditions trigger a rapid and dramatic decrease in miR-520b-3p and a corresponding increase in ATF5 mRNA over time in HeLa cells. (A) Endogenous miR-520b-3p expression levels were measured by qPCR in HeLa cells undergoing a time-course treatment of 0.5 μ M ThG. (B) Matched samples were analyzed for ATF5 mRNA expression via qPCR. (C) Endogenous miR-520b-3p expression was assessed by qPCR in HeLa cells under amino acid deprivation. (D) ATF5 mRNA expression was measured in matched samples by qPCR. Expression levels are shown relative to non-treated control. miRNA expression was quantified against the SNORD44 endogenous control, while ATF5 mRNA expression was adjusted to the endogenous control gene RPLP0. Data points represent the mean \pm S.E. of three independent replicates. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

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Discussion

This study is the first to investigate the post-transcriptional regulation of ATF5 by miRNA in the context of cellular stress, and only the second to examine its regulation by miRNA overall. Here, the ability of multiple miRNA candidates to regulate the dynamic expression of ATF5, a stress-responsive transcription factor, was investigated under cellular stress conditions. Results from the present study show that miR-520b-3p is able to selectively bind the ATF5 3' UTR *in vitro*, and its expression is inversely related to ATF5 mRNA levels in HeLa cells under both ER stress and amino acid deprivation. Moreover, miR-520b-3p can suppress endogenous ATF5 protein expression under diverse cellular stress conditions *in vitro*. This was seen in the case of heat stress, amino acid deprivation, and ER stress in HeLa cells, with the effects under amino acid deprivation and ER stress replicated in MCF-7 cells.

Post-transcriptional regulation of ATF5 by miR-520b-3p occurred in a significant and consistent manner, with a reduction in ATF5 protein of approximately 40% across all stress conditions in HeLa cells. Similarly, ATF5 was significantly decreased by 40–50% in MCF-7 cells transfected with precursor miR-520b under similar cellular stress conditions. These results were supported by a rapid and dramatic decrease in endogenous miR-520b-3p of greater than 70% as early as 0.5 h after treatment in HeLa cells under both ER stress and amino acid deprivation, which was inversely related to an increase in ATF5 mRNA over time of treatment in matched samples. Taken together, these findings suggest miR-520b-3p to be an important and novel contributor to the modulation of ATF5 expression under diverse stress conditions and

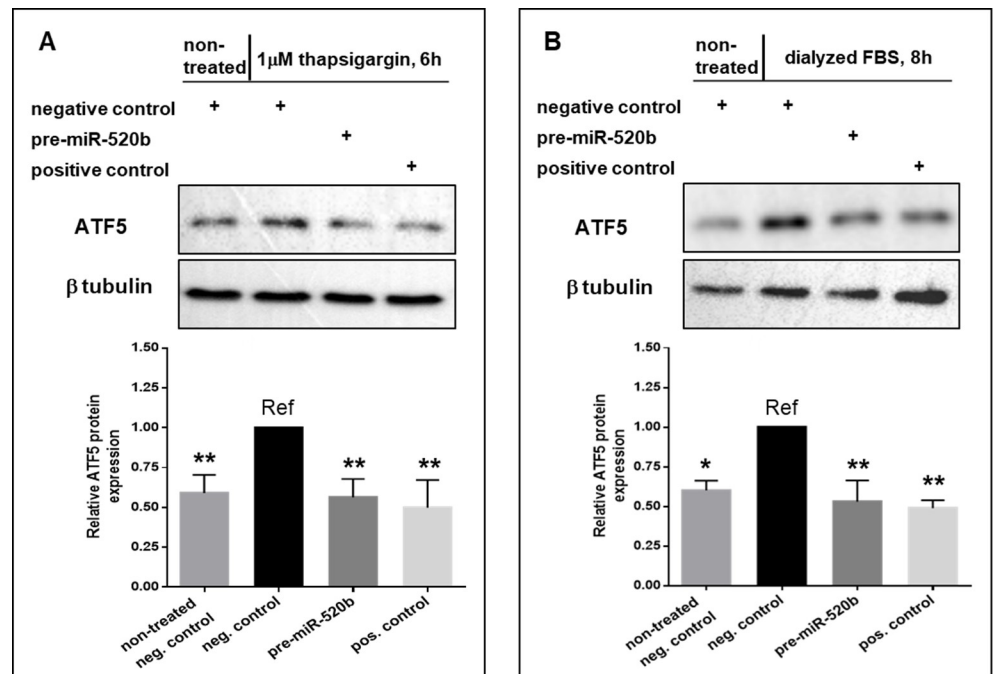


Fig 6. miR-520b-3p suppresses ATF5 upregulation under diverse stress conditions in MCF-7 cells. (A) MCF-7 cells were transfected with 1 μ g of either negative control (shRNA against luciferase), pre-miR-520b, or a positive control (shRNA against ATF5 3' UTR) and then treated with 1 μ M ThG for 6 h prior to collection of cell lysates, with the exception of non-treated negative control. (B) MCF-7 cells were transfected as in (A) and treated with glutamine-, methionine-, and cysteine-free media supplemented with dFBS for 8 h prior to collection of cell lysates, with the exception of non-treated negative control. Blots are representative images of 3 independent experiments. Columns represent the mean \pm S.E. of ATF5 protein expression normalized to β -tubulin and compared the reference (Ref). * $P < 0.05$ and ** $P < 0.01$.

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across different cancer cell types. Recently, miR-141-3p was found to inhibit ATF5 protein synthesis and lead to a decrease in tumor size *in vivo* in glioma [47]. It would be useful to investigate miR-141-3p in the context of cellular stress, which was not examined in the previous study, as well as whether miR-520b-3p suppression of ATF5 expression could also lead to decreased tumor growth.

The ability of accurate prediction of miRNA-mRNA targeting is imperative to understanding miRNA regulation of genetic expression [36]. However, the extent to which miRNA act upon target mRNA translation and/or stability once bound is variable [34]. *In silico* algorithms attempt to gauge the potential of miRNA-mRNA binding and the expected efficacy of repression. However, Pinzon et al. (2017) cited a high rate of false positives by *in silico* modeling programs and a lack of reporting thereof [48]. It is important to note that the ability of miRNA to regulate expression of a gene is dependent on the context of the cell or tissue type and other conditions such as developmental or disease state [39]. Therefore, experimental confirmation of biological functions of miRNA is essential. The results presented in this study clearly illustrate this point. Luciferase assay data confirmed the ability of not only miR-520b-3p, but also miRNA candidates miR-433-3p and miR-129-5p, to interact with the ATF5 3' UTR. Deletion of 3–4 nt sequences within the MRE sequences for the candidate miRNA reversed these effects, suggesting specificity of action, while confirming the binding of the mature miRNA candidates to the ATF5 3' UTR in our system. However, *in vitro*, neither miR-433-3p nor miR-129-5p showed a significant effect on ATF5 protein levels. This is in contrast to the *in silico* data, which predicted miR-520b-3p to have the least efficacy for ATF5 post-transcriptional

regulation out of the three candidates. The lack of effect from miR-129-5p was particularly surprising as it is known that microRNA with binding sites near either end of the 3' UTR are more likely to affect protein translation and miRNA with multiple binding sites can have enhanced efficacy in target suppression [34]. As miR-129-5p did show a greater reduction in luciferase activity (about 20% over that of miR-433-3p), this may indeed account for some enhanced binding capacity. However, in the cellular context, there may be changes in mRNA 3D structure that do not allow efficient binding of miR-129-5p to the ATF5 3' UTR, and which are not accounted for in our reporter system. Additionally, the RNA binding protein HUR, which binds AU-rich elements to stabilize mRNA in stress conditions, has been implicated in making target sites inaccessible for miRNA binding [49]. As the ATF5 3' UTR has been reported to have at least one HUR binding site, it is possible that this mechanism could be negatively impacting the activity of miR-433-3p or miR-129-5p observed in the present study. Functional assays revealed that miR-520b-3p significantly suppressed endogenous ATF5 protein expression *in vitro* under varied cellular stress conditions. Hoffman et al. (2016) highlighted a greater potential impact of sites in the 3' UTR closer to the stop codon and, as mentioned previously, it is known that miRNA post-transcriptional regulation is highly context specific [38, 39].

It is known that multiple miRNA can act in concert for an enhanced effect. Though it was expected that co-transfection of the three miRNA candidates would generate a cumulative suppression on ATF5 expression, this was not the case. The distance between miRNA binding sites has been investigated as an important factor in the ability of multiple miRNA to have a combined effect [34, 35]. A recent study determined that, overall, the majority of miRNA modules, or groups of miRNA that repressed a common mRNA target more than the individual miRNA, exhibited a preferred binding distance within one another of 130–360 nucleotides [37]. The average distance between binding sites for the three miRNA candidates is 213 nucleotides, which is well within this range. However, previous studies have shown preferred binding distances on the 3' UTR to be less than 130 nucleotides [35]. This should be studied further for ATF5, as synergistic activity could enhance potential pharmacologic intervention.

Supporting the relevance of our findings that exogenously introduced miR-520b-3p reverses the upregulation of ATF5 protein expression in cancer cells under stress, we report a significant decrease in endogenous miR-520b-3p levels in HeLa cells exposed to both ER stress and amino acid deprivation. This likewise corresponds to a decrease in ATF5 mRNA over time of stress treatment. Although a lag time was observed in ATF5 mRNA upregulation as compared to the rapid miR-520b-3p decrease, this would be expected to some extent, as ATF5 is also post-transcriptionally regulated by upstream open reading frames in the 5' UTR, which is known to impact ATF5 mRNA levels and is relieved upon stress-induced phosphorylation of eIF2 α [16, 17]. Additionally, it is likely that miR-520-3p is acting primarily to inhibit translation, and the decrease of miR-520b-3p levels under cellular stress effectively primes the system, thus enabling the rapid increase in ATF5 protein translation seen upon stress induction. Further investigation into the mechanisms involved in the interplay between miRNA-520b modulation of ATF5 and its regulation at the 5' UTR is warranted.

Evasion of apoptosis is one of the hallmarks of cancer [8]. ATF5 is a widely expressed transcription factor that is upregulated under diverse cellular stress conditions and typically leads to enhanced cell survival [18, 27, 28, 50]. With prolonged cellular stress, the adaptive stress response switches from pro-survival to activate apoptosis. However, cancer cells can become resistant to stress-induced apoptosis through upregulation of BCL-1 and MCL-2, both downstream targets of ATF5. Previous studies have found ATF5 is upregulated in a wide variety of cancers, and have linked ATF5 expression to a malignant phenotype, citing resistance to apoptosis, increased invasive capacity, and treatment resistance [20, 51–53]. Therefore,

understanding the relationship between the upregulation of ATF5 due to cellular stress inherent to cancer cells and the effects of ATF5 on tumor progression and metastasis could have great therapeutic potential. Additionally, the use of cell stress to trigger apoptosis may enhance the function of various chemotherapeutic agents if the resistance to apoptosis can be overcome [54]. Thus, elucidating the mechanisms of ATF5 regulation under cellular stress could aid in the development of novel or enhanced cancer therapeutics. Further, miRNAs hold great promise for use as biomarkers for disease diagnosis and prognosis as well as potential targets or modes of treatment [55].

miRNA dysregulation leads to the impairment of fundamental cellular processes and is, therefore, associated with a number of different disease states, including cancer. While the mechanisms are not completely understood, both the expression and activity of miRNA can be altered. Potential scenarios include changes in the levels and activity of key proteins involved in miRNA biogenesis or function, miRNA sequestration into extracellular vesicles, and stress-dependent transcriptional changes in miRNA expression. Additionally, the RNA binding protein HUR has been shown to bind and sequester miRNA under cellular stress [49, 56]. It would be interesting to investigate further the specific mechanisms behind the suppression of miR-520b-3p seen under multiple stress conditions. Notably, miR-520b-3p is part of the miR-302-3p/372-3p/373-3p/520-3p miRNA family. Future studies could include assessing whether any members of this family may act as a redundant mechanism to aid in the suppression of ATF5 at the translational level.

In summary, a novel mechanism for modulation of ATF5 expression was identified in the present study. Specifically, the post-transcriptional regulation of ATF5 by miR-520b-3p, with a release of translational repression occurring via a reduction in miR-520b-3p levels upon exposure to diverse stress conditions is described. The findings presented in these studies are consistent with the role of miRNA acting as a buffer to reduce unwanted variation in protein levels under normal states, and to finely tune genetic expression in a rapid manner when conditions change, and with the potential role of ATF5 in stress-induced apoptosis and cancer progression. This study highlights a role for miR-520b-3p suppression in the regulation of ATF5 protein levels under cellular stress. The cellular stress response is adaptive and involves a complex network of systems. The data from the present study suggest an interplay between miR-520b-3p regulation of ATF5 expression at the 3' UTR and the previously reported post-transcriptional suppression at the 5' UTR. The extent to which these mechanisms cooperate to influence the dynamic regulation of ATF5 in stress and physiological disease states remains to be determined. Further studies are warranted to investigate the potential utility of miR-520b-3p as a therapeutic agent to re-sensitize cancer cells to chemotherapeutics as well as impact tumor growth via reversal of ATF5 expression in cancer cells.

Supporting information

S1 Raw Images. Full length Western blots for Figs 3, 4 and 6.
(PDF)

Author Contributions

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Formal analysis: Kari A. Gaither.

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Methodology: Bhanupriya Madarampalli, Philip Lazarus.

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Supervision: Christy J. W. Watson, Philip Lazarus.

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Visualization: Kari A. Gaither.

Writing – original draft: Kari A. Gaither.

Writing – review & editing: Kari A. Gaither, Christy J. W. Watson, Philip Lazarus.

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