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RESEARCH LETTER

miR-4734 conditionally suppresses ER stress-associated proinflammatory responses

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(Received 17 July 2022, revised 7 November 2022, accepted 20 November 2022, available online 11 December 2022)

doi:10.1002/1873-3468.14548

Edited by Nicola Gray

Prolonged metabolic stress can lead to severe pathologies. In metabolically challenged primary fibroblasts, we assigned a novel role for the poorly characterized miR-4734 in restricting ATF4 and IRE1-mediated upregulation of a set of proinflammatory cytokines and endoplasmic reticulum stress-associated genes. Conversely, inhibition of this miRNA augmented the expression of those genes. Mechanistically, miR-4734 was found to restrict the expression of the transcriptional activator NF-kappa-B inhibitor zeta (NFKBIZ), which is required for optimal expression of the proinflammatory genes and whose mRNA is targeted directly by miR-4734. Concordantly, overexpression of NFKBIZ compromised the effects of miR-4734, underscoring the importance of this direct targeting. As the effects of miR-4734 were evident under stress but not under basal conditions, it may possess therapeutic utility towards alleviating stress-induced pathologies.

Keywords: ATF4; ER stress; inflammation; IRE1a; miR-4734; NFKBIZ

Metabolic dysregulation may lead to inflammation and cancer. In addition, metabolic perturbations such as nutrient deprivation, and glucose deprivation in particular, can trigger continuous accumulation of misfolded proteins in the endoplasmic reticulum (ER), leading to ER stress [1–3]. ER stress is characterized by suboptimal folding efficiency of ER-resident proteins and is in fact a result of a functional compromise in a system referred to as ER protein quality control (PQC). Molecular chaperones and folding enzymes [4,5] take part in the adaptive ER stress response that is required to establish protein folding homeostasis (proteostasis). BiP (encoded

Abbreviations
ECL, enhanced chemiluminescent; ER, endoplasmic reticulum; FDR, false discovery rate; GS, glucose and serum; HFF, human foreskin fibroblasts; NFKBIZ, NF-kappa-B inhibitor zeta; NF-κB, nuclear factor kappa B; PD, pulldown; PQC, protein quality control; UPR, unfolded protein response.

by the *HSPA5* gene) is a major ER chaperone, which functions during ER-targeted protein translocation, protein folding and ER-associated degradation of misfolded proteins (ERAD) [6,7]. During ERAD, BiP collaborates with ERdj4 (encoded by *DNAJB9*) and ERdj5 (encoded by *DNAJC10*), enabling the proteasomal degradation of misfolded proteins in the cytosol [8–10].

IRE1a (IRE1, encoded by human *ERN1*), PERK and ATF6 sense the increase in the load of aberrantly folded proteins in the ER and function as signal transducers, which serve to activate signalling networks that lead to profound transcriptional and translational changes that constitute the unfolded protein response (UPR) [3,11–13]. The UPR target genes are required to meet the increasing demand for PQC components [13]. Some of the canonical genes turned on by these factors code for major ER chaperones, including BiP, and proteins required during ERAD [13]. In concert, the UPR response leads to decreased synthesis of a subset of proteins and preferential translation of a subset of mRNAs, including the mRNA encoding the ATF4 transcription factor [14–18]. ATF4 regulates a broad group of unique prosurvival and adaptation-geared targets, including genes involved in protection from stress and the *DDIT3* gene encoding the transcriptional modulator and ATF4 collaborator CHOP [19,20]. Importantly, adaptation to ER stress may be accompanied by a variety of physiologically relevant secretory responses [21,22] such as the production of proinflammatory cytokines, which in turn may further augment ER stress [3,23–26]. Under certain circumstances, proinflammatory gene expression is IRE1-dependent [27–29] as well as ATF4 dependent [30,31]. Importantly, adaptation following ER stress may not always be successful, and in some instances chronic ER stress may persist. Such failure to maintain proteostasis may lead various UPR arms to collaborate in the induction of cell death [32,33]. Finally, ER stress-induced inflammation and cell death may severely damage numerous organs, leading to chronic diseases [3,34].

As microRNAs (miRNAs) have been implicated in the regulation of UPR [35,36], in this study we wished to explore the role of additional miRNAs, especially in normal cells in which ER stress may be induced under progressive and prolonged challenge. Employing primary human foreskin fibroblasts (HFF), our analysis revealed a pivotal role of the under-investigated miRNA miR-4734 under chronic stress conditions. We report that this miRNA can suppress ER stress-associated proinflammatory responses and ablate IL-8 secretion. Furthermore, our study implicates NF-kappa-B inhibitor zeta (NFKBIZ), a potent transcriptional activator of proinflammatory genes [37–39], as a co-regulator of gene expression and a direct target of miR-4734 in HFF. Because attenuation of ER stress can ameliorate UPR-related chronic diseases [40], we propose that miR-4734 mimics may potentially serve as new tools against diseases associated with ER stress and inflammation.

Materials and methods

Detailed description of the materials and methods can be found in Appendix S1.

Cell culture

Human foreskin fibroblasts (AG14608) were obtained from the NIA Repository, administered by the Coriell Institute for Medical Research, Camden, NJ. BJ cells were obtained from American Type Culture Collection, Manassas, VA, USA (CRL-2522). The sequences of the RNA molecules used for transfection are listed in Table S5. Following transfection, cultures were grown for 40–46 h, until confluency was reached. At this stage, cells were either collected ($t = 0$) or treated as indicated. For starvation, cultures were replenished with GS starvation medium (Appendix S1). Tunicamycin (Sigma T7765; St. Louis, MO, USA) was added to the fresh medium at the onset of the treatment.

Expression arrays and analysis of high-throughput mRNA expression data

For the first mRNA expression microarray analysis (Affymetrix, Santa Clara, CA, USA) was used. For the second analysis Clariom™ S, human (Thermo Fisher Scientific, Waltham, MA, USA; 902917) was used. Statistical analysis of microarray data was performed using the PARTEK® GENOMICS SUITE software (Partek Inc., St. Louis, MO, USA). Data were preprocessed and normalized using the robust multichip average algorithm [41]. To identify differentially expressed genes, analysis of variance was applied. Contrasts were calculated for each of the three pairwise comparisons. False discovery rate (FDR) was used to correct for multiple comparisons using the procedure of Benjamini and Hochberg [42].

For Hierarchical Clustering, Log_2 transformed normalized intensity values were used. Samples were clustered using Pearson Dissimilarity distance measure and Ward's linkage.

For Heatmap of Partitioning Clustering in the first expression array experiment, genes exhibiting an absolute fold change in at least 2 and FDR of 0.05 or less between any two pairwise comparisons were chosen. Functional analysis was performed using the Ingenuity Pathway Analysis (IPA) (https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis/).

Real-time PCR

Cellular RNA was purified using the miRNeasy kit (Qiagen, Hilden, Germany; 217004) and in most RT-qPCR

assays RPL8 was used as a normalizing (reference) gene. Relative quantification was derived using standard curves according to the MIQE guidelines [43]. For real-time PCR analysis of relative miRNA expression, PerfeCTA reagents were used (Quanta Biosciences, Beverly, MA, USA; 95054). PCR amplification was done in a StepOnePlus thermocycler (Applied Biosystems, Waltham, MA, USA).

Biotinylated-miR-mRNA pulldown experiments

The pulldown (PD) assay, designed to enrich for high-specificity miRNA targets, was performed essentially as previously described [44]; a detailed description can be found in Appendix S1. In short, biotinylated miRNA mimic (bt-miRNA) was transfected and cells were processed or starved for 48 h before harvesting. Streptavidin Dynabeads M-280 were employed to capture mRNA bound to the transfected bt-miR. To increase the sensitivity and reproducibility of the assay, we introduced an RT-pre-amplification step prior to final qPCR. qPCR results were considered valid only when amplification plots were within the dynamic range and met all other criteria set by the MIQE guidelines [43].

Generation of NFKBIZ-overexpressing HFF

Using standard recombinant DNA procedures, the cDNA encoding for IκB-ζ_L, the long variant of IκB-ζ (isoform a), was cloned and inserted into the pLVX-EF1a-IRES-mCherry lentiviral vector (TakaraBio/Clontech, Kusatsu, Japan), downstream to the EF1a promoter and upstream to the IRES-mCherry encoding sequence. Following transfection into 293FT cells, virus-containing supernatants were used for reverse transduction.

Immunoblotting and IL-8 enzyme-linked immunosorbent assay

Protein extracts were separated on 10% polyacrylamide gels and transferred onto a nitrocellulose membrane. Processed blots were immersed in enhanced chemiluminescent (ECL) substrate solution. Digital images were captured using myECL imager (Thermo, Waltham, MA, USA). To quantify secreted IL-8, growth medium samples from cells were frozen and secreted IL-8 was quantified with the Human IL-8 ELISA MAX Deluxe kit (BioLegend, San Diego, CA, USA).

Statistical analysis

Unless otherwise stated, statistical analysis of RT-qPCR results was performed with PARTEK® GENOMICS SUITE software (Partek Inc.). Log_2 transformed relative expression values or ΔC_t values were imported for mRNA and miRNA statistical analysis respectively. For all assays, three-way ANOVA was performed using starvation time, the relevant RNA agent and experimental batch variables. Contrasts were calculated to compare pairwise between the given parameters. FDR was used to correct for multiple comparisons. For secretion assays, log_2 concentration of IL-8 was used.

Results

GS starvation leads to ATF4 and IRE1-dependent proinflammatory and ER stress responses

Metabolic perturbations and glucose starvation, in particular, lead to ER stress. This prompted us to investigate sequential molecular adaptation processes in primary HFF challenged by combined glucose and serum (GS) starvation (Appendix S1). Following GS starvation, gene expression microarray analysis enabled us to cluster the 1723 differentially expressed informative genes into seven clusters (Fig. 1A). We focused our attention on clusters 2 and 7, which were the only ones displaying increased gene expression at the late phase of starvation (48 h) and included genes of pathological relevance (see below); the most prominent changes in mRNA levels are shown in Table S1. Ingenuity Pathway Analysis (IPA) of genes in these two clusters revealed enrichment for the UPR and the ER stress pathways (Fig. 1B; Table S2). Notably, according to the IPA several ATF4-dependent and IRE1-dependent genes were upregulated following starvation (Fig. S1), prompting us to further investigate the roles of ATF4 and IRE1 in our experimental model. Thus, we performed a reverse transcriptase quantitative PCR (RT-qPCR) analysis after 48 h of starvation. This analysis confirmed that the expression of multiple proinflammatory genes increased strongly following starvation (Fig. 1C; Fig. S2A,B). Moreover, we also observed a similar pattern for several genes involved in the ER stress response, including *DNAJB9* (encoding for ERdj4) and Bip (also known as GRP78; gene name *HSPA5*) (Fig. 1C; Fig. S2B). ERdj4 collaborates with BiP/GRP78 in ER-associated degradation of misfolded proteins [5], underscoring that GS-starved HFF are experiencing ER stress. Interestingly, expression of ERO1LB (also known as ERO1B and Ero1beta), best characterized in insulin-producing β cells where it contributes to oxidative maturation/folding of pro-insulin [45], also increased in a similar manner (Fig. 1C; Fig. S2B).

Next, we asked whether the expression changes summarized in Fig. 1C are dependent on ATF4 and IRE1. Knockdown of ATF4 or IRE1 (encoded by the *ERN1* gene) by siRNA showed that, in cells starved for 48 h, ATF4 and IRE1 expression was indeed required for induction of proinflammatory cytokines and chemokines and of a subset of ER stress-related genes (Fig. 1D; Fig. S2A,B). The fact that not all ER stress genes

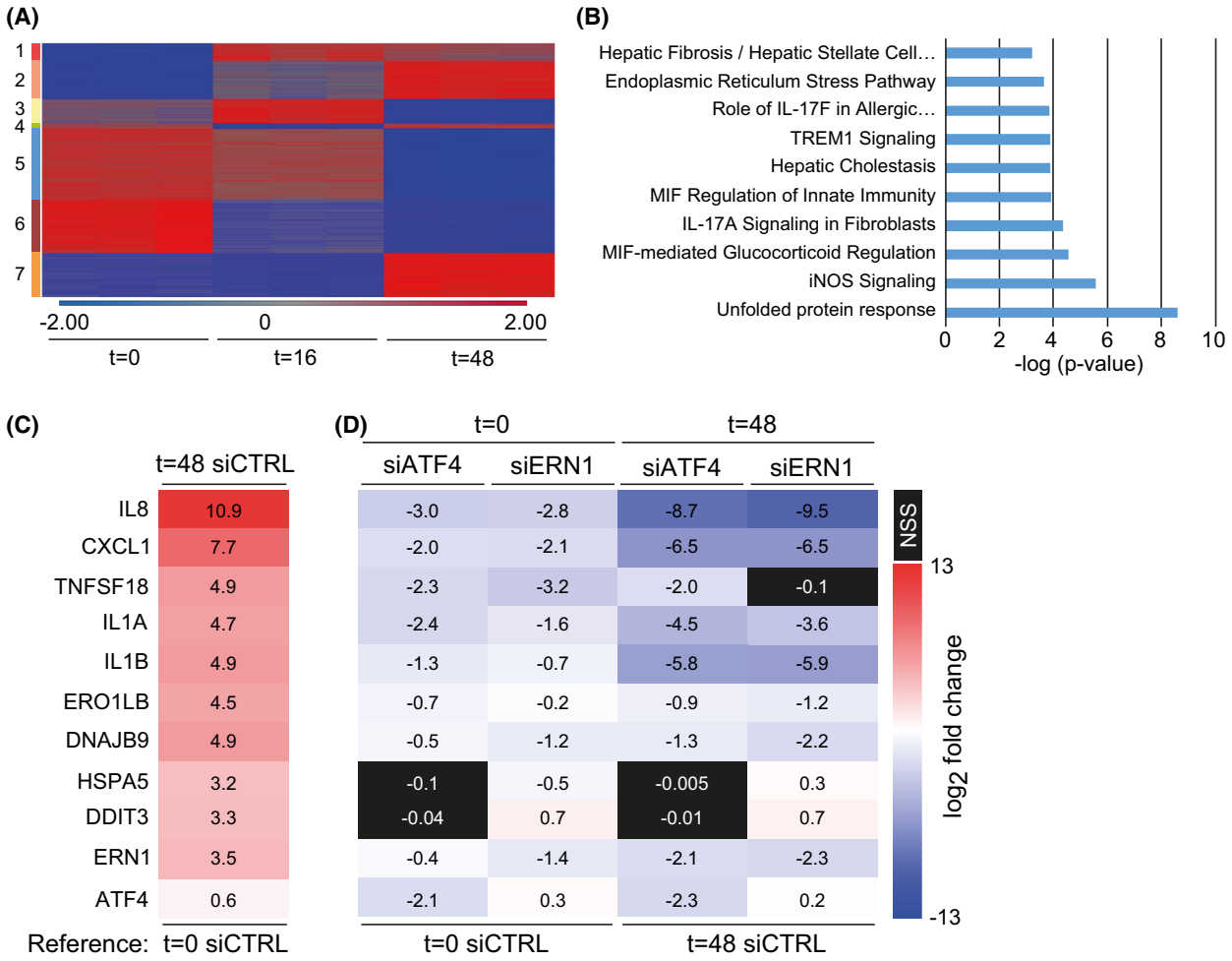

Fig. 1. ATF4 and ERN1-dependent ER stress associated proinflammatory response in GS-starved cells. (A) Heatmap partitioning of global differential gene expression in non-starved vs. GS-starved cells (16 and 48 h). Partitioning Clustering divided the 1723 informative genes, characterized by an absolute fold change in at least 2 and a FDR of < 0.05 between two pairwise comparisons, into seven clusters, according to differential expression patterns (Appendix S1). (B) Ingenuity Pathway Analysis of the genes in clusters 2 (260 differentially expressed genes) and 7 (301 differentially expressed genes) (combined; 48 h starvation vs. non-starved); both these clusters were characterized by upregulation of gene expression only at $t = 48$. Top 10 statistically significant Igenuity Canonical Pathways are listed. Full pathway names are listed in Table S2. (C, D) HFF cell cultures were transfected with either control siRNA (siCTRL), siRNA against *ERN1* (siERN1) that encodes IRE1a or siRNA against *ATF4* (siATF4), and 48 h later ($t = 0$) were subjected to GS starvation for the indicated durations. Shown are relative changes in gene expression, as determined by reverse transcriptase quantitative PCR (RT-qPCR) analysis. Numbers represent average $\log_2$ fold change relative to cells pre-transfected with miR-CTRL, obtained from three biological repeats. (C) The fold change in expression of each indicated mRNA was deduced by comparing its relative expression at the different time points to its basal expression at $t = 0$ (non-starved cells). (D) The listed fold changes reflect the ratio between expression of each mRNA in the siERN1 sample or the siATF4 sample versus the corresponding siCTRL sample. The raw average data taken for statistical analysis and the FDRs, determined by statistical analysis of variance (ANOVA) contrasts, are listed under Appendix S2. Coloured rectangles indicate FDR < 0.05, black rectangles denote not statistically significant (NSS) fold change. The data in C and D were obtained in parallel from the same experiments.

responded identically suggests that at least some of these genes may be co-regulated by additional transcription factors other than ATF4. Notably, IL8 mRNA expression was affected to a similar extent by four different ATF4-directed siRNAs and three ERN1-directed siRNAs (Fig. S3), ruling out off-target effects. Of note, ATF4 was found to augment IL-6 expression [30] and, in another system, ATF4 and NF-κB were found to co-regulate the expression and secretion of various cytokines and chemokines following starvation [31]. In conclusion, here we show that in our experimental conditions both ATF4 and IRE1-dependent signalling pathways

were necessary, to a similar extent, for the manifestation of an ER-stress-associated proinflammatory response.

miR-4734 tunes down the proinflammatory and ER-stress responses

To characterize additional regulatory circuits operating in our experimental system, we investigated microRNA (miRNA) expression patterns in control and 48 h–starved HFF, and observed that the expression of several miRNAs was modulated upon starvation. Of those, we chose to focus on miR-4734 (Genome coordinates derived from chr17: 38702262–38702331 [−]), since its cellular and molecular roles had not been studied before. Given that the GC content of this miRNA is 81.8%, quantification of its expression required rigorous RT-qPCR analysis. Using a miR-seed-annealed primer, miR-4734 expression was found to be reduced upon prolonged starvation, concurrently with the enhanced induction of cytokine and chemokine genes (Fig. 2A). This suggested that miR-4734 might play a role in limiting the stress-induced expression of those genes, such that the reduction in its levels at 48 h might enable their more pronounced induction. Indeed, introduction of miR-4734 mimics into HFF significantly attenuated the induction of most tested cytokine and chemokine genes (with the exception of *TNFSF18*) upon starvation (Fig. 2B,C; Fig. S4A). Of note, the overexpressed miR-4734 tuned down the induction of the ER stress-associated genes *HSPA5*, *DNAJB9*, *DDIT3* and *ERO1LB* only at 24 h, but not at 48 h (Fig. 2B,C; Fig. S4B). Moreover, expression of *ERN1* was upregulated at both time points, but this upregulation was attenuated by miR-4734. Thus, miR-4734 restricts the proinflammatory and ER stress responses in GS-starved HFF.

Given the above inhibitory effects of miR-4734 on gene expression, we asked whether it could also tune down the induction of ATF4 and IRE1 at the protein level. Indeed, introduction of this miRNA suppressed the increase in ATF4 protein (particularly at 24 h), as well as of IRE1 protein (particularly at 48 h) (Fig. 3A–C), supporting a role of miR-4734 in ameliorating the proinflammatory response that is accompanied by ER stress.

Next, we sought to determine whether the observed effects of miR-4734 on IL8 mRNA were also manifested at the level of IL-8 secretion. As shown in Fig. 3D, following 48 h of starvation the amount of IL-8 secreted into the culture medium was massively increased, relative to non-starved cells, in line with the extensive upregulation of IL8 mRNA. Remarkably, even though miR-4734 only partly attenuated the increase in IL8 mRNA (Fig. 2B,C; Fig. S4A), it completely abolished the increase in secreted IL-8 (Fig. 3D). The IL8 mRNA sequence does not contain any predicted target sites for miR-4734. Therefore, we believe that it is unlikely that miR-4734 directly targets IL8 mRNA for degradation or directly restricts its translation. However, we cannot exclude the possibility that miR-4734 may target other mRNAs whose protein products affect the translation of IL8 mRNA or the stability of the IL-8 protein.

Next, we used miRNA inhibitors to ascertain that the proinflammatory and ER stress response can be tuned down also by endogenous miR-4734. While we observed starvation-induced upregulation of cytokine and chemokine mRNAs in the presence of a hairpin negative control (Fig. 4A; Fig. S5A), inhibition of miR-4734 further increased the expression of several of them at the early phase of the stress response (t = 24) (Fig. 4A,B; Fig. S5A). Notably, the ERAD-associated *DNAJB9* and *HSPA5* mRNAs displayed similar patterns (Fig. 4A,B; Fig. S5B), further supporting a linkage between the ER stress and the cytokine and chemokine response. Remarkably, such pronounced effect of miR-4734 inhibition was not observed at 48 h. This is consistent with the observation that endogenous miR-4734 levels drop substantially by 48 h (Fig. 2A), presumably diminishing its contribution to the cell state; thus, its neutralization by the anti-miRNA at this time may not be expected to have much impact. Taken together, the data in Fig. 4A,B corroborate the suggestion that the main role of miR-4734 in the endogenous setting is to delay, rather than prevent, the cytokine and chemokine response.

To examine the effect of miR-4734 on the response to a different type of ER-stress inducer, HFF were treated with tunicamycin, which blocks the initial steps of glycoprotein biosynthesis in the ER, causing the accumulation of unfolded proteins in the ER [1]. As expected, this resulted in induction of inflammatory and ER-stress related genes (Fig. 4C; Fig. S6). However, miR-4734 overexpression silenced markedly this transcriptional response (Fig. 4C,D; Fig. S6). Moreover, miR-4734 exerted a similar effect also on tunicamycin-treated BJ fibroblasts (Fig. S7). Altogether, our findings suggest that miR-4734 restricts the induction of proinflammatory and several ER stress response genes under different experimentally induced ER stress conditions and in different cell lines.

NFKBIZ expression governs the cytokine-chemokine and ER stress responses

To identify candidate direct targets of miR-4734 that might account for the modulation of the cytokine-

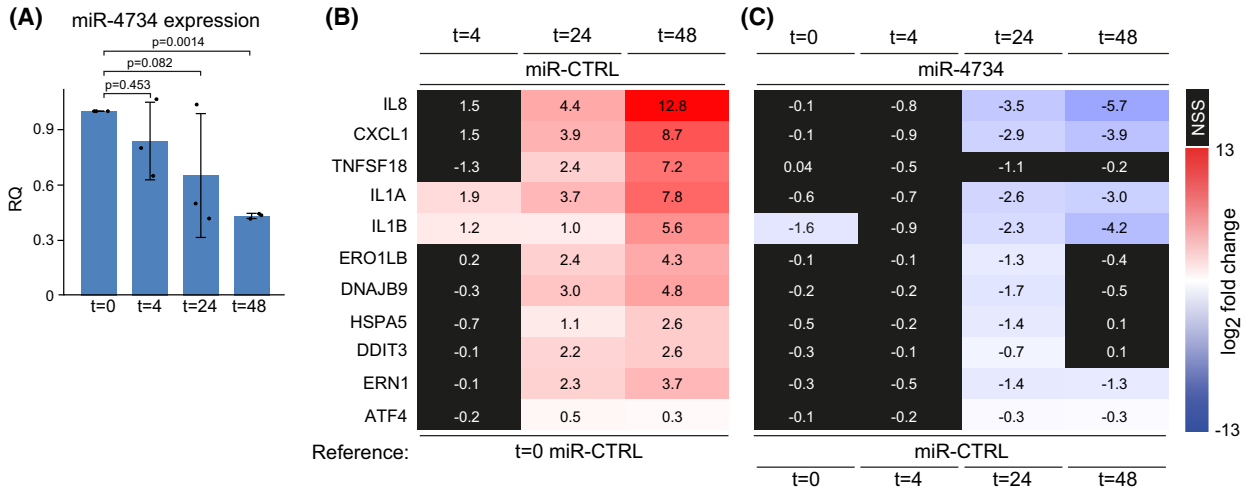

Fig. 2. miR-4734 attenuates the induction of inflammation and ER stress-related genes. (A) miR-4734 levels are reduced upon prolonged starvation. HFF were subjected to SG starvation for 48 h. Expression of miR-4734 was determined by pre-amplification followed by RT-qPCR, analysis in which SNORD44 served as the endogenous control. The data originated from three biological replicates. Relative quantity (RQ) was calculated by normalizing the relative expression of the miRNA in starved cells to its expression in control cells (t = 0). ANOVA was used to calculate the significance of the changes at different time points against time = 0. ΔC_t values of three replicates were used as the input for ANOVA, and the replicates were added as a random factor. RQ values with standard deviations as well as P values are shown. (B, C) HFF were transfected with miR-CTRL or miR-4734 mimic and subjected to SG starvation. Relative expression was determined by RT-qPCR. Numbers represent average $\log_2$ fold change obtained from three biological repeats. (B) Relative expression levels in starved miR-CTRL-transfected cells, compared to t = 0. (C) Relative expression levels in cells transfected with miR-4734, compared to miR-CTRL-transfected cells (used as a reference) starved for the same duration. For statistical analysis and significance see legend in Fig. 1C,D. The data in B and C were obtained in parallel from the same experiments.

chemokine and ER stress responses, we used an expression array to match the results described in Fig. 1A,B. Thus, we analysed the transcriptome of miR-4734 transfected HFF that underwent starvation (a heat map summarizing the analysis is shown in Fig. 5A). Out of 2018 differentially expressed informative genes, we focused on clusters 3 and 4, comprising genes that were upregulated upon stress (t = 48 miR-CTRL, vs. t = 0 miR-CTRL) and modestly downregulated by miR-4734 (t = 48 miR-4734 vs. t = 48 miR-CTRL), as expected for a miRNA effect. These clusters were combined and evaluated as explained under Appendix S1, yielding 125 genes for further study (Table S3; genes are listed according to the effect of miR-4734 on their expression). Notably, this list comprises many proinflammatory genes, suggesting that a common upstream transcriptional regulator might be targeted by miR-4734.

One of the genes downregulated in stressed cells by miR-4734 was *NFKBIZ* (Table S3), which encodes the IκB-ζ protein. IκB-ζ is an atypical nuclear member of the IkB family [46], which is rapidly induced by several proinflammatory stimuli [37,47,48] and by specific cytokines [46]. IκB-ζ can interact with the p50 subunit of nuclear factor kappa B (NF-κB) [39] and has been shown to account for proinflammatory cytokine expression in various innate immune-related pathways [37–39]. Its transcriptional effects involve the recruitment of SWI/SNF chromatin remodeller complexes, highlighting its role as a transcriptional coactivator for NF-κB [49]. Of note, previous studies in other systems have indicated that *NFKBIZ* expression is either reduced upon ER stress [20] or is correlated with ER stress [50]. We therefore wished to examine the role of *NFKBIZ* in our experimental system. As shown in Fig. S8A–D, NFKBIZ silencing suppressed the induction of IL1B and IL8 mRNA upon starvation, implying that it contributes positively to this response. Expression of *HSPA5* and *DNAJB9* was also compromised, suggesting a role for NFKBIZ also in ER stress, either directly or as a consequence of elevated proinflammatory gene expression.

NFKBIZ mRNA is a direct target of miR-4734

To determine whether miR-4734 regulates NFKBIZ post-transcriptionally, we compared the effect of this miRNA on the steady-state levels of *NFKBIZ* mRNA to its effect on *NFKBIZ* pre-mRNA levels, which were monitored by using intronic primers. As seen in Fig. S9,

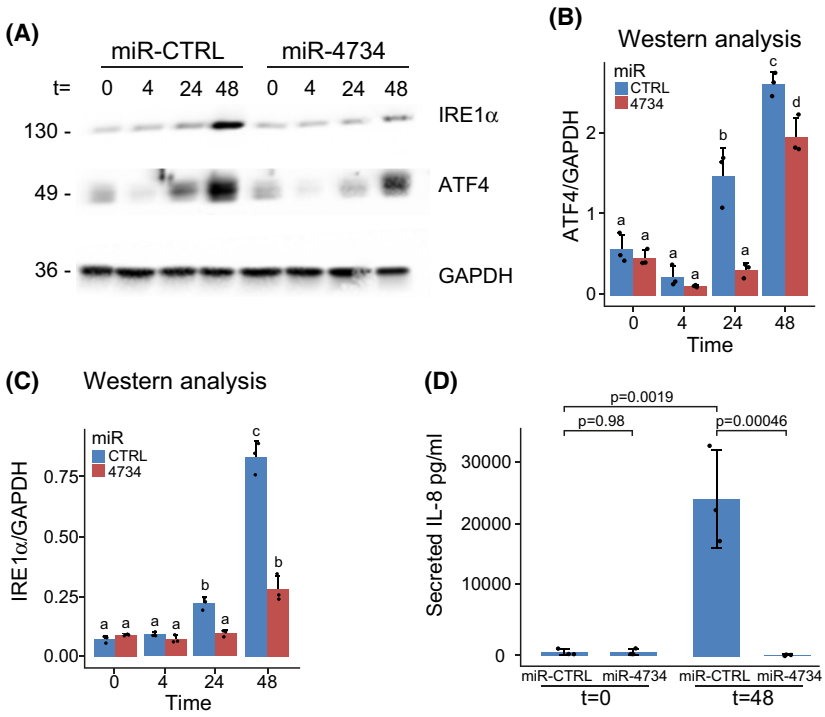

Fig. 3. miR4734 restricts ATF4 and IRE1a protein levels and completely blocks the secretion of IL-8. (A–C) miR-4734 reduces the expression of ATF4 and IRE1α proteins following starvation. HFF were transfected with miR-CTRL or miR-4734 mimic and subjected to SG starvation. Levels of ATF4 and IRE1α proteins were determined by western blot analysis and digital images were acquired using myECL® imager. (B, C) Three biological repeats were subjected to western analysis and linear quantitative information was obtained using the myECL® software. Expression values were compared using ANOVA (with time, miR, batch and time:miR interaction as independent factors), followed by Tukey's HSD test. Treatments marked with different letters are significantly different ($P < 0.05$) while those that are marked by the same letter are not statistically significant. SD are shown. (D) IL-8 secreted from pre-transfected cells, either non-starved or starved, was quantified by an enzyme-linked immunosorbent assay (ELISA). Error bars represent standard deviation.

at the 48 h starvation time point the suppressive effect of miR-4734 on *NFKBIZ* mature RNA levels was more than twice stronger than its effect on *NFKBIZ* primary transcript levels, consistent with the involvement of a post-transcriptional mechanism. Inspection of the *NFKBIZ* sequence revealed several candidate miR-4734 targets sites, including a putative canonical binding site within the coding region (exon 5), as well as several non-canonical potential binding sites in both the coding sequence and the 5′UTR of *NFKBIZ* mRNA. Of note, binding of miRNAs within coding sequences or 5′UTRs has already been implicated in post-transcriptional regulation [51,52]. To probe for direct binding of miR-4734 to *NFKBIZ* mRNA, we next performed a PD experiment using transfected biotinylated miR-4734 [44]. As a reference, we tested the binding of miR-4734 to the mRNA encoding the eukaryotic translation elongation factor 2 (gene name: EEF2), predicted by TargetScanHuman7.1 [53] to be one of the top hits for binding by miR-4734. Remarkably, we found that *NFKBIZ* mRNA was selectively bound by miR-4734 upon stress (Fig. 5C), but not under basal conditions (Fig. 5B).

To further assess the relevance of NFKBIZ for the anti-inflammatory effect of miR-4734, we performed a rescue experiment. Specifically, we stably expressed in HFF the long variant of NFKBIZ (isoform a), whose transcription emanates from the proximal promoter 2 [54], fused in-frame with mCherry (expression analysis shown in Fig. S10A). Expression of proinflammatory mRNAs was then compared between control (expressing only mCherry) and mCherry-NFKBIZ expressing cells. As seen in Fig. 5D,E and Fig. S10B, overexpression of NFKBIZ quenched the ability of miR-4734 to reduce the expression of IL8, CXCL1, IL1A and IL1B mRNAs in starved cells, supporting the importance of NFKBIZ as a relevant target of miR-4734 in this setting.

Discussion

Inflammation is a significant contributor to metabolic and degenerative disorders as well as to cancer [3,55–57]. In this study, we describe an experimental system based on primary cells, which responds to a metabolic challenge

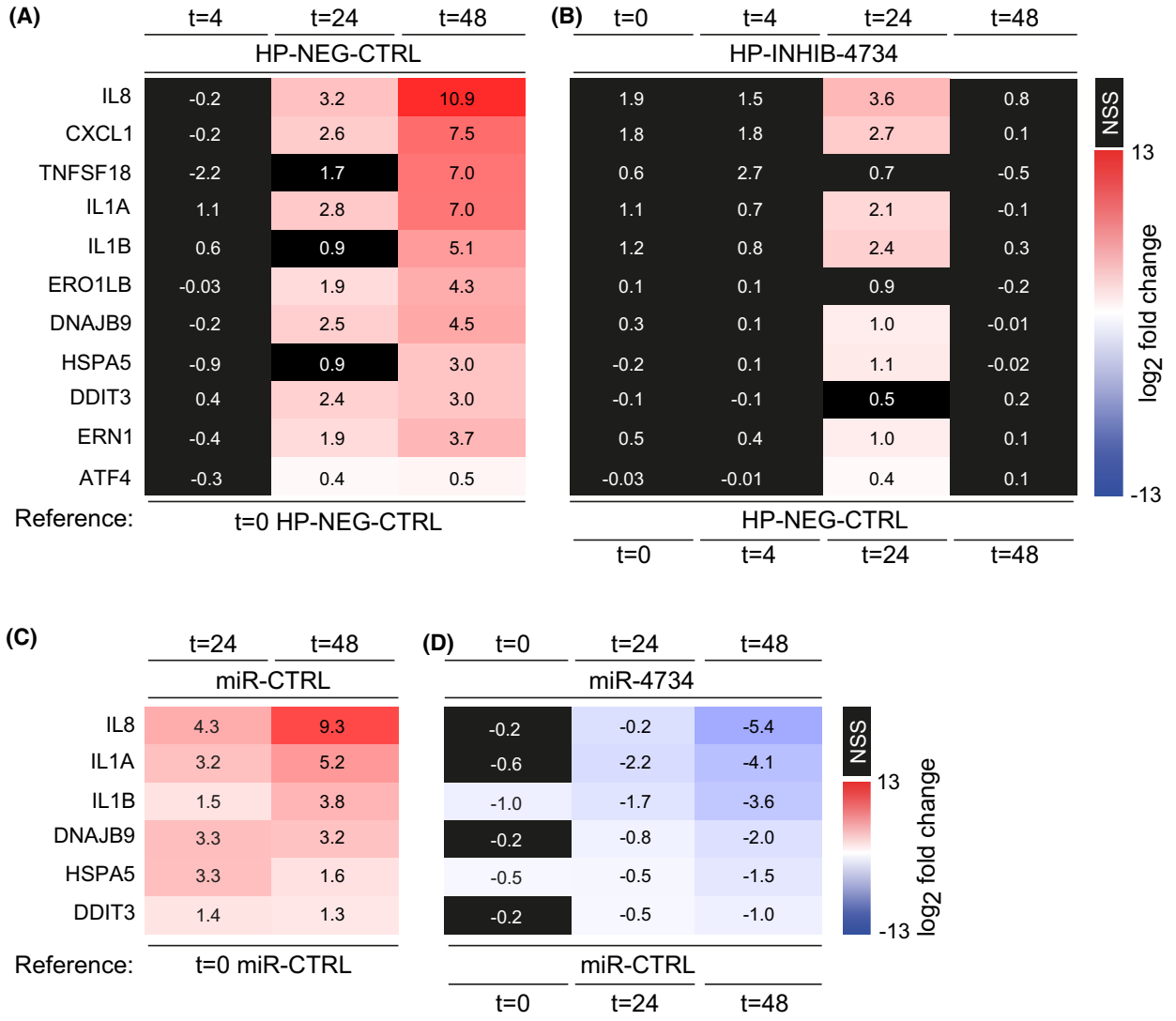

Fig. 4. Hairpin inhibitor of miR-4734 augments the expression of inflammation and ER stress-related genes and miR-4734 silences, the induction of the ER stress-associated proinflammatory response in HFF subjected to chemically-induced ER stress. (A, B) HFF were transfected with either hairpin negative control (HP-NEG-CTRL) or hairpin inhibitor of miR-4734 (HP-INHIB-4734), and then subjected to GS starvation. RT-qPCR analysis is shown. Numbers represent average $\log_2$ fold change obtained from three biological repeats. (A) Expression levels in starved HP-NEG-CTRL-transfected cells, relative to $t = 0$. (B) Expression levels in cells transfected with the hairpin inhibitor, relative to HP-NEG-CTRL-transfected cells starved for the same duration ($\log_2$ fold change). For statistical analysis and significance, see legend in Fig. 1C,D. The data in A and B were obtained in parallel from the same experiments. (C, D) HFF were transfected with miR-CTRL or miR-4734 mimic, and 48 h later subjected to 0.3 $\mu g \cdot mL^{-1}$ tunicamycin for various durations. Gene expression was determined by RT-qPCR. Numbers represent average $\log_2$ fold change obtained from three biological repeats. (C) Relative mRNA levels in miR-CTRL-transfected tunicamycin-treated cells, compared to non-treated cells ($t = 0$). (D) Relative expression levels in cells transfected with miR-4734, either non-treated ($t = 0$) or tunicamycin-treated for the indicated duration, compared to miR-CTRL-transfected cells harvested in parallel. For statistical analysis and significance, see legend in Fig. 1C,D. The data in C and D were obtained in parallel from the same experiments.

in an ATF4, ERN1 and NFKBIZ-dependent manner (Fig. 1C,D; Fig. S8A,B). Although it may seem counterintuitive that starved cells dedicate resources to the synthesis and secretion of cytokines and chemokines, this may be beneficial at the organismal level in order to recruit cells to subserve physiological processes that are required for tissue maintenance. However, under prolonged stress, overexpression of inflammatory mediators may further exacerbate the pathology of the inflicted tissue. As shown here, challenged fibroblasts may activate an amplification loop wherein early induction of a subset of cytokines augments the UPR response, resulting in the further

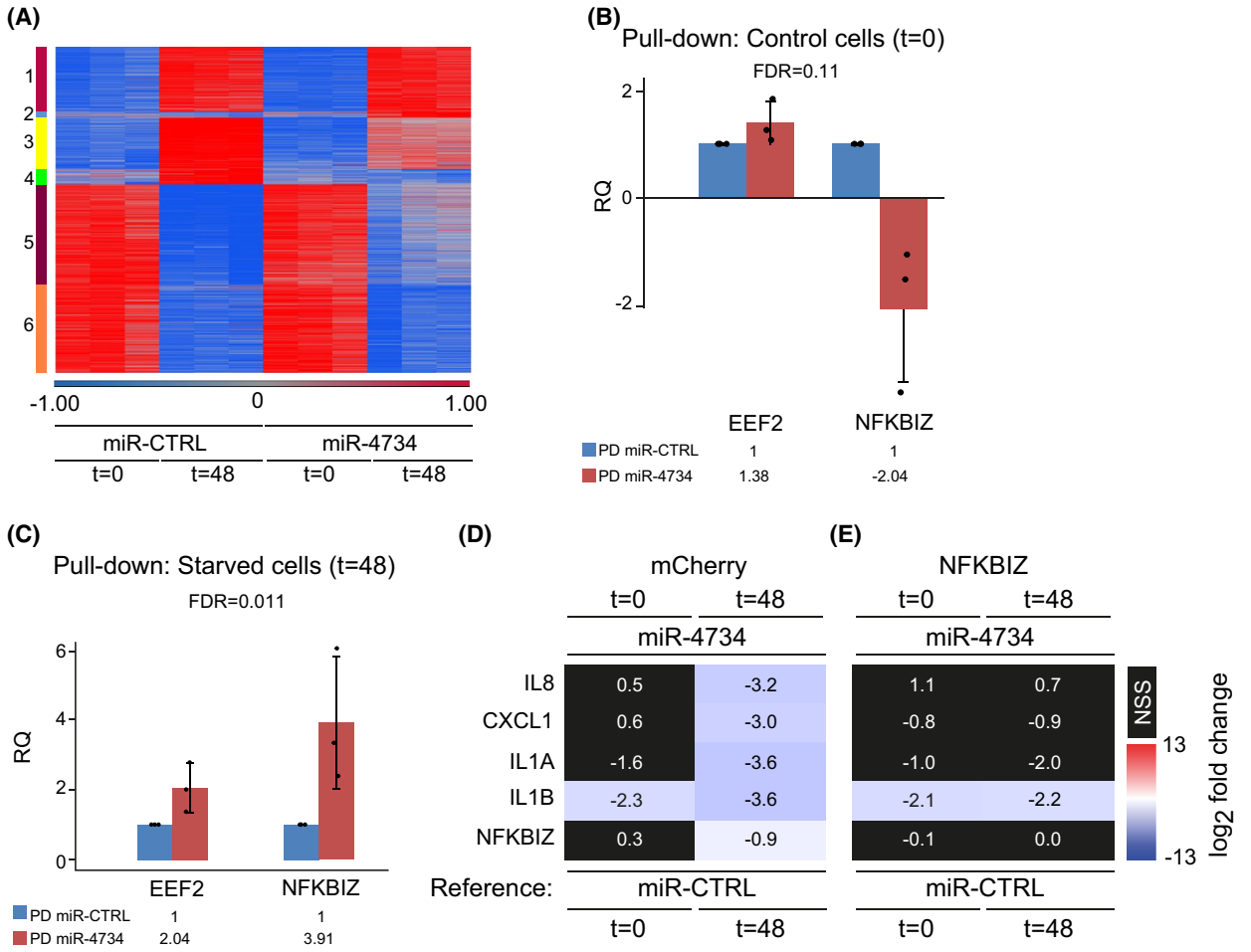

Fig. 5. NFKBIZ mRNA is a direct target of miR-4734. (A) Global effects of miR-4734 on mRNA expression in stressed cells. Heatmap partitioning of global differential gene expression in non-starved and GS-starved cells ($t = 48$). A total of 2018 genes, characterized by an absolute fold change in at least 2 and a FDR of 0.05 between at least two pairwise comparisons, were divided into six clusters (see Materials and methods), according to differential expression patterns. In clusters 3 and 4 (combined), the expression of 125 genes was downregulated by at least 2 fold. (B, C) HFF were transfected with biotinylated (bt) miR control or bt-miR-4734, after which cells were either harvested ($t = 0$) or starved for 48 h ($t = 48$). Cell extracts were subjected to PD analysis as described in Appendix S1, followed by RT-qPCR analysis with primers derived from the indicated genes. Numbers were obtained from three biological repeats. Error bars represent standard deviation. While the fold change at $t = 0$ for both comparisons was not significant (FDR = 0.11), it was significant at $t = 48$ for both comparisons (FDR = 0.011). (D, E) In a rescue experiment, HFF stably overexpressing mCherry or mCherry-NFKBIZ were transfected with either miR-CTRL (used as a reference) or miR-4734 and harvested ($t = 0$) or after starvation ($t = 48$). RNA was subjected to RT-qPCR analysis of the indicated genes. Numbers represent average $\log_2$ fold change obtained from three biological repeats. For statistical analysis and significance see the legend in Fig. 1C,D. The data in D and E were obtained in parallel from the same experiments.

production and secretion of an expanded panel of cytokines and chemokines. This conjecture is supported by the fact that *IL1A* and *IL1B* can be induced already within 4 h of starvation, a time at which ATF4 protein levels tend to decrease, and prior to the upregulation of prominent UPR components (Figs 2B and 3A–C).

In this study, we highlight the roles of the understudied miRNA miR-4734 in restricting the aforementioned ER stress-associated proinflammatory responses. Interestingly, while ectopically introduced miR-4734 quenched to a large extent the stress-induced expression of several proinflammatory and ER stress genes, it did not affect the basal expression of those genes, with the exception of *IL1B* (Figs 2B,C and 4C,D; Fig. S7). Thus, this miRNA selectively restricts the transcriptional response to metabolic stress signals. In fact, miRNAs have been proposed to buffer against stochastic fluctuations in mRNA accumulation, which may occur throughout a cell population [58]. Buffering the mRNA availability for translation may in turn ensure 'canalization' of the response at the

relevant time and the relevant magnitude. Thus, at the early stages of a metabolic challenge and upon the initial stochastic accumulation of proinflammatory mRNAs, there may be a decision window or a threshold, which may place these transcripts under miR-4734 regulation to prevent a premature full-blown cytokine response. In this manner, oscillations in inflammatory and stress-associated gene expression may be prevented, especially prior to the phase of enhanced stress (Fig. 4A,B). In addition to changes at the transcriptional level, we found that miR-4734 can also ablate the secretion of IL-8 (Fig. 3D). Therefore, a role for this miRNA in maintaining a tight secretion threshold may be envisioned as well.

We also report here the identification of NFKBIZ mRNA as a direct target of miR-4734. In fact, links of NFKBIZ to inflammation-associated diseases, including cancer [59,60] and psoriasis [61,62], have been described. Interestingly, the binding of miR-4734 to NFKBIZ is stress-dependent (Fig. 5B,C), which might in part explain the conditional silencing profile of miR-4734 (Fig. 2B,C). The exact mechanism underlying this conditional binding deserves further investigation. Furthermore, the significance of NFKBIZ as a target was highlighted by the fact that its stable overexpression ablated part of the miR-4734-dependent anti-inflammatory response (Fig. 5D,E). Importantly, NF-κB and NFKBIZ expression are linked [47], and ER stress has been linked to activation of NF-κB [30,63–65]. Remarkably, NF-kB activation was found to be IRE1-dependent [66,67]. Therefore, given the roles of NFKBIZ in NF-kB-dependent regulation of inflammation-associated genes [68–70], it is conceivable that upon initial ER stress, cells respond by IRE1-dependent activation of proinflammatory genes. This appears to be mediated by NF-κB and NFKBIZ, and at a later phase it becomes greatly dependent also on ATF4 (Fig. 1C,D). Remarkably, we found that ATF4 and IRE1 are both critical but not sufficient on their own for maximal response (Fig. 1C,D). Thus, targeting of NFKBIZ by miR-4734 could explain how a full-blown cytokine and chemokine response can be silenced by this miRNA (Figs 2B,C, 4A,B, and 5B–E; Fig. S8).

The importance of modulating ER stress by potential therapeutic agents has already been tested in several settings [71,72]. Our findings raise the possibility of taking advantage of miR-4734 mimics to treat a variety of disorders characterized by UPR and inflammatory responses. In that regard, since an individual miRNA typically targets multiple mRNAs [73], it is likely that the anti-inflammatory activity of miR-4734 may stem from targeting additional relevant mRNAs besides NFKBIZ, potentially in the ERN1 and/or ATF4 pathways (Fig. 3A), thus making this miRNA a potential novel pathway-centred multi-target therapeutic tool. As the effects reported here for miR-4734 seem to be restricted to stress settings, with minimal apparent impact on non-stressed cells, this gives hope that administration of this miRNA may harness its potential to act as therapeutic agent while sparing the remainder of the non-stressed, homeostatic tissue and the body.

Acknowledgements

The authors thank and Gilgi Friedlander for analysis of the mRNA expression array data. This work was supported in part by the Dr. Miriam and Sheldon G. Adelson Medical Research Foundation, a grant from Anat and Amnon Shashua, and the Moross Integrated Cancer Center. MO is incumbent of the Andre Lwoff chair in molecular biology.

Author contributions

DM involved in research design and experimental planning; DM, AA, CG, NF, FG, AM, OH, AT, AB-K, AB-D, NH and DB performed the experiments; EF and RR performed the statistical analysis; MO and DM involved in supervision and data analysis; IU provided the experimental advice; DM and MO involved in manuscript writing; NF, IU and GB involved in manuscript editing.

Data accessibility

The data that support the findings of this study are available in the Supporting Information (Figs S1–S10, Tables S1–S5 and Appendix S2) as well as in Appendix S1. The functional genomics data that support the findings of this study are openly available in NCBI's Gene Expression Omnibus and are accessible through GEO accession GSE214963 https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE214963.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Appendix S1. Supplementary materials and methods.
Appendix S2. Supporting statistical analysis.
Fig. S1. Analysis of starvation-induced mRNA expression changes.
Fig. S2. ATF4 and IRE1-dependent ER stress-associated proinflammatory response in GS starved cells.
Fig. S3. ATF4 or ERN1 silencing by different single siRNAs reduces IL8 expression to a similar extent.
Fig. S4. miR-4734 attenuates the induction of inflammation and ER stress-related genes.
Fig. S5. Hairpin inhibitor of miR-4734 augments the expression of inflammation and ER stress-related genes.
Fig. S6. miR-4734 silences the induction of ER stress-associated proinflammatory response in HFF subjected to chemically-induced ER stress.
Fig. S7. Expression of ER stress-associated genes and proinflammatory genes in miR-4734-transfected BJ fibroblasts exposed to tunicamycin.
Fig. S8. NFKBIZ is required for induction of cytokine and ER stress genes under starvation.
Fig. S9. miR-4734 reduces preferentially the mature NFKBIZ mRNA as compared to its pre-mRNA.
Fig. S10. NFKBIZ overexpression rescues the effects of miR-4734.
Table S1. Stress and inflammation-related genes upregulated in HFF following combined glucose and serum (GS) starvation for 48 hours.
Table S2. Upregulated gene expression in starved HFF classified according to Ingenuity Canonical Pathways.
Table S3. miR-4734 downregulated gene expression in GS stressed HFF.
Table S4. Cloning primers and primers used to detect mRNA and non-coding RNA expression.
Table S5. Small RNA molecules transfected into cells.