**JKTBP1 mediates NRF mRNA stability and IRES dependent translation by
binding to 5' and 3' untranslated regions**

Deike Johanne Omnus^{b1}, Sarah Mehrtens^b, Birgit Ritter^{b2}, Klaus Resch^b, Michiyuki Yamada^c, Ronald Frank^a, Mahtab Nourbakhsh^a and Marc René Reboil^{a*}

Helmholtz Centre for Infection Research, Department of Chemical Biology,
Braunschweig, Germany ^a, Institute of Pharmacology, Hannover Medical School,
Hannover, Germany ^b, Yokohama University, Yokohama, Japan ^c

Present addresses:

Wenner-Gren Institute, Department of Cell Biology, Stockholm University, Stockholm,
Sweden¹, Institute of Virology, Hannover Medical School, Hannover, Germany²

*Corresponding author:

Mailing address: Helmholtz Centre for Infection Research,

Department of Chemical Biology, Inhoffenstrasse 7, 38124 Braunschweig, Germany

Phone: (531) 6181-3413, Fax: (531) 6181-3499

E-mail: Marc.Reboil@helmholtz-hzi.de

Coauthors E-mail:

Deike Johanna Omnus: deike.omnus@wgi.su.se

Sarah Mehrtens: sarah-mehrtens@o2online.de

Birgit Ritter: Ritter.Birgit@MH-Hannover.de

Klaus Resch: Resch.Klaus@MH-Hannover.de

Michiyuki Yamada: myamada@yokohama-cu.ac.jp

Ronald Frank: Ronald.Frank@helmholtz-hzi.de

Mahtab Nourbakhsh: Mahtab.Nourbakhsh@helmholtz-hzi.de

ABSTRACT

Heterogeneous nuclear ribonucleoprotein D-like protein 1 (JKTBP1) was implicated in cap-independent translation by binding to the internal ribosome entry segment (IRES) in the 5'UTR of NF-kappaB repressing factor (NRF). Two different NRF mRNAs have been identified so far, both sharing the common 5' IRES but different length of 3' UTRs. Here, we used a series of DNA and RNA luciferase reporter constructs comprising either 5', 3' or both NRF UTRs to study the effect of JKTBP1 on translation of NRF mRNA variants. The results indicate that JKTBP1 regulates the level of NRF protein expression by specific binding to both, NRF 5' and 3' UTRs. Using successive deletion and point mutations as well as RNA binding studies, we define two distinct JKTBP1 binding elements in NRF 5' and 3' UTRs. Furthermore, JKTBP1 requires two distinct domains to interact with NRF UTRs and a short C-terminal region for its effect on NRF expression. Together, our study shows that JKTBP1 augments NRF protein expression via two disparate mechanisms, mRNA stabilization and cap-independent translation. By binding to 5' UTR, JKTBP1 increases the internal translation initiation in both NRF mRNA variants, whereas its binding to 3' UTR enhances the stability of the major NRF mRNA. Thus, JKTBP1 is a key regulatory factor linking two pivotal control mechanisms of NRF gene expression, the cap-independent translation initiation and mRNA stabilization mechanisms.

KEYWORDS: JKTBP1; NRF; UTRs; RNA stability; ITAF; ARE

Abbreviations used: UTR, untranslated regions; IRES, internal ribosome entry site; NRF, NFkappaB repressing factor; ITAF, IRES transacting factor; JKTBP1, JKT binding protein 1 or heterogeneous nuclear ribonucleoprotein D-like protein 1; GST, glutathione-S-transferase; EGFP, enhanced green fluorescent protein; HRP, horseradish peroxidase; EMSA, electrophoretic mobility shift assay; siRNA, short interfering RNA; ARE, AU-rich element; TNF α , tumor necrosis factor alpha; hnRNP, heterogeneous nuclear ribonucleoprotein

INTRODUCTION

The heterogeneous nuclear ribonucleoprotein D-like protein 1 (JKTBP1) was identified as a member of DNA- and RNA-binding protein family hnRNPs.¹ JKTBP1 is abundant in cell lines and tissues. However, its expression level is highly elevated in epithelial cells in response to proinflammatory cytokines, interferon- γ , interleukin-6 and interleukin-1 β .^{2, 3} JKTBP1 was shown to bind to an AU-rich RNA element in 3' untranslated region (UTR) of the pro-inflammatory factor TNF- α .⁴ As an important regulatory mode of gene expression, AU-rich elements (AREs) in 3' UTRs have been implicated in both, degradation and stabilization of mRNAs.⁵ AREs of human mRNAs have been divided into three classes regarding the number of copies of an AUUUA motif. Whereas class I AREs contain a single AUUUA pentamer, class II AREs enclose multiple copies of this motif and are subdivided into four clusters depending on the frequency of AUUUA motifs.^{6, 7} All other AREs lacking an intact AUUUA motif have been classified as class III AREs. Following binding of ARE-binding proteins (ARE-BPs) to an ARE, RNAs are targeted for translation or degradation. RNAs targeted for degradation undergo deadenylation, decapping, and degradation in a large multiprotein complex, the exosome, or in cytoplasmic compartments known as GW bodies or processing bodies (P-bodies).⁸

We have previously shown that JKTBP1 also binds to 5'UTR of the NF-kappaB repressing Factor (NRF) which regulates Interferon- β and Interleukin-8 genes.⁹ NRF 5' UTR was identified as an internal ribosome entry segment (IRES)¹⁰ which is able to enhance the internal translation initiation at a downstream open reading frame (ORF) in bicistronic constructs.⁹ Despite JKTBP1 binding, the comparison of NRF IRES sequence and TNF- α 3' UTR revealed no significant homology. Furthermore, possible binding sites of JKTBP1 were identified from pools

of 20 nt random sequence RNAs *in vitro* showing no significant homology with NRF IRES or TNF- α 3' UTR. Thus, we suggested that JKTBP1 might be implicated in disparate regulatory mechanisms by binding to different type of functional elements, like NRF IRES or TNF- α 3'UTR.

Two different length of NRF encoding cDNAs were identified in HeLa cells, both share the common 5' untranslated as well as coding regions. They carry long 3' poly(T) stretches indicating that both mRNAs are polyadenylated by different signal sequences.¹¹ However, the NRF mRNAs markedly differ in the length of their 3'UTRs. The negligible 67 nt-long NRF 3'UTR exhibits no relevant RNA sequence motifs whereas the major 3'UTR (1007 nt) contains a class II cluster IV ARE according to the ARE 3.0 database (<http://brp.kfshrc.edu.sa/ARED>). Interestingly, the expression level of NRF transcripts is not equal in cells suggesting a possible regulatory effect of 1007 nt 3'UTR on NRF mRNA stability.¹¹ Previous siRNA experiments demonstrated that down-regulation of endogenous JKTBP1 significantly decreases the level of endogenous NRF protein, but has a less prominent effect on NRF IRES activity.⁽⁹⁾ Together, these observations led to the hypothesis that JKTBP1 might link two disparate regulatory mechanisms by binding to 5' and 3'UTRs within the same mRNA.

RESULTS

The regulatory function of NRF 5' and 3' UTRs on protein expression level

In general, 5' and 3' untranslated regions (UTRs) are known to be involved in the regulation of gene expression by affecting mRNA stability or translational activity. Two distinct NRF coding mRNAs have been identified containing identical 5' UTR and coding sequences but different 3' UTRs. To study the effects of NRF 5' and 3' UTRs on gene expression level, we utilized a reporter gene assay in human epithelial HeLa cells. The reporter gene constructs used in this study contain firefly luciferase coding sequences under control of SV40 and T7 promoters. In addition, they contain either NRF 5', 3' or both UTRs as demonstrated in figure 1A. To monitor general effects of NRF UTRs on reporter gene expression, we transfected identical amounts of pT7FL, pT75UFL, pT7FL3U or pT75UFL3U reporter plasmids together with internal control plasmid pRL-Tk into HeLa cells. pRL-Tk encodes *Renilla* luciferase under control of Tk and T7 promoters. Following transfection, firefly and *Renilla* luciferase activities were analyzed and the ratio of firefly luciferase activity to *Renilla* luciferase activity was calculated in order to estimate a relative reporter gene activity (Fig.1B). Compared to pT7FL, pT75UFL contains additional 653 nt of the NRF 5' UTR upstream to luciferase AUG start codon. However, pT7FL and pT75UFL reveal similar reporter gene expression. Compared to pT7FL or pT75UFL containing SV40 3' UTR, pT7FL3U or pT75UFL3U show a significant lower expression suggesting a distinct function of the major NRF 3' UTR. In general, 3' UTRs were commonly implicated in post transcriptional regulation mechanisms. To confirm a post-transcriptional effect on NRF 3' UTR, we transcribed FL-RNA and FL3U-RNA *in vitro* using T7 promoter and transfected identical amounts of transcribed, capped and

polyadenylated mRNAs (FL and FL3U) as well as control RL-RNA into HeLa cells (Supplemental Fig. 1). The data show that FL3U reveals a lower relative reporter activity compared to FL (Fig. 1C). Thus, the presence of NRF 3'UTR sequences has a significant effect on the level of translated protein.

JKTBP1 binds to NRF 5' and 3'UTRs *in vitro*

Using RNA electrophoretic mobility shift assays (EMSA), we previously identified the heterogeneous nuclear ribonucleoprotein D-like protein 1 (JKTBP1) binding to the IRES module in NRF 5'UTR (nt 507-653).⁹ To further isolate a functional JKTBP1 binding site, we introduced various mutations into the NRF IRES and performed additional RNA EMSA experiments as described earlier.⁹ As shown in figure 2B, GST-JKTBP1 forms a predominant complex with NI0 RNA (Fig. 2B, lane 3). A slower migrating and unspecific complex was irregularly detected caused by co-purified bacterial proteins (Figure 2B lane 3, 6, indicated with an asterisk). As control, no complex was obtained with NI0 RNA or with GST domain alone, indicating the specificity of the detected complex with GST-JKTBP1 (Fig. 2B, lane 1,2). Furthermore, GST-JKTBP1 entirely fails to bind to NIH0 RNA missing 58 nt between nt 579-636 of NRF IRES (Fig. 2B, lane 6). To further define the JKTBP1 binding site in NRF IRES module, we introduced point mutations between the nt 579-636 in NIMsp0 RNA (nt 625-628 UUAA to CCGG, Fig. 2A). As shown in figure 2B (lanes 7-9), these point mutations strongly reduce the binding affinity of JKTBP1 to NRF 5'UTR, indicating that nt 625-628 are essential for binding of JKTBP1 to NRF 5'UTR. To examine the binding of JKTBP1 to NRF 3'UTR, this region was dissected in three fragments (Fig. 3A) and each was separately tested in RNA EMSA. The data show that JKTBP1 binds to nt 3194-3416 (3UTR-2B) of NRF 3'UTR (Fig. 3B, lane 6), but fails to form a complex with 3UTR-1, 3UTR-3 and 3UTR-2A (Fig. 3B, lane 9, 12, 15).

The JKTBP binding domain in 3'UTR (nt 3194-3416) contains a class II ARE cluster group IV (AUUUUAUUUA). We introduced 6 point mutations into the ARE sequence to examine its binding to JKTBP1 (nt 3254-3263: AUUUUAUUUAU to AUcgcUcgAg). Strikingly, these point mutations (3UTR-2BMut) lead to strong decrease of JKTBP1 binding to NRF 3' UTR(Fig. 3B, lane 18). Together, these data indicate that JKTBP1 is able to bind to two distinct elements in NRF UTRs, a regulatory motif in 5'UTR and an ARE class II in 3'UTR.

JKTBP1 affects reporter expression via NRF 3'UTR

To analyze the impact of the class II ARE in NRF 3'UTR, we mutated the ARE in reporter vector pT7FL3U (pT7FL3UMut, Fig. 4A). Identical amounts of pT7FL3U and pT7FL3UMut reporter plasmids were transfected into HeLa cells together with pRL-Tk as an internal control. Additionally, JKTBP1 siRNA was simultaneously introduced into HeLa cells to reduce the endogenous level of JKTBP1 expression (Supplemental Fig. 2). Figure 4B shows that mutation of the class II ARE in pT7FL3UMut reduces the relative reporter gene expression compared to pT7FL3U. Similarly, down-regulation of JKTBP1 significantly reduces the relative level of pT7FL3U expression. Most importantly, the point mutations that inhibit JKTBP1 binding (Fig. 3B, lane 18) lead to the reduction of reporter gene expression in HeLa cells (pT7FL3UMut).

JKTBP1 requires both RNA Binding Domains (RBDs) to bind NRF UTRs

To further analyse the molecular interaction of JKTBP1 with NRF UTRs, we used several truncated mutants of JKTBP1 described earlier.¹², Supplemental Figure 3) . The fusion proteins GST-RBD1 and GST-RBD2 contain RBD1 or RBD2 accordingly. GST-RBD12Q contains both RBDs and a short Q-rich motif. All three

proteins lack the C-terminal region of JKTBP1 (Fig 5A, hatched domain). As shown in figure 5B, GST-JKTBP1 and GST-RBD12Q form a RNA-protein complex with NRF 5' UTR (Fig. 5B. Lanes 3 and 6). Compared to GST-JKTBP1 full length protein, however, GST-RBD12Q seems to have a lower affinity to the 5' UTR. In contrast, GST-RBD1 and GST-RBD2 fail to bind to NRF 5'UTR (lanes 4 and 5). Thus, we suggest that both RBDs are required for efficient binding of JKTBP1 to NRF 5'UTR.

Further examination of JKTBP1 binding to NRF 3'UTR indicates that GST-JKTBP1 and GST-RBD12Q form a complex with NRF 3' UTR (Fig. 5B, lane 9). However, neither GST-RBD1 nor GST-RBD2 are able to form a complex with NRF 3' UTR (Fig. 5B, lanes 10, 11). Together, the results indicate that both RNA binding domains are required for binding of JKTBP1 to NRF 5' and 3'UTRs. In contrast to the results obtained with NRF 5' UTR, GST-RBD12Q shows a significant higher binding affinity to NRF 3' UTR than GST-JKTBP1 full length protein (Fig. 5B, lane 12). Thus, the GST-RBD12Q protein exhibits different binding properties to NRF 5' and 3' UTRs. This might suggest a differential mode of JKTBP1 binding to NRF 5' or 3' UTRs (Fig. 5B, lanes 6 and 12).

The RBDs and the C-terminal domain of JKTBP1 are indispensable for its function

Eukaryotic expression plasmids encoding truncated JKTBP1 fusion proteins were previously used to study their cellular expression pattern.¹³ We used these constructs pEGFP-JKTBP1, pEGFP-JKTBP1RBD2 and pEGFP-JKTBP1Δ6 (Fig. 6A) to examine whether binding of JKTBP1 to NRF 5' and 3' UTRs is a prerequisite for its regulatory function *in vivo*. These JKTBP1 expression plasmids were transfected into HeLa cells together with reporter vectors pT75UFL, pT7FL3U and internal control vector pRL-Tk. We note that the N-terminal fusion of recombinant tags, like Strep and

EGFP, do not interfere with JKTBP1 enhancing activity *in vivo* (Supplemental Fig. 4). As demonstrated in figure 6B, EGFP-JKTBP1 full length protein is able to increase the expression of pT75UFL containing NRF 5'UTR (Fig. 6B). EGFP-JKTBP1-RBD2 lacking RBD1 is not able to promote reporter gene expression. Interestingly, the splice variant JKTBP1 Δ 6 containing RBD1 and 2 failed to elevate the expression of pT75UFL. Compared to JKTBP1, JKTBP1 Δ 6 variant lacks a short C-terminal domain (aa 222-279) which seems to be important for JKTBP1 function. As summarized in figure 6C, co-transfection of reporter gene plasmid pT7FL3U reveals similar results. For instance, pT7FL3U expression is enhanced by EGFP-JKTBP1 full length protein, but not by EGFP-JKTBP1RBD2 and EGFP-JKTBP1 Δ 6. Together, the data strongly suggest that JKTBP1 RBDs are required for binding to NRF 5' or 3' UTRs whereas the C-terminal domain of JKTBP1 might be important for its enhancing effect on the reporter gene expression.

JKTBP1 acts on both NRF UTRs

Next we determined the impact of endogenous JKTBP1 on both NRF UTRs. Therefore, HeLa cells were transfected with reporter gene constructs pT7FL, pT75UFL or pT75UFL3U as well as internal control vector pRL-Tk. In addition, cells were transfected with siRNA to inhibit the endogenous expression of JKTBP1 (Fig. 7). JKTBP1 down-regulation has no effect on control pT7FL. However, the reporter activities of pT75UFL and pT75UFL3U were reduced by JKTBP1 down-regulation to 58% and 42%, respectively. The data confirm the results obtained with the recombinant protein EGFP-JKTBP1 in figure 6 and show that the endogenous JKTBP1 acts on NRF 5' and 3' UTRs.

JKTBP1 stabilizes the major NRF mRNA

In order to elucidate the regulatory function of JKTBP1 on NRF mRNAs stability, HeLa cells were transfected with the JKTBP1 expression plasmid (pEXPR-JKTBP1) or an empty vector as control (pEXPR). The level of JKTBP1 expression in cells was adjusted by western blot analysis (Supplemental Figure 5). Additionally cells were treated with actinomycin D to inhibit *de novo* RNA synthesis (Fig. 8). Following indicated times, polyA(+)-RNA was purified from transfected cells and subjected to northern blot analysis to estimate the stability of NRF mRNAs. In addition to NRF mRNAs, transferrin receptor (TFR) mRNA was detected as loading control. As obtained in non transfected HeLa cells earlier, the major NRF mRNA is three times higher expressed than the minor mRNA in cells transfected with control vector.¹¹ Following actinomycin D treatment, the level of the major NRF mRNA continuously decreases over time, but the level of the minor variant remains constant. This demonstrates the higher stability of the minor variant compared to the major NRF mRNA variant in HeLa cells. By overexpression of JKTBP1 in HeLa cells, the stability of both NRF mRNA variants is increased. However, the stabilization effect of JKTBP1 on the major NRF mRNA is significantly higher compared to its effect on the minor NRF variant. Most strikingly between 60 and 90 minutes of actinomycin D treatment, the level of the major mRNA reduces to 60%, but only to 22% by JKTBP1 overexpression. These results demonstrate the predominant effect of JKTBP1 on the stabilization of the major NRF mRNA. Thus, we suggest that JKTBP1 is able to increase the stability of NRF mRNA by acting on 3' UTR.

DISCUSSION

Several studies suggested that IRES trans-acting factors (ITAFs) can also interact with 3' UTR binding proteins establishing a RNA/protein bridge between the IRES and the 3' end of the transcript.^{14, 15} This circularization model so far presumed the interaction of two distinct sets of 5' IRES and 3' UTR binding proteins. In this study, we demonstrate that the hnRNP protein JKTBP1 directly binds to 5' IRES and 3' UTR of the major NRF mRNA variant. hnRNPs are known to form homo- or heterodimers through their Gly-rich domains which is also present in the C-terminal domain of JKTBP1.^{16, 17} Thus, it is tempting to speculate that JKTBP1 alone or in concert with other interacting proteins might constitute a circular mRNA complex which remains to be confirmed experimentally. Nevertheless, binding of JKTBP1 to NRF 5' and 3' UTRs is implicated in two disparate regulatory mechanisms, cap-independent translation and mRNA stabilization. Both mechanisms ensure the efficient translation of NRF mRNA.

In a previous study, we elucidated the role of NRF 5' UTR and JKTBP1 in internal translation initiation using bicistronic reporter constructs.⁹ However, the regulatory function of JKTBP1 in the expression of endogenous NRF gene was not studied yet. In the current report, the use of monocistronic reporter constructs pT7FL and pT75UFL and overexpression of JKTBP1 confirms that internal translation initiation by NRF 5' IRES is mediated by binding of JKTBP1. Notably, the length of NRF 5' UTR and the binding of JKTBP1 do not interfere with an efficient translation initiation as demonstrated by the equal expression level of pT7FL and pT75UFL reporter genes.

In this study, we focused mainly on the regulatory function of major NRF 3' UTR. Initial cloning of NRF cDNAs revealed that the polyadenylation signals in NRF 3' UTR are functional and both corresponding mRNAs are polyadenylated.¹¹ Our

current data strongly suggest that the differential expression level of NRF mRNAs in HeLa cells is based on the regulation of mRNA stability by NRF 3' UTR. It is important to note that the level of reporter expression in presence of NRF 3' UTR is significantly lower compared to SV40 late 3' UTR (Fig. 1B). Although less potent than SV40 3' UTR, several observations indicate that NRF 3' UTR increases the stability of the endogenous major NRF mRNA by binding of JKTBP1. Mutation of JKTBP1 binding site in NRF 3' UTR and down-regulation of JKTBP1 decreases the level of pT7FL3U expression. Interestingly, JKTBP1 knockdown has a more significant effect on reporter expression than the point mutations in 3' UTR. This might indicated a residual binding of JKTBP1 to the mutant 3' UTR as shown in RNA EMSA experiments (Fig. 3B, lane 18). Finally, increased JKTBP1 expression enhances predominantly the stability of the major NRF mRNA compared to the minor NRF mRNA. However, JKTBP1 overexpression slightly enhances the stability of the minor NRF mRNA. We assume that this might be based on increased recruitment of ribosomes to the 5' UTR which was shown to protect mRNAs against random mRNA degradation.¹⁸ The binding of JKTBP1 to NRF 5'UTR was abolished by mutation of 4 bases. In addition, we introduced these four point mutations (nt 625-628, UUAA to CCGG) in a dicistronic reporter gene vector containing the NRF IRES module (p0RLNI0FL). Reporter gene analysis revealed that these mutations reduce IRES activity to 50% of the wild type activity and that overexpression of JKTBP1 did not elevate IRES activity anymore (data not shown). These findings suggest that nt 625-628 are essential for binding of JKTBP1 to the NRF 5' UTR. By sequence comparison, however, JKTBP1 binding sequences in NRF 5' and 3'UTRs exhibit no sequence homology. An *in vitro* purification approach from pools of 20 nt random RNA sequences was previously used to identify a consensus binding sequence for JKTBP1.¹² Again, the suggested consensus sequence shows no homology with NRF

UTRs. Thus, we assume that JKTBP1 might have a variable binding sequence specificity *in vivo* and *in vitro*.

In agreement with previous reports, our data show that JKTBP1 requires both RNA binding domains (RBD1 and RBD2) to bind to NRF UTRs *in vitro*.¹² Moreover, both RBDs of JKTBP1 are required to enhance reporter gene expression through NRF 5' and 3' UTRs *in vivo* (Fig. 6B,C). Interestingly, the C-terminal deletion mutant RBD12Q shows a differential binding affinity to NRF 5' and 3' UTRs *in vitro*. *In vivo*, JKTBP1Δ6 shows no significant effect on NRF 5' or 3' UTR in reporter gene expression (Fig. 6B,C). Thus, the C-terminal domain might have a significant effect on JKTBP binding affinity to a certain RNA sequence. Notably, JKTBP1 Δ6 lacks a part of Gly-rich domain which is common among a number of hnRNPs.^{1, 19, 20} This domain was previously implicated in regulatory protein-protein interactions between hnRNPs.¹⁶ Therefore, it is likely that the interaction of other hnRNPs with JKTBP1 is crucial for the regulatory function of JKTBP1 in NRF expression.

Although NRF was implicated in transcriptional regulation of several cytokines^{11, 21}, little was known about the regulation of NRF gene itself. The identification of NRF 5' and 3' UTRs in HeLa cells presumed unique regulatory mechanisms at post-transcriptional level. We have previously shown that the endogenous expression of NRF protein is regulated by JKTBP1.⁹ Both NRF mRNAs contain the NRF 5' IRES element and were found to be associated with polysomes when cap-dependent translation was inhibited by polio virus infection (data not shown). The data presented here assume that JKTBP1 regulates NRF expression through disparate mechanisms. By binding to NRF 5' UTR, JKTBP1 is able to enhance the internal translation of both NRF mRNAs. Through its binding to the NRF 3' UTR, however, JKTBP1 can specifically enhance the stability of the major NRF mRNA. It is conceivable that the two NRF mRNA variants and the differential effects

of JKTBP1 constitute a complex regulation mechanism which allows an adequate level of NRF expression by different cellular conditions. An inducer of pro-inflammatory genes, neutrophils elastase (NE), was recently suggested to increase the level of NRF protein expression at the posttranscriptional level.²² However, further analysis is required to clarify the role of JKTBP1 in NE induced expression of NRF.

MATERIALS AND METHODS

Plasmid construction. Plasmids for *in vitro* transcription were constructed using amplified PCR fragments containing desired NRF 5'UTR or NRF 3'UTR sequences (accession number NM_017544.1) cloned to *EcoRI* and *XhoI* sites of pBluescript SK+ or pBluescript KS- (Stratagene, accession number X52327), respectively. Mutagenesis of NRF-UTR sequences was performed using the QuickChange site-directed mutagenesis kit (Stratagene). Monocistronic reporter gene vector p5UFL was obtained from pRL001-653FL⁹ by deletion of *Renilla* luciferase coding sequence using *XhoI* and *SalI* sites. Additional deletion of NRF 5'UTR using *Ascl* sites results in pFL. NRF 3'UTR was cut from pBluescript KS- vector using *ScaI* and *NotI* sites and inserted in p5UFL, creating p5UFL3U. pFL3U was obtained by deletion of 3'UTR from p5UFL3U using *PvuI* and *Ascl* sites. For *in vitro* transcription, we integrated T7 promoter in all four reporter vectors using QuickChange site-directed mutagenesis kit (Stratagene). pEXPR-JKTBP1 was created by amplifying JKTBP1 coding sequence from pEGFPC1-JKTBP1¹³. The fragment was cut with *Eco31I* and inserted in MCS of pEXPR-IBA105 (IBA). JKTBP1 deletion mutants were constructed as described earlier^{12, 13} as well as dicistronic reporter plasmid p0RLNI0FL.⁹ The control vector pRL-Tk was obtained from Promega.

Cell lines and DNA transfection. HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (BIO-Whittaker), 2mM glutamine, penicillin, and streptomycin, and were transfected by calcium phosphate co-precipitation. A total of 5µg DNA containing 3µg of reporter plasmid, and a suitable complementing amount of empty vector were transfected per 7.5×10^4 cells. Cells were harvested after 48 hours and pooled for reporter gene assays.

RNA transfection. A total amount of 2.5µg RNA were transfected per 7.5×10^4 cells using DMRIE-C reagent (Invitrogen). Cells were harvested 6 hours after transfection and subjected to reporter gene assays.

siRNA transfection. HeLa cells were transfected by calcium phosphate co-precipitation. A total of 5µg DNA containing 2.5µg of reporter plasmid and 113nM siRNAs (JKTBP1-siRNA 5'GUUGUAGACUGCACAUAUAdTdT3';3' dTdTCAACAUCUGACGUGUAU 5' or negative control siRNA (obtained from Eurogentec)) were transfected per 7.5×10^4 cells. Cells were harvested after 48 hours and pooled for reporter gene assays.

Reporter gene assay. Firefly and *Renilla* luciferase activities were determined using the Dual-LuciferaseTM reporter system (Promega) as described in the manufacturer's protocol. Luciferase activity was measured in a Lumat LB 9501 luminometer (Berthold), reported as relative light units (RLUs).

Northern blot analysis. Total RNA from cells was isolated using TRIZOL reagent (Gibco/BRL, Life Technologies) according to the manufacturer's instructions. Poly(A)+ RNA was isolated from total RNA using Dynabeads Oligo (dT)25 (Dyna) according to the manufacturer's instructions. Northern blot analysis was performed as described earlier.¹⁰ NRF specific probe was described earlier containing coding sequences of human NRF (aa 18-398), respectively.^{11, 20} The TFR specific fragment was obtained by digestion of pT7AS-TFR with *XbaI* and *HindIII* enzymes. The fragment containing whole TFR coding sequence was analyzed and isolated from 1% Agarose gel.

Western blot analysis. Cellular extracts were prepared as described in the Dual Luciferase assay protocol. 20µg of extracts were loaded onto a 10% polyacrylamide gel. Western blot analysis was performed as described earlier¹⁰ using 1:500 diluted rabbit polyclonal antibodies against JKTBP1.^{3, 22} The monoclonal

antibody against p65 (sc8008) was obtained from Santa Cruz Biotechnology Inc.. Strep-Tag fusion proteins were detected using 1:4000 diluted strepMAB classic HRP (IBA).

***In vitro* transcription.** Templates for *in vitro* transcription were prepared by linearization of pBluscript SK+ plasmids (Stratagene) with restriction enzymes as indicated in figures. *In vitro* transcription reactions with the purified linearized templates were carried out with the T7-Riboprobe Kit (Promega) using radioactive labeled rUTP (as indicated) following the manufacturer's instructions.

Templates for monocistronic reporter RNA were prepared by linearization of pT7FL, pT75UFL, pT75UFL3U, pT7FL3U and pRL-Tk with restriction enzymes as indicated in the figures. Templates were transcribed with RiboMAX T7 Kit (Promega) following the manufacturer's instructions. For capping 40 mM Ribo m7G Cap Analog (Promega) was added.

Polyadenylation. *In vitro* transcribed RNAs were polyadenylated prior RNA transfection using A-Plus Poly(A) Polymerase Tailing Kit (Epicentre Biotechnologies) following the manufacturer's instructions. After polyadenylation RNA was analyzed on a 1% agarose gel.

Expression and purification of GST fusion proteins. Recombinant proteins were expressed and purified as fusion proteins with GST in *Escherichia coli* BL21 cells as described earlier.^{1, 23}

RNA-shift analysis. 5×10^5 cpm of *in vitro* synthesized radioactive transcript were incubated with 1 μ g of purified GST or different GST-JKTBP1 proteins in 20mM Hepes pH 7.9, 10mM KCl, 1mM CaCl₂, 2mM MgCl₂, 2 μ g tRNA competitor from *E. coli* (Roche), and 2% Ficoll for 10min at 37°C. 30U of RNase T1 (Roche) was added to the mixture and incubated for 30min at 37°C. The complexes were then analyzed

by 6% native polyacrylamide gel electrophoresis and autoradiography of the dried gel as described earlier.⁹

Accession numbers. NRF cDNA sequence and pBluescript vector sequences are deposited at accession numbers **NM 017544.1** and **X52327**, respectively.

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FIGURE LEGENDS

FIGURE 1. The NRF UTRs differentially affect reporter gene expression. **(A)** A schematic diagram of the transfected reporter constructs is shown on the *top*. pT7FL contains a SV40 early promoter/enhancer (SV40), a minimal T7 promoter (T7), the firefly luciferase gene (Firefly Luc) and a SV40 polyadenylation signal. pT75UFL, pT7FL3U and pT75UFL3U additionally contain the NRF 5'UTR (nt 1-653), the NRF 3'UTR (nt 2728-3701) including a polyadenylation signal or both UTRs, respectively. pRL-Tk served as internal control containing a Thymidine kinase promoter (Tk), the *Renilla* luciferase gene (*Renilla* Luc) and a SV40 polyadenylation signal. **(B)** HeLa cells were transfected with reporter constructs pT7FL, pT75UFL, pT7FL3U or pT75UFL3U and internal control vector pRL-Tk. Two days after transfection, the cells were harvested and luciferase activities were determined. Firefly luciferase activities were normalized to the corresponding *Renilla* luciferase activities to obtain the relative firefly luciferase activity shown here as mean $\pm$ SEM of three independent transfection experiments. Relative firefly luciferase activity of cells transfected with pT7FL was set to 1. A p -value < 0.05 is indicated (*). **(C)** The T7 promoter of pT7FL, pT7FL3U and pRL-Tk was used to express reporter RNAs *in vitro*. HeLa cells were transfected with 2.5 μ g reporter RNAs FL, FL3U and RL-RNA. Six hours following transfection, the cells were harvested and luciferase activities were determined. The relative firefly luciferase activity was obtained as described above. Relative firefly luciferase activity of cells transfected with FL RNA was set to 1. A p -value < 0.05 is indicated (*).

FIGURE 2. JKTBP1 binds to nt 578-637 in the NRF 5'UTR. **(A)** A schematic diagram of the constructs used to create the wild-type NRF IRES module RNA (NI0),

the deletion mutant (NIH0) or the mutated IRES module (NIMsp0, nt 625-628 UUAA to CCGG) is shown on the *top*. Constructs were digested with *NdeI* as indicated. **(B)** GST and GST-JKTBP1 protein were expressed in bacteria and purified. Each protein (1 µg) was incubated with *in vitro* transcribed and radioactively labeled NRF IRES module RNA (NI0), the deletion mutant (NIH0) or the mutated NRF IRES module (NIMsp0) as indicated on the top of each lane. Complexes were analyzed on native 6% polyacrylamide gel and then subjected to autoradiography. An unspecific, bacterial complex is indicated with an asterisk (*). The JKTBP1 complex and the unbound RNA are indicated on the *left*.

FIGURE 3. JKTBP1 binds to the ARE in NRF 3' UTR. **(A)** The isolated sections of NRF 3'UTR were inserted into pBLKS to generate 3UTR-1, 3UTR-2, 3UTR-3 RNA, 3UTR-2A, 3UTR-2B, and mutant 3UTR-2BMut RNA as indicated. Constructs were digested with designated restriction enzymes for precise termination of the transcripts. **(B)** GST or GST-JKTBP1 proteins (1 µg) were incubated with *in vitro* transcribed and radioactively labeled 3'UTR segments 3UTR-1, 3UTR-2, 3UTR-3, 3UTR-2A, 3UTR-2B as well as 3UTR2BMut as indicated on the top of each lane. Complexes were analyzed on native 6% polyacrylamide gel and then subjected to autoradiography. An unspecific, bacterial complex is indicated with an asterisk (*). The JKTBP1 complex and the unbound RNA are indicated at the left.

FIGURE 4. JKTBP1 binding to the ARE in the NRF 3'UTR affects reporter gene expression. **(A)** A schematic diagram of the reporter gene constructs pT7FL3U and pT7FL3UMut is shown. Point mutations in pT7FL3UMut are indicated in lower-case letters. **(B)** HeLa cells were transfected with reporter gene constructs pT7FL3U or pT7FL3UMut and internal control vector pRL-Tk. Additionally, JKTBP1 siRNA (+) or

control siRNA (-) was transfected. 48 hours following transfection, cells were harvested and luciferase activities were determined. Firefly luciferase activities were normalized to corresponding *Renilla* luciferase activities. The relative firefly luciferase activity is shown as mean $\pm$ SEM of three independent transfection experiments. Relative firefly luciferase activity of cells transfected with pT7FL3U was set to 100 %. A *p*-value < 0.01 is indicated (**).

FIGURE 5. JKTBP1 requires both RBDs to bind to NRF UTRs. **(A)** A schematic diagram of recombinant GST fusion proteins is shown. The numbers indicate the JKTBP1 amino acids. GST-JKTBP1 contains two RNA binding domains (RBDs), a short glutamine stretch (6xQ) and a glycine rich C-Terminus (hatched box). GST-RBD1 just contains the first RBD, GST-RBD2 the second RBD. GST-RBD12Q lacks the whole C-Terminus of JKTBP1, but contains both RBDs and the Q-stretch. **(B)** GST, GST-JKTBP1, GST-RBD1, GST-RBD2 or GST-RBD12Q protein (1 μ g) was incubated with *in vitro* transcribed and radioactively labeled NRF IRES module (NI0) or NRF 3'UTR (3UTR-2B). These RNAs were obtained as described in the legends of Figures 2A and 3A, respectively. Complexes were analyzed on native 6% polyacrylamide gel and then subjected to autoradiography. The unspecific, bacterial complex is indicated with an asterisk (*). The JKTBP1 complex and the unbound RNA are indicated on the *left*.

FIGURE 6. The binding to RNA is a prerequisite for JKTBP1 action. **(A)** A schematic overview of EGFP fusion proteins is presented. The numbers indicate the JKTBP1 amino acids. EGFP-JKTBP1 consists of the full length JKTBP1 protein and a N-terminal EGFP tag. In EGFP-JKTBP1-RBD2 the first RBD is replaced by a GST tag. EGFP-JKTBP1 Δ 6 is composed of the EGFP tag and the full sequence of the

JKTBP slice variant 1Δ6. **(B)** HeLa cells were transfected with reporter construct pT75UFL, internal control vector pRL-Tk and identical amounts of expression plasmids pEGFP, pEGFP-JKTBP1, pEGFP-JKTBP1-RBD2 and pEGFP-JKTBP1Δ6. Two days after transfection, the relative firefly luciferase activity was determined as described in the legend of Figure 1B. Relative firefly luciferase activity is shown as mean $\pm$ SEM of three independent transfection experiments. Relative firefly luciferase activity of cells transfected with pT7FL was set to 1. A p -value < 0.01 is indicated (**). **(C)** The experiments were performed as described in B. Instead of pT75UFL the reporter gene construct pT7FL3U was used here. A p -value < 0.05 is indicated (*).

FIGURE 7. JKTBP1 acts on both NRF UTRs. HeLa cells were transfected with reporter constructs pT7FL, pT75UFL, pT75UFL3U as indicated on the top and internal control vector pRL-Tk. Additionally, JKTBP1 siRNA (siRNA) or control siRNA (control) were transfected as indicated on the bottom. 48 h after transfection, the relative reporter activity was determined as described in the legend of Figure 1B. Relative reporter activity is shown here as mean $\pm$ SEM of three independent transfection experiments and was set to 100% for cells transfected with pT7FL and control siRNA. p -values < 0.05 (*) and < 0.01 (**) are indicated.

FIGURE 8. JKTBP1 increases the stability of the major NRF mRNA. HeLa cells were transfected with JKTBP1 expression vector (pEXPR-JKTBP1) or empty vector (pEXPR). Two days after transfection, cells were treated with actinomycin D (5 μ g/ml) to interrupt the de novo transcription in cells. The poly(A)-RNA fractions were purified from transfected cells at indicated times after actinomycin D treatment and subjected to Northern Blot analysis. NRF and Transferrin receptor (TFR) mRNAs were detected using corresponding radioactively labeled NRF and TFR probes and

subsequent autoradiography. On the *top*, data are representative for at least three independent experiments. NRF and TFR mRNAs are indicated by arrows on the right. The diagram summarizes the densitometric analysis of detected bands $\pm$ SEM from three independent Northern blot experiments. NRF signals were quantified and normalized to corresponding TRF signals. For better comparison, the relative level of the larger NRF mRNA was set to 1.

FIGURES

Fig. 1A and 1B

C

Fig. 1C

A

B

Fig. 2A and 2B

A

Fig. 3A

B

Fig. 3B

A

B

Fig. 4A and 4B

A

B

Fig. 5A and 5B

A

B

C

Fig. 7

Fig. 8

SUPPLEMENTAL MATERIAL

SUPPLEMENTAL FIGURE LEGENDS

SUPPLEMENTAL FIGURE 1. A schematic presentation of the constructs used to express FL, FL3U and RL RNAs *in vitro* is shown on the *top*. For precise termination, the constructs were digested with *NotI*, *NdeI*, or *BamHI* as indicated. For *in vitro* transcription, the cap analogue [m⁷G(5')ppp(5')G] was added. Subsequently, RNAs were purified and except RL-RNA were subjected to polyadenylation reaction. After additional purification, the quality and quantity of transcribed RNAs were analyzed on a 1% agarose gel.

SUPPLEMENTAL FIGURE 2. Mutation of nt 625-628 in NRF IRES module reduces IRES activity to 50% of wild type activity. HeLa cells were transfected with dicistronic reporter plasmids p0RLFL (containing no intercistronic sequences), p0RLNI0FL (containing wild type NRF IRES module), p0RLNIH0FL (NRF IRES module lacking nt 579-636) and p0RLNIMsp0FL (NRF IRES module with point mutations at nt 625-628). Two days after transfection, cells were harvested and were subjected to firefly and *Renilla* Luciferase assay. The relative *Renilla* luciferase activity of cells transfected with the dicistronic plasmids $\pm$ S.E.M. (white bars) are shown on primary axis. The *Renilla* luciferase activity in cells transfected with p0RLNI0-FL was set to 1 in each experiment. The relative IRES activity is shown as the mean ratio of firefly and *Renilla* luciferase activities $\pm$ S.E.M. from three independent experiments (grey bars) on secondary axis. The IRES activity of cells transfected with p0RLNI0-FL was set to 1 in each experiment.

SUPPLEMENTAL FIGURE 3. Control of JKTBP1 down regulation on protein level. HeLa cells were transfected with JKTBP1 siRNA or control siRNA. Two days after transfection, cell lysates were subjected to western blot analysis with rabbit polyclonal serum against JKTBP1.

SUPPLEMENTAL FIGURE 4. Expression of GST fusion proteins for RNA EMSA analysis. Proteins were expressed in *E.coli* BL21 and purified using GSH sepharose. Identical amounts of GST, GST-JKTBP1, GST-RBD1, GST-RBD2 and GST-RBD12Q were analyzed on 10% denaturing SDS-polyacrylamide gel. Protein bands were detected by Coomassie blue staining.

SUPPLEMENTAL FIGURE 5. N-terminal tags do not interfere with JKTBP1 function. HeLa cells were transfected with dicistronic reporter plasmid p0RLNI0FL, containing minimal NRF IRES module as described earlier.⁹ Simultaneously, 4 µg of expression plasmids pEGFP-JKTBP1, pEXPR-JKTBP1 and corresponding empty vectors pEGFP and pEXPR were transfected. Two days after transfection, cells were harvested and were subjected to firefly and *Renilla* Luciferase assay. Relative reporter activity was determined as ratio of firefly luciferase activities and *Renilla* luciferase activities. Relative IRES activity is shown as mean ± SEM of three independent transfection experiments and was set to 1 for control vector pEGFP and pEXPR.

SUPPLEMENTAL FIGURE 6. Overexpression of Strep-JKTBP1. HeLa cells were transfected with indicated amounts of pEXPR-JKTBP1 and suitable complementing amount of empty vector pEXPR. Two days after transfection cell

extracts were subjected to western blot analysis with monoclonal HRP conjugated antibody against strep-tag or monoclonal antibody against NF κ B p65 as control.

SUPPLEMENTAL FIGURES

Supplemental Fig. 1

Supplemental Fig. 2 and Fig.3

Supplemental Fig. 4 and Fig. 5

Supplemental Fig. 6