Human NF-κB repressing factor acts as a stress-regulated switch for ribosomal RNA processing and nucleolar homeostasis surveillance

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The nucleolus, a dynamic nuclear compartment long regarded as the cell ribosome factory, is emerging as an important player in the regulation of cell survival and recovery from stress. In larger eukaryotes, the stress-induced transcriptional response is mediated by a family of heat-shock transcription factors. Among these, HSF1, considered the master regulator of stress-induced transcriptional responses, controls the expression of cytoprotective heat shock proteins (HSPs), molecular chaperones/cochaperones constituting a major component of the cell protein quality control machinery essential to circumvent stress-induced degradation and aggregation of misfolded proteins. Herein we identify human NF-κB repressing factor (NKRF) as a nucleolar HSP essential for nucleolus homeostasis and cell survival under proteotoxic stress. NKRF acts as a thermosensor translocating from the nucleolus to the nucleoplasm during heat stress; nucleolar pools are replenished during recovery upon HSF1-mediated NKRF resynthesis. Silencing experiments demonstrate that NKRF is an unconventional HSP crucial for correct ribosomal RNA (rRNA) processing and preventing aberrant rRNA precursors and discarded fragment accumulation. These effects are mediated by NKRF interaction with the 5′-to-3′ exoribonuclease XRN2, a key coordinator of multiple pre-rRNA cleavages, driving mature rRNA formation and discarded rRNA decay. Under stress conditions, NKRF directs XRN2 nucleolus/nucleoplasm trafficking, controlling 5′-to-3′ exoribonuclease nucleolar levels and regulating rRNA processing. Our study reveals a different aspect of rRNA biogenesis control in human cells and sheds light on a sophisticated mechanism of nucleolar homeostasis surveillance during stress.

heat shock factor 1 | NF-kappaB | nucleolus | proteotoxic stress | rRNA processing

Protein homeostasis is essential for life in eukaryotes (1). A critical consequence of proteotoxic stress is the activation of the heat shock response (HSR), a fundamental cell defense mechanism, regulated by a family of heat shock transcription factors (HSFs) that are expressed and maintained in an inactive state under nonstress conditions (1–3). Mammalian genomes encode three homologs of HSF (HSF1, HSF2, and HSF4); among these, HSF1 is considered the paralog responsible for regulating stress-induced transcriptional responses (3, 4).

HSF1 is generally found as an inert monomer in unstressed cells (4). Upon exposure to proteotoxic stress, HSF1 is derepressed in a stepwise process that involves HSF1 trimerization, nuclear translocation, phosphorylation/sumoylation, and binding to DNA sequences (heat shock elements, HSEs), characterized by inverted repeats of a "nGAAn"-pentameric motif (4). Upon stress-signal removal, the response attenuates rapidly with HSF1 reconversion to monomers (4). HSF1 binding to HSE triggers a rapid shift in the transcriptional program, resulting in the expression of cytoprotective heat shock proteins (HSPs), which include molecular chaperones of the HSP70 and HSP90 families, HSP27, and other proteins of the network (2, 3). HSF1-binding sites have been described also in genes encoding proteins with nonchaperone function (5). We have recently identified the human zinc-finger AN1-type domain-2a gene as a canonical HSF-1 target gene (6). During these studies, gene expression profile analysis of HSF1 knockdown (HeLa-HSF1i) (7) versus wild-type HeLa cells under stress conditions revealed an increase in the expression of NF-κB repressing factor (NKRF) selectively in wild-type cells.

NF-κB transcription factors comprise a family of critical regulators of the innate and adaptive immune response, playing an important role in promoting inflammation and in the control of cell proliferation and survival (8). NF-κB normally exists as an inactive cytoplasmic complex, whose predominant form is a heterodimer composed of p50 and p65 (RelA) subunits, bound to inhibitory proteins of the IκB family, and is induced in response to a variety of pathogenic stimuli, including exposure to proinflammatory cytokines, mitogens, and viral infection (8, 9). NKRF is known as a silencer protein binding negative regulatory elements (NRE) specific for suppression of NF-κB/Rel-binding element basal activity in several NF-κB–regulated genes (10–13). NKRF has also been shown to interact with NF-κB/p65 through a minimal-core sequence, differentially controlling NF-κB–driven transcription under basal and/or stimulated conditions (14). The fact that we previously described a cross-talk between HSF1 and NF-κB (15–17) prompted us to investigate whether NKRF could be HSF1-regulated and induced by heat exposure. The results unexpectedly show that human NKRF is an unconventional HSP strictly controlled by HSF1, essential for correct ribosomal RNA

Significance

Quality control of ribosomal RNA (rRNA) processing is critical for ribosome biogenesis, nucleolar homeostasis, and cell survival; however, the molecular mechanisms governing rRNA processing under stress conditions are poorly understood. This study identifies human NF-κB repressing factor (NKRF) as a HSF1 target gene essential for nucleolar homeostasis during proteotoxic stress. Rather than preventing protein misfolding and/or aggregation, this unconventional stress protein has a critical role in preventing aberrant rRNA precursors and discarded fragment accumulation and directing rRNA processing dynamics. The findings highlight a key aspect of the human cell response to proteotoxic stress, opening new scenarios on ribosome biogenesis regulation.

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(rRNA) processing and nucleolar homeostasis under proteotoxic stress conditions.

Results and Discussion

To investigate whether HSF1 is implicated in NKRF gene regulation, we first analyzed the effect of heat treatment on NKRF expression. Heat-induced HSF1 activation is strictly dependent on both the temperature increase above physiological conditions and exposure duration. When HeLa cells were exposed to 43 °C for 40 min, HSF1 phosphorylation and DNA-binding activity were detected during heat stress, continuing during recovery at 37 °C for 1.5 h and declining rapidly thereafter; under these conditions, heat stress induced NKRF-mRNA expression with a kinetics parallel to HSF1 activation (Fig. 1*A*). A temperature-dependent increase in NKRF-mRNA levels after short (2 h) heat exposure was detected starting at 39 °C (Fig. 1*B*), indicating that NKRF expression can be induced also under febrile temperature conditions.

NKRF induction represents a general response of human cells to temperature increase. In addition to HeLa cells, heat exposure induced NKRF expression in human cancer cells of different origin, including breast adenocarcinoma, colon carcinoma, T-cell lymphoma and melanoma, and primary cells, including peripheral-blood monocytes, endothelial cells, and keratinocytes (Fig. S1 *A*–*C*). Interestingly, human monocytes showed the highest level of heat-induced NKRF expression also at febrile temperatures (Fig. 1*B*).

Notably, heat stress did not affect NKRF expression in HSF1i-silenced cells (Fig. 1*A* and Fig. S1*A*).

Heat-induced NKRF-mRNA increase is abolished by actinomycin-D (Fig. S1*D*), suggesting de novo gene transcription. NKRF promoter nucleotide sequence analysis revealed two putative HSEs located at –474 (HSE1) and –689 (HSE2) from the transcription start site (TSS) (Fig. 1*C*). The NKRF promoter HSE-comprising region was cloned and used for reporter analysis, which confirmed heat-induced NKRF promoter transcription in wild-type but not in HSF1-silenced cells (Fig. 1*D*). Next, a G-to-T mutation in the

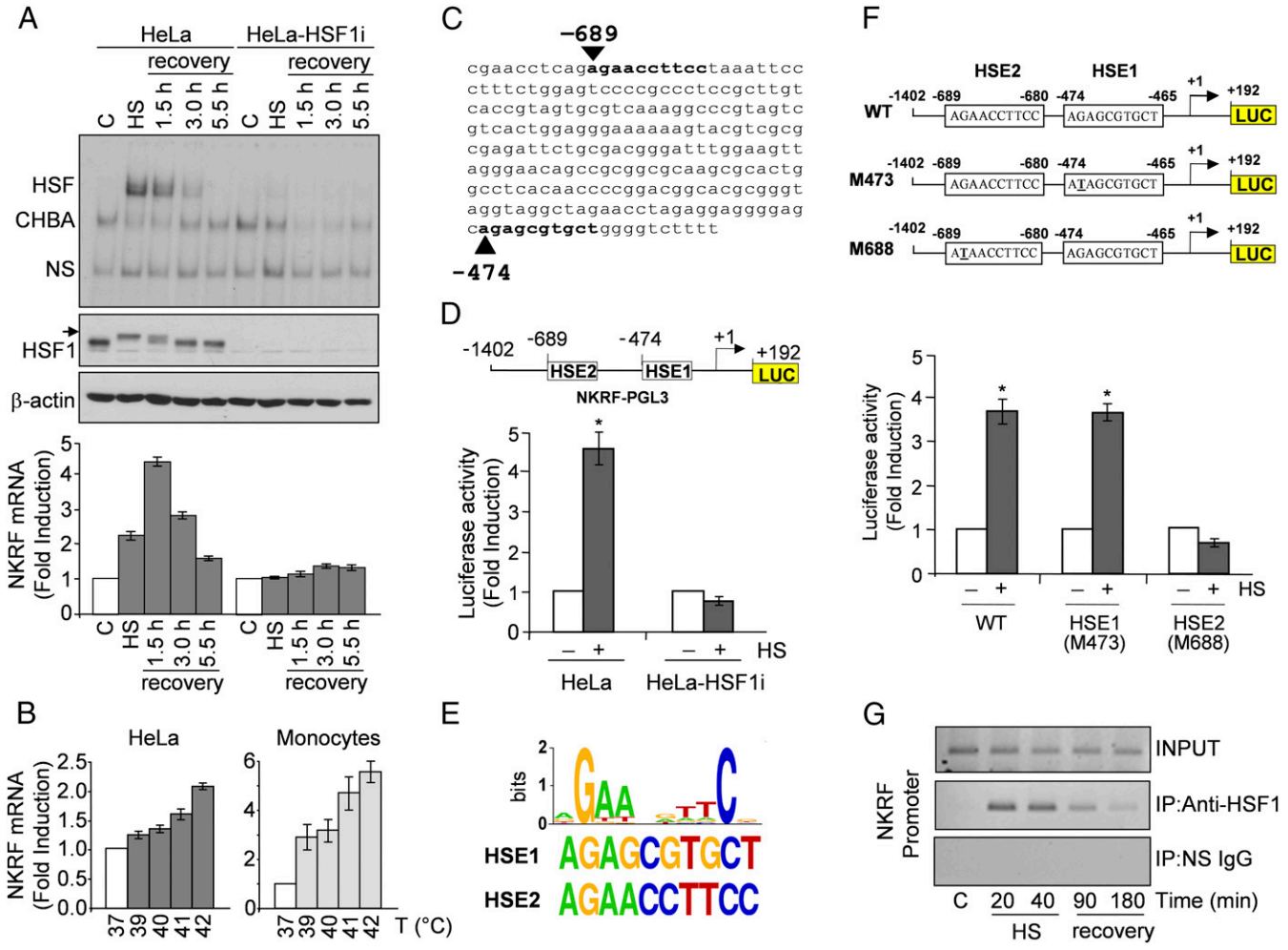

Fig. 1. HSF1 directly regulates NKRF expression during temperature increase. (*A*) HSF1 DNA-binding activity (gel-shift analysis, *Top*) and phosphorylation (immunoblot, IB, *Middle*) and NKRF-mRNA levels (qPCR, *Bottom*) of whole-cell protein or RNA extracts from HeLa and HeLa-HSF1i cells exposed to heat stress (HS, 43 °C, 40 min) and allowed to recover at 37 °C for the indicated times. CHBA, constitutive HSE-binding activity; HSF, HSF/DNA complexes; NS, nonspecific protein–DNA interactions. Arrow indicates hyperphosphorylated HSF1. (*B*) NKRF mRNA levels in HeLa cells or human peripheral-blood monocytes incubated at febrile temperatures (2 h). (*C*) NKRF promoter putative HSF1-binding sites (bold) identified by TFSearch. (*D*) NKRF promoter reporter analysis in HeLa and HeLa-HSF1i cells transfected with NKRF–PGL3 (*Top*) and heat stressed (6 h recovery). Putative HSF1-binding sites (HSE1, HSE2) identified in *C* are shown; numbers indicate positions relative to TSS (+1). (*E*, *Top*) WebLogo-generated HSF1 consensus motif. (*Bottom*) NKRF promoter HSE1 and HSE2 sequences. (*F*) Wild-type (WT) and point-mutated (G-to-T) (M473, M688) constructs (*Top*) were used for reporter analysis in HeLa cells treated as in *D* (*Bottom*). (*G*) ChIP analysis of HSF1 binding to the NKRF promoter in HeLa cells treated as in *A*. ChIP-enriched DNAs using preimmune (IP:NS-IgG) or anti-HSF1 (IP:anti-HSF1) serum and input DNAs are shown. Error bars indicate ±SD. **P* < 0.05.

second nucleotide of the HSE nGAAn unit, known to reduce HSF1 DNA binding (6), was inserted in HSE1 or HSE2 (Fig. 1 *E* and *F*). Mutating HSE1 did not alter heat-induced NKRF promoter activity, whereas mutating HSE2 prevented transcription (Fig. 1*F*), identifying HSE2 as the critical element for heat-induced NKRF transcription. Finally, ChIP analysis revealed that HSF1 binds directly to the NKRF promoter in vivo starting 20 min after heat stress (Fig. 1*G*), confirming a critical role of HSF1 in heat-regulated NKRF transcription. Analysis of genome-wide ChIP-seq data (Gene Expression Omnibus accession no. GSE43579) also confirmed that NKRF is included in the 1,242 HSF1 target genes identified during heat stress in a different type of human cell (K562 erythroleukemia) (18). Interestingly, in addition to heat, HSF1 activation during proteotoxic stress induced by arsenite or proteasome inhibition also triggered NKRF expression, whereas endoplasmic reticulum (ER) stress inducers thapsigargin and tunicamycin had no effect (Fig. S2). Differently from human cells, heat stress did not induce NKRF expression in murine fibroblasts; in silico analysis revealed substantial differences in murine versus human NKRF promoter structure, including lack of HSEs at position –689, which may account for the differential responses observed (Fig. S1*E*).

Endogenous or exogenous NKRF protein analysis surprisingly revealed that NKRF levels decreased during heat stress (Fig. 2 *A*–*C* and Fig. S3*A*) to recover thereafter, an effect largely due to newly synthesized NKRF accumulation (Fig. 2*D*). Notably, NKRF-mRNA contains a type-I Internal Ribosome Entry Site (IRES), allowing cap-independent translation under stress conditions (19). NKRF synthesis was not detected in HSF1i-silenced cells (Fig. 2 *A* and *D*). NKRF reduction during heat stress was not prevented by proteasome or autophagy inhibitors (Fig. S4 *A*–*C*), suggesting an effect independent of proteasome- or autophagy-mediated degradation; instead, we found that NKRF is heat-sensitive, converting from a soluble to insoluble state selectively during heat stress (Fig. S4 *D* and *E*) but not after arsenite- or bortezomib-induced proteotoxic stress or ER stress (Fig. S3 *B* and *C*) and was rescued by tertiary-structure stabilizer glycerol during heat exposure (Fig. S4*F*). One explanation for these observations is that NKRF may undergo temperature-induced conformational changes and/or become associated with specific structures following heat treatment. NKRF binds to DNA (10) and RNA (20); however, RNase, DNase, and benzonase treatment did not restore soluble NKRF levels (Fig. S4 *G* and *H*).

To investigate whether heat causes intracellular redistribution of the factor, NKRF localization was determined by cell fractionation and confocal immunomicroscopy studies. Under normal conditions, endogenous or exogenous NKRF is predominantly localized in nucleoli of human cells (Fig. 2 *E*–*G* and Fig. S5), as described in murine cells (20). In HeLa cells, NKRF is distributed throughout the three structural nucleolar compartments: fibrillar and dense-fibrillar centers, where rRNA transcription and posttranscriptional

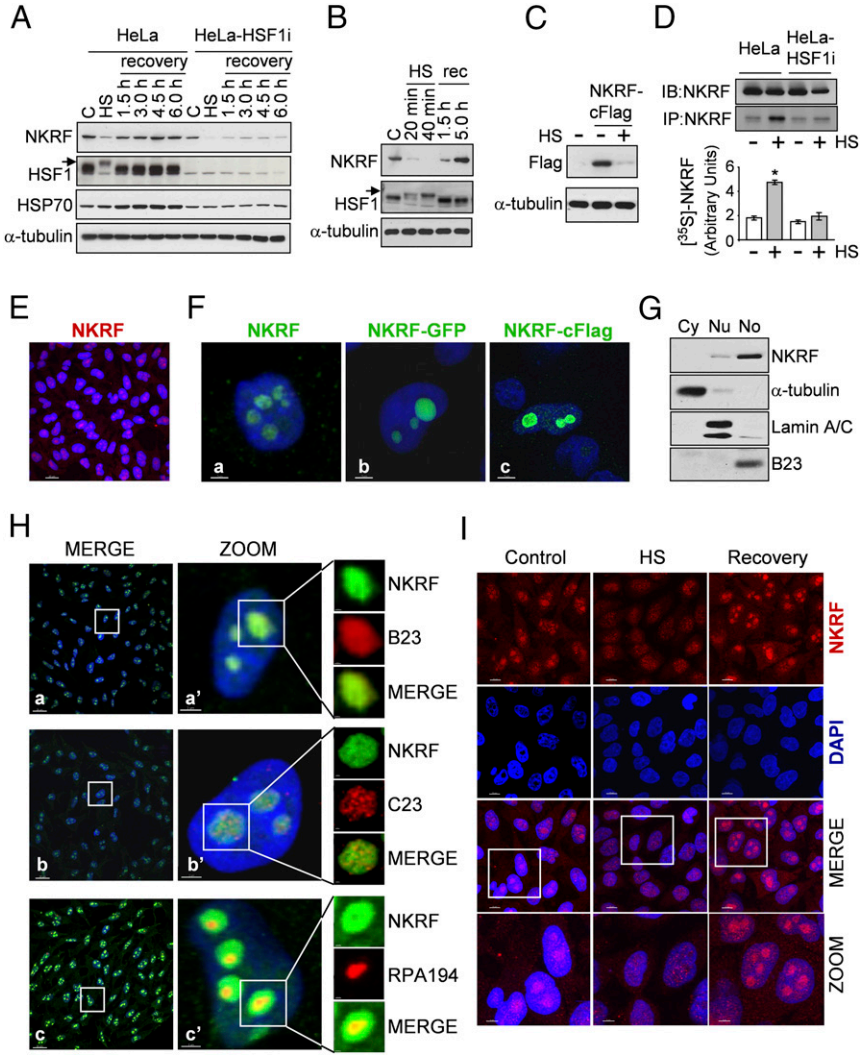

Fig. 2. NKRF dynamic nucleolar–nuclear trafficking and synthesis in response to heat. (*A* and *B*) NKRF, HSF1, HSP70, and α-tubulin levels from HeLa and HeLa-HSF1i cells exposed to HS (43 °C, 40 min) and recovered (rec) at 37 °C for the indicated times. Arrows indicate hyperphosphorylated HSF1. NKRF levels decrease during HS, due to protein insolubilization (described in Fig. S4). (*C*) Flag-tagged NKRF levels from NKRF-cFlag–expressing HeLa cells after HS (40 min). (*D*, *Top*) IB (IB:NKRF) and autoradiography of immunoprecipitated endogenous NKRF (IP:NKRF) from HeLa and HeLa-HSF1i cells [^{35}S]methionine-labeled (3 h pulse) during recovery after HS. (*Bottom*) [^{35}S]-NKRF quantification. Error bars indicate ±SD. *$P < 0.05$. (*E* and *F*) NKRF nucleolar localization detected by immunoconfocal microscopy in nontransfected (*E* and *F*, *a*), NKRF-GFP–transfected (*F*, *b*), or NKRF-cFlag–transfected (*F*, *c*) HeLa cells. Nuclei are stained with DAPI (blue). [Scale bars, 20 μm (*E*) and 2 μm (*F*).] (*G*) IB of NKRF in HeLa cytoplasmic (Cy), nuclear (Nu), and nucleolar (No) fractions. α-Tubulin, lamin-A/C, and nucleophosmin/B23 proteins were used as cytoplasm, nucleus, and nucleolus markers. (*H*) Confocal images of NKRF (green) distribution in nucleolar compartments. Merge (*a*, *b*, and *c*) and zoom (*a′*, *b′*, and *c′*) images are shown. Markers for granular (nucleophosmin/B23), dense-fibrillar (nucleolin/C23), and fibrillar-center (Pol-I RPA194 subunit) compartments are indicated. [Scale bars, 20 μm (zoom, 2 μm; nucleoli, 0.5 μm).] (*I*) Confocal images of NKRF (red) localization in HeLa cells untreated (control), heat stressed (40 min, HS), or recovered 3 h at 37 °C. [Scale bar, 10 μm (zoom, 5 μm).]

maturation occurs, and granular component, the site of final rRNA processing and preribosomes assembly (21) (Fig. 2*H*). Interestingly, heat exposure caused NKRF relocalization to the nucleoplasm; however, NKRF nucleolar levels were completely restored after 3 h of recovery (Figs. 2*I* and 3*A* and Fig. S5*B*).

NKRF is known to bind specific DNA sequences in several NF-κB–regulated genes and to interact with NF-κB/p65, differentially controlling NF-κB–driven transcription under basal and/or stimulated conditions (10–14). Therefore, proteotoxic stress-induced NKRF expression and dynamic nucleolar–nuclear movement may contribute to NF-κB–driven transcription regulation, with important implications in inflammation and cancer. However, as NKRF was mainly localized in human nucleoli, organelles emerging as important players in the recovery from stress (22), we focused our attention on its nucleolar function during heat stress. The recently described sequence homology of NKRF with *Caenorhabditis elegans* PAXT-1 protein (23), known to interact and stabilize the 5′-to-3′ exoribonuclease XRN2 (24, 25), prompted us to investigate a possible NKRF/XRN2 interaction in human nucleoli. Cell fractionation, immunoprecipitation (IP), proximity ligation assay (PLA), and confocal immunomicroscopy colocalization studies clearly show that NKRF interacts with XRN2 in human nucleoli under normal conditions (Fig. 3). During heat stress XRN2 follows NKRF fate, transiently redistributing to the nucleoplasm during heat exposure and returning to nucleoli after 3 h of recovery (Fig. 3 *A–D* and Figs. S5*B* and S6*A*). Interestingly, XRN2 was unable to relocalize to the nucleolus in NKRF-silenced cells (Fig. 3 *E* and *F* and Fig. S6*B*), whereas NKRF nucleolar localization was not affected by XRN2 silencing (Fig. S7), indicating that NKRF directs XRN2 nucleolus/nucleoplasm trafficking.

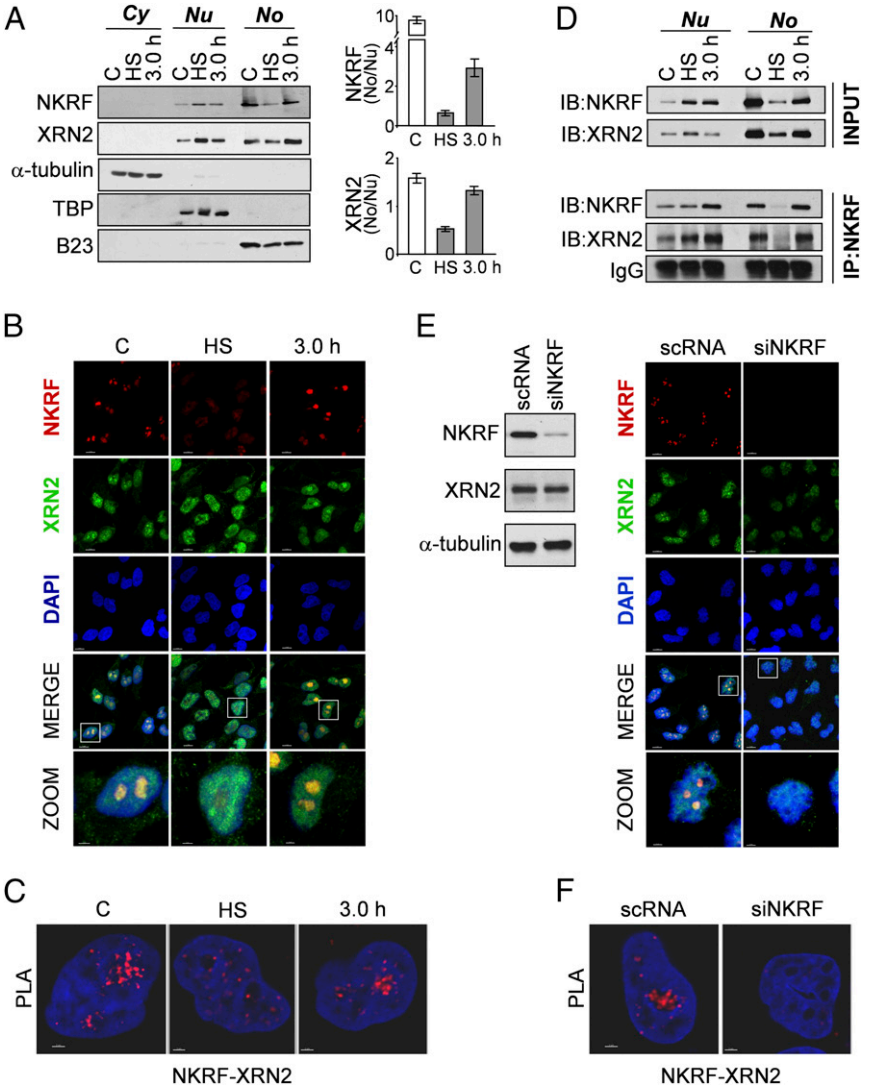

Fig. 3. NKRF interacts with 5′-to-3′ exoribonuclease XRN2 and controls XRN2 nucleolar localization. (*A*) IB of NKRF and XRN2 levels in cytoplasmic (Cy), nuclear (Nu), and nucleolar (No) fractions of HeLa cells untreated (*C*), heat stressed (40 min, HS), or recovered 3 h at 37 °C (*Left*). Cytoplasm (α-tubulin), nucleus (TATA-binding protein, TBP), and nucleolus (B23) markers are indicated. (*Right*) Quantification of NKRF and XRN2 No/Nu ratios in the same samples, as described in *Materials and Methods*. (*B*) Confocal images of HeLa cells treated as in *A* stained for NKRF, XRN2, and DNA (DAPI). Colocalization is shown in merge and zoom. [Scale bar, 7 μm (zoom, 2 μm).] (*C*) NKRF–XRN2 interactions (visualized as red spots) detected by PLA in samples treated as in *B*. (Scale bar, 2 μm.) (*D, Bottom*) Co-IP of NKRF and XRN2 in nuclear (Nu) and nucleolar (No) fractions of HeLa cells treated as in *A*. IgG are shown as control. Antibodies for IP and IB analyses are indicated. Relative inputs are shown (*Top*). (*E, Right*) Confocal images of HeLa cells transfected (48 h) with scramble-RNA (scRNA) or NKRF-siRNA (siNKRF) and stained as in *B*. (*Left*) IB for NKRF, XRN2, and α-tubulin of parallel samples. [Scale bar, 7 μm (zoom, 2 μm).] (*F*) NKRF–XRN2 interactions detected by PLA in samples described in *E*. (Scale bar, 2 μm.)

In mammalian cells, the main structural ribosome components (small-subunit 18S rRNA and large-subunit 5.8/28S rRNAs) are transcribed by RNA polymerase-I (Pol-I) from 300 to 400 rDNA head-to-tail tandem repeats as a single large polycistronic precursor, 47S pre-rRNA, that contains internal (ITS1, ITS2) and external (5′ETS, 3′ETS) transcribed spacers (Fig. 4*A*) (26–28). rRNA biogenesis is a highly energy-consuming process requiring a complex series of endonucleolytic cleavages within spacers–regions followed by exonucleolytic trimming to form mature rRNA 5′ and 3′ ends (26). This process was mainly characterized in yeast and, surprisingly, only recently investigated in human cells, revealing that pre-rRNA processing pathways are notably different in metazoan: ∼27% of human factors have distinct or additional functions in pre-rRNA processing compared with their yeast orthologs, and several pre-RNA processing factors have no yeast homolog (26, 29).

XRN2 (homolog of yeast XRN2/Rat1) plays a major role in rRNA maturation, coordinating the optimal order of multiple pre-rRNA cleavages (25); it is essential for degradation of 5′-extended 45.5S and 34.5S pre-rRNAs forms, it removes ITS-1–derived extensions to generate 32S from 32.5S pre-rRNA, and it promotes decay of 5′-01, 5′-A0, and E2 fragments, generated by endonucleolytic cleavages in the 5′ETS/ITS1 region in human cells (27–29) (Fig. 4*A*). As the pre-rRNA transcription rate is high, discarded fragment decay is important for nucleolar homeostasis; in addition to nucleolar toxicity, aberrant pre-rRNA and excised spacer-region turnover is also important for cellular nucleotide level maintenance (25).

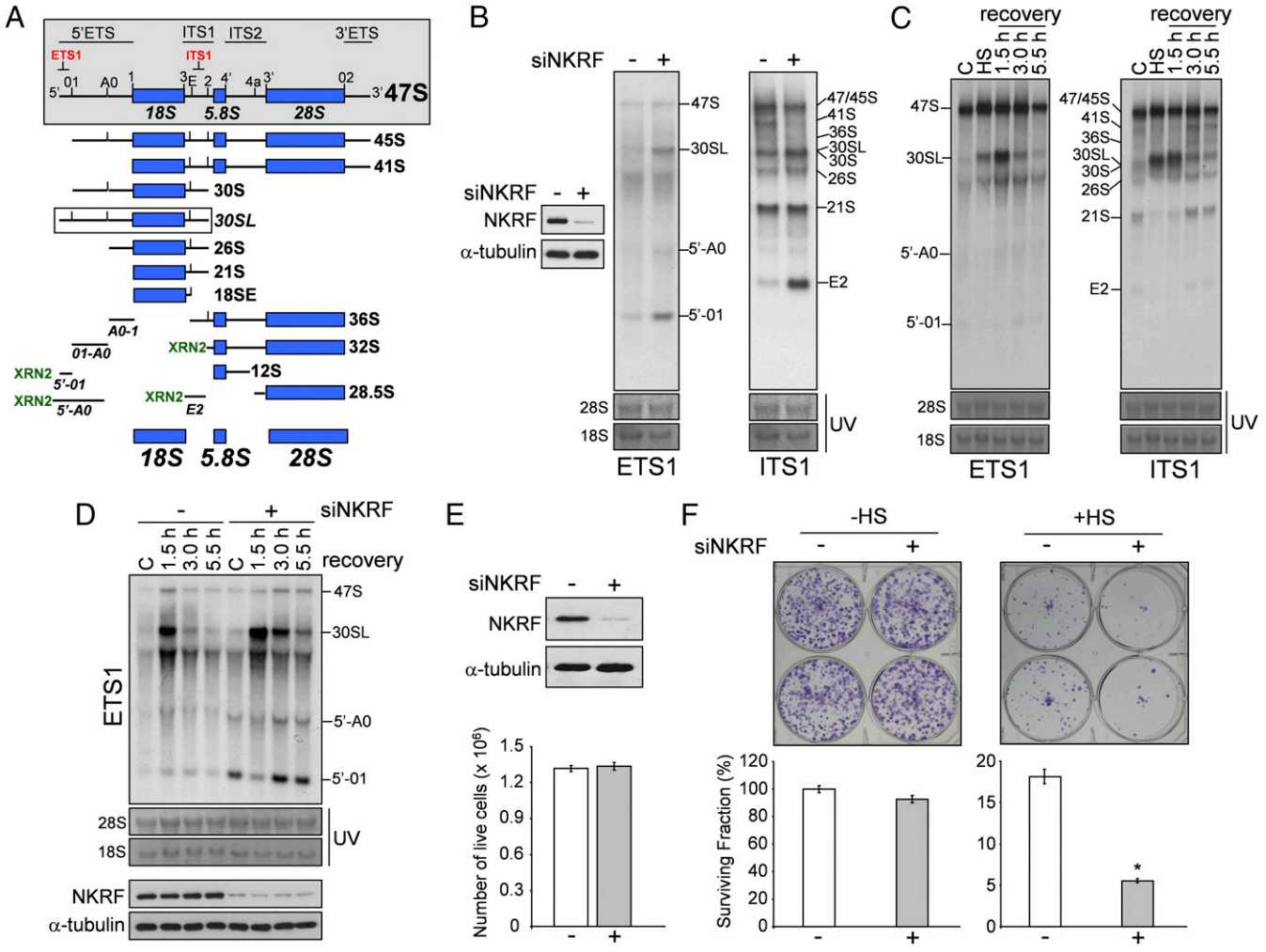

Fig. 4. NKRF is essential for XRN2-driven pre-rRNA processing and aberrant fragment turnover. (*A*) Schematic representation of human 47S pre-rRNA processing. ETS,ITS: external- and internal-transcribed spacers. The 47S pre-rRNA cleavage sites and oligonucleotides for Northern hybridization (red) are indicated (gray box). Aberrant 30SL pre-rRNA, accumulated during 01-processing inhibition, is shown (white box). Steps requiring XRN2 exonuclease activity are shown (XRN2); discarded fragments 5′-01, 5′-A0, and E2 are indicated. (*B*) Northern blot of HeLa cells transfected (48 h) with scRNA (–) or NKRF-siRNA (+). rRNA intermediates and discarded fragments recognized by ETS1 or ITS1 probes are indicated. Hybridizations were performed on the same membrane after stripping and reprobing. The same 28S and 18S loading control is shown for ETS1 and ITS1 probes. IB of NKRF in the same samples is shown. (*C*) Northern blot (ETS1,ITS1 probes) of HeLa cells heat stressed (HS, 43 °C, 40 min) and recovered at 37 °C for the indicated times. Hybridizations were performed on the same membrane after stripping and reprobing. The same 28S and 18S loading control is shown for ETS1 and ITS1 probes. (*D*, *Top*) Northern blot (ETS1 probe) of cells transfected (48 h) with scRNA or NKRF-siRNA and treated as in *C*. (*Bottom*) IB of NKRF in the same samples. (*E* and *F*) NKRF silencing enhances cell sensitivity to heat. IB for NKRF and α-tubulin in HeLa cells transfected for 48 h with scramble RNA (–) or NKRF-siRNA (+); live cell numbers in the same samples are shown (*E*). HeLa cells described in *E* were left untreated (–HS) or subjected to sublethal hyperthermic treatment (43 °C, 120 min) (+HS); cell survival was analyzed by clonogenic assay after 10 d (*F*). Representative images (*Top*) and quantification of clonogenic assay (*Bottom*) are shown. Data are expressed as percentage of surviving fractions. Error bars indicate ±SD. $*P < 0.01$.

We postulated that NKRF may participate in rRNA processing via XRN2 interaction and investigated the effect of NKRF silencing on 5′-ETS and ITS1 pre-rRNA regions. NKRF silencing suppressed 45S/47S pre-rRNA processing and resulted in accumulation of aberrant 30SL pre-rRNA and 5′-01, 5′-A0, and E2 fragments (Fig. 4*B*). Next we investigated rRNA processing during heat stress, a phenomenon still poorly characterized in human cells. Heat exposure (43 °C, 40 min) caused transient halting of rRNA processing characterized by accumulation of 30SL precursor and XRN2 target fragments, with rRNA processing returning to normal functions at 3 h of recovery (Fig. 4 *C* and *D*), parallel to the kinetics of NKRF-driven XRN2 nucleolus–nucleoplasm trafficking (Fig. 3). Interestingly, recovery was impaired in NKRF-silenced cells, where accumulation of 30SL precursor and 5′-01 and 5′-A0 discarded fragments was evident for several hours after stress (Fig. 4*D*). XRN2 silencing resulted in rRNA processing alterations comparable to NKRF silencing during heat stress (Fig. S8). In addition, transient (14 h) NKRF silencing, preventing NKRF resynthesis after heat stress, was sufficient to impair rRNA processing recovery (Fig. S9), indicating an important role of newly synthesized NKRF for reestablishment of rRNA metabolism. These results are summarized in the NKRF cycle model proposed in Fig. S10.

Notably, mammalian XRN2 is also present in the nucleoplasm and is implicated in Pol-II transcription termination, intron degradation, and pre-RNA and microRNA metabolism (24). Because NKRF was lately implicated in XRN2 function in the nucleoplasm (30), our results suggest the intriguing possibility that proteotoxic stress may also affect these other aspects of RNA metabolism via NKRF–XRN2 trafficking control.

As ribosome biogenesis is directly linked to protein synthesis and therefore to cell proliferation and survival control (31), we postulated that NKRF silencing may sensitize cancer cells to heat stress. In fact, whereas transient (48 h) NKRF silencing did not affect the viability of HeLa cells under nonstress conditions, it greatly hindered their ability to recover from sublethal heat stress (Fig. 4 *E* and *F*), indicating an important role of NKRF in cell survival after proteotoxic stress. NKRF was reported to participate in pancreatic cancer growth control via NF-κB regulation (32); however, because dysregulated rRNA synthesis is common in cancer cells (31), the NKRF rRNA controlling function described in this study may inspire novel therapeutic strategies against cancers addicted to accelerated ribosome biogenesis.

Our study identifies NKRF as a stress protein acting as a guardian of rRNA biogenesis and nucleolus homeostasis in human cells. We propose that NKRF is part of a dynamic nucleolar multitasking protein network contributing to orchestrate nuclear functions under stress conditions.

Materials and Methods

The establishment of HeLa cells stably transfected with pSUPER-HSF1i/pcDNA (HeLa-HSF1i) or control (HeLa wild-type) plasmids was described previously (7). All cell lines; culture conditions; source of antibodies, reagents, and plasmids; and methods are described in *SI Materials and Methods* and Table S1.

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