

The long non-coding RNA NEAT1 and nuclear paraspeckles are upregulated by the transcription factor HSF1 in the heat shock response

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Running title: *NEAT1 is a novel HSF1 target gene in the heat shock response*

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## ABSTRACT

The long non-coding RNA (lncRNA) NEAT1 is the architectural component of nuclear paraspeckles, and has recently gained considerable attention as it is abnormally expressed in pathological conditions such as cancer and neurodegenerative diseases. NEAT1 and paraspeckle formation are increased in cells upon exposure to a variety of environmental stressors, and believed to play an important role in cell survival. The present study was undertaken to further investigate the role of NEAT1 in cellular stress response pathways. We show that NEAT1 is a novel target gene of heat shock transcription factor 1 (HSF1), and upregulated when the heat shock response pathway is activated by Sulforaphane (SFN) or elevated temperature. HSF1 binds specifically to a newly identified conserved heat shock element (HSE) in the NEAT1 promoter. In line with this, SFN induced the formation of NEAT1-containing paraspeckles via a HSF1-dependent mechanism. HSF1 plays a key role in the cellular response to proteotoxic stress by promoting the expression of a series of genes, including those encoding molecular chaperones. We have found that the expression of HSP70, HSP90, and HSP27

is amplified and sustained during heat shock in NEAT1-depleted cells compared to control cells, indicating that NEAT1 feeds back via an unknown mechanism to regulate HSF1 activity. This interrelationship is potentially significant in human diseases such as cancer and neurodegenerative disorders.

NEAT1 (Nuclear Enriched Abundant Transcript 1) is a highly abundant long non-coding RNA (lncRNA) that is essential for the formation of specific nuclear bodies called paraspeckles (1-3). There are two overlapping isoforms of NEAT1 transcribed from the same promoter: NEAT1\_1 of 3.7 kb and NEAT1\_2 of 22.3 kb (2-4). NEAT1\_2 is indispensable for paraspeckle formation and is generated when the polyadenylation signal, and thus termination of the NEAT1\_1 transcript, is suppressed by an hnRNPK-dependent mechanism (4). Unlike NEAT1\_1, the 3' end of NEAT1\_2 is not polyadenylated, but processed by RNase P cleavage and subsequently stabilized through formation of a triple helical structure (3,5,6).

Whereas NEAT1\_1 is highly expressed in many tissues in mice, the expression pattern of NEAT1\_2, and consequently the presence of paraspeckles, are more restricted (7). Recently, NEAT1 was found to be required for mammary gland development and lactation in mice (8). NEAT1 has also a critical role in corpus luteum formation (9). Even though the function of NEAT1 is still not fully understood, several reports have suggested that increased NEAT1 expression regulates the expression of certain genes by sequestering specific mRNAs and proteins into paraspeckles (10-12). NEAT1 expression is upregulated in response to different cellular stresses including viral infections, proteasome inhibition, oncogene-induced replication stress, and hypoxia (11-17). Emerging evidences suggest that NEAT1 plays a cytoprotective role, as cells deficient of NEAT1 display increased sensitivity towards stress-induced cell death (11,15). In line with this, NEAT1 was found to be transcriptionally activated by HIF2 $\alpha$  in response to hypoxia in cancer cells, and more recently, reported as a p53 target gene that prevents replication stress and DNA damage induced by mutagenic agents and oncogenes (13,15,18,19). Interestingly, high levels of NEAT1 are associated with tumorigenic characteristics and poor clinical outcome in several human cancers (13,15,20).

Cells are constantly subjected to extrinsic and intrinsic stressors that might have detrimental effects unless neutralized by specific cytoprotective mechanisms. The heat shock response is a universal cellular defense mechanism towards agents causing proteotoxic stress (21,22). Elevated temperatures, as well as wide range of oxidative and electrophilic agents, cause misfolding and damage of cellular proteins that will lead to cellular dysfunction or death unless repaired and/or removed. The heat shock transcription factor 1 (HSF1) plays a key role in this response mechanism (21-24). Under normal conditions, HSF1 is kept in an inactive form in the cytoplasm by a multichaperone complex consisting of Hsp90, Hsp70, Hsp40, and TriC (23,25-29). Upon activation, HSF1 is released from the repressive complex, undergoes a series of posttranslational modifications, and forms homotrimers that accumulates in the nucleus (21,23,30). Here, HSF1 stimulates the transcription of genes encoding proteins involved in repair and

clearance of damaged proteins (21,23,31). HSF1 specifically binds to heat shock elements (HSE), inverted repeats of nGAAn where “n” is any nucleotide, in the upstream regulatory regions of its target genes (32,33). Among the best-studied target genes of HSF1 are those encoding protein chaperones including Hsp70 and Hsp90 that restore proteostasis by regulating folding, activity, and degradation of proteins (34,35). The heat shock response is attenuated when HSF1 is released from the promoters of its target genes, and either degraded or re-engaged into the HSF1-repressive multichaperone complex by a negative feedback mechanism (21,36).

Here, we report that the isothiocyanate compound sulforaphane (SFN) induces NEAT1 expression and paraspeckle formation in MCF7 cells. This is not dependent on the Keap1-NRF2 pathway, but on binding and transcriptional activation of the NEAT1 promoter by HSF1. We have identified a HSE site in the NEAT1 promoter that is highly conserved among vertebrates. Moreover, we show that NEAT1 is upregulated in response to heat shock demonstrating that upregulation of NEAT1 is a general event in the heat shock response. Finally, we demonstrate that the expression of HSP70, HSP90, and HSP27 is enhanced and sustained in the heat shock response in NEAT1 knockdown cells, compared to control cells.

## **RESULTS**

### *SFN induces NEAT1 expression and paraspeckle formation*

Several lines of evidence clearly point towards NEAT1 being a stress-induced lncRNA that is involved in cytoprotection (11,13,15). NEAT1 expression has recently been shown to be induced by hypoxia and confers protection to hypoxia-induced cell death in breast cancer cells (15). To further determine the role of NEAT1 in oxidative stress, MCF7 cells were treated with the isothiocyanate sulforaphane (SFN), which triggers an antioxidative response in cells by modifying thiol groups in several proteins, including Keap1 in the Keap1-NRF2 pathway (37,38). NEAT1 expression was assessed by RT-qPCR using two different primer sets; one recognizing both isoforms and one solely recognizing the long NEAT1\_2 isoform (Fig. 1A). SFN potently and

rapidly induced the expression of NEAT1 in MCF7 cells (Fig. 1A). Pretreatment of cells with N-acetylcysteine, a strong antioxidant and precursor of cellular glutathione, counteracted the effect of SFN on NEAT1 expression (Fig. 1B).

Paraspeckles are dynamic ribonucleoprotein complexes that form around the NEAT1\_2 isoform in the nucleus (4). To determine if SFN-induced NEAT1 expression is associated with increased paraspeckle formation, we performed RNA-fluorescence in-situ hybridization (RNA-FISH) on untreated and SFN-treated MCF7 cells using probes recognizing the long NEAT1\_2 isoform. Whereas NEAT1\_2-containing punctas appeared small and scarcely distributed in the nucleus of untreated MCF7 cells, SFN treatment potently increased the numbers and the overall signal intensity of the paraspeckles (Fig., 1C and D).

#### *SFN-induced NEAT1 expression is not dependent on NRF2*

SFN stimulates several stress signaling pathways in cells, of which the Keap1-NRF2 pathway is the most prominent. To determine if NRF2 is involved in SFN-induced NEAT1 expression, MCF7 cells were transfected with an siRNA towards NRF2 and stimulated with SFN for 6 hours. The NRF2 protein accumulated after 6h SFN treatment, but its depletion did not interfere with the induction of NEAT1 (Fig. 2A). We also assessed the NEAT1 expression in control and NRF2-depleted cells after a prolonged treatment with SFN for 24 hours. Elevated levels of NEAT1 were observed in both control and siNRF2-transfected cells (Fig. 2B). In contrast, SFN-mediated induction of NQO1 mRNA, a well-established target of NRF2, was severely reduced in NRF2-depleted cells (Fig. 2C). We conclude that SFN-induced NEAT1 expression is not dependent on the Keap1-NRF2 pathway.

#### *SFN-induced NEAT1 expression and paraspeckle formation are dependent on HSF1*

SFN, as well as other oxidants, have recently been shown to stimulate HSF1, the key transcription factor conferring cellular protection to agents causing protein misfolding (39,40). We therefore sought to determine if SFN-induced NEAT1 expression is dependent on a mechanism involving HSF1. SFN treatment indeed induced a mobility shift of HSF1, which is associated with its

activation, and nuclear accumulation of the protein (Fig. 3, A and B). Consistent with the observed shift and nuclear translocation of HSF1, SFN potently induced the expression of the HSP70 mRNA, a prominent target gene of HSF1 (Fig. 3C). We next transfected MCF7 cells with two different siRNAs specifically silencing HSF1 expression, and determined the effect on SFN-induced NEAT1 expression. Both siRNAs significantly reduced the increase in NEAT1 levels observed after SFN treatment (Fig. 3D). The same was observed when HSF1 expression was silenced in SFN-treated HeLa cells (Fig. 3E). To determine if SFN-induced paraspeckle formation is dependent on HSF1, we performed co-immuno-FISH analyses on control and HSF1-depleted cells using an HSF1 antibody and probes specifically binding to NEAT1\_2. In line with the observations described above, SFN enhanced the nuclear staining of HSF1 (Fig. 4, A and B) and the formation of NEAT1\_2 containing paraspeckles (Fig. 4, A and C). Importantly, SFN-induced paraspeckle formation was severely compromised in HSF1-depleted cells (Fig. 4, A and C). Taken together, our data clearly demonstrate that HSF1 is essential for increased NEAT1 expression and paraspeckle formation as response to SFN-treatment in MCF7 cells.

#### *NEAT1 is transcriptionally regulated by HSF1*

Having established that SFN induces NEAT1 expression by an HSF1-dependent mechanism, we next asked if SFN treatment leads to transcriptional activation of the NEAT1 gene. A luciferase reporter vector containing nucleotides -4040 to +144 of the NEAT1 upstream regulatory region was generated and transfected into MCF7 cells. Reporter gene assays were performed in extracts from untreated and SFN-treated cells. SFN significantly stimulated the NEAT1 promoter-driven luciferase activity (Fig. 5A). This stimulation was severely compromised upon co-transfection with an HSF1-directed siRNA, demonstrating that SFN-induced activation of the NEAT1 promoter is dependent on HSF1 (Fig. 5B). HSF1 binds to heat shock elements (HSE) within its target genes that are composed of alternating inverted repeats of 5 base pairs, nGAAn where “n” is any nucleotide (32,33). We carefully inspected the NEAT1 promoter, and identified three putative HSEs. One of these, located between nucleotides -

445 and -431 specifically caught our attention as it is highly conserved between species (Fig. 5C). To determine if this region is involved in SFN-activated NEAT1 transcription, a truncated construct of the NEAT1 promoter reporter vector was made containing nucleotides -470 to +144. We also made a mutated version where we introduced four point mutations in the predicted HSE core, and both constructs were transfected into MCF7 cells. SFN potently stimulated transcription from the truncated NEAT1 promoter (Fig. 5D). This stimulation was absolutely dependent on an intact HSE core, as point mutations in this region totally abolished the SFN-induced increase in NEAT1 promoter driven luciferase activation. To analyze if HSF1 can bind to the NEAT1 promoter in vivo, ChIP experiments were conducted on untreated and SFN treated MCF7 cells using an antibody against HSF1 and RT-qPCR primers amplifying a 100 base pair fragment of the NEAT1 promoter encompassing the HSE site. HSE-containing NEAT1 promoter fragments co-precipitated with the HSF1 antibody (Fig. 5E). Importantly, SFN robustly increased HSF1 binding to NEAT1 HSE fragments. Primers amplifying a GAPDH fragment and a region of the NEAT1 promoter upstream of the HSE site (“upstr”) were used as controls. Control ChIPs with IgG gave very high Ct values compared to that of the HSF1 antibody and, importantly, showed no differences upon SFN stimulation.

#### *NEAT1 is induced by heat shock*

Having established that NEAT1 levels are enhanced by an HSF1-dependent mechanism upon SFN treatment, we next sought to determine if NEAT1 is induced as response to heat shock (HS). MCF7 cells were incubated at 43°C for 30 min, and either harvested directly, or after recovery at 37°C for the indicated periods. HSF1 was rapidly activated during HS as assessed by a mobility shift in western blot (Fig. 6A). This was accompanied by increased expression of the HSP70 mRNA (Fig. 6B). Importantly, HS rapidly and transiently stimulated the expression of NEAT1 (Fig. 6C). This indicates that elevated NEAT1 expression is a general mechanism in the heat shock response pathway.

#### *Proliferation is compromised and expression of HSF1 target genes is amplified in NEAT1-depleted cells*

Elevated NEAT1 levels and paraspeckle formation in response to cellular stress are widely observed, and believed to play a pro-survival role by regulating the expression of specific genes. To start unravelling the function of NEAT1 in the heat shock response, we measured the sensitivity of control and NEAT1-depleted cells to heat shock by cell confluence proliferation assays. MCF7 cells were transfected with NEAT1-specific gapmeR antisense oligonucleotides (ASOs), which generally reduced the NEAT1 expression by 70-80 % for up to 120 hours, or a control gapmeR. Cell confluence was then monitored for 96 hours using the IncuCyte® live cell analysis system. After the first 48 hours, half of the cells were subjected to heat shock for 30 min, and then returned to IncuCyte system for another 48 hours. Strikingly, NEAT1-depletion severely decreased the confluency of MCF7 cells, indicating that NEAT1 is necessary for their proliferation or survival (Fig. 7A). The proliferation rate was not further decreased after heat shock compared to cells kept at 37°C over the whole monitoring period (Fig. 7A and B). Taken together, this suggests that NEAT1 is generally required for the proliferation or survival of MCF7 cells, and that an additional stress such as heat shock, does not further affect the already growth-inhibited cells. Control-transfected cells generally recovered well after heat shock with only a slight reduction in confluency (Fig 7A and B).

To further analyze the role of NEAT1 in the heat shock response, we assayed the expression of the HSF1 target genes HSP70, HSP90, and HSP27 in control and NEAT1-depleted cells. MCF7 cells were transfected with two different gapmeR ASOs, which either targeted both isoforms of NEAT1, or solely the long NEAT1\_2 isoform. Transfected cells were exposed to heat shock and HSP70, HSP90, and HSP27 expression was assessed by RT-qPCR. Interestingly, the expression of all target genes was repeatedly amplified and sustained in cells where NEAT1 was silenced, compared to cells transfected with a control gapmeR (Fig. 8). Moreover, the background expression in unstressed cells was slightly enhanced. Of note, a stronger effect on the HSF1



target genes was observed for cells transfected with the gapmeR targeting both isoforms of NEAT1, compared to those transfected with the gapmeR only silencing the NEAT1\_2 isoform. Taken together, our data suggest that NEAT1-depletion, by some mechanism, potentiates the HSF1 activity by either creating additional proteotoxic stress in the cells, or by regulating the turnover or the activity of the HSF1 protein.

## **DISCUSSION**

High-throughput RNA-sequencing has demonstrated that most cells express a plethora of long non-coding transcripts (41,42). During the last few years, huge efforts have been made to reveal their biological function, and many of them now appear as important contributors to gene regulation at different levels. NEAT1 is the architectural component of nuclear ribonucleoprotein complexes called paraspeckles, and has recently gained considerable attention as several reports have shown that the transcript is abnormally expressed in human diseases including cancer (13,15,20). The function of NEAT1 remains elusive, but emerging evidences suggest that NEAT1 and paraspeckles have a role in cytoprotection. Here, we show that NEAT1 is induced at the transcriptional level by the isothiocyanate compound sulforaphane (SFN). This is accompanied with increased paraspeckle formation. SFN mimics oxidative stress in cells by modifying thiol groups in cellular proteins, and induces antioxidative response pathways of which Keap1-NRF2 is the most prominent (37,38). We demonstrate that SFN-induced NEAT1 expression is not dependent on NRF2. In contrast, depletion of HSF1 severely abrogates SFN-induced NEAT1 expression and paraspeckle formation. Several reports have shown that SFN and other sulfhydryl-reactive compounds can stimulate the heat shock response pathway in cells by activating HSF1 (39,40,43,44). The mechanism for how SFN activates HSF1 is somewhat obscure, but previous studies have shown that oxidative compounds might promote the DNA-binding activity of HSF1 by modifying cysteine residues in the DNA-binding domain (45,46). SFN has also been shown to modify Hsp90 and thereby disrupt complex formation between Hsp90 and its protein partners (47,48). Recently, Naidu et al. reported that phenethyl isothiocyanate (PEITC) indeed modified

cysteine residues within Hsp90 leading to dissociation and activation of HSF1 (44).

Our results show that HSF1 accumulates in the nucleus upon SFN treatment and binds to the NEAT1 promoter *in vivo*. We have identified a conserved HSE in the NEAT1 promoter that is critical for SFN-induced transcriptional activation of the NEAT1 gene. Intriguingly, this site overlaps with a recently reported NF- $\kappa$ B binding site, which is necessary for LPS-induced NEAT1 expression in lung cancer cells (49). An overlapping NF- $\kappa$ B and HSF1 binding site has been identified previously in the promoter of the gene encoding MHC Class I Chain-Related Protein A (MICA) (50). Here, HSF1 and NF- $\kappa$ B bind mutually exclusive to the site, and overexpression of a truncated version of HSF1 containing only the DNA-binding domain outcompetes NF- $\kappa$ B binding and abolishes TNF $\alpha$ -induced MICA expression. If the overlapping HSF1/NF- $\kappa$ B site in the NEAT1 promoter represents a regulatory hub, coordinating outputs from different signaling pathways, remains to be resolved.

In the present study we show that NEAT1, as well as being induced by SFN, is also induced upon heat shock. This clearly suggests that NEAT1 upregulation is a general phenomenon in the heat shock response. This is supported by a study by Hirose et al., demonstrating that NEAT1 expression and paraspeckle formation are induced by inhibition of the 26S proteasome by MG132 or Bortezomib (11). Proteasome inhibition causes a proteotoxic stress in the cells as proteins that are destined for degradation form aggregates in both the cytoplasm and the nucleus (11,51). Activation of HSF1 to induce expression of molecular chaperones, is a general cellular response mechanisms to proteasome inhibition (52-54). Thus, we envision that NEAT1 induction upon proteasome inhibition might be mediated by HSF1-mediated transcriptional activation of the NEAT1 promoter.

Several reports have shown that NEAT1-depletion sensitizes cells to a variety of stressors. Thus, we hypothesized that knock down of NEAT1 expression would make cells more susceptible to heat shock. However, we repeatedly observed that transient transfection with NEAT1 antisense oligos by itself, dramatically reduced the proliferation of MCF7 cells, and that this tendency was not

reinforced by heat shock. This shows that MCF7 cells cultivated *in vitro*, are highly dependent on NEAT1. To further dissect the function of NEAT1 in the heat shock response, we knocked down NEAT1 expression by antisense oligos and assessed the effect on the expression of three HSF1 target genes including HSP70, HSP90, and HSP27. Interestingly, knockdown of NEAT1 amplified and prolonged the expression of these target genes. The mechanism for this is still obscure. NEAT1-depletion abrogates the formation of paraspeckles (4). This might lead to mislocalization of paraspeckle-associated proteins that disturbs proteostasis in the cells, and thereby contribute to the activation of HSF1. Alternatively, NEAT1 might regulate the turnover of the HSF1 protein or activity by a negative feedback mechanism. Interestingly, the effect of NEAT1-depletion on HSF1 target genes, was significantly stronger when cells were transfected with a gapmeR targeting both isoforms compared to one only reducing NEAT1\_2 expression. This indicates that the short NEAT1\_1 isoform has an important function in the regulation of the heat shock response.

HSF1 plays a critical role in the cellular defense to proteotoxic stress. Many neurodegenerative diseases including amyotrophic lateral sclerosis (ALS), Huntington's disease, and Alzheimer are associated with the formation of protein aggregates (31,55). Loss of HSF1 expression or activity is frequently observed in these diseases (55-58). Our results demonstrate that HSF1 activates the expression of NEAT1 during the heat shock response. Interestingly, several reports have shown that NEAT1 is abnormally expressed in ALS and Huntington's disease (59-61). Moreover, mislocalization of two paraspeckle proteins, FUS (Fused in sarcoma) and TDP-43 (TAR DNA-binding protein-43) is well-known to be associated with ALS (62). It has been speculated that NEAT1 expression and paraspeckle formation might have a protective role in neuronal cells in early stages of ALS and Huntington's disease (60,61,63). In line with this, Hirose et al. showed that mouse embryonic fibroblasts from NEAT1 knockout cells displayed an increased sensitivity to proteasome inhibitors causing formation of protein aggregates, compared to wild-type cells (11). The crosstalk between NEAT1, paraspeckle formation, sub-cellular localization of FUS and TDP-43, and

HSF1 in these devastating diseases should be a focus of future research.

Constitutive activation of HSF1 and abnormal expression of NEAT1 are both frequently observed in human cancers (13,15,20,64-66). There are clear evidences that both HSF1 and NEAT1 have cytoprotective roles in tumors and are associated with poor prognosis. In the present study, we demonstrate that NEAT1 is a novel target gene of HSF1. It remains to be determined if there is any correlation between HSF1 activation and NEAT1 expression in cancer.

## EXPERIMENTAL PROCEDURES

### *Cell culture and treatments*

MCF7 (ATCC® HTB-22™) and HeLa (ATCC® CCL-2™) cells were purchased from American Type Culture Collection and maintained in minimal essential medium (MEM, Sigma-Aldrich) supplemented with 10% fetal bovine serum (Biochrom) and 1% penicillin-streptomycin (Sigma-Aldrich). MCF7 cells were cultured in the presence of 0.01 mg /ml insulin (Sigma-Aldrich). All Cells were grown at 37°C in humidified condition containing 5% CO<sub>2</sub>. Sulforaphane (SFN, cat# S4441) and N-acetyl cysteine (NAC, cat# A9165) were purchased from Sigma-Aldrich. SFN was added to the cells at a final concentration of 20 μM for short-term treatments up to 8 hours, and at a final concentration of 10 μM for long-term treatment (24 hours). When included, NAC (5 mM) was added to the media 1 hour before SFN treatment. To induce a cellular heat shock response, cells were incubated at 43°C for 30 minutes, and then either harvested directly or returned to 37° for recovery.

### *Plasmid constructions*

The human NEAT1 promoter (-4040/+144) was cloned from genomic DNA by performing two PCR amplification reactions using PrimeSTAR®GXL DNA Polymerase (Takara Bio Inc, R050Q) generating fragments of 1756 bp (primers NP1.1F/NP2.1R) and 2414 bp (nested PCR, outer primer set NP2.1F/NP3.1R; inner primer set NP2.2F/NP3.2R). The 1756 bp fragment was digested with NheI (provided in primer) and HindIII (internal) and cloned into corresponding sites in pGL3-Basic (Promega). This was followed by insertion of the 2414 bp fragment into the

HindIII site using internal HindIII sites. The resulting pNEAT1(-4040/+144)-Luc plasmid was verified by sequencing. pNEAT1(-470/+144)-luc was generated from a promoter construct containing the 2414 PCR-product (pNEAT1(-2384bp/+144)-luc) by cutting with KpnI and PstI followed by religation. pNEAT1(-470/+144)-HSEmut-luc was made by site-directed mutagenesis according to the QuickChange II Site-Directed Mutagenesis kit protocol (Agilent Technologies). All primer sequences are provided in Table 1.

#### *RNA interference*

siNRF2 (siGENOME SMART pool Human NFE2L2, DM-003-755-02) was purchased from Dharmacon, and siHSF1\_#1 (Silencer® Select, s6950), siHSF1\_#2 (Silencer® Select, s6952), and Silencer® Select Negative Control No.2 were obtained from Thermo Fisher Scientific. Locked nucleic acid (LNA)-GapmeR NEAT1 antisense oligos and control GapmeRs were purchased from Exiqon. All sequences are provided in Table 1. Cells were transfected using Lipofectamine 2000 according to the reverse transfection protocol provided by the manufacturer (Thermo Fisher Scientific). Successful knock down was verified by RT-qPCR or Western blot analyses.

#### *Reverse transcription and quantitative PCR*

Cells were lysed in 300 µl Tri Reagent, and total RNA was isolated with Direct-zol RNA MiniPrep (Zymo Research) according to the manufacturer. RNA concentration was measured by NanoDrop 2000 (Thermo Fisher Scientific), and cDNA synthesis of total RNA was performed with SuperScript™ IV Reverse Transcriptase (Thermo Fisher Scientific). 2.5 µM of random hexamer primer (Thermo Fisher Scientific) and approximately 250 ng of template was used for the reaction. Total RNA was denatured at 65°C for 5 min, and cDNA was synthesized at 50 °C for 10 minutes. Quantitative PCR was run on a LightCycler 96 (Roche Life Science) with the SYBR green reaction mix FastStart Essential DNA Green Master (Roche Life Science) and 0.25 µM forward and reverse primer. (Thermal cycle conditions; 95°C 10 minutes and 40 cycles of 95°C 10 seconds, 60°C 10 seconds and 72°C for 10 seconds). All primers sequences are provided in

Table 1. Experiments were done in triplicates, and the  $\Delta\Delta Cq$  method was used for fold change calculations. GAPDH was used as reference gene.

#### *Immunoblotting*

Whole-cell extracts (WCE) were made by lysing cells directly in 2 x NuPAGE LDS Sample Buffer (Thermo Fisher Scientific). Nuclear extracts (NE) were isolated using the NE-PER™ Nuclear and Cytoplasmic Extraction kit (Thermo Fisher Scientific) according to manufacturer's instruction. In brief, cells were resuspended in Cytoplasmic Extraction reagent I and II and nuclei were pelleted by centrifugation at 16 000 g. The pellet was resuspended in ice-cold Nuclear Extraction Reagent, vortexed for 1 minute and incubated on ice for 10 minutes. This step was repeated 3 more times before centrifugation at 16 000 g for 10 minutes. Proteins were resolved on SDS-PAGE gels and transferred to nitrocellulose membranes. Equal loading of proteins was verified by probing the membranes with an antibody recognizing actin (WCE) or lamin B (NE). The following primary antibodies were used, all at 1:000 dilution: Rabbit anti-NRF2 (Abcam, cat# ab62359), rabbit anti-HSF1 (Cell Signaling, cat# 4356), rabbit anti-Lamin B (Proteintech, ca# 12987-1-AP), mouse anti-Actin (Millipore, MAB1501). The blots were detected with IRDye®-conjugated secondary antibodies (LI-COR Biosciences) at a 1:10 000 dilution (800CW goat anti-rabbit, cat# 926-32211; 680LT goat anti-mouse, cat# 926-68020), and the Odyssey® CLx Infrared Imaging System.

#### *RNA-fluorescence in situ hybridization and immunofluorescence staining*

Stellaris® NEAT1 RNA FISH probes recognizing the NEAT1\_2 isoform (VSMF-2251-5, Quasar® 670-conjugated) were purchased from LGC Biosearch Technologies. Preparation of cells, hybridization, and mounting were performed according to the Stellaris® RNA FISH Probes manuals. In brief, cells were seeded onto circular coverslips in 12-well dishes and allowed to attach for 2-3 days. They were fixed with 4% freshly made formaldehyde at room temperature, and permeabilized with 70% ethanol. Hybridization was done at 37°C in a humidifying chamber overnight. For co-immuno-FISH experiments, the hybridization was performed as described above

and cells were subsequently incubated in 1% RNase-free BSA for 30 minutes, and then stained with anti-HSF1 antibody for 1 hour (1:50, Cell Signaling, cat# 4356A). Cells were incubated with goat anti-rabbit Alexa 488-conjugated secondary antibody (1:500, Thermo Fisher Scientific, cat# A11070), and mounted using Vectashield® Antifade Mounting Medium containing DAPI (Vector Laboratories, H-1200). Images were generated using a Zeiss LSM780 confocal microscope (Carl Zeiss Microscopy GmbH, Jena, Germany). In all samples, Z-stacks (5 slices, 2.5 µm total height) images were taken at 40x magnification. For all images, the middle Z slice was positioned at DAPI's best focus. The same treatment and setting were applied to all replicates, and for each slide at least ten pictures were taken for velocity analysis. The Volocity software (PerkinElmer, version 6.3) was used to measure signals intensity for both NEAT 1\_2 and HSF1 signals. At least 250 cells in each group of treatment were analyzed by volocity software. The mean intensity of NEAT 1\_2 or nuclear HSF1 signals in the SFN-treated group were normalized against CTRL.

#### *Reporter gene assays*

Sub-confluent MCF7 cells in 12-well plates were transfected with 150 ng of luciferase reporter plasmids using Lipofectamine®2000 reagent (Thermo Fisher Scientific) according to the manual provided by the manufacturer. After 24 hours, cells were either left untreated or treated with SFN (20 µM) for 8 hours. Cells were harvested and luciferase assays were performed using the Dual-Light® Luciferase & β-Galactosidase Reporter Gene Assay System (Thermo Fisher Scientific). Of note, cells were initially co-transfected with luciferase reporter plasmids and an expression vector for β-galactosidase, but as SFN repeatedly interfered with the β-galactosidase activity in the cells, the expression vector was omitted from the transfections and only the luciferase activity was included in the analyses. Co-transfections with siRNA and plasmid DNA were performed in two steps using Lipofectamine®2000. First, siRNAs were introduced into the cells by reverse transfection. After 48 hours, plated cells were re-transfected with plasmid DNA and left for another 24 hours.

#### *Chromatin immunoprecipitation (ChIP) assays*

MCF7 cells were seeded at a density of 6 million cells per 10 cm dish the day before use. The cells were left untreated or treated with SFN (20 µM) for 6 hours before harvesting. Two 10 cm dishes were used per condition. The “iDeal ChIP-seq kit for Transcription Factors” (Diagenode, C01010055) was used for harvest and ChIP according to the manufacturers instruction. The two dishes for each treatment were combined, and the approximate cell number was estimated to be 15 mill of cells. Volumes of buffers used in the kit was adjusted to this. Cells were fixed for 15 minutes. Sonication was performed in ice cold water on a Bioruptor UCD-200 (Diagenode), 30 sec pulses on/off for 3 x 10 min. Samples run on an agarose gel showed majority of DNA with size from 100-400 bp after shearing. For immunoprecipitation, 10 µl of anti HSF1 antibody (Cell Signaling, 4356) or 1 µl of IgG (provided with the kit) was used with 200 µl sheared chromatin. Two µl (1%) of input chromatin was set aside. The eluate had a volume of 25 µl, which was diluted 1/10 before 5 µl was used in a qPCR reaction. qPCR was performed in triplicates on a LightCycler 96 (Roche Life Science). The relative amount of immunoprecipitated (IP) DNA compared to input DNA was calculated using the “percent input method” as follows: Since the input chromatin was 1%, a dilution factor of 100 (6,644 cycles, log<sub>2</sub> of 100) was subtracted to adjust input Ct value to 100%. To calculate the percentage of specific chromatin co-immunoprecipitated with the HSF1 antibody or the IgG control, the triplicate average Ct values, Ct(IP), for the specific qPCR primers (HSE, “upstream”, and GAPDH) were used in the equation  $100 \cdot 2^{-(\text{Adjusted input} - \text{Ct}(\text{IP}))}$ . Primer sequences are given in Table 1.

#### *Cell confluence proliferation assay*

MCF7 cells were transfected in solution with indicated LNA-GapmeR antisense oligoes and seeded in 96 well plates at an initial confluency of approximately 30% (20 000 cells per well) and immediately placed in an IncuCyte® S3 live-cell analysis system, which is equipped a fully automated microscope for cell confluence monitoring. Three phase contrast images were acquired from each well at 120 minute intervals over a period of 96 hours, using a 20x objective. For each condition, five wells were monitored.



Data was analyzed using the IncuCyte® S3 Software.

#### Statistics

GraphPad software (Prism version 7, Mac OS X) was used to analyze quantitative data. Statistical significance was evaluated with unpaired student t-

Test or one-way ANOVA followed by the Dunnett multiple comparison test. The data were considered statistically significant when  $p \leq 0.05$ . For all experiments significance is expressed as \*\*\*,  $p \leq 0.001$ , \*\*,  $p \leq 0.01$ , and \*,  $p \leq 0.05$ . The error bars indicate  $\pm$  S.D. in all figures. All the experiments were performed at least three times.

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**Conflict of interest:** The authors declare that they have no conflicts of interest with the contents of this article.

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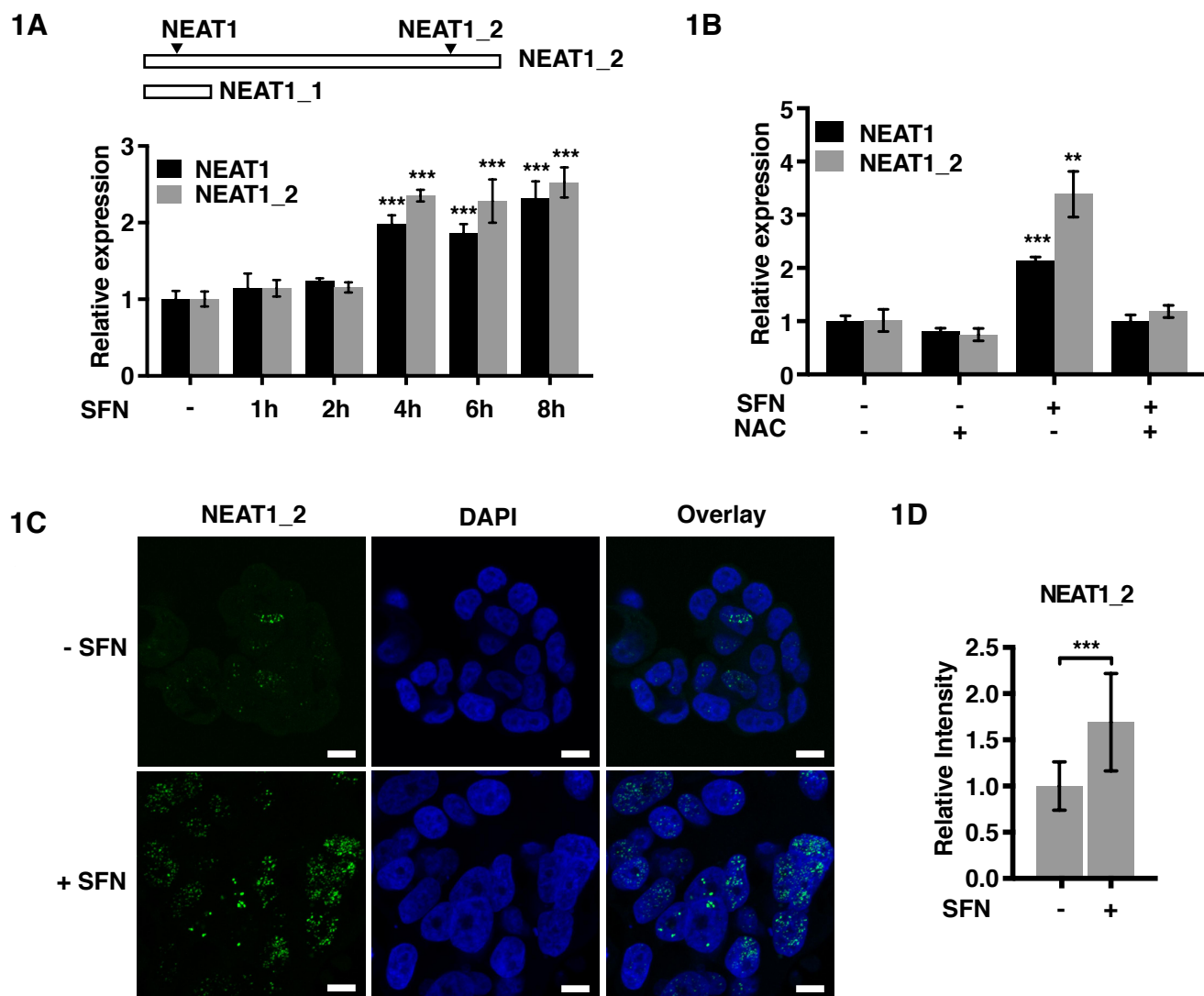
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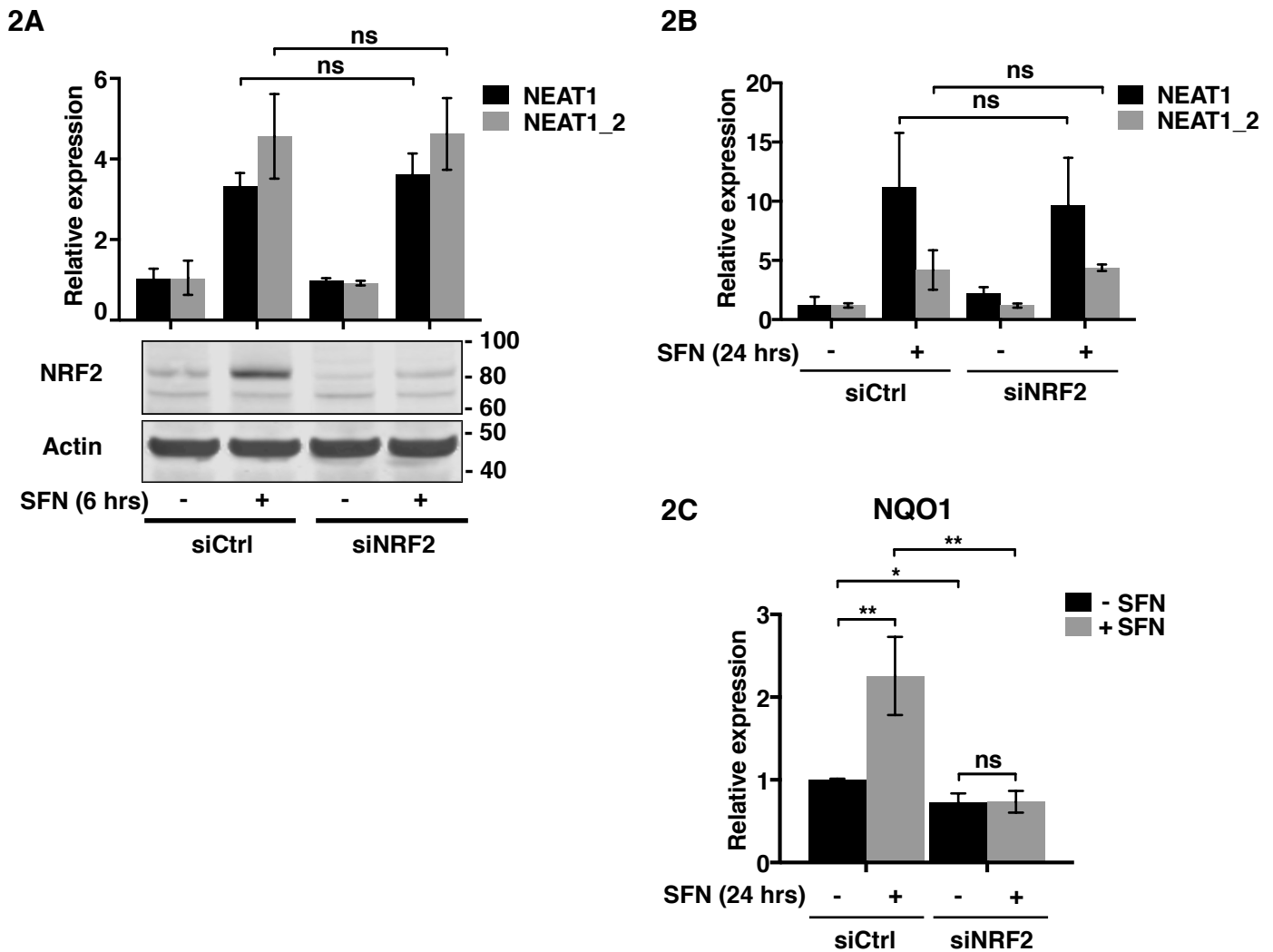
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Table 1. Primer and siRNA/ASO sequences

Name	Primer sequences (5'→3')
<b>RT-qPCR</b>	
<i>GAPDH</i>	F- GAGCGAGATCCCTCCAAAAT
	R- AAATGAGCCCCAGCCTTCT
<i>HSP90AA1 (HSP90)</i>	F- GAGCTTGACCAATGACTGGGA
	R- AGCACGTCGTGGGACAAAATA
<i>HSPA1A (HSP70)</i>	F- GGGCCTTTCCAAGATTGCTG
	R- TGCAAACACAGGAAATTGAGAACT
<i>HSPB1 (HSP27)</i>	F- TTCACGGGAAATACACGCT
	R- TTGGACTGCGTGGCTAGCTT
<i>NEAT1</i>	F- TCGGGTATGCTGTTGTGAAA
	R- TGACGTAACAGAATTAGTCTTACCA
<i>NEAT1_2</i>	F- CGGAGGGTCTTGTAACACCAG
	R- AGTCCGGGCAACACAGAAAG
<i>NQO1</i>	F- GTTGCTGAAAAATGGGAGA
	R- AAAAACACCAGTGCCAGTC
<b>Cloning</b>	
<i>NP1.1F</i>	F- GGACGCTAGCCTCCCTTCCTCAGTCAGTCCACAA
<i>NP2.1R</i>	R- CCAAGTCTCCTTTGTGCCCTTGAT
<i>NP2.1F</i>	F- GTAGAGGAAGAGAGCAGAACCAG
<i>NP3.1R</i>	R- CTGACTCCTCCACCCTTCTACCT
<i>NP2.2F</i>	F- AACGAGCTGTGTGGAAGTGGAGG
<i>NP3.2R</i>	R- CTAGACCTAGTCTCCTTGCCAAGCT
<b>Site-directed mutagenesis</b>	
<i>HSEmut</i>	F- CTCCGCCGCCCTGCGTTTGTCCAGATGTCCTGCCGG
	R- CCGGCAGGACATCTGGACAAACGCAGGCGGCGCGGAG
<b>RT-qPCR/ChIP</b>	
<i>HSE</i>	F- GAACCACGCCCGAAAGT
	R- CCGGCAGGACATCTGAAAA
<i>GAPDH</i>	F- GACTCACCTCGCCCTCAATA
	R- AAAGCACTCCTGGAACT
<upstr>	F- GGAACCTCCTTCTCAGTCAG
	R- TAAAGCGCCGCCCAACT
Name	siRNA and ASO sequences
<b>siRNA</b>	
<i>siNRF2 (sense strand)</i>	CCAAAGAGCAGUCAAUGA
<i>siHSF1_#1 (sense strand)</i>	GGACAAGAAUGAGCUCAGUtt
<i>siHSF1_#2 (sense strand)</i>	CUGGUGCAGUCAACCCGGAit
<i>siCtrl</i>	Silencer Select Negative Control No.2 siRNA (ThermoFisher Scientific, 4390847)
<b>Antisense LNA GapmeR Standard</b>	
<i>NEAT1 (described in ref 13)</i>	TAAGCACTTTGGAAAG
<i>NEAT1_2 (described in ref 13)</i>	CTCACAGTCCATCT
<i>Negative Control</i>	AACACGTCTATACGC

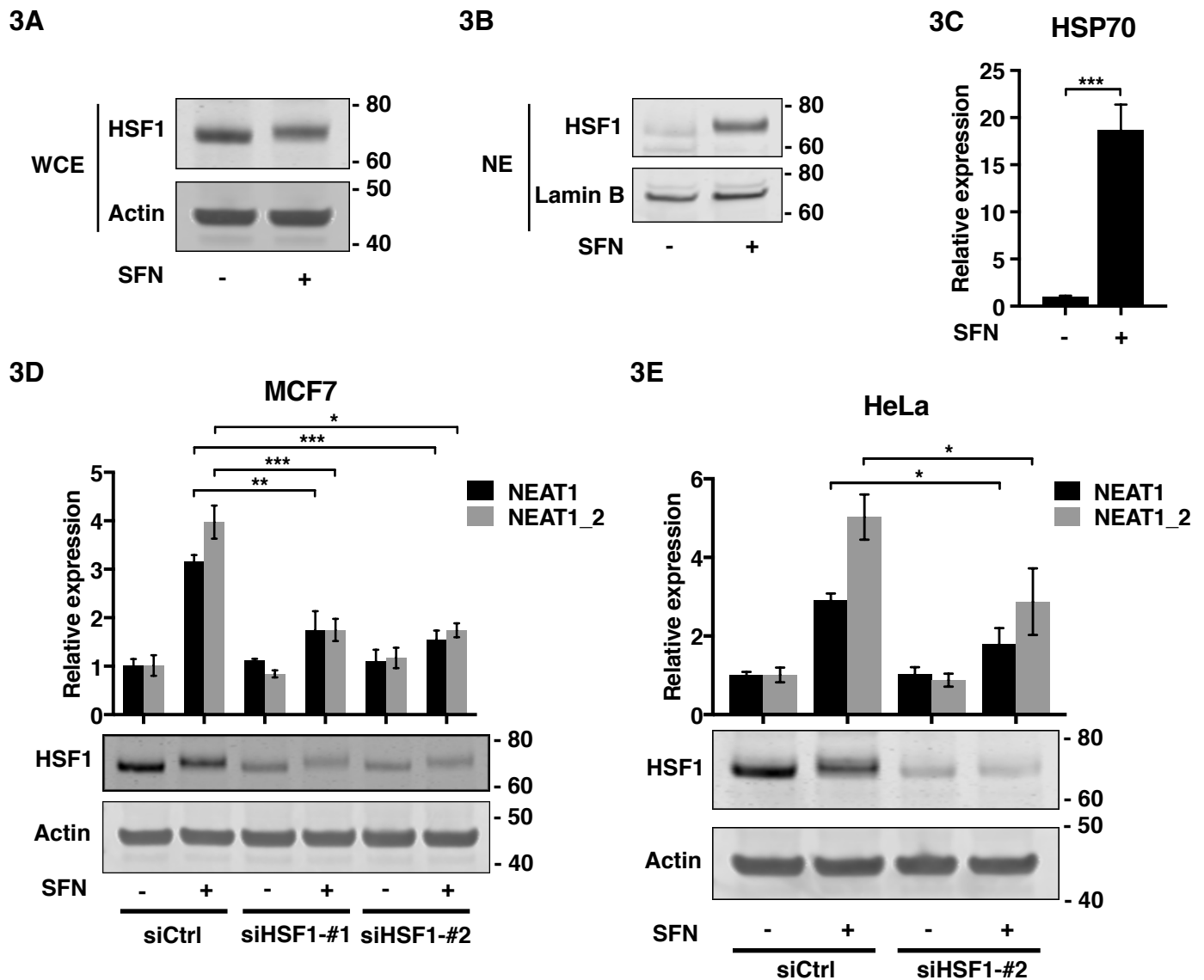


**FIGURE 1. NEAT1 expression and paraspeckle formation are induced by SFN.** *A*, MCF7 cells were treated with SFN (20  $\mu$ M) for the indicated time points. RNA was isolated and the expression of NEAT1 (both isoforms) and NEAT1\_2 was determined by RT-qPCR. The mean value  $\pm$  SD of three biological replicates in one experiment is presented as fold change relative to untreated cells. The results are representative of three independent experiments. *B*, MCF7 cells were pre-incubated with N-acetylcysteine (NAC, 5 mM) and then treated with SFN for 6 h. NEAT1 expression was determined as described in *A*. *C*, MCF7 cells were left untreated or treated with SFN for 6 h, fixed and subjected to RNA-fluorescent in situ hybridization (RNA-FISH) using probes recognizing the NEAT1\_2 isoform. DAPI was used to visualize the nuclei. *Bars*, 10  $\mu$ m. *D*, The overall intensity of the dots in at least 250 cells were quantitated using the Volocity software. Mean values  $\pm$  SD of three biological replicates are shown and presented as fold change relative to untreated cells. P values were calculated using ANOVA (*A*) or student's T-test (*B*, *D*) with  $p < 0.05$  considered statistically significant. (\*\*\*,  $p \leq 0.001$ , \*\*,  $p \leq 0.01$ , \* $p \leq 0.05$ ).



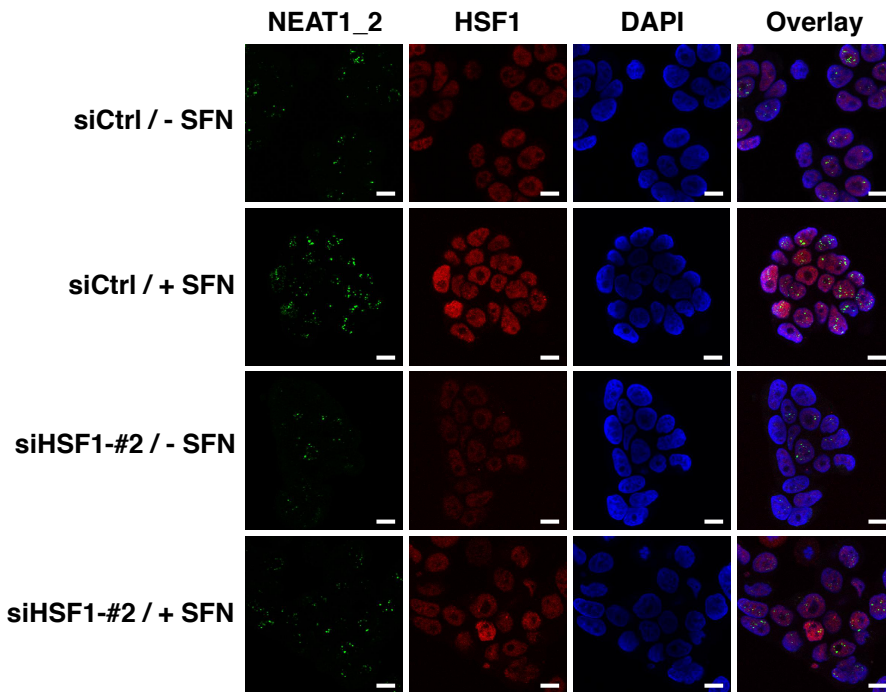
**FIGURE 2. NEAT1 induction by SFN is not dependent on NRF2.** *A*, MCF7 cells were transfected with an siRNA specifically targeting NRF2 (siNRF2) or control siRNA (siCtrl). Twenty-four h post-transfection, cells were either left untreated or treated with SFN (20  $\mu$ M) for 6 h. NEAT1 expression was determined by RT-qPCR as described in Fig 1. Depletion of NRF2 expression in whole cell extracts was verified by western blot analyses using an NRF2 antibody. The membrane was re-probed with an anti-actin antibody to ensure equal loading. *B*, *C*, MCF7 cells were transfected as described in *A*, and subjected to a long-term treatment with SFN (10  $\mu$ M) for 24 h. The expression of NEAT1 and NEAT1\_2 (*B*), and NQO1 (*C*) was determined by RT-qPCR. Experiments were performed in triplicates and the graph is representative of three independent experiments. (\*\*,  $p \leq 0.01$ , \* $p \leq 0.05$ ).



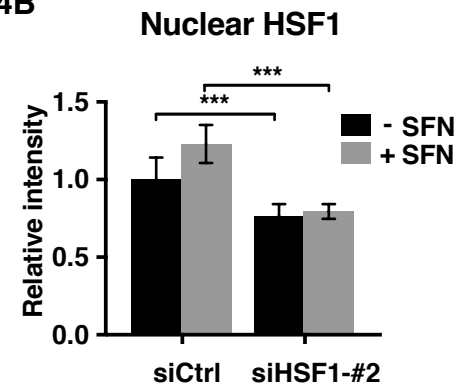


**FIGURE 3. SFN-induced NEAT1 expression is dependent on HSF1.** *A, B*, MCF7 cells were left untreated or treated with SFN (20  $\mu$ M) for 6 h. HSF1 expression in whole cell extracts (WCE) (*A*) and nuclear extracts (NE) (*B*) was determined by immunoblot analyses. Equal loading was verified by re-probing the membranes with actin (*A*) or lamin B (*B*) antibodies. *C*, Cells were treated with SFN as described above, and HSP70 expression was determined by RT-qPCR. *D*, MCF7 cells were transfected with two different siRNAs targeting HSF1, siHSF1\_#1 and siHSF1\_#2, or a control siRNA. Forty-eight hours post-transfection, cells were left untreated or treated with SFN for 6 h. NEAT1 expression was assessed by RT-qPCR. SiRNA-mediated HSF1 depletion was verified by immunoblot analyses. *E*, HeLa cells were transfected with siHSF1\_#2 or control siRNA and after 48 h SFN-induced NEAT1 expression was determined by RT-qPCR. HSF1 expression was determined by immunoblot analyses using actin as loading control. (\* $p \leq 0.05$ , \*\*\* $p \leq 0.001$ ).

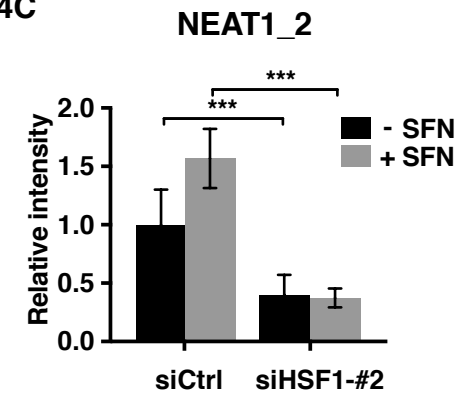
4A



4B

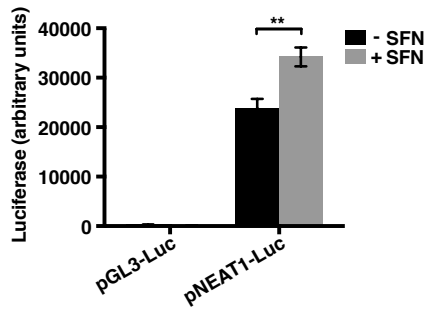


4C

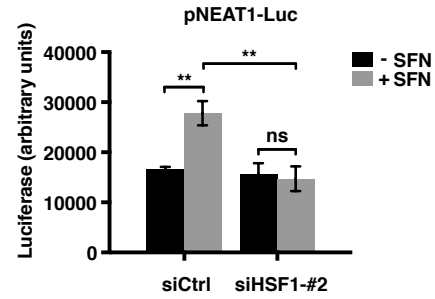


**FIGURE 4. HSF1-depletion abrogates SFN-induced paraspeckle formation.** *A*, MCF7 cells were transfected with an HSF1-specific siRNA or a control siRNA. After 48 h, cells were left untreated or treated with SFN for 6 h, fixed and subjected to co-immuno-FISH analyses by confocal microscopy using an antibody recognizing HSF1 (red) and fluorescent probes binding to NEAT1\_2 (green). Nuclei were visualized with DAPI (blue). All experiments were performed in triplicates. Bars, 10  $\mu$ m. *B*, *C*, The intensities of NEAT1\_2 containing paraspeckles and nuclear HSF1 staining in at least 250 cells were quantitated using Volocity software. (\*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$ ).

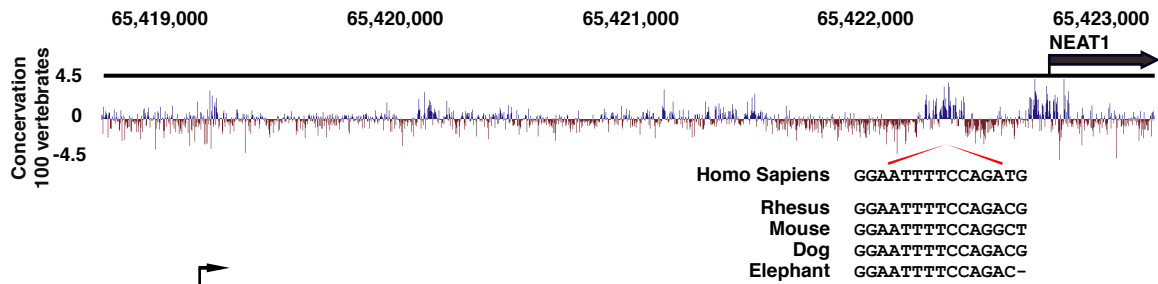
5A



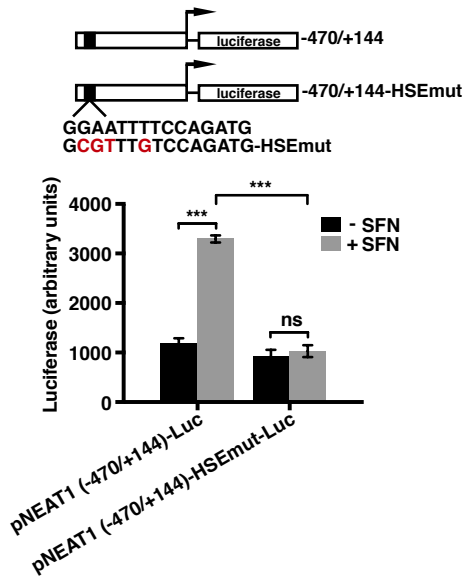
5B



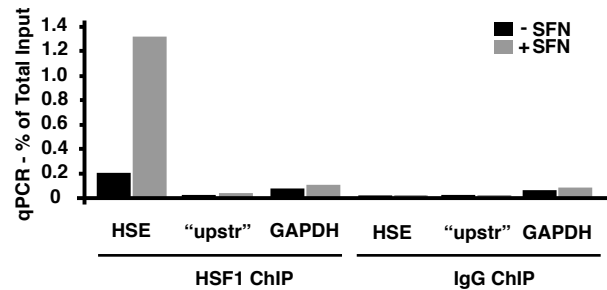
5C



5D

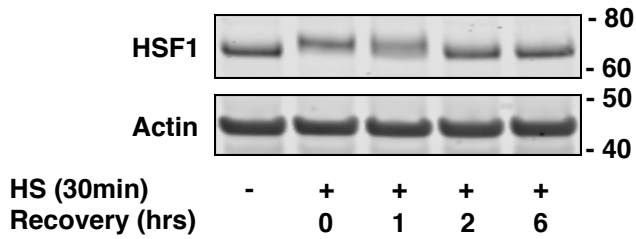


5E

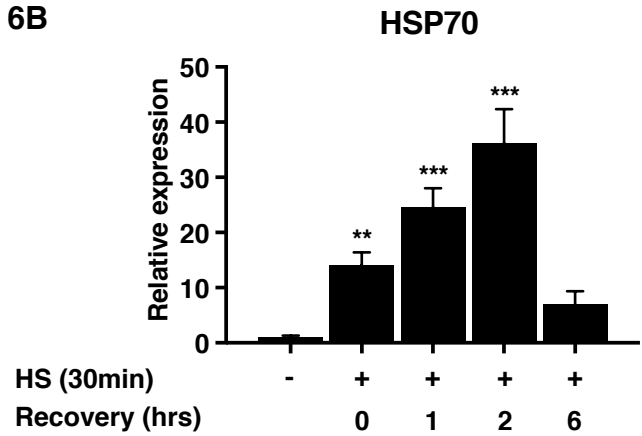


**FIGURE 5. HSF1 binds to and transcriptionally activates the NEAT1 promoter.** *A*, MCF7 cells were transfected with a luciferase reporter vector containing 4040 bp of the NEAT1 upstream region (pNEAT1-luc) or empty control vector. After 24 h, cells were left untreated or treated with SFN (20  $\mu$ M) for 8 h and luciferase assays were performed. The experiments were performed in triplicates and mean values  $\pm$  SD are shown. The result is representative of three independent experiments. *B*, MCF7 cells were co-transfected with pNEAT1-luc and siHSF1\_#2 as described in experimental procedures. Cells were left untreated or treated with SFN for 8 h and luciferase assays were performed. *C*, sequence conservation within NEAT1 upstream regions is illustrated by PhyloP Basewise Conservation scores from 100 vertebrates (USCS Genome Browser). An alignment of conserved HSE core sequences from human, rhesus, mouse, dog, and elephant is shown. *D*, A truncated mutant of the NEAT1 promoter luciferase reporter construct encompassing the putative HSE site was generated and transfected into MCF7 cells along with a version harboring 4 point mutations within the HSE consensus sequence. SFN-induced luciferase activity was measured 24 h post-transfection. *E*, MCF7 cells were left untreated or treated with SFN (20  $\mu$ M) for 6 h and ChIP assays were performed using an anti-HSF1 antibody. RT-qPCR was performed with primers flanking the HSE site. Primers flanking a region further upstream in the NEAT1 promoter ("upstr"), as well as primers amplifying a region of the GAPDH promoter, were used as negative controls. The relative amount of immunoprecipitated DNA compared to input DNA for each primer set is shown for the HSF1 ChIP. The values obtained by the IgG ChIP was less than 0.003% for the HSF1 and control primers. The result is representative of three independent ChIP experiments, where qPCR reactions were done as triplicates. (\*\*\*) $p \leq 0.001$ , (\*\*) $p \leq 0.01$ ).

6A



6B



6C

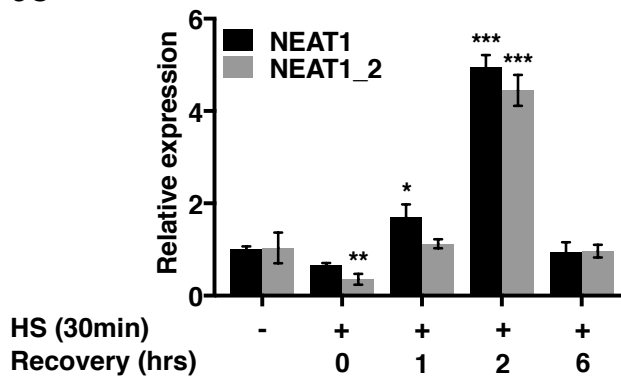
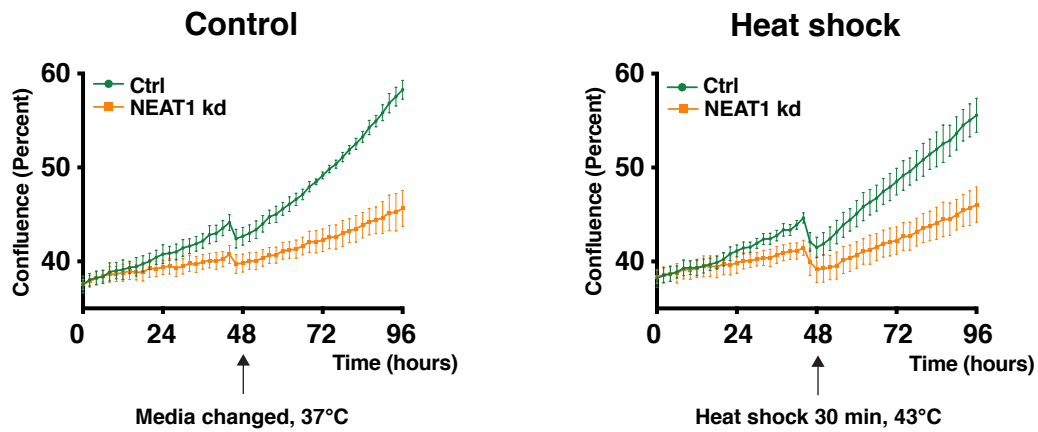


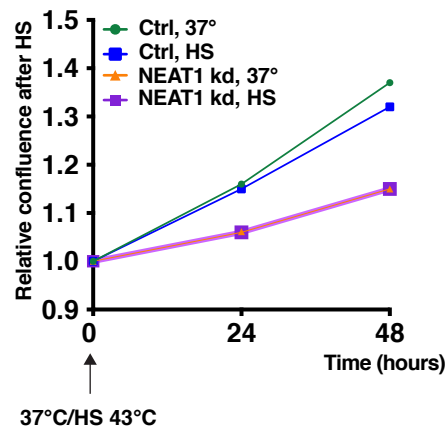
FIGURE 6. **NEAT1 is induced by heat shock.** *A* and *B*, MCF7 cells were subjected to heat shock by incubation at 43° for 30 minutes, and then returned to 37° to recover for the indicated time periods. Activation of HSF1 was verified by shifted migration in western blot analyses (*A*) and by induction of HSP70 mRNA expression (*B*). *C*, Cells were treated as above and expression of NEAT1 and NEAT1\_2 were assessed by RT-qPCR. (\*\*\*) $p \leq 0.001$ , (\*\*) $p \leq 0.01$ , (\*) $p \leq 0.05$ ).



7A



7B



**FIGURE 7. Proliferation is compromised in NEAT1-depleted cells.** *A*, MCF7 cells were transfected with two LNA-gapmeR antisense oligos targeting NEAT1, or a negative control oligo, and immediately placed in a IncuCyte® live cell analysis system for cell confluence monitoring. After 48 h, cells were removed from the incubator, and for half of the cells the media was changed at 37°C, whereas the other half was subjected to heat shock at 43°C for 30 minutes. All the cells were then returned to the IncuCyte® live cell analysis system and monitored for another 48 hours. Confluency (%) was calculated using the IncuCyte® S3 software. Mean values  $\pm$  SD of 15 images (3 images from each well of 5 wells in total) are shown. The results are representative for three independent experiments. *B*, The relative confluency of cells over the last 48 hours of the experiment described in *A*, is shown.

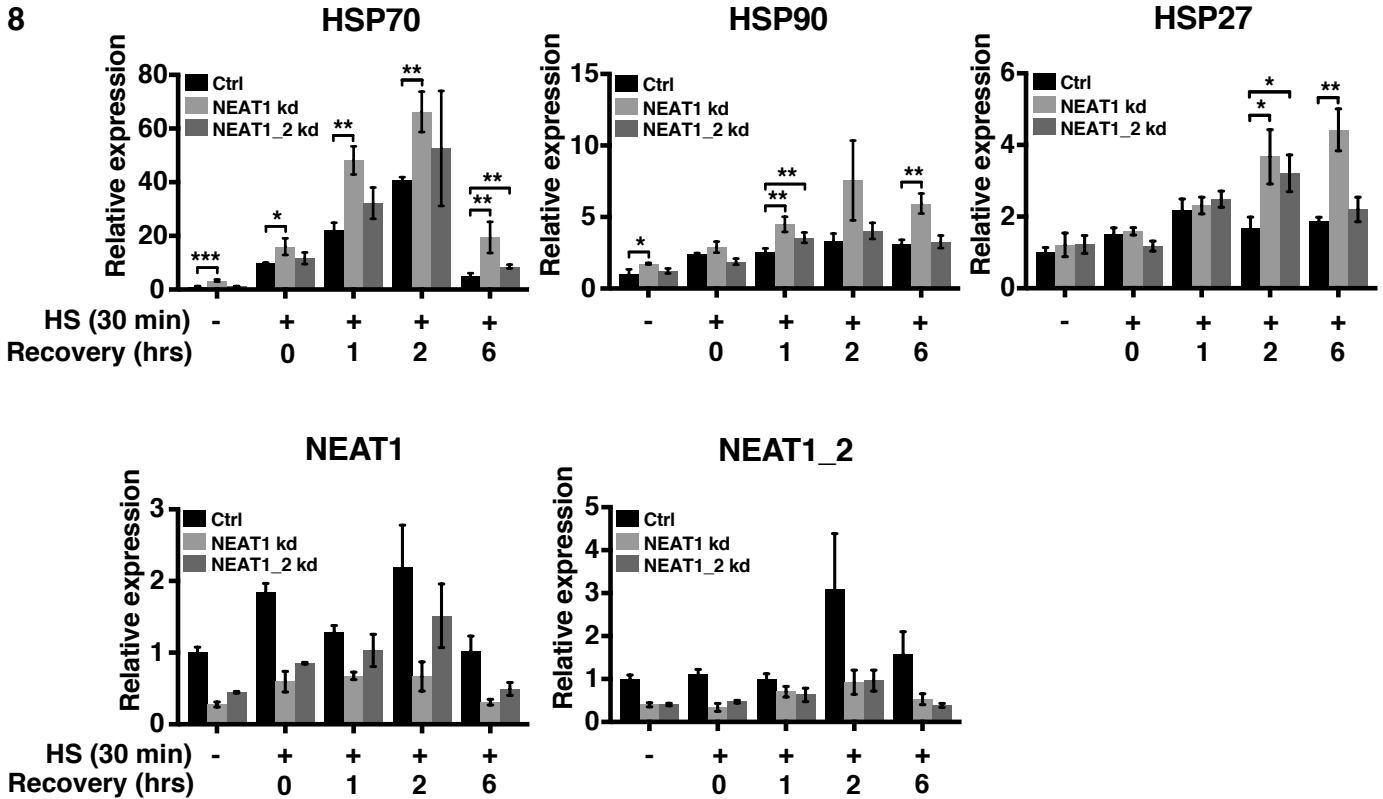


FIGURE 8. NEAT1 knockdown amplifies the expression of HSF1 target genes upon heat shock. MCF7 cells were transfected with two different LNA-gapmeR NEAT1 antisense oligos either targeting both isoforms of NEAT1 or solely the long NEAT1\_2 isoform, and a negative control oligo. After 48 hours, cells were subjected to heat shock and recovered for the indicated time periods. The expression of HSP70, HSP90, and HSP27 was determined by RT-qPCR. Knockdown of NEAT1 and NEAT1\_2 was verified by RT-qPCR. (\*\* $p \leq 0.01$ , \*\* $p \leq 0.01$ , \* $p \leq 0.05$ ).

**The long non-coding RNA NEAT1 and nuclear paraspeckles are upregulated by the transcription factor HSF1 in the heat shock response**

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