

The bromodomain protein BRD4 regulates splicing during heat shock

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ABSTRACT

The cellular response to heat stress is an ancient and evolutionarily highly conserved defence mechanism characterised by the transcriptional up-regulation of cyto-protective genes and a partial inhibition of splicing. These features closely resemble the proteotoxic stress response during tumor development. The bromodomain protein BRD4 has been identified as an integral member of the oxidative stress as well as of the inflammatory response, mainly due to its role in the transcriptional regulation process. In addition, there are also several lines of evidence implicating BRD4 in the splicing process. Using RNA-sequencing we found a significant increase in splicing inhibition, in particular intron retentions (IR), following heat treatment in BRD4-depleted cells. This leads to a decrease of mRNA abundance of the affected transcripts, most likely due to premature termination codons. Subsequent experiments revealed that BRD4 interacts with the heat shock factor 1 (HSF1) such that under heat stress BRD4 is recruited to nuclear stress bodies and non-coding *SatIII* RNA transcripts are up-regulated. These findings implicate BRD4 as an important regulator of splicing during heat stress. Our data which links BRD4 to the stress induced splicing process may provide novel mechanisms of BRD4 inhibitors in regard to anti-cancer therapies.

INTRODUCTION

The response to cellular stress is regulated by complex control mechanisms on transcriptional-, translational-, mRNA-processing-, mRNA-degradation and -export levels, resulting in the inhibition of global protein synthesis and the selective up-regulation of stress response proteins (1,2). One of the best investigated and most ancient stress response mechanisms is the heat shock (HS) response, which shares molecular features with the response to proteotoxic stress. During malignant transformation the heat shock factor 1 (HSF1), the main regulator of the HS response, is activated and the expression of individual heat shock proteins (HSPs) is elevated (3,4). Besides changing gene expression levels, the HS response induces a shift in the exon–intron composition of transcripts (5). The mechanism underlying the heat-induced splicing alterations is still not well understood and may play an important role in adapting the cellular transcriptome under stress conditions. The major alternative splicing events are (i) alternative 5' splice sites (5'SS), (ii) alternative 3' splice sites (3'SS), (iii) mutually exclusive alternative exons, (iv) cassette exon inclusion or skipping and (v) intron retention (IR) (6). IR often results in a premature termination codon (PTC), leading to loss of function through nonsense-mediated decay (NMD) or to a truncated protein with detrimental effects on its function (7–10). Interestingly, this mechanism is frequently observed in tumor-suppressor gene inactivation (11). Extending the knowledge of heat-induced splicing regulation on a global level, Shalgi and colleagues performed a detailed study investigating exon usage, IR and splice site changes under se-

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vere as well as mild HS conditions and observed a frequent retention of introns in post-transcriptionally spliced pre-mRNAs (12). One explanation for the heat-induced splicing inhibition could be the assembly of various splicing factors, including SR (serine ‘S’ and arginine ‘R’ containing)-proteins, hnRNPs, and satellite III RNAs (*SatIII* RNA) in nuclear stress bodies (nSB) (5). This assembly could ensure a functional splicing of vital genes on the expense of the correct processing of non-vital genes (13,14). nSB are unique subnuclear foci that were originally identified as the main site of HSF1 accumulation (15). Besides their role in mRNA splicing, nSB also participate in epigenetic and transcriptional control of gene expression (16).

The epigenetic sensor BRD4 (bromodomain protein 4), an acetylated histone binding protein, has been identified as a regulator of the interferon- and oxidative stress response (17,18). By interacting with a subunit of the pTEFb (positive transcription elongation factor b) complex BRD4 plays a critical role during the transition from abortive to productive elongation of polymerase II (Pol II) (19,20). Besides its well established role in transcriptional elongation, there are several lines of evidence that BRD4 might also be involved in alternative splicing. As such, BRD4 interacts with the JmjC domain-containing protein 6 (JMJD6), which mediates the 5-hydroxylation of U2AF65 (U2 small nuclear RNA auxiliary factor 65) (21,22). Furthermore, a study with lipopolysaccharide (LPS) stimulated macrophages showed that BRD4 is important for the production of mature spliced transcripts of primary response genes (PRG) (23). Further implications of BRD4 in mRNA splicing arise from its homologue in *Saccharomyces cerevisiae* *ddf1* where chromatin immunoprecipitation data revealed a decrease in U1 snRNP recruitment at intron containing genes in the *ddf1* Δ strain (24,25).

Since BRD4 functions in different stress response pathways, we wondered if BRD4 may also participate in the heat shock response, and if so, if it might modulate gene splicing patterns under elevated temperatures. We used RNA-Seq experiments to calculate alternative splicing events under HS, with and without BRD4 knock down (BRD4k.d.). We found BRD4 to prevent cells from heat stress-induced splicing inhibition, represented by an increase of IR under BRD4k.d. Transcripts with IR were concomitantly down-regulated. Furthermore, functional analyses revealed that BRD4 is recruited to nSB by HSF1, enhances *SatIII* RNA transcription and presumably thereby influences heat induced splicing regulation.

MATERIALS AND METHODS

Immunofluorescence and quantitative high-content screening microscopy

Analyzes were performed according to Kaehler *et al.* with slight modifications (26). Additional details can be found in the Supplementary Data.

RNA-Sequencing (RNA-Seq) and quantitative PCR

Library preparations were performed with the TrueSeq RNA Sample Preparation Kit (Illumina) and sequencing was performed on the Illumina HighSeq 2500 with 50 bp

paired end sequencings. Additional details can be found in the Supplementary Data.

Co-Immunoprecipitations and proximity ligation assay (PLA)

Additional experimental procedures can be found in the Supplementary Material and Methods. Complete lists of oligonucleotides used are provided in Supplementary Table S5.

Bioinformatics and statistical analysis

Fastq files were obtained after demultiplexing using Illumina CASAVA v1.8.2 pipeline with default parameters. Reads were mapped against the human genome GRCh37/hg19 using bwa v0.5.9-r16 with default parameters. Read coverages were obtained with coverageBed v2.17.0 using exon-intron coordinates of the Ensembl database v69. In detail: gene expression was calculated as all reads that overlapped exon coordinates; exon-intron junction expression was calculated from uniquely mappable reads that flawlessly mapped with minimum six bases across the junction (‘intron-exon-junction’ method); intron expression was calculated for intron intervals of minimal size 100 bp that did not overlap with any other ENSEMBL database entity (‘intron-only’ method); Percent spliced in (PSI) values were calculated by dividing the RPKM value of a feature by the RPKM value of its associated gene. Delta PSI values were obtained analogous to log₂ ratios by dividing PSI values in log space. Global changes of alternative splicing events (delta PSI values) by treatment were determined with the Mann-Whitneys rank sum test. Further information on the bioinformatics data analysis can be found in the supplementary data.

RESULTS

Intron retention is significantly increased under BRD4 depletion and heat shock

BRD4 plays an important role in many cellular processes, such as DNA repair, proliferation and transcriptional regulation and participates in several stress related pathways, such as the oxidative stress response (18,27). Besides the oxidative stress response, the heat shock response is one of the most important inducible systems to protect cellular homeostasis in eukaryotes. We therefore wondered whether BRD4 may also play a role in the regulation of transcription during HS. We exposed BRD4 knock down (k.d.) and control WI38 cells to mild HS at 42°C for 4 h and analysed the transcriptomes of three independent experiments. We investigated the main effects of HS and BRD4k.d. as well as their interaction using the edgeR framework (28,29). We used HS conditions with a robust HS answer as indicated by the induction of HSP70 (Supplementary Figure S1A). Furthermore, in all replicates the expression of BRD4 was efficiently diminished down to 16–40% and major HSPs were sufficiently and comparably expressed (Figure 1A, Supplementary Figure S1B–E). BRD4 depletion has neither a significant influence on the major stress response regulation, represented by an equal up-regulation of

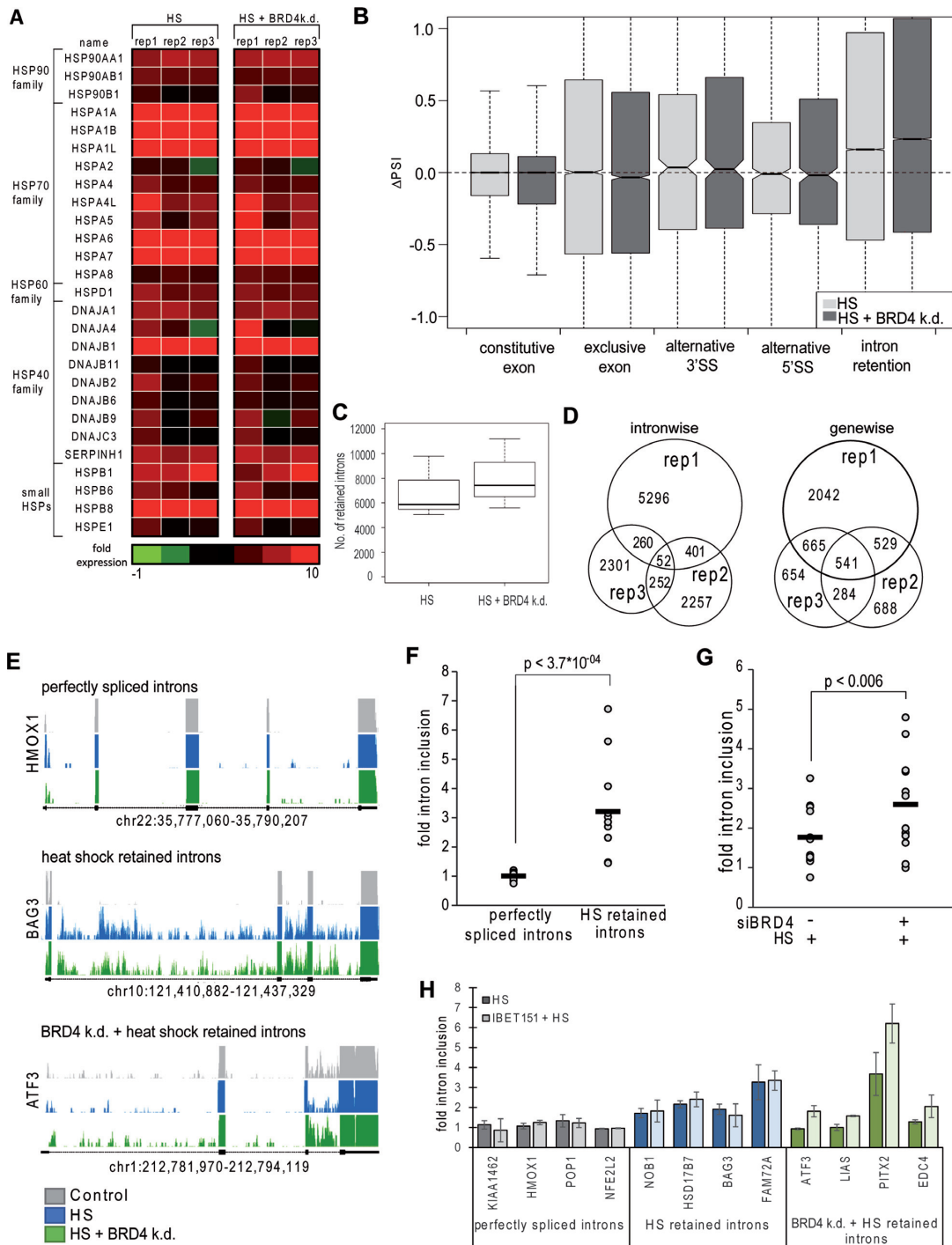


Figure 1. BRD4 knock down increases heat shock-mediated intron retention. (A) Gene expression analysis of the RNA transcriptome data of the main heat shock proteins (HSPs) for each replicate in HS cells (left) as well as in HS + BRD4k.d. cells (right) treated at 42°C for 4 h. (B) Bioinformatic alternative splicing analysis from RNA transcriptome data of HS (42°C for 4 h) treated cells (HS) and HS treated cells with BRD4k.d. (HS + BRD4k.d.) compared to untreated control cells. Inclusion and exclusion of constitutive and exclusive exons and introns as well as alternative 3' and 5' splice sites were calculated using the 'percent spliced in' (PSI) index corresponding to Wang et al. (C) Number of introns with an IR of more than 2-fold compared to untreated cells. (D) Venn diagram depicting the overlap between the identified intron retentions in three HS + BRD4k.d. replicates (intron-wise) and of the corresponding genes with retained introns (gene-wise). (E) Visualization of perfectly spliced transcripts (*HMOX1*), transcripts with increased IR following HS (*BAG3*) and transcripts with an increased IR in HS + BRD4k.d. cells (*ATF3*). Exons are depicted as black squares, introns by a black line. (F–H) qPCR validation of intron inclusion with PCR products that span the exon/intron junction and as control the corresponding exon/exon junction of the same transcript. The values of the exon/intron PCR-product were set into relation to the expression values generated by the exon/exon primers. (P-values were calculated by a two-tailed, paired *t*-tests; *n* = 4). Genes were selected from the RNA-Seq experiments. (F) Validation of HS retained introns (*BAG3*-group in E) compared to perfectly spliced introns (*HMOX1*-group in E) in HS samples. (G) Analysis of introns that were retained in HS+BRD4k.d. cells and spliced in HS treated cells (*ATF3*-group in E). (H) Analysis of IR using qPCR in WI38 cells treated with 1 μM IBET151 for 72 h and exposed to HS at 42°C for 4 h. IR was analyzed in at least three independent experiments.

HSPs, nor on the global gene expression profile after HS (Supplementary Figure S1G).

However, besides transcriptional regulation, pre-mRNA splicing is another important mechanism in the adaptation to and survival of stress. Since it is known that BRD4 participates in the production of mature spliced transcripts after LPS stimulation (23), we analysed the role of BRD4 in the heat-induced splicing process using the RNA-Seq derived transcriptome data. We first ruled out that technical factors, such as genomic DNA contamination, interfered with our analyses and counted the aligned reads on exons, introns as well as on intergenic regions in all experiments and found a decreased averaged read density on intergenic regions (0.04 RPKM) compared to the averaged read density found on introns (0.08 RPKM) or on exons (8.0 RPKM) (Supplementary Figure S1H, I and Supplementary Tables S2 and S3). Subsequently, to calculate differential splicing alterations we used the 'percent spliced in' (PSI) value according to Wang *et al.* (30). First, we analysed the global changes of several alternative splicing events. We calculated the individual Δ PSI values for the HS-treated samples (PSI (HS) versus PSI (control)) and the HS + BRD4k.d. samples (PSI (HS + BRD4 k.d.) versus PSI (control)). HS alone resulted in a significant increase in IR ($P = 7.71 \times 10^{-265}$) as already shown by Shalgi *et al.* 2014. Interestingly, a simultaneous BRD4k.d. in HS-treated cells further enhanced the observed effect of IR, but hardly altered other splicing events (Figure 1B). The majority of introns with IR after HS treatment and BRD4k.d. are so far not annotated in the ENSEMBL database as common IR introns (Supplementary Figure S1J). In HS samples we detected on median a number of 5879 retained introns with an Δ PSI value greater than 2-fold that increased in HS + BRD4k.d. samples up to 7,421 (Figure 1C). To extract IR events that are affected by BRD4 depletion under HS conditions, we computed the ratio ($\Delta\Delta$ PSI) between the intron expression value Δ PSI (HS) and Δ PSI (HS + BRD4k.d.) for each intron. A $\Delta\Delta$ PSI > 1.5 in at least two experiments was observed for 965 introns, accounting for 824 genes, indicating that in most instances only one intron of a transcript is affected (Figure 1D and Supplementary Table S1). Since an increase in intron retention upon heat stress was observed to be a gene-level rather than an intron-level phenomenon (12), we asked if this effect is also present upon BRD4 depletion. Therefore, we performed an intersection analysis on a gene-level with genes that present with at least one intron retained in BRD4k.d. + HS independently of the position and identified 541 genes affected in all three replicates, indicating that the influence of BRD4 on the splicing process under heat shock is rather a gene-wise phenomenon than an intron-wise regulation and that BRD4 is required for the correct splicing regulation of a subset of genes. Interestingly, depletion of BRD4 alone, without HS, did not show significant changes in splicing events (Supplementary Figure S1K).

To validate our bioinformatic data analyses, quantitative PCR experiments were performed in four additional biological replicates with HS and HS+BRD4k.d. (Supplementary Figure S1F). The investigated genes were classified into three groups according to their IR pattern (Figure 1E): (i) genes that are perfectly spliced under all conditions (e.g. *HMOX1*), (ii) genes exhibiting IR under HS, irrespectively

of their expression pattern in HS + BRD4k.d. (e.g. *BAG3*) and (iii) genes with introns that showed an even enhanced IR in BRD4k.d. cells following HS compared to HS alone (e.g. *ATF3*). qPCRs were performed with oligonucleotides spanning over the exon/intron junction and, as control, oligonucleotides spanning over the exon/exon junction. Retained introns under HS conditions alone showed, comparable to the sequencing data, a significant splicing inhibition ($P < 3.7 \times 10^{-04}$) with a median increase of IR of more than 3-fold (Figure 1F). For the third group 12 introns were selected that showed in at least two sequencing replicates a significant increase of intron inclusion under HS + BRD4k.d. compared to HS treatment alone. The mean IR in BRD4-depleted and HS treated cells (HS + BRD4k.d.) displayed a significant increase of 50% ($P < 0.006$) compared to the heat treated and mock transfected cells (HS) (Figure 1G).

To further validate the increased IR after HS + BRD4k.d. we took advantage of the bromodomain inhibitor, IBET151 (31). Indeed, treatment with IBET151 significantly increased, similar to BRD4k.d., the HS mediated IR of introns that had been already identified as introns affected by HS + BRD4k.d. ($P < 0.0001$, Figure 1H, Supplementary Figure S1L). Notably, IBET151 treatment did not influence the splicing modality of perfectly spliced genes nor of genes with IR in solely HS treated samples.

IR negatively correlates with mRNA abundance of affected transcripts

Retention of introns often results in premature termination codons (PTC), which lead to loss of protein function through either the generation of truncated proteins or nonsense-mediated mRNA decay (7,8,10). As such, Jung *et al.* recently showed that in cancer 97% of SNVs at splice sites, which create IR, cause a PTC, and that these transcripts are frequently delivered to NMD pathways (11). We therefore asked whether the observed enhanced IR results in a decrease of functional mRNA of the affected transcripts. For this, we integrated the intron expression changes of a transcript into a single value, the integrated intron ratio (IIR). In a similar way we further assumed that (i) a strong increase of IR will override a small increase and (ii) enhanced splicing (ES) will only matter if no IR is present. We omitted transcripts without IR or ES from the correlation analysis. Figure 2A summarizes our integrative bioinformatics strategy. For all experiments we found a significant (all $P < 1.02 \times 10^{-6}$) negative Pearson correlation between the IIR and the expression changes of the corresponding genes ranging from -0.24 to -0.76 depending on the chosen ratio cut-off value to detect IR (Supplementary Table S4). Our results showed further that not only IR had an effect on the gene expression levels but ES did so as well. We found a significant (all $P < 2 \times 10^{-16}$) enrichment of genes (a) with IR which were down-regulated as well as (b) with ES which were up-regulated. We found a strong negative Pearson correlation of -0.24 ($P < 3.38 \times 10^{-102}$) between IIR and transcript expression without using any fold-change cutoffs (Figure 2B). This finding holds true across a range of different cutoff values to detect IR or ES, with Fisher-test odds ratios increasing exponentially with increasing stringency as well as for different experimental

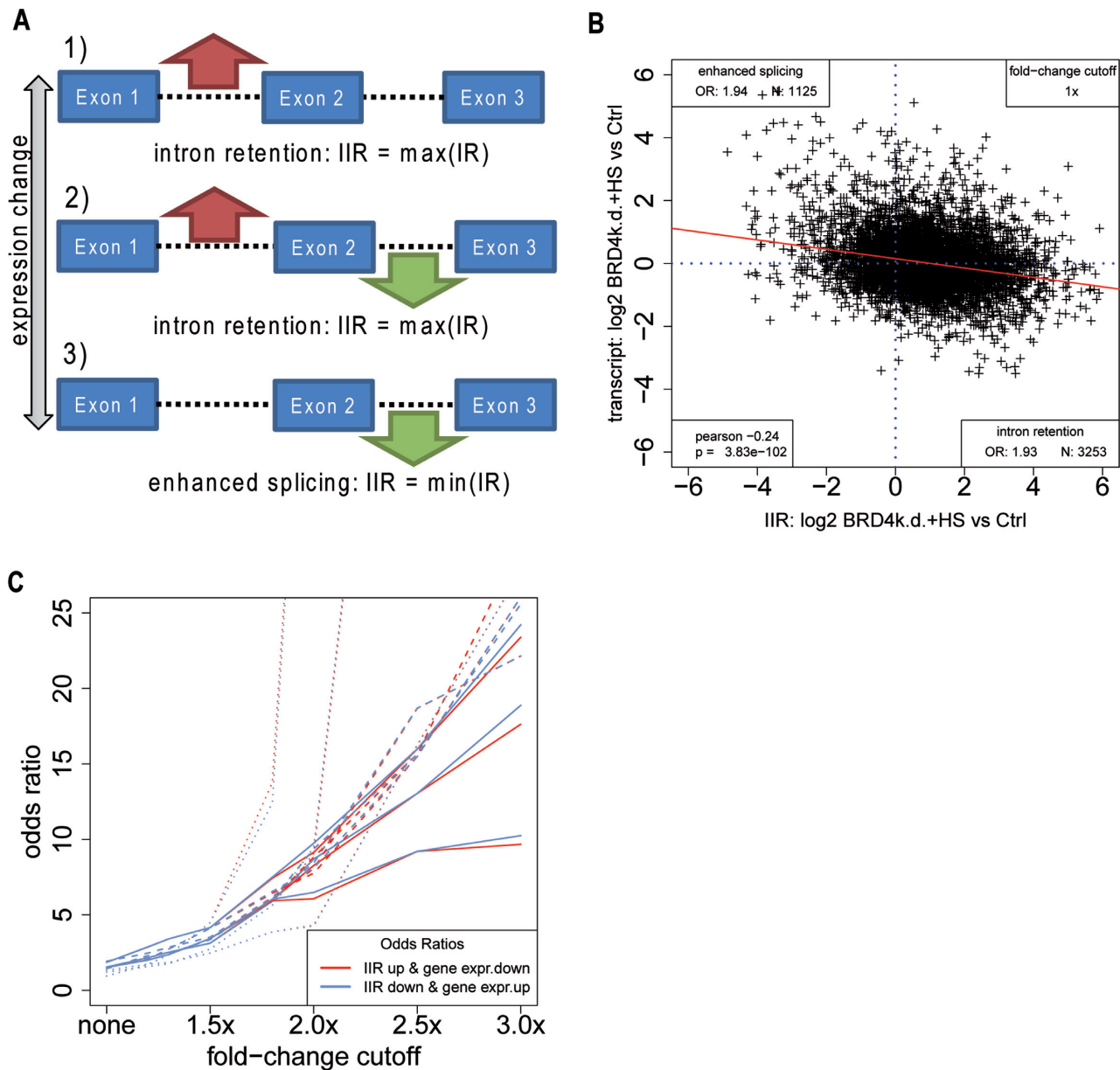


Figure 2. Increased IR following HS+BRD4k.d. negatively correlates with gene expression. **(A)** Strategy to integrate intron expression changes into a single value per transcript: If IR is present, the most extreme IR value represents the transcript (1) even if ES is present (2). The strongest ES represents the transcript if no IR is present (3). Transcripts without IR or ES are omitted from the analysis. **(B)** Application of our strategy described in **(A)** to compare HS+BRD4k.d. induced integrated intron expression ratios (IIR) to the coding transcript expression changes. The red line depicts a linear trend to visualize the Pearson correlation. Odds ratios (OR) were calculated by Fisher's exact test for the upper left and lower right quadrant. Numbers of the affected transcripts with IR or ES and corresponding lower/higher expression were counted (N). **(C)** Enrichment of genes with a transcriptional impact of IR or ES for different detection cut-offs. Depicted are the odds ratios of nine experiments (3× HS versus Ctrl., 3× HS+BRD4k.d. versus Ctrl., 3× HS+BRD4k.d. versus HS). Dashed lines depict BRD4HS versus Ctrl, dotted lines represent BRD4HS versus HS and continuous lines show HS versus Ctrl.

comparisons such as HS versus control, HS+BRD4k.d. versus control and HS+BRD4k.d. versus HS (Figure 2C, Supplementary Figure S2, Table S4). In conclusion, our data suggests that HS leads to increased IR, which in turn is correlated to a decreased gene expression level of the affected genes, and that additional BRD4k.d. enhances this effect. Interestingly, pathway analyses of the identified genes with IR revealed cancer-relevant pathways, such as molecular mechanisms of cancer ($P = 5.46 \times 10^{-3}$), ERK/MAPK

signaling ($P < 9.79 \times 10^{-3}$), FAK Signaling ($P < 2.56 \times 10^{-2}$) and endoplasmic reticulum stress pathway ($P < 3.1 \times 10^{-2}$) (Table 1) as the most significantly affected signaling cascades. Moreover, we found ~15% of the IR events to affect tumor suppressors, oncogenes and cancer hallmark genes.

Table 1. Pathway analysis of the 964 genes with increased IR in BRD4-depleted cells following HS

Ingenuity canonical pathways	<i>P</i> -value ^a	Ratio ^b	<i>Z</i> -score ^c
Sonic Hedgehog Signaling	0.003	0.17	NaN
TNFR1 Signaling	0.005	0.12	2.449
Molecular Mechanisms of Cancer	0.005	0.06	NaN
ERK/MAPK Signaling	0.010	0.07	2.496
Protein Kinase A Signaling	0.019	0.05	NaN
FAK Signaling	0.026	0.08	NaN
Adipogenesis pathway	0.026	0.07	NaN
Nucleotide Excision Repair Pathway	0.028	0.11	NaN
Endoplasmic Reticulum Stress Pathway	0.031	0.14	NaN

^aThe *P*-value was calculated using the right-tailed Fisher's Exact test.

^bNumber of molecules in a given pathway that passed the cut-off criteria, divided by the total number of molecules encompassed in that pathway.

^cActivation score of the given canonical pathway.

Data were analyzed with the Ingenuity[®] Pathway Analysis Software (IPA[®], QIAGEN Redwood City, www.qiagen.com/ingenuity). The most significant canonical pathways are listed according to their *P*-value. The ratio gives the number of genes identified in our analysis and assigned to a pathway in relation to the total number of genes in that particular pathway.

BRD4 is recruited to nuclear stress bodies following heat stress in an HSF1 dependent manner

We next wondered what the cause for the increased IR might be. We performed gene expression and Western blot analyses for selective splicing factors, but did not find evidence for an effect of BRD4k.d. on these factors (Supplementary Figure S3). Previous work has determined that treatment of cells with the CDK9 inhibitor flavopiridol results in an increased co-localization of the active BRD4/P-TEFb complex with SRSF2 within nuclear splicing speckles (32). Therefore, we performed additional immunofluorescence experiments of SRSF2 and BRD4 in HeLa cells treated with flavopiridol in presence and absence of HS and observed no change in the localisation of SRSF2 and BRD4 upon treatment (data not shown). Upon thermal stress, a subset of hnRNP and various other splicing factors, including SAFB, KHDRBS1, SRSF1, RSF7 and SRSF9 are recruited to specific nuclear sites which are known as nuclear stress bodies (nSB) (14,33–37). nSB are marked by the main regulators of HS, heat shock factors HSF1 and HSF2, and are only present under certain stress conditions (38). Since we did not detect splicing alterations in BRD4k.d. cells in the absence of stress and the expression level of BRD4 was unchanged upon heat induction, we wondered whether the distribution and localization of BRD4 might be influenced by alterations in the stress induced nuclear organization. Indeed, immunofluorescence experiments under mild (42°C) as well as severe (44°C) HS conditions showed a clear localization of BRD4 in specific nuclear foci. Co-staining of BRD4 and HSF1 depicted a co-localization of these proteins under both HS conditions, with an increase after exposure to severe HS (Figure 3A). Similar results were obtained in HeLa cells by overexpressing pTL-FLAG-BRD4 and pTL-HA-HSF1 or pTL-HA-HSF2, respectively (Supplementary Figure S4A).

To investigate the interplay of BRD4 and HSF1 in nSB we performed co-immunoprecipitation experiments. We transiently overexpressed HSF1 and heat treated the cells (42°C for 4 h) or incubated them for 4 h at 37°C as control. The precipitation was performed with an antibody against endogenous BRD4 protein and revealed a specific pulldown of the overexpressed HSF1 (Figure 3B).

The interaction is present in HS as well as in non-HS conditions. This indicates that the interaction between BRD4 and HSF1 is already present independent of heat. However, the localization of BRD4 and HSF1 to nSBs is only detected upon heat treatment (Figure 3A). Furthermore, we confirmed the interaction between BRD4 and HSF1 by a reciprocal immunoprecipitation with an overexpressed BRD4 and precipitation with an antibody targeting endogenous HSF1 (Supplementary Figure S4B). To specify the localization of this interaction we used proximity ligation assays (PLA) to study the protein–protein association simultaneously with the cellular localization of the proteins (Figure 3C). HeLa cells were subjected to 44°C for 1 h and the protein interaction was analyzed using antibodies directed against HSF1 and BRD4. Simultaneously, HSF1, as marker for nSB, was counter-stained using an additional antibody. The assay revealed a close proximity of BRD4 and HSF1 that co-localized with the immunostaining of HSF1. Additionally, due to the high specificity and sensitivity of the assay, which permits single molecule resolution, we also observed signals outside of nSB that might not be detectable by the less sensitive immunostaining.

Next, we asked if this interaction is required for the recruitment of BRD4 and/or HSF1 to nSB and subjected BRD4k.d., HSF1k.d. and control HeLa cells for 1 h to 44°C HS to analyze the localization of BRD4 and HSF1. Interestingly, a diminished BRD4 expression did not influence the localization of HSF1, indicating that BRD4 is not necessary for HSF1-dependent nSB formation (Figure 4A). In contrast, a reduced HSF1 expression abolished the recruitment of BRD4 to specific nuclear foci, indicating an essential role of HSF1 for the translocation of BRD4 to nSB.

Recruitment of BRD4 to nSB is mediated by its bromodomains

nSB are specifically enriched in acetylated histones (H4K8ac and H4K16ac), which are markers of active gene transcription, as well as several transcriptional regulators such as CREBBP (CREB-binding protein) and RNA Polymerase II (Pol II) (34,39). BRD4, as a member of the BET family, contains two bromodomains that allow BRD4 to bind to acetylated histones, with

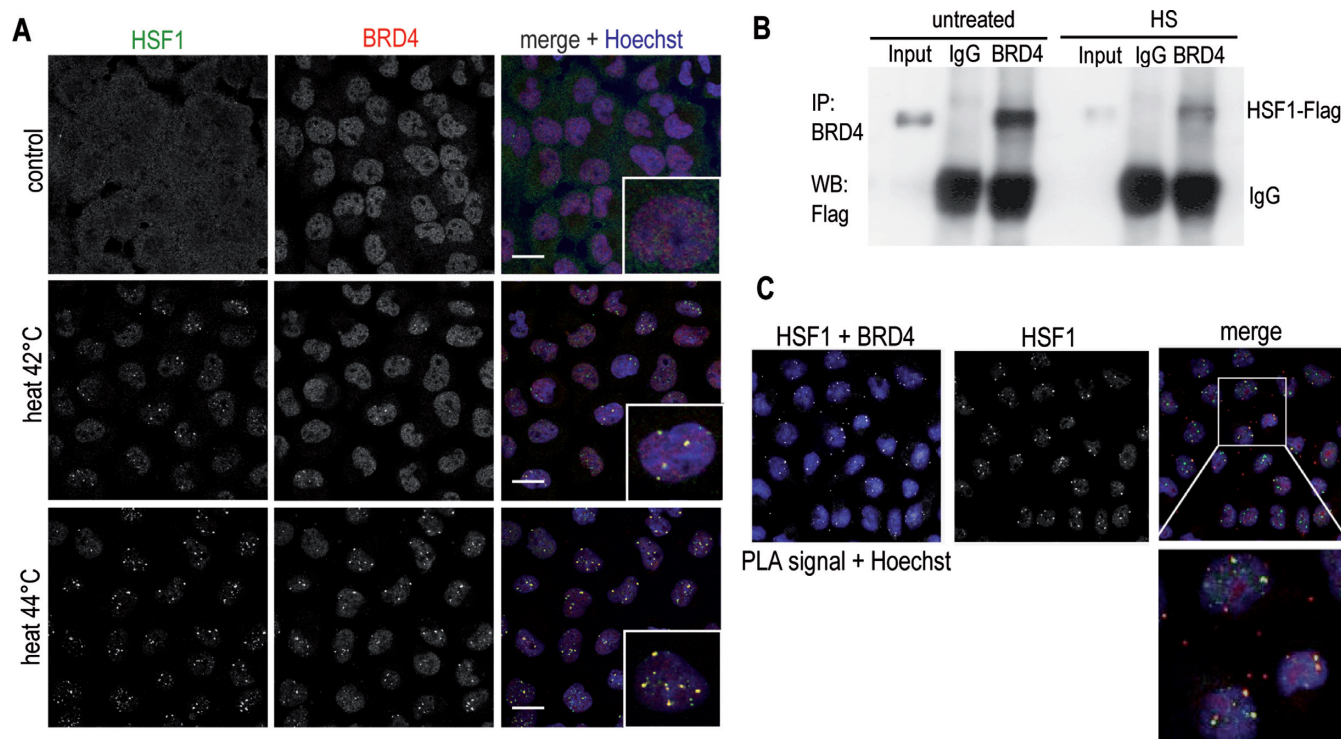


Figure 3. BRD4 is recruited to nuclear stress bodies following HS in an HSF1 dependent manner. (A) HeLa cells were exposed to HS at 42 or 44°C for 1 h or left untreated (control) and processed for confocal microscopy to analyze the localization of BRD4 and HSF1. Nuclei were stained with Hoechst. Scale bars represent 20 μm . (B) WI38 cells were transfected with pTL-FLAG-HSF1 and either exposed to 42°C for 4 h (HS) or incubated at 37°C (untreated). Co-IPs were performed using an antibody against the endogenous BRD4 protein and analyzed on Western Blot using an antibody against the FLAG-tag to detect HSF1. (C) Proximity-Ligation Assay (PLA) of the BRD4/HSF1 interaction. PLA was performed with anti-BRD4 (rabbit) and anti-HSF1 (mouse) antibodies. Interactions are visualized by fluorescent dots and co-stained with an anti-HSF1 (rabbit) antibody. The overlay of the PLA signal for a BRD4-HSF1 interaction (red) and the co-staining signal of HSF1 (green), revealed an interaction of BRD4 with HSF1 in a subset of HSF1 positive nuclear stress bodies.

preference for acetylated lysine 5 and 12 on histone H4 (H4K5 and H4K12) (23,40). The binding to acetylated peptides can be abolished by BET inhibitors (IBET151, JQ1) that reversibly block the bromodomains and thereby cause a displacement of BRD4 from chromatin. Next, we asked whether the capability of BRD4 to bind acetylated chromatin is required for the localization of BRD4 to nSB. Treatment with bromodomain inhibitors reduced the formation of BRD4-containing nSB, but had no influence on the formation of the nSBs themselves, at 42°C as well as at 44°C (Figure 4B, Supplementary Figure S4C). Using an automated microscopy approach based on a Cellomics ArrayScan VTI high-content screening platform, we measured the number of HSF1 and BRD4 foci in IBET151 as well as JQ1 treated and control cells upon HS. Here, we used severe HS as we found the strongest induction of nSBs under these conditions. The analysis confirmed a significant decrease of BRD4 positive foci in IBET151 or JQ1 treated cells after HS compared to the DMSO control, whereas the number of HSF1 foci remained unaffected (Figure 4C, Supplementary Figure S4D). In particular the number of cells without any BRD4 positive foci increased with IBET151 treatment.

BRD4 regulates the expression of *SatIII* RNA in an HSF1-dependent manner

The localisation of HSF1 to nSB is responsible for the activation of the transcription of satellite III repeats into stable non-coding RNAs (*SatIII*) that are supposed to play a role in the formation of nSB (34,39). In another context, a stimulating function of BRD4 on the transcription of noncoding enhancer RNAs (*eRNAs*) as well as on the noncoding RNA *HOTAIR* has been recently described (41,42). This prompted us to ask if BRD4 is involved in the regulation of *SatIII* RNA transcription in nSB.

For this, total RNA from untreated cells, HS treated cells, HS+BRD4k.d. and HS+HSF1k.d. cells was isolated and the induction of the non-coding *SatIII* transcripts upon mild HS (42°C, 4h) was analysed using *SatIII* RNA specific oligonucleotides (38). As expected, a reduced expression of HSF1 diminished the heat-induced upregulation of the *SatIII* RNA transcripts up to 4-fold (Figure 5A). A BRD4k.d. reduced the upregulation of *SatIII* RNA under HS as well, albeit to a lesser extent (Figure 5A and Supplementary Figure S5A). This was confirmed by inhibition of BRD4 using IBET151 (Figure 5B). The specificity of these results was further substantiated when we found *HSP70* mRNA, another target of HSF1 not to be affected by BRD4 depletion or inhibition (Supplementary Figure S5A).

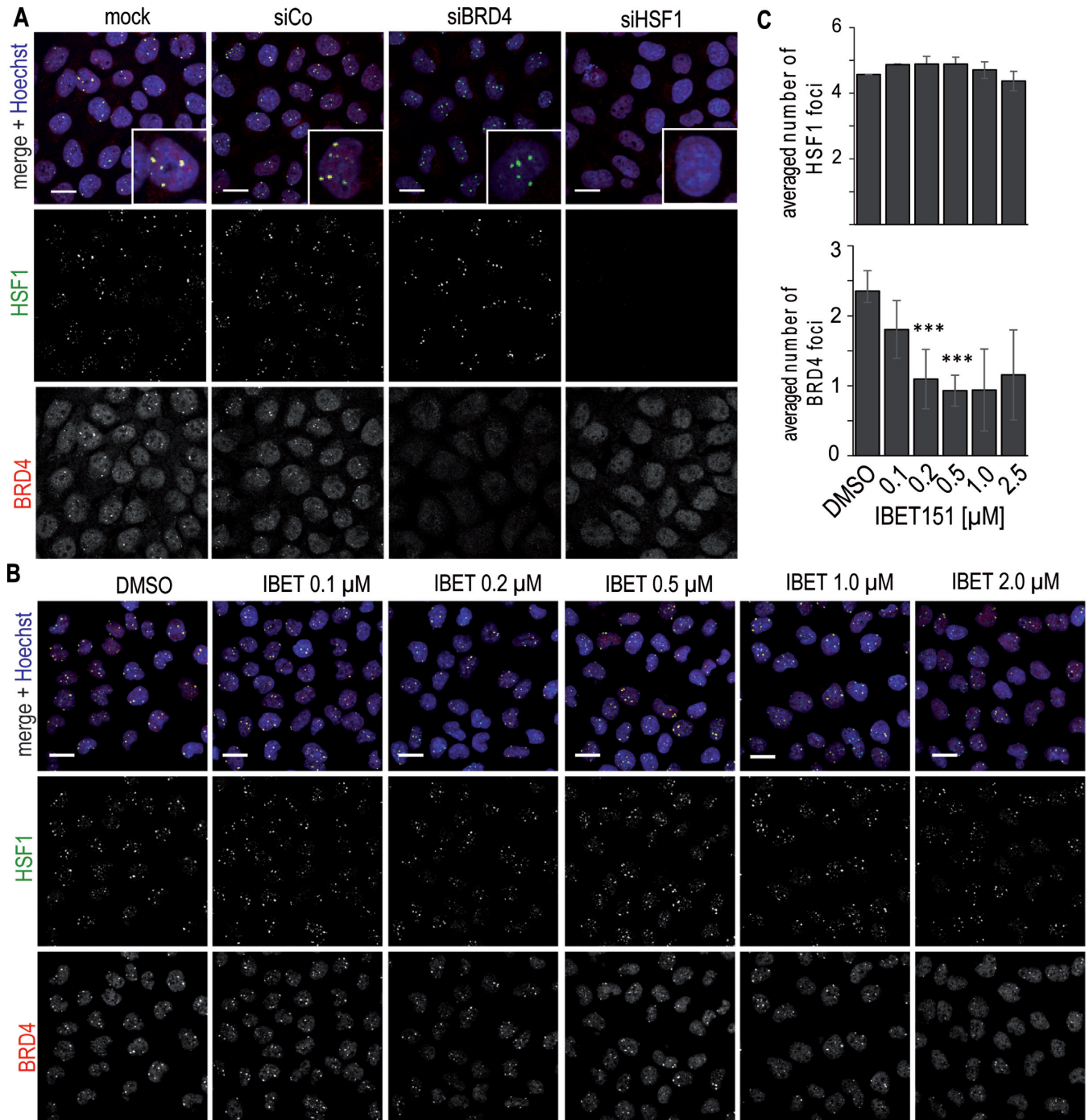


Figure 4. (A) HSF1 is required for the recruitment of BRD4 to nSB. HeLa cells were transfected with siRNAs against BRD4 (siBRD4) or HSF1 (siHSF1) transcripts, or non-targeting control siRNA (siCo). 72 h post transfection cells were exposed to HS at 44°C, fixed and processed for immunostaining of BRD4 and HSF1. Nuclei were stained with Hoechst. Scale bars represent 20 μm. (B) BRD4 inhibitors impair the localization of BRD4 to nSB. HeLa cells were incubated with the indicated concentrations of the BET inhibitor IBET151 for 24 h. Subsequently, cells were exposed to HS at 44°C for 1 h, fixed and stained with antibodies directed against BRD4 and HSF1. Nuclei were stained with Hoechst. Scale bars represent 20 μm. (C) Quantitative high-content screening microscopy of HeLa cells exposed to heat stress at 44°C for 1 h with prior treatment of IBET151 for 24 h with the indicated concentrations. Cells were fixed and stained with antibodies directed against BRD4 and HSF1. Nuclei were stained with Hoechst. Number of HSF1 foci as well as of BRD4 foci were measured using a Cellomics ArrayScan VTI high-content screening platform (***) *P*-values < 0.01, unpaired two-tailed *t*-test).

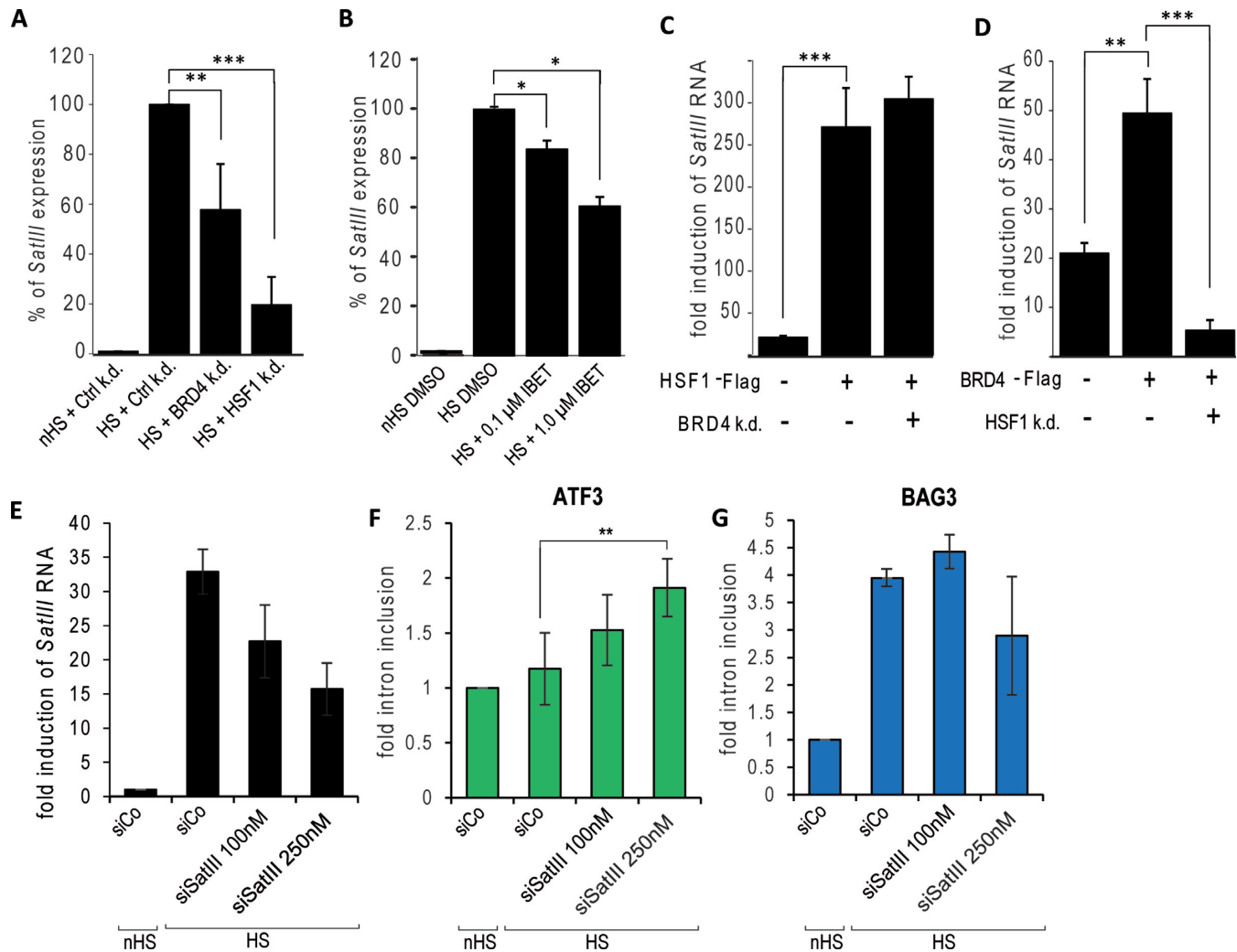


Figure 5. BRD4 induces *SatIII* RNA expression in an HSF1 dependent manner. (A–D) Expression analysis of the non-coding *SatIII* RNA. (A) WI38 cells were transfected with siBRD4 (BRD4k.d.), siHSF1 (HSF1k.d.) or a non-targeting control siRNA and 72 h after transfection. Cells were subjected to either HS at 42°C for 4 h (HS) or incubated at 37°C (nHS). *SatIII* transcripts were reverse transcribed and analyzed by qPCR as previously described (Valgardsdottir *et al.* 2008). Additionally, the same amount of RNA was reverse transcribed with random hexamer oligonucleotides and amplified with primers specific for TUBB as control. *SatIII* RNA expression was normalized to TUBB and normalized to nHS conditions. HS + Ctrl k.d. were set to 100%. (B) WI38 cells were treated with 0.1 and 1 μM IBET151/DMSO for 72 h and subsequently exposed to HS (4 h at 42°C). As controls, cells were incubated at 37°C (nHS DMSO) or exposed to HS (4 h at 42°C) in the presence of DMSO (HS DMSO). Total RNA was prepared from the cells and *SatIII* transcripts were reverse transcribed, amplified and analyzed as described above. (C) Expression analysis of *SatIII* RNA cells in HSF1 overexpressing and BRD4-depleted cells. WI38 cells were transfected with siBRD4 (BRD4k.d.) or a non-targeting control siRNA and 24 h posttransfection cells were transfected with pTL-FLAG-HSF1. After an additional 48 h cells were subjected to HS at 42°C for 4 h. Subsequently, *SatIII* transcripts were reverse transcribed, amplified and analyzed as described above. (D) WI38 cells were transfected with siHSF1 (HSF1k.d.) or a non-targeting control siRNA. 24 h post-transfection, cells were transfected with pTL-FLAG-BRD4. After an additional 48 h cells were subjected to HS at 42°C for 4 h. *SatIII* transcripts were reverse transcribed, amplified and normalized as described above. (E–G) *SatIII* RNA k.d. increases IR of BRD4 regulated genes under HS. (E) Knock down efficiency of *SatIII* RNA. WI38 cells were transfected with different concentrations of *SatIII*-siRNA (siSatIII), as indicated, and as control, with a non-targeting siRNA (siCo). 24 h post-transfection, cells were subjected to HS at 42°C for 4 h (siCo HS and siSatIII) or incubated at 37°C (siCo nHS). Subsequently, total RNA was isolated and the expression of *SatIII* RNA was determined as described in (A). (F) Intron retention analysis of *ATF3* in *SatIII*-depleted cells. Fold IR of *ATF3* was calculated using oligonucleotides that detect the exon/intron junction and as control the corresponding exon/exon junction of the same transcript. The expression values generated by the exon/intron oligonucleotides were set into relation to the expression values generated by the exon/exon oligonucleotides. (G) Intron retention analysis of *BAG3* in *SatIII*-depleted cells of BRD4. Fold IR of *BAG3* was calculated as described in (F) (**P*-values = 0.05, ***P*-values < 0.05, ****P*-values < 0.01, unpaired two-tailed *t*-tests)

To further elucidate the interplay of BRD4 and HSF1 in regard to the transcriptional regulation of *SatIII* RNA, we analyzed the expression of *SatIII* RNA after simultaneous overexpression of HSF1 and knock down of BRD4 and vice versa. Both, HSF1 and BRD4 overexpression alone resulted in a significant activation of *SatIII* RNA transcription. An abolished BRD4 expression did not influence HSF1-overexpression mediated enhanced induction of *SatIII* RNA (Figure 5C, Supplementary Figure S5B). In contrast, simultaneous HSF1 knock down and BRD4 overexpression resulted in a diminished *SatIII* RNA expression (Figure 5D, Supplementary Figure S5C). These data suggest an HSF1-dependent transcriptional role of BRD4 in nSB.

SatIII transcripts are also known to be responsible for the recruitment of several splicing factors to nSB and are thought to play a role in the altered splicing pattern during cellular stress (33,43). Thus, we asked whether the effect of BRD4 on the splicing machinery during HS might be mediated by *SatIII* RNA. We used custom designed siRNAs against *SatIII* to downregulate *SatIII* RNA. We then performed qPCR experiments on siRNA control and siRNA-*SatIII* cells to determine the amount of retained introns in a subset of previously validated genes. We used *ATF3* as well as *EP300* and *SOD2* as prototypes of genes with increased IR after HS+BRD4k.d., and *BAG3*, *HSD17B7* and *FAM72A* as genes with increased IR after HS and no further enhanced IR after HS+BRD4k.d.. *SatIII*-siRNA knock down diminished the otherwise HS-induced *SatIII* RNA transcription by ~50% at 42°C with 250nM *SatIII* siRNA (Figure 5E). Interestingly, even though the *SatIII* siRNA knock down had no influence on BRD4 expression (Supplementary Figure S5D), the *ATF3*, *EP300* and *SOD2* introns were increasingly retained with decreased *SatIII* RNA, a situation we have described before for these introns in HS+BRD4 k.d. conditions (Figure 5F and Supplementary Figure S5E). Notably, the *SatIII*-siRNA did not result in a significant increase in *BAG3* (similarly for *HSD17B7* and *FAM72A*) intron retention (Figure 5G and Supplementary Figure S5E) and suggests that the observed splicing deregulation in BRD4 depleted cells under HS might be a consequence of a reduced *SatIII* expression induced by BRD4 depletion.

DISCUSSION

The heat shock response is a highly conserved, well-ordered and regulated process through which an organism attempts to overcome cellular stress, triggered by elevated temperature, exposure to heavy metals or infections. It is regulated on multiple levels, including the transcriptional up-regulation of cyto-protective genes, inhibition of global protein synthesis and the splicing machinery. The mechanism underlying the global effect of HS on the splicing regulation is not well understood and may play a major role in the adaptation to stress. Recently, Shalgi and colleagues reported intron retention, altered 3' and 5' splice sites and exon skipping events after HS. We have now shown that BRD4 is involved in intron retention under HS. BRD4, an acetylated histone-binding protein, has been identified as an important factor for the generation of mature spliced transcripts

after LPS stimulation (23). In addition, we have shown that BRD4 participates in the oxidative stress response and thus may participate in general stress-regulatory processes including the HS response (18).

We now found, using RNA-Seq analysis, that a BRD4 knock down further enhances the splicing inhibition of 965 introns after mild HS conditions (42°C) resulting in a decrease of gene expression. This implicates BRD4 in the maintenance of splicing under stress. Shalgi et al report a slightly increased expression of splicing-inhibited genes under primarily severe HS (44°C for 2 and 8 h). The discrepancy to our data might be due to different heat shock conditions used within the two studies. Using immunofluorescence analyses we further showed that under HS the typically diffuse located BRD4 protein is recruited to nSB and co-localizes with HSF1. The number of BRD4 co-stained nSB clearly increased at severe HS conditions (44°C), suggesting that our observed effect on IR in BRD4 depleted cells should be even stronger at higher temperatures (Figure 4B in comparison to Supplementary Figure S4C). In contrast, we did not find a significant effect of BRD4 knock down under non-HS conditions on the global splicing pattern. We cannot exclude that this effect is masked by remaining BRD4 functions due to the siRNA technology used, but this effect seems to be marginal in comparison to the IR effect under HS and BRD4k.d. As shown in previous work, under severe as well as mild heat shock conditions, the global splicing inhibition occurs mainly in transcripts which are spliced post-transcriptionally, whereas transcripts which undergo the splicing process co-transcriptionally remained almost unaffected. Having in mind the role of BRD4 in transcriptional elongation, the observed effects on splicing could be due to an altered transcription rate and defects in the co-transcriptional splicing process. Even though we cannot completely exclude an effect of an altered transcription rate on splicing in our setting, we think that it is a minor effect, because we would expect to also see an increase in exon inclusion, the main effect of altered transcription rates. Furthermore, we did not find an increased IR rate in BRD4k.d. samples without heat stress further arguing against a sole effect of transcriptional elongation rates. On the other side, our finding of the interaction between BRD4 and HSF1 further supports the model that the function of nSBs in HS is to recruit the splicing machinery and protect genes from splicing-inhibition which is in accordance to previous studies (5,12,44). Disruption of this complex leads to inhibition of splicing and increased IR which would argue for an involvement of BRD4 in splicing of co-transcriptionally spliced genes. However, for a final conclusion, additional experiments are required. Additionally, it has been reported that a depletion of BRD4 or its interaction partner NSD3 (Nuclear SET Domain-Containing Protein 3) - reduces histone H3 lysine 36 tri-methylation (H3K36me3) (21). A decrease of H3K36me3 in turn has been associated with an increase in IR in renal carcinomas (45). Thus, the effect of BRD4 inhibition on IR levels might also be indirect over a reduction in H3K36me3 levels.

nSB are unique subnuclear foci which were originally identified as the main sites of HSF1 accumulation and are places of active transcription at pericentric heterochromatic

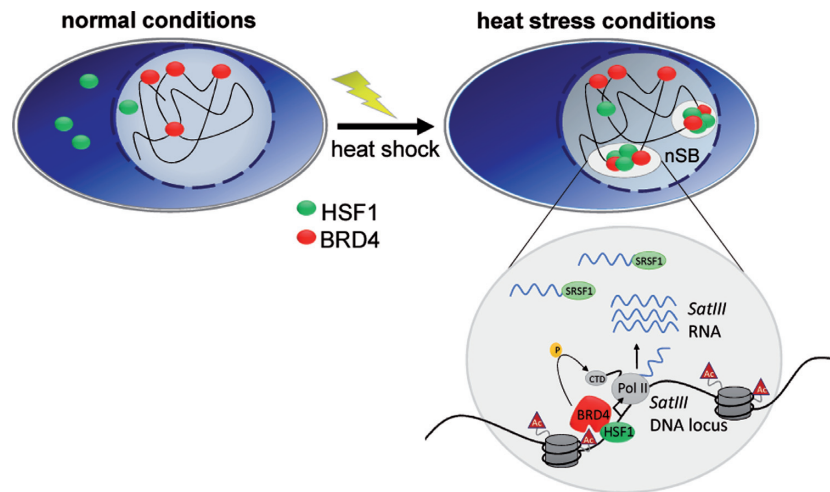


Figure 6. Schematic representation of BRD4's function in the splicing-process during the heat shock response. Upon heat stress BRD4 is recruited to sub-nuclear structures, the so-called 'nuclear stress bodies' that are the main sites of HSF1 accumulation in response to HS. The recruitment of BRD4 by HSF1 may enhance the elongation rate of Pol II and thereby increases the transcription of heat-induced *SatIII* RNA. Subsequently, *SatIII* transcripts recruit several RNA-binding proteins, such as the splicing factors SRSF1 to nSB that may induce the splicing process. PolII: RNA-Polymerase II, CTD: Carboxy-terminal domain of PolII, Ac: acetylated histone tail, SRSF1: pre-mRNA-splicing factor SF2/alternative splicing factor 1.

tandem repeats of *SatIII* DNA sequences (15,34,39). The additional recruitment of splicing factors to these sites under HS is supposed to protect the splicing machinery from splicing-inhibition under HS (5,35,46). Furthermore, after HS, the heterochromatic regions in nSB convert to euchromatin as shown by the presence of acetylated histone H4, especially H4K8ac and H4K16ac, and the lack of HP1 (heterochromatin protein 1) (34,39). The hyperacetylation in nSB, triggered by HSF1, is accompanied by a global deacetylation of chromatin in the rest of the nucleus following HS (47). This would also be a conceivable mechanism for the HS induced recruitment of BRD4 to nSB since BRD4 binds to acetylated H4 peptides (40) and might thus be recruited to sites of increased acetylation in nSB. Pointing towards this model, treatment with the bromodomain inhibitor IBET 151 blocks this interaction and de-localizes BRD4 from nSB.

As mentioned, nSB are thought to be the place of active *SatIII* RNA transcription (34,39). Indeed, besides an increased IR we found under BRD4 depletion or IBET151 treatment an attenuation of the induction of *SatIII* RNA transcription which is HSF1-dependent. It was previously reported that a downregulation of *SatIII* RNA transcripts blocks the recruitment of splicing factors to nSB what might, in turn, result in splicing defects similar to BRD4k.d. (Figure 6) (5,33).

Alternative splicing has been found to be associated with various monogenic and complex diseases including cancer (48,49). It is worth noting that abnormal RNA splicing and in particular IR seems to be a common characteristic of cancers even in the absence of mutations in the splicing machinery (48). Interestingly, when we looked at IR events in lung adenocarcinomas, a tumor type which frequently responds to BRD4 inhibitors, we found, out of a set of 2340 tumor-specific IR, 136 also in our HS + BRD4k.d. dataset (50). The overlap contains eight oncogenes including *BCR* and *EZH2*, where an IR effect might be further enhanced with

BRD4 inhibitors and might lead to a tumor growth regression. Thus, an over-activation of IR might at least partially explain the tumor growth inhibitory effect of BRD4 inhibition. However, future work will reveal the effect of BRD4 inhibition in cancer therapy on epigenetic patterns and alternative splicing events. If, similar to HS, splicing under proteotoxic stress, one of the major stress factors in cancer cells, is partially maintained by BRD4's recruitment to nuclear stress bodies, this would not only shed light on important cancer pathways, but also give rise to new therapeutic considerations.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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