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RESEARCH ARTICLE

Human satellite-III non-coding RNAs modulate heat-shock-induced transcriptional repression

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ABSTRACT

The heat shock response is a conserved defense mechanism that protects cells from physiological stress, including thermal stress. Besides the activation of heat-shock-protein genes, the heat shock response is also known to bring about global suppression of transcription; however, the mechanism by which this occurs is poorly understood. One of the intriguing aspects of the heat shock response in human cells is the transcription of satellite-III (Sat3) long non-coding RNAs and their association with nuclear stress bodies (nSBs) of unknown function. Besides association with the Sat3 transcript, the nSBs are also known to recruit the transcription factors HSF1 and CREBBP, and several RNA-binding proteins, including the splicing factor SRSF1. We demonstrate here that the recruitment of CREBBP and SRSF1 to nSBs is Sat3-dependent, and that loss of Sat3 transcripts relieves the heat-shock-induced transcriptional repression of a few target genes. Conversely, forced expression of Sat3 transcripts results in the formation of nSBs and transcriptional repression even without a heat shock. Our results thus provide a novel insight into the regulatory role for the Sat3 transcripts in heat-shock-dependent transcriptional repression.

KEY WORDS: Heat shock response, Non-coding RNA, Transcription factor, Nuclear stress bodies

INTRODUCTION

The heat shock response (HSR) is a ubiquitous cell-defense mechanism and is conserved throughout the eukaryotes (Akerfelt et al., 2010). The HSR is induced when the cell is under physiological stress that alters the protein folding, such as a heat shock, and is therefore characterized by the increased expression of chaperones known as the heat shock proteins (HSPs) (Lindquist, 1986). The HSR is mediated by the heat shock transcription factor (HSF1 in mammals), which is activated upon exposure of cells to the stress and is essential for the stress-induced transcription of genes coding for HSPs (Wu, 1984; Parker and Topol, 1984). Thus, HSF1 is considered to be the master regulator of the HSR (McMillan et al., 1998; Xiao et al., 1999; Pirkkala et al., 2000; Zhang et al., 2002). In addition to its role in the activation of the HSPs, the HSR is also involved in global suppression of transcription and translational processes and associated changes in the cellular physiology (Lindquist, 1986). Besides the heat shock, the HSR is also activated by a variety of stressors and therefore the HSR is considered to be a generic stress-response mechanism to protect cells against stress-induced damages (Courgeon et al., 1984; Heikkila et al., 1982; Michel and Starka, 1986; Yura et al., 1984).

An intriguing observation with regard to the HSR in humans is the heat-induced expression of a class of long non-coding RNAs (lncRNAs) known as the satellite-III transcripts (hereafter referred to as Sat3 transcripts) (Rizzi et al., 2004; Jolly et al., 2004). Sat3 transcripts are coded by the satellite-III family of repetitive sequences harboring a consensus GGAAT motif and localized in the peri-centromeric region of acrocentric chromosomes (Valgardsdottir et al., 2005; Jolly et al., 2002). The heat-shock-induced expression of Sat3 is HSF1-dependent, the polyadenylated Sat3 transcripts vary in length (ranging from 2 to over 5 kb), and the transcripts tend to accumulate at the site of transcription to form what is known as nuclear stress bodies (nSBs), primarily at the 9q12 locus (Jolly et al., 2004; Sengupta et al., 2009; Rizzi et al., 2004; Metz et al., 2004). The Sat3-positive nSBs colocalize with HSF1, CREB-binding protein (CREBBP; also known as CBP), RNA polymerase II, RNA-binding proteins such as SRSF1 (serine/arginine-rich splicing factor 1; also known as SF2 or ASF), KHDRBS1 (also known as Sam68), and several heterogeneous nuclear ribonucleoproteins (hnRNPs) (Weighardt et al., 1999; Denegri et al., 2001; Metz et al., 2004; Chiodi et al., 2004; Jolly et al., 2004). The recruitment of HSF1 and CREBBP onto the nSBs is thought to be necessary for the transcription of Sat3 loci (Jolly et al., 2004; Sengupta et al., 2009). The association of several splicing factors with nSBs possibly suggests a role for Sat3 transcripts in sequestration of these proteins into an inactive compartment during the thermal stress (Jolly et al., 2002; Chiodi et al., 2004; Jolly and Lakhotia, 2006). However, the specific functions of Sat3 transcripts in the HSR and the cellular pathways that are regulated by Sat3 transcripts are not very well understood. Here, we show that the Sat3 transcripts recruit critical factors involved in the transcriptional processes, thereby contributing to the heat-induced transcriptional silencing. We also provide evidence to suggest that Sat3-mediated recruitment of the transcription factors during a heat shock is required to provide full protection against the heat-shock-induced cell death. Our results thus uncover a newly identified regulatory role for the Sat3 transcripts in the cellular heat shock response.

RESULTS

SRSF1 facilitates the recruitment of CREBBP on to the Sat3 transcripts

The stress-induced Sat3-positive nSBs are known to colocalize with several RNA-binding proteins and a few transcription factors (reviewed in Jolly and Lakhotia, 2006). Prominent among them are

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the transcription factors HSF1 (Cotto et al., 1997) and CREBBP (Jolly et al., 2004), the splicing factors SRSF1 and SRSF9 (also known as SRp30c) (Metz et al., 2004), and several other RNA-binding proteins such as hnRNPs and SAFB (scaffold attachment factor B) (Chiodi et al., 2000; Denegri et al., 2001; Weighardt et al., 1999). We have validated the colocalization of CREBBP and SRSF1 with Sat3-positive nSBs by immuno-fluorescence *in situ* hybridization (immuno-FISH) (Fig. 1A,B). With the exception of HSF1, which is required for the heat-shock-induced expression of Sat3 transcripts (Jolly et al., 2004; Sengupta et al., 2009), the functional significance of the other factors that are recruited to the nSBs is not fully understood. We therefore wanted to test whether loss of CREBBP would destabilize the nSBs or not. As shown in Fig. 1C,D, the RNAi-mediated knockdown of CREBBP did not affect the heat-shock-induced, HSF1-positive or the Sat3-positive nSBs, whereas the loss of HSF1 did affect the nSB formation by arresting the transcription of Sat3 loci (Fig. 1E), as reported earlier (Jolly et al., 2004; Sengupta et al., 2009). Intriguingly, knockdown of Sat3 transcripts, using the phosphorothioate-modified antisense oligos, led to the loss of CREBBP on the nSBs (Fig. 1F; Fig. S1A,D), whereas HSF1 continued to form nSB-like structures even in the absence of the Sat3 transcripts (Fig. 1F,G), implying that the Sat3 transcripts serve as a scaffold for the recruitment of CREBBP to the nSBs.

As expected, the loss of HSF1 abolished the formation of CREBBP-positive nSBs, confirming that HSF1 is essential for the formation of nSBs during a heat shock (Fig. 2A). Intriguingly, knockdown of SRSF1 led to the loss of CREBBP on the HSF1-positive nSBs, suggesting SRSF1 to be essential for the recruitment of CREBBP to HSF1-positive nSBs (Fig. 2A; Fig. S1A,D). However, the partial loss of SRSF1 did not affect the formation of Sat3-positive nSBs (Fig. 2B) or the HSF1-positive nSBs (Fig. 2C). SRSF1 is known to physically interact with the Sat3 transcripts (Metz et al., 2004); therefore, and as expected, the loss of Sat3 transcripts resulted in the loss of SRSF1 on the nSBs (Fig. 2C). Knockdown of SRSF1, however, did not affect the formation of Sat3-positive nSBs (Fig. 2B). Similarly, knockdown of CREBBP did not affect the SRSF1-positive nSBs (Fig. 2D). The knockdown efficiency of Sat3 antisense oligos, and the RNAi constructs for the knockdown of HSF1, CREBBP and SRSF1 are shown in Fig. S2A-C.

Having seen a change in the localization pattern of CREBBP upon loss of Sat3 transcript or the SRSF1, we next wanted to see whether CREBBP would get recruited to the Sat3-positive nSBs even when the Sat3 transcripts are overexpressed using the viral promoter. For this, we have created a mammalian expression construct in which the 158 base pair (bp) fragment harboring the Sat3 repeats was cloned under a viral promoter and confirmed its expression (see Fig. S2D). As shown in Fig. 3A,B, the pcDNA-mediated expression of Sat3 transcripts resulted in the formation of nSB-like Sat3-positive bodies in the majority of the transfected cells; in a small proportion of transfected cells, however, the overexpressed Sat3 transcripts showed a diffused pattern in the nuclei. Interestingly, the overexpression of Sat3 repeats resulted in the recruitment of SRSF1 and CREBBP, but not HSF1, to the nSBs in the majority of the transfected cells even when the cells were not exposed to a heat shock (Fig. 3C-E; Fig. S1F). This observation was similar to that seen in the nSBs resulting from the expression of Sat3 endogenous transcripts (Fig. 1A,B), strengthening the notion that Sat3 transcripts are required for the recruitment of CREBBP on to the nSBs. Transient transfection of a bacterial expression vector (pGEM2-98) bearing the same repeat fragment did not yield nSB-like signals upon *in situ* hybridization (Fig. S2D), suggesting that the signals obtained for the overexpressed Sat3 repeats are indeed from the transcripts and not from cross hybridization with the DNA.

Because the overexpressed Sat3 formed nSBs in the majority of the transfected cells, and because the nSBs were positive for SRSF1 and CREBBP, we wanted to test whether the overexpressed Sat3 transcripts associate with the endogenous Sat3 chromosomal loci. For this, we carried out a combined DNA–RNA FISH with a DNA probe to detect the 9q21 locus, a non-overlapping region adjacent to the Sat3 locus on chromosome 9 (Moyzis et al., 1987) devoid of the satellite III repeats (see Fig. 3F) and an oligo probe to detect the Sat3 transcripts. In the heat-shocked cells, the signals for the 9q21 region were seen in close proximity to the nSBs positive for the Sat3 transcripts in the majority (~80%) of the cells that expressed the Sat3 transcripts (Fig. 3G). Intriguingly, a similar pattern was also seen for the overexpressed Sat3 transcripts in the transfected cells not exposed to a heat shock (Fig. 3H). However, nearly 50% of the overexpressed Sat3-positive nSBs did not cluster around the 9q21 locus (Fig. 3H).

Knockdown of Sat3 transcripts partially relieves the heat-shock-induced transcriptional repression

Having observed the dynamic nature of the Sat3 expression and the recruitment of CREBBP on Sat3 transcripts, we reasoned that nSBs might regulate the heat-shock-induced transcriptional repression. To investigate this, we selected 18 genes whose expression levels are known to go down during heat shock in HeLa cells (Pandey et al., 2011). Because the transcription process is known to be suppressed during the HSR, we used the nuclear RNA to accurately quantify the expression difference. For this we used the real-time PCR approach using the Fluidigm's microfluidic device (Livak et al., 2013). As observed previously (Pandey et al., 2011), all 18 genes tested in the present study showed a significant reduction in the expression level upon exposure to heat shock (Fig. 4A). As shown in Fig. 4A, a significant increase in the expression level of 14 genes in heat-shocked cells was observed upon partial knockdown of Sat3 transcripts. However, four genes did not show a change in the expression level upon partial loss of Sat3 transcripts (Fig. 4A). No such change in the expression level was found when Sat3 was silenced in cells not exposed to the heat shock (see Fig. S3A). We next tested whether ectopic expression of Sat3 transcripts would alter the expression level of these 18 genes. For this, the mammalian expression construct harboring the Sat3 repeat (pcDNA-Sat3) was transiently transfected in HeLa cells and the expression level of the 18 genes was quantified. Cells transfected with a bacterial expression construct harboring the Sat3 repeat (pGEM-Sat3) was used as the control. As shown in Fig. 4B, a significant reduction in the expression level was observed for 14 genes in cells that overexpressed the Sat3 transcripts. Intriguingly, the same set of genes showed an increase in the expression level upon loss of Sat3 in cells exposed to the heat shock (Fig. 4A), suggesting a direct role for Sat3 transcripts in the transcriptional regulation. The expression levels of the remaining four genes were unaltered upon Sat3 overexpression (Fig. 4B), as was seen upon knockdown of endogenous Sat3 transcript during a heat shock (Fig. 4A). We next wanted to test whether the Sat3-transcript-mediated transcriptional repression is due to the recruitment of CREBBP and/or SRSF1 on to the Sat3 transcripts. For this, pcDNA-Sat3 was co-expressed with an expression construct coding for CREBBP or SRSF1, and the expression levels of nine genes in the control set was tested. As shown in Fig. 5A, co-expression of CREBBP or

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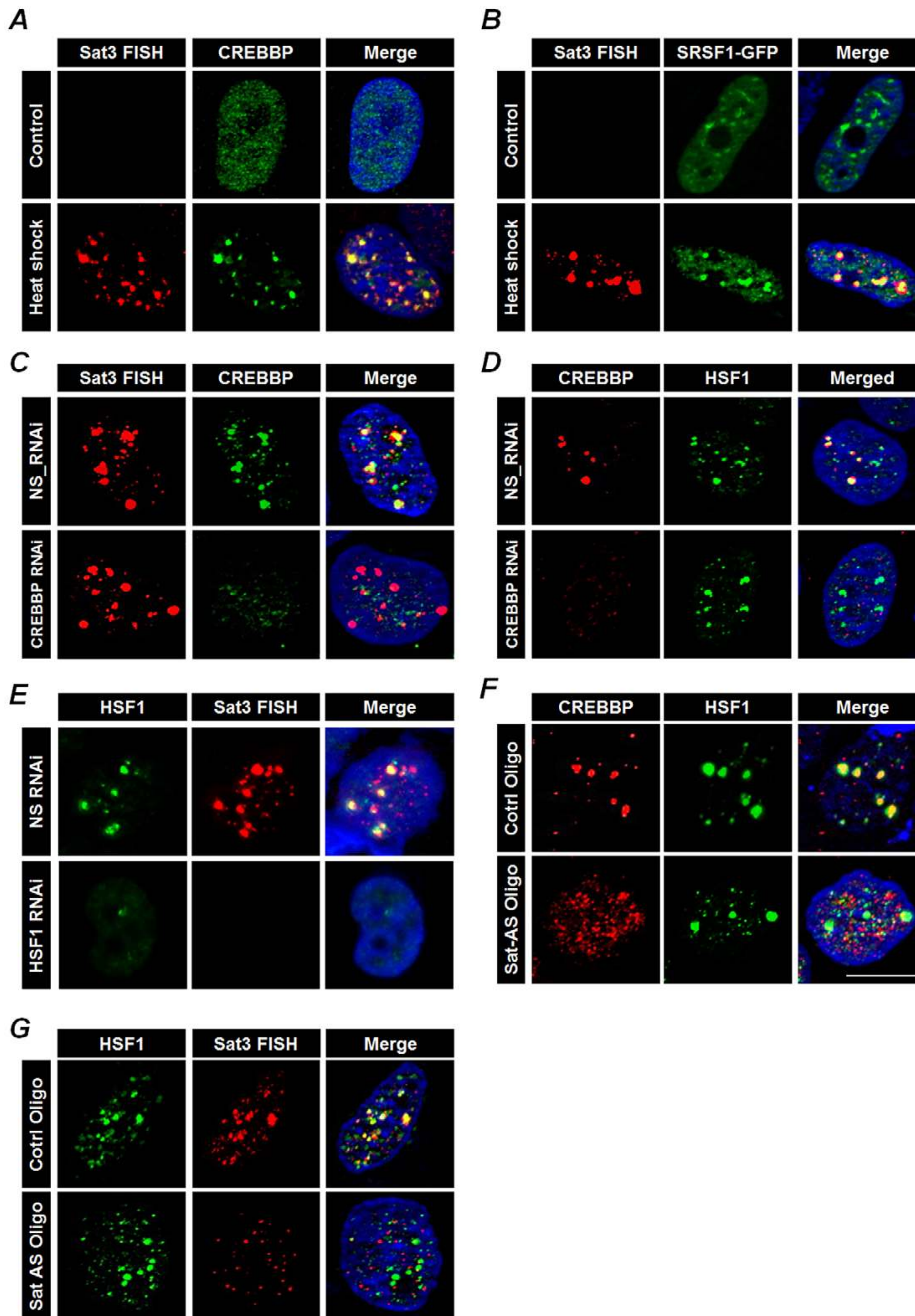

Fig. 1. The heat-shock-induced Sat3 transcripts sequester CREB-binding protein (CREBBP) to nSBs. (A,B) Representative immuno-FISH images showing colocalization of Sat3 transcripts with endogenous CREBBP (A) or with transiently expressed GFP-tagged SRSF1 (SRSF1-GFP) (B) in HeLa cells; control (upper panels) or heat-shocked (lower panels) cells, as indicated. (C,D) Images of immuno-FISH or immunofluorescence stainings showing the continued presence of Sat3 transcripts (C) or HSF1-positive nSBs (D) upon knockdown of CREBBP in cells exposed to a heat shock. Note the colocalization of nSBs positive for Sat3 transcripts or HSF1 with the CREBBP foci in the control cells (upper panels). (E) Immuno-FISH images showing the absence of Sat3-posititve nSBs upon loss of HSF1 in heat-shocked cells. The upper panels represent the control set, whereas the lower panels show the HSF1 knockdown. (F) Immunofluorescence images showing the absence of CREBBP in HSF1-positive nSBs when Sat3 was knocked down in heat-shocked cells. The upper panels represent the control set, whereas the lower panels represent the Sat3-knockdown set. (G) Immuno-FISH images showing the presence of HSF1-positive nSBs even in the absence of Sat3 transcripts in the heat-shocked cells. The upper panels represent the control set showing the colocalization of Sat3 transcripts with HSF1. The efficiency of Sat3 antisense oligos used for the knockdown of Sat3 transcripts was validated by FISH and semi-quantitative PCR (Fig. S2A,B). The efficiency of the knockdown construct for HSF1 was validated by semi-quantitative PCR and by immunoblotting (Fig. S2C). Scale bar: 10 µm.

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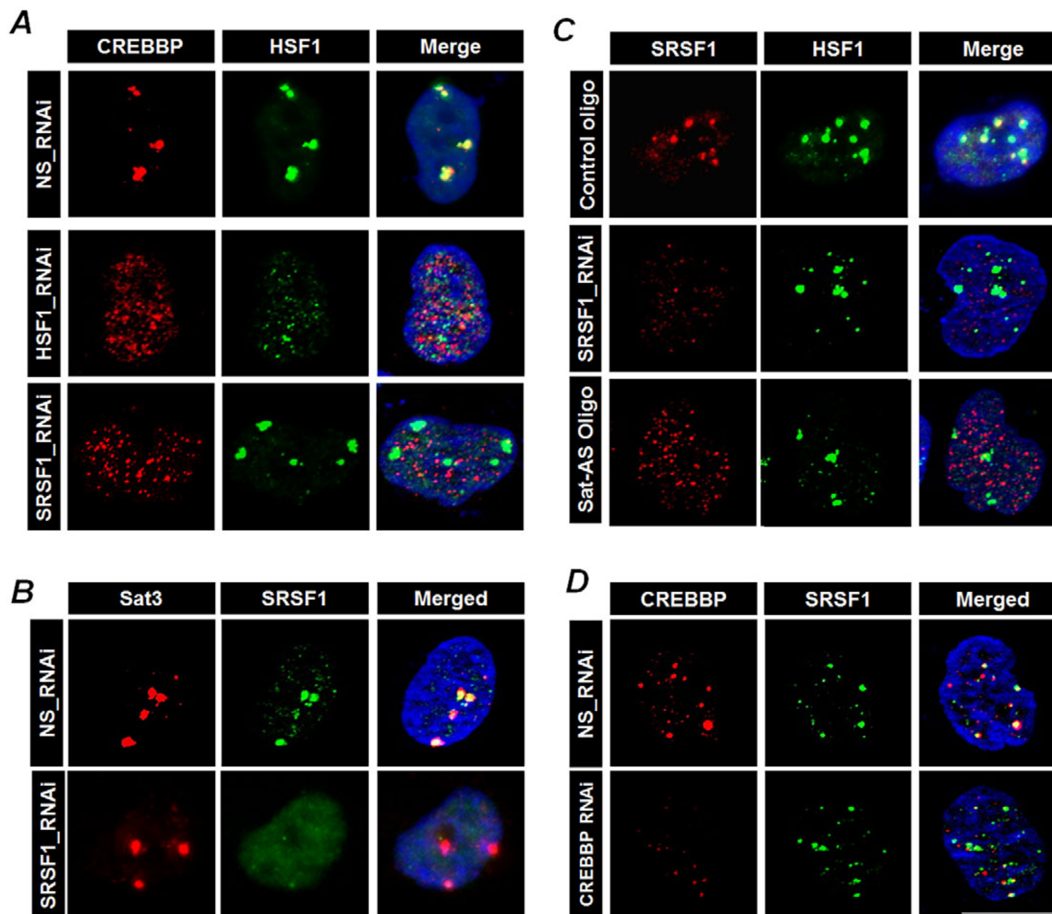

Fig. 2. SRSF1 facilitates the recruitment of CREBBP to Sat3 transcripts. (A) Immunofluorescence images showing the absence of CREBBP in HSF1-positive nSBs when SRSF1 was knocked down in heat-shocked cells. The upper panels represent the control set [non-silencing RNAi duplex (NS_RNAi)], whereas the lower panels represent the HSF1 and SRSF1 knockdown sets. (B) Immuno-FISH images showing the presence of Sat3-transcript-positive nSBs upon SRSF1 knockdown in cells exposed to a heat shock. Note the colocalization of Sat3 transcripts with SRSF1 in the control cells (upper panels). (C) Immunofluorescence images showing the absence of SRSF1 in the HSF1-positive nSBs upon loss of Sat3 transcripts. The upper panels represent the control set, whereas the lower show SRSF1 and Sat3 knockdown. (D) Immunofluorescence images showing the presence of SRSF1-positive nSBs upon CREBBP knockdown in cells exposed to a heat shock. Upper panels show the colocalization of CREBBP and SRSF1 in the control condition, whereas lower panels represent the CREBBP knockdown set. The efficiency of the knockdown constructs for CREBBP and SRSF1 was validated by semi-quantitative PCR and by immunoblots (Fig. S2C). For quantified colocalization data, see Fig. S1A-E. Scale bar: 10 µm.

SRSF1 partially relieved the Sat3-mediated transcriptional suppression of the nine genes tested. Overexpression of either of these two proteins alone resulted in an increase in the expression levels of the target genes upon heat shock (Fig. 5B) as well as at the physiological temperatures (Fig. S3B). Depletion of Sat3 transcripts in cells overexpressing CREBBP did not significantly alter the expression levels of the target genes in the heat-shocked cells (Fig. S4A); their expression profile was similar to the cells that overexpressed CREBBP and were exposed to a heat shock (Fig. 5B). Conversely, partial knockdown of CREBBP resulted in reduced expression of the same set of genes in the heat-shocked cells (Fig. 5C), further strengthening the role of CREBBP in the heat-shock-induced transcriptional regulation. Because the recruitment of CREBBP to nSBs requires the presence of SRSF1 (Fig. 2A), we next tested whether knockdown of SRSF1 would relieve the heat-shock-induced transcriptional repression. As shown in Fig. 5D, partial loss of SRSF1 did increase the expression level of target genes during the heat shock. This could possibly mean that the Sat3 transcripts sequester CREBBP via SRSF1 to bring about transcriptional repression of target genes during the heat shock. Indeed, partial loss of SRSF1 in cells that overexpressed Sat3 transcripts led to a significant increase in the expression level of target genes (Fig. 5E), possibly due to the increased ‘availability’ of CREBBP for transcription. However, no such change was seen when HSF1 was knocked down (Fig. 5E), suggesting that HSF1 is not involved in overexpressed-Sat3-mediated transcriptional silencing. Intriguingly, knockdown of SRSF1 in cells not exposed to heat shock also showed some suppression in the transcript level of target genes (see Fig. S4B), suggesting a direct role for SRSF1 in the transcriptional process. Thus, the loss of either the Sat3 transcripts or SRSF1 is likely to increase the availability of CREBBP for transcriptional activity even during heat shock. Conversely loss of CREBBP during the heat shock is expected to further reduce the expression level of target genes and indeed that was observed (Fig. 5C). To test whether these genes are indeed the direct targets of CREBBP, we queried the data available from a genome-wide study that looked at the CREBBP occupancy in over 16,000 promoter sites *in vivo* (Ramos et al., 2010). Interestingly, we found the occupancy rate for CREBBP to be much higher in the regulatory regions of the 14 genes that showed an increased expression upon loss of Sat3 transcripts as compared with the four genes that did not show a

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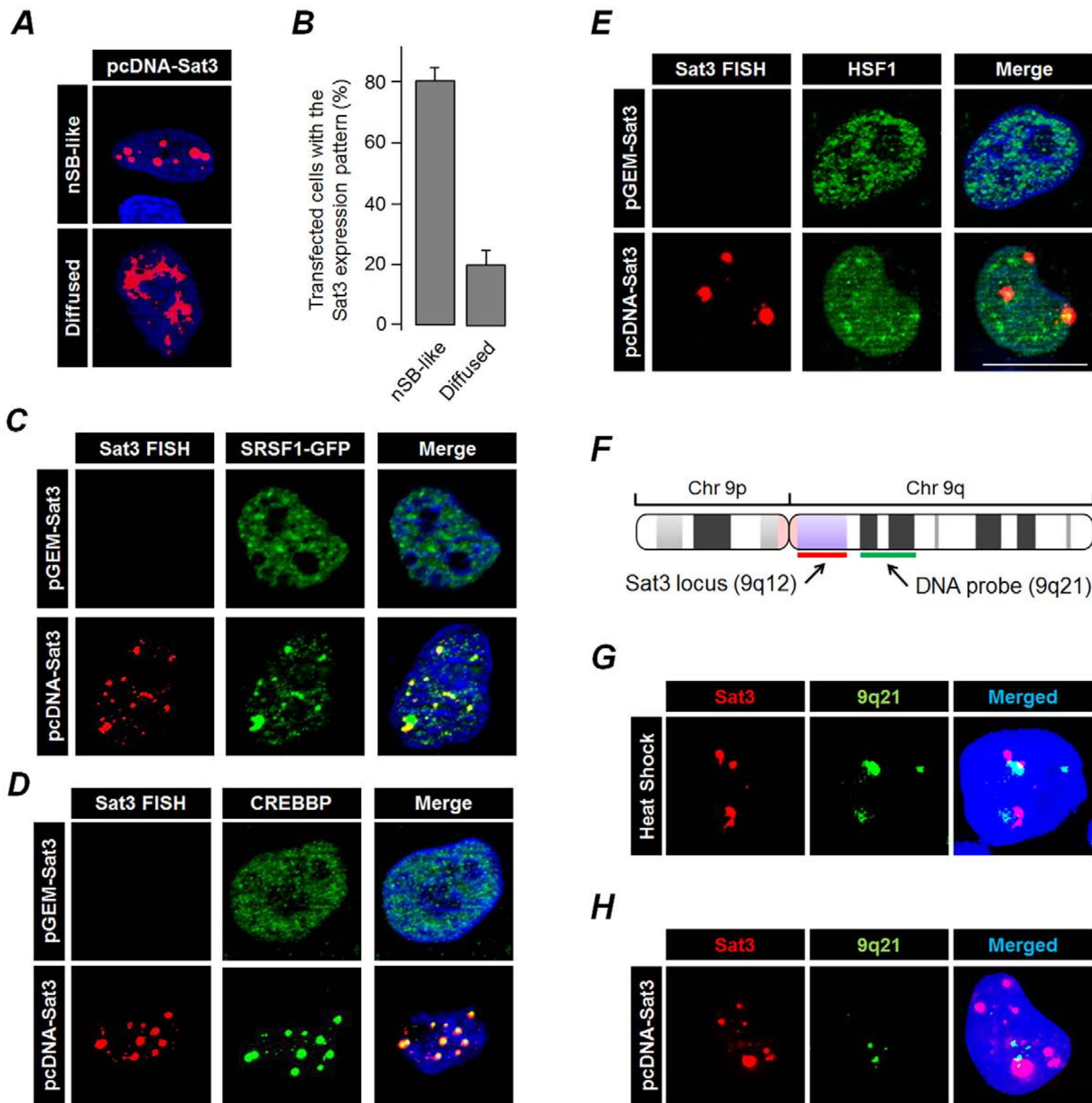

Fig. 3. Ectopic overexpression of Sat3-repeat-bearing transcripts form nSBs in non-heat-shocked cells. (A) FISH images showing the nSB-like (top) and the diffused (bottom) pattern of localization for the Sat3 transcripts when overexpressed using a mammalian expression construct (pcDNA-Sat3) in HeLa cells. (B) Bar diagram representing the percentage of cells showing the different expression patterns of pcDNA-Sat3-derived Sat3 transcripts, as depicted in A (*n*=3). (C) Immuno-FISH images showing colocalization of transiently expressed SRSF1-GFP with Sat3-repeat-bearing transcripts in cells transfected with the Sat3 mammalian expression construct (pcDNA-Sat3) but not in cells transfected with the Sat3-repeat-bearing bacterial expression construct (pGEM-Sat3). (D,E) Similarly, the transiently expressed Sat3-repeat-bearing transcripts colocalized with endogenous CREBBP (D) but not with HSF1 (E). Note the lack of any Sat3 signal in cells transfected with pGEM-Sat3. For quantification of colocalization data, see Fig. S1F. (F) Ideogram of chromosome 9 showing the position of the Sat3 locus (9q12; identified in purple on the ideogram and a red line below), and the physical position of the genomic probe (9q21; identified by a green line below the ideogram) used for the DNA FISH. (G) Representative image of the combined DNA-RNA FISH showing the fluorescent signals obtained for the endogenous Sat3 transcripts (red) and for the 9q21 genomic locus (green) in a cell exposed to a heat shock. The nucleus is stained with DAPI (blue). (H) Similarly, a representative image for the combined DNA–RNA FISH showing the fluorescent signals obtained for the overexpressed Sat3 transcripts (pcDNA-Sat3; red) and for the 9q21 genomic locus (green). The nucleus is stained with DAPI. Note the overlapping as well as non-overlapping signals for the overexpressed Sat3 transcripts with the 9q21 locus. Scale bar: 10 µm.

change in their expression level (Table S1). Thus, there seems to be a direct correlation between the CREBBP occupancy on the promoter and a change in the Sat3-mediated expression level of the genes tested.

It is known that SRSF1, an RNA-binding protein, physically interacts with the Sat3 transcripts (Metz et al., 2004). Because CREBBP requires Sat3 transcripts and SRSF1 for its recruitment to the nSBs, we wanted to test whether CREBBP interacts with Sat3 transcripts through SRSF1. For this, we carried out an RNA immunoprecipitation to pull-down CREBBP and tested the presence of Sat3 transcripts in the immunoprecipitate. As shown in Fig. 6, Sat3 transcripts were detected in the precipitate derived from cells exposed to the heat shock, and their level was lower when the SRSF1 was knocked down. No amplification was observed in samples that were not exposed to a heat shock or in samples that were immunoprecipitated with a non-specific antibody, establishing the specificity of the pull-down assay used.

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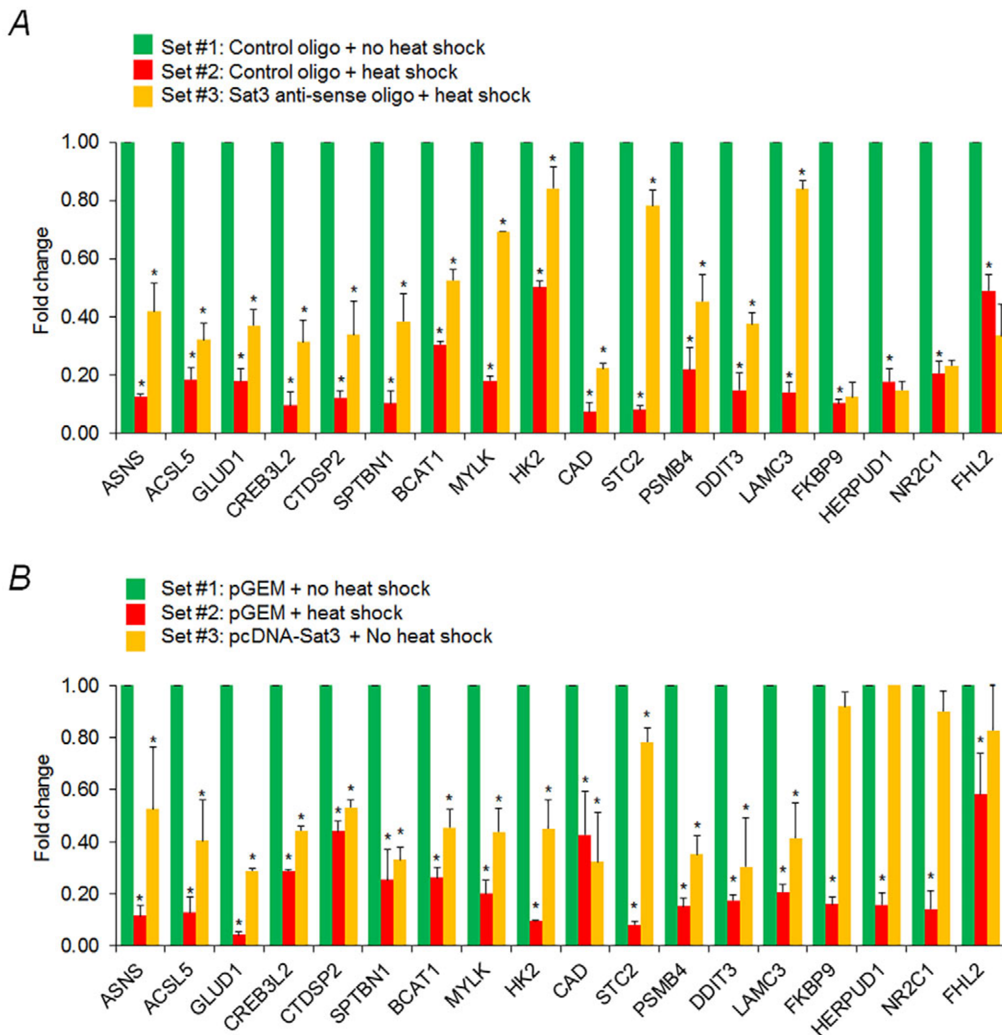

Fig. 4. Knockdown of Sat3 transcripts partially relieves heat-shock-induced transcriptional repression. (A) Bar diagram showing fold change in the expression levels of 18 genes evaluated by quantitative PCR. The cells were transfected with the anti-sense Sat3 oligos or the control oligos, either exposed or not exposed to heat shock, and the gene expression was evaluated by the Fluidigm's microfluidic devices, as indicated. All values are normalized to the samples transfected with control oligos and not given a heat shock (identified as 'Set #1'). For calculating statistical significance, Set #2 was compared with Set #1, and Set #3 was compared with the Set #2. (B) Bar diagram showing the fold difference in the expression levels of the indicated genes in cells transfected with the bacterial expression construct having the Sat3 repeat (pGEM-Sat3) and given heat shock or no heat shock, or cells transfected with the mammalian expression construct bearing the Sat3 repeat (pcDNA-Sat3) and given no heat shock, as indicated. All values are normalized to the samples transfected with the pGEM-Sat3 construct and not given a heat shock (identified as 'Set #1'). For calculating statistical significance, Set #3 was compared with Set #1. For both figures, each bar represents mean values of three biological replicates, each of which were processed for two experimental replicates. Unpaired Student's *t*-test, $^{*}P\leq0.05$.

Sat3 transcripts are required for full protection against heat-shock-induced cell death

Because transcription of Sat3 loci is regulated by HSF1, and HSF1 is a critical player in the heat shock response, we wanted to check whether the Sat3 transcripts are required for human cells to confer full protection against thermal stress. For this, HeLa cells were transiently transfected with antisense oligos for the knockdown of Sat3 transcript, the cells exposed to a heat shock (42°C for 1 hour) and cell viability measured by an MTT assay at the end of the heat shock. The loss of Sat3 transcripts led to a significant reduction (∼70%) in cell survival as compared to cells that were transiently transfected with the control oligos and exposed to a heat shock (Fig. 7A). However, the Sat3 antisense oligos did not affect the survival of cells that were not exposed to the heat shock, suggesting that the Sat3 transcripts are required for survival only under thermal stress condition (Fig. 7A). We validated these findings using a different approach, wherein the transfected cells were counted for fragmented nuclei, indicating apoptotic cell death (see Fig. S2E). As shown in Fig. 7B, around 60% of the cells transfected with the antisense oligo showed an increase in the apoptotic nuclear phenotype as compared to cells transfected with the control oligos. We next wanted to assess whether forced expression of Sat3 transcripts would alter the viability of cells that are not exposed to a heat shock. Transient expression of the Sat3 mammalian expression construct (pcDNA-Sat3) resulted in a lower survival rate as compared to cells transfected with an empty vector or the bacterial expression vector Sat3 repeats (pGEM2-Sat3) (Fig. 7C,D). Intriguingly, cells that expressed the cloned Sat3 repeats exhibited a significantly lower survival rates when exposed to a heat shock, suggesting a toxic effect of Sat3 transcripts when ectopically expressed (Fig. 7C). As stated earlier, transient expression of pcDNA-Sat3 resulted in the formation of Sat3-positive nSBs even when the cells were not exposed to a heat shock, whereas the bacterial expression vector bearing the same repeat fragment did not yield nSB-like signals upon *in situ* hybridization (Fig. S2D), suggesting the specific involvement of the Sat3-repeat-bearing transcripts in the formation of nSBs and the associated cytotoxicity.

DISCUSSION

Crucial roles for non-coding regulatory RNAs (ncRNAs) in cellular physiology are being uncovered, and studies in the last decade have highlighted the importance of ncRNAs in transcriptional regulation (Ponting et al., 2009). These include a causal role for ncRNAs in chromatin remodeling (Hirota et al., 2008) and gene silencing (Chaumeil et al., 2006; Green et al., 2007; Martianov et al., 2007). Our current findings provide a novel insight into the role of the Sat3 ncRNAs in the heat shock response. The heat-shock-induced expression and localization of Sat3 transcripts in the nSBs have been known for a while, but their specific functions in HSR was not known. We show here that Sat3

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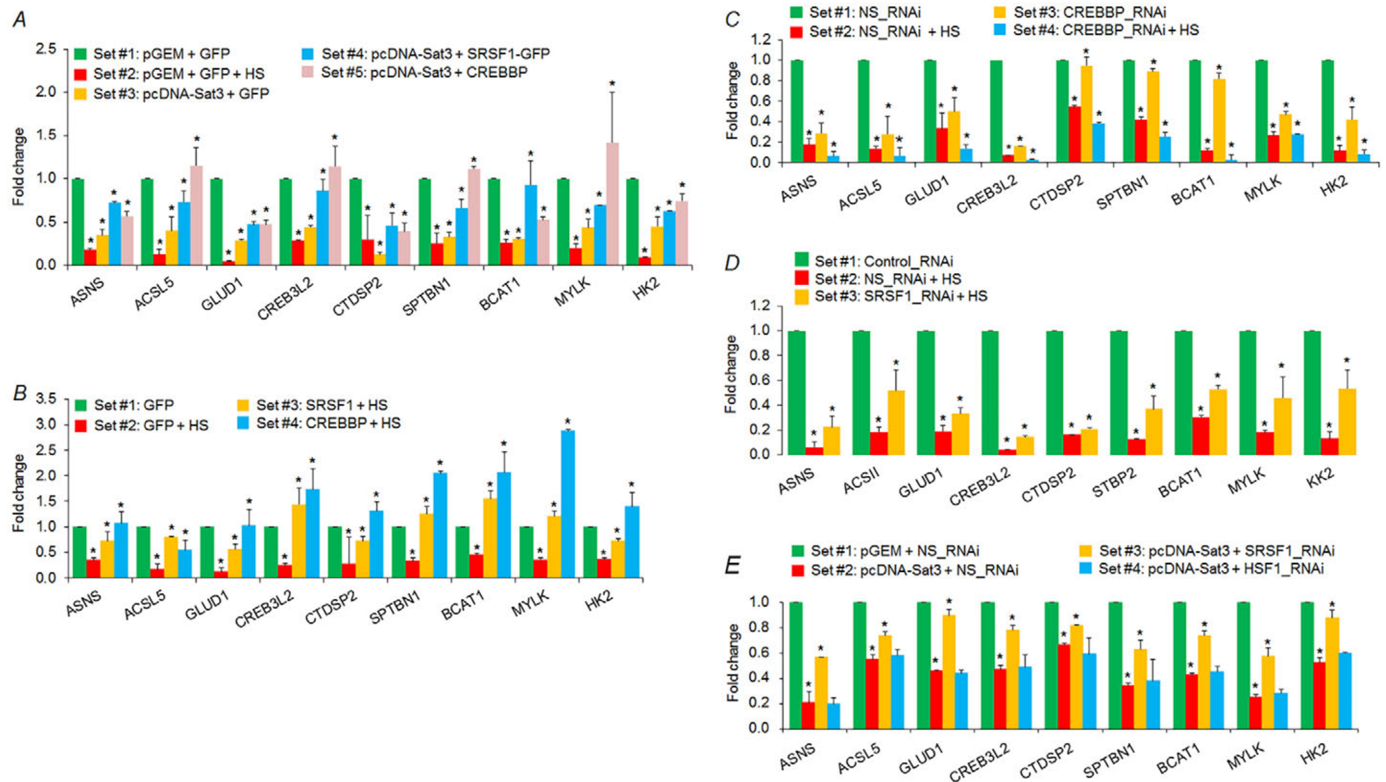

Fig. 5. Overexpression of Sat3-repeat-bearing transcripts bring about SRSF1-dependent transcriptional suppression of genes even when the cells are not exposed to a heat shock. (A) The cells were transfected with the indicated expression construct for the Sat3 repeat (mammalian expression construct pcDNA-Sat3 or the bacterial expression construct pGEM-Sat3) along with the constructs coding for GFP, CREBBP or SRSF1, and the expression level of nine genes was evaluated by the Fluidigm's microfluidic devices, as indicated. All values are normalized to the samples co-transfected with the pGEM-Sat3 and GFP expression constructs and not given a heat shock (HS) (Set #1). For calculating statistical significance, both Set #2 and Set #3 were compared with Set #1, whereas Set #4 and Set #5 were compared with Set #3. (B) Cells transfected with the expression constructs coding for GFP, CREBBP or SRSF1 were exposed or not exposed to HS as indicated, and the expression levels of the genes were evaluated by qPCR. All values are normalized to the samples co-transfected with the construct coding for GFP and not given HS (Set #1). For calculating statistical significance, Set #2 was compared with Set #1, whereas Set #3 and Set #4 were compared with the Set #2. (C) Cells transfected with the RNAi duplex for the knockdown of CREBBP were exposed or not exposed to HS as indicated, and the expression levels of the nine genes were evaluated by qPCR. All values are normalized to Set #1, and fold change in the expression level in the remaining sets was plotted. For calculating statistical significance, both Set #2 and Set #3 were compared with Set #1, whereas Set #4 was compared with Set #2. (D) Cells transfected with the RNAi duplex for the knockdown of SRSF1 were exposed or not exposed to HS as indicated, and the expression levels of the nine genes were evaluated by qPCR. All values are normalized to Set #1, and fold change in the expression level in the remaining sets was plotted. For calculating statistical significance, Set #2 was compared with Set #1, whereas Set #3 was compared with Set #2. (E) Bar diagram showing the fold difference in the expression levels of the indicated genes in cells transfected with pGEM-Sat3 or pcDNA-Sat3 along with siRNA for *SRSF1* or the *HSF1*-knockdown construct, as indicated. All values are normalized to Set #1, and fold change in the expression level in the remaining sets was plotted. For calculating statistical significance, Set #2 was compared with Set #1, whereas both Set #3 and Set #4 were compared with Set #2. For all panels (A-E), each bar represents mean values of three biological replicates, each of which were processed for two experimental replicates. Unpaired Student's *t*-test, $^{*}P\leq0.05$.

ncRNAs are essential for the cells to mount an effective HSR, and that the Sat3 transcripts possibly serve as a 'sink', as originally proposed (Jolly and Lakhotia, 2006), to recruit critical factors of the transcriptional machinery and thus aid in heat-shock-induced gene silencing – at least for the 14 genes tested in the present study. Chronic heat stress is known to induce cell death (Stankiewicz et al., 2009). Perhaps the prolonged ectopically expressed Sat3-repeat-bearing transcripts mimic such a chronic condition, and induce cell death.

Heat shock is known to alter the transcriptional status of the cell and much attention has been paid to the transcriptional upregulation of genes coding for the heat shock proteins, with their mechanisms being well elucidated (Akerfelt et al., 2010). However, the mechanism behind heat-shock-induced global transcriptional repression is not very well understood. One line of evidence suggests heat-shock-induced release of RNA polymerase II from the DNA as a possible mechanism (Hieda et al., 2005). Consistent with this notion, a causal role for Alu transcripts in lowering the affinity between the RNA polymerase complex and the promoter elements has been established (Allen et al., 2004; Mariner et al., 2008). Expression of Alu transcripts is regulated by RNA polymerase III and its level is known to go up during the heat shock (Li et al., 1999; Liu et al., 1995). By contrast, the expression of Sat3 transcripts is under the control of HSF1 and is RNA-polymerase-II-dependent (Jolly et al., 2004; Sengupta et al., 2009; Rizzi et al., 2004). Secondly, Sat3 transcripts are required only during a heat stress (Valgardsdottir et al., 2008). Long ncRNAs are thought to regulate cellular processes by serving as docking sites for proteins and protein complexes (Willingham et al., 2005; Mariner et al., 2008; Sasaki et al., 2009; Souquere et al., 2010). Our findings that the Sat3-mediated recruitment of transcription factors might underlie heat-induced transcriptional silencing of a set of genes studied uncover a newly identified role of these transcripts and mechanism of transcriptional control. Indeed, Sat3-positive nSBs were known to recruit several proteins, including the transcription factors HSF1

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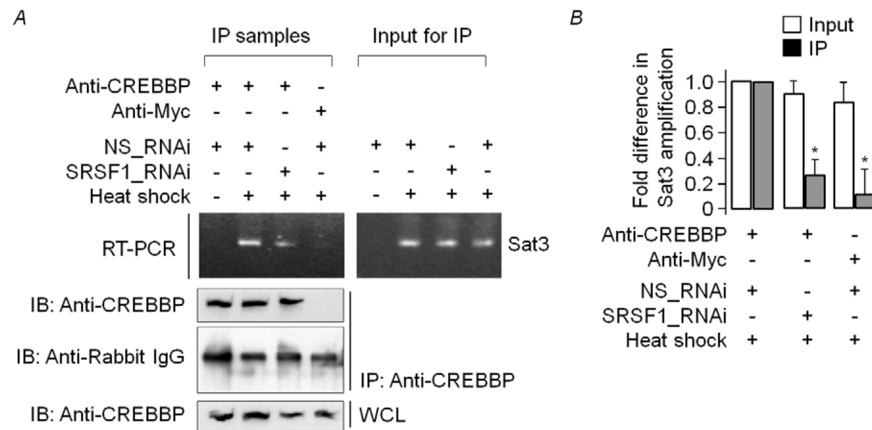

Fig. 6. SRSF1 is required for the recruitment of CREBBP to Sat3-positive nSBs. (A) RNA-immunoprecipitation assay (IP) establishing the presence of endogenous Sat3 transcripts in the immunoprecipitates pulled-down by anti-CREBBP. The cells were transiently transfected with the non-silencing RNAi duplex (NS_RNAi) or the RNAi for *SRSF1* and the cells were exposed or not exposed to heat shock, and processed for the IP using anti-CREBBP or the control antibody, anti-Myc (both raised in rabbit). RNA from the IP was processed for RT-PCR, and a semi-quantitative RT-PCR was carried out for the Sat3 transcripts. An aliquot of the pull-down samples were probed with anti-CREBBP to establish the CREBBP pull-down and with anti-rabbit IgG to serve as loading control. (B) Bar diagram representing the fold difference in the intensity of the RT-PCR amplicons, a representative image of which is shown in A. Each bar represents mean values of three biological replicates. WCL, whole-cell lysate. Unpaired Student's *t*-test, $^*P \le 0.05$.

and CREBBP, and RNA-binding proteins such as SRSF1 and hnRNPs (Jolly and Lakhotia, 2006). Although HSF1 is distinctly required for Sat3 expression, our observations that knockdown of CREBBP did not affect heat-shock-induced Sat3 expression was intriguing. On the contrary, loss of Sat3 transcripts led to the loss of CREBBP recruitment to the nSBs, suggesting a role of Sat3 transcripts as a scaffold for CREBBP's recruitment to nSBs, and that CREBBP is not required for Sat3 expression. The presence of SRSF1, a protein that is known to bind to Sat3 transcripts (Chiodi et al., 2004), might be crucial in order for other factors to be recruited on Sat3 transcripts, because CREBBP is dependent on the presence of SRSF1 for its colocalization with Sat3-positive nSBs. Thus, CREBBP's association with the nSBs could be a consequence and not a cause of Sat3 expression. It is interesting to note in this regard that loss of non-coding hsromega transcripts – the suggested functional homolog of Sat3 transcripts in *Drosophila* (Jolly and Lakhotia, 2006) – results in increased activity of CREBBP under neurological stress in the fly model of Huntington disease (Mallik and Lakhotia, 2010). Similar to Sat3, the *hsr-omega* transcripts in fly are enriched near their site of transcription, recruit several RNA-binding proteins, and these transcripts are essential for heat tolerance (Jolly and Lakhotia, 2006). Unlike Sat3, however, the hsromega transcripts are expressed in physiological conditions as well, but their expression level increases manifold upon exposure to a heat shock (Jolly and Lakhotia, 2006; Bendena et al., 1989), suggesting a similar mode of action for Sat3 transcripts in the heat shock response (Jolly and Lakhotia, 2006). Indeed, stress-induced ncRNAs are known in mammalian cells to 'immobilize' proteins within subnuclear structures and regulate the transcriptional process (Prasanth, 2012). For example, the lncRNA *NEAT1*, an essential component of the nuclear subcompartment known as paraspeckles, is known to be upregulated upon proteasomal blockade and to facilitate transcriptional repression by sequestering critical proteins to the paraspeckles (Hirose et al., 2014). A recent report demonstrates that the assembly of both paraspeckles and nSBs is compromised upon knockdown of essential components of the SWI/SNF chromatin remodeling complex, (Kawaguchi et al., 2015), suggesting a common pathway for the assembly of lncRNA-dependent nuclear bodies in mammalian cells. Intriguingly, a good number of nSBs formed by the overexpressed Sat3 transcripts localize closer to the Sat3 locus on chromosome 9, suggesting the possible presence of a cellular process that 'anchors' the Sat3 transcripts to their genomic locus. Indeed, lncRNAs are known to interact with DNA and serve as scaffolds to recruit protein complexes on the chromatin (Rinn and Chang, 2012).

CREBBP is a co-activator that is believed to form a complex with a variety of transcription factors that aid in positioning the complex near the promoters of the target genes (Sterner and Berger, 2000). This complex is believed to recruit general transcription factors and RNA polymerase II to activate the target genes (Vo and Goodman, 2001). More than 400 potential target sites for CREBBP are known in the human genome (Zhang et al., 2005); therefore, modulation of CREBBP activity is likely to have cascading effects on secondary and tertiary targets. Thus, our findings on CREBBP's recruitment to Sat3 transcripts suggest a global role of CREBBP in heat-shock-induced transcriptional suppression. Our assays are limited to 18 genes, among which only 14 (75%) of them showed an upregulation upon loss of Sat3 transcripts; the observed trend therefore could not be generalized and extrapolated to a global level. Nonetheless, our observation that either the loss of Sat3 transcript under heat stress, or the overexpression of CREBBP in cells maintained at physiological temperature, led to a significant increase in the expression level of the genes tested suggests that the availability of CREBBP could modulate the expression of the target genes (Fig. 8). Support for the model comes from the observation that the CREBBP occupancy rate was higher for genes that increased their expression upon partial loss of Sat3 transcript (Table S1). It is, however, interesting to note that the majority of CREBBP target genes seem to function in transcriptional regulation (Ramos et al., 2010). Thus, the 'availability' of CREBBP during heat shock could have a cascading effect on the transcriptional process. Therefore, it would be interesting to test for the altered expression levels of CREBBP targets in cells that are partially deficient for Sat3 transcripts and to compare with the CREBBP occupancy on their promoters. Pending such genome-wide studies, our results are strong enough to suggest that the heat-shock-induced recruitment of CREBBP onto the Sat3 transcripts could be one of the mechanisms by which the cell could bring about transcriptional suppression of at least a subset of genes.

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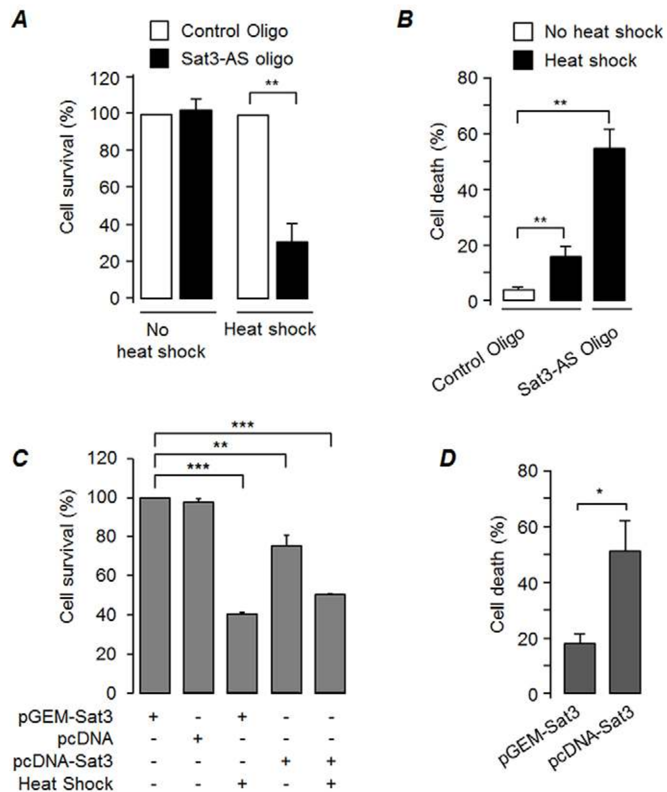

Fig. 7. Sat3 transcripts are required for the full protection against heat-shock-induced cell death. (A) Bar diagram showing loss in cell survival upon anti-sense (AS)-oligonucleotide-mediated knockdown of Sat3 transcripts during a heat shock or in the 'no heat shock' (control) conditions, as measured by an MTT assay. (B) Bar diagram showing frequency of cell death as measured by scoring abnormal/apoptotic nuclei in cells transfected with the AS oligos for the Sat3 or control oligos and exposed or not exposed to a heat shock. A representative image for the abnormal, apoptotic nuclei can be seen in Fig. S2A,E. (C) Bar diagram showing the survival of cells transiently transfected with Sat3-bearing mammalian (pcDNA-Sat3) or bacterial (pGEM-Sat3) expression constructs, or an empty vector (pcDNA), when exposed or not exposed to a heat shock. The cell survival was measured by an MTT assay. (D) Bar diagram showing the percent of apoptotic nuclei in cells transiently transfected with the Sat3-bearing mammalian (pcDNA-Sat3) or bacterial (pGEM-Sat3) expression constructs as indicated. Unpaired Student's *t*-test, *$P \leq 0.05$; **$P \leq 0.005$; ***$P \leq 0.0005$.

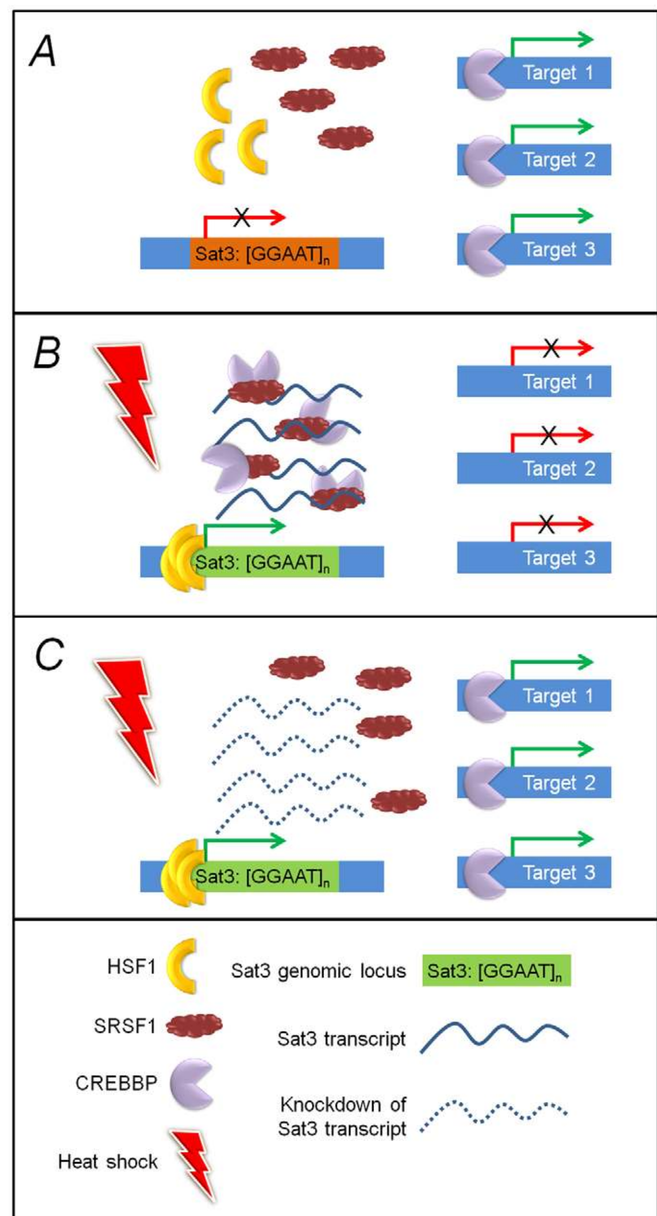

Fig. 8. Schematic diagram depicting the proposed model for the role of Sat3 transcripts in heat-shock-induced transcriptional repression. In unstressed cells (A), the Sat3 locus is transcriptionally silent and, hence, CREBBP and SRSF1 are available for the transcription of target genes. During a heat shock (B), HSF1 activates the transcription of Sat3 loci, leading to the formation of Sat3-transcript-dependent nSBs, which sequester CREBBP and SRSF1 and, as a consequence, there is a loss of transcription of target genes. However, the experimental loss of Sat3 transcripts in a heat-shocked cell (C) releases CREBBP and SRSF1 from nSBs, leading to the transcriptional activation of target genes even under the thermal stress.

The RNA-binding protein SRSF1 is a well-known splicing factor; therefore, it is intriguing to note that the loss of SRSF1 led to transcriptional repression during heat shock. Although this is likely to be an indirect effect, due to CREBBP not being able to be recruited to the Sat3 transcripts in the absence of SRSF1, it is intriguing to note that a couple of reports have also established a direct role for SRSF1 in transcriptional regulation. For example, the transcription factor PPARγ is known to interact with SRSF1 to form a functional complex to activate the target genes (Kim et al., 2009). Similarly, loss of SRSF1 was shown to result in RNA polymerase II accumulation on the genes and diminished transcription elongation (Lin et al., 2008), and SRSF1 was required for the transcriptional activation of promoter-associated nascent RNA (Ji et al., 2013). Thus, SRSF1-like serine- and arginine-rich (SR) proteins are believed to be involved in multiple levels of transcriptional control (Howard and Sanford, 2015). Therefore, a direct role for SRSF1 in Sat3-mediated transcriptional control could not be ruled out. Our observation that overexpression of SRSF1 enhanced the expression of the target genes tested, and conversely its loss results in a significant reduction in the expression of the same set of genes, strongly supports this notion. Because Sat3-positive nSBs are known to recruit several other factors (Jolly and Lakhotia, 2006), it would be interesting to test their contributions to the transcriptional process and beyond during the heat shock response. Taken together, our leads are strong enough to suggest that Sat3 transcripts are essential for the cells to provide full protection against heat-shock-induced cell death. Our results also uncover a newly identified insight into the regulatory role for the Sat3 transcripts in heat-shock-dependent transcriptional repression.

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MATERIALS AND METHODS

Cell culture, transfection and cell treatment

All experiments were carried out using the HeLa cell line obtained from the national repository, the National Centre of Cell Science, Pune, India. The cells were grown in Dulbecco's modified Eagle's medium (Sigma Aldrich Chemicals Pvt. Ltd., New Delhi, India) supplemented with 10% (v/v) fetal calf serum, 100 units/ml penicillin and 100 mg/ml streptomycin. Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific Pvt. Ltd., Mumbai, India) was used for transfection according to the manufacturer's protocol. For the heat shock treatment, cells grown on gelatin-coated dishes were allowed to float in a water bath maintained at 42°C for 1 h and were harvested at the end of the heat shock.

Knockdown approach

For the knockdown of Sat3 transcripts, a phosphorothioate-modified antisense oligo (5′-CCATTCCATTCCATTCCATT-3′) was used as reported (Valgardsdottir et al., 2005), and a non-specific oligo 5′-GTCTTCCAGCCACTGCTCAT-3 was used as a control, and the knockdown efficiency was evaluated by *in situ* hybridization and also by a semi-quantitative PCR. The *CREBBP* and *SRSF1* transcripts were knocked down using commercially available siRNA duplexes (cat. no. EHU075681 and EHU132201, respectively; Sigma Aldrich Chemicals Pvt. Ltd., India). The shRNA construct for the knockdown of *HSF1* was purchased from Open Biosystem, CO, and have been validated previously in our studies (Sengupta et al., 2011). The efficiency of knockout was confirmed by RT-PCR and immunoblots/FISH, as shown in Fig. S2A-C.

Expression constructs used

The Sat3 overexpression construct was created by subcloning the 158-bp fragment harboring the Sat3 repeat derived from the 9q12 locus and cloned in pGEM2-98 (a kind gift from Dr. Caroline Jolly, INSERM, France), into the mammalian expression construct pcDNA3.1. The expression construct coding for GFP-tagged SRSF1 (pGFPC1-SF2) was a kind gift from Dr. J. Caceres (Cold Spring Harbor Laboratory, NY). The pcDNA3β-FLAG-CBP-HA construct for the CREBBP expression was obtained from Addgene, MA (plasmid #32908).

Antibodies used

The following antibodies were used for the experiments: anti-CREBBP (cat. no. 7389S; Sigma Aldrich Chemicals Pvt. Ltd., India; dilution 1:100), anti-HSF1 (cat. no. 4356S; Cell Signaling Technology, MA; dilution 1:400); anti-SF2/ASF (cat. no. 32-4600; Invitrogen Inc.; dilution 1:100); anti-digoxin (cat. no. 200-062-156; dilution 1:250), and all secondary antibodies were from Jackson ImmunoResearch, PA. The specificity of the antibodies was validated by the RNAi-mediated knockdown approach (see Fig. S2C).

Immunocytochemistry

HeLa cells grown on gelatin-coated sterile glass coverslips were processed for immunofluorescence microscopy, as reported earlier (Sengupta et al., 2009). Fluorescence images were captured using an epifluorescence microscope (Axiovision) with the ApoTome module attached (Carl Zeiss, Bangalore, India) and the images were assembled using Adobe Photoshop.

Image quantification

Quantification for the colocalization analysis was performed for about 30 cells for each set by the Green and Red Puncta Co-localization macro of ImageJ (created by D. J. Swiwarski, modified by R. K. Dagda and co-approved by Charleen T. Chu) (Pampliega et al., 2013).

Fluorescence RNA and DNA *in situ* hybridization (FISH) and immuno-FISH

The 158-bp Sat3 repeat cloned in the pGEM2-98 construct was PCR-amplified and *in vitro* transcribed to generate anti-sense probes using the digoxigenin (DIG) labeling kit (Roche Products Pvt. Ltd, Mumbai, India). Fixed cells were processed for RNA *in situ* hybridization and immuno-FISH as reported earlier (Sengupta et al., 2009). For the combined DNA–RNA FISH, cells were fixed and processed first for RNA *in situ* hybridization as discussed above. Post-hybridization, the RNA signals were fixed using 4% paraformaldehyde and the slides were treated with RNAse A. The cells were then denatured at 73°C in a denaturation buffer (70% formamide, 2× SSC, 50 mM sodium phosphate, pH 7.0) for 5 min, followed by dehydration in ice-cold 70% ethanol for 2 min. Dehydration of the cells was continued with 80% and 100% ice-cold ethanol for 2 min each and then the slides were allowed to air-dry. A chromosome-9 DNA probe specific to the 9q21 region lacking the Sat3 repeats and fluorescently labeled with green 5-fluorescein dUTP (Empire Genomics, New York, NY; cat. no. CHR9-10-GR) was denatured and hybridized. The cells were processed for post-hybridization washes and detection as per the manufacturer's protocol.

Cell viability assays

Two different methods were used to assess the cell viability, as reported earlier (Sengupta et al., 2011). For the MTT method, cells were incubated with 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 6 h at 37°C prior to harvesting and the metabolic product measured at 570 nm using a spectrophotometer. For the cell-counting method, transfected cells (GFP-positive) were scored for the fragmented nuclei morphology, as shown in Fig. 7B,D. Cell counting was done in a blinded manner.

Nuclear/cytoplasmic fractionation

The nuclear and cytoplasmic fractions of cells were obtained using the nuclear/cytosol fractionation kit (BioVision Inc., CA), as per the protocol of the manufacturer.

RNA isolation and RT-PCR

For the heat shock experiments, the total RNA was extracted from the nuclear fraction using Trizol reagent (Invitrogen, USA). Around 5 µg of total RNA was used to synthesize cDNA and the efficiency of cDNA was checked by RT-PCR with primers for the amplification of housekeeping genes. The primer sequences used for the PCR are given in the Table S2. The oligonucleotides were commercially synthesized (Sigma Aldrich Chemicals Pvt. Ltd., India).

Quantitative PCR using Fluidigm qPCR methodology

Quantitative PCR (qPCR) was carried out using the Biomark™ HD system with a fast ramp rate (5.5°C/s) on a 48.48 Dynamic Array™ integrated fluidic circuit (IFC) from Fluidigm, CA (Livak et al., 2013). The cDNA used for the qPCR was checked for its efficiency using housekeeping genes using a conventional PCR. Prior to the qPCR reactions, the specific target amplification (STA) protocol (manufacturer's protocol) was used to increase the number of copies of target cDNA. Post-STA, samples were treated with exonuclease 1 to eliminate the carryover of unincorporated primers. The resultant product was diluted tenfold for making pre-mix to be used for qPCR using the Kapa SYBR FAST (Kapa Biosystems). Rox dye was used as a passive reference with SYBR as the probe type. The data was collected using Biomark HD data collection software v.3.1.2 and analysis was done using Fluidigm Real-Time PCR analysis software v.3.0.2.

RNA immunoprecipitation (RIP)

The RIP protocol was adapted from Rinn et al. (2007) as follows: cells were trypsinized and resuspended in 2 ml PBS, 2 ml nuclear isolation buffer (1.28 M sucrose; 40 mM Tris-HCl pH 7.5; 20 mM $MgCl_2$; 4% Triton X-100) and 6 ml water on ice for 20 min (with frequent mixing). Nuclei were pelleted and resuspended in 1 ml RIP buffer [150 mM KCl, 25 mM Tris pH 7.4, 5 mM EDTA, 0.5 mM DTT, 0.5% NP40, 1× PIC, 1× PhIC, 100 U/ml RiboLock RNase Inhibitor (Fermentas)]. Resuspended nuclei were split into two fractions of 500 µl each (for Mock and IP) and were mechanically sheared. Nuclear membrane and debris were pelleted by centrifugation. Antibody to CREBBP (cat. no. 7389S; Sigma) or the Myc epitope (Mock IP, Cell Signaling Technology, cat. no. 2272S) was added to the supernatant and incubated overnight at 4°C with gentle rotation. Thirty-five µl of pre-cleared protein A/G beads were then added and incubated for 5 h at 4°C with gentle rotation. Beads were pelleted at 600 ***g*** for 1 min, the supernatant was removed and beads were washed in 500 µl RIP buffer three times followed

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by one wash in PBS. Beads were then re-suspended in 1 ml of Trizol. Co-precipitated RNAs were isolated and RT-PCR for the Sat3 mRNA was performed.

Statistical analysis

An unpaired Student's *t*-test was used for analysis of differences between groups. Error bars represent mean±s.d. A *P*-value of ≤ 0.05 was considered as statistically significant.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

A.G., S.S., R. Pandey, R. Parihar and G.C.M. performed the experiments; A.G., S.S., R. Pandey, M.M. and S.G. designed and analyzed the experiments; A.G., S.S. and S.G. conceived the study; A.G. and S.G. co-wrote the paper. S.G. coordinated the study. All authors analyzed the results and approved the final version of the manuscript.

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Supplementary information

Supplementary information available online at http://jcs.biologists.org/lookup/doi/10.1242/jcs.189803.supplemental

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