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Trans-activation of small EDRK-rich factor 2 (*SERF2*) promoter by Heat Shock Factor 1

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ABSTRACT

Heat shock response is an adaptive mechanism of cells characterized by rapid synthesis of a class of proteins popularly known as heat shock proteins (HSPs) by heat-induced activation of Heat Shock Factor 1 (HSF1). In course of our earlier study to show that HSF1 regulates transcription of HYPK (Huntingtin Yeast two-hybrid protein K), a chaperone-like protein, we observed presence of few other genes within 10 kb of *HYPK* promoter. In an attempt to understand whether adjacent genes of *HYPK* are co-regulated, we identified that *SERF2* (small EDRK-rich factor 2), an upstream neighboring gene of *HYPK*, is also regulated by heat stress and HSF1. We also showed that *SERF2* promoter can be *trans*-activated by HSF1 due to the presence of functional heat shock element (HSE). Strikingly, *HYPK* is linked with *SERF2* through a Conjoined Gene (CG) albeit the respective proteins have opposite effect on mutant Huntingtin aggregates and subsequent toxicity. Our study provides the first report on regulation of *SERF2* expression and thereby depicts a paradigm where two parent genes of a CG are regulated by a common transcription factor despite the fact that they code for proteins having opposite cellular function in a given context.

1. Introduction

Heat Shock Factor 1 (HSF1) is an evolutionary conserved transcription factor capable of inducing transcription of heat shock protein (HSP)-coding genes in response to stress by directly binding to specific DNA sequences (heat shock elements)-a process broadly known as heat shock response [1,2]. We earlier showed that Huntingtin (HTT) interacting protein HYPK is induced by heat shock and transcriptionally regulated by HSF1 [3]. HYPK is an intrinsically unstructured protein [4] and can suppress aggregates of mutant HTT and subsequent toxicity by virtue of its chaperone-like activity [5,6].

The genomic organization of *HYPK* revealed presence of putative promoter region of two other protein-coding genes *viz. SERF2* and *SERINC4* within10kb upstream or downstream of human *HYPK* promoter. Intriguingly, a Conjoined Gene (CG) containing exons of *SERF2* and *HYPK* was also identified in the vicinity of *HYPK* promoter. We attempted to understand whether any of the neighboring genes of *HYPK* are co-regulated with heat-inducible gene *HYPK* and subsequently identified *SERF2*, an upstream neighboring gene of *HYPK*, as a heat-inducible gene and novel transcriptional target of HSF1. Our study shows that two neighboring genes *HYPK* and *SERF2* are co-regulated at transcription level.

2. Materials and methods

2.1. Antibodies

Anti-HSF1 and anti-acetylated histone H4 antibodies were obtained from Abcam. Anti-RNA polymerase II antibody was purchased from Imgenex and anti-SERF2 antibody was purchased from Novus Biologicals. Anti-β-actin antibody was procured from Sigma. Anti-mouse and anti-rabbit secondary antibodies conjugated with horseradish peroxidase were purchased from Bangalore Genei (India).

2.2. Cell culture and treatments

HeLa cells were obtained from National Cell Science Centre, India and grown in Minimal Essential Medium (Himedia) supplemented with 10% fetal bovine serum (Biowest) at 37 °C in 5% CO_2 atmosphere under humified conditions. To induce heat shock

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Abbreviations: CG, Conjoined Gene; HS, heat shock; HSE, Heat Shock Element; HSF1, Heat Shock Factor 1; HYPK, Huntingtin Yeast-Two-Hybrid Protein K; SERF2, small EDRK-Rich Factor 2

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response, cells were subjected to standard heat shock treatment at 42 °C for 60 min and kept at 37 °C for 4 h or unless indicated otherwise. Cells were treated with 5 μM 17-AAG (Sigma Chemicals) or DMSO (control) for 24 h. Transfection of cells was performed using Lipofectamine 2000 (Invitrogen).

2.3. *Construction of plasmids*

Human *hsf1* gene cloned in pcDNA3.1 vector was kindly gifted by Dr. Richard Voellmy (HSF Pharmaceuticals, Switzerland). Empty pSUPER vector and pSUPER constructs for HSF1 siRNA and scrambled RNA were provided by Dr. L Sistonen (Åbo Akademi University, Finland).

For luciferase reporter assay, *SERF2* promoter encompassing putative HSF1-binding site (−535 to −526) was cloned in pGL3 vector (Promega) between the restriction sites of MluI and BglII (NEB) and designated as SERF2_ups. Primer sequences used for cloning are given in Supplementary Table S1. Promoter sequence of human *hsp70* gene containing functionally active HSEs (designated as Hsp70_ups) was used as positive control as described previously [3].

2.4. *Site-directed mutagenesis*

The HSE present in *SERF2* promoter was destroyed by mutagenesis. Three conserved nucleotides (‘GAA’ at position −534 to −532) from HSE present in *SERF2* promoter were deleted from the parental (wild-type) promoter sequence (SERF2_ups) and the resulting mutated promoter was designated as SERF2_ups_ΔHSE. QuickChange site-directed mutagenesis kit from Stratagene was used for mutagenesis. Primer sequences are given in Supplementary Table S2.

2.5. *Luciferase Assay*

Luciferase assay was performed as earlier described [7,8]. Briefly, HeLa cells expressing empty pGL3 vector or other reporter constructs (600 ng) and empty pcDNA vector or HSF1-pcDNA (400 ng) were either grown at 37 °C (control) or subjected to heat shock at 42 °C for 60 min followed by recovery at 37 °C for 4 h. Twenty four hours after transfection, luciferase assay was carried out using luciferase reporter assay system (Promega) according to manufacturer's protocol and detected by Sirius Luminometer (Berthold Detection Systems). The experiments were carried out in triplicates.

2.6. *RNA preparation*

Total RNA was prepared from cultured cells using TRIzol reagent (Invitrogen) following manufacturer's protocol.

2.7. *Semiquantitative RT-PCR (sqRT-PCR) and Quantitative Real-time PCR (qRT-PCR)*

Two microgram of total RNA was reverse transcribed using random hexamer primer and MuLv-reverse transcriptase (Fermentas). Semiquantitative RT-PCR (sqRT-PCR) was carried out using Red TaqDNA polymerase (Bioline). Quantitative Real-time PCR (qRT-PCR) was carried out using 7500 Real time PCR system (Applied Biosystems) as described in [9]. Fold changes were calculated in accordance with SDS software V2.0 (Applied Biosystems). Primer sequences used for gene expression by qRT-PCR are given in Supplementary Table S3.

2.8. *Knockdown of HSF1 by siRNA*

The method used for siRNA-mediated knockdown of HSF1 was as described in [10]. Knockdown of endogenous HSF1 in HeLa cells was earlier confirmed by sqRT-PCR and immunoblot [3].

2.9. *Western blot*

Western blot was performed as described previously [3]. Membrane was probed with anti-SERF2 antibody. β-actin was used as loading control. Integrated optical density (IOD) of each band was calculated using Image Master VDS software (Amersham Biosciences).

2.10. *Chromatin immunoprecipitation (ChIP) and Transient ChIP assay*

Chromatin immunoprecipitation (ChIP) was performed in HeLa cells as described earlier [3,8]. Briefly, HeLa cells expressing endogenous HSF1 were either grown unstressed (No HS) or exposed to heat shock at 42 °C for 1 h, followed by recovery at 37 °C for 4 h. Then cells were cross-linked with 1.1% formaldehyde for 10 min at room temperature. The cross-linking reaction was stopped by 125 mM glycine. Cells were washed with ice-cold PBS and harvested at 300*g* for 3 min at 4 °C. Cytosol was extracted with cytoplasm extraction buffer (20 mM HEPES, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA and 1 mM PMSF). Nuclei were harvested at 13,000*g* for 10 min at 4 °C, and the pellet was resuspended in breaking buffer (50 mM Tris–HCl, pH 8.0, 1 mM EDTA, 150 mM NaCl, 1% SDS and 2% Triton-X-100) and sonicated twice (two pulses of 10 s each). Contents were then centrifuged. Triton buffer (50 mM Tris–HCl, pH 8.0, 1 mM EDTA, 150 mM NaCl, and 0.1% Triton-X-100) was added to the nuclear extract. The immunoprecipitation reaction was carried out using anti-HSF1, anti-RNA polymerase II and anti-acetylated histone H4 (AcH4) antibodies followed by the addition of BSA-soaked Protein G-agarose beads. The immunoprecipitated complex was washed, followed by decross-linking, phenol-chloroform extraction and ethanol precipitation of the DNA. Amplification of the eluted DNA was carried out by semi-quantitative (sqRT-PCR) and quantitative RT-PCR (qRT-PCR) using primers specific for human *SERF2* promoter. A portion of the genome having no putative HSF1-binding site was amplified along with *SERF2* promoter and this non-specific sequence (NS seq) was used as control. PCR amplification of input and immunoprecipitated DNA was carried out using primers flanking the putative HSF1-binding site present in *SERF2* promoter.

For transient ChIP assay, HeLa cells were transiently transfected with SERF2_ups, SERF2_ups_ΔHSE and Hsp70_ups constructs and 24 h after transfection, cells were either kept unstressed or exposed to standard HS treatment. Cells were then cross-linked, harvested and nuclear fraction was prepared and sonicated as described for ChIP assay. Immunoprecipitation was done using anti-HSF1 antibody and protocol used for ChIP assay was followed until elution of input and immunoprecipitated DNA. Next, instead of using primers specific for upstream sequence of human *SERF2* and *hsp70* promoter for amplification of DNA, a special pair of primers was used. The forward primer was specific for human *SERF2* or *hsp70* promoter whereas the reverse primer was specific for the firefly (*Photinus pyralis*) *luciferase* gene present in the plasmid (pGL3 basic vector) backbone. PCR amplification of input and immunoprecipitated DNA using this primer pair ensures selective amplification of the synthetic or recombinant *SERF2* and *hsp70* promoter cloned in pGL3 basic vector, leaving native *SERF2* and *hsp70* promoter in the genome unamplified. Primers used for ChIP and transient ChIP assays are given in Supplementary Table S4.

2.11. Use of databases and web tools

Primers used for cloning were designed using Primer3 software (http://primer3.ut.ee/), whereas primers used for gene expression study (semiquantitative RT-PCR and quantitative Real-time PCR) and Chromatin Immunoprecipitation or Transient Chromatin Immunoprecipitation were designed by online software Primer Express (http://home.appliedbiosystems.com/support/tutorials/taqman/taqman_probes_121502.cfm). Primers used in site-directed mutagenesis were designed by Primer X software (http://www.bioinformatics.org/primerx/). UCSC Genome Browser (https://genome.ucsc.edu/) was used for identifying promoter or other regulatory regions of respective genes. In-house search tool (http://www.bioinformatics.org/grn/npb1) was used for identifying putative HSF1-binding site in *SERF2* promoter. All information about conjoined gene was retrieved from ConjoinG database (http://metasystems.riken.jp/conjoing/index).

2.12. Statistical analysis

For statistical analysis, unpaired *t* test was performed to compare the means of two experimental groups using the software GraphPad QuickCalcs. *Error bars* indicate ± SD. The statistical significance level between various experimental pairs is indicated (*, $p < 0.05$;**,$p < 0.01$;***,$p < 0.001$).

3. Results and discussion

3.1. HYPK and its adjacent genes in human genome

In an interesting study conducted on yeast, Arnone et al. observed that among the heat shock genes, immediately adjacent gene pairs often exhibit a tighter degree of transcriptional co-regulation than the genes distantly located across the genome [11]. *HYPK* is induced by heat shock and transcriptionally regulated by HSF1 [3]. To investigate whether regulation of adjacent genes of *HYPK* are similar to that of *HYPK*, we used UCSC Genome Browser (https://genome.ucsc.edu/) and observed presence of three protein-coding genes, namely *SERF2*, *SERINC4* and *MFAP1* within 10 kb of *HYPK* promoter (Supplementary Figs. S1 & S2). Of these, the putative promoter of *MFAP1*, as revealed by UCSC, is beyond 10 kb of *HYPK* promoter (Supplementary Table S5) and hence it was excluded from further analysis. In addition to these protein-coding genes, one Conjoined Gene (CG) (*SERF2-C15orf63*) and microRNA (miR-1282) gene were also present in the vicinity (10 kb) of *HYPK* promoter (Supplementary Figs. S1 & S2). It was further noted that direction of *HYPK* transcription is same as that of *SERF2* and the CG while opposite to that of *SERINC4* and miR-1282 (Supplementary Figs. S1 & S2).

Interestingly, apart from being a neighboring gene of *HYPK*, *SERF2* is also linked to *HYPK* through the CG *SERF2-C15orf53* (Supplementary Figs. S1 & S2). A CG is defined as a gene which gives rise to transcripts by combining at least part of one exon from each of two or more parent genes, which lie in same orientation on the same chromosome and may translate independently into different proteins [12] (Supplementary Fig. S3). At present, about 800 CGs have been identified in the entire human genome [12; 13; 14; 15]. Two parent genes of the CG *SERF2-C15orf63* are *SERF2* (5′ parent gene) and *HYPK* (previously known as *C15orf63*; 3′ parent gene). The CG is about 10 kb long and encompasses the entire genomic length of two parent genes *viz.*, *SERF2* and *HYPK* (Supplementary Table S5**)**. From UCSC database, it appears that the putative promoter of the CG overlaps with *SERF2* promoter **(Supplementary** Fig. S1, Supplementary Table S5). Notably, presence of this CG is also documented in ConjoinG, a database of around 800 CGs identified in human [12], having the symbol *SERF2-HYPK* and ConjoinG ID CGHSA0117 (Supplementary Fig. S5). It was first identified by Akiva and his colleagues [13] without any experimental validation. Later, one of the two transcripts (AK095876) of this CG was reported to be expressed in pancreas [12]. The function and regulation of this CG is yet unknown.

Therefore, *SERF2* is an upstream neighboring gene of *HYPK* and also connected to *HYPK* via a CG. Intriguingly, an earlier study showed that overexpression of SERF2 increases aggregates of mutant HTT and cell death and knockdown of SERF2 has opposite effect in mammalian cells [16]. On the other hand, HTT-interacting protein HYPK can suppress aggregates of mutant HTT and subsequent toxicity by virtue of its chaperone-like activity [5]. Therefore, SERF2 and HYPK have opposite functional effect on mutant HTT aggregates and toxicity. We attempted to determine whether any of the two neighboring genes *(SERF2 and SERINC4)* are co-regulated with *HYPK*.

3.2. Hyperthermia increases SERF2 expression in HeLa cells

To determine the effect of thermal stress on *SERF2* and *SERINC4* expression, HeLa cells were either grown at 37 °C (control) or were exposed to standard HS treatment (HS at 42 °C for 1 h followed by recovery at 37 °C for different time points). Total RNA was extracted from each sample and analyzed for *SERF2*, *SERINC4* and *β-actin* expression by quantitative RT-PCR (qRT-PCR).

As evident from Fig. 1A, heat shock-driven induction of *SERF2* expression was first detected during 2 h recovery ($p=0.003$, $n=3$) and continued till 4 h recovery ($p<0.001$, $n=3$), followed by a significant drop during 6 h recovery ($p=0.002$, $n=3$). *SERINC4*, on the other hand, showed no induction in response to HS treatment (Fig. 1B). We used *hsp70*, a *bona fide* transcriptional target of HSF1, as a marker of effective induction of heat shock response pathway. As expected, HS treatment resulted in robust induction of *hsp70* in HeLa cells (Fig. 1C), suggesting that the treatment was adequate to induce heat shock response in host cells. Notably, upon same HS treatment, *HYPK* expression was significantly induced in HeLa cells [3]. Perplexingly, *SERF2* and *HYPK* exhibited similar response in heat-shocked cells in spite of the fact that the respective proteins have opposite effect on mutant HTT aggregates and toxicity. We failed to detect the expression of the CG in unstressed and stressed HeLa cells (data not shown), possibly due to its faster degradation or tissue-specific expression.

To determine whether the effect of hyperthermia on *SERF2* transcript holds true also at the protein level, SERF2 expression was measured by immunoblot in all unstressed and stressed samples described above. As presented in Fig. 1**D**, no significant change in SERF2 protein level was observed during heat shock and subsequent recovery time. It is difficult to explain this observation at this stage of our knowledge. We speculate that the basal SERF2 level might be sufficient for its yet unexplored function(s) in stressed cells. Further investigation on *in vivo* functions of SERF2 and regulation of its metabolism in cells is required to draw definitive conclusions.

3.3. Identification and functional validation of Heat Shock Element (HSE) present in SERF2 promoter

Our observation that *SERF2* is transcriptionally induced by heat shock motivated us to investigate the molecular mechanism underlying such induction. To understand whether HSF1, the evolutionary conserved transcription factor and master regulator of heat shock proteins, has any effect on *SERF2* expression, *SERF2* promoter was searched for presence of any putative HSE using the search tool described earlier [17]. Our search identified a putative HSE

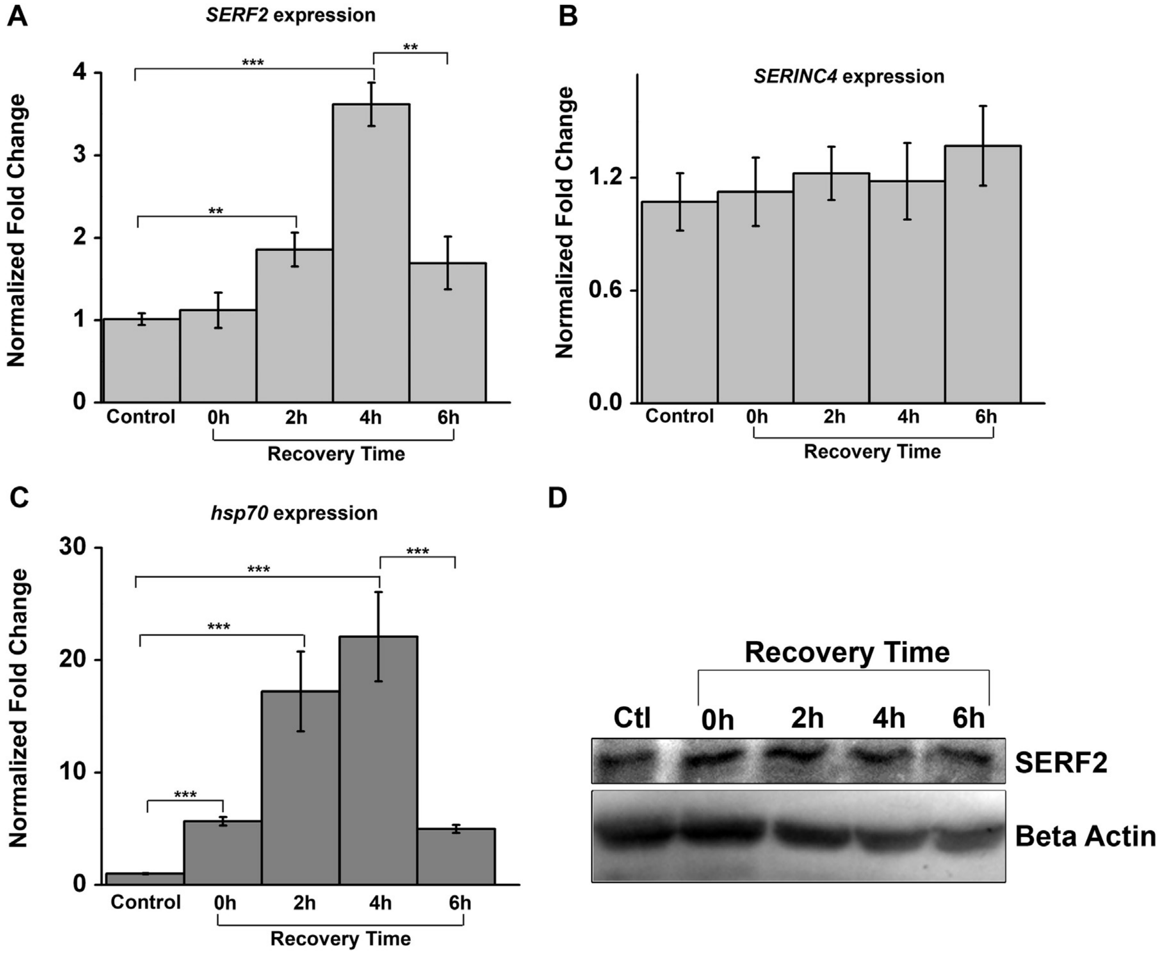

Fig. 1. Hyperthermia increases SERF2 expression in HeLa cells. Bar graph representative of three independent experiments (n=3) for qRT-PCR of **(A)** *SERF2*, **(B)** *SERINC4* and **(C)** *hsp70* expression in HeLa cells undergoing no heat shock (HS) treatment (control) or subjected to HS treatment at 42 °C for 60 min followed by recovery at 37 °C for 0, 2, 4 and 6 h. Expression of *β-actin* was taken as endogenous control. **(D)** Western Blot analysis for the expression of SERF2 in three independent experiments (n=3) in HeLa cells in absence or presence of HS treatment. β-actin was used as loading control. *Error bars* indicate ± SD. The statistical significance level between various experimental pairs is indicated (*,$p < 0.05$;**,$p < 0.01$;***,$p < 0.001$).

(AGAAGGGTCG) at −535 to −526 region of human *SERF2* promoter. To verify the functionality of this HSE, part of *SERF2* promoter encompassing the HSE was cloned in pGL3 reporter vector and designated as SERF2_ups. A mutant *SERF2* promoter was also generated by deleting two consensus nucleotides of HSE (‘G’ at position −534 and ‘A’ at position −533) by mutagenesis and the mutant promoter was designated by SERF2_ups_ΔHSE. A part of *hsp70* promoter carrying multiple functional HSEs (designated as Hsp70_ups) was used as a positive control as also used earlier [3,7].

HeLa cells were transfected with empty pGL3 vector (control), SERF2_ups, SERF2_ups_ΔHSE and Hsp70_ups and cells were either grown at 37 °C or were allowed to undergo standard HS treatment. Luciferase assay performed 24 h after transfection clearly showed induced reporter activity of SERF2_ups construct (p=0.006, n=3) whereas SERF2_ups_ΔHSE remained unaffected by thermal stress (Fig. 2**A**), indicating that heat shock could specifically induce *SERF2* promoter fragment. To determine the direct effect of HSF1 on *SERF2* promoter, HeLa cells were transfected with aforementioned reporter constructs together with empty pcDNA vector (control) or HSF1-pcDNA and luciferase activity of reporter constructs was measured in presence of standard HS treatment. Result showed that ectopic HSF1 upon heat shock could increase reporter activity of SERF2_ups (p=0.005, n=3) in HeLa cells (Fig. 2**B**). The mutant promoter (SERF2_ups_ΔHSE) having deleted HSE showed no response to ectopic HSF1 (Fig. 2**B**). Not surprisingly, reporter activity of Hsp70_ups was induced in response to thermal stress as well as exogenous HSF1 (Fig. 2**, A and B**). Our finding thus showed that *SERF2* promoter fragment containing HSE is responsive to both heat shock as well as exogenous HSF1. It should be noted here that we cloned about one third of the ~1600 bp long *SERF2* promoter as predicted from UCSC Genome Browser. No other putative HSE was identified in the entire *SERF2* promoter; however, it can be *trans*-activated by other transcription factors through distinct regulatory elements located anywhere in the entire *SERF2* promoter.

We earlier reported that human *HYPK* promoter is similarly *trans*-activated by HSF1 in response to same HS treatment in HeLa cells [3]. However, both thermal stress and ectopic HSF1 had stronger effect on *HYPK* promoter (induced by ~4.6 and ~4.3 fold respectively) [3] compared to *SERF2* promoter (~2.6 and ~2.4 fold respectively) (Fig. 2, **A and B**). This quantitative difference in the extent of induction of two promoters is possibly due to the difference in the composition of two HSEs present in respective promoters. The HSE responsible for heat-induced activation of *HYPK* promoter is composed of three tandem copies of the pentameric motif (nGAAn) [3], whereas the HSE present in *SERF2* promoter contains two copies of the consensus motif which is considered as the minimal requirement for inducible binding of HSF1 [18; 19]. This might have contributed to the weaker induction of *SERF2* promoter compared to *HYPK* promoter in response to thermal stress. Since expression of a single gene is often regulated

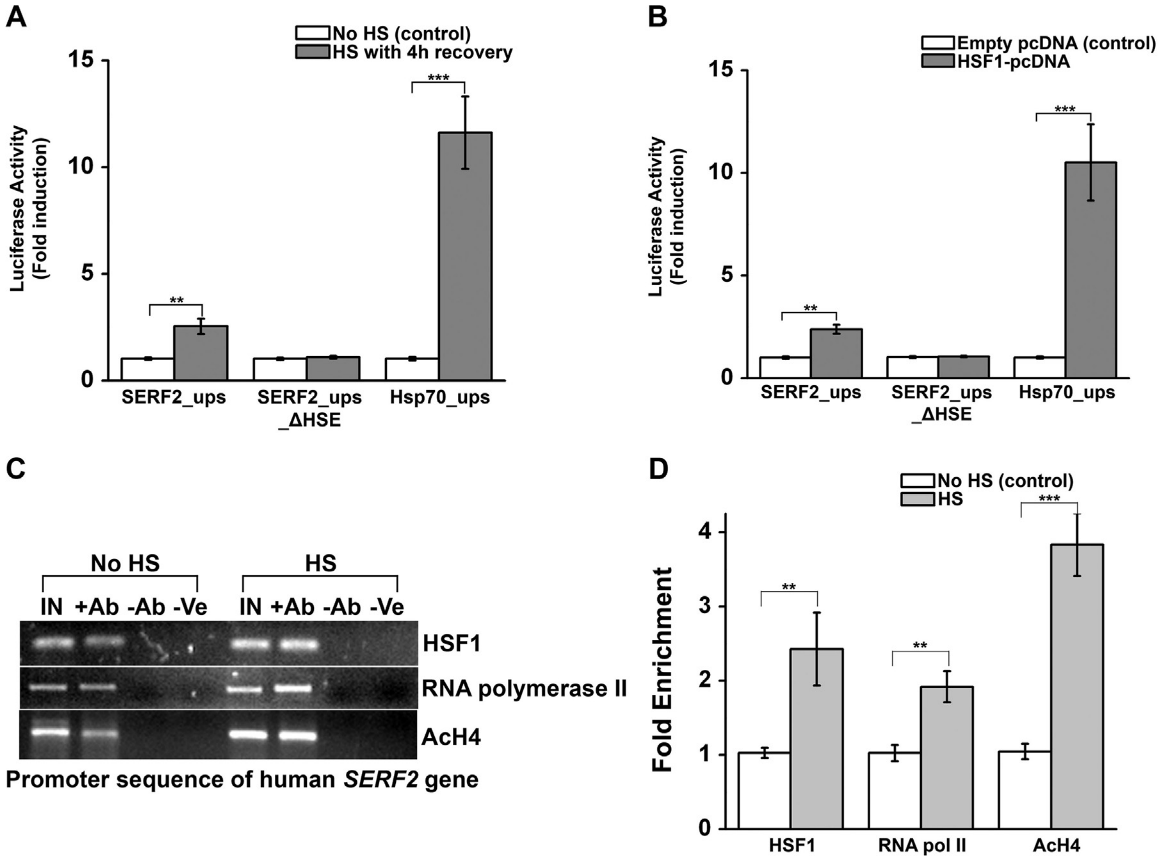

Fig. 2. Functional validation of the HSE present in *SERF2* promoter. **A.** Luciferase reporter assay (n=3) of different reporter constructs (SERF2_ups, SERF2_ups_ΔHSE and Hsp70_ups) in HeLa cells undergoing no HS treatment (control) and standard HS treatment. **B.** Luciferase reporter assay (n=3) of three reporter constructs mentioned in A in HeLa cells transiently transfected with empty pcDNA vector (control) and HSF1-pcDNA in presence of standard HS treatment. **C.** Chromatin immunoprecipitation showing *in vivo* interaction of HSF1 and RNA polymerase II with the *SERF2* promoter and acetylation of histone H4 (AcH4) at the same site in HeLa cells in absence or presence of HS treatment. Input and immunoprecipitated DNA was PCR-amplified using primers flanking the HSF1-binding site present in *SERF2* promoter. Lane IN: PCR amplification was carried out using DNA isolated from HeLa cells subjected to no HS or HS treatment. Lane +Ab: PCR amplification was carried out using chromatin immunoprecipitated by anti-HSF1, anti-RNA polymerase II and anti-AcH4 (acetylated histone H4) antibodies. Lane IgG: PCR amplification was carried out using chromatin immunoprecipitated by IgG alone. Lane –Ve: PCR amplification was carried out without adding any template DNA. **D.** Quantitative analysis of ChIP assay. DNA from different samples described in C was amplified by qRT-PCR using mouse *SERF2* promoter-specific primers. The immunoprecipitated DNA was normalized by respective input DNA in each sample and fold enrichment was calculated by considering the normalized immunoprecipitated DNA in unstressed cells (control) as 1. *Error bars* indicate ± SD. The statistical significance level between various experimental pairs is indicated (*,$p < 0.05$;**,$p < 0.01$;***,$p < 0.001$).

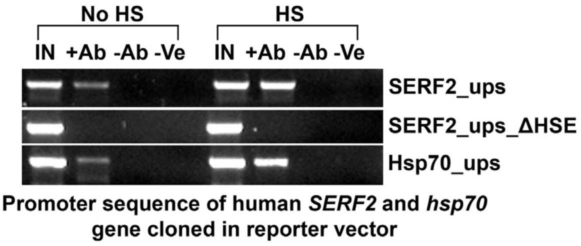

Fig. 3. *In vivo* interaction of HSF1 with recombinant *SERF2* promoter. Transient chromatin immunoprecipitation assay showing *in vivo* interaction of HSF1 with *SERF2* and *hsp70* promoter cloned in pGL3 vector. HeLa cells were transiently transfected with SERF2_ups, SERF2_ups_ΔHSE and Hsp70_ups constructs and transfected cells were either left untreated (no HS treatment) or subjected to HS treatment. Immunoprecipitation was carried out using anti-HSF1 antibody and precipitated DNA was PCR-amplified using primers described in Methods section. The lane markings are as described in Fig. 2C.

by the interplay of many transcription factors and co-factors, the quantitative difference between induction of *HYPK* and *SERF2* promoter could also be due to other regulatory proteins.

To further understand the regulation of *SERF2* promoter by HSF1, we performed chromatin immunoprecipitation (ChIP) in HeLa cells in presence or absence of HS treatment. As demonstrated in Fig. 2**, C and D**, heat shock increased the occupancy of HSF1 (p=0.008, n=3) in the HSE present in *SERF2* promoter. It was accompanied by simultaneous increase in the occupancy of RNA polymerase II (p=0.003, n=3) at the same locus (Fig. 2**, C and D**), strengthening our notion that *SERF2* is transcriptionally regulated by HSF1. Interestingly, ChIP assay also revealed hyperacetylation of histone H4, a typical epigenetic change characteristic of inducible HSF1 binding [20], at *SERF2* promoter ($p < 0.001$, n=3) following heat shock treatment (Fig. 2**, C and D**). Therefore, functionality of the HSE present in *SERF2* promoter is induced by HSF1 in heat shock-dependent manner and HSF1-mediated *trans*-activation of *SERF2* promoter involves chromatin remodeling. Occupancy of HSF1 and RNA polymerase II was detected in *SERF2* promoter even in absence of heat shock without significant acetylation of histone H4 at the same genomic locus (Fig. 2**C**). We assume that it might have contributed to basal *SERF2* expression in unstressed HeLa cells. Intriguingly, it has been reported earlier that HSF1 can bind to promoters of canonical and non-canonical HSP-coding genes in unstressed cells without subsequent expression of respective genes or other functional outcomes [18].

3.4. *In vivo interaction of HSF1 with recombinant SERF2 promoter*

To further confirm the specificity of *in vivo* interaction of endogenous HSF1 with its cognate binding site in *SERF2* promoter,

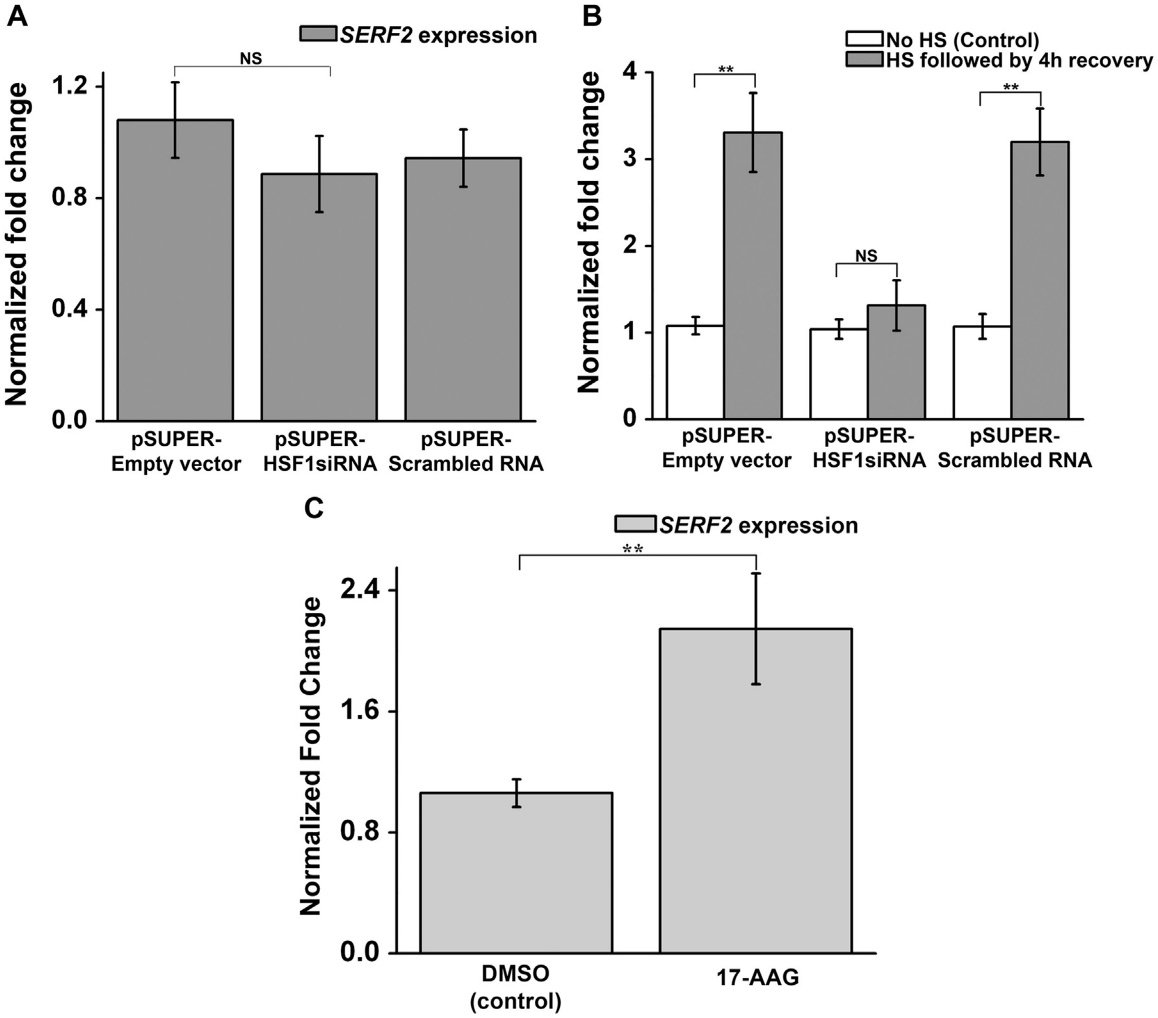

Fig. 4. Effect of HSF1 on *SERF2* expression. **A.** Average (n=3) expression of *SERF2* in HeLa cells transiently expressing empty pSUPER vector (control), HSF1-siRNA containing pSUPER vector and scrambled RNA containing pSUPER vector. **B.** Average (n=3) expression of *SERF2* in HeLa cells expressing different pSUPER constructs mentioned in A in absence (control) or presence of HS treatment. **C.** Bar graph showing mean (n=3) *SERF2* expression in DMSO-treated (control) and 5 μM 17-AAG treated HeLa cells. Expression of *β-actin* was used as endogenous control in all experiments. *Error bars* indicate ± SD. The statistical significance level between various experimental pairs is indicated (*,$p < 0.05$;**,$p < 0.01$;***,$p < 0.001$).

we performed transient ChIP assay. HeLa cells were transiently transfected with SERF2_ups, SERF2_ups_ΔHSE and Hsp70_ups constructs and twenty four hours after transfection, cells were harvested in absence or presence of HS treatment. Anti-HSF1 antibody was used for immunoprecipitation and the immunoprecipitated DNA was amplified using forward primer specific for 5′-end of *SERF2* or *hsp70* promoter and reverse primer specific for *luciferase* gene present in vector backbone (downstream of the multiple cloning site). This ensures selective amplification of synthetic or recombinant *SERF2* promoter (*SERF2* promoter cloned in pGL3 basic vector) leaving native *SERF2* promoter in the genome unamplified. As presented in Fig. 3, occupancy of endogenous HSF1 in recombinant or synthetic *SERF2* promoter increased significantly in response to hyperthermia in HeLa cells. As evident from Fig. 3, mutant *SERF2* promoter (SERF2_ups_ΔHSE) showed no occupancy of endogenous HSF1 even in presence of heat shock whereas recombinant *hsp70* promoter showed heat shock-driven increased occupancy of HSF1. Therefore endogenous HSF1 could interact *in vivo* with recombinant *SERF2* promoter and deletion of putative HSE by mutagenesis abolished this interaction. Transient ChIP assay thus strengthened our conclusion from luciferase reporter assay by showing that the truncated *SERF2* promoter cloned in reporter vector is sufficient to recruit endogenous HSF1 to its cognate binding site which subsequently leads to heat shock-dependent *trans*-activation of *SERF2* promoter by HSF1. This result also shows that despite being composed of two pentameric (nGAAn) repeats, the HSE present in *SERF2* promoter is sufficient for HSF1-mediated *trans*-activation of *SERF2* promoter.

3.5. Effect of HSF1 loss-of-function and gain-of-function on SERF2 expression

We next attempted to determine the effect of HSF1 on *SERF2* expression. To understand the effect of HSF1 knockdown, HeLa cells were transfected with HSF1-specific siRNA as described earlier [3]. Upon knockdown of endogenous HSF1, no significant change in endogenous *SERF2* level was observed (Fig. 4**A**). We then wanted to investigate the effect of HSF1 knockdown on heat shock-driven induction of *SERF2*. HeLa cells transiently expressing empty pSUPER vector or HSF1-siRNA containing pSUPER vector or scrambled RNA-containing pSUPER vector were either grown at 37 °C (control) or were exposed to standard HS treatment and *SERF2* and *β-actin* expression was measured by qRT-PCR. Unlike HeLa cells expressing empty vector and scrambled RNA, cells expressing HSF1-siRNA showed no significant induction in *SERF2* level in response to HS treatment (Fig. 4**B**). We conclude that knockdown of HSF1 has no effect on endogenous *SERF2* expression, however; it abolishes the ability of host cells to induce *SERF2* expression in response to thermal stress. Importantly, the observation that endogenous *SERF2* expression was not affected by knockdown of HSF1 suggests that HSF1 may not have any effect on basal *SERF2* expression as we thought earlier. Therefore, the interaction of endogenous HSF1 with *SERF2* promoter in unstressed HeLa cells, as depicted in Fig. 2**C**, possibly has no functional outcome and recapitulates earlier observation made by Trinklein and his colleagues [18].

Our final aim was to check the effect of HSF1 activation on *SERF2* expression. 17-AAG is a small molecule activator of HSF1 and has been shown beneficial in the context of many proteinopathies primarily because of its ability to boost the expression of multiple heat shock proteins [21]. HeLa cells were either treated with DMSO (control) or 17-AAG and *SERF2* expression was measured by qRT-PCR. As presented in Fig. 4**C**, 17-AAG treatment augmented *SERF2* expression significantly ($p=0.007$, $n=3$) in HeLa cells. Therefore, both loss-of-function and gain-of-function studies revealed that *SERF2* expression is regulated by HSF1. It is noteworthy that SERF2 protein level was found unaltered in response to 17-AAG treatment in HeLa cells (data not shown) which is in line with the observation that thermal stress had no significant effect on SERF2 protein level (Fig. 1**D**). Therefore, both thermal stress and 17-AAG were effective in upregulating *SERF2* expression only at transcription level.

To conclude, this is the first report focusing on regulatory mechanism of poorly characterized protein SERF2. Our findings clearly show that *SERF2* expression is transcriptionally regulated by HSF1 and *SERF2* promoter is *trans*-activated by HSF1 in heat shock-dependent manner by virtue of a functional HSE. To the best of our knowledge, this also represents existence of a unique CG whose two parent genes are regulated by a common transcription factor despite the fact that proteins translated from these two parent genes have opposite cellular function in a given context.

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Appendix A. Transparency document

Transparency document associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2016.04.003.

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