The role of Mediator kinase module in the heat shock response

（熱ショック応答におけるメディエーターキナーゼモジュールの役割）

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By

PRATIBHA SRIVASTAVA

YAMAGUCHI UNIVERSITY

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Abbreviations

AZC	l-Azetidine-2-carboxylic acid
CCNC	Cyclin C
ChIP	Chromatin Immunoprecipitation
CKM	CDK8 kinase module
CTD	Carboxylic terminal domain
DNA	Deoxyribonucleic acid
GTFs	General transcription factors
HSF1	Heat shock transcription factor 1
HSP	Heat shock protein
HSR	Heat shock response
PIC	Pre-initiation complex
Pol II	RNA polymerase II
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RT-PCR	Teverse transcriptase polymerase chain reaction
TBP	TATA binding protein
Snx A	Senexin A

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1. Abstract

Heat shock response, an adaptive and evolutionary conserved mechanism, plays a pivotal role in balancing proteostasis capacity via induction of heat shock proteins (HSPs) and non-HSPs that help in coping up the stress alterations by facilitating the correct folding of misfolded proteins. At the transcriptional level, activated and promoter-bound heat shock transcription factor 1 (HSF1) induces RNA polymerase II recruitment upon heat shock, and this is facilitated by the core Mediator in *Drosophila* and yeast. Another Mediator module, CDK8 kinase module (CKM), consisting of four subunits including MED12 and CDK8, plays a negative or positive role in the regulation of transcription; however, its involvement in HSF1-mediated transcription remains unclear. We herein demonstrated that HSF1 interacted with MED12, and recruited MED12 and CDK8 to the *HSP70* promoter during heat shock in mammalian cells. CDK8 (and its paralog CDK19) kinase activity promoted *HSP70* expression partly by phosphorylating HSF1-S326 and maintained proteostasis capacity. These results indicate an important role for CKM in the protection of cells against proteotoxic stress.

2. Introduction

Environmental or metabolic stress induces protein misfolding and challenges the capacity for proteostasis or buffering against protein misfolding [1,2]. One of the adaptive mechanisms for protein misfolding is the heat shock response (HSR), which is characterized by the induction of a set of heat shock proteins (HSPs) including HSP70, which facilitates protein folding and a large number of non-HSP proteins [3] (Fig. 1). HSR is regulated at the transcriptional level by evolutionarily conserved heat shock transcription factors (HSFs) that bind to HSR elements (HSEs) in eukaryotes. Among the four HSF family members in mammals, HSF1, an ortholog of *Drosophila* HSF or yeast HSF1, is a master regulator of HSR [4,5]. HSF1 mostly remains as an inactive monomer, but is converted to an active trimer that binds to HSE in response to heat shock. A small amount of the HSF1 trimer has been shown to constitutively bind to the *HSP70* promoter in complex with replication protein A (RPA) and the histone chaperone FACT (facilitates chromatin transcription) [6], and recruits poly(ADP-ribose) polymerase PARP13-PARP1 complex in advance [7]. During heat shock, activated HSF1 heavily occupies HSEs in the promoter, facilitating the redistribution of PARP1 within the *HSP70* locus [7], and also recruits coactivators, including BRG1 [8], p300 [9], ASC-2 [10], and [11] MLL1, which promote the establishment of an active chromatin state and the transcription of *HSP70* in mammals. Promoter-bound HSF1 also recruits other coactivators, including shugoshin 2 (SGO2) and the central coactivator complex Mediator [12], which facilitate the formation of the pre-initiation complex (PIC) containing general transcription factors (GTFs) and RNA polymerase II (Pol II) [13]. Mediator and TBP, a component of GTFs, were also found to be recruited, directly or indirectly, by *Drosophila* HSF during heat shock [14,15].

Mediator is an evolutionarily conserved multi-subunit protein complex comprising 25 subunits in yeast and 33 subunits in mammals, and plays an essential role in the regulation of Pol II transcription [16,17]. Mediator acts as a functional bridge between transcription factors (TFs) and the basal transcriptional machinery, including GTFs and Pol II, and promotes the assembly of PIC. It also transmits functional information from enhancer-bound

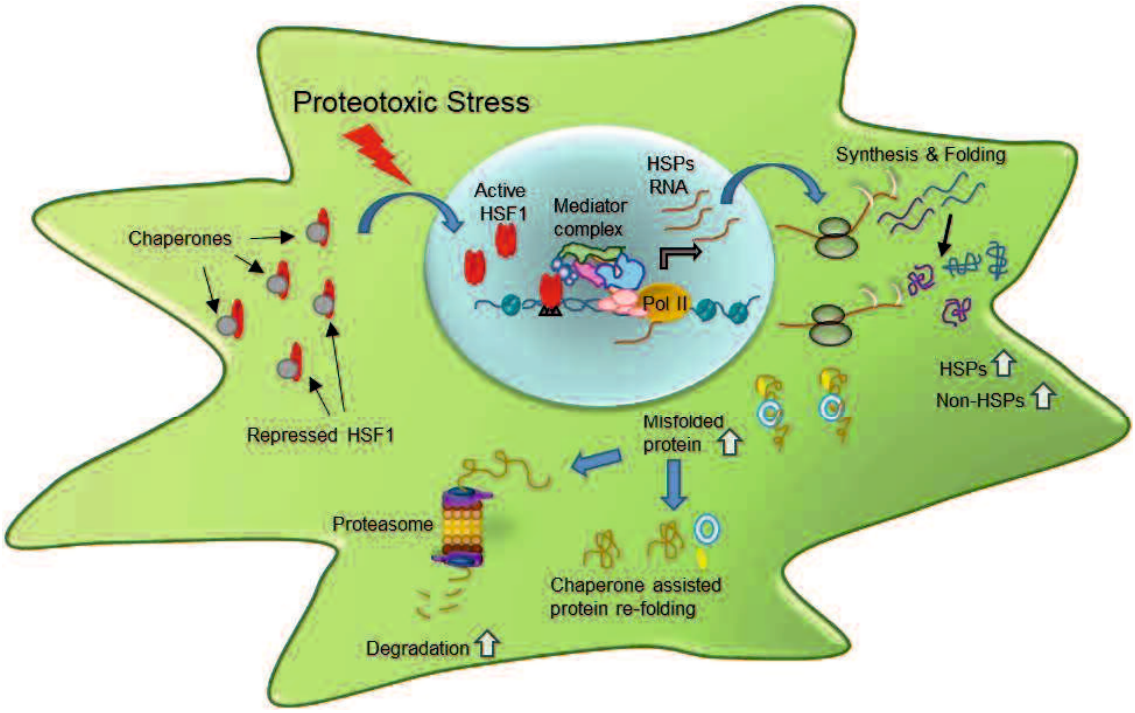


Figure 1. Schematics of cellular *proteostasis*. Under the proteotoxic stress stimuli, the shift in chaperone equilibrium allow the conformational changes in monomeric HSF1 (repressed) to convert to trimeric state (active), translocate to the nucleus, bind to HS gene promoters and induce the transcription of the HS genes. The HSF1 then recruits various of GTFs, co-activators (including Mediator complex) and Pol II, that alters the chromatin modification and forms PIC to initiate transcription of HSPs and non-HSPs gene. Proteotoxic stress causes increase in abundance of misfolded protein, which follows one of the two fates, chaperone-assisted protein folding or degradation via proteasomes and lysosomes.

transcription factors to GTFs and Pol II, and regulates Pol II pausing and elongation (Fig. 2A). Mediator is divided into the following four modules: the head, middle, tail, and CDK8 kinase module (CKM), and the core Mediator consists of the head, middle, and tail modules. The head and middle modules form an essential core that is responsible for its interaction with Pol II, whereas the regulatory tail generally binds to TFs (Fig. 2B). Another regulatory module CKM, which consists of MED12, MED13, Cyclin C (CCNC), and CDK8 kinase, is reversibly associated with the core Mediator through its MED13 subunit [18,19]. CDK19, a paralog of CDK8, and paralogs of MED12 and MED13 (MED12L and MED13L, respectively)

exist in vertebrates. MED12 and CCNC are required for CDK8 kinase activity [20,21]. Previous studies reported that CKM functioned as a negative regulator during transcription [22,23], and physically prevented the incorporation of the Pol II into PIC [18]. CKM was subsequently shown to play a positive role in regulating the transcription of genes that preferentially respond to environmental cues, including DNA damage [24], serum stimulation [25], hypoxia [26], inflammation [27], and viral infection [28,29]. CKM and the core Mediator were found to be independently recruited to the promoters of *HSP* genes in response to heat shock in yeast [30]. For example, the depletion of MED14 decreased the occupancy of core Mediator subunits, but did not affect that of MED12. Although the depletion of core Mediator

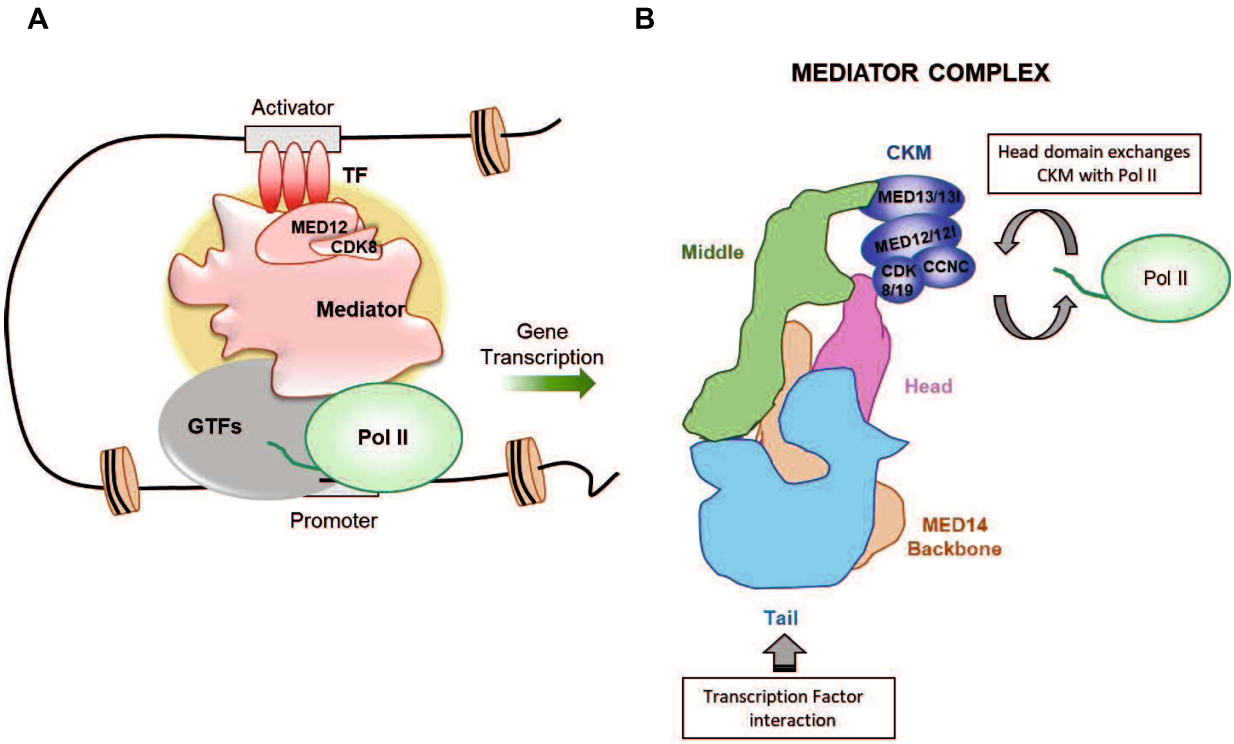


Figure 2. Mediator complex in mammals. **(A)** Mediator complex bridges the activator and promoter thereby transferring the signal to aid in PIC recruitment, transcription initiation and Pol II pause release [16, 17]. **(B)** The four modules of mediator complex, head, middle, tail and CKM are assembled to form the large multi-subunit mediator complex. The tail module can interact with Transcription factors whereas Head module exchanges CKM for Pol II.

subunits, including the tail subunits MED15 and MED16, markedly reduced the induction of *HSP* expression, that of the CKM subunit CDK8 did not affect its expression [30-32]. In mammals, the depletion of the core Mediator subunit MED26 reduced heat shock-induced *HSP70* expression [33]; however, the roles of other Mediator subunits in the regulation of *HSP* expression have not yet been elucidated. Therefore, it is currently remains unclear whether CKM contributes to the regulation of HSR.

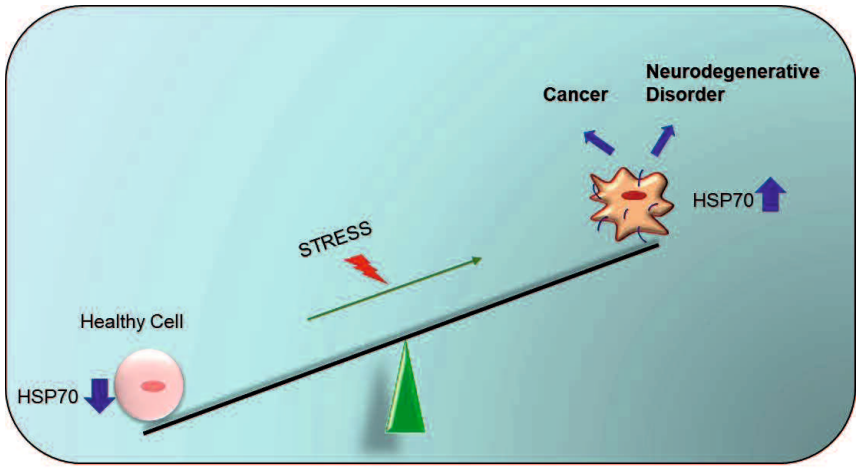


Figure 3. Dysregulation in proteostasis capacity causes accumulation of protein aggregates formed as a result from protein misfolding in the cell. The protein aggregates are toxic to cell and related with diseases including neurodegenerative diseases and cancers.

We previously identified the components of HSF1-transcription complexes using a DNA pull-down assay coupled with mass spectrometry [12]. In the present study, we re-evaluated the numbers of peptides identified as well as the intensities of all Mediator subunits and found that MED12 was one of the subunits closely associated with HSF1 upon heat shock. HSF1 recruited not only MED12, but also CDK8 to the *HSP70* promoter in mouse and human cells. The knockdown (KD) of MED12 and CDK8 or the inhibition of CDK8 (and CDK19) kinase activity significantly down-regulated *HSP70* expression. Unexpectedly, HSF1 at Ser326 was identified as a CDK8/19 kinase target during heat shock. Furthermore, CDK8/19 kinase activity supported cell survival and maintained the capacity for proteostasis during heat shock.

3. Materials and Methods

3.1 Cell cultures and treatments

Immortalized HSF1$^{+/+}$ (stock no. 10) and HSF1$^{-/-}$ (stock no. 4) mouse embryonic fibroblasts (MEFs) [12], C2C12 (ATCC, CRL-1772), Neuro2a (ATCC, CCL-131), human HEK293 (ATCC, CRL-1573), OUMS-36T-3F (JCRB1006.3F), HeLa (ATCC, CCL-2), and U2OS (ATCC, HTB-96) cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) or DMEM/Ham's F-12 (Gibco) (1:1) (for Neuro2a) containing 10% fetal bovine serum (Sigma-Aldrich) at 37°C in a humidified incubator with 5% CO_2. Cell culture dishes were submerged in a water bath at 42°C for the indicated periods to expose cells to heat shock. To monitor cell viability under heat shock conditions, cell culture dishes were incubated in a CO_2 incubator at 45°C for longer periods. Cells were treated with 5 mM L-azetidine-2-carboxylic acid (AZC; Tokyo Chemical Industry) for 3 h. To examine the effects of the inhibition of CDK8/CDK19, cells were pretreated with 2.5 or 10 μM Senexin A (HY-15681, MedChem Express, New Jersey, USA) for 1 h before heat shock.

3.2 Assessment of mRNA

Total RNA was isolated from cells using TRIzol (Ambion). First-strand cDNA was synthesized using PrimeScript II Reverse Transcriptase and the oligo dT primer in accordance with the manufacturer's instructions (Takara Bio Inc.). RT-PCR for kinase module component genes was performed using the primers summarized in Table S1. A real-time quantitative polymerase chain reaction (qPCR) was performed using StepOnePlus (Applied Biosystems) with the Power SYBR Green PCR Master Mix (Applied Biosystems) using primers for the mouse *HSP* genes [12] and human *HSP70* [7]. The relative quantities of mRNAs were normalized against β-actin or GAPDH mRNA levels. All reactions were performed in triplicate with samples derived from three independent experiments.

Table 1. Primer sequences used for RT-PCR

Gene	Forward primer	Reverse primer
mMED12	5'-GCGGCTTTCGGGATCTTGAGCT-3'	5'-AACCTCGAGAACGTTCCGGGAGACCTGAGCCGTG -3'
mMED13	5'-AGTTCCTCCTTCGTGTCGAACG-3'	5'-AAGAACTTCCACCGCAGCCAAAGAA-3'
mCCNC	5'-GCAGGGAACTTCTGGCAGAGCT-3'	5'-GCAGCACGTCTTCCTGGCCCATGTC-3'
mCDK8	5'-GACTATGACTTTAAAGTGAAGC-3'	5'-TCTGAAATCTTTCATTAATGTTGAA-3'
mMED14	5'-GCCCCAGTGCAGCTGGACAACC-3'	5'-TCTCCTGTTTCCTTATCCTCAACTA-3'
mMED17	5'-TCGGGCGTGCGAGCCGTGCGCA-3'	5'-CCAATGTCTGGAGCCTGTTTCTGAA-3'
mp300	5'-GCCAAGTATGCCAACCCTAA-3'	5'-TGTTCATTTGCTGAGCTTGG-3'
mCBP	5'-TGGAGTGAACCCCCAGTTAG-3'	5'-TTGCTTGCTCTCGTCTCTGA-3'
mBag3	5'-CGAGAGCCTCCACCTGTTAC-3'	5'-TTCCACTTTCAGCACACCTG-3'
mSlc5a3	5'-GGGGCTTTTTATGGTGGAAT-3'	5'-CAGACTTCCCGTTGGGAATA-3'
mHemt1	5'-GCCATTGAGCCTAGATGGAG-3'	5'-GAGTTCTGGAGGCACGTGTT-3'
mOciad2	5'-GGACTCGTCCACCAAGGTTA-3'	5'-ATCCACGCCTGGTTTTACAG-3'
mRrgd	5'-CTGAGGTGAAGCCCAGAATC-3'	5'-CTGAGGTGAAGCCCAGAATC-3'
mUspl1	5'-TCCTGTGTTTGCACTTCCTG-3'	5'-CGCTCTTTCCAACACCATTT-3'
mACTIN	5'-GACAGGATGCAGAAGGAGAT-3'	5'-TTGCTGATCCACATCTGCTG-3'

3.3 RNA interference

To generate adenovirus vectors expressing short hairpin RNAs, oligonucleotides containing each target sequence (Table S2) were annealed and inserted into pCR2.1-hU6 at the BamHI/HindIII sites, and then XhoI/HindIII fragments containing hU6-shRNA were inserted into a pShuttle-CMV vector (Stratagene). Regarding MED12 KD, MEF cells were infected with Ad-sh-mMED12-KD1, Ad-sh-mMED12-KD2, Ad-sh-mMED12-KD3, or one of the other adenoviruses (1×10^8 pfu/ml) in serum free medium for 2 h, and then maintained in normal medium for 70 h. As a control, cells were infected with adenovirus vector expressing scrambled RNA (Ad-sh-SCR). The KD of MED13, CCNC, CDK8, MED14, MED17, p300, and CBP was performed using a similar approach. Concerning CDK8 KD, CDK19 KD, or MED12 KD in human OUMS-36 cells, cells were infected with Ad-sh-hMED12-KD1, Ad-sh-hMED12-KD2, Ad-sh-hCDK8-KD1, or Ad-sh-hCDK19-KD1 (1×10^8 pfu/ml) in the same manner.

Table 2. Nucleotide sequences of shRNAs used for gene knockdown

shRNA	Sense strand	Anti-sense strand
Scrambled (SCR)	5'-GATCCATGTACTGCGCGTGGAGACTTCAAGAGA GTCTCCACGCGCAGTACATTC TTTTGGAAA-3'	5'-AGCTTTTCCAAAA GAATGTACTGCGCGTGGAGAC TCTCTTGAA ATCAGTCGTATTTCTCTTC G-3'
mMED12-KD1	5'-GATCC AGCAATGTCTGAGACTAAG TTCAAGA GACTTAGTCTCAGACATTGCTTT TTTTGGAAA-3'	5'-AGCTTTTCCAAAA AAAGCAATGTCTGAGACTAAG TCTCTTGAA CTTAGTCTCAGACATTGCT G-3'
mMED12-KD2	5'-GATCC GCACTCATCTCATGTCATAG TTCAAGAGA CTATGACATGAGATGAGTGCTT TTTTGGAAA-3'	5'-AGCTTTTCCAAAA AAGCACTCATCTCATGTCATAG TCTCTTGAA CTATGACATGAGATGAGTGCG-3'
mMED12-KD3	5'-GATCC GCCCAGCCTTTCTGCTTAA TTCAAGAGA TTAAGCAGAAAGGCTGGGCTT TTTTGGAAA-3'	5'-AGCT TTTCCAAAA AAGCCCAGCCTTTCTGCTTAA TCTCTTGAA TTAAGCAGAAAGGCTGGGC G-3'
mMED13-KD1	5'-GATCC AGCACTATGTTAATACTGT TTCAAGAGA ACAGTATTAACATAGTGCTTT TTTTGGAAA-3'	5'-AGCTTTTCCAAAA AAAGCACTATGTTAATACTGT TCTCTTGAA ACAGTATTAACATAGTGCT G-3'
mMED13-KD2	5'-GATCC GCGAAGAAGATGCTATGTCA TTCAAGAGA TGACATAGCATCTTCTTCGCTT TTTTGGAAA-3'	5'-AGCTTTTCCAAAA AAGCGAAGAAGATGCTATGTCA TCTCTTGAA TGACATAGCATCTTCTTCGC G-3'
mCCNC-KD1	5'-GATCC GTGGACTGTTGCTTGATAGT TTCAAGAGA ACTATCAAGCAACAGTCCACTT TTTTGGAAA-3'	5'-AGCTTTTCCAAAA AAGTGGACTGTTGCTTGATAGT TCTCTTGAA ACTATCAAGCAACAGTCCAC G-3'
mCCNC-KD2	5'-GATCC ACAGGATGAATCATATACT TTCAAGAGA AGTATATGATTCATCCTGTTT TTTTGGAAA-3'	5'-AGCTTTTCCAAAA AAACAGGATGAATCATATACT TCTCTTGAA AGTATATGATTCATCCTGT G-3'
mCDK8-KD1	5'-GATCC GTGGACTGTTGCTTGATAGT TTCAAGAG AACTATCAAGCAACAGTCCACTT TTTTGGAAA-3'	5'-AGCTTTTCCAAAA AAGTGGACTGTTGCTTGATAG TTCTCTTGAA ACTATCAAGCAACAGTCCAC G-3'
mCDK8-KD2	5'-ATC CACAGGATGAATCATATACT TTCAAGAGA AGTATATGATTCATCCTGTTT TTTTGGAAA-3'	5'-AGCTTTTCCAAAA AAACAGGATGAATCATATACT TCTCTTGAA AGTATATGATTCATCCTGT G-3'
mMED14-KD1	5'- GATCC GTGCAATTCGCTTATTAAAGATTC TTCAAGAGA GAATCTTTAATAAGCGAATTGCACTT TTTTGGAAA-3'	5'-AGCTTTTCCAAAA AAGTGCAATTCGCTTATTAAAGATTC TCTCTTGAA GAATCTTTAATAAGCGAATTGCAC G-3'
mMED17-KD1	5'-GATCC AGAGATGGTCGGGTAATCA TTCAAGAGA TGATTACCCGACCATCTCTTT TTTTGGAAA-3'	5'-AGCTTTTCCAAAA AAAGAGATGGTCGGGTAATCA TCTCTTGAA TGATTACCCGACCATCTCT G-3'
mp300-KD	5'-GATCC AGGTGAGGATGTTAAAGTA TTCAAGA GATACTTTAACATCCTCACCTT TTTTTGGAAA-3'	5'-AGCTTTTCCAAAA AAAGGTGAGGATGTTAAAGTA TCTCTTGAA TACTTTAACATCCTCACCT G-3'
mCBP-KD	5'-GATCC ACAGAGCATGGTCAATAGT TTCAAGA GAACTATTGACCATGCTCTGTT TTTTTGGAAA-3'	5'-AGCTTTTCCAAAA AAACAGAGCATGGTCAATAGT TCTCTTGAA ACTATTGACCATGCTCTGT G-3'
mHSF1-KD1	5'-GATCC GTGATCACCTGGATGCCAT TTCAAGAGA ATGGCATCCAGGTGATCACTT TTTTGGAAA-3'	5'-AGCTTTTCCAAAA AAGTGATCACCTGGATGCCAT TCTCTTGAA ATGGCATCCAGGTGATCAC G-3'
hCDK8-KD	5'-GATCC GCTTCCAAGTTGTACCTATTT TTCAAGAGA AAATAGGTACAACTTGGAAGCTT TTTTGGAAA-3'	5'-AGCTTTTCCAAAA AAGCTTCCAAGTTGTACCTATTT TCTCTTGAA AAATAGGTACAACTTGGAAGC G-3'
hCDK19-KD	5'- GATCC ACCAGCAAATATCCTAGTAATG TTCAAGAGA CATTACTAGGATATTTGCTGGTTT TTTTGGAAA-3'	5'- AGCTTTTCCAAAA AAACCAGCAAATATCCTAGTAATG TCTCTTGAA CATTACTAGGATATTTGCTGGT G-3'
hMED12-KD1	5'-GATCC GCGTCCTGTGCGCTTACCAA TTCAAGAGA TTGGTAAGCGCACAGGACGCTT TTTTGGAAA-3'	5'-AGCTTTTCCAAAA AAGCGTCCTGTGCGCTTACCAA TCTCTTGAA TTGGTAAGCGCACAGGACGC G-3'
hMED12-KD2	5'-GATCC GTACCATGACTCCAATGAG TTCAAGAGA CTCATTGGAGTCATGGTACTT TTTTGGAAA-3'	5'-AGCTTTTCCAAAA AAGTACCATGACTCCAATGAG TCTCTTGAA CTCATTGGAGTCATGGTAC G-3'

*Red, Sense target sequences; Blue, Antisense target sequences.

3.4 Western blotting

Cell pellets were lysed with NP-40 lysis buffer (150 mM NaCl, 1.0% Nonidet P-40, and 50 mM Tris, pH 8.0) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml pepstatin) on ice for 10 min. After centrifugation at 16,000 × g for 10 min, supernatants were subjected to SDS-polyacrylamide gel electrophoresis. Proteins were transferred onto nitrocellulose membranes, which were then blocked in phosphate-buffered saline (PBS)/5% non-fat dry milk at room temperature for 1 h, followed by blotting using primary antibodies diluted in PBS/2% non-fat dry milk at room temperature for 1 h or at 4°C overnight. The following antibodies were used; rabbit antibodies against MED12 (anti-mMED12ΔQ-1; dilution 1:1000) [12], MED1 (A300-793A, Bethyl Laboratories; 1:5000), CDK8 (A302-501A, Bethyl Laboratories; 1:1000), CDK19 (A16109, ABclonal; 1:1000), HSF1 (anti-hHSF1j or ABE1044, Merck Millipore; 1:1000), HSF1 phospho-S326 (ab76076, Abcam), HSP110 (anti-HSP110a; 1:1000), HSP40 (anti-hHSP40a; 1:1000), and HSP25 (anti-mHSP27c; 1;1000) [12], and mouse antibodies for HSP70 (W27, Santa Cruz; 1:1000) and β-actin (A5441, Sigma-Aldrich; 1:1000). Membranes were washed three times with PBS for 5 min, and incubated with a secondary antibody, either peroxidase-conjugated goat anti-rabbit or anti-mouse IgG at room temperature for 1 h. After washing with PBS/0.1%Tween-20 three times, chemiluminescent signals from ECL detection reagents (GE Healthcare) were captured on X-ray films (Super RX, Fujifilm).

3.5 Co-immunoprecipitation assay

MEF or HeLa cells were infected with Ad-hHSF1-HA (MEF, 2×10^7 pfu/ml; HeLa, 2×10^6 pfu/ml) in serum free medium for 2 h, and then maintained in normal medium for 46 h. Cells were lysed in NP-40 lysis buffer and centrifuged at 16,000 × g for 10 min. Supernatants containing 10 mg of protein were incubated with 2.5 μl of anti-MED12 antiserum (anti-mMED12ΔQ-1) [12] or preimmune serum on ice for 3 h, and then mixed with 20 μl protein A-Sepharose beads (GE Healthcare) by rotating at 4°C for 1 h. Complexes were washed five times with NP-40 lysis buffer, and were subjected to immunoblotting using antibody for HA (3F10, Roche) or MED12.

3.6 GST pull-down assay

To generate expression vectors for mMED12 deletion mutants fused to glutathione S-transferase (GST), we initially generated pGEX-2T-MCS vector by inserting a DNA fragment containing KpnI and XhoI restriction sites into pGEX-2T vector at the BamHI/EcoRI site (GE Healthcare) [12]. cDNA fragments encoding mMED12 regions were amplified by PCR using mouse cDNA, and were then inserted into the pGEX-2T-MCS vector at the KpnI/XhoI sites and BamHI/XhoI sites for the region 1-4 and region 7, respectively. cDNA fragments encoding mMED12 regions 5 and 6 were also amplified by PCR and were inserted into the pGEX-2T vector at the SmaI/EcoRI sites. The expression of recombinant GST fusion proteins in *Escherichia coli* BL21 (DE3) was induced by 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 20°C for 16 h, 25°C for 6 h, or 37°C for 3 h, and proteins were then purified using Glutathione Sepharose 4B (GE Healthcare). A mixture of each purified GST protein (1 μg) and the nuclear extract (2 mg) of heat-shocked (at 42°C for 30 min) HeLa cells expressing hHSF1-HA in binding buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 1.0% Nonidet P-40) was rotated at 4°C for 4 h, and was then incubated with 20 μl of Glutathione-Sepharose beads at 4°C for 1 h. After the beads has been washed five times with washing buffer (50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 5 mM EDTA, and 0.5% Nonidet P-40), bound proteins were eluted using elution buffer (50 mM Tris-HCl, pH 8.0, 120 mM NaCl, and 20 mM reduced glutathione). The eluates were subjected to immunoblotting using a rat anti-HA monoclonal antibody (3F10, Roche) and rabbit anti-GST antibody [12].

3.7 Chromatin immunoprecipitation (ChIP) analysis

Chromatin immunoprecipitation (ChIP) experiments were performed using a kit in accordance with the manufacturer's instructions (EMD Millipore). The following antibodies were used for ChIP assays: anti-HSF1 (anti-mHSF1j, Millipore ABE1044), anti-MED12 (anti-mMED12ΔQ-1) [12], anti-MED1 (A300-793A, Bethyl Laboratories), and anti-Flag (F3165, Merck). Real-time qPCR of ChIP-enriched DNAs in the proximal HSE (pHSE) (-54 to -151), pausing region (+10 to +106), and intergenic region (+3138 to +3218) of the *HSP70.3* (*HSPA1A*) locus was conducted using previously reported primers [6]. Percentage input was

determined by comparing the cycle threshold value of each sample to a standard curve generated from a 5-point serial dilution of genomic input and compensated for by the values obtained using normal IgG. IgG-negative control immunoprecipitation at all sites yielded < 0.05% input. All reactions were performed in triplicate with samples derived from three experiments.

Table 3. Primer sequences used for CHIP assay

CHIP-qPCR	Forward sequence	Reverse sequence
mHSP70.3 promoter (pHSE)	5'-GATTACTCAAGGGAGGCGGG-3'	5'-TCCGCTGGGCCAATCA-3'
mHSP70.3 promoter (pausing)	5'-TGACAGCTACTCAGAACCAAATCTG-3'	5'-TGGTCCTGGCCGAGGAT-3'
mHSP70.3 promoter (intergenic region)	5'-GTGGCGCATGCCTTTGAT-3'	5'CTTTGTAGAACAGGCTGACCTTGA 3'

3.8 In vitro phosphorylation assay

In vitro phosphorylation was performed using purified recombinant hHSF1-His [6] and GST-CDK8/CycC (CCNC) (#04-109, Carna Biosciences, Kobe, Japan). Briefly, hHSF1-His (2 μg) was untreated or treated with heat shock at 42°C for 15 min, and then mixed with GST-CDK8/CCNC (0.1 μg) in a phosphorylation assay buffer (20 mM HEPES-KOH, pH 7.9, 10 mM $MgCl_2$, 1 mM DTT, 20 mM β-glycerophosphate, and 0.05 mM Na_3VO_4). ATP was added to the reaction at a final concentration of 40 μM, and the reaction was incubated at 30°C for 2 h. In some reactions, 2.5 μM Senexin A was added to inhibit CDK8/19 kinase activity. Proteins in the reactions were then subjected to immunoblotting using the antibody for HSF1 or HSF1 phospho-S326.

3.9 Statistical analysis

Data were analyzed using the Student's t-test or an analysis of variance (ANOVA). Asterisks in figures indicate that differences were significant (P < 0.001, 0.01, or 0.05). Error bars represent the standard deviations of at least three independent experiments.

4. Results

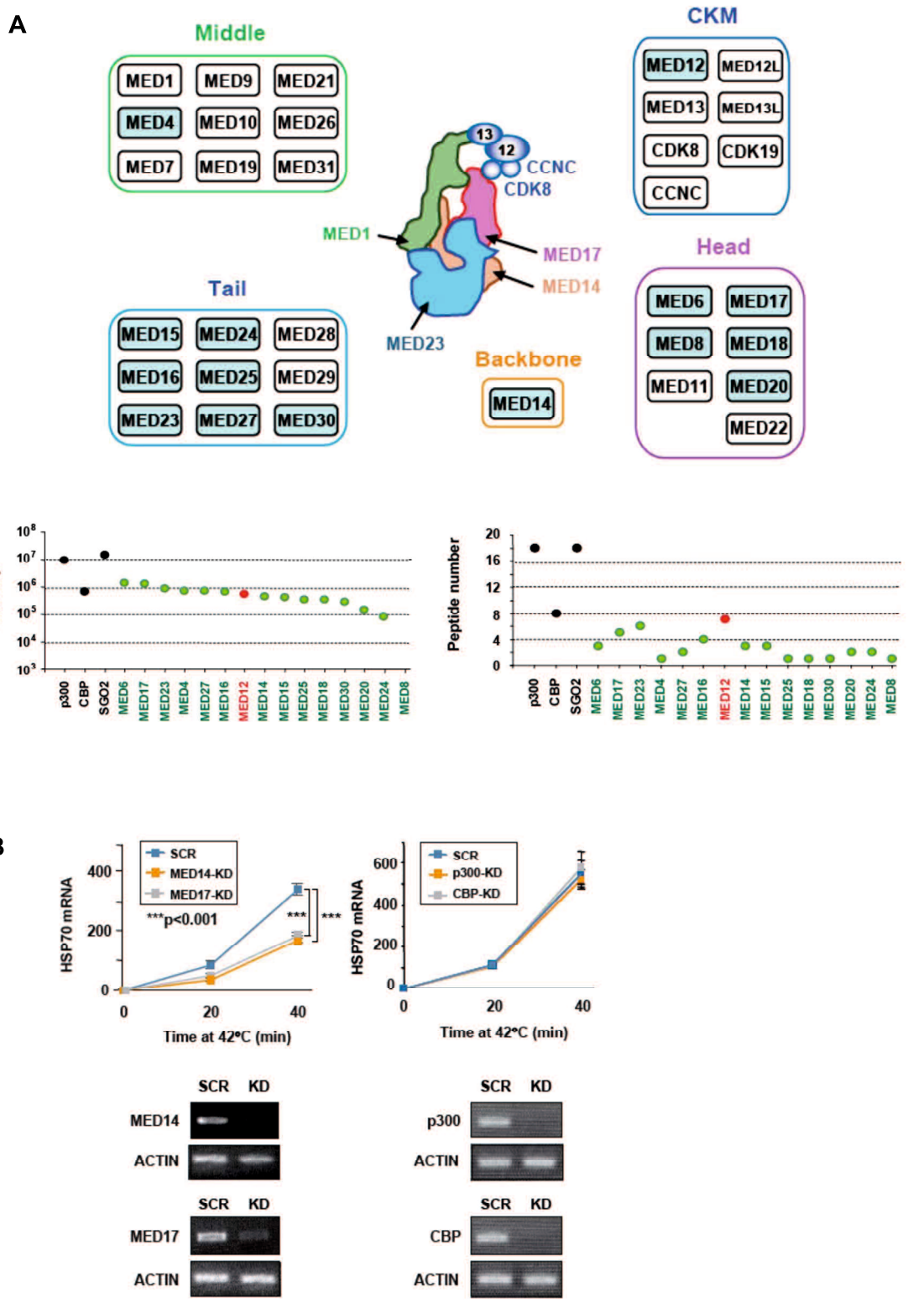
4.1 MED12 promotes HSR in mammalian cells.

The core transcriptional mechanisms of HSR are expected to be evolutionarily conserved because HSF1 in one species can be replaced with those of other species [34-36]. We previously identified coregulators of anole lizard (*Anolis sagrei*) HSF1 (AsHSF1) in heat-shocked MEFs by analyzing complexes bound to AsHSF1 on the human *HSP70* promoter using a biotin-labeled DNA pull-down assay [12]. This was essentially an in vitro assay that was conducted using nuclear extracts derived from AsHSF1-overexpressing cells exposed to heat shock. We selected 10 newly identified proteins from the major HSE- and activity-dependent components, and found that the depletion of five proteins, including MED12, significantly reduced HSP70 mRNA expression levels during heat shock [12]. This finding prompted us to examine the role of CKM, including MED12, in HSR. We searched 33 subunits of the mammalian Mediator [37] among 676 HSE-dependent components, and identified many subunits of the tail module (7 out of 9), which generally interact with transcription factors, and the head module (5 out of 7) as well as MED14, which acts as the backbone connecting the tail, head, and middle modules (Fig. 4A). Among the protein intensities of the head subunits, that of MED17, the *Drosophila* ortholog of which binds to HSF [14], was relatively strong (Fig. S1A). In contrast, we only identified MED4 in the middle module (1 out of 9) and MED12 in CKM (1 out of 7) (Fig. 4A). CDK19, MED12L, and MED13L were not detected in these complexes. Therefore, among the CKM subunits examined, MED12 uniquely interacted with AsHSF1 on the human *HSP70* promoter in vitro.

We then investigated whether heat shock-induced *HSP70* expression was enhanced by CKM and the core Mediator. MEF cells were initially infected with an adenovirus expressing short hairpin RNA for MED14 or MED17, and HSP70 mRNA was estimated during heat shock at 42°C for 20 and 40 min. The results obtained showed that heat shock-induced HSP70 mRNA expression was down-regulated by MED14 KD and MED17 KD (Fig. 4B), which is consistent with previous findings obtained using *Drosophila* and yeast models

[30,38]. As a control, we confirmed that it was unaffected by the KD of the functionally redundant histone acetyltransferases, p300 and CBP [39]. These results suggested that the core Mediator also promotes *HSP70* expression during heat shock in mouse cells. We then examined *HSP70* expression after the KD of each CKM subunit and found that heat shock-induced HSP70 mRNA expression was down-regulated by the KD of MED12, MED13, CCNC, and CDK8 (Fig. 4C).

Based on the strong association between HSF1 and MED12 and its effects on *HSP* expression, we focused our analysis on the role of MED12 in HSR. MED12 KD reduced the mRNA expression levels of major HSPs, including HSP110, HSP40, and HSP25 as well as HSP70, during heat shock at 42°C for 40 min in MEF cells (Fig. 4D). The constitutive expression of HSP mRNAs was not affected by MED12 KD. We investigated the protein levels of HSPs, and found that MED12 KD mildly reduced HSP110 and HSP25 levels at 3 and 6 h, HSP70 levels at 1 and 3 h, and HSP40 levels at 1 h during recovery from heat shock (Fig. 4E). HSP expression was induced by a treatment with another proteotoxic stress inducer, proline analogue AZC, and elevated mRNA levels were also reduced by MED12 KD (Fig. 4F). Furthermore, MED12 KD decreased heat shock-induced HSP70 mRNA levels in other mouse cells including C2C12 myoblasts and Neuro2a neuroblastoma cells (Fig. 4G). As expected from the conservation of the MED12 amino acid sequence between mice and humans (96% identity), MED12 KD also reduced HSP mRNA levels in different human cell lines including OUMS-36T-3F immortalized fibroblasts and U2OS osteosarcoma cells (Fig. 4H). These results indicated that MED12 promotes proteotoxic stress responses, including HSR, in mammalian cells.


A
Middle
MED1
MED9
MED21
MED4
MED10
MED26
MED7
MED19
MED31
CKM
MED12
MED12L
MED13
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CDK8
CDK19
CCNC
13
12
CCNC
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MED17
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MED15
MED24
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MED16
MED25
MED29
MED23
MED27
MED30
Backbone
MED14
Head
MED6
MED17
MED8
MED18
MED11
MED20
MED22
Intensity
Peptide number
p300
CBP
SGO2
MED6
MED17
MED23
MED4
MED27
MED16
MED12
MED14
MED15
MED25
MED18
MED30
MED20
MED24
MED8
B
SCR
MED14-KD
MED17-KD
***p<0.001
HSP70 mRNA
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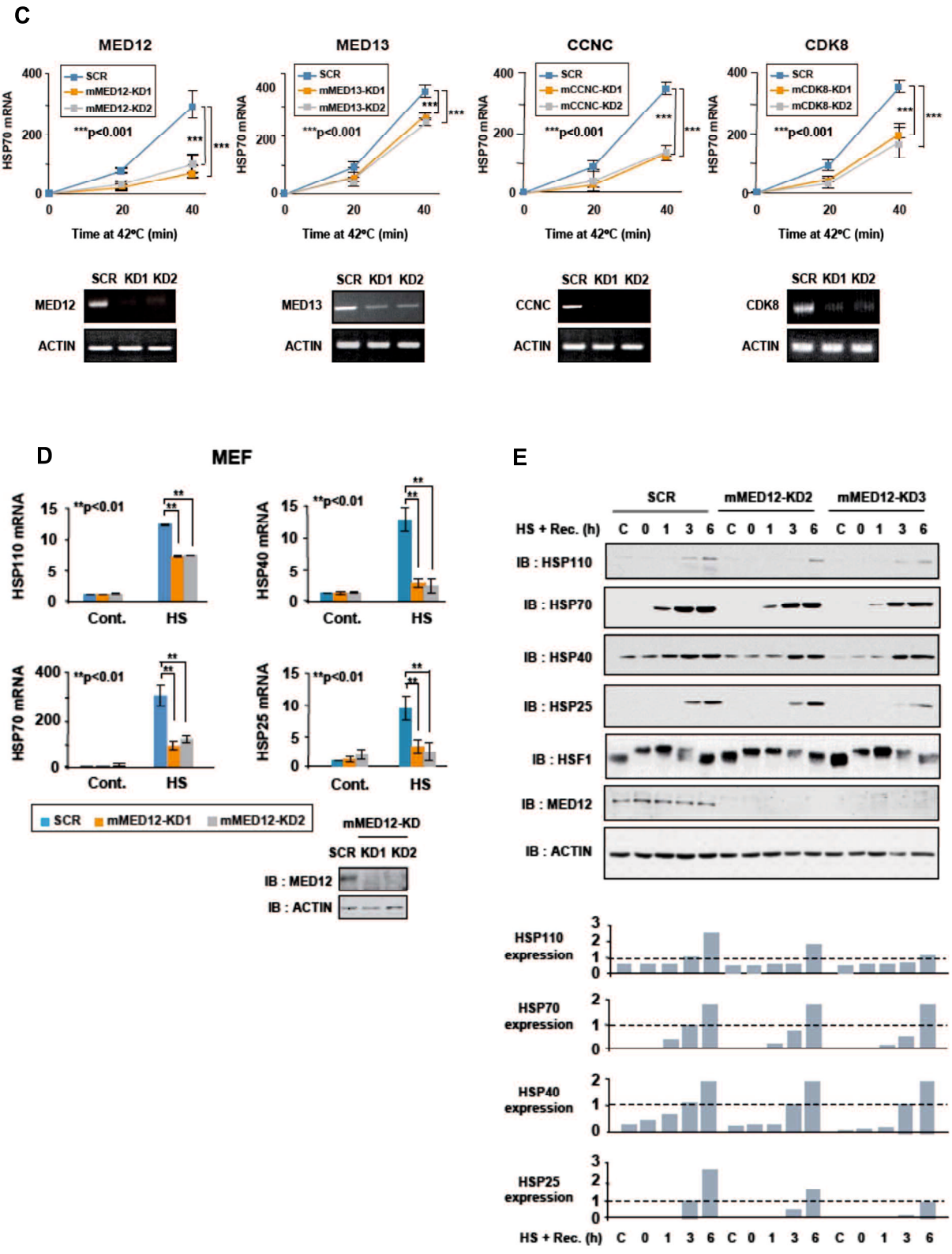
SCR
p300-KD
CBP-KD
SCR KD
MED14
ACTIN
p300
MED17
CBP


C
MED12
MED13
CCNC
CDK8
HSP70 mRNA
SCR
mMED12-KD1
mMED12-KD2
mMED13-KD1
mMED13-KD2
mCCNC-KD1
mCCNC-KD2
mCDK8-KD1
mCDK8-KD2
***p<0.001
Time at 42°C (min)
SCR KD1 KD2
ACTIN
D
MEF
HSP110 mRNA
HSP40 mRNA
HSP25 mRNA
**p<0.01
Cont.
HS
mMED12-KD
IB : MED12
IB : ACTIN
E
HS + Rec. (h)
IB : HSP110
IB : HSP70
IB : HSP40
IB : HSP25
IB : HSF1
IB : MED12
IB : ACTIN
HSP110 expression
HSP70 expression
HSP40 expression
HSP25 expression

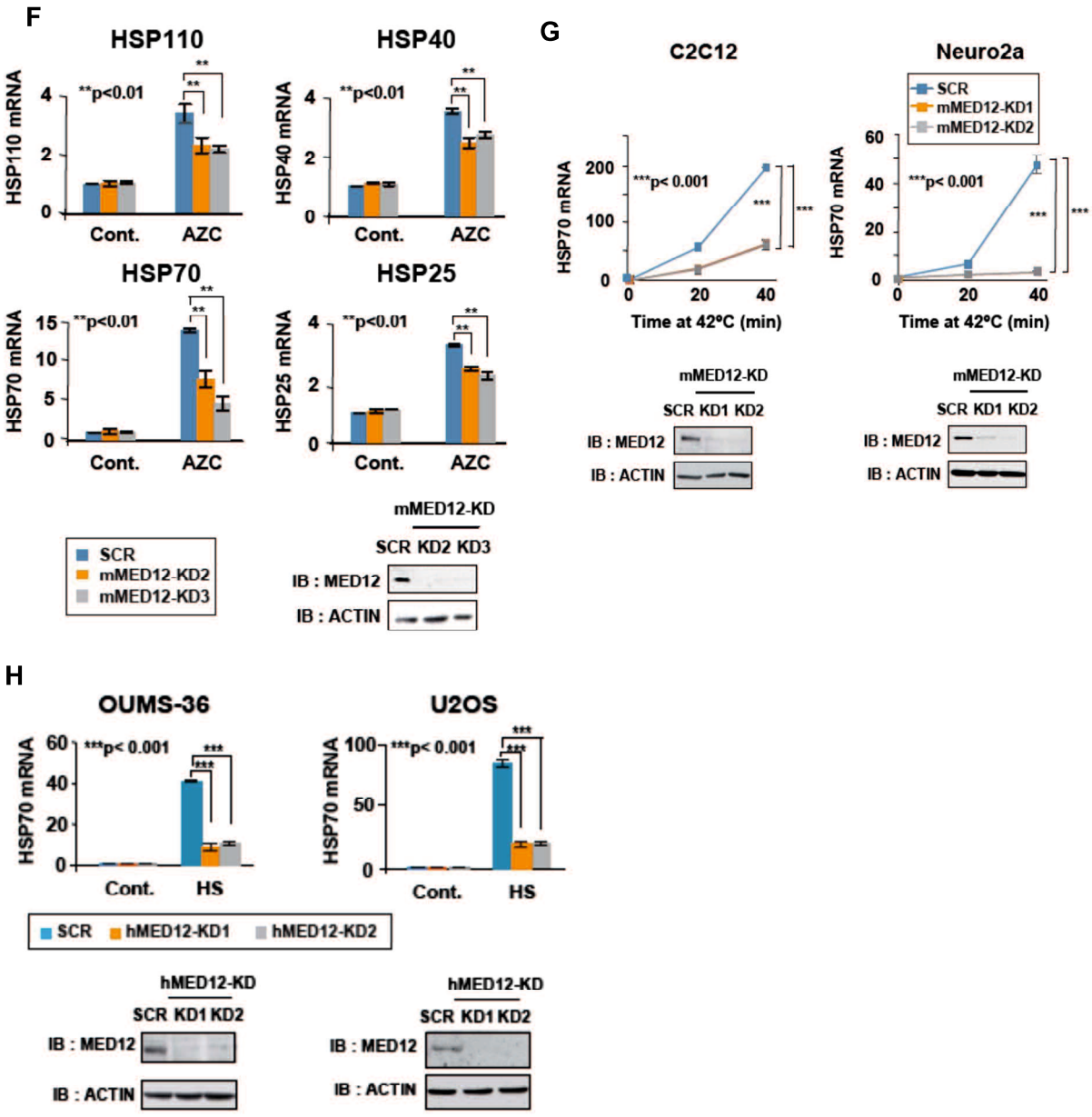


Figure 4. MED12 promotes HSR in mammalian cells. (A) HSE-dependent Mediator subunits complexed with AsHSF1 on the human *HSP70* promoter. A biotin-labeled human *HSPA1A (HSP70-1*) promoter fragment (wt70P) containing the proximal and distal HSE sequences or its HSE-mutated promoter fragment (mu70P) was incubated with nuclear extracts from heat-shocked MEF cells expressing AsHSF1, and bead-bound proteins were identified using mass spectrometry [12]. Among 676 HSE-dependent components, the peptide numbers of which identified in the AsHSF1:wt70P complex were higher than those in the AsHSF1:mu70P complex (difference in peptide numbers > 1),

all of the Mediator subunits are listed and subunits identified are indicated (blue boxes). Identified peptide numbers and LFQ intensity values calculated by MaxQuant software are shown in Fig. 4A. Schematics of core Mediator modules (head, middle, and tail modules) and CKM are shown [37]. Subunits of CKM (CDK8, CCNC, MED12, MED13) and some core Mediator subunits (MED1, MED14, MED17, and MED23) are indicated (lower). HSE-dependent Mediator subunits complexed with AsHSF1 on the human *HSP70* promoter. A biotin-labeled human *HSPA1A (HSP70-1*) promoter fragment (wt70P) containing the proximal and distal HSE sequences or its HSE-mutated promoter fragment (mu70P) was incubated with nuclear extracts from heat-shocked MEF cells expressing AsHSF1, and bead-bound proteins were identified using mass spectrometry [12]. Among 676 HSE-dependent components, the peptide numbers of which identified in the AsHSF1:wt70P complex were higher than those in the AsHSF1:mu70P complex (difference in peptide numbers > 1), all of the Mediator subunits (green) are listed and subunits identified are indicated (blue boxes). MED12 is indicated in red. p300, CBP, and SGO2 are listed as a reference (black) [12]. LFQ intensity values calculated by MaxQuant software (https://www.maxquant.org) (upper) and peptide numbers (lower) are shown. The intensity value of MED8 was not obtained using this software. The intensity values of Mediator subunits are lower than those of p300 and SGO2. **(B)** Expression of HSP70 mRNA during heat shock upon the KD of some core Mediator subunits. Immortalized MEF cells were infected for 72 h with an adenovirus vector expressing shRNA against mMED14, mMED17, or scrambled RNA (SCR). Alternatively, cells were infected with an adenovirus vector expressing shRNA against mouse p300 or CBP as negative controls [39]. These cells were then treated with heat shock at 42°C for 0, 20, or 40 min. HSP70 mRNA levels were quantified using RT-qPCR (n=3). After normalization with β-actin mRNA levels, levels relative to that in control SCR-treated cells (fold induction) were calculated. Mean ± S.D. is shown (upper). Asterisks indicate ***P < 0.001 by ANOVA. KD efficiency was assessed by RT-PCR using a primer set for each gene (lower). **(C)** Expression of HSP70 mRNA during heat shock upon KD of Mediator kinase module subunits. Cells were infected with an adenovirus vector expressing shRNA against mMED12, mMED13, mCCNC, or mCDK8 (KD1 or KD2) or scrambled RNA (SCR), and were then treated with heat shock. HSP70 mRNA levels were quantified using RT-qPCR (n=3) and calculated as described in B (upper). KD efficiency was assessed by RT-PCR (lower). **(D)** Expression of HSP110, HSP70, HSP40 and HSP25 mRNAs upon

MED12 KD. MEF cells were infected with an adenovirus vector expressing shRNA against mMED12 (KD1 or KD2) or scrambled RNA (SCR), and were then untreated (Cont.) or treated with heat shock at 42°C for 0 or 40 min. HSP70 mRNA levels were quantified using RT-qPCR (n=3). Mean ± S.D. is shown (upper). Asterisks indicate $^{**}P < 0.01$ by the Student's t-test. KD efficiency was assessed by immunoblotting (IB) using an antibody for MED12 or β-actin (lower). **(E)** Expression of HSP proteins in MED12 KD cells during heat shock. Cells were infected with adenovirus vector expressing shRNA against mMED12 (KD2 or KD3) or scrambled RNA (SCR). Control cells (C), cells treated with heat shock at 42°C for 30 min (0), or cells recovered at 37°C after 1 (1), 3 (3), or 6 (6) h from heat shock at 42°C for 30 min were harvested. Cell extracts were prepared using NP40-lysis buffer and subjected to immunoblotting (upper). The intensity of HSP bands in representative blots was quantified using NIH ImageJ and normalized to the intensity of each actin loading control. Expression levels relative to those in SCR-expressing cells recovered at 3 h after heat shock are shown (lower). **(F)** Expression of the mRNAs of various *HSP* genes during the AZC treatment in MED12 KD cells. Scrambled RNA-treated and MED12 KD (KD2 or KD3) MEF cells were untreated (Cont.) or treated with 5 mM AZC for 3 h. HSP mRNA levels were quantified using RT-qPCR (n=3) (upper). Mean ± S.D. is shown. Asterisks indicate $^{**}P < 0.01$ by the Student's t-test. KD efficiency was assessed using immunoblotting (lower). **(G)** Expression of HSP70 mRNA upon MED12 KD in various mouse cells. C2C12 or Neuro2a cells were infected with adenovirus vector expressing shRNA against mMED12 or scrambled RNA, and were then treated with heat shock at 42°C for 0, 20, or 40 min. HSP70 mRNA levels were quantified using RT-qPCR (n=3). Mean ± S.D. is shown (upper). Asterisks indicate $^{***}P < 0.001$ by ANOVA. KD efficiency was determined using immunoblotting (lower). **(H)** Expression of HSP70 mRNA upon MED12 KD in various human cell lines. OUMS-36T-3F and U2OS cells were infected with an adenovirus vector expressing shRNA against hMED12 (KD1 or KD2) or scrambled RNA, and were then treated with heat shock at 42°C for 0, 20, or 40 min. HSP70 mRNA levels were quantified and shown as described in (A) (upper). KD efficiency was assessed by immunoblotting (lower).

4.2 HSF1 interacts with MED12 during heat shock.

To investigate the interaction between MED12 and HSF1, extracts of cells overexpressing hHSF1-HA were prepared and subjected to a co-immunoprecipitation assay using anti-MED12 serum or preimmune serum (Fig. 5A). The results obtained showed that hHSF1-HA was co-precipitated with endogenous MED12 from extracts of heat-shocked MEF and human HeLa cells, but did not or was only slightly co-precipitated from unstressed cell extracts (Fig. 5A). This result suggested that the formation of the HSF1-MED12 complex was induced during heat shock. We then searched for an HSF1-binding region in a large mMED12 protein (amino acids 1 to 2,190) using a GST pull-down assay with purified GST-fused mMED12 fragments (regions 1 to 7) and nuclear extracts of heat-shocked HeLa cells overexpressing hHSF1-HA. It was revealed that hHSF1-HA was precipitated with GST-mMED12-region 5 (amino acids 1,401 to 1,821), which is located upstream of the PQL domain that interacts with SOX9 and catenin transcription factors [40,41] (Fig. 5B). Therefore, the mMED12 region 5 functioned as an HSF1-binding region under heat shock conditions.

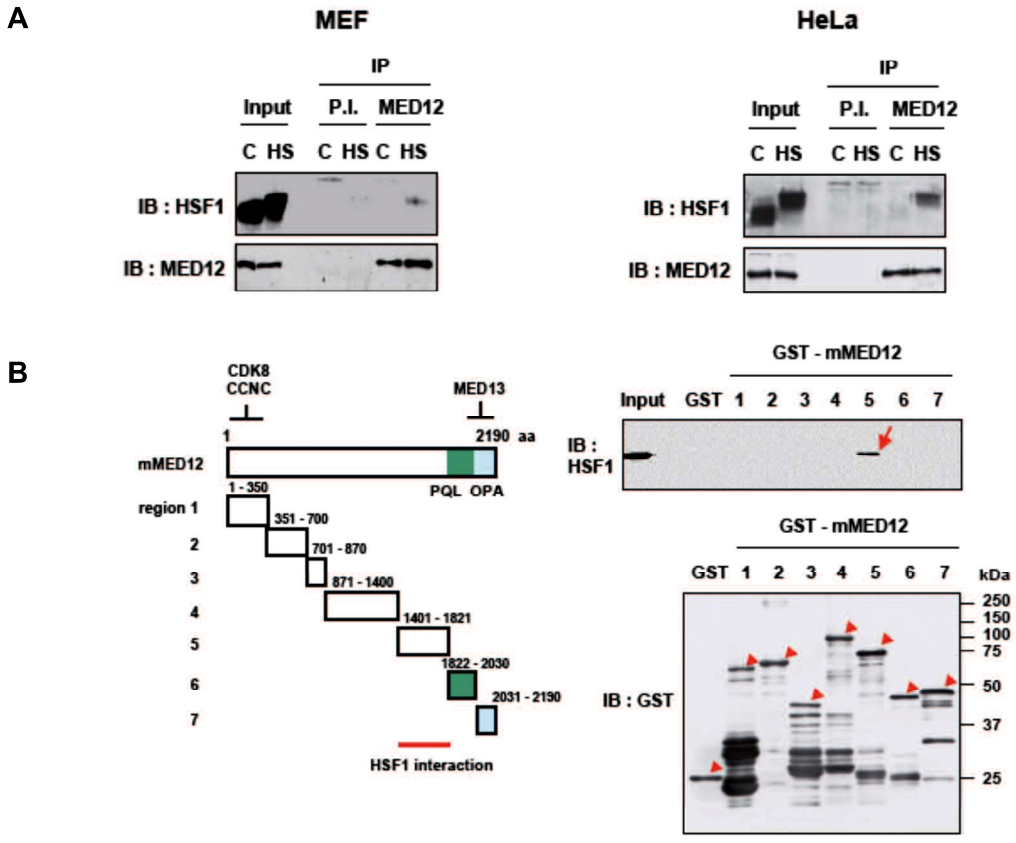


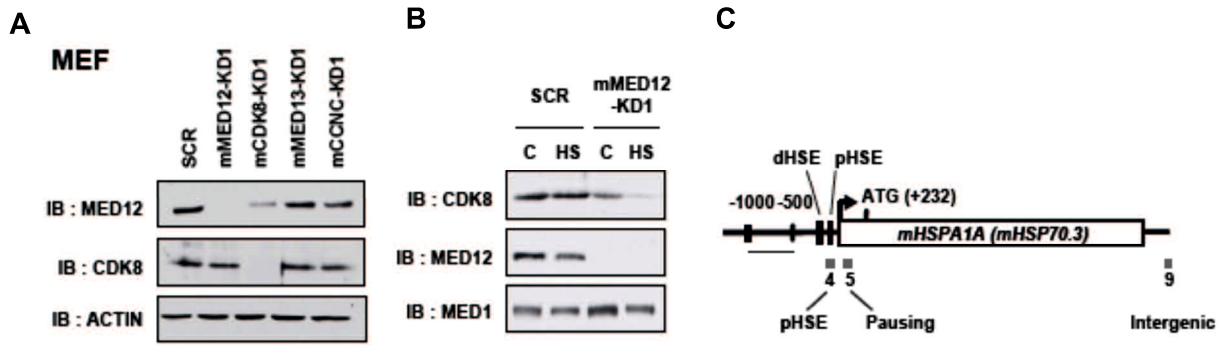
Figure 5. HSF1 interacts with MED12. (A) Interaction between HSF1 and MED12. MEF cells (left) or human HeLa (right) cells were infected for 48 h with Ad-hHSF1-HA. Extracts of control cells (C) and cells treated with heat shock at 42°C for 30 min (HS) were prepared using NP-40 lysis buffer, and soluble fractions were subjected to co-immunoprecipitation assay. Complexes co-immunoprecipitated using anti-MED12 serum or preimmune serum (P.I.) were blotted with an HA or MED12 antibody. **(B)** Identification of the MED12 region required for the interaction with HSF1. Schematic representation of mMED12 fragments fused to GST (left). GST pull-down assays were performed with mixtures of each purified GST fusion protein and nuclear extracts of heat-shocked (42°C for 30 min) HeLa cells overexpressing hHSF1-HA, and blotted with an HA or GST antibody (right). An red arrow indicates the hHSF1-HA band. Red arrowheads indicate GST or GST-MED12 fragments. The mMED12 region required for the interaction with hHSF1 is indicated as a red bar at the bottom. PQL, proline-, glutamine-, and leucine-rich domain; OPA, glutamine-rich domain. The binding regions of CDK8/CCNC and MED13 are indicated [60,61].

4.3 HSF1-dependent recruitment of MED12-CDK8 in *HSP70* promoter.

Since CDK8 kinase activity is regulated by CCNC and MED12, we assessed the stability of CDK8 following the KD of CKM subunits in MEF cells. CDK8 protein levels were slightly reduced by MED12 KD or CCNC KD, but not by MED13 KD (Fig. 6A), and were markedly decreased by MED12 KD during heat shock (42°C for 30 min) (Fig. 6B), which is consistent with previous findings [19,42,43]. MED12 protein levels were markedly reduced by CDK8 KD even under normal conditions [25, 42]. Therefore, CDK8 and MED12 stabilized each other under normal or heat shock conditions in MEF cells.

We next examined the occupancy of CDK8 and MED12 in the *HSP70* (*HSPA1A*) promoter. HSF1 binds to HSEs upon heat shock, and PIC containing Mediator and Pol II is assembled at the core promoter and DNA sequences ~50 bp downstream of the transcription start site [16,17]. Therefore, we performed a ChIP assay using primer sets that amplify the promoter proximal region (pausing region, +10 to +106) and one of the two HSEs

(pHSE region, -54 to -151) (Fig. 6C). Since the occupancy of endogenous mouse CDK8 was not detected using commercially available anti-CDK8 antibodies, we prepared MEF cells overexpressing mCDK8-FLAG and performed a ChIP assay using an anti-FLAG antibody. HSF1 occupancy levels were on pHSE in the *HSP70* promoter were low under normal conditions [6,39], and gradually increased in pHSE (region 4), but not in the Pol II pausing region (region 5), during heat shock at 42°C for 5, 10, and 30 min (Fig. 6D). The occupancy levels of CDK8-FLAG and MED12 as well as the core Mediator subunit MED1 were elevated in both pHSE and the pausing region during heat shock. We then investigated the effects of HSF1 KD, and found that MED12 occupancy was inhibited by HSF1 KD during heat shock at 42°C for 10 min (Fig. 6E). The core Mediator has been shown to recruit CKM, including MED12 [16,17]; therefore, it was completely blocked by the core Mediator subunit MED14 KD or MED17 KD in pHSE and the pausing region in the present study (Fig. 6F). Furthermore, CDK8 occupancy in the pHSE region was suppressed by HSF1 KD or MED12 KD (Fig. 6G). It is important to note that the occupancy of the core Mediator subunit MED1 was inhibited under the same conditions [25]. These results suggested that HSF1 and the core Mediator were required for the heat shock-induced recruitment of MED12, while MED12 was required for that of CDK8 in the *HSP70* promoter.



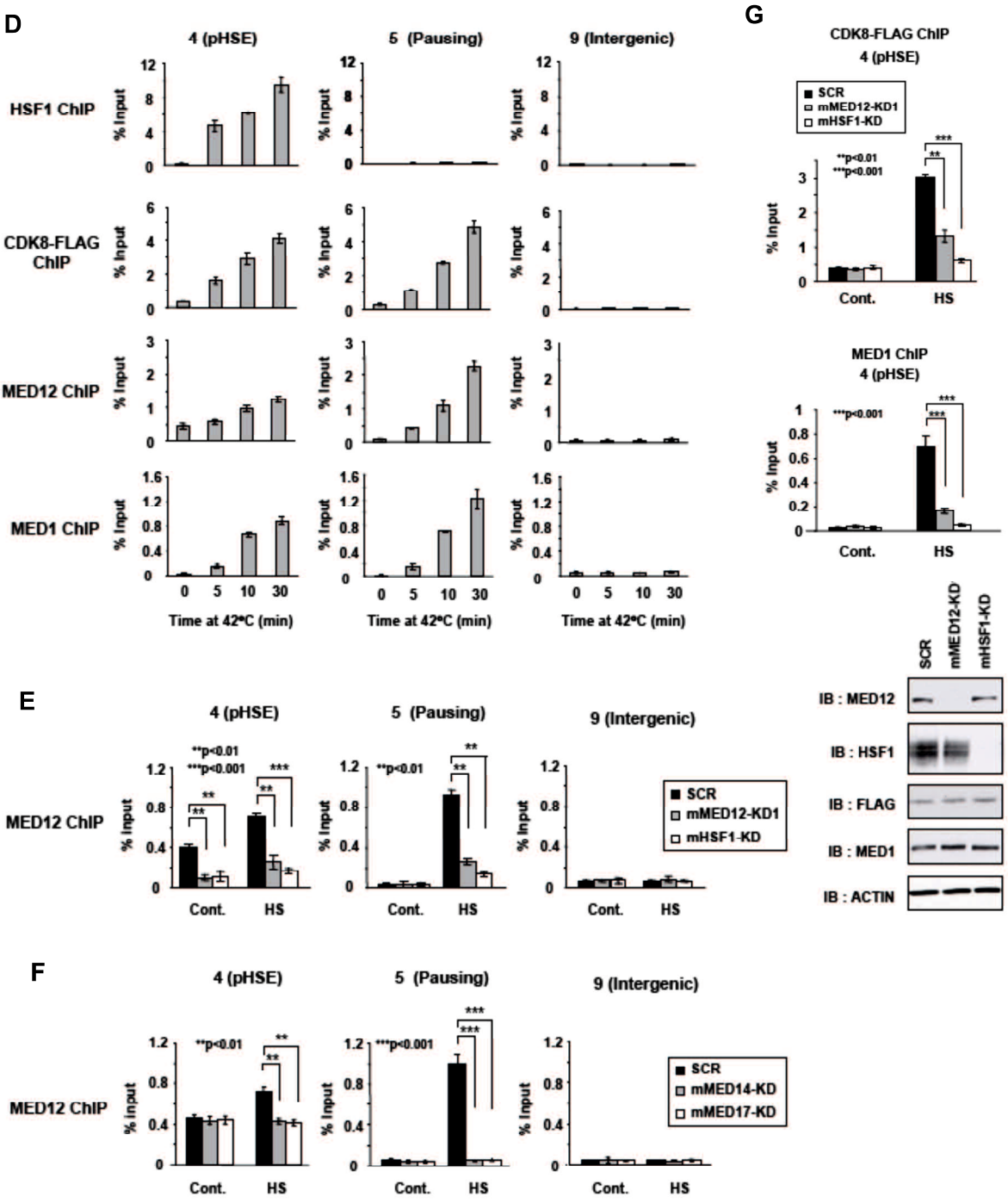


Figure 6. HSF1-dependent recruitment of MED12-CDK8 in the *HSP70* promoter. (A) Stability of MED12 and CDK8 upon the KD of CKM subunits. MEF cells were infected for 72 h with an adenovirus vector expressing shRNA against each CKM subunit or scrambled RNA (SCR). Cell extracts were prepared using NP40-lysis buffer, and soluble fractions were subjected to immunoblotting. **(B)** Stability of CDK8 upon MED12 KD during heat shock. MEF cells, infected for 72

h with Ad-sh-mMED12-KD1 or scrambled RNA (SCR), were untreated (C) or treated with heat shock at 42°C for 30 min (HS). Cell extracts were prepared and subjected to immunoblotting. **(C)** Schematic view of the mouse *HSP70* (*HSPA1A* or *HSP70.3*) locus. Proximal HSE (pHSE, -91 to -120) and distal HSE (dHSE, -172 to -216) are indicated in the promoter. Shaded boxes indicate DNA regions amplified using qPCR, which include pHSE (region 4, -54 to -151), the Pol II pausing region (region 5, +10 to +106), and intergenic region (region 9, +3138 to +3218) [12]. ATG, translation start site at +232. **(D)** Occupancy of HSF1, MED12, CDK8-FLAG, and MED1 in the *HSP70* promoter. MEF cells were infected with Ad-mCDK8-FLAG for 48 h and then treated with heat shock at 42°C for 0, 5, 10, or 30 min. ChIP-qPCR analyses in pHSE, the pausing region, and intergenic region on the *HSP70* locus were performed using an antibody for HSF1, FLAG, MED12, or MED1. Mean ± S.D. is shown. **(E)** Occupancy of MED12 in the *HSP70* promoter upon HSF1 KD. Cells were infected for 72 h with Ad-sh-mMED12-KD1, Ad-sh-mHSF1-KD, or Ad-sh-SCR, and were then untreated (Cont.) or treated with heat shock at 42°C for 10 min (HS). ChIP-qPCR analyses were performed (n=3). Mean ± S.D. is shown. Asterisks indicate **P < 0.01 or ***P < 0.001 by the Student's t-test. **(F)** Occupancy of MED12 in the *HSP70* promoter upon the KD of MED14 or MED17. Cells were infected with Ad-sh-mMED14-KD, Ad-sh-mMED17-KD, or Ad-sh-SCR, and were then untreated (Cont.) or treated with heat shock at 42°C for 10 min (HS). ChIP-qPCR analyses were performed (n=3) and data are shown as described in E. **(G)** Occupancy of CDK8-FLAG and MED1 in the *HSP70* promoter upon the KD of HSF1 or MED12. Cells were infected with Ad-sh-mHSF1-KD, Ad-sh-mMED12-KD1, or Ad-sh-SCR, and were then untreated (Cont.) or treated with heat shock at 42°C for 10 min (HS). ChIP-qPCR analyses of CDK8-FLAG and MED1 were performed (n=3) and data are shown as described in E (upper). KD efficiency was assessed by immunoblotting (lower).

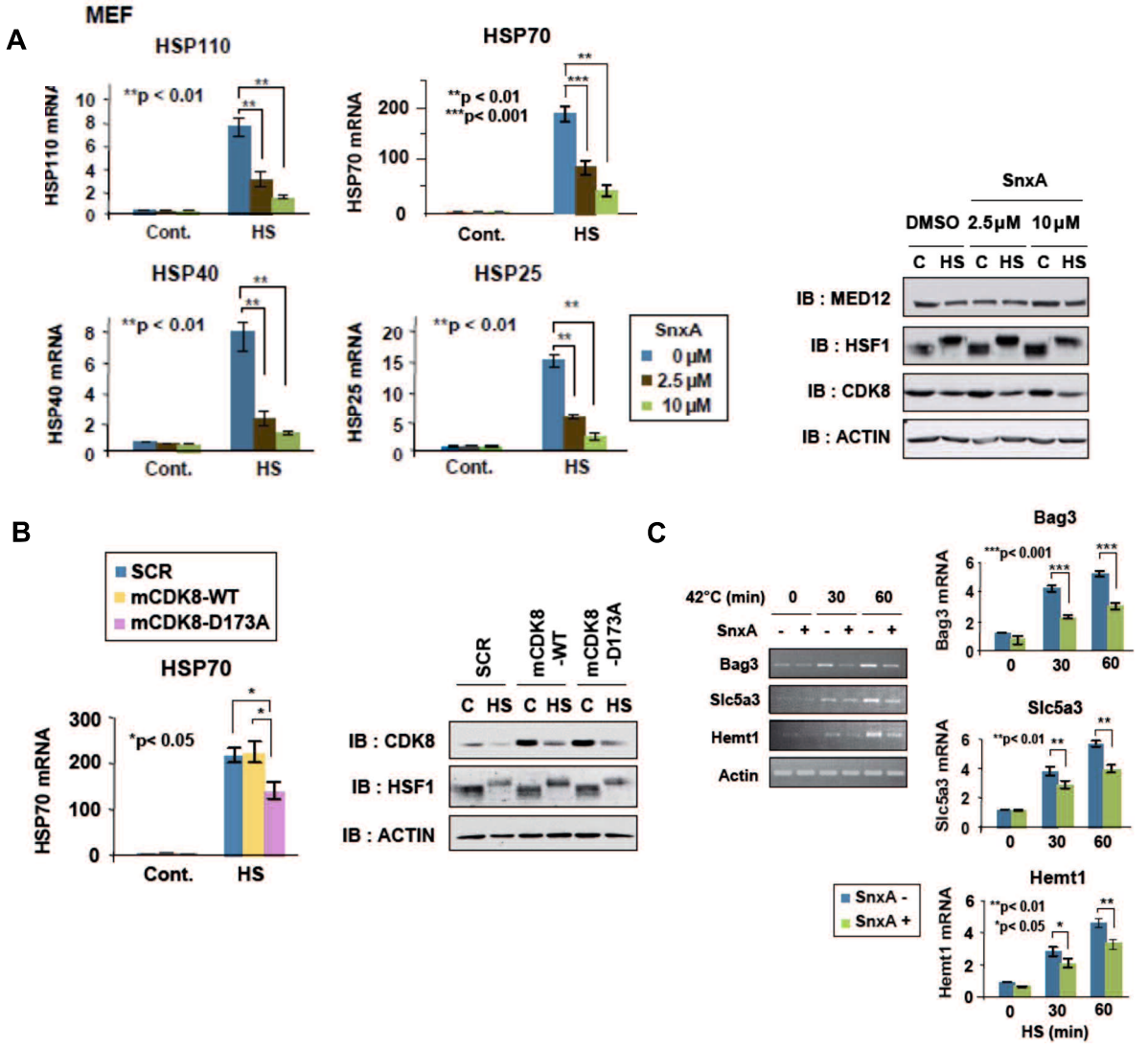
4.4 CDK8 promotes *HSP70* expression in part by phosphorylating HSF1.

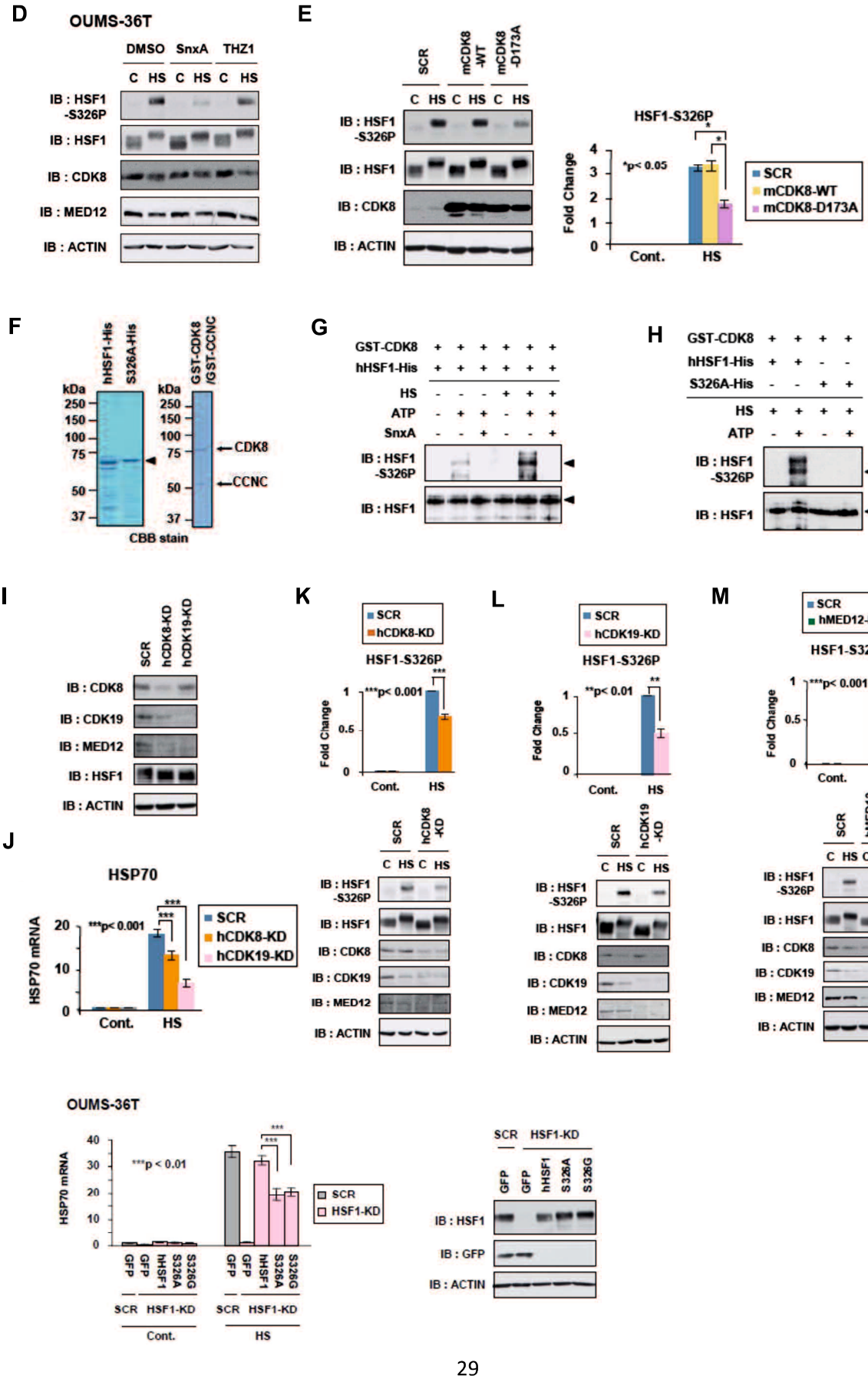
To establish whether CDK8 kinase activity is required for enhanced HSR, we treated MEF cells with the selective CDK8/19 inhibitor Senexin A [44]. The results obtained showed that the mRNA levels of HSP70 and other major HSPs including HSP110, HSP40 and HSP25 during heat shock at 42°C for 30 mins were markedly reduced by the treatment with Senexin

A at a concentration of 2.5 or 10 μM (Fig. 7A). Elevated HSP70 mRNA levels were also reduced by the overexpression of the kinase-dead mutant mCDK8-D173A [45] (Fig. 7B), indicating that CDK8 kinase activity promotes *HSP70* expression. We also examined the effects of Senexin A on the expression of other HSF1-dependent *non-HSP* genes [39], and found that the up-regulated expression of *Bag3*, *Slc5a3*, and *Hemt1* was significantly reduced by Senexin A (Fig. 7C). These results suggested that CDK8 (and CDK19) kinase activity promoted the expression of heat-inducible genes, including *HSP* and *non-HSP* genes.

CDK8/19 phosphorylates sequence-specific transcription factors as well as components of the general transcription machinery [46]. Therefore, we investigated whether CDK8/19 phosphorylates HSF1. The retardation of HSF1 bands in heat-shocked cells was not altered in the presence of Senexin A (Fig. 7A), suggesting that CDK8/19 was not involved in HSF1 hyperphosphorylation. We then examined its phosphorylation at a single site, such as Ser326, which enhances transcriptional activity [47]. Since an antibody to detect phosphorylated Ser326 in mouse HSF1 is not currently available, the phosphorylation of this residue was examined using human cells [12]. It was revealed that the phosphorylation of HSF1-S326 was strongly inhibited by Senexin A, but not by the CDK7 inhibitor THZ1, in human OUMS-36T-3F cells during heat shock (Fig. 7D). The phosphorylation of HSF1-S326 was reduced by the overexpression of the kinase-dead mutant mCDK8-D173A (Fig. 7E). To address if HSF1-S326 is a direct target for CDK8, we performed an *in vitro* kinase assay using recombinant wild-type hHSF1-His, phosphorylation site mutant hHSF1-S326A-His, and GST-CDK8/CCNC (Fig. 7F). We found that GST-CDK8/CCNC phosphorylated heat-shocked wild-type hHSF1-His much more than non-heat-shocked one, and incubation of wild-type hHSF1-His with Senexin A inhibited the phosphorylation of wild-type hHSF1-His (Fig. 7G). Furthermore, the mutant hHSF1-S326A-His was not phosphorylated by GST-CDK8/CCNC (Fig. 7H). These results suggested that CDK8/19 directly phosphorylated HSF1-S326.

We also analyzed HSF1-S326 phosphorylation and *HSP70* expression following the KD of CDK8, CDK19, or MED12 in OUMS-36T-3F cells. MED12 protein levels were reduced by both CDK8 KD and CDK19 KD, and CDK19 protein levels were also decreased by CDK8 KD (Fig. 7I). CDK8 KD or CDK19 KD decreased heat shock-induced HSP70 mRNA levels (Fig. 7J), similar to MED12 KD (Fig. 4H). KD of CDK8, CDK19, or MED12 simultaneously reduced the phosphorylation of HSF1-S326 (Fig. 7K-M). Furthermore, the substitution of endogenous HSF1 with the mutant hHSF1-S326A or hHSF1-S326G inhibited the induction of HSP70 mRNA expression during heat shock (Fig. 7N). Collectively, these results indicated that CDK8/19 promoted *HSP70* expression during heat shock in part by directly phosphorylating HSF1-S326.

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OUMS-36T
DMSO
SnxA
THZ1
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IB : HSF1
IB : CDK8
IB : MED12
IB : ACTIN
E
SCR
mCDK8 -WT
mCDK8 -D173A
C HS C HS C HS
IB : HSF1 -S326P
IB : HSF1
IB : CDK8
IB : ACTIN
HSF1-S326P
*p< 0.05
Fold Change
Cont.
HS
SCR
mCDK8-WT
mCDK8-D173A
F
hHSF1-His
S326A-His
GST-CDK8 /GST-CCNC
kDa
250
150
100
75
50
37
CDK8
CCNC
CBB stain
G
GST-CDK8
hHSF1-His
HS
ATP
SnxA
IB : HSF1 -S326P
IB : HSF1
H
GST-CDK8
hHSF1-His
S326A-His
HS
ATP
IB : HSF1 -S326P
IB : HSF1
I
SCR
hCDK8-KD
hCDK19-KD
IB : CDK8
IB : CDK19
IB : MED12
IB : HSF1
IB : ACTIN
J
HSP70
HSP70 mRNA
***p< 0.001
SCR
hCDK8-KD
hCDK19-KD
Cont.
HS
K
SCR
hCDK8-KD
HSF1-S326P
***p< 0.001
Fold Change
Cont.
HS
SCR
hCDK8 -KD
C HS C HS
IB : HSF1 -S326P
IB : HSF1
IB : CDK8
IB : CDK19
IB : MED12
IB : ACTIN
L
SCR
hCDK19-KD
HSF1-S326P
**p< 0.01
Fold Change
Cont.
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SCR
hCDK19 -KD
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IB : HSF1 -S326P
IB : HSF1
IB : CDK8
IB : CDK19
IB : MED12
IB : ACTIN
M
SCR
hMED12-KD1
HSF1-S326P
***p< 0.001
Fold Change
Cont.
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SCR
hMED12 -KD1
C HS C HS
IB : HSF1 -S326P
IB : HSF1
IB : CDK8
IB : CDK19
IB : MED12
IB : ACTIN
N
OUMS-36T
HSP70 mRNA
***p < 0.01
SCR
HSF1-KD
GFP
GFP
hHSF1
S326A
S326G
SCR HSF1-KD
SCR HSF1-KD
Cont.
HS
SCR HSF1-KD
GFP
GFP
hHSF1
S326A
S326G
IB : HSF1
IB : GFP
IB : ACTIN

Figure 7. CDK8 promotes *HSP70* expression in part by phosphorylating HSF1. (A) Effects of Senexin A on *HSP110, HSP70, HSP40 and HSP25* expression during heat shock. MEF cells were pre-treated for 1 h with 0, 2.5, or 10 μM Senexin A (SnxA), and were then untreated (Cont.) or treated with heat shock at 42°C for 30 min (HS). HSP110, HSP70, HSP40 and HSP25 mRNA levels were quantified using RT-qPCR (n=3). After normalization with β-actin mRNA levels, levels relative to that in untreated cells (fold induction) were calculated. Mean ± S.D. is shown (left). Asterisks indicate $^{**}P < 0.01$ or ***P < 0.001 by the Student's t-test. Cell extracts were prepared using NP-40 lysis buffer, and soluble fractions were subjected to immunoblotting using each antibody (right). **(B)** Down-regulation of *HSP70* expression in cells overexpressing the CDK8 kinase-dead mutant. MEF cells were infected with an adenovirus expressing wild-type mCDK8-FLAG or the kinase-dead mutant mCDK8-D173A-FLAG for 48 h, and were then untreated (Cont.) or treated with heat shock at 42°C for 30 min (HS). HSP70 mRNA levels were quantified using RT-qPCR as described in A (n=3). Asterisks indicate $^{*}P < 0.05$ by the Student's t-test (left). Cell extracts were prepared and subjected to immunoblotting using each antibody (right). **(C)** Effects of Senexin A on heat-inducible *non-HSP* genes. Cells, pre-treated with 10 μM Senexin A (SnxA), were treated with heat shock at 42°C for 30 or 60 min (HS). The mRNA levels of heat shock-inducible genes and the *β-actin* gene were analyzed using RT-PCR, and representative images are shown (left). The intensities of bands were quantified using NIH ImageJ, and normalized with those of β-actin mRNA levels (n=3). Mean ± S.D. is shown (right). Asterisks indicate $^{*}P < 0.05$, $^{**}P < 0.01$ or $^{***}P < 0.001$ by the Student's t-test. **(D)** Effects of Senexin A on the phosphorylation of HSF1-S326. Human OUMS-36T-3F fibroblasts were pre-treated with 2.5 μM Senexin A (SnxA) for 1 h or 1 μM THZ1 for 2 h, and were then untreated (Cont.) or treated with heat shock at 42°C for 30 min (HS). Cell extracts were prepared and subjected to immunoblotting using each antibody. **(E)** Reduced phosphorylation of HSF1-S326 in cells overexpressing the CDK8 kinase-dead mutant. OUMS-36T-3F cells were infected with an adenovirus expressing wild-type mCDK8-FLAG or mCDK8-D173A-FLAG for 48 h, and were then untreated (Cont.) or treated with heat shock at 42°C for 30 min (HS). Cell extracts were prepared and subjected to immunoblotting. A representative image is shown (left). The phosphorylation levels of HSF1-S326 were quantified using NIH ImageJ, and normalized with β-actin levels (n=3). Mean ± S.D. is shown (left). Asterisks indicate $^{**}P < 0.01$ by the Student's t-test (right). (**F-H**) Phosphorylation of HSF1-S326 in vitro. Recombinant

wild-type hHSF1-His and phosphorylation site mutant hHSF1-S326A-His (S326-His) was purified from *Escherichia coli*. Aliquots (2 μg each) were subjected to 8% SDS-PAGE and stained with Coomassie Brilliant Blue (left). Recombinant GST-CDK8/CCNC complex (1 μg), purified from baculovirus-infected Sf9 insect cells, was similarly subjected to SDS-PAGE and stained (right) (F). Purified HSF1-His (2 μg) was untreated or treated with heat shock (HS) and mixed with GST-CDK8/CCNC (0.1 μg) in phosphorylation buffer. ATP was added to the reactions at a final concentration of 40 μM, and this was followed by an incubation at 30°C for 2 h in the presence or absence of Senexin A (SnxA). Proteins in the reactions were subjected to immunoblotting using an antibody for HSF1 or HSF1 phospho-S326 (G). Purified HSF1-His and hHSF1-S326A-His (2 μg each) were treated with heat shock (HS) and mixed with GST-CDK8/CCNC (0.1 μg) in phosphorylation buffer. These were incubated at 30°C for 2 h in the presence or absence of ATP. Proteins in the reactions were subjected to immunoblotting as described in G (H). **(I-J)** Down-regulation of *HSP70* expression in CDK8 KD or CDK19 KD cells. OUMS-36T-3F cells were infected with Ad-sh-hCDK8-KD1, Ad-sh-hCDK19-KD1, or scrambled RNA (SCR). Cell extracts were prepared and subjected to immunoblotting using each antibody (I). Cells infected with each adenovirus were then untreated (Cont.) or treated with heat shock at 42°C for 30 min (HS). HSP70 mRNA levels were quantified using RT-qPCR as described in A (n=3). Asterisks indicate ***P < 0.001 by the Student's t-test (J). **(K-M)** Reduced phosphorylation of HSF1-S326 in CDK8, CDK19, or MED12 KD cells. OUMS-36T-3F cells were infected with Ad-sh-hCDK8-KD1 (K), Ad-sh-hCDK19-KD1 (L), Ad-sh-hMED12-KD1 (M), or scrambled RNA (SCR), and were then untreated (Cont.) or treated with heat shock at 42°C for 30 min (HS). Cell extracts were subjected to immunoblotting, and a representative image is shown (lower). The phosphorylation levels of HSF1-S326 were quantified (n=3), and Mean ± S.D. is shown (upper). Asterisks indicate ***P < 0.001 or **P < 0.01 by the Student's t-test. **(N)** *HSP70* expression in the presence of hHSF1-S326 mutants. OUMS-36T cells were infected with Ad-sh-hHSF1-KD or Ad-sh-SCR (2×10^7 pfu/ml) for 2 h and maintained in normal medium for 22 h [7]. The cells were then infected with Ad-hHSF1-FLAG, Ad-hHSF1-S326A-FLAG, Ad-hHSF1-S326G-FLAG, or Ad-GFP (2×10^5 pfu/ml) for 2 h and maintained in normal medium for a further 46 h [12]. These cells were untreated (Cont.) or treated with heat shock at 42°C for 30 min (HS). HSP70 mRNA levels were quantified by RT-qPCR. After normalization with GAPDH mRNA levels, levels relative to that in control scrambled RNA-treated cells

(fold induction) were calculated. Mean ± S.D. is shown (left). Asterisks indicate ***P < 0.001 by the Student's t-test. Cell extracts were subjected to immunoblotting (right).

4.5 Inhibition of CDK8 impairs proteostasis capacity during heat shock.

We investigated whether the CDK8 and CDK19 mediated up-regulation of *HSP70* expression is associated with cell survival and the capacity for proteostasis during heat shock. We found that the survival of MEF cells was significantly reduced by the treatment with Senexin A (2.5 or 10 μM) in a dose dependent manner during heat shock at 45°C until 3 h (Fig. 5A). We then performed the luciferase refolding assay using MEF cells stably expressing luciferase (MEF-Fluc) [12]. It was revealed that the refolding of heat-denatured luciferase was severely impaired by the treatment with Senexin A, even at a concentration of 2.5 μM (Fig. 5B). Therefore, the inhibition of CDK8 reduced cell survival and impaired proteostasis capacity during heat shock.

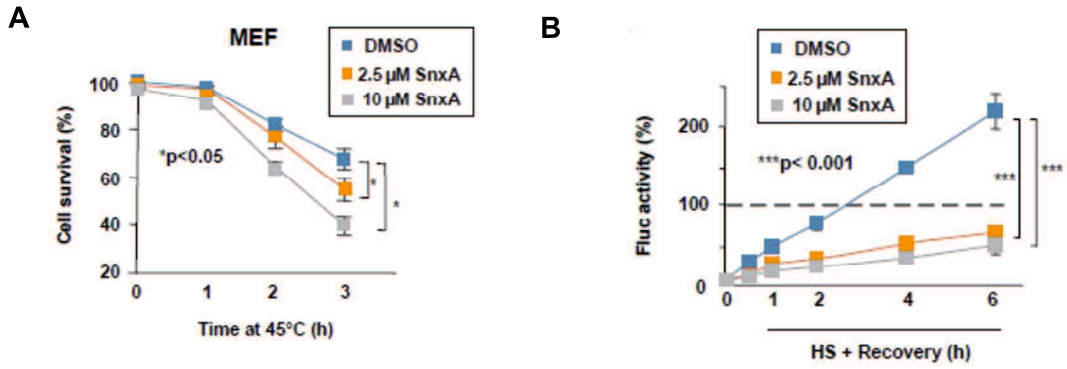

Figure 8. Inhibition of CDK8 impairs proteostasis during heat shock. (A) Senexin A sensitize cells to heat shock. MEF cells were pretreated with Senexin A (2.5 or 10 μM) or DMSO for 1 h, and then treated with heat shock at 45°C until 3 h. The numbers of viable cells with the ability to exclude trypan blue was counted. Mean ± S.D. is shown (n=3). Asterisks indicate *P < 0.05 by ANOVA. **(B)** Senexin A inhibited the refolding of the luciferase sensor protein during recovery from heat shock. MEF-Fluc cells, pretreated with Senexin A (2.5 or 10 μM) or DMSO for 1 h, were treated with heat shock at 42°C until 2 h, and were then recovered at 37°C for the indicated periods. Luciferase activity values were measured, and relative activities were calculated. The value of control cells was set to 100% (dotted line). Mean ± S.D. is shown (n=3). Asterisks indicate ***P < 0.001 by ANOVA (left).

5. Discussion

HSF1-mediated HSR is one of major inducible gene expression systems, and is mainly controlled by the recruitment of Pol II to *HSP* promoters and the release of paused Pol II for elongation [48]. On the other hand, Mediator is a central coactivator complex that facilitates the formation of PIC containing GTFs and Pol II by specifically interacting with TFIIB and TFIIH, and also regulates Pol II pausing and elongation by transmitting the information of transcription factors to GTFs and Pol II [13,17]. Previous studies reported that HSR was promoted at least by the core Mediator in *Drosophila* and yeast cells [31,32,38]. We herein demonstrated that the KD of the pivotal subunits MED14 and MED17 in the core Mediator markedly reduced HSR in mammalian cells (Fig. 1A). In contrast, the depletion of CKM through the cytoplasmic anchoring of CDK8 did not influence *HSP* expression during heat shock in yeast [30], while the yeast CKM subunit CycC (yeast ortholog of CCNC) down-regulated the expression of SSA1, a member of HSP70 family, under normal growth conditions [49]. Therefore, it was unclear whether CKM plays a role in mammalian HSR. To answer this question, we examined *HSP70* expression following the KD of each of the four CKM subunits, and showed that its expression was inhibited by each KD (Fig. 4). This result demonstrated that CKM promotes *HSP70* expression in response to heat shock, and suggested that this function is mediated through CDK8 kinase activity. Heat shock-induced expression of HSPs and non-HSPs were inhibited by treatment with the selective CDK8/19 inhibitor Senexin A or the overexpression of the kinase-dead mutant mCDK8-D173A (Fig. 7A-C). Thus, CDK8 kinase activity is required to enhance HSR in mammalian cells, in contrast to that in yeast [30]. Our results are consistent with CKM potentially playing a positive role in regulating the transcription of genes that respond to environmental stress [46, 50].

We then investigated the mechanisms by which CKM and CDK8 positively affect heat shock-induced *HSP70* transcription. The results obtained showed that the occupancy levels of CDK8 and MED12 in the *HSP70* promoter were markedly elevated in a manner that depended on the core Mediator as well as HSF1 during heat shock (Fig. 6). The unique

interaction between HSF1 and MED12, which was elucidated using extracts of cells overexpressing HSF1 (Figs. 4A, 5), may facilitate the recruitment of CKM (Fig. 6C). We also showed that the occupancy of core Mediator subunit MED1 during heat shock was inhibited by MED12 KD (Fig. 6G), suggesting that CKM regulates the recruitment of core Mediator [25, 51]. Since the core Mediator interacts with Pol II or CKM in a mutually exclusive manner [18, 37], CKM may transiently interact with the core Mediator and stabilize it in the *HSP70* promoter, and is then substituted with Pol II to facilitate the formation of PIC [16].

CDK8 kinase activity was previously shown to be stimulated by forming a complex with MED12 and CCNC [20, 21], and promoted transcription by phosphorylating target proteins, possibly within the promoter region. CDK8 target proteins include GTFs, such as TFIID, Pol II, Mediator itself, and chromatin regulators [51]. Sequence-specific transcription factors,

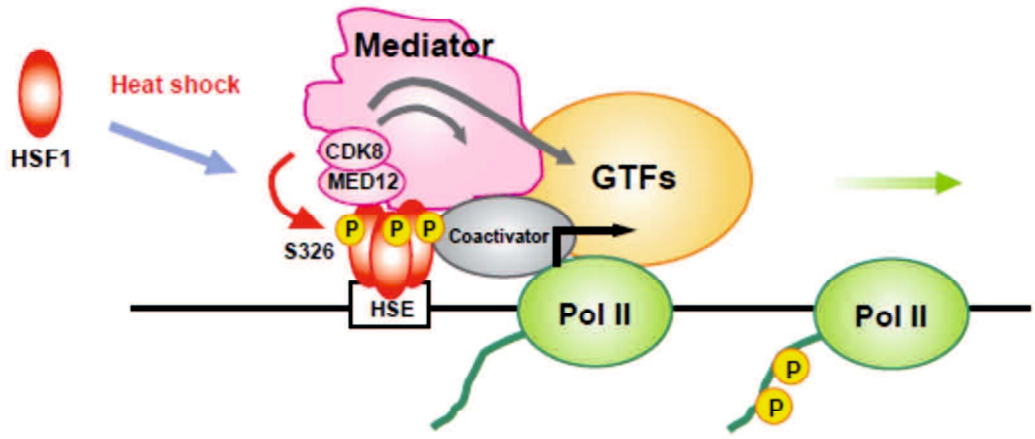

Figure 9. Schematic model depicting the role of MED12-CDK8 in mammalian HSR. In response to heat shock, HSF1 binds to HSEs in the *HSP70* promoter and recruits MED12 and CDK8. CDK8 not only phosphorylates the components of Mediator and general transcription factors (GTFs) that facilitates the formation of the preinitiation complex [45], but also phosphorylates HSF1-S326, which enhances its transcriptional activity by promoting the recruitment of coactivators [12], and promotes *HSP70* transcription by Pol II.

including STAT1 and SREBP, are also important target proteins, and their activity and stability were shown to be regulated by CDK8-mediated phosphorylation [52,53]. Therefore, we investigated the phosphorylation of HSF1-S326, one of the major active modifications in HSF1 [4,5]. The results obtained revealed that the phosphorylation of HSF1-S326 was markedly reduced upon affecting the mediator CKM module in any possible ways such as treatment with Senexin A, KD of Mediator CKM subunits (CDK8, CDK19, or MED12) (Fig. 7K-M) or overexpression of the CDK8 kinase-dead mutant in human cells (Fig. 7E). These results identified HSF1-S326 as a target of CDK8/19 during heat shock; however, this site is also phosphorylated by MEK kinase [54]. CDK8/19-mediated phosphorylation may stabilize coactivators, such as SGO2 [12] and the core Mediator including MED1 (Fig. 6H), which interact with Pol II in the *HSP70* promoter, thereby facilitating the formation of PIC during heat shock (Fig. 9). Transcriptional activation mediated by HSF1-S326 phosphorylation is conserved in vertebrate species, except in the chicken, but is lacking in yeast [12]. This may be the reason for the different requirements of yeast CKM in HSR [30].

'*Mediatorpathy*' is associated with *de novo* mutations occurring in Mediator complex, mainly in the CKM module (MED12, MED13 or CDK8/19). MED12, MED13 and CDK8 are often reported to be mutated in the numerous cancer and syndromic disabilities [63]. Mutation in CDK8/CDK19 is associated in cancers including colon cancer [63], melanoma (64), Prostate cancer (65) and breast cancer (66). MED12 loss of function is associated with activation the TGF-βR signaling, which suffices the drug resistance [67]. This suggests CKM to be an important regulatory part in progression of the disease. Similarly, a shift in HSF1 protein expression was observed contributing to neurodegenerative diseases and cancers, by a reduction and elevation of protein expression, respectively. An elevated level of HSF1 has been reported in numerous cancers including, hepatocellular carcinoma [68, 69], breast cancer [70], sporadic colorectal cancer [71], endometrial carcinoma, oral squamous cell carcinoma [72], prostate cancer [73]. Together, these studies conclude that both HSF1 and Mediator-CKM play an important role in disease onset and progression. HSF1 is activated in part through the phosphorylation of S326 in malignant tumor cells, and enhances

proteostasis capacity to prevent the formation of toxic protein aggregates and amyloids [54]. Therefore, these cells are addicted to HSF1 for their proliferation. As CDK8 and MED12 have been associated with many types of cancers, and, thus, the therapeutic usefulness of CDK8 inhibitors is now being intensively investigated [55]. CDK8/19 inhibition using combination therapies with Senexin B and EGFR-targeting small molecules (gefitinib and erlotinib) or monoclonal antibody (cetuximab) has a potential by preventing the adaptive resistance against cancer drugs [74]. This present study suggest that the interaction between HSF1 and MED12 is a more specific therapeutic target for some cancer types.

6. Summary

In the present study, we have shown the importance of Mediator CKM module and its role during Heat shock. Knockdown studies targeting core Mediator and CKM module components were shown compromised HSPs gene induction, which therefore suggests that Mediator complex is required for the HSF1 activation, PIC- and Pol II- recruitment to initiate transcription in mammalian cells. We found MED12 as an important co-factor, which interacts with HSF1 directly and majorly during heat shock. MED12 recruitment to proximal promoter and pausing site, guided by HSF1, is necessary for the recruitment of core Mediator and CKM module following the heat shock. Given that, MED12-CCNC is required to activate CDK8 kinase activity and CKM subunit is required to maintain each-other stability. Our in vitro study suggests that CDK8 or CDK19 kinase activity is required to phosphorylate HSF1-S326 site, which is essential for full activation of HSF1 and its interaction with the other co-activators, such as SGO2. Furthermore, inhibition of CDK8 impairs the proteostasis capacity of a cell during heat shock. Together, the Mediator CKM is required to regulate the proteostasis capacity of a cell.

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