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Permalink
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Journal
Proceedings of the National Academy of Sciences of the United States of America, 112(26)

ISSN
0027-8424

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Publication Date
2015-06-30

DOI
10.1073/pnas.1509658112

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Arginine methylation of HSP70 regulates retinoid acid-mediated *RARβ2* gene activation

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Contributed by Michael G. Rosenfeld, May 19, 2015 (sent for review January 21, 2015; reviewed by Michael R. Stallcup)

Although "histone" methyltransferases and demethylases are well established to regulate transcriptional programs and to use nonhistone proteins as substrates, their possible roles in regulation of heat-shock proteins in the nucleus have not been investigated. Here, we report that a highly conserved arginine residue, R469, in HSP70 (heat-shock protein of 70 kDa) proteins, an evolutionarily conserved protein family of ATP-dependent molecular chaperone, was monomethylated (me1), at least partially, by coactivator-associated arginine methyltransferase 1/protein arginine methyltransferase 4 (CARM1/PRMT4) and demethylated by jumonji-domain–containing 6 (JMJD6), both in vitro and in cultured cells. Functional studies revealed that HSP70 could directly regulate retinoid acid (RA)-induced retinoid acid receptor *β*2 (RAR*β*2) gene transcription through its binding to chromatin, with R469me1 being essential in this process. HSP70's function in gene transcriptional regulation appears to be distinct from its protein chaperon activity. R469me1 was shown to mediate the interaction between HSP70 and TFIIH, which involves in RNA polymerase II phosphorylation and thus transcriptional initiation. Our findings expand the repertoire of nonhistone substrates targeted by PRMT4 and JMJD6, and reveal a new function of HSP70 proteins in gene transcription at the chromatin level aside from its classic role in protein folding and quality control.

heat-shock proteins | arginine methylation | gene transcription

Nucleosome is the basic unit of chromatin, which consists of ~146 bp of DNA wrapped around an octamer comprised of two copies of the core histone proteins (H3, H4, H2A, and H2B). A central mechanism for regulating chromatin structure and therefore gene expression is achieved via covalent posttranslational modifications (PTMs) on histone proteins, which include but are not limited to phosphorylation, acetylation, ubiquitylation, sumoylation, and methylation (1, 2). Histone methylation can be found on all basic residues, including arginines, lysines, and histidines (3, 4), which was originally believed to be an irreversible modification that could only be removed by histone eviction or by dilution during DNA replication; however, the discovery of histone demethylase LSD1 has changed this notion, and the subsequent discovery of the Jumonji C (jmjC)-domain–containing demethylases by a number of laboratories supports this conclusion (5, 6). Histone methylation dynamics is known to have important roles in many biological processes, including developmental control, cell-fate decision, aging, cancer, and other diseases (7, 8).

Protein arginine methylation results in the addition of one or two methyl groups to the guanidino nitrogen atoms of arginine (9), which is catalyzed by a family of enzymes called protein arginine methyltransferases (PRMTs). The PRMT family has been shown to include at least 11 members in mammalian genomes, designated as PRMT 1 to 11 based on difference in primary sequences and substrate specificity. Arginine methylation has also been found on hundreds of nonhistone proteins in both the nucleus and the cytoplasm (10, 11). In conjunction with the expanding number of proteins that are known to be arginine methylated, a growing list of biological processes is being shown to involve arginine methylation, which include transcription, RNA processing and transport, translation, signal transduction, DNA repair, and apoptosis, among others (10–14). Arginine methyltransferases have been linked to diseases, including prostate and breast cancers, cardiovascular diseases, viral pathogenesis, and spinal muscular atrophy, suggesting these enzymes might be promising drug targets (15). Arginine methylation has been considered to be an irreversible posttranslational modification until recently. Two groups showed that arginine methylation of histones can be reduced in vitro by deimination with peptidyl-arginine deiminase 4, which converts both unmodified arginine and monomethylarginine, but not dimethylarginine, to citrulline (16, 17). However, peptidyl-arginine deiminases are not real demethylases because citrulline lacks the positively charged guanidine group, and thus it cannot functionally substitute for arginine. Recently, it was shown that a JmjC-domain–containing protein named JMJD6 functions as an arginine demethylase, which specifically demethylates both H3R2me and H4R3me (18). More recently, we reported the dual enzymatic activities of JMJD6 targeting both histone (H4R3me) and RNA (methyl group on the γ-phosphate of 7SK snRNA's first 5′

Significance

HSP70 proteins are well known as molecular chaperones involved in protein folding and quality control. Whether they also function in gene transcription on chromatin, and if so, how they are regulated, remains elusive. Here we report that HSP70 can also regulate gene transcription through its association with chromatin, distinct from its "classic" function as a molecular chaperone. The function of HSP70 in gene transcription is subject to regulation of an arginine methylation on a highly conserved residue in HSP70, which modulates the recruitment of a key component in the preinitiation complex, and thus transcription initiation. The present study reveals an additional, previously overlooked function of HSP70 chaperone proteins, and links arginine methylation of nonhistone proteins to gene transcriptional regulation.

Author contributions: M.G.R. and W.L. designed research; W.-w.G., R.-q.X., B.-l.P., H.-t.X., H.-f.S., M.-f.H., T.-t.S., J.Y., W.-j.Z., X.-n.W., X.G., and W.L. performed research; X.-z.L. and P.C.D. contributed new reagents/analytic tools; W.-w.G., R.-q.X., B.-l.P., H.-t.X., H.-f.S., M.-f.H., T.-t.S., J.Y., W.-j.Z., X.-n.W., X.G., and W.L. analyzed data; and W.L. wrote the paper with contributions from M.G.R.

Reviewers included: M.R.S., University of Southern California.

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1509658112/-/DCSupplemental.

Fig. 1. HSP70 methylation in cultured cells. (*A*) Cell lysates collected from HEK293T cells were subjected to IP with control IgG or anti-HSP70 antibody followed by IB with anti-methyl-lysine (Kme) (*Left*), antimethyl-arginine (Rme) (*Center*), or anti-HSP70 (*Right*) antibody. IgG H.C: IgG heavy chain. (*B*) HEK293T cells were treated with or without AdOx (20 μM) for 6 h before IP and IB experiments as described in *A*. (*C*) HEK293T cells were transfected with Flag-tagged HSP70 and then treated with or without AdOx (20 μM) for 6 h before IP with anti-Flag antibody, followed by IB with anti-Kme, anti-Rme, or anti-Flag antibody. Input was shown on the far left panel. N.S: nonspecific bands. (*D*) In vivo labeling of HSP70 proteins. HEK293T cells were maintained in DMEM without methionine for 24 h before adding NTM [cycloheximide (100 μg/mL) and chloramphenicol (40 μg/mL)] for another hour, followed by adding [^{35}S]-methionine (20 μCi/mL) (lanes 1 and 2) or L-[*methyl*-^{3}H]-methionine (20 μCi/mL) (lanes 3 and 4) in the presence or absence of AdOx (20 μM) for 4 h. Cells were then lysed and subjected to IP with anti-HSP70 antibody, followed by SDS/PAGE gel and autoradiogram. (*E*) HEK293T cells were transfected with Flag-tagged HSP70 before in vivo labeling with L-[*methyl*-^{3}H]-methionine as described in *D*. (*F*) HSP70 proteins purified from HEK293T cells were subjected to Coomassie blue staining (C.B.S). IgG H.C: IgG heavy chain; IgG L.C: IgG light chain. (*G* and *H*) Sequence alignment of the region surrounding arginine 469 (boxed) in multiple HSP70 isoforms (*Homo sapiens*) (*G*) or in paralogous HSP70 genes in various organisms (*H*) by using Clustal Omega. Asterisk (*) indicates positions which have fully conserved residue; Colon (:) indicates conservation between groups of strongly similar properties; Period (.) indicates conservation between groups of weakly similar properties. *At*, *Arabidopsis thaliana*; *Dm*, *Drosophila melanogaster*; *Dr*, *Danio rerio*; *Hs*, *Homo sapiens*; *Mm*, *Mus musculus*; *Pf*, *Plasmodium falciparum*; *Rn*, *Rattus norvegicus*; *Sc*, *Saccharomyces cerevisiae*; *Xt*, *Xenopus (Silurana) tropicalis*.

nucleotide), which are important for its function in Pol II promoter-proximal pause release of a substantial list of genes (19). Interestingly, JMJD6 also targets to arginine methylation on nonhistone proteins, such as estrogen receptor alpha (ERα) and RNA helicase A (RHA) (20, 21).

HSP70 (heat-shock protein of 70 kDa) proteins constitute an evolutionarily conserved protein family of ATP-dependent molecular chaperones (22). Humans have 13 genes encoding HSP70s, among which some are induced by heat shock and other types of stress, and others are constitutively expressed (23, 24). HSP70s share a domain architecture consisting of an amino (N)-terminal nucleotide-binding domain, followed by a substrate-binding domain, which can be further subdivided into a peptide-binding domain and a carboxyl (C)-terminal "lid" domain (25). At the extreme C-terminal end of most cytoplasmic HSP70 chaperones lies an EEVD motif that is known to interact with certain tetratricopeptide repeat-containing proteins (26, 27). In addition to their function in client protein folding, HSP70 chaperones play an important role during the translocation of a number of proteins, such as nuclear receptors and kinases, to their designated subcellular compartments (28).

Mammalian HSP70 proteins are subject to various post-translational modifications, including phosphorylation, acetylation, ubiquitination, and methylation (29, 30). Interestingly, despite the fact that lysine and arginine methylation on HSP70 chaperones was first reported in the early 1980s, the exact residues being modified and the methyltransferases catalyzing such modification are only now being identified. A novel family of lysine methyltransferases, including METTL21A, was found to trimethylate multiple HSP70 isoforms on K561, a conserved lysine residue (31). Trimethylation of this residue was reported to affect binding of HSP70 chaperones to their client protein, the Parkinson disease-associated protein α-synuclein (32). A third group reported that the same residue was dimethylated by the SETD1A methyltransferase, which led to the translocation of HSP70 from the cytoplasm to the nucleus, and stimulated the enzymatic activity of Aurora kinase B on histone H3, thus causing proliferation of cancer cells (33).

Recent research efforts have primarily focused on HSP70 lysine methylation, but little is known about its arginine methylation and the functional consequences of such modification. Using anti-methyl-lysine or arginine antibodies combined with proteomics analysis, we have identified several lysine and arginine methylation sites on HSP70. Further investigation of methylated arginine 469 (R469), which was mediated at least partially by PRMT4 in vitro and in vivo, revealed that, in contrast to HSP70 lysine methylation reported to be indispensable for its ATPase activity or client protein binding, the R469 methylation was crucial for HSP70-regulated gene transcription, exemplified by HSP70 binding to the retinoid acid (RA) receptor $\beta2$ (*RAR$\beta2$*) gene promoter and inducing *RAR$\beta2$* gene transcription in a R469 methylation-dependent fashion. Mechanistically, HSP70 proved to be required for the effective recruitment of TFIIH to the *RAR$\beta2$* gene promoter, and thus transcription initiation through physical interaction with TFIIH occurred in a HSP70 R469 methylation-dependent manner.

Results

HSP70 Is Lysine and Arginine Methylated. Initially, we sought to identify nonhistone proteins that are targeted for lysine or arginine methylation through immunoprecipitation (IP) with antibody against the protein of interest followed by immunoblotting (IB) with anti-methyl-lysine (Kme) or arginine (Rme) antibody. Consistent with previous reports, it was found that HSP70 was abundantly methylated on both lysine and arginine residues, among the many proteins tested (Fig. 1*A*). Specifically, cell lysates from HEK293T were subjected to IP using anti-HSP70 antibody followed by IB using either anti-methyl-lysine or arginine antibody. To confirm the methylation signals detected by these antibodies were specific, similar experiments were performed in cells treated with or without adenosine-2′, 3′-dialdehyde (AdOx), a methyltransferase inhibitor. It was found that HSP70 methylation was nearly blocked in the presence of AdOx (Fig. 1*B*). To further demonstrate the specificity of HSP70 methylation detected, HEK293T cells were transfected with a Flag-tagged HSP70, and then treated with or without AdOx followed by IP with anti-Flag antibody. It was again found that HSP70 methylation was attenuated in the presence of

Table 1. Arginine and lysine methylation sites identified through mass spectrometry analysis

Identified peptides with arginine or lysine methylation	Type of methylation	Position	Hsp70 isoforms containing the modified peptides
R.FELSGIPPAPR+14.G	Rme1	469	HSPA1A, HSPA1B, HSPA6
K.FELTGIPPAPR+14.G	Rme1	469	HSPA8
R.K+14FGDPVVQSDMK.H	Kme1	77	HSPA1A, HSPA1B
R.K+28FGDPVVQSDMK.H	Kme2		
R.K+42FGDPVVQSDMK.H	Kme3		
K.FGDPVVQSDMK+14.H	Kme1	88	HSPA1A, HSPA1B
R.FDDAVVQSDMK+14.H	Kme1	88	HSPA8
K.AK+14IHDIVLVGGSTR.I	Kme1	332	HSPA1L
K.AK+28IHDIVLVGGSTR.I	Kme2		
K.AK+42IHDIVLVGGSTR.I	Kme3		
K.YK+14AEDEVQR.E	Kme1	526	HSPA1A, HSPA1B, HSPA1L
K.YK+28AEDEVQR.E	Kme2		
K.YK+42AEDEVQR.E	Kme3		
K.NALESYAFNMK+14.S	Kme1	550 (HSPA1A, HSPA1B) 552 (HSPA1L)	HSPA1A, HSPA1B, HSPA1L
K.NSLESYAFNMK+14.A	Kme1	550	HSPA8
K.SAVEDEGLKGK+14.I	Kme1	561	HSPA1A, HSPA1B
K.LQGK+42INDEDKQK.I	Kme3	561	HSPA8

Tryptic peptides containing methylated arginine or lysine residues were shown (+14, monomethylation, Rme1, or Kme1; +28, dimethylation, Kme2; +42, trimethylation, Kme3). HSP70 isoforms containing these tryptic peptides, and the positions of the methylated lysine or arginine were shown as indicated.

AdOx (Fig. 1*C*). Alternatively, HSP70 methylation was shown by in vivo labeling with L-[*methyl*-^{3}H]-methionine in the presence of protein translation inhibitors [cycloheximide/chloramphenicol mixture, no-translation mixture (NTM)], followed by IP with anti-HSP70 antibody. It was found that a 70-kDa band was robustly labeled with ^{3}H, signal of which decreased significantly when AdOx was added (Fig. 1*D*, compare lane 3 to lane 4). Inhibition of protein translation by NTM was examined through labeling of cells with [^{35}S]-methionine in parallel, finding that radioactive signal disappeared in the presence of NTM (Fig. 1*D*, compare lane 1 to lane 2). Therefore, the radioactive signal detected with L-[*methyl*-^{3}H]-methionine labeling was a result of posttranslational methylation instead of residual translational incorporation of L-[*methyl*-^{3}H]-methionine. Similarly, in vivo labeling experiments were repeated with cells transfected with Flag-tagged HSP70 and followed by IP with anti-Flag antibody, which confirmed that HSP70 indeed was methylated in cultured cells (Fig. 1*E*).

Next, we sought to identify the potential methylation sites in HSP70 protein purified from HEK293T cells by mass spectrometry analysis (Fig. 1*F*). Consistent with previous reports, tryptic peptides containing methylated lysine 561 were identified (Table 1 and Dataset S1, *J* and *K*). Meanwhile, several other lysine (K) and arginine (R) residues were found to be methylated with high confidence as well, with K77 and K526 being mono-, di-, and trimethylated, and K88, K550, and R469 being monomethylated (Table 1 and Dataset S1, *A–E* and *G–I*). In addition, K332, a lysine residue unique to HSPA1L among all isoforms of HSP70 protein family, was also targeted for mono-, di-, and trimethylation (Table 1 and Dataset S1*F*). In particular, R469 was found to be highly conserved among different isoforms of HSP70 proteins (Fig. 1*G*), and conserved during evolution (Fig. 1*H*), suggesting that methylation on this residue might be functionally important. In the present study, we have focused on investigating the biological function of monomethylation on R469 (R469me1) in HSP70 protein.

HSP70 Is Methylated by CARM1/PRMT4 and Demethylated by JMJD6 at R469. To elucidate the biological function of methylation on R469 in HSP70 protein, an antibody specifically targeting R469me1 was generated and its specificity was examined in multiple ways: first, total lysates from HEK293T cells were subjected to IB with anti-R469me1 antibody preincubated with Flag-peptide (negative control) or HSP70 peptide with unmodified R469 (R469un) or monomethylated R469 (R469me1). It was found that anti-R469me1 antibody detected a specific band with a molecular weight at around 70 kDa, which presumably corresponded to HSP70 protein (Fig. 2*A*). Preincubation with peptide containing R469me1, but not unmodified R469, significantly decreased the chemiluminescent signal compared with control, suggesting the anti-R469me1 antibody specifically recognized the monomethylated form of HSP70 (Fig. 2*A*). Second, total lysates from HEK293T cells transfected with Flag-tagged wild-type or mutant HSP70 with substitution of arginine 469 to alanine (R469A) were subjected to IP with anti-Flag antibody, followed by IB with anti-R469me1 antibody. As expected, mutation of R469 completely abolished the chemiluminescent signals, further confirming the specificity of this antibody toward R469 methylated-HSP70 (Fig. 2*B*).

Next, we sought to identify the PRMT, which might be responsible for modifying this residue in cultured cells by transfecting short interfering RNA (siRNA) specifically targeting each individual member of the PRMT family (PRMT1 to -11), followed by examination of R469me1 levels. It was found that knock-down of either coactivator-associated arginine methyltransferase 1 (CARM1)/PRMT4 or PRMT7 led to a significant reduction of R469me1 levels compared with control sample, whereas other members exerted no apparent effects, suggesting that CARM1/PRMT4 or PRMT7 may methylate R469 (Fig. 2*C*). The knock-down efficiency of each PRMT was examined through quantitative RT-PCR (RT-qPCR) (Fig. S1). To test which member of the PRMT family can methylate HSP70 directly in vitro, an in vitro methylation assay was performed mixing purified bacterially expressed wild-type or mutant HSP70 (R469A) with individual PRMT (PRMT1 to -11). It was found that PRMT1, -4, -6, and -8 were capable of methylating HSP70 in vitro (Fig. 2*D*). Consistent with previous reports, automethylation of PRMT1, -4, and -6 was also observed. Interestingly, the methylation of HSP70 mediated by PRMT4 was nearly abolished when arginine 469 was substituted to alanine, suggesting R469 was the primary site methylated by PRMT4 in vitro (Fig. 2*D*). The expression of wild-type and mutant HSP70 (R469A) was comparable when examined by Coomassie blue staining (Fig. S2*A*). The expression of all PRMTs was also shown (Fig. S2*B*). Of note, although members in the PRMT family, except PRMT10 and PRMT11, have been shown to possess

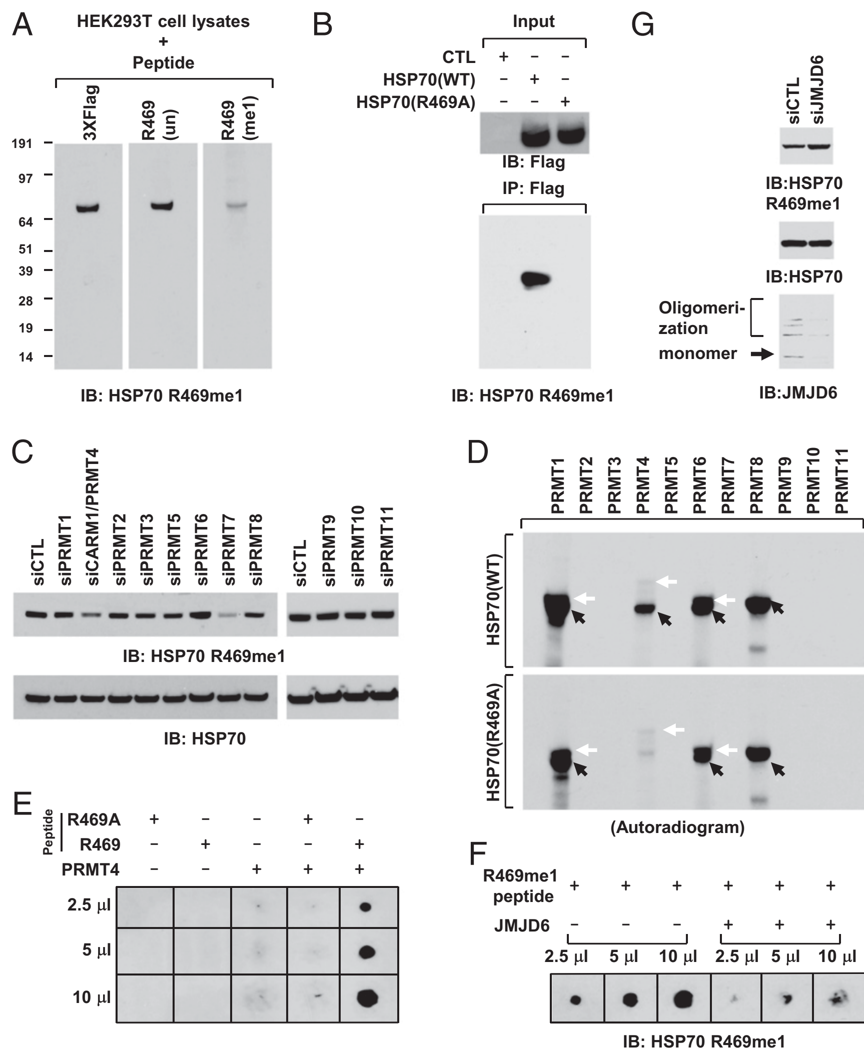

Fig. 2. Methylation and demethylation of arginine 469 in HSP70 by PRMT4 and JMJD6, respectively. (*A*) Anti-HSP70R469me1 antibody was preincubated with 3XFlag (*Left*), HSP70R469 unmodified (*Center*), or HSP70R469me1 (*Right*) peptide for 30 min before probing HEK293T total cell lysates. (*B*) HEK293T cells transfected with control vector, Flag-tagged wild-type (WT) or mutant HSP70 (R469A) were subjected to IP with anti-Flag antibody, followed by IB with anti-HSP70R469me1 antibody. (*C*) HEK293T cells were transfected with control siRNA or siRNA specifically against each individual member of PRMT protein family for 72 h, followed by IB with anti-HSP70R469me1 (*Upper*) or anti-HSP70 (*Lower*) antibody. (*D*) In vitro methylation assay was performed by mixing purified bacterially expressed PRMTs with wild-type (*Upper*) or mutant HSP70 (R469A) (*Lower*) proteins. White arrows indicate automethylation of PRMT1, PRMT4, and PRMT6; Black arrows indicate methylation of HSP70. (*E*) In vitro methylation assay was performed by mixing purified bacterially expressed PRMT4 proteins with R469 or R469A peptide. Increased amount of each reaction was taken for dot blot as indicated and followed by autoradiogram. (*F*) In vitro demethylation assay was performed by mixing R469me1 peptide with or without purified bacterially expressed JMJD6 protein. Increased amount of each reaction was taken for dot blot as indicated and followed by IB with anti-HSP70R469me1 antibody. (*G*) HEK293T cells were transfected with control siRNA or siRNA specifically against *JMJD6* for 72 h, followed by IB with antibodies as indicated. The ratio of R469me1 levels between the siCTL and siJMJD6 sample was 0.45 quantified using ImageJ.

methyltransferase activity (10), only PRMT1, -3, -4, -6, -8, and -9 seemed to be active when purified from bacterial cells and histones were serving as substrates under current conditions tested (Fig. S2 *C* and *D*). Because many of the histone-modifying enzymes purified from bacterial cells are inactive, Flag-tagged PRMTs were overexpressed and purified from HEK293T cells, followed by in vitro methylation assay using histones, HSP70 (wild-type) or HSP70 (R469A) as substrates. It was found that all PRMTs except PRMT10 were capable of methylating histones, indicating they were active (Fig. S2*E*). Consistent with what we observed by using PRMT proteins purified from bacterial cells, PRMT1, -4, -6, and -8 purified from overexpressed HEK293T cells were capable of methylating HSP70 in vitro (Fig. S2*F*). In addition, PRMT7 was capable of methylating HSP70 as well (Fig. S2*F*). However, the observed methylation signal in the presence of PRMT4 was completely abolished when R469 was substituted to alanine, whereas there was no significant change for PRMT1, -6, -7, and -8 when comparing HSP70 (wild-type) to HSP70 (R469A) (Fig. S2*F*). The expression of all Flag-tagged PRMTs was shown (Fig. S2*G*). To further confirm that PRMT4 is capable of methylating HSP70 at R469, short-peptide containing arginine 469 (R469), or with substitution of arginine 469 to alanine (R469A) was incubated with or without PRMT1 or PRMT4, finding that PRMT4 methylated the R469 peptide robustly, but not the R469A peptide (Fig. 2*E*). Meanwhile, PRMT1 displayed no significant activity toward either R469 or R469A peptide (Fig. S2*H*). Taking these data together, despite several members in the PRMT protein family being capable of methylating HSP70 in vitro, we find that R469 was the primary site in HSP70 methylated by PRMT4. PRMT4 appeared to be at least partially responsible for R469 methylation in cultured cells. Meanwhile, although it targeted HSP70 at sites other than R469 in vitro, PRMT7 influenced HSP70R469me1 levels in cultured cells.

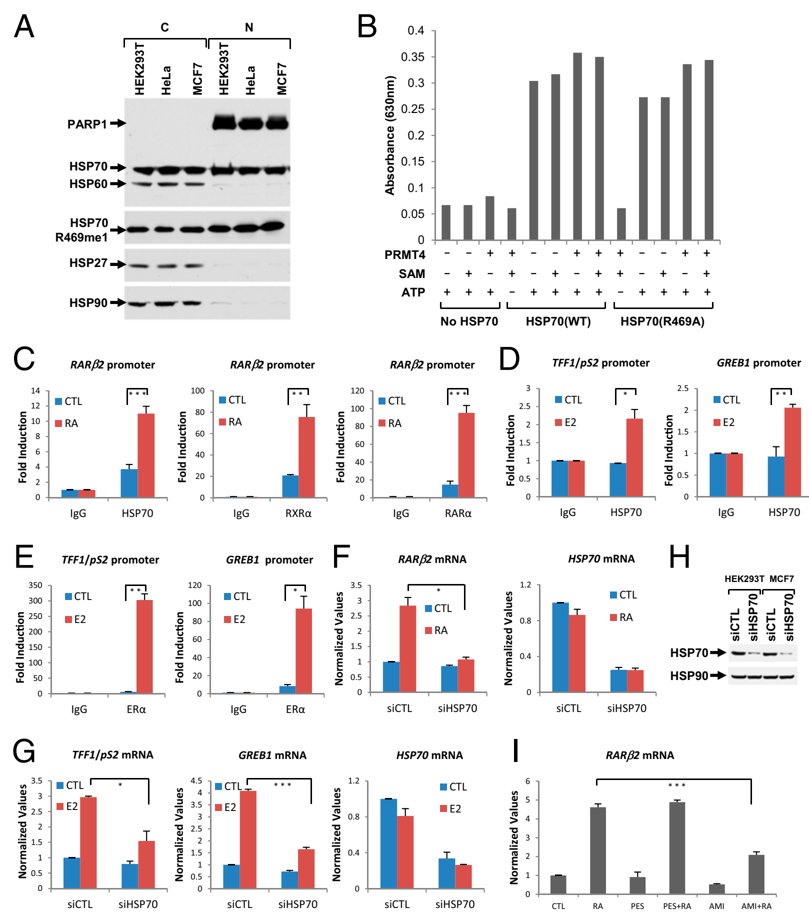

Fig. 3. HSP70 association with chromatin in gene transcriptional regulation. (*A*) Cellular fractionation was done in three independent cell lines, HEK293T, HeLa, and MCF7, followed by IB with various antibodies, as indicated. (*B*) HSP70 ATPase activity assay was performed by mixing PRMT4 (0.04 μg/μL), HSP70 (wild-type or R469A mutant) (0.05 μg/μL), SAM (1 mM), and ATP (100 μM), as indicated. (*C*) HEK293T cells were treated with or without RA (10^{-7} M) for 1 h and then subjected to ChIP with control IgG, anti-HSP70, RXRα, or RARα antibody. Binding of HSP70, RXRα, and RARα on the *RARβ2* gene promoter region were examined through qPCR. ChIP signals were presented as fold-enrichment over that of IgG (±SEM, $^{**}P < 0.01$, $^{***}P < 0.001$). (*D* and *E*) MCF7 cells were treated with or without E_2 (10^{-7} M) for 1 h and then subjected to ChIP with control IgG, anti-HSP70 (*D*) or ERα (*E*) antibody. Binding of HSP70 (*D*) and ERα (*E*) on *TFF1/pS2* or *GREB1* gene promoter regions were examined through qPCR (±SEM, $^{*}P < 0.05$, $^{**}P < 0.01$). (*F* and *G*) HEK293T (*F*) or MCF7 (*G*) cells were transfected with control siRNA or siRNA specifically targeting *HSP70* for 72 h, followed by treatment with RA (10^{-7} M) (*F*) or E_2 (10^{-7} M) (*G*), respectively, for 6 h before RT-qPCR analysis to examine mRNA levels of genes as indicated. Data shown was the relative fold-change compared with control siRNA transfected samples after normalization to actin (±SEM, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). (*H*) Cell lysates as described in *F* and *G* were subjected to IB with antibodies, as indicated. (*I*) HEK293T cells were pretreated with or without PES (20 μM) or AMI (100 μM) for 1 h before treatment with or without RA (10^{-7} M) for 6 h, followed by RT-qPCR analysis to examine the mRNA levels of *RARβ2*. Data shown is the relative fold-change compared with control sample after normalization to actin (±SEM, $^{***}P < 0.001$).

We speculate that there is a cross-talk between PRMT7-mediated methylation and R469 methylation. Alternatively, PRMT7 might also target to HSP70 R469 directly despite it displayed no such activity in vitro, which certainly remains as an intriguing topic for future investigation.

JMJD6, which belongs to the JmjC-domain–containing demethylase family, is the only member in this family possessing arginine demethylase activity reported so far. It can demethylate both mono- and di-methylated arginine on both histones and nonhistone proteins (18–21). We tested directly whether HSP70R469me1 might serve as a substrate for JMJD6 by performing in vitro demethylation assay mixing short-peptide containing monomethylated R469 (R469me1), with or without in vitro purified bacterially expressed JMJD6, followed by either immunoblotting with anti-R469me1 antibody (Fig. 2*F*) or MALDI-TOF MS analysis (Fig. S3). It was found that R469me1 signal decreased dramatically in the presence of JMJD6 based on IB (Fig. 2*F*). MALDI-TOF MS analysis further revealed that the decrease of R469me1 signal was because of the removal of one methyl-group by JMJD6 (Fig. S3). To demonstrate JMJD6 demethylation of HSP70R469me1 in cultured cells, HEK293T cells were transfected with control siRNA or siRNA specific against *JMJD6* followed by IB with anti-R469me1 antibody, finding that knock-down of JMJD6 consistently led to a mild yet significant increase of R469me1 levels (Fig. 2*G*). Lack of dramatic effects when knocking down of JMJD6 might be because it only functions locally to demethylate HSP70R469me1, or because there are other arginine demethylases yet to be discovered (19).

R469 Methylated-HSP70 Is Localized in Both Cytoplasm and Nucleus, with an Implication in Nuclear Function. Most of the molecular chaperones are believed to localize predominantly in the cytoplasm, whereas others are more concentrated in specific cellular compartments, such as endoplasmic reticulum (ER)-resident HSPA5/GRP78, and mitochondrial HSPA9/GRP75 and HSPA14/HSP60 (23, 24, 28). We examined cellular localization of HSP70 through cellular fractionation experiments in three independent cell lines, HEK293T, HeLa, and MCF7, and found that it distributed equally in both cytoplasm and nucleus, whereas other chaperones, such as

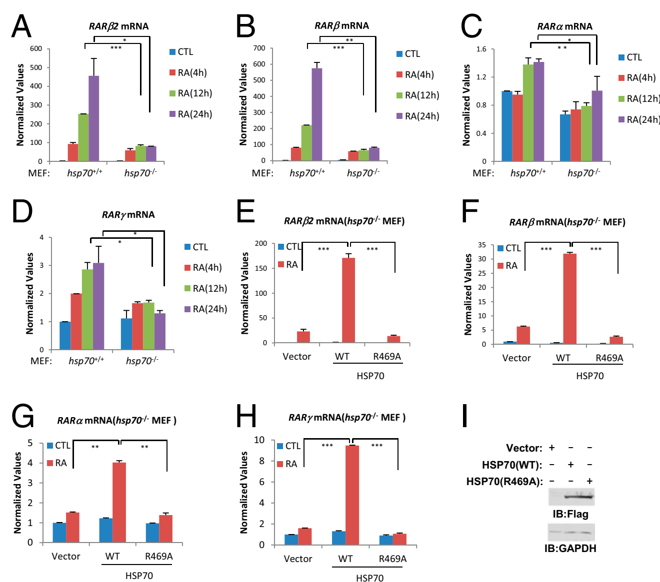

Fig. 4. HSP70 is required for RA-induced *RAR* gene transcriptional activation. (*A–D*) Wild-type (*hsp70.1*$^{+/+}$) and *hsp70*-deficient (*hsp70.1*$^{-/-}$) MEFs were treated with or without RA (10^{-7} M) for 4, 12, and 24 h, followed by RT-qPCR analysis to examine the mRNA levels of *RARβ2* (*A*), *RARβ* (*B*), *RARα* (*C*), and *RARγ* (*D*). Data shown was the relative fold-change compared with control sample in wild-type MEFs after normalization to actin (±SEM, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$). (*E–H*) *hsp70.1*$^{-/-}$ MEFs were transfected with control vector, Flag-tagged wild-type or mutant HSP70 (R469A) for 48 h and then treated with or without RA for 4 h, followed by RT-qPCR analysis to examine the mRNA levels of *RARβ2* (*E*), *RARβ* (*F*), *RARα* (*G*), and *RARγ* (*H*) (±SEM, $**P < 0.01$, $***P < 0.001$). (*I*) Expression of wild-type and mutant HSP70 (R469A) as described in *E–H* was examined through IB with anti-Flag antibody. GAPDH was served as loading control.

HSP90 and HSP27, were predominantly localized in the cytoplasm, suggesting that HSP70 might play a distinct role in the nucleus (Fig. 3*A* and Fig. S4*A*). Similarly, R469 monomethylated-HSP70 was found in both the cytoplasm and nucleus (Fig. 3*A*). Poly(ADP-ribose) polymerase 1 (PARP1) and HSP60 served as purity controls of nuclear and cytoplasmic fractions, respectively (Fig. 3*A*). The localization of HSP70 and its methylated form were further confirmed in the other three cell lines examined (Fig. S4*B*).

HSP70 facilitates the folding process by transient association of its substrate-binding domain with short hydrophobic peptide segments within their "client proteins." The client protein binding and release cycle is driven by ATP binding and hydrolysis, which requires the ATPase activity from HSP70 nucleotide-binding domain (28). To assess whether R469me1 plays a functional role in regulating ATP hydrolysis rate, HSP70 ATPase activity was assayed in the presence or absence of PRMT4. Notably, it was found that ATP hydrolysis was not affected by adding PRMT4, suggesting that R469me1 was not involved in modulating HSP70 ATPase activity (Fig. 3*B*). Meanwhile, ATPase activity of mutant HSP70 (R469A) was very similar to that of wild-type HSP70 (Fig. 3*B*). Considering its unique nuclear localization compared with most other molecular chaperones, and its presence in a wide range of protein complexes involved in histone modifications and chromatin remodeling, we sought to examine whether HSP70, and hence R469 monomethylation, might exert a functional role in gene transcriptional regulation at the chromatin level.

HSP70 Associates with Chromatin and Regulates Gene Transcription. The binding of HSP70 with promoter regions of target genes, such as RA-induced *RARβ2* gene in HEK293T cells and *homeotic* (*Hox*) genes in NTera2 cells, 17β-Estradiol (estrogen, E2)-induced *trefoil factor 1 precursor* (*TFF1*/*pS2*) and *growth regulation by estrogen in breast cancer 1* (*GREB1*) genes in MCF7 cells, androgen [5α-Dihydrotestosterone (DHT)]-induced *prostate specific antigen* (*PSA*/*KLK3*) and *NK3 homeobox 1* (*NKX3.1*) genes in LNCaP cells, and TNF-α–induced *IL6* and *IL8* genes in HeLa cells, was examined using ChIP assay. Interestingly, RA-dependent recruitment of HSP70 to *RARβ2* and selected Hox, and E_2-dependent recruitment to *TFF1*/*pS2* and *GREB1* gene promoters were observed, whereas HSP70 displayed no binding upon DHT or TNF-α treatment (Fig. 3*C*, *Left*, Fig. 3*D*, and Fig. S5*A*). The recruitment of HSP70 to the *RARβ2* gene promoter was accompanied with that of retinoid X receptor α (RXRα) and RARα, the two nuclear receptors that drive the activation of *RARβ2* gene (Fig. 3*C*, *Center* and *Right*). Similarly, the recruitment of HSP70 to *TFF1*/*pS2* and *GREB1* gene promoter regions was accompanied with that of ERα, which mediates the activation of these genes (Fig. 3*E*). These data suggested that HSP70 might involve in gene transcriptional activation, such as *RARβ2*, *Hox*, and E_2-target genes. Indeed, knock-down of HSP70 attenuated RA-induced *RARβ2* and selected Hox, and E_2-induced *TFF1*/*pS2* and *GREB1* gene activation (Fig. 3 *F* and *G* and Fig. S5*B*). Consistent with the lack of HSP70 binding on promoter regions, the induction of DHT- or TNF-α target genes was not affected by HSP70 knock-down (Fig. S5 *C* and *D*), indicating that knock-down of HSP70 did not induce a nonspecific defect in gene transcription. The knock-down efficiency of HSP70 was shown by qPCR and IB with primers and antibody specifically targeting HSP70, respectively (Fig. 3 *F–H* and Fig. S5). To determine whether regulation of transcription by HSP70 is coupled with its ATPase activity, a key component of HSP70 protein chaperone function, we measured the effects of 2-phenylethynesulfonamide (PES), a HSP70 chaperone inhibitor (34), on RA-induced *RARβ2* gene activation. It was found that PES exhibited no apparent effects, suggesting that HSP70's function in gene transcription is distinguished from its role in protein folding and quality control (Fig. 3*I*, the four columns on the left).

To further confirm the functional significance of HSP70 in RA-induced *RARβ2* gene activation, wild-type (*hsp70.1*$^{+/+}$) and *hsp70.1*-deficient (*hsp70.1*$^{-/-}$) mouse embryonic fibroblasts (MEFs) were treated with or without RA for 4, 12 or 24 h, followed by examining *RARβ2* expression level. Surprisingly, RA-induced *RARβ2* expression was comparable in wild-type and *hsp70.1*$^{-/-}$ MEFs at 4 h of treatment, with the induction in *hsp70.1*$^{-/-}$ MEFs a little weaker than that in wild-type (Fig. 4*A*). However, further activation of *RARβ2* with RA treatment for 12 or 24 h was observed in a time-dependent manner in wild-type MEFs, which was not detectable in *hsp70.1*$^{-/-}$ MEFs (Fig. 4*A*). We reasoned that the other members of the *hsp70* protein family might compensate for the loss of *hsp70.1* initially, whereas full activation of *RARβ2* required the presence of *hsp70.1*. Because all four *RARβ* isoforms are inducible by RA (35), we examined their expression by using a primer set common to all, with similar effects as seen for *RARβ2* observed when *hsp70.1* was not present (Fig. 4*B*). The *RAR* family consists of three receptor subtypes, α, β, and γ, which exhibit functional redundancy (36). We then tested if *hsp70* is required for *RARα* and *RARγ* gene expression, finding that the expression of both receptors was induced by RA to a much less extent compared with *RARβ*, which was abrogated in *hsp70.1*$^{-/-}$ MEFs (Fig. 4 *C* and *D*). Finally, the functional significance of *hsp70* in RA-induced *RARβ2* gene activation was demonstrated by transfecting *hsp70.1*$^{-/-}$ MEFs with a control vector or vectors expressing wild-type HSP70 followed by treatment with or without RA. In accordance with its role in *RAR* gene activation, overexpression of wild-type HSP70 led to a dramatic increase in the fold-induction by RA for all RAR receptor subtypes (Fig. 4 *E–H*, four columns on the left).

HSP70R469me1 Is Involved in RA-Induced *RARβ2* Gene Activation. Next, using RA-induced *RARβ2* gene activation as a model, we began to test whether PRMT4-mediated R469me1 on HSP70 might play a functional role in gene transcription. First, arginine

N-methyltransferase inhibitor (AMI), a general PRMT inhibitor, was shown to inhibit *RARβ2* promoter- and retinoid acid response element (RARE)-driven luciferase activity, as well as *RARβ2* gene expression, in the presence of RA, suggesting that PRMT-mediated arginine methylation might be important for RA-induced *RARβ2* promoter activity, and hence *RARβ2* gene activation (Fig. 3*I*, the two columns on the right, and Fig. 5 *A* and *B*). To test whether HSP70 R469 is involved in RA-induced *RARβ2* gene activation, HEK293T cells were transfected with control siRNA or siRNA specifically targeting *HSP70* in the presence or absence of wild-type or mutant HSP70 (R469A), followed by treatment with or without RA. It was found that wild-type, but not mutant HSP70 (R469A), rescued the activation of *RARβ2* gene despite they bound to the *RARβ2* gene promoter equally well, indicating HSP70 R469 is important for *RARβ2* gene activation (Fig. 5*C* and Fig. S6). As shown above, overexpression of wild-type HSP70 in *hsp70.1*$^{-/-}$ MEFs led to a dramatic increase in the fold-induction by RA for all RAR receptor subtypes (Fig. 4 *E–H*, four columns on the left), whereas the R469A mutant displayed no such effects (Fig. 4 *E–H*, two columns on the right). The expression of wild-type and mutant HSP70 (R469A) was comparable, as examined by IB (Fig. 4*I*).

Consistently, PRMT4, the arginine methyltransferase capable of catalyzing R469me1, was found to increase significantly on *RARβ2* promoter region upon RA treatment when examined by ChIP in HEK293T cells (Fig. 5*D*). Furthermore, knock-down of PRMT4 attenuated *RARβ2* promoter- and RARE-driven luciferase activity as well as *RARβ2* gene expression in the presence of RA (Fig. 5 *E* and *F*). More importantly, introducing a vector-expressing wild-type mouse PRMT4, but not its enzymatically dead mutant (E267Q), rescued the expression of *RARβ2*, suggesting that PRMT4 enzymatic activity was crucial for RA-induced *RARβ2* expression (Fig. 5*F*). PRMT4 levels were examined by RT-qPCR (Fig. S7*A*). Conversely, *RARβ2* promoter- and RARE-driven luciferase activity, as well as *RARβ2* gene expression, was further stimulated when cells were transfected with a PRMT4 expression vector (Fig. 5 *G* and *H*). Taken together, these data suggested that PRMT4 is required for RA-induced *RARβ2* gene activation, and is consistent with the fact that PRMT4 is capable of methylating HSP70 R469.

We previously reported that JMJD6 is capable of demethylating mono- and di-methylated arginines, both symmetrical (s) and asymmetrical (a), and that it functions in both gene activation and repression (19). Particularly, it was found that JMJD6 regulates transcriptional pause release, and thus gene activation partially through its demethylase activity toward H4R3me2(s), a repressive mark associated with gene repression. However, the role of JMJD6 in gene repression and the potential substrates, through which it works, remain to be explored. As JMJD6 demethylase activity toward HSP70R469me1 was observed in vitro and in cultured cells, its role in *RARβ2* gene expression was tested. It was found that knock-down of JMJD6 further stimulated *RARβ2* promoter- or RARE-driven luciferase activity as well as *RARβ2* gene expression (Fig. 5 *I* and *J*). Knock-down efficiency of JMJD6 was examined through RT-qPCR (Fig. S7*B*). Taken together, these findings show that PRMT4 and JMJD6, and possibly their-regulated HSP70R469me1 are important for RA-induced *RARβ2* gene activation.

HSP70 Is Essential for Transcription Initiation Through Regulating TFIIH Recruitment. Next, we sought to understand the molecular mechanism underlying HSP70 regulation of RA-induced *RARβ2* gene activation. It was reported that the sequential events occurring on *RARβ2* promoter and involved in *RARβ2* gene activation include but are not limited to: (*i*) upon ligand binding, recruited-RARα undergoes a conformation change, leading to the dismissal of corepressor complexes and the recruitment of coactivator complexes, such as histone-modifying enzymes and chromatin remodeling complexes, which prepare chromatin for transcriptional activation; (*ii*) the MED1/TRAP220 subunit of mediator complexes, in conjunction with RARα, PARP1, and other subunits in the mediator complexes, triggers the release of the repressive CDK8 module, thus converting mediator into transcriptionally active state;

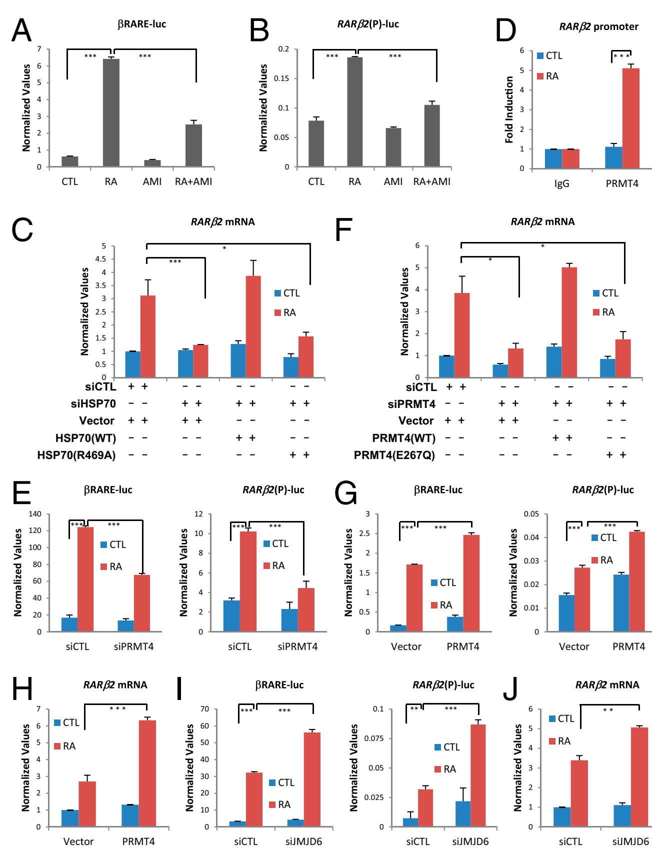

Fig. 5. R469me1 is involved in RA-induced *RARβ2* gene transcriptional activation. (*A* and *B*) HEK293T cells were transfected with luciferase reporter construct containing retinoid acid response element (βRARE-luc) (*A*) or mouse *RARβ2* gene promoter sequence [*RARβ2*(P)-luc] (*B*) for 24 h, and pretreated with or without AMI (100 μM) for 1 h before RA treatment (10^{-7} M) for another 12 h, followed by luciferase reporter activity measurement (±SEM, ****P* < 0.001). (*C*) HEK293T cells were transfected with control siRNA or siRNA specifically against *HSP70* together with or without control vector, wild-type, or mutant HSP70 (R469A) before RA (10^{-7} M) treatment for 6 h, followed by RT-qPCR analysis to examine mRNA levels of *RARβ2* (±SEM, **P* < 0.05, ****P* < 0.001). It should be noted that siRNA against *HSP70* here was targeting to the 5′UTR region of HSP70. (*D*) HEK293T cells were treated with or without RA (10^{-7} M) for 1 h and then subjected to ChIP with control IgG or anti-PRMT4 antibody. Binding of PRMT4 on the *RARβ2* gene promoter region was examined through qPCR (±SEM, ****P* < 0.001). (*E* and *I*) HEK293T cells were transfected with control siRNA or siRNA specifically against *PRMT4* (*E*) or *JMJD6* (*I*) for 24 h before transfection with luciferase reporter construct, βRARE-luc (*Left*) or *RARβ2*(P)-luc (*Right*) for another 48 h, followed by RA treatment for 12 h. Cells were then subjected to luciferase reporter activity measurement (±SEM, ****P* < 0.001). (*F*) HEK293T cells were transfected with control siRNA or siRNA specifically against *PRMT4* together with or without control vector, wild-type or enzymatically dead mutant (E267Q) PRMT4 (mouse) before RA (10^{-7} M) treatment for 6 h, followed by RT-qPCR analysis to examine mRNA levels of *RARβ2* (±SEM, **P* < 0.05). (*G*) HEK293T cells were transfected with control or PRMT4 expression vector together with or without luciferase reporter constructs, βRARE-luc (*Left*) or *RARβ2*(P)-luc (*Right*), for 48 h, followed by RA treatment for 12 h. Cells were then subjected to luciferase reporter activity measurement (±SEM, ****P* < 0.001). (*H*) HEK293T cells were transfected with control or PRMT4 expression vector for 48 h, followed by treatment with RA (10^{-7} M) for 6 h before RT-qPCR analysis to examine mRNA levels of *RARβ2* (±SEM, ****P* < 0.001). (*J*) HEK293T cells were transfected with control siRNA or siRNA specifically targeting *JMJD6* for 72 h, followed by treatment with RA (10^{-7} M) for 6 h before RT-qPCR analysis to examine mRNA levels of *RARβ2* (±SEM, ***P* < 0.01).

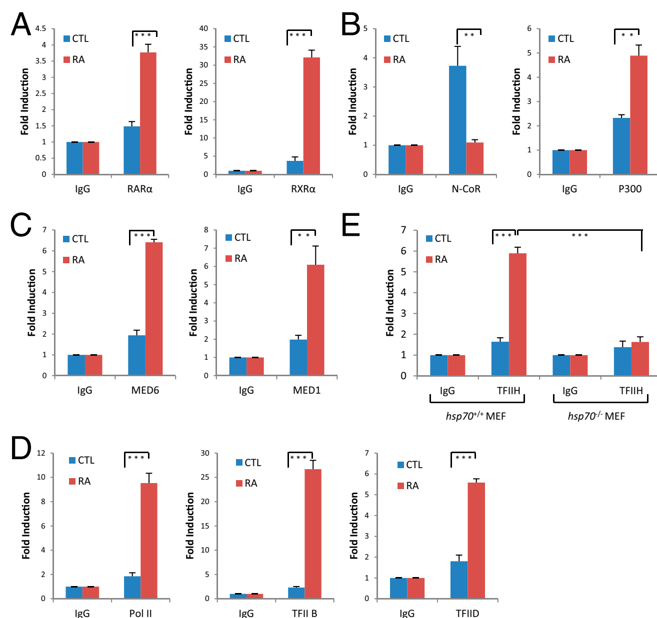

Fig. 6. HSP70 is required for the recruitment of TFIIH during RA-induced *RARβ2* gene transcriptional activation. (*A*–*D*) Wild-type (*hsp70.1*$^{+/+}$) MEFs were treated with or without RA (10^{-7} M) for 1 h and then subjected to ChIP with control IgG or RARα, RXRα (*A*); NCoR, p300 (*B*); MED6, MED1 (*C*); Pol II, TFIIB, TFIID (TBP) (*D*) antibody. Binding of these factors on the *RARβ2* gene promoter region was examined through qPCR (±SEM, $**P < 0.01$, $***P < 0.001$). (*E*) Wild-type (*hsp70.1*$^{+/+}$) and *hsp70*-deficient (*hsp70.1*$^{-/-}$) MEFs were treated with or without RA (10^{-7} M) for 1 h and then subjected to ChIP with control IgG or TFIIH (ERCC3) antibody. Binding of TFIIH (ERCC3) on the *RARβ2* gene promoter region was examined through q-PCR (±SEM, $***P < 0.001$).

(*iii*) change in the conformation of mediator leads to a further change in preinitiation complex (PIC) containing RNA Pol II, TFIIA, TFIIB, TFIID, TFIIE, TFIIF, resulting in the recruitment of TFIIH, and subsequent phosphorylation of Pol II at serine 5 and transcriptional initiation (37). To investigate the molecular mechanism underlying HSP70's regulation of RA-induced *RARβ2* gene activation, the binding of transcription factors (RARα/RXRα heterodimer), corepressor complexes (represented by NCoR), chromatin modifying enzymes/coactivator complexes (represented by p300), mediator complexes (represented by MED1/TRAP220 and MED6), and PIC (represented by Pol II, TFIIB, TFIID, and TFIIH) on the *RARβ2* promoter region were examined through ChIP in the presence or absence of RA in wild-type or *hsp70.1*$^{-/-}$ MEFs. As reported previously, binding of both RARα and RXRα on the *RARβ2* promoter was observed even without RA treatment, which were further increased in the presence of RA in wild-type MEFs (Fig. 6*A*). Upon ligand binding, RARα undergoes a conformational change, resulting in the dissociation of corepressor complexes and the recruitment of coactivator complexes (38, 39). Accordingly, it was found that NCoR was displaced from the promoter, concomitant with the recruitment of the histone acetyltransferase p300 (Fig. 6*B*). The binding of representative components in the mediator complex including MED1/TRAP220 and MED6 were also examined, with a binding pattern similar to that observed for RARα and RXRα (Fig. 6*C*). Interestingly, *hsp70.1*$^{-/-}$ MEFs exhibited similar binding profiles as wild-type MEFs for all factors tested above (Fig. S8 *A*–*C*). Next, we explored the binding profiles of PIC, with Pol II found to be present in the absence of RA, which increased with RA treatment in both wild-type and *hsp70.1*$^{-/-}$ MEFs (Fig. 6*D* and Fig. S8*D*, *Left*). As expected, the factors required for Pol II recruitment including TFIIB and TFIID (TBP) displayed a similar binding profiles as Pol II (Fig. 6*D* and Fig. S8*D*, *Center* and *Right*). However, RA-induced binding of TFIIH (ERCC3), which is dispensable for Pol II recruitment but essential for phosphorylation of Pol II carboxyl-terminal domain at serine 5 (Pol II Ser5 pho) and thus transcription initiation, was only observed in wild-type, but not *hsp70.1*$^{-/-}$ MEFs, suggesting HSP70 function in the step of initiation during *RARβ2* transcriptional activation (Fig. 6*E*).

HSP70 R469 Methylation Is Involved in TFIIH Recruitment. The requirement of HSP70 for TFIIH recruitment to the *RARβ2* promoter promoted us to test whether an interaction occurs between HSP70 and PIC. HEK293T cell lysates were subjected to IP with control IgG or anti-HSP70 specific antibody, followed by IB with antibodies specific against components in PIC, including TFIIB, TFIID (TBP), and TFIIH (ERCC3). Surprisingly, HSP70 specifically pulled down TFIIH, but not TFIIB or TFIID (Fig. 7*A*). Given the potential role of R469me1 in *RARβ2* gene transcriptional activation, we further tested whether the interactions detected between HSP70 and TFIIH is R469 methylation-dependent. HEK293T cell lines stably expressing Flag-tagged wild-type or mutant HSP70 (R469A) were established (Fig. S9), and lysates collected from these cells as well as parental cells were subjected to affinity purification with anti-Flag antibody followed by IB with TFIIB, TFIID (TBP), and TFIIH (ERCC3). As expected, wild-type HSP70 specifically interacted with TFIIH (Fig. 7*B*). More importantly, the observed interaction seemed to be regulated by R469 as R469A mutation attenuated the interaction between HSP70 and TFIIH, suggesting an important role of HSP70 R469 in the recruitment of TFIIH during *RARβ2* gene activation (Fig. 7*B*). Consistent with a role of R469me1 in the recruitment of TFIIH, knock-down of PRMT4 in HEK293T, which led to a decrease of HSP70R469me1 levels (Fig. 2*C*), resulted in a decrease of TFIIH (ERCC3) binding induced by RA (Fig. 7*C*). Finally, *hsp70.1*$^{-/-}$ MEFs were transfected with a control vector or vectors expressing wild-type or mutant HSP70 (R469A), treated with or without RA followed by ChIP for TFIIH (ERCC3). It was found that ectopic expression of wild-type HSP70, but not the R469A mutant, induced the binding of TFIIH (ERCC3) to the *RARβ2* promoter upon RA treatment (Fig. 7*D*). Wild-type and mutant HSP70 (R469A) were expressed equally well examined by IB (Fig. 7*E*).

Discussion

Emerging evidence suggest that histone methyltransferases and demethylases use both histones and nonhistone proteins as substrates, exerting functional roles in a multitude of biological processes (40–44). HSP70 proteins have been known to be methylated on both arginine and lysine residues for decades, but the exact methylation sites, their methyltransferases and demethylases, and the biological functions of these modifications remain incompletely understood (29). In the present study, we provide evidence that a highly conserved arginine residue in HSP70 proteins is methylated, which can be reciprocally regulated by PRMT4 and JMJD6 both in vitro and in cultured cells. Further functional studies have suggested that HSP70 involves in gene transcription through its binding on chromatin, revealing a novel function of HSP70 aside from its classic role in protein folding and quality control. Specifically, HSP70 is indispensable for RA-induced *RARβ2* gene activation, during which PRMT4 and potentially PRMT4-mediated HSP70 R469me1 are required for the recruitment of TFIIH, and thus transcription initiation. Mechanistically, HSP70 interacts with TFIIH in an R469me1-dependent manner.

Methylation: An Emerging Piece of the "Nonhistone Code" Involving Chaperone Proteins. In the past few years, it seemed evident that PTMs found on chaperones become an important regulatory strategy in terms of modulating their ATPase activity, affinity with cochaperone, translocation, and stability, among other activities (29, 31–33, 36, 45–50). Interestingly, in the present study it was found that HSP70 was targeted for methylation at a

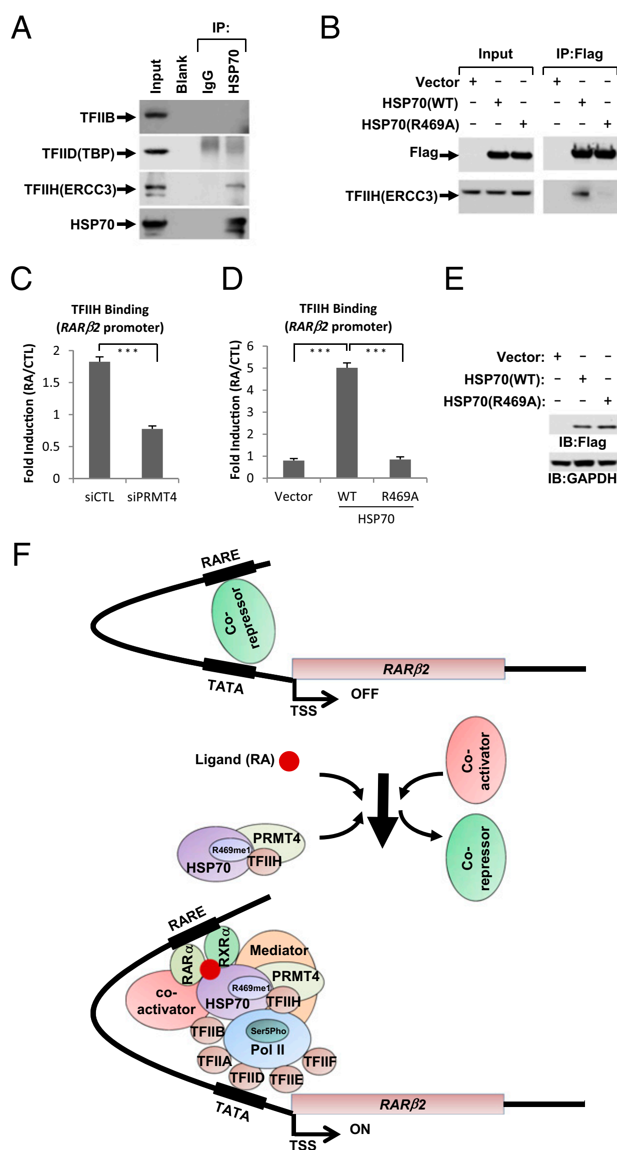

Fig. 7. HSP70 R469 methylation involved in TFIIH recruitment. (*A*) Cell lysates collected from HEK293T cells were subjected to IP with control IgG or anti-HSP70 antibody followed by IB with antibodies as indicated. (*B*) Cell lysates collected from HEK293T cells stably expressing control vector, Flag-tagged wild-type, or mutant HSP70 (R469A) were subjected to IP with anti-Flag antibody followed by IB with antibodies as indicated. (*C*) HEK293T cells were transfected with control siRNA or siRNA specifically against *PRMT4* for 72 h and then treated with RA (10^{-7} M) for 1 h, followed by ChIP with TFIIH (ERCC3) antibody. Binding of TFIIH (ERCC3) on the *RARβ2* gene promoter region was examined through qPCR. ChIP signals were presented as fold-induction by RA compared with control after normalization to input (±SEM, $***P < 0.001$). (*D*) *hsp70.1*$^{-/-}$ MEFs were transfected with control vector, Flag-tagged wild-type or mutant HSP70 (R469A) for 48 h, and then treated with RA (10^{-7} M) for 1 h, followed by ChIP with TFIIH (ERCC3) antibody. Binding of TFIIH (ERCC3) on *RARβ2* gene promoter region was examined through qPCR (±SEM, $***P < 0.001$). (*E*) The expression of HSP70 vectors as described in *D* was examined by IB. (*F*) Model: HSP70 arginine methylation in RA-induced *RARβ2* gene transcriptional activation. Upon RA stimulation, the binding of receptors (RARα and RXRα), mediator complex (such as MED1 and MED6), preinitiation complex (PIC, such as Pol II, TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH), HSP70, and PRMT4 were further increased on *RARβ2* gene promoter, accompanied by the exchange of corepressor complexes (such as NCoR) and coactivator complexes (such as p300). During this process, PRMT4-mediated monomethylation of R469 in HSP70 was required for the recruitment of TFIIH and transcription initiation. For simplicity, other factors shown to be essential for RA-induced *RARβ2* gene transcriptional activation were not included.

couple of lysine and arginine residues, which have never been revealed or studied before, including lysine methylation on K77, K88, K332 (unique to HSPA1L), K526, and K550, and arginine methylation on R469. The high conservation of R469 among different isoforms of HSP70 proteins and during evolution prompted us to pursue the functional significance of methylation on this residue, revealing HSP70 has the potential to regulate gene transcription, such as RA-induced *RARβ2* gene activation, through its association with chromatin, with R469 methylation being indispensable for transcription initiation. R469 methylation was regulated, at least partially, by PRMT4 and JMJD6 both in vitro and in vivo. Despite the controversial role of JMJD6 functioning as arginine demethylase (51–53), we and others found this enzyme can target to methylated arginine in both histone and nonhistone proteins (18–21). The biological meaning of the multiple methylated lysine residues identified here—and the cross-talk among the PTMs found on HSP70 so far—certainly remains as a great area to explore. Overall, given the large number of PTMs detected on chaperone proteins and their potential to be dynamically regulated by enzymes called "writer" (enzymes adding modifications) and "eraser" (enzymes erasing modifications), we envision that, similar to what has been proposed for histones, there is a "nonhistone" code existing on chaperone proteins. Functionally, we further propose that such a code may define the specificity of chaperone proteins toward their substrates and cofactors, and regulate the diversified cellular processes in which chaperone proteins are involved.

HSP70 Function in Gene Transcription. Unlike many other chaperones, several isoforms in the HSP70 protein family were found to localize in both nucleus and cytosol of cells. In the cytosol of cells, it serves as a guardian for protein folding and quality control through its binding with stretches of hydrophobic residues in a variety of proteins. In the present study, we provide evidence showing that HSP70 is recruited to the *RARβ2* gene promoter region in the presence of RA, and is required for the recruitment of TFIIH. Given its presence in a large number of protein complexes involved in gene regulation, we speculate that HSP70 might also regulate other transcriptional events, such as E2-induced ERα target genes and RA-induced *Hox* genes, using the same mechanism. However, the molecular basis underlying the observed R469me-dependent interaction between HSP70 and TFIIH remains elusive. It could be either that one of subunits in TFIIH directly reads R469me1 as a variety of functional domains are present in these proteins. Alternatively, an unknown R469me1 "reader" mediates the interaction between HSP70 and TFIIH. Both possibilities certainly remain as intriguing topics for future investigation.

Implications of HSP70 Arginine Methylation. HSP70 is often seen to be overexpressed in various types of cancers, and its expression correlates with increased tumor grade, poor prognosis, and drug resistance (54). Whereas the precise mechanisms linking HSP70 expression to tumor development are largely unknown, currently available data indicate that elevated HSP70 levels can alter multiple cell signaling and survival pathways, including apoptosis, senescence, autophagy, and HSP90 chaperone activity. HSP70 is therefore believed to play a causal role in cancer initiation (54). Consistently, di-methylated-K561 of HSP70 was found to be significantly enriched in various types of human cancers compared with corresponding normal tissues (33). Given that HSP70 was also required for the transcription of E_2-induced target genes, it will be valuable to investigate whether HSP70 R469me1 is enriched and therefore serving as a biomarker in breast cancer, and if so, what the molecular mechanism underlying HSP70 function in breast cancer initiation and progression is. Finally, although initially greatly anticipated to have clinical effects, the development and clinical efficacy of HSP70 inhibitors have been generally disappointing, which

we suggest is largely because of the fact that these inhibitors were designed based on HSP70 chaperone activity (54). As proposed above, targeting PRMT4, the methyltransferase modifying R469 of HSP70 might represent a more promising therapeutic strategy for cancer treatment.

Materials and Methods

The in vitro methylation assay was performed by mixing purified bacterially expressed wild-type or mutant HSP70 (R469A) with PRMT proteins in methylation buffer (50 mM Tris·HCl, pH 8.0, 20 mM KCl, 5 mM DTT, 4 mM EDTA) in the presence of 2 μCi L-[*methyl*-^{3}H]-methionine at 37 °C for 1 h. The reaction was stopped by adding SDS sample buffer followed by SDS/PAGE gel and autoradiogram. For in vitro methylation assay using peptides as substrates, enzymes (PRMTs) were removed by adding glutathione agarose before dot assay and autoradiogram. For in vitro demethylation assay, purified bacterially expressed JMJD6 protein was incubated with HSP70 R469me1 peptide in demethylation buffer (50 mM Hepes, pH 8.0, 20 μM $[NH_4]_2Fe[SO_4]_2$, 1 mM α-ketoglutarate, and 500 μM ascorbic acid) for 4 h at 37 °C. Similarly, enzyme (JMJD6) was removed by adding Ni-NTA agarose before dot assay and autoradiogram.

Methods for other experiments are detailed in *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank Dr. Jeong Sun Seo (Seoul National University) for kindly providing *hsp70.1* wild-type and deficient mouse embryonic fibroblasts. This work was supported by the Fundamental Research Funds for the Central University Grant 2013121036, National Natural Science Foundation of China Grants 31371292, 31422030, and 91440112, and Regional Demonstration of Marine Economy Innovative Development Project 12PYY001SF08 (to W.L.); and China Postdoctoral Science Foundation 2015M571969 (to W.-w.G).

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