A Novel Histone H4 Arginine 3 Methylation-sensitive Histone H4 Binding Activity and Transcriptional Regulatory Function for Signal Recognition Particle Subunits SRP68 and SRP72*§

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Arginine methylation broadly occurs in the tails of core histones. However, the mechanisms by which histone arginine methylation regulates transcription remain poorly understood. In this study we attempted to identify nuclear proteins that specifically recognize methylated arginine 3 in the histone H4 (H4R3) tail using an unbiased proteomic approach. No major nuclear protein was observed to specifically bind to methylated H4 tail peptide. These proteins were identified as the SRP68 and SRP72 heterodimers (SRP68/72), the components of the signal recognition particle (SRP). Only SRP68/72, but not the SRP complex, bound the H4 tail peptide. SRP68 and SRP72 bound the H4 tail in vitro and associated with chromatin in vivo. The chromatin association of SRP68 and SRP72 was regulated by PRMT5 and PRMT1. Both SRP68 and SRP72 activated transcription when tethered to a reporter via a heterologous DNA binding domain. Analysis of the genome-wide occupancy of SRP68 identified target genes regulated by SRP68. Taken together, these results demonstrate a role of H4R3 methylation in blocking the binding of effectors to chromatin and reveal a novel role for the SRP68/SRP72 heterodimer in the binding of chromatin and transcriptional regulation.

Arginine methylation, a post-translational modification catalyzed by a family of protein arginine methyltransferases (PRMT),3 is commonly observed in cytoplasmic and nuclear proteins including core histones (1–3). Multiple arginine (Arg) residues in histone tails, including Arg-2, -8, -17, and -26 in H3 and Arg-3 in H4 have been shown to be mono (me1)- or dimethylated (me2), with the latter in a symmetrical or an asymmetrical configuration (me2s or me2a) (1, 4). For example, PRMT5 has been shown to catalyze the symmetrical dimethylation of the Arg-3 residue in H4 N-terminal tail (H4R3me2s) (5, 6), whereas PRMT1 can catalyze the asymmetrical dimethylation of the same residue (H4R3me2a) (7). Extensive studies have correlated H4R3me2s catalyzed by PRMT5 with transcriptional repression of associated genes (8–10) and H4R3me2a catalyzed by PRMT1 with transcriptional activation (7, 11).

Like histone lysine methylation (12–14), in principle histone arginine methylation can regulate transcription either by effect in cis on other histone modifications and/or by serving as histone code to influence the binding of histone-interacting effector proteins. In this regard, H4R3me2a catalyzed by PRMT1 has been shown to promote subsequent histone acetylation by CBP/p300 (7, 15); this in cis effect explains at least in part the role of H4R3me2a in transcriptional activation. In support of the histone code hypothesis, an increasingly large number of proteins has been shown to specifically bind various methylated lysine residues in histone N-terminal tails and plays diverse roles in epigenetic regulation (14, 16, 17). In contrast, so far only a few proteins including Tudor domain-containing protein 3 (TDRD3), DNA methyltransferase 3a (Dnmt3a), RNA polymerase-associated protein 1 (PAF1) complex, and p300/CBP-associated factor (PCAF) have been implicated in binding of

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H4R3me2 Inhibits SRP68/72 Chromatin Association

methylated arginine residues in histone tails (18–21), and among them only the binding of H3R17me2a and H4R3me2a by TDRD3 is supported by biochemical and structural evidences (22). TDRD3 binds H3R17me2a and H4R3me2a via a Tudor domain that has been recognized as a structural motif for binding of arginine-methylated non-histone proteins (23). The limited number of arginine-methylated histone-binding proteins identified so far raises the possibility for the existence of large number of arginine-methylated histone-specific effectors that remain to be identified. Alternatively, it may underscore a major mechanistic difference in the action of arginine and lysine methylation.

Mammalian signal recognition particle (SRP) is a ribonucleoprotein complex composed of six SRP proteins (SRP9, SRP14, SRP19, SRP54, SRP68, and SRP72) and a RNA molecule known as 7 S RNA or 7 SL RNA (24, 25). The SRP complex is conserved in evolution and plays a central role in the co-translational targeting of secretory and membrane proteins to the endoplasmic reticulum (ER). SRP binds nascent signal peptide sequences of proteins as they emerge from the ribosome. The resulting targeting complex then docks to ER via interaction with the SRP receptor in a GTP-dependent manner (26). Previous studies have shown that SRP68 and SRP72 exist predominantly as a stable SRP68/72 heterodimer that is essential for SRP-mediated ER-targeting of proteins (27).

In this study we used an unbiased proteomic approach to screen for proteins that bind specifically to H4R3me2s and H4R3me2a. Instead of identifying new methyl-H4R3-binding proteins, we found two proteins, SRP68 and SRP72, whose binding to the H4 tail was inhibited by arginine methylation. Our study illustrates a novel function of H4R3 methylation in inhibiting binding of chromatin effectors and reveals a novel transcriptional function for SRP68 and SRP72.

EXPERIMENTAL PROCEDURES

Plasmids, Antibody, Cell Lines, Transfection, and Luciferase Assay—The expression plasmids pcDNA3/SRP54, pcDNA3/SRP68, pcDNA3/SRP72, pGEX-4T-1/SRP68, and pGEX-4T-1/SRP72 were constructed by cloning the full-length human SRP68 and SRP72 into pcDNA3.0 and pGEX4T-1 vectors, respectively. The CFP-Lac-H4t plasmid was generated by cloning 2 tandem copies of oligonucleotides encoding the first 20 amino acids of human H4 N-terminal tail. The plasmids for in vitro synthesis of [35S]Met-labeled SRP54, SRP68, and SRP68 and their respective deletion mutants have been described previously (27–29). To express SRP68 or SRP72 and their deletion mutants as Gal4(DBD) fusion proteins, the corresponding cDNAs were cloned into pCMV-Gal4(DBD) vector. The 4xUAS–TK–luc luciferase reporter was as described (30). Commercially available antibodies directed toward H3, H4, and H4R3me2s were from Abcam (Cambridge, MA); HA was from Roche Applied Science; FLAG was from Sigma; SRP54, SRP19, SRP14, and SRP9 were from eBiosciences (San Diego, CA). SRP68 and SRP72 antibodies were generated in the laboratory by immunizing rabbits with GST-SRP68 and GST-SRP72.

HeLa and 293T cell lines were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Transient transfections in 293T and HeLa cells were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Luciferase reporter assay was essentially as described (30).

Isolation of Binding Proteins from HeLa Nuclear Extracts Using Biotinylated H4 Tail Peptides—Nuclear extracts were prepared from HeLa cells by the protocol of Dignam et al. (31). The C-terminal biotinylated H4 tail peptides (amino acids 1–16) without or with either a H4R3me2a or H4R3me2s were synthesized and purified by Beijing Scilight Biotechnology Ltd. Co. Purification of corresponding H4 peptide-binding proteins from HeLa nuclear extracts was carried out essentially as described (32).

Mass Spectrometry and Western Blot Analysis—Both methods were performed as described previously (32).

Pulldown Assay with in Vitro Synthesized Proteins and Recombinant Proteins—Radiolabeled proteins were generated with the TNT coupled reticulocyte lysate system (Promega). In vitro pulldown assays were carried out by incubating the in vitro translated products with 1 μg of immobilized histone tail peptides as described (33). For binding of purified recombinant GST-SRP68, GST-SRP68–(436–620), GST-SRP72, and GST-SRP–(529–659), the recombinant proteins (10 μg) were incubated with immobilized H4 tail peptides in the binding buffer (20 mM HEPES, pH 7.9, 150 mM KCl, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 10% glycerol, 0.1% Nonidet P-40, protease inhibitors) for 2 h at 4 °C. Unbound proteins were removed by washing the beads with washing buffer (20 mM HEPES, pH 7.9, 200 mM KCl, 1 mM DTT, 1 mM PMSF, 0.1% Nonidet P-40, protease inhibitors) 4 times for 5 min each. The proteins that remained bound to the peptides were separated by SDS-PAGE followed by Coomassie Blue staining.

Preparation of Cytosol, Nuclear Extract, and Chromatin and Chromatin Immunoprecipitation (ChIP)-Western Blot—To fractionate cellular contents to cytosol and nuclear and chromatin fractions, cultured cells were collected by centrifugation and washed twice with ice-cold PBS. The pellets were resuspended in 2 packed cell volumes of solution A (20 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1% Nonidet P-40, 1 mM DTT, and protease inhibitors), incubated on ice for 10 min, and centrifuged at 4000 rpm for 5 min at 4 °C. The resulting supernatants were designated as cytosol and the pellets were designated as cytosol. The pellets were resuspended with solution A containing 0.4 M NaCl and incubated in ice for 20 min. The samples were centrifuged at 12,000 rpm for 10 min, and the supernatants were designated as nuclear extracts. The pellets were washed once with solution A, resuspended in 2 volumes of 1× SDS loading buffer, and designated as chromatin fractions.

For ChIP-Western blot analysis, HeLa or 293T cells were treated with 1% formaldehyde for 15 min in culture medium. The cells were lysed as above, and the pellets containing nuclei were resuspended in solution A plus 3 mM CaCl2 and 5 units of micrococcal nuclease. After incubation on ice for 1 h, the samples were sonicated, and the soluble chromatin was prepared. Immunoprecipitation was carried out with or without the addition of anti-SRP68 antibody, and Western blot analyses were performed using the antibodies as indicated.
RESULTS

**H4R3 Methylation Blocks the Binding of SRP68/72 to the H4 Tail Peptide**—In the attempt to identify proteins that specifically recognize the H4R3me2s and H4R3me2a codes, we employed an unbiased in vitro affinity purification approach using immobilized histone tail peptides and HeLa nuclear extracts. We have previously used this approach to identify chromatin effectors specific for methylated H3K4 or H3K9 (32, 35, 36). Three biotinylated H4 N-terminal tail peptides (amino acids 1–16) containing either no modification or R3me2s or R3me2a were synthesized. These peptides were immobilized on streptavidin-agarose beads through a C-terminal biotin moiety and used for affinity purification of specific binding proteins obtained from HeLa nuclear extracts. Bound polypeptides were resolved by electrophoresis on a 4–20% SDS-PAGE gel and visualized by silver staining (Fig. 1A). In multiple experiments no prominent polypeptides that bound specifically to either the H4R3me2s or H4R3me2a peptides but not to the H4 peptide was observed. Instead, two polypeptides with molecular masses in the range of 70–75 kDa were reproducibly observed to be enriched in the control H4 peptide sample as compared with the H4R3me2s and H4R3me2a peptides (Fig. 1A). We sequenced the two protein bands by mass spectrometry and determined their identities to be SRP68 and SRP72. Subsequent Western blot analyses using antibodies specific for SRP68 and SRP72 confirmed that both proteins bound with higher affinity to the H4 peptide than to the H4R3me2s and H4R3me2a peptides (Fig. 1B). Thus, we identified SRP68 and SRP72 as novel H4 tail-binding proteins whose binding activity is inhibited by H4R3 methylation.

**Knockdown of SRP68 by siRNA**—The siRNAs against human SRP68 were synthesized and purified by Shanghai GenePharma Co., Ltd. The sequences of three SRP68 siRNAs are as follow: GGCUGGUGCUGUAUAAACCAATT (siSRP-(68–722)), GAGAUUCCUCAGAUUUAUATT (siSRP-(68–216)) and GCUCUGAAGCAGUAUAGATT (siSRP-(68–383)). The knockdown of SRP68 in 293T cells for expression profiling was carried out using 1:1 mixture of siSRP-(68–722) and siSRP-(68–216) according to the manufacturer’s instructions. The sequence of control siRNA is UUCUCGGAACGUGUCAGUUTT.

Chromatin Immunoprecipitation and High-throughput Sequencing—Chromatin immunoprecipitation assays were performed with or without immunoaﬃnity-puriﬁed SRP68 antibody using chromatin prepared from 293T cells. After immunoprecipitation, the puriﬁed DNAs were subjected to sequencing on an Illumina Genome analyzer. SOAP 2.20 was used to align reads with two mismatches for each sample using the updated Human genome databases available on line at Human (Homo sapiens) Genome Browser Gateway. Sequences with greater than 83% identity were used for further analyses. To identify ChIP peaks, ChIP seq data were analyzed using the MACS program available at with the SRP68 ChIP-Seq data as background. Default parameters were set for human genome, and the input and the control ChIP-seq data as background. Default p value was set 0.00001 or 1/P. The binding of SRP68/72 to the H4 tail by co-localization in DG44 CHO cells was assessed as described previously (34).

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**RESULTS**

H4R3 Methylation Blocks the Binding of SRP68/72 to the H4 Tail Peptide—In the attempt to identify proteins that speciﬁcally recognize the H4R3me2s and H4R3me2a codes, we employed an unbiased in vitro afﬁnity puriﬁcation approach using immobilized histone tail peptides and HeLa nuclear extracts. We have previously used this approach to identify chromatin effectors speciﬁc for methylated H3K4 or H3K9 (32, 35, 36). Three biotinylated H4 N-terminal tail peptides (amino acids 1–16) containing either no modiﬁcation or R3me2s or R3me2a were synthesized. These peptides were immobilized on streptavidin-agarose beads through a C-terminal biotin moiety and used for afﬁnity puriﬁcation of speciﬁc binding proteins obtained from HeLa nuclear extracts. Bound polypeptides were resolved by electrophoresis on a 4–20% SDS-PAGE gel and visualized by silver staining (Fig. 1A). In multiple experiments no prominent polypeptides that bound speciﬁcally to either the H4R3me2s or H4R3me2a peptides but not to the H4 peptide was observed. Instead, two polypeptides with molecular masses in the range of 70–75 kDa were reproducibly observed to be enriched in the control H4 peptide sample as compared with the H4R3me2s and H4R3me2a peptides (Fig. 1A). We sequenced the two protein bands by mass spectrometry and determined their identities to be SRP68 and SRP72. Subsequent Western blot analyses using antibodies speciﬁc for SRP68 and SRP72 conﬁrmed that both proteins bound with higher afﬁnity to the H4 peptide than to the H4R3me2s and H4R3me2a peptides (Fig. 1B). Thus, we identiﬁed SRP68 and SRP72 as novel H4 tail-binding proteins whose binding activity is inhibited by H4R3 methylation.

The SRP68/72 Heterodimer but Not the SRP Complex Binds to the H4 Tail—The observed binding of SRP68 and SRP72 to the H4 N-terminal tail peptide raised the question if these proteins bind to the H4 tail in the form of heterodimers or associated with the SRP complex (25, 27). Western blot analysis demonstrated that, unlike SRP68 and SRP72, SRP9, SRP14, SRP19, and SRP54 did not bind to the H4 peptide (Fig. 1B), suggesting that SRP68 and SRP72 bind the H4 tail peptide independently of the other SRP proteins. To substantiate this further, we utilized Superose 6 gel ﬁltration chromatography to separate SRP68 and SRP72 in HeLa nuclear extract into a different complex(es). Western blot analysis revealed at least two protein complexes that contained SRP68 and SRP72. The larger molecular weight complex cofractionated with SRP54 and probably represented the fully assembled SRP (Fig. 1C, lanes 3–5). A smaller complex(es) also contained SRP68/72 but lacked SRP54 (Fig. 1C, lanes 6–9). When these fractions were assayed with regard to their binding of H4 tail peptide, we observed that only SRP68 in the smaller complex bound the H4 tail peptide (Fig. 1D). In addition, among all four histone tail peptides tested, SRP68 and SRP72 bound only the H4 tail peptide (Fig. 1E). The binding of SRP68 and SRP72 to H4 peptide was insensitive to RNase A treatment (Fig. 1F), further supporting the conclusion that SRP68 and SRP72 bind in the form of heterodimers in the absence of 7SL SRP RNA. Taken together, these data provide compelling evidence that SRP68/72 heterodimers, but not the SRP complex, are the histone H4 tail-speciﬁc binding proteins.

**SRP68 and SRP72 Associate with Chromatin in Vivo**—SRP68 and SRP72 are well known for their exclusive protein targeting function within the SRP complex. Our ﬁnding that SRP68 and SRP72 bind speciﬁcally to the unmodiﬁed H4 tail peptide suggests a new function of SRP68 and SRP72 in chromatin regulation. In agreement with previous reports (37, 38), immuno- staining of HeLa cells revealed a predominantly cytoplasmic localization for SRP9, SRP19, and SRP54 (Fig. 2A and data not shown). However, the endogenous SRP68 in HeLa cells was detected predominantly in the nucleus, whereas SRP72 was present both in the nucleus and cytoplasm (Fig. 2A). Similarly, we observed that ectopically expressed SRP68 and SRP72 were mainly nuclear in HeLa cells (Fig. 2B).

To investigate if the nuclear SRP68 and SRP72 associated with chromatin, cytosol, nuclear, and chromatin fractions were prepared from HeLa cells. A substantial amount of SRP68 and SRP72 was found to associate with chromatin (Fig. 2C). In contrast, SRP54 did not associate with chromatin under the same condition (Fig. 2C). As markers for appropriate cellular fractionation, β-actin was detected only in the cytosol, whereas the core histone H3 was detected only in the chromatin fraction (Fig. 2C). To test further the chromatin association of SRP68
and SRP72, HeLa cells were treated with 1% formaldehyde, and nuclei were prepared and subjected to chromatin digestion with micrococcal nuclease. After centrifugation to remove insoluble nuclei pellets, the soluble chromatin-containing fraction was immunoprecipitated with anti-SRP68 antibody and assayed for the presence of core histones by Western blot analysis. Fig. 2D shows the presence of core histones H3 and H4 in immunoprecipitation of soluble chromatin with anti-SRP68 antibody. Significantly, the chromatin that co-precipitated with SRP68 was devoid of H4R3me2s (Fig. 2D). This was in agreement with the in vitro peptide binding data that showed that H4R3 methylation blocks the binding of SRP68/72 to the H4 tail. Similar results were observed when immunoprecipitation was performed with anti-SRP72 antibody and chromatin derived from HeLa cells (data not shown).

To further demonstrate that SRP68 binds the H4 N-terminal tail within cells, we made use of a CHO cell line, which contains a large number of Lac operator sequences stably integrated in a single chromosomal site (39). Expression of control CFP-tagged Lac proteins or CFP-Lac fused with a tandem H4 tail peptide (designated as CFP-Lac-H4t) in these cells generated bright foci due to the binding of integrated Lac sequences by CFP-Lac fusion proteins. We observed co-localization of ectopically expressed HA-SRP68 with CFP-Lac-H4t but not the control CFP-Lac (Fig. 2E), indicating a tandem H4 tail dependent interaction with SRP68. Similar results were observed for SRP72 (data not shown). Together these data demonstrate that SRP68 binds H4 tail peptide in cells and that a portion of the SRP68 and SRP72 proteins is intracellularly associated with chromatin.

SRP68 and SRP72 Directly Interact with the H4 Tail in a H4R3 Methylation-sensitive Manner—To investigate if SRP68 and SRP72 directly interact with the H4 tail, in vitro translated polypeptides were assayed for binding to the four histone tail peptides using pulldown assays. In vitro synthesized SRP68 and SRP72 bound H4 but not other histone tail peptides (Fig. 2F). Under the same conditions, SRP54 did not bind to the H4 tail peptide (Fig. 2F). Using a series of SRP68 deletion mutants, we further mapped the H4 tail binding activity to the C-terminal region (amino acids 436–620) of SRP68 that is known to also bind to SRP72 (Fig. 2G). We observed that both the N-terminal region (amino acids 1–356) and C-terminal region (amino acids 529–659) of SRP72 were able to bind the H4 tail peptide (Fig. 2H).

To test if SRP68 and SRP72 bind directly the H4 tail peptide, we expressed and purified GST fusions of full-length SRP68 and SRP72 and their C-terminal H4 binding domains. In pulldown assays, these recombinant proteins bound specifically the H4 tail peptides with CFP-Lac-H4t but not the control CFP-Lac (Fig. 2E), indicating a tandem H4 tail dependent interaction with SRP68. Similar results were observed for SRP72 (data not shown). Together these data demonstrate that SRP68 binds H4 tail peptide in cells and that a portion of the SRP68 and SRP72 proteins is intracellularly associated with chromatin.
tail peptide (Fig. 2I). Furthermore, H3R4 methylation abolished the binding of recombinant SRP68 and SRP72 to the H4 tail (Fig. 2I, compare lane 5 with lane 4). Together these data demonstrate that both SRP68 and SRP72 bind directly to the H4 tail in a Arg-3 methylation-sensitive manner.

PRMT5 Regulates SRP68/72 Chromatin Association and Subcellular Localization—Having established that H4R3 methylation blocks the binding of SRP68 and SRP72 to the H4 tail peptide and that SRP68 and SRP72 associate with chromatin in cells, we next investigated the effect of H4R3 methylation on...
the intracellular association of SRP68 and SRP72 with chromatin. For this purpose, we overexpressed FLAG-PRMT5 and its enzymatic inactive mutant in 293T cells and analyzed the effect on SRP68 and SRP72 subcellular localization and chromatin association by cellular fractionation. We found that overexpression of wild type (Fig. 3A, compare lanes 5 and 6 with lanes 2 and 3) but not the mutant PRMT5 (Fig. 3A, compare lanes 8 and 9 with lanes 2 and 3) reduced the levels of nuclear and chromatin-associated SRP68 and SRP72. Western blot analysis also detected increased levels of H4R3me2s in chromatin derived from FLAG-PRMT5 but not FLAG-PRMT5m-expressed cells (see Fig. 5A, compare lane 6 with lanes 3 and 9). As controls for proper cellular fractionation, β-actin was detected mainly in the cytosol and core histone H3 in the chromatin. These results indicate that PRMT5 regulates SRP68 and SRP72 chromatin association in an enzymatic activity-dependent manner. Furthermore, PRMT5 appears to promote nuclear to cytoplasmic translocation of SRP68 and SRP72.

To further examine the effect of PRMT5 on SRP68 and SRP72 subcellular localization and chromatin association, we cotransfected FLAG-PRMT5 with HA-SRP68 or HA-SRP72 into HeLa cells and analyzed the subcellular localization of HA-SRP68 and HA-SRP72 by immunofluorescent staining. Although HA-SRP68 was primarily nuclear in cells expressing HA-SRP68 alone, it was predominantly localized in the cytoplasm in cells co-expressed wild type but not mutant PRMT5 (Fig. 3B). Similar results were observed for SRP72 (Fig. 3C). Together with the cellular fractionation experiments described above, these results suggest that PRMT5 inhibits the binding of SRP68/72 to chromatin and sequesters SRP68 and SRP72 from the nucleus toward the cytosol in an enzymatic activity-dependent manner.

In our in vitro binding assays both H4R3me2a and H4R3me2s modifications inhibited the binding of SRP68 and SRP72 to H4 tail peptide (Fig. 1, A and B). As H4R3me2a and H4R3me2s are known to have distinct transcriptional regulatory functions, we were eager to determine if overexpression of PRMT1, the enzyme that catalyzes H4R3me2a modification, also influences the chromatin association and subcellular localization of SRP68 and SRP72. We thus overexpressed PRMT1 in 293T cells and carried out cellular fractionation experiments to...
determine the effect on SRP68 and SRP72 chromatin association and subcellular fractionation. As shown in Fig. 3D, we found that overexpression of PRMT1 led to the dissociation of SRP68 and SRP72 from chromatin. Western blot analysis confirmed an increased H4R3me2a level upon ectopic expression of HA-PRMT1. This result is in agreement with our in vitro H4 tail peptide binding data showing that H4R3me2a modification also inhibits the binding of H4 tail by SRP68 and SRP72. Unlike the case of PRMT5 overexpression, PRMT1 overexpression did not appear to reduce the nuclear fraction of SRP68 and SRP72 (Fig. 3D, compare lane 5 and lane 2). Indeed, unlike PRMT5, ectopic expression of PRMT1 did not affect the nuclear localization of SRP68 and SRP72 as shown by immunofluorescent staining (Fig. 3E). Together these results show that both PRMT5 and PRMT1 regulate SRP68/72 chromatin association, presumably through its ability to catalyze H4R3me2s and H4R3me2a, respectively, which in turn interferes with the binding of SRP68/72 to histone H4 tail in chromatin. The mechanism by which PRMT5 and PRMT1 differentially affect the subcellular localization of SRP68/72 remains to be investigated.

Both SRP68 and SRP72 Appear to Possess a Transcriptional Activation Activity—The above findings that both SRP68 and SRP72 associate with chromatin in cells and that their chromatin association is regulated by H4R3 methylation raise the possibility that SRP68 and SRP72 are involved in transcriptional regulation. To this end, we investigated if SRP68 and SRP72 possess transcriptional activity. We generated fusion proteins of SRP68 and SRP72 with a heterologous DNA binding domain (DBD amino acids 1–147) from yeast transcription factor Gal4. When cotransfected with a minimal TK promoter-driven luciferase reporter containing four tandem Gal4 binding sites (UAS) upstream of the TK promoter (4xUAS-TK-luc) into 293T cells, we found that expression of Gal-SRP68 or Gal-SRP72 led to transcriptional activation in a dose-dependent manner (Fig. 4A). The correct expression of Gal-SRP68 and Gal-SRP72 was verified by Western blot analysis using a Gal4(DBD)-specific antibody (Fig. 4A, lower panel). These results suggest that SRP68 and SRP72 have a transcriptional activation function.

To map the potential transcriptional activation domain(s) in SRP68 and SRP72, we fused the different regions of SRP68 and SRP72 to Gal4(DBD) and tested their ability to activate transcription in luciferase reporter assay as above. We found that the transcriptional activity of SRP68 mainly resided in the C-terminal region amino acids 436–620 (Fig. 4B). The differences in the transcriptional activity for different regions of SRP68 were not due to variation in protein expression, because Western blot analysis revealed a similar expression level for various Gal-SRP68 fusion proteins (Fig. 4B, lower panel). For SRP72, the major transcriptional activation domain was mapped to the N-terminal region, amino acids 1–356. Given that Gal-(1–356) exhibited only half of the transcriptional activity of the full-length SRP68, the contribution of the additional C-terminal region to the transcriptional activity could not be excluded. Fig. 4D summarizes the transcriptional activa-
tion domain mapping results of SRP68 and SRP72. Although the precise transcriptional activation domain(s) and mechanism(s) by which they activate transcription remains to be determined, these data nevertheless support a transcriptional regulatory function for both SRP68 and SRP72.

Identification of Potential SRP68 Target Genes by ChIP-Seq Analysis—We next attempted to identify potential endogenous SRP68 target genes using ChIP followed by high throughput sequencing (ChIP-seq). 293T cells were fixed by formaldehyde, and the chromatin was fragmented by sonication and immunoprecipitated using purified specific anti-SRP68 antibodies or no antibodies as the ChIP negative control group. A total of 1166 SRP68 binding peaks with a \( p \) value 1.00e-004 was identified, and 681 of the 1166 peaks can be annotated to 638 unique genes using a parameter of maximum distance of 100 kb up- and downstream of the transcription starting sites (TSS) (Fig. 5, A and B). The representative SRP68 binding profiles were shown in Fig. 5C for the CD1E and TCTA genes. The SRP68 binding sites were not enriched in particular genomic regions (e.g. promoters or introns) (supplemental Fig. S1A) but interestingly were enriched in chromosomes 1, 3, 13, and X (supplemental Fig. S1B). We randomly selected 18 SRP68 peaks and validated the binding of SRP68 to these regions by ChIP followed by quantitative PCR analysis (Fig. 5D). As negative controls, the binding of SRP68 was not observed in the promoter regions of NKX3.1 and PSA genes (data not shown). The results suggested that most if not all peaks identified by our ChIP-seq analysis are authentic SRP68 binding sites. GO analysis revealed that the 638 unique SRP68 binding site-containing genes are slightly enriched for cytoskeleton organization, cell adhesion, DNA catabolic, and apoptosis processes (supplemental Fig. S2).

SRP68 May Regulate Target Gene Expression in a Context-dependent Manner—Having identified the potential SRP68 target genes, we next investigated if SRP68 had a role in their expression. We knocked down SRP68 in 293T cells by RNAi and verified the efficient down-regulation of SRP68 protein by Western blotting (Fig. 5E). We then analyzed the effect of SRP68 knockdown on mRNA levels of the 18 genes that had been verified for binding of SRP68 by quantitative RT-PCR. We found that knockdown of SRP68 resulted in the substantial up-regulation of DDIT3, DIDO1, CUL1, HNRNPA3, SLC37A3, YEATS4, and DRD2, in a significant down-regulation of TMEM110, TCTA, NF1, ODC43, CD1E, and CDKN1A, and in insignificant changes of the remaining genes. This effect on target gene expression was reproducible in three independent experiments. Thus, although the reporter assays clearly suggested a transcriptional activation function for SRP68, the knockdown of SRP68 differentially affected the expression of its associated target genes, suggesting a context-dependent transcriptional function for SRP68.

DISCUSSION
In this study we attempted to use an unbiased proteomic approach to identify nuclear proteins that selectively bind histone H4 N-terminal tail peptides with H4R3me2a or H4R3me2s. Despite extensive effort, we did not observe any prominent nuclear protein that binds the H4 tail peptide in an H4R3me2a- or H4R3me2s-dependent manner (Fig. 1A). Instead, we consistently observed that both H4R3me2a and H4R3me2s inhibited the binding of two nuclear proteins that were subsequently identified as the SRP68/72 heterodimers. It is noteworthy that the same experimental approach has permitted us previously
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FIGURE 6. A model of H4R3 methylation in regulating chromatin association of SRP68/72. SRP68/72 has dual roles either in the SRP complex participating in protein targeting (left) or as histone modifier in transcription (right). (A) The association of SRP68/72 with chromatin is likely to influence transcription either directly and/or indirectly. (B) The role of histone arginine methylation in inhibiting rather than recruiting effector proteins is not unique to H4R3 methylation. It was shown previously that H3R2me2a catalyzed by PRMT6 antagonizes H3K4 methylation by interfering with the binding of H3K4 methyltransferase mixed lineage leukemia (MLL) complexes and other proteins to chromatin (40–42). More recently, H3R2 methylation has been shown to inhibit the binding of UHRF1/ICBP90 to histone H3 tail (43). In addition, by using the same unbiased peptide pulldown approach as employed in this study, we also failed to detect any prominent nuclear proteins that bind the H3 tail peptides containing either H3R17me2a or H3R26me2a (44). Instead, we uncovered an about 10-fold increase in binding of proteins such as TDRD3 (19). Neverthe-
less, the limited number of arginine-methylated histone-bind-
ing proteins identified so far raises the possibility that this type of histone modification mainly functions to inhibit rather than to recruit the effector proteins.

H4R3me2a and H4R3me2s are catalyzed, respectively, by PRMT1 and PRMT5 and have been linked to transcriptional activation and repression, respectively (7, 9–11). Given their opposite roles in transcription, we initially expected to identify distinct sets of proteins that bind H4R3me2a or H4R3me2s, respectively, or whose binding to H4 tail are differentially affected. Although we could not rule out the possibility that we failed to observe these proteins due to technical limitations in our experiments, it is equally possible that H4R3me2a or H4R3me2s may exert opposite effect on transcription through their distinct cis-effect on other histone modifications. For example, the presence of H4R3me2a has been shown to facilitate in cis histone acetylation catalyzed by CBP/p300 (7) and PCAF (20). On the other hand, H4R3me2s catalyzed by PRMT5 has not been shown to enhance in cis histone acetylation. Thus, the effect of histone arginine methylation on transcription is likely due to the combinatorial effect of histone arginine methylation on the binding of effectors and/or other histone modifications in cis.

A surprising finding in this study is the identification of SRP68 and SRP72 as major H4-binding proteins. SRP68 and SRP72 are part of the SRP, a particle critically important for targeting secretory and membrane proteins to ER. SRP68 and SRP72 were previously shown to form heterodimers independent of the SRP complex and are released from the SRP as a stable SRP68/72 that is essential for SRP-mediated protein targeting (27, 29). Our pulldown and gel filtration assays provide compelling evidence that the SRP68/72 heterodimers, but not the SRP complex, binds the H4 tail. Multiple lines of evidence support a direct binding of SRP68/72 to H4 tails, including a H4 tail-dependent recruitment (co-localization) of SRP68 in CHO cells (Fig. 2E) and binding of H4 tail peptide in vitro by recombinant SRP68 and SRP72 (Fig. 2F). The ability for both SRP68 and SRP72 to bind H4 tail may allow SRP68/72 heterodimers to bind chromatin with high affinity. It is noteworthy that SRP68 and SRP72 do not share sequence similarity. Exactly how these proteins bind the H4 tail peptide remains to be determined.

Consistent with an inhibitory role of H4R3me2a and H4R3me2s on binding of SRP68/72 to H4 tail peptide, ectopic expression of PRMT5 or PRMT1 all resulted in the dissociation of SRP68/72 from chromatin. Interestingly, ectopic expression of PRMT5 also drives SRP68/72 out of the nucleus, whereas ectopic expression of PRMT1 does not. At this stage we do not...
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know how PRMT1 and PRMT5 differentially regulate SRP68/72 subcellular localization. One possibility is that PRMT5 not only methylates H4R3, which leads to dissociation of SRP68/72 from chromatin, but also methylates SRP68/72, which results in nuclear export of SRP68/72. On the other hand, PRMT1 may only methylate H4R3 to affect SRP68/72 chromatin association. Further work is needed to elucidate the mechanisms by which PRMT5 regulates SRP68/72 subcellular localization.

By tethering SRP68 and SRP72 to a luciferase reporter through a heterologous Gal4 DNA binding domain, we demonstrated that both SRP68 and SRP72 possess a transcriptional activation activity (Fig. 4A). The transcriptional activity can be mapped primarily to the C-terminal region of SRP68 and N-terminal region of SRP72 (Fig. 4, B and C). Although the mechanism by which tethering SRP68 or SRP72 to DNA leads to transcriptional activation remains to be investigated, it nevertheless suggests that the chromatin-associated SRP68 and SRP72 may have a transcriptional regulatory function. In support of this notion, we carried out ChIP-seq analysis and identified 1166 SRP68-associated regions and 638 potential SRP68 target genes using a parameter of maximum SRP68 peak distance of 100 kb up- and downstream of the transcription starting sites (TSS) (Fig. 5, A and B). As the enrichment of SRP68 in the SRP68 peaks was confirmed by ChIP-quantitative PCR analysis for 18 randomly selected genes (Fig. 5D), most of the SRP68 peaks identified in this study are likely the authentic SRP68-associated regions in 293T cells. Given that SRP68 and SRP72 form heterodimers, the SRP68-associated regions are most likely the SRP68/72-associated regions, although this remains to be tested experimentally. Interestingly, although SRP68 possesses a transcriptional activation activity in the reporter assay, knockdown of SRP68 affects positively or negatively the expression of its directly associated genes (Fig. 5E), suggesting that the effect of SRP68 on target gene expression is likely context-dependent. Although the underlying mechanism remains to be fully investigated, many transcription factors or epigenetic regulators possess a context-dependent transcriptional function. For example, stem cell factor Oct4 can activate or repress its target gene expression (45), in part depending on the coregulators it interacts with (46, 47).

Taken together, our study has identified SRP68 and SRP72 as novel H4 tail-binding proteins whose binding of H4 tails is inhibited by H4R3 methylation, and thus we uncovered a novel transcriptional regulatory function for SRP68/72 (Fig. 6). The identification of potential SRP68/72 target genes by ChIP-seq substantiates the histone binding activity of SRP68/72 and sets up the stage for characterization of their transcription and potentially other chromatin-related function.

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