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A Dual Role for the Histone Methyltransferase PR-SET7/SETD8 and Histone H4 Lysine 20 Monomethylation in the Local Regulation of RNA Polymerase II Pausing

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RNA polymerase II (Pol II) promoter-proximal pausing plays a critical role in postinitiation transcriptional regulation at many metazoan genes. We showed recently that histone H4 lysine 16 acetylation (H4K16Ac), mediated by the MSL complex, facilitates the release of paused Pol II. In contrast, H4 lysine 20 trimethylation (H4K20me3), mediated by SUV420H2, enforces Pol II pausing by inhibiting MSL recruitment. However, how the balance between H4K16Ac and H4K20me3 is locally regulated remains unclear. Here, we demonstrate that PR-SET7/SETD8, which monomethylates histone H4 lysine 20 (H4K20me1), controls both H4K16Ac and H4K20me3 and in doing so, regulates Pol II pausing dynamics. We find that PR-SET7-mediated H4K20me1 is necessary for the recruitment of the MSL complex, subsequent H4K16Ac, and release of Pol II into active elongation. Although dispensable for SUV420H2 recruitment, PR-SET7-mediated H4K20me1 is required for H4K20me3. Although depletion of SUV420H2 is sufficient to deplete H4K20me3 and relieve an H4K20me3-induced pause, pausing is maintained in the absence of PR-SET7 despite H4K20me3 depletion because of an inability to recruit the MSL complex in the absence of H4K20me1. These findings highlight the requirement for PR-SET7 and H4K20me1 in establishing both the H4K16Ac and H4K20me3 marks and point to a dual role in the local regulation of Pol II pausing.

Proper cell growth and differentiation are dependent on the appropriate regulation of gene expression. Abnormal gene expression underlies many diseases, including cancer. Having mechanisms in place that allow prompt induction of transcription, in response to stimuli arising from alterations in the environment and during development, is one way in which this homeostasis is maintained. In metazoans, promoter-proximal pausing represents a critical point of postinitiation transcriptional regulation wherein initiated and transcriptionally engaged RNA polymerase II (Pol II)2 pauses within the first 60 nucleotides downstream of the transcription start site. Pol II in this state is said to be “poised” for rapid firing, and the rate of release of paused Pol II into elongation is a critical determinant of ultimate transcriptional output. Initially discovered at stress-induced heat shock genes in Drosophila, genome-wide analyses indicate that Pol II pausing is likely a common feature of transcription at most human and Drosophila genes, with local factors and cellular signals determining the extent to which this step becomes rate-limiting (1, 2).

At the molecular level, Pol II pausing is regulated by the negative elongation factor (NELF) complex, a four-subunit complex that collaborates with the 5,6-dichloro-1-B-D-ribofuranosylbenzimidazole (DRB) sensitivity-inducing factor (DSIF) to pause Pol II and inhibit productive elongation (3, 4). Positive transcription elongation factor b (pTEFb), a cyclin-dependent kinase, relieves NELF/DSIF-mediated pausing by phosphorylating serine 2 in the C-terminal domain of the largest Pol II subunit as well as NELF and DSIF, allowing dissociation of NELF, and the transition into elongation (5–8). A number of factors contribute to the recruitment of pTEFb to different subsets of genes, thereby aiding in Pol II release, including the c-Myc and NF-kB transcription factors and the bromodomain protein BRD4, which binds acetylated histones (9–14).

Local chromatin architecture at gene promoters also plays an essential role in the regulation of transcription. DNA methylat-

References

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2 The abbreviations used are: Pol II, RNA polymerase II; NELF, negative elongation factor; DSIF, DRB sensitivity-inducing factor; pTEFb, positive transcription elongation factor b; H4K16Ac, histone H4 lysine 16 acetylation; H4K20me3, histone H4 lysine 20 trimethylation; SUV420H2, suppressor of variegation 4-20 homolog 2; MSL, male-specific lethal; hMOF, human males absent on the first; H4K20me2, histone H4 lysine 20 dimethylation; H4K20me1, histone H4 lysine 20 monomethylation; DAC, 5-aza-2′-deoxycytidine; TMS1, target of methylation-mediated silencing 1.
tion and posttranslational histone modifications are epigenetic modifications that have a major influence in dictating the local chromatin state. CpG-dense regions called CpG islands encompass most human gene promoters, and their unmethylated status relative to the remainder of the genome is thought to maintain promoter chromatin in a transcriptionally permissive state, whereas the methylation of CpG islands is associated with gene silencing in the context of genomic imprinting, X-chromosome inactivation, and tumor suppressor gene silencing in cancers. Histone modifications act in concert to mutually repress or inhibit transcription, with histone H3/H4 acetylation (H3/4Ac) and H3 lysine 4 methylation (H3K4me) generally associated with active transcription and histone H3 lysine 9 and 27 methylation (H3K9/27me) generally linked to gene repression (15–18). Nucleosome occupancy also affects promoter activity. Active promoters tend to be depleted of nucleosomes, which enhances the accessibility of DNA to sequence-specific transcription factors (19, 20). Genome-wide analyses have also revealed a relationship between the pattern of nucleosome occupancy upstream of gene promoters and transcriptional plasticity (21). Dynamically positioned nucleosomes directly upstream of the transcription start site were correlated with genes that had a higher capacity to alter their expression, whereas well-positioned nucleosomes located more distantly upstream of the transcription start site were associated with genes that had a lower ability to alter their expression.

There is also cross-talk between both Pol II transcriptional dynamics and chromatin architecture. For example, Pol II paused at promoters affects local chromatin structure and helps to maintain a permissive chromatin landscape at the promoter. The presence of paused Pol II can occlude nucleosomes and promote histone H3 lysine 4 trimethylation (H3K4me3), thereby maintaining promoter accessibility to transcription factors and the open chromatin structure (22). In addition, Pol II promoter occupancy correlates with a lack of DNA methylation and excludes remethylation of CpG island promoters after drug-induced demethylation, thereby preventing gene silencing (23, 24).

Recently, we identified a novel epigenetic switch involving H4K16Ac and H4K20me3 that controls the release of Pol II from promoter-proximal pausing and, ultimately, regulates gene expression. We found that the local deposition of H4K20me3 by suppressor of variegation 4–20 homolog 2 (SUV420H2) invokes Pol II pausing at select genes by inhibiting the recruitment of the male-specific lethal (MSL) complex. This prevents the acetylation of H4K16 by human males absent on the first (hMOF) in the MSL complex, which is necessary for the association of BRD4 and pTEFb and the release of Pol II into productive elongation. Diminished SUV420H2 and H4K20me3 levels allow for the reassociation of MSL with chromatin, subsequent H4K16Ac, and release of Pol II into active elongation (25). However, the precise mechanism that regulates the local balance between these marks and the pausing process is currently unknown.

In this study, we show that the histone H4K20 monomethyltransferase PR-SET7/SETD8 plays a key role in modulating Pol II pausing through the local regulation of both H4K16Ac and H4K20me3. We found that PR-SET7-mediated H4K20me1 is necessary for the release of Pol II into active elongation, and it does so by promoting the local recruitment of the MSL complex and acetylation of H4K16. In the absence of PR-SET7, MSL recruitment and H4K16Ac levels are locally down-regulated, Pol II pausing is induced, and gene expression is down-regulated. Interestingly, H4K20me1 by PRSET7 also imposes Pol II pausing by serving as a substrate for SUV420H2-mediated H4K20me3. Down-regulation of PR-SET7 results in the local depletion of H4K20me2/3 at genes controlled by H4K20me3-mediated pause but does not result in relief of paused Pol II because of an inability to recruit MSL in the absence of H4K20me1. Taken together, these studies indicate that PRSET7-mediated H4K20me1 can function in both gene activation and repression and points to its central role in Pol II promoter-proximal pausing.

**EXPERIMENTAL PROCEDURES**

**Cell Lines**—MCF7 and MDA-MB231 are breast cancer cell lines that were obtained from the ATCC. The cells were maintained in DMEM with 2 mM glutamine and 10% fetal bovine serum. When required, cells were selected in 1.0 μg/ml puromycin and 0.5 μM 5-aza-2′-deoxycytidine (DAC).

**RNAi and DAC Treatment**—Retroviral pSUPERIOR retro. puro vectors were obtained from Judd Rice (26). For transfection with the retroviral vectors, 2 × 10⁶ cells were plated on 15-cm plates. The next day, cells were transfected with either an empty pSUPERIOR retro.puro vector or a pSUPERIOR retro. puro vector containing shRNA against PR-SET7 using FuGENE (Roche) according to the instructions of the manufacturer. Twenty-four hours later, the transfection medium was removed, and cells were incubated in medium containing puromycin with or without DAC. Cells were replenished with fresh medium containing puromycin (and DAC when required) every other day. Cells were maintained in selection for 4 days and then harvested for subsequent assays.

For siRNA transfaction, 1 × 10⁶ cells were plated per 10-cm plates. The next day, cells were transfected with 100 nm scrambled siRNA or siGENOME siRNA against SUV420H2 (Thermo Scientific, catalog no. D-018622-23-0005) using Oligofectamine (Invitrogen) according to the instructions of the manufacturer. Twenty-four hours after transfection, cells were replenished with fresh medium containing puromycin with or without DAC. Medium was exchanged every other day. Cells were incubated in selective medium for 4 days and, subsequently, harvested for further assays.

**Western Blot Analysis**—Protein lysates prepared by radioimmunoprecipitation assay buffer extraction (25) were resolved by SDS-PAGE electrophoresis and transferred onto a PVDF membrane. The membrane was immunoblotted with the following primary antibodies: PR-SET7 (Millipore, catalog no. 06-1304), TMS1 (ProteinTech, catalog no. 10500-1-AP), JUND (Santa Cruz Biotechnology, catalog no. 74), GAPDH (Abcam, catalog no. 8245), hMOF (a gift from Edwin Smith, Emory University), and hMSL3 (Abnova, catalog no. H00010943-B01). Horseradish peroxidase-conjugated goat anti-rabbit (Thermo Scientific, catalog no. 31460) or goat anti-mouse (Thermo Scientific, catalog no. PI31430) secondary antibodies were used.
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and immunocomplexes were detected with chemiluminescence reagent (Thermo Scientific).

Quantitative Reverse Transcriptase PCR—Total RNA was prepared from cells using the RNeasy mini kit (Qiagen). RNA (3 μg) was DNase I-digested and reverse-transcribed using random hexamer primers and Moloney murine leukemia virus reverse transcriptase as described previously (27). The cDNA was amplified with primers specific for PR-SET7, TMS1, JUND or 18 S rRNA (internal control) using IQ SYBR Green Supermix (Bio-Rad) in real-time PCR. Starting RNA quantities were determined relative to a common standard curve generated with MCF7 cDNA. Primer sequences are available upon request.

ChIP and real-time PCR—ChIP using antibodies directed against histone H4 and histone H4 modifications were done as outlined in the acetyl-histone H3 immunoprecipitation assay kit from Millipore (catalog no. 17-229); SUV420H2, hMOF, hMSL1, and PR-SET7 ChIPs were carried out as described in the acetyl-histone H3 immunoprecipitation assay kit, with the exception that 1 × 10⁶ cells were resuspended in 100 μl of lysis buffer and then diluted 10-fold in 0.5× dilution buffer before immunoprecipitation. ChIPs with Pol II antibodies were done exactly as described previously (25). Before immunoprecipitation of chromatin from each treatment condition, a portion of the sonicated samples was removed for input DNA and to determine sonication efficiency. DNA immunoprecipitated from the ChIP assays was analyzed by real-time PCR using IQ SYBR Green Supermix and primer sets that amplify sequences from the ChIP assays was analyzed by real-time PCR using IQ SYBR Green Supermix and primer sets that amplify sequences upon request. Starting amounts of precipitated DNA were quantified relative to a standard curve generated with MCF7 genomic DNA. Antibodies used for ChIP were as follows: IgG (Santa Cruz Biotechnology, catalog no. 2027); pan-histone H4 (Millipore, catalog no. 05-858), H4K16Ac (Millipore, catalog no. 07-329), H4K20me1 (Millipore, catalog no. CS200569), H4K20me2 (Active Motif, catalog no. 39173), H4K20me3 (Abcam, catalog no. 9053), PR-SET7, SUV420H2 (Abcam, catalog no. 91224), hMOF, hMSL1 (a gift from Edwin Smith, Emory University), total Pol II (Santa Cruz Biotechnology, catalog no. 9001X), Pol II phosphorylated at serine 5 (Abcam, catalog no. 5131), and Pol II phosphorylated at serine 2 (Abcam, catalog no. 5095).

Peptide Pulldown Assay—C-terminal biotinylated peptides representing amino acids 11–27 of histone H4 that were unmodified or mono-, di-, or trimethylated at Lys-20 were synthesized by Epicypher, Inc. (The Woodlands, TX). Streptavidin-Sepharose beads (10 μl) (GE Healthcare) were saturated with peptide by incubation with 4 μg of peptide in 100 μl of binding buffer (150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 0.5 mM DTT, 0.2% Nonidet P-40, and protease inhibitors) for 2 h at 4 °C. MCF7 nuclear extracts were prepared using a standard high salt extraction protocol (28), and the salt concentration was adjusted to 150 mM NaCl by dilution with NaCl-free binding buffer. For each pulldown, 400 μg of nuclear extract was incubated with 10 μl of peptide-saturated beads or beads alone (control) in 600 μl of total volume in binding buffer overnight at 4 °C. Peptide complexes were washed three times in binding buffer, resuspended in 2× SDS buffer, and subjected to Western blot analysis as described above.

RESULTS

PR-SET7-mediated H4K20me1 Promotes the Release of Pol II into Productive Elongation by Allowing for the Association of the MSL Complex with Chromatin—To understand the mechanisms that govern the local regulation of Pol II promoter-proximal pausing, we asked whether PR-SET7, a histone methyltransferase that specifically monomethylates H4K20, plays a role in this process. For these studies, we examined the TMS1 and JUND genes, both of which have been shown previously to be controlled by hMFO-dependent relief from Pol II pausing in MCF7 cells. In contrast, the promoters of both genes are negatively regulated by H4K20me3-induced pausing in MDA-MB231 cells, and the TMS1 gene is further silenced by hypermethylation of the Cpg island DNA (25).

We first examined the effect of PR-SET7 down-regulation on the TMS1 and JUND genes in MCF7 cells. Both PR-SET7 and the H4K20me1 mark were present across the TMS1 and JUND loci in MCF7 cells (Fig. 1, A and D). Upon PR-SET7 depletion, PR-SET7 and H4K20me1 levels were decreased across both loci (Fig. 1D). Interestingly, JUND and TMS1 expression was reduced in the absence of PR-SET7, both at the protein and mRNA level (Fig. 1, B and C). These results support the role established previously of PR-SET7 as a H4K20 monomethylase and indicate a positive regulatory role for PR-SET7 and H4K20me1 in TMS1 and JUND expression.

We next determined the impact of PR-SET7 down-regulation on Pol II pausing dynamics. Both the TMS1 and JUND loci were associated with Pol II in MCF7 cells, with the initiated form of Pol II (phosphorylated at serine 5, Pol II (S5P)) concentrated around the transcription start site and the elongating form of Pol II (phosphorylated at serine 2, Pol II (S2P)) localized throughout the gene body (Fig. 1D). Upon PR-SET7 down-regulation, Pol II at both loci became paused, as evident by the shift of total Pol II (Pol II (T)) from along the gene body to a peak around the transcription start site (Fig. 1D). Consistent with this interpretation, Pol II (S5P) was accumulated at the transcription start site, whereas Pol II (S2P) was depleted along the gene body in the absence of PR-SET7 (Fig. 1D). Thus, PR-SET7 and/or its product H4K20me1 promote Pol II elongation.

Acetylation of H4K16 by hMFO and the MSL complex mediates the recruitment of BRD4 and pTEFb to chromatin and is responsible, in part, for the release of paused Pol II to active elongation at the TMS1 and JUND loci (25). In the absence of hMFO or other components of the MSL complex (hMSL1), Pol II pauses downstream of the gene promoter much in the same way as observed when PR-SET7 is silenced (Ref. 25 and Fig. 1D). We therefore determined the effect of PR-SET7 down-regulation on H4K16Ac and the recruitment of the MSL complex. Although H4K16Ac, and the MSL components hMFO and hMSL1, were associated with the TMS1 and JUND loci in the presence of PR-SET7, their levels were decreased in the absence of PR-SET7 (Fig. 2A). Like TMS1, the ESR1 and CDH1 loci are expressed in MCF7 cells and epigenetically silenced by CpG hypermethylation in MDA-MB231 cells, but, unlike TMS1, they are not regulated by H4K16Ac-mediated release from
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**A**

- **TMS1**
  - bp: -2000 to 2500
  - CpG Island
  - Segments I, II, III

- **junD**
  - bp: -1000 to 2000
  - CpG Island

**B**

- **shPR-SET7:**
  - **Control** shPR-SET7
  - **+** shPR-SET7
  - **PR-SET7**
  - **TMS1**
  - **junD**
  - **GAPDH**

**C**

- **Fold Expression**
  - **PR-SET7**
  - **TMS1**
  - **junD**

**D**

- **IgG**
  - Control shPR-SET7
  - **FR-SET7**
  - **Pol (T)**
  - **Pol (S5P)**
  - **Pol (S2P)**
  - **Relative H4**
  - **Relative H4K20me1**

- **TMS1 Locus**
  - **junD Locus**
pausing (25). Interestingly, neither H4K20me1 nor PR-SET7 were observed at ESR1 and CDH1 (data not shown). These results indicate that the local recruitment of the MSL complex and H4K16Ac is dependent on PR-SET7 and further suggest that these events are locally regulated in a gene-specific manner.

The dependence of local MSL recruitment and H4K16Ac on PR-SET7 could be due to a direct requirement for H4K20me1 itself or potentially other PR-SET7 targets. To test this, we performed pulldown assays with histone H4 peptides unmodified or methylated at Lys-20 (Fig. 2B).

FIGURE 2. The MSL complex binds H4K20me1 in vitro, and PR-SET7-mediated H4K20me1 allows for MSL recruitment and H4K16 acetylation. A, chromatin from cells treated as in Fig. 1 was immunoprecipitated with the indicated antibodies. DNA isolated from the immunoprecipitation was subjected to real-time PCR with primer sets that span the TMS1 and JUND loci (Fig. 1A) and is expressed as a percent of input DNA or fraction of DNA immunoprecipitated with the histone H4 antibody. Shown is the mean ± S.D. of triplicate determinants from a representative experiment. Each experiment was carried out twice with reproducible results. B, MCF7 nuclear extracts were incubated with streptavidin-Sepharose beads (−) or beads conjugated to C-terminal biotinylated peptides representing amino acids 11–27 of histone H4 (H4K20 peptides) that were unmodified (me0) or mono-, di-, or trimethylated at lysine 20 (me1–3). Protein-bead complexes were isolated by centrifugation, solubilized in 2 × SDS buffer, loaded on SDS-PAGE gels, and analyzed by Western blot analysis with antibodies against hMOF or hMSL3. Ten percent of input nuclear extract was also loaded.

The dependence of local MSL recruitment and H4K16Ac on PR-SET7 could be due to a direct requirement for H4K20me1 itself or potentially other PR-SET7 targets. To test this, we performed pulldown assays with histone H4 peptides unmodified or methylated at Lys-20 (Fig. 2B). We found that components of the MSL complex (hMOF and hMSL3) bind H4K20me1 in vitro, and PR-SET7-mediated H4K20me1 allows for MSL recruitment and H4K16 acetylation.
hMSL3) were selectively precipitated from MCF7 nuclear extracts by H4 peptides monomethylated at Lys-20 (H4K20me1) but not the unmethylated (H4K20me0) or di- or trimethylated (H4K20me2/3) forms (Fig. 2B). These data suggest that recruitment of the MSL complex is specifically dependent on PR-SET7-mediated H4K20me1.

Taken together, our results support the hypothesis that PR-SET7-mediated H4K20me1 mediates productive elongation by promoting the recruitment of the MSL complex and subsequent H4K16 acetylation and, in doing so, allows for the release of Pol II from promoter-proximal pausing. The MSL complex is unable to associate with chromatin in the absence of PR-SET7, and the lack of H4K16 acetylation results in paused Pol II and subsequent gene repression.

PR-SET7-mediated H4K20me1 Is Necessary for H4K20me3, but Depletion of H4K20me3 upon PR-SET7 Inhibition Is Insufficient for Pause Release—Recent studies suggest that mono-, di-, and trimethylation of H4K20 occurs in a progressive manner (29–31). Thus, we determined the effect of PR-SET7 and H4K20me1 down-regulation on higher H4K20me forms. For these studies, we used the MDA-MB231 cell line, in which both TMS1 and JUND are transcriptionally repressed because of H4K20me3-induced pausing (25). ChIP analysis showed that PR-SET7 was localized throughout TMS1 and JUND in MDA-MB231 cells, albeit at levels significantly lower than that observed in MCF7 cells (compare Figs. 1D and 3), and negligible levels of H4K20me1 were observed (Fig. 3). H4K20me3 and SUV420H2 were enriched around the transcription start sites of both genes in MDA-MB231 cells (Fig. 3). Upon PR-SET7 down-regulation, H4K20me3 levels at both genes were depleted (Fig. 3). H4K20me2 was also present at these loci and was decreased considerably in the absence of PR-SET7 (Fig. 3). Interestingly, down-regulation of PR-SET7 had little effect on SUV420H2 localization, which remained enriched around the transcription start site even in the absence of PR-SET7 and H4K20me1 (Fig. 3). These data suggest that PR-SET7-mediated H4K20me1 is necessary for the local conversion to H4K20me2/3 and that its absence results in depletion of these higher modified forms.

FIGURE 3. The PR-SET7 enzyme is required for the di- and trimethylation of H4K20 but is not required for the association of SUV420H2 with chromatin. MB231 cells transfected with either the empty pSUPERIOR.retro.puro vector (Control, DAC) or a pSUPERIOR.retro.puro vector expressing shRNA against PR-SET7 (shPR-SET7, DAC + shPR-SET7) were grown under puromycin selection, with or without DAC, for 4 days. Cells were harvested and used for ChIP with the antibodies shown. DNA isolated from ChIP was quantified by real-time PCR with primers that span the TMS1 (1–9) or JUND (1–6) regions shown in Fig. 1A. Quantified DNA is shown as a percentage of input DNA or normalized to DNA isolated from histone H4 ChIP. The mean ± S.D. of triplicate determinants from a sample experiment is shown. Each experiment was performed twice and yielded similar results.
forms not through effects on the recruitment of SUV420H2 but, rather, through the local loss of their substrate, H4K20me1.

We demonstrated previously that H4K20me3-mediated gene repression at the TMS1 and JUND genes in MDA-MB231 cells can be relieved upon depletion of SUV420H2 and H4K20me3 by allowing for the reassociation of the MSL complex with chromatin and the local restoration of H4K16Ac. Thus, it is not the loss of MSL function that drives pausing at these loci in MDA-MB231 cells but, rather, the exclusion of the MSL complex recruitment by H4K20me3 (25). Because H4K20me3 levels at TMS1 and JUND were also diminished upon PR-SET7 silencing in MDA-MB231 cells (Fig. 3), we asked whether this was similarly associated with changes in gene expression and Pol II pausing dynamics. Given that TMS1 is aberrantly methylated at the CpG island and, consequently, devoid of Pol II in these cells, demethylation by DAC treatment was first necessary for the association of Pol II with the TMS1 promoter (Ref. 25 and Fig. 4). Despite significant H4K20me3 depletion, down-regulation of PR-SET7 had no effect on gene expression or Pol II pausing dynamics (Fig. 4). Pol II remained paused in its initiated form downstream of the gene promoter, and TMS1 and JUND gene expression remained unchanged (Fig. 4). Unlike SUV420H2 inhibition, PR-SET7 down-regulation did not allow for the reassociation of the MSL complex nor the recetylation of H4K16Ac at the TMS1 and JUND loci (Fig. 4C). Taken together, the data indicate that, although PR-SET7 is necessary for H4K20me2/3 and, thus, plays an important role in H4K20me3-driven Pol II promoter-proximal pausing, depletion of H4K20me3 in and of itself is not sufficient to alleviate Pol II pausing or the associated gene repression in the absence of H4K20me1 because of an inability to recruit the MSL complex.

Local H4K20me2 Levels Are Dependent on PR-SET7 but Independent of SUV420H2—We established previously that SUV420H2 mediates the local deposition of H4K20me3 at genes regulated by H4K20me3-associated Pol II pausing and that its depletion allows for the reestablishment of H4K16Ac (25). The above data indicate that PR-SET7, by mediating H4K20me1, is also necessary to maintain local H4K20me2 and H4K20me3 levels and is required for the restoration of local H4K16Ac (Fig. 4). However, whether H4K20me2 plays a role in the local balance between H4K16Ac and H4K20me3 has not been addressed. We therefore asked whether loss of SUV420H2 and H4K20me3 had an impact on H4K20me2 in MDA-MB231 cells. We found that although SUV420H2 RNAi led to a drastic decrease in H4K20me3 levels and a slight increase in H4K20me1 levels at the TMS1 and JUND loci, consistent with the role of H4K20me1 as a local substrate for conversion to H4K20me3, there was little impact on local levels of H4K20me2 (Fig. 5). The data indicate that H4K20me2 at these genes is independent of SUV420H2 and, further, that the local conversion of H4K20me1 to H4K20me2 or H4K20me3 may be regulated independently.

**DISCUSSION**

Here we established that PR-SET7 and H4K20me1 play two important roles in the regulation of Pol II promoter-proximal pausing. We show that PR-SET7-mediated H4K20me1 is required for the association of the MSL complex with chromatin and subsequent H4K16 acetylation, leading to the release of paused Pol II into productive elongation and up-regulation of full-length transcription. PR-SET7-mediated H4K20me1 is also required for the di- and trimethylation of H4K20, and the local conversion of H4K20me1 to H4K20me3 promotes Pol II pausing by blocking MSL recruitment and H4K16Ac, resulting in gene repression. In the absence of PR-SET7, H4K20me3 is down-regulated at H4K20me3-marked genes, but H4K16Ac levels are not reestablished, and Pol II remains paused because of the inability to recruit the MSL complex in the absence of H4K20me1. This is the first study to demonstrate a role for PR-SET7-mediated H4K20me1 in pausing and to outline a novel epigenetics-based mechanism for the regulation of Pol II pausing involving the local regulation of histone H4 modifications.

The Drosophila MSL complex mediates dosage compensation by catalyzing the 2-fold up-regulation of genes on the single male X chromosome and is thought to bind chromatin using a two-step mechanism where the complex is initially targeted via sequence-specific high-affinity sites and then spreads to lower-affinity sites within active X genes (32–36). Binding of the MSL3 subunit to histone H3 lysine 36 trimethylation (H3K36me3) via its chromodomains and association of the MOF chromodomain directly to DNA are two mechanisms implicated in MSL spreading on the male X chromosome (36–38). In comparison, very little is known about the recruitment mechanism of the human MSL complex, which mediates autosomal gene up-regulation. Recently, the isolated chromodomains of Drosophila and human recombinant MSL3 proteins have been shown to preferentially bind H4K20me1 and H4K20me2 peptides in vitro (40, 41). This study provides biological evidence that PR-SET7-mediated H4K20me1, but not H4K20me2, specifically required for the targeting of the MSL complex in human cells. We found that hMOF and the MSL complex components (hMSL3) were precipitated selectively from cell extracts by H4K20me1 peptides but not H4K20me2 (or H4K20me0 or H4K20me3). Furthermore, we found that there is little correlation between MSL recruitment and H4K20me2 levels. MSL is localized to the TMS1 locus despite the absence of H4K20me2 in MCF7 cells (data not shown), and although depletion of H4K20me3 in MDA-MB231 cells allows for the reassociation of MSL, this is not only dependent on PRSET7 and H4K20me1 but independent of H4K20me2 levels, which remain unchanged after SUV420H2 depletion. Thus, our evidence suggests that the role of H4K20 methylation in MSL recruitment and exclusion from chromatin is quite specific for H4K20me1 and H4K20me3, respectively. Consistent with a role for PR-SET7 and H4K20me1 in MSL targeting, a recent study in flies demonstrated that loss of PR-SET7 leads to perturbations in MSL localization on the male X chromosome (42).

Other modifications have also been implicated in MSL recruitment in humans. hMOF is recruited to the FOS1 gene via the 14-3-3 adaptor protein, which binds chromatin through H3 serine 10 phosphorylation, but whether hMOF is in the MSL complex in this context is not known (39). The association of MSL3 with H4K20me1 and association of MOF with 14-3-3 may serve as two independent mechanisms for MSL recruitment functioning in a gene-specific and/or cell type-specific.
manner. Alternatively, MSL recruitment may involve more than one MSL component and, thus, require both (MSL3/H4K20me1 and MOF/14-3-3) for efficient anchoring to chromatin and subsequent acetylation by MOF.

Previous studies have yielded conflicting results regarding the relationship between H4K20me1 and H4K16Ac. Histone H4 tail peptides containing H4K16Ac cannot be monomethylated at H4K20, and H4K20me1 and H4K16Ac levels are

FIGURE 4. Down-regulation of PR-SET7 does not lead to Pol II pause release and gene up-regulation because MSL and H4K16Ac are not reestablished in the absence of PR-SET7. A, MB231 cells treated as in Fig. 3 were used to determine protein expression levels of PR-SET7, TMS1, JUND, and GAPDH (loading control) as described in Fig. 1B. B, MB231 cells treated as in Fig. 3 were used to determine RNA expression levels of PR-SET7, TMS1, and JUND as indicated in Fig. 1C. C, ChIP with the indicated antibodies, followed by real-time PCR of immunoprecipitated DNA with TMS1 or JUND genomic primers, was conducted with MB231 cells used in A and B. DNA quantity is expressed as a percentage of input DNA or a fraction of histone H4 ChIP. Each experiment was carried out twice with reproducible results, and shown are the means ± S.D. of triplicate determinants from a representative experiment.
inversely correlated on *Caenorhabditis elegans* hermaphrodite X chromosomes, suggesting that the two marks are mutually exclusive (43, 44). However, mass spectrometry studies have identified histone H4 tails containing both H4K20me1 and H4K16Ac, and genome-wide studies indicate that the two marks have similar distributions across genes, suggesting that the two marks can coexist (29, 45, 46). We found that H4K20me1 and H4K16Ac have similar distributions across the TMS1 and JUND loci, occupying both the promoter and gene body regions, suggesting that, although H4K20me1 and H4K16Ac may be mutually exclusive under certain conditions, they can coexist at individual genes. Furthermore, our data indicate that the coexistence reflects the requirement of H4K16 acetylation on prior H4K20me1 in the regulation of Pol II pause release and gene transcription. No doubt, the relationship between H4K20me1 and H4K16Ac is complex, and whether they coexist or are mutually exclusive is likely dependent on influences from and interactions with other chromatin pathways and the specific functional context.

Likewise, the role of PR-SET7 and H4K20me1 in the regulation of gene transcription has been controversial. A repressive role has been proposed for PR-SET7. This stems from studies showing that PR-SET7-mediated H4K20me1 is necessary to maintain chromatin compaction and to recruit the transcriptional repressor L3MBTL1 to chromatin (47–49). Furthermore, down-regulation of PR-SET7 leads to up-regulation of H4K20me1-associated genes (50). However, other studies argue against the role of PR-SET7-mediated H4K20me1 in gene repression and, instead, suggest that it is involved in gene activation. For instance, genome-wide ChIP analyses and studies at individual loci have demonstrated that H4K20me1 is enriched on active genes and that the levels of H4K20me1 correlate with expression levels (45, 46, 51). Also, the activation of estrogen- and wnt3a-responsive genes is associated with an increase in local H4K20me1 levels (52, 53). Here we have shown that PR-SET7 and H4K20me1 play a dual role in gene regulation in both the promotion and repression of transcription. At individual genes, PR-SET7-mediated H4K20me1 facilitates transcription by promoting the release of Pol II from promoter-proximal pausing. At the same genes, PR-SET7 also plays a role in repression as H4K20me1 acts as a substrate for local conversion to H4K20me3, which enforces Pol II pausing and gene repression. Indeed, we find that the locus-specific accumulation of H4K20me3 at individual genes is dependent on PR-SET7-mediated H4K20me1. Similarly, mass spectrometry studies showed that H4K20me1 serves as a substrate for H4K20me2/3 and that down-regulation of PR-SET7 leads to a loss of H4K20me1 as well as H4K20me3 both globally and at imprinted control regions (29–31, 54, 55). Thus, the conflicting interpretations regarding the role of PR-SET7 and H4K20me1
Role of PR-SET7-mediated H4K20me1 in Pol II Pausing

![Diagram showing active elongation and pausing of Pol II](image)

**FIGURE 6. Dual functions of H4K20me1 in the local regulation of Pol II pausing.** H4K20me1 by PR-SET7 allows for active elongation at genes regulated by Pol II promoter-proximal pausing via the recruitment of the MSL complex. The MSL complex acetylates H4K16, which, in turn, allows for the association of the BRD4/pTEFb complex, subsequent phosphorylation of serine 2 in the C-terminal domain of the initiated and paused Pol II, and, ultimately, pause release. Alternatively, PR-SET7-dependent H4K20me1 also promotes Pol II pausing at the same genes because it serves as a template for SUV420H2-mediated (SUV) H4K20me3. The presence of H4K20me3 inhibits MSL recruitment, acetylation of H4K16Ac, and, consequently, active elongation.

in gene regulation may arise, in part, from an inability to attribute a particular phenotype to an effect on H4K20me1 versus H4K20me3.

Apart from transcription, PR-SET7 is also involved in other cellular processes that contribute to the overall maintenance of genomic integrity. PR-SET7 and H4K20me1 play a role in the DNA damage response and repair pathways in part through the recruitment of 53BP1 to sites of dsDNA breaks (48, 56, 57). PR-SET7 and H4K20me1 also function as positive regulators of replication origin licensing by affecting the chromatin structure at replication origins and through the recruitment of the origin recognition complex (55, 58–61). The cellular functions of PR-SET7 and H4K20me1 are coupled to and dependent on their tight regulation during the cell cycle, with their levels highest during G2/M and early G1 phase and lowest during S phase (56), controlled via ubiquitination-mediated degradation of PR-SET7 by the E3 ubiquitin ligase complex CRL4(CDT2) during S phase and stabilization during G2/M and G1 phase by CRL1(FBXO11)-mediated degradation of CRL4(CDT2) (56, 62–65). Like other PR-SET7 functions, Pol II pausing may also be linked to the cell cycle. Indeed, it has been suggested that pausing may play a “bookmarking” function specifically during mitosis, when PR-SET7 and H4K20me1 levels are high, to “remember” prior gene states (66). The role of H4K20me1 in MSL recruitment versus conversion to H4K20me3 may also underlie the regulation of other cellular processes by PR-SET7. For example, hMOF-mediated H4K16Ac is implicated in the DNA damage response and 53BP1 recruitment, raising the question whether the role of PR-SET7 in these processes is mediated through an effect on MSL recruitment (67). Likewise, it has been shown that the function of PR-SET7-mediated H4K20me1 in replication origin firing via ORC recruitment and cell cycle progression is dependent on its conversion to H4K20me3 (58).

Our research demonstrates that the balance between H4K16Ac and H4K20me3 acts as a means of local fine-tuning of transcriptional output by regulating the Pol II pausing checkpoint. Although PR-SET7 regulates both H4K16Ac and H4K20me3, the mechanism(s) that dictate whether its product H4K20me1 ultimately results in MSL recruitment and subsequent H4K16Ac or becomes a substrate for conversion to H4K20me3 is unknown. In this regard, it is the targeting of SUV420H2 to chromatin (which, we show here, is independent of PR-SET7) that likely plays a critical role in establishing the transcriptional state of genes regulated by H4K16Ac–H4K20me3-mediated Pol II pausing. One can envision that local binding of SUV420H2 to chromatin, either directly or indirectly, pushes the balance toward the conversion of H4K20me1 to H4K20me3, thus preventing the association of the MSL complex and resulting in Pol II pausing. Consistent with this, we find that TMS1 and JUND are associated with negligible levels of H4K20me1 in MDA-MB231 cells despite the presence of some PR-SET7 (Fig. 3). A plausible explanation is that, in these cells, H4K20me1 is mostly converted to the H4K20me2 and H4K20me3 forms. In support of this interpretation, down-regulation of SUV420H2 and depletion of H4K20me3 led to a slight increase in H4K20me1 (Fig. 5). Alternatively, in the absence of SUV420H2, H4K20me1 is free to associate with the MSL complex, which pushes the balance toward H4K16Ac and release from pausing.

At present, the cell type-specific mechanism by which SUV420H2 is targeted to particular genes remains unknown. SUV420H2 is targeted to heterochromatin via the HP1 protein, which binds chromatin through H3K9me3, but whether this is also the mechanism that directs SUV420H2 to individual genes in euchromatin is not clear (68). Although H4K20me1 has a broad distribution across individual genes, H4K20me3 has a more limited distribution, peaking ~1 kb upstream of the transcription start site (Ref. 50 and Fig. 3). Thus, one would expect that factor(s) that have a similar genomic distribution might serve as good candidates for SUV420H2 targeting.

Our data also provide new insight into the local regulation of H4K20 methylation. Previous studies using *Suv420h1*- or *Suv420h2*-null mouse cells have suggested that H4K20me2, the most abundant form of H4K20me in cells, is mostly mediated by Suv420h1 (30). Other studies have suggested a sequential model for conversion from H4K20me1 to H4K20me2 to H4K20me3 (29, 31). We find that, although all three H4K20me forms are dependent on PR-SET7, only H4K20me3 is dependent on SUV420H2. In MB231 cells, down-regulation of SUV420H2 results in severe depletion of H4K20me3 and an associated increase in H4K20me1, whereas H4K20me2 levels remain unchanged. Although we cannot rule out an effect on an as yet unknown H4K20me2/3 demethylase, these data are most consistent with the interpretation that, although PRSET7-mediated H4K20me1 can serve as a local substrate for conversion
to both H4K20me2 and H4K20me3, the two pathways appear to be regulated independently. Importantly, only the latter is dependent on SUV420H2. Maintenance of H4K20me2 is not dependent on SUV420H2, nor does its correlate with Pol II pausing (above), thus establishing an important and specific role for SUV420H2 targeting and H4K20me3 in the regulation of Pol II pausing.

Interestingly, a number of factors that regulate Pol II pausing are themselves misregulated in human cancers. For example, c-Myc is frequently amplified in a large fraction of human tumors and acts as a transcriptional "amplifier" to directly recruit pTEFb to its gene targets (12, 69, 70). BRD4 is translocated in NUT midline carcinomas and amplified in breast cancers (71). Ectopic expression of BRD4 suppresses breast cancer tumor growth and metastasis in vivo, and a BRD4-activated gene signature predicts a better prognosis in breast cancer patients (72). BRD4 is a target of therapeutic agents in cancers (71). Ectopic expression of BRD4 suppresses breast cancer recruitment domain protein, is a target of methylation-induced gene silencing (73). In addition, the histone H4 modifications (H4K16Ac and H4K20me3) and their respective modifying enzymes (hMOF and SUV420H2) are down-regulated in human cancers and have been linked to a poor prognosis in medulloblastomas and lung cancers (74–76). PR-SET7 is also overexpressed in many cancers, such as pancreatic cancer, leukemia, and hepatocellular carcinoma, and overexpression of PR-SET7 is associated with an increased invasive potential of breast cancer cells and metastatic potential in primary breast tumors (77, 78). Given the potential involvement of altered Pol II pausing dynamics in tumorigenesis, further understanding of the precise mechanisms that govern Pol II pausing is important in the development of novel therapeutic targets to inhibit and treat human cancers.

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