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Journal Title: Molecular and Cellular Biology
Volume: Volume 25, Number 21
Publisher: American Society for Microbiology | 2005-11-01, Pages 9175-9188
Type of Work: Article | Final Publisher PDF
Publisher DOI: 10.1128/MCB.25.21.9175-9188.2005
Permanent URL: https://pid.emory.edu/ark:/25593/s66dg

Final published version: http://dx.doi.org/10.1128/MCB.25.21.9175-9188.2005

MOLECULAR AND CELLULAR BIOLOGY, Jan. 2006, p. 387 Vol. 26, No. 1
0270-7306/06/$08.00+0 doi:10.1128/MCB.26.1.387.2006

ERRATUM

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Volume 25, no. 21, p. 9175–9188. Page 9180, Fig. 3A: The middle lane should be labeled hMOF-TAP, and the right lane should be labeled hMSL3L1-TAP.

MOLECULAR AND CELLULAR BIOLOGY, Nov. 2005, p. 9175–9188 Vol. 25, No. 21
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A Human Protein Complex Homologous to the *Drosophila* MSL Complex Is Responsible for the Majority of Histone H4 Acetylation at Lysine 16

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Received 23 April 2005/Returned for modification 20 May 2005/Accepted 26 July 2005

We describe a stable, multisubunit human histone acetyltransferase complex (hMSL) that contains homologs of the *Drosophila* dosage compensation proteins MOF, MSL1, MSL2, and MSL3. This complex shows strong specificity for histone H4 lysine 16 in chromatin in vitro, and RNA interference-mediated knockdown experiments reveal that it is responsible for the majority of H4 acetylation at lysine 16 in the cell. We also find that hMOF is a component of additional complexes, forming associations with host cell factor 1 and a protein distantly related to MSL1 (hMSL1v1). We find two versions of hMSL3 in the hMSL complex that differ by the presence of the chromodomain. Lastly, we find that reduction in the levels of hMSLs and acetylation of H4 at lysine 16 are correlated with reduced transcription of some genes and with a G_2/M cell cycle arrest. This is of particular interest given the recent correlation of global loss of acetylation of lysine 16 in histone H4 with tumorigenesis.

The dynamic regulation of chromatin structure can be brought about by a complex series of posttranslational modifications to histones, particularly on the amino-terminal histone tails (33, 48). Among the lysines that can be acetylated, lysine 16 of histone H4 appears to be uniquely targeted in a number of organisms. Studies of telomeric silencing in *Saccharomyces cerevisiae* have shown that lysine 16-specific acetyltransferase and deacetylase activities determine the boundaries of silenced chromatin (32, 59). Furthermore, chromatin immunoprecipitation and site-directed mutagenesis in budding yeast clearly indicate that acetylation of H4 lysine 16 has an independent specific function in relation to gene transcription when compared to other histone acetylation sites (16, 34). In *Drosophila*, lysine 16 acetylation is targeted to the hypertranscribed male X chromosome in the process of dosage compensation (8, 35). Biochemical analysis of bulk histones in mammalian cells has indicated that most of the monoacetylated H4 is acetylated at lysine 16, as is most of the di- and triacetylated forms, leading to the suggestion that this is the first acetylation mark on H4 or possibly the last to be taken off (61, 69). One rationale for a special role for lysine 16 acetylation is that it is the only known site of acetylation in the basic patch of H4, a region from amino acids 16 to 20 that is implicated in the formation of higher-order chromatin structure (17). Recently, loss of acetylation at lysine 16 of histone H4 has been identified as a common hallmark of human cancer (22).

In *Drosophila*, dosage compensation—the equalization of X-linked gene products in males and females—is achieved by enhancing the transcriptional level of X-linked genes in males. Five genes involved in this process were identified due to male-specific lethality when mutated (4, 5, 26). These genes are male-specific lethal 1 (*msl1*), *msl2*, *msl3*, maleless (*mle*), and males-absent on the first (*mof*). All five gene products form the MSL complex, which harbors histone acetyltransferase (HAT) activity with specificity toward histone H4 lysine 16 (56). The MOF subunit responsible for this activity is a member of the MYST (named for yeast and human members MOZ, YBF2, SAS2, and Tip60) HAT family (62). The MSL complex associates at hundreds of sites along the X chromosome in male somatic cells and results in the hyperacetylation of lysine 16 of histone H4 (8, 35, 56). In addition to the MSL proteins, the complex contains one of two noncoding RNAs, roX1 and roX2 (3, 39), that are essential for its formation and proper targeting (reviewed in reference 30). A tandem kinase (JIL-1) is enriched on the male X chromosome but, in contrast to the MSL proteins and roX RNAs, JIL-1 is also found on the X chromosomes of females as well as on the autosomes of both sexes, where it is responsible for phosphorylation of histone H3 on serine 10 (29, 63).

While the roX RNAs are not recognizable by standard homology searches even in closely related species of *Drosophila* (47), homologs of the MSL proteins can be readily identified in other species (37, 45). Here, we report the existence of a human complex (hMSL) that includes at least four of the human homologs of the *Drosophila* MSLs and exhibits the same specificity for acetylating lysine 16 on histone H4. In contrast to the *Drosophila* complex, the hMSL complex targets all chromosomes in vivo and is responsible for the majority of H4-lysine 16 acetylation in the cells.

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MATERIALS AND METHODS

Antisera. Rabbit antisera recognizing hMOF and hMSL3L1 and guinea pig antiserum recognizing hMSL1 were raised in animals that were immunized with the peptides CLKWAPPKHKQVKLSKK for hMOF, NPSTPQSTESQPTTGE PAT(G/C) for hMSL3, and KKGIQESEPEVTS(G/C) for hMSL1 conjugated to Imject Activated Supercarrier (Pierce). Later boosts were done with the same peptide conjugated to keyhole limpet hemocyanin. Antisera were affinity purified with peptides coupled to Sulfolink resin (Pierce) according to the manufacturer's instructions. Rabbit antisera recognizing hMSL1 were obtained with a bacterially expressed His-tagged polypeptide corresponding to amino acids 336 to 614 of hMSL1 (accession number XP_496217). Monoclonal anti-HA.11 (where HA is hemagglutinin) and polyclonal anti-TAP were obtained from Covance and Open Biosystem, respectively. Anti-H4Ac16 (histone H4 acetylated at lysine 16) was obtained from Serotec, while anti-H4Ac8 and anti-H4Ac12 were from Upstate Biotechnology (Lake Placid, NY). Anti-H3MeK36 (histone H3 dimethylated at lysine 36) antibody was provided by David Allis and was described previously (58). Rabbit anti-HCF-1^N (where HCF is host cell factor) antibody was obtained from Winship Herr and was described previously (23).

Cell lines and cell culture. K562 cells were obtained from the American Type Culture Collection and grown in RPMI medium supplemented with 10% fetal bovine serum. HeLa cells were provided by Paul Wade (National Institute of Environmental Health Sciences), and Raji cells were obtained from Jeremy Boss (Emory University). HeLa S3 cells expressing the tetracycline-responsive transactivator were obtained from Clontech. K562 cells expressing hMSL2-HA were made by cloning hMSL2 cDNA (obtained from the Kazusa DNA Research Institute) into pUB vector (Invitrogen) modified with a single HA tag at the C terminus instead of the V5 epitope and six-His tag. Cells were transfected using Effectene (QIAGEN), and a stable cell line was selected with blasticidin at 4 μg/ml. For the TAP-tagged cell lines, hMOF cDNA was subcloned by PCR in the BamH1 site of the pRevTre-TAP retroviral vector previously described (20). hMSL3L1 cDNA was first cloned by PCR in the Kpn1/BamH1 sites of pcDNA3-FLAG. The FLAG-hMSL3L1 construct was then subcloned in the BamH1 site of pRevTre-TAP. TAP-tagged hMOF and hMSL3 were expressed from transduced HeLa cells grown in suspension in Joklik medium supplemented with 10% fetal bovine serum (tetracycline free) as previously described (20).

Coimmunoprecipitation and complex purification. Nuclear extracts were prepared from K562 cells as follows: 5×10^8 cells were pelleted, washed in phosphate-buffered saline (PBS), swollen in hypotonic buffer on ice (20 mM HEPES, pH 7.2, 0.3 M sucrose, 3 mM $MgCl_2$, 3 mM beta-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride), and homogenized with 20 strokes of a loose-fitting Dounce pestle. Homogenate was layered on a 0.65 M sucrose cushion and pelleted at $1,000 \times g$ for 5 min. Nuclei were resuspended in DNase 1 buffer and digested as previously described (44). DNase extracts were added to protein A agarose beads that were prebound with antibody. Immunoprecipitates were washed with immunoprecipitation wash buffer: 20 mM HEPES, pH 7.2, 10% glycerol, 0.35 M NaCl, 1 mM $MgCl_2$, 0.5 mM phenylmethylsulfonyl fluoride. For tandem affinity purification (TAP) of native complexes, nuclear extracts and fractionation on immunoglobulin G (IgG)-Sepharose and calmodulin resin were carried out according to the methods described previously (20, 38). Stable components of purified complexes were identified by silver staining, Western blotting, and tandem mass spectrometry as previously described (21).

Histone acetyltransferase assays. Histone acetyltransferase activity was assayed with ^{3}H-labeled acetyl coenzyme A (acetyl-CoA) and core histones or H1-depleted mono- and oligonucleosomes purified from HeLa cells (14). For sequencing of H4 acetylated by hMOF immunoprecipitates, reactions were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 13.75% gel and transferred to polyvinylidene difluoride, and the band was subjected to amino-terminal deblocking and Edman degradation as previously described (56). Recombinant histone H4 was a kind gift of Luc Gaudreau.

RT-PCR. RNA was isolated from K562, Raji, or HeLa cells with the RNeasy kit according to the manufacturer's instructions. Human tissue total RNA was purchased from Clontech. Reverse transcription-PCR (RT-PCR) was performed using 125 ng of total RNA in a 25-μl reaction mixture using QIAGEN's one-step SYBR Green RT-PCR kit. Forward and reverse primers, respectively, were as follows: GAPDH (glyceraldehyde-3-phosphate dehydrogenase), 5′-CCATGGG GAAGGTGAAGGTCGGAGTC-3′ and 5′-GGTGGTGCAGGCATTGCTGA TG-3′; hMSL3L1 isoform a, 5′-GAGCCTGACCCCACCAAG-3′ and 5′-TCCT CAGGCGAGCTACAGCT-3′; hMSL3L1 isoform c, 5′-GGCTGTCGGTCTAA AAGAGGAG-3′ and 5′-TCCTCAGGCGAGCTACAGCT-3′; and hMSL3L2, 5′-CCAGGCCGCAGTCTACAGA-3′ and 5′-TGGGCACACTGGTGGACAT-3′. Gene expression analysis of RNA interference (RNAi)-treated cells was measured with prevalidated TaqMan probes and primers from Applied Biosystems, Taqman One-step PCR Master Mix from Applied Biosystems, and a Bio-Rad iCycler.

RNAi. RNA duplexes targeting hMSL1 and hMOF were synthesized by Dharmacon. Duplexes targeting the following sequences were used: hMOF-1, UGC UGUACAGAAGAACUCA; hMOF-2, GCAGCCUGAGCGCAAGAUC; and hMSL1, AAGGCGGAUGCAGCUGGUA. Cells were transfected on coverslips for immunofluorescence or in six-well dishes for Western analysis. At 48 to 72 h after transfection, cells were processed for immunofluorescence, Western blotting, fluorescence-activated cell sorting (FACS) analysis, or TaqMan RT-PCR.

FACS. Cell-cycle analysis was carried out on 293T cells after transfection with hMOF (hMOF-1 and hMOF-2) and hMSL1 small interfering RNAs (siRNAs) against a luciferase control. At 72 h posttransfection, cells were washed once in PBS and fixed in 70% ethanol. The fixed cells were washed again once with PBS and treated with RNase A at 37°C for 30 min. Finally, the cells were stained with propidium iodide and incubated in the dark for 30 min before analysis. The samples were analyzed through flow cytometry using a Coulter EPICS XL-MCL. The proportion of cells in the different phases of the cell cycle were quantified using MultiCycle software.

RESULTS

Human homologs of the *Drosophila* MSL proteins physically interact with each other in vivo. Database searches revealed the existence of human orthologs of *Drosophila* proteins MSL1 and MSL2 (37). MSL1 orthologs have an amino-terminal coiled-coil region and a conserved C-terminal domain implicated in binding to hMOF and hMSL3 (37, 41, 54) (Fig. 1A). MSL2 orthologs are characterized by an amino-terminal RING finger domain and a novel C-terminal metal-binding motif (37, 70). The coiled-coil of MSL1 interacts with the RING finger of MSL2 (12, 54). The conservation of MSL1 and MSL2 in humans, where homologs of MOF and MSL3 were previously described (42, 49), suggests that an MSL-like complex exists in humans. One hallmark feature of *Drosophila* MOF is its specificity toward nucleosomal histone H4. When hMOF immunoprecipitates from the human erythroleukemia K562 cell nuclear extracts are assayed with HeLa mononucleosomes, histone H4 is specifically labeled (Fig. 1B). To determine if the other human MSL homologs associate with hMOF, we performed coimmunoprecipitation experiments using antibodies to hMSLs and a K562 cell line that expresses HA-tagged hMSL2. Antibodies to hMSL1, hMOF, and hMSL3L1, but not preimmune sera, can immunoprecipitate hMSL1 and hMSL2 as shown by Western analysis (Fig. 1C). hMSL immunoprecipitates frequently revealed two classes of hMSL3 proteins, "long" and "short" forms (Fig. 1D). To confirm that the smaller protein was related to hMSL3, the hMOF immunoprecipitates were subjected to peptide elution and a second immunoprecipitation with hMSL3 antibodies. Eluates from this tandem immunoprecipitation reveal prominent bands corresponding to the sizes of the known hMSLs, including the short form of hMSL3 (Fig. 1E). The results of these coimmunoprecipitation studies support the contention that a complex similar to the *Drosophila* MSL complex exists in humans.

Alternative forms of MSL3 are found in humans. The longer form of hMSL3 most likely corresponds to the previously characterized hMSL3L1 protein, based on its migration on SDS-PAGE (49) (Fig. 1D and 2A), while the shorter form is consistent with the predicted sizes of the hMSL3L1 isoform 'c' protein identified from cDNA sequencing projects and hMSL3L2 (accession number XP087225), an expressed retrogene of the hMSL3L1c isoform. hMSL3L1c and hMSL3L2

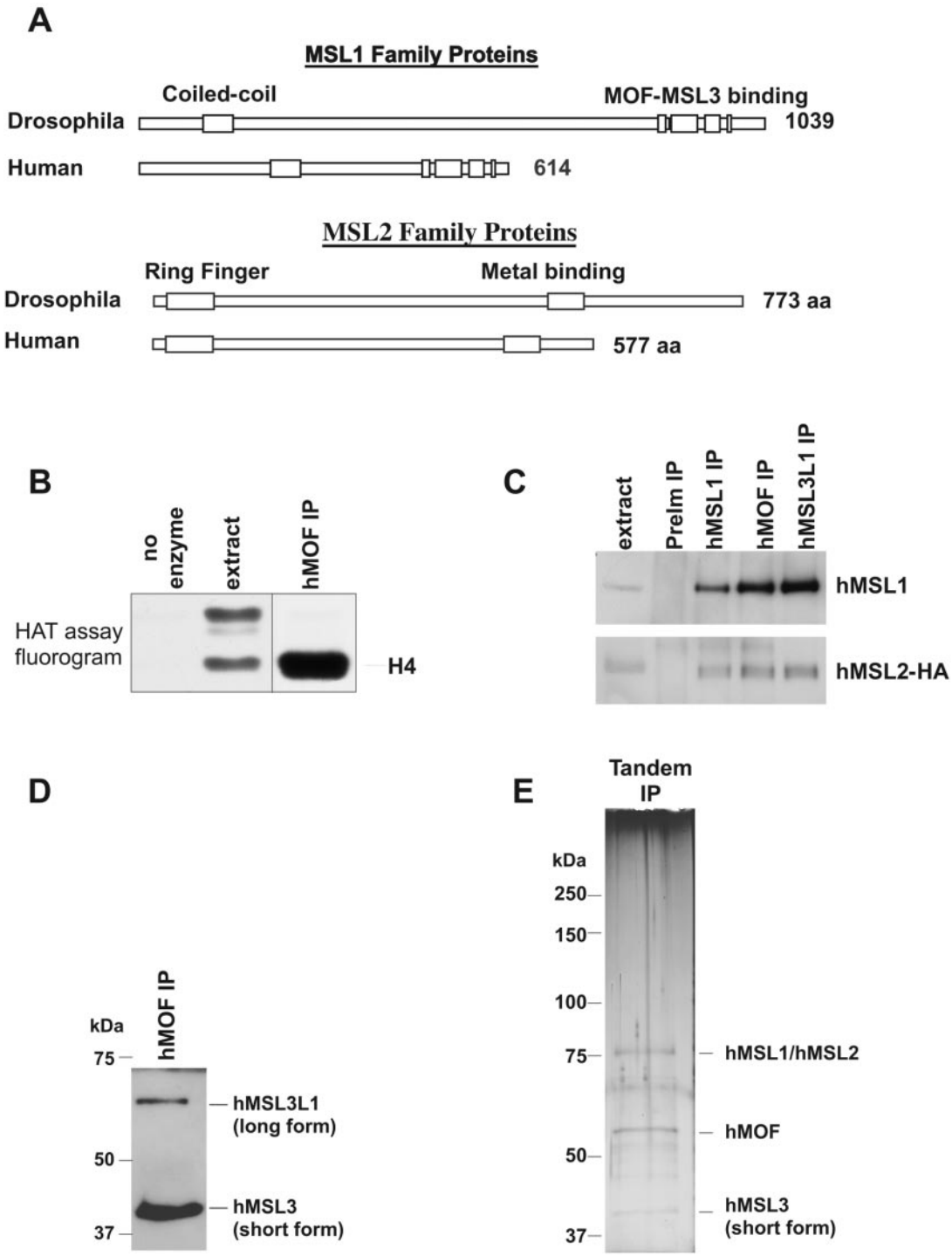


FIG. 1. Conservation of the *Drosophila* MSL complex in human cells. (A) Schematic representation of MSL1 and MSL2 in *Drosophila* and humans. *Drosophila* and human MSL1 proteins have a coiled-coil motif near their N termini and a conserved C terminus implicated in binding to MOF and MSL3. MSL2 proteins are characterized by an N-terminal RING finger and a novel metal-binding domain at their C termini. (B) Histone H4 acetyltransferase activity of human MOF complexes. hMOF immunoprecipitates and control nuclear extract were assayed for histone acetyltransferase activity towards HeLa nucleosomal substrates with ^{3}H-labeled acetyl-CoA. Reactions were separated by SDS-PAGE and processed for fluorography. (C) Coimmunoprecipitation of human homologs of the MSL proteins. Rabbit polyclonal antisera recognizing hMSL1, hMSL3, and hMOF or preimmune (PreIm) serum were used in immunoprecipitation experiments from a K562 cell line that expresses an HA-tagged hMSL2. Western blots were probed with guinea pig anti-hMSL1 and mouse anti-HA. (D) Western analysis of hMOF immunoprecipitates reveals the presence of two forms of hMSL3. The longer form is the expected size of hMSL3L1, while the size of the shorter form is consistent with an alternatively spliced form of hMSL3L1. (E) Successive immunoprecipitation of hMOF and hMSL3 from nuclear extract reveals a limited number of associated polypeptides. hMOF immunoprecipitates were eluted with immunizing peptides, and the eluate was subjected to immunoprecipitation with an antibody recognizing hMSL3L1, which was then also eluted. Proteins were separated by SDS-PAGE and silver stained. Major bands correspond to the predicted size of hMSL1 (comigrating with hMSL2), hMOF, and the short form of hMSL3L1.

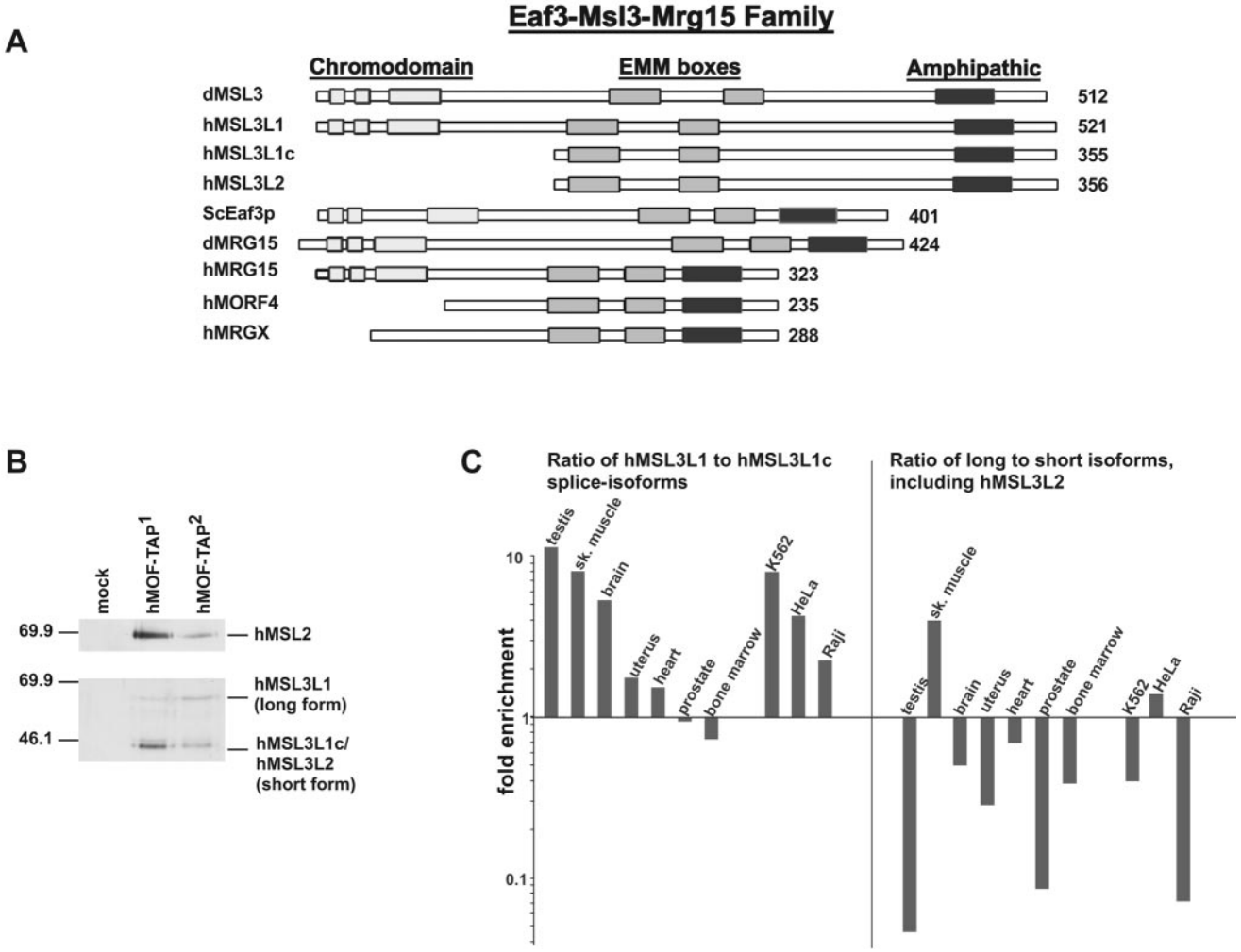


FIG. 2. Multiple forms of MSL3-like proteins in humans. (A) Schematic diagram of the MSL3-related proteins shows conservation of an amino-terminal chromodomain, two boxes of similarity that are unique to the Eaf3, MSL3, and MRG15 (EMM) family of proteins (21) and a putative amphipathic helix or leucine zipper at the C terminus. The X-chromosomal hMSL3L1 gene undergoes alternative splicing to generate two isoforms that differ by inclusion of the chromodomain (hMSL3L1 and hMSL3L1c). A retrogene similar to hMSL3L1c is present on the second chromosome (hMSL3L2). The MRG15 subfamily also has multiple genes in humans, two of which lack an amino-terminal chromodomain (6). (B) Anti-hMSL3L1 Western analysis of two different preparations of hMOF affinity purified from HeLa cells (with TAP tag) shows variability in the relative ratios of hMSL3L1 (long form) and hMSL3L2 (short form). Anti-hMSL2 signals are shown as a control as well as a mock purification from untagged cells. (C) The ratio of long to short forms of hMSL3 are set by both alternative splicing of the hMSL3L1 gene and differential transcription of the hMSL3L1 and hMSL3L2 genes. The left panel shows RT-PCR analysis of the ratio of hMSL3L1 long and short (hMSL3L1c) isoforms in a sampling of different tissues and tissue culture cells. The right panel shows the ratio of long and short forms of hMSL3 when cDNA levels of the hMSL3L2 gene are taken into account.

lack an amino-terminal chromodomain characteristic of MSL3 and its related proteins human MRG15 and yeast Eaf3p (EMM protein family) (21) (Fig. 2A). Although both long and short forms of hMSL3 protein were consistently observed, the ratios varied from cell line to cell line and even in different preparations from the same cell line (Fig. 2B). Additionally, RT-PCR measurements indicate that the ratios of the chromodomain containing hMSL3L1, the shorter splice isoform, and the product of its retrogene vary widely among different cell lines and tissues (Fig. 2C). Together, these results indicate that the presence or absence of the chromodomain in hMSL3 is a highly regulated phenomenon most likely implicated in controlling the association of the hMSL proteins to specific chromatin regions. Interestingly, the related human protein MRG15 has also been shown to have splice variants lacking the chromodomain region as well as two shorter paralogs with the same feature (Fig. 2A). hMRG15 and chromodomain-less MRGX proteins have both been found associated with a distinct human HAT protein, Tip60 (11, 19).

Affinity purification of hMOF and hMSL3L1 identifies a stable human MSL HAT complex. To determine if the hMSL proteins are components of a stable HAT complex and if there are additional proteins associated with them, we used transduced cell lines carrying either TAP-tagged hMOF or hMSL3L1. Both purified hMOF and hMSL3 proteins were found stably associated with the other hMSLs as shown by Western analysis (Fig. 3A). Furthermore, both preparations had HAT activity with specificity toward nucleosomal histone

H4 (Fig. 3A, bottom panel). Proteins copurifying with hMSL3L1 through the two affinity steps were separated by SDS-PAGE and stained with SYPRO Ruby, and gel slices were subjected to in-gel digestion and tandem mass spectrometry (Fig. 3B). Strikingly, the three major bands detected by staining were identified by mass spectrometry as hMSL1, hMSL2, and hMOF, in addition to hMSL3L1. A previous report suggested that hMOF was present in a complex with another MSL3-related protein, MRG15, a known component of the TIP60 HAT complex (20, 46). This suggests that MSL3 family proteins could interact with more than one MYST family histone acetyltransferase. To determine if hMOF was the only histone acetyltransferase associated with hMSL3L1, partially purified hMSL3L1 preparations were subjected to immunoprecipitation with hMOF antibodies. HAT activity was observed in the partially purified hMSL3L1 fractions and in the sequential hMOF immunoprecipitate but not in the unbound fraction (Fig. 3C), indicating that hMOF is the only HAT protein associated with hMSL3L1.

TAP-tagged hMOF was also subjected to TAP purification and identification of associated proteins by Western blotting and tandem mass spectrometry. In contrast to purified hMSL3L1, the pattern of bands obtained on stained gels was less clear, with hMOF-TAP being by far the most prominent band (Fig. 3D). This result suggests that hMOF can associate with polypeptides other than hMSLs, for example, with HCF-1 (Fig. 3D). HCF-1 was originally identified as a cellular protein that associates with VP16 to activate immediate early genes during the lytic cycle of herpes simplex virus infection (reviewed in reference 64). In addition, HCF-1 associates with a Sin3 histone deacetylase complex as well as with ASH1 and MLL1 histone methyltransferase complexes (65, 68), including an MLL1 complex containing MOF (18). We confirmed that HCF-1 associates specifically with hMOF by performing Western analysis with anti-HCF antibodies on hMOF-TAP, hMSL3L1-TAP, and mock TAP-purified samples (Fig. 3E). These data clearly indicate that hMOF associates with HCF-1 in a complex distinct from the hMSL complex obtained with hMSL3L1.

Another protein specifically identified in the hMOF-TAP preparation, but not with hMSL3L1-TAP, is distantly related to hMSL1: LOC284058 (which we will refer to as hMSL1v1 (Fig. 3D and F). hMSL1v1 and the closely related hMSL1v2 (FLJ23861) share similarity with hMSL1 at their C termini (37) (Fig. 3F). The MSL1 C terminus was shown to mediate binding to MOF and MSL3 in *Drosophila* (41, 54). The interactions between MSL1 with MOF and MSL3 were extensively mapped, and distinct regions of the C-terminal domain were shown to be responsible for binding to MOF or MSL3 (41) (Fig. 3F). While the region of similarity between the MSL1 variants and the true MSL1 orthologs does not extend to the region of MSL1 that is implicated in binding to MSL3, significant homology is found within the putative MOF-interacting region (Fig. 3F). Importantly, hMSL1v1 was not found in TAP-purified hMSL3L1 preparations, reflecting at least one functional difference between hMSL1 and hMSL1v.

Nucleosomal HAT activity of the human MOF complexes is specific for histone H4 lysine 16. A notable feature of the *Drosophila* MSL complex is its specificity for acetylation of H4 at lysine 16 (1, 56). hMSL3L1-TAP and hMOF-TAP complexes were assayed with free histones and nucleosomes. hMSL3-TAP and hMOF-TAP complexes specifically labeled histone H4 in the context of chromatin, while also acetylating histone H3 when presented with a mixture of free histones (Fig. 4A). The site specificity of the hMOF-TAP complexes was tested on recombinant histone H4, and acetylation was detected with site-specific antisera. While purified yeast NuA4 complex acetylated both lysine 12 and 16 of H4 as expected (44), the hMOF-TAP complexes had a strong preference for lysine 16 (Fig. 4B). To confirm this specificity for lysine 16 in the context of nucleosomes, hMOF immunoprecipitates were assayed with mononucleosomes and ^{3}H-labeled acetyl-CoA. The histones were separated, and the band corresponding to histone H4 was subjected to Edman degradation as previously described (57). Incorporation of acetate was deduced by scintillation counting of the product from each cycle of Edman degradation. As shown in Fig. 4C, the majority of the released [^{3}H]acetate can be attributed to acetylation of lysine 16. The post-lysine 16 trailing pattern is likely due to sequencing lag, a result previously observed when sequencing of H4 was attempted from membrane supports (56).

The human MSL complex is responsible for the majority of H4 lysine 16 acetylation in vivo. To determine if the hMSL complex acetylates histone H4 at lysine 16 in vivo, we compared acetylation levels in HeLa cells that had been transfected with control or hMSL-directed siRNAs. At 48 h post-transfection, cells were lysed in SDS-PAGE sample buffer for Western analysis with antibodies to H4AcK16, H4AcK8, and H3MeK36 (Fig. 5A). The length of treatment was chosen to avoid the lethality we observed with longer incubations. While hMOF siRNAs did not significantly affect in vivo levels of H4 acetylated at lysine 8 or H3 methylated at lysine 36, they provoked a strong specific decrease of H4AcK16 signals. These results demonstrate that hMOF is responsible for the majority of histone H4 acetylation at lysine 16 in these cells. Furthermore, siRNA treatment against hMSL1 created a similar if not better effect, which indicates that the bulk of histone H4 acetylation at lysine 16 is performed by the hMSL complex in vivo (in contrast to other hMOF-containing complexes, e.g., hMSL1v). hMOF siRNA-treated cells were also fixed and processed for immunofluorescence (Fig. 5B). In order to obtain a rough estimate of the relative change (*n*-fold) in H4Ac16 levels, different exposures of the hMOF-siRNA samples are shown adjacent to two exposures of control cells. Once again, significant reductions in H4Ac16 levels are also observed using this assay. HeLa cells exhibit two levels of H4Ac16, with cells in S phase having significantly higher levels (51). When treated with siRNAs that target either hMSL1 or hMOF, both populations appear to show reductions in lysine 16 acetylation (Fig. 5C). Altogether these data demonstrate that the human MSL complex is a major regulator of chromatin acetylation in vivo since it is responsible for the majority of histone H4 acetylation at lysine 16.

Phenotypic consequences of RNAi-mediated knockdown of hMSL function. RNAi-treated cells were assayed for levels of expression of several genes, including GAPDH and HOXA9, which have recently been shown to be unchanged and downregulated in hMOF-knockdown cells, respectively (18). In addition we analyzed a sample of genes that appeared to be affected by reduced hMSL1 or hMOF levels in preliminary

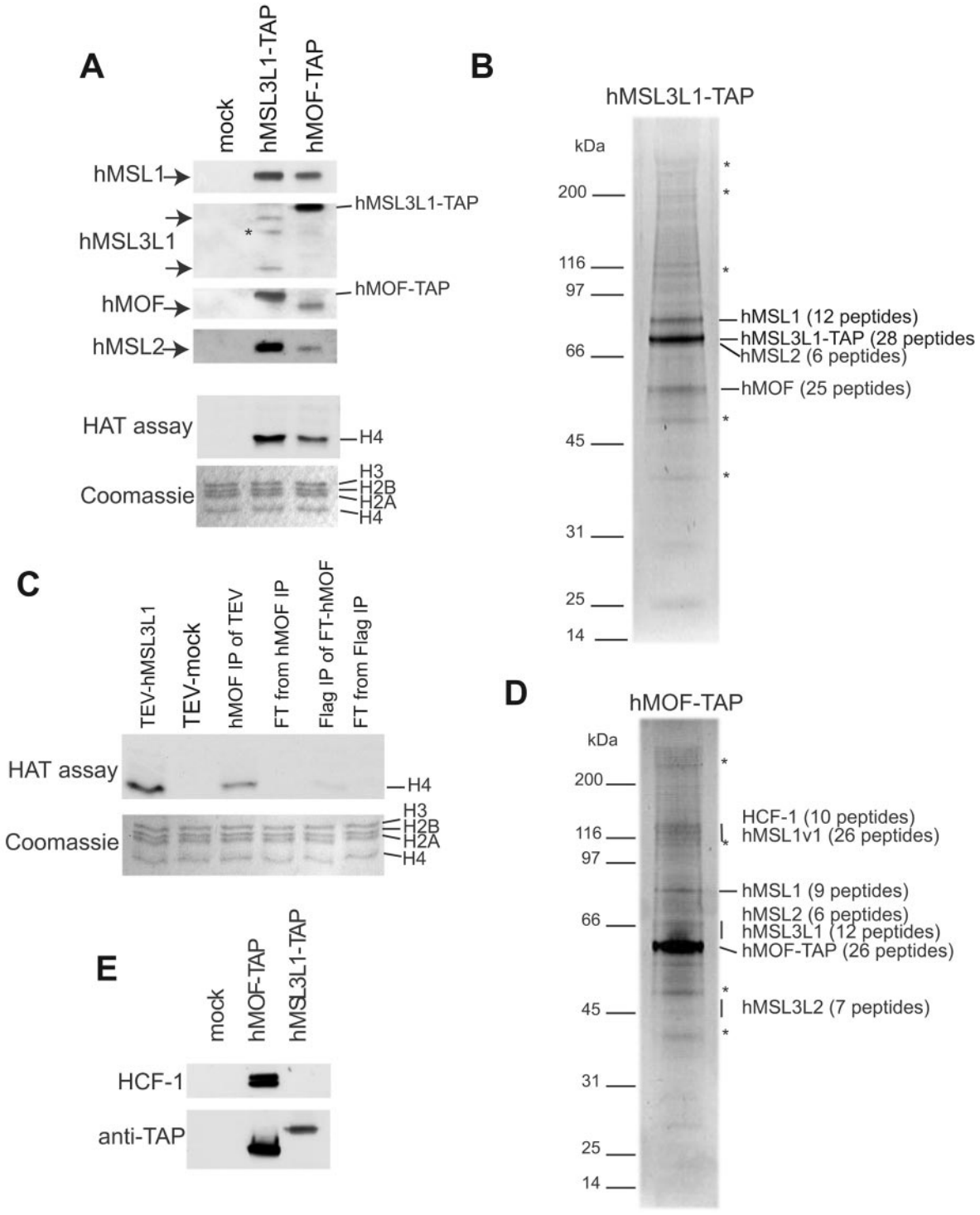
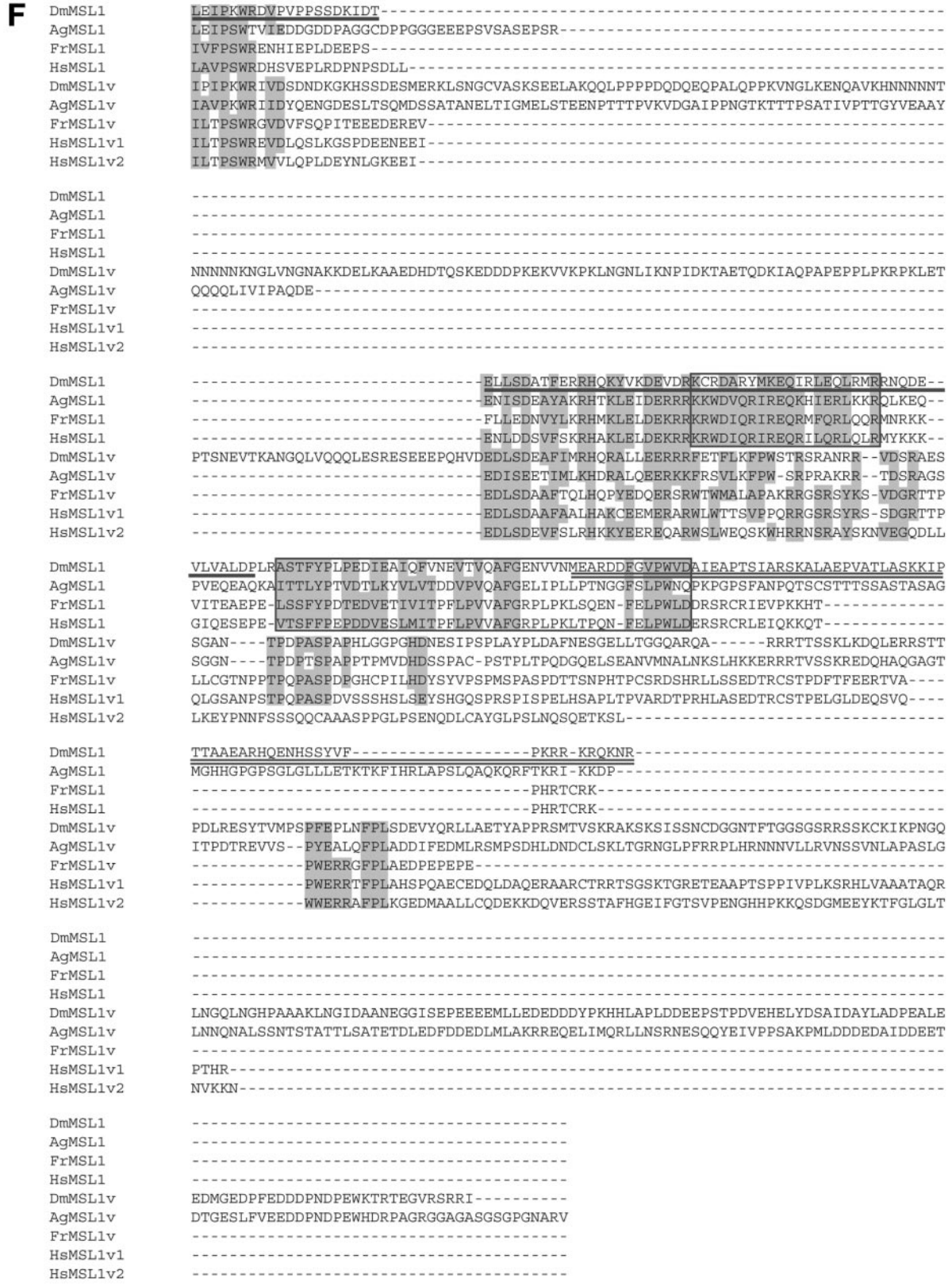


FIG. 3. Human MSL proteins are found together in a stable multisubunit HAT complex. (A) Tandem affinity purification of hMSL3L1 and hMOF or a mock preparation from untagged HeLa cells was prepared by fractionation of nuclear extract on IgG-Sepharose and calmodulin resin and analyzed by Western blotting with the indicated antibodies and HAT assay on oligonucleosomes. hMSL3L1-TAP and hMOF-TAP migrate more slowly than the endogenous proteins. (The asterisk in the hMSL3 Western blot marks a nonspecific band left over from a previous probing of the blot). (B) MSL3L1-TAP is purified as a stable complex that contains three major protein bands corresponding to hMOF, hMSL1, and hMSL2. The calmodulin fraction of MSL3L1-TAP was separated by SDS-PAGE and stained with Sypro Ruby Red, and gel slices were subjected to in-gel digestion and tandem mass spectrometry. Peptides corresponding to hMSL1, hMSL2, and hMOF were identified along with hMSL3L1 (number of peptides shown in parentheses). Nonspecific bands found in other TAP preparations are labeled with asterisks. (C) hMOF is the major, if not only, HAT present in hMSL3 complexes. hMSL3L1-TAP was released from IgG-Sepharose with TEV protease and immunoprecipitated with hMOF antibodies, followed by elution with immunizing peptides. Supernatant from the hMOF immunoprecipitation was incubated with anti-FLAG beads to recuperate hMOF-depleted MSL3L1 complex after elution with FLAG peptides (hMSL3L1-TAP is also tagged at its N terminus with FLAG epitope). (D) Tandem affinity-purified hMOF was also analyzed by SDS-PAGE and mass spectrometry as in panel B. Besides hMOF, peptide hits were also obtained for hMSL1, hMSL2, and hMSL3L1 and hMSL3L2. Peptides hits not previously obtained in purified hMSL3L1 preparations identified a distantly related MSL1-like protein, hMSL1v1, and HCF-1. (E) HCF-1 is specifically associated with hMOF but not the



human MSL complex. hMOF-TAP, hMSL3L1-TAP, and mock TAP-purified fractions were assayed for the presence of HCF-1 by Western blotting. (F) Sequence alignment of the C-terminal domain of the MSL1 family. *Drosophila* MSL1 orthologs from the mosquito *Anopheles gambiae*, *Fugu rubripes*, and humans are aligned with MSL1-like variants from *Drosophila* (DmMSL1v or CG4699), and human (HsMSL1v1 and HsMSL1v2 or LOC284058 and FLJ23861, respectively). Sequences corresponding to the MOF and MSL3 interaction domains are underlined and double-underlined, respectively. Two boxes indicate regions conserved in MSL1 orthologs but not in the MSL1 variants.

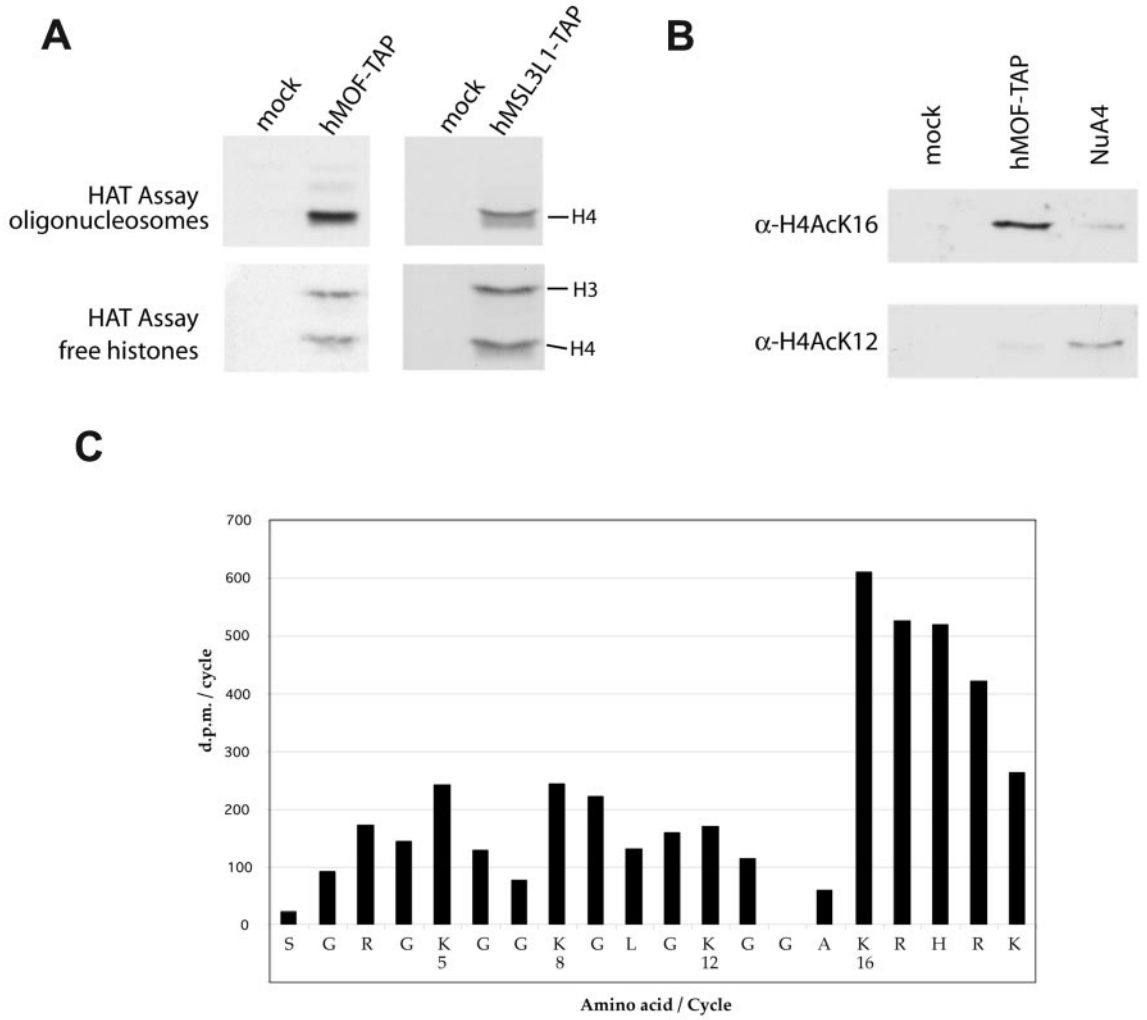


FIG. 4. The human MSL complex specifically acetylates lysine 16 of histone H4 on nucleosomes. (A) hMOF-TAP- and hMSL3L1-TAP-purified complexes were assayed for HAT activity toward oligonucleosomes or free histones along with mock purification from HeLa cells (mock). While HAT activity on free histones is directed toward both H3 and H4, activity is restricted to H4 in the context of nucleosomes. (B) Purified hMOF specifically acetylates lysine 16 of histone H4. Purified human hMOF-TAP and yeast NuA4 complexes were used in a HAT assay with recombinant histone H4. The sites of acetylation were determined by Western blotting, using acetylation site-specific antibodies for H4 lysine 12 or 16. The yeast NuA4 complex is used as control as it was shown to target all four lysines of H4 in vitro (44). (C) hMOF complexes acetylate nucleosomal histone H4 with a strong preference for lysine 16. hMOF immunoprecipitates from K562 cells were used in a HAT assay with mononucleosomes. Histone H4 residues that were acetylated were determined by Edman degradation of purified and deblocked H4 and scintillation counting of each cycle. [^{3}H]acetate released after cycle 16 likely reflects sequencing lag.

microarray studies being carried out by our laboratories. RNA was isolated at 48 and 72 h posttransfection, and expression levels were measured by real-time PCR (Fig. 6). As expected, GAPDH is relatively unchanged in hMSL1- and hMOF-knockdown cells, and we confirmed that HOXA9 was down-regulated in MOF-knockdown cells (18). HOXA9 was also down-regulated by MSL1 knockdown, suggesting that some genes may be subjected to both the global acetylation mediated by the hMSL complex as well as a more targeted and specific acetylation mediated by other hMOF-bearing complexes. HIP1 and KIAA0657 appear to have lower expression levels in both MOF- and MSL1-treated cells, while calreticulin and UCP2 appear to be down-regulated in the absence of either hMSL1 or hMOF but not both. Five other genes that we tested (RCHY1, PMAIP1, PTEN, DICER1, and KIAA1102) showed no consistent or significant changes (data not shown), while one gene, the transcriptional repressor IFI16, showed about a threefold increase in expression in hMSL1- and hMOF-knockdown cells. Together, these data indicate a modest reduction in transcription of a number of genes in the absence of the hMSL complex, suggesting that its normal transcriptional effect is on a par with the twofold increase in transcription mediated by the *Drosophila* MSL complex. A smaller number of genes may increase in transcription in hMSL-depleted cells. Interestingly, induction of IFI16 has been shown to cause cell cycle arrest in G_1 (36), as does mutation of HCF-1 (23). To determine if the cell cycle was altered in hMSL1- or hMOF-depleted cells, we measured the distribution of cells in various cell cycle stages by comparing their DNA content to that of control cells. As shown in Fig. 7, by 72 h after treatment, cells transfected with

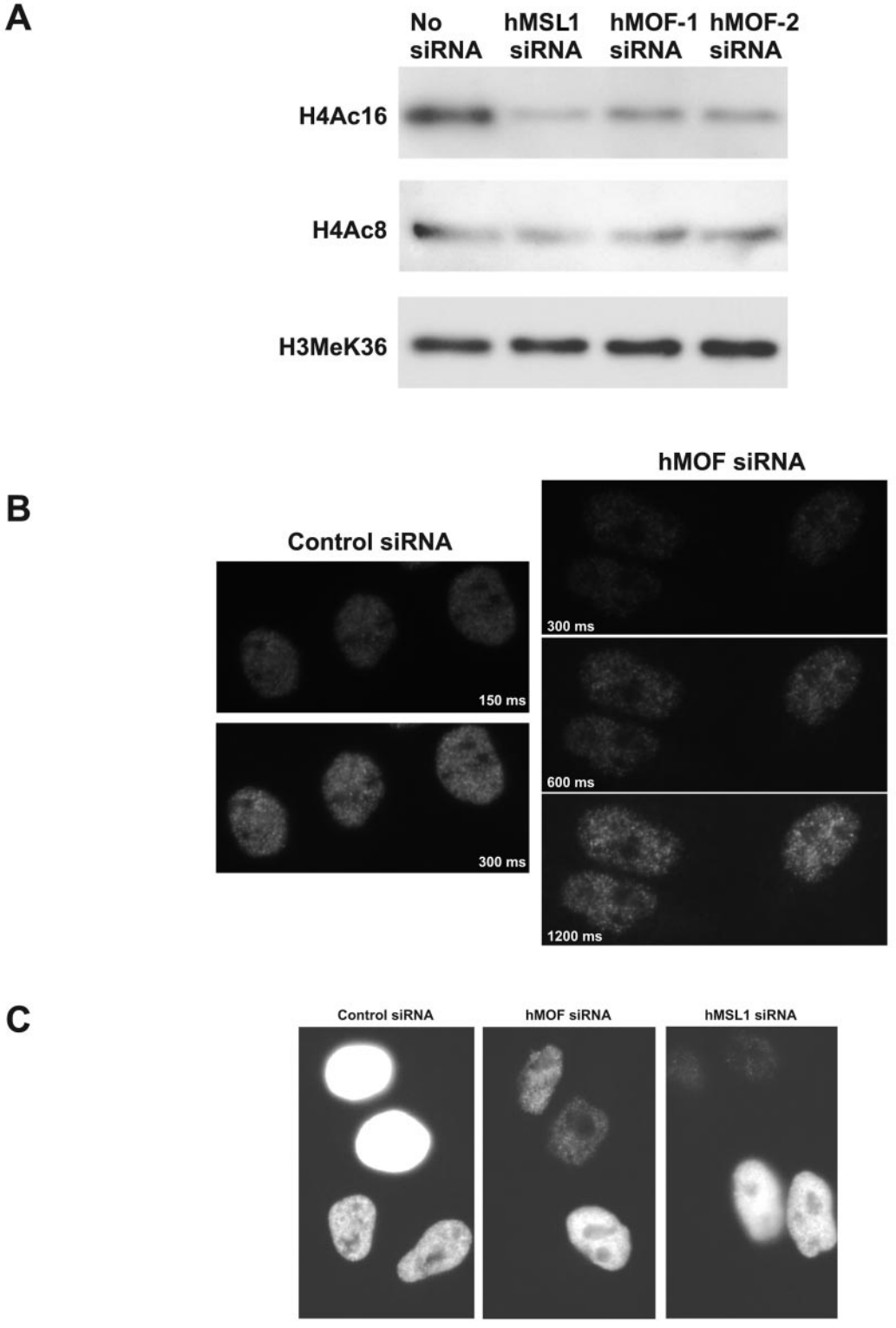


FIG. 5. The human MSL complex is responsible for most of histone H4 acetylation at lysine 16 in vivo. HeLa cells were transfected with control, hMSL1-specific siRNAs, or two different hMOF-specific siRNAs. At 48 h posttransfection, cells were processed for Western analysis or immunofluorescence. (A) siRNA-directed knockdowns of hMOF and hMSL1 provoke a significant loss of histone H4 AcK16 isoform in vivo while H4 AcK8 is unaffected. Total cell lysates were subjected to Western blotting with specific antibodies against H4Ac8, H4Ac16, and H3MeK36 as a loading control. (B and C) Reductions in levels of H4Ac16 were also apparent by immunofluorescence in both hMOF- and hMSL1-knockdown cells. (B) Three different exposures of H4Ac16 staining in hMOF-knockdown cells, 300 to 1,200 milliseconds, are compared to exposures of 150 and 300 milliseconds of cells transfected with a control siRNA. (C) H4Ac16 levels can vary significantly in a population of asynchronously growing cells. Shown are examples of cells with the brightest staining (presumed S phase [51]) along with the more common level of H4Ac16 staining as shown in panel B. Knockdown of hMSL1 and hMOF cause a similar strong reduction of H4Ac16 levels in all populations of cells (identical time of exposure).

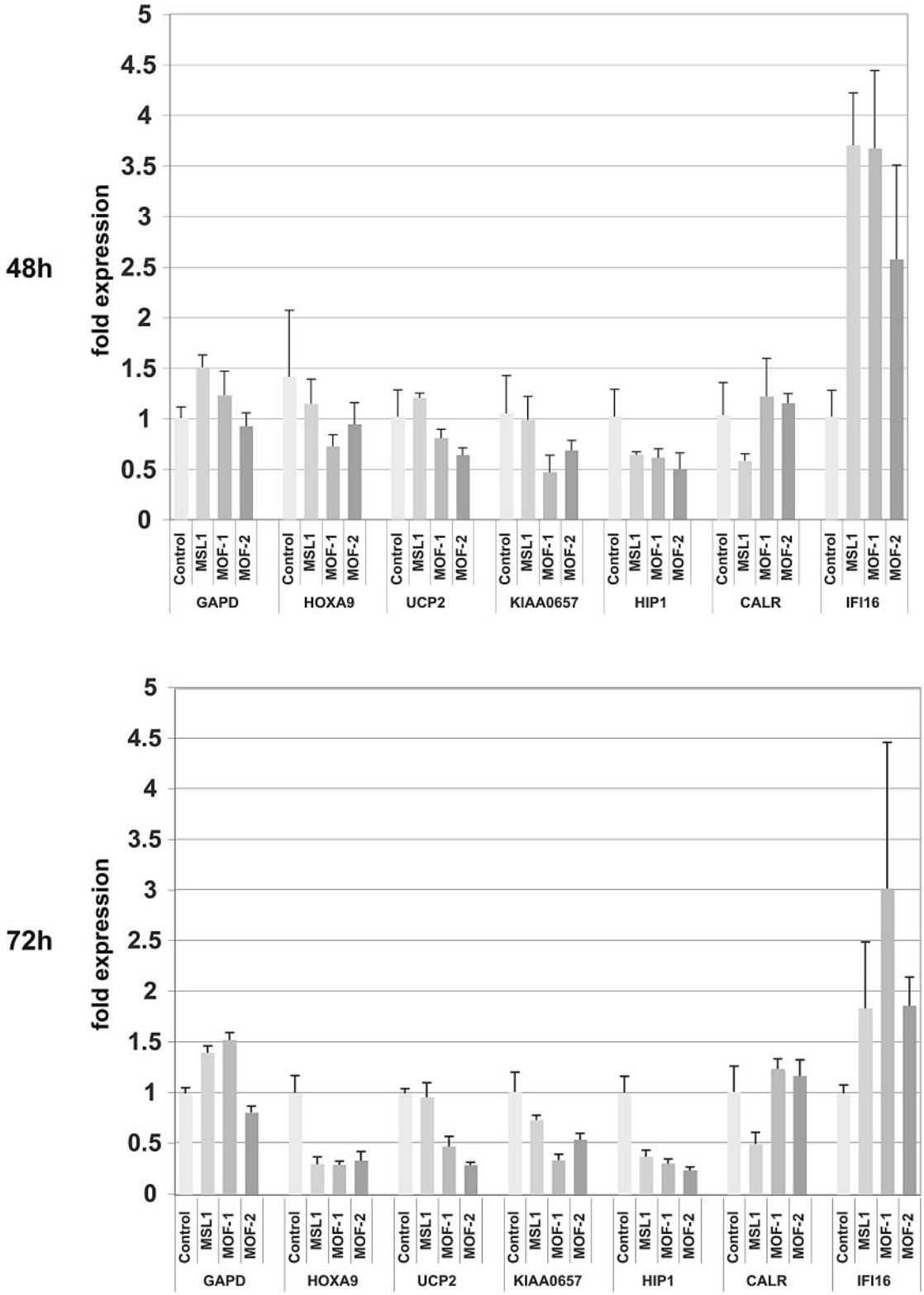


FIG. 6. Transcriptional effects of hMSL1 and hMOF depletion. HeLa cells were transfected with control, hMSL1-specific siRNAs, or two different hMOF-specific siRNAs. At 48 and 72 h posttransfection, RNA was isolated and levels of expression of several genes were tested. Expression levels are normalized to the control siRNA (set at 1). Changes in expression of several genes are apparent at the 48- and 72-h time points. Error bars represent the standard deviation of the mean expression obtained from three different samples.

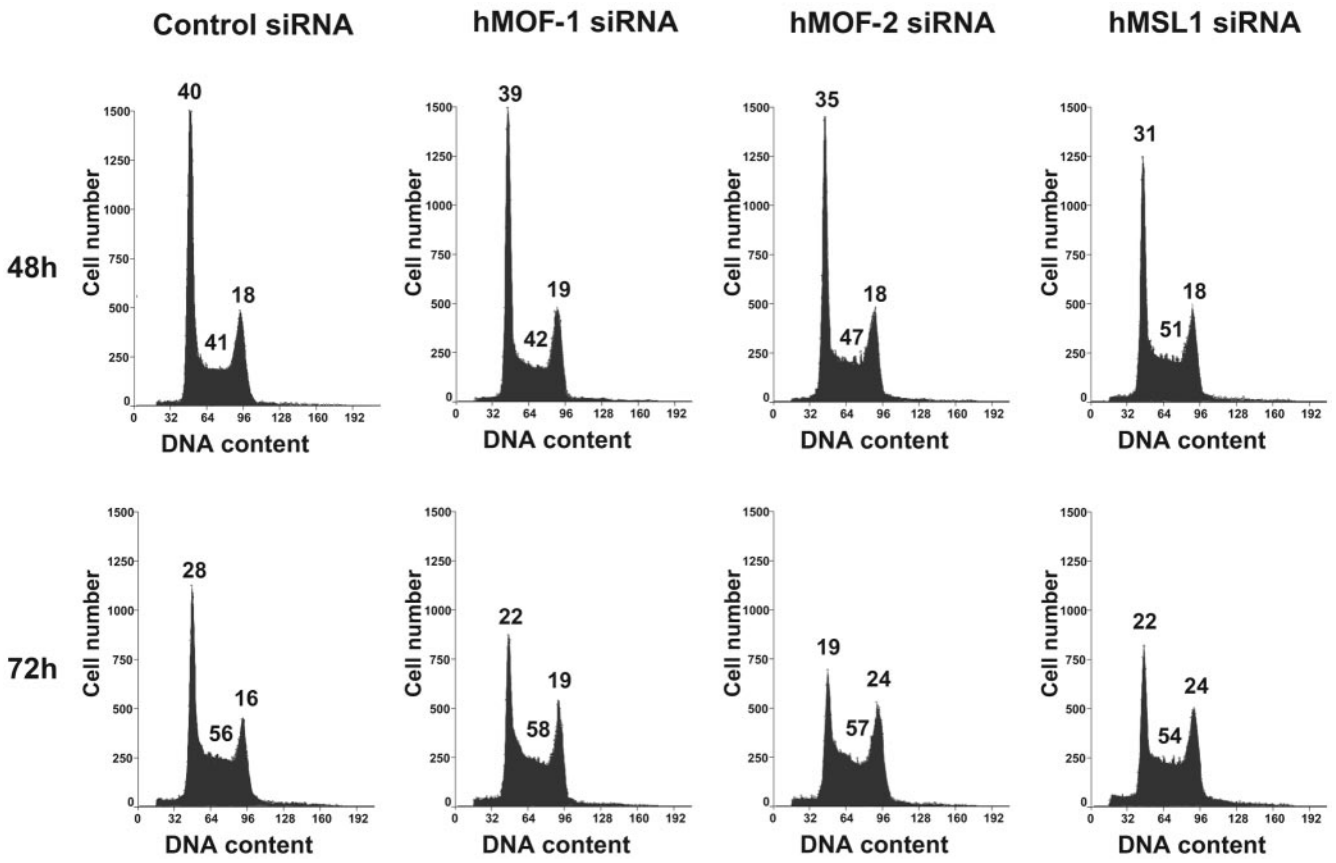


FIG. 7. Depletion of hMOF and hMSL1 affects cell cycle progression. 293T cells were transfected with control siRNA, two different hMOF siRNAs, or an hMSL1 siRNA. At 48 and 72 h posttransfection, cells were processed for flow cytometric cell cycle analysis. The percentages of cells in G_1/G_0, S, and G_2/M stages of the cell cycle are indicated. siRNA-directed knockdowns of hMOF and hMSL1 provoke a significant increase of cells in G_2/M stage by 72 h posttransfection.

hMOF-1/2 or hMSL1 siRNAs clearly accumulated at G_2/M. These data indicate that the hMSL complex is required for normal cell cycle progression in a manner that is distinct from the cell cycle control exerted by HCF-1 and IFI16 and is consistent with the requirement of lysine 16 acetylation for DNA repair (25).

DISCUSSION

Conservation of the MSL complex from *Drosophila* to humans. In flies, the mechanism of dosage compensation consists of enhancing the transcriptional activity of the X chromosome in males; in humans, dosage compensation occurs by a different, independently evolved mechanism of X inactivation in females. In light of these facts, the level of conservation of the MSL complex in flies and humans is remarkable. In *Drosophila* this complex is found exclusively on the X chromosome in males, where it targets activated genes (53) and enhances the level of gene expression by acetylating entire transcriptional domains (55). The forced ectopic relocation of the complex to autosomal sites can lead to spreading and acetylation of adjacent areas (31), indicating that the normally exclusive location of the complex on the male X is not a characteristic of its molecular function; rather, it is the result of its synthesis on or near the X chromosome and of interactions with particular X chromosome sequences. In humans, the complex does not associate with a specific chromosome and appears to have a more dispersed and ubiquitous genomic distribution. This difference in distribution of the complex in flies and humans is mirrored by the different distribution of H4 lysine 16 acetylation in these organisms (28, 61). It is reasonable to expect that at these many sites of association, this modification plays a role in transcriptional regulation that is similar to the one responsible for the enhancement of gene expression in *Drosophila*. Furthermore, a recent report that linked loss of H4 acetylation at lysine 16 to the tumorigenic process also found loss of hMOF association at specific chromosomal loci (22).

Not surprisingly, we have not been able to identify RNA helicase A or MSK1/2 kinase in our purified fractions or in coimmunoprecipitation studies (data not shown). In *Drosophila*, MLE, the counterpart of RNA helicase A, appears to associate with the complex solely by binding to one of the roX RNAs; during purification, MLE is easily lost, binding more transiently to the RNA than the other MSLs (2; our unpublished observations), and is released from the complex with RNase and high salt (56). MLE can also be released from larval salivary gland chromosomes by RNase treatment (52) and from S2 cultured cells by permeabilization with detergent (2). Similarly, it was not surprising that MSK1/2, the human

counterpart of *Drosophila* JIL-1 kinase, was not found in our hMSL preparations. Although one report indicated that JIL-1 coimmunoprecipitated with the MSL complex (29), we have not been able to find evidence that it is a stable stoichiometric component of the *Drosophila* MSL complex (our unpublished observations). Our working model is that JIL-1's enrichment on the *Drosophila* male X chromosome is a consequence of the increased transcription and/or more open chromatin structure rather than its being a component of the MSL complex.

Additional hMOF-containing complexes. In addition to hMOF and hMSL3L1 (or hMSL3L2), the only other major components that copurify with hMSL complexes are hMSL1 and hMSL2 (Fig. 1E and 3A and B). A number of non-MSL proteins associate with hMOF. Recently, the association of hMOF with the ataxia telangiactesia mutated protein has been reported in cells that have or have not been exposed to ionizing radiation (25), and hMOF was found as a component of an MLL1 complex containing HCF-1 (18). In the experiments described in the present paper, we found several other proteins that associate uniquely with hMOF (Fig. 2D and data not shown) and MSL3L1 (data not shown) but not with both; these associations are currently being subjected to further validation and characterization. hMOF had previously been reported to associate with the MRG15-containing complex MAF2, providing an early indication that human MOF was associated with more than one complex (46). However, we could not detect any MRG15 protein in our hMOF-purified fractions (data not shown). This apparent contradiction could be explained by the fact that MRG15 was overexpressed in the previous study, possibly driving interaction with hMOF due to the homology between MRG15 and hMSL3. Since then, MRG15 has been clearly shown to be a stable component of the human Tip60 HAT complex (11, 20). The extent to which MRG15 and hMSL3 subunits are exchanged between hMOF and Tip60 complexes in different cell types is presently unknown.

We have confirmed the association of hMOF with the non-MSL-related protein HCF-1 (Fig. 3E). HCF-1 is present in a number of complexes, including histone deacetylase and histone methyltransferase complexes. Affinity-purified hMOF preparations also included hMSL1v1, a protein that is distantly related to hMSL1 (37) (Fig. 3D and F). hMSL1v1 and the highly similar human hMSL1v2 protein are likely to be orthologs of an uncharacterized *Drosophila* protein dMSL1v (Celera Genomics predicted protein CG4699) (37). Although the vast majority of H4Ac16 in *Drosophila* is found on the male X chromosome, it is interesting that significant levels of MOF protein are present in *Drosophila* females (24). While the hMSL1v1/hMOF complex needs further characterization, it is likely to be found in other regulatory complexes with HCF-1 and other components of the MLL-MOF complex (18). Sequence comparison with the hMSL1 protein suggests that hMSL1v1/v2 is not expected to associate with hMSL3 or hMSL2 due to lack of conservation of the appropriate regions in hMSL1v1 (41, 54, 70) (Fig. 3F).

Regulation of MSL3 proteins. Chromodomains have been implicated in binding methylated lysine residues and nucleic acids (reviewed in reference 9), and the chromodomains from the MSL3 and MRG15 proteins could form an aromatic cage implicated in methyl-lysine binding (27, 43) and may be required for MSL3 binding to RNA or single-stranded DNA (40). In light of these considerations, the existence of truncated forms of hMSL3L1 that lack an amino-terminal chromodomain was unexpected. This is reminiscent of the observations of Bertram and colleagues, who reported that two genes related to MRG15 (MRGX and MORF4) are missing the amino-terminal chromodomain (6, 7) (Fig. 2). The existence of full-length and chromodomain-less forms of hMSL3 and MRG15 in hMSL and Tip60 complexes, respectively, argues for a role of this variation in regulating the targeting and function of the different HAT complexes (Fig. 2) 10, 11).

One possible role for the chromodomains of EMM family proteins is to facilitate the spreading of their associated histone modifying activities along chromatin, whether HAT or histone deacetylase complexes (50). Interestingly, MRG15 and chromodomain-less MRGX/MORF4 were also found to associate with Sin3A and histone deacetylase 1 (20, 60, 67), supporting the notion that deacetylase activities may also be regulated by the presence or absence of the chromodomain of some EMM family members.

Histone acetyltransferase activity in the human MSL complex. Our data strongly suggest that a conserved multiprotein assembly, the MSL complex, is the major histone H4 K16 acetyltransferase activity in a wide range of higher eukaryotes. Acetylation of lysine 16 might be particularly important for the regulation of chromatin folding. For example, mutation of some chromatin regulators including the ISWI ATP-dependent nucleosome remodeling protein and the JIL-1 serine 10 kinase leads to the apparent total disorganization of the male X chromosome, while the morphology of autosomes in males and all female chromosomes is unaffected (13, 15, 63). Antagonism between ISWI remodeling complexes and MSL-dependent acetylation of lysine 16 is also apparent from the enhancement of mutant ISWI phenotypes by overexpression of the lysine 16-specific MOF acetyltransferase (13). A similar phenomenon is observed in yeast, where the SIR2 deacetylase and the SAS2 acetyltransferase regulate H4Ac16 levels at the boundaries of silenced chromatin (32, 59). Together with the in vitro folding data, the yeast and *Drosophila* studies indicate the importance of lysine 16 acetylation status for higher-order chromatin structure. It will be very interesting to analyze the interplay between the hMSL complex and ISWI or Sir2 homologs in human cells and their effect on local chromatin structure.

We showed that the hMSL complex, formed by hMSL1/2/3-hMOF proteins, is responsible in vivo for the bulk of histone H4 acetylation at lysine 16 (Fig. 5). This is based on RNAi experiments that led to significant reductions of cellular H4AcK16 signals when hMOF or hMSL1 proteins are depleted. In comparison, other hMOF-containing complexes must be targeted to specific, localized pathways such as the MLL-MOF complex (18) or induced at specific times such as in response to ionizing radiation (25) so that their contribution to H4 lysine 16 acetylation in vivo would normally represent a relatively small proportion of the total of this histone isoform present in cells.

Our initial studies of the transcriptional effects of loss of function of the hMSL complex are consistent with a global distribution of acetylation with relatively modest effects on transcription that are similar in magnitude to those mediated by the *Drosophila* complex. A reduction in the level of cell cycle

regulators could contribute to the G_2/M arrest phenotype observed in hMOF- and hMSL1-knockdown cells. Alternatively, a reduction in lysine 16 acetylation, a modification that normally reaches its highest level during S phase (51), could adversely affect some aspect of DNA replication. For example, a defective S phase checkpoint can lead to accumulation of cells at G_2/M (66). Interestingly, H4 lysine 16 acetylation by hMOF is required for ataxia telangiactesia mutated protein-dependent repair of DNA after ionizing radiation (25), establishing that lysine 16 acetylation is critical for maintaining genome integrity. Furthermore, a recent study has correlated global loss of H4 lysine 16 acetylation to the tumorigenic process (22). We can speculate, therefore, that the hMSL complex we have identified in the present work is a major target of regulation during the process of cell transformation and carcinogenesis.

ACKNOWLEDGMENTS

We thank Winship Herr, Paul Wade, Jeremy Boss, Luc Gaudreau, Nicolas Lacoste, and David Allis for providing reagents; Richard Cook and the microchemical facility at the Baylor College of Medicine for sequencing the site of acetylation within H4; and Valerie Côté for FACS analysis. We are grateful to Antonio Pannuti for helpful discussions, Yannick Doyon for advice on TAP purification, and Yali Dou and Robert Roeder for communicating results prior to publication.

We thank David Allis for support to E.S. Research support was provided by grants from the National Institutes of Health and the A. G. Candler Endowment to J.C.L. and from GenomeCanada/GenomeQuebec and the Canadian Institutes of Health Research (CIHR) to J.C. J.C. is a CIHR Investigator.

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