

Supplementary Information

Supplementary Materials and Methods

Plasmids

The following plasmids were described previously: MCK-Luc (Mal et al., 2001); myogenin-Luc (Xu and Wu, 2000); 4RE-luc (Weintraub et al., 1990); Gal4-Luc (Wu et al., 2000); pCSA-MyoD (Novitch et al., 1999); pGEX-MyoD constructs plasmids encoding GST-MyoD fusion proteins (Mal et al., 2001); pGEX-4T-3-H3 (N) plasmid expresses GST fusion protein encoding amino acid 1-57 of histone H3 (Tachibana et al., 2001); the in vitro transcription plasmid pSP64(polyA)/F-Suv39H1 and pSP6(polyA)/F-Suv39H1 (H324K) encode a Flag-tagged wild type and mutant version H324K of Suv39H1, respectively (Li et al., 2002); retroviral vectors encoding Flag-Suv39h1 (Rice et al., 2003) and MyoD (Novitch et al., 1999), self-inactivating retroviral vector pUSTdS-mCMV-LacZ (Razorenova et al., 2005); pCMVdelta8.2, pVSV-G, pLV-bleo-siRNA (EGFP was replaced by bleomycin gene) (Budanov et al., 2004). The SV40-Luc was purchased from Promega. Plasmid encoding β -galactosidase (CMV- β -gal) and Gal4 fusion ATF2 (Gal4-ATF2) were purchased from Clontech and Stratagene, respectively.

Recombinant Constructs

pcDNA3/F-Suv39H1 and pcDNA3/F-Suv39H1 (H324K) were constructed by subcloning the fragment from the respective pSP64(polyA) plasmid into pcDNA3 (Invitrogen). To obtain the construct pUSTdS-mCMV-4RE-LacZ, the MyoD binding 4RE sites (oligos 5' - AGCAGGTGTTGGGAGGCAGCAGGTGTTGGGAGGCAGCAGGTGTTGGGAGGCAGCAGGTGT - 3' , (Weintraub et al., 1990)), were cloned into the Spe1-Xho1 sites of pUSTdS-mCMV-LacZ. To obtain the constructs expressing siRNA against luciferase (pLV-bleo-Luc-siRNA) and Suv39h (pLV-bleo-Suv39h-siRNA), the following oligos representing 21 bp and 19 bp of the mRNA of luciferase (Razorenova et al., 2005) or Suv39h (both

Suv39h1 and Suv39h2 (Ait-Si-Ali et al., 2004)), respectively, were cloned into pLV-bleo-siRNA) such a way that the oligos were present in the hairpin transcript.

For luciferase RNA, 5' -CACTTACGCTGAGTACTTCGA-3' ; for Suv39h RNA, 5' -ACCTCTTTGACCTGGACTA-3' .

Antibodies

The following antibodies were purchased: MyoD (M318 or C20), p21 (C19), Myogenin (M225), Cyclin A2 (C19); GST, and normal rabbit IgG (NRIgG) antibodies (Santa Cruz Biotechnology); MyoD (5.8A), and myogenin (FD9) antibodies (Pharmingen); Suv39h1 (#2291) antibodies (Cell Signaling); Suv39h1 (#07-550, #05615), dimethylated-H3-K9 (#07-212), and trimethylated-H3-K9 (#07-442) antibodies (Upstate); MHC (MF-20) antibodies (Developmental Studies Hybridoma Bank, University of Iowa); GAPDH (H86504) antibodies (BIODESIGN); β -Actin (A5441), Flag-M2 (F3165), and Flag-M2-conjugated-POD (A8592) antibodies (Sigma)

Transfection

One day prior to transfection, cells were plated in 6 wells plate for luciferase and β -galactosidase assays, and in 10 cm plate for immunoprecipitation and immunoblot analysis. The cells were transfected using Fugene 6 according to manufacturer instruction. Each well of a 6-well plate and each 10 cm plate received 1.5 μ g and 7.5 μ g of total DNA, respectively.

Purification of ³⁵S-labeled Flag-Suv39h1

³⁵S-labeled Flag-Suv39H1 was purified after generating the labeled protein from pSP6(polyA)/F-Suv39H1 plasmid by using the TNT kit (Promega) followed by binding with Flag-M2 agarose beads and elution by flag peptide (Sigma) as described previously (Mal et al., 2001).

Retroviral/lentiviral production, transduction and generation of polyclonal population of cells

For the production of retroviruses, phoenix-ampho packaging cells (Grignani et al., 1998) (provided by Andrei V. Gudkov) were transfected with experimental retroviral vectors using Lipofectamine-Plus reagent (Invitrogen) according to the manufacturer's instruction. Supernatant containing viral particles were harvested 36-48 h post-transfection, and filtered. High-titer stock of recombinant lentivirus pseudotyped with VSV-G protein was produced as described (Boiko et al., 2006). Briefly, 293T cells were cotransfected with experimental pLV-bleo-Luc-siRNA/pLV-bleo-Suv39h-siRNA constructs together with the pCMVdelta8.2 and pVSV-G by lipofectamin-Plus reagent. Supernatant containing viral particles were harvested 12, 24, 36, 48, and 60 h post-transfection, pooled, and filtered. Stable polyclonal population of cells were generated by infecting target growing cells with retrovirus-containing supernatant supplement with 4µg/ml polybrene (Sigma) for 12 h and selecting G418-resistance (for retroviral vector encoding with or without Flag-Suv39h1) or puromycin-resistance (retroviral vectors encoding with or without MyoD, and pUSTdS-mCMV-LacZ with or without 4RE sites) clones. I combined individual retroviral-transduced antibiotic-resistance colonies to yield a polyclonal population of cells. Transduction of lentiviruses expressing siRNA into 70-80% confluent C2C12 cells was performed every 8 h for 2 times by incubating virus stock with 6 µg/ml polybrene. Three days after viral transduction, cells were induced to differentiate by replacing growth medium with differentiation medium.

Chromatin Immunoprecipitation (ChIP) and Re-Immunoprecipitation (Re-ChIP)

Assays (Detail procedure was described previously (Mal and Harter, 2003)).

Briefly, for ChIP assay, C2-C and C2-h1 cells, cultured in GM or DM for 48 h, were cross-linked with 1% formaldehyde (HCHO) for 10 min at room temperature. Chromatin was prepared from these HCHO-cross-linked cells and purified through CsCl as

described previously (Mal and Harter, 2003). Equivalent amount (50 μ g) of CsCl-purified chromatin (normalized by A_{260}) were immunoprecipitated with anti-dimethylated-H3-K9 antibodies or control NR1gG. The DNA derived from these immunoprecipitate was subjected for PCR amplification. In case of Re-ChIP assay, chromatin was prepared from HCHO cross-linked growing C3-C and C3-MyoD cells, and C2-C and C2-h1 cells grown in GM or DM for 48 h. One hundred (100 μ g) microgram of CsCl-purified chromatin was initially immunoprecipitated with anti-Suv39h1 (upstate, #07-550) antibodies or control NR1gG. The associated materials were then released from the initial immunoprecipitation by incubating with 10 mM DTT at 37^oC for 30 min and the supernatant was collected. Afterwards, the supernatant was diluted with appropriated dilution buffer and reimmunoprecipitated with anti-MyoD (M318) antibodies. The precipitated DNA from the MyoD immunoprecipitate was then PCR amplified. PCR products were fractionated on 8% native polyacrylamide gel and visualized by autoradiography. For amplification of myogenin promoter region, primers were designed that amplify the two E-box sites (E1 and E2) located in the proximal region of this gene promoter. As an input control for ChIP, PCR was performed with DNA from chromatin prior to immunoprecipitation (0.04% total chromatin used for immunoprecipitation) using primers that amplify the coding region of GAPDH gene. For Re-ChIP assay, amylase promoter region was used. The primer sets used for amplification have been described previously: for myogenin and GAPDH (Mal and Harter, 2003) and amylase (Bergstrom et al., 2002). The PCR amplification condition was also described in our previous work (Mal and Harter, 2003). The number of cycles and the amount of DNA template were varied to ensure that the results were within the PCR linear range.

Chromatin Immunoprecipitation (ChIP) and Re-Immunoprecipitation (Re-ChIP)

Assays

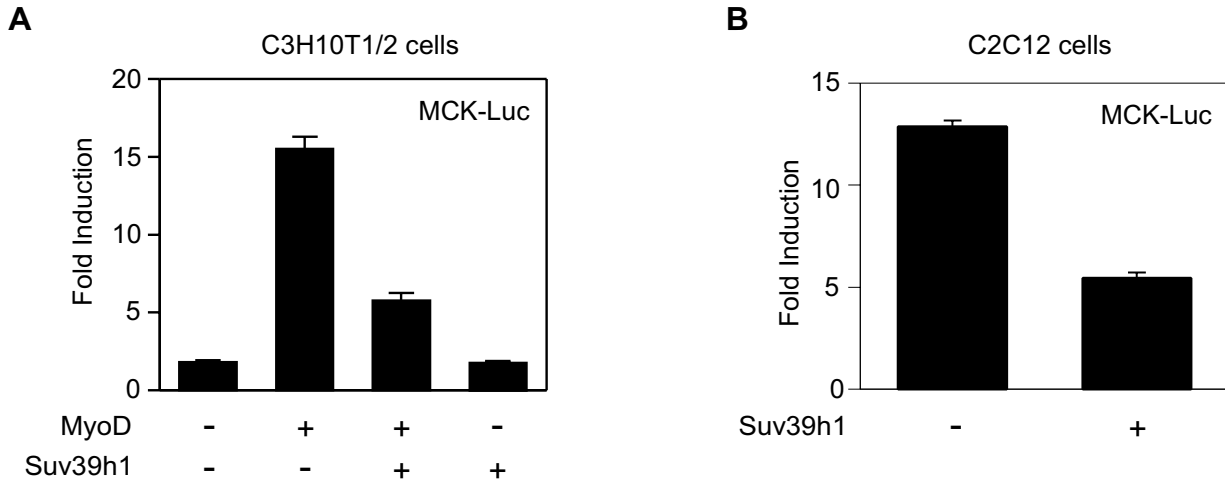
ChIP assay was performed as described previously (Mal and Harter, 2003). Briefly, equivalent amount (50 µg) of chromatin (normalized by A₂₆₀) were immunoprecipitated with the indicated antibodies. Sample derived from the immunoprecipitate was subjected for PCR amplification. Procedure for Re-ChIP assay was also described previously (Mal and Harter, 2003). Briefly, 100 µg of chromatin was initially immunoprecipitated in parallel with an antibody specific for Suv39h1 or control NR1gG. The immunoslective DNA-protein complex was then eluted from the primary immunoprecipitate by incubation with 10 mM DTT at 37°C for 30 min. Afterwards, the supernatant was diluted 20 fold in IP dilution buffer and used for an immunoprecipitation using anti-MyoD antibody. Sample derived from the second immunoprecipitate was then subjected for PCR amplification. PCR products were fractionated on 8% native polyacrylamide gel and visualized for autoradiography.

For amplification of myogenin promoter region, primers were designed that amplify the two E-box sites (E1 and E2) located in the proximal region of this gene promoter. As an input control for ChIP, PCR was performed with DNA from chromatin prior to immunoprecipitation using primers that amplify the coding region of GAPDH gene. For Re-ChIP assay, amylase promoter region was used. The primer sets used for amplification have been described previously: for myogenin and GAPDH (Mal and Harter, 2003) and amylase (Bergstrom et al., 2002).

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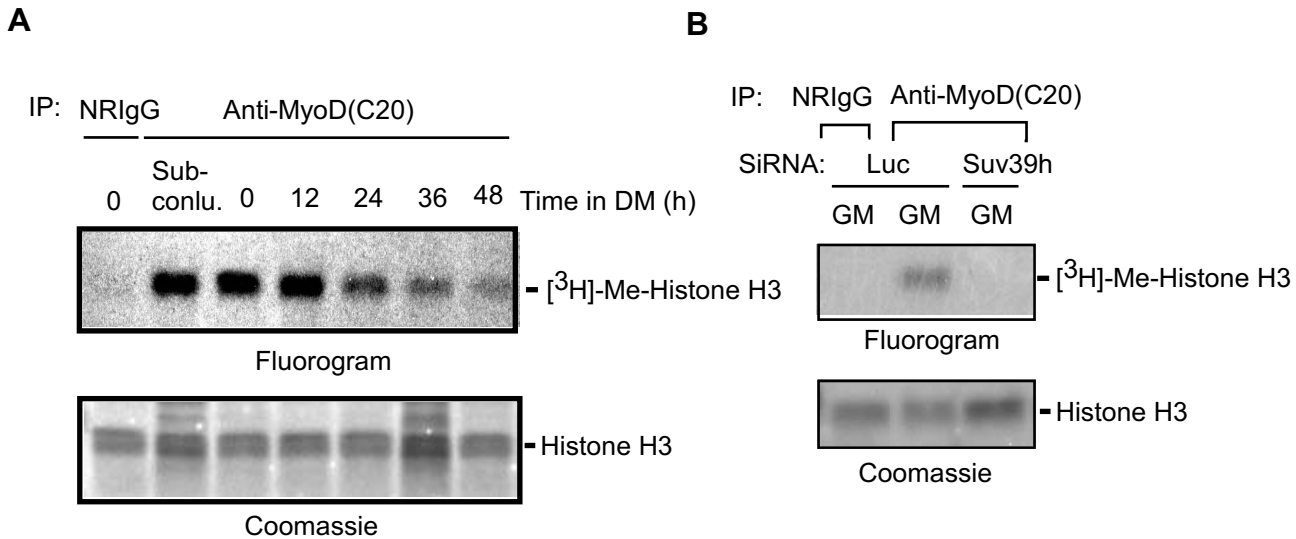
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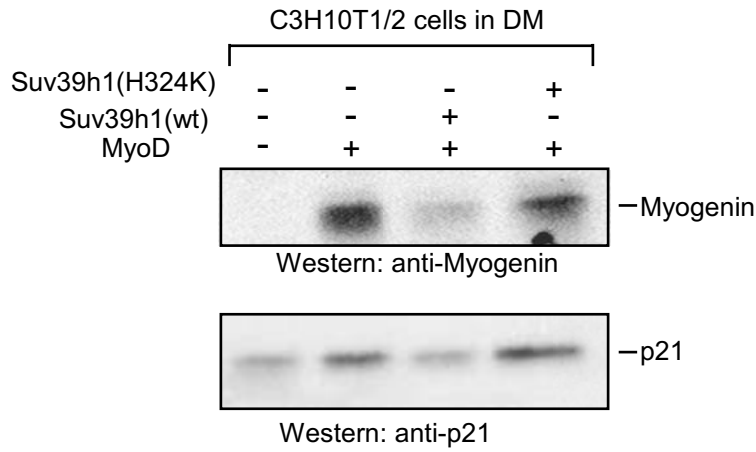
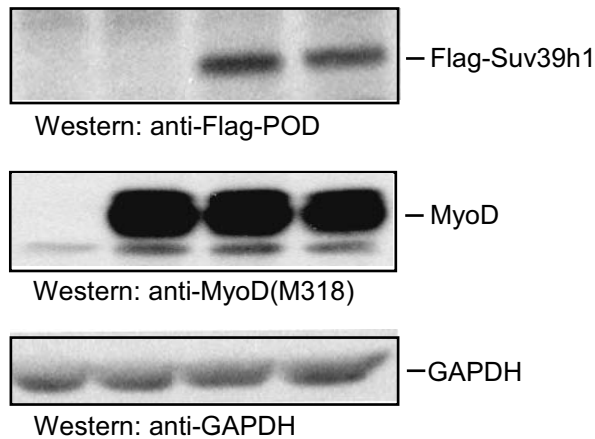
Supplementary Fig. S1.

Histone Methyl Transferase Suv39h1 inhibits MyoD-dependent MCK-luciferase (MCK-Luc) reporter gene activation. (A) C3H10T1/2 cells were cotransfected with MCK-Luc and vectors encoding MyoD and Suv39h1. (B) C2C12 cells were cotransfected with MCK-Luc reporter plasmid and vector expressing Suv39h1. The (-) sign indicates that an empty expression vector has been added instead of the corresponding expression vector. Values are expressed as fold activation after normalization.

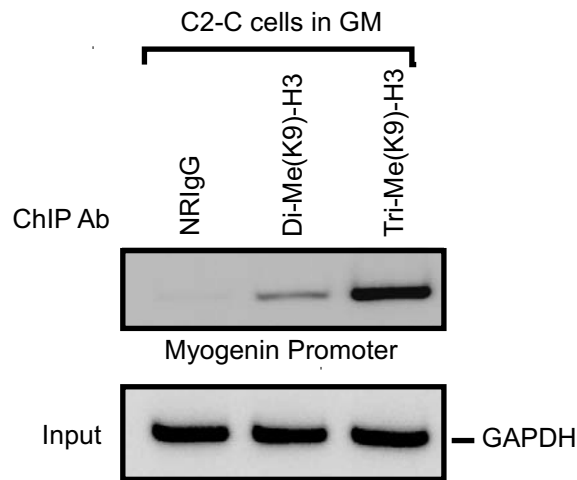


Supplementary Fig. S2.

(A) Kinetics of MyoD-associated H3 HMT activity during the differentiation of C2C12 cells. Nuclear extracts derived from sub-confluent cells cultured in GM media, and near confluent cells cultured in GM or DM media for indicated period of times, were immunoprecipitated with either MyoD antibodies or control NR1gG. These immunoprecipitates were then subjected to H3 HMT activity assay and methylated-H3 was detected by fluorography (upper panel). Equal amount of H3 added in each reaction was verified by coomassie staining (lower panel). **(B) MyoD-associated H3-HMT activity in control (Luc) and Suv39h-deficient C2C12 cells.** MyoD immunoprecipitates retrieved from nuclear extracts of C2C12 cells, infected with lentivirus expressing siRNA against luciferase or Suv39h, cultured in GM medium, were subjected to H3 HMT activity assay. Methylated H3 was detected by fluorography (upper panel) and equal loading of H3 was verified by coomassie (lower panel).

A**B****Supplementary Fig. S3.**

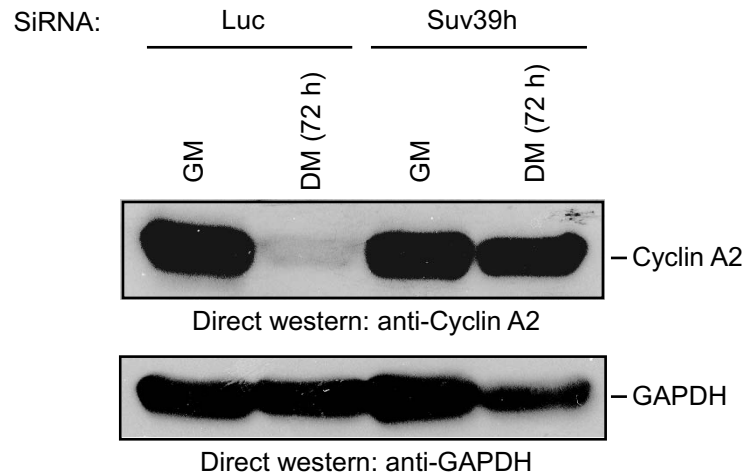
(A) C3H10T1/2 cells were cotransfected with the indicated expression vectors. Twenty-four hours post-transfection, the cells were switched to DM media for 48 h. Total cell extracts were then analyzed by western blotting for the detection of myogenin and p21 expression with antibodies specific for myogenin and p21, respectively. **(B)** The same amount extracts as depicted in (A) was analyzed by western blotting for the detection of Flag-Suv39h1 and MyoD expression using anti-Flag(M2) conjugated POD and anti-MyoD antibodies, respectively. Equal loading of the cell extracts was confirmed by re-probing the membrane with an antibody for GAPDH. The (-) sign indicates that an empty expression vehicle has been added.



Supplementary Fig. S5.

Myogenin promoter surrounding MyoD binding sites is enriched in trimethylated K9 on H3 in C2C12 derived control C2-C cells under growth conditions.

ChIP with control NR1gG, anti-dimethylated-H3-K9 and anti-trimethylated-h3-K9 antibodies on chromatin obtained from C2-C myoblasts. The precipitated DNA was then subjected to amplify myogenin promoter regions surrounding MyoD binding sites by PCR. The PCR products were visualized by autoradiography (upper panel). Lower panel represents the input (0.04% total chromatin used for ChIP) control (GAPDH).



Supplementary Fig. S6.

Cyclin A2 protein level in control (Luc) and Suv39h-deficient C2C12 cells cultured in GM and 3 days in DM media. Total cell extracts obtained from C2C12 cells infected with lentivirus expressing siRNA against control or Suv39h were subjected to western blot analysis for the detection of cyclin A2 protein level using anti-cyclin A2 antibodies (upper panel). Loading control was revealed by western analyses with GAPDH antibodies (lower panel).