Supporting Information

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SI Materials and Methods.

Cell Culture. 293T and 293FT cells were from American Type Culture Collection (ATCC) and maintained in DMEM supplemented with 10% (vol/vol) fetal bovine serum (Hyclone). For generation of viruses, cells were seeded a day before transfection. The mouse myoblast cell line C2C12 was obtained from ATCC and maintained at less than 60% confluence in DMEM containing 15% (vol/vol) fetal bovine serum (growth medium) at 37 °C with 5% CO_2. To induce differentiation into myotubes, cells were incubated in DMEM containing 2% (vol/vol) horse serum (differentiation medium) when they reached 80% confluence. All media were supplemented with 1% penicillin and streptomycin.

Plasmids and Stable Cell Lines. H3.1 or H3.3 fused with Flag-HA in the C terminus (eH3) was prepared from pOZ H3.1 and H3.3 (gift from Dr. Y. Nakatani, Dana-Farber Cancer Institute, Boston, MA), respectively. The eH3s were cloned into pBABE puro. To produce stable cell lines, cloned DNA was used for transfection with a retroviral packaging system into 293T cells. After 36 h, retrovirus-containing medium was collected, filtered (0.45 μm), and applied to C2C12 cells with 5 μg/mL of polybrene (Sigma). Cells were then selected in medium containing puromycin (2.5 μg/mL, Sigma). A clone that expresses eH3 at about 10% of the level of endogenous H3 was selected for each. To generate HIRA shRNA or Asf1b shRNA stable cells, a pLKO1 shRNA construct was transfected into 293FT cells with the lentiviral packaging vectors (pLP1, pLP2, and pLP-VSVG) to produce lentiviruses. Recovered virus solution was used for infection of C2C12 cells (selection with puromycin). Knockdown efficiency of endogenous target genes was monitored by immunoblotting and RT-PCR. In case of shHIRA expressing cells, to avoid clonal bias with exacerbated differentiation phenotype, puromycin resistant clones with affected HIRA expression were pooled together and maintained in the growth medium. Cells with stable expression of shAsf1b showed no difference in proliferation and differentiation compared to control shRNA cells. For a complementation experiment, HA-human HIRA was amplified from pcDNA HA-HIRA by PCR and was cloned into pBABE hygro vector to use for infection of shHIRA C2C12 cells. After culture in the medium with hygromycin (200 μg/mL, Sigma) independent three clones were isolated and checked for human HIRA expression.

RNA interference. Transfection of siRNA was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The siRNAs were purchased from either Invitrogen or Samchully Pharm. The shRNA constructs were purchased from Open Biosystems. The target sequences of siRNA and shRNA were as follows: mHIRA shRNA ; 5′ CTC AAG CTG ATG ATC GAA GTT 3′, mHIRA siRNA-1 (used in Fig. 3*B*, Fig. S4 *B* and *C*, and Fig. S7*B*); 5′ GAC CAA GTT TGC AAC TGG AGG ACA A 3′, mHIRA siRNA-2 (used in Fig. 3*C*, Fig. S2*D*, and Fig. S4*B*); 5′ CCG GAA AGC TGT GAC TGT TGT GAA A 3′, mAsf1a siRNA; 5′ GGC ATA TGT TTG TGT TTC AGG CTG A 3′, mAsf1b shRNA; 5′ TGT GGG CTA CTA TGT CAA CAA 3′, mCHD1 siRNA; 5′ ACA TTA TGA TGG AGC TAA A 3′, mH3.3a siRNA-1; 5′ TGA AGA AAC CTC ATC GTT A 3′, mH3.3a siRNA-2; 5′ GAG AAA TTG CTC AGG ACT T 3′, mH3.3b siRNA; 5′ CAG AGA TTG GTG AGG GAG A 3′.

RNA Extraction and quantitative RT-PCR (qRT-PCR). Total RNA was purified using TRizol reagent (Invitrogen) according to the manufacturer's instructions. One microgram of RNA was reverse transcribed with cDNA synthesis kit (Promega). Real-time PCR was performed using the CFX96 System (BioRad). PCR amplification was carried out using KAPA SYBR FAST Master Mix (KAPA Biosystem) and relative quantification was done with the 2^{-CT} (Livak) method. The sequences of primers used for RT-PCR are listed in Table S1.

Chromatin Immunoprecipitation. C2C12 cells were cross-linked with 1% formaldehyde at 37 °C for 15 min. Crosslinking was quenched by addition of 0.125 M glycine for 5 min. Cells were washed with PBS and nuclei were isolated with Lysis buffer A [10 mM Tris HCl (pH 7.5), 10 mM KCl, 5 mM $MgCl_2$, 0.5% NP40], resuspended in SDS lysis buffer [50 mM Tris HCl (pH 7.9), 10 mM EDTA, 0.5% SDS], and sonicated at 0 °C to produce DNA fragments ranging between 200 and 1,000 bp using the Bioruptor (Cosmo Bio). After centrifugation, antibodies and beads were added to the chromatin samples and incubated overnight at 4 °C with rotation. After washing and de-cross-linking, DNA was purified using a PCR purification kit (Qiagen). The relative amount of immunoprecipitated DNA was represented as the percentage of input DNA (IP DNA/input).

Microarray Analysis. RNA was prepared with RNeasy Plus Mini kit (Qiagen). RNA purity and integrity were evaluated by denaturing gel electrophoresis, OD 260/280 ratio, and analysis on Agilent 2100 Bioanalyzer (Agilent Technologies). The array signal was detected using Amersham fluorolink streptavidin-Cy3 (GE Healthcare Bio-Sciences) and scanned with an Illumina bead array reader confocal scanner. Array data export processing and analysis was performed using Illumina BeadStudio v3.1.3 (Gene Expression Module v3.3.8).

Fusion Index. Differentiated cells were immunocytochemically stained with MHC (myosin heavy chain) antibody and then cell fusion was measured by calculating the percentage of nuclei positioning in a consecutive manner in MHC-positive cells.

Chromatin Accessibility Assay. Nuclei isolated from C2C12 cells were resuspended in 1× reaction buffer and incubated with different units of either *Pvu*II or micrococcal nuclease (New England Biolabs) at 37 °C. Samples were phenol-extracted and ethanol-precipitated and subjected to real-time PCR analysis. Each sample was normalized to nondigested input control.

Immunoblot Analysis of Histones. For detection of histones, cell lysates were briefly sonicated with Bioruptor (Cosmo Bio) to release histones from chromatin. Equal amounts of protein (20–50 μg) were separated on 15% SDS-PAGE gel and transferred onto 0.2-μm nitrocellulose membranes. The membranes were blocked with 5% skim milk and incubated with primary antibodies diluted in the range of 1:10,000–1:1,000. After wash with TBST [5 mM Tris HCl (pH 7.4), 150 mM NaCl, 0.01% Tween 20], secondary antibodies were added and incubated for 1 h at RT. Signals were visualized by ECL detection (Abfrontier).

Immunoprecipitation. Cells were harvested and lysed in NETN buffer [25 mM Tris HCl (pH 8.0), 1 mM EDTA, 150 mM NaCl, 0.5% (vol/vol) NP40] containing protease inhibitors. Following brief sonication and clarification, anti-FLAG antibody was added

to 0.5–1 mg whole cell lysates. Immunoprecipitates were separated by SDS-PAGE and immunoblotted with respective antibodies.

Immunofluorescence. Cells were fixed with 4% (wt/vol) paraformaldehyde and permeabilized with 0.5% Triton X-100 in PBS. The cells were then blocked with 2% (wt/vol) bovine serum albumin in PBS and incubated with primary antibody. Cy3-conjugated AffiniPure goat anti-rabbit IgG and Fluorescein (FITC)-conjugated AffiniPure goat anti-mouse IgG (Jackson Immunoreaearch) were used as secondary antibodies. DNA in the nuclei was stained with DAPI (2 μg/mL, Molecular Probe). Immunofluorescence was examined using a LSM-510 Confocal microscope (Carl-Zeiss).

Antibodies. Antibodies used in this study: anti-HIRA [WC15 for chromatin immunoprecipitation (ChIP) (10–15 μg/IP) and WC119 for immunoblotting assay (1:100), a gift from P. Adams, Beatson Institute, Glasgow, UK], anti-Asf1a [Cell Signaling, 2990 for ChIP (1:100) and for immunoblotting (1:1000)], anti-CAF1 p150 (Abcam, ab7655), anti-CHD1 (BETHYL, A301-218A), anti-MyoD (Santa Cruz, sc-304), anti-myogenin (Santa Cruz, sc-576), anti-MHC (Upstate, 05-716), 8WG16 (Covance, MMS-126R), anti-Ezh2 (Cell Signaling, 4905), anti-SIRT1 (Upstate, 07-131), anti-H1 (Upstate, 05-457), anti-H3 (Abcam, ab1791), anti-H3K4me2 (Upstate, 07-030), anti- H3K4me3 (Upstate, 07-473), anti-H3K9me3 (Abcam, ab8898), anti-H3K27me3 (Upstate, 05-851), anti-HA (Roche, 11 666 606 001), anti-FLAG (Sigma, F 3165), anti-α-tubulin (Abfrontier, LF-PA0146), anti-GAPDH (Santa Cruz, sc-25778). The specificity of HIRA antibodies (WC15 and WC119) has been described in Hall et al. (1)

1. Hall C, et al. (2001) HIRA, the human homologue of yeast Hir1p and Hir2p, is a novel cyclin-cdk2 substrate whose expression blocks S-phase progression. *Mol Cell Biol* 21:1854–1865.

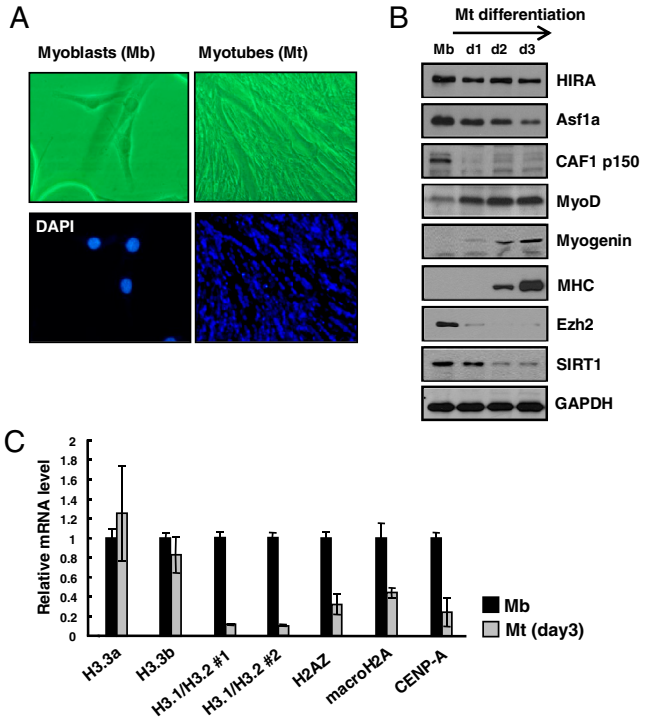

Fig. S1. HIRA is maintained during skeletal myogenesis. (*A*) C2C12 cells were induced to differentiate for 3 d, and myotube formation was observed by phase contrast microscopy. DAPI was used to stain nuclei. (*B*) Whole cell extracts prepared from each differentiation day were subjected to immunoblotting assay with indicated antibodies. GAPDH was used as a loading control. MHC, MyoD, Ezh2, myogenin, and SIRT1 are used as myogenesis markers. (*C*) The mRNA extracted from C2C12 Mb (myoblasts) or Mt (myotubes) was analyzed by quantitative RT-PCR. GAPDH was used for normalization.

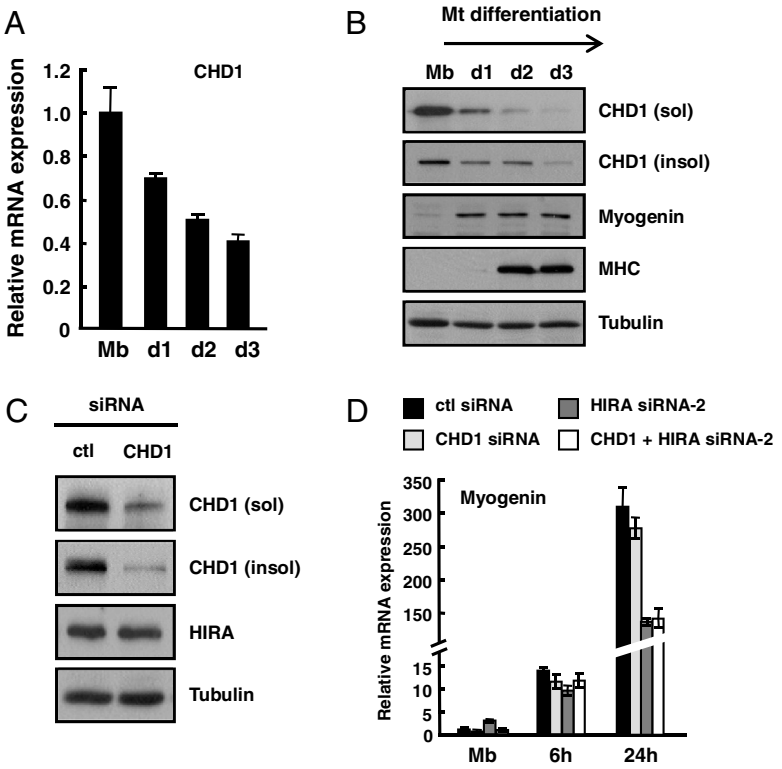

Fig. S2. *CHD1* expression decreases during skeletal myogenesis. (*A*) The mRNA level of *CHD1* was analyzed by quantitative real-time RT-PCR. (*B* and *C*) Soluble (sol) and insoluble (insol) fractions were analyzed to monitor CHD1. The levels of individual proteins were monitored by immunoblotting analysis. (*D*) quantitative RT-PCR analysis of *myogenin* mRNA levels in control (ctl), siCHD1, or siHIRA-2 treated C2C12 cells at each differentiation time point.

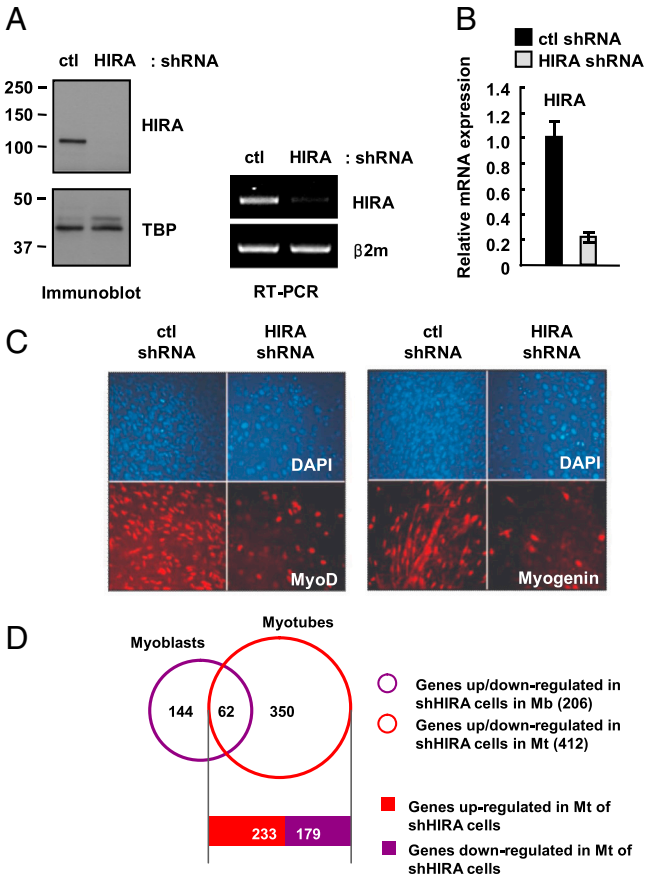

Fig. S3. HIRA has a role in gene regulation during skeletal myogenesis. (*A* and *B*) The efficiency of knockdown of HIRA was measured by immunoblotting analysis (*A*, *Left*), semiquantitative (*A*, *Right*) or quantitative RT-PCR (*B*). TATA binding protein (TBP) and β2m were used as controls. (*C*) ctl and HIRA shRNA C2C12 cells were stained with anti-MyoD or anti-myogenin to monitor myogenic differentiation. (*D*) Genome-wide analysis shows genes up- or down-regulated by HIRA knockdown, greater than 1.5-fold in Mb (myoblasts) or in Mt (myotubes). Two hundred six and 412 genes were changed in Mb and Mt, respectively, by HIRA depletion, with 62 genes overlapped. We focused on the 412 genes changed in Mt to identify a link between HIRA and the myogenic differentiation process. Of 412 genes, 233 genes were up-regulated and 179 genes were down-regulated upon HIRA depletion.

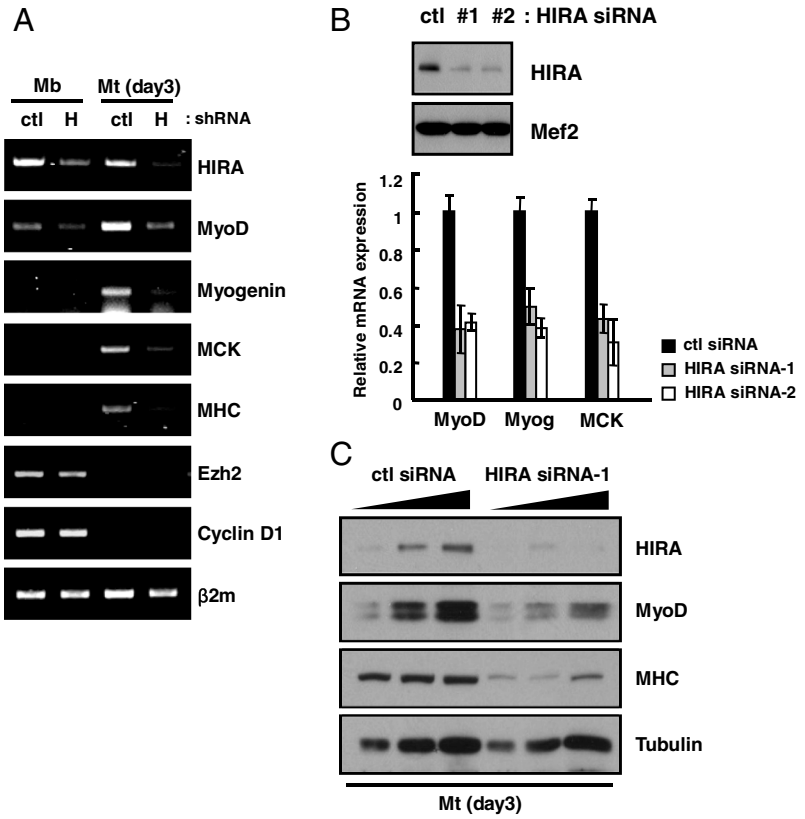

Fig. S4. HIRA knockdown attenuates myogenic differentiation. (*A*) cDNA prepared from ctl and shHIRA C2C12 myoblasts or myotubes was subjected to semiquantitative RT-PCR for indicated genes. (*B*) C2C12 cells were transfected with ctl or two independent HIRA siRNAs (#1 and #2) and allowed to differentiate for 3 d. Their effect on expression of muscle marker genes was examined by quantitative RT-PCR. (*C*) Whole cell extracts prepared from the control and HIRA siRNA-1(#1)-treated cells were loaded in three different amounts and immunoblotted with the indicated antibodies to analyze protein levels of target genes. The β2m (*A*) and tubulin (*C*) were used as loading controls.

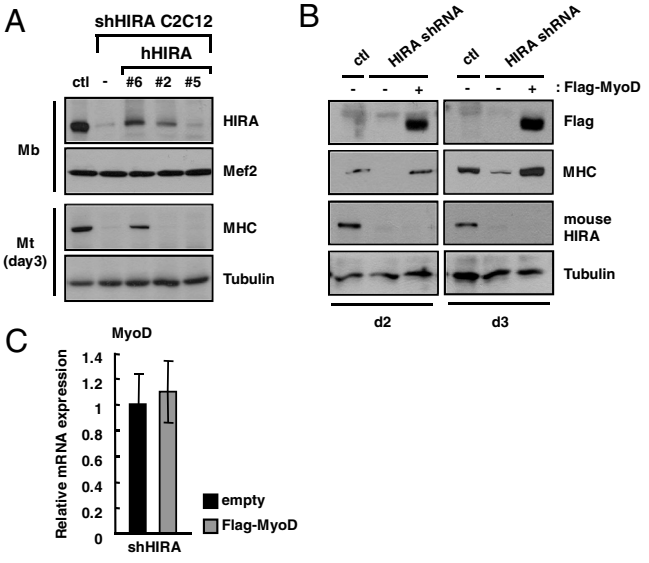

Fig. S5. HIRA regulates *MyoD* at the level of transcription. (*A*) Normal C2C12 cells (ctl) and rescued clones were subjected to immunoblotting analysis (anti HIRA Ab WC119) as indicated. (*B*) FLAG-tagged MyoD was ectopically expressed in the shHIRA C2C12 cells. Expression of FLAG-MyoD and MHC was monitored by immunoblotting (on differentiation day2 and day3). (*C*) The mRNA level of endogenous *MyoD* of shHIRA C2C12 cells was not affected by an ectopic expression of FLAG-MyoD. The mRNA level of endogenous *MyoD* was analyzed by specific qRT-PCR primers with the RNAs prepared from shHIRA cells that express empty or FLAG-MyoD.

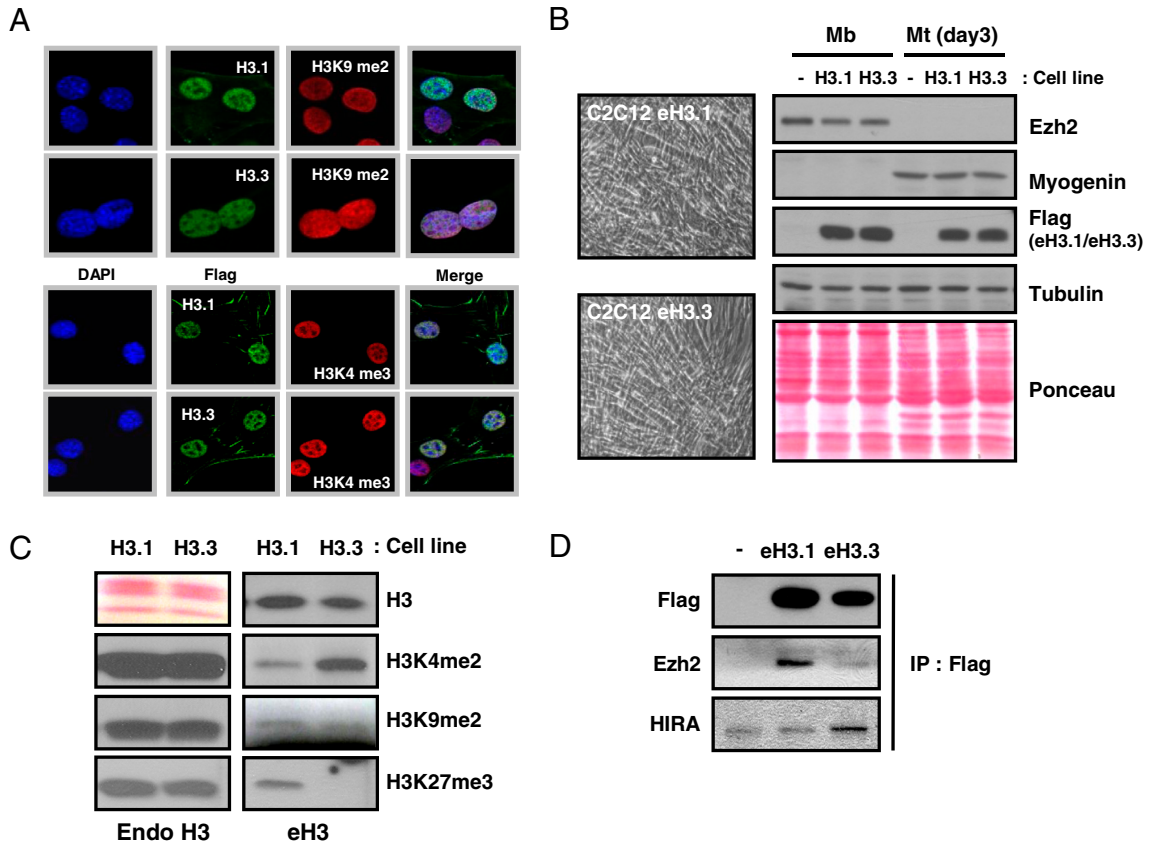

Fig. S6. Epitope-tagged H3 (eH3) C2C12 cells differentiate normally. (*A*) C2C12 myoblasts stably expressing Flag-H3.1 or H3.3 were stained with anti-FLAG, anti-H3K9me, or anti-H3K4me. eH3 was appropriately localized in nucleus and did not perturb distribution patterns of methylated H3. (*B*) ctl and eH3 C2C12 cells were differentiated for 3 d, and the efficiency of myogenic differentiation was monitored by examining the expression patterns of Ezh2 (myoblast specific) and myogenin (myotubes specific). Myotube formation (*Left*) and the levels of Ezh2 and myogenin (*Right*) were evaluated by phase contrast microscopy and immunoblotting, respectively. (*C*) Posttranslational modifications of endogenous H3 and FLAG-HA tagged H3 (eH3, ~20 KDa) in eH3.1- or eH3.3-expressing C2C12 cells were analyzed by immunoblotting with the indicated antibodies. Total histones were visualized by Ponceau stain. (*D*) Cell extracts prepared from eH3 C2C12 cells were immunoprecipitated with anti-FLAG antibody and then subjected to immunoblotting with the indicated antibodies.

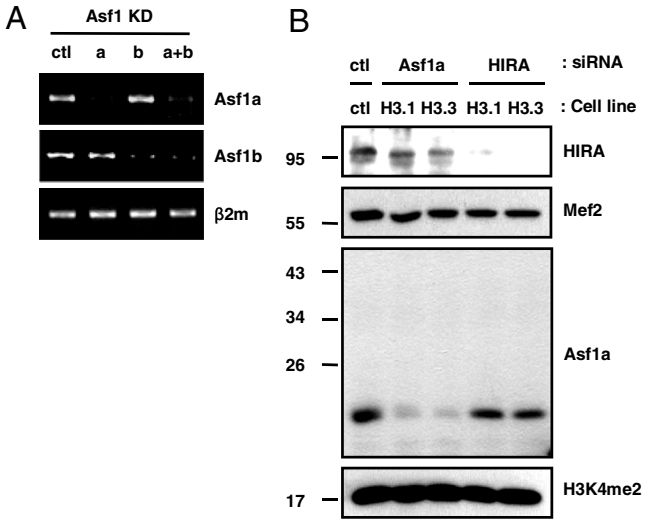

Fig. S7. RNA interference to target Asf1a/b or HIRA (*A*) Asf1a, Asf1b, or both were knocked down with specific siRNA or shRNA. C2C12 cells stably expressing control or Asf1b shRNA were constructed using the lentiviral RNAi system. These cells were used for siRNA transfection to target Asf1a. Isotype-specific knockdown efficiency was analyzed by semiquantitative RT-PCR. (*B*) eH3 C2C12 cells were transfected with siRNA targeting either Asf1a or HIRA(siRNA #1). Immunoblotting was performed to evaluate levels of the indicated proteins.

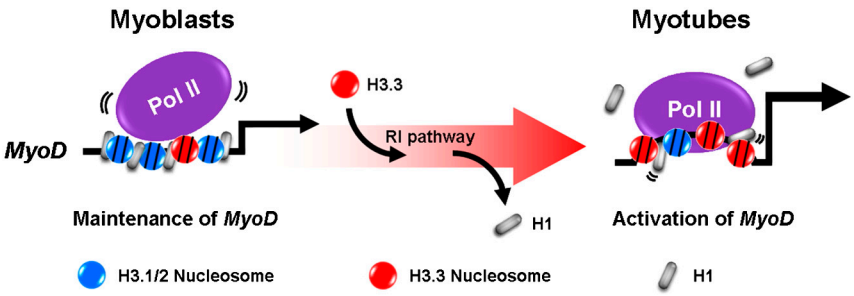

Fig. S8. A model for *MyoD* activation by RI histone deposition pathway. Repressive histone H1 binds CER and PRR to minimally maintain *MyoD* expression during myoblast proliferation. Upon differentiation, RI pathway actively mediates H3.3 deposition into regulatory regions of *MyoD* to drive *MyoD* activation with concomitant loss of H1.

Table S1. Primers used for RT-PCR and ChIP

RT-PCR

Primer name	Direction	Sequence
HIRA	Forward	CTC CAT CTT GTC AGG AAG TGA T
	Reverse	GTT CCT GGC ACT CAG TAA AGA G
Asf1a	Forward	CAC CGA ATG CAG GAC TCA TC
	Reverse	GCA TCT GTT GAA AGA AGG GAC TG
Asf1b	Forward	CCC AAA CCC ATC CCT CAT TC
	Reverse	GAA AGG CTG AAG TCC ACA TTG G
CAF1 p150	Forward	GCA ATG TGA ATG GGA GCA AG
	Reverse	GGT GGT GAT GTA GGT CCA CTG
CHD1	Forward	ATC ACA AGG ACC GCC ATC AGG
	Reverse	TCT CGA CTC CTG TGG TCA TCC AGT
H3.1/H3.1 #1	Forward	GAA GAA GCC TCA CCG CTA CCG
	Reverse	GGT TGG TGT CCT CAA ACA GAC CC
H3.1/H3.2 #2	Forward	CTA AGC AGA CCG CTC GCA AGTC
	Reverse	CTT GAA GTC CTG CGC GAT CTC
H3.3a	Forward	GGC TCG TAC AAA GCA GAC TGC C
	Reverse	GGG AGC TTG CGG ATC AGA AG
H3.3b	Forward	CCA AGC AGA CCG CTA GGA AGT C
	Reverse	CGA TGG CTG CAC TTT GAA ACC
Ezh2	Forward	GCA CTT ACT GCT GGC ACC GTC
	Reverse	GCC TAT CCT GTG GTC ACC ATT AAC C
cyclin D1	Forward	GCT CAA GAC GGA GGA GAC CTG TG
	Reverse	CAG TGT AGA TGC ACA ACT TCT CGG G
MyoD	Forward	ACT TTC TGG AGC CCT CCT GGC A
	Reverse	TTT GTT GCA CTA CAC AGC ATG
p21	Forward	TCA CTC TGT GTG TCT TAA TTA
	Reverse	AGG ACT GTT CCT CCG GTA TAG G
Myogenin	Forward	CTA AAG TGG AGA TCC TGC GCA GC
	Reverse	GCA ACA GAC ATA TCC TCC ACC GTG
MHC	Forward	AGG GAG CTT GAA AAC GAG GT
	Reverse	GCT TCC TCC AGC TCG TGC TG
MCK	Forward	CTT CAT GTG GAA CGA GCA CCT G
	Reverse	GCG TTG GAG ATG TCG AAC ACG
GAPDH	Forward	CCA ATG TGT CCG TCG TGG ATC T
	Reverse	GTT GAA GTC GCA GGA GAC AAC C
β2m	Forward	GTC TTT CTG GTG CTT GTC TCA
	Reverse	GGC GTA TGT ATC AGT CTC AGT
ChIP & ChART-PCR		
MyoD CER	Forward	GGG CAT TTA TGG GTC TTC CT
	Reverse	CTC ATG CCT GGT GTT TAG GG
MyoD DRR	Forward	TCA GGA CCA GGA CCA TGT CT
	Reverse	CTG GAC CTG TGG CCT CTT AC
MyoD PRR	Forward	GAG TAG ACA CTG GAG AGG CTT GG
	Reverse	GAA AGC AGT CGT GTC CTG GG
MyoD CD1	Forward	CAT CTG ACA CTG GAG TCG CTT TG
	Reverse	CAA GCA ACA CTC CTT GTC ATC AC
MyoD CD2	Forward	GTG AGC CTT GCA CAC CTA AGC C
	Reverse	GTT GCA CTA CAC AGC ATG CCT G
MyoD −15 Kb	Forward	TGC CCA GAG CCT AGA ATC AT
	Reverse	TCA TGC ATC CTT GCT GGA TA
MyoD −7 Kb	Forward	GGC ATG GGA GGT TTA TAG CA
	Reverse	ATG CCA CTA TGC AAT CCA CA
GAPDH −1.5 Kb	Forward	GAC TCT GAA TCT GCC ATG CCT C
	Reverse	CCA GAG CCA AGG CTG TGT TAG