SUPPLEMENTARY DATA

Essential role of p18^{Hamlet}/SRCAP-mediated histone H2A.Z chromatin incorporation in muscle differentiation

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Supplementary Materials and Methods

Chromatin immunoprecipitation (ChIP)

Cells were fixed for 15 min in medium containing 1% formaldehyde, and the cross-linking reaction was stopped by adding glycine to 125 mM final concentration. Cells were washed twice with ice-cold PBS containing protease inhibitors cocktail (Roche), scrapped on ice and harvested by centrifugation at 2,000 rpm at 4ºC. The resulting pellet was then lysed in an appropriate volume (10^6 cells per 100 ml) of lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8.1 plus protease inhibitor cocktail (Roche) and chromatin was sheared in a bath sonicator (Diagenode Bioruptor) to an average length of 0.2-0.8 kb. Samples were then centrifuged at 13,000 rpm for 10 min at 13˚C, and supernatants containing the fragmented chromatin were collected. Fragmented DNA (200 μg per antibody and condition, including a no-antibody control sample) was diluted in 10x dilution buffer (1% Triton X-100, 2 mM EDTA pH 8.0, 150 mM NaCl, 20 mM Tris-HCl pH 8.1), pre-cleared for 1 h at 4ºC with 60 μl of a 50% slurry of salmon sperm DNA/protein A or protein G agarose (Upstate), and incubated overnight at 4ºC with 2-5 μg of the following antibodies: anti-Histone H3 (1791, Abcam), anti-Histone H2A.Z (05-594, Upstate), anti-MyoD (M-318, Santa Cruz), anti-SRCAP (sc-133312) and anti-p18^{Hamlet} rabbit serum. An aliquot of each sample (20-50 μg, INPUT) was frozen and kept at -80˚C until the reverse cross-linking step. After 14 h at 4ºC in a rotating platform, samples were incubated for 1h more in the presence of 60 μl of salmon

sperm DNA/protein A/G agarose 50% slurry. Immune complexes were then washed once each in the following buffers: low salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA pH 8.0, 20 mM Tris-HCl pH 8.1, 150 mM NaCl), high salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA pH 8.0, 20 mM Tris-HCl pH 8.1, 500 mM NaCl) LiCl wash buffer (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1mM EDTA, 10 mM Tris-HCl pH 8.1), and twice in TE. Immune complexes were eluted after two rounds of incubation in 250 μl of freshly prepared elution buffer (1% SDS, 0.1 M $NaHCO_3$), and the cross-linking was reverted by addition of 20 μl 5M NaCl followed by incubation at 65º C for 4 h. After 1 h of digestion with proteinase K, DNA was recovered by phenol/chloroform extraction and ethanol precipitation.

Supplementary Figure legends

Supplementary Figure S1. C2C12 myoblasts were cultured in GM (0) or incubated in DM for the indicated times, either in the presence or absence of SB203580 (SB), and the binding of H2A.Z to the region "C" of the myogenin promoter (see Fig. 2A) was analysed by ChIP and normalised to histone H3 binding.

Supplementary Figure S2. Wt and p38α-/- primary myoblasts, infected with either control or p38α-expressing retrovirus, were cultured in GM and then shifted to DM for 14 h. H2A.Z binding to the myogenin TATA-box containing region was analysed by ChIP and qPCR (*upper panel*). Relative binding values are referred to the p38α expression levels, which were determined by immunoblotting using tubulin as a loading control (*lower panel*). The binding in wt myoblasts was given the value of 1. Myogenin expression was determined by immunoblotting.

Supplementary Figure S3. C2C12 myoblasts were incubated with either p18^{Hamlet} or H2A.Z siRNAs and then cultured in GM or incubated in DM for 14 h. MyoD binding to the myogenin TATA box region was quantified by qRT-PCR. The histogram shows values of triplicate qPCR reactions. Error bars represent s.d.

Supplementary Figure S4. C2C12 myoblasts were incubated with either p18^{Hamlet} or H2A.Z siRNAs and 48 h later p18^{Hamlet} and H2A.Z protein levels were analysed by immunoblotting. Tubulin was used as a loading control.

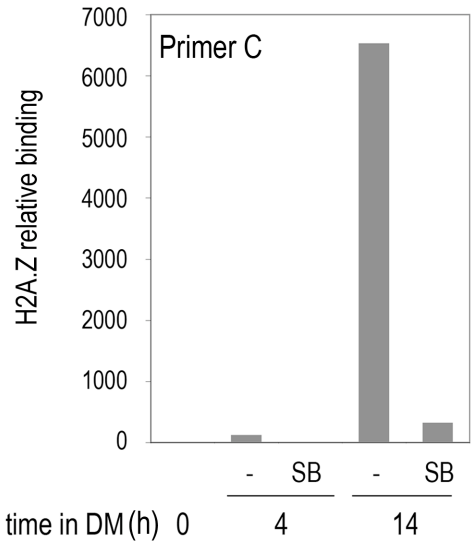
Supplementary Figure S5. C2C12 myoblasts were transfected with 100 nM of cyclophylin B (control) or two different p18^{Hamlet} siRNAs and, 48 h later, p18^{Hamlet} mRNA levels were analysed by qRT-PCR and normalised to GAPDH.

Supplementary Figure S6. Myogenin protein levels induced by p18^{Hamlet} overexpression are very similar to those observed upon DM treatment. C2C12 myoblasts were transfected with a p18^{Hamlet} expression construct and clones were selected with G418. Myogenin protein levels were analysed in C2C12 myoblasts incubated in DM for the indicated times and compared with those present in p18^{Hamlet} overexpressing clones maintained in GM. Tubulin was used as a loading control.

Supplementary Figure S7. RNA was purified from C2C12 myoblasts that were transfected with control or p18^{Hamlet}-oligo2 siRNAs and then maintained in DM for the indicated times or in GM. Myogenin and MHC mRNA levels were analysed by qRT-PCR. GAPDH mRNA levels were used for normalization. The histogram shows values of triplicate qRT-PCR reactions. Error bars represent s.d.

Supplementary Figure S8. Primers used for mRNA amplification in qRT-PCR analysis.

Supplementary Figure S9. Commercial antibodies used for immunoblotting.



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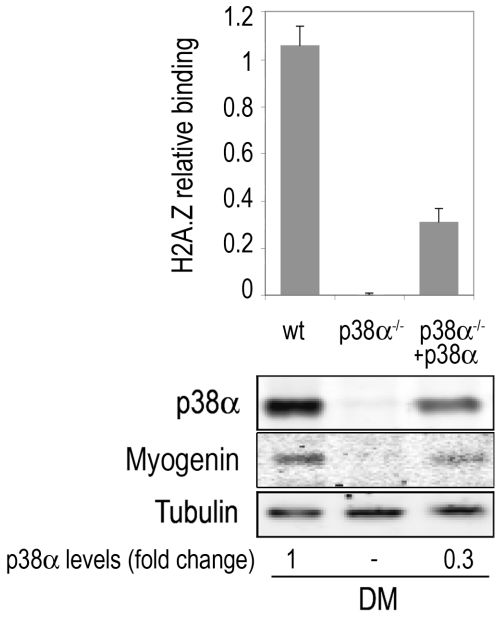
Supplementary Figure S1



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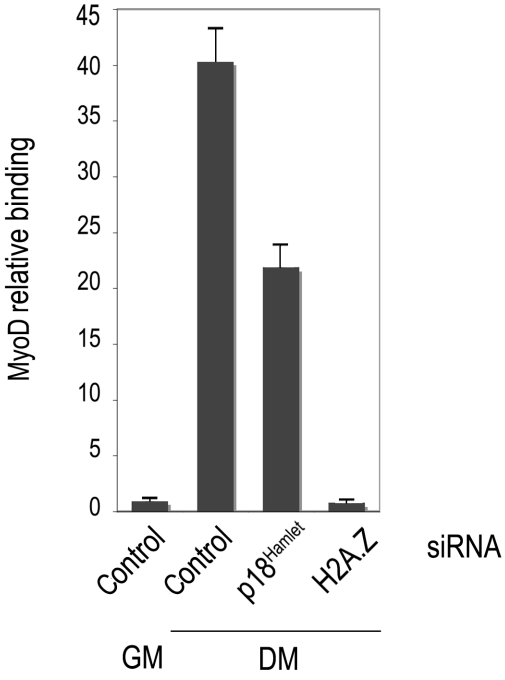
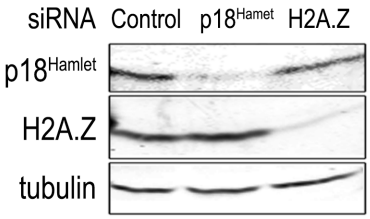
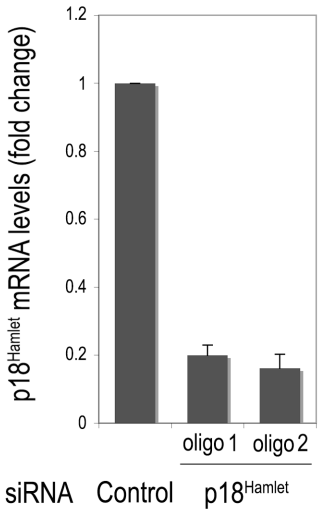
Supplementary Figure S2



Cuadrado et al. Supplementary Figure S3



Cuadrado et al. Supplementary Figure S4



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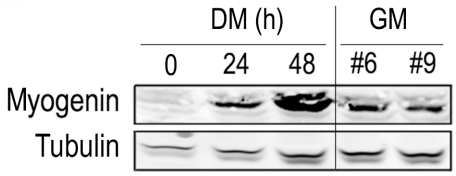

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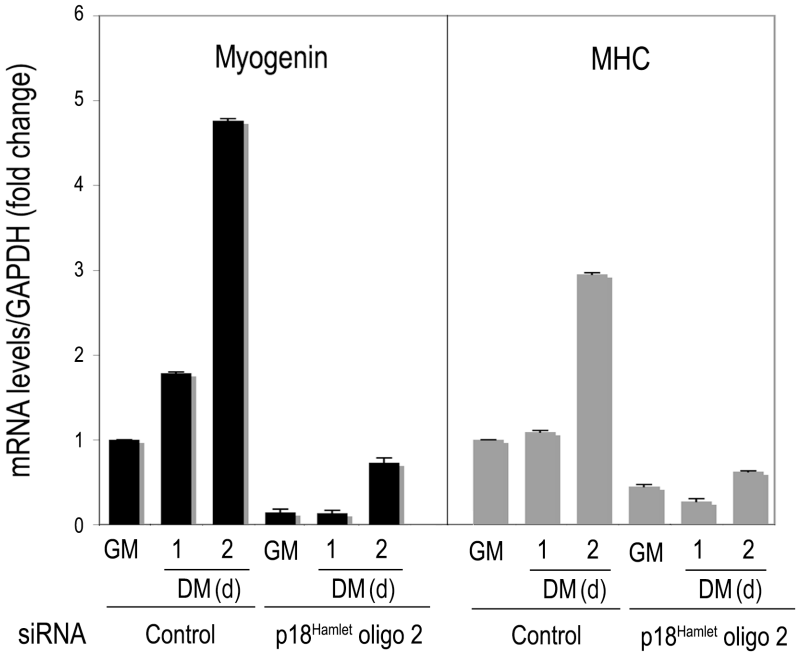

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mRNA	Primer sequence (5'-3')
MCK	fw: AGGCATGGCCCGAGAC rev: AGATCACGCGAAGGTGGTC
MHC	fw: CTCCAGGCTGCTTTAGAGGAA rev: CCTGCTCCTAATCTCAGCATCC
Myogenin	fw: GGTGTGTAAGAGGAAGTCTGTG rev: TAGGCGCTCAATGTACTGGAT
p18^{Hamlet}	fw: CTCGAGTTACACGGTCCACTTCAGACA rev: AAGCTTATGCAGACGGCGAGACAAGTT
H2A.Z	fw: CGTATCACCCCTCGTCACTT rev: AAGCCTCCAACTTGCTCAAA
YL-1	fw: GACCAGTCTGATACGGAG rev: AGCGTCTTCTCCTCTCTG
SRCAP	fw: AACAGGGGTCAGAAGTGGG rev: CCTGAGCAAAGTCAGCAGA
p400	fw: CAGTTGTGGGAGGAAACCA rev: GTTGCTGCATATGCCCAG
GAPDH	fw: GAGGGGCCATCCACAGTCTTC rev: GACCCCTTCATTGACCTCAAC

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Myogenin	F5D. Santa Cruz Biotechnology
p38	C20. Santa Cruz Biotechnology
MHC	MF20. Developmental Studies Hybridoma Bank
eMHC	F1.652. Developmental Studies Hybridoma Bank
phospho-p38	9211. Cell Signaling
phospho-Thr	9381. Cell Signaling
H2A.Z	07-1594. Upstate
YL-1	28708-50. Abcam
α-tubulin	DM1A. Sigma
SRCAP	sc-133312 (F-15). Santa Cruz Biotechnology sc-133310 (C-13). Santa Cruz Biotechnology

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