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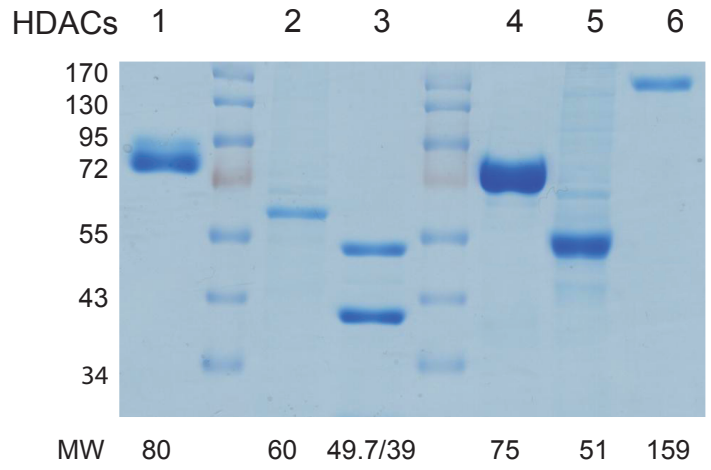

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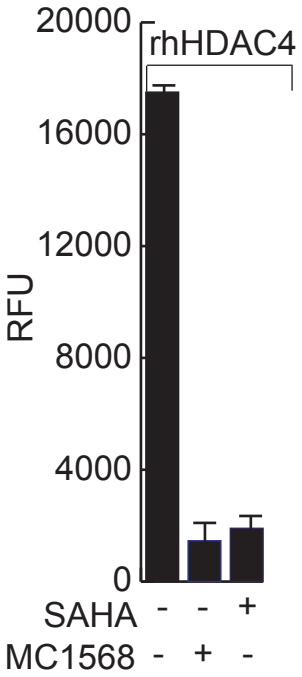
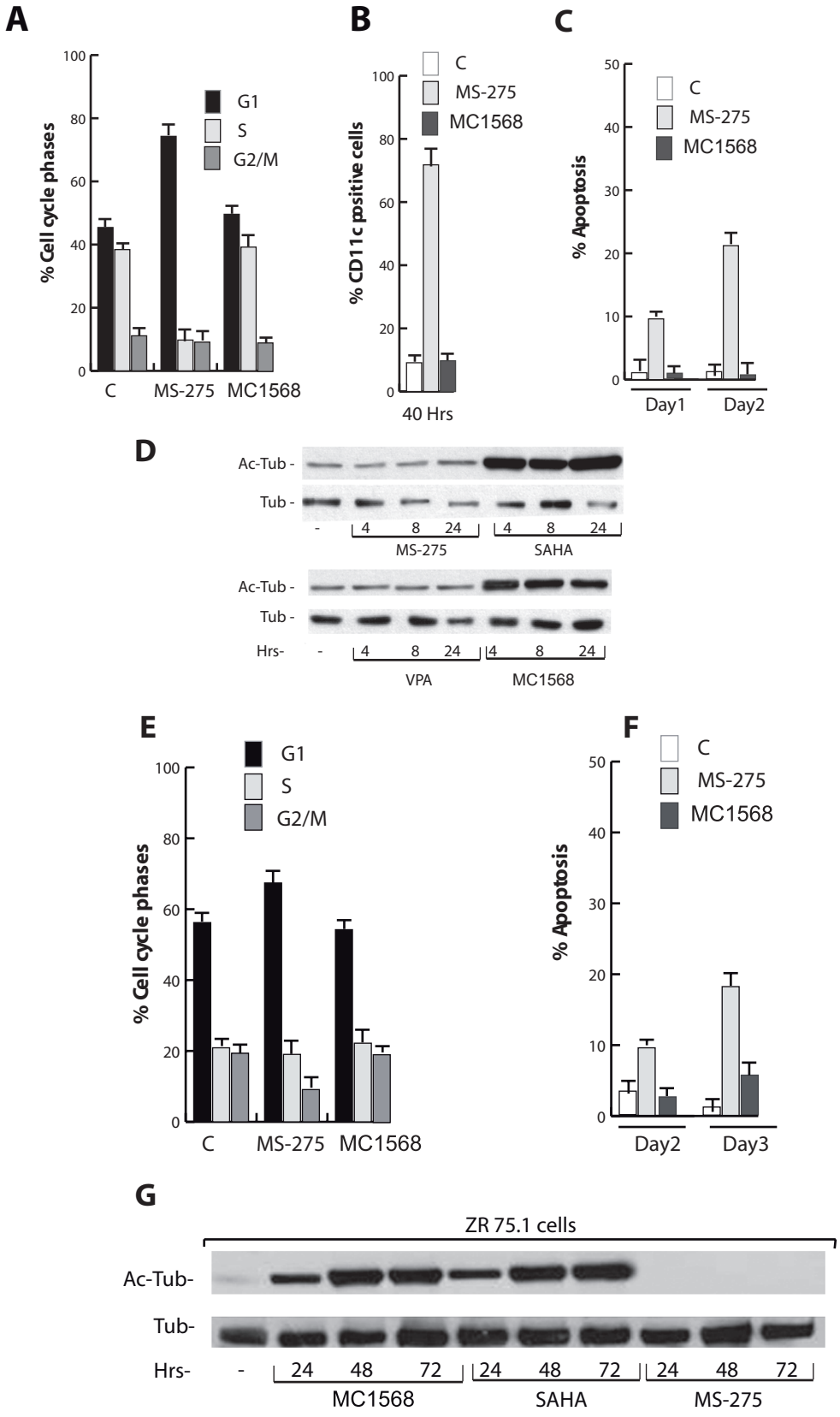
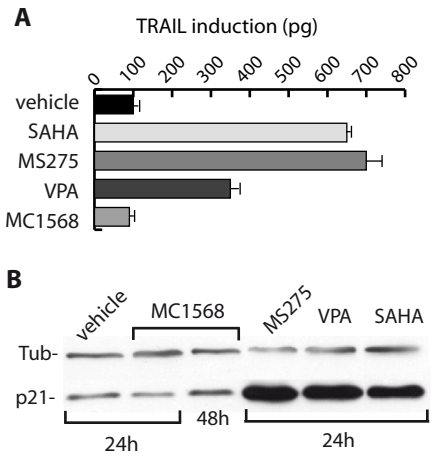


Supplementary Figure 1: Purity of the recombinant HDAC proteins.
(A) Recombinant HDACs have been visualised on a coumassie coloured acrylamide gel; Note that NCoR (39KD) is present together with HDAC3; 3-7 μg have been loaded.
(B) rhHDAC4 assay on non-histone substrate after 2 h of incubation.
Saha and MC1568 have been used at 5 μM.



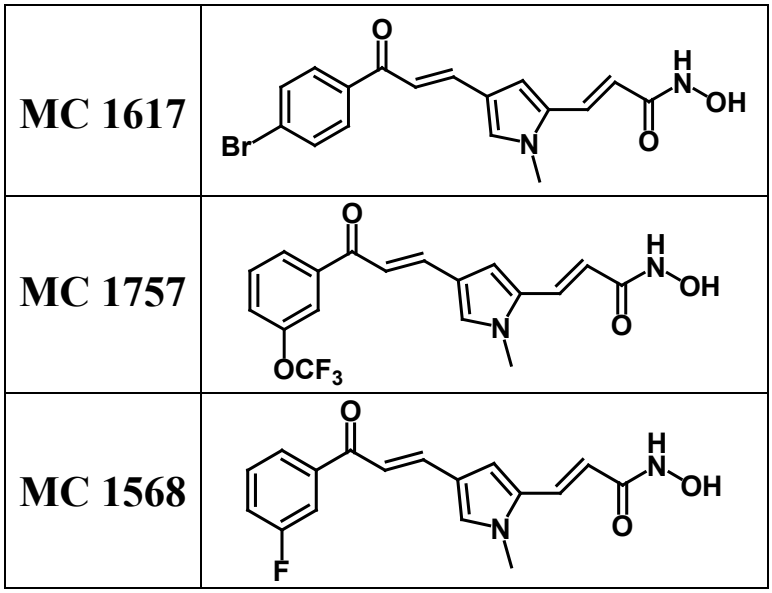
Suppl- Figure 2. Biological activity of class specific HDAC inhibitors in hematological and solid cancer models
(A) cell cycle analysis at 40 hours,
(B) granulocytic differentiation and
(C) apoptosis assay in U937 cells after treatment with 5μM MS275 or 5μM MC1568;
(D) α-tubulin acetylation levels compared with tubulin expression levels in U937 cells upon stimulation with the indicated treatments at the indicated time course;
(E) cell cycle analysis at 48 hours and
(F) apoptosis assay in breast cancer cells ZR75.1 after 48 and 72 hours of treatment with 5μM MS275 or 5μM MC1568;
(G) α-tubulin acetylation levels compared with tubulin expression levels in ZR75.1 cells upon stimulation with the indicated treatments at the indicated time course;

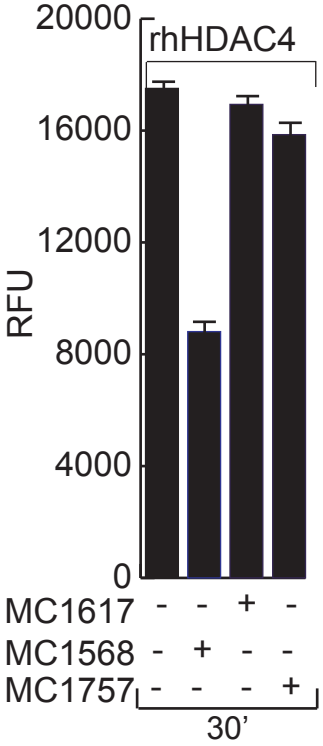


Nebbioso et al, Suppl. Fig 3

Suppl. Figure 3. The specific inhibitor MC1568 does not induce known target of pan and class I HDAC inhibitors.
(A) ELISA assay for TRAIL expression at 16 hours upon induction with the indicated HDAC inhibitors;
(B) Western blot to reveal p21 induction by HDAC blocking class I HDACs (MS275, SAHA, VPA);

A





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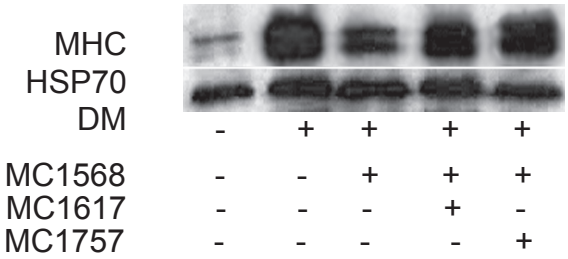

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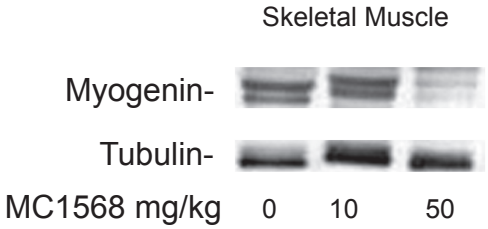
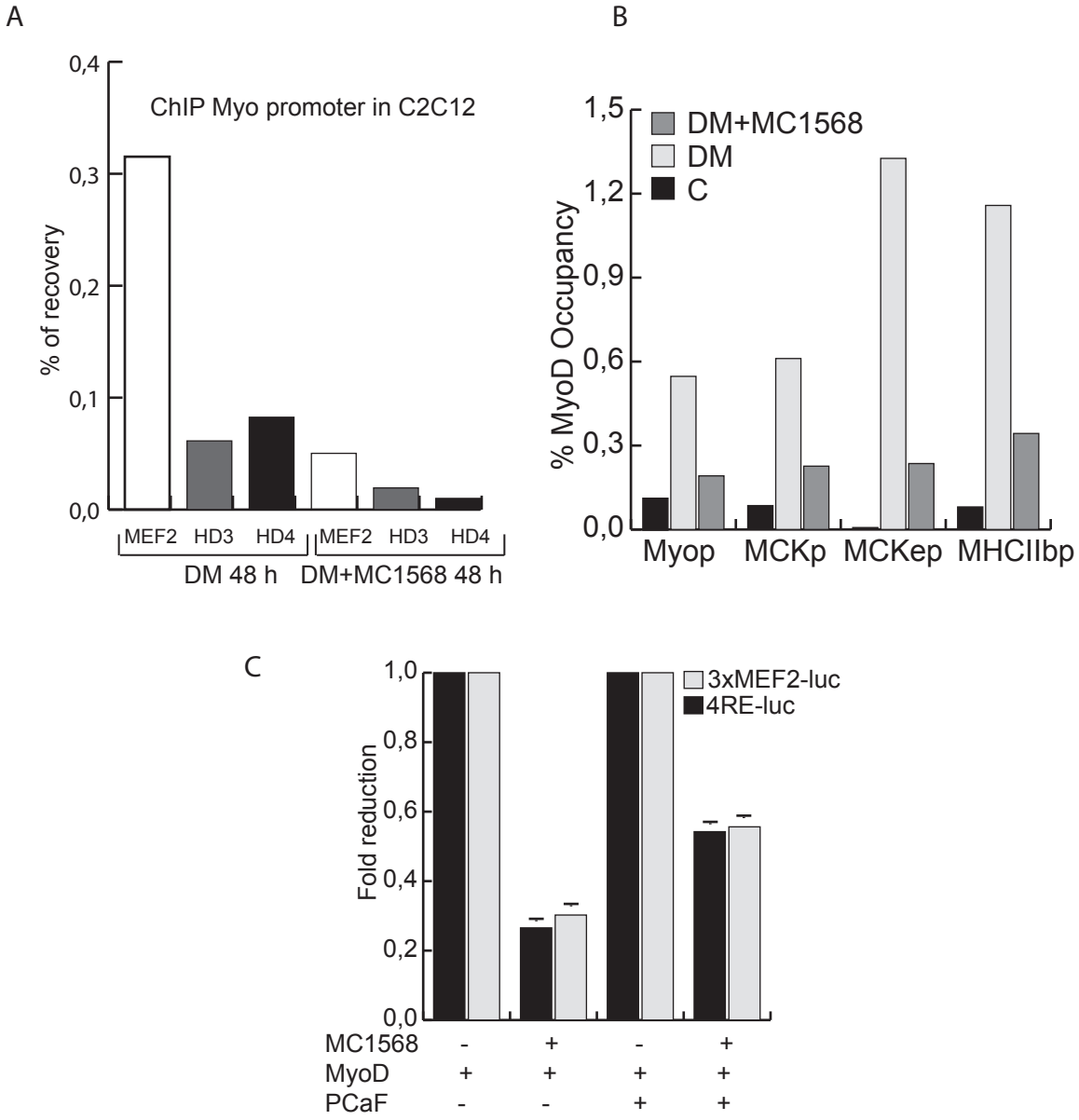
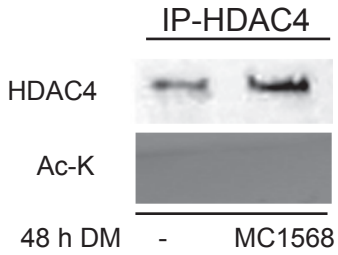


Supplementary Figure 4. Chemical structure of MC1568 analogues and their activity.
(A) Chemical structure and activity on rhHDAC4 on non histone substrate at the concentration of 5 μM;
(B) Western blot analyses of MHC expression levels in C2C12 cells after 48 h of incubation with differentiation medium (DM) alone or in presence of the indicated compounds used at 5 μM.
(C) Western blot analyses of myogenin expression levels in skeletal muscles of CD1 mice treated at the indicated concentrations with MC1568 for 2 days.



Suppl. Figure 5. MEF2D, HDAC3, HDAC4 and Myo D occupancy on responsive promoters after MC1568 treatment.
A. ChIP experiments carried out in C2C12 cells after 48 h of treatment with MC1568 at 5 μM show that MEF2D, HDAC3 and HDAC4 are not regulating the Myogenin promoter. Differently with the sole DM MEF2D is enriched on the promoter.
B. ChIP experiments in C2C12 cells after 48 h of treatment with MC1568. MyoD occupancy on the indicated promoters is enriched during differentiation, but MC1568 counteract this increase.
C. Transfection experiments in C2C12 cells of the reporters 4REluc and 3xMEFluc in presence of MyoD show that the MC1568 is able to reduce the reporter activity. When PCaf is added the reduction occurs to a lower extent.



Supplementary Fig. 6 HDAC4 seems not to be directly acetylated in differentiating C2C12 cells upon treatment with MC1568. HDAC4 has been IP*ed* after 48 h of the indicated treatments. The immunoprecipitated have been tested for HDAC4 (upper panel) and for the signal due to acetylated lysines (lower panel). Note that the signal due to acetylated lysines is over-exposed to assure that no signal is detected in this system.

Mice treatment & Tissue homogenization.

3 months old CD1 mice were housed in cages (29 x 17.5 x 12.5 cm) at constant temperature (22° ± 1°C) and maintained on a 12/12h light/dark cycle, with food and water *ad libitum*. Experiments were in conformity with protocols approved by the Italian Ministry of Health and in accordance to the ethical and safety rules provided by EU directives (n. 86/609/EC). Efforts were made to minimize animal's suffering. Mice were injected with MC1568 at the indicated doses every 2 days for 10 days or in acute administration for 6h. Animals were sacrificed, organs dissected and frozen in N_2. For homogenization tissues have been cut into small pieces, resuspended into in 5 volumes of RIPA Buffer.

Immunoprecipitation assay. C2C12 cells or tissue extracts were lysed in TAP buffer (Tris-HCl pH 7.0, 50 mM NaCl 180 mM, NP-40, 0.15% glycerol, 10% $MgCl_2$, 1.5 mM $NaMO_4$ 1 mM NaF) with protease inhibitors (Sigma), 1 mM DTT and 0.2 mM PMSF. 1 mg was pre-cleared with 20µl A/G plus Agarose (Santa Cruz). Antibodies [anti-Mef2D, HDAC4 or purified IgG] were added and IP was proceeded overnight at 4°C. 10 µl A/G plus Agarose were added and incubated for 2h. Beads were washed. 20 µl of 2X concentrated electrophoresis sample buffer were added.

Coupling. IgG were bound to A/G plus Agarose (Santa Cruz) in PBS for 1h. MEF2D (BD) and HDAC3 (abcam) were used. Beads have been washed into 10 V of Na Borate buffer and re-suspended in 10 V of buffer with dimethylpinelimidate (DMP) to 20 mM. The last coupling was stopped with glycine pH 7.0 for 30'. Beads were washed in 10 V of glycine 100 mM pH 2.5 and in PBS and used in IP.

ChIP assay. Acetyl H3 (Upstate), MEF2D (BD), MyoD and purified IgG (Santa Cruz) were used. ChIP assays were as described (Denissov et al., 2007; Nebbioso et al., 2005). Amplifications were done by Q-PCR (BIORAD). Primers for the myogenin promoter (-143/-5) are: (set 1) forward 5' GAATCACATGTAATCCACTGGA 3', reverse 5' ACGCCAACTGCTGGGTGCCA 3'; (set 2)

forward: 5’ CCCTGCCCCACAGGGGCTGTG 3’, Reverse: 5’ACGCCACAGAAACCTGAGCCC 3’. Primers for the MCK enhancer (-1210/-1048) are: 5’ GACACCCGAGATGCCTGGTT 3’ (forward); 5’ GATCCACCAGGGACAGGGTT 3’ (reverse); (set 2) forward: 5’ AGGGATGAGAGCAGCCACTA 3’, reverse: 5’ CAGCCACATGTCTGGGTTAAT 3’. Primers for the MCK muscle creatine kinase promoter are: forward: 5’ CGCCAGCTAGACTCAGCACT 3’, reverse: 5’ CCCTGAGAGCAGATGAGCTT3’. Primers for the myosin heavy chain (MHCMHCIIb) promoter are: forward: 5’ CACCCAAGCCGGGAGAAACAGCC 3’, reverse: 5’ GAGGAAGGACAGGACAGAGGCACC 3’.

Fluorimetric human recombinant HDAC1, 2, 3, 4, 5, 6 assays. GST-HDAC1, 4, 5, 6 have been cloned into the pAcG2T baculovirus transfer vector (BD) and purified by using glutathione beads. The BD BaculoGold transfection system (BD) has been used in Sf9 insect cells for expression following supplier’s instructions. HDAC3/NCoR complex and HDAC2 proteins have been purchased (BPS; truncated proteins). Purity of all proteins (commercial and our purified proteins) has been verified as shown in Suppl. Figure 1.

The HDAC assay has been carried out as follows: the HDAC Fluorescent Activity Assay is based on the Fluor de Lys Substrate and Developer combination (BioMol) and has been carried out according to supplier’s instructions. Briefly, the Fluor de Lys Substrate, which comprises an acetylated lysine side chain, has been incubated with the purified recombinant HDAC enzymes in presence or absence of the inhibitors, for 0.5 h at 37 °C. When a different incubation time has been used, it is specified into the text. Deacetylation of the substrate sensitizes the substrate so that, in the second step, treatment with the Developer for 30 minutes produces a fluorophore. The fluorophore is excited with a 360 nm light and the emitted light (460 nm) has been quantified with a TECAN Inphinite M200 station. For different substrates please see (Lahm et al., 2007) and (Heltweg et al., 2004).

Cell based HDAC1 and 4 assay. 1000µg of total protein extracts (RIPA buffer) were immunoprecipitated with 6µg of HDAC4 (Sigma) or HDAC1 antibody (Abcam) over night at 4°C in slow rotation. As negative control the same amount of protein extracts were immunoprecipitated with purified IgG (Santa Cruz). The day after the samples were incubated with 40µl of protein A/G plus agarose (Santa Cruz) at 4°C in slow rotation for 2 hrs. The samples were than washed 6 times with RIPA buffer and twice in PBS and re-suspended in 20 µl of sterile PBS. The HDAC assay was carried out according to suppliers instructions (Upstate). Briefly, all samples immunoprecipitated with the HDAC4 and 1 or with purified IgG were pooled respectively to homogenize all samples. 10µl of the IP was incubated with a previously labelled ^{3}H-Histone H4 peptide linked with streptavidine agarose beads (Upstate). In details, 100.000 CPM of the H4-^{3}H-acetyl-peptide was used for each tube and incubated in 1X HDAC buffer with 10µl of the sample in presence or absence of the HDACi's with a final volume of 200µl. Those samples were incubated over night at 37°C in slow rotation. The day after 50µl of a quenching solution were added and 100µl of the samples were counted in duplicate after a brief centrifugation in a scintillation counter.

Transfections: C2C12 cells at a density of 2 x 10^{5} cells per well in 24-well plates were plated one day before trasfection so that cells will be 90-95% confluent at the time of trasfection. C2C12 were transfected by using lipofectamine^{TM} 2000 (INVITROGEN). The complexes contained: 200 ng of 3xMEF2-luc or 4RE-luc; 20 ng of full-length MyoD and/or 20 ng of PCAF and 100 ng of cytomegalovirus-β-galactosidase (CMV-β-Gal, used as an internal control to normalize variations in the transfection efficiency). The total quantity of DNA was adjusted at 1 µg with pBluescript. Luciferase and βgal assay, after 48 hours of differentiation, were measured using standard protocols (PROMEGA) at the TECAN inphinite200.