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Heterochromatin protein (HP)1γ is not only in the nucleus but also in the cytoplasm interacting with actin in both cell compartments

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ABSTRACT

Confocal and electron microscopy images, and WB analysis of cellular fractions revealed that HP1γ is in the nucleus but also in the cytoplasm of C2C12 myoblasts, myotubes, skeletal and cardiac muscles, N2a, HeLa and HEK293T cells. Signal specificity was tested with different antibodies and by HP1γ knockdown. Leptomycin B treatment of myoblasts increased nuclear HP1γ, suggesting that its nuclear export is Crm-1-dependent. HP1γ exhibited a filamentous pattern of staining partially co-localizing with actin in the cytoplasm of myotubes and myofibrils. Immunoelectron microscopic analysis showed high-density immunogold particles that correspond to HP1γ localized to the Z-disk and A-band of the sarcomere of skeletal muscle. HP1γ partially co-localized with actin in C2C12 myotubes and murine myofibrils. Importantly, actin co-immunoprecipitated with HP1γ in the nuclear and cytosolic fractions of myoblasts. Actin co-immunoprecipitated with HP1γ in myoblasts incubated in the absence or presence of the actin depolymerizing agent cytochalasin D, suggesting that HP1γ may interact with G-and F-actin. In the cytoplasm, HP1γ was associated to the perinuclear actin cap that controls nuclear shape and position. In the nucleus, re-ChIP assays showed that HP1γ-actin associates to the promoter and transcribed regions of the house keeping gene *GAPDH*, suggesting that HP1γ may function as a scaffold protein for the recruitment of actin to control gene expression. When HP1γ was knocked-down, myoblasts were unable to differentiate or originated thin myotubes. In summary, HP1γ is present in the nucleus and the cytoplasm interacting with actin, a protein complex that may exert different functions depending on its subcellular localization.

1. Introduction

Heterochromatin proteins (HP)1 belong to the chromodomain superfamily that in mammals correspond to HP1α, -β and –γ. HP1 proteins have an N-terminal globular chromodomain (CD) and a C-terminal globular chromoshadow domain (CSD) linked by a less conserved and flexible hinge region [1,2]. HP1s are conserved throughout evolution from yeast to humans [1]. Trimethylated lysine 9 of histone H3 provides a binding site for HP1s, which in turn recruit modifier factors to heterochromatic loci and stabilize its structure [3–6]. The CD is responsible for HP1 recruitment to methylated histone H3, and the CSD is required for the homo- and heterodimerization between the different isoforms. HP1 proteins were initially implicated in gene silencing [7,8]. Later on it was demonstrated that they also participate in the control of cell fate, DNA repair, DNA replication, telomere stability, active transcription and elongation, and RNA splicing. Homozygous *Cbx5*-/- (HP1α gene) mutants are indistinguishable from wild-type littermates, indicating that its function is redundant [9]. On the other hand, the HP1β null mutation in mice leads to perinatal lethality, indicating that HP1α and HP1γ cannot compensate for the loss of the other HP1, in spite of their high degree of homology [9]. The loss of HP1β causes defective neuromuscular and cerebral cortex development [9]. In the case of HP1γ, by using gene-targeted mice for *Cbx3* (HP1γ gene), it was reported that only 1% of homozygotes for the hypomorphic allele reach adulthood [10]. Adult males and females exhibit a severe hypogonadism that is associated with a loss of germ cells [10,11]. Thus, small differences in sequence among HP1s may result in biochemical differences such as specific post-translational modifications and conformational versatility that ultimately lead to their divergent biological functions (reviewed in [12]).

Many studies were undertaken to better understand the role of each HP1 in different models of cell differentiation. We have reported that

Abbreviations: HP1γ, heterochromatin protein 1γ; CD, chromodomain; CSD, chromoshadow domain; LINC, linker of nucleoskeleton and cytoskeleton complex; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; WB, Western blot

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HP1α is a negative regulator of the transcription factor C/EBPβ during the process of adipogenesis [13]. Using bimolecular fluorescence complementation in living cells, we showed that the differential subnuclear distribution of C/EBPβ forms mirrors the site for their interaction with HP1α. Thus, the equilibrium among different pools of C/EBPβ-HP1α associated with chromatin or the nucleoskeleton plays key roles in the regulation of C/EBPβ target genes during adipogenesis [13]. We then decided to extend our study to the process of myogenesis. In C2C12 myoblasts, HP1α and –β, but not HP1γ, interact with MyoD, a key transcription factor during the process of myogenesis [14]. It has been shown that knockdown of HP1γ, with small interfering RNAs results in a reduced efficiency of differentiation of C2C12 myoblasts [14]. Another recent report showed that knockdown of HP1α, but not HP1β and HP1γ, blocks C2C12 myoblasts differentiation [15]. Therefore, it is still unclear what the requirement of each HP1 isoform is in the process of myogenesis. This prompted us to focus our attention on HP1γ, mainly because it is the only isoform that interacts with RNA pol II and is associated to active transcribed genes [16]. We found that knockdown of HP1γ compromised the capacity of C2C12 myoblasts to properly differentiate, exhibiting different phenotypes depending on the level of interference of HP1γ expression. Unexpectedly, when we analyzed the subcellular distribution of HP1γ during the differentiation of C2C12 cells, we observed that HP1γ was not exclusively detected in the nucleus but surprisingly also in the cytoplasm of C2C12 myoblasts and myotubes. HP1γ exhibited a striated pattern of staining in mature myotubes, isolated myofibrils and in skeletal and cardiac muscles of mice. Importantly, HP1γ interacts with actin in both the cytoplasm and the nucleus, showing how broad the protein network of HP1γ could be and, consequently, its functions that are not only restricted to the nucleus but also take place in the cytoplasm.

2. Materials and methods

2.1. Materials and antibodies

Mouse monoclonal IgG against the HP1γ (clone 42s2), mouse monoclonal IgG against HP1α (clone 15.19s2) were from Upstate - Millipore (Billerica, MA, USA). Rabbit polyclonal IgG against HP1γ were from Cell Signaling (Cat # 2619 - Beverly, MA, USA) and Abcam (Cat # ab10480; Cambridge, UK), respectively. Goat polyclonal IgG against Lamin B (cat # sc-6217) and anti-ERK2 (c-14) (cat # sc-154) were from Santa Cruz Biotechnology Inc., anti-histone H3 was from Millipore and anti-sarcomeric actinin was from Abcam (Cat # ab109776; Cambridge, UK). Mouse IgG anti-β tubulin, myosin heavy chain (clone MY-32), the HRP-conjugated anti-mouse, latrunculin B, cytochalasin D and leptomycin B were from Sigma Chemical Co. (St. Louis, MO, USA). Secondary antibodies labeled with Alexa-Fluor (488, 546 or 647) dyes were purchased from Molecular Probes (Eugene, OR, USA). HRP-conjugated goat anti-rabbit was from Pierce (Rockford, IL, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and horse serum were from Gibco Life Technologies – Invitrogen, (Carlsbad, CA, USA).

2.2. Cell culture

Murine C2C12 myoblasts, N2a cells, human embryonic kidney HEK293T and HeLa cells were obtained from American Type Culture Collection (Manassas, VA, USA). C2C12 cells were grown in DMEM 4.5 g/l glucose and supplemented with 10% v/v FBS in a humidified 5% CO_2 atmosphere at 37 °C. C2C12 myoblasts were grown until confluence, media was changed for DMEM supplemented with 2% horse serum and samples were obtained at the indicated time point post-induction of differentiation.

2.3. Indirect immunofluorescence assays

Indirect immunofluorescence (IIF) was performed as previously described [13,17,18]. Briefly, C2C12 myoblasts were grown on coverslips, induced or not to differentiate for the indicated periods of time, and then fixed with 4% PFA in PBS and permeabilized upon incubation 10 min with 0.5% Triton X-100 in PBS. Coverslips were washed three times with PBS, and inverted onto a 50μl drop of 1% BSA in PBS with the antibody as indicated in figure legends. All IIF conditions were tested to avoid non-specific reactions. Nuclei were stained with DAPI and coverslips were mounted in Vectashield mounting medium. Laser-scanning confocal microscopy was performed with LSM5 Pascal (Carl Zeiss, Oberkochen, Germany), using C-Apochromat 63 × /1.4 NA Oil-immersion objective and images were taken in the middle section of the cell nucleus except in experiments to determine whether HP1γ localizes in the perinuclear actin cap. For this purpose images were taken in the apical plane of the cell as previously described by Kathau et al. [19].

2.4. Image analysis

Software Fiji-Image J program (v.1, 42) from the NIH was utilized for all image analyses. Profiles of the fluorescence intensity of HP1γ antibodies, or ERK were obtained using the *Plot profile* plug-in of the *Fiji-Image J program*. The width of myotubes was measured from randomly selected microscope fields from three samples. At least two measures were taken in each myotubes and at least 50 myotubes were measured per sample using a *Analize* > *Measure* plug-in of the *Image J program*.

2.5. Immunohistochemistry

Male BALB-c mice (8 weeks old) were sacrificed humanely following the Animal Care Committee of the Instituto de Biología y Medicina Experimental guidelines (in accordance with the Division of Animal Welfare, Office for Protection from Research Risks, National Institutes of Health, A#5072–01). Frozen sections of hind leg muscles and heart of mice were placed onto Superfrost Plus glass slides and fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. They were washed, permeabilized with 0.5% Triton X-100 in PBS, and incubated in blocking solution (10% horse sera, 0.2% Triton X-100 in PBS) followed by incubation with antibodies. Sections were mounted in Vectashield and visualized by confocal microscopy. The isolation of myofibrils was performed as previously described [20]. Briefly, hind legs from BALB-c mice were incubated in rigor buffer (RB, 75 mM KCl, 10 mM Tris, 2 mM $MgCl_2$, 2 mM EGTA, 0.5% Triton X-100, pH 6.8 and protease inhibitors) overnight at 4 °C. Skeletal muscle was minced in 5 volumes (v/w) of ice-cold RB II (rigor buffer without Triton X-100), samples were homogenized on ice, and myofibrils were collected by centrifugation at 1500 × *g* for 10 min at 4 °C, washed twice with cold RB II, and resuspended in RB II with glycerol (1:1). For immunohistochemistry 100 μl of myofibrils were aliquoted onto Superfrost slides, allowed to adhere and samples were ready to follow the steps for immunohistochemistry described above.

2.6. Immunoelectron microscopy

It was performed at Laboratorio Nacional de Investigación y Servicios de Microscopía Electrónica (LANAIS-MIE) of the Instituto de Biología Celular y Neurociencia Prof. E. De Robertis, Facultad de Medicina UBA-CONICET. Briefly, hind leg muscles obtained from euthanized mice were cut into pieces with a maximum size of 1 mm^3, fixed for 24 h at room temperature in a mixture of 2% formaldehyde and 0.25% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). Tissue pieces were incubated in 0.15 M glycine solution in PB and then incubated in 10% sucrose solution in PB overnight. Specimens were rinsed in 0.1 M PB, dehydrated and embedded in LR White (Sigma Chemical Co., St. Louis, MO, USA). Then, the samples were cut at a

thickness of 60 nm with a Porter Blum Sorvall Ultra Microtome MT-1 (LKB-Pharmacia, Bromma, Sweden). Sections were picked up on 300 mesh nickel grids then blocked and labeled using a HP1γ antibody (Millipore, Billerica, MA, USA). After being washed, they were incubated with anti-mouse IgG conjugated to colloidal gold particles of 10 nm (Sigma Chemical Co., St. Louis, MO, USA). For contrast staining, the grids were incubated on drops of uranyl acetate solution (1% in water) for 10 s. For control, samples were incubated only in the presence of anti-mouse IgG conjugated to colloidal gold particles. Samples were examined using a Transmission Electron Microscope Zeiss EM 109 T equipped with digital camera Gatan ES1000W.

2.7. Cell fractionation and Western blot (WB) analysis

Cell fractionation was performed as previously described [18,21]. Briefly, cells were resuspended in CLB buffer (10 mM HEPES, 10 mM NaCl, 1 mM KH_2PO_4, 5 mM $NaHCO_3$, 5 mM EDTA, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$ and protease inhibitors), dounced 50 times with a loose pestle, sucrose was added to reach a final concentration of 0.25 M, and then samples were centrifuged at 6300*g* for 5 min at 4 °C. The supernatant, which corresponded to the cytosolic fraction, was kept. The pellet was washed once with CLB buffer and 0.25 M sucrose, and then nuclei were lysed using TSE buffer (10 mM Tris-HCl, 300 mM sucrose, 1 mM EDTA, 0.1% IGEPAL-CA 630 (v/v), pH 7.5). Total, cytoplasmic and nuclear fractions were resolved by SDS-PAGE and analyzed by immunoblotting, as previously described [13,18]. The same amount of protein was loaded in all cases.

2.8. Immunoprecipitation assays

Immunoprecipitations were performed as we previously described [18].

2.9. Chromatin immunoprecipitation assay (ChIP)

ChIP was performed as previously described [13]. The primers for *GAPDH* gene used for the PCR amplification were previously reported by Vackoc et al. [16].

Re-ChIP assays were performed as described [22] with the following modifications: after the first immunoprecipitation with anti-HP1γ, the beads were eluted with 100 μl ChIP SDS lysis buffer (50 mM Tris–HCl, pH 8.1, 10 mM EDTA, and 1% SDS) containing protease inhibitors and 10 mM DTT, and were rotated at room temperature for 15 min with vortexing. Eluates were diluted (1:10) with ChIP dilution buffer (20 mM Tris–HCl, pH 8.1, 150 mM NaCl, 2 mM EDTA, and 1% Triton X-100) containing protease inhibitors as described above. Each eluted sample was divided into two for the second immunoprecipitation (re-ChIP) with 4 μg of either anti-actin (rabbit), or normal rabbit IgG as control. Then immunoprecipitated DNA was purified and 35 cycles of PCR were performed with the same conditions and primers used for the ChIP experiments.

2.10. RNA interference

HP1γ was knockdown by infecting C2C12 myoblasts with retroviral particles harboring plasmids encoding specific shRNAs for HP1γ (Origene, USA, cat. no. TG5000712) or scrambled non-target shRNA as previously described [18].

3. Results

3.1. HP1γ is present in the cytoplasm of C2C12 myoblasts and myotubes

To gain insight into the role of HP1γ in the process of myogenesis, we analyzed the subcellular distribution of HP1γ during the differentiation of C2C12 myoblasts by indirect immunofluorescence (IIF) and confocal microscopy. HP1γ was present in the nucleus of C2C12 myoblasts (Fig. 1A), myocytes (Fig. 1B–C, see insert in gray scale) and myotubes (Fig. 1D–E, see insert in gray scale), as expected. Surprisingly, we found that HP1γ was also present in the cytoplasm of the myoblasts (Fig. 1A), myocytes (Fig. 1B–C), and myotubes 4 days after induction of differentiation (Fig. 1D), as well as in mature myotubes kept in culture for seven days (Fig. 1E). Interestingly, in myotubes (4 days), HP1γ pattern of staining was in longitudinal filaments (Fig. 1D), while in mature myotubes HP1γ acquired a transversal striated pattern (Fig. 1E). To test the specificity of the antibody, the expression of HP1γ was knocked down with specific shRNA. The fluorescent signal of HP1γ was almost absent both in the nucleus and the cytoplasm of myoblasts with knock-down HP1γ (Fig. S1B) compared to myoblasts transfected with scrambled shRNA (Fig. S1A), and the decrease in the expression of HP1γ was also observed by WB (Fig. S1C), demonstrating the specificity of the antibody. The presence of HP1γ in the cytoplasm was also observed by using different procedures of cell fixation/permeabilization (Fig. S1D, F and G), and secondary antibody (Fig. S1E). Furthermore, IIF was performed using different commercially available antibodies for HP1γ. The antibody from Abcam detected HP1γ in both the nucleus and the cytoplasm of myoblasts (Fig. 1F–H) and myotubes (Fig. 1I–K), results similar to those obtained with the Millipore antibody (Fig. 1A and D). When we used anti-HP1γ from Cell Signaling, HP1γ was detected in the nucleus of C2C12 myoblasts (Fig. 1L–N) and myotubes (Fig. 1O–Q), and only a weak signal of HP1γ was observed in the cytoplasm of myotubes (Fig. 1O–Q). According to the information obtained from the manufacturers' datasheets, the different antibodies were raised with peptides that encompass the amino terminal domain of HP1γ. For example, the Millipore antibody was raised against a peptide corresponding to residues 17–173 of human HP1γ protein, and the antibody from Abcam was generated using a synthetic peptide conjugated to KLH comprising the residues 1 to 100 of murine HP1γ. In this case, it is possible that in each cell compartment HP1γ may be present in complexes that make its epitope more or less available for the binding of the different antibodies used for IIF, in spite of the similar peptides that the companies used to generate them. To evaluate this possibility, we analyzed images obtained by double staining of HP1γ using a mix of anti-HP1γ from Millipore and Cell Signaling in C2C12 myoblasts. The former detected HP1γ in both the nucleus and cytoplasm (Fig. 2A) while the latter detected HP1γ only in the nucleus (Fig. 2B). Notice that a low co-localization signal was observed between both antibodies in the nucleus (Fig. 2c, and arrowheads insert). Analysis of fluorescence intensity in the section of the cell indicated by the arrow in panel c evidenced the partial overlap of the signals of the two antibodies in the nucleus (Fig. 2D). Taken together these results support the fact that antibodies from different sources, Millipore and Cell Signaling, have the capacity to interact with different pools of nuclear HP1γ, and only the former could detect the population of HP1γ present in the cytoplasm of C2C12 myoblasts.

The presence of HP1γ in the cytoplasm was an unexpected result, thus to obtain more evidence for this finding, we performed cell fractionation of C2C12 myoblasts and myotubes, and the samples were analyzed by WB. Regardless of the antibody used for WB, a band corresponding to HP1γ was found not only in the nuclear (Fig. 2E, lanes 3, 6, 9 and 12) but also in the cytosolic fractions (lanes 2, 5, 8 and 11) of myoblasts and myotubes. Furthermore, HP1α was only present in the nuclear fractions of myoblasts and myotubes (Fig. 2E, lanes 3 and 6) and absent in the cytosolic fractions (lanes 2 and 5), results that are in agreement with the images obtained by IIF that showed HP1α exclusively present in the nucleus of C2C12 myoblasts (Fig. S2A–C), myotubes (Fig. S2D–F), and as previously reported [13,23,24], among many other works. Detection of lamin B or histone H3 and β tubulin were used as control of the correct isolation of the nuclear and cytosolic fractions, respectively.

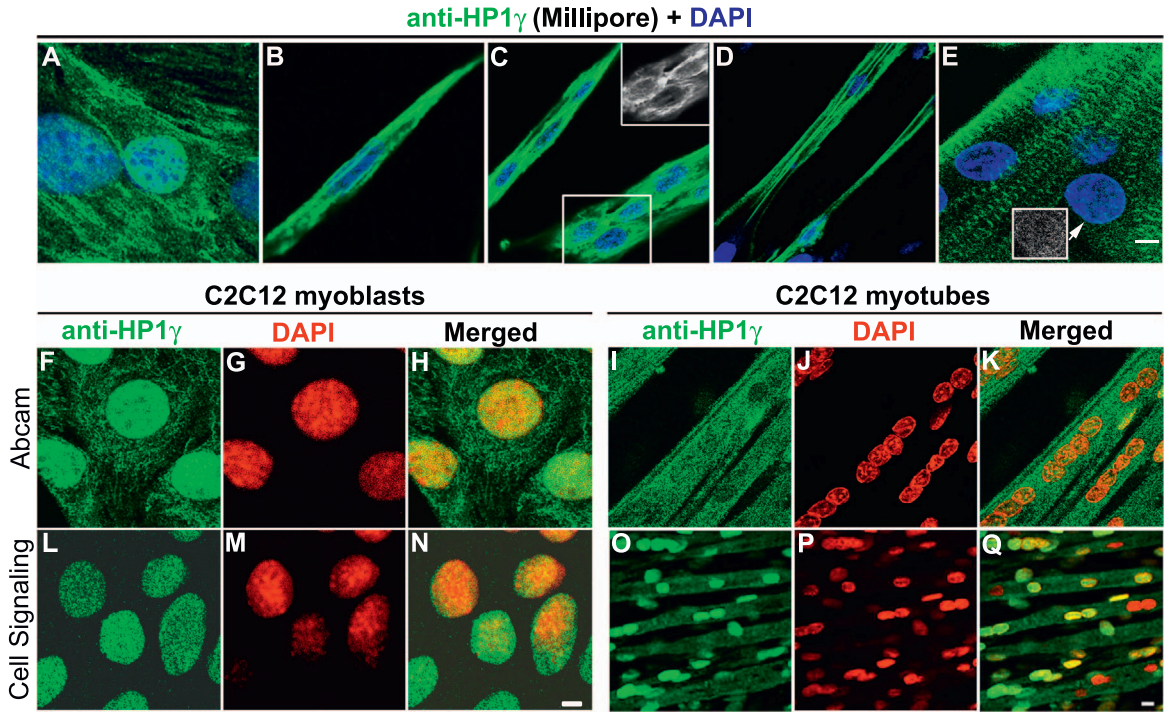

Fig. 1. HP1γ localizes in both the nucleus and the cytoplasm of C2C12 myoblasts and myotubes. Images of confocal microscopy of HP1γ in myoblasts (A), and after 1 (B–C), 4 (D) and 7 (E) days post induction of differentiation using the antibody from Millipore. Nuclei were counterstained with DAPI (blue). Insets in panels C and E correspond to images in gray scale. C2C12 myoblasts or myotubes were grown on coverslips, fixed, permeabilized and stained with anti-HP1γ from Abcam (F–K) or from Cell Signaling (L–Q), and images were analyzed by confocal microscopy. Nuclei were counterstained with DAPI (red). Results are representative of 5 independent experiments. Scale bars: 5 μm.

3.2. HP1γ is in the cytoplasmic compartment of N2a, HEK293T and HeLa cells, and is exported from the nucleus in a Crm-1-dependent manner

Next, we investigated whether the localization of HP1γ in the cytoplasm is exclusive of C2C12 cells or takes place in other cell types. As shown in Fig. 2, HP1γ localizes in both the nucleus and the cytoplasm of N2a cells, a murine neuroblastoma cell line (Fig. 2F–H), and in the human cells HEK293T (Fig. 2I–K) and HeLa (Fig. 2L–N). WB analysis of the cell fractions corroborated that HP1γ is present in the nuclear (Fig. 2O, lanes 3, 6 and 9) as well as in the cytosolic (Fig. 2O, lanes 2, 5 and 8) fractions of N2a, HEK293T and HeLa cells. In contrast, HP1α was exclusively detected in the nuclear fractions (Fig. 2O, lanes 3, 6 and 9) of all the cell types tested. Detection of histone H3 and β-tubulin were used as control of the correct isolation of the nuclear and cytosolic fractions, respectively.

HP1γ is a small protein (25 kDa) with a nuclear localization signal [24]; therefore once it is synthesized in the endoplasmic reticulum HP1γ has no impediment to enter the nucleus where it interacts with chromatin and nuclear factors that contribute its nuclear retention. The existence of a cytoplasmic pool of HP1γ led us to investigate whether HP1γ is actively exported from the nucleus by incubating C2C12 myoblasts in the presence of Leptomycin B (LMB), a Crm1 (Chromosomal regional maintenance-1) inhibitor [25]. LMB treatment efficiently inhibited Crm1 as ERK accumulated in the nucleus (Fig. 2P), as previously reported [26]. Interestingly, nuclear HP1γ also increased in C2C12 myoblasts incubated in the presence of LMB, suggesting that Crm-1 may regulate HP1γ active nuclear export. Taken together, we show for the first time that HP1γ not only localizes in the cytoplasm of C2C12 cells, but also in all murine and human cell lines tested in this study. Importantly, HP1γ may dynamically shuttle between the nucleus and the cytoplasm in Crm-1-dependent manner.

3.3. HP1γ is present in the sarcomere of skeletal and cardiac muscles

The C2C12 cell line is a very useful tool for studying myogenesis, but our results prompted us to analyze the localization of HP1γ in samples of skeletal and cardiac muscles obtained from mice. As expected, HP1γ was present in the nuclei of skeletal and cardiac muscles (Fig. 3A and B, respectively). Importantly, HP1γ was also detected in the cytoplasm of skeletal (Fig. 3A) and cardiac muscles cells (Fig. 3B), exhibiting a striated pattern of staining. This is the same pattern of staining observed when sarcomeric proteins (i.e. α-actinin, obscurin, among others) that form the Z disk with actin are detected by IIF [27,28]. Next, we performed immunoelectron microscopic analysis to confirm the IIF results and analyze HP1γ localization in skeletal muscle at the ultrastructural level. In the nuclei of skeletal muscle, HP1γ was associated with euchromatin and heterochromatin (Fig. 3C, and magnification in Fig. 3D). Importantly, high-density immunogold particles that correspond to HP1γ were observed associated mainly to the A-band and Z-disk of the sarcomere (Fig. 3C and E). No signal was detected when only the secondary antibody, anti-mouse IgG conjugated to colloidal gold particles of 10 nm, was used (data not shown). Furthermore, WB analysis of the subcellular fractions of murine skeletal and cardiac muscles, showed a band corresponding to HP1γ in both the cytosolic (Fig. 3F, lanes 2 and 5), and the nuclear (Fig. 3F, lanes 3 and 6) fractions. Detection of β tubulin and histone H4 were used as control of the correct isolation of the cytosolic and nuclear fractions, respectively. Taken together these results provide more evidence of the presence of HP1γ in the cytoplasm, in this case in murine muscle tissue, and show for the first time that HP1γ is associated to the A-band and Z-disk of the sarcomere.

3.4. Actin interacts with HP1γ both in the cytoplasm and the cell nucleus

The striated pattern of HP1γ staining observed in mature myotubes (Fig. 1E), and its localization in the sarcomere in muscle cells (Fig. 3A–C) led us to investigate whether HP1γ co-localizes with actin, the building block of microfilaments and one of the main components of the sarcomere. For this purpose the subcellular distributions of HP1γ and actin were analyzed by IIF and confocal microscopy in C2C12

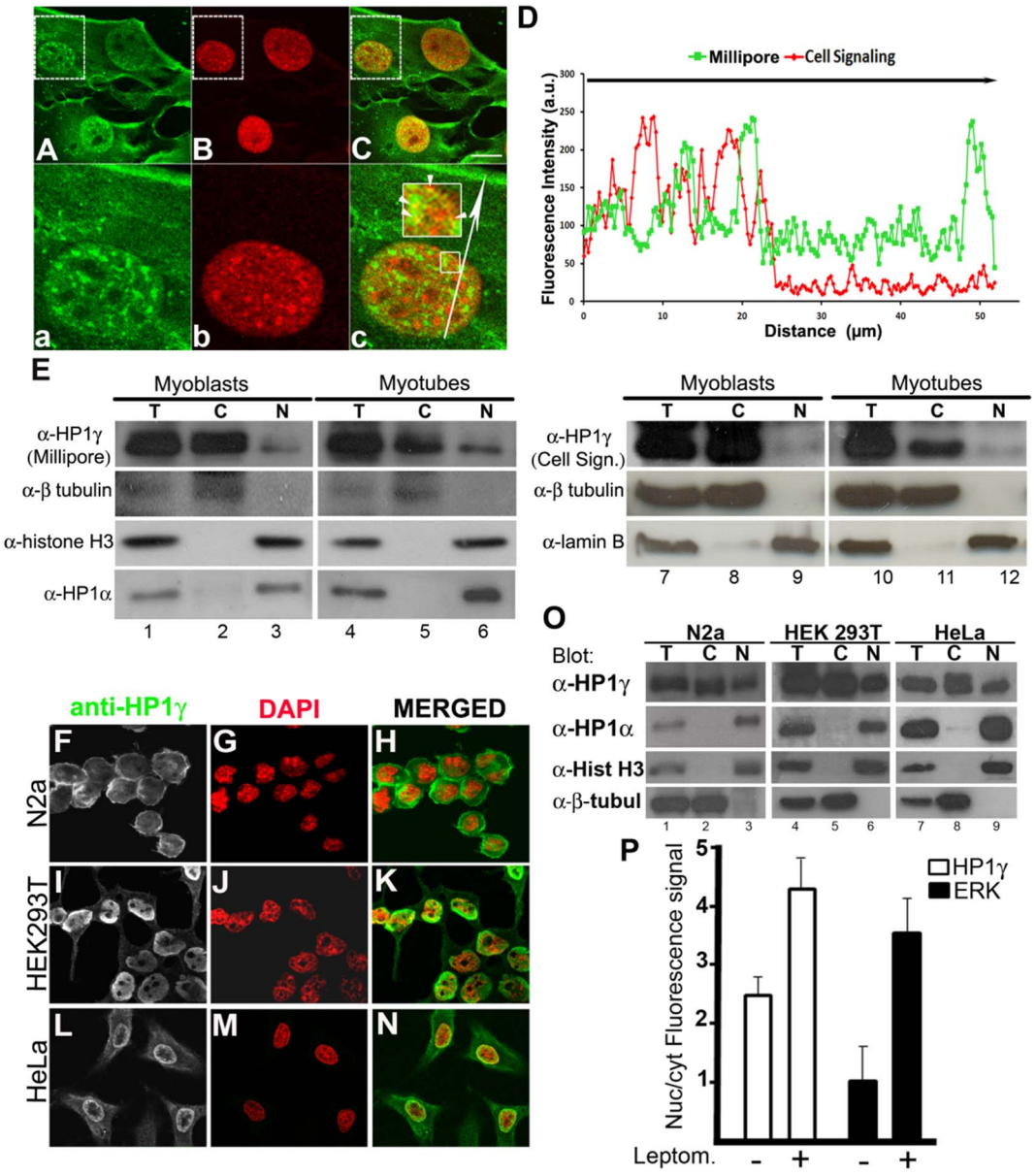

Fig. 2. HP1γ is not exclusively a nuclear protein. (A–C) C2C12 myoblasts were grown on coverslips, fixed, stained with a mix of anti-HP1γ from Millipore (green) and from Cell Signaling (red), and images were analyzed by confocal microscopy. The cell in the inset was magnified for better observation (panels a–c). Images are representative of three independent experiments. Scale bar: 5 μm. (D) Profile of the fluorescence intensity of HP1γ for each antibody in the section indicated by the arrow in panel c. (E) Total (T), cytosolic (C) and nuclear (N) fractions from C2C12 myoblasts or myotubes were resolved by SDS-PAGE, and analyzed by immunoblotting with the indicated antibodies. Detection of lamin B or histone H3 and β tubulin were used as control of the correct isolation of the nuclear and cytosolic fractions, respectively. Blots are representative of four independent experiments. (F–N) N2a, HEK293T and HeLa cells were grown on coverslips, fixed, subjected to IIF with anti-HP1γ (Millipore) and samples analyzed by confocal microscopy. Images of HP1γ staining in panels A, D and G were shown in gray scale for better observation. Nuclei were counterstained with DAPI. Scale bar: 10 μm. Results are representative of three independent experiments. (P) Total (T), cytosolic (C) and nuclear (N) fractions from the indicated cell types were resolved by SDS-PAGE, and analyzed by immunoblotting with the indicated antibodies. Blots are representative of three independent experiments. (Q) C2C12 myoblasts grown on coverslips were incubated 3 h in the presence or the absence of 10 ng/ml LMB. Cells were fixed, HP1γ or ERK were stained by IIF as described in Materials and Methods, images were taken by confocal microscopy and the ratio of nuclear and cytoplasmic (Nuc/cyt) fluorescence intensities was determined by ImageJ. Data represent mean fluorescent intensity signal ratios + std (n = 40–50).

myotubes 4 and 7 days after induction of myoblast differentiation. It has been reported that myotubes progressively undergo changes in their cytoarchitecture as the sarcomere is formed. At early stages of sarcomere formation (1–4 days post induction of myoblasts differentiation) actin is observed as continuous filaments extending the longitudinal axis of the cell [28–30]. We observed such pattern of actin staining in myotubes of 4 days (Fig. 4B). HP1γ staining also exhibited a longitudinal filamentous pattern (Figs. 4A, 1D) that partially co-localized with actin (Fig. 4C, and magnification in Fig. 4c see arrowheads). On the other hand, at late stages of sarcomere formation (5–7 days post induction of differentiation), the continuous longitudinal actin filaments are remodeled, generating a striated banding pattern across the lateral axis of the myotube [28,29]. As reported, when myotubes matured in culture for 7 days, we observed a striated pattern of actin in which the brightest staining corresponds to Z bands (Fig. 4E versus B), as reported [28]. Interestingly, HP1γ staining also adquired a striated pattern of staining (Fig. 4D versus A) exhibiting partial co-localization with actin in Z lines (Fig. 4F, and magnification in Fig. 4f, see arrowheads). Furthermore, when HP1γ (Fig. 4G) and actin (Fig. 4H) were labeled in isolated myofibrils obtained from the posterior leg of mice, they also rendered a striated pattern of staining with a partial co-localization in Z lines (Fig. 4I, and magnification panel I, see

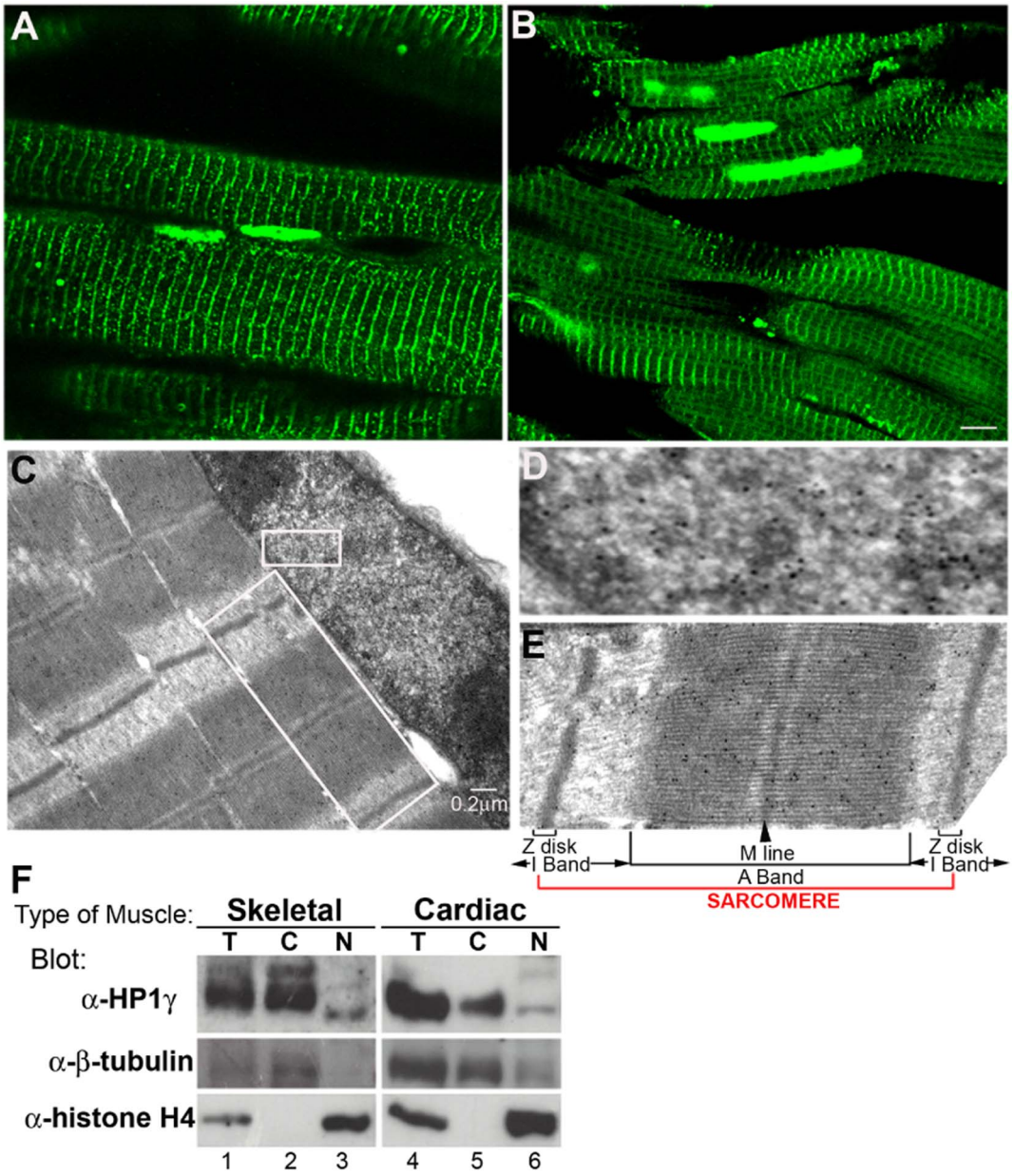

Fig. 3. HP1γ is in the nucleus as well as in the cytoplasm of skeletal and cardiac muscles of mice. (A–B) Samples of muscle of the posterior leg and heart of mice were processed for immunohistochemistry analysis of HP1γ using antibodies from Millipore, as described in Material and Methods. Representative images of confocal microscopy of HP1γ in skeletal and cardiac muscles from three independent experiments are shown. Scale bar: 5 µm (C) Electron microscopy of skeletal muscle. Samples were incubated with anti-HP1γ (Millipore) followed by incubation with gold-conjugated anti-mouse IgG. The magnification is 50,000 ×. (D–E) The insets were magnified for better observation. (F) Total (T), cytosolic (C) and nuclear (N) fractions from murine skeletal and cardiac muscle were resolved by SDS-PAGE, and analyzed by immunoblotting with the indicated antibodies. Detection of histone H4 and β tubulin were used as control of the correct isolation of the nuclear and cytosolic fractions, respectively. Blots are representative of three independent experiments.

arrowheads), as observed in mature C2C12 myotubes (Fig. 4F). This pattern of HP1γ staining was also observed in isolated myofibrils when the antibody from Abcam was used (data not shown). Importantly, actin co-immunoprecipitated with HP1γ in lysates of C2C12 myotubes (Fig. 5A, lane 3) showing that these two proteins interact. Since HP1γ is a small protein with a nuclear localization signal [24]; it has no impediment to enter the nucleus where it interacts with chromatin and nuclear factors that contribute to its nuclear retention. Thus, we hypothesized that the localization of HP1γ in the cytoplasm may be favored at least in part, by its interaction with actin filaments. To test this possibility, actin filaments were disassembled by treatment of C2C12 myotubes or myoblasts with latrunculin B, which sequesters G-actin monomers preventing its polymerization [31], and the subcellular distribution of HP1γ was assessed by IIF and confocal microscopy. Latrunculin B treatment caused the disassembly of actin filaments (Fig. 5F versus C), as reported. Interestingly, latrunculin B also caused the loss of the filamentous pattern of HP1γ staining (Fig. 5E versus B), and HP1γ exhibited a partial co-localization with actin (Fig. 5G) in the cytoplasm. Similar results were obtained when C2C12 myoblasts were incubated in the presence of Latrunculin B (Fig. 5H–J) and when cytochalasin D was used as an actin depolymerizing agent (data not shown). Importantly, actin co-immunoprecipitated with HP1γ in C2C12 myotubes incubated in the presence of cytochalasin D (Fig. 5A, lane 5). Taken together these results suggest that HP1γ interacts with actin, independently of the integrity of actin filaments, and it is possible that the interaction of HP1γ with actin may facilitate, at least in part, the localization of HP1γ in the cytoplasm.

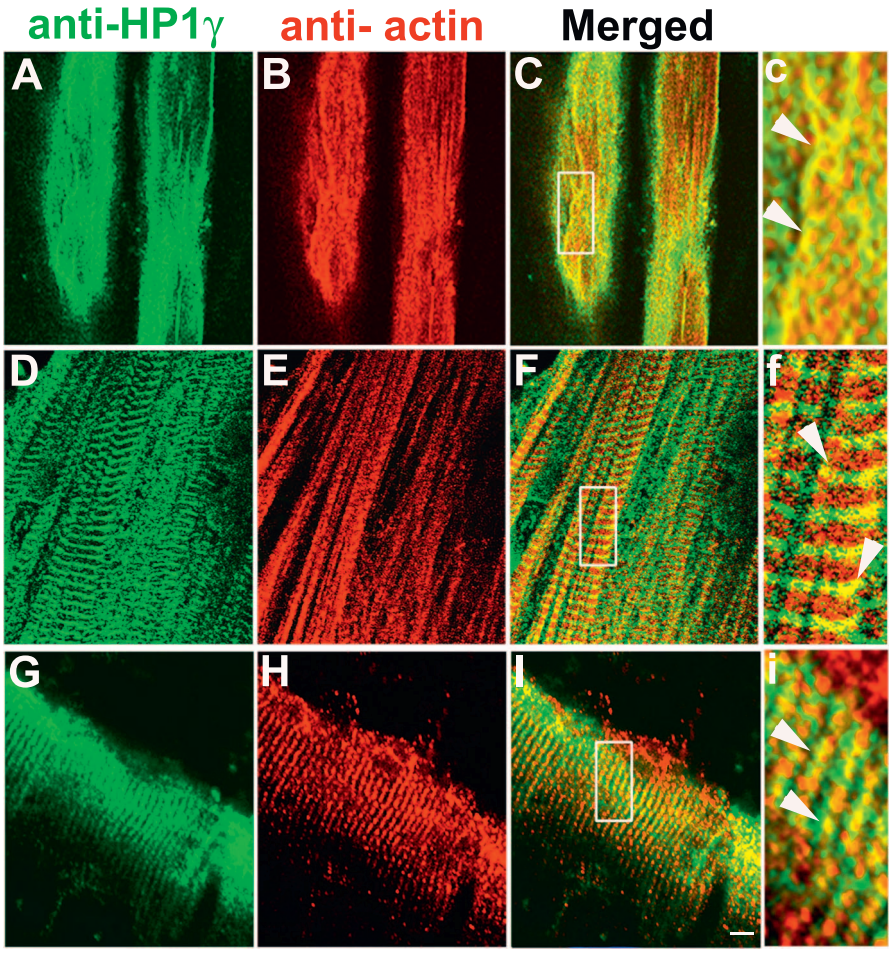

Fig. 4. HP1γ co-localizes with actin in C2C12 myotubes and skeletal muscle. (A–F) C2C12 myoblasts grown on coverslips were induced to differentiate 4 (panels A–C, and magnification in c) and 7 days (panels D–F, and magnification in f), subjected to IIF with anti-HP1γ (Millipore) and anti-actin followed by confocal microscopy analysis. (G–I and magnification in i) Myofibrils were fixed and subjected to IIF for HP1γ (Millipore) and actin, followed by confocal microscopy analysis. Images are representative of three independent experiments. Scale bar: 5 μm.

3.5. HP1γ is in the perinuclear actin cap and forming a complex with actin bound to chromatin

Actin is an essential building block of the microfilaments present in the cytoplasm that provide mechanical support to the cell, tracks for movement of macromolecular protein complexes and organelles, and mechanical force to drive cell movement [32]. It is now accepted that actin is also present in the cell nucleus, where it participates in chromatin remodeling and the control of gene transcription (reviewed in [33–35]). Since both actin and HP1γ are present in the cytoplasm and the nucleus, we tested whether their interaction takes place in both cell compartments. As shown in Fig. 6A, actin co-immunoprecipitated with HP1γ in the cytosolic and nuclear fractions of C2C12 myoblasts; the absence of tubulin demonstrated that the nuclear fraction was not contaminated with cytoplasmic content rich in actin. In order to gain insight into the importance of HP1γ-actin interaction in the cytoplasmic compartment, we analyzed by IIF and confocal microscopy whether HP1γ may be present in the perinuclear actin cap. This recently described structure corresponds to a group of parallel actin filaments located above the apical surface of the nucleus present in different cell types including C2C12 myoblasts [19]. The perinuclear actin cap participates in the control of nuclear shape [19], cell differentiation [36], mechanosensing [37] and mechano-transduction [38] since the actin cap fibers are directly connected to the nuclear envelope through the linker of nucleoskeleton and cytoskeleton (LINC) complexes and to the extracellular matrix by associating with focal adhesions. HP1γ was labeled and confocal microscopy images were taken at the apical focal plane of the cell (Fig. 6B, see schematic representation), as previously done for actin by Kathau et al. to demonstrate the existence of the perinuclear actin cap [19]. HP1γ filaments were observed at the apical plane of C2C12 myoblasts (Fig. 6B, and magnification of the inset in panel C). These apical HP1γ filaments (Fig. 6D, arrowheads) co-localized with actin (Fig. 6E, arrowheads) showing that HP1γ is present in the perinuclear actin cap possibly through its interaction with actin.

At the nuclear level, it has been reported that HP1γ interacts with RNA polymerase II and is present in the transcribed region of active genes (i.e. *GAPDH*) [16]. On the other hand, nuclear actin also interacts with RNA polymerase II [39,40], and is present at the promoter and coding regions of constitutively expressed genes, such as *GAPDH* [41]. Thus, we analyzed whether HP1γ and actin were associated in complex at *GAPDH* in C2C12 myoblasts by using ChIP assays. We found HP1γ associated with the promoter and transcribed region of the *GAPDH* gene (Fig. 6G, lane 3), as previously reported [16]. Actin was also observed in these regions (Fig. 6G, lane 2), as reported [41], thus overlapping with HP1γ. This observation raised the question as to whether HP1γ and actin were present in complex associated with the promoter and transcribed region of the *GAPDH* gene. To examine this possibility, re-ChIP experiments were performed. Chromatin fragments from C2C12 myoblasts were immunoprecipitated first with anti-HP1γ, and eluted samples were subjected to a second immunoprecipitation with anti-actin followed by purification of chromatin fragments prior their PCR analysis, as described in Materials and Methods. We found enrichment of chromatin associated with both proteins at the promoter and transcribed regions of the *GAPDH* gene compared to control non immune IgG (Fig. 6H, lane 2), indicating that a complex containing both HP1γ and actin occupies the same GAPDH promoter and transcribed region DNA. Taken together, these results show for the first time that HP1γ and actin in complex may play different roles in each cell compartment. In the cytoplasm, the HP1γ-actin interaction may facilitate the former to be present in the perinuclear actin cap, while in the nucleus they are

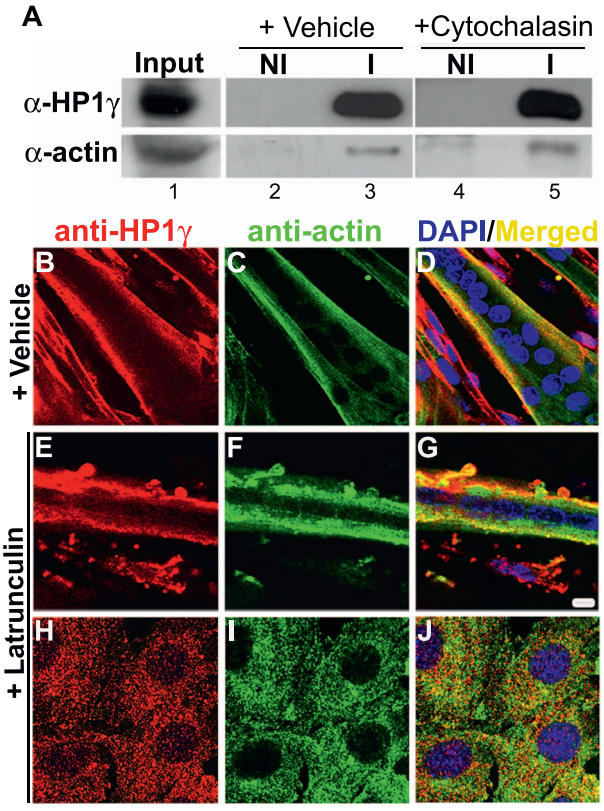

Fig. 5. HP1γ interacts with actin. (A) C2C12 myotubes were incubated 1 h in the absence or the presence of 1 μg/ml cytochalasin D, cells were lysated, HP1γ was immunoprecipitated, and then complexes were resolved by SDS-PAGE and analyzed by immunoblotting with the indicated antibodies. NI, non-immune antibody; I, anti-HP1γ (Millipore). Results are representative of four independent experiments. (B–J) Myotubes and myoblasts C2C12 were incubated 35 min in the absence or the presence of 0.1 μM Latrunculin B, cells were fixed, subjected to IIF with anti-HP1γ (Millipore) and anti-actin, and analyzed by confocal microscopy. Nuclei were stained with DAPI. Images are representative of two independent experiments. Scale bar, 10 μm.

present in a complex in actively transcribed genes, as shown here with *GAPDH*.

3.6. HP1γ is required for the differentiation of C2C12 myoblasts

To evaluate the requirement of HP1γ in the process of myogenesis, the expression of HP1γ was knocked-down in C2C12 myoblasts by using specific shRNA, and we then tested the capacity of the cells to differentiate. We found that knockdown of HP1γ may result in different outcomes depending on the level of HP1γ interference. We observed that myoblasts with knocked-down HP1γ (labeled as KD1-HP1γ) differentiated into myotubes, but they were thinner than those obtained from myoblasts transfected with scrambled shRNA (Fig. 7B versus A). In addition, KD2-HP1γ, another set of myoblasts with decreased expression of HP1γ, did not differentiate (Fig. 7C). HP1γ level of expression was markedly decreased in both KD1- and KD2-HP1γ cells compared to control cells (Sc) (Fig. 7D). Densitometric analysis showed that the average level of expression of HP1γ in KD1- and KD2-HP1γ myoblasts was 45 ± 4% and 10 ± 5%, respectively, of HP1γ expression in Sc myoblasts. Therefore, depending on the level of HP1γ interference, myoblasts give rise to thin myotubes or lose their capacity to differentiate. Myotubes derived from KD1-HP1γ myoblasts had a diameter 65 ± 3% lower than myotubes derived from cells transfected with scrambled shRNA. In KD1-HP1γ myotubes, the expression of the myogenic marker α-actinin was markedly reduced (Fig. 7D, *lane 4* vs. *2*) and the level of expression of myosin heavy chain was lower compared to myotubes differentiated from myoblasts transfected with scrambled

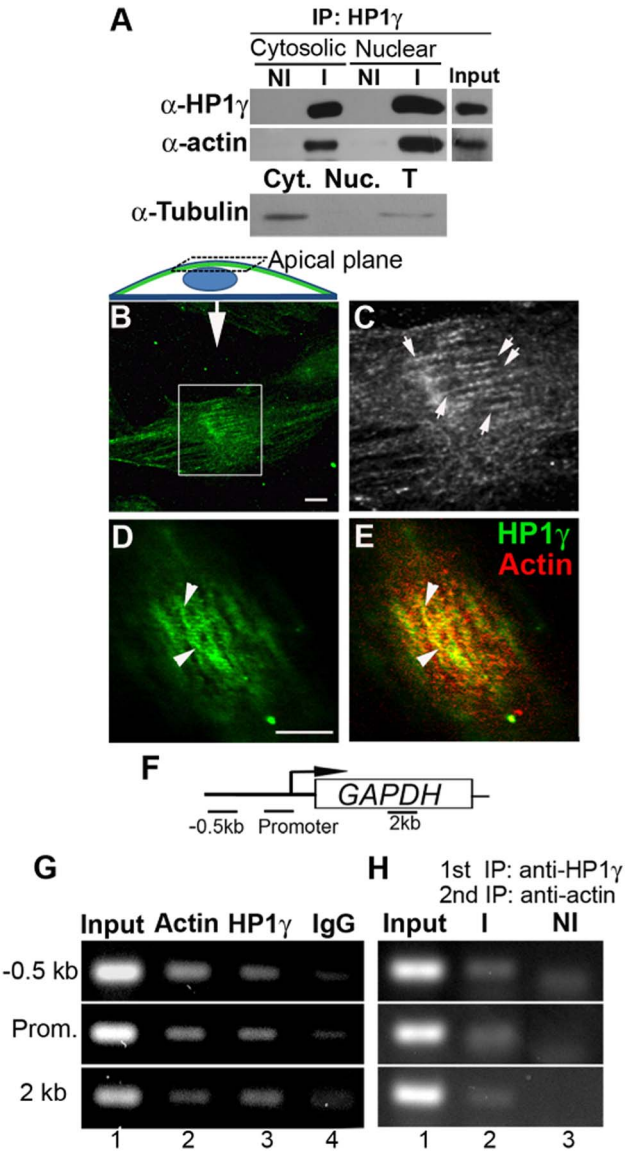

Fig. 6. HP1γ is associated to actin in the perinuclear actin cap and in actively transcribed genes. (A) HP1γ immunoprecipitated from cytosolic and nuclear fractions of C2C12 myoblasts was resolved by SDS-PAGE and analyzed by immunoblotting with the indicated antibodies. NI, non-immune antibody; I, anti-HP1γ (Millipore). Results are representative of three independent experiments. (B–E) Images of HP1γ (green) and actin (red) were taken by confocal microscopy at the apical plane of C2C12 myoblasts (see the schematic representation) as described by Kathau et al. [19]. (F) Schematic representation of the position of primers pairs used in ChIP and re-ChIP assays. (G) ChIP was performed using antibodies for actin, HP1γ (Abcam) or non-immune IgG as described in Materials and Methods. Representative results of PCR with the primers pairs for *GADPH* gene are shown. Results are representative of four independent experiments. (H) For re-ChIP assay the beads from the first IP with anti-HP1γ (Abcam) were washed, eluted and subjected to a second IP with anti-actin (I) or non-immune IgG (NI). Representative results of PCR with the primers pairs for *GADPH* gene are shown.

shRNA (Fig. 7D, *lane 4* vs. *2*). When myogenic markers were analyzed in KD2-HP1γ cells, the expression of α-actinin and myosin heavy chain were not induced (Fig. 7D, *lane 6* versus *2*), in line with lack of differentiation. Notice that the level of expression of myosin heavy chain in KD2-HP1γ is almost undetectable compared to the level reached by KD1-HP1γ cells (Fig. 7D, *lane 6* versus *4*).The reduction of the capacity of myoblasts to differentiate depended specifically on the decreased expression of HP1γ since the shRNA used to knockdown HP1γ did not affect the expression of HP1α or HP1β (Fig. 7E). These results suggest that depending on the level of HP1γ interference, myoblasts differentiate in an anomalous manner giving rise to thin myotubes or their

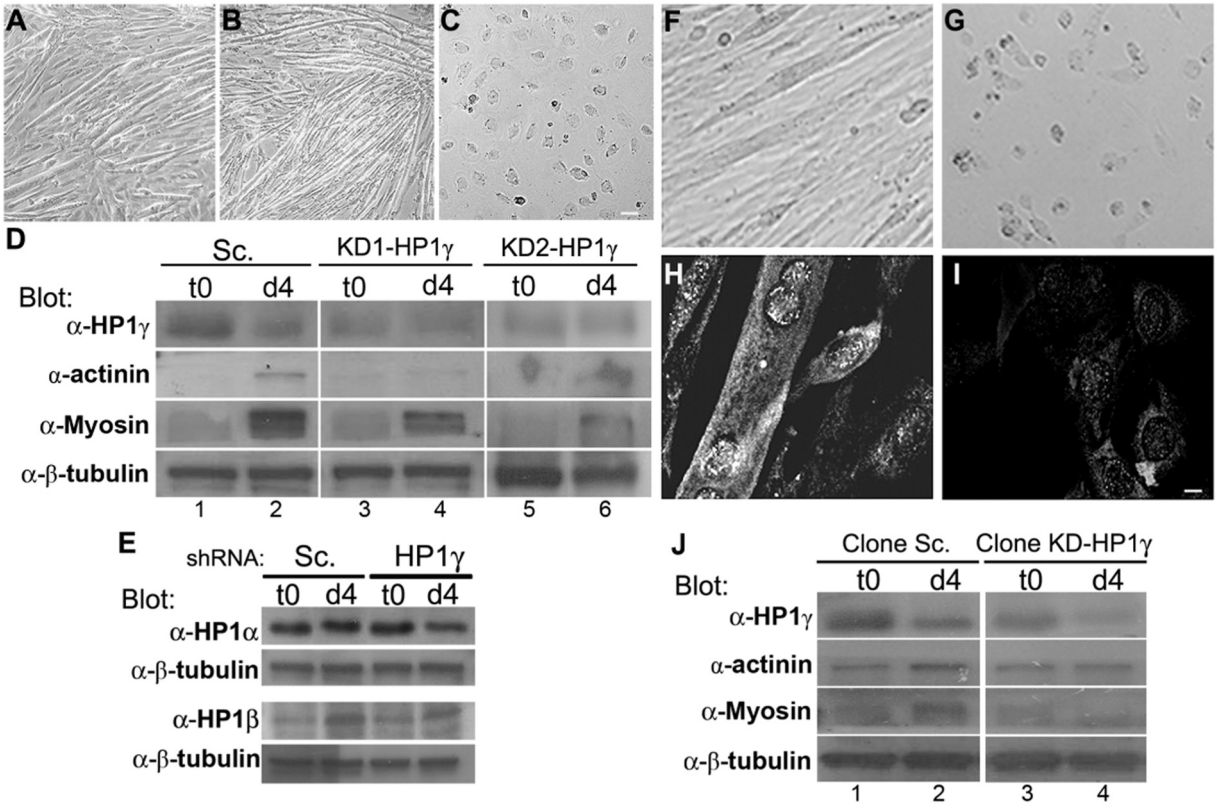

Fig. 7. HP1γ is required for myogenic differentiation. (A–C) C2C12 myoblasts were retroviraly transduced with scrambled non-target shRNA (panel A) or specific shRNA for HP1γ (panels B and C) to knockdown its expression, and after 7 days of puromycin selection, myoblasts were induced to differentiate during 4 days and images of living cells were taken (40 ×) prior to their harvest for WB analysis. Images are representative of three independent experiments. (D–E) Representative WBs of C2C12 cells transduced with scrambled (Sc.) or specific HP1γ shRNA (KD1- and KD2-HP1γ) prior (t0) and after 4 days (d4) of myogenic differentiation. (F–G) Clonal cells from scrambled shRNA or HP1γ shRNA grown on coveslipps were induced to differentiate during 4 days and their capacity to differentiate into myotubes was evaluated by brightfield microscopy. (H–I) Clonal cells from scrambled shRNA or HP1γ shRNA grown on coveslipps were induced to differentiate during 4 days, and then subjected to IIF for HP1γ (Millipore) and images taken by confocal microscopy. (J) Representative WB with the indicated antibodies of samples from scrambled or HP1γ shRNA clonal C2C12 cells, harvested prior (t0) and 4 days (d4) post induction of differentiation.

capacity to differentiate is blocked when HP1γ expression is almost negligible.

In order to have a population of cells with a homogeneous level of interference of HP1γ expression, we generated clones of KD-HP1γ by dilution cloning, as previously described [42]. In parallel, we also generated clones of myoblasts from cells transduced with scrambled shRNA. Clonal myoblasts with interfered expression of HP1γ did not differentiate (Fig. 7G), while clones from myoblasts transduced with scrambled shRNA differentiated in myotubes (Fig. 7F). HP1γ expression was markedly reduced in the clone KD-HP1γ, as shown by indirect immunofluorescence (Fig. 7I versus H) and WB (Fig. 7J, *lane 3* versus *1*). Densitometric analysis showed that the average level of expression of HP1γ in clonal myoblasts with interfered expression of HP1γ was 12 ± 8% of that found in control myoblasts. The degree of interference of HP1γ in clonal myoblasts was similar to the interference in KD2-HP1γ myoblasts that were not able to differentiate. The lack of differentiation was accompanied by no increase in the expression of myogenic markers α-actinin and myosin (Fig. 7J, *lane 4* versus *2*). Taken together, these results showed that HP1γ is required for myogenesis to proceed. Interestingly, depending on the degree of interference of HP1γ expression achieved, myoblasts could differentiate into anomalous thin myotubes or myoblasts could not differentiate at all.

4. Discussion

In the present study we show for the first time that HP1γ is not exclusively present in the nucleus but is also in the cytoplasm of C2C12 myoblasts and myotubes (Figs. 1, 2, 4, and Supplementary Fig. 1), as well as in skeletal and cardiac murine muscle tissues in which we add HP1γ as a new member of the large list of sarcomeric proteins (Fig. 3). In addition, a population of HP1γ was also present in the cytoplasm of all murine and human cell lines we tested (Fig. 2). We used different strategies, i.e. IIF and confocal microscopy using different commercial antibodies, electron microscopy, as well as cell fractionation and WB analysis, and in all cases we found HP1γ present in both the nucleus and the cytoplasm. In IIF assays, antibodies from Millipore and Abcam detected cytoplasmic and nuclear HP1γ; in contrast the antibody from Cell Signaling mainly detected HP1γ in the cell nucleus. According to the information obtained from the manufacturers' datasheets, the antibody from Millipore was raised against a peptide corresponding to residues 17–173 of human HP1γ protein, the antibody from Abcam was generated using a synthetic peptide conjugated to KLH comprising the residues 1 to 100 of murine HP1γ and the Cell Signaling was raised with a synthetic peptide corresponding to the amino terminus of human HP1γ while. In other words, there are not significant differences between the immunogens used to generate the antibodies. We observed a low co-localization signal in the nucleus when a mix of anti-HP1γ from Millipore and Cell Signaling was used in the IIF assay. Therefore, it is possible that in each cell compartment, HP1γ may be present in different complexes that make its epitope more or less available for its recognition by the different antibodies. The subcellular distribution of HP1γ contrasts with the exclusive presence of HP1α in the nucleus (Fig. 2, and Fig. S2). The existence of a cytoplasmic pool of HP1γ was an absolutely unexpected and surprising finding. We performed a deep search in the literature and, to our knowledge there are only five studies in which data also show HP1γ in the cytoplasm. Blanc et al. studied the subcellular distribution of the retroviral protein ENS-1/ERN1 that controls the activity of HP1γ in pluripotent stem cells isolated from

chick embryonic epiblast. In this study, the presence of HP1γ in the nucleus and the cytoplasm was evidenced by IIF and cell fractionation experiments [43]. HP1γ staining was also observed in the cytoplasm of S2 cells, in a study that analyzed the interactome of HP1 homologs in *Drosophila* [44]. Endogenous HP1γ as well as the flag-tagged protein overexpressed in HeLa cells were detected in the cytosolic fraction in a study that showed the differential interaction of HP1α with the chromatin-remodeling factor BRG1 [45]. None of these studies was focused on the analysis of the subcelular distribution of HP1γ. We think that for this reason, the authors did not make any special emphasis on the presence of HP1γ in the cytoplasm of avian, fly or mammalian cells. By using a tissue microarray and VECTRA™ immunofluorescent analysis, Slezak et al. performed a quantitative compartmentalization analysis between nuclear versus cytoplasmic HP1γ in samples of different types of prostate cancer compared to normal tissue [46]. They reported that a population of HP1γ is in the cytoplasm and proposed cytoplasmic HP1γ as a novel prognostic marker for predicting recurrence of the disease following prostatectomy [46]. They suggested that its presence in the cytoplasm may represent altered protein processing, or may have some as of yet unknown functional significance [46]. Last but not least, Ruddock-D'Cruz et al. reported that HP1γ showed dynamic changes in its subcellular distribution during early time points of bovine embryo development. HP1γ exhibits a diffuse cytoplasmic/nuclear localization in oocytes, as well as at the two- and eight-cell stages of embryos developed in vitro, distribution that is predominantly nuclear at the four- and sixteen-cell stage onwards [47]. In our present study, we show the presence of HP1γ in both the nucleus and cytoplasm of different murine and human cell lines, and murine muscle tissue. HP1γ has always been considered a nuclear protein; however, all this body of evidence suggests that this concept needs to be revised.

Molecular modeling has revealed that HP1γ behaves as an intrinsically disordered protein with dynamic flexibility and the ability to integrate signals from different signaling pathways [48]. Functional SLiM algorithms demonstrated that HP1γ has a bipartite nuclear localization signal (NLS) in an intrinsically disordered region that comprises the hinge region [24,48]. Albeit HP1γ is a low molecular protein that has no impediment to enter the nucleus, it has the ability to form an NLS-α-importin complex to translocate in an active manner into the nucleus [48]. Now the question is how does HP1γ shuttle from the nucleus to the cytoplasm? HP1γ does not possess any leucine-rich nuclear export signal. However, treatment of C2C12 myoblasts with the Crm-1 inhibitor LMB increased nuclear HP1γ, suggesting that Crm-1 regulates HP1γ active nuclear export (Fig. 8). Our results are in line with the study by Ruddock-D'Cruz and coworkers. They concluded that at early time points of bovine embryo development, the dynamic HP1γ shuttling between the nucleus and the cytoplasm appears to depend on active nuclear export via Crm-1 [47]. The authors proposed that HP1γ, a nuclear Crm-1 cargo critical for bovine embryogenesis, exits the nucleus just prior to the maternal to embryonic transition, possibly to facilitate specific chromatin remodeling [47]. We also found that Crm-1 is responsible for HP1γ nuclear export in C2C12 cells, and future studies will allow to gain insight in how HP1g nuclear-cytoplasmic shuttling impinges in the regulation of HP1γ functions in both cell compartments.

The unexpected finding of HP1γ in the cytoplasm brought to our attention another protein, actin; in particular, because of HP1γ's striated pattern of staining in matured myotubes and because by immunoelectron microscopy we evidenced that in skeletal muscle HP1γ is present in the A-band and Z-disk of the sarcomere (Fig. 3). We found that HP1γ interacts with actin (Figs. 5–6) in the cytosolic fraction of C2C12 cells. HP1γ is a small protein (25 kDa) with a nuclear localization signal [24]; therefore HP1γ has no impediment to enter the nucleus once it is synthesized in the endoplasmic reticulum. Thus, we hypothesized that HP1γ could be retained in the cytoplasm upon its interaction with actin filaments (Fig. 8). It has been reported that cytoplasmic actin, in addition to its structural role in the cytoskeleton, also contributes to the subcellular localization of transcription factors, for

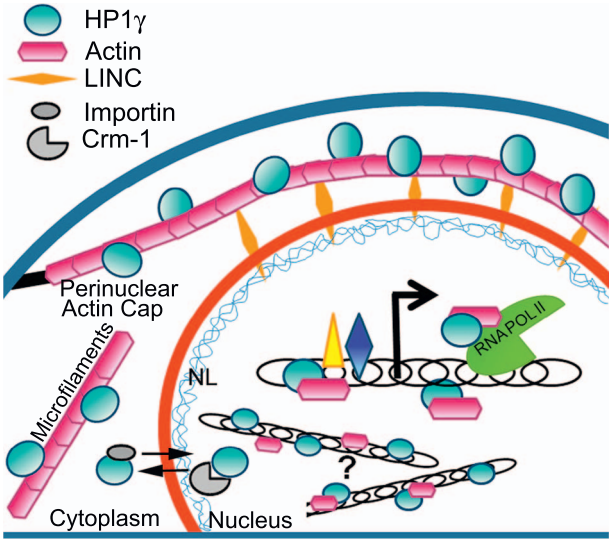

Fig. 8. Schematic representation of the model of HP1γ in the cytoplasmic and nuclear cell compartments. In the cytoplasm HP1γ localizes in microfilaments and the perinuclear actin cap, a group of parallel actin filaments located above the apical surface of the nucleus that participates in the control of nuclear shape [19], mechanosensing [37], mechano-transduction [38] as well as cell differentiation [36]. In the nucleus, HP1γ associates to chromatin via H3K9Me3 and RNApolII, and may facilitate the recruitment of actin.

example Nrf2 that is sequestered in the cytoplasm by Keap1 that is bound to the actin cytoskeleton [49]. The actin cytoskeleton provides scaffolding that is essential for the function of Keap1 to control Nrf2-dependent gene expression. Treatment with the actin depolymerizing agent latrunculin B, that sequesters G-actin monomers, caused the loss of the filamentous pattern of HP1γ staining (Fig. 5). Importantly, actin co-immunoprecipitated with HP1γ in C2C12 myotubes incubated in the presence of cytochalasin D (Fig. 5), another actin depolymerizing agent that induces dimerization of G-actin, promotes ATP hydrolysis and caps filament barbed ends [50]. Therefore, these results suggest that HP1γ has the capacity to interact with G- and F-actin, and it is likely that this interaction could mediate, at least in part, its retention in the cytoplasm. This could be an important regulatory mechanism that may control the bioavailability of HP1γ in the nucleus for exerting its actions in this cell compartment. In this regard, acetylation on K730 present in the NLS of the non-receptor tyrosine kinase c-Abl drives its accumulation in the cytoplasm during myogenic differentiation [51]. We speculate that acetylation of a lysine present in NLS of HP1γ may possibly participate in the control of the cytoplasmic pool of HP1γ. Future studies are required to gain insight in the active exchanged of HP1γ between the nucleus and the cytoplasm, studies that will uncover whether post-translational modifications of HP1γ may modulate its nuclear-cytoplasmic distribution and function.

During myogenesis, like in any cellular differentiation process, certain subsets of genes are activated while others are silenced, generating a pattern of gene expression necessary for cells to differentiate. These gene expression patterns are not only regulated by post-translational modifications of transcriptional regulators or changes in their nuclear localization, but also by epigenetic changes, chromatin remodeling as well as dynamic modifications in nuclear compartmentalization, favoring either transcriptional activation or silencing [52,53]. These events are controlled with precision by the integration of not only biochemical signals (hormones and other soluble factors) but also mechanical forces [54]. It has been recently reported the existence of a cytoskeletal structure known as the perinuclear actin cap, which is formed by actomyosin fibers located at the apical surface of the interphase nucleus, and that is physically connected to the nuclear envelope through the LINC complex [19]. The perinuclear actin cap participates

in the control of nuclear shape [19], mechanosensing [37], mechanotransduction [38] as well as cell differentiation [36]. Cell lineage specification, as shown for muscle stem cells, is influenced by the mechanical properties of the extracellular matrix [55], and the actin cap through its associated focal adhesions is important for mechanosensing the extracellular matrix during cell differentiation. When human embryonic stem cells (hESCs) undergo differentiation, the perinuclear actin cap is formed for the proper shaping of the nucleus [36]. Muscle cells possess multiple nuclei and constitute one of the few syncytia present in mammals. Thus, controlling transcriptional activity of nuclei at specific locations, such as neuromuscular synapses and myotendinous junctions, is a challenge for myofibers. It is a peculiarity that muscle nuclei are located at the cell surface and in an orderly manner, probably to minimize transport distances from nuclei to various locations in the cell [56]. C2C12 cells possess a perinuclear actin cap, as shown here and in a previous study [19]. We found that HP1γ is associated to the perinuclear actin cap, possibly through its interaction with actin (Fig. 6, model Fig. 8). Importantly, depending on the level of HP1γ interference in C2C12 myoblasts, their differentiation was blocked, or compromised, in a manner that thin myotubes or disorganized fibers developed. Since the perinuclear actin cap participates in the control of nuclear shape and position [57], it is possible that interfering HP1γ expression may affect the actin cap compromising the correct distribution of nuclei and/or the mechanosensing capacity of the cells, and future experiments are required to uncover the role of HP1γ within this cytoskeletal structure.

Although actin was thought to be present and function only in the cytoplasm, the presence of actin in the nucleus was first reported more than 30 years ago [58–60] and for a long time scientists were very skeptical about the existence of nuclear actin. Nowadays, its presence in the nucleus is well accepted exerting key roles in the control of transcription, chromatin remodeling, RNA processing and nuclear export [61]. As we mentioned above, we found that the interaction between HP1γ and actin takes place not only in the cytoplasm but also in the nucleus of C2C12 cells. Methylation of histone H3 on lysine 9 (H3K9me2 and H3K9me3) is the recognition site for the association of HP1s to chromatin, and has been related with heterochromatin and repressed genes within euchromatin. However, this epigenetic mark has also been found in the transcribed region of active genes. In fact, H3K9me3 even increases at those sites during activation of transcription, allowing for the recruitment of HP1γ that is also associated with RNA pol II to favor transcription [16]. Nuclear actin also interacts with RNA polymerase II for regulating transcription [39,62]. In the present study, we showed that HP1γ and actin are present in complex in actively transcribed genes (Fig. 6H, model Fig. 8) in C2C12 myoblasts. Both HP1γ and actin interact with RNA polymerase II; therefore they may be part of a complex with RNA polymerase II at actively transcribed region of the expressed gene to control transcription. Since HP1γ is also distributed across the transcribed regions of genes overlapping with trimethyl H3K9 [16], it is also possible that HP1γ bound to H3K9me3 may function as a scaffold protein for the recruitment of actin to chromatin, and consequently facilitating actin dependent scaffolding function to increase local concentrations of regulatory factors of transcription.

The importance of HP1γ for myogenesis is a controversial matter. It has been shown that knockdown of HP1γ with small interfering RNAs results in almost no differentiation of C2C12 myoblasts [14]; however another study did not find the expression of HP1γ to be critical for this process [15]. In our present work, we found that myoblasts with interfered expression of HP1γ gave origin to thin myotubes or did not differentiate, depending on the level of efficiency of HP1γ interference (Fig. 7). In fact, C2C12 myoblasts with 23 ± 1% of decrease in HP1γ expression fully differentiated like cells transfected with scrambled shRNA, and properly expressed actinin and myosin heavy chain (data not shown). Then, it is possible that there is a critical level of HP1γ expression below which cells are no longer able to differentiate into myotubes. We show for the first time that HP1γ is a sarcomeric protein. Myofibrilogenesis is a complex event that is under intensive study [63]. However, since HP1γ is present in the cytoplasm and the nucleus, it is not possible to determine to what extent the decrease of each pool of HP1γ contributed to the observed defects in C2C12 differentiation. It is likely that differences between studies that investigated the requirement of HP1γ in myogenesis may be related to the level of interference of the protein, which ultimately could affect the equilibrium between the nuclear and cytoplasmic pools of HP1γ. Since HP1γ is present in the A-band and Z-disk of the sarcomere associated with actin and possibly other sarcomeric proteins, and in the nucleus associated to RNA pol II and actively transcribed genes, knockdown of HP1γ may affect sarcomere assembly and/or the level of expression of myogenic genes, causing the development of thin myotubes or myotubes with abnormal nuclei repartition, phenotypic characteristics observed in myotubes derived from isolated satellite cells from patients with genetically confirmed diagnosis of facioscapulohumeral muscular dystrophy [64].

5. Conclusions

In the present study we show that HP1γ is not exclusively present in the nucleus but it is also in the cytoplasm of all cells we tested. HP1γ interacts with actin in both cell compartments. In the cytoplasm of C2C12 myoblasts, HP1γ is in the perinuclear actin cap, and in murine muscle tissue HP1γ is in the sarcomere. In the nucleus, HP1γ- actin are associated at the promoter and transcribed region of the house keeping gene *GAPDH*, suggesting that HP1γ may function as a scaffold protein for the recruitment of actin to control gene expression. Therefore, HP1γ-actin complex may exert different functions depending on its subcellular localization. Future studies will continue to uncover the biological functions of HP1γ in the nucleus, but undoubtedly it is now necessary to extend them to a different cell compartment, the cytoplasm.

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Transparency document

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