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Graphical Abstract

Intrabody-mediated diverting of HP1β to the cytoplasm induces CO-aggregation of H3–H4 histones and lamin-B receptor

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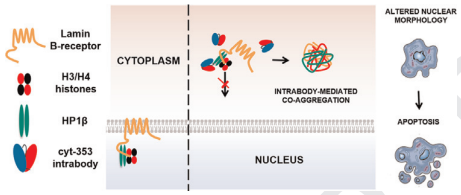
Alessio Cardinale, Ilaria Filesi, Prim B. Singh, Silvia Biocca



Highlights

- Intrabodies against heterochromatin protein 1 β (HP1β) inhibit its traffic to the nucleus.
- Anti-HP1β intrabodies sequester HP1β in cytoplasmic aggregates.
- Anti-HP1β scFv causes co-aggregation of LBR and H3–H4 histones in the cytoplasm.
- Methylated histone H3 at K9 (Me9H3) is not affected by anti-HP1β scFv expression.
- Anti-HP1β scFv induces altered nuclear morphology and apoptosis.

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Research Article

Intrabody-mediated diverting of HP1β to the cytoplasm induces CO-aggregation of H3–H4 histones and lamin-B receptor

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ABSTRACT

Diverting a protein from its intracellular location is a unique property of intrabodies. To interfere with the Q2 intracellular traffic of heterochromatin protein 1β (HP1β) in living cells, we have generated a cytoplasmic targeted anti-HP1β intrabody, specifically directed against the C-terminal portion of the molecule. HP1β is a conserved component of mouse and human constitutive heterochromatin involved in diverse nuclear functions including gene silencing, DNA repair and nuclear membrane assembly. We found that the anti-HP1β intrabody sequesters HP1β into cytoplasmic aggregates, inhibiting its traffic to the nucleus. Lamin B receptor (LBR) and a subset of core histones (H3/H4) are also specifically co-sequestered in the cytoplasm of anti-HP1β intrabody-expressing cells. Methylated histone H3 at K9 (Me9H3), a marker of constitutive heterochromatin, is not affected by the anti-HP1β intrabody expression. Hyper-acetylating conditions completely dislodge H3 from HP1β:LBR containing aggregates. The expression of anti-HP1β scFv fragments induces apoptosis, associated with an alteration of nuclear morphology. Both these phenotypes are specifically rescued either by overexpression of recombinant full length HP1β or by HP1β mutant containing the chromoshadow domain, but not by recombinant LBR protein. The HP1β-chromodomain mutant, on the other hand, does not rescue the phenotypes, but does compete with LBR for binding to HP1β. These findings provide new insights into the mode of action of cytoplasmic-targeted intrabodies and the interaction between HP1β and its binding partners involved in peripheral heterochromatin organisation.

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1. Introduction

The gene-based approach of intracellular antibodies (intrabodies) relies on the manipulation and expression of recombinant antibodies in different intracellular compartments to ablate or modulate the function of target molecules [1,2]. Intrabodies can hamper the function of the target antigen by direct binding to the functional domain [3] or by diverting the antigen to a different subcellular location. Examples of diverting include cytoplasm [4], nucleus [5,6], endoplasmic reticulum [7] and the proteasome degradation pathway [8,9]. These molecules have found applications as therapeutics in infectious diseases, in cancer and in neurodegenerative disorders [2,10].

We have previously demonstrated that nuclear and cytoplasmic targeting of intrabodies against different epitopes of the chromodomain (CD) of heterochromatin proteins 1 (HP1) in mammalian cells induces cell death [11]. HP1 proteins were originally identified as crucial factors involved in heterochromatin formation and gene silencing [12], but subsequent studies revealed that these proteins have additional nuclear functions including chromosome segregation, telomere maintenance, DNA repair, transcriptional activation and sister chromatid cohesion [13 and references herein]. In mammals, three subtypes of HP1 have been identified: HP1α, HP1β and HP1γ [14]. HP1 proteins consist of two highly conserved regions, the N-terminal chromodomain (CD) and the C-terminal chromoshadow domain (CSD), linked by the "hinge" flexible sequence [15]. The HP1 CD binds to methylated lysine 9 of histone H3 [16,17], a property consistent with the histone-code hypothesis [18]. The CSD is required for homo- and

Abbreviations: HP1, heterochromatin protein 1; CD, chromodomain; CSD, chromoshadow domain; Pc, polycomb; CAF-1, chromatin assembly factor 1; TIF-1β, transcriptional intermediary factor 1β; Suvar, Suppressor of variegation; INCENP, inner centromere protein; NE, nuclear envelope; LBR, lamin B receptor; scFv, single-chain variable fragment; PCR, polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; BSA, bovine serum albumin; TSA, trichostatin A; NaB, sodium butyrate; GFP, green fluorescent protein; FBS, foetal bovine serum; PMSF, phenyl-methyl-sulfonyl-fluoride; IDP, intrinsically disordered proteins

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hetero-dimerisation of HP1 proteins and interaction with specific protein ligands such as CAF-1 subunit p150, nuclear auto-antigen SP100, Su(var)3–7 and LBR *via* the PXVXL motif [19,20]. It is also involved in the recruitment of HP1 proteins to DNA damage sites under different stimuli [21]. The less conserved hinge region is implicated in binding of different molecular partners including the inner centromere protein (INCENP), histone deacetylases, RNA and DNA [22–25].

Mammalian HP1 associate with components of the nuclear envelope (NE) and, in particular, with the lamin *B* receptor (LBR) [26,27]. LBR is a type II inner nuclear transmembrane protein whose *N*-terminus faces the nucleoplasm [28]. This protruding domain binds *in vitro* to *B*-type lamins [26] and to HP1. Moreover, microinjection of mouse recombinant HP1 into living cells have shown a transient interaction of HP1 with the nuclear envelope and dominant negative mutants of HP1 inhibit nuclear reassembly in an *in vitro* assay [29]. Molecular characterisation of the HP1-NE interaction has revealed that HP1 proteins form, *in vitro*, a tight complex with two core histones (H3/H4) and LBR [30,31]. These interactions have been correlated with a possible role of HP1 in peripheral heterochromatin organisation and in regulating nuclear membrane re-assembly at the end of the mitosis [30,31]. However, a direct involvement of HP1 in the process of NE formation in mammalian cells has not been yet demonstrated *in vivo*.

Despite their structural similarity and high sequence homology, HP1 isotypes segregate into distinct nuclear domains [32] and display diverse and multiple functions [13]. Thus, mice deficient for each HP1 isotype manifest different phenotypes [33,34]. In particular, HP1β deficient mice show perinatal lethality associated with defective neuromuscular and cerebral cortex development, and genomic instability, indicating that HP1β is essential for organismal survival and that HP1 proteins are not functionally redundant [33]. However, mice expressing only 10% of HP1β protein levels are indistinguishable from wild-type littermates [35], suggesting that the amount of HP1β required for survival is small [36]. Consistent with these results, knockdown of HP1β by specific RNAi in different cell types neither induces cell death nor alters cell cycle progression [37,38].

Here we aimed at specifically interfering with HP1β by intrabody-mediated retargeting of HP1β from its nuclear physiological location to the cytoplasm. To this aim, we have cloned a scFv fragment specifically directed against the CSD domain of HP1β into a vector optimised for cytoplasmic expression.

2. Materials and methods

2.1. Engineering of the 353-scFv fragment

Hybridoma V regions of the rat monoclonal antibody MAC 353 [39], directed against the C-terminal sequence of the HP1β have been cloned using a phage display system as previously described [40]. Since sequences of non-functionally rearranged variable light (VL) regions (pseudogenes) were obtained by PCR of the MAC 353 hybridoma cDNA, we used an antisense directed RnaseH digestion of the myeloma derived VL pseudogene mRNA, as described [41]. The following antisense oligonucleotide directed against the pseudogene CDR 3 region of the myeloma light chain was designed: 5′ TGG GCA TCT ACT AGG CAA TCT GGT 3′. Briefly, mRNA obtained from 5×10^5 hybridoma cells was suspended in 20 µl 1 mM EDTA pH 7.4, incubated 10 min at 70 °C for breakage the secondary structure and digested (30 min at 37 °C) with 1.6 U of RnaseH (Promega) in the presence of 120 pmol of antisense oligonucleotide. The remaining non digested VL region was PCR amplified and cloned into pDAN plasmid [42]. The variable heavy (VH) gene was PCR amplified using selected oligos [43].

2.2. ELISA assay

Supernatants containing the scFv-expressing phages were analysed in a phage-enzyme-linked immunosorbent assay (phage-ELISA) for binding to coated recombinant HP1β and lysozyme (Sigma) as a negative control. Positive clones were further analysed by fingerprinting and sequencing. Selected anti-HP1β scFvs and the anti-lysozyme scFv D1.3 [44] were expressed in *E. coli* HB2151 non-suppressor strain and purified by affinity chromatography using Ni-NTA agarose (Quiagen). ELISA assay was carried out by coating the plate with 1 µg of recombinant HP1α, HP1β, HP1γ GST-fusion proteins and 300 µg of lysozyme, as described [11].

2.3. DNA constructs

For the expression in mammalian cells, the anti-HP1β 353-scFv was subcloned into the NcoI/NotI sites of pscFvexpress-cyt and pscFvexpress-nuc [45]. These vectors direct the expression of the scFv fragments under the transcriptional control of EF-BOS (elongation factor 1A) promoter. For PCR amplification of anti-HP1β scFv fragment from the bacterial pDAN-scFv vector, the following degenerate primers were designed: 5′-CTTGCCATGG-GATTGTTATTACTC-3′ and 5′-TTTAGCGGCCGCTGGGATTGGTTT-3′. The pscFvexp-cyt R4 (anti-βgal), which was derived from pPM163-R4 (kindly provided by P. Martineau) and pscFvexp-cyt αD11 were subcloned as described previously [11]. HP1 (α, β and γ) were epitope-tagged in an expression vector by sub-cloning the HP1α/β/γ open reading frames into pFLAG-CMV-1 (Clontech). GFP-tagged HP1β mutants that contain the CSD (GFP-CSD), the CD (GFP-CD) and the hinge region alone (GFP-HP1h) were constructed as described [46].

2.4. Antibodies

The detection of scFv fragments was carried out using the mouse anti-myc IgG 9E10 (Invitrogen) and the affinity-purified rabbit anti-myc IgG (Santa-Cruz). MAC 353 [39] and a polyclonal rabbit antiserum (Upstate) were used to identify HP1β. HP1α was detected with a polyclonal rabbit antiserum (M235) [39], HP1γ by the rat anti-HP1γ IgM (MAC 385) [47], methylated histone H3 in lysine 9 (Me9H3) with a rabbit anti-Me9H3 IgG [48], Suvar39h1 with a polyclonal rabbit anti-Suvar39h1 (Santa Cruz) and LBR with affinity-purified rabbit anti-LBR antibodies. All anti-histones antibodies were purchased from Cell Signalling Technology. Flag-tagged recombinant proteins were identified by mouse anti-Flag IgG (Sigma).

2.5. Cell lines, transfection, drug treatments and western blotting

NIH-3T3 fibroblasts and HeLa cells were grown in DMEM medium (Euroclone) supplemented with 10% foetal bovine serum (FBS). Cells were grown to 85% confluency and transiently transfected with Superfect (Qiagen) following the manufacturer's instructions. Cells were harvested and analysed 24–48 h after transfection. For rescue experiments, cells were co-transfected with 1 µg of cyt-353 intrabody plasmid and increasing amount of constructs (0.25, 0.5 and 1 µg) encoding for HP1α, β and γ, LBR and GFP-tagged HP1 mutants containing the CSD (GFP-CSD), the CD (GFP-CD) or the hinge region (GFP-h). For drug treatments, just after transfection, cells were cultured for 16 h in complete medium containing 200 ng/ml TSA (Sigma) and 1 or 5 mM Na Butyrate (Sigma).

Extraction of proteins from transfected cells and Western blotting were performed as previously reported [11]. Horseradish peroxidase-linked anti-mouse IgG (Amersham Pharmacia Biotech),

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anti-rabbit IgG (Amersham Pharmacia Biotech) and anti-rat IgG (Pierce) were used as secondary antibodies.

2.6. Preparation of nuclear and cytoplasmic fractions

3×10^6 transfected cells were washed twice in PBS, scraped and collected. The nuclear and cytoplasmic fractions were prepared by resuspending cells in 0,3 ml hypotonic isolation buffer (IB) (10 mMTris–HCl pH 7.6, 10 mMNaCl, 1.5 mM $MgCl_2$, protease inhibitor cocktail [1:1000] (Calbiochem) and 0.1 mM phenyl-methyl-sulfonyl-fluoride (PMSF)). Cells were passed through an ice-cold cylinder cell homogenizer and nuclei isolated by centrifuging at 4 °C for 15 min at $290 \times g$. Nuclear pellet was washed twice with 0.3 ml of IB, incubated for 30 min on ice in modified IB with 1% Triton X-100 and centrifuged at $12{,}000 \times g$ for 15 min to separate the soluble and the insoluble nuclear fractions. The cytoplasmic supernatant, after two subsequent centrifugations for clearing from cell debris, was detergent extracted by adding 0,5% Nonidet P40 (Sigma) for 30 min on ice and centrifuged twice ($12{,}000 \times g$ at 4 °C for 15 min) to separate the soluble and insoluble fractions. The cytoplasmic pellet was washed twice in NP40 enriched IB buffer by subsequent centrifugations at $12{,}000 \times g$ for 15 min. Soluble (S) and insoluble (I) proteins from nuclei and cytoplasm were separated on SDS-PAGE and analysed by Western blot.

2.7. Immunofluorescence

Immunofluorescence, including *in situ* identification of apoptotic cells, was carried out as previously described [11]. For LBR detection, cells were fixed and permeabilised with cold methanol at −20 °C for 7 min, rinsed in PBS and incubated over night at 4 °C with the primary antibody. Cy™2-conjugated*AffiniPure Donkey anti-rabbit IgG, Cy™2-conjugated AffiniPure rabbit anti-goat IgG (Jackson Immunoresearch) and Texas Red anti-mouse IgG (Calbiochem) were used as secondary antibodies. Hoechst 33342 dye was used at 300 ng/ml. Samples were examined with a Leica fluorescence microscope and CCD camera, equipped with oil immersion lens.

Confocal images were acquired by using PCM-2000 Confocal Microscope (Nikon) and processed by the PCM-2000 Software (Nikon).

2.8. Statistical data analysis

Data are reported as mean ± SEM. Results were analysed using one-way ANOVA and were considered statistically significant when p value < 0.05.

3. Results

3.1. Anti-HP1β intrabodies specifically sequester HP1β in the cytoplasm of mammalian cells

We used the phage display system to clone the V regions of MAC 353, an anti-HP1β monoclonal antibody, specifically directed against the C-terminal portion of the protein, in a scFv intrabody format. MAC 353 is a rat IgG and specifically recognises both human and mouse HP1β [39]. Fig. 1A shows the aminoacid sequence of MAC 353 scFv fragment as deduced from the nucleotide sequence. Recombinant anti-HP1β scFv was expressed in bacteria, purified and characterized for its binding specificity by ELISA. As shown in Fig. 1B, the scFv maintains the immunogenic properties of the original monoclonal antibody MAC 353.

In order to retarget the nuclear protein HP1β to the cytoplasm, the 353 scFv was subcloned into vectors optimised for cytoplasmic expression in mammalian cells (cyt-353 scFv/intrabody) [40]. Myc-tagged cyt-353 anti-HP1β scFv was transiently transfected in NIH 3T3 cells and analysed by Western blot. Analysis of soluble and insoluble proteins extracted from transfected cells shows that the cyt-353 scFv fragment is insoluble and Triton-resistant (Fig. 1C). Thus, a single immunoreactive band of the expected size is present in the insoluble fraction (lane 6). The expression level of the cyt-353 scFv is comparable to that found for other insoluble scFvs that we have used as controls, as the anti-NGF scFv αD11

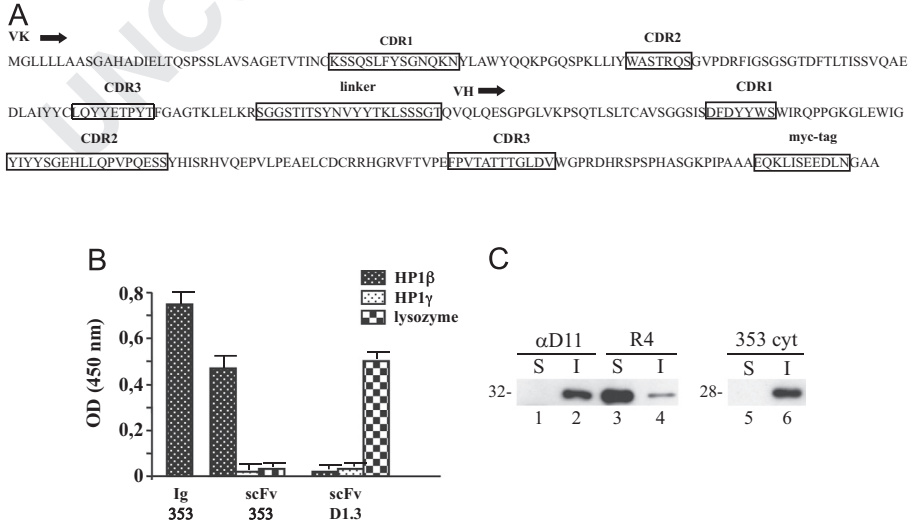

Fig. 1. Amino-acid sequence and expression of anti-HP1β 353 scFv in *E. coli* and in mammalian cells. (A) The deduced amino-acid sequence of the VK and VH variable regions assembled in a scFv format is shown. Complementary determining regions (CDRs), the linker peptide and the myc-tag are boxed. (B) ELISA assay to measure and compare the binding activity of purified 353 scFv fragment and the parental Ig. HP1β-GST (black columns), HP1γ-GST (light columns) and lysozyme (squared columns) were used for coating. As a negative control, a purified anti-lysozyme D1.3 scFv fragment was used. Values obtained by coating the wells with GST alone have been subtracted. (C) Western Blot analysis of soluble (*S*) and insoluble (*I*) proteins extracted from NIH 3T3 cells transfected with αD11 (lanes 1 and 2), R4 (lanes 3 and 4) and 353 (lanes 5 and 6) intrabodies. ScFv fragments were detected with Mab anti-Myc 9E10. Differences in electrophoretic mobility between the scFvs are due to their respective primary sequences. Molecular weight markers (in kDa) are shown on the left.

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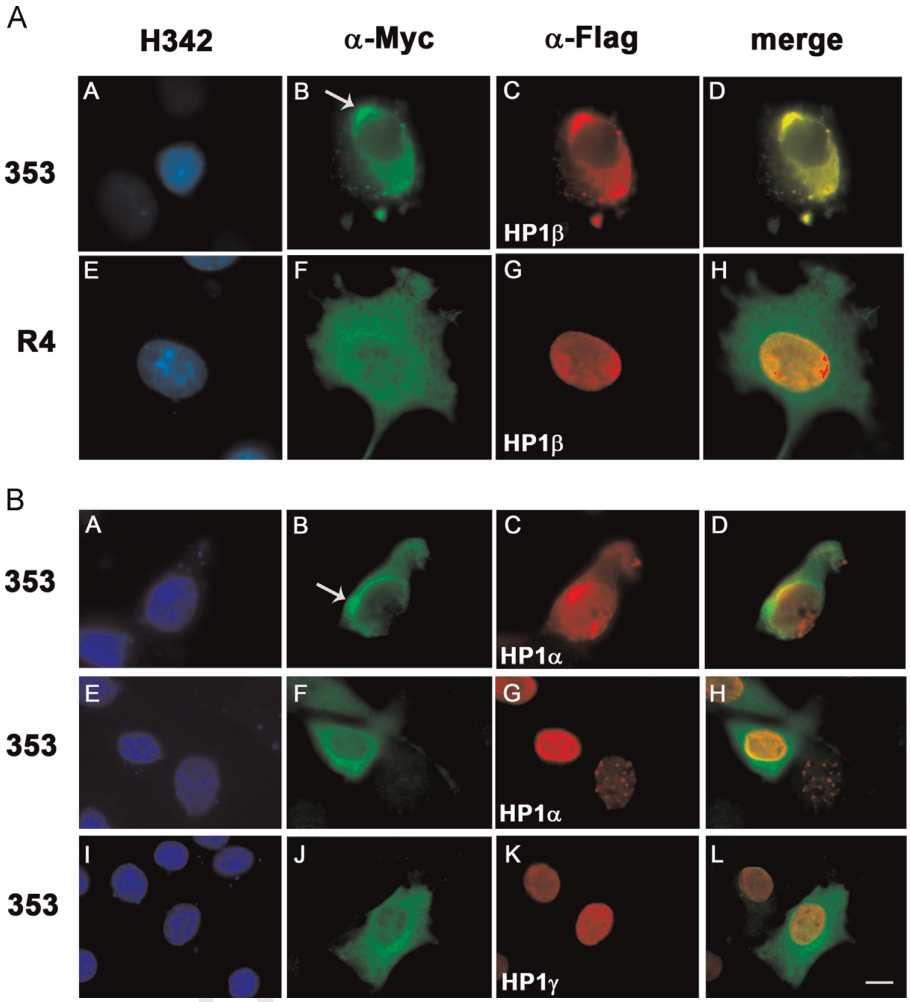

Fig. 2. Co-localisation analysis of cyt-353 scFv with HP1β, HP1α and HP1γ. (A) Cells were co-transfected with scFv fragments and recombinant HP1β. The intracellular distribution of scFvs was determined using rabbit anti-Myc antibodies (panels B and F) and HP1β by mouse anti-Flag antibodies (panels C and G). Panels D and H represent the merged images. Panels A and E depict nuclei stained with Hoechst 33342. (B) Immunofluorescence analysis of NIH 3T3 cells co-transfected with cyt-353 scFv fragment and the recombinant HP1α and HP1γ proteins. Panels C and G represent the distribution of Flag-tagged HP1α and panel K that of Flag-tagged HP1γ. Panels B, F and J represent the distribution of 353 intrabodies. Panels A, E and I depict nuclei stained with Hoechst 33342 and panels D, H and L are the merged images. Bar, 5 μm.

(lane 2). The mostly soluble anti-βGal scFv R4 is more efficiently expressed and only a minor amount of R4 intrabody is detected in the insoluble fraction (lanes 3 and 4). It is worth noting that the molecular weight of cyt-353 intrabody is lower compared to αD11 and R4 due to differences in primary sequence length (28 kD vs 32 kD).

To test whether the cyt-353 scFv specifically interacts with HP1β in the cytoplasm of living cells, we co-transfected myc-tagged cyt-353 intrabody and recombinant Flag-tagged HP1β into NIH 3T3 cells and undertook double immunofluorescence analysis. Fig. 2A shows the staining using anti-Myc antibodies to detect scFv fragments (panels B and F) and anti-Flag antibodies to detect HP1β (panels C and G). Merged images are shown in panels D and H. The intracellular localisation of the cyt-353 intrabody was studied by confocal analysis. As shown in Fig. 2A (panel B) and Fig. 2B (panels B, F and J) the myc-tagged cyt-353 intrabody is highly expressed and localizes in the cytoplasm of transfected cells. In most cells the signal is condensed in the perinuclear region or in large cytoplasmic aggregates (see arrows in panels B). In 20–30% of cells the staining is more reticular and diffusely widespread in the cytoplasm (see panel J).

HP1β distribution is altered by the cyt-353 intrabody. As shown in the merged image, anti-HP1β intrabody co-localises with HP1β in the cytoplasm (panel D). Intracellular expression of the control cytoplasmic scFv fragment (R4-anti-βGal) (panel F) does not influence the nuclear intracellular localisation of HP1β (panels G and H).

To investigate the specificity of retargeting, we then investigated the effect of cyt-353 scFv intrabody on the localisation of Flag-tagged HP1α and γ, the other two isoforms of HP1 (Fig. 2B). HP1α is partly diverted to the cytoplasm (panel C) and delocalizes from its heterochromatic localisation only in a small number of cells (~10%). Where delocalisation of HP1α takes place, it accumulates around the nuclear envelope or in smaller defined perinuclear regions (panels C and G). In these regions there is only a partial co-localisation between cyt-353 scFv and HP1α (merged images, panels D and H). In contrast, the distribution pattern of HP1γ does not change in cells expressing the intracellular cyt-353 scFv (Fig. 2B, panels K and L). Expression of the control cytoplasmic R4 intrabody does not affect intracellular distribution of HP1α and HP1γ molecules. It is worth noting that the distribution of endogenous HP1α and γ in cyt-353 scFv expressing cells parallels the results obtained with Flag-tagged recombinant constructs (Fig. 5B and data not shown).

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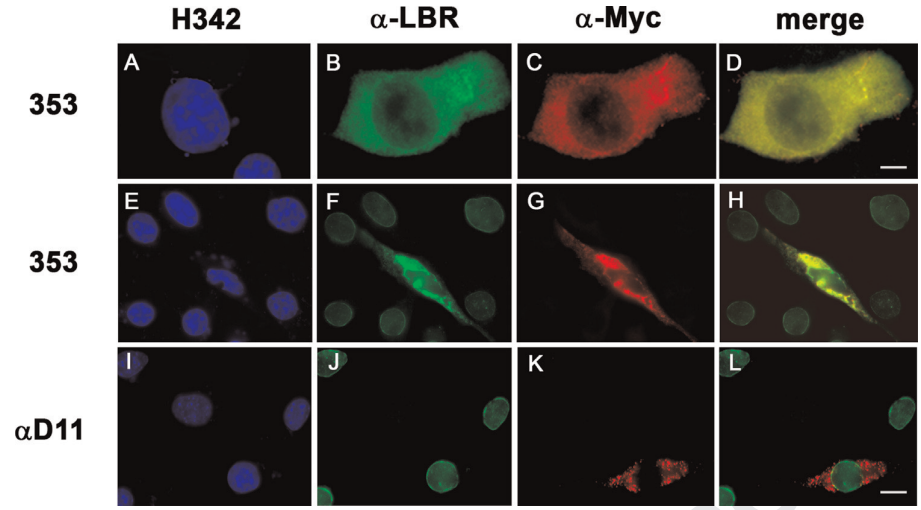

Fig. 3. Co-localisation analysis of cyt-353 scFv with LBR. NIH 3T3 cells, transfected with cyt-353 and αD11 scFv fragments, were double stained with Mab 9E10 IgG (panels C, G, and K) and with rabbit anti-LBR IgG (panels B, F and J). Merged images are shown in panels D, H and L. Panels A, E and I depict nuclei stained with Hoechst 33342. Bar, 5 μm in panels A–D. Bar, 10 μm in panels E–L.

3.2. *Intrabody-mediated re-targeting of HP1β causes co-aggregation of HP1β with LBR and H3–H4 histones in the cytoplasm*

Several studies have highlighted the importance of the association of the HP1 proteins with components of the nuclear envelope (NE) and, in particular, with LBR, a major constituent of the inner nuclear membrane [26,29]. LBR associates with HP1 proteins *via* histones H3/H4 [30], although other studies showed a direct binding of LBR to HP1 [26,27].

To test whether diversion of HP1β to the cytoplasm by the intrabody also affects endogenous LBR localisation, NIH 3T3 cells were transfected with cyt-353 or the control αD11 scFvs, and analysed by double immunofluorescence with anti-LBR (Fig. 3, panels B, F and J) and anti-myc antibodies (Fig. 3, panels C, G and K). Remarkably, in cyt-353 scFv transfected cells, LBR was predominantly found in the cytoplasm (panel B) or in large cytoplasmic aggregates (panel F), where it co-localises with the 353 intrabody (merged images panels D and H). In cells expressing the control αD11 scFv, LBR maintains its typical nuclear localisation, as in non-transfected cells (panel J).

We then studied the distribution of endogenous histones H3 and H4 in cyt-353 scFv transfected cells. Fig. 4A shows the results of a double staining experiment performed on cells transfected with the anti-HP1β intrabody (panels C, G and K) and cells transfected with the control αD11 scFv (panel O). Both intrabodies mostly localise in the cytoplasm. We find that the expression of cyt-353 scFv intrabody results in a dramatic altered distribution of histone H3 (panel B) and histone H4 (panel F) that are found in the cytoplasm of all transfected cells. A strong co-localisation with cyt-353 intrabody is evident in the merged images (panels D and H respectively). We never observed changes in the intracellular distribution of histone H2A (panel J), indicating a specific interaction between HP1β and H3/H4 histones. Interestingly, endogenous methylated histone H3 at K9 (Me9H3) (Fig. 4B, panel B), which is a marker of constitutive heterochromatin, is not affected by cyt-353 intrabody and presents its typical nuclear localisation. The expression of the control αD11 scFv (Fig. 4A, panel O) does not affect the distribution of H3 (panel N), as well as Me9H3, H4 and H2A histones and Suvar39h1 (not shown).

It is worth noting that a much higher fluorescence signal of LBR (Fig. 3, panels B and F), H3 and H4 (Fig. 4A, panels B and F) is evident in cyt-353 scFv transfected cells compared with cells transfected with control scFvs (Fig. 3, panel J and Fig. 4A, panel N). This finding may suggest inhibition of degradation when these proteins aggregate in the cytoplasm [8]. The absence of endogenous nuclear staining of histones H3 and H4 in the nucleus in 353 intrabody expressing cells is due to the fact that to acquire images of cells harbouring aggregates, a short exposure time is necessary to avoid overexposure of the images, drawing the H3 and H4 nuclear signals below the detection limit in Fig. 4B and F.

Since it has been reported that the interaction of core histones with HP1 and LBR are regulated by acetylation [29,30], we analysed the distribution of H3 in cyt-353 scFv transfected cells treated with different concentration of two deacetylase inhibitors, TSA and Na butyrate [49]. As shown in Fig. 4C, after treatment with 5 mM Na butyrate, H3 histone (panel B) is confined in the nucleus of cyt-353 scFv transfected cells (as in non-transfected cells), indicating that 353-scFv:HP1β:H3 association in the cytoplasm is abolished under hyper-acetylating conditions. On the contrary, the cytoplasmic localisation of HP1β and LBR in cyt-353 scFv transfected cells was not affected by treatment with deacetylase inhibitors (not shown).

We further analysed the biochemical features of cytoplasmic aggregates detected in cyt-353 scFv expressing cells. We fractionated NIH 3T3 transfected cells by separating nuclear and cytoplasmic fractions in the absence of detergent. Subsequently, these two fractions were detergent-extracted and centrifuged to isolate soluble and insoluble pools and analysed by Western blot. We examined the distribution of endogenous HP1β, LBR, H3, Me9H3, H4, H2A histones and the histone H3 methyl-transferase Suvar39h1 in the four separated pools derived from intrabody-transfected and non-transfected cells. In non-transfected cells (Fig. 5 A) HP1β is mostly in the nucleus, both in the soluble and insoluble fractions (lanes 1 and 2) although a consistent amount is also present in the detergent soluble cytoplasmic fraction (lane 3). LBR, a membrane protein, is completely detergent extractable in our experimental conditions and partitioned between the soluble nuclear and cytoplasmic fractions (lanes 1 and 3). Histones H3, Me9H3, H4, and H2A are present, as expected, only in nuclear pellets, highly enriched in chromatin (lane 2). Suvar39h1 distributes mostly in the nucleus (lanes 1 and 2) and, as HP1β, is also found in the detergent soluble cytoplasmic fraction (lane 3). Importantly, none of these molecules is found in the cytoplasmic detergent insoluble fraction (Fig. 5A, lane 4).

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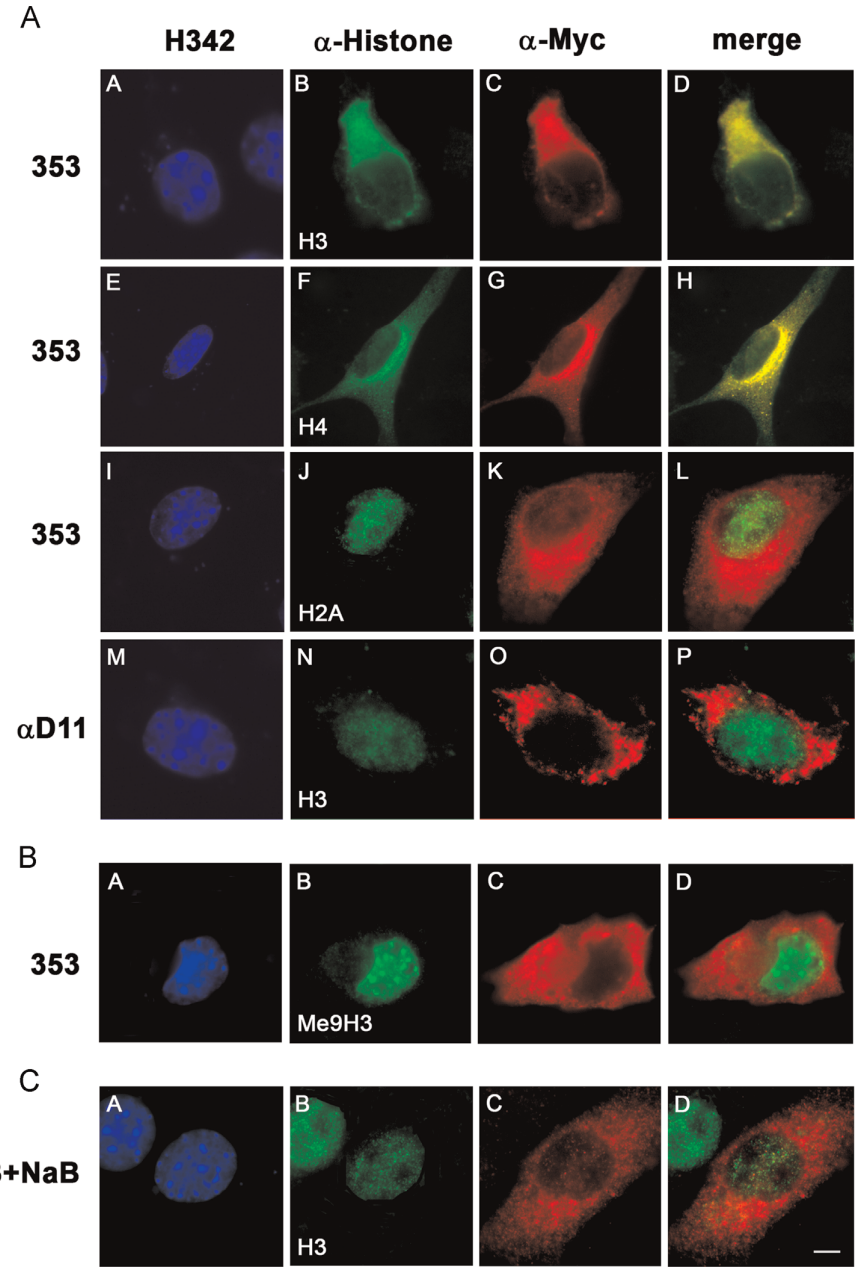

Fig. 4. Co-localisation analysis of cyt-353 scFv with H3, H4, H2A, Me9H3 and acetylated H3 histones. (A) Double immufluorescence analysis with rabbit anti-histone H3 (panels B and N), rabbit anti-histone H4 (panel F), rabbit anti-histone H2A (panel J) and Mab anti-myc 9E10 IgG (panels C, G, K, and O) of cells expressing cyt-353 and αD11 scFv fragments as indicated. (B) NIH-3T3 cells transiently transfected with cyt-353 scFv fragment and stained with rabbit anti-Me9H3 IgG (panel B) and with Mab anti-myc 9E10 IgG (panel C). (C) Double immunofluorescence analysis of cyt-353 scFv transfected cells treated with 5 mM Na-butyrate and stained with rabbit anti-histone H3 (panel B) and Mab anti-myc 9E10 IgG (panel C). Panels A, E, I, and M in (A), and A in (B) and (C) depict nuclei stained with Hoechst 33342 and panels D, H, L, and P in (*A*), and D in (B) and (C) represent the merged images. Bar, 5 μm.

To investigate the ability of intrabody-mediated diversion of HP1β, LBR, H3, Me9H3, H4, H2A histones and the histone H3 methyl-transferase Suvar39h1 molecules into cytoplasmic aggregates, we examined the partitioning of the nuclear and cytoplasmic insoluble fractions. A consistent amount of endogenous HP1β is present in the cytoplasmic insoluble pool in cyt-353 scFv transfected cells (Fig. 5B, compare lane 6 with lanes 2 and 4). LBR protein is also in the cytoplasmic insoluble fraction (lane 6). Since LBR is detergent soluble in non-transfected cells (Fig. 5A, lanes 1 and 3), the shift of this molecule towards the insoluble pool is particularly salient. The same shift is also detectable for H3 and H4 histones, which are present in the insoluble cytoplasmic fraction only in cyt-353 scFv transfected cells (Fig. 5B, lane 6). It is worth noting that in cyt-353 scFv transfected cells there is no change in the distribution of H2A, Me9H3 and Suvar39h1.

As described in Fig. 4C, panel B, hyper-acetylated histone H3 does not interact with HP1β in the cytoplasm. We, therefore, wanted to verify whether deacetylase inhibitors could affect the partitioning of H3 in nuclear and cytoplasmic insoluble fractions. We examined, by Western blot, H3 distribution in cyt-353 scFv transfected cells treated with different concentration of TSA and Na butyrate. Both inhibitors were equally effective. As shown in

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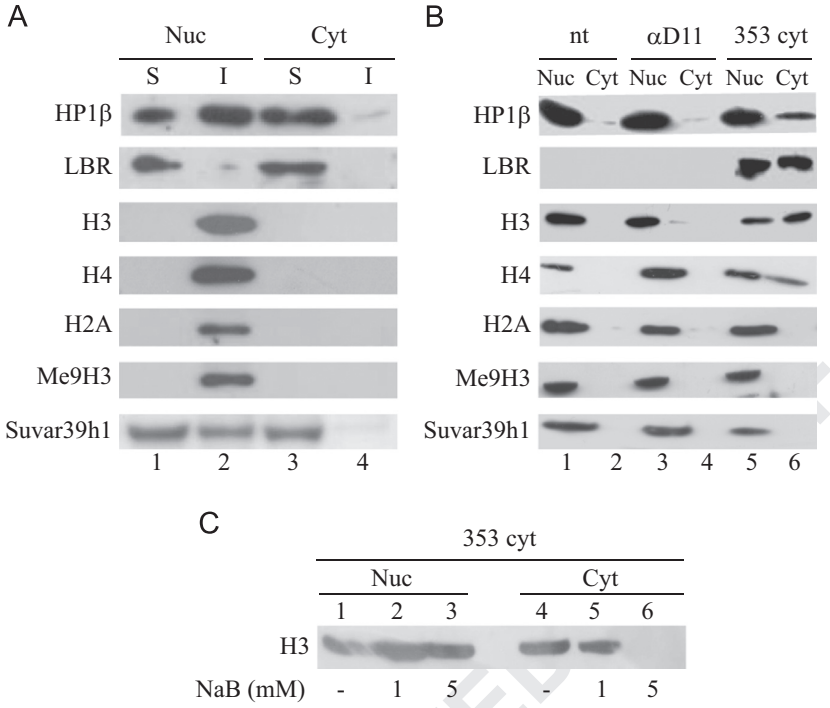

Fig. 5. Co-sequestration of HP1β, LBR and H3:H4 core histones in 353 scFv cytoplasmic aggregates. (A) Western blot analysis of soluble (lanes 1 and 3) and insoluble (lanes 2 and 4) proteins extracted from nuclei (Nuc) and cytoplasm (Cyt) of non transfected NIH 3T3 cells. (B) Western blot analysis of insoluble pool derived from nuclei (Nuc) and cytoplasm (Cyt) of non transfected (nt, lanes 1 and 2), cells transfected with αD11 scFv (lanes 3 and 4) and cyt-353 scFv intrabodies (lanes 5 and 6). (C) Western blot analysis of insoluble pools extracted from nuclei (Nuc) and cytoplasm (Cyt) of NIH 3T3 cells transfected with the cyt-353 scFv fragment and cultured in the presence of 1 mM (lanes 2 and 5) and 5 mM Na butyrate (lanes 3 and 6) for 16 h. Detection of blots in A and B was performed by using specific antibodies for HP1β, LBR, Suvar39h1, histones H3, H4, H2A and Me9H3. Blot in C was detected using rabbit anti-H3 IgG. Equivalent amount of soluble and insoluble proteins was loaded for each lane. The blots shown are representative of three different experiments.

Fig. 5C, the presence of 1 mM Na butyrate (lanes 3 and 4), reduces the amount of H3 present in the cytoplasmic insoluble fraction and 5 mM Na butyrate (lanes 5 and 6) completely abolishes the presence of histone H3 in the cytoplasmic insoluble fraction.

3.3. Altered nuclear morphology and apoptosis in cyt-353 intrabody expressing cells

Immunofluorescence analysis of transfected NIH 3T3 cells revealed that cyt-353 scFv alters nuclear morphology. To explore this phenomenon in more detail, we analysed cyt-353 scFv and R4-scFv expressing cells by immunofluorescence with anti-myc antibodies and counterstaining with Hoechst 33342, 24 h after transfection. As shown in Fig. 6A many nuclei of cyt-353 intrabody-expressing cells have an irregular shape consisting of invaginations of the nuclear envelope (see arrows in panels A and D). By counting Annexin V positive cells and condensed/fragmented nuclei stained with Hoechst 33342-positive cells, we found a cyt-353 intrabody dose-dependant apoptotic effect (from 0.5 to 2 μg of DNA plasmid) with a peak of 60–65% of transfected cells undergoing apoptosis at the highest amount of DNA plasmid (Fig. 6B). Similar results were observed 48 h after transfection (68 ± 2% apoptotic cells, not shown). Parallel experiments in HeLa cells confirmed the toxicity of cyt-353 intrabody that leads to 58 ± 2% of apoptotic cells by using 2 μg of DNA plasmid (not shown). It is worth noting that in the same experimental conditions, the control R4 intrabody does not induce apoptosis or altered nuclear morphology (Fig. 6B and Fig. 2A, panel E).

3.4. Overexpression of HP1β rescues altered nuclear morphology and apoptosis

HP1 isoforms exhibit differential patterns of subnuclear localisation [32], suggesting that, despite their sequence similarities, HP1α, β and γ interact with chromatin and NE components in different ways. Notwithstanding this fact, the possibility of redundancy in function between the three isoforms is still an open question. To examine this possibility, we co-transfected NIH 3T3 cells with increasing amounts of DNA plasmids encoding recombinant HP1α, β and γ and constant amount of cyt-353 scFv (ratios HP1s/scFv1:4; 1:2; 1:1) and inspected the effect of their overexpression on nuclear morphology and apoptosis. Remarkably, the ectopic expression of mouse recombinant HP1β results in an almost complete dose-dependant rescue of the cyt-353 scFv-induced phenotype (apoptosis/altered nuclear morphology was reduced from 60% to 6–8%) (Fig. 6C). Expression of recombinant HP1α leads to a 20% rescue of the altered nuclear morphology and of the apoptotic phenotype at the ratio 1:1. On the contrary, no significant rescue is observed upon expression of recombinant HP1γ. It is worth noting that recombinant HP1α, β and γ are expressed at similar levels and the expression level of cyt-353 scFv remains constant when increasing amount of HP1α, β and γ plasmids are co-transfected (Fig. S1, Supplementary material).

We then investigated whether LBR overexpression could rescue the nuclear phenotype observed in cyt-353 scFv transfected cells. We reasoned that Flag-LBR could stabilise the peripheral organisation of heterochromatin at the nuclear envelope, making it harder for the cyt-353 scFv to destabilise the structure. As shown in the histogram of Fig. 6C, recombinant LBR is not able to restore the 353-scFv-induced phenotype.

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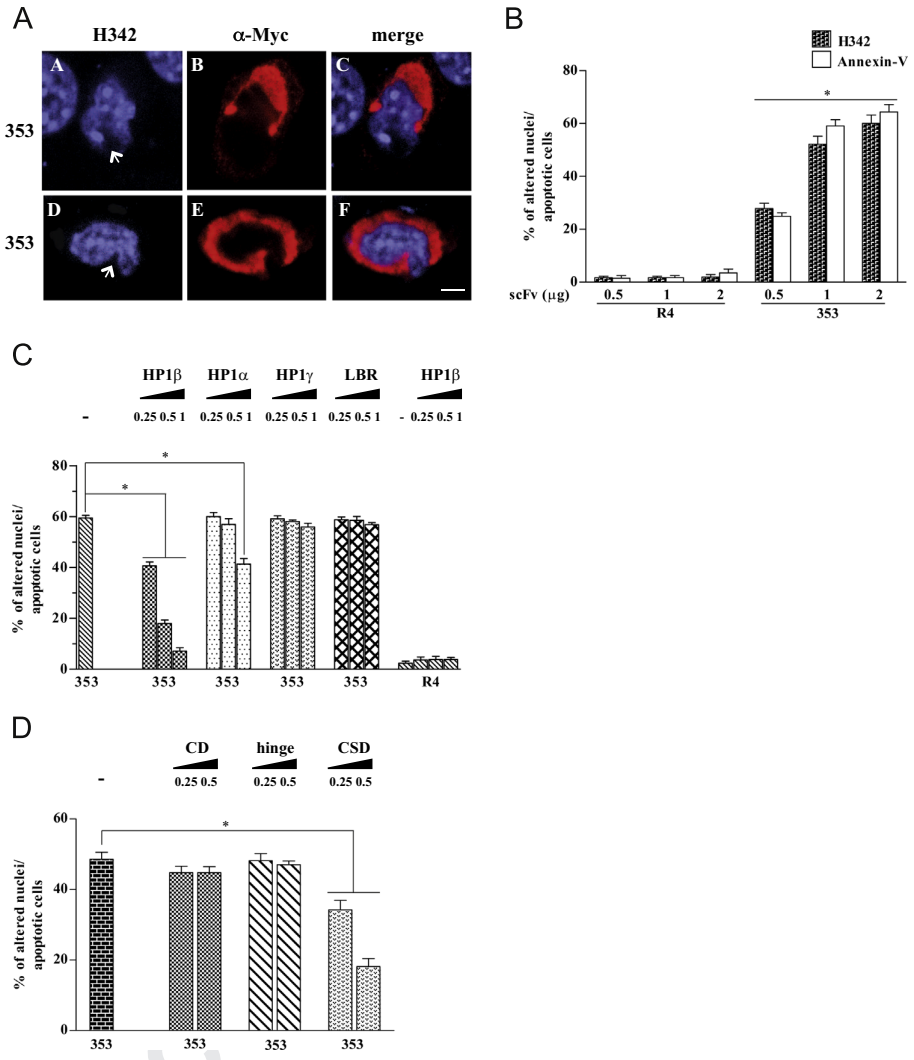

Fig. 6. Rescue of the nuclear morphology and apoptotic phenotype by recombinant HP1β or CSD mutant. (A) NIH 3T3 cells were transiently transfected with cyt-353 scFv fragment. Intrabody localisation was determined using anti-Myc antibodies (panels B and E). Panels A and D show the same cells counterstained with Hoechst 33342. Panels C and F represent the merged images. Bar, 5 μm. (B) Nuclei with altered morphology and apoptotic cells (visualised either with Annexin V and with Hoechst 33342) of intrabody transfected cells were counted. A dose-dependant effect of intrabody expression (0.5–2 μg DNA plasmid) was analysed. (C) NIH 3T3 fibroblasts were co-transfected with constant amount of cyt-353 fragment and increasing concentrations of recombinant Flag-tagged HP1s and Flag-tagged LBR constructs to assess the percentage of cells with altered nuclei/apoptotic phenotype. As control we used R4 scFv intrabody. (D) NIH 3T3 were co-transfected with cyt-353 and GFP-HP1β mutants (CD, Hinge and CSD) to evaluate the percentage of cells with altered nuclear morphology and apoptotic phenotype. At least 150 positively co-transfected cells for each plasmids were analysed. The results shown are the average of three different experiments.

In control experiments, co-expression of the control R4 scFv (Fig. 6C) or αD11 (not shown) with increasing amount of recombinant HP1α, HP1γ or LBR does not cause an overt nuclear alteration. As monitored by Western blot, recombinant HP1α, β, γ and LBR are expressed at similar levels in these experimental conditions (not shown).

In order to define the region of HP1β responsible for the rescue of nuclear damages, we examined the contribution of the N-terminal domain CD, the C-terminal CSD and the hinge region of HP1β protein. To this aim, NIH 3T3 cells were co-transfected with increasing amounts of GFP-tagged HP1β mutants that contain the CSD (GFP-CSD), the CD (GFP-CD) or the hinge region (GFP-HP1h) and with a constant amount of cyt-353 scFv (ratios HP1 mutants/scFv1:4; 1:2; 1:1). As shown in Fig. 6D, the percentage of cells with altered nuclear morphology/apoptotic phenotype decreases in a dose dependant way only when cells are co-transfected with the GFP-CSD mutant, the C-terminal portion of HP1β that contains the epitope recognised by the cyt-353 intrabody (apoptosis/altered nuclear morphology was reduced from 50% to 18–20%). On the contrary, the GFP-CD and GFP-HP1h mutants do not have any effect on the phenotype of cyt-353 scFv transfected cells. In control experiments, GFP-tagged HP1β mutants are expressed at similar levels as verified by Western blot analysis (not shown). Altogether these results confirm that, in cells, the cyt-353 scFv intrabody binds to the CSD of HP1β and maintains its immunospecificity and that this specific interaction brings to the accumulation of aggregates containing HP1β, LBR and H3/H4 histones in the cytoplasm with the concomitant effects on nuclear morphology and cell viability.

3.5. Overexpression of HP1β-CD mutant displaces LBR from cytoplasmic HP1β-LBR aggregates

We next wanted to define the domain of HP1β that is involved in the formation of cytoplasmic aggregates containing H3–H4 and

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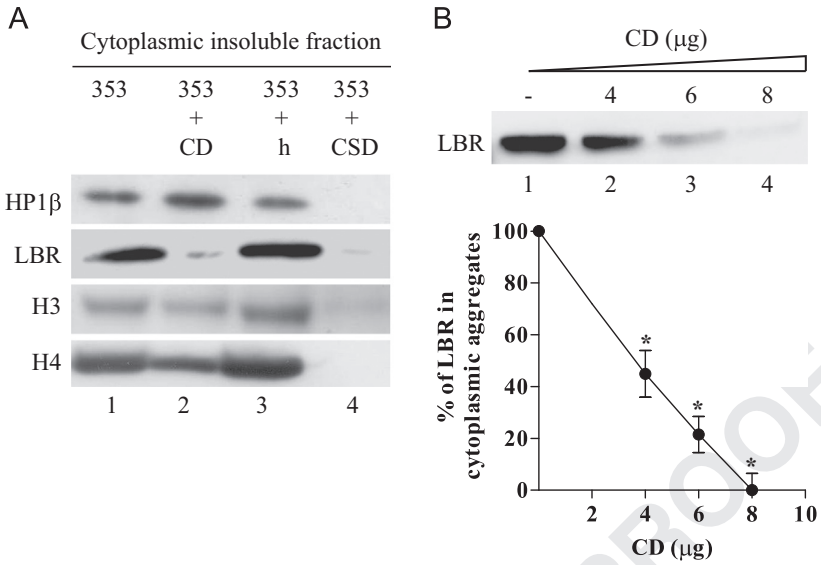

Fig. 7. LBR interacts with the CD region of HP1β. (A) NIH 3T3 fibroblasts were co-transfected with DNA plasmids encoding for cyt-353 scFv fragment (lane 1) or cyt-353 scFv and GFP-CD (lane 2), GFP-HP1β hinge (lane 3) and GFP-CSD (lane 4) mutants. Western blot of insoluble cytoplasmic proteins was performed by using specific antibodies for HP1β, LBR, histones H3 and H4. Equal amount of proteins was loaded for each lane. (B) Western blot analysis of insoluble cytoplasmic proteins derived from cells transfected with cyt-353 scFv (lane 1) or cyt-353 scFv and increasing concentrations of DNA plasmid encoding for GFP–CD mutant (lanes 2–4) using rabbit anti-LBR antibodies (upper panel). Lower panel shows the densitometric quantitation of LBR immunoreactive band in the cytoplasmic insoluble fraction. 100% refers to the amount of LBR in cytoplasmic aggregates of cyt-353 scFv transfected cells. The results shown are representative of three different experiments.

LBR. To that end, we performed competition assays. In these experiments a fixed amount of cyt-353 scFv DNA plasmid was co-transfected with increasing concentration of DNA encoding the GFP tagged HP1β-CSD, HP1β-CD and HP1β-hinge (h) mutants (ratios HP1 mutants/scFv1:4; 1:2; 1:1). By Western blot we examined the presence of HP1β, LBR and histones H3 and H4 in the cytoplasmic insoluble fraction (Fig. 7A). Overexpression of the C-terminal domain of HP1β (GFP-CSD) leads to the complete displacement of HP1β, histones H3 and H4, and LBR proteins (Fig. 7A, lane 4), confirming that the CSD mutant competes with the binding site of the cyt-353 scFv, thus abolishing the intrabody-mediated formation of the cytoplasmic aggregates containing LBR and H3/H4 histones. The GFP-CD mutant, on the contrary, does not displace HP1β, but does compete with LBR for binding to HP1β (Fig. 7A, lane 2). Histone H3 and H4 binding is affected, albeit only slightly, by the expression of the GFP-CD mutant; there is approximately 20% decrease of the intensity of H3 and H4 bands in the blots (Fig. 7A, lane 2). By contrast, co-transfection of the GFP-HP1-hinge does not affect the aggregated HP1β, LBR and H3/H4 histones that remain in the insoluble cytoplasmic fraction (Fig. 7A, lane 3).

The effect of increasing concentrations of the GFP-CD mutant on the binding between LBR and HP1β was also verified and is shown in Fig. 7B. We performed densitometric analysis to quantify LBR in cytoplamic aggregates and found that a ratio of 1:1 between cyt-353 scFv and GFP-CD plasmids (6 μg:6 μg) reduces the LBR band of $78 \pm 5\%$. These data confirmed that the CD mutant inhibits LBR binding in a dose dependant fashion.

4. Discussion

Here we report that anti-HP1β intrabodies targeted to the cytoplasm of mammalian cells inhibit the traffic of HP1β to the nucleus and, consequently, its function. The intracellular expression of cyt-353 intrabody causes the intrabody-mediated sequestration of HP1β in the cytoplasm, where it often accumulates around the external part of the nuclear envelope. Biochemical analysis of cyt-353 scFv transfected cells indicates that the intrabody-HP1β complex is present as intracellular cytoplasmic aggregates. Since cyt-353 scFv is totally recovered in the cytoplasm as insoluble aggregates, we hypothesise that the HP1β intrabody interaction occurs in the cytoplasm and that the aggregation promotes the sequestration of newly-synthetised HP1β in large aggregates, physically impairing HP1β traffic to the nucleus. Consistent with this result the nuclear version of 353scFv also tends to aggregate and localizes in the cytoplasmic compartment of transfected cells, despite the presence of a dominant NLS signal, leading to co-aggregation of HP1β in the cytoplasm (data not shown). As reported for other intrabodies, the intrinsic insolubility of cyt-353 scFv in the cytoplasm might be related to its net overall charge and/or hydropathicity [50]. Control experiments showing that another insoluble scFv does not lead to HP1β aggregation suggest that even though the intrabody folding kinetics are sub-optimal, there is still enough of a binding site present that the effect appears to be specific, although direct evidence of this mechanism is still lacking.

Biochemical and immunofluorescence analysis of cyt-353 intrabody-transfected cells reveals that LBR and two core histones (H3/H4) are also specifically sequestered in the cytoplasm, together with HP1β. The N-terminal portion of LBR is probably responsible for the binding to HP1β [26–28]. This domain is in an open conformation free to interact with molecules in the mitotic cytoplasm during nuclear reassembly. Competition experiments with various HP1β mutants has allowed mapping of the LBR-binding site within the N-terminal region of HP1β, which includes the chromodomain. Thus, we find a total displacement of LBR when HP1-CD mutant is overexpressed. This is in accordance with *in vivo* studies on the dynamic association between HP1 proteins and elements of the nuclear membrane that have provided evidence for a specific association of the CD region of HP1 with the nuclear membrane [29] and, more specifically, with LBR through H3/H4 histones [30,31]. We suspect that most of the LBR molecules directly interact with the CD of HP1β rather than indirectly through H3/H4 histones because overexpression of HP1β CD mutant results in only 20% displacement of H3/H4. The interaction of

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HP1β with H3/H4 histones may take place *via* the H3:CSD dimer interaction, as described for HP1α and γ [51].

Notwithstanding the fact that histones H3/H4 are diverted to the cytoplasm in 353-scFv expressing cells, the intracellular distribution of Me9H3 and its specific methyltransferase Suvar39h1 is not affected. We propose two different explanations for this result. First, since methylation of H3 occurs in the nucleus [52], cyt-353 intrabody is not able to intercept methylated pool of H3 (i.e. Me9H3) bound to HP1β, but may interact only with the neosynthetised and unmethylated H3 molecules. Of note, Dialynas et al. demonstrated that HP1β is able to directly interact with H3, regardless of its methylation state [31]. A second possibility is that the methylated pool of H3 interacting with HP1β might form part of the small, very-slow immobile HP1β fraction already incorporated in the heterochromatin, thus not accessible to the interaction with cyt-353 intrabody even during mitosis [53].

Treatment of cyt-353 scFv expressing cells with histone deacetylase inhibitors TSA or sodium butyrate impairs the sequestration of H3/H4 in cytoplasmic aggregates in a dose dependant manner. The most likely explanation is that histone deacetylase (HDAC) inhibitors disrupt the interaction of histones with HP1 proteins that are incorporated into the cytoplasmic aggregates by the intrabody and thereby lead to the dispersion of the histones. This is consistent with previous work which has shown that histone:HP1 interaction is disrupted by HDAC inhibitors [54]. However, it is also possible that the neutralization of charge that results from deacetylation may also influence the incorporation of the histones into the intrabody aggregates.

In cyt-353 scFv transfected cells, most nuclei have irregular shape with lobules and deep invaginations of the envelope and a high percentage of cells undergo apoptosis. Our observation that ablation of HP1β induces altered nuclear morphology supports a role for HP1β in maintaining the nuclear lamina reassembly [55]. In particular, HP1-NE associations regulate the NE reassembly at the end of mitosis [29] and LBR and H3–H4 histones are involved in this function [30,31].

Overexpression of recombinant full-length HP1β or C-terminal (CSD) fragment of HP1β, which represents the intrabody-binding site, abolishes the formation of intracellular cytoplasmic aggregates, rescues the altered nuclear morphology and protects from apoptosis. Interestingly, a moderate rescue of these phenotypes is obtained by overexpression of recombinant HP1α, but only at highest concentration, suggesting a functional redundancy between HP1β and HP1α [13]. In contrast, over-expressing recombinant LBR or HP1β-CD mutant, which displaces endogenous LBR from cyt-353 scFv cytoplasmic complexes, does not rescue the phenotype. Taken together, these findings raise the possibility that an association involving components of condensed chromatin and inner nuclear membrane proteins in the cytoplasm might provide the basis for organising peripheral heterochromatin and regulating nuclear envelope assembly. A future line of research will be to investigate the relationship between the LBR/H3–H4/HP1β complex and regulation of genome organisation and expression at the nuclear periphery [56,57].

We describe two major phenotypes in cyt-353 intrabody expressing cells: an altered nuclear morphology and cell death. Similar results were obtained with anti-chromodomain aggregating intrabodies which are able to divert HP1β in aggregates, inducing cell death phenotypes associated with pronounced invaginations and lobules formation of the nuclear membrane [11]. In contrast, intrabody-mediated diverting of Ras oncoprotein to cytoplasmic aggregates induces apoptosis without altering nuclear morphology [8]. These results, together with the present study, support the notion that HP1β is important in maintaining nuclear membrane integrity. Of note, in mandibuloacral dysplasia, a rare form of laminopathy due to a mutation in *LMNA* gene encoding lamin A/C, the delocalisation of HP1β and LBR is associated to a marked alteration of nuclear membrane and chromatin organisation [58]. It is not clear, however, whether HP1β is required for cell survival or whether intrabody aggregation is responsible for cell death observed in about 60% of transfected cells. Thus, although we find that intrabody-mediated HP1β aggregation in the cytoplasm induces cell death, knockdown of HP1β by specific RNAi in different cell types neither induces cell death nor alters cell cycle progression [37,38]. Moreover, mice expressing only 10% of HP1β protein levels are indistinguishable from wild-type littermates [35] and displacement of HP1β from constitutive heterochromatin by using dominant negative HP1 constructs does not affect cell viability, suggesting that the amount of HP1β required for survival is small [59,53].

We envisage different explanations for the cell death phenotype observed in cyt-353 scFv expressing cells. One possibility is that intrabody-mediated aggregation of HP1β, a well-known adaptor protein able to recruit several binding partners, leads to knockdown of these proteins, including LBR and H3/H4 histones, which could be required for cell survival. Alternatively, cell death may result from the formation of cytoplasmic inclusions, which derive from abnormal accumulation and aggregation of proteins prone to misfold observed in different pathological conditions, including neurodegenerative diseases and cancer [60,61]. Accordingly, intrabodies may act as amyloid-like proteins promoting, beyond the described specific interactions, widespread aberrant interactions with other unrelated proteins having essential cellular functions, leading to cell toxicity [62], or intrabody-mediated cytoplasmic HP1β aggregation could induce an apoptotic phenotype strictly related to the impairment of proteasome activity, as we have previously reported for anti-Ras intrabodies [8,63]. Regarding the latter, it has been recently shown that aggregation of proteins such as Tau, α-synuclein and p53, also termed as intrinsically disordered proteins (IDPs), is able to trigger a cytotoxic response associated with proteasome inhibition. IDPs may also promote aberrant interactions with other proteins, leading to diversion from their functional sites and sequestration into aggregates, thereby enhancing their cytotoxic potential [64,65]. In this context, aggregating intrabodies such as 353-anti-HP1β, anti-CD, anti-Ras and anti-E6 viral protein [8,11,66] could act as IDPs, inducing cell death through co-aggregation and accumulation of crucial proteins and consequent engulfment of the ubiquitin–proteasomal system. The property of intrabodies to specifically interfere with the aggregation kinetics and on modulating the severity of cellular dysfunction could be further exploited in therapy of pathological conditions where it is necessary to induce cell death, such as, for example, in tumours.

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.yexcr.2015.09.006.

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