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► **To cite this version:**

Didier Fesquet, David Llères, Charlotte Grimaud, Cristina Viganò, Francisca Méchali, et al.. The 20S proteasome activator PA28 γ controls the compaction of chromatin. 2020. hal-03023078

HAL Id: hal-03023078

<https://hal.science/hal-03023078>

Preprint submitted on 25 Nov 2020

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The 20S proteasome activator PA28 γ controls the compaction of chromatin

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Running title: PA28 γ controls chromatin compaction

Key words: PA28 γ / Proteasome/ HP1/ Heterochromatin/ FLIM-FRET.

Abstract

PA28 γ , a nuclear activator of the 20S proteasome, is involved in the degradation of several proteins regulating cell proliferation, but its precise cellular functions remain unclear. Here, we show that PA28 γ is crucial for chromatin compaction. In human cells, we find that a small fraction of PA28 γ co-localizes with HP1 β , and PA28 γ is present at HP1 β -containing repetitive-DNA regions. PA28 γ -depletion induces a decompaction of pericentromeric heterochromatin, as observed upon HP1 β -knockdown. Using a quantitative FLIM-FRET based microscopy assay monitoring close proximity between nucleosomes in living cells, we show that PA28 γ controls chromatin compaction more broadly. Importantly, HP1 β on its own is unable to drive chromatin compaction without the presence of PA28 γ . At the molecular level, PA28 γ is necessary to maintain the level of H3K9 tri-methylation, as well as H4K20 mono- and tri-methylation, modifications required for heterochromatin establishment. Overall, our findings demonstrate the implication of a proteasome regulator in chromatin organization.

Introduction

Proteasome-mediated protein degradation is a central pathway that controls the stability and the function of numerous proteins in most cellular processes (Collins & Goldberg, 2017). The various functions of the proteasome are actually performed by a family of proteasome complexes resulting from the association of different regulators/activators with the catalytic core-called the 20S proteasome (Rechsteiner & Hill, 2005; Coux *et al.*, 2020). Among the proteasome family, the best-characterized member is the 26S proteasome, composed of the 20S core proteasome and the 19S regulator, which degrades poly-ubiquitylated proteins in an ATP-dependent manner (Collins & Goldberg, 2017; Bard *et al.*, 2018). In the nucleus, two other regulators, PA200 (Ustrell *et al.*, 2002; Rechsteiner & Hill, 2005) and the homoheptamer PA28 γ (also known as REG γ or Ki antigen) (Ma *et al.*, 1992; Wilk *et al.*, 2000; Mao *et al.*, 2008), are present and function in an ATP- and ubiquitin- independent manner. Among their many functions, it is now well established that proteasome complexes are associated with chromatin and enriched at specific sites in the genome (Geng & Tansey, 2012; Kito *et al.*, 2020). Notably, they are specifically recruited during transcription or in response to DNA damage, thereby suggesting a direct role for chromatin-associated proteasome complexes in genomic processes (McCann & Tansey, 2014).

Chromatin exists mainly in two distinct compaction states: whereas euchromatin is a relaxed state that is generally transcriptionally active, heterochromatin corresponds to a highly compacted and often repressed state, rich in repetitive sequences such as satellite repeats, transposable elements and ribosomal DNA (Lippman *et al.*, 2004; Nishibuchi & Nakayama, 2014; Saksouk *et al.*, 2015; Janssen *et al.*, 2018). Heterochromatin is paramount to the stability of eukaryotic genomes. Indeed, loss of control over these repetitive DNA regions can lead to transcriptional perturbation and DNA recombination, all of which events are at the root of oncogenic transformation (Ayarpadikannan & Kim, 2014; Klement & Goodarzi, 2014).

Multiple evidence from genetic and cell biology point to an important involvement of Heterochromatin Protein-1 (HP1) (Maison & Almouzni, 2004; Verschure *et al.*, 2005) and trimethylation of histone H3 lysine 9 (H3K9me3) (Martin & Zhang, 2005; Saksouk *et al.*, 2015) and of histone H4 lysine 20 (H4K20me3) (Schotta *et al.*, 2004; Oda *et al.*, 2009; Beck *et al.*, 2012; Bosch-Presegue *et al.*, 2017) in establishing and maintaining heterochromatic states. Two proteins of the HP1 family (Nielsen *et al.*, 2002; Thiru *et al.*, 2004), HP1 α and β , are recruited through their binding to H3K9me3, and participate in the folding of chromatin

into higher-order structures (Bannister *et al.*, 2001; Lachner *et al.*, 2001; Maison & Almouzni, 2004; Machida *et al.*, 2018). Recent reports identify a liquid phase-like HP1 population that generates a dynamic phase transition compartment surrounding the stable chromatin-bound HP1 fraction (Larson *et al.*, 2017; Strom *et al.*, 2017). Such a compartmentalization mechanism may facilitate the access of proteins and their rapid exchange, necessary for the dynamic structural changes of heterochromatin during cell cycle progression and in the DNA damage response. Thus, it has been suggested that HP1 would act as a platform that selectively favors local concentration of different proteins to fulfill their chromatin-related functions (Grewal & Jia, 2007). However, the mechanism by which HP1 folds H3K9me3-containing chromatin into higher-order structures, and the requirement of other factors have not been fully elucidated.

The roles of the different proteasome complexes in chromatin organization remain still elusive as well. Although PA28 γ -20S proteasome complexes constitute only a minor fraction of the whole proteasome population (below 5%) (Fabre *et al.*, 2014), PA28 γ is important for cell growth and proliferation. Indeed, among the limited number of identified proteins whose degradation is mediated by PA28 γ -20S proteasome complexes, many of these such as CKIs (p21, p19, p16) and Myc (Chen *et al.*, 2007; Li *et al.*, 2007; Li *et al.*, 2015) are involved in the control of cell proliferation. Consistent with this, PA28 γ -knockout mice show a decrease in body size (Murata *et al.*, 1999; Barton *et al.*, 2004). In addition, PA28 γ plays a role in intranuclear organization, since it is involved in the dynamics of various nuclear bodies, including Cajal bodies (Cioce *et al.*, 2006; Jonik-Nowak *et al.*, 2018), nuclear speckles (Baldin *et al.*, 2008), and promyelocytic leukemia protein bodies (PML) (Zannini *et al.*, 2009). A potential role of PA28 γ in chromatin has also been proposed, since it has been linked to chromosome stability (Zannini *et al.*, 2008) and DNA repair (Levy-Barda *et al.*, 2011).

In this study, we highlight a still unsuspected function of PA28 γ in the control of chromatin compaction. By studying the localization of the 20S proteasome in human cells, we found that a fraction of the 20S proteasome localizes in nuclear foci, together with its regulator PA28 γ and HP1 β . Subsequent investigations revealed that PA28 γ is associated with repetitive DNA sequences abundant in heterochromatin, and is necessary to sustain the compaction of chromatin. This function of PA28 γ , possibly independent of the 20S proteasome, depends on its ability to maintain appropriate levels of H3K9me3 and H4K20me3, histone modifications that are both involved in heterochromatin formation.

Results

A fraction of PA28 γ -20S proteasome complexes co-localizes with HP1 proteins.

To investigate the subcellular location of the 20S proteasome in human cells, we established a stable U2OS cell line expressing an inducible alpha 4 (α 4) protein, one of the constitutive subunits of the 20S proteasome, fused with GFP (α 4-GFP). Biochemical characterization of this cell line demonstrated that the α 4-GFP subunit was correctly incorporated into proteolytically active proteasomes (Fig S1A). As reported for the endogenous α 4 subunit, ectopic α 4-GFP was detected throughout the cell, with an accumulation in the nucleoplasm (Fig 1A). However, it was also present in nuclear foci of a fraction of asynchronously growing cells (~ 25-30%) (Fig 1A-B). A similar location to nuclear foci was observed in U2OS cells expressing an ectopic untagged α 4 subunit, thus excluding a potential mis-localization due to the fusion with the GFP moiety (Fig S1B). Co-immunostaining experiments showed that other subunits of the 20S proteasome, such as α 6, were detected in α 4-GFP foci as well (Fig 1B), reflecting the presence of the entire 20S proteasome. In addition to the 20S proteasome, we detected its PA28 γ regulator (Fig 1B), but not the 19S complex, as shown by the absence of accumulation of the 19S subunit Rpt6 within α 4-GFP foci (Fig 1B). Interestingly, PA28 γ -containing α 4-GFP foci were resistant to an extraction-fixation buffer treatment (Fig 1C), used to reveal chromatin-associated proteins by immunofluorescence (Britton *et al.*, 2013). These observations suggest that α 4-GFP foci are closely linked to chromatin. Combined, these findings reveal a particular nuclear localization of PA28 γ -20S proteasome complexes upon ectopic expression of α 4 subunit.

Visualization of α 4-GFP in living U2OS cells by time-lapse video microscopy revealed that these α 4-GFP nuclear foci are dynamic, as their presence fluctuates throughout the cell cycle (Movie-1). α 4-GFP displayed a diffused localization during mitosis, after which it started to assemble into foci, whose number and size gradually increased during interphase. Detection of nuclear α 4-GFP foci in synchronized cells indicated that the percentage of cells with α 4-GFP foci reached a peak in late-S/early-G2 phases (Fig S1C).

Next, we investigated the nature of the α 4-GFP nuclear foci. Among different proteins forming nuclear foci, we focused our attention on a member of the HP1 family, HP1 β . Newly synthesized HP1 β was reported to also accumulate into nuclear foci with a characteristic peak in late-S/early-G2 phases when GFP-HP1 β was overexpressed (Dialynas *et al.*, 2006). By

performing immunostaining against HP1 β protein, we revealed the presence of endogenous HP1 β in α 4-GFP foci (Fig 1D). Immunoblot analyses of PA28 γ and GFP immunoprecipitation experiments performed in U2OS- α 4-GFP cell extracts showed that HP1 β is present in PA28 γ - and 20S proteasome-containing complexes (Fig 1E). We then wondered whether HP1 β plays a role in the formation of PA28 γ -20S proteasome foci. After depletion of HP1 β by siRNAs in U2OS- α 4-GFP cells (Fig 1F), we observed a significant decrease of the percentage of cells containing α 4-GFP foci (~14%) compared to the control cells (~36%) (Fig 1F, bar graph). We also noted that PA28 γ was no longer detected at identifiable nuclear foci in the absence of HP1 β . Importantly, PA28 γ depletion significantly decreased the percentage of cells containing α 4-GFP foci (~9.7%) (Fig 1F), suggesting that PA28 γ is required for their formation. First, these results indicate that HP1 β plays a role in the recruitment and/or retention of PA28 γ -20S proteasome to these foci when α 4 is over-expressed. Second, these data reveal that PA28 γ is required to recruit the 20S proteasome to nuclear foci.

Altogether, the above findings demonstrate that ectopic α 4 overexpression promotes the enrichment of PA28 γ -20S proteasome complexes in HP1 β -containing nuclear foci.

Without α 4 overexpression, a fraction of PA28 γ co-localizes also with HP1 β .

Immunostaining analyses of PA28 γ in U2OS cells revealed only a diffuse distribution of PA28 γ within the nucleus. We hypothesized that the presence of foci was due to the amplification of a natural process that most likely occurs in cells, but was only easily detectable when exacerbated by α 4 overexpression. To test this hypothesis, we asked whether PA28 γ co-localizes with HP1 proteins in U2OS cells without α 4 ectopic expression.

Since both PA28 γ and HP1 β proteins are abundant in the nucleus, we pre-extracted the soluble nuclear proteins by treating the U2OS cells with 0.5% Triton X-100/PBS (Guillot *et al.*, 2004), and performed immunostaining of HP1 β and PA28 γ proteins. Analysis of widefield microscope images (Figs 2A and S2A) showed a potential co-localization between HP1 β and PA28 γ in some discrete areas of the nucleus (Fig 2A, merge image and higher magnifications). Z-stack acquisition on an Airyscan confocal microscope (Fig 2B, left panel), followed by image analysis with Imaris software module, confirmed the co-localization between both proteins in U2OS cells (~32 co-localization sites per nucleus) (Fig 2B, right panel).

To strengthen this result, we used the *in situ* Proximity-Ligation Assay (*is*-PLA), which allows the detection of the close proximity between two proteins within cells (less than 40 nm, i.e. likely to be an interaction) (Soderberg *et al.*, 2006). Using both PA28 γ and HP1 β antibodies, *is*-PLA revealed a characteristic dotted pattern throughout the nuclei of U2OS cells (Fig 2C, upper middle panel), strongly supporting the close proximity of the two proteins. Silencing of PA28 γ expression with siRNAs (Fig 2C, left panel), used as a negative control, abolished the PLA dots, demonstrating the specificity of the PLA signal (Fig 2C, lower middle panel). Quantification of the number of PLA dots per nucleus (see Materials and methods) indicated on average 37 and 1.4 dots per nucleus, in the presence and absence of PA28 γ , respectively (Fig 2C, bar graph). We also evidenced that PA28 γ co-localizes in part with HP1 α (Fig S2B). Finally, we also found a close proximity between the 20S proteasome and HP1 β by *is*-PLA, using antibodies against the α 4 subunit of the 20S proteasome (Fig 2D).

Recently, we identified an important regulator of PA28 γ , the protein PIP30 (PSME3IP1), which affects PA28 γ interactions and localization in Cajal bodies (Jonik-Nowak *et al.*, 2018). Since PIP30 was detected in α 4-GFP foci, we tested whether PIP30 modulates the co-localization of PA28 γ and HP1 β . We found that PIP30 had no effect on this process since no difference in the number of PLA-dots between PA28 γ and HP1 β was observed in wild-type and PIP30-knockout (KO-PIP30) U2OS cells (Fig S2D).

Taken together, these results demonstrate that a fraction of PA28 γ and the 20S proteasome is in close physical proximity (and thus is likely to interact either directly or indirectly) to a fraction of the heterochromatin-binding protein HP1 β .

PA28 γ controls pericentromeric heterochromatin compaction.

The co-localization of PA28 γ and HP1 β prompted us to investigate whether PA28 γ was associated with chromatin comprising repetitive sequences characteristic of heterochromatin, such as interspersed (HERV-K), pericentromeric (Satellite II and α Satellite) and Major Satellite (L1 LINE) DNA repetitive sequences (Padeken *et al.*, 2015). Quantitative chromatin immunoprecipitation (ChIP-qPCR) experiments performed on parental (WT) *versus* KO-PA28 γ U2OS cells revealed that PA28 γ was associated with all four heterochromatin sequences tested (Fig 3A), as was HP1 β (Fig S3). Note that PA28 γ is also associated with a DNA sequence corresponding to Cyclin E2 promoter, located in euchromatin (Fig 3A).

Based on these results, we then investigated whether PA28 γ could regulate heterochromatin compaction. For this purpose, we used a previously described U2OS cell clone (F42B8) carrying *lacO* DNA repeats stably integrated within constitutive heterochromatin, at a pericentromeric region (Jegou *et al.*, 2009). This *lacO* array forms a single heterochromatic locus that can be visualized in cells following the transient expression of the GFP-LacI construct. The GFP signal allows us to measure the area occupied by the *lacO* locus and thus to quantify the variations of its compaction state. We first examined the effect of HP1 β depletion on *lacO* array compaction by transfecting these cells with siRNAs directed against HP1 β (si-HP1 β) or luciferase (si-Luc), and with a GFP-LacI-expressing construct. The efficiency of si-HP1 β was verified by immunoblot (Fig 3B, upper panel), and changes in heterochromatin compaction state were monitored 48 hours post-transfection by fluorescence microscopy (Fig 3B, lower panel). *LacO* locus appeared as a small dot with a surface area that was not significantly affected by the transfection of si-Luc ($0.390 \pm 0.045 \mu\text{m}^2$ vs $0.370 \pm 0.052 \mu\text{m}^2$ in control cells). Upon HP1 β knockdown, we observed a significant increase of the GFP-LacI dot surface area ($0.730 \pm 0.069 \mu\text{m}^2$). This corresponds to an expansion of the surface area occupied by the *lacO* DNA repeats due to heterochromatin decompaction (Fig 3C). We then examined the effect of PA28 γ knockdown (Fig 3D-E). Upon PA28 γ -depletion we observed a significant increase in the GFP-LacI dot surface area ($0.636 \pm 0.014 \mu\text{m}^2$ vs $0.370 \pm 0.052 \mu\text{m}^2$ and $0.443 \pm 0.011 \mu\text{m}^2$ in control cells and si-Luc treated cells, respectively) (Fig 3E). Thus, as observed in absence of HP1 β , the pericentromeric heterochromatin is significantly decompacted in the absence of PA28 γ .

PA28 γ controls chromatin compaction in living cells, independently from its interaction with the 20S proteasome.

To complete and expand the results obtained on fixed cells and at a specific genome locus, we performed quantitative FLIM-FRET (Fluorescence Lifetime Imaging Microscopy-Förster Resonance Energy Transfer) measurements of chromatin compaction at the nanometer-scale in a single living cell. For this study, we used HeLa cells that stably co-expressed histone H2B-GFP and mCherry-H2B (HeLa^{H2B-2FPs}). FRET was measured between the fluorophore-tagged histones incorporated into the chromatin and in this assay an increase in FRET efficiency corresponds to an increase in the occurrence of close proximity (<10 nm) between nucleosomes (Lleres *et al.*, 2009).

We first confirmed the co-localization between PA28 γ and HP1 β proteins at some nuclear sites in HeLa cells as seen in U2OS cells, by using *is*-PLA (Fig S4). Then, we established a stable CRISPR/Cas9 PA28 γ knockout HeLa cell line (Fig 4A), expressing either H2B-GFP alone (HeLa^{H2B-GFP}-KO-PA28 γ) or both H2B-GFP and mCherry-H2B (HeLa^{H2B-2FPs}-KO-PA28 γ). We verified that PA28 γ depletion affected neither H2B-GFP nor mCherry-H2B expression levels by immunoblot and microscopy analysis (Fig S5A-B).

Next, we analyzed by FLIM-FRET microscopy the levels of nanoscale chromatin compaction in asynchronous interphase parental and KO-PA28 γ cells. In wild-type (WT) HeLa^{H2B-2FPs} cells, a heterogeneous FRET efficiency map was apparent throughout nuclei on representative images using continuous pseudocolors (Fig 4B, left panel). We found that the areas with highest FRET signal (red-orange population) decreased in KO-PA28 γ cells (Fig 4B, left panel). This effect was confirmed by the determination of the mean FRET efficiency percentage, which shows a major reduction in the level of chromatin compaction compared to the WT cells (Fig 4B, right panel). As a positive control for chromatin decompaction, we treated HeLa^{H2B-2FPs} cells with Trichostatin A (TSA), an inhibitor of histone deacetylases used to provoke large-scale chromatin decompaction (Lleres *et al.*, 2009; Otterstrom *et al.*, 2019). As expected, after 24 hours of TSA treatment, the mean FRET efficiency percentage dropped drastically, consistent with a massively decompacted interphase chromatin (Fig S5C). By extracting the FRET efficiency distribution curves related to the FRET efficiency map of individual nuclei in both WT and KO-PA28 γ cell lines, we observed upon loss of PA28 γ a marked reduction of the high FRET population corresponding to high levels of chromatin compaction (Fig 4C, black curve *vs* blue curve). In contrast, the low-FRET population corresponding to chromatin regions with the lowest degree of chromatin compaction remained poorly affected. Therefore, PA28 γ seems to impact preferentially, if not exclusively, the most condensed chromatin domains during interphase. To ascertain that the relaxation of the chromatin was due to the absence of PA28 γ in the above experiments, we re-expressed PA28 γ in PA28 γ -KO cells (two different clones named KO/KI-WT#6 and # 8 were selected) at a level comparable to that of the endogenous protein (Fig S5D). Remarkably, for KO/KI-WT#6 and # 8 clones, the FRET efficiency was restored to values similar to WT cells (Fig 4D) indicating the re-establishment of normal chromatin compaction. Thus, these results show that PA28 γ plays an important role in regulating the compaction of chromatin in cells, with a particular impact on the highly structured chromatin.

An important question was to determine whether the 20S proteasome is required for this function of PA28 γ . For this, we used a mutant of PA28 γ deleted of its last C-terminal 14 amino acids (called Δ C), which is unable to bind and therefore to activate the 20S proteasome (Ma *et al.*, 1993; Zannini *et al.*, 2008). We stably expressed this mutant in HeLa^{H2B-2FPs}-KO-PA28 γ cells (named KO/KI- Δ C) at an expression level comparable to that observed in WT cells (Fig S5E). The inability of this PA28 γ mutant to bind the 20S proteasome was confirmed by co-immunoprecipitation experiments from cell extracts treated (Fig 4E) or not (Fig S5F) with the proteasome inhibitor MG132, known to increase the association between PA28 γ and the 20S proteasome (Welk *et al.*, 2016). As shown in Fig 4E and S5F, the 20S proteasome was detected by the presence of its α 4 subunit in PA28 γ immunoprecipitations from HeLa^{H2B-2FP} WT and KO/KI-WT cell extracts, but not in KO/KI- Δ C and KO-PA28 γ cells. Chromatin compaction was then analyzed by FLIM-FRET in living asynchronous cells. Surprisingly, we found that the expression of PA28 γ - Δ C mutant restored the level of chromatin compaction in PA28 γ -KO cells to a FRET efficiency value (24.7%) similar to the one observed in WT cells (23.03%) (Fig 4F). These results demonstrate that the compaction of chromatin requires PA28 γ , but not its interaction with the 20S proteasome.

PA28 γ is required for HP1 to mediate chromatin compaction.

To further characterize the PA28 γ /HP1 β connection, we performed siRNA-mediated depletion of both PA28 γ and HP1 β proteins (Fig 5A), and analyzed the effects on chromatin compaction levels by FLIM-FRET. FRET measurements revealed a marked decompaction of chromatin upon PA28 γ -knockdown that was even stronger than upon HP1 β depletion (Fig 5B). This decompaction was correlated with the clear disappearance of the most compacted states of the chromatin within nuclei (Fig 5C, left panel). To complete these data, the quantitative analysis of the FRET distribution profiles revealed a less pronounced decompaction of the chromatin with still some remaining high-FRET values upon si-HP1 β depletion than after si-PA28 γ depletion (Fig 5C right panel, compare red and black curves). Most likely the lower effect of HP1 β knockdown can be explained by the presence of HP1 α and/or by the redundancy of the HP1 isoforms in regulating chromatin compaction (Bosch-Presegue *et al.*, 2017). Importantly, these results indicate that even in the presence of HP1 proteins the lack of PA28 γ results into a strong decompaction of chromatin. We noted that the transfection of the siRNA-Luc only (blue curve) caused an increase in the high-FRET population (Fig 5C right panel) as compared to parental cells (Fig 4C right panel).

Intriguingly, we noted that PA28 γ -knockdown by siRNA had a stronger effect than the PA28 γ -knockout. This difference can be explained by potential compensatory mechanisms developed by the PA28 γ -KO cell line to maintain homeostasis. Finally, depletion of both PA28 γ and HP1 β had no additional effect compared to PA28 γ alone. Altogether, these results demonstrate that PA28 γ and HP1 β control the compaction of the chromatin, and that PA28 γ is required for this process to occur properly.

In order to determine the impact of chromatin decompaction induced by PA28 γ depletion on physiological events, we next investigated the consequences of PA28 γ depletion on the cell cycle. Analysis of cell-cycle profiles of KO-PA28 γ cells by fluorescence-activated cell sorting (FACS) revealed a minor but significant increase of 10% in the number of G1-phase cells, and a decrease of 7% in the number of S-phase cells relative to WT cells (Fig S6A), as previously described in PA28 γ ^{-/-} MEFs (Murata *et al.*, 1999). To obtain more insight into these perturbations, we analyzed the progression of both cell lines during the cell cycle after their release from a G1/S boundary block induced by double thymidine treatment. As shown in Fig S6B, the depletion of PA28 γ in U2OS cells caused an early exit from S phase (left panel) correlated to an early entrance in G2 phase as compared to the parental U2OS cells (right panel). This significant shortening of S phase (\approx 1 hour) in KO-PA28 γ cells was also confirmed by an immunoblot using cell cycle markers including Cyclin E and the phosphorylation of histone H3 on serine 10 (Fig S6C). Together, these results indicate that the duration of S phase is reduced in the absence of PA28 γ , suggesting that the chromatin decompaction induced by the lack of PA28 γ could facilitate the accessibility to DNA for the replication machinery.

PA28 γ contributes to the maintenance of heterochromatin marks.

Besides the recruitment of HP1 proteins, methylations of histone H3 on lysine 9 (H3K9me) (Maison & Almouzni, 2004; Trembecka-Lucas *et al.*, 2013; Saksouk *et al.*, 2015) and histone H4 on lysine 20 (H4K20me) (Shoab *et al.*, 2018) have been shown to be important for chromatin compaction. Since tri-methylation of histone H4 (H4K20me₃) is also an evolutionarily-conserved mark of heterochromatin (Schotta *et al.*, 2004; Balakrishnan & Milavetz, 2010; Beck *et al.*, 2012), we tested whether PA28 γ regulates these heterochromatin-enriched modifications. By analyzing the levels of H3K9me₃ in total cell extracts by immunoblotting, no change in the steady-state level of H3K9me₃ was observed in KO-PA28 γ

cells compared to WT cells (Fig 6A). By contrast, PA28 γ depletion led to a decrease (~20%) in the steady-state level of H4K20me3 (Fig 6B). This result was particularly interesting since it has recently been suggested that HP1 β mediates a direct functional link with H4K20me3 (Bosch-Presegue *et al.*, 2017). As H4K20me3 depends on the mono-methylation of histone H4 lysine K20 (H4K20me1) (Tardat *et al.*, 2007), we also analyzed the steady-state level of H4K20me1. We observed a strong reduction of H4K20me1 (~40%) (Fig 6B). To examine the variation of H3K9me3 and H4K20me3 at specific heterochromatin sequences, we carried out ChIP assays on parental (WT) and KO-PA28 γ U2OS cells using antibodies against H3K9me3 and H4K20me3, and performed qPCR using the same primers as in Fig 3A. We observed a significant decrease in H3K9me3 precipitation levels at these specific sequences (Fig 6C) that was not detected by immunoblot analyses on total cell extract (Fig 6A). Note that the difference observed between immunoblot and ChIP assay could result from the use of different antibodies in the two experiments and/or be due to the highly enrichment of H3K9me3 in pericentric heterochromatin. We confirmed the decrease of H4K20me3 ($\geq 60\%$) in KO-PA28 γ *versus* WT U2OS cells (Fig 6D). As expected, this effect was stronger than the one found in total extract. As summarized in Fig 6E, our data suggest that PA28 γ participates to the regulation of histones H3K9 and H4K20 methylation states that are required for heterochromatin establishment.

Discussion

The key finding of this study is that PA28 γ , known as a nuclear activator of the 20S proteasome, is an essential factor in the process of chromatin compaction.

We demonstrate that PA28 γ plays a key role in this process by showing that: i) the lack of PA28 γ causes the decompaction of *lacO* DNA repeats that are stably integrated into pericentromeric heterochromatin, as does the loss of HP1 β , ii) the depletion of PA28 γ induces a decompaction of the highly structured fraction of the chromatin, even in the presence of HP1 proteins, as visualized in living cells with the quantitative FLIM-FRET measurements of chromatin compaction. In line with these observations, we also found that PA28 γ knockout decreases significantly the level of H3K9 and H4K20 tri-methylation, as well as mono-methylation of H4K20.

Importantly, our data show that in interphase PA28 γ and HP1 β are both required to control the most condensed states of chromatin as defined by our experimental approaches. Whether PA28 γ and HP1 β act independently or in concert will need further investigation. Nevertheless, based on our data obtained in α 4-GFP expressing cells, HP1 β seems to be required for the recruitment of PA28 γ -20S proteasome complexes in nuclear foci. We show that a fraction of PA28 γ and HP1 β co-localize in normal cells, although this co-localization is difficult to detect, probably because they are located in much more dynamic and/or transient structures than when α 4 is overexpressed. It is thus tempting to speculate that the formation of these foci is an early step in the chromatin condensation process.

Using cells overexpressing α 4-GFP, we found that PA28 γ -20S proteasome complexes gradually concentrate into foci through mid-to-late S-phase to G2-phase, which is reminiscent of the accumulation of newly-synthesized GFP-HP1 β at heterochromatin foci, whose formation has been shown to require passage through S-phase (Dialynas *et al.*, 2006). Interestingly, we found that the loss of PA28 γ decreases the S phase duration by one hour. Considering this point, it is tempting to hypothesize that, following the progression of replication forks, PA28 γ might regulate the re-establishment of modifications on the newly incorporated histones on each of the daughter DNA strands. To support this hypothesis, in the absence of PA28 γ , a significant decrease of H3K9me3 and H4K20me3 marks was observed by ChIP-qPCR experiments at specific heterochromatin regions. Furthermore, an important decrease in the steady-state level of H4K20me1 was found. In addition, the longer G1-phase observed upon the loss of PA28 γ is a cell cycle effect comparable to the G1-to-S phase

transition delay reported upon a drop in H4K20me levels (Everitts *et al.*, 2013) or upon the depletion of the methyltransferase PR-Set7 (Liao *et al.*, 2018). Altogether these data argue for a possible role of PA28 γ in the regulation of these histone marks. At this stage it is important to note that the 20S proteasome, which bears the proteolytic activity, is not necessary for PA28 γ -mediated chromatin compaction; this precludes any direct regulation of this process by a PA28 γ -dependent proteolysis event. PA28 γ could potentiate the function of the lysine methyltransferases Suv39h, PR-Set7 or Suv4-20h, which are responsible for H3K9 trimethylation, H4K20 mono-methylation and H4K20 tri-methylation, respectively. It cannot be excluded that PA28 γ could facilitate the action of specific histone deacetylases (HDACs) (Almouzni & Cedar, 2016) and/or the recruitment of chromatin remodeling factors.

Interestingly, recent studies show that HP1 proteins have the capacity to form liquid-like droplets resulting from a liquid-liquid phase separation (LLPS) mechanism (Larson *et al.*, 2017; Strom *et al.*, 2017). This property could facilitate the enrichment of transient supramolecular complexes/machineries that could rapidly assemble and disassemble, and exchange their components. This plasticity in their composition allows them to efficiently respond to the structural changes of heterochromatin, and might thus stimulate PA28 γ 's interactions with other proteins required for controlling chromatin compaction. In this context, it is important to underline that PA28 γ is involved in the dynamics of various membraneless nuclear bodies (Cioce *et al.*, 2006; Baldin *et al.*, 2008; Zannini *et al.*, 2009; Jonik-Nowak *et al.*, 2018), also considered to be liquid-like protein droplet organelles (Erdel & Rippe, 2018; Sawyer *et al.*, 2019).

The reason behind the recruitment of 20S proteasome into foci is still an intriguing open question, since its interaction with PA28 γ is not required for PA28 γ -mediated chromatin compaction. To explain the 20S proteasome recruitment, one possibility is that the catalytic activity of PA28 γ -20S proteasome complexes is involved in specific steps of chromatin compaction, such as the control of heterochromatin spreading, as it was shown for the 26S proteasome in yeast (Seo *et al.*, 2017). Another possibility, in line with a possible link with LLPS droplet organelle formation (Sawyer *et al.*, 2019), is that the recruitment of the 20S proteasome by PA28 γ could constitute a reservoir of active proteasome that can rapidly be mobilized upon cellular stress. In the cellular response to DNA double-strand breaks (DSBs), PA28 γ is required for the rapid mobilisation of the 20S proteasome at DNA damage sites and contributes to the repair-pathway choice (Levy-Barda *et al.*, 2011). In this regard, HP1 β was also shown to be recruited at DSB sites and to favor the initiation of DNA damage response

(DDR) by promoting chromatin changes (Ayoub *et al.*, 2008; Luijsterburg *et al.*, 2009; Bartova *et al.*, 2017), it would therefore be interesting to investigate whether the mobilization of PA28 γ to DNA damage sites is also dependent on the presence of HP1 β .

It is noteworthy that, over the years, PA28 γ has been implicated in several chromatin-related processes, such as maintenance of chromosomal stability (Zannini *et al.*, 2008), DNA repair (Levy-Barda *et al.*, 2011) and control of rDNA transcription (Sun *et al.*, 2016). Our present observations suggest that the role of PA28 γ in the regulation of chromatin compaction could be the common mechanism that links these processes to PA28 γ . Although much remains to be understood regarding the biological functions of PA28 γ in this process, our data reveal that PA28 γ is a crucial factor in the regulation of chromatin compaction and this discovery undoubtedly opens new perspectives for a deeper understanding of the proteasome functions and the complex mechanisms that control chromatin organization, particularly during cell cycle.

Materials and methods

Plasmids

A human cDNA encoding the full length of 20S proteasome subunit $\alpha 4$ (*PSMA7*), with or without a final stop codon, was PCR amplified from a human fibroblast cDNA library and inserted into pcDNA₃ or pML1-EGFP (Baldin *et al.*, 2008; Farras *et al.*, 2008; Le Feuvre *et al.*, 2009). The cDNA encoding $\alpha 4$ -EGFP was then inserted into the pTRE2 vector (Clontech). The human HP1 β (*CBX1*) cDNA was PCR-amplified from pDONR223 (provided by the Montpellier Genomic Collections facility, IGMM, Montpellier, France) and cloned into pEGFP-C1 (Clontech). For Cas9-mediated gene disruption, guide RNA (GGAAGTGAAGCTCAAGGTAGCGG) targeting PA28 γ (*PSME3*) was selected using ChopChop (<https://chopchop.rc.fas.harvard.edu/index.php>) and oligonucleotides were subcloned into pMLM3636 (a gift from Keith Joung, Addgene plasmid #43860) and pUC57-U6 (a gift from Edouard Bertrand's laboratory, IGMM, Montpellier, France). For rescue experiments, PA28 γ ORF WT or delta C-terminal 14 amino acids (called ΔC) were cloned in pSBbi-Pur (gift from E. Kowarz addgene plasmid #60523) according to (Kowarz *et al.*, 2015). The resulting vector was co-transfected with pCMV(CAT)T7-SB100 (gift from Zsuzsanna Izsvak, Addgene plasmid #34879) into recipients cells, and puromycin-resistant single colonies were selected for re-expression of PA28 γ WT or ΔC proteins. pEGF-LacI (Jegou *et al.*, 2009) was a generous gift from Prof. K. Rippe (DKFZ, Heidelberg, Germany).

Antibodies

The following antibodies were used at 1:1000 dilution for immunoblotting and 1-3 $\mu\text{g/ml}$ for immunoprecipitation: anti-PA28 γ (rabbit polyclonal BML-PW8190), anti- $\alpha 4$ (1:2000), anti- $\alpha 6$ (1:2000), and anti-Rpt6/Sug1 (mouse monoclonal, BML-PW8120, BML-PW8100 and BML-PW9265, ENZO Life Sciences, respectively); anti-PA28 γ (mouse monoclonal, 611180, BD Transduction); anti-HP1 α (rabbit polyclonal, 2616S, Cell Signaling); anti-HP1 β (rabbit monoclonal (D2F2), 8676S, Cell Signaling, mouse monoclonal (1MOD-1A9) 39979, Active Motif); anti-MCM7 (monoclonal, sc-9966, Santa-Cruz); anti-PIP30 (*PSME3IP1*) (Jonik-Nowak *et al.*, 2018); anti-GFP (mouse monoclonal (Clone 7.1), 11814460001, ROCHE, Sigma); anti-RFP (rat monoclonal, 5F8, Chromotek); anti- β -actin (rabbit monoclonal, 13E5, Cell Signaling); anti-H3K9me3 (mouse monoclonal (clone 2AG-6F12-H4) 39285, Active Motif); anti-H3 (rabbit polyclonal, ab1791, Abcam); anti-H4K20me1 (rabbit polyclonal,

#9724, Cell Signaling Technology); anti-H4K20me3 (rabbit monoclonal, #5737, Cell Signaling Technology); anti- α -tubulin (mouse monoclonal, T9026, Sigma-Aldrich, 1:6,000). Fluorescent secondary antibodies conjugated either to Alexa Fluor 488, 594 and 680 (1:1,000), or to DyLight 680 and 800 (1:10,000) were purchased from Thermo Fisher Scientific. Secondary antibodies conjugated to HRP were purchased from Bio-Rad SA (1:10,000). GFP-TRAP-A® beads were from ChromoTek.

Cell culture, transfections, cell synchronization and FACS analysis

HeLa (CCL-2) and U2OS (HTB-96) cells, obtained from ATCC, were grown in DMEM (Lonza) containing 4.5 g/L glucose, 10% heat-inactivated fetal bovine serum (Biowest), 2 mM glutamine, 100 U/ml penicillin and 10 μ g/ml streptomycin (Lonza). A U2OS Tet-Off stable inducible cell line expressing α 4-GFP was established as previously described for α 7-GFP (Baldin *et al.*, 2008), and cultured in medium supplemented with 250 μ g/ml G418 (Sigma), 200 μ g/ml hygromycin B (Calbiochem) and 2 μ g/ml tetracycline (Sigma-Aldrich) to inhibit the expression of the exogenous protein. Single clones were then expanded and analyzed by western blotting using GFP or α 4 antibodies. After cell washing with PBS (4 times), α 4-GFP expression was induced for 24 to 36 hours in fresh medium, in the absence of tetracycline. For positive clones, the functionality of α 4-GFP was controlled by testing its incorporation into the 20S, and the activity of these chimeric 20S proteasomes in a proteasome activity assay (see Appendix Fig S1 B-D). U2OS-LacO (F42B8) cells (a generous gift of Prof. K. Rippe, DKFZ, Heidelberg, Germany) were grown in the same media as U2OS but containing G418 (500 μ g/ml) (Jegou *et al.*, 2009). Establishment and characterization of parental HeLa^{H2B-GFP} and HeLa^{H2B-2FPs} (H2B-GFP and mCherry-H2B) cell lines were previously described (Lleres *et al.*, 2009). Of note: after thawing, cells were cultured for one week before seeding, for all experiments.

For transient PA28 γ and HP1 β knockdown experiments, U2OS and/or HeLa (H2B-GFP or 2FPs) cells were transfected with 20 nM of Luciferase targeting siRNA (si-Luc, 5'-CGTACGCGGAATACTTCGA-3') used as negative control, or -PA28 γ (*PSME3*), and -HP1 β (*CBX1*) targeting siRNA (si-PA28 γ : 5'-GAAUCAUAUGUCACUCUA-3'; si-HP1 β : 5'-AGGAAUAUGUGGUGGAAAA-3') purchased from Eurofins Genomics, using Lipofectamine RNAiMAX (Thermo Fisher Scientific) and examined after 2 days. When indicated, cells were transiently transfected with 0.5 μ g/ml DNA using JetPEI™ (Ozyme), according to the manufacturer's instructions and analyzed after one day. Stable U2OS (Jonik-

Nowak *et al.*, 2018), HeLa^{H2B-GFP}- and HeLa^{2FPs}-KO-PA28 γ cell lines were generated by co-transfection of PSME3/PA28 γ sgGuide and pX459 vectors (a gift from Feng Zhang, Addgene plasmid #62988), and cells were selected with puromycin (1 μ g/ml). Single clones were then expanded and analyzed by western blotting using PA28 γ antibodies. Synchronization of cells at G1/S phases transition was performed by hydroxyurea treatment (10 mM, Sigma-Aldrich) for 16 hours. For Fluorescence-Activated Cell Sorting (FACS) analysis, cells were fixed with 70% ethanol and conserved at -20°C. Before analysis, cells were washed with PBS, resuspended in PBS containing RNase A (1 mg/ml, Sigma-Aldrich) and propidium iodide (10 μ g/ml, Sigma-Aldrich) and incubated for 30 min at room. Samples were run on a FACS Calibur (Becton-Dickinson), and data analysis was performed using CellQuest Pro software (Beckton-Dickinson).

Immunofluorescence and is-PLA assays

Cells on coverslips were fixed in 3.7% paraformaldehyde/PBS at room temperature then permeabilized with 0.25% Triton X-100 in PBS for 5 min, followed by an incubation in methanol (100%) at -20°C for 10 min. Note that in some specific experiments, cells were first pre-extracted CSK buffer (10 mM Pipes, pH 7.0, 100 mM NaCl, 300 mM sucrose, and 3 mM MgCl₂, 0.7% Triton X-100) to release soluble proteins, then washed with PBS and fixed with 2% PFA (Britton *et al.*, 2013). After washes with PBS, cells were blocked with 1% FCS/PBS for 15 min. Incubation with primary antibodies (anti-PA28 γ 1:6,000 for BML-PW8190 or 1:1,000 for 611180); anti- α 4, anti- α 6, and anti-Rpt6/Sug1 (1:4,000 BML-PW8120, 1:1,000 BML-PW8100 and 1:1,000 BML-PW9265, respectively; anti-HP1 α (1:8,000, 2616S); anti-HP1 β (1:1,000 8676S and 1MOD-1A9)) was carried out at 37°C for 1 hour in a humidified atmosphere. After washes, cells were incubated with Alexa Fluor conjugated secondary antibodies for 40 min at RT. DNA was stained with 0.1 μ g/ml DAPI (4',6-diamidino-2-phenylindole, dihydrochloride, Sigma-Aldrich) solution 5 min at RT, cells were washed twice in PBS and finally once in H₂O. Coverslips were mounted on glass slides using ProLong Gold anti-fade reagent (Thermo Fisher Scientific). For *in situ* proximity ligation assays (*is-PLA*), cells on coverslips were fixed and permeabilized as above. Coverslips were then blocked in a solution provided by the Duolink® kit (Sigma-Aldrich). Cells were then incubated with antibodies as described above. Duolink® *In Situ* PLA Probe Anti-Rabbit MINUS and Anti-Mouse PLUS and Duolink® *In Situ* Detection Reagents (Sigma-Aldrich) were used, according to the manufacturer's instructions. In some specific experiments, cells were

permeabilized prior the fixation with 0.5% Triton X-100 in PBS for 5 min at 4°C for the co-localization between endogenous PA28 γ and HP1 β in U2OS cells.

Images and Z-stack images were acquired with 63X/1.32 NA or 100X/1.4 NA oil immersion objective lenses using a DM 6000 microscope (Leica). Microphotographs were taken with a 12-bit CoolSnap HQ2 camera. Images were acquired as TIFF files using MetaMorph imaging software (Molecular Devices). For quantitative analyses of PLA dots, Z-stacks were acquired every 0.3 μm (Z step) with a range of 6-7.5 μm . For endogenous detection, images (as a Z stack, slices every 200 nm) were also acquired on a Zeiss LSM 880 point scanning confocal microscope equipped with a 63x Plan-Apochromat 1.4NA oil immersion objective (Zeiss) and using the 488 nm and 561 nm laser lines with the Airyscan detector. The Zeiss Zen black software was used to process the Airyscan raw Images. Co-localization in 3D, between PA28 γ and HP1 β , was analyzed using the Imaris (Bitplane) co-localization module.

The number of PLA-dots and the size of GFP-LacI dots were detected with ImageJ (1.49v). Specific macros were created to automatically quantify these different parameters. The script allows the creation of a mask of DAPI image to isolate the nucleus of each cell and create a maximum intensity projection (MIP) of the Z-stacks or the image. The mask is used in the MIP to count the number of PLA-dots of each nucleus via an appropriate threshold. The “Analyze Particles” tool of ImageJ was used to calculate the size of each GFP-LacI dots.

FLIM-FRET Microscopy.

FLIM-FRET data were acquired with a Zeiss LSM 780 laser scanning microscope coupled to a 2-photon Ti:Sapphire laser (Chameleon Ultra II tunable 680–1080 nm, Coherent) producing 150-femtosecond pulses at 80 MHz repetition rate and a Time Correlated Single Photon Counting (TCSPC) electronics (SPC-830; Becker & Hickl GmbH) for time-resolved detection. Enhanced green fluorescent protein (EGFP) and mCherry fluorophores were used as a FRET pair. The two-photon excitation laser was tuned to 890 nm for selective excitation of the donor fluorophore. The LSM780 microscope is equipped with temperature- and CO₂-controlled environmental black wall chamber. Measurements were acquired in live cells at 37°C, 5% CO₂ with 63x/1.4 oil Plan-Apochromat objective lens. A short-pass 760-nm dichroic mirror was used to separate the fluorescence signal from the laser light. Enhanced detection of the emitted photons was afforded by the use of the HPM-100 module (Hamamatsu R10467-40 GaAsP hybrid PMT tube). The FLIM data were processed using SPCimage software (Becker & Hickl GmbH).

FLIM-FRET analysis

FLIM-FRET experiments were performed in HeLa cells stably expressing H2B-GFP alone (HeLa^{H2B-GFP}) or with mCherry-tagged histone H2B (HeLa^{H2B-2FPs}). 5×10^4 cells were seeded in a Fluorodish 35 (FD35-100, World Precision Instruments). For siRNA experiments, 24 hours after seeding, cells were transfected with 20 nM of siRNA (against Luciferase, PA28 γ or HP1 β) and FLIM-FRET experiments were performed 48 hours later. 30 min prior to imaging, the culture medium was changed to complete DMEM medium without phenol red. An acquisition time of 90 s was set up for each FLIM experiment. The analysis of the FLIM measurements was performed by using SPCImage software (Becker & Hickl, GmbH). Because FRET interactions cause a decrease in the fluorescence lifetime of the donor molecules (EGFP), the FRET efficiency was calculated by comparing the FLIM values obtained for the EGFP donor fluorophores in the presence (HeLa^{H2B-2FPs}) and absence (HeLa^{H2B-GFP}) of the mCherry acceptor fluorophores. FRET efficiency (E FRET) was derived by applying the following equation:

$E \text{ FRET} = 1 - (\tau_{DA} / \tau_D)$ at each pixel in a selected region of interest (nucleus) using SPCImage software. τ_{DA} is the mean fluorescence lifetime of the donor (H2B-EGFP) in the presence of the acceptor mCherry-H2B in HeLa^{H2B-2FPs} cells and τ_D is the mean fluorescence lifetime of H2B-EGFP (in the absence of acceptor) in HeLa^{H2B-GFP} cells. The FRET distribution curves from nuclei were displayed from the extracted associated matrix using SPCImage and then normalized and graphically represented using Microsoft Excel and GraphPad Prism software. For each experiment, FLIM was performed on multiple cells from several independent experiments (see figure legends).

Time-lapse video microscopy

U2OS- α 4-GFP cells seeded on a 6-well plate were induced to express α 4-GFP 24 hours previously, and imaged on an Olympus IX83 microscope with a 40x objective and equipped with an Andor Zyla 4.2 sCMOS camera (van Dijk *et al.*, 2018). Both systems were driven by MetaMorph software. Images were then processed with the imageJ package and saved in Avi Format Schneider, C.A., (Schneider *et al.*, 2012).

Immunoprecipitation and immunoblotting

For immunoprecipitation of GFP-fusion or endogenous proteins, cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM MgCl₂, 1% IGEPAL CA630, 0.5% DOC, 0.1% SDS, 1 mM DTT, 5 mM EDTA, 50 mM NaF and 1 mM Na₃VO₄) in the presence

of complete EDTA-free protease inhibitor cocktail (Roche Life Science) for 20 min at 4°C. Lysates were clarified by centrifugation for 10 min at 10,000 x g and the protein concentration of the supernatant was determined using BSA as a standard (CooAssay protein dosage reagent, Interchim). Total lysate (200 µg) was pre-cleared for 30 min, and immunoprecipitations were performed using either GFP-TRAP beads or the antibodies indicated and protein A or G magnetic beads (Dynal, Lake Success, NY) for 2 hours at 4°C with constant gentle stirring. After several washes, bead pellets were boiled in 2x Laemmli buffer, separated by SDS-PAGE, and subjected to immunoblotting. Note that proteasome activity assay, cells were homogenized in a modified lysis buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM MgCl₂, 0.5 mM EDTA, 1 mM ATP, 1 mM DTT, 10% Glycerol, 0.5% IGEPAL CA630, 0.3% Triton X-100) in the same.

ChIP-qPCR

ChIP experiments with U2OS were performed as described previously (Brustel *et al.*, 2017). Briefly, cells were fixed with 1% formaldehyde (10 min) and quenching was performed with 125 mM Glycine. After a PBS wash, cells were resuspended in buffer A (10 mM Tris-HCl pH 8, 10 mM KCl, 0.25% Triton X-100, 1 mM EDTA, 0.5 mM EGTA) for 5 min on ice. After centrifugation, nuclei were extracted with buffer B (10 mM Tris pH 8, 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA for 10 minutes on ice. To extract chromatin, nuclei were resuspended in Lysis buffer (10 mM Tris-HCl pH 8, 140 mM NaCl, 0.1% SDS, 0.5% Triton X-100, 0.05% Na-Deoxycholate, 1 mM EDTA, 0.5 mM EGTA). After sonication with EpiShear probe sonicator (Active Motif) to obtain chromatin fragments less than 800 bp, ChIP was performed with 15-30 µg of sheared chromatin incubated with protein A magnetic beads (Invitrogen, Thermo Fisher) coupled with the appropriate antibody, as follows: anti-H3pan (1 µl/ChIP, C15310135 Diagenode), anti-H3K9me3 (2 µl/ChIP, C15410056 Diagenode), anti-H4K20me3 (2 µl/ChIP, C15410207, Diagenode), anti-PA28γ (0.5 µl /ChIP, ENZO Life Sciences). ChIP experiments were performed at least three times from independent chromatin preparations and quantitative PCR analyses of ChIP DNAs were performed using a SYBR green quantitative PCR kit (Invitrogen, Thermo Fisher) and a LightCycler 480 II instrument (Roche) under conditions standardized for each primer set. The amount of DNA in ChIP samples was extrapolated from standard curve analysis of chromatin DNA before immunoprecipitation (input), and values were represented as the ratio between the percentage of input obtained for each antibody to the ones obtained for H3. Primer sets used for qPCR: HERV-K For 5'-TGCCAAACCTGAGGA AGAAGGGAT-3' and HERV-K

Rev 5'-TGCAGGCATTA AACATCCTGGTGC-3', Sat-II For 5'-CCAGAAGGTAATAA GTGGCACAG-3' and Sat-II Rev 5'-CCCTCCTTGAGCATTCTAACTACC-3', α -Sat For 5'-GAAACACTCTTTCTGCACTACCTG-3' and α -Sat Rev 5'-GGATGGTTCAACACT CTTACATGA-3' (Djeghloul *et al.*, 2016), L1 Line 5'UTR For 5'-CAGCTTTGAAGA GAGCAGTGG-3' and L1 LINE 5'UTR Rev 5'-GTCAGGGACCCACTTGAGG-3' (Filipponi *et al.*, 2013) and CCNE2: For 5'- AAGCGTTAGAA ATGGCAGAAAG-3' and Rev 5'- TCTCTCCCTAATTTACCTGTAGGA-3'.

Statistics

Error bars represent standard deviations unless otherwise noted. Different tests were used to determine significance, and noted in the legend.

Author contribution

VB conceived the project and supervised the study. DF, DL, CG, CV, SB, CBA, and VB performed experiments and analyzed data. FM generated reagents. OC and RF provided conceptual advice on study and interpretation of the data. CBA and VB wrote the article with input from all of the authors.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgements

We thank Prof. Karsten Rippe (Deutsches Krebsforschungszentrum, Heidelberg, Germany) for providing U2OS-LacO (F42B8) cell and the pEGFP-LacI vector, Dr. Philippe Fort for help with statistical analysis, Dr. Nathalie Morin for Airyscan acquisition and helpful discussions and Dr. Eric Julien for helpful discussions; and the Montpellier Ressources Imagerie (MRI) platform, a member of the National Infrastructure France-BioImaging supported by the French National Agency (ANR-10-INSB-04, Investments for the Future). We thank Dr. James Hutchins for checking the scientific English.

Funding

Institutional support was provided by the Centre National de la Recherche Scientifique (CNRS) and the University of Montpellier. This work was also supported by grants from the People Programme (Marie Curie Actions) of the EU Seventh Framework Programme (FP7 REA agreement 290257, UPStream, to OC), Comité de l'Aude et Comité du Gard de la Ligue Nationale Contre le Cancer (2014 and 37-2015, to VB), Fondation ARC pour la Recherche

sur le Cancer (SFI20111203984, to SB/ PJA20181207962, to DL), Institut National du Cancer (INCa) (PLBIO18-KiMec, to RF).

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Figure legends

Figure 1. PA28 γ and HP1 β co-localize in α 4-GFP-20S proteasome foci.

A. Stable asynchronously growing U2OS (Tet-Off) α 4-GFP cells were induced for the expression of GFP-tagged α 4 subunit of the 20S proteasome (α 4-GFP, green) for 24 hours in the absence of tetracycline, then fixed and stained with DAPI (blue). Scale bar, 10 μ m.

B. Induced U2OS- α 4-GFP cells (α 4-GFP, green), as in A, were immunostained with antibody raised against alpha 6 subunit of the 20S proteasome (α 6, left panel), the regulatory complex PA28 γ (PA28 γ , middle panel) and a subunit of the 19S regulatory complex (Rpt6, right panel), all in grey. Scale bar, 10 μ m.

C. α 4-GFP foci are associated with chromatin. Induced U2OS- α 4-GFP cells (α 4-GFP, green), as in A, were not treated (- CSK buffer) or treated (+ CSK buffer) with an extraction CSK buffer, then fixed and immunostained with anti-PA28 γ antibodies (red). Scale bar, 5 μ m.

D. Induced U2OS- α 4-GFP cells (α 4-GFP, green) were immunostained with anti-HP1 β antibodies (grey). A merge image of GFP and HP1 β signal is shown. Scale bar, 10 μ m. Arrows indicate sites of co-localization between HP1 β and α 4-GFP.

E. Co-immunoprecipitation of PA28 γ and HP1 β in asynchronous induced U2OS- α 4-GFP cells. Induced U2OS- α 4-GFP cells, as in A, were lysed and subjected to pull-down with either an antibody raised against PA28 γ or GFP-TRAP, or the appropriate isotype control (CTL). An immunoblot of the pull-down (IP) and supernatant (SN) from whole-cell extracts (WCE) was probed with the antibodies indicated.

F. U2OS- α 4-GFP cells were transfected with si-Luc, si-PA28 γ or si-HP1 β . One day later, the expression of α 4-GFP was induced, and cells were recovered 48 hours after siRNA treatment. Immunostaining was performed to detect PA28 γ (red) and HP1 β (magenta) in cells treated with the siRNA indicated. Representative images are shown (left). Arrows indicate cells with α 4-GFP and PA28 γ foci. Scale bar, 10 μ m. The percentage of cells with α 4-GFP foci is shown in the bar graph (right). Error bars derived from 3 independent experiments represent the mean \pm SD, $n \geq 47$ cells per condition. One-way ANOVA analysis, $p = 0.0001$ (****) for siRNA-PA28 γ and -HP1 β versus WT.

Figure 2. Endogenous HP1 β , PA28 and the 20S proteasome co-localize in U2OS cells.

A. Nuclear localization of endogenous HP1 β (left panel) and PA28 γ (middle panel) by immunofluorescence in asynchronous U2OS cells after pre-permeabilization with 0.5% Triton

X-100. A representative merged image of HP1 β (green) and PA28 γ (red), and higher-magnification views are shown (right panel). Scale bars, 10 μ m.

B. A representative Airyscan confocal Z-projected image showing the co-detection of HP1 β (green) and PA28 γ (red) (left). Co-localizations of both proteins along the cross is shown. Scale bars, 5 μ m. Using the co-localization module of Imaris, a representative image of HP1 β (green), PA28 γ (red) and co-localization spots (white/grey) corresponding to 3-D image (middle panel) is shown with the corresponding image showing only co-localization spots (right panel). Scale bars, 5 μ m.

C. Immunoblot analysis of PA28 γ expression level in total extracts of U2OS cells treated or not with si-PA28 γ , used for *in situ* Proximity Ligation Assay (*is*-PLA) (left panel). Tubulin was used as a loading control. The relative abundance of PA28 γ was quantified using ImageJ software. Control (CTL) or si-PA28 γ treated U2OS cells were subjected to *is*-PLA using primary antibodies directed against HP1 β and PA28 γ , and DNA stained with DAPI. Positive PLA signals appear as green dots and higher magnification views of a nucleus are shown (middle panel). Scale bars, 10 μ m. Quantification of PLA dots was carried out using an ImageJ plugin (see Materials and Methods). The number of PLA dots per nucleus for HP1 β /PA28 γ interaction in control (CTL) or si-PA28 γ treated cells is shown graphically (right panel). Data represent the means \pm SD from 3 independent experiments, the number of analyzed cells is $n = 78$ and $n = 45$ in control and si-PA28 γ treated cells, respectively. The p -value was determined with Student's T-test, **** ($p = 0.0001$).

D. Immunoblot analysis of HP1 β expression level in total extracts from U2OS cells treated or not with si-HP1 β (upper left panel). Tubulin was used as a loading control. The relative abundance of HP1 β proteins was quantified using ImageJ software. Control (CTL) or si-HP1 β treated U2OS cells were subjected to *is*-PLA using primary antibodies directed against HP1 β and $\alpha 4$ (a subunit of the 20S proteasome), and DNA was stained with DAPI. Positive PLA signals appear as green dots. A higher magnification view of a nucleus is shown (lower left panel). Scale bar, 10 μ m. The number of PLA dots per nucleus for HP1 β / $\alpha 4$ interaction in control (CTL) or si-HP1 β treated cells is shown on the bar graph (right panel). Data represent the means \pm SD from 3 independent experiments, the number of cells analyzed is $n = 48$ and $n = 46$ in control cells and si-HP1 β treated cells, respectively. p -value was determined with Student's T-test, **** ($p < 0.0001$).

Figure 3. PA28 γ is present on heterochromatin and its depletion induces a decompaction of pericentromeric heterochromatin.

A. ChIP-qPCR analysis of PA28 γ levels at different repetitive elements located in heterochromatin or euchromatin (as indicated on the x-axis) in wild type (WT) *versus* KO-PA28 γ U2OS cells (right panel). Data are represented as relative enrichment of PA28 γ antibody *versus* H3 control as shown on the y-axis. Data are means \pm SEM ($n = 5$). Significance was calculated by Student's T-test, ** $p < 0.01$ ($p = 0.0046$, L1 LINE), *** $p < 0.001$ ($p = 0.00011$, Sat II), **** $p < 0.0001$ ($p = 2.09E-05$, $p = 5.15E-05$ and $p = 2,54318E-06$), HERV-K, α -Sat and CCNE2 respectively).

B. U2OS-LacO cells, treated or not with si-HP1 β or si-Luc, were transiently transfected with GFP-LacI construct the same day and were recovered 48 hours later. Proteins were analyzed by immunoblotting. The relative abundance of HP1 β in the extracts was quantified using ImageJ software and normalized to tubulin (upper panel). Cells on coverslips were immunostained with anti- HP1 β (red) and the GFP signal was imaged in parallel (green). DNA was stained with DAPI (cyan). Representative fluorescence and immunofluorescence images of Z-stack projections of U2OS-LacO cells are shown. Magnified views of GFP-LacI spot are shown in inserts. Scale bars, 10 μ m.

C. Quantitative analysis of the decompaction of the LacO array. Z-stacks images were acquired on U2OS-LacO cells treated as in (B) and the area of the GFP-LacI signal was quantified on a Z-projection using the ImageJ software (see Materials and Methods). Data represent the means \pm SD from three biological repeats, numbers of analyzed nuclei with GFP-LacI spot were $n = 30$, $n = 28$ and $n = 27$ in control cells (CTL), si-HP1 β or si-Luc treated cells, respectively. (ns, no significant $p = 0.2503$, *** $p = 0.0003$; ** $p = 0.001$, p -values were determined by a 2-way ANOVA test).

D. U2OS-LacO cells, treated or not with a si-PA28 γ or si-Luc, were transiently transfected with GFP-LacI construct the same day, recovered 48 hours later and cells were analyzed as in B. Immunostaining was performed against PA28 γ (red). Representative fluorescence and immunofluorescence images of Z-stack projections of U2OS-LacO cells are shown. Magnified views of the GFP-LacI spot are shown in inserts. Scale bars, 10 μ m.

E. Quantitative analysis of the decompaction of the LacO array. Z-stack images were acquired on U2OS-LacO cells treated as in D and the area of the GFP-LacI signal was quantified as in C. Data represent the means \pm SD from three biological repeats, numbers of

analyzed nuclei with GFP-LacI spot were $n = 30$, $n = 31$ and $n = 29$ in control cells (CTL), si-PA28 γ or si-Luc treated cells, respectively. (ns, not significant *** $p = 0.0002$; ** $p = 0.0013$, values were determined by Tukey's multiple comparisons test.

Figure 4. PA28 γ controls chromatin compaction, independently of its interaction with the 20S proteasome.

A. Immunoblot analysis of PA28 γ expression level in total extracts from parental (WT) and PA28 γ -knockout (KO-PA28 γ) HeLa^{H2B-2FPs} cells. Tubulin was used as a loading control.

B. FRET analysis in asynchronous parental (WT) and PA28 γ -knockout (KO-PA28 γ) HeLa^{H2B-2FPs} cells. FLIM-FRET measurements were performed and the spatial distribution of the FRET efficiency is represented in a continuous pseudo-color scale ranging from 0 to 30 % (left panel). Scale bars, 10 μ m. Right panel, statistical analysis of the mean FRET efficiency percentage in WT and KO-PA28 γ HeLa^{H2B-2FPs} nuclei, presented as box-and-whisker plots. The thick line represents median, the boxes correspond to the mean FRET values upper and lower of the median, with the whiskers covering the 10-90 percentile range. Data represent the means \pm SD from 4-6 independent experiments, the total number of analyzed cells is $n = 154$ nuclei (WT) and $n = 132$ nuclei (KO-PA28 γ), **** $p < 0.0001$ (Student's T-test).

C. Spatial distribution of the FRET efficiency (percentage) in representative WT and KO-PA28 γ HeLa^{H2B-2FPs} nuclei. The FRET percentage distribution is depicted in a continuous pseudo-color scale ranging from 0 to 30% (left panel). Scale bars, 10 μ m. FRET distribution graph shows distinct populations of FRET efficiency in WT and KO-PA28 γ cells (blue and black curves, respectively) (right panel).

D. Spatial distribution of the FRET efficiency (percentage) in representative WT, KO-PA28 γ and KO/KI-WT #6, KO/KI-WT #8 HeLa^{H2B-2FPs} nuclei. The FRET percentage distribution is depicted as in C. Scale bars, 10 μ m. Quantification of the mean FRET efficiency was represented as box-and-whisker plots. Data represent the means \pm SD from 3 independent experiments, the total number of analyzed cells is $n = 102$ nuclei (WT), $n = 90$ (KO-PA28 γ), $n = 53$ (KO/KI-WT #6), $n = 54$ (KO/KI-WT #8). n.s not significant, **** $p < 0.0001$ (Student's T-test).

E. Cell extracts from parental HeLa^{H2B-2FPs} (WT), PA28 γ -knockout (KO-PA28 γ) cells and KO cells re-expressing the wild-type form (KO/KI-WT) or the Δ C-mutant (KO/KI- Δ C) of PA28 γ , treated for 2 hours with 25 μ M of MG132, were subjected to immunoprecipitation using anti-

PA28 γ antibodies. Immunoblots of the supernatant (SN-IP) and the pull-down (IP- PA28 γ) from whole cell extracts were probed with the antibodies indicated.

F. Spatial distribution of the FRET efficiency (percentage) in representative WT, KO-PA28 γ and KO cells re-expressing the Δ C-mutant (KO/KI- Δ C) HeLa^{H2B-2FPs} nuclei. The FRET percentage distribution is depicted as in B (left panel). Scale bars, 10 μ m. Quantification of the FLIM-FRET measurements. Data represent the means \pm SD from 3 independent experiments, the total number of analyzed cells is $n = 102$ nuclei (WT), $n = 90$ nuclei (KO-PA28 γ), $n = 83$ nuclei (KO/KI- Δ C). n.s, not significant, **** $p < 0.0001$, (Student's T-test).

Figure 5. PA28 γ is a crucial factor for chromatin compaction.

A. HeLa^{H2B-2FPs} cells (WT) were transfected with control si-Luc, si-PA28 γ , si-HP1 β or a mix of both siRNAs (si-PA28 γ /HP1 β) for 48 hours. Immunoblot analysis of PA28 γ and HP1 β protein levels in HeLa^{2FPs} following siRNA treatments were performed. Tubulin and anti- β actin antibodies were used as loading controls. The relative abundance of PA28 γ and HP1 β proteins was quantified using ImageJ software.

B. Quantification of the mean FRET efficiencies were presented as box-and-whisker plots where the thick line represents median, the boxes correspond to the mean FRET values upper and lower of the median, with the whiskers covering the 10-90 percentile range. Data represent the means \pm SD from 4 independent experiments, the total number of analyzed cells is $n = 152$ (si-Luc), $n = 85$ (si-PA28 γ), $n = 73$ (si-HP1 β), $n = 61$ (si-PA28 γ /HP1 β). ns = not significant, *** $p < 0.001$, **** $p < 0.0001$ (Student's T-test).

C. Representative images of the spatial distribution of the FRET efficiency (percentage) in representative control si-Luc, si-PA28 γ , si-HP1 β , or both siRNAs (si-PA28 γ /HP1 β) treated HeLa^{H2B-2FPs} nuclei. Scale bars, 10 μ m. Mean FRET distribution graph showing distinct populations of FRET efficiency in si-Luc (blue curve), si-PA28 γ (black), si-HP1 β (red), or both si-PA28 γ /HP1 β (green) treated HeLa^{H2B-2FPs} (right panel).

Figure 6. PA28 γ contributes to the maintenance of heterochromatin marks.

A. Representative immunoblots of whole cell extracts from U2OS (WT and KO-PA28 γ) cells, using anti-H3K9me3 antibodies. Histone H3 was used as loading control. Graphical representation of the relative abundance of the tri-methylation (H3K9me3) mark on histone H3 normalized to histone H3. The mean \pm SD is from four independent experiments. The p -value was determined with a Student's T-test, ns = not significant ($p = 0.9354$).

B. Immunoblots of whole cell extracts from U2OS (WT and KO-PA28 γ) cells, using anti-H4K20me3 and anti-H4K20me1 antibodies. Histone H3 was used as loading control. Graphical representation of the relative abundance of the mono-methylation (H4K20me1) and the tri-methylation (H4K20me3) marks on histone H4 normalized to histone H3. The mean \pm SD is from four independent experiments. The *p*-value was determined with a Student's T-test, **** ($p \leq 0.0001$).

C and D. ChIP-qPCR analysis of H3K9me3 and H4K20me3 levels in WT *versus* KO-PA28 γ U2OS cells. Data are represented as relative enrichment of each specific antibody *versus* H3 control as shown on the y-axis. Data are means \pm SEM ($n = 5$ for H3K9me3 and H3, $n = 3$ for H4K20me3). Significance was calculated by Student's T-test, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. *p*-values are presented in Table 1.

E. Schematic summary of findings. Upon certain physiological events, decompaction of the chromatin is required and involves a change in H3K9me3 and H4K20me1/3. After this event, chromatin and heterochromatin need to be recompact. Our results show that in the absence of PA28 γ , H3K9me3 and H4K20me3 are reduced and chromatin retains a decompacted form.

SUPPLEMENTARY DATA

Supplementary Methods

Proteasome Activity Assay

Proteasome peptidase activity was measured using black flat-bottom 96-well plates (Nunc) by incubating beads of immunopurified proteasome in 50 μ l of activity buffer (20 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 1 mM ATP, 1 mM DTT, 10% Glycerol) containing 100 μ M succ-LLVY-AMC, in the presence or absence of 25 μ M MG132 (Enzo Life Science), for 20 min at 37°C. Proteasome activity was determined by the detection of the free AMC fluorescence using a FLx800 microplate fluorescence reader (excitation 380 nm, emission 440 nm, Bio-Tek Instruments).

Figure Supplementary

Figure S1. α 4-GFP is incorporated into active proteasome and α 4-GFP foci fluctuate during the cell cycle.

A. GFP-pull-downs from U2OS and U2OS- α 4-GFP cell lysates were subjected to proteasome activity measurements using an exogenous peptide (suc-LLVY-AMC) in the absence or the presence of an inhibitor of the 20S proteasome activity (MG132, 25 μ M).

B. U2OS cells, transfected (right panel) or not (left panel) with a vector allowing the expression of α 4 without any tag protein. After 24 hours, both cell lines were immunostained with anti-alpha 4 antibodies. Representative images are shown. Scale bars, 10 μ m. Arrows indicate α 4 foci.

C. Asynchronous induced U2OS- α 4-GFP cells (AS) were synchronized at the G1/S phase boundary by hydroxyurea treatment (10 mM, 16 hours) (HU) then released from HU-block for 4, 6, 8 10 and 11 hours (R4h, R6h, R8h, R10h and R11h, respectively). Cells were subjected to GFP microscopy detection and flow cytometry analysis. Upper panel: bar graph indicates the percentage of cells with nuclear α 4-GFP foci observed by fluorescence microscopy. Error bars derived from 3 independent experiments represent the mean \pm SD. (ns = no significant, ** $p < 0.001$; * $p < 0.5$, p -values were determined by One-way ANOVA test and are presented in Table 2. Lower panel: bar graph representing the percentage of cells in G1, S and G2/M phases of the cell cycle, obtained by FACS analysis.

Figure S2. Co-localization of PA28 γ with HP1 α and HP1 β /PA28 γ co-localization is independent of PIP30, a regulator of PA28 γ .

A. Immunoblot of whole cell extract (30 μ g) from asynchronous parental (WT) and KO-PA28 γ (KO-PA28 γ) U2OS cells, using anti-PA28 γ . Tubulin was used as a loading control (left panel). Asynchronously-growing U2OS-KO-PA28 γ cells were pre-permeabilized with 0.5 % Triton-X100 before fixation and the detection of endogenous HP1 β (left panel) and PA28 γ (middle panel) by indirect immunofluorescence using anti-HP1 β and PA28 γ antibodies. A representative merged image of HP1 β (green) and PA28 γ (red) is shown (right panel). Scale bars, 10 μ m.

B. *In situ* Proximity ligation assay (*is*-PLA) was carried out in asynchronous U2OS cell line. Fixed cells were treated with primary antibodies directed against PA28 γ (mouse monoclonal) and HP1 α (rabbit polyclonal) (CTL) or with only PA28 γ antibodies (w/o anti-HP1 α) and DNA was stained with DAPI (left panel). A higher magnification view of a nucleus is shown. Scale bars, 10 μ m. The number of PLA dots per nucleus in cells treated with both antibodies (CTL) or with only PA28 γ antibodies (w/o anti-HP1 α) is shown on the bar graph (right panel). Data represent the mean \pm SD from 3 independent experiments, the number of cells

analyzed was $n = 40$ and $n = 41$ in control cells and cells treated without primary HP1 α antibody, respectively. The p -value was determined with Student's T-test, **** ($p \leq 0.0001$).

C. Whole-cell extracts (30 μ g) of parental (WT), PA28 γ -knockout (KO-PA28 γ) and PIP30-knock-out (KO-PIP30) U2OS cells used for the *is*-PLA were analyzed by SDS-PAGE and immunoblotted with the antibodies indicated (left panel). *Is*-PLA was carried out using primary antibodies directed against HP1 β and PA28 γ , and DNA was stained with DAPI. Representative images of parental (WT) and KO-PIP30 U2OS cells are presented and higher magnification views are shown (middle panel). Scale bars, 10 μ m. Quantification of PLA-dots was performed as in Fig 2C. The number of PLA-dots per nucleus is shown on the bar graph (right panel). Data represent the mean \pm SD from 3 independent experiments; the number of cells analyzed was $n = 59$ (WT), $n = 40$ (KO-PIP30) and $n = 42$ (KO-PA28 γ). Statistical significance was evaluated based on one-way ANOVA analysis (ns = not significant, and *** $p < 0.0001$ were obtained for KO-PIP30 and KO-PA28 γ versus WT).

Figure S3. HP1 β is present at the same repetitive elements than PA28 γ .

Immunoblot analysis of HP1 β expression level in total extracts from U2OS cells treated or not with si-HP1 β (left panel). Tubulin was used as a loading control. The relative abundance of HP1 β proteins was quantified using ImageJ software. ChIP-qPCR analysis of HP1 β levels at different repetitive elements (as indicated on the x-axis) in U2OS cells treated with si-Luc or si-HP1 β . Data are represented as relative enrichment of HP1 β antibody *versus* H3 control as shown on the y-axis (right panel). Data are means \pm SEM ($n = 5$). Significance was calculated with Student's T-test, ns = not significant $p \geq 0.05$, ** $p < 0.01$ and *** $p < 0.001$. p -values are presented in Table 3.

Figure S4. Endogenous PA28 γ and HP1 β co-localize in HeLa cells.

Immunoblot analysis of PA28 γ expression level in total extracts from HeLa cells, treated or not with si-PA28 γ (left panel). Tubulin was used as loading control (left panel). The relative abundance of PA28 γ proteins was quantified using ImageJ software. Control (CTL) or si-PA28 γ (si- PA28 γ) treated HeLa cells were subjected to *is*-PLA using primary antibodies directed against HP1 β and PA28 γ , and DNA was stained with DAPI. Positive PLA signals appear as green dots, and a higher magnification view of a nucleus is shown (middle panel). Scale bars, 10 μ m. Quantification of PLA dots was carried out using an ImageJ plugin (see Materials and Methods). The number of PLA dots per nucleus for HP1 β /PA28 γ interaction in

control (CTL) or si-PA28 γ treated cells is shown graphically (right panel). Data represent the means \pm SD from 3 independent experiments, the number of analyzed cells is $n = 40$ and $n = 34$ in control and si-PA28 γ treated cells, respectively. p value was determined with Student's T-test, ** $p = 0.0016$.

Figure S5. PA28 γ -depletion does not alter the expression level of H2B-GFP or mCherry-H2B, and analysis of re-expression of WT- and Δ C- PA28 γ in HeLa^{H2B-2FPs} cells.

A. Immunoblot analysis of H2B-GFP and mCherry-H2B expression level in total extracts from parental (WT) and KO-PA28 γ HeLa^{H2B-2FPs} cells (left panel). Tubulin was used as a loading control. The relative abundance of HP1 β proteins was quantified using ImageJ software. Graphical representation of the relative abundance of H2B-GFP and mCherry-H2B normalized to tubulin (right panel). The mean \pm SD is from four independent experiments. Statistical significance was evaluated based on Student's T-test, ns = not significant. ($p = 0.2027$ and 0.4024 for H2B-GFP and mCherry-H2B, respectively)

B. Quantification of the H2B-GFP and mCherry-H2B fluorescence intensities in WT and KO-PA28 γ HeLa^{H2B-2FPs} cells. The total number of analyzed cells is $n = 172$ (WT), $n = 183$ (KO-PA28 γ). Statistical significance was evaluated with Student's T-test, ns= not significant.

C. FRET analysis in WT, KO-PA28 γ HeLa^{H2B-FPs} cells, and WT HeLa^{H2B-FPs} cells treated with Trichostatin A (TSA, 200ng/ml, 24h). The statistical analysis of the mean FRET efficiency percentage is presented as box-and-whisker plots. The thick line represents median, the boxes correspond to the mean FRET values upper and lower of the median, with the whiskers covering the 10-90 percentile range. The total number of analyzed nuclei is $n = 154$ (WT), $n = 132$ (KO-PA28 γ), and $n = 33$ (WT + TSA), **** $p < 0.0001$ (Student's T-test).

D. Immunoblot analysis of PA28 γ expression level in total extracts from parental (WT), PA28 γ -knockout (KO-PA28 γ) HeLa^{H2B-2FPs} cells and two independent clones of HeLa^{H2B-2FPs} cells knocked out for PA28 γ in which wild-type PA28 γ was stably re-expressed (KO/KI-WT #6, KO/KI-WT #8). Tubulin was used as a loading control.

E. Whole-cell extracts from parental HeLa^{H2B-2FPs} (WT), PA28 γ -knockout (KO-PA28 γ) cells and KO cells re-expressing the wild-type (KO/KI-WT#8) form or the Δ C-mutant (KO/KI- Δ C) of PA28 γ . Cells were treated or not for 2 hours with MG132 (25 μ M), and whole cell extracts analyzed by immunoblot using the antibodies indicated.

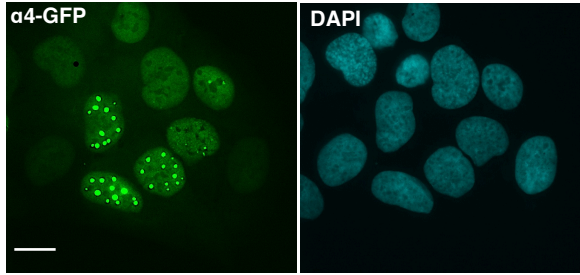
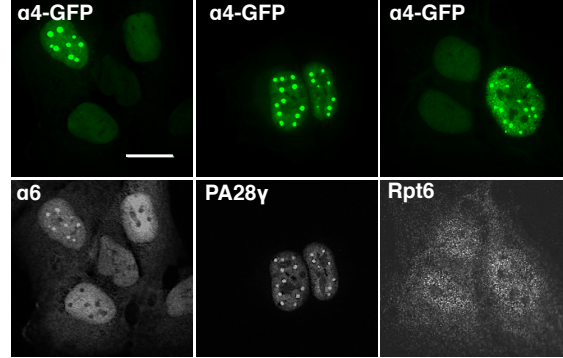
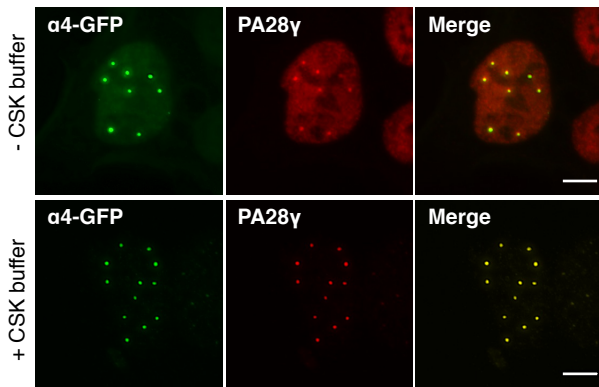
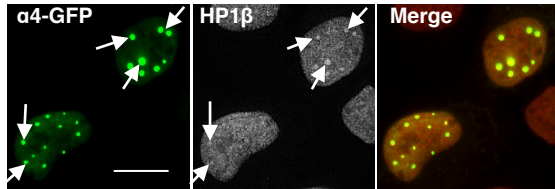
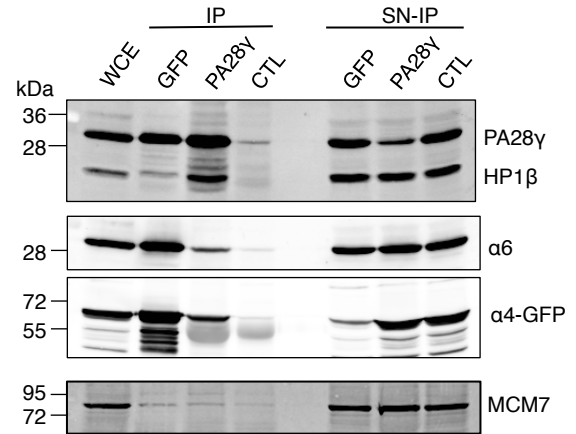
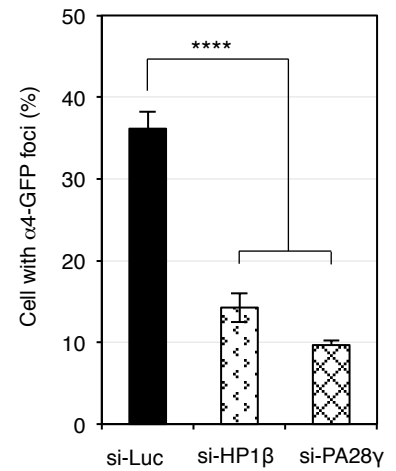
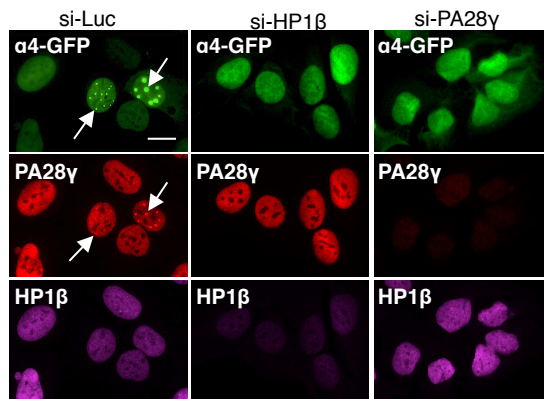
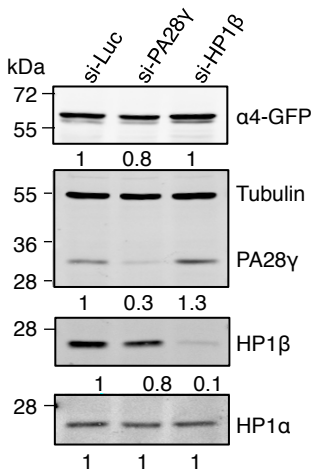
F. Co-immunoprecipitation of PA28 γ and the 20S proteasome from whole-cell extracts without MG132 treatment was analyzed by immunoblotting of the pull-down (IP- PA28 γ) and the supernatant (SN-IP 1/10^{eme}) with the antibodies indicated.

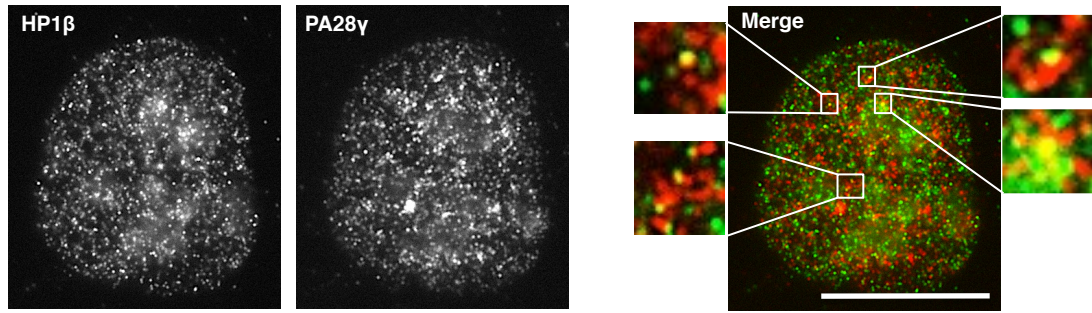
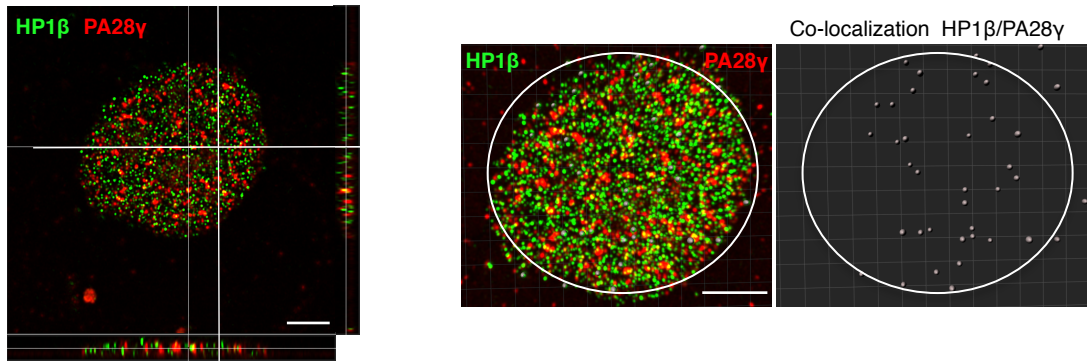
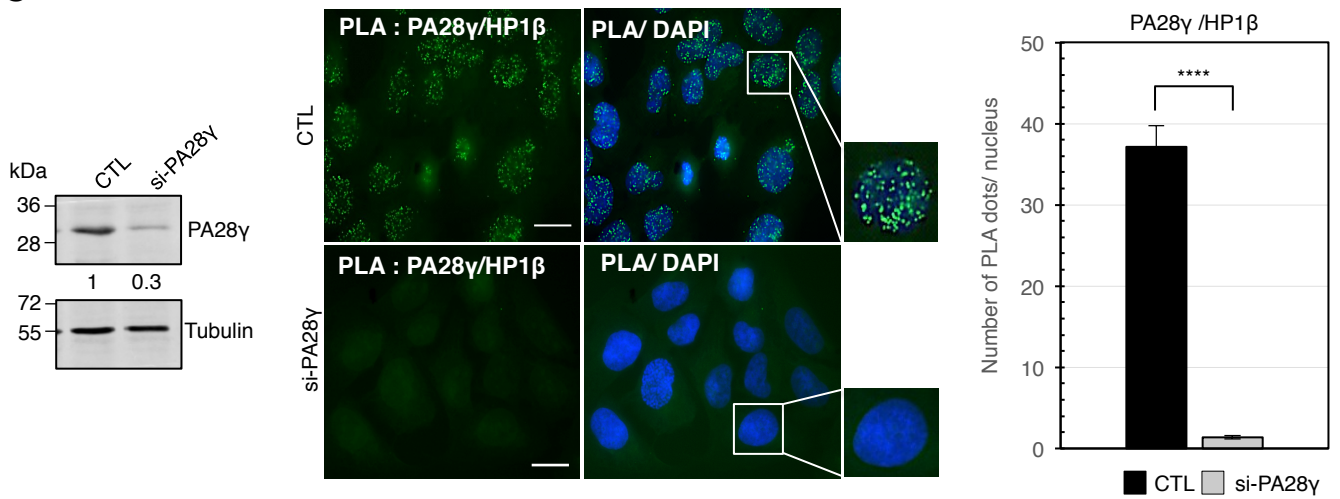
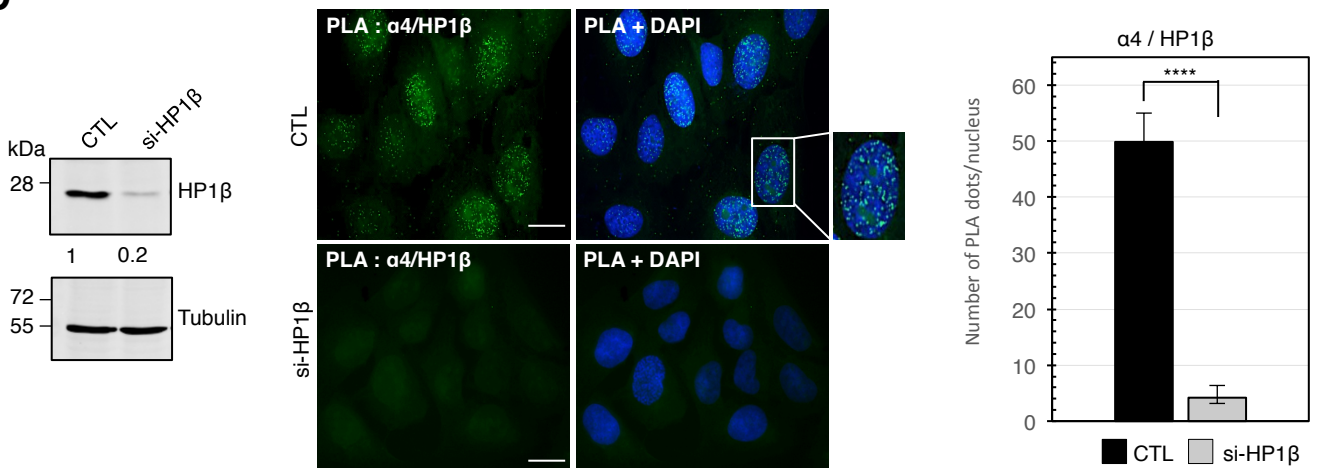
Figure S6. PA28 γ depletion decreases the S phase duration.

A. Asynchronous parental (WT) and KO-PA28 γ U2OS cells were fixed and stained with propidium iodide and then subjected to flow cytometry analysis. The histogram presents the repartition of the cells in the G1, S, and G2/M phases of the cell cycle. Data represent the means \pm SD from four independent biological repeats. *p*-values were determined by 2-way ANOVA test, ns = not significant (*p* = 0.2407), **p* < 0.05 (*p* = 0.0312) and ****p* < 0.001 (*p* = 0.0006).

B. Asynchronous parental U2OS and U2OS-KO-PA28 γ cells, synchronized at the G1/S phase transition by a double thymidine block, then were released for the times indicated and subjected to FACS analysis. Histograms representing the percentage of the cells in S (left panel) and G2/M (right panel) phases of the cell cycle are shown. Data represent the means \pm SD from three biological repeats. *p* values were determined with a 2-way ANOVA for each time point. *p*-values are presented in Table 4.

C. Total cell extracts of WT- or -KO-PA28 γ U2OS cells, asynchronous (AS) and synchronized at the G1/S phase transition by a double thymidine block and then release procedure were analyzed by immunoblot using the antibodies indicated. β -actin was used as a loading control.

A**B****C****D****E****F****Figure 1**

A**B****C****D****Figure 2**

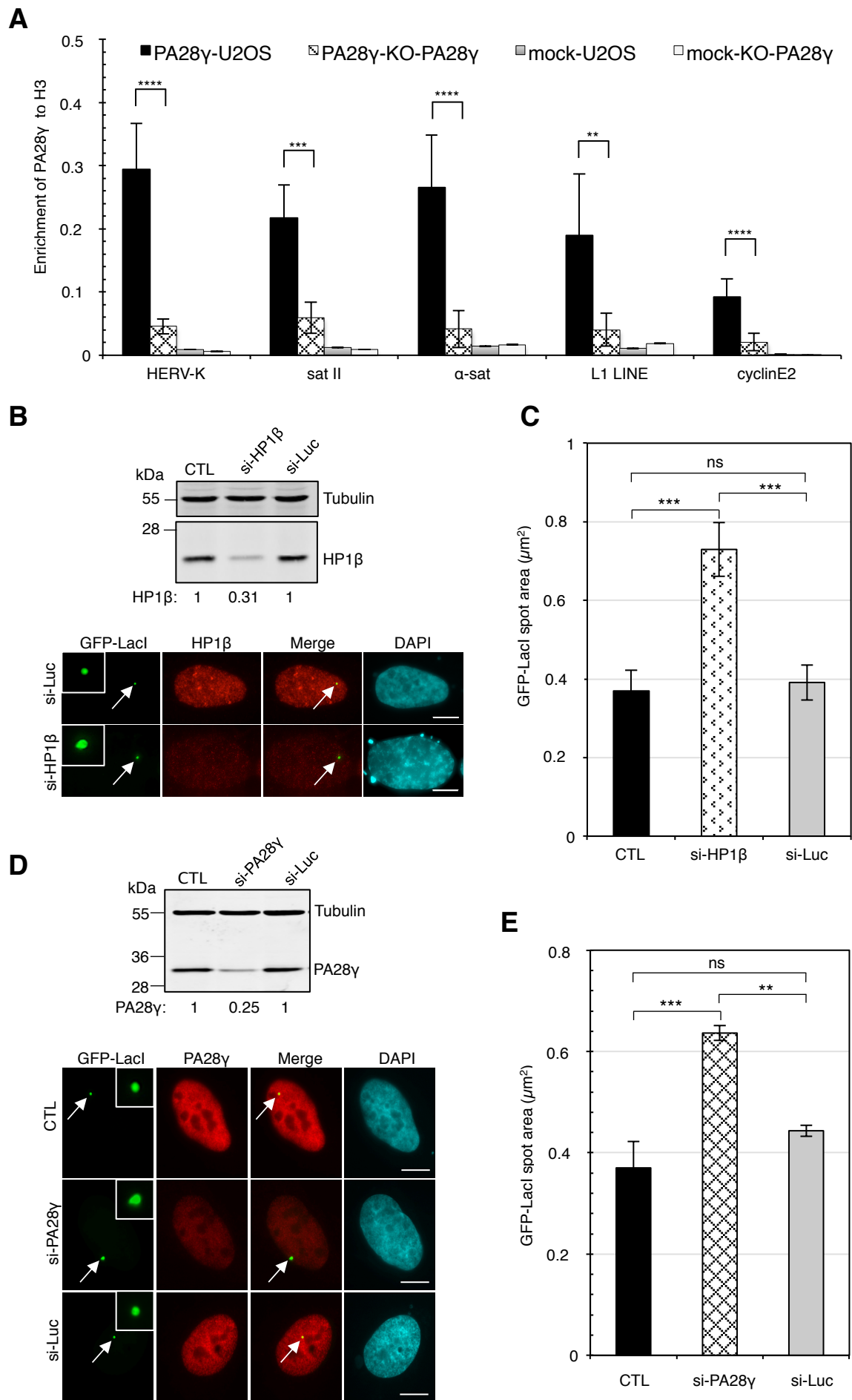


Figure 3

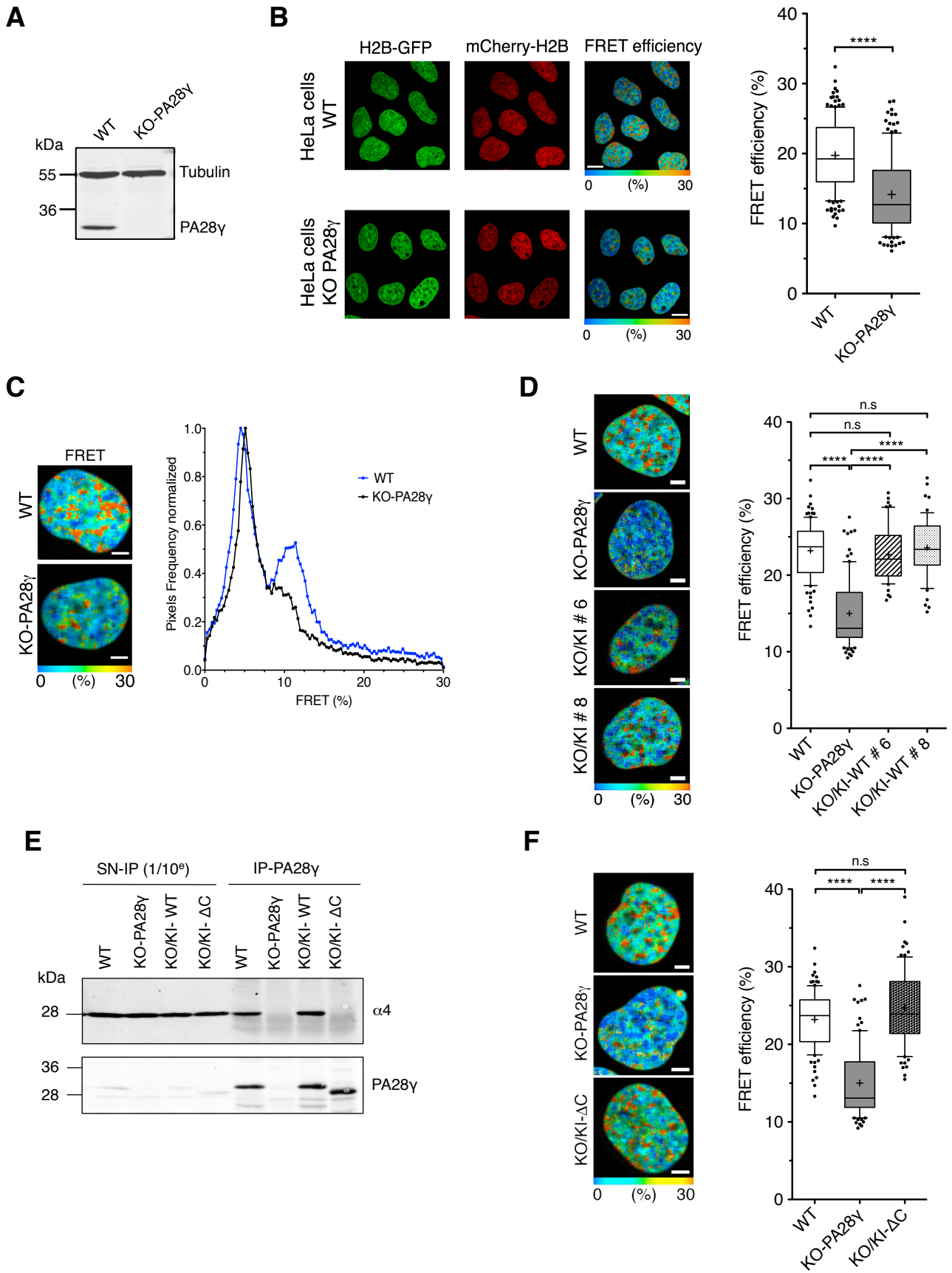
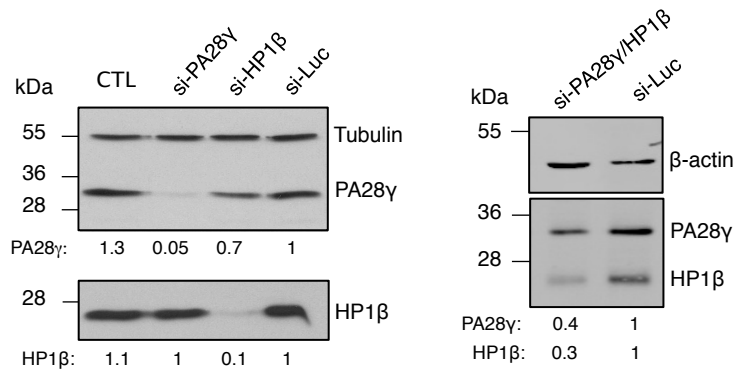
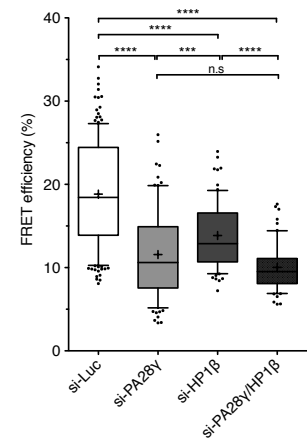
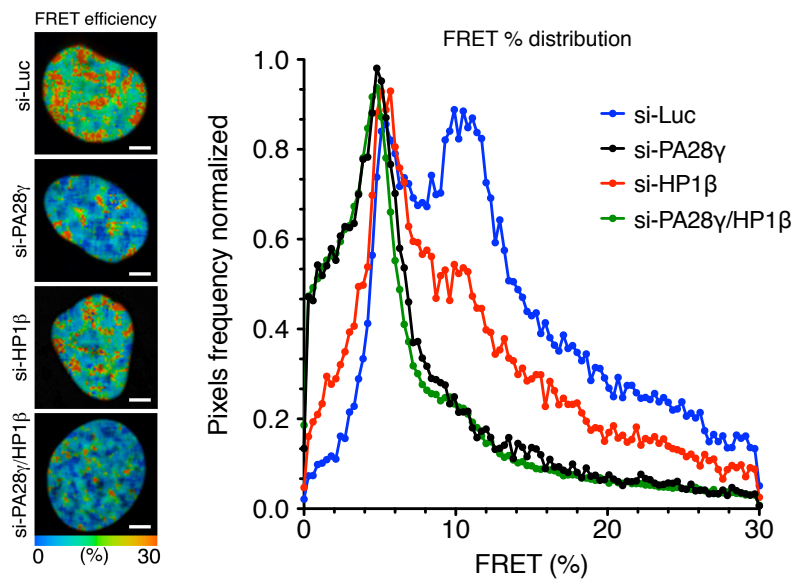


Figure 4

A**B****C****Figure 5**

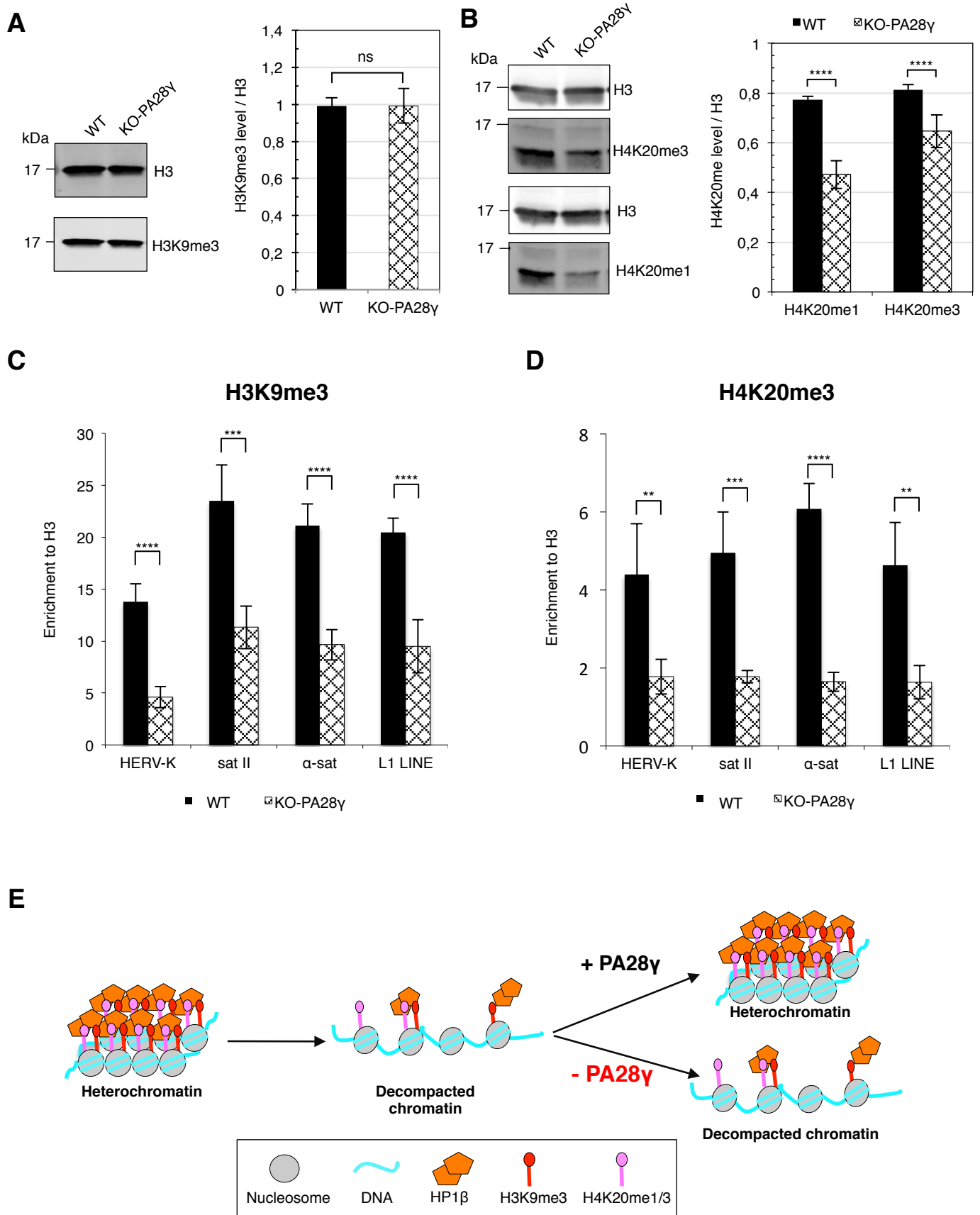


Figure 6

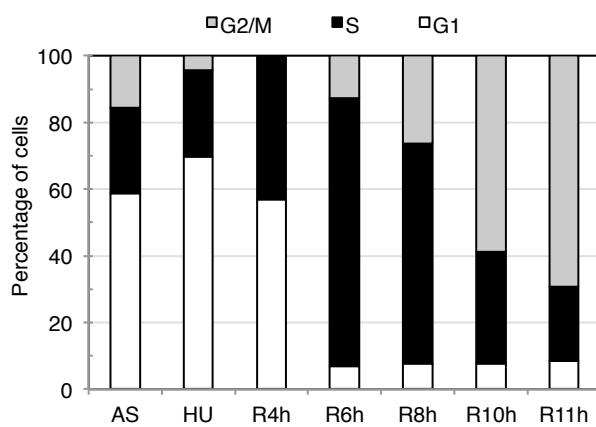
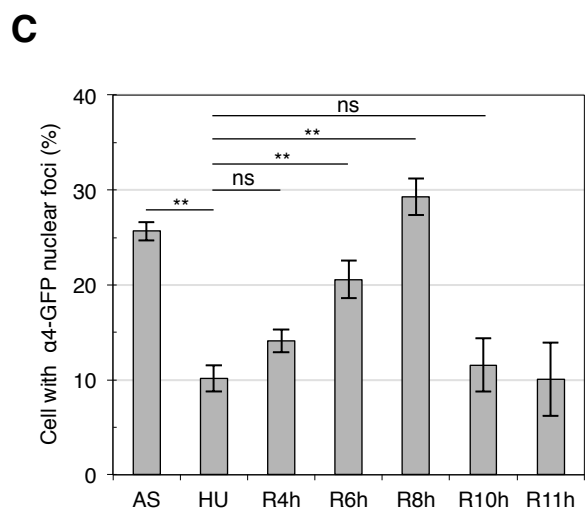
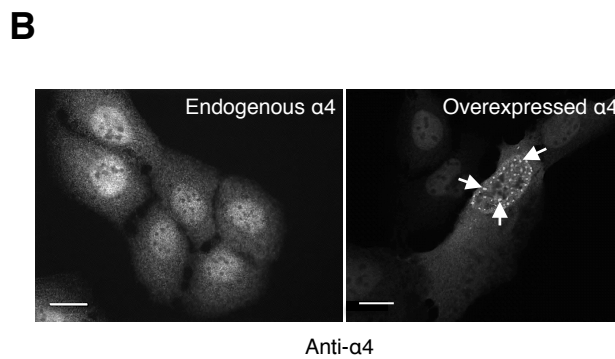
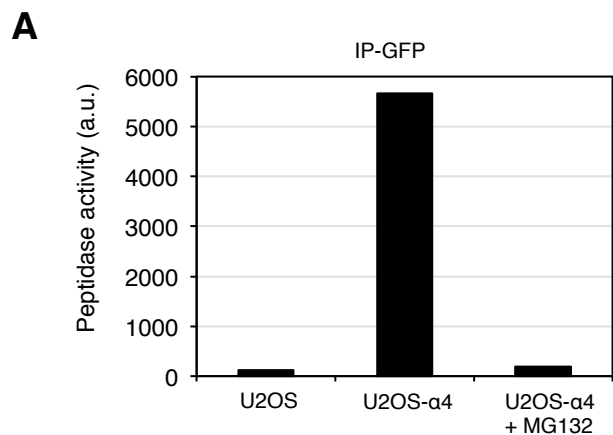


Figure S1

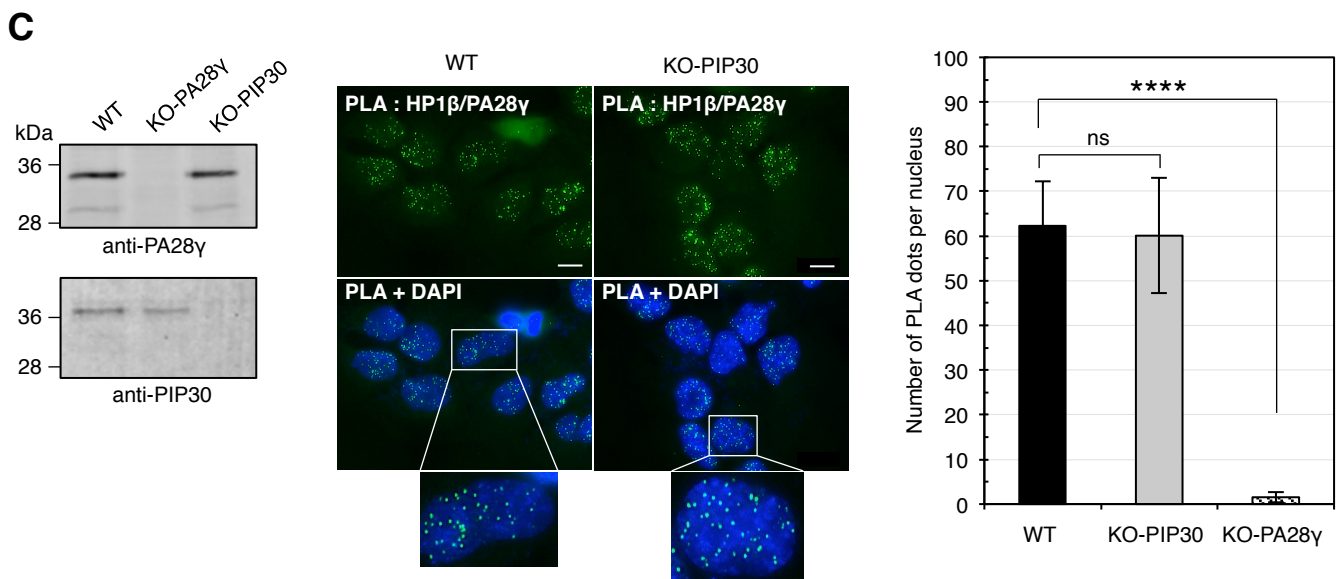
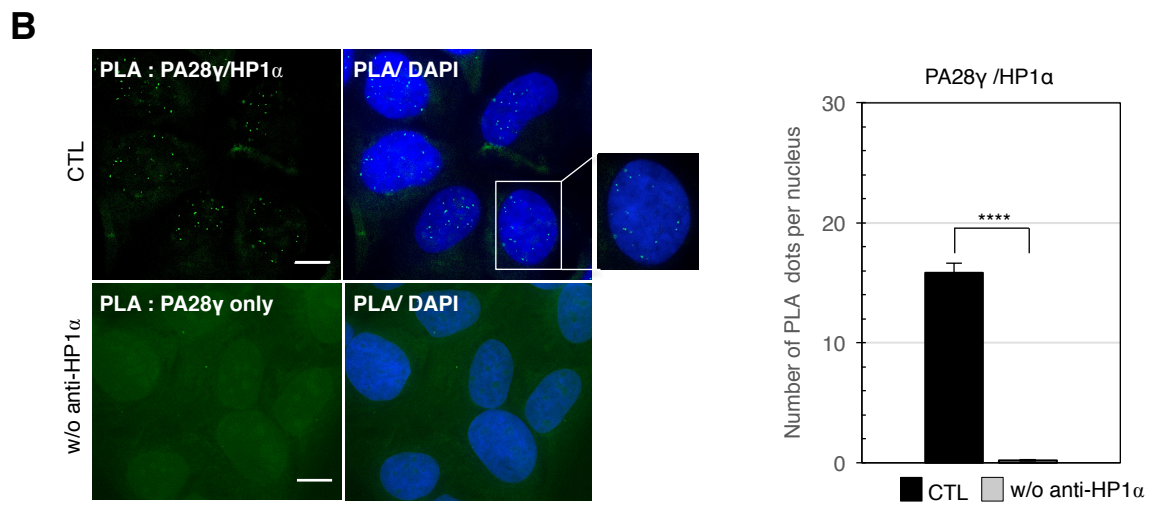
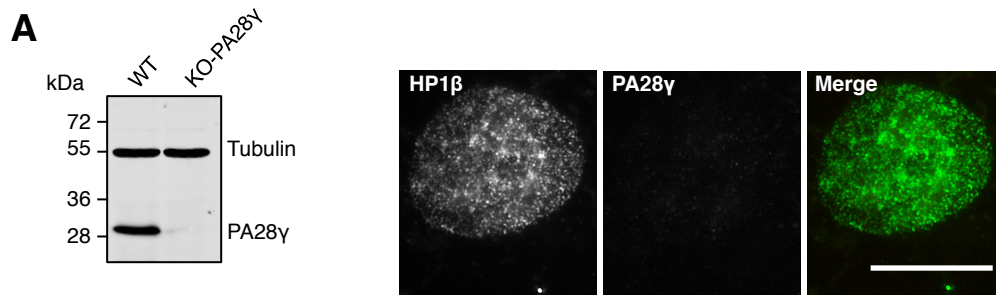


Figure S2

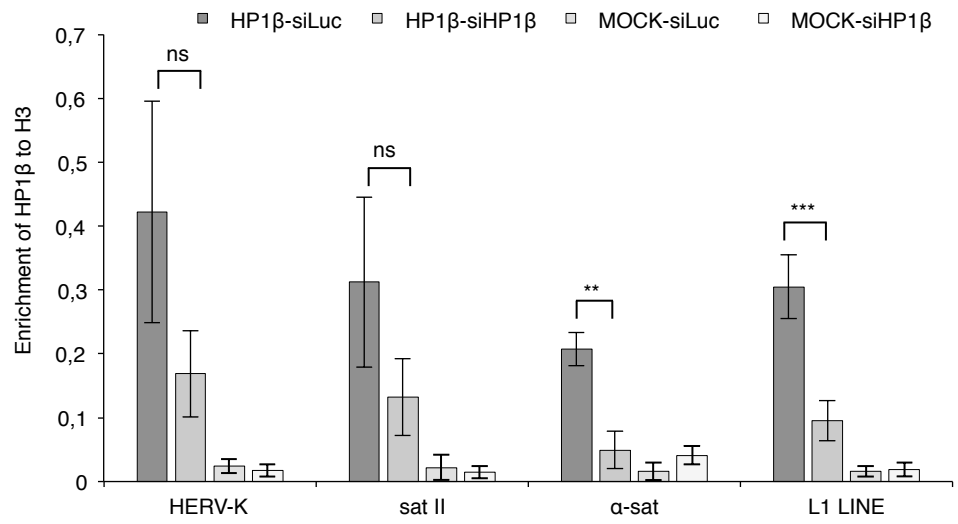
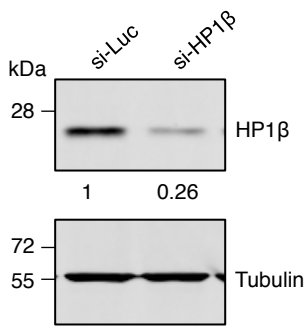


Figure S3

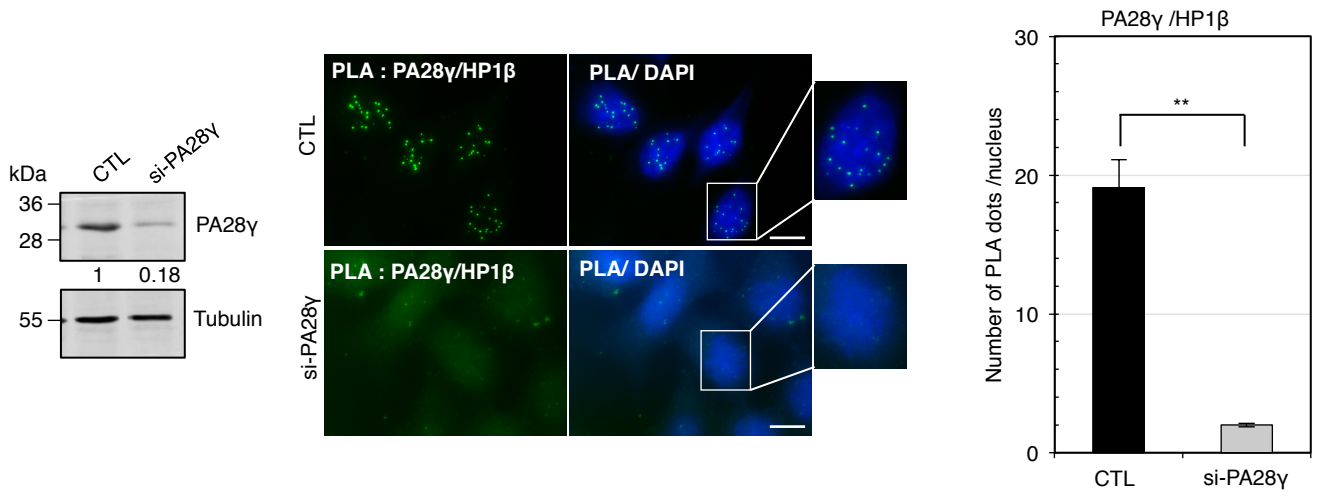


Figure S4

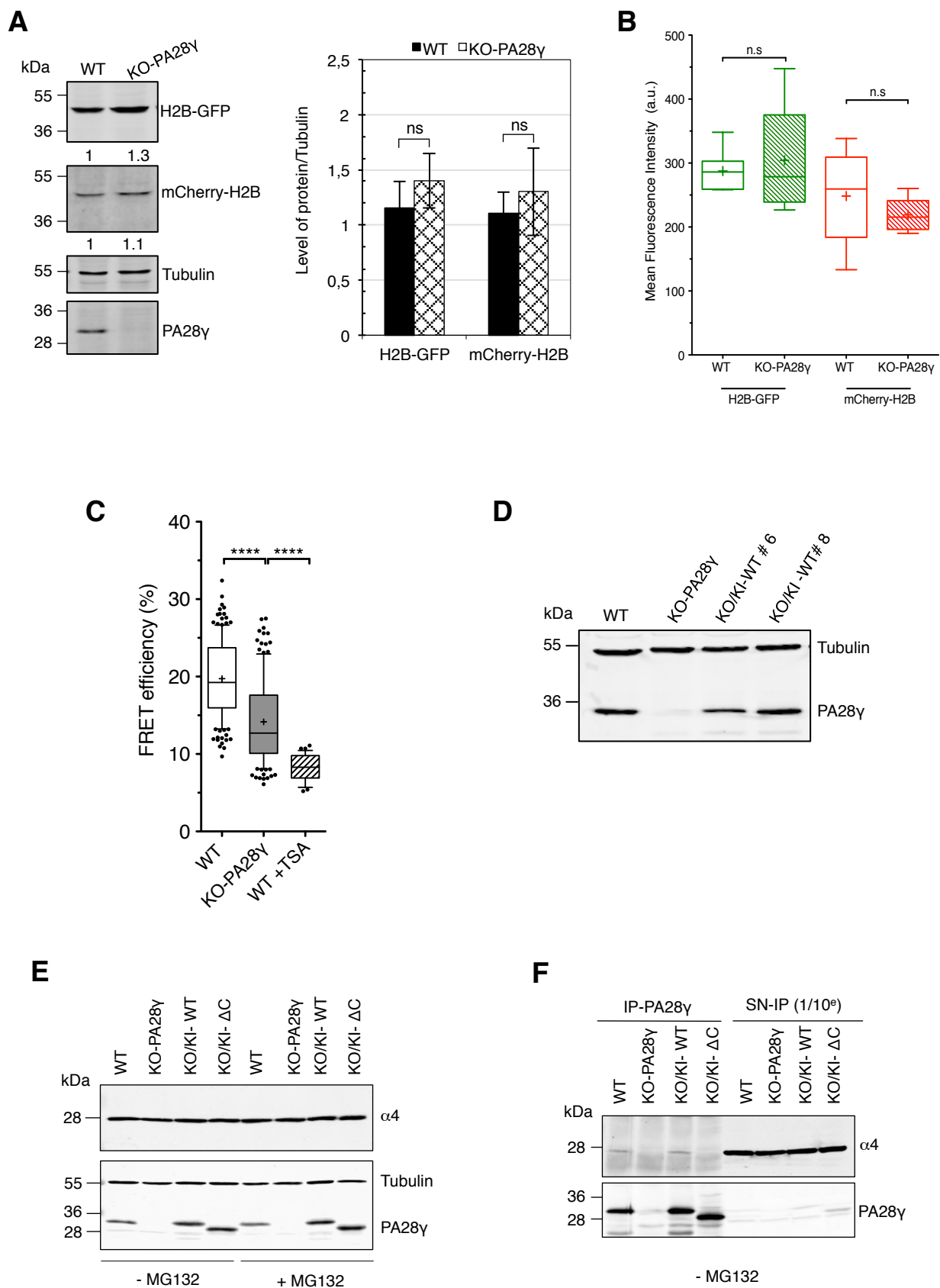


Figure S5

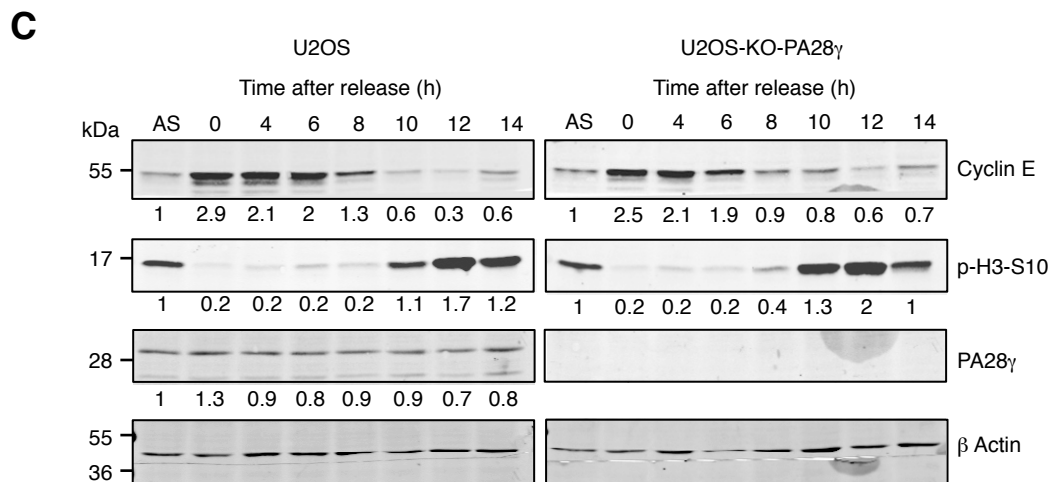
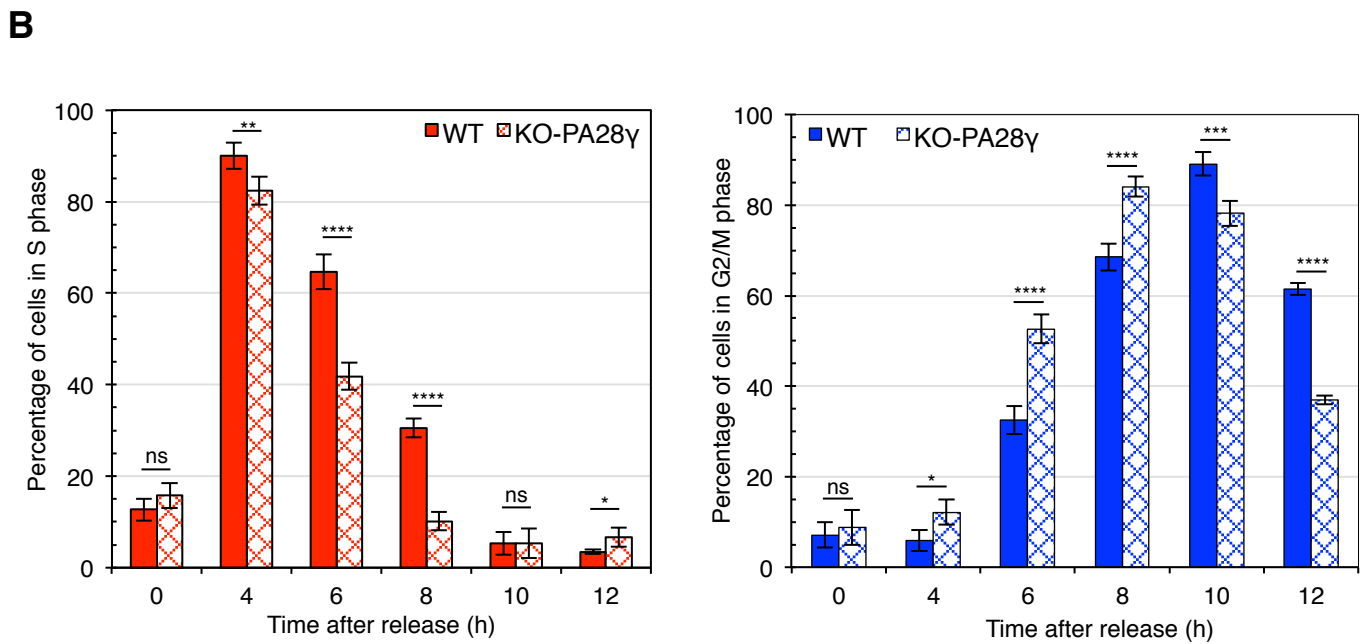
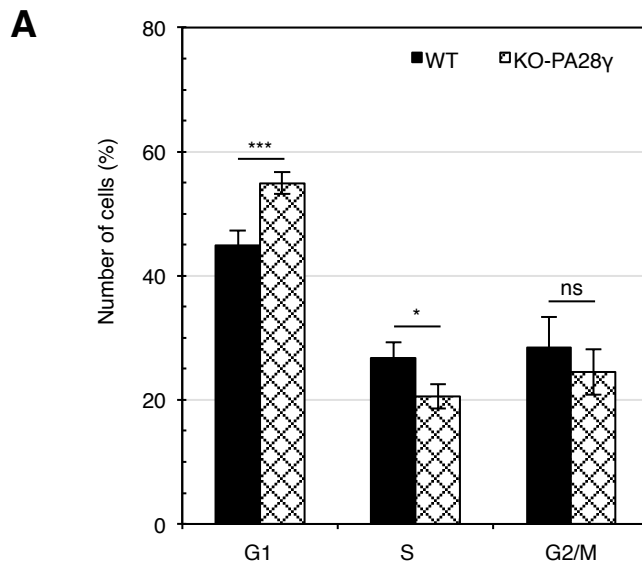


Figure S6

Table 1: *p* values of the figure 6C-D (ChIP)

	T-test (H3K9me3/H3) U2OS vs KO PA28γ	T-test (H4K20me3/H3) U2OS vs KO PA28γ
HERV-K	6,47871E-06	0,009044059
α sat	0,00014585	0,001004765
Sat II	8,6538E-06	1,37015E-05
L1 LINE	2,80855E-05	0,002330735

Table 2: *p* values of the figure S1C

	AS	HU	R4h	R6h	R8h	R10h	R11h
AS		0,0011	0,0099	0,0723	0,0264	0,0047	0,0127
HU			0,1957	0,0017	0,0013	0,7166	>0.9999
R4h				0,0938	0,0124	0,827	0,6832
R6h					0,0286	0,021	0,021
R8h						0,0011	0,0041
R10h							0,4511
R11h							

Table 3: *p* values of the figure S3 (ChIP)

	T-test (si-Luc vs si-HP1β)
HERV-K	0,057078569
Sat II	0,066693611
α sat	0,005250998
L1 LINE	0,000382203

Table 4: *p* values of the figure S6 (determined with the 2-way ANOVA)

PHASE	Time after release (hours)					
	0	4	6	8	10	12
G1	0.9919	0.8713	0.5848	0.2726	0.0002	< 0.0001
S	0.6759	0.0052	< 0.0001	< 0.0001	> 0.9999	0.0377
G2/M	0.9946	0.0187	< 0.0001	< 0.0001	< 0.0002	< 0.0001