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EXTRA VIEW

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Nucleoplasmic Lamin A/C and Polycomb group of proteins: An evolutionarily conserved interplay

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ABSTRACT

Nuclear lamins are the main components of the nuclear lamina at the nuclear periphery, providing mechanical support to the nucleus. However, recent findings suggest that lamins also reside in the nuclear interior, as a distinct and dynamic pool with critical roles in transcriptional regulation. In our work we found a functional and evolutionary conserved crosstalk between Lamin A/C and the Polycomb group (PcG) of proteins, this being required for the maintenance of the PcG repressive functions. Indeed, Lamin A/C knock-down causes PcG foci dispersion and defects in PcG-mediated higher order structures, thereby leading to impaired PcG mediated transcriptional repression. By using ad-hoc algorithms for image analysis and PLA approaches we hereby show that PcG proteins are preferentially located in the nuclear interior where they interact with nucleoplasmic Lamin A/C. Taken together, our findings suggest that nuclear components, such as Lamin A/C, functionally interact with epigenetic factors to ensure the correct transcriptional program maintenance.

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Introduction

The nuclear lamina, the inner part of the nuclear envelope (NE), is made up of a complex meshwork of proteins, collectively called lamins, which provide the mechanical support to the nucleus and the entire cell.[1] Lamins are classified as type V filament proteins and in vertebrates are divided into A and B types, based on sequence homologies. In particular, Lamin A and C, the major isoforms of A-type lamins, are encoded by the LMNA gene as alternative splice variants, while LMNB1 and LMNB2 genes encode the 2 major B-type lamins: Lamin B1 and Lamin B2, respectively.[1]

Intriguingly, in the last few years, in addition to their well-established role in structural stability, lamins have been found to be involved in several cellular processes from cell differentiation to spatial organization of chromatin and gene expression.[2] Seminal studies have described lamin proteins as being tightly associated with heterochromatic and transcriptionally repressed large genomic regions, called Lamina-associated domains (LADs). These domains show variable lengths from 0.1 to 10 Mb and generally create an environment that maintains genes repressed[3,4] and marked by H3K9me2, H3K9me3 or H3K27me3.[5-8] Although A- and B-type lamins share common protein structural features, recent studies have revealed that they differ in several aspects from gene expression to nuclear distribution and function. In mammals the expression of A-and B-type lamins is developmentally regulated, resulting in cell type-specific arrangement of lamins. In particular, while at least one B-type lamin is expressed in every cell, A-type lamins are principally expressed after birth in differentiated cells.[9,10] Interestingly, recent findings have indicated that lamin A:B ratio, more than their individual concentration, plays a pivotal role in cell differentiation.[11] One hypothesis suggests that mechanical signals from the extracellular environment can be physically transmitted by the cytoskeleton to the nucleus by changing the lamin A:B ratio[12] and, in turn, cell-specific gene expression.[1] This hypothesis is supported by the observation that A and B-type lamins also differ in

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their nuclear distribution, where they probably bind and regulate distinct genomic regions. In fact, although both A and B-type lamins are predominantly present at the inner nuclear membrane, Lamin A also exists in lower concentration as a detergent-soluble pool within the nucleoplasm.[13,14] Nucleoplasmic lamins have been also identified within whole tissues,[15] excluding that nuclear interior localization of Lamin A was an artifact of *in vitro* cell cultures. Despite such evidence, in recent years, the nucleoplasmic localization of A-type lamins has been much debated, with some scientists proposing a nucleoplasmic pool of Lamin A composed of intermediates of Lamin A complexes after disassembly of nuclear lamin in mitosis.[16] Live imaging studies in cells at different cell cycle stages have solved this controversy by showing a stable, nucleoplasmic GFP-Lamin A in interphase cells.[17] More recently, several efforts have been made to understand the molecular role of nucleoplasmic Lamin A. Experimental limitations such as the low abundance and the difficulty in separating nucleoplasmic from peripheral Lamin A have made these studies highly complex. However, researchers have managed to avoid these obstacles by focusing their studies on the nuclear interior components interacting with nucleoplasmic Lamin A. One example is the Lamina-associated polypeptide 2alpha (LAP2 α), the only isoform of LAP2 protein lacking the transmembrane domain (reviewed in[18]). LAP2 α directly interacts with Lamin and is a key factor in regulating the nucleo-plasmic localization of A-type lamins.[19] In fact, knock out mice lacking LAP2 α show an absence of nucleoplasmic A-type lamins. Re-expression into LAP2 α-deficient cells of full length LAP2 α but not of a lamin binding-defective LAP2 α mutant rescues the nucleoplasmic Lamin A/C.[20] Functionally, lack of LAP2 α and the subsequent delocalization of Lamin A leads to hyperproliferation of progenitor tissue cells, ultimately leading to cardiac and muscle differentiation defects.[20-23] This suggests that nucleoplasmic Lamin/Lap2alpha complexes play a key role in tissue homeostasis maintenance, regulating the balance between proliferation and differentiation.[24] Recent genome wide studies have further supported the nucleoplasmic Lamin A's functional role, revealing that Lamin A/C enriched DNA sequences are not always confined to the nuclear periphery and suggesting that Lamin A dependent transcriptional regulation can also take place in the nuclear interior.[5,7,25] Furthermore, microscopy and combined chip-DamID experiments have revealed that the same Lamin Associated Domain (LAD) exhibits distinct epigenetic signatures when located in the nuclear interior or in the periphery,[5] suggesting that nucleoplasmic or peripheral Lamin A can regulate the same LAD and that the engagement of different epigenetic factors is dependent on the nuclear localization. Although the scientific community is making great efforts to elucidate the role of Lamin A/C in the chromatin organization, it has still not explored how A-type lamins interact with the network of epigenetic players involved in the regulation of chromatin conformation. Our research provided new insights into Lamin studies, describing for the first time a functional interaction between Lamin A and key epigenetic regulators governing essential cellular processes: the Polycomb group (PcG) of proteins.

The Polycomb group of proteins (PcG)

PcG proteins are transcriptional repressors that play central roles in cell differentiation, development and cell-identity maintenance.[26] They are present predominantly in the nucleus as multimeric protein complexes named Polycomb repressive complexes (PRCs), able to post-translationally modify histones and silence target genes. In mammals, the best-characterized complexes are PRC1 and PRC2, that can act synergistically or independently of each other.[26] The PRC1 complex contains a mix of proteins from distinct families including Chromobox homolog (Cbx), Polyhomeotic (PH), Ring1, PcG RING fingers (PcGFs), YY1-associated factor 2 (YAF2) and RYBP;[27] PRC2 is composed of Enhancer of Zeste1/2 (Ezh1/2), retinoblastoma-associated protein 46 and 48 (RbAp46/48), Embryonic Ectoderm Development (EED) and Suppressor of Zeste 12 (SUZ12).[28] PRC1 is responsible for lysine 119 mono-ubiquitination of histone H2A (H2A119Ub1), catalyzed by the subunits Ring1a and b; while the histone methyl-transferase Ezh2 di- and tri- methylates the Lys 27 of histone H3 (H3K27me2/3). Despite the traditional hierarchical model of PRC recruitment on chromatin depicting PRC2 dependent deposition of H3K27me3 as the epigenetic mark recognized and bound by the PRC1 complex, emerging findings have shown that PRC recruitment relies on several

alternative locus-specific mechanisms involving other subunits and noncoding RNA.[26]

In the nucleus Polycomb proteins form microscopically visible foci called Polycomb bodies, localized close to the pericentric heterochromatin.[29] PcG bodies are formed in the early embryos and progressively increase in size and number during embryogenesis, mirroring the repressive function of PcG proteins.[30] This suggests that the formation of PcG bodies is necessary for a correct maintenance of PcG repressive programs. Consistently, it has been shown that the coordinated action of PcG proteins is evolutionarily required to form specific, cell cycle regulated, multi-looped DNA structures where all the PcG targets are clustered.[31-36] The formation of PcG dependent higher order chromatin structures and their influence on transcriptional repression were recently confirmed by super-resolution microscopy.[37]

We found a previously unanticipated functional and evolutionary conserved interplay between intra-nuclear Lamin A/C and PcG proteins, this being required for a correct PcG-mediated transcriptional repression and PcG nuclear compartmentalization.[38]

Nucleoplasmic Lamin A/C interacts with PcG proteins

By co-immunoprecipitation (co-IP) and proximity ligation assay (PLA) experiments we previously showed that Lamin A/C interacts with Polycomb Repressive Complexes (PRC) in murine muscular cells.[38] Here we show that these interactions are evolutionary conserved. By performing co-IP in human 293 cells, we found that the endogenous Lamin A/C co-precipitated members of both Polycomb complexes PRC1 and PRC2 (Fig. 1A). Similarly, by immunoprecipitating components of both PRC1 and PRC2 complexes, we were able to detect Lamin A/C (Fig. 1A). Of note, the stoichiometry of the identified interactions indicated that only a small portion of the PcG complexes is associated with Lamin A/C, suggesting that nucleoplasmic Lamin A/C interacts with Polycomb complexes. Proximity ligation assay (PLA) analysis in 293 cells corroborated these findings, showing several spots of protein-protein interactions in the nuclear interior obtained by using different combinations of antibodies against Lamin A/C and PRC components (Fig. 1B and C). Importantly, knock-down of PcG subunits or Lamin A/C strongly reduces the detected associations, proving the reliability of the assay.

PcG proteins are preferentially localized in the nuclear interior

To further investigate the putative preferential positioning of PcG proteins in the nuclear interior, we evaluated the sub-nuclear localization of PcG aggregates relative to the nuclear periphery on single plane images of C2C12 nuclei, by measuring the area occupied by PcG foci in 3 concentric nuclear zones of equal surface area (Fig. 2A). We used our MCV algorithm to select nuclei and to automatically segment PcG foci on a large number of images.[38] We found that PcG foci are mostly localized in the interior part of the nucleus, being excluded from nuclear periphery (Fig. 2B); this confirms that PcG-Lamin A/C interplay takes place in the nucleoplasm.

Lamin A/C-Polycomb axis in muscle differentiation

We previously showed that reduction of Lamin A/C determines an "erosion" of PcG bodies due to dispersion of PcG proteins in the nucleus; this, in turn, is accompanied by an aberrant transcriptional reactivation of some PcG targets, ultimately leading to an anticipation of the myogenic program.[38] Interestingly, after knock down of Lamin A/C, although we were able to observe a higher reduction of nucleoplasmic Lamin A/C in respect to peripheral Lamin A/C (Fig. 2), no differences in the nuclear localization of the remaining PcG foci were found, further suggesting that peripheral Lamin A/C does not interact with PcG proteins (Fig. 2). Our work reveals the importance of intra-nuclear PcG architecture for PcG function, underlying that nucleoplasmic LaminA/C acts as a scaffold for PcG foci stability. It remains to be elucidated what is the determinant of the PcG foci localization in the nucleoplasm. One possible candidate is the Lap2 α protein, whose expression is physiologically downregulated during myoblast differentiation. This event determines a reduction in nucleoplasmic Lamin A/C,[23] in line with the concomitant PcG target activation.[38] This hypothesis was recently supported by an interesting work showing that nucleoplasmic Lamin A/C and Lap2 α co-occupy genomic regions enriched with the PcG-dependent histone mark H3K27me3.[39] Importantly, in Lap2alpha KO, Lamin A/C is redistributed in the nucleus and the H3K27me3 marks increased in regions which gained Lamin A/C binding,[39] suggesting a coordinated Lamin A/C -PcG proteins crosstalk guided, in wt, by Lap2 α. In line with

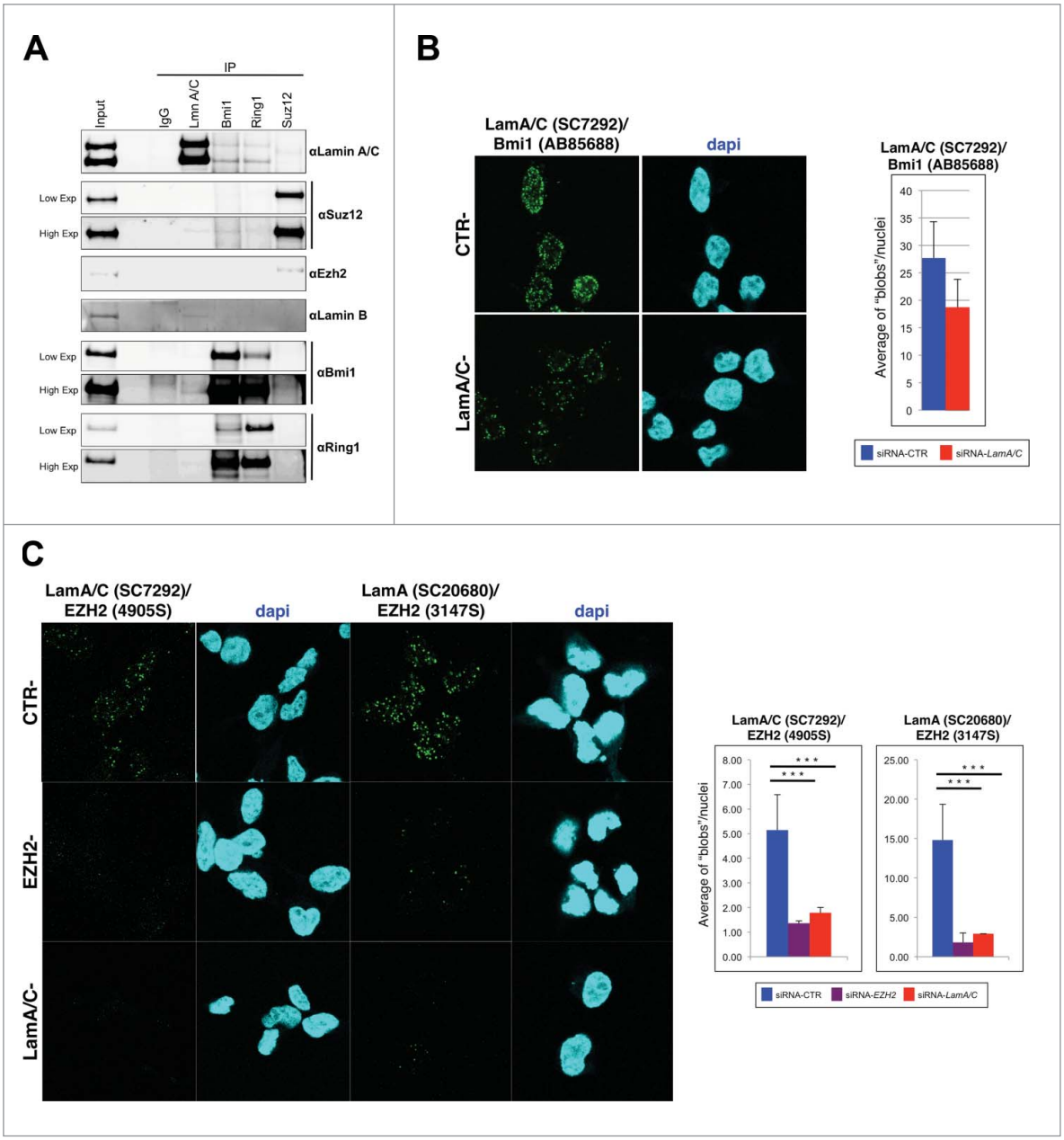

Figure 1. Polycomb proteins and Lamin A/C interact endogenously. (A) Western blot analysis of co-IP performed in 293. Nuclear extracts immunoprecipitated with Suz12, Bmi1, Ring1b or Lamin A/C antibodies together with inputs were immunoblotted and hybridized with indicated antibodies. An unrelated antibody (murine IgG) was used as negative control. Two panels with low and high exposures of Suz12, Ring1b and Bmi1 were shown. (B and C) Representative fields of confocal microscopy images of PLA experiments performed on 293 cells transfected with indicated siRNAs. Each fluorescent dot, 'blob', represents the co-localization of Lamin A/C and Bmi1 (B) or Ezh2 (C). Data are reported for 3 different combinations of antibodies. Quantification of the blobs is represented in the graphs beside. The average of blobs/nuclei in the diagram corresponds to the quantification of 2 independent experiments, n > 244, 91 and 267, respectively (from left to right). Two-tailed t-test was applied for statistical analysis. Standard error of the mean is indicated. Asterisks indicate statistically relevant differences: ($\alpha = 0.05$). * $p < 0.05$, *** $p < 0.001$.

this hypothesis, we think that the interplay between Lamin A/C-PcG-Lap2 α could dynamically regulate physiological skeletal muscle differentiation. We found that the reduction of endogenous Lamin A/C levels accelerates the myogenic program in an evolutionary conserved manner, phenocopying Ezh2 depletio[38,40] (Fig. 3A-C). Hence, the Lamin dependent premature muscle differentiation is due to a detachment of Ezh2 from chromatin and the concomitant transcription of PcG regulated muscle genes (Fig. 3D). Interestingly, it has been reported that the processing rate and localization of Lamin A/C are regulated

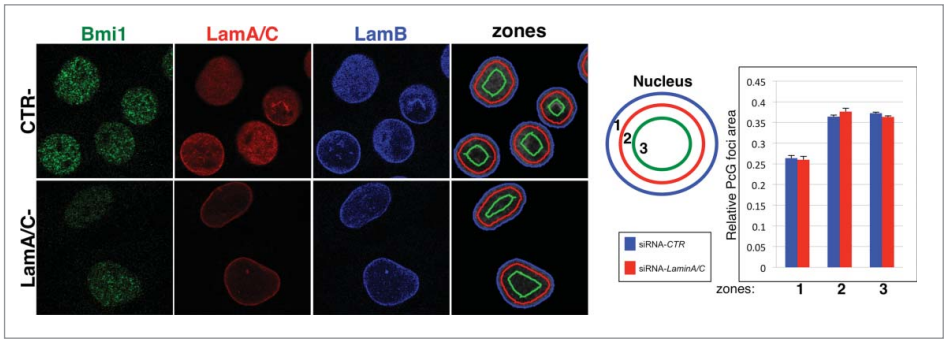

Figure 2. Intra-nuclear localization of PcG proteins depend on Lamin A/C. (A) Representative confocal microscopy images of C2C12 myoblasts transfected with indicated siRNA. PcG foci distribution is evaluated in a 3-zone assay using the focal plane in which the PcG foci have the highest intensity. Each cross-section was divided into 3 concentric zones of equal surface. A random distribution gives 33% per zone. (B) Quantification of PcG distribution described in.[27] PcG foci area in the indicated zone relative to total area of PcG foci measured in the whole nucleus. The average in the graph corresponds to the quantification of 4 independent experiments. n > 352.

during myoblast differentiation, dropping when myoblasts achieve the confluence, at the onset of muscle differentiation.[41] On the other hand, the downregulation of Lap2 α during myogenesis is accompanied by a reduction of the nucleoplasmic pool of A-type lamins.[21] These dynamics suggest a physiological role for the

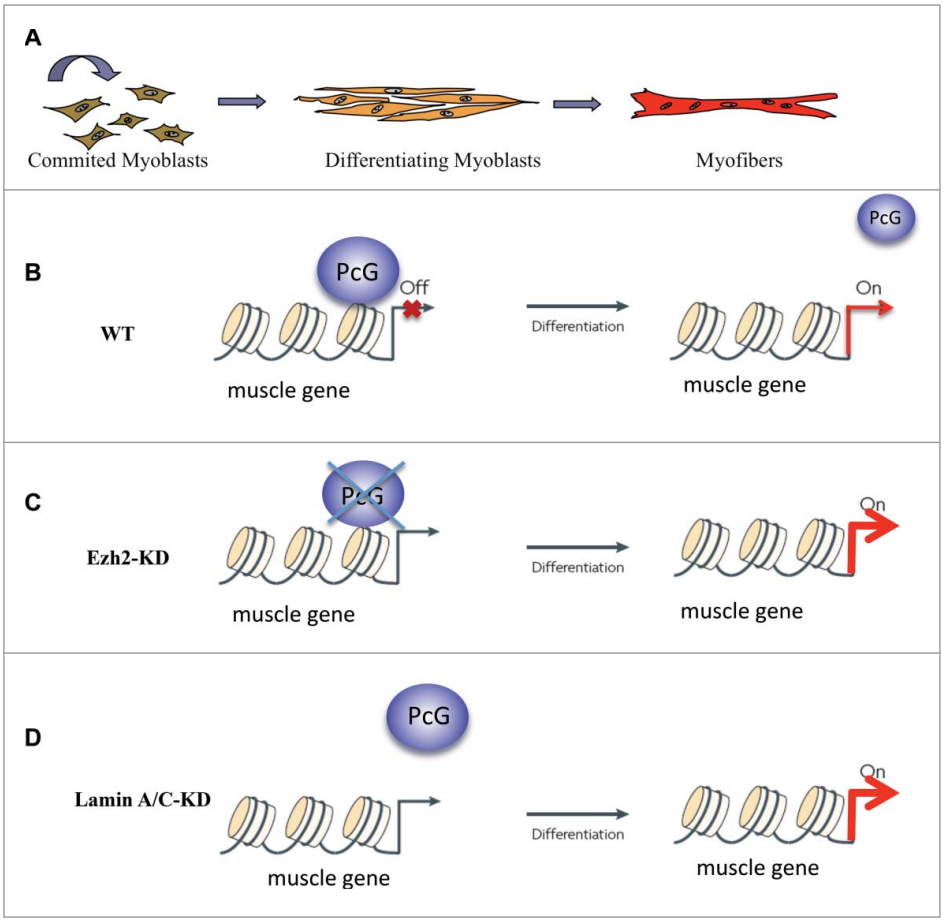

Figure 3. Nucleoplasmic LaminA/(C) affects muscle differentiation through PcG functions. (A) Schematic representation of muscle differentiation in vitro. (B) In physiological conditions PcG proteins total levels are reduced at the onset of myogenesis. This determines a displacement from muscle promoters and a consequent transcriptional activation of target genes. (C) Depletion of PcG proteins induces de-repression of muscle genes and premature muscle differentiation. (D) Depletion of Lamin A/C, although does not change overall PcG proteins levels, leads to an acceleration of myogenesis due to a premature displacement of PcG proteins from muscle-specific genes promoters.

reduction of Lamin A/C and Lap2alpha in the induction of the differentiation process. In line with this evidence, the enforced reduction of Lamin A/C before the onset of myogenesis, which mimics physiological conditions, is probably responsible for the delocalization of PcG proteins from muscle specific genes and consequent transcriptional activation of the myogenic program.

Future directions

Recently, super-resolution imaging studies have shown that the chromatin is organized in different 3D conformations, depending on its epigenetic state.[42] Intriguingly, PcG repressed domains show a special chromatin folding, with a high degree of chromatin intermixing and exclusion of neighboring active chromatin, suggesting that PcG proteins play an active role in the packaging of repressed chromatin. These observations were confirmed and corroborated by other studies showing that PcG protein aggregation facilitates long-range interactions.[35,37] We provided new insights into PcG biology, describing for the first time a functional interaction between Lamin A and PcG proteins and demonstrating that an intact intranuclear Lamin A/C is necessary to provide a framework for the stability of PcG proteins aggregations and to ensure the maintenance of PcG-mediated higher order nuclear structures and epigenetic transcriptional programs[38] (Fig. 4). In the near future, studies aimed at understanding the role of lamin proteins in regulating chromatin higher order structures and epigenetic functions could open up alternative approaches in the cure of lamin-associated diseases, where Lamin A shows a nuclear mislocalization leading to aberrant cellular processes.[43-45]

Experimental procedures

Co-immunoprecipitation (co-IP)

coIP on nuclear extracts was performed using standard procedures. In brief, nuclear extracts were prepared after cytosolic extraction with cytosolic lysis buffer (10 mM Hepes, pH 7.5, 1.5 mM MgCl2, 10 mM KCl, 10% glycerol, 0.2% NP-40, 1 mM DTT, and protease inhibitors) for 10 min on ice and a 3/8-in syringe with a 25G needle. Nuclei pellets were then lysed in nuclei lysis buffer (10 mM Hepes, pH 7.5, 1.5 mM MgCl2, 300 mM KCl, 10% glycerol, 0.2% NP-40, 1 mM DTT, and protease inhibitors) for 30 min at 4°C. 0.5 to 1 mg of nuclear extract was immunoprecipitated overnight at 4°C with 5 μg corresponding

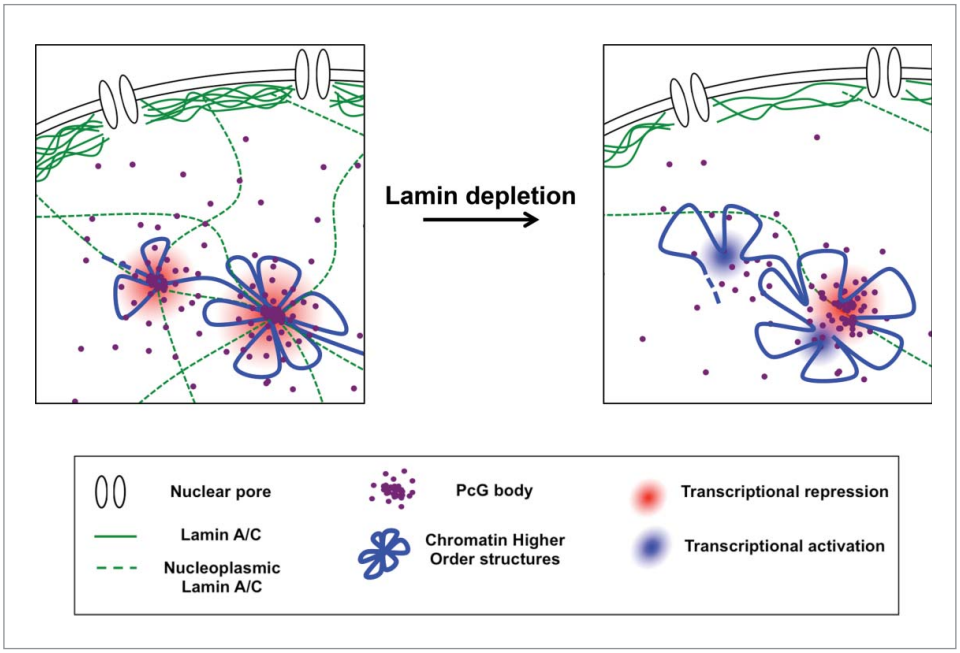

Figure 4. Molecular mechanism underlying PcG/Lamin A interplay. An intact intra-nuclear Laminv A/C is required for the stability of PcG foci aggregation. Reduction of Lamin A/C levels determines an erosion of PcG foci caused by PcG protein dispersion. This is accompanied by a relaxation of PcG-mediated higher-order chromatin structure that acquires a conformation more prone to transcriptional reactivation.

antibodies at a final concentration of 150 mM KCl in nuclei lysis buffer. Immuno-precipitates were incubated with magnetic protein G beads (10004D; Dynabeads; Invitrogen) for 2 h at 4°C. Beads were extensively washed with wash buffer (10 mM Hepes, pH 7.5, 1.5 mM MgCl2, 150 mM KCl, 0.2% NP-40, and 10% glycerol) and eluted in Tris-EDTA. Finally, beads were re-suspended in Laemmli buffer and boiled at 95°C for 5 min, and the supernatant was analyzed by Western blot.

PLA experiments

Coverslips were fixed with 4% paraformaldehyde in PBS for 10 min. Cells were permeabilized with 0.5% Triton X-100 in PBS and blocked with 1% BSA in PBS for 1 h at RT. Detection of protein interactions was performed using the Duolink system (Sigma-Aldrich) according to the manufacturer's instructions. PLA blob quantification was performed using Cell Profiler 2.0. Nuclei were detected using the Otsu method with a global 2-class threshold strategy, and foci were detected using the Otsu method with a per-object 3-class threshold strategy.

PcG foci quantification

PcG foci quantification was performed with an automated pipeline described in.[38] For the analysis of PcG localization respect to the nuclear periphery, each nucleus area was divided in 3 concentric regions of the same size using matlab (bwmorph shrink operation). The amount of polycomb proteins in each region was measured by counting the pixels contained.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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