PRR14 organizes H3K9me3-modified heterochromatin at the nuclear lamina

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Abstract

The eukaryotic genome is organized in three dimensions within the nucleus. Transcriptionally active chromatin is spatially separated from silent heterochromatin, a large fraction of which is located at the nuclear periphery. However, the mechanisms by which chromatin is localized at the nuclear periphery remain poorly understood. Here we demonstrate that Proline Rich 14 (PRR14) protein organizes H3K9me3-modified heterochromatin at the nuclear lamina. We show that PRR14 dynamically associates with both the nuclear lamina and heterochromatin, and is able to reorganize heterochromatin in the nucleus of interphase cells independent of mitosis. We characterize two functional HP1-binding sites within PRR14 that contribute to its association with heterochromatin. We also demonstrate that PRR14 forms an anchoring surface for heterochromatin at the nuclear lamina where it interacts dynamically with HP1-associated chromatin. Our study proposes a model of dynamic heterochromatin organization at the nuclear lamina via the PRR14 tethering protein.

Introduction

The eukaryotic genome is organized at multiple scales in the nucleus. The genome is spatially arranged in subnuclear compartments so that transcriptionally active euchromatin is spatially segregated from silent heterochromatin [1–4]. One example of spatially organized chromatin is transcriptionally silent heterochromatin sequestered at the periphery of the nucleus [5,6]. This precise organization of heterochromatin at the nuclear periphery contributes to many cellular functions such as mechanical force response, cell migration, signaling, transparency to light, and transcription control (reviewed in [1–4,7,8]). Multiple studies have shown that chromatin localization in the nucleus is often correlated with its transcriptional state: more often active when located in the nuclear interior and more often silenced when sequestered to the nuclear periphery [9–11]. During development and lineage commitment, chromatin in the nucleus undergoes notable spatial reorganization with lineage-specific genes moving toward the nuclear interior while genes that are no longer required move to the nuclear lamina and become transcriptionally silenced [5]. It has been demonstrated that correct spatial organization of heterochromatin at the nuclear lamina is essential for cell differentiation and lineage maintenance [12–14], however we know surprisingly little about the cellular mechanisms (epigenetic modifications, protein complexes, signaling pathways, etc.) that orchestrate three-dimensional chromatin positioning within the nucleus in mammalian cells.

To date, only a few proteins have been shown to tether chromatin to the nuclear periphery [15]. Lamin B Receptor (LBR) has been identified as a transmembrane protein that binds heterochromatin through the adapter protein Heterochromatin Protein 1 (HP1) [16–18]. Later studies identified Proline Rich 14 (PRR14) as a nuclear lamina-binding protein that also binds heterochromatin through the HP1 adapter [19]. An example of non-mammalian protein is CEC-4, identified as a heterochromatin tether in C. elegans [13]. While mammalian LBR and PRR14 proteins bind heterochromatin through HP1, CEC-4 itself encodes an
HP1-like domain [13], thereby obviating the need for an HP1 adapter. It is notable that all of the known peripheral heterochromatin tethers utilize a similar mechanism for heterochromatin binding.

Mammalian cells employ three isoforms of HP1 – HP1α, HP1β, and HP1γ – which vary in their function and localization in the nucleus [20–23]. HP1α and β isoforms are largely found in association with heterochromatin [24], while HP1γ is found in both heterochromatin and euchromatin compartments throughout the nucleus [25,26]. All HP1 isoforms are known to bind methylated Lys9 residue on Histone H3 tail (H3K9me) [27,28].

In contrast to the transmembrane protein LBR, PRR14 is a nuclear lamina-associated protein that is found primarily at the nuclear lamina with a much smaller fraction observed in the nucleoplasm. The observation that PRR14 localization is not restricted to the nuclear periphery [19,29] indicates that PRR14 association with the nuclear lamina is dynamic, and that the mechanism of chromatin tethering to the nuclear lamina via PRR14 could be different from that of LBR.

PRR14 was originally identified as an epigenetic repressor of gene expression [30]. Further studies demonstrated that PRR14 localizes at the nuclear lamina, specifically in association with Lamin A/C [19]. PRR14 has a 120-residue lamina-binding domain (LBD) that includes two independent lamina-binding motifs, both of which have been reported to be phosphoregulated [29]. PRR14 also contains an HP1-binding motif in the N-terminal portion of the protein, and the PRR14-HP1 interaction has been shown to be required for PRR14 to bind chromatin [31]. The C-terminus of the protein has a proposed regulatory function and includes a Tantalus domain with a Protein Phosphatase 2 Subunit Alpha (PP2A) binding motif. Interaction with PP2A is understood to regulate phosphorylation of the LBD and thus association of the protein with the nuclear lamina. While the domain structure of PRR14 has been described and several interacting proteins identified, the spatial dynamics of PRR14 in the nucleus and its ability to organize heterochromatin at the nuclear periphery have yet to be investigated.

Here, we demonstrate that PRR14 functions to organize heterochromatin at the nuclear lamina. Our experimental results show that PRR14 associates dynamically with the nuclear lamina and heterochromatin, and is capable of reorganizing H3K9me3-modified heterochromatin in interphase, independent of mitosis. We assessed the ability of PRR14 to interact with the individual isoforms of heterochromatin protein 1 (HP1) and found an in vivo preference for HP1α and HP1β. In addition, we have identified a second HP1-binding motif in the N-terminus of PRR14 and show that it contributes to the association of PRR14 with heterochromatin, albeit to a lesser degree than the first PRR14 HP1-binding motif. Fluorescent recovery after photobleaching (FRAP) experiments demonstrated that PRR14 interactions with the nuclear lamina are relatively stable as compared with the more dynamic interactions of PRR14 with heterochromatin via HP1. Finally, our super-resolution imaging of PRR14 localization in the nucleus indicates that PRR14 is predominantly located at the nuclear lamina where, we predict, it provides an anchoring surface for HP1-associated heterochromatin. Combined, our results demonstrate the molecular mechanism of heterochromatin organization by PRR14 and suggest a dynamic model of heterochromatin tethering to the nuclear periphery.

Results

**PRR14 tethers H3K9me3-modified heterochromatin to the nuclear lamina**

PRR14 encodes a protein with modular domains that bind to heterochromatin, via HP1, or to the nuclear lamina (Figure 1a). Given this domain structure, it has been postulated that PRR14 functions to tether heterochromatin to the nuclear lamina (Figure 1b) [19]. It has not yet been determined whether PRR14 shows specificity for heterochromatin with particular histone modifications to be bound and positioned at the nuclear lamina. We tested the ability of PRR14 to tether H3K9me2- and H3K9me3-modified heterochromatin to the nuclear lamina in light of recent reports showing separate functions for H3K9me2 and H3K9me3 chromatin modifications [12,32,33].
First, we performed gain-of-function experiments by expressing exogenous GFP-tagged PRR14 in two different cell types, murine NIH/3T3 and human IMR-90. Overexpression of PRR14, observed to be about 1.5 times higher than endogenous PRR14 levels (Fig. S1), altered the localization of H3K9me3-modified heterochromatin in the nucleus, significantly increasing that found at the nuclear lamina (Figure 1c-d, Fig. S2A-B). This repositioning of H3K9me3-modified heterochromatin to the nuclear lamina was greater in NIH/3T3 cells, probably due to the lower
baseline amount of H3K9me3 at the nuclear periphery prior to overexpression of PRR14.

To determine if the relocalization of H3K9me3-modified heterochromatin depends on the HP1-binding domain of PRR14, we used a mutant form of PRR14 in which the HP1-binding motif (LAVVL) residues V54 and V55 were substituted to glutamic acid (54-55EE) [19]. In contrast to exogenous expression of wild-type PRR14, expression of the mutant PRR14 54-55EE construct did not alter the localization of H3K9me3-marked heterochromatin; rather cells expressing PRR14 54-55EE were similar to mock-transfected cells (Figure 1c-d). Expression levels of PRR14 WT and mutant constructs were comparable (Fig. S3). Further, the HP1-binding motif-dependent ability of PRR14 to reposition heterochromatin was specific to that modified with H3K9me3. In cells exogenously expressing PRR14, we also observed repositioning of H3K9me2-marked heterochromatin, but it was independent of this PRR14 HP1-binding site: we observed approximately the same relocation of H3K9me2-modified chromatin in cells expressing both WT and PRR14 mutant 54-55EE constructs (Fig. S4A-B). This suggests that overexpression of PRR14 has an as yet undefined and perhaps nonspecific effect on H3K9me2-marked chromatin. These results demonstrate that PRR14 overexpression results in an increase in the H3K9-methylated heterochromatin associated with the nuclear lamina. At the same time, overexpression of PRR14-GFP constructs showed no significant effects on the total amount of H3K9me3- or H3K9me2-modified heterochromatin (Fig. S5).

It has been reported previously that loss of PRR14 in HeLa cells results in release of H3K9me3-modified heterochromatin from the nuclear lamina [19]. To complement our gain-of-function experiments in NIH/3T3 and IMR-90 cells, we examined the effect of loss of PRR14 on localization of H3K9me3- and H3K9me2-marked chromatin. Following small interfering RNA (siRNA) knockdown of PRR14, we observed a decrease in the fraction of H3K9me3-modified heterochromatin at the nuclear lamina in both NIH/3T3 and IMR-90 cells compared to cells treated with control siRNAs (Figure 1e-f). The knockdown of PRR14 was confirmed by RT-qPCR (Fig. S6). Treatment of cells with PRR14 siRNAs also resulted in a significant reduction of H3K9me2-marked chromatin at the nuclear lamina (Fig. S4C-D). Since the lamina-associated domains are known to be enriched for both H3K9me3 and H3K9me2 [32], PRR14 loss could affect heterochromatin regions marked with both modifications and indirectly impact the fraction of H3K9me2 at the nuclear lamina. Combined, our PRR14 overexpression and knockdown results demonstrate that PRR14 functions to organize heterochromatin at the nuclear lamina.

**PRR14 organizes heterochromatin at the nuclear lamina in interphase nuclei independent of mitosis**

PRR14 was previously reported to bind chromatin at early anaphase onset prior to nuclear lamina formation [19]. Later in telophase, chromatin bound by PRR14 was observed to associate with newly formed nuclear lamina, and it was therefore proposed that PRR14 might play a role in reestablishing peripheral heterochromatin at mitotic exit [31]. We sought to test whether mitosis is required for PRR14 to reorganize H3K9me3-modified heterochromatin in the nucleus.

NIH/3T3 cells expressing PRR14-GFP were assayed for changes in H3K9me3-modified heterochromatin localization following a double thymidine block to arrest the cell cycle and prevent mitosis (Fig. S7, see Methods). PRR14 overexpression in both cycling and arrested cells resulted in a significant increase in the fraction of H3K9me3-modified heterochromatin at the nuclear lamina with no significant difference between untreated and thymidine-treated cells (Figure 2). Similar levels of H3K9me3 at the nuclear periphery of both arrested and cycling cells with PRR14 overexpression suggests that PRR14 can tether heterochromatin in interphase nuclei and organize heterochromatin at the nuclear lamina independent of mitosis.

**PRR14 N-terminus includes two conserved HP1-binding motifs**

The LAVVL motif at the N-terminus of PRR14 (aa 52–56) is a variation of HP1-binding motif LxVxL. Substitution of key residues within this motif – V54E, V55E (LAEEL) – results in
a dramatic decrease of PRR14 association with chromatin (Figure 1). Through motif scanning of the remainder of the protein, a second evolutionarily conserved LxVxL sequence was identified within PRR14: LVVML, at amino acids 153–157 (Figure 3a), just beyond the region that was originally implicated as the chromatin-binding domain (aa 1–135 [19]). We sought to determine if this site could also contribute to heterochromatin binding.

To compare the functions of the first and putative second HP1-binding motifs of PRR14, we made GFP-tagged PRR14 constructs encoding only the N-terminal 212 amino acids of the protein that terminate before the lamina-binding domain (LBD) (Figure 3b). In addition to a wild-type version, we made GFP-tagged PRR14 1–212 constructs coding for amino acid substitutions in the first (V54E, V55E), the second (V154E, V155E), or both HP1-binding motifs (V54E, V55E, V154E, V155E), denoted 54–55EE; 154–155EE; and double mutant (DM), respectively (Figure 3b).

We introduced the wild-type and mutant 1–212 fragments of PRR14 into NIH/3T3 cells, and as expected, the wild-type fragment showed consistent colocalization in the nucleus with chromocenters and other heterochromatic regions known to be marked with the H3K9me3 histone modification [34] (Figure 3c). Further, the PRR14 1–212 fragment, which lacks the Lamina-Binding Domain (LBD) was, as predicted, not observed at the nuclear periphery (Figure 3c).

Compared with WT PRR14 1–212 fragment, expression of the HP1 binding site 1 mutant version of PRR14 1–212 (54-55EE) resulted in a dramatic decrease, but not complete loss of PRR14 co-localization with heterochromatin as visualized by Hoechst staining. We observed a diffuse localization of this 54-55EE mutant in the nucleus and minimal colocalization with heterochromatin (Figure 3c-d). In contrast, the HP1-binding site 2 mutant (154-155EE) retained association with heterochromatin, but it was significantly reduced compared with WT (Figure 3c-d). PRR14 1–212 with mutations of both HP1 binding sites (DM) showed a complete absence of colocalization with heterochromatin (Figure 3c-d). Taken together, these results demonstrate that HP1-binding site 1 plays the major role in heterochromatin binding, while HP1-binding site 2 contributes to a lesser degree to PRR14 interactions with heterochromatin. This is consistent with the observations that PRR14 54-55EE lacks the ability
to efficiently tether H3K9me3-modified heterochromatin to the nuclear lamina (Figure 1c-d). Overexpression of full-length PRR14 DM construct also showed no effect on heterochromatin tethering, except when expressed in NIH/3T3 cells where we observed a minor, but statistically significant effect on H3K9me2-modified heterochromatin localization (Fig. S8).

To further examine the influence of PRR14 HP1 binding sites on heterochromatin interactions, we performed fluorescent recovery after photobleaching (FRAP) experiments. We probed the association of the overexpressed PRR14-GFP 1–212 fragment with the large, H3K9me3-marked heterochromatin regions found at chromocenters in murine cells [34]. There was a rapid recovery of the GFP-PRR14 signal following photobleaching of a single chromocenter in cells expressing the wild-type PRR14 1–212 fragment (Figure 4a, Fig. S9). Normalized fluorescent recovery in the
photobleached regions was very high, with only about 15% average immobile fraction of WT PRR14 1–212 fragment on heterochromatin (Figure 4b-d). The average recovery half-time for WT PRR14 1–212 fragment was 4.5 seconds (Figure 4c) indicating a rapid exchange and dynamic association of PRR14 with heterochromatin.

While the 1–212 PRR14 constructs with HP1-binding site mutations (54–55EE and DM) show little to no enrichment at heterochromatin loci (Figure 3), we were able to measure those of the HP1-binding site 2 mutant (154–155EE) and compare its dynamics with the WT PRR14 fragment. The 1–212 PRR14 154–155EE mutant displayed heterochromatin association similar to that of WT but with a significantly faster average recovery half-time of 2.5 seconds (Figure 4, Fig. S9). Thus, the interaction of 1–212 PRR14 154–155EE with heterochromatin is even more dynamic than that of WT 1–212 PRR14, consistent with a proposed role for the second HP1-binding site in stabilizing the overall PRR14-chromatin association. The observed dynamics of PRR14 interaction with heterochromatin through its two HP1-binding sites is similar to that reported for another HP1-
binding protein, SENP7, which displays evidence of two HP1-binding sites shown to contribute to the association of SENP7 with heterochromatin [35].

**PRR14 binding preference for HP1 isoforms**

Given PRR14 chromatin binding is mediated by its interactions with HP1 proteins, we next sought to determine whether PRR14 interacts preferentially with any of the HP1 isoforms. Since the exogenous expression of full-length PRR14 resulted in relocation of H3K9me3-modified heterochromatin to the nuclear lamina, we asked whether overexpression of full-length PRR14 led to an increase in the amount of any of the HP1 isoforms at the nuclear periphery. In murine NIH/3T3 cells transfected with full-length PRR14, we examined the localization of each of the HP1 isoforms. We observed a significant increase of both HP1α and HP1β isoforms at the nuclear lamina in PRR14-overexpressing cells as compared to mock transfected cells (Figure 5a, b, Fig. S10). PRR14 overexpression also resulted in a change of HP1γ localization, even though HP1γ is less tightly associated with heterochromatin and known to organize euchromatin [25,26] (Figure 5a, b).

We then compared the repositioning of HP1 isoforms by different mutant forms of PRR14. PRR14 54-55EE overexpression did not reposition HP1α or HP1β (Figure 5a-b), consistent with the minimal interaction of this mutant with H3K9me3-modified heterochromatin presented previously (see Figure 1). PRR14 54-55EE overexpression had an observable and statistically significant effect on HP1γ (Figure 5a-b), and no relocalization of HP1γ was seen in cells overexpressing the DM construct (Figure 5a-b). Given the measurable effect of WT PRR14 overexpression on HP1γ, we used siRNA knockdown of HP1γ to determine whether HP1γ contributes to PRR14-chromatin interactions. In this assay, we observed a statistically significant decrease of PRR14 association with chromatin (Fig. S11). Overall, our data demonstrate that PRR14 HP1-binding site 1 (LAVVL, aa 52–56) binds all 3 HP1 isoforms and is required for PRR14-dependent tethering of H3K9me3-modified heterochromatin to the nuclear lamina (Figures 1 and 5). PRR14 HP1-binding site 2 (LVVML, aa 153–157) may contribute to strengthen overall PRR14 association with chromatin (Figure 4).

We next assayed the PRR14 interactions with HP1 isoforms through immunoprecipitation followed by Western blot. Given the low solubility of full-length PRR14, we used PRR14 1–212 fragments to assess the PRR14-HP1 isoform interactions. Using anti-GFP antibodies, GFP-PRR14 was immunoprecipitated from cells expressing WT GFP-PRR14 1–212 and the co-immunoprecipitated proteins were subjected to Western blot analysis of individual HP1 isoforms. All three HP1 isoforms were detected following immunoprecipitation which, consistent with the relocalization experiments, indicated that PRR14 can interact with each of these proteins (Figure 5c, d, Fig. S12).

To determine whether the two HP1 binding sites of PRR14 showed any differences in terms of HP1 isoform interaction, we quantified the amounts of each HP1 protein that co-precipitated with WT PRR14 1–212 compared with the 54-55EE, 154-155EE and DM forms when expressed in cells at comparable levels (Figure 5c, Fig. S12A). PRR14 was immunoprecipitated in Lenti-X 293 T cells due to low transfection efficiency of NIH/3T3 and IMR-90 cells. Expression of HP1 isoforms in NIH/3T3 and 293 T cells were observed to be similar (Fig. S13). The interactions between PRR14 HP1-binding site 1 mutant (54-55EE) and all 3 HP1 isoforms were significantly reduced compared with WT, although a small amount of residual binding to HP1γ was retained (Figure 5c-d). We could not detect any significant difference between WT and PRR14 HP1-binding site 2 (154-155EE) mutant in co-precipitating any of the 3 HP1 isoforms (Figure 5c-d). Analysis of the double mutant (DM) showed a complete lack of HP1-PRR14 interaction (Figure 5c-d). These results are consistent with a primary role for HP1 binding site 1 as a mediator of PRR14 interaction with all three HP1 isoforms, and an apparently small contribution of HP1 binding site 2 to the interaction of PRR14 with HP1.

**PRR14 association with the nuclear lamina is independent of heterochromatin binding**

PRR14 association with heterochromatin through HP1 is highly dynamic, as described above
Next, we sought to characterize PRR14 interactions with the nuclear lamina, which have been shown to occur between the PRR14 lamin-binding domain (LBD) and Lamin A/C [19]. The relative contributions of PRR14-nuclear lamina and PRR14-heterochromatin binding for nuclear lamina localization were investigated using FRAP. We assayed the dynamics of PRR14 at the nuclear lamina for both the full-length protein and the isolated PRR14 LBD (residues 231–351) (Figure 6a). Full-length WT PRR14 protein exhibited a high level of immobile fraction at the nuclear lamina.
Figure 6. PRR14 association with the nuclear lamina is dynamic and independent of PRR14-heterochromatin binding. (a) Representative confocal images of fluorescence recovery after photobleaching (FRAP) assay of WT GFP-PRR14 full-length (PRR14 FL) and lamina-binding domain (LBD) constructs. White boxes indicate bleached area. Grayscale images show magnified bleached areas indicated by white arrows. (b) Line graph shows normalized FRAP signal in the areas indicated with boxes in (A) for PRR14 full-length (blue) and PRR14 LBD-only fragment (purple). (c) Box plots show distributions of recovery half-time (left) and immobile fraction (right) for indicated constructs. (d) Line graph shows normalized FRAP signal in the areas indicated with boxes (Fig. S6) for PRR14 FL WT (blue) and mutant full-length constructs 54-55EE (red), or double mutant (DM; yellow). (e) Box plots show distributions of recovery half-time (left) and immobile fraction (right) for indicated constructs. n ≥ 12 cells per condition. Line graphs show mean values with standard deviations displayed as shading. Box plots show median, 25th and 75th percentiles; whiskers show minimum to maximum range. Statistical analysis was performed using Mann–Whitney test and ANOVA Kruskal–Wallis test with Dunn’s multiple comparisons. ****p < 0.0001, ns: not significant. Scale bars 5 μm.
lamina (55% on average), and the recovery half-time of PRR14 at the nuclear lamina averaged 25 seconds (Figure 6b-c). By way of comparison, the recovery half-time for Lamin A/C at the lamina has been reported to be from 10 to 60 minutes [36], suggesting that PRR14 interactions with the lamina are relatively dynamic.

In contrast to full-length PRR14, the LBD fragment demonstrated measurably different dynamics with only a small immobile fraction (<15%) and an average recovery halftime of 15 seconds (Figure 6a-c). The LBD fragment lacks both the N-terminal chromatin-binding region and the C-terminal regulatory region. This suggests that the full-length protein is stabilized at the nuclear lamina through additional interactions that result in a significant immobile fraction.

We tested if interaction with HP1 proteins and/or chromatin contribute to PRR14 retention at the nuclear periphery by assaying the dynamics at the nuclear lamina of full-length PRR14 constructs with HP1-binding site mutants (54-55EE and DM). We found no significant differences between WT and these mutant forms of PRR14 in terms of recovery half-time or amount of immobile fraction (Figure 6d-e, Fig. S14). This suggests that PRR14 dynamics at the nuclear periphery are independent of PRR14-HP1 interaction.

Direct comparisons of PRR14 association with heterochromatin (Figure 4) and with the nuclear lamina (Figure 6) indicate that PRR14 association with the nuclear periphery is less dynamic than the association of PRR14 with heterochromatin (Fig. S15). Since the PRR14 1–212 fragment is significantly smaller than the full-length protein, which could affect the protein dynamics, we performed FRAP with the PRR14 Δ231-351 mutant which lacks the LBD and is therefore unable to bind the nuclear lamina. Consistent with the results observed for PRR14 1–212, loss of lamina interaction in the context of the larger construct (PRR14 Δ231-351) also results in a much faster recovery compared to the full-length WT protein. Thus, our results support a model in which PRR14 primarily occupies its position at the nuclear lamina where it performs transient attachment to HP1-associated heterochromatin.

**PRR14 at the nuclear lamina creates a surface for heterochromatin anchoring**

We used super-resolution microscopy to visualize the location of PRR14 in the nucleus, examining both the nuclear lamina and the nucleoplasm. Localization of PRR14, Lamin A/C and H3K9me3 were examined by Stochastic Optical Reconstruction Microscopy (STORM) and Figure 7 shows representative images of a plane through the middle of the nucleus (Figure 7a) and a bottom plane at the periphery of the nucleus (Figure 7b). PRR14 is observed predominantly at the inner nuclear membrane (INM) forming a similar meshwork as Lamin A/C on the INM surface (Figure 7b), consistent with analyses of our confocal images. A much smaller fraction of PRR14 was observed in the nucleoplasm (Figure 7a). Nucleoplasmic PRR14 is almost uniformly distributed, and we did not observe clusters specific for H3K9me3-modified heterochromatin (Figure 7a, b). In comparison, H3K9me3 is apparent as aggregated clusters of heterochromatin both at the nuclear lamina and in the nucleoplasm (Figure 7a, b). No such clustering was observed for PRR14, suggesting that the nucleoplasmic fraction of PRR14 does not strongly associate with heterochromatin away from the nuclear lamina surface. The localization of PRR14 at the nuclear periphery creates a layer of molecules that are capable of binding H3K9me3-modified heterochromatin associated with HP1 as it contacts the nuclear periphery.

The results presented here suggest a model in which PRR14 creates a meshwork at the nuclear lamina that organizes H3K9me3-modified heterochromatin at the nuclear periphery through dynamic association with HP1 (Figure 7c). This PRR14 meshwork might be thought of as a ‘sticky’ surface, or 'Velcro’, that allows H3K9me3-modified heterochromatin bound by HP1 to become transiently attached to the nuclear lamina.

**Discussion**

In this study, we determined the mechanism of PRR14 in organizing heterochromatin at the nuclear lamina. Results presented here demonstrate
that PRR14 overexpression results in HP1 binding domain-mediated repositioning of H3K9me3-modified chromatin to the nuclear lamina. This further supports a role for PRR14 as an organizer of H3K9me3-modified heterochromatin in the nucleus. Structure-function analyses indicate that PRR14 primarily organizes H3K9me3-modified chromatin. While overexpression of PRR14 resulted in a modest increase of H3K9me2-modified chromatin at the nuclear lamina, we have not identified a specific domain of PRR14 that mediates H3K9me2 localization. Thus, we propose that

Figure 7. PRR14 is predominantly localized at the nuclear lamina where it creates a surface for anchoring HP1-associated H3K9me3-modified heterochromatin. (a) Representative super-resolution stochastic optical reconstruction microscopy (STORM) images from cells overexpressing PRR14-GFP show localization of Lamin A/C, PRR14 and H3K9me3 in the middle plane of nuclei of NIH/3T3 cells. White boxes (top panels) show zoomed areas (bottom panels). (b) Representative STORM images from cells overexpressing PRR14-GFP show localization of Lamin A/C, PRR14 and H3K9me3 at the bottom plane of nuclei of NIH/3T3 cells. (c) A model illustrating the mechanism for PRR14 tethering H3K9me3-modified chromatin to the nuclear lamina through interaction with HP1. Scale bars: 5 μm (top panels) and 1 μm (bottom panels).
PRR14 may affect H3K9me2 through an indirect mechanism. We have shown that PRR14 acts on H3K9me3-marked chromatin through its interactions with heterochromatin protein 1 (HP1) to tether chromatin to the nuclear lamina, primarily via HP1α and HP1β isoforms, although our experiments indicate that PRR14 can bind the HP1γ isoform as well. We have also mapped a second evolutionarily conserved HP1-binding motif in the N-terminus of the PRR14 protein. This site is not sufficient for independent chromatin tethering but may function to strengthen PRR14 association with chromatin or promote the dense nucleosome compaction characteristic of heterochromatin. Analysis of the dynamics of PRR14 association with the nuclear lamina and chromatin, combined with super-resolution imaging of PRR14 localization in the nucleus and the ability of PRR14 to reorganize heterochromatin localization independent of mitosis, have led us to propose a refined model of heterochromatin tethering at the nuclear periphery.

Our results suggest a mechanism through which PRR14 organizes heterochromatin at the nuclear lamina and provide new insights into our understanding of the organization of heterochromatin at the nuclear periphery. Further studies are needed to determine the cellular mechanisms that might alter PRR14 tethering function to regulate heterochromatin association with the nuclear periphery. Notably, the majority (up to 90%) of overexpressed PRR14 localizes at the nuclear periphery, consistent with our previous studies [29] and with the lack of dominant-negative effects observed in cells overexpressing mutant PRR14 constructs. This observation also suggests that the cell can tolerate an excess of PRR14 at the nuclear lamina and might adjust PRR14 levels and thereby chromatin organization.

Only a few chromatin tethers have been described to date (reviewed in [15]). The list of widely expressed tethers is limited to LBR and PRR14 in mammals [16,19] and CEC-4 in C. elegans [13]. These tethers are expressed in most cell types and have a similar mechanism of action: all of them interact with chromatin bearing H3K9 methyl-modified histones. The complete list of chromatin tethers is not restricted to these proteins as other cell-type specific tethers have been reported [15].

A notable feature of PRR14 is the duplication of key functional domains. The protein has conserved residues for two NLS signals, two adjacent lamina-association motifs [19,29], and, shown in this study, two HP1-binding sites. As is frequently seen in evolution, these duplicated regions are not identical. We have yet to determine whether each of these regions provides functional redundancy, modified function, or has already lost their original function through mutational drift. Such protein redundancy might ensure protein function in multiple cell types as well as additional levels of regulation of protein function(s). Our earliest PRR14 study demonstrated that each NLS is independently sufficient to transport PRR14 into the nucleus, but with lesser efficiency singly than when combined [19]. Therefore, two NLS sequences might serve to efficiently deliver PRR14 in the nucleus. A similar function is observed for the LBD where each lamina-associated domain is able to position the protein to the nuclear lamina independently, but much less efficiently than that observed when both are intact [29]. Furthermore, LBD-1 region has been shown to be regulated by phosphorylation, whereas this has not been observed for LBD-2 [29]. Such complex organization of the lamina-binding domain allows for regulation of PRR14 association with the nuclear lamina, thus regulating heterochromatin tethering capabilities of PRR14. In this study, we identified a second HP1-binding motif (LVVML, aa 153–157), located near the first HP1-binding site (LAVVL, aa 52–56) in the N-terminus of the protein. Both motifs are a variation of LxVxL sequence which binds the chromo-shadow domain (CSD-CSD) pocket of HP1 [37] and are not identical (Figure 3a). The first HP1-binding motif (LAVVL) of PRR14 was described previously and experiments with substitution of the key amino acid residues in this motif showed a dramatic reduction of PRR14-chromatin association [19]. Here, we have demonstrated, through a combination of immunoprecipitation and FRAP imaging, that the LAVVL motif of binding site 1 is primarily responsible for PRR14 chromatin binding and tethering (Figures 1, 3, and 5), while the second LVVML motif of binding site 2 has a supportive function in PRR14 association with heterochromatin (Figures 3–4). Machida et al.,
recently demonstrated that HP1 dimers can bind H3K9me3 histone modifications at two distinct nucleosomes, and thus function to compact heterochromatin by bringing nucleosomes together [38]. We speculate that the ability of PRR14 to bind two HP1 dimers could also result in chromatin compaction. If this hypothesis is correct, HP1-bound heterochromatin would undergo further compaction when it interacts with PRR14 at the nuclear periphery. Further, the two HP1-binding sites of PRR14 may have an HP1-locking function, similar to that previously described for SENP7 [35], in which two HP1 interaction motifs restrict HP1 mobility at heterochromatin, thereby locking HP1 molecules docked on H3K9me3-modified nucleosomes to promote stable HP1 accumulation. Future studies will be aimed at elucidating the role of HP1 in specifying chromatin regions for localization at the nuclear periphery.

While PRR14 was previously reported to bind HP1α [19,39], here we demonstrate that PRR14 can bind all HP1 isoforms. Tethering to the nuclear periphery in the context of PRR14 overexpression was observed largely for HP1α and HP1β isoforms (Figure 5b). This agrees with the accepted function for HP1α and HP1β in organizing heterochromatin, in contrast to the HP1γ isoform which has been found in both heterochromatin and euchromatin regions [25,26]. Previous studies demonstrated that HP1 isoforms can form heterodimers as well as homodimers [26,40,41]. This feature of HP1 isoforms makes it difficult to determine whether a homodimer of a single isoform or a heterodimer is bound by a single PRR14 protein. Specificity of PRR14 for chromatin binding via HP1 could be further complicated by various HP1 dimers and post-translational modifications of HP1 (reviewed in [23]). Some HP1 interactions with other binding partners utilize the same PxVxL-binding pocket or position the HP1 dimer deep inside the nucleosome, thus making HP1 unavailable for interaction with PRR14 [23]. This agrees with the relatively moderate effect of PRR14 overexpression on HP1 relocalization to the nuclear lamina (Figure 5). Our results provide evidence that PRR14 functions to organize H3K9me3-modified heterochromatin associated with HP1α/β at the nuclear lamina. While the specific genomic regions that are organized at the nuclear periphery by PRR14 are currently unknown, such specificity could be cell typespecific and dictated by a combination of multiple factors including relative levels of HP1 isoforms and PRR14.

Our data demonstrate that PRR14 functions to tether heterochromatin regions enriched for the H3K9me3 histone modification, which is known to mark silent genes and promoters, but is more abundant in repetitive heterochromatin regions such as major and minor satellites and peripheral heterochromatin, including lamina-associated domains (LADs) [6,34,42]. Localization of heterochromatin at the nuclear periphery is an additional mechanism to efficiently repress expression of cellular genes and repetitive elements. We originally identified PRR14 as an epigenetic repressor and demonstrated that PRR14 knockdown can activate epigenetically silent genes [30]. We hypothesize that PRR14 can serve as an epigenetic repressor by tethering and thereby silencing genes at the nuclear lamina. To date, the detailed mechanisms of LAD formation and maintenance remain unclear. Both PRR14 and HP1 were recently identified as proteins in the LAD interactome [43]. PRR14/HP1 likely functions to maintain association of the H3K9me3-marked heterochromatin of LADs with the nuclear lamina. Such PRR14 function could be regulated by PRR14 phosphorylation [29] and might be a mechanism for release of genes from LADs.

Recent studies have proposed that histone modifications, specifically H3K9 methylation, can guide chromatin localization in the nucleus [32,44]. We have previously suggested a function for PRR14 to reestablish heterochromatin localization at the nuclear lamina at mitotic exit [31]. We propose that the function of PRR14 in binding H3K9me3 heterochromatin and organizing it at the nuclear periphery is an example of spatial chromatin organization via histone modification and protein tethers.

The results presented here suggest a dynamic model of heterochromatin organization at the nuclear periphery. H3K9me3-modified heterochromatin is observed at perinucleolar heterochromatin and chromocenters, in addition to the nuclear periphery (Figures 1 and 7). Several studies have described the
dynamic behavior of heterochromatin, including that seen at the nuclear periphery, as it is exchanged between the peripheral and perinucleolar compartments [33]. This dynamic feature of H3K9me3-modified chromatin is consistent with observations that peripheral heterochromatin can be relocated to another heterochromatin compartment after mitosis [45]. The fact that mitosis is not required for tethering further supports the idea that PRR14 may play a role in heterochromatin dynamics during interphase. Based on our results, we propose that PRR14 does not form a sustained bond between heterochromatin and the nuclear lamina, but rather creates a surface at the nuclear lamina that is capable of anchoring heterochromatin with which it comes into contact (Figure 7c).

Materials and methods

Cell lines

Mouse NIH/3T3 (ATCC, cat# CRL-1658), human IMR-90 (ATCC, cat# CCL-186) and Lenti-X™ 293 T (Takara Bio, cat# 632180) cell lines were cultured in full-DMEM (Corning, cat# 10013CV) supplemented with 10% FBS (Atlanta Biologicals, cat# S11150) and 1x Penicillin–Streptomycin solution (ThermoFisher Scientific/Invitrogen, cat# 15140122). All cultured cell lines were routinely tested for mycoplasma contamination every 3 months using an e-Myco VALID Mycoplasma PCR Detection Kit (iNtRON Biotechnology, Inc., cat# 25239).

Plasmids

The human wild-type PRR14 expression construct pCMV6-AN-mGFP-PRR14 was previously described [19,29]. pCMV6-AN-mGFP-PRR14 wild-type and HP1-binding domain mutants (site 1 mutant (54-55EE), site 2 mutant (154-155EE), and double mutant (54-55EE and 154-155EE)) were made using site-directed mutagenesis. PRR14 1–212 aa fragments (wild-type and the HP1-binding domain mutants, as described above) were amplified from the corresponding pCMV6-AN-mGFP-PRR14 full-length vectors using Q5 Hot-start High-Fidelity Polymerase 2x Mix (New England Biolabs, cat# M0494S) and the following primers containing HindIII and RsrII restriction sites (Forward (HindIII): 5’-GCTTTCGAAGCTTGACCATTCC ATGGGACTTGCCCGG-3’; Reverse (RsrII): 5’ -TGGGATCGTCCGGGTAGTCTGCAGGCA-GAGC-3’). Then, pRR14 1–212 PCR products were sub-cloned into the expression vector pCMV6-AN-mGFP using T7 ligase (New England Biolabs, cat# M0318S) according to the manufacturer’s instructions.

To make Doxycycline-inducible vectors, PRR14-GFP full-length or 1–212 wild-type and the HP1-binding domain mutants were amplified from the corresponding pCMV6-AN-mGFP-PRR14 vectors using Q5 Hot-start High-Fidelity Polymerase 2x Mix (New England Biolabs, cat# M0494S) and the following primers containing EcoRI and AgeI restriction sites (1–212 PRR14 – Forward (EcoRI): 5’ – ACTATAGGGCGGCCGG GAATTCTCGACT – 3’; Reverse (AgeI): 5’ – GATGTCGGACCGTTATAGTCTGCAGGCA-GAGCAGA –3’; full-length PRR14 – Forward (EcoRI): 5’ – GCTTTCGAAGCTTGACCATTCC TGCGATCGGTCCGCGCTTAGTCTGCAGGCA-3’; Reverse (AgeI): 5’ – GATGTCGGACCGTTATAGTCTGCAGGCA-GAGCAGA –3’). After that, various PRR14-GFP PCR products were sub-cloned into pLVX-TetOne-Puro (Takara Bio, cat# 631847) vector using T7 ligase (New England Biolabs, cat# M0318S) according to the manufacturer’s protocol.

Lentivirus production

Lenti-X 293 T cells were plated on a 6-well dish and grown to 80% confluent before transfection. To make the lentiviruses containing Doxycycline-inducible full-length or 1–212 PRR14-GFP (WT and HP1-binding site mutants) cell lines, 1.2 μg of lentiviral vectors were premixed with 1.2 μg of packaging psPAX2 vector (Addgene, cat# 12260), and 0.75 μg of envelope pMD2.G vector (Addgene, cat# 12259) and transfected into Lenti-X 293 T cells using Lipofectamine 3000 (ThermoFisher Scientific/Invitrogen, cat# L3000008) transfection reagent. Supernatant containing lentiviruses was harvested 48- and 72 h post-transfection, passed through 0.45 μm filter to eliminate contamination with Lenti-X 293 T cells and cell debris, and pooled together. The lentiviruses were used immediately to
infect NIH/3T3 cells or stored at +4°C for no longer than 48 hours.

**Stable cell line production**

To generate a stable cell line expressing Doxycycline-inducible full-length or 1–212 PRR14-GFP, NIH/3T3 cells were infected with the corresponding lentiviruses (1/5 – lentivirus/cell culture medium) in the presence of 4 μg/mL polybrene (Sigma-Aldrich, cat# 107689). Four days post-infection, cells were split and selected for positive clones using 2 μg/mL puromycin for 5 days.

**Transient DNA and siRNA transfection**

Lipofectamine 3000 or FuGENE 6 transfection reagents (ThermoFisher Scientific/Invitrogen, cat# L3000008; and Promega, cat# E2691) were used for transient delivery of PRR14 plasmids, in accordance with the manufacturer’s guidelines. Expression levels of GFP-tagged PRR14 full-length WT and mutant constructs, as well as 1–212 PRR14 constructs were observed to be at comparable levels as measured by GFP-signal intensity in flow cytometry (Fig. S3). For super-resolution and confocal imaging cells were plated on 8-well ibidi µ-slides (ibidi, cat# 80826) or on 12 × 12 mm GOLD SEAL® glass coverslips (Electron Microscopy Sciences, cat# 63786–01) correspondingly, then transfected at 50% confluence and fixed 48 hours post-transfection (for STORM or IF) or imaged live (for FRAP or PRR14/DNA co-localization analysis).

For siRNA transfection, lipofectamine RNAiMAX transfection reagent (ThermoFisher Scientific/Invitrogen, cat# 13778075) was used according to the manufacturer’s protocol. siGENOME pools of four siRNAs against Mouse Hp1γ, Prr14, and human PRR14, or non-targeting siRNA control (Horizon Discovery/Dharmacon, cat# M-044218-01-0005, M-042451-00-0005, M-018060-00-0005, and D-001206-13-05) at the final concentration of 50 nM were used. For Figure S11 A-B, Doxycycline-inducible NIH/3T3 cells expressing WT or 54–55 EE mutant of 1–212 PRR14-GFP, described above, were plated on 8-well ibidi µ-slides. The next day, PRR14-GFP expression was induced by adding 2 ug/mL Doxycycline (Sigma-Aldrich, cat# D9891-25 g), and at the same time, cells were transfected with the siRNAs (siHP1γ or siControl). Live cell imaging was performed 72 hours post-siRNA transfection and the addition of Doxycycline. Right before the imaging, cells were counterstained with 1 μg/mL of Hoechst 33342 (ThermoFisher Scientific/Invitrogen, cat# 62249) for 10 min. The imaging was performed using a 1x Live Cell Imaging Solution (ThermoFisher Scientific/Invitrogen, cat# A14291DJ) and in a temperature and humidity-controlled microscope chamber set at 37°C.

**RNA isolation and quantitative RT-PCR**

For RT-PCR experiments in Figure S6, total RNA from NIH/3T3 and IMR-90 cells post siControl or siPRR14 transfection was isolated using Direct-zol™ RNA MicroPrep Kit (Zymo Research, cat# R2061) and Zymo-Spin™ IC Columns (Zymo Research, cat# C1004-50) at 72 h time point. The on-column DNase I digestion was also performed for the samples. Then, first-strand DNA was synthesized using SuperScript™ III Reverse Transcriptase (ThermoFisher/Invitrogen, cat# 18080051), following the manufacturer’s instructions. Quantitative RT-PCR was performed in triplicate using Power SYBR Green PCR Master Mix (Applied Biosystems, cat# 4367659). Human or mouse GAPDH was used as a reference control gene. Quantitative RT-PCR data is presented as ‘Relative gene expression’ and reflects a fold change of siPRR14 versus siControl.

Quantitative RT-PCR primer sequences used in the present study are listed here:  
Ms (NM_001289726.1, Gapdh) FW: 5’ – AACAGCACTCCACTCTTC – 3’  
Ms (NM_001289726.1, Gapdh) RV: 5’ – CCTGTTGCTGTAGCCGTATT – 3’  
Ms (NM_145589.2, Prr14) FW: 5’ – ACACAGTCACCTGCTTTACC – 3’  
Ms (NM_145589.2, Prr14) RV: 5’ – GCTGCCCTTGATCTCATTCT – 3’.  
Hu (NM_001256799.3, GAPDH) FW: 5’ – GGTGTGACCATGAGATGATGA – 3’  
Hu (NM_001256799.3, GAPDH) RV: 5’ – GAGTCCTTCCACGATACCAAAG – 3’  
Hu (NM_001320464.3, Prr14) FW: 5’ – CAAGGGAAGGAGCCAAGAG – 3’
Thirteen ethanol

**Cell cycle synchronization by double thymidine block**

Cells were synchronized at the G1/S border using a double thymidine block as described previously [46]. In brief, NIH/3T3 cells were plated on 12 × 12 mm coverslips in 6-well plates (ThermoFisher Scientific, cat# 140675). The next day cells were pre-treated with 2 mM Thymidine (Sigma-Aldrich, cat# T9250-1G) and 5 hours later transfected with pCMV6-AN-mGFP-PRR14-WT or pCMV6-AN-mGFP-PRR14-site 1 mutant (54-55EE) using FuGENE 6 transfection reagent in the presence of 2 mM Thymidine in the culture medium to arrest cells in G1/S phase. Thirteen hours post-transfection, Thymidine was removed from the medium, and the cells were ‘released’ for 9 hours by adding fresh medium to initiate cell-cycle progression. To increase the percentage of G1/S-arrested cells, a second round of 2 mM Thymidine treatment was performed for another 13 hours, and after that the cells were either kept with Thymidine for additional 12 hours (‘Arrested Cells’) or released and fixed 12 hours post-release (‘Cycling Cells’).

**Flow cytometry**

NIH/3T3 and IMR-90 cells were transfected with PRR14-GFP constructs as described above. At 48 hours post-transfection, live cells were collected and analyzed using BD Accuri C6 flow cytometer (Becton, Dickinson and Company) to assess levels of PRR14-GFP signal intensity.

For the thymidine block experiment, NIH/3T3 cells were trypsinized using 1x Trypsin-EDTA (Invitrogen, cat# 25200056), washed two times in PBS by centrifugation, and fixed with 70% ice-cold ethanol. The cells were stored at −20°C in ethanol for 1 week, then washed twice in PBS by centrifugation, stained with 1 µg/mL final concentration of propidium iodide (Sigma-Aldrich, cat# P4864) for 20 min and processed by flow cytometry/cell cycle analysis.

**Fluorescence-activated cell sorting**

For FACS analysis, NIH/3T3 cells were transfected with pCMV6-AN-mGFP-PRR14, as described above, and 48 hours post the transfection the cells were trypsinized and resuspended in 2 mL of full-DMEM at the final concentration of 15 million/mL. The cells then were sorted into two population based on the PRR14-GFP expression (e.g., ‘GFP-negative’ and ‘GFP-positive’) using BD Influx™ Cell Sorter (BD Biosciences, NJ) with assistance from the UPenn Flow Core sorting operator.

**Immunofluorescence**

Immunofluorescence assays were performed as described previously [29,44]. NIH/3T3 and IMR-90 cells were fixed with 2% paraformaldehyde (PFA) (Electron Microscopy Sciences, cat# 15710) for 8–10 min at room temperature, washed 3 times with DPBS (Gibco, cat# 14190-136), and then permeabilized with 0.25% Triton X-100 (ThermoFisher Scientific/Invitrogen, cat# 28314) for 10 min. Then, cells were washed 3 times with DPBS for 5 min and blocked in 1% BSA (Sigma-Aldrich, cat# A4503) in PBS-T (DPBS with 0.05% Tween 20, pH 7.4 (ThermoFisher Scientific/Invitrogen, cat# 28320)) for 60 min. Next, primary antibodies diluted in 1% BSA/TBS-T were added for 1 h, then samples were washed three times with PBS-T for 5 min and incubated with secondary antibodies in 1% BSA/TBS-T for 60 min followed by washing twice with PBS-T and one time with PBS for 5 min. Samples were mounted using Duolink® In Situ Mounting Medium with DAPI (Sigma-Aldrich, cat# DUO82040-5ML). All steps were performed at room temperature.

**Image acquisition and analysis**

All confocal immunofluorescent images were taken using a Leica SP8 laser scanning confocal system using 63X/1.40 HC PL APO CS2 objective and HyD detectors in the standard mode with 100% gain. 3D images of the nuclei middle Z-planes were taken as Z-stacks with 0.1 µm intervals with a range of 1 µm per nucleus. Confocal 3D images were deconvoluted using Huygens Professional software by utilizing the microscope parameters, standard PSF and automatic settings for background estimation. Image analysis was...
performed using ImageJ software (National Institutes of Health, MD). To determine whether PRR14 over-expression or knockdown alters the amount of H3K9me3, H3K9me2 or HP1 isoforms found at the nuclear lamina, we measured the immunofluorescence signal that overlaps a nuclear lamina mask, created with the Lamin A/C or Lamin B1 signal, and we report this as a fraction of the total signal in the nucleus (in Figures 1, 2, 5, S4, S10, and S8). Analysis of PRR14 colocalization with heterochromatin was performed using the Colocalization Threshold tool in ImageJ with automatic parameters (in Figure 3 and Figure S11). Pearson’s above threshold coefficient was measured. Analysis of H3K9me3 and H3K9me2 levels in cells transfected with PRR14 constructs was performed using ImageJ software (Figure S5D). The total fluorescence intensities of H3K9me3 or H3K9me2 in individual nuclei were measured using a mask created based on DAPI signal. Confocal images for this analysis were taken with exactly the same parameters. Line intensity profiles (in Figure S2) were created using the Plot Profile tool in ImageJ.

**Fluorescence recovery after photobleaching**

FRAP imaging was performed on a Leica SP8 laser scanning confocal system equipped with a temperature control incubation chamber and an 63X/1.40 HC PL APO CS2 objective. NIH/3T3 cells were plated on 8-well ibidi μ-slides and transfected with GFP-tagged human PRR14 constructs. FRAP was performed 48 hours post-transfection using LAS X software (Leica Microsystems, Buffalo Grove, IL) with a built-in FRAP wizard at 488 nm laser and a PMT detector. Live cells were imaged using a 1x Live Cell Imaging Solution (ThermoFisher Scientific/ Invitrogen, cat# A14291DJ) and in a temperature and humidity-controlled microscope chamber set at 37°C. FRAP assays for PRR14 1–212aa constructs (Figure 4) were performed on the same day and under the same conditions. Cells expressing WT and 154-155EE constructs were plated on the same 8-well ibidi μ-slide to minimize sample-to-sample variations. FRAP assays for PRR14 full-length constructs (Figure 6) were also performed on the same day and under the same conditions. Cells expressing WT, 54-55EE, 154-155EE and LBD constructs were plated on the same 8-well ibidi μ-slide to minimize sample-to-sample variations. For the benefit of data representation, fluorescent recovery curves of WT and LBD, and WT and mutants were presented as two panels (Figure 6 (b-d)), where the WT sample is the same.

Briefly, a region of interest (ROI) was bleached for 4 seconds, then recovery of GFP-PRR14 signal was measured for 40–120 seconds at 0.75–1.5 s intervals. Fluorescence intensity of the bleached ROI and total fluorescence intensity of the nucleus was measured using ImageJ software (National Institutes of Health, MD). Out-of-focus frames were excluded from the analysis. To compensate for the inherent baseline photobleaching over time, the overall recovery signal was normalized to the inherent photobleaching rate of whole nucleus region. Fluorescent recovery curve fitting and extension was performed using GraphPad Prism 9 software (GraphPad Software, Inc.), as well as calculation of a recovery half-time and an immobile fraction. Immobile fraction was calculated as a complement to one of fluorescent recovery plateau/maximum recovery.

**Super-resolution microscopy**

Super-resolution imaging was performed using Stochastic Optical Reconstruction Microscopy (STORM). Images were obtained using ONI Nanoimager (ONI Inc., CA). Cells were plated on 8-well ibidi μ-slides and transfected with pCMV6-AN-mGFP-PRR14. Forty-eight hours post-transfection, samples were fixed, immunostained as described above, and kept in DPBS until image acquisition. Secondary antibodies were conjugated with Alexa 647 dye. Imaging acquisition was performed in fresh imaging buffer (50 mM Tris-HCl, pH 8.0, 10 mM NaCl, 10% (w/v) glucose (Sigma-Aldrich, cat# G8270), 1.5 mg MEA (Sigma-Aldrich, cat# 30070), 170 AU Glucose oxidase (Sigma-Aldrich, cat# G2133), 1400 AU Catalase (Sigma-Aldrich, cat# C40)). Images were processed using ONI Nanoimager™ Software version: 1.16 (ONI Inc., CA).

**Immunoprecipitation experiments**

GFP-tagged PRR14 1–212 fragments (wild-type, site 1 mutant (54-55EE), site 2 mutant (154-155EE), and DM (54-55EE and 154-155EE)) were transfected into Lenti-X 293 T cells and used to assess interactions with HP1 isoforms. Protein A/G Magnetic Beads (ThermoFisher Scientific, cat# 88,802) preincubated
overnight at +4°C with anti-GFP antibodies or Normal Rabbit IgG were used for pull-down of GFP-tagged proteins or as a mock pull-down. Forty-eight hours post-transfection, cells were washed in PBS, then scraped off the plates in 500 µL ice-cold NP-40 lysis buffer (VWR Life Science, cat# J619-500ML) containing protease and phosphatase inhibitors (Roche, cat# 11697498001 and cat# 4906845001) and 1x benzozane (Sigma-Aldrich, cat# E1014-5KU). The lysates were then incubated for 30 min at +4°C with constant agitation and cleared by centrifugation at 12,000 rpm for 20 min at +4°C. The supernatants were collected and mixed with GFP antibody pre-conjugated beads, which were pre-washed with NP-40 lysis buffer with protease/phosphatase inhibitors (Roche, cat# 11697498001 and cat# 4906845001) and incubated for 2.5 hours at +4°C with gentle agitation. The beads were collected, washed with lysis buffer, resuspended in lysis buffer containing 1x NuPAGE Sample Reducing Agent (ThermoFisher Scientific/Invitrogen, cat# NP0004), 1x Laemmli Sample Buffer (Bio-Rad, cat# 1610747) and heated for 5 min at 95°C to dissociate immunocomplexes from the beads. The beads were then cleared on a magnet, and the supernatants were collected. Proteins were separated on 4–12% SDS–PAGE gels (ThermoFisher Scientific/Invitrogen, cat# NP0335) and Western blotting of a 0.2 µm nitrocellulose membrane (ThermoFisher Scientific/Invitrogen, cat# LC2000) was performed to detect GFP-PRR14 and HP1 isoforms by probing the membrane with the corresponding antibodies. The membranes were developed using ECL Prime Western Blotting Detection Reagents (Amersham/Cytiva cat# RPN2232).

Image analysis was performed using ImageJ Software. In brief, raw RGB Western Blot images were converted into 8-bit, and the colors were inverted. Each band was then selected using an ImageJ Rectangular Tool, and the signal from the selected areas was measured as a mean intensity value. The mean intensity value of the ‘IP:WT-IgG’ condition was then subtracted from the values measured for the rest of the experimental conditions. Then, the values corresponding to HP1 proteins were normalized to the values of total HP: GFP-PRR14 signal following with a second normalization to the maximum values within each experimental condition.

**Western blotting**

Cell lysates were prepared using 1x RIPA buffer (Cell Signaling, cat# 9806) supplemented with 1% SDS, 1x Halt™ Protease and Phosphatase Inhibitor (ThermoFisher Scientific, cat# 78442) and 1x benzozane (Sigma-Aldrich, cat# E1014-5KU). The lysates were incubated on ice for 30 min and cleared at 12,000 g for 20 min at +4°C. Before loading on a gel, the lysates were premixed with 1x NuPAGE Sample Reducing Agent (ThermoFisher Scientific/Invitrogen, cat# NP0004), 1x Laemml Sample Buffer (Bio-Rad, cat# 1610747) and heated for 5 min at 95°C. Proteins were separated on 4–12% or 10% SDS–PAGE gels (ThermoFisher Scientific/Invitrogen, cat# NP0335 or cat# NP301) and Western blotting of a 0.2 µm PVDF membrane (ThermoFisher Scientific/Invitrogen, cat# LC2002) was performed to detect PRR14, H3K9me3, or HP1 isoforms by probing the membrane with the corresponding antibodies. Image analysis for Figure S5 A–B was performed as described in the section above, except the mean intensity values for H3K9me3 were first normalized to the total Histone H3 signal and then to the ‘PRR14 WT>Tet no Dox’ condition; and, similarly, for Figure S1, PRR14 signal was first normalized to GAPDH and then to the endogenous PRR14 signal of the ‘GFP-negative’ sample.

**Antibodies**

The following primary antibodies were used in this study: anti-H3K9me3 (Abcam, cat# ab8898; IF 1:1000, WB 1:2000), anti-H3K9me2 (Active Motif, cat# 39239, IF 1:1000), anti-Lamin A/C (Santa Cruz, cat# sc-376248; IF 1:500), anti-Lamin B1 (Abcam, cat# ab16048; IF 1:1000), anti-GFP (Abcam, cat# ab290; WB 1:1000, IP 1:200), Normal Rabbit IgG (Cell Signaling, cat# 2729; IP 1:200), anti-HP1α (Abcam, cat# 77256; IF, WB 1:1000), anti-HP1β (Abcam, cat# ab10478; IF, WB 1:1000), anti-HP1γ (Santa Cruz, cat# sc-398562; IF 1:500, WB 1:1000), anti-PRR14 (Proteintech, cat# 22819-1-AP; WB 1:1000), anti-β-Actin (8H10D10) (Cell Signaling, cat# 3700S; WB 1:5000), anti-Histone H3 (Abcam, cat# ab1791; WB 1:10,000), and anti-GAPDH (D4C6R) (Cell Signaling, cat# 51332; WB 1:1000).
The following secondary antibodies from ThermoFisher Scientific/Invitrogen were used: Donkey anti-Rabbit Alexa Fluor 568 (cat# A10042, IF 1:1000), Donkey anti-Mouse Alexa Fluor 488 (cat# A10037, IF 1:1000), Donkey anti-Rabbit Alexa Fluor 647 (cat# A31573, IF 1:1000), Donkey anti-Mouse Alexa Fluor 647 (cat# A31571, IF 1:1000); and HRP-linked anti-Rabbit IgG and HRP-linked anti-Mouse IgG (Cell Signaling, cat# 7074 and 7076; WB 1:5,000). IF – immunofluorescence, WB – Western Blot, IP – immunoprecipitation.

**Statistical analysis**

For immunofluorescence assays, 10 to 40 individual cells were imaged per condition. These numbers exceed the sample size calculated using power analysis. We considered each imaged individual cell/nucleus as a biological replicate. The numbers of cells per each analysis are indicated in the figure legends. Statistical analysis was performed with GraphPad Prism 9.3.1 software (GraphPad Software, Inc.) using one-way Analysis of Variation (ANOVA) Brown-Forsythe and Welch test with Dunnett’s multiple comparisons (for samples with Gaussian distribution of residuals), paired ANOVA test with Geisser-Greenhouse correction for immunoblot assay, or non-parametric ANOVA Kruskal–Wallis test with Dunn’s multiple-comparison test. For comparison of two samples an unpaired non-parametric Student’s t-test (Mann–Whitney test) was used.

**Acknowledgments**

We thank Jonathan A. Epstein and Rajan Jain for support (NIH R35 HL140018 and DP2 HL147123) and for discussions and comments on the manuscript; Kelly Dunlevy, Jason Wasserman, Jade Wilson, Valentina Medvedeva, and Trinity Pellegrin for help with making certain constructs; Andrea Stout from the Penn CDB Microscopy Core for help with confocal imaging; Melike Lakadamylali for help with super-resolution imaging; Center for Engineering and Mechanobiology (CEMB) and NSF Science and Technology Center Pilot Award (CMMI 15-48571) for access to ONI super-resolution microscope and the Penn Flow Cytometry Core for technical support.

**Disclosure statement**

No potential conflict of interest was reported by the author(s).

**Funding**

This work was supported by the National Institutes of Health (NIH) R35 HL140018 and DP2 HL147123 supported A. P. and C.L.S., R21 AG054829 to R.A.K., and P30 CA006927 supported R.A.K., American Heart Association (AHA) 827458 to A.A.K.

**Author contributions**

Conceptualization: A.A.K., R.A.K. and A.P. Methodology: A. A.K., Y.C., C.L.S., and A.P. Formal analysis: A.A.K. and A.P. Investigation: A.A.K., Y.C., and A.P. Visualization: A.A.K. and A.P. Data curation: A.A.K. and A.P. Resources: R.A. K. Supervision: A.P. Writing (original draft): A.P. Writing (review and editing): A.A.K, R.A.K., C.L.S., and A.P.

**Data and materials availability**

All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Raw western blot images are available as source data.

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