On the mechanism of induction of heterochromatin by the RNA-binding protein vigilin

JING ZHOU, QIAOQIAO WANG,¹ LING-LING CHEN, and GORDON G. CARMICHAEL

Department of Genetics and Developmental Biology, University of Connecticut Stem Cell Institute, University of Connecticut Health Center, Farmington, Connecticut 06030-3301, USA

ABSTRACT

Vigilin is an RNA-binding protein localized to both the cytoplasm and the nucleus and has been previously implicated in heterochromatin formation and chromosome segregation. We demonstrate here that the C-terminal domain of human vigilin binds to the histone methyltransferase SUV39H1 *in vivo*. This association is independent of RNA and maps to a site on vigilin that is not involved in its interaction with several other known protein partners. Cells that express high levels of the C-terminal fragment display chromosome segregation defects, and ChIP analyses show changes in the status of pericentric β -satellite and rDNA chromatin from heterochromatic to more euchromatic form. Finally, a cell line with inducible expression of the vigilin C-terminal fragment displays inducible alterations in β -satellite chromatin. These and other results lead us to present a new model for vigilin-mediated, RNA-induced gene silencing.

Keywords: RNA editing; gene silencing; heterochromatin; histone methyltransferase

INTRODUCTION

Vigilin is conserved from yeast to mammals. All vigilins contain 14 tandem and related, but nonidentical, type I KH (hnRNP K homology) domains, which are involved in nucleic acid binding and protein–protein interaction. Mutation or depletion experiments have demonstrated the role of vigilin in establishing heterochromatin in the nucleus. Both yeast Scp160p mutants and *Drosophila* DDP1 knockdown in insect cells show chromosome segregation defects, likely the result of aberrant pericentromeric heterochromatin formation (Wintersberger et al. 1995; Huertas et al. 2004). Suppression of heterochromatin-induced position effect variegation, reduction of H3K9 methylation, and deposition of heterochromatin protein 1 (HP1) at the chromocenter were also observed in DDP1 mutants (Huertas et al. 2004). Recently, our laboratory identified by inosine-containing RNA (ADAR-edited RNA) chromatography a vigilin nuclear complex that is likely involved in heterochromatin formation through an RNA-mediated pathway

(Wang et al. 2005). This complex includes the following proteins: ADAR1, the well-known DNA double strand break repair factor Ku70/86 heterodimer (Koike 2002), DNA-dependent protein kinase DNA-PKcs, RNA helicase A (RHA) (Zhang and Grosse 2004), the histone variant H2AX (Paull et al. 2000), and the heterochromatin protein HP1 α (Wang et al. 2005). Importantly, the complex also contains an RNA-dependent protein kinase activity (presumably DNA-PKcs) that can phosphorylate RHA, H2AX, and HP1 α (Wang et al. 2005).

To pinpoint a direct connection between the vigilin nuclear complex and heterochromatin, we asked whether the vigilin complex recruits the key component in initiating heterochromatin formation, SUV39H1. SUV39H1 is a histone methyltransferase that methylates H3 on lysine 9 and creates a binding site for HP1 (Aagaard et al. 2000; Melcher et al. 2000; Eskeland et al. 2007). *Drosophila* HP1 alone binds only weakly to methylated chromatin *in vitro*, yet the addition of Su(var)3-9 (SUV39H1 homolog in *Drosophila*) strongly facilitates this binding (Eskeland et al. 2007). SUV39H1 also associates with HP1 *in vivo*, and this interaction plays an important role in propagating the heterochromatin status to nearby regions by spreading H3K9 methylation (Krouwels et al. 2005). We show that the KH13–14 region at the C terminus of vigilin strongly associates with SUV39H1 *in vivo*. Expression of this small vigilin fragment in cells gives rise to a dominant negative phenotype and disrupts heterochromatin.

¹**Present address:** Yale Stem Cell Center and Department of Cell Biology, Yale University School of Medicine, New Haven, CT 06509, USA.

Reprint requests to: Gordon G. Carmichael, Department of Genetics and Developmental Biology, University of Connecticut Stem Cell Institute, University of Connecticut Health Center, Farmington, CT 06030-3301, USA; e-mail: carmichael@nso2.uchc.edu; fax: (860) 679-8345.

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RESULTS

The vigilin KH13–14 domain interacts with the histone methyltransferase SUV39H1

Association of nuclear vigilin with SUV39H1 and HP1 α was initially determined in immunoprecipitation experiments (Fig. 1A). HEK293 nuclear lysates were used for immunoprecipitation with anti-SUV39H1 (Fig. 1A, lane 2), anti-HP1 α (Fig. 1A, lane 3), or anti-Flag (Fig. 1A, lane 4, control) antibodies. Western blotting with anti-vigilin antibody indicated that each of these heterochromatin-associated factors could be found in vigilin complexes (Fig. 1A). In addition, both anti-SUV39H1 and anti-HP1 α immunoprecipitations indicated an association of Ku70 with at least a subset of the nuclear complexes. These results are consistent with previous work showing that Ku70 could also be found associated with nuclear vigilin complexes (Wang et al. 2005). Next, to confirm whether vigilin associates with SUV39H1 *in vivo*, Flag-tagged vigilin was expressed in HEK293 cells followed by immunoprecipitation with anti-SUV39H1 antibody. Western blotting with anti-flag antibody revealed not only the full-length recombinant protein in transfected cells, but also numerous smaller fragments, presumably generated by proteolysis in the cells or extracts

(Fig. 1B). Since the Flag tag is at the N terminus of the constructs, all smaller fragments lack the C terminus. Interestingly, anti-SUV39H1 only immunoprecipitated full-length Flag-vigilin but not the shorter fragments (Fig. 1B), suggesting that the C terminus of vigilin is required for interaction with SUV39H1.

To confirm the site of interaction with SUV39H1, cDNA fragments expressing KH domains 1–7, 8–14, 10–14, 12–14, and 13–14 were each cloned into the vector pcDNA-DEST53, which encodes a GFP tag at the N terminus of the proteins, and were transfected into HEK293 cells (Fig. 1D). All fragments were expressed at similar levels and of the expected sizes (Fig. 1C).

The various vigilin fragments expressed in HEK293 cells were next examined for interaction with SUV39H1. Immunoprecipitation using antibodies against SUV39H1 showed that the GFP-KH8–14 fragment associates with SUV39H1, while the GFP-KH1–7 fragment does not (Fig. 2A). Figure 2B further maps the interaction to the C-terminal KH13–14 region, consistent with the results of Figure 1. After RNase treatment, the vigilin KH13–14 domain still interacts strongly with SUV39H1, indicating that the interaction between KH13–14 and SUV39H1 is RNA independent (Fig. 2C).

During the course of the binding studies described above, we noticed that the KH13–14 region consistently

FIGURE 1. (A) Vigilin associates with SUV39H1 and HP1 α *in vivo*. HEK293 nuclear lysates were used for immunoprecipitation with the indicated antibodies. Western blotting was with anti-vigilin antibody and anti-Ku70 antibody. (B) SUV39H1 interacts with the C-terminal domain of vigilin. HEK293 cells were transfected with N-terminal Flag-tagged vigilin. Seventy-two hours after transfection, nuclear lysates were used for immunoprecipitation with anti-SUV39H1 antibody. Western blotting was with anti-flag antibody. (C) Seventy-two hours after transfection of HEK293 cells with GFP-tagged vigilin constructs, whole-cell lysates were used for Western blotting with anti-GFP antibody. Equal amounts of extracts were examined, showing that all fragments were expressed at comparable levels. (D) Full-length vigilin and mutant constructs.

FIGURE 2. Mapping the SUV39H1 binding domain of vigilin. (A) HEK293 cells were transfected with GFP-tagged KH1–7 or KH8–14, IP was carried out with anti-SUV39H1 antibody as in Figure 1, and Western blotting was with anti-GFP antibody. (B) Cells were transfected with vigilin KH10–14 or KH13–14 followed by IP and Western blotting as in A. (C) The interaction of SUV39H1 with the vigilin KH13–14 region is independent of RNA. Cells were transfected with vigilin GFP-KH13–14. The same experiments were carried out as in B, except that cell lysates were treated with 200 μ g/mL RNase A at 4°C for 2 h before immunoprecipitation. (D) SUV39H1 specifically associates with GFP-tagged vigilin KH13–14 (lane 4) but not GFP alone (lane 2) in vivo. Beads alone cannot pull down vigilin KH13–14 (lane 5). Cells were transfected with plasmid expressing GFP or GFP-KH13–14 followed by IP with anti SUV39H1 antibody and Western blotting with anti-GFP antibody.

appeared to bind more strongly to SUV39H1 than any of the other fragments, including longer ones containing the KH13 and KH14 domains (Fig. 2A,B; data not shown). As seen in Figure 2B, lanes 5 and 6, the KH10–14 fragment appears to have a lower affinity for SUV39H1 than does the KH13–14 fragment. The subcellular localization data of Figure 3 offer an explanation for this. Full-length vigilin is primarily localized to the cytoplasm, with a small but significant fraction localized in the nucleus (Fig. 3a–c). GFP-KH1–7 (Fig. 3d–f), GFP-KH8–14 (Fig. 3g–i), and GFP-KH10–14 (Fig. 3j–l) show a similar cytoplasmic abundance. In contrast, however, GFP-KH12–14 (Fig. 3m–o) and GFP-KH13–14 (Fig. 3p–u) are almost equally distributed between the nucleus and cytoplasm. These data suggest that the C-terminal region of human vigilin may harbor a nuclear localization signal, but this signal might be masked or not exposed in constructs containing the KH10–12 region, such as full length vigilin, GFP-KH8–14, and GFP-KH10–14.

Vigilin KH13–14 has a dominant negative phenotype

Since the C-terminal region of vigilin interacts with SUV39H1 and expression of the KH13–14 fragment leads to accumulation in the nucleus, we asked whether over-expressed KH13–14 might compete with endogenous vigilin

for a limited supply of SUV39H1, and might therefore exhibit dominant negative effects. Figure 4 shows that this is the case. Flow cytometry showed that, compared to control cells expressing GFP only (Fig. 4A), the percentage of GFP-KH13–14 transfected cells in G2 phase was dramatically increased (Fig. 4C). Cells expressing KH13–14 also displayed a higher average DNA content (Fig. 4B, cf. average contents, indicated by filled triangles). Importantly, this phenotype was not observed in cells expressing the cytoplasmic fragment GFP-KH10–14 (Fig. 4B).

Expression of vigilin KH13–14 destabilizes heterochromatin

Heterochromatin formation in the centromeric region is critical for sister chromatid synapsis, spindle formation, and chromosome segregation (Dobie et al. 1999; Sullivan et al. 2001; Sharp et al. 2003). As DDP1 was found to associate with pericentromeric heterochromatin in *Drosophila* (Cortes et al. 1999; Cortes and Azorin 2000), we asked whether vigilin KH13–14 might disrupt the heterochromatin status in the pericentromeric region. Chromatin immunoprecipitation (ChIP) was carried out using antibodies against the characteristic heterochromatin

FIGURE 3. Immunolocalization of vigilin and vigilin fragments. (a–c) Immunolocalization with a polyclonal anti-vigilin antibody. DAPI is pseudocolored in red. For panels d–x the indicated GFP-tagged constructs were transfected into HEK293 cells and GFP expression monitored and localized by fluorescence. Note that GFP-KH1–7, GFP-KH8–14, and GFP-KH10–14 are mostly in the cytoplasm, while GFP-KH12–14 and GFP-KH13–14 are equally distributed between the cytoplasm and nucleus.

marker trimethylated histone 3 lysine 9 (H3K9Me3) and euchromatin marker acetylated histone 4 (H4Ac), followed by quantitative PCR with primers specific for pericentromeric β -satellite sequences (Fig. 4D). Fold enrichment of PCR signals obtained from H4Ac IP was corrected for the fold enrichment of PCR signals obtained from H3K9Me3 IP. Compared to control cells, the ratio of fold enrichment from H4Ac IP to that from H3K9Me3 IP in vigilin KH13–14 transfected cells increased fourfold, while transfection with vigilin fragment KH10–14 had virtually no effect. In contrast, transfection of KH13–14 did not alter the chromatin status of the euchromatic GAPDH gene region. These results support our hypothesis that KH13–14 titrates SUV39H1 away from heterochromatin or from full-length vigilin. To determine whether vigilin might also be involved in the formation of heterochromatin in other regions, we performed real-time PCR

with primers specific for rDNA. Again, we observed a fourfold increased association of rDNA with H4Ac relative to H3K9Me3 (Fig. 4D).

The vigilin KH13–14 region does not bind RNA helicase A and Ku86

Nuclear vigilin interacts with a number of factors other than SUV39H1. Our previous results showed that vigilin binds to RHA and Ku86 and exhibits an RNA-dependent protein kinase activity that phosphorylates RHA, HP1 α , and histone H2AX, likely by the Ku-mediated recruitment of DNA-PKcs (Wang et al. 2005). We therefore used the constructs shown in Figure 1B to map the sites of interaction of RHA and Ku86 in vigilin. Results shown in Figures 5 and 6 confirm that these other factors bind to vigilin, but upstream of the KH13–14 region. GFP-KH8–14 interacts with RHA, while GFP-KH1–7 does not (Fig. 5A, lanes 5–8), mapping the interaction domain to the C-terminal half of vigilin. However, although we could detect interaction between GFP-KH10–14 and RHA, we never observed any binding of RHA to GFP-KH13–14, which interacts with SUV39H1 (Fig. 5A, lanes 12–14). This interaction is independent of RNA (Fig. 5C).

The Ku complex appears to associate with vigilin in the same region as RNA helicase A. GFP-KH10–14 binds well to Ku86 (Fig. 6A, lane 9), but GFP-KH13–14 shows no interaction (Fig. 6A, lane 10). Again, this interaction is independent of RNA, as reported previously (Wang et al. 2005).

Construction of a cell line with inducible vigilin KH13–14

Finally, we produced a transgenic line in which the expression of vigilin KH13–14 domain is tetracycline regulated. HEK293 cells were cotransfected with plasmids expressing Flag-tagged vigilin KH13–14 and hygromycin resistance, and hygromycin-resistant clones were selected. The expression of Flag-KH13–14 in clone H4 was found to be tightly regulated; without induction, no Flag-KH13–14 could be detected (Fig. 7A). Doxycycline induced the appearance of Flag-KH13–14 as detected with an anti-vigilin antibody that recognizes the C terminus (Fig. 7B), although the level of expression was lower than that typically seen in transient transfections (Fig. 7C). While the induced H4 cells did not show a clear defect in chromosome segregation, ChIP analysis revealed inducible changes in β -satellite chromatin (Fig. 7D). Curiously, the extent of chromatin alteration seen in Figure 7D appeared to be greater than that observed in Figure 4D, yet no chromosome defect was seen. A likely explanation for this is that in the earlier experiments (Fig. 4D), the observed ratios may have been smaller because ChIP assays were carried out on cell populations where transfected cells were mixed with untransfected cells,

FIGURE 4. (A–C) The KH13–14 fragment exhibits dominant negative effects on chromosome segregation. HEK293 cells were transfected with plasmids expressing GFP, GFP-KH10–14, or GFP-KH13–14. Seventy-two hours after transfection, GFP-positive cells were collected and analyzed by FACS. Compared to control cells transfected with GFP alone (A) or GFP-KH10–14 (B), cells transfected with GFP-KH13–14 (C) are mostly arrested at the G2/M phase, with an increased average DNA content indicated by the intensity of PI staining of DNA (black arrows). The data shown are representative results from three independent experiments. (D) Antibodies against trimethylated H3K9 or acetylated H4 were used for chromatin immunoprecipitations with sheared chromatin from untransfected HEK293 cells or cells transfected with GFP or GFP-tagged vigilin mutants. Purified DNAs were used for real time PCR with primers specific for pericentromeric β -satellite, rDNA, or GAPDH sequences. The chromatin status of these regions is expressed as the ratio of PCR signal obtained for H4Ac (euchromatin marker) IP to that obtained for H3K9Me3 (heterochromatin marker) IP. The ratios from transfected cells were normalized to those from untransfected cells. The results represent mean $\pm$ SD signal of PCRs performed in triplicate.

while in the latter experiments (Fig. 7D) all cells expressed vigilin KH13–14.

DISCUSSION

Assembly of heterochromatin requires both CpG DNA methylation and specific histone methylation (Jenuwein 2001). Trimethylation of H3K9 mediated by SUV39H1 is essential for the establishment of constitutive heterochromatin at pericentromeric and telomeric regions in the genome (Peters et al. 2001; Maison et al. 2002; Peters et al. 2003; Garcia-Cao et al. 2004). HP1 binds to methylated H3K9 (Bannister et al. 2001; Lachner et al. 2001) and recruits SUV39H1 for further histone methylation (Stewart et al. 2005). We show here that vigilin also associates with SUV39H1 in vivo and that this association influences chromatin.

Vigilin has different functions in the cytoplasm and nucleus. Consistent with a role in mRNA stability and

translation, cytoplasmic vigilin is associated with ER and polyribosomes (Klinger and Kruse 1996). In the nucleus, vigilin is enriched in heterochromatic regions (Klinger and Kruse 1996; Huertas et al. 2004; Wang et al. 2005). It was reported that a substantial fraction of SUV39H1 is in a dynamic state (Krouwels et al. 2005), and maintenance of stable heterochromatin domains involves the transient binding and dynamic exchange of HP1 from chromatin in a histone methyltransferase-dependent manner (Cheutin et al. 2003). Taken together with our data showing the functional interaction of vigilin and SUV39H1, we speculate that vigilin, like HP1 and SUV39H1, is dynamically associated with heterochromatin.

Intriguingly, while an RNA component has been shown to be indispensable for the integrity of heterochromatin in mammalian cells (Maison et al. 2002; Muchardt et al. 2002), the nature of this component is still unknown, and the connection between RNA and heterochromatin in

FIGURE 5. Mapping the RHA binding domain of vigilin. (A) RHA binds to the KH10–12 region of vigilin. HEK 293 cells were cotransfected with Flag-tagged RHA and GFP-tagged vigilin mutants. Seventy-two hours after transfection, whole cell lysates were used for immunoprecipitation with Flag antibody conjugated beads. The presence of vigilin mutants in the precipitates was detected by Western blotting with anti-GFP antibody. Vigilin KH8–14 (lane 6) and KH10–14 (lane 10) regions, but not KH1–7 (lane 5) and KH13–14 (lane 14) regions associate with RHA. (B) The same samples were used for Western blotting with anti-flag antibody to confirm that the tagged constructs could be immunoprecipitated. (C) Association is independent of RNA. Experiments were carried out as in A except that cell lysates were treated with 200 μ g/mL RNase A at 4°C for 2 h before immunoprecipitation. After RNase treatment, vigilin KH10–14 still associates with RHA (lane 4).

mammalian cells remains an enigma. We suggest that one class of RNAs involved in silencing includes those with high affinity to vigilin. Both DDP1 and vigilin prefer to bind long unstructured single-stranded DNAs or RNAs (Ferrer et al. 1995; Kanamori et al. 1998; Cortes et al. 1999; Cortes and Azorin 2000). This is congruent with our findings that these proteins, as well as Scp160p, bind to hyperedited RNAs, which also have low secondary structure (Wang et al. 2005; data not shown).

Since no nucleic-acid binding activity has been found in SUV39H1, the protein–protein interaction must play an essential role in RNA-mediated silencing. Heterochromatic regions are often associated with repetitive elements, and these regions are not transcriptionally inert. In mouse ES cells, pericentric heterochromatin can give rise to transcripts spanning the major satellite repeat (Lehnertz et al. 2003), and a significant fraction of the human genome consists of

retrotransposons. Bidirectional transcription of these repetitive elements could produce dsRNA, which would be edited by ADAR in the nucleus (Wang and Carmichael 2004). We propose that Vigilin recognizes these genomic loci through RNA binding and thus recruits SUV39H1.

Phosphorylation events also play a critical role in heterochromatin formation. Mutations in the phosphorylation site of HP1 can reduce or completely eliminate its silencing activity. On the other hand, constitutive phosphorylation introduced by site-directed mutation can also abolish its silencing activity (Norwood et al. 2004). SUV39H1 is also a phosphoprotein (Aagaard et al. 2000). Phosphorylation of SUV39H1 specifically occurs at mitotic G1/S cell cycle transition, modulating both its nuclear localization and silencing effect (Firestein et al. 2000). Since the nuclear vigilin complex has an associated kinase activity (DNA-PKcs) (Wang et al. 2005), it may also participate in higher order chromatin organization through regulating these phosphorylation events.

The available evidence leads us to propose an integrated model for vigilin function (Fig. 8). Vigilin recognizes single-stranded nucleic acid regions of low secondary structure. When bound to its RNA or DNA target, the vigilin complex (which contains Ku70/86, RHA, and SUV39H1) recruits DNA-PKcs and perhaps other kinases. The activated complex then phosphorylates

nearby protein targets, including HP1 α , as well as methylates histone H3K9. These events separately or together facilitate the establishment of heterochromatin. Although the reported RNA-dependent kinase activity of the vigilin complex is consistent with a role of phosphorylation in gene silencing, it is possible that the primary mode of silencing is only via SUV39H1.

MATERIALS AND METHODS

Plasmids and cloning

Plasmid pTXL12, which contains the full-length vigilin cDNA sequence, was used as a PCR template for cloning vigilin KH1–7, KH8–14, KH10–14, KH12–14, and KH13–14 into the Gateway system (Invitrogen). PCR products were directly cloned into the pENTR/D-TOPO vector by topo cloning followed

FIGURE 6. Mapping the Ku86 binding domain of vigilin. (A) Ku86 binds to the KH10–12 region of vigilin. HEK 293 cells were transfected with GFP-tagged vigilin mutants. Seventy-two hours after transfection, whole cell lysates were used for immunoprecipitation with anti-Ku antibody. The presence of vigilin mutants in the precipitates was detected by Western blotting with anti-GFP antibody. Vigilin KH1–7 (lane 7), KH8–14 (lane 8), and KH10–14 (lane 9) regions associate strongly with RHA. (B) The association of KH10–14 with Ku86 is independent of RNA. Experiments were carried out as in A except that cell lysates were treated with 200 μ g/mL RNase A at 4°C for 2 h before immunoprecipitation. After RNase treatment, vigilin KH10–14 still associates with Ku86 (lane 4).

by sequencing. The vigilin fragments were then subcloned into pcDNA-DEST53 containing the GFP coding sequence at the 5'-end of the recombination site by LR recombination. The resulting plasmids were subsequently used to transfect HEK293 cells to express the vigilin fragments with N-terminal GFP-tags.

Cell culture and transfection

HEK293 cell lines were cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, and antibiotics. For coimmunoprecipitations, transfections were done by the standard calcium phosphate precipitation method using 3×10^6 HEK293 cells and a total of 10 μ g of expression vectors.

Coimmunoprecipitation and immunoblotting

At 48–72 h post-transfection, cells were lysed in hypotonic buffer (10 mM HEPES at pH 7.5/pH 6.4, 10 mM NaCl, 10 mM EDTA, 0.1% Triton X-100, 1 mM DTT, and protease inhibitor cocktail). Nuclear extracts were clarified by high-speed centrifugation followed by brief sonication in IP buffer (20 mM HEPES at pH 7.5/pH 6.4, 150 mM NaCl, 10 mM EDTA, 0.5% NP-40, 0.5 mM PMSF). After centrifugation, supernatants were incubated with 40 μ L Protein A/G PLUS-Agarose beads (Santa Cruz) and 2.5 μ L mouse anti-SUV39H1 monoclonal

FIGURE 7. (A) Western blotting with anti-Flag antibody on lysates from expanded hygromycin resistant clones from stable cell lines expressing flag-KH13–14. Ku86 was used as loading control. Clone H4 expresses flag-KH13–14, while clone H5 does not. (B) Expression of flag-KH13–14 in the H4 cell line was confirmed by Western blotting with anti-vigilin antibody that recognizes the C-terminal portion of vigilin. Expression of endogenous vigilin was used as loading control. HEK293 cells (“293”) were used as control. (C) Lysates from H4 cells or from HEK293 cells transiently transfected with pcDNA-DEST53-KH13–14 were analyzed by Western blotting with anti-vigilin antibody. Expression of endogenous vigilin was used as loading control. (D) Chromatin changes in cells with inducible KH13–14. Equal amounts of lysates from uninduced H4 cells, induced H4 cells, or induced H4 cells removed from doxycycline treatment were used for ChIP assays with antibodies against trimethylated H3K9 and acetylated H4, followed by PCR with primers specific for β -satellite or GAPDH sequences. Chromatin status is presented as the ratio of fold enrichment of PCR signals from H4Ac IP to that from H3K9Me3 IP. The ratios obtained from induced stable cell ChIP analyses were normalized to those from uninduced stable cells.

FIGURE 8. A model for vigilin-induced silencing. (A) In the nucleus, vigilin binds to hyperedited RNA or other unstructured RNAs that reside in heterochromatic regions and recruits a protein complex including the Ku70/Ku86 heterodimer, RHA, and the histone methyltransferase, SUV39H1. SUV39H1 methylates histone 3 on lysine 9, creating binding sites for HP1, which further recruits SUV39H1. In the presence of RNA, DNA-PKcs is also recruited by Ku heterodimer, which can phosphorylate HP1 α and other proteins. (B) Overexpressed vigilin KH13–14 interacts with SUV39H1 and tritrates it away from heterochromatic regions, resulting in the disruption of the normal function of vigilin in heterochromatin formation.

antibody (Upstate) at 4°C for 2.5 h. The beads were washed five times with IP buffer, resuspended in 2 $\times$ SDS sample buffer and boiled for 5 min before loading on SDS PAGE. Anti-GFP antibody (1:2000) was used as the first antibody to detect the association of SUV39H1 with a GFP tagged vigilin fragment. Anti-mouse immunoglobulin was used as the secondary antibody. To examine the expression of vigilin fragments and the interaction of vigilin fragments with Ku86 or RHA in HEK293 cells, whole cell lysate were used for Western blotting or immunoprecipitation.

Immunofluorescence

To detect the subcellular localization of vigilin, rabbit anti-vigilin antibodies generated in rabbits using purified full-length vigilin were generously provided by Dr. A. Das (University of Connecticut Health Center). HEK293 cells were fixed with 4% paraformaldehyde for 15 min at room temperature, followed by permeabilization with 0.1% Triton X-100. Cells were incubated with anti-vigilin antibody (1:100) for 1 h, followed by washing with PBS and incubation with fluorescence labeled secondary antibody FITC (anti-rabbit antibody; Jackson ImmunoResearch Lab) for 1 h. Fluorescence signals were detected using fluorescence microscopy (Axiovision; Carl Zeiss).

Flow cytometry

Seventy-two hours post-transfection, 2 $\times$ 10⁷ HEK293 cells were collected and washed twice with PBS followed by fixation in 1% formaldehyde for 1 h and permeabilization with 70% ethanol overnight at 4°C. Cells were spun down, resuspended, and incubated in 1 mL solution containing 40 μ g/mL of propidium iodide and 100 μ g/mL RNase A at 37°C in the dark for 30 min. Samples were filtered through a nylon mesh to remove clumps and kept on ice until analysis on two color FACScalibur with Argon and Red diode lasers (Becton-Dickinson).

Chromatin immunoprecipitation and quantitative PCR

ChIP assays were performed as described (Nelson et al. 2006). Basically, cells were fixed directly by adding 37% formaldehyde to the overlaying medium to a final concentration of 1.42% and incubated for 15 min at room temperature. After quenching formaldehyde with 125 mM glycine, 1 $\times$ 10⁶ cells were washed twice in cold PBS and lysed in IP buffer (150 mM NaCl, 50 mM Tris-HCl at pH 7.5, 5 mM EDTA, 0.5% NP-40, 1% Triton X-100, 0.5 mM PMSF, 10 μ g/mL leupeptin). Nuclear pellets from 10⁶ cells were sonicated in 1 mL IP buffer using an Ultrasonics sonifier W220-F (three rounds of 15 sec with 3.5 power output, each cycle including a 2-min rest interval). For immunoprecipitation, 200 μ L sheared chromatin were incubated with anti-trimethylated H3K9 or anti-acetylated H4 antibodies (Upstate) at 4°C for 5 h. Precipitated DNA was isolated with Chelex100 resin (Bio-Rad) and resuspended in 180 μ L MilliQ H₂O,

5 μ L of which was used for quantitative PCR in 20- μ L reactions with the Bio-Rad real-time PCR system. Data analysis was done with the iCycler software. Primers for PCR are as follows:

Forward β satellite primer (5'-AGGGGCTTTATCCTCATTTTCACAA-3');
Reverse β satellite primer (5'-GGCCTCCATATTCCTAACTTCC-3');
Forward rDNA primer (5'-ACCTGGCGCTAAACCATTTCGT-3');
and
Reverse rDNA primer (5'-GGACAAACCCCTTGTGTCGAGG-3').

Generation of a stable cell line with inducible vigilin KH13–14

Flag-tagged KH13–14 was cloned into the BamHI/XbaI site of plasmid pTre-tight (Clontech), which contains a Tet-responsive promoter, and linearized with ScaI. HEK293 cells were grown in 35-mm dishes and cotransfected with 0.5 μ g linearized pTre-tight-KH13–14, 0.5 μ g pUHRt62-1 (linearized with ScaI) expressing rtTA2S-M2 (Urlinger et al. 2000) and 50 ng linearized hygromycin selection marker (Clontech) by the lipofectamine (Invitrogen) method. After 12 h, cells were transferred to 10-cm dishes and maintained in medium containing 200 μ g/mL hygromycin. Resistant clones were isolated, expanded separately, and analyzed for expression of flag-KH13–14 48 h after induction with 1 μ g/mL doxycycline by Western blotting with anti-Flag and anti-vigilin antibody (a gift from Dr. D.J. Shapiro, University of Illinois at Urbana-Champaign). The vigilin KH13–14 H4 stable cell line was induced with 1 μ g/mL doxycycline for 2 mo and used for ChIP analyses as above. In order to reverse the effects of induction, doxycycline was removed from the induced stable cell line for 2 wk before ChIP analysis.

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