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The SUVR4 Histone Lysine Methyltransferase Binds Ubiquitin and Converts H3K9me1 to H3K9me3 on Transposon Chromatin in Arabidopsis

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Abstract

Chromatin structure and gene expression are regulated by posttranslational modifications (PTMs) on the N-terminal tails of histones. Mono-, di-, or trimethylation of lysine residues by histone lysine methyltransferases (HKMTases) can have activating or repressive functions depending on the position and context of the modified lysine. In Arabidopsis, trimethylation of lysine 9 on histone H3 (H3K9me3) is mainly associated with euchromatin and transcribed genes, although low levels of this mark are also detected at transposons and repeat sequences. Besides the evolutionarily conserved SET domain which is responsible for enzyme activity, most HKMTases also contain additional domains which enable them to respond to other PTMs or cellular signals. Here we show that the N-terminal WIYLD domain of the Arabidopsis SUVR4 HKMTase binds ubiquitin and that the SUVR4 product specificity shifts from di- to trimethylation in the presence of free ubiquitin, enabling conversion of H3K9me1 to H3K9me3 *in vitro*. Chromatin immunoprecipitation and immunocytological analysis showed that SUVR4 *in vivo* specifically converts H3K9me1 to H3K9me3 at transposons and pseudogenes and has a locus-specific repressive effect on the expression of such elements. Bisulfite sequencing indicates that this repression involves both DNA methylation–dependent and –independent mechanisms. Transcribed genes with high endogenous levels of H3K4me3, H3K9me3, and H2Bub1, but low H3K9me1, are generally unaffected by SUVR4 activity. Our results imply that SUVR4 is involved in the epigenetic defense mechanism by trimethylating H3K9 to suppress potentially harmful transposon activity.

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Introduction

In eukaryotes, gene expression and chromatin structure is specified by the combinatorial pattern of posttranslational modifications (PTMs) on the histone tails, which include phosphorylation, acetylation, methylation, SUMOylation and ubiquitination [1,2]. These PTMs are interdependent, thus providing regulatory cross-talk, and established at the histone tails in a coordinated manner by different classes of highly specific chromatin modifying enzymes.

The combination of PTMs constitutes the so-called histone code, and their downstream effect on chromatin organization and gene expression is mediated by nonhistone effector proteins that contain domains that bind or "read" this code in order to specify epigenetic function. Such domains show specificity for particular modified residues (e.g. acetylation or methylation of lysine) in the context of its surrounding amino acid sequence, and for the state of the modification (e.g. H3K9me1 vs H3K9me3) [1,3]. For example, domains belonging to the Royal Superfamily, including the chromodomain, Tudor domain and MBT domain and members of the PHD finger family, bind methylated lysine residues on the histone tails [4]. More specifically, the PHD finger of the ORC1 protein in Arabidopsis binds H3K4me3, but not H3K4me1 or H3K4me2 at target genes, and this mediates H4K20 trimethylation and activates transcription [5].

Lysine ubiquitination of histones and other target proteins is a three step process involving Ub (ubiquitin)-activating (E1), Ub-conjugating (E2) and Ub-ligating (E3) enzymes, eventually leading to monoubiquitination, multi-monoubiquitination or polyubiquitination [6,7]. Ubiquitin binding domains (UBDs) represent a new class of motifs that enable proteins to bind non-covalently to the PTM ubiquitin. More than twenty families have been identified to date, and they differ in structure and the type of ubiquitin modification they recognize [6,7]. Poly-Ub chains linked via the K48 residue of ubiquitin are largely recognized by UBDs of receptors that target proteins for proteosomal degradation, while monoubiquitin is recognized by UBDs of proteins involved in processes like DNA repair, regulation of protein activity, chromatin remodeling and transcription [6–8].

Author Summary

The characteristics of the diverse cell types in multicellular organisms result from differential gene expression that is dependent on the level of DNA packaging. Genes that are essential for the function of the cell are expressed; while unessential genes, and DNA elements (transposons or "jumping genes") that can move from one position to another within a genome and potentially cause deleterious mutations, are repressed. The mechanisms evolved in eukaryotes to avoid unwanted gene expression and transposon movement include DNA methylation and specific combinations of post translational modifications (PTMs) of the histones that package DNA. Here we show that the SUVR4 enzyme binds the signaling protein ubiquitin and that ubiquitin enables the enzyme to trimethylate lysine 9 (H3K9me3) of histone H3. In contrast to other reports demonstrating an activating role on expressed genes, we show that H3K9me3 has a locus-specific repressive effect on the expression of transposons. The specificity is maintained by the communication with other PTMs on transposons and euchromatic genes, which has a stimulating or repressing effect on enzyme activity, respectively. Our results demonstrate how repression of transcription can be restricted to specific targets and demonstrate that this repression involves a context-dependent read-out of different PTMs.

The cross-talk between H2B monoubiquitination (H2Bub1) and histone methylation has been extensively studied and is highly conserved from yeast to human. These studies show that monoubiquitination of H2B recruits proteins that direct histone H3K4 di- and trimethylation but not monomethylation by activation of the Set1 histone lysine methyltransferase (HKMTase) of the COMPASS complex (reviewed in [9,10]). In Arabidopsis, H2B monoubiquitination at K143 coincides with active transcription [11–13]. Deubiquitinating enzymes (DUBs) oppose the function of E3 ligases by deubiquitinating Ub-conjugated proteins. Increased H2Bub1 caused by a mutation in the DUB SUP32/UBP26, leads to reduced H3K9me2 and increased H3K4me3 at transposons that correlate with increased transcription [11]. A key function for DUBs is to generate a pool of free ubiquitin monomers from ubiquitin precursors synthesized from Ub-encoding genes, and from polyubiquitin chains and ubiquitin conjugates [14]. Free monomeric ubiquitin is required under stress conditions, and organisms defective in ubiquitin precursor proteins or DUBs are more sensitive to stress. In yeast, heat stress stimulates the production and activation of the Doa4 deubiquitinase which increases the supply of free monomeric ubiquitin by cleaving polyubiquitin [15].

HKMTases contain SET domains with specificities for different lysine residues on the histone tails, and may be involved in either gene activation or gene repression depending on which lysine residue is methylated [16]. In general, methylation of H3K9, H3K27 and H4K20 has been associated with heterochromatin and gene repression, while H3K4, H3K36 and H3K79 methylation has been related to euchromatin and gene activation [1]. The downstream effect of histone methylation also depends on the number of methyl groups at each lysine residue. Histones mono-, di-, or trimethylated at lysines are differently distributed within eu- and heterochromatin, each potentially indexing a specific biological outcome [17,18]. For example, in Arabidopsis, H3K36 trimethylation, but not H3K36 monomethylation, shows a strong positive correlation with transcription of MADS box genes involved in flowering-time and flower development [19,20].

Although lysine methylation to a large extent is conserved between eukaryotes, the distribution and biological outcome of the methylation may be different. H3K9me1, H3K9me2 and H3K27me2 are for instance predominantly found in the chromocenters of Arabidopsis but not in mouse chromocenters (reviewed in [21,22]). Conversely, H3K9me3 and H4K20me3 that localize to heterochromatin in mouse are mainly associated with euchromatin in Arabidopsis. Additionally, recent results suggest that in contrast to other eukaryotes, H3K9me3 methylation correlates with gene transcription and might have a slight activating function in Arabidopsis [23,24].

H3K9 methylation is carried out by proteins of the SU(VAR)3-9 subgroup which consists of 14 proteins in Arabidopsis; the SU(VAR) 3-9 HOMOLOGs SUVH1-SUVH9, and the more distantly related SU(VAR) 3-9 RELATED proteins SUVR1-5 [25]. In addition to the SET domain the SUVH proteins contain the YDG/SRA domain that has been shown to bind methylated DNA and might direct SUVH mediated H3K9me2 to heterochromatin or stimulate its activity [26]. Thus in Arabidopsis, the SUVH proteins link the epigenetic gene-silencing marks H3K9me2 and DNA-methylation and work as transcriptional repressors of transposons or inverted repeat sequences, for instance by directing CHG methylation via the CMT3 DNA methyltransferase (reviewed in [27]). In contrast to the SUVH proteins, the SUVR1, SUVR2 and SUVR4 proteins do not contain an YDG/SRA domain, but an N-terminal WIYLD domain of unknown function [28], suggesting another mode of action for these proteins. SUVR proteins associate with the nucleolus or euchromatin, and we have earlier shown that SUVR4 can dimethylate H3K9 when this position is monomethylated [28].

In the present study we show that the WIYLD domain of SUVR4 specifically binds ubiquitin, demonstrating a close connection between ubiquitin binding and histone H3K9 methylation. We have furthermore revealed that ubiquitin stimulates the enzyme activity of SUVR4 and converts SUVR4 from a strict dimethylase to a di/trimethylase *in vitro*. Chromatin Immunoprecipitation (ChIP) analysis of Arabidopsis lines with reduced or enhanced expression of *SUVR4*, demonstrate that SUVR4 localizes to both euchromatin and heterochromatin *in vivo*, but only converts H3K9me1 to H3K9me3 at transposons and pseudogenes. SUVR4 dependent H3K9 trimethylation correlates with locus specific transcriptional repression of transposable elements intercalated within euchromatin of the Arabidopsis genome.

Results

The WIYLD domain is a ubiquitin-binding domain

To address the function of the SUVR4 WIYLD domain, a construct encompassing only this domain (Figure 1A) was used in a yeast two-hybrid screen to identify interacting proteins. One positive clone identified in this screen, contained the full-length coding sequence (CDS) of UBIQUITIN EXTENSION PROTEIN 1 (UBQ1, AT3G52590) (Figure 1B). The UBQ1 protein consists of an N-terminal ubiquitin moiety and the C-terminal ribosomal protein L40 [29]. These moieties were subcloned and tested separately for their interaction with SUVR4-WIYLD. Clones containing the ubiquitin moiety, but not clones containing the L40 moiety, supported growth on selective media when transformed into yeast cells and mated with cells containing SUVR4-WIYLD, suggesting that SUVR4 specifically interacts with ubiquitin (Figure 1B). This was confirmed in an *in vitro* pull-down experiment, where SUVR4-WIYLD pulled down full-length UBQ1 and ubiquitin but not L40 (Figure 1C).

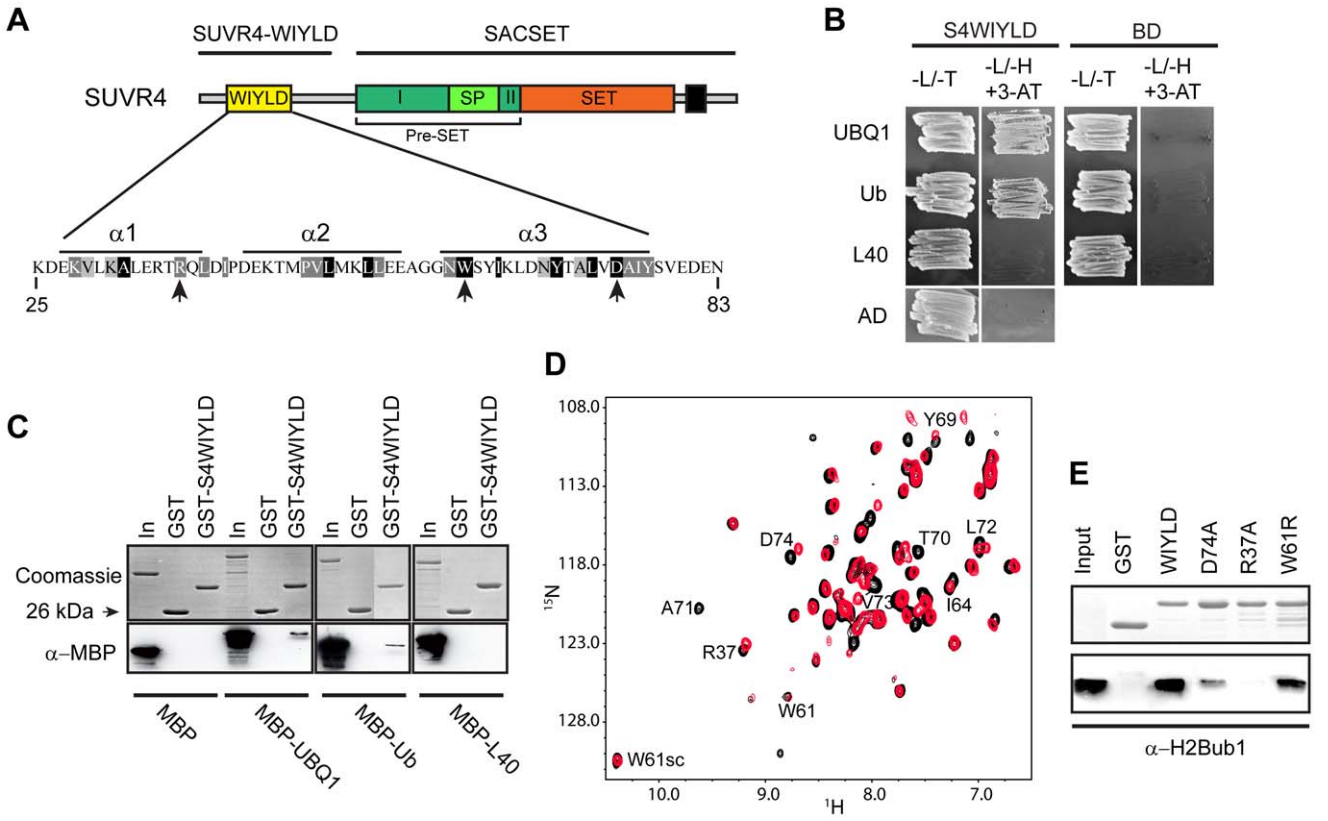

Figure 1. The WIYLD domain is a ubiquitin-binding domain. (A) Layout of the SUVR4 full-length protein, showing the different domains/motifs and the regions included in the two constructs SUVR4-WIYLD and SACSET. SP, SUVR pre-SET; I, pre-SET I; II, pre-SET II; SET, SET domain. Black box indicates the post-SET domain [28].The amino acid sequence of SUVR4 from N_{25} to K_{83} encompassing the WIYLD domain, with conserved residues shaded in black, and residues mutated in this work indicated with arrowheads. (B) Yeast two-hybrid interaction test between SUVR4-WYILD and the full-length CDS of UBQ1, as well as the N-terminal ubiquitin (Ub) and the C-terminal ribosomal L40 moieties of UBQ1. –L/-T – medium selective for diploid colonies; -L/-T/-H +3 AT – medium selective for protein-protein interactions. AD, control mating with empty prey vector; BD, control matings with empty bait vector. (C) GST-SUVR4-WIYLD immobilized on glutathione sepharose beads were used to pull down MBP, MBP-UBQ1, MBP-Ub or MBP-L40 from bacterial lysate. Pull-down reactions were separated on SDS-PAGE, blotted onto a PVDF membrane and detected with an anti-MBP antibody. IN, input (5%); GST, GST negative control. (D) [^{1}H,^{15}N]-HSQC spectrum of SUVR4-WIYLD in its free form (black), and after the addition of excess ubiquitin to a molar ratio of 1:3 (red). The assigned amino acid residues are indicated. (E) GST pull-down of H2Bub1. GST-SUVR4-WIYLD was mutated at positions D74, R37 and W61 and used for GST pull-down of core histones from calf thymus. The pull down reactions were blotted onto a PVDF membrane and probed with an antibody against ubiquitinylated H2B (H2Bub1).
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To address whether the WIYLD domain binds ubiquitin in its unconjugated form and to identify residues directly involved in the interaction between WIYLD and ubiquitin, an NMR analysis was performed. The [^{1}H,^{15}N]-HSQC spectrum of ^{15}N-isotopically labeled SUVR4-WIYLD is well-dispersed demonstrating that the protein domain is folded (Figure 1D). Upon titration of ubiquitin, chemical shift perturbations were observed for a number of residues including the six consecutive amino acids Y_{69}TALVD_{74} of helix 3 (Figure 1D), indicating that they are involved in binding. Alignment of SUVR4-WIYLD with WIYLD domains in other proteins have earlier shown that many of these residues are highly conserved (Figure 1A and [28]).

SUVR4 binds and efficiently methylates calf thymus histone H3 as well as H3K9me1 peptides *in vitro*, but shows only weak activity against recombinant histones, arguing that SUVR4 cross-talks to premodified histones [28]. Since the WIYLD domain binds ubiquitin, and SUVR4 binds and methylates histones, we tested whether the WIYLD domain binds H2B monoubiquitinated on lysine 143 (H2Bub1), which is the only ubiquitination on core histones reported so far in Arabidopsis [11,30]. In these experiments the WIYLD domain indeed was able to pull down H2Bub1, however, when R37 and D74 were mutated, the interaction was strongly reduced (Figure 1E). This supports the chemical shift perturbations shown by the NMR analysis, arguing that these residues are directly involved in ubiquitin binding. Interestingly, the invariant W61 residue that showed no shift in the NMR analysis, only weakly affected the WIYLD-ubiquitin interaction when mutated, confirming that this position is not crucial for ubiquitin binding.

The WIYLD domain enhances the HKMTase activity of SUVR4 through binding of ubiquitin

As the WIYLD domain was able to bind ubiquitin (Figure 1D), we asked whether ubiquitin could stimulate SUVR4 enzyme activity, as previously shown for the deubiquitinase USP5 [31]. To this end, we compared the activity of a SUVR4 protein without the WIYLD domain to a full-length SUVR4 protein, both in fusion with the Maltose Binding Protein (MBP-SACSET and MBP-SUVR4, Figure 1A), with and without the addition of ubiquitin. In both cases the full-length protein showed higher enzymatic activity than the truncated SACSET fragment (Figure 2A, B), suggesting that the WIYLD domain has a positive

effect on the catalytic activity of SUVR4 although the domain itself does not contain HMTase activity (Figure S1C). The difference in activity was more pronounced when ubiquitin was added to the reaction. With ubiquitin the full-length protein was stimulated 2-3 fold whereas the SACSET construct was only weakly affected, suggesting that most of the ubiquitin response is mediated through the WIYLD domain (Figure 2A, B). Addition of free ubiquitin only stimulates enzymatic activity of the SUVR4 protein on histone H3 but does not affect its specificity as no other core histones becomes methylated (Figure 2C).

Ubiquitin converts SUVR4 from a strict dimethylase to a di/trimethylase

Using H3K9me1 and H3K9me2 peptides we tested whether the increased SUVR4 enzyme activity after the addition of ubiquitin also affected the product specificity. As expected from previous results [28], H3K9me1 peptides were the preferred substrate as unmethylated peptides were only weakly methylated (Figure S1A), and no activity against H3K9me2 peptides was observed in the absence of ubiquitin. Methylation of H3K9me1 modified peptides was increased 2.5–3 fold when ubiquitin was added to the reaction (Figure 2D). Unexpectedly we also observed methylation of the H3K9me2 peptide in the presence of ubiquitin, suggesting that ubiquitin converted the SUVR4 protein to a histone H3K9 trimethylase (Figure 2D, Figure S1B). The activity on H3K9me2 peptides was however several folds lower than when H3K9me1 peptides were used. No activity was observed on H3K9me3 peptides either with or without ubiquitin, excluding the possibility that any other lysine of histone H3 1-21 was methylated by SUVR4, underscoring the specificity against H3K9 (Figure 2D).

The products from the enzyme reactions using peptide substrates were analyzed by peptide mass fingerprinting. After 3 hours incubation, the reactions containing SUVR4 only converted 40.9% of the H3K9me1 peptide to H3K9me2, while 0% was converted to H3K9me3 (Figure 2E, upper middle panel). In the reactions containing ubiquitin, 90.2% of the H3K9me1 peptide was converted to H3K9me2 while 3.5% was converted to H3K9me3 (Figure 2E, upper right panel). When H3K9me2 peptides were used as substrate, we did not see any conversion to H3K9me3 above background level in the absence of ubiquitin (3%

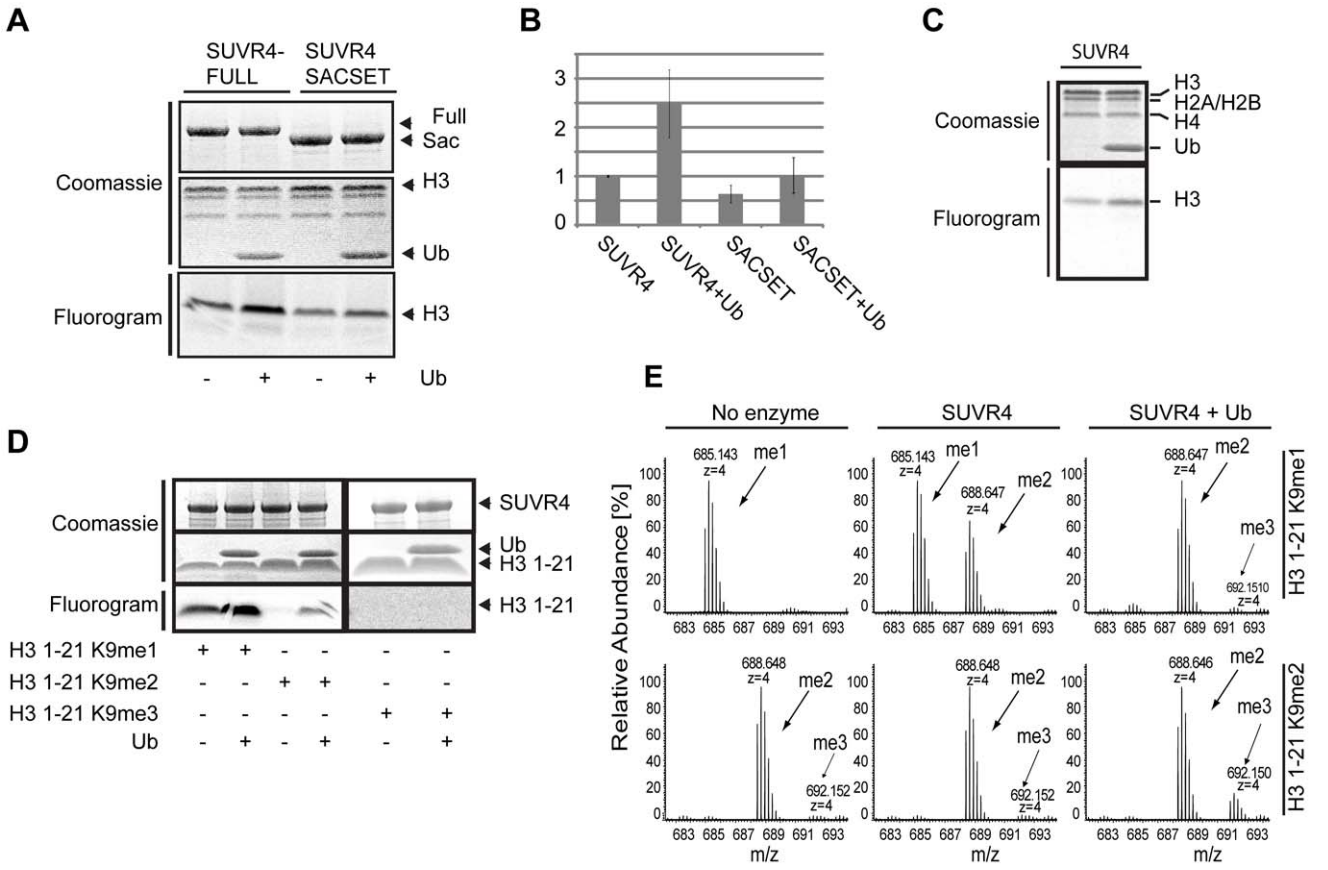

Figure 2. SUVR4 HKMTase activity is stimulated by free ubiquitin *in vitro*. (A) HKMTase assay on core histones using a construct encompassing the SACSET domain of SUVR4 or the full-length SUVR4 protein without and with the addition of free ubiquitin. (B) Quantification of band intensity from fluorogram in A, relative to the reaction with SUVR4 without adding ubiquitin. The graph represents the average of four independent assays. (C) HKMTase assay with SUVR4 full-length using core histones from calf thymus as substrate, without (left) and with (right) the addition of 5 μg free ubiquitin, respectively. (D) The same assay as (C) but using histone H3 1-21 K9me1, H3 1-21 K9me2 or H3 1-21 K9me3 peptides with and without the addition of 5 μg free ubiquitin. (E) Peptide mass fingerprints of the products of an identical HKMTase assay as in C, using unlabelled SAM as methyl donor and H3 1-21 K9me1 (upper panel) or H3 1-21 K9me2 peptides as substrate (lower panel). Products from assays without (left) the addition of SUVR4 enzyme, containing SUVR4 protein (middle) and SUVR4 protein with the addition of 5 μg ubiquitin (right), were analyzed. The mass spectra of each peptide are shown as bars representing the mass-to-charge ratio (m/z), and the most abundant m/z is set to 100%. The length of the bars indicates abundance of the m/z relative to the most abundant. All enzyme assays were repeated at least 4 times with independent protein samples.
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background H3K9me3, versus 3.5% when SUVR4 was added to the reaction) (Figure 2E, lower middle panel), however when ubiquitin was present together with SUVR4, a 16.4% conversion from H3K9me2 to H3K9me3 was found (Figure 2E, lower right panel). This suggests that ubiquitin stimulates the catalytic activity of SUVR4 and alters the product specificity in that it converts SUVR4 from a strict dimethylase to a di/trimethylase.

SUVR4 directs H3K9 trimethylation to transposon chromatin

As SUVR4 converts H3K9me1 to H3K9me2/3 *in vitro*, we asked how these modifications were affected by SUVR4 *in vivo*. Since no SUVR4 T-DNA knock-out insertion lines were available, knock-down RNAi lines for SUVR4 were established. We also generated GFP overexpression (OE) lines where SUVR4-GFP expression was driven by the strong constitutive 35S promoter, giving a uniform SUVR4-distribution in the nucleus in addition to accumulation in the nucleolus or in foci of unknown function (Figure S2). A weaker glucocorticoid-inducible construct has earlier been reported to give an almost exclusive nucleolar localization of SUVR4 [28]. We did not observe any phenotypes under the tested growing conditions for neither the SUVR4-GFP line, nor the SUVR4 RNAi line.

H3K9me1-3 display different nuclear distributions, with high H3K9me1/2 in chromocenters and pericentric heterochromatin, whereas H3K9me3 is distributed more uniformly in the nucleoplasm with highest concentration in euchromatin and at expressed genes [32]. Immunocytological analysis on seedling leaves using specific antibodies against H3K9me showed a strong reduction in H3K9me1 and a corresponding increase in H3K9me3 in nuclei with high SUVR4-GFP expression (Figure 3A). Nuclei from lines with a low SUVR4-GFP expression did not show this effect on H3K9me1 and H3K9me3 methylation, suggesting that the global changes in H3K9me1 and H3K9me3 correlated with SUVR4-GFP expression (Figure 3A).

To analyze this effect at individual genes, ChIP experiments were performed with the same antibodies as used for immunocytological analysis and an antibody specific for GFP, respectively. Different classes of transposon sequences were selected for ChIP analysis, as these sequences are likely targets of SUVR4 because of their high H3K9me1 level (Figure 3B and Table 1). These experiments confirmed that SUVR4 is associated with transposons and genes both in eu- and heterochromatin, but a significantly higher amount of SUVR4-GFP is found at euchromatic genes like *TUB8* and *ACTIN2* (Figure S3). However, only transposon and pseudogenes like *AtSN1, AtGP1, AtMU1, AtCOPIA4* and *MULE At2g15810* were affected by overexpression of SUVR4, resulting in a drastic increase in H3K9me3 and reduction of H3K9me1 (Figure 3B). We did not see any effect of SUVR4 OE for highly expressed genes like *TUB8* or *ACTIN2*, or for the moderately expressed transposon At4g13120, all with an already low level of H3K9me1. Although having a dramatic effect on H3K9me3 at transposons, SUVR4 OE did not affect the distribution of the euchromatic mark H2Bub1 at any of the tested sequences (Figure S4A).

As the *35S* driven SUVR4-GFP construct could lead to unspecific downstream effects due to ectopic and elevated SUVR4 expression, we complemented the OE data with ChIP analysis of two of the transposons in knock-down SUVR4 RNAi plants. The RNAi lines showed a 90% reduction of the SUVR4 expression level compared to wild type (Figure S5 A). In contrast to the OE line, there was an increase of H3K9me1 on *AtSN1* and *MULE At2g15810* (Figure 3C). Furthermore, there was a corresponding reduction of H3K9me3, suggesting that SUVR4 directs H3K9me3 methylation on transposons. The weak reduction of H3K9me3 could reflect the residual SUVR4 expression in the RNAi line and possibly redundancy with other H3K9me3 methyltransferases at these sequences. Together, these data suggest that although SUVR4 is localized in both eu- and heterochromatin, it is active only on target sequences with a high level of H3K9me1, where its activity increases H3K9me3 at the expense of the H3K9me1 level.

H3K4me3 reduces SUVR4 HKMTase activity

Recent studies suggest that in Arabidopsis H3K9me3 associates with euchromatin and transcriptional activation of genes [23,24,32]. In contrast, H3K9me1 is a mark mainly associated with repetitive sequences in chromocenters and pericentric heterochromatin in Arabidopsis [21]. The specific activity of SUVR4 on transposon chromatin although associated with both transposons and euchromatic genes (Figure 3, S3), made us speculate that the lack of SUVR4 activity on euchromatic genes was due to cross-talk to PTMs characteristic for euchromatin. We thus tested histone tail peptides that were mono- or trimethylated at H3K4 but devoid of H3K9me in an *in vitro* HKMTase assay (Figure 4). SUVR4 activity was not affected by monomethyl H3K4, whereas trimethyl H3K4 reduced SUVR4 activity significantly (Figure 4 A, B), arguing that chromatin associated with genes like *TUB8* and *ACTIN2*, with a high level of this mark, might not be good substrate for SUVR4 activity.

SUVR4 is a transcriptional repressor of transposable elements

To evaluate the effect of SUVR4 mediated H3K9me3 methylation on transposon transcription we investigated the expression of three of the ChIP-analyzed transposons, *MULE At2g15810*, *AtIS112A* (At4g04293) and *AtCOPIA4*, which all had a high level of H3K9me1 and were expressed in wild type plants (Figure 3B, C, Figure S5 B and Table 1). In the OE line, all the studied transposons showed significant reduction in expression compared to wild type (60%, 80% and 35%, respectively, Figure 5A), suggesting that SUVR4 acts as a repressor of these transposable elements. As a control, we used the At4g13120 transposable element of intermediate expression with a very low H3K9me1 level which is not a target of SUVR4 methylation (Figure 3, Figure 5A and Table 1). This transposon was also unaffected in its transcription level in SUVR4-GFP overexpression lines.

In the RNAi line we did not see a corresponding release of repression for the *AtCOPIA4* and *AtIS112A* elements, however, the *MULE At2g15810* element was induced 2.5 to 3- fold in the RNAi line compared to wild type (Figure 5A). Interestingly, the gene *Cyp40* which is known to be regulated by MULE [33] showed the same expression response to SUVR4 as *MULE At2g15810*, although weaker (Figure 5A). The *AtSN1* repeat interspersed within euchromatin, and the heterochromatin localized *AtMU1* that are silent in wild type plants (Table 1 and Figure S5 B), were examined in both the RNAi and OE line but we did not detect any signal above the –RT control reaction, arguing that these transposons were not reactivated in any of the lines (data not shown).

SUVR4 shows a locus-specific effect on DNA methylation

H3K9me2 directed by SUVH proteins regulates non-CG methylation in Arabidopsis [34]. To determine if there was a similar correlation between DNA methylation and the H3K9me3 methylation directed by SUVR4, bisulfite sequencing was

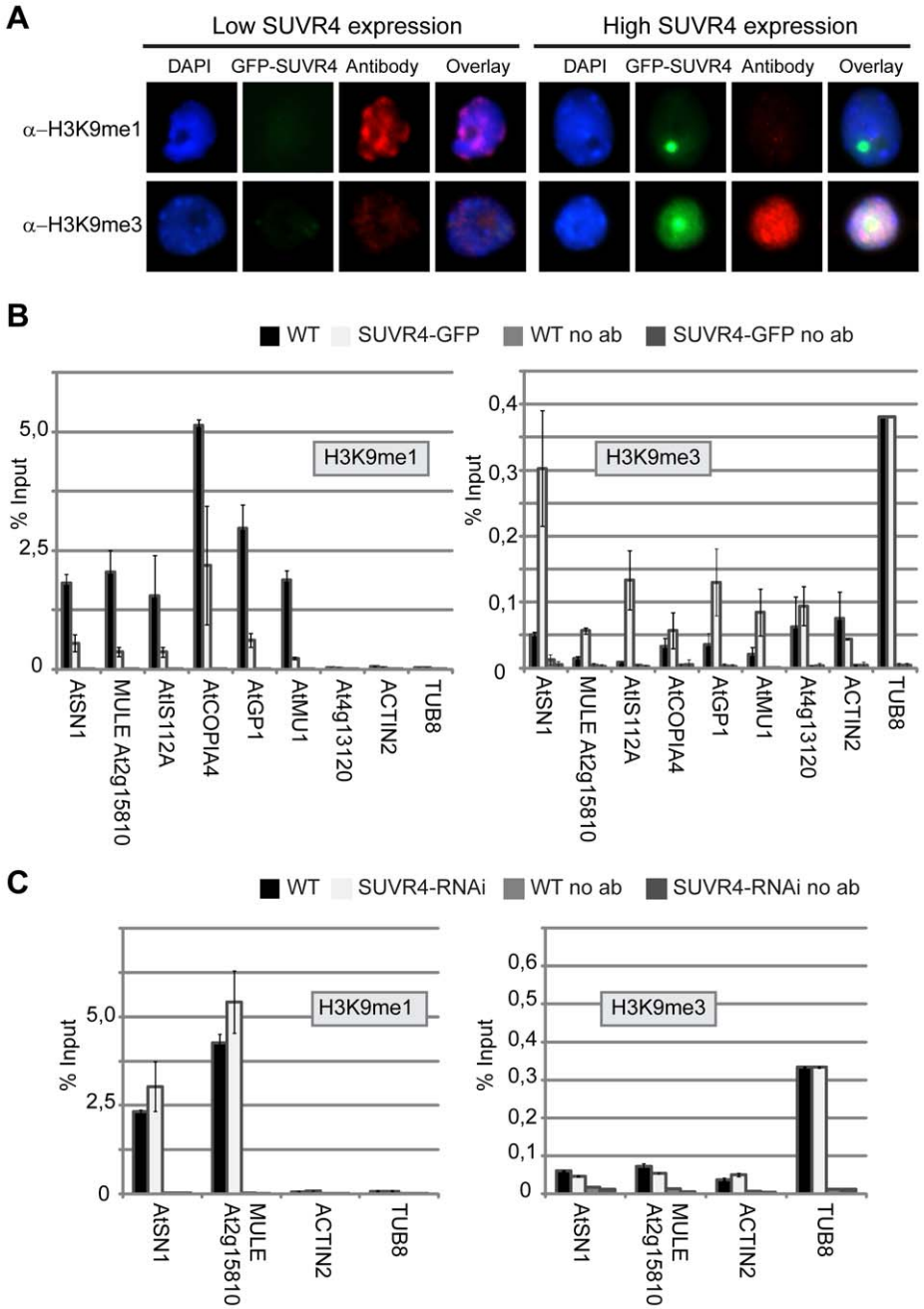

Figure 3. SUVR4 directs H3K9me3 on transposon and repeat sequences. (A) Immunostaining of nuclei from SUVR4-GFP^{OE} seedlings with low expression (left panel) or high expression (right panel) of SUVR4-GFP with antibodies against H3K9me1 or H3K9me3. ChIP analysis of (B) SUVR4-GFP^{OE} and (C) SUVR4 RNAi lines using antibodies against H3K9me1 (left) or H3K9me3 (right). DNA levels from the ChIP experiments (B, C) relative to the input reactions were quantified using real time PCR and normalized to *TUB8*. The bars represent the average of two independent biological replicates.
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performed on two of the transposons that are targets of SUVR4 histone lysine methylation. We did not detect an effect of SUVR4 activity on DNA methylation of the *MULE At2g15810* transposon for CG, CHG or CHH in neither SUVR4 OE nor SUVR4 RNAi lines (Figure 5B). This suggests that the repressive effect of H3K9me3 added by SUVR4 is not mediated by DNA methylation. In contrast, the *AtSN1* transposon showed an increase in CHH methylation (Figure 5C) in the OE line. The CG and CHG methylation levels were unaffected. There was, however, no corresponding reduction of CHH methylation in the RNAi-line.

The ubiquitin protease UBP26 regulates the H3K9me2 and H3K9me3 level on transposons

The ubiquitin binding properties of the SUVR4 WIYLD domain and the ubiquitin-enhanced H3K9me3 activity of SUVR4 *in vitro* led us to look for links between ubiquitin and H3K9 trimethylation *in vivo*. Interestingly, deubiquitination of H2BUb1 by the nuclear UBP26/SUP32 ubiquitin protease, is required for repression of transposons [11], which also are targets of SUVR4. Therefore we investigated the H3K9me levels in the *ubp26-1/sup32* mutant (Figure S6). No effect was seen on highly expressed

Table 1. Transposon expression in various mutant backgrounds.

Gene/transposon	Agi Code	Type	Localization	*mom1*[a,b]	*kyp* [c, d]	*dc/ddc*[a]	*met1*[c,d]	K27 me3[c]
AtSN1	At3g44000/5	Retrotransposon	Euchromatin	-	X/UP	nd	X/UP	yes
MULE	At2g15810	DNA transposon	Euchromatin	UP	X/NoE	-	X/UP	yes
AtIS112A	At4g04293	DNA transposon	Euchromatin	UP	nd	UP	nd	nd
ATCOPIA4	At4g16870	Retrotransposon	nd	nd	X/Up	nd	X/UP	nd
ATGP1	At4g03650	Retrotransposon	Heterochromatin	nd	-	nd	X/UP	nd
AtMu1	At4g08680	DNA transposon	Heterochromatin	-	X/NoE	nd	X/UP	nd
AT4G13120	AT4g13120	DNA transposon	Euchromatin	nd	nd	nd	nd	nd
ACTIN2	AT3g18780	Non-TE control	Euchromatin	nd	-	-	-	nd
TUB8	AT5g23860	Non-TE control	Euchromatin	nd	-	-	-	yes

a) Numa et al., 2010 [52],
b) Habu et al.,2006 [33],
c) Mathieu O, Probst AV, Paszkowski J (2005) Distinct regulation of histone H3 methylation at lysines 27 and 9 by CpG methylation in Arabidopsis. EMBO J 24: 2783-2791
d) Lippman Z, Gendrel AV, Black M, Vaughn MW, Dedhia N, et al. (2004) Role of transposable elements in heterochromatin and epigenetic control. Nature 430: 471-476.
NoE = No expression, UP = increased transcription, X = affected in histone or DNA methylation, - = not affected, nd = not determined.
doi:10.1371/journal.pgen.1001325.t001

genes like *TUB8* and *ACTIN2* (Figure 6), and consistent with earlier findings [11], our ChIP analysis showed a reduction of H3K9me2 on transposons and repeat sequences (Figure 6A). Similarly, H3K9me3 was also reduced on transposons in the mutant compared to the wild type (Figure 6B). Although mutation in the *UBP26/SUP32* gene has been reported to lead to a global accumulation of H2Bub1 [35], the H2Bub1 level on transposons was only weakly affected by the mutation (Figure 6C), and the level of free ubiquitin monomers in the nuclei of *ubp26-1/sup32* was similar to the level in the wild type (Figure 6D).

We next tested the effect of global reduction of H2Bub1 on H3K9me3 level on transposon chromatin using the *hub2-2* mutant. This mutant is defect in the HISTONE MONOUBIQUITINATION2 E3 ligase, which acts non-redundantly with HUB1 to monoubiquitinate histone H2B [13]. The *hub2-2* mutant showed an almost complete lack of H2Bub1 at the *TUB8* gene, while the effect was absent or negligible on the *AtGP1* transposon. As reported for H3K9me2 [13,36], the H3K9me3 level was not affected either on *TUB8* or on transposon chromatin (Figure S7).

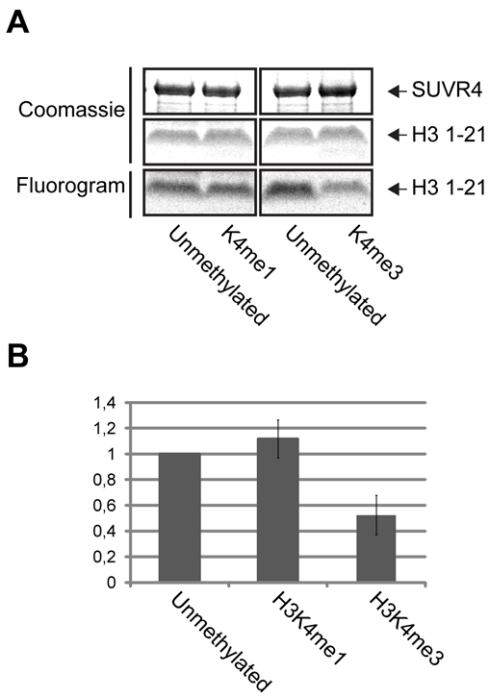

Figure 4. SUVR4 HKMTase activity is inhibited by H3K4me3. (A) HKMTase assay showing SUVR4 activity on peptides covering the first 1-21 aa of histone H3, that are unmodified, monomethylated or trimethylated on K4. (B) Quantification of band intensity from fluorogram in A, relative to the reaction with unmodified H3 1-21 peptide. The bars represent the average of three independent HKMTase assays.
doi:10.1371/journal.pgen.1001325.g004

Discussion

H3K9me3 has only recently been confirmed as a histone modification present in Arabidopsis, and its significance in gene regulation has only been indicative [23,24]. The presented work identifies SUVR4 as the first histone H3K9me3 methyltransferase in Arabidopsis and demonstrates how it cross-talks to ubiquitin and chromatin modifications like H3K9me1 and H3K4me3 to repress transposon transcription.

The WIYLD domain is a ubiquitin-binding domain pivotal for the HKMTase activity of the SUVR4 protein

Our experiments have identified the WIYLD domain of the SUVR4 HKMTase as a new ubiquitin interacting domain, demonstrating a direct link between ubiquitin binding and H3K9 methylation. Ubiquitin is extensively distributed in the eukaryotic proteome, and exists as free ubiquitin monomers, ubiquitin extension proteins, polyubiquitin, or ubiquitin conjugates [14]. The interactions with free ubiquitin, the ubiquitin moiety of the ubiquitin extension protein UBQ1 and the ubiquitin conjugate H2Bub1 (Figure 1), indicate that the SUVR4 WIYLD domain can target ubiquitin either in its free or conjugated form.

The interaction between the WIYLD domain of SUVR4 and ubiquitin is further supported by the WIYLD-dependent positive effect of ubiquitin on enzymatic activity (Figure 2). Free ubiquitin stimulated the HKMTase activity of the full-length SUVR4 protein without compromising the substrate specificity because no histones other than H3 were methylated (Figure 2C). However,

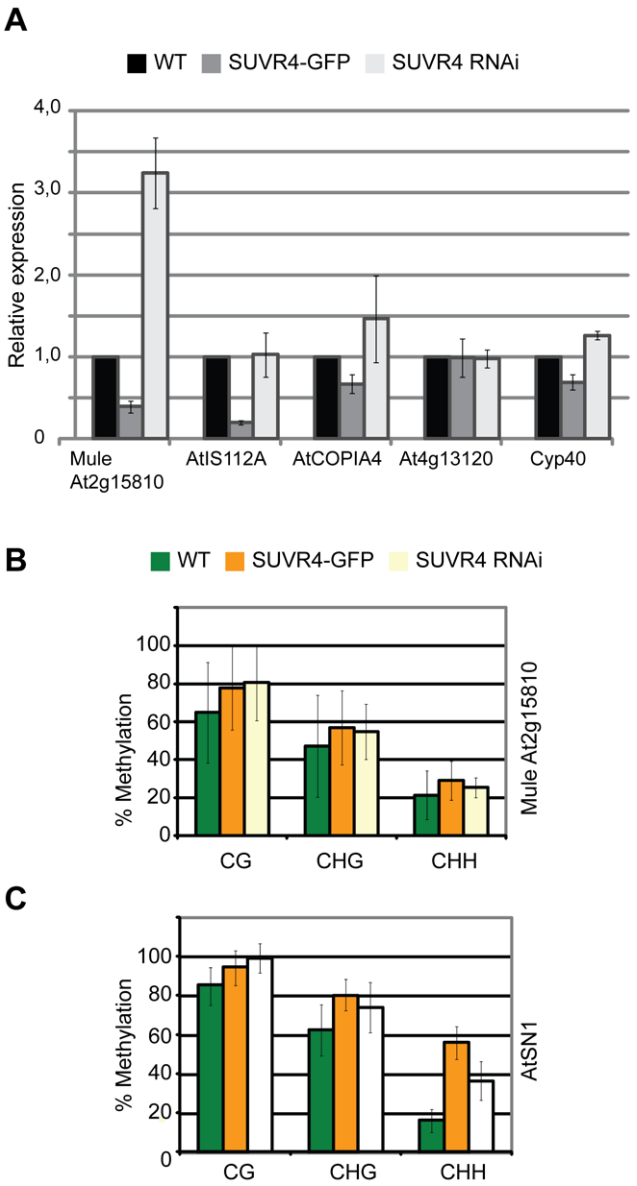

Figure 5. SUVR4 represses transcription of transposons. (A) Real time RT-PCR quantification of transcripts reversely transcribed from mRNA isolated from 14 day old SUVR4-GFP^{OE} and SUVR4-RNAi seedlings, respectively. The data were normalized to *ACTIN2* and shown relative to wild type. (B, C) Quantification of bisulfite treated DNA from wt, SUVR4^{OE} and SUVR4 RNAi seedlings for *MULE At2g15810* (B) and *AtSN1* (C) respectively.
doi:10.1371/journal.pgen.1001325.g005

the addition of free ubiquitin (Ub) converted the protein from a strict H3K9me2 to a H3K9me2/me3 methyltransferase (Figure 2D, 2E), suggesting that ubiquitin either in its free form or conjugated to other proteins like H2B can act as a signal for H3K9 trimethylation. We only observed 3% conversion of H3K9me1 to H3K9me3 after a 3 hour reaction time in our *in vitro* HKMTase assay while most of the H3K9me1 was converted to H3K9me2 (Figure 2E). In contrast, a massive shift from H3K9me1 to H3K9me3 was seen *in vivo* when over-expressing SUVR4 (Figure 3A, 3B). Together this implies the need for another component in addition to ubiquitin for SUVR4 to efficiently convert H3K9me1 to H3K9me3 *in vitro*, as shown for the murine ESET HKMTase [37]. In recombinant form *in vitro* ESET only catalyzes mono- and dimethylation of H3K9, but in complex with the transcriptional repressor mAM the enzyme generates H3K9me3.

Interestingly, the truncated SUVR4 SACSET protein showed a lower HKMTase activity compared to the full-length SUVR4 protein on core histones (Figure 2A), arguing that the N-terminal WIYLD domain is essential for normal activity of the C-terminal SET domain. Furthermore, the activity of the SUVR4 SACSET was only weakly enhanced by ubiquitin (Figure 2A, 2B), demonstrating that ubiquitin in its free form stimulates SUVR4 activity mainly through the WIYLD domain. Several enzymes that are involved in Ub pathways have shown to be regulated by ubiquitin. Recently, the activity of the mammalian deubiquitination enzyme ataxin-3 was shown to be enhanced by ubiquitination [38], and binding of free ubiquitin to the N-terminal ZnF-UBP domain of the deubiquitinase USP5 led to a conformational change that stimulated enzyme activity [31].

SUVR4 converts H3K9me1 to H3K9me2/me3 at transposons

In Arabidopsis H3K9me3 methylation broadly marks 40% of all genes within euchromatin [39]. In addition a low but detectable level of H3K9me3 methylation is found in regions with silenced transposons and pseudogenes [24] (Figure 3 and Figure 6). Our ChIP results suggest that although associated with both eu- and heterochromatin, SUVR4 has no HKMTase activity on euchromatic genes, but specifically targets transposons and repeat sequences where it converts H3K9me1 to H3K9me3 (Figure 3B, 3C). This is perfectly in line with our *in vitro* HKMTase results, which show that SUVR4 preferably uses H3K9me1 as substrate (Figure 2D). Together the *in vivo* and *in vitro* data indicate that SUVR4 only methylates transposons with a high H3K9me1 level although the protein might also associate with regions with a low level of this modification (Figure S3).

SUVR4 methylates unmethylated H3 poorly, and the level of H3K9me1 decreases in the OE line (Figure S1A and Figure 3B). This suggests that SUVR4 does not itself monomethylate H3K9 *in vivo*. Both SUVH4 and SUVH6 are efficient monomethyl transferases *in vitro* [40], which together with SUVH5 control the deposition of the majority of H3K9me1 at transposons and repeat sequences [41]. As SUVR4 targets the same type of sequences, it is likely that SUVR4 uses the monomethylated histone substrates created by the SUVH proteins to trimethylate H3K9. In mammalian cells, the SUV39H1 HKMTase depends on a monomethylase as it preferably converts H3K9me1 of H3.1, but not H3K9me2 of H3.3, to H3K9me3. [42]. Similarly, SUVR4 is stimulated by H3K9me1, but is only active on H3K9me2 if ubiquitin is added to the *in vitro* reaction.

The SUVH2 HKMTase has a strong impact on centromeric and pericentromeric heterochromatinization and gene silencing and reduces the level of H3K9me3 when overexpressed [32]. In contrast, overexpression of SUVR4 leads to increased H3K9me3 levels, and no changes in heterochromatinization could be observed (Figure 3A). Pericentromeric regions contain high levels of H3K9me1 and H3K9me2 in plants, but also H3S10 phoshporylation during mitosis and meiosis II [22]. The cell cycle dependent H3S10ph modification generated by Aurora kinase 1 inhibits SUVR4 activity *in vitro* [43]. This and the uninterrupted regions of high levels of H3K9me2 associated with the many transposons and pseudogenes located in pericentromeric and centromeric heterochromatin [44], may contribute to repress SUVR4 activity in these regions in dividing cells. Alternatively, SUVR4 might be able to methylate histones in pericentric heterochromatin before H3S10ph is added as Aurora kinase 1 is

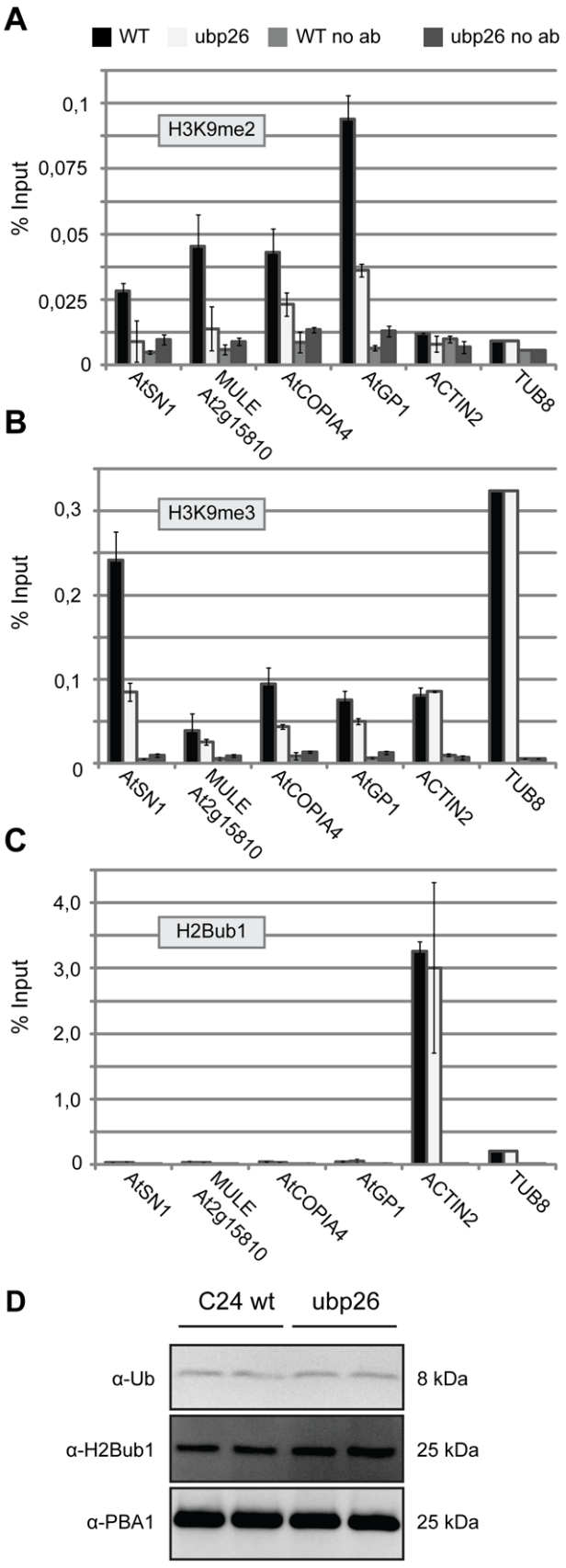

Figure 6. UBP26 directs H3K9me2 and H3K9me3 on transposon sequences. ChIP analysis of *ubp26-1* lines using antibodies against (A) H3K9me2, (B) H3K9me3 or (C) H2Bub1. DNA levels from the ChIP experiments relative to the input reactions were quantified using real time PCR and normalized to *TUB8*. The bars represent the average of two independent biological replicates. (D) Western blot of nuclear proteins isolated from *ubp26-1* and wild type, probed with antibodies against ubiquitin (ub), H2Bub1 or PBA1 (loading control).
doi:10.1371/journal.pgen.1001325.g006

active on methylated histones. Although pericentric heterochromatin most likely is not the preferred target of SUVR4 activity because of the high level of uninterrupted H3K9me2 [44], SUVR4 could potentially methylate transposons in these regions under certain conditions when ubiquitin levels are high, as demonstrated by the ability of SUVR4 to methylate H3K9me2 peptides when ubiquitin is added (Figure 2D, 2E, Figure S1B, and Figure 7B).

Mutation in the SUP32/UBP26 deubiquitinating enzyme that removes the ubiquitin conjugate from H2Bub1 has been reported to lead to reduction in H3K9me2 [11]. Using ChIP analysis we found low levels of H2Bub1 at all tested transposons, which were only weakly altered in the *ubp26* mutant line (Figure 6C). A reduction of both H3K9me2 and H3K9me3 was, however, observed on the same sequences targeted by SUVR4 (Figure 3B, 3C and Figure 6A, 6B). We therefore suggest that SUVR4 and UBP26 act in the same pathway leading to repression of transposon activity, and speculate that the reduction of H3K9me3 in *ubp26-1* mutant background can be due to reduced SUVR4 activity. Thus UBP26 can repress transposon transcription by lowering the H2Bub1 level at these sequences to maintain repressive H3 methylation as suggested by Sridhar et al. [11], and/or by maintaining a high local level of free ubiquitin which stimulates SUVR4-mediated H3K9me3 (Figure 7). Possibly UBP26/SUP32 can also cleave the ubiquitin extension protein UBQ1 initially found in our yeast two-hybrid screen to obtain free ubiquitin, as it has been shown to also be active on the human homologue CEP52 [11] which has 92% sequence identity with UBQ1. We did not however observe any reduction of free ubiquitin in the nuclear extracts of *ubp26-1* mutants (Figure 6D) that might have affected SUVR4 activity, and there was no effect on H3K9me3 or H2Bub1 at transposon sequences in the *hub2-2* line (Figure S7). Thus, HUB2 seems not to be involved in regulation of H2Bub1 or H3K9me2/3 or to be the counterpart of UBP26 on transposon chromatin. The minor reduction of H2Bub1 at transposons and the ability of UBP26/SUP32 to deubiquitinate the CEP52 *in vitro*, opens the possibility that UBP26 regulates SUVR4-dependent H3K9me2/3 by additional mechanisms, for instance transient changes in the levels or subnuclear distribution of free ubiquitin.

Highly transcribed euchromatic genes like *ACTIN2* and *TUB8* were unaffected by SUVR4, and the *in vitro* assay implies that SUVR4 activity is inhibited by H3K4me3 which is abundant in euchromatin (Figure 4). Furthermore, the *in vivo* data shows that the targets for SUVR4 activity have low levels of H3K4me3, H3K9me3 and H2Bub1 (Figure 3, Figure 6, and Figure S4). Intercalary heterochromatic sequences located within euchromatin are associated with intermediate amounts of opposing histone marks like H3K4me2 and H3K9me2 [33,44], but have comparable levels of H3K9me1 as heterochromatin (Figure 3B, 3C). As depicted in the model in Figure 7, this suggests that SUVR4 cross-talks to other PTMs and preferably targets transposons outside pericentric and centromeric heterochromatin, with low H3S10ph, H3K9me2, H3K4me3 and H2Bub1 and high H3K9me1 in order to trimethylate H3K9.

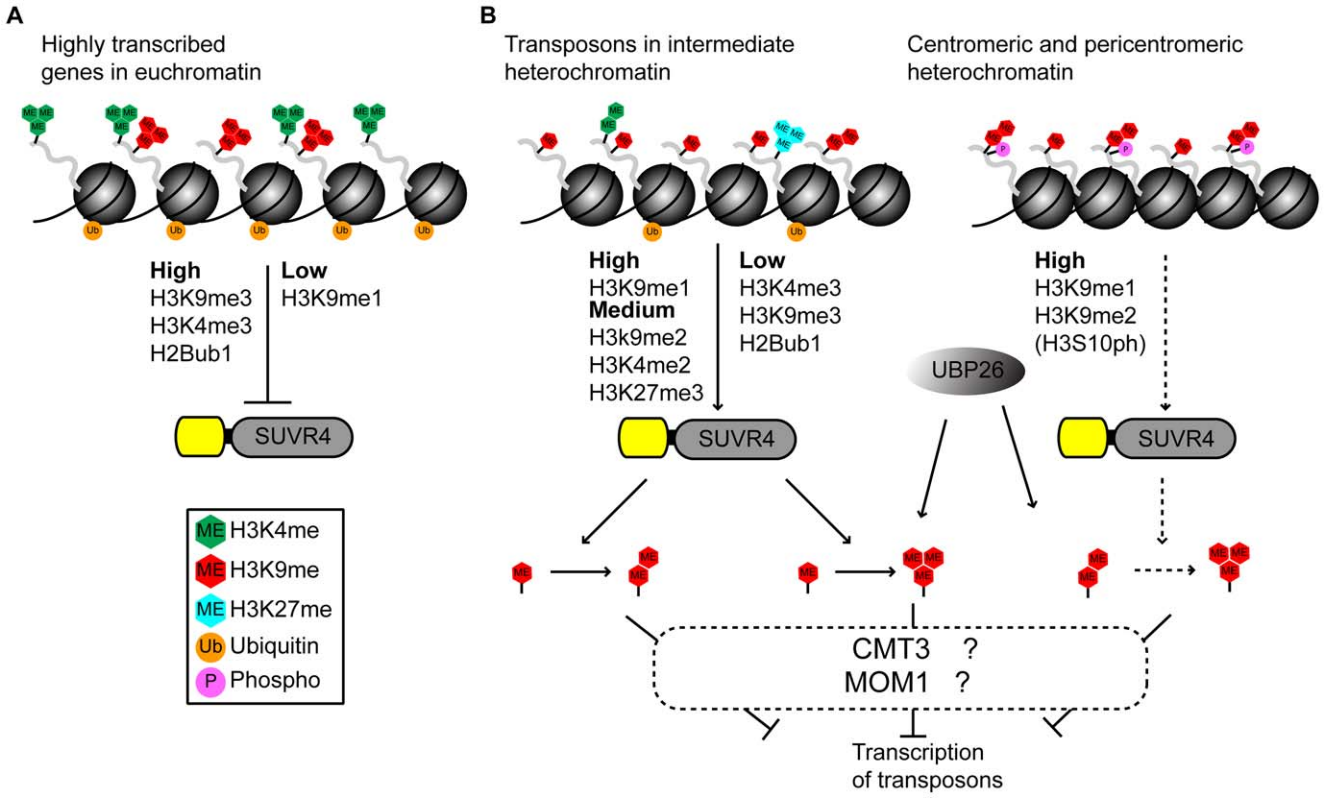

Figure 7. Model describing the relationship between free ubiquitin and SUVR4 activity on transposons. (A) SUVR4 is repressed by H3K4me3 *in vitro*, and has no activity on genes with high H3K4me3, H3K9me3, H2Bub1 and a low level of H3K9me1. (B) SUVR4's preference for heterochromatic transposons intercalated within euchromatin is maintained by its specificity for H3K9me1 which is highly enriched at transposons, and its repression by activating marks like H3K4me3. The deubiquitinase UBP26 regulates H3K9me2/me3 at the same targets as SUVR4, and might produce free ubiquitin that stimulates the H3K9me2/me3 activity of SUVR4 at target transposons. Although SUVR4 normally is repressed by H3K9me2 and H3S10ph which is high in pericentric heterochromatin, these regions may be targets for SUVR4 activity when ubiquitin levels are high. Since the transposons also contain a medium level of H3K27me3 in addition to H3K9me3, this could possibly create a binding site for CMT3 in order to repress transcription in a DNA methylation-dependent manner at some transposons. At other transposons, transcription may be repressed in a DNA methylation- independent manner by the MOM transcriptional repressor (See text for details).
doi:10.1371/journal.pgen.1001325.g007

SUVR4-mediated conversion of H3K9me1 to H3K9me3 represses transposon transcription in a locus specific manner

For transposon sequences with a low or intermediate expression level in wild type plants, increase in H3K9me3 levels mediated by SUVR4 is associated with repression of transcription (Figure 3, Figure 5, and Figure 7). In the RNAi line only the *MULE At2g15810* transposon, localized in euchromatin outside the typical pericentric heterochromatin or centromeric regions [33], showed relief of repression (Figure 5A), suggesting it to be a normal target of SUVR4 activity. However, *AtIS112A*, another transposon intercalated in euchromatin with an intermediate expression level, was only affected in the OE line. The heterochromatin localized *AtMU1* and the euchromatin localized *AtSN1*, both silent in wild type plants, were also targets for SUVR4 methylation but showed no reactivation in the RNAi line. This suggests that SUVR4-directed H3K9me3 regulates transposon activity in a locus specific manner, where SUVR4 activity alone is sufficient for repression of *MULE At2g15810*, while it works redundantly with an unknown HKMTase at other elements like *AtIS112A, AtMU1* and *AtSN1*. A similar regulation can be seen for the SUVH2 and SUVH9 SET domain proteins that act redundantly at some loci but independently at others [45]. Thus different transposons are regulated by different combinations of epigenetic marks (Table 1).

Genes in euchromatin have a much higher level of H3K9me3 than transposons, and in these regions this modification seems to correlate with activation of transcription and the deposition of other activating marks [23,24]. This argues for a combinatorial readout where the context of other PTMs with which H3K9me3 appears decides the biological outcome (Figure 7). In contrast to genes, transposon and repeat sequences contain a high level of H3K9me1 and low levels of H3K4me3 and H2Bub1 (Figure 3B, 3C, and Figure S4) and in this context H3K9me3 may lead to repression of transcription.

H3K9me1 on transposon chromatin seems to be a prerequisite and the preferred substrate for SUVR4 activity, as the control transposon At4g13120, with very low H3K9me1, was not methylated or affected at the transcriptional level (Figure 5A). Several studies have reported the accumulation of H3K9me1 in heterochromatin (reviewed in [22]) but little is known about the function of this mark. Our data supports a model where H3K9me1 is associated with both pericentric and centromeric heterochromatin and transposons intercalated in euchromatin, but does not act as a repressive signal, but rather a template for other methyltransferases. This is supported by the observation that increased H3K9me1 level correlated with increased transcription in the SUVR4 RNAi line and inversely correlated with increased H3K9me3 and repression of transcription in the SUVR4-GFP^{OE} line (Figure 3A–3C and Figure 5A).

H3K9me3 by SUVR4 may promote methylation-dependent and -independent repression of transposons

The level of DNA methylation of the *MULE At2g15810* transposon did not correlate with SUVR4 expression. At the *AtSN1* transposon, however, increased H3K9me3 mediated by SUVR4 overexpression coincided with an increase of CHH while no effect was seen for CG methylation (Figure 5B, 5C). Pericentric H3K9me2 shows a strong correlation with CHG methylation but a weaker correlation with CG and CHH methylation [44], while transposons located outside pericentric or centromeric heterochromatin have shorter patches of H3K9me2 at lower levels. Together with the repressive effect of H3K9me2 on SUVR4 activity this argues that the main DNA methylation regulated by SUVR4 is CHH.

The DRM2 methylase is the main regulator of asymmetric CHH methylation, while CHROMOMETHYLASE3 (CMT3) is the main regulator of CHG methylation in Arabidopsis, but at some loci they work together [46,47]. At dispersed repeats within euchromatin like *AtSN1*, DRM1, DRM2 and CMT3 act redundantly to maintain CHH and CHG methylation [48]. At such loci we suggest that the H3K9me3 methylation by SUVR4 might mark the underlying transposon sequence for CHH methylation by DRM2/CMT3 (Figure 7B). Interestingly, many transposon sequences contain both H3K27me3 and H3K9me3, a combination that CMT3 has been shown to bind *in vitro* (Table 1, [24,30,49,50]). The redundant regulation of *AtSN1* by CMT3 and DRM1 might thus explain the lack of reactivation and DNA methylation upon reduction of SUVR4 H3K9me3 methylation in the SUVR4 RNAi line.

Although a target of SUVR4-directed H3K9me3 and repression, the *MULE* transposon was not affected at the DNA methylation level (Figure 5B). In contrast to *AtSN1*, this transposon has been shown earlier to be activated only in *mom1* mutants, and not in mutants with reduced non-CG methylation and *kyp/suvh4* mutants (Table 1). MOM1 is a transcriptional repressor that regulates transcriptional gene silencing of loci outside centromeric and pericentromeric heterochromatin, with only small effects on epigenetic marks [33,51,52]. This suggests that non-CG methylation is not involved in silencing of *MULE*. The similar relief of silencing without any effect on DNA methylation between SUVR4 RNAi and *mom1* makes it tempting to speculate that SUVR4 recruits MOM1 to its targets in order to repress transcription at this locus (Figure 7B). The intermediately expressed *AtIS112A* is repressed in SUVR4 OE lines but did not show any relief of expression in the RNAi line. As for *AtSN1*, this transposon is regulated by non-CG methylation, but also by MOM1. This argues that SUVR4 mediated repression might act via DNA methylation-independent mechanisms such as for *MULE At2g15810*, but also by DNA methylation-dependent mechanisms as seen for *AtSN1*, or possibly both as seen for *AtIS112A*.

DUBs are important to maintain ubiquitin homeostasis by recycling ubiquitin from free ubiquitin chains, ubiquitin conjugates and ubiquitin fusion proteins [14,15]. UBP26 regulates H3K9me2 and H3K9me3 methylation as well as non-CG methylation at the same sequences as SUVR4 [11]. We hypothesize that UBP26 acts in concert with SUVR4 to trimethylate transposons with a high level of H3K9me1 and low level of H3K4me3 and H2Bub1 (Figure 7). The H3K9me3 methylation thus directs locus-specific methylation-dependent or -independent repression of transposon activity.

Methods

Plant material

Arabidopsis plants, ecotype Columbia (Col), were grown under long day greenhouse conditions at 18°C. Transgenic *Arabidopsis* plants were generated by the floral dip method [53] using the *Agrobacterium tumefaciens* strain C58 pCV2260. Transgenic plants containing the pEG104 [54] or pART27 [55] vectors were selected on MS-2 medium (1x Murashige and Skoog salts, 0.05% 2-N-morpholino/ethanesulfonic acid, 2% sucrose, 0.8% agar) containing 10 μg/ml basta or 50 μg/ml kanamycin, respectively. For ChIP, RT-PCR and cytology experiments, Col wild type plants and non-segregating lines containing the respective T-DNA constructs were grown on MS-2 without antibiotic selection. The *ubp26*-mutant [11] and the *hub2-2* [13] mutant lines have been described earlier.

RNA extraction, cDNA synthesis, and real-time PCR

RNA was isolated from approx. 100 mg of 14 day old seedlings using the Spectrum Plant Total RNA Kit with on-column DNase treatment (Sigma). cDNA synthesis and Real time RT-PCR experiments were performed as described previously [20] using gene specific primers (Table S1), except that 4 μg of total RNA was used to synthesize first strand cDNA with Superscript III Reverse Transcriptase and random primers (Invitrogen).

DNA constructs

SUVR4-Full (At3g04380), SUVR4-SACSET, SUVR4-WIYLD, UBQ1, ubiquitin moiety of UBQ1 and L40 moiety of UBQ1 were PCR amplified from cDNA using gene specific *attB* gateway primers (Table S1) and *Pfu* DNA polymerase (Fermentas). The attB PCR products were recombined into the pDONR/Zeo vector using the Gateway BP Clonase II Enzyme Mix (Invitrogen) according to the manufacturer's instructions. The resulting pDONR/Zeo entry clones were recombined into destination vectors using the Gateway LR Clonase Enzyme Mix (Invitrogen). All constructs were verified by sequencing. The knock-down SUVR4 RNAi construct was made by cloning a unique fragment from the SUVR4 5′end as an inverted repeat on each side of an intron into the binary vector pART27. Cloning procedures are described in detail (Text S1).

Yeast two-hybrid screening

Two-hybrid interactions were screened by mating the yeast strain Y187 carrying the pGBKT7-SUVR4-WIYLD bait construct with the strain AH109 carrying a cDNA library (Matchmaker library construction and screening kit, Clontech) at 30°C ON. The cDNA library was created from Columbia wt 14 day old seedlings and recombined into the pGADT7-Rec vector to create an AD-fusion library. Selective media for the nutritional reporter genes ADE2, HIS3 and MEL1 (QDO) containing 20 mg l-1 X-alpha-Gal, was used to identify positive two-hybrid interactions according to the suppliers suggestions. To confirm interaction with SUVR4-WIYLD, the pGADT7-UBQ1, pGADT7-ubiquitin and pGADT7-L40 were mated separately with the pGBKT7-SUVR4-WIYLD or the empty pGBKT7 vector (BD control). Diploid colonies were selected on SD –L/-T, and then streaked out on SD –L/-T/-H +3 AT medium selective for protein-protein interactions.

Expression of recombinant proteins for enzyme assays

pHMGWA-SUVR4-Full and pHMGWA-SUVR4-SACSET constructs were transformed into *E. coli* BL21-Star DE3 and grown at 150 rpm, 37°C in LB-medium with 1% Glucose and 100 μg/ml ampicillin. At an OD_{600} 0.6–0.8, the cells were induced with 1 mM IPTG overnight at 20°C. The cells were lysed with Express and then resuspended in pre-cooled lysis Buffer: 20 mM Tris–HCl, pH 7.5, 400 mM NaCl, 100 mM KCl, 1 mM

EDTA, 1 mM DTT, 0.05% Triton X-100 and Protease inhibitor. After centrifugation (15,000 rpm), the supernatant containing recombinant protein was filtered through 0.45 µm filters and prepared for affinity chromatography.

Purification of recombinant proteins

Recombinant proteins SUVR4-Full and SUVR4-SACSET were purified by Ni-NTA affinity chromatography using HisTrap FF 5 ml (GE Healthcare) column in the ÄKTA purifier. Binding buffer or Buffer A and Elution Buffer or Buffer B in the purification step were as follows, Buffer A: 20mM Tris–HCl, pH 7.5, 500mM NaCl, 1 mM EDTA, 1 mM DTT, 20 mM Imidazole and Buffer B: 20 mM Tris–HCl, pH 7.5, 500 mM NaCl, 1 mM EDTA, 1 mM DTT, 500 mM Imidazole.

HKMTase assays

HKMTase assays were essentially performed as described in [28]. Twenty µg of MBP-SUVR4 protein was incubated in reaction buffer (50 mM Tris pH 8.5, 20 mM KCl, 20 mM $MgCl_2$, 10 mM β-mercaptoethanol and 250 mM sucrose) with 7.5 µl µCi 14C S-adenosyl methionine (SAM) (Amersham/Perkin Elmer) or 100 µM unlabelled SAM (New England Biolabs) as methyl donor. Twenty µg of core histones from calf thymus (Roche), or 5 µg histone H3 peptides were used as substrate. Reactions were incubated at 30°C for 3 hours, and each experiment was repeated at least 4 times. Core histones from calf thymus (Roche), unmodified histone H3 peptide (#12-403, Millipore), monomethyl-histone H3 (Lys9) peptide (#12-569, Millipore), dimethyl-Histone H3 (Lys9) Peptide (#12-430, Millipore), Trimethyl-Histone H3 (Lys9) Peptide (#12-568, Millipore), Trimethyl-Histone H3 (Lys4) Peptide (#12-564, Millipore), monomethyl histone H3 (Lys 4) peptide (gift from Thomas Jenuwein) and ubiquitin (U6253, Sigma) were used in the assays.

GST pull-down

Recombinant proteins were expressed in BL21 cells, lysed in 1 X PBS with 0.1 mg/ml lysozyme, 0.2–1% Triton X-100 and protease inhibitor cocktail (Roche), and immobilized on glutathione sepharose beads (Amersham). 3 µg of GST-S4WIYLD was incubated with MBP protein lysates at 4°C for 2.5 hours or 10 µg of GST-SUVR4-WIYLD with 20 µg of precleared core histones (Roche) at 4°C for 3 hours, following a series of washes. Pull-down reactions were run on SDS-PAGE gels, blotted onto a PVDF membrane (Machery Nagel) and probed with either anti-MBP (1:10000, New England Biolabs, #E8030S) or anti-H2Bub1 (1:1000, MediMabs, MM-0029). Detection of primary antibody was performed with peroxidase-conjugated secondary antibody; goat anti-rabbit HRP for pulldown of MBP-proteins (1:10000, Thermo Scientific, PA1-74361) and anti-mouse HRP for pull-down of core histones (1:10000, Abcam, ab6728) using the ECL kit (GE HealthCare, RPN2135).

MS analysis of peptides from HKMTase reaction mixtures

Reverse phase (C18) nano online liquid chromatographic MS/MS analyses of proteolytic peptides from HKMTase reactions using unlabelled SAM were performed using a HPLC system as described [56].

Nuclear magnetic resonance spectroscopy

Uniformly ^{15}N- or ^{15}N,^{13}C-labeled SUVR4-WIYLD (residues 1-89) was expressed as a GST-fusion (pGEX4T3) in minimal media containing $^{15}NH_4Cl$ and ^{13}C-glucose as the sole nitrogen and carbon sources, respectively, after induction at 18°C for 18 hours. Protein was purified by glutathione sepharose affinity and size-exclusion chromatography and thrombin digestion to remove the affinity tag. NMR samples contained 0.5 mM protein in PBS at pH 7.4, 5 mM d_{10}-DTT and 10% D_2O. All spectra were acquired at 25°C on a 500MHz or 600MHz Bruker spectrometer.

Chromatin immunoprecipitation

For each experiment 2-3 g of fifteen day old seedlings was crosslinked in 1% formaldehyde under vacuum until the tissue was translucent. Chromatin immunoprecipitation was done as described in [57]. The antibodies used for immunoprecipitation were anti-H2Bub1 (#MM-0029, Medimabs), anti-H3K9me1 (#07-450, Millipore), anti-H3K9me2 (#07-212, Millipore) anti-H3K9me3 (#07-442, Millipore), anti-H3K4me3 (#07-473, Millipore) and anti-GFP (#ab290-50, Abcam). Immunoprecipitated chromatin was eluted in a total of 250 µl elution buffer (1% SDS, 0.1 M NaHCO3) and after reversion of crosslinking, DNA was extracted using the Qiaquick PCR purification kit (Qiagen) and eluted in 100 µl elution buffer. 5 µl of a 4 X dilution was used as a template for real-time PCR in a Lightcycler (Roche). Typically a program of: 1 cycle 95°C 10 min, 45 cycles of 95°C 20 s, 52° 30 s and 72°C 30 s was used to amplify target sequences with gene specific primers (Table S1). PCR was performed on ChIP DNA isolated from two independent experiments, each quantified two separate times.

Western blotting

Nuclear protein extracts were isolated from a chromatin preparation as described [57]. The protein lysate obtained after sonication was separated on a 10-20% SDS-PAGE (Invitrogen, catalog no. EC6625BOX) and transferred to a PVDF membrane (Machery Nagel). Nuclear protein levels were determined using the following antibodies; anti-ubiquitin (1:4000, Millipore, 07-375), anti-H2Bub1 (1:1000, MediMabs, MM-0029) and anti-PBA1 (1:1000, abcam, ab98999).

Immunostaining of nuclei

Leaves from 14 day old seedlings were chopped in 4% formaldehyde on slides, covered with coverslips and flash frozen in liquid N2. The coverslips were removed from the slides when the material was still frozen, and then the slides were washed three times 5 minutes in 1 X PBS. The material was then blocked for 30 min at 37°C in blocking solution (1% BSA in PBS), and incubated with primary antibody (anti H3K9me1, 1:200; antiH3K9me3, 1:100) diluted in blocking solution for one hour at 37°C. After a series of washes in PBS, the slides were incubated with goat-anti rabbit Alexa 555 (Invitrogen) secondary antibody (1:200). Before microscopy the slides were washed in PBS and counterstained in DAPI and inspected with a Zeiss Axiovision2 microscope equipped with epifluorescence attachment. All images were captured using the same exposure times and at 100X magnification.

Bisulfite sequencing

2 µg of genomic DNA, prepared from leaf material using the Invisorb Spin Plant Kit (INVITEK Berlin), was restricted with ApaI and PstI and used in the bisulfite reaction with the EpiTect Bisulfite Kit (Quiagene Hilden). Bisulfite treated DNA was used as template in a PCR with specific primers. The PCR-Fragments are ligated into pGEMT-vector (Promega) and transformed in DH5alpha cells. Plasmid DNA from several colonies was sequenced with the ABI Prism 310.

Supporting Information

Figure S1 HKMTase activity of SUVR4. (A) HKMTase assay with MBP-SUVR4 full-length using unmethylated histone H3 1-21 or histone H3 K9me1 peptides as substrate. (B) Second independent replica of the HKMTase assay in Fig. 2 D. MBP-SUVR4 full-length activity on histone H3 1-21 K9me2 peptides without and with the addition of 5 µg of free ubiquitin. (C) HKMTase assay with MBP-SUVR4 full length, MBP-SUVR4-SACSET, MBP-SUVR4-WIYLD and no protein on core histones without and with the addition of 5 µg of free ubiquitin.
Found at: doi:10.1371/journal.pgen.1001325.s001 (2.43 MB TIF)

Figure S2 SUVR4 subcellular localization. Fluorescence microscopy of interphase nuclei from seedlings expressing SUVR4-GFP fusion proteins, demonstrating varying subcellular localization. (A) Nucleus showing uniform SUVR4 localization to the nucleoplasm and nucleolus (no), with high accumulation in an unknown focus (uf). (B) Uniform SUVR4-GFP distribution in the nucleoplasm, with strong localization in nucleolar associated foci and weaker localization to the nucleolus. (C) Strong SUVR4 localization to the nucleolus and weaker association to the nucleoplasm. (D) SUVR4 localization to the nucleoplasm, with stronger accumulation in the nucleolus and an unknown focus.
Found at: doi:10.1371/journal.pgen.1001325.s002 (0.99 MB TIF)

Figure S3 SUVR4-GFP associates with eu- and heterochromatin. ChIP analysis of SUVR4-GFP^{OE} lines using an antibody against GFP. DNA levels from the ChIP experiments relative to the input reactions were quantified using real time PCR and normalized to *TUB8*. The bars represent the average of two independent biological replicates.
Found at: doi:10.1371/journal.pgen.1001325.s003 (0.34 MB TIF)

Figure S4 H2Bub1 levels on transposons. (A) ChIP analysis of SUVR4-GFP^{OE} lines using antibodies against H2Bub1. (B) ChIP analysis of SUVR4 RNAi lines using antibodies against H3K4me3. DNA levels from the ChIP experiments relative to the input reactions were quantified using real time PCR and normalized to TUB8. The bars represent the average of two independent biological replicates.
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Figure S5 Expression levels of *SUVR4* and transpsons. (A) Real time RT-PCR quantification of transcripts reversely transcribed from mRNA isolated from 14 day old SUVR4-RNAi seedlings, using *SUVR4* primers. The data were normalized to *ACTIN2* and the mutant expression is relative to wild type. Error bars represent standard deviation according to three biological replicates (n = 3). (B) Real time quantification of transposon expression in wild type. The expression of each transposon is relative to *ACTIN2* which is set to 1. Reactions without the addition of reverse transcriptase (-RT) is used as a negative control.
Found at: doi:10.1371/journal.pgen.1001325.s005 (0.17 MB TIF)

Figure S6 Genotyping of the *ubp26*-mutant. PCR on wild type and ubp26 mutant plants using the primer combinations P1 (*ubp26-1* F) primer with P2 (*ubp26-1* R), or P1 (*ubp26-1* F) with LB, on two biological replicas b1 and b2 (upper panel). Layout of the the *ubp26* gene indicating the position of the T-DNA insertion and the primer annealing sites (lower panel).
Found at: doi:10.1371/journal.pgen.1001325.s006 (1.20 MB TIF)

Figure S7 ChIP analysis of *hub2-2* plants. ChIP analysis of *hub2-2* and wild type plants using antibodies against H3K9me3 (A) or H2Bub1 (B). DNA levels from the ChIP experiments relative to the input reactions were quantified using real time PCR and normalized to *TUB8*. The data for H2Bub1 is not normalized to *TUB8* because the chromatin at this gene is affected by the *hub2-2* mutation. The bars represent the average of two independent biological replicates.
Found at: doi:10.1371/journal.pgen.1001325.s007 (0.64 MB TIF)

Table S1 Oligos used in this study.
Found at: doi:10.1371/journal.pgen.1001325.s008 (0.03 MB XLS)

Text S1 Cloning of DNA constructs.
Found at: doi:10.1371/journal.pgen.1001325.s009 (0.04 MB DOC)

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Author Contributions

Conceived and designed the experiments: KLY AF WEJ TT. Performed the experiments: SVV MAR KLY AF WEJ TT. Analyzed the data: SVV MAR KLY AF WEJ TT. Contributed reagents/materials/analysis tools: GR MMZ RBA. Wrote the paper: TT. Contributed to paper writing: SVV KLY GR MMZ RBA.

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