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Ash1 counteracts Polycomb repression independent of histone H3 lysine 36 methylation

Eshagh Dorafshan[1], Tatyana G Kahn[1], Alexander Glotov[1], Mikhail Savitsky[1], Matthias Walther[2,3], Gunter Reuter[2] & Yuri B Schwartz[1,*]

Abstract

Polycomb repression is critical for metazoan development. Equally important but less studied is the Trithorax system, which safeguards Polycomb target genes from the repression in cells where they have to remain active. It was proposed that the Trithorax system acts via methylation of histone H3 at lysine 4 and lysine 36 (H3K36), thereby inhibiting histone methyltransferase activity of the Polycomb complexes. Here we test this hypothesis by asking whether the Trithorax group protein Ash1 requires H3K36 methylation to counteract Polycomb repression. We show that Ash1 is the only *Drosophila* H3K36-specific methyltransferase necessary to prevent excessive Polycomb repression of homeotic genes. Unexpectedly, our experiments reveal no correlation between the extent of H3K36 methylation and the resistance to Polycomb repression. Furthermore, we find that complete substitution of the zygotic histone H3 with a variant in which lysine 36 is replaced by arginine does not cause excessive repression of homeotic genes. Our results suggest that the model, where the Trithorax group proteins methylate histone H3 to inhibit the histone methyltransferase activity of the Polycomb complexes, needs revision.

Keywords Ash1; *Drosophila*; H3K36 methylation; Polycomb; Trithorax
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Introduction

Polycomb repression is essential to maintain cell type specific gene expression programmes in a wide range of multicellular animals including *Drosophila*, mice and humans [1–3]. It is potent and once established tends to repress target genes for many cell generations. Polycomb proteins, the building blocks of the repressive system, are ubiquitous but the set of genes they repress varies between different cell types. For example, homeotic selector (*HOX*) genes of the bithorax complex are repressed by Polycomb in the anterior half of the fly body but remain transcriptionally active in the posterior half [4,5]. The genetic evidence indicates that Trithorax (Trx) and Absent, small or homeotic discs 1 (Ash1) proteins are critical to safeguard *Drosophila* Polycomb target genes from erroneous repression in cells where they have to remain active [6–8]. Importantly, neither Trx nor Ash1 are responsible for transcriptional activation of the Polycomb target genes, which is done by appropriate enhancers and associated transcription factors. Instead, the two, in some way, specifically antagonize Polycomb repression [9,10].

Polycomb proteins act as multisubunit complexes that affect chromatin organization in multiple ways, which includes posttranslational modification of histone proteins [2,11,12]. Of those, tri-methylation of lysine 27 of histone H3 (H3K27me3) by Polycomb Repressive Complex 2 (PRC2) is essential for repression [13]. *In vitro* experiments indicate that the catalytic activity of PRC2 is inhibited by prior methylation of histone H3 tail at lysine 4 (K4) and lysine 36 (K36) [14–16]. Trx and Ash1 both have SET domains that can methylate H3K4 and H3K36, respectively [16–20]. From this, it was proposed that Trx and Ash1 counteract Polycomb repression by inhibiting PRC2 catalytic activity via H3K4 and H3K36 methylation [14–16].

The model provides a simple mechanistic explanation of the antagonism between Trx/Ash1 and Polycomb repression. However, several observations do not easily fit to the model. First, there are other *Drosophila* histone methyltransferases, Set1 and Trr [21–23] that can methylate H3K4 and two histone methyltransferases NSD and Set2 [24,25] that can methylate H3K36. Why these are not redundant with Trx and Ash1 is unclear. Second, methylated H3K4 or H3K36 have to be present on the same H3 tail to inhibit PRC2 activity [15]. Therefore, nucleosomes have to be extensively methylated by Trx and Ash1 to block Polycomb repression efficiently. However, recent quantitative mass spectrometry study indicates that in *Drosophila* cells only a very small fraction of histone H3 is methylated at K36 (H3K36me1 = 2.5% of total, H3K36me2 = 0.5% of total and H3K36me3 = 1.5% of total) [26]. Since these modifications are widely spread over the *Drosophila* genome [27], their density at any given site is expected to be very low. Third, transgenic experiments of Hödl and Basler [28] indicate that flies in which all zygotic histone H3 molecules carry arginine or alanine instead of lysine 4

1 Department of Molecular Biology, Umeå University, Umeå, Sweden
2 Institute of Developmental Genetics, Martin-Luther University of Halle-Wittenberg, Halle, Germany
3 Max Planck Institute of Immunobiology and Epigenetics, Freiburg, Germany
*Corresponding author. Tel: +46 90 785 6784; E-mail: yuri.schwartz@umu.se

(K4) have no ectopic repression of *HOX* or other developmental genes. The latter cannot be ascribed to the redundancy with Ash1-mediated H3K36 methylation as individual loss-of-function mutations in *trx* and *ash1* both cause stochastic loss of *HOX* gene expression [6–9]. Finally, chromatin immunoprecipitation studies in *Drosophila* embryos and cultured cells indicate that, when Polycomb-regulated genes are transcriptionally active, they often lose PRC2 binding [29–31]. The loss of methyltransferase would automatically cause the loss of H3K27me3 leaving no need to invoke special mechanisms to inhibit catalytic activity of PRC2.

HOX genes specify anterior–posterior axis of multicellular animals. In *Drosophila*, the *HOX* genes are grouped in two clusters: the Antennapedia complex that encompasses genes responsible for the identity of the segments that form the head and the anterior thorax [32] and the bithorax complex, which groups genes that specify the third thoracic and all abdominal segments [4]. Both clusters are classical targets of Polycomb/Trithorax regulation and changes in their gene expression patterns proved to be one of the best readouts to detect defects in the Polycomb or Trithorax functions [6,33–36]. For example, in mutants lacking any of the core Polycomb proteins, the expression of the *HOX* genes is not confined to appropriate segments, which leads to transformations of multiple segments towards the more posterior neighbours [6,33–35]. On the other hand, impaired *trx* or *ash1* function causes stochastic loss of *HOX* gene expression and partial transformation of corresponding segments towards the anterior fate [6,36].

Here we investigated whether Ash1 counteracts Polycomb repression by methylating K36 on histone H3. Unlike the Trx protein, which binds Polycomb target genes both when they are repressed and when they are transcriptionally active [31,37], Ash1 is a hallmark of the de-repressed state [31,38,39]. Using a combination of genetic and biochemical approaches, we, for the first time, showed that Ash1 is the only *Drosophila* H3K36-specific methyltransferase required to prevent excessive Polycomb repression of the *HOX* genes. Surprisingly, our experiments demonstrated that complete substitution of the zygotic histone H3 with a variant in which lysine 36 is replaced by arginine does not lead to ectopic repression of homeotic genes. Altogether, our results suggest that the model, where the Trithorax group proteins methylate histone H3 to inhibit the histone methyltransferase activity of PRC2, may need to be reconsidered.

Results

If Ash1 counteracts Polycomb repression by methylating H3K36, other H3K36-specific histone methyltransferases may also contribute to the process. To address this question, we examined stochastic loss of the homeotic gene expression in flies with mutations in the *ash1*, *NSD* and *Set2* genes. As previously reported [7,8,40,41], flies with combinations of mutant *ash1* alleles lose expression of the bithorax complex genes and display transformations of thoracic and abdominal segments. The $ash1^{22}$ allele is a point mutation that converts Gln 129 into a stop codon (Fig 1A, [8]). The truncated open reading frame of $ash1^{22}$ encodes for a short non-functional polypeptide that lacks all conserved domains. The $ash1^{21}$ allele is a substitution of Glu 1365 to Lys within the Associated With SET (AWS) domain (Fig 1A, [8]). The $ash1^{22}/ash1^{21}$ heterozygotes develop to pharate adult stage and about 12% survive as adults. The mutant adult flies show haltere to wing and third leg towards second leg transformations, characteristic of partial loss of the *Ubx* gene expression (Fig 1B and C). In addition, they show transformations of the 5th and 6th abdominal segments towards more anterior fate (Fig 1B and C), which reflect partial loss of the *Abd-B* gene expression. The $ash1^{Df(3L)Exel9011}$ allele (hereafter referred to $ash1^{9011}$) is a large deletion that removes the entire *ash1* and several other genes [42]. Nearly all $ash1^{22}/ash1^{9011}$ animals die at early pupal stage before the adult cuticle is formed. The single $ash1^{22}/ash1^{9011}$ male with the adult cuticle developed enough to examine its morphology showed clear posterior to anterior transformations of the third thoracic and abdominal segments.

No loss-of-function alleles for the *NSD* gene have been described to date. Therefore, we used CRISPR/Cas9-mediated mutagenesis [43] to replace the entire *NSD* open reading frame with a *DsRed* transgene driven by a synthetic eye-specific *3xP3* promoter (Fig EV1). Flies homozygous for resulted NSD^{ds46} allele are viable, fertile and show no homeotic transformations (Fig 1C). Both Ash1 and NSD were said to di-methylate H3K36 [24,44]. Nevertheless, the NSD^{ds46} allele does not reduce the viability or enhance the homeotic transformations of *ash1* mutants (Fig 1C). These observations indicate that *NSD* is not required to counteract Polycomb repression of the homeotic genes.

The reported mutation of the *Drosophila Set2* gene ($Set2^1$) removes the N-terminal half of its open reading frame, which includes the catalytic SET domain [25]. Most of the $Set2^1$ homozygous flies die during metamorphosis. The lethality can be complemented by a transgenic copy of the *Set2* genomic region, which indicates that the mutant chromosome does not contain second site lethal mutations [25]. In our hands, from 300 $Set2^1$ homozygous larvae picked at the first instar and grown separately from their heterozygous siblings, 221 formed pupal cases but only five males and three females formed some chitin structures, including one male with fully developed adult cuticle (Appendix Fig S1). None of the cuticle structures showed signs of homeotic transformations suggesting that the Set2 protein is not essential to counteract Polycomb repression of the *HOX* genes. To test this further, we compared the haltere imaginal discs from the wild-type, $ash1^{22}/ash1^{21}$ and $Set2^1$ third instar larvae stained with antibodies against the Ubx protein. Consistent with previous reports, the $ash1^{22}/ash1^{21}$ discs showed clonal patches of cells lacking Ubx immunostaining [9,40]. In contrast, the discs from the wild-type and the $Set2^1$ larvae displayed uniform Ubx staining (Fig 1D), supporting the conclusion that Set2 is not required to counteract Polycomb repression of the *HOX* genes.

To summarize, our observations suggest that, from the three *Drosophila* H3K36-specific histone methyltransferases, only Ash1 is critical to prevent the unscheduled Polycomb repression of the homeotic genes.

Ash1 SET domain is required to counteract Polycomb repression

If Ash1 is the only H3K36-specific methyltransferase critical to counteract Polycomb repression, something in its mode of action must differ from that of NSD and Set2. Ash1 may be specifically targeted to Polycomb-regulated genes, it may methylate some non-histone substrates, or it could have functions unrelated to methyltransferase activity.

The latter option seems unlikely considering recent reports that Ash1 interacts with Mrg15 and that this interaction enhances the

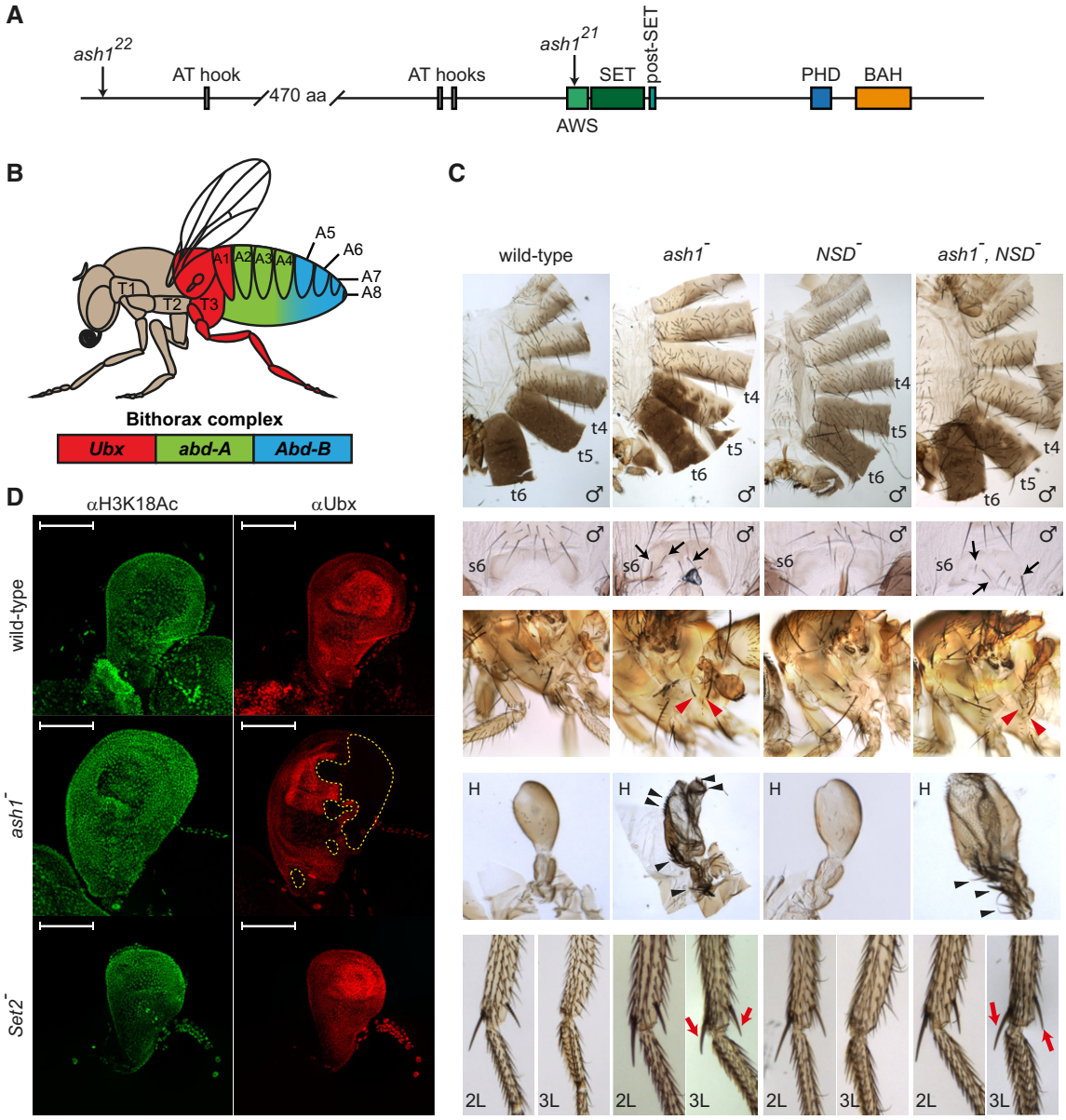

Figure 1. Ash1 is the only H3K36-specific methyltransferase critical to counteract Polycomb repression of the *Drosophila HOX* genes.

A The schematic of the *Drosophila* Ash1 protein organization. Ash1 is 2,226 amino acid long and contains eight domains (indicated by coloured rectangles). The SET domain together with the AWS (Associated With SET) and the post-SET domains are necessary and sufficient for Ash1 histone methyltransferase (HMTase) activity. The functions of the BAH (Bromo Adjacent Homology), PHD (Plant homeodomain) and AT-hook domains are unknown. The positions of $ash1^{22}$ and $ash1^{21}$ point mutations are indicated by arrows.

B Segmental expression of the *Drosophila* bithorax complex genes. The three genes of the complex, *Ubx*, *abd-A* and *Abd-B*, are shown as coloured rectangles. The expression of *Ubx* gives identity to the third thoracic (T3) and the first abdominal (A1) segments, the expression of *abd-A* defines the second, third and fourth abdominal segments (A2–A4), and the expression of *Abd-B* gives identity to the rest of the abdominal segments (illustrated with corresponding colour code).

C Adult phenotypes of the *ash1* and *NSD* mutants. In $ash1^{22}/ash1^{21}$ mutants (designated as $ash1^{-}$), the loss of *Abd-B* expression results in partial transformation of abdominal segments 6 and 5 towards segments 5 and 4, which is visible from the partial loss of pigmentation on tergites 5 and 6 (t5 and t6) and appearance of bristles on sternite 6 (s6, marked with black arrows). The loss of *Ubx* expression causes transformation of the third thoracic to the second thoracic segment visible as partial haltere (H) to wing and third leg (3L) to second leg (2L) transformations. The former is evident from the change in the haltere shape and the appearance of multiple bristles (black arrowheads). The latter is indicated by the apical and pre-apical bristles (red arrows) on the tibia of the third leg of *ash1* mutants. These are normally present on 2L but absent on 3L (compare to wild-type). Also note the appearance of additional hypopleural bristles on the third thoracic segment of the $ash1^{-}$ flies (red arrowheads), which indicate its transformation towards the second thoracic segment. Phenotype of the NSD^{ds46}/NSD^{ds46} (NSD^{-}) flies is indistinguishable from wild-type and the phenotype of the double $ash1^{22},NSD^{ds46}/ash1^{22},NSD^{ds46}$ ($ash1^{-},NSD^{-}$) flies is no more severe than that of the single $ash1^{22}/ash1^{21}$ ($ash1^{-}$) mutants.

D *Ubx* expression in the haltere imaginal discs. The expression was assayed by immunostaining with antibodies against Ubx (red) and acetylated H3K18 (green, positive control). While $ash1^{22}/ash1^{21}$ ($ash1^{-}$) larvae show stochastic clonal loss of the Ubx immunostaining in haltere discs (yellow dashed lines), $Set2^{-}$ larvae have uniform expression of *Ubx* throughout the haltere disc, resembling that in the wild-type larvae. Scale bars indicate 100 μm.

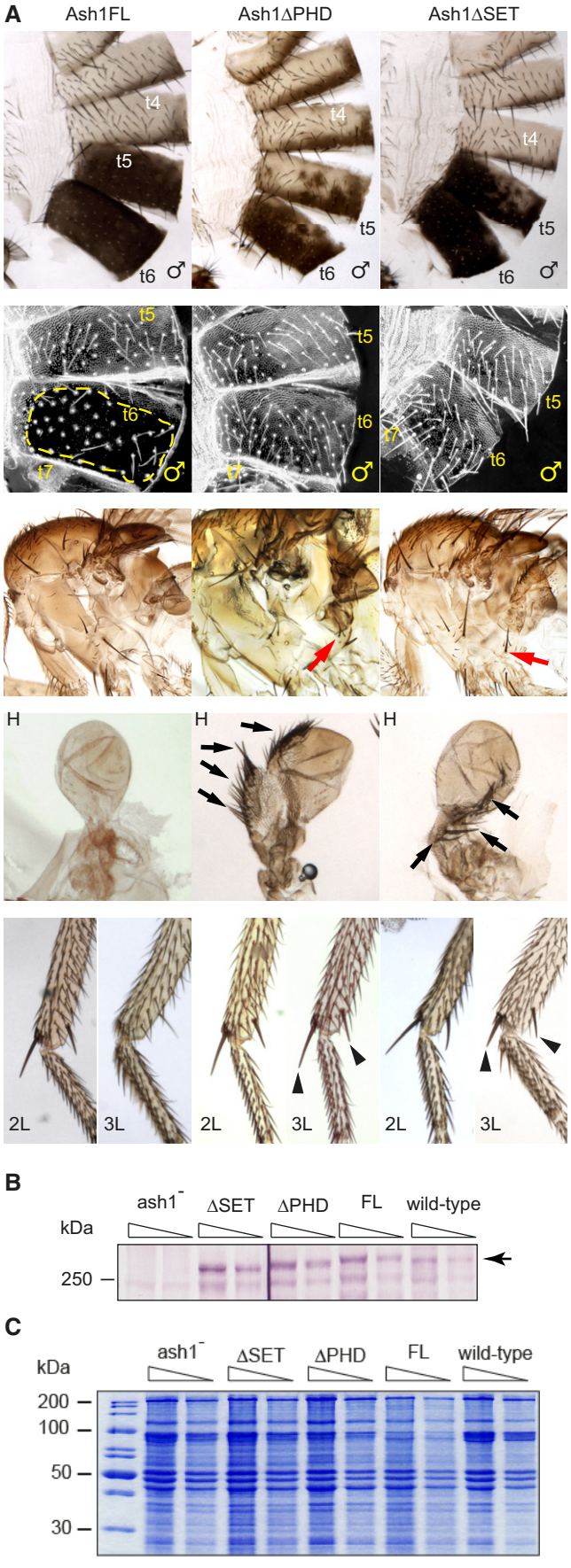

Figure 2. Ash1 SET domain is required to counteract Polycomb repression.

A Adult phenotypes of the *ash1*^{22}/*ash1*^{9011} flies supplemented with transgenic constructs expressing either the full-length Ash1 (Ash1FL) or the truncated variants lacking the PHD (Ash1ΔPHD) or the SET (Ash1ΔSET) domains. Note extra hypopleural bristles (red arrows), the third leg (L3) to second leg (L2), haltere (H) to wing, t5–t4 and t6–t5 transformations in the *Ash1ΔPHD* and the *Ash1ΔSET* but not in the *Ash1FL* flies. The latter are evident from the partial loss of pigmentation in t6 and t5, or the appearance of small bristles (trichomas) on t6 of the *Ash1ΔPHD* and the *Ash1ΔSET* flies in the area that is normally naked (*Ash1FL*, yellow dashed line). The transformed L3 acquire apical and pre-apical bristles on the tibia (black triangles) while halteres change shape and acquire rows of bristles (black arrows).

B Twofold dilutions of total nuclear protein extracts from the third instar larvae of the *ash1*^{22}/*ash1*^{9011} mutants supplemented with various transgenic constructs and wild-type flies were analysed by Western blot with anti-Ash1 antibodies. Arrow indicates the position of Ash1 protein. Note that transgenic proteins are expressed at comparable level.

C Coomassie staining of SDS–PAGE separated protein extracts from (B) was used to control the loading.

Source data are available online for this figure.

catalytic activity of Ash1 and helps to antagonize Polycomb repression [40,41]. To investigate this option further, we generated transgenic fly strain that expressed the full-length Ash1 (Ash1-FL) protein as well as strains that expressed the Ash1 variants lacking either the SET domain (Ash1ΔSET) or the PHD domain (Ash1ΔPHD), all under control of the *Ubi-p63E* promoter [45]. The flies carrying either of the transgenic constructs along with a copy of the endogenous wild-type *ash1* are fully viable, fertile and display no homeotic transformations. This indicates that the deletion of the SET or PHD domains, or potential overexpression of the transgenic Ash1 proteins, does not have adverse dominant effects. When introduced into the *ash1*^{22}/*ash1*^{9011} background, one or two copies of the transgene expressing full-length Ash1 restore the viability and yield flies with no homeotic transformations (Fig 2A, Appendix Fig S2). Somewhat surprisingly, the *ash1*^{22}/*ash1*^{9011} strain supplemented with two copies of the *ash1ΔSET* or the *ash1ΔPHD* transgene also yields viable adult flies (Appendix Fig S2). These flies, however, have low fitness and no stable stocks could be established. Importantly, all *ash1*^{22}/*ash1*^{9011} flies expressing the Ash1ΔSET or Ash1ΔPHD proteins show homeotic transformations indicating that the expression of *HOX* genes is still stochastically lost (Fig 2A). Since transgenic Ash1-FL, Ash1ΔSET or Ash1ΔPHD are expressed at comparable levels (Fig 2B and C), this argues that the SET and the PHD domains of Ash1 are required to counteract Polycomb repression of the *HOX* genes.

Western blot analyses showed no major difference in the overall levels of H3K36me1, H3K36me2 and H3K36me3 between the wild-type and *ash1*^{22}/*ash1*^{9011} third instar larvae (Fig 3A and C). However, we noted mild (~ 2-fold) reduction of H3K36me1, also visible in the *ash1*^{22}/*ash1*^{9011}, *NSD*^{ds46} double mutants but not in the *Set2*^{1} mutants (Fig 3A). Consistent with previous reports, the *Set2*^{1} mutant larvae displayed tenfold reduction in H3K36me3 [25] as well as slight loss of H3K36me2 (Fig 3B and C). Neither mutant showed increase in the level of bulk di- or tri-methylated H3K27 (Fig EV2). Altogether, these results suggest that Ash1 is not solely responsible for any of the H3K36 methylation states and that aside from H3K36me3, which is produced predominantly by Set2, all three methylases contribute to the H3K36me1 and H3K36me2 pools.

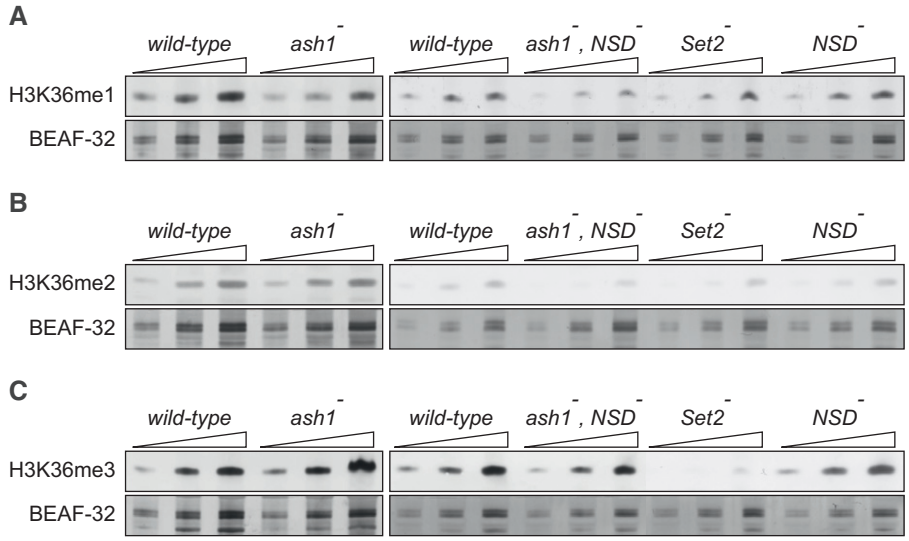

Figure 3. Western blot analysis of the bulk H3K36 methylation in larval tissues of various mutants.

A–C Twofold serial dilutions of the total protein extracts from the wild-type, $ash1^{22}/ash1^{9011}$ ($ash1^{-}$), $ash1^{22},NSD^{ds46}/ash1^{9011},NSD^{ds46}$ ($ash1^{-}$, NSD^{-}) and $Set2^{1}$ ($Set2^{-}$) larval brains, imaginal discs and salivary glands were analysed by Western blot with antibodies against H3K36me1 (A), H3K36me2 (B) and H3K36me3 (C). Note the strong (> 10-fold) reduction of H3K36me3 signal in the $Set2^{-}$ extract and the slight (~ 2-fold) reduction of H3K36me1 signal in the $ash1^{-}$ and $ash1^{-}$, NSD^{-} extracts. The protein extracts from the wild-type, double $ash1^{-}$, NSD^{-} and single NSD^{-} and $Set2^{-}$ mutants (right panels) were analysed together on the same membrane; however, the images of the H3K36me1 and H3K36me3 Western blots were modified to splice out the marker lane between the $ash1^{-}$, NSD^{-} and the $Set2^{-}$ extracts. Western blots with constitutively expressed BEAF-32 protein were used as loading controls.

Source data are available online for this figure.

Although its contribution to the bulk H3K36 methylation is limited, Ash1 may be critical for the appropriate level of H3K36 methylation at de-repressed Polycomb target genes. To address this question, we assayed the presence of Ash1, H3K36me1 and H3K36me2 at five genomic sites using Chromatin Immunoprecipitation coupled with quantitative PCR (ChIP-qPCR). Studies in cultured *Drosophila* cells suggested two modes of Ash1 binding to the genome. The first mode produces numerous weak binding sites biased towards long 5′ introns of transcriptionally active genes [38,41]. The second mode results in few dozens of strongly bound regions many of which represent Polycomb-regulated genes captured in transcriptionally active state [31,38,41]. Here we selected three developmental genes *no ocelli* (*noc*), *homothorax* (*hth*) and *extra macrochaetae* (*emc*), which displayed the strong Ash1 binding in multiple cultured cell lines [31,38]. One of the genes, *hth*, was previously shown to bind Polycomb proteins when transcriptionally inactive [31]. We also included the two well-known Polycomb target genes *Su(z)2* and *Ubx*. Both were shown to bind Ash1 when transcriptionally active [31,39] and *Ubx* is one of the genes whose erroneous repression causes some of the homeotic transformations seen in the *ash1* mutants. Finally, we assayed an intergenic region on chromosome 3R and the constitutively active *TBP-associated factor 4* (*Taf4*) gene. Neither of them is known to bind Ash1 [31,38] and, therefore, can serve as negative controls.

Chromatin immunoprecipitation (ChIP) and quantitative PCR analysis (ChIP-qPCR) indicates that, following the Ash1 depletion in the $ash1^{22}/ash1^{9011}$ third instar larvae, H3K36me1 is mildly (~ 2-fold) reduced at some of the selected genes (Figs 4A and EV3), while the H3K36me2 levels are not measurably affected (Fig 4B). The detected reduction of H3K36me1 is small. Nevertheless, the partial loss of H3K36me1 is completely reversed by the re-introduction of the transgenes expressing the wild-type and the PHD-deficient but not the SET domain-deficient Ash1 proteins (Fig 4A). This suggests that the partial loss of H3K36me1 from the Ash1-bound genes is caused by the loss of Ash1 catalytic activity. Importantly, while the Ash1ΔSET and Ash1ΔPHD proteins are expressed (Fig 2B) and bind the chromatin with comparable efficiency (Fig 4C) and the Ash1ΔPHD protein restores the wild-type levels of H3K36me1 (Fig 4A), the transgenic $ash1ΔPHD$, $ash1^{22}/ash1^{9011}$ flies still have homeotic transformations (Fig 2A). Altogether, our observations indicate that Ash1 mono-methylates a measurable fraction of the nuclear H3K36 and that Ash1 SET domain is required to counteract Polycomb repression of the *HOX* genes. Yet, there seems to be no correlation between the levels of methylated H3K36 (bulk or at specific loci) and the extent of the erroneous Polycomb repression.

Loss of zygotic H3K36 methylation does not affect the expression of *HOX* genes

The unexpected lack of clear anti-correlation between the amount of methylated H3K36 and the extent of the erroneous Polycomb repression made us wonder whether H3K36 methylation is required to counteract the repression. To address this issue, we turned to the transgenic flies that combine the deletion of the histone gene cluster $Df(2L)HisC^{ED1429}$ (hereafter *ΔHisC*) [46] with the BAC-based transgenic construct carrying twelve copies of the 5 kb histone repeat unit in which H3K36 is mutated to arginine ($12x^{H3K36R}$) (Fig 5A, [47]). The transgene carrying wild-type histone repeats ($12x^{WT}$) inserted in the same genomic location was used as a control. The $12x^{WT}$ transgene fully complements the *ΔHisC* deletion yielding viable, fertile

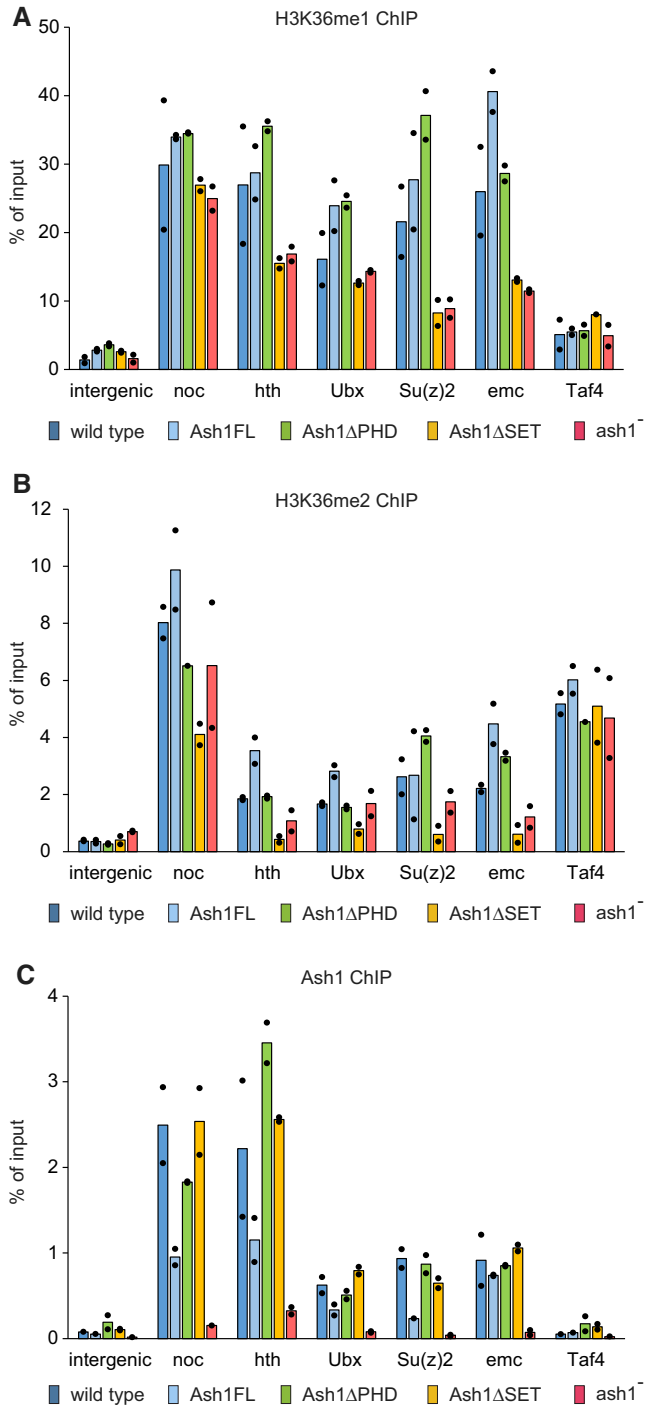

Figure 4. ChIP and quantitative PCR (ChIP-qPCR) analysis of H3K36 methylation and Ash1 binding.

A–C Chromatin from the wild-type (dark blue bars), *ash1*^{22}/*ash1*^{9011} (ash1^{-}, red bars) and transgenic *ash1*^{22}/*ash1*^{9011} (*Ash1FL*, light blue bars; *Ash1ΔPHD*, green bars; *Ash1ΔSET*, orange bars) larvae was subjected to immunoprecipitation with the antibodies against H3K36me1 (A), H3K36me2 (B) and Ash1 (C). Histograms show the mean of the two independent experiments (n = 2) with dots indicating individual experimental results. An intergenic region on chromosome 3R (intergenic) and the constitutively active *TBP-associated factor 4* (*Taf4*) gene serve as controls. The loss of Ash1 ChIP signal in the *ash1*$^{-}$ larvae indicates that the selected genes are the genuine Ash1 binding sites.

and morphologically normal flies [47]. In contrast, the combination of the homozygous *ΔHisC* deletion with one copy of the *12x*^{H3K36R} transgene is lethal [47]. In our hands, when separated from their heterozygous siblings at the first instar larval stage, the *ΔHisC, 12x*^{H3K36R} animals survive till pharate adult stage with some adults escaping from the pupal case to die shortly after (Fig EV4). Strikingly, the *ΔHisC, 12x*^{H3K36R} flies display no homeotic transformations (Fig 5B), indicating that zygotic replacement of the histone cluster with the *12x*^{H3K36R} transgene does not cause erroneous repression of the *HOX* genes. The simplest explanation of this finding is that H3K36 methylation is not required to maintain the *HOX* gene expression. However, more complex alternative explanations are possible. First, the H3K36 methylation by Ash1 may be needed to prevent the erroneous repression by the Polycomb proteins, not as the modification that inhibits the histone methyltransferase activity of PRC2 but as a placeholder, which prevents some other modification of this lysine 36. Replacing this lysine with arginine would automatically prevent such modification and thereby bypass the need for the Ash1-mediated H3K36 methylation. Second, in addition to the *HisC* gene cluster, which contains twenty-three gene copies encoding the H3.2 histone variant, *Drosophila* has two distinct loci, one on the first and one on the second chromosomes, which encode the variant H3.3 histones (Fig 5A). In the *ΔHisC* flies, both *H3.3* genes remain intact. Conceivably, the Ash1-mediated methylation of H3.3 is sufficient to counteract Polycomb repression.

If the Ash1-mediated H3K36 methylation is a placeholder and the K36R replacement bypasses the need to have it, we expect the *12x*^{H3K36R} transgene combined with the *ΔHisC* deletion to suppress some of the adverse effects of the Ash1 loss. For example, such flies would live longer than the *ash1*^{22}/*ash1*^{9011} mutants, till pharate adult stage as the *ΔHisC, 12x*^{H3K36R} flies do. To test this, we recombined the *ash1*^{22} allele with the *12x*^{H3K36R} transgenic insertion and introduced the *ΔHisC* and the *ash1*^{9011} chromosomes (Fig 5A, also see Appendix Figs S3–S5 for details). As illustrated by Appendix Fig S4, the *ΔHisC; ash1*^{22}, *12x*^{H3K36R}/*ash1*^{9011} flies die at early pupal stage, exactly as the single *ash1*^{22}/*ash1*^{9011} mutants, lending no support for the placeholder hypothesis.

The H3.2 histone is produced only during the S phase when it is used to package newly replicated DNA [48]. In most cells, H3.2 comprises the bulk of the nuclear histone H3. The variant histone H3.3 is synthesized in smaller quantities but in a replication independent manner. Because of its availability outside the S phase, H3.3 gets incorporated at places where nucleosomes are lost throughout the cell cycle, often within the chromatin of actively transcribed genes, which have higher nucleosome turnover [49,50]. Flies deficient for both *H3.3B* and *H3.3A* genes are viable and show no homeotic transformations [51,52]. The latter indicates that H3.3 is not essential to counteract Polycomb repression of the *HOX* genes and, if H3K36 methylation is important for the process, Ash1 must be able to use H3.2 as a substrate.

To test whether the presence of the wild-type H3.3 can explain the lack of homeotic transformations in the *ΔHisC, 12x*^{H3K36R} flies, we generated animals that combined deletions of both *H3.3* genes, the *ΔHisC* deletion and the *12x*^{H3K36R} transgene (hereafter *ΔH3.3B, ΔH3.3A, ΔHisC, 12x*^{H3K36R} (Figs 5A and EV5 and Appendix Figs S6–S10 for detailed description of the crosses). The *ΔH3.3B, ΔH3.3A, ΔHisC, 12x*^{H3K36R} animals die at the first instar larval stage, which precludes examination of adult cuticles or larval imaginal discs.

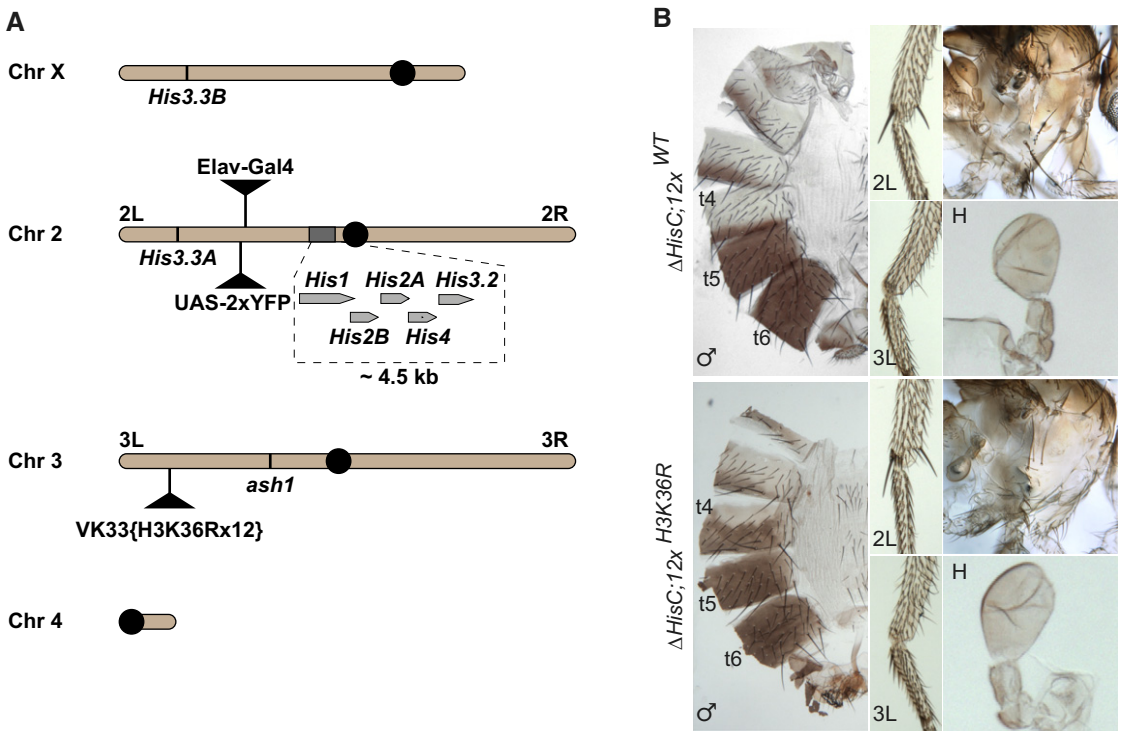

Figure 5. Zygotic replacement of replication coupled H3.2K36 with H3.2R36 does not cause ectopic repression of *HOX* genes.

A Chromosomal positions of various histone H3 genes, *ash1* and marker transgenes. Twenty-three histone gene repeat units, each containing single *His1*, *His2B*, *His2A*, *His4* and *His3.2* gene, are clustered near the centromere (black circle) of chromosome 2. These histone genes are removed by the *ΔHisC* deletion. To select the animals homozygous for the deletion, the *ΔHisC* chromosomes are marked with insertions of either *Elav-Gal4* or *UAS-2xYFP* transgenes (black triangles on chromosome 2L). The *His3.3A* and *His3.3B* genes encode the same protein, but reside on chromosomes 2L and X. The transgenes carrying twelve copies of either the wild-type histone repeat unit ($12x^{WT}$) or the unit in which *H3* gene is altered to have K36 replaced with arginine ($12x^{H3K36R}$) are inserted in the same *attP* site (black triangle on chromosome 3L) [47]. The *ash1* gene is located on the same chromosome arm.

B The *ΔHisC;* $12x^{H3K36R}$ and control *ΔHisC;* $12x^{WT}$ flies show no homeotic transformations and are indistinguishable from the wild type.

When we looked at larval denticle bands, which have shapes specific for each abdominal segment, we could see no signs of posterior to anterior homeotic transformations [6]. At embryonic stages, the misexpression of the *HOX* genes is most easily detectable in cells of the segmented embryonic Central Nervous System (CNS). The loss of *Abd-B* expression from CNS parasegments 10–12 of the $ash1^{22}$ homozygous embryos derived from $ash1^{22}$ mutant germ line cells was reported previously [9]. It is also readily visible in the $ash1^{22}$/$ash1^{9011}$ embryos produced by heterozygous mothers (Fig 6A). However, when we immunostained the *ΔH3.3B, ΔH3.3A, ΔHisC,* $12x^{H3K36R}$ embryos, we saw no differences in the *Abd-B* expression compared to the control embryos heterozygous for *ΔH3.3A, ΔHisC* chromosome (Fig 6B and C). Consistently, RT–qPCR measurements of the *Abd-B* mRNA level in the whole *ΔH3.3B, ΔH3.3A, ΔHisC,* $12x^{H3K36R}$ stage 16 embryos showed no difference compared to the heterozygous control or the wild-type strain (Fig 6D). Altogether, it appears that the replacement of all zygotic H3 with H3K36R does not cause the erroneous repression of the *HOX* genes.

Discussion

Three main conclusions could be drawn from the results presented here. First, the examination of homeotic gene expression in *ash1*, *NSD* and *Set2* mutants suggests that Ash1 is the only *Drosophila* H3K36-specific histone methyltransferase critical to counteract Polycomb repression. Second, the transgenic experiments with truncated Ash1 variants indicate that while the SET domain of Ash1, and likely its catalytic activity, are essential for the anti-Polycomb function, the levels of methylated H3K36 (bulk and at specific loci) are not correlated with the ability to antagonize Polycomb repression. Third, the H3K36R replacement experiments show that the zygotic loss of methylatable H3K36 does not phenocopy the loss of *ash1* function. Below we shall discuss one potential caveat of the latter experiments. Although the *ΔH3.3B, ΔH3.3A, ΔHisC,* $12x^{H3K36R}$ embryos lack zygotic synthesis of the wild-type H3, they derive from heterozygous females, which supply histone proteins and mRNAs to the embryo [53]. *Drosophila* development starts with 13 rapid mitotic divisions where nuclei divide within single cytoplasm without cytokinesis. Two hours after fertilization, the first extended interphase (interphase 14) occurs, the zygotic genes start to be transcribed and cell membranes form around embryonic nuclei. Homozygous *ΔHisC* embryos complete the first 14 cell divisions but cannot progress past the S phase of 15th cycle since maternal histones and their mRNA get degraded during the G2 phase of cycle 14 [46,54]. As development continues, most cells of the *ΔH3.3B, ΔH3.3A, ΔHisC,* $12x^{H3K36R}$ embryos undergo two more rounds of cell division but the CNS cells, where we assayed the *Abd-B* expression,

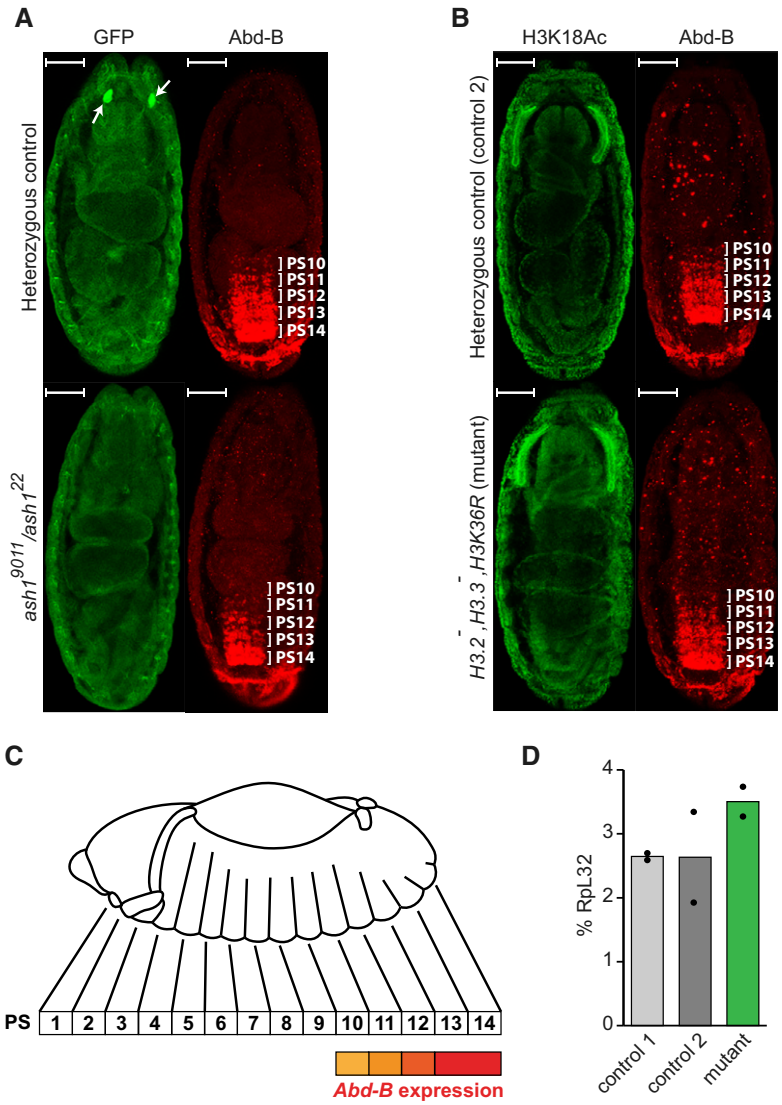

Figure 6. Zygotic replacement of H3.3K36 and H3.2K36 with H3.2R36 does not cause ectopic repression of *Abd-B* in the central nervous systems.

A The *Abd-B* expression in the Central Nervous System (CNS) of stage 16 embryos was assayed by immunostaining with antibodies against Abd-B (red). In the $ash1^{22}$/*TM3, Sb, e Kr::GFP* or $ash1^{9011}$/*TM3, Sb, e Kr::GFP* embryos (heterozygous control), *Abd-B* is expressed in parasegments 14–10 in a gradient that recedes from the posterior to anterior parasegment. In the $ash1^{22}/ash1^{9011}$ mutants, the Abd-B gradient is much steeper, with reduced staining of parasegments 13 and 12 and at the edge of the detection in parasegments 11–10. Heterozygous control and $ash1^{22}/ash1^{9011}$ mutant embryos were stained together and separated by strong GFP expression (green) in the Bolwig's organs (white arrows). Here and in (B) the embryos are oriented with anterior pole facing the top and scaling bars correspond to 50 μm.

B Unlike *ash1* mutants, embryos homozygous for *His3.3A, His3.3B, ΔHisC* deletions and supplemented with $12x^{H3K36R}$ transgene ($His3.2^{-}$,$His3.3^{-}$,*H3K36R*) display the wild-type Abd-B immunostaining pattern (red), the same as the control embryos heterozygous for *His3.3A*, and *ΔHisC* deletions. In this experiment, immunostaining with antibodies against acetylated H3K18 (green) served as positive control.

C The schematic of the *Abd-B* expression in the *Drosophila* embryo. Although homeotic transformations are often described according to the adult segments affected, the expression of the bithorax complex genes is regulated at the level of embryonic parasegments (PS), which are directly related to adult segments but slightly shifted relative to one another [5,64].

D Reverse transcription and quantitative PCR (RT–qPCR) measurement of *Abd-B* expression in $His3.2^{-}$,$His3.3^{-}$,*H3K36R* embryos. Expression of *Abd-B* in stage 16 embryos, which are homozygous for *His3.3A, His3.3B* and Δ*HisC* deletions and carry $12x^{H3K36R}$ transgene (mutant), is not reduced compared to their wild-type counterparts (control 1) or embryos heterozygous for *His3.3A*, and *ΔHisC* deletions (control 2). Histograms show the mean of the two independent experiments (n = 2) with dots indicating individual experimental results.

divide five times [55]. During each cell division, the pool of maternal histones within chromatin is diluted twofold. Hence, by stage 16 (13–16 h after fertilization) most cells of the embryo have their pools of maternal histones diluted fourfold (2^2) and, in the CNS, diluted 32-fold (2^5). Assuming random incorporation of zygotic and maternal histones in the nucleosome, in the CNS cells of the $\Delta H3.3B$, $\Delta H3.3A$, $\Delta HisC$, $12x^{H3K36R}$ embryos, only 1 in 32 nucleosomes will have an H3 tail that can be methylated at lysine 36 and only 1 in 1,024 nucleosomes will have both tails that can be methylated at this position. If an H3 tail methylated at K36 can no longer

be methylated at K27, could a single nucleosome with one "K27-unmethylatable" H3 tail placed randomly within 6 kb long stretch of the fully "K27-methylatable" chromatin prevent Polycomb repression? Although we cannot formally exclude this, it seems highly unlikely. For example, after the DNA replication, every second H3 molecule within H3K27me3 domains of Polycomb-repressed genes becomes un-methylated and it takes hours until the H3K27me3 density is fully restored [56]. Yet, Polycomb repression endures as cells continue to proliferate.

To summarize, our experiments argue that Ash1 counteracts Polycomb repression in a way that does not involve the direct inhibition of PRC2 activity by prior methylation of H3K36. This surprising conclusion parallels the findings of Hödl and Basler [28] who replaced all zygotic H3K4 with arginine or alanine and saw no ectopic Polycomb repression of *HOX* and other developmental genes. From this, they concluded that H3K4 methylation is not critical for Trithorax function at developmental genes. Taken together, our results and the results of Hödl and Basler argue that the model where the methylation of H3K4 and/or H3K36 directly inhibits the catalytic activity of PRC2 is a poor fit to explain how the Trithorax group proteins safeguard the expression of the Polycomb-regulated developmental genes. Not investigated here, the inhibition of Polycomb complexes by methylated H3K4 and H3K36 may still help to antagonize pervasive hit-and-run di-methylation of H3K27 throughout the genome [57,58].

If Ash1 does not act via H3K36 methylation, how does it antagonize Polycomb repression of developmental genes? Since the SET domain is required for this aspect of Ash1 function, methyltransferase activity is likely to be involved. It is possible that Ash1 methylates histones at positions other than H3K36 although these are probably not H3K4, H3K9 and H4K20 [16,17,19]. More likely, the relevant substrate of Ash1 activity is a non-histone protein. Conceivably, it is Trithorax or one of the Polycomb group proteins. Exciting times lie ahead as we start to explore these possibilities.

Materials and Methods

Fly strains

To generate the *ash1*^{22} (*w/+; ash1*^{22}*,P{w*$^{+mW.hs}$*=FRT(w*^{hs}*)2A}/TM3, Ser, e,Act-GFP[+mW]*) fly strain, the *w;ash1*^{22}*,P{w*$^{+mW.hs}$*=FRT(w*^{hs}*)2A}/TM6C,Sb*^{1}*,Tb*^{1} (Bloomington Drosophila Stock Center, #24161) flies were re-balanced over the *TM3, Ser, e, Act-GFP[+mW]* balancer. To generate the *w*^{1118}*; Df(3L)Exel9011/TM3,Ser,e,Act-GFP[+mW]* strain, the *w*^{1118}*; Df(3L)Exel9011/TM6B, Tb*^{1} flies (Bloomington Drosophila Stock Center, 7945) were re-balanced over the *TM3,Ser,e,Act-GFP[+mW]* balancer. The *ash1*^{21} (*y*$^{-}$*,w*^{1}*; ash1*^{21}*/TM3,Ser*) [59] strain was re-balanced over the *TM3,Ser,e,Act-GFP[+mW]* balancer. *Oregon R* flies were used as wild-type in all experiments unless stated otherwise. To generate histone H3 mutant strains, the fly strains *w,ΔHis3.3B (w*$^{+}$*), hsp-Flp; Df(2L)His3.3A/SM6B,Cy* [51], *yw; Elav-Gal4,ΔHisC/CyO; VK33{H3K36Rx12}/TM6B,Tb* and *w; UAS-2xYFP,ΔHisC/CyO,Ftz, lacZ; +/+* (gift from Dr. Gregory Matera, [47]) were used. Derivation of flies lacking zygotic *His3.3* and *His3.2* genes is described in Fig EV5, Appendix Figs S6 and S7. Generation of the *ash1*^{22}, NSD^{ds46}*/TM3,Ser,GFP* together with *ash1*^{9011},NSD^{ds46}*/TM3,Ser,GFP* and *ash1*^{21},NSD^{ds46}*/TM3,Ser,GFP* fly strains is described in Appendix Figs S11 and S12. Generation of the *Elav-Gal4,ΔHisC/CyO; ash1*^{22}*,VK33{H3K36Rx12}/TM6B,Tb* and *UAS-2xYFP,ΔHisC/CyO; ash1*^{9011}*/TM6B,Tb* fly strains is described in Appendix Fig S3. The fly strain *y,w,rox1[ex6],rox2[4–9]/FM7i,ActGFP; nos[4L],sco[rv9R] b[1]/CyO,ActGFP; +/+* was a gift from Dr. Maria Kim.

Plasmid construction and generation of Ash1 transgenes

The *Ubi*-One-STrEP::Ash1FL, *Ubi*-One-STrEP::Ash1ΔPHD and *Ubi*-One-STrEP::Ash1ΔSET plasmids were generated using Gateway LR reaction (Invitrogen) between the entry vector containing 5′ One-STrEP-tag fused with one of the three variants of Ash1 Open Reading Frame (ORF) and a destination vector which contained the *mini-white* marker gene, the *attB* site and the *Ubi-p63E* promoter (pWattB-*Ubi*-DEST) upstream of the recombination sites. To generate pENTR1A-One-STrEP-Ash1FL entry vector, Ash1C (404 bp) and Ash1N (391 bp), DNA fragments were amplified from the Pmt-V5-HIS-ASH1 plasmid (gift from Dr. Vincenzo Pirrotta) using the ASH1CN_Cfwd, ASH1CN_Crev, ASH1CN_Nfwd and ASH1CN_Nrev primers. The nucleotide sequences of all PCR primers used to generate the transgenic constructs are listed in Appendix Table S1. pENTR1A no ccDB (w48-1) (Addgene plasmid #17398) was digested with KpnI and XhoI. The Ash1N and Ash1C fragments were cloned in the linearized vector using InFusion HD (Clontech) to obtain the pENTR1A-Ash1NC plasmid. To introduce the One-STrEP-tag N-terminally into pENTR1A-Ash1NC, the plasmid was linearized with KpnI. The One-STrEP_fwd and One-STrEP_rev oligos were annealed in a thermocycler using the following program: 95°C 30 s, 72°C 2 min, 37°C 2 min, 25°C 2 min. The linear pENTR1A-Ash1NC plasmid and the annealed One-STrEP oligos were used in an InFusion reaction to obtain the pENTR1A-One-STrEP-Ash1NC plasmid. To clone the full-length Ash1 CDS, the Pmt-V5-HIS-ASH1 plasmid was digested by BglII and the middle fragment of Ash1 CDS (5.9 kb) was extracted from the gel and ligated into pENTR1A-One-STrEP-Ash1NC, linearized with BglII, to obtain pENTR1A-One-STrEP-Ash1. pENTR1A-One-STrEP-Ash1 was sequenced using the primers ASH1_seq2, ASH1_seq3, ASH1_seq4, ASH1_seq5, ASH1_seq6, ASH1_seq7, ASH1_seq8, ASH1_seq10, ASH1_seq11, ash1-22.2, ash1-22.R, ASH1CN_Cfwd and ASH1_mE_Fwd to confirm the integrity of the Ash1 ORF and the One-STrEP-tag.

To construct pWattB-DEST destination vector, the pWattB plasmid [60] was linearized with BamHI and the ends of the resulted fragment blunted using T4 DNA Polymerase (Fermentas). The destination cassette (DEST) was retrieved from pLentiX1PuroDEST (694-6) (Addgene plasmid #17297) by the EcoRV digestion and extraction of the 1.7 kb band from the agarose gel. Linear pWattB and the destination cassette were ligated to obtain pWattB-DEST.

The *Ubi-p63E* promoter was amplified as described by [60] using the Ubi-1.1 and Ubi-1.2 primers. The pWattB-DEST plasmid was linearized with SpeI (right before the destination cassette), and the *Ubi-p63E* promoter was cloned in the linear vector using InFusion to generate pWattB-*Ubi*-DEST.

To generate the plasmids for expression of truncated Ash1 variants, the deletions were introduced to the pENTR1A-One-STrEP-Ash1 entry vector and the modified entry cassette was shuffled into pWattB-*Ubi*-DEST using Invitrogen Gateway™ LR Clonase™ II. To construct pENTR1A-One-STrEP-Ash1ΔSET, the two fragments (upstream deltaSET_AB and downstream deltaSET_CD) flanking the

SET domain were amplified from pENTR1A-One-STrEP-Ash1 using the primers deltaSET_A, deltaSET_B, deltaSET_C and deltaSET_D. pENTR1A-One-STrEP-Ash1 was digested with BstZ17I and SphI restriction enzymes. The linearized vector (8.7 kb) was extracted from the agarose gel and used in the InFusion reaction together with the fragments deltaSET_AB and deltaSET_CD to obtain pENTR1A-One-STrEP-Ash1ΔSET. The ASH1_seq7 and ASH1_seq8 primers were used to sequence pENTR1A-One-STrEP-Ash1ΔSET.

To generate pENTR1A-One-STrEP-Ash1ΔPHD, the two fragments (upstream deltaPHD_AB and downstream deltaPHD_CD) flanking the PHD domain were amplified from pENTR1A-One-STrEP-Ash1 using the primers deltaPHD_A, deltaPHD_B, deltaPHD_C and deltaPHD_D. pENTR1A-One-STrEP-Ash1 was digested with KpnI and SphI restriction enzymes. The linearized vector (8.9 kb) was extracted from the agarose gel and used in the InFusion reaction together with the fragments deltaPHD_AB and deltaPHD_CD to obtain pENTR1A-One-STrEP-Ash1ΔPHD. The primers ASH1_seq8, ASH1_seq9 and ASH1_seq12 were used to sequence pENTR1A-One-STrEP-Ash1ΔPHD.

The *Ubi*-One-STrEP::Ash1FL, *Ubi*-One-STrEP::Ash1ΔPHD and *Ubi*-One-STrEP::Ash1ΔSET constructs produced by corresponding Gateway LR reactions were injected into y^1 *M[vas-int.Dm]ZH-2A* w^-*; M[3xP3-RFP.attP']ZH-51C* embryos by BestGene Inc.

Generation of the *NSD* null mutation

Most of the *Drosophila NSD* Open Reading Frame (ORF) was replaced with the *DsRed* ORF and placed under control of the 3xP3 promoter, using CRISPR/Cas9-mediated Homology-Directed Repair. To this effect, target-specific gRNAs (1gRNA_NSD_sens, 1gRNA_NSD_asen, 2gRNA_NSD_sens and 2gRNA_NSD_asen) were synthesized as 5′-phosphorylated oligonucleotides, annealed and ligated into the BbsI site of the pU6-BbsI-chiRNA vector [43], Addgene plasmid #45946. DNA of the pU6-1gRNA_NSD-chiRNA and pU6-2gRNA_NSD-chiRNA plasmids was purified and sequenced before injection. To insert DsRed by Homology-Directed Repair (HDR), the donor plasmid pHD-DsRed-attP-HDR^{NSD} was used. To generate pHD-DsRed-attP-HDR^{NSD}, the pHD-DsRed-attP vector was used to clone homology arms flanking the CRISPR/Cas-9 cleavage sites. The HA-1 region was amplified (Phusion polymerase, Thermo Fisher Scientific) with a forward primer HA-1-SpeI containing the SpeI site and a reverse primer HA-1-PstI containing the PstI site. The HA-2 region was amplified with a forward primer HA-2-NotI containing the NotI site and a reverse primer HA-2-EcoRI containing the EcoRI site. The amplified fragments were inserted into the SpeI/PstI sites and the NotI/EcoRI sites of the pHD-DsRed-attP vector (Addgene plasmid #51019), respectively, to generate the donor plasmid pHD-DsRed-attP-HDR^{NSD} [43]. pDsRed-attP was a gift from Melissa Harrison, Kate O'Connor-Giles and Jill Wildonger. The donor plasmid pHD-DsRed-attP-HDR^{NSD}, together with the guide-RNA-Plasmids, pU6-1gRNA_NSD-chiRNA, and pU6-2gRNA_NSD-chiRNA, were co-injected into embryos of the fly strain y^2,cho^2,v^1*; Sp/CyO,P{nos-Cas9,y+,v+}2A* [NIG-FLY, CAS-0001] [61], which expresses Cas9 in the germline. Injected flies were balanced and tested for incorporation of *DsRed* into the *NSD* locus. Resulting *NSD* loss-of-function mutants were verified by sequencing and RT–PCR using the primers NSD-Rt-1f and NSD-Rt-1r. The sequences of the oligonucleotide primers are shown in Appendix Table S1.

Imaginal disc fixation and immunostaining

Haltere imaginal discs from the third instar larvae were dissected in cold 1× PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4) and fixed for 30 min at room temperature with 4% formaldehyde diluted in 1× PBS. The discs were washed three times for 15 min in 1× PBS, 0.3% Triton X-100 (PBST) and then incubated for 30 min in the blocking solution (5% normal goat serum in PBST) and overnight at 4°C with the primary antibodies (anti-H3K18Ac and anti-Ubx, Appendix Table S2) diluted in the blocking solution. The discs were further washed three times for 15 min in PBST, incubated for 1 h at room temperature with secondary antibodies (Appendix Table S2) diluted in PBST, washed three times for 15 min in PBST and mounted on glass slides in Vectashield mounting media (Vector Laboratories). The mounted discs were imaged with Leica TCS SPE confocal microscope.

Embryo fixation and immunostaining

Fly crosses (Fig EV5) were set up in cages and embryos collected after 24 h. Embryos homozygous for the *ΔHisC* deletion (YFP$^+$) and the control (YFP$^-$) embryos were selected under the fluorescent stereomicroscope prior to fixation. Embryos were dechorionated for 5 min in 3% bleach. The dechorionated embryos were transferred to a scintillation vial containing 5 ml heptane. 5 ml of the 4% formaldehyde solution diluted in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4) was added to the vial, and it was shaken for 20 min at 350 rpm on the Unimax 2010 shaker (Heidolph). The lower phase (formaldehyde) was removed, and 5 ml of cold methanol was added to the vial. The vial was shaken vigorously for 30 s. The upper phase (heptane) and most of the methanol were removed. Embryos were transferred to a 1.5 ml tube and washed three times for 5 min, in methanol.

Before staining, the embryos were rehydrated in 50% methanol. Then, they were incubated three times for 5 min in 1× PBS, 0.1% Tween-20 (PBSTW) followed by three times for 15-min incubation in PBSTW. Next, the embryos were incubated at room temperature for 1 h in the blocking solution (5% normal goat serum in PBST) and then overnight at 4°C in primary antibody diluted in the blocking solution. The embryos were washed four times 15 min in PBSTW, incubated with a secondary antibody diluted in PBSTW and washed again four times 15 min in PBSTW. Then, the embryos were dehydrated by 5-min incubations in 1 ml of the 30, 50, 70, 95, 100% ethanol solutions. Ethanol was removed, 500 µl of methyl salicylate added, and the embryos incubated overnight at 4°C. After this, the embryos were mounted on a glass slide, covered by 18 × 18 coverslip and imaged with Leica TCS SPE confocal microscope.

Western blot

For analysis of the bulk H3K36 methylation, total extracts prepared from hand-dissected brains, imaginal discs and salivary glands of the third instar larvae were separated on a 15% SDS–PAGE and blotted to PVDF membrane for 60 min at 200 mA. For analysis of Ash1 protein levels, nuclear extracts from whole 3rd instar larvae were separated on a 6% SDS–PAGE and blotted to PVDF membrane for 3 h at 200 mA. The same extracts were separated on a 15%

SDS–PAGE and stained with Coomassie to be used as loading control. Primary and secondary antibodies were diluted in 1× PBS with 1% BSA and 0.05% Tween-20. For the list of antibodies, see Appendix Table S2.

Chromatin immunoprecipitation

Chromatin immunoprecipitation and qPCR analysis were done essentially as described [62] except that crosslinked material was sonicated in 4 ml of 10 mM Tris–HCl pH8.0, 1 mM EDTA pH8.0 with Branson sonicator for 45 min (45 cycles of 20 s ON–40 s OFF, amplitude 40%). Isolated ChIP DNA was re-suspended in 400 µl of DNase free water, and 4 µl was used for each PCR product. The antibodies used are listed in Appendix Table S2, and ChIP amplicons are listed in Appendix Table S3. The nucleotide sequences of corresponding PCR primers are listed in Appendix Table S1.

Reverse transcription and quantitative PCR

Drosophila embryos were staged and collected is described by [63]. For each replicate, 10 embryos were homogenized in TRIzol (SIGMA) and RNA was extracted with chloroform and precipitated with isopropanol using glycogen (Invitrogen) as a carrier. cDNA was synthesized using Random Hexamer primers and RevertAid Minus RT enzyme (Thermo Scientific) according to manufacturer's protocol. Quantitative PCR was performed with Bio-Rad Real-Time System CFX Connect using qPCRBIO SyGreen Mix No-Rox (Pcrbio) and primer pairs Abd-B_F_ex and Abd-B_R_ex; RpL32-ex1.1 and RpL32-ex1.2. The PCR conditions were as follows: 10 min at 95°C, 40 cycles of 10 s at 95°C, 20 s at 55°C and 30 s at 72°C. The melting curve was recorded at the end of the cycling program. Six dilutions of wild-type genomic DNA were used as calibration curve to calculate the starting number of template molecules.

Fly cuticle preparation

Flies were boiled in 10% KOH for 10 min and then incubated in distilled water for 30 min. Fly cuticles were dehydrated in 70% ethanol and 99% ethanol for 10 min each. Ethanol was replaced with glycerol and incubated for 30 min. Cuticle were dissected under the stereomicroscope and mounted on glass slide in glycerol.

Single fly genomic DNA preparation

Single flies were placed in 200-µl PCR tubes containing 50 µl of the homogenization buffer (10 mM Tris–HCl pH 8.0, 1 mM EDTA, 25 mM NaCl, 200 µg/ml proteinase K). Flies were crushed with a 200-µl pipette tip, and the resulting fly lysates were incubated for 30 min at 37°C followed by 2 min at 95°C. 3 µl of the lysate was used for each PCR product.

Embryo genomic DNA extraction

One embryo was placed in a 1.5-ml tube containing 50 µl of sonication buffer (10 mM Tris–HCl pH 8.0, 1 mM EDTA, 25 mM NaCl) and sonicated for three cycles (30 s ON, 30 s OFF) in Bioruptor® Pico (Diagenode). Proteinase K (0.2 µg/µl) was added to the embryo lysate and incubated 30 min at 37°C followed by 2 min at 95°C. 3 µl of the lysate was used for each PCR product.

Expanded View for this article is available online.

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Author contributions

ED and YBS conceived the project. ED performed all genetic and immunostaining experiments. ED and MS analysed homeotic phenotypes. TGK performed ChIP experiments and characterized the expression of *Ash1* transgenes. AG measured bulk methylated histones by Western blot and characterized *Abd-B* expression by qPCR. MW generated the *NSD* null allele under the supervision of GR. ED and YBS wrote the manuscript with inputs from all authors.

Conflict of interest

The authors declare that they have no conflict of interest.

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