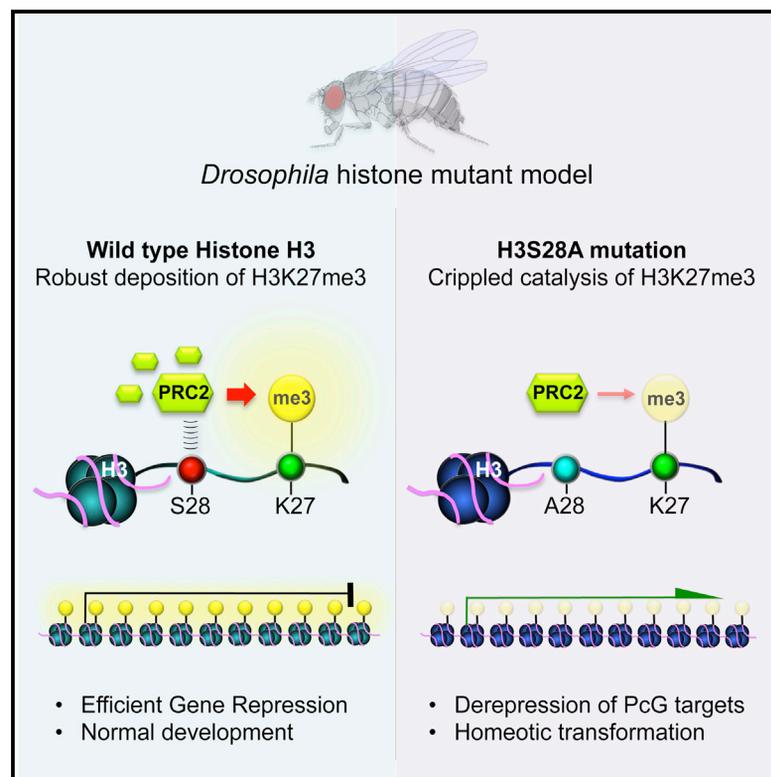


Histone H3 Serine 28 Is Essential for Efficient Polycomb-Mediated Gene Repression in *Drosophila*

Graphical Abstract



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In Brief

Yung et al. establish a *Drosophila* histone mutant carrying the H3S28A substitution, which depletes H3S28ph without affecting mitosis. In contrast, H3K27 methylation levels decrease and polycomb silencing is strongly compromised. A combination of genetics and biochemical approaches reveal a key role of the H3S28 residue in promoting PRC2-dependent methylation at H3K27.

Highlights

- *Drosophila* H3S28A mutation supports normal mitosis despite the lack of H3S28ph
- H3S28A mutant shows reduced H3K27 methylation and compromises polycomb silencing
- H3S28A defects are not caused by active H3K27 demethylation or by loss of H3S28ph
- H3S28A reduces the ability of PRC2 to methylate H3K27 on nucleosomal substrates



Histone H3 Serine 28 Is Essential for Efficient Polycomb-Mediated Gene Repression in *Drosophila*

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SUMMARY

Trimethylation at histone H3K27 is central to the polycomb repression system. Juxtaposed to H3K27 is a widely conserved phosphorylatable serine residue (H3S28) whose function is unclear. To assess the importance of H3S28, we generated a *Drosophila* H3 histone mutant with a serine-to-alanine mutation at position 28. *H3S28A* mutant cells lack H3S28ph on mitotic chromosomes but support normal mitosis. Strikingly, all methylation states of H3K27 drop in *H3S28A* cells, leading to Hox gene derepression and to homeotic transformations in adult tissues. These defects are not caused by active H3K27 demethylation nor by the loss of H3S28ph. Biochemical assays show that H3S28A nucleosomes are a suboptimal substrate for PRC2, suggesting that the unphosphorylated state of serine 28 is important for assisting in the function of polycomb complexes. Collectively, our data indicate that the conserved H3S28 residue in metazoans has a role in supporting PRC2 catalysis.

INTRODUCTION

Polycomb group (PcG) proteins are epigenetic regulators essential for repression of key developmental genes. Canonical targets of PcG proteins include the Hox genes, which specify the identities of body segments along the anterior to posterior axis. PcG mutants fail to maintain repressive chromatin states, leading to derepression of Hox genes and to homeotic transformations (Di Croce and Helin, 2013; Grossniklaus and Paro, 2014; Margueron and Reinberg, 2011; Schuettengruber et al., 2007; Simon and Kingston, 2013). *Drosophila* PcG proteins assemble into at least five different multiprotein complexes, namely polycomb repressive complex 1 (PRC1) (Shao et al., 1999), PRC2 (Cao et al., 2002; Czermin et al., 2002; Kuzmichev et al., 2002; Müller et al., 2002), Pho-repressive complex (Pho-RC) (Klymenko et al., 2006), dRing-associated factor (dRAF) (Lagarou et al., 2008), and polycomb repressive deubiquitinase (PR-DUB) (Scheuermann et al., 2010). Enhancer of zeste, E(z), the catalytic subunit of PRC2 methylates the lysine 27 residue of histone H3

(H3K27me) on target chromatin regions. dRing is an active E3 ligase subunit in dRAF that monoubiquitinates nucleosomal H2A. In the canonical model, H3K27me3 binding by the chromo-domain of polycomb helps to recruit PRC1 to target genes (Cao et al., 2002; Fischle et al., 2003; Kuzmichev et al., 2002; Min et al., 2003). Recent studies suggest an alternative recruitment hierarchy whereby H2Aub initiates PRC2 chromatin targeting and the establishment of H3K27me3 domains (Blackledge et al., 2014; Cooper et al., 2014; Kalb et al., 2014).

The local chromatin environment modulates the repressive functionality of PcG complexes (Müller and Verrijzer, 2009; O'Meara and Simon, 2012). For example, both H3K27me3 (Margueron et al., 2009) and H2Aub (Kalb et al., 2014) stimulate PRC2 activity, whereas active histone marks such as H3K4me3 and H3K36me3 (Schmitges et al., 2011) inhibit its function. Notably, the serine residue juxtaposed to H3K27, H3S28, is widely conserved and present in every species that has a canonical PRC2-dependent silencing system (Figure S1A). In contrast, it is absent in species like fission yeast that do not possess PRC2. H3S28 is mainly phosphorylated during mitosis (Giet and Glover, 2001), but whether this mitotic phosphorylation is required for mitosis in metazoans is unknown. Interphase H3S28ph has also been detected in mammals, where it was shown to counteract mammalian PRC1 and PRC2 chromatin binding. This mark derepresses PcG target genes in response to stress and developmental signaling (Gehani et al., 2010; Lau and Cheung, 2011) and activates stress response genes via displacement of HDAC corepressor complexes (Sawicka et al., 2014). However, biochemical studies showed that H3K27me3S28ph is refractory to demethylation by UTX (Sengoku and Yokoyama, 2011) and JMJD3 (Kruidenier et al., 2012). Hence, on one hand, H3S28ph might evict PcG proteins, and on the other hand, it might preserve H3K27me3. H3S28ph is established by the mitotic Aurora B kinase and, like H3S10ph, it is highly enriched during the course of mitosis (Giet and Glover, 2001). As such, H3S28ph might play a role in PcG repression and epigenetic inheritance. However, whether this is the only function of H3S28 remains unknown. In particular, the putative in vivo function of its non-phosphorylated state has not been investigated.

To assess the importance of the highly conserved H3S28 residue and to investigate the physiological relevance of mitotic H3S28ph in modulating PcG repression, we have established an in vivo *Drosophila* model where the endogenous source of

histone H3 is replaced by a non-phosphorylatable serine 28 to alanine mutation. Surprisingly, this mutation did not significantly affect mitosis. However, methylation of H3K27 was severely impaired and polycomb-mediated silencing of Hox genes was partially lost, resulting in homeotic transformations in the adults. The deregulation of PcG silencing associated with *H3S28A* mutation is independent of active demethylation and cannot be recapitulated upon the loss of Aurora B kinase. These observations are consistent with in vitro experiments showing that PRC2 activity is impaired upon H3 serine to alanine mutation at position 28 (*H3S28A*). Collectively, our data suggest that one main function of H3S28 is to support polycomb-mediated silencing via PRC2-dependent methylation of H3K27.

RESULTS

All H3K27 Methylation States Are Reduced in the *H3S28A* Mutant

In the *Drosophila* genome, canonical core and linker histones are encoded by the histone gene unit (HisGU), which is organized as a multicopy gene array residing at a single locus known as the histone gene cluster (*HisC*). Previous studies showed that embryos carrying homozygous *HisC* deletion (Δ *HisC*) die in late blastoderm, after exhaustion of maternally deposited histone reserves (Günesdogan et al., 2010; McKay et al., 2015). A histone replacement genetic platform has been developed based on the fact that reintroduction of a minimum of 12 copies of wild-type transgenic HisGU is sufficient to rescue lethality of the Δ *HisC* mutant. Likewise, HisGU carrying different point mutations can be introduced in this system to study the biological functions of specific histone residues and modifications thereof (Günesdogan et al., 2010). In a previous study, an *H3K27R* mutant was analyzed (McKay et al., 2015; Pengelly et al., 2013). It was reported that the limited number of cell divisions during late embryogenesis was not sufficient to completely replace wild-type, chromatinized maternal histones, resulting in chromatin with a mixture of wild-type and mutant histones. To circumvent this limitation, mosaic analysis of histone mutants was developed with the use of FLP-FRT-mediated recombination (Figure S1B). This way, homozygous Δ *HisC* clones are detected when they are supplemented with 12×HisGU of either WT or mutated histones in larval tissues (Hödl and Basler, 2012; Pengelly et al., 2013). We adopted the same system to generate a *H3S28A* mutant. In parallel, histone replacement lines carrying WT and *H3K27R* histone mutants were established as controls. This system replaces all canonical histones, leaving the endogenous genes coding for the histone variant H3.3 (*His3.3A* and *His3.3B*) intact. To simplify the nomenclature, we refer to homozygous Δ *HisC* clones supplemented with different transgenic 12×HisGU as WT, *H3S28A*, and *H3K27R* lines, unless otherwise stated.

Consistent with previous reports (Hödl and Basler, 2012; Pengelly et al., 2013), homozygous Δ *HisC* clones (lacking GFP) induced with a heat shock pulse of FLP died (data not shown). Reintroducing 12×HisGU in the form of WT-H3, *H3S28A*, or *H3K27R* mutants displayed DAPI and H3 staining patterns similar to the neighboring GFP-positive tissues (Figures 1 and S1C). Importantly, *H3S28A* clones specifically lost the mitotic H3S28ph signal, whereas they retained normal levels of

H3S10ph (Figure 1B). Likewise and as previously reported (McKay et al., 2015; Pengelly et al., 2013), *H3K27R* clones almost completely lost the H3K27me3 mark, without affecting neighboring mitotic phosphorylation at H3S28 (Figure S1D). These results imply that the native pool of histone variant H3.3 neither supports mitotic H3S28ph nor H3K27me3. This may be linked to the transcription-coupled deposition pathway of H3.3, which restricts its distribution to active promoters, resulting in genomic distributions that might not be compatible with PRC2 catalysis. Importantly, mitotic H3S10ph, H3S28ph, and H3K27me3 signals were not affected in WT clones (Figures 1A, 1C, and S1D).

Because H3K27 and H3S28 are adjacent to each other, and as H3S28ph was shown to evict PcG components (Gehani et al., 2010; Lau and Cheung, 2011) and to prevent H3K27me demethylation (Kruidenier et al., 2012; Sengoku and Yokoyama, 2011), we asked whether the loss of H3S28ph in *H3S28A* clones affects H3K27 methylation. Strikingly, H3K27me3 and H3K27me1 were strongly reduced in *H3S28A* clones (Figure 1D). Considerable reduction of H3K27me2 was also detected. This was in stark contrast to active chromatin marks, where H3K27ac was only mildly decreased and H3K4me3 was unaffected in *H3S28A* clones. As a control, WT clones showed normal staining levels indistinguishable from neighboring tissues for all histone marks examined (Figure 1C). These findings show that H3S28 is specifically required for PRC2 function in vivo.

H3S28A and *H3K27R* Mutants Both Cause Derepression of Hox Genes

In various PRC2 mutants (Beuchle et al., 2001; Birve et al., 2001; Müller et al., 2002), as well as in the *H3K27R* histone mutant clones where H3K27me3 levels are compromised (McKay et al., 2015; Pengelly et al., 2013), canonical PcG targets such as Hox genes are derepressed. Hence, we analyzed Hox gene expression in the *H3S28A* mutant in comparison to the *H3K27R* mutant.

Endogenous expression of *Scr* is limited to a patch of cells located at the base of antennal discs (asterisks) and is absent within the antennal disc proper (Abzhanov et al., 2001). *Scr* was derepressed across the entire antennal disc in both *H3S28A* and *H3K27R* mutant clones (Figure 2A). In line with a previous report (Pattatucci and Kaufman, 1991), low levels of endogenous *Scr* expression were observed in the ad epithelial cells of the second and third leg discs (Figure S2A, asterisks). We observed strong derepression of *Scr* in *H3S28A* and *H3K27R* mutant clones of these tissues (Figure S2A). Moreover, *Scr* derepression was occasionally detected in the notum region of wing discs in both histone mutant clones (Figure S2A), similar to what was previously reported in *Pc^{3/+}* heterozygous mutant background (Pattatucci and Kaufman, 1991).

We also detected strong derepression of *Ubx* in antennal and wing discs of both *H3S28A* and *H3K27R* mutant clones (Figures 2A and S2A). *Ubx* derepression was mainly restricted to the wing pouch region at a level comparable to its native expression in the haltere disc. For *Abd-B*, strong derepression was detected in *H3K27R* clones across the entire eye-antennal and wing discs (Pengelly et al., 2013), whereas *H3S28A* clones showed no obvious derepression in the same tissues (Figures 2A and S2A), suggesting that the partial loss of H3K27me3 was not

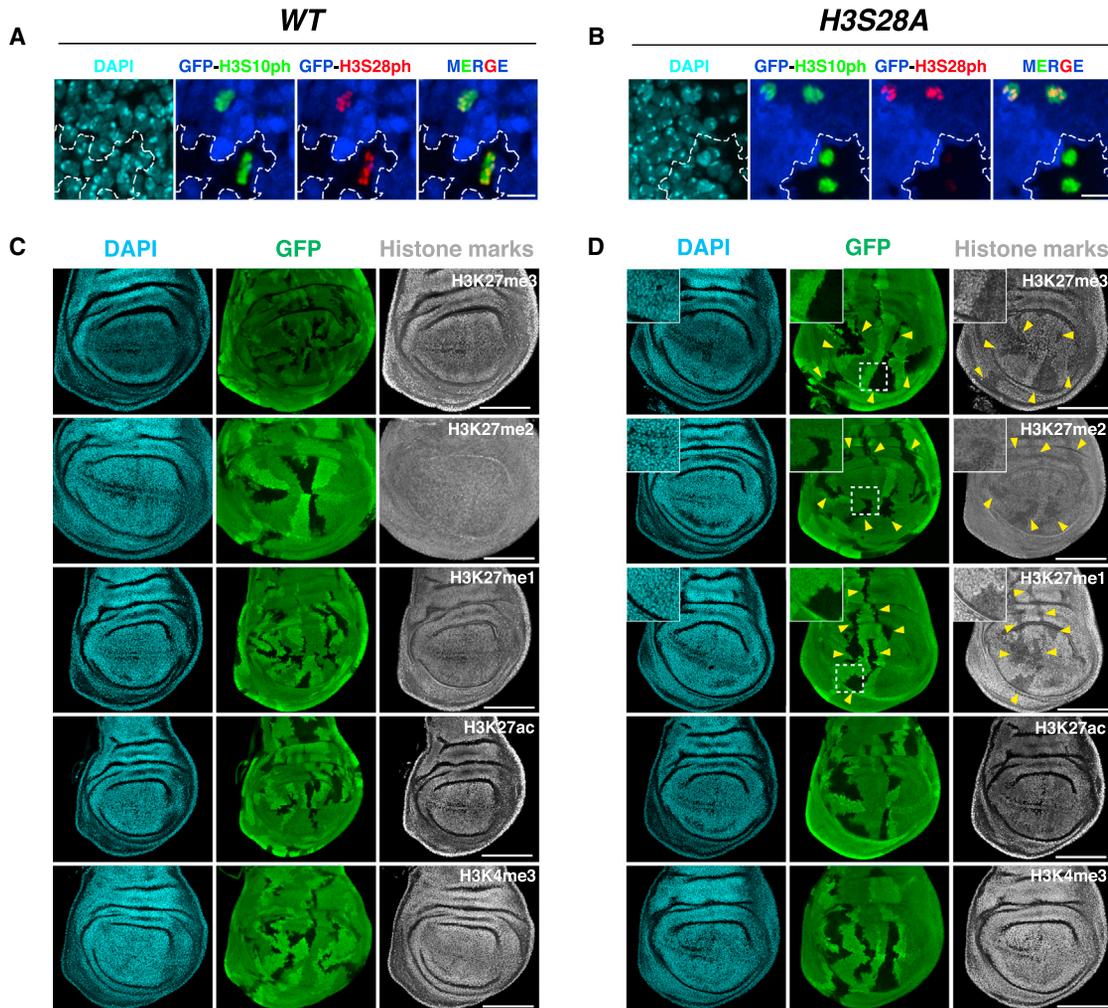


Figure 1. *H3S28A* Mutant Specifically Depletes Mitotic *H3S28ph* and Compromises All *H3K27* Methylation States

Eye-antenna (A and B) or wing (C and D) imaginal discs of the indicated histone replacement genotype. Immunostaining with the indicated antibodies is shown; DNA was stained with DAPI. Clones of interest were marked by the lack of GFP signal and are indicated by dashed lines. (A and B) *H3S10ph* (green) and *H3S28ph* (red) levels are shown in mitotic cells from GFP-negative (histone replacement) clones as well as GFP-positive normal tissue. (C) and (D) Arrowheads highlight *H3S28A* clones with considerable drop in *H3K27* methylation levels. Insets represent the magnified region indicated by dashed lines. For clarity of composite micrographs, GFP is pseudocolored to blue in (A) and (B). In order to capture mitotic cells at different focal planes, a Z projection is applied in (A) and (B). The scale bars in (A) and (B) correspond to 5 μm , whereas those in (C) and (D) correspond to 100 μm . See also Figure S1.

sufficient to induce Abd-B derepression in *H3S28A* clones. No changes in expression of Hox genes were detected in the imaginal discs of WT-H3 clones.

In addition to the aforementioned Hox genes, we observed derepression of *Antp* in the antennal discs of *H3S28A* and *H3K27R* mutant clones (Figures 2B and S2A). Similar to the *Antp^{Ns}* mutant with ectopic expression of *Antp* in the antennal disc (Figure S2B), GAL4-driven overexpression of various Hox genes in the antennal disc is known to silence the antennal selector gene *Hth* and cause antenna-to-leg transformation (Yao et al., 1999). In agreement with this observation, the *H3S28A* and *H3K27R* clones also showed *Hth* silencing, which was most prominent in the *H3K27R* mutant (Figure 2B).

Because Hox gene derepression can induce homeotic transformations, we investigated the phenotypic consequence of the in-

duction of *H3S28A* or *H3K27R* clones in adults. Consistent with the Hox derepression and *Hth* silencing phenotypes in larval tissues, adult flies developing from clonal *H3S28A* and *H3K27R* mutant backgrounds displayed antenna-to-leg transformation. Wild-type *Drosophila* adults showed distinct antenna segmentation into a1–a3 and arista, as annotated on *w¹¹¹⁸* in Figure 2C. Notably, the *H3K27R* mutant displayed an enlarged a3 antennal segment, from which massive outgrowth developed with leg-like features. This resembled the *antennapedia* phenotype of the *Antp^{Ns}* mutant. The *H3S28A* mutant showed milder transformations, with smaller a3 segment protrusions and thickening of arista resembling the *aristapedia* phenotype. In contrast, WT histone replacement flies showed normal antenna structures (Figure 2C).

In summary, the partial loss of *H3K27me3* in the *H3S28A* mutant induces loss of polycomb silencing, although the effects

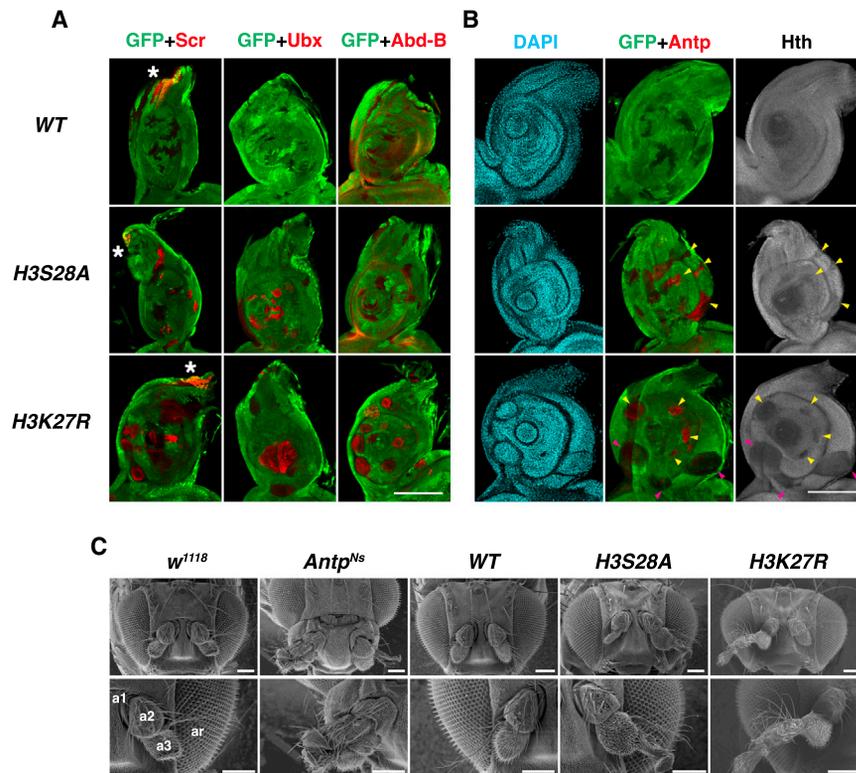


Figure 2. Deregulation of Hox and Antenna Selector Genes in *H3S28A* and *H3K27R* Mutants and Their Associated Transformation Phenotypes

(A and B) Antennal discs with WT, *H3S28A*, and *H3K27R* clones were immunostained with anti-GFP and the indicated antibodies. DNA was stained with DAPI. Mutant clones are marked by the absence of GFP signal. The scale bars in antennal discs represent 100 μ m. (A) The expression of Scr (left), Ubx (center), and Abd-B (right) in the antennal discs is shown in the indicated histone mutant background. Endogenous expression of Scr is marked by asterisks. (B) The expression of Antp and Hth in the antennal discs is shown in the indicated histone mutant background. Clones with derepressed Antp and silenced endogenous Hth expressions are marked by yellow arrowheads; those with silenced Hth but without Antp derepression are marked by magenta arrowheads.

(C) Electron micrographs of adult *Drosophila* heads derived from *w¹¹¹⁸*, *Antp^{Ns}*, and the indicated mosaic histone replacement lines. Normal segmented antenna structures are annotated as a1, a2, a3, and ar (arista). *Antp^{Ns}* was shown as a positive control for antenna-to-leg transformation. The scale bars correspond to 100 μ m. See also Figures S2 and S4.

were milder than those of the *H3K27R* mutant, as indicated by Abd-B staining, which only shows derepression in *H3K27R* clones (Figures 2A and S2A).

Deregulation of PcG Silencing in *H3S28A* Mutant Is Independent of Active Demethylation and Cannot Be Recapitulated by Depletion of Aurora B Kinase

We investigated the mechanistic basis for the loss of silencing induced by the *H3S28A* mutation. Because H3S28ph was shown to evict PcG protein binding to H3K27me3 but also to prevent H3K27me3 demethylation, one explanation for the observed deregulation in PcG silencing in *H3S28A* mutant could be active H3K27me demethylation upon the loss of H3S28ph. If this was correct, combining *dUtx*-null background with the *H3S28A* mutation should alleviate the reduction of H3K27me levels and PcG-silencing defects. By recombining *dUtx Δ* mutation (Copur and Müller, 2013) with Δ *HisC*, we generated double homozygous mutants by recombination over a single FRT element. As shown in Figure S3A, the *dUtx Δ* , *H3S28A* mutant clone is depleted of dUtx. However, all H3K27 methylation states are not rescued under such condition (Figure 3A). We also tested the possibility that the *H3S28A* phenotype is a consequence of the absence of H3S28 phosphorylation. We therefore induced RNAi against the Aurora B kinase in the wing imaginal disc. This resulted in robust depletion of Aurora B (Figure S3B) and mitotic H3S10ph and H3S28ph (Figure S3C). Consistent with previous reports, loss of Aurora B caused aneuploidy and enlarged nuclei (Giet and Glover, 2001). If the decrease in H3K27me and the loss of Hox gene silencing observed in the

H3S28A mutant was depending on the absence of H3S28 phosphorylation, one would expect to observe these phenotypes upon loss of Aurora B. In contrast, we did not observe reduction of H3K27me3 levels or *Ubx* derepression in the knockdown region of wing discs (Figure 3B). Taken together, these data suggest that demethylation by dUtx and the loss of H3S28 phosphorylation per se do not compromise polycomb silencing in the *H3S28A* mutant.

H3S28A Impairs H3K27 Methylation of Nucleosomes by PRC2

Together, the results described above suggested the possibility that the serine-to-alanine substitution per se might affect PRC2 activity. To directly test this hypothesis, we performed in vitro histone methyltransferase (HMT) assays using a reconstituted, four-component core *Drosophila* PRC2 complex that includes Esc, Su(z)12, E(z), and Nurf55 (Müller et al., 2002; Schmitges et al., 2011). As shown by previous reports (Margueron et al., 2009; Schmitges et al., 2011), when assayed on WT nucleosomes supplied with a *trans*-acting histone peptide carrying H3K27me3, PRC2 activity was robustly enhanced in a dose-dependent manner (Figure 4). Only basal HMT activities were detected when H3S28ph, unmodified, or no histone peptides were added to the reaction mixtures. Interestingly, the stimulatory effect of H3K27me3 was retained when combined with the *H3S28A* mutation (H3K27me3S28A; Figure 4A). By contrast, the PRC2-stimulatory activity was abolished in context of the H3K27me3S28ph “double mark” (Figure 4A). Available structural data show that the stimulatory effect relies on the binding

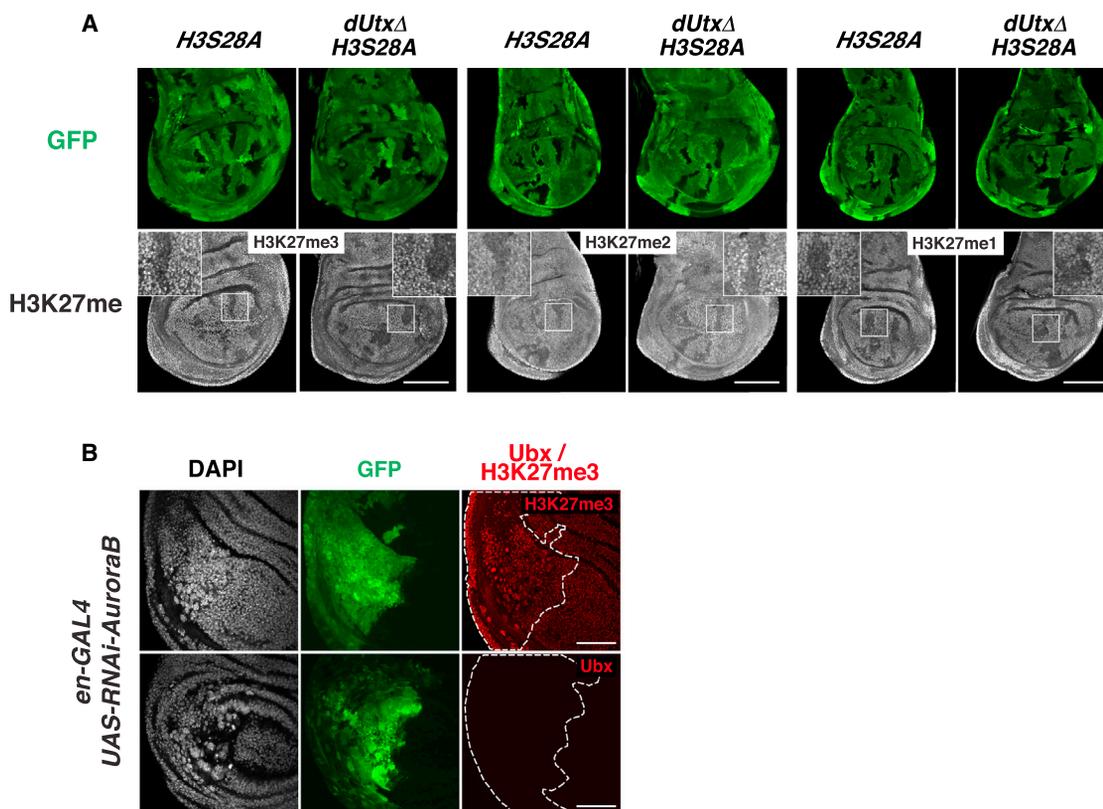


Figure 3. *dUtxΔ*, *H3S28A* Double Mutant Does Not Rescue H3K27 Methylation Levels, and Loss of Aurora B Kinase Does Not Perturb Polycomb Silencing

(A) Wing discs carrying clones with either *H3S28A* alone or in combination with *dUtxΔ* were immunostained with the indicated antibodies.

(B) Wing discs of L3 larvae with *en-GAL4* directed RNAi against Aurora B at the posterior compartment (knocked down cells are in the region marked by GFP and dotted lines) were immunostained with the indicated antibodies. DNA was stained with DAPI. The scale bars correspond to 100 μ m.

See also Figure S3.

of H3K27me3 to the beta-propeller structure of Esc with the side chain of H3S28 pointing away from the binding side (Margueron et al., 2009; O'Meara and Simon, 2012). Apparently, this interaction is not affected by the loss of the hydroxyl group in the serine-to-alanine substitution in H3K27me3S28A peptides. However, the introduction of the more-bulky and charged phosphate group on H3S28 prevents any stimulatory effect. We assume that this is due to hampered binding of the H3K27me3S28ph peptide to Esc.

We then performed PRC2 HMT assays with H3S28A mutant nucleosomes. In this context, enzymatic activity was considerably reduced, with or without supplementation of stimulatory H3K27me3-containing peptides in trans (Figure 4B). Similar to WT nucleosomal substrates, the stimulatory effect of H3K27me3 and H3K27me3S28A peptides was comparable on H3S28A nucleosomes. Therefore, the H3S28A effect can be ascribed to inhibition of PRC2-dependent methylation of H3K27.

DISCUSSION

In this report, we have established a *H3S28A* histone mutant in *Drosophila*. In theory, this mutation could have two different ef-

fects on the polycomb system. (1) It could be that PcG proteins are not evicted from H3K27me3-binding sites in the absence of H3S28ph, and thus, PcG target genes might become ectopically repressed or (2) the mutation at H3S28 or the absence of H3S28ph could compromise PcG functions, resulting in derepression of PcG target genes. We found no evidence for the first possibility, although it is formally possible that H3S28 is phosphorylated under certain developmental conditions or in response to particular stimuli to counteract polycomb silencing. Instead, our data point to an inhibition of PRC2 activity by the *H3S28A* mutation. This inhibition is independent of active H3K27 demethylation by dUtx. Besides, RNAi against Aurora B kinase and hence depletion of H3S28ph did not hamper polycomb silencing. On the other hand, H3S28A nucleosomes proved to be a suboptimal substrate for in vitro PRC2 HMT activity. Although a 3D structure of the human Ezh2 SET domain is available (Antonysamy et al., 2013), the exact contribution of the hydroxyl group of H3S28 for H3K27 methylation is difficult to deduce from the available data. vSET, the only other protein capable of H3K27 methylation in the absence of PRC2 subunits, does not require H3S28 for catalysis, whereas it does use H3A29 to define substrate specificity (Wei and Zhou, 2010). Clearly,

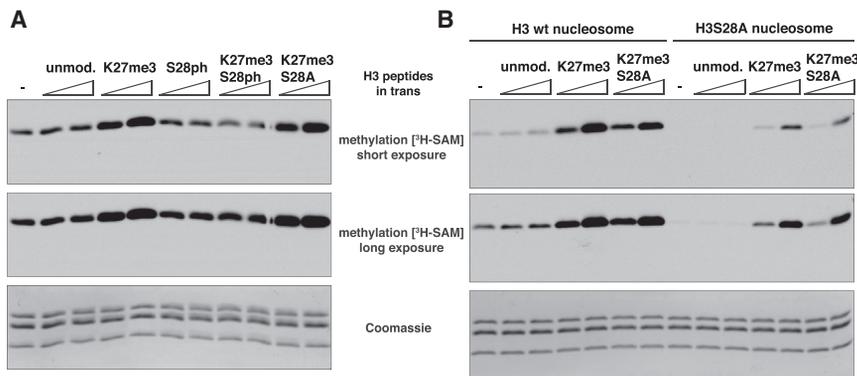


Figure 4. H3S28A Impairs H3K27 Methylation of Nucleosomes by PRC2

(A) In vitro HMT assay using reconstituted *Drosophila* PRC2 complex, WT nucleosomes, and ³H-SAM as substrates. Reactions were supplied in trans with the indicated histone peptides at concentrations of 5 and 40 μM.

(B) In vitro PRC2 HMT assay as in (A) but using nucleosomal substrates assembled with either WT-H3 or H3S28A mutant protein. Reactions were separated by SDS-PAGE and analyzed by fluorography. Fluorographs with different exposures are shown. Coomassie staining of the histones serves as loading control.

more work will be required to determine the exact structural and biochemical role of H3S28 in PRC2 catalysis. Consistent with the in vitro HMT assays, in vivo the *H3S28A* mutant exhibits defects in H3K27 methylation and shows similar, though milder, Hox derepression profiles and transformation phenotypes to those observed in *H3K27R* mutant flies.

Interestingly, the “KS” module is frequently found in Ezh2 substrates other than K27S28 of histone H3. These include K26S27 of human histone H1 variant H1b (H1.4), K38S39 of the nuclear orphan receptor ROR α , and K180S181 of STAT3 (Kim et al., 2013; Kuzmichev et al., 2004; Lee et al., 2012; Pasini et al., 2004). Whether these serine residues act similarly to H3S28 to support methylation of the adjacent lysine residue remains unknown. Of note, some other Ezh2 substrates can be methylated despite the lack of a “KS” module. These include K26 of mouse histone H1 variant H1e, K49 of STAT3, and K116 of Jarid2 (Dasgupta et al., 2015; Kuzmichev et al., 2004; Sanulli et al., 2015), where the lysine residue is followed by an alanine, glutamate, and phenylalanine, respectively. Moreover, the link between peptide sequence and enzymology of Ezh2 was shown to differ in non-histone substrates (Lee et al., 2012). Hence, the role of serine following the Ezh2 methylation target amino acid might not be extrapolated to all other Ezh2 substrates and should be tested individually.

Previous reports revealed discrepancies in *Drosophila* PcG protein localization on mitotic chromosomes depending on staining protocols and tissue types (Buchenau et al., 1998; Fanti et al., 2008; Follmer et al., 2012; Fonseca et al., 2012). Nonetheless, live imaging of Pc-GFP, Ph-GFP, and E(z)-GFP in early *Drosophila* embryos (Cheutin and Cavalli, 2012; Steffen et al., 2013) all suggested that the majority of these PcG components are dissociated from mitotic chromosomes. Because stress-induced H3S28ph evicts PcG complexes during interphase (Lau and Cheung, 2011; Schmitges et al., 2011), one might expect re-binding of PcG proteins on mitotic chromosomes depleted of H3S28ph. Whereas we did observe loss of Ph from mitotic chromosomes in WT background, we did not observe significant Ph association in *H3S28A* mutant condition (Figure S4). The reduced levels of H3K27me3 in the *H3S28A* mutant could contribute to this observation. Alternatively, other mechanisms might operate to dissociate the majority of PcG proteins during mitosis.

The establishment of the histone replacement system in *Drosophila* has proven to be an important tool to complement

functional characterization of chromatin modifiers (Hödl and Basler, 2012; McKay et al., 2015; Pengelly et al., 2013). Whereas depletion of H3K27 methylation, either by mutation of the histone mark writer *E(z)* or by mutation of the histone itself in the *H3K27R* mutant, leads to similar loss of polycomb-dependent silencing (McKay et al., 2015; Pengelly et al., 2013), other histone mutations revealed different phenotypes than the loss of their corresponding histone mark writers. For example, *H3K4R* mutations in both H3.2 and H3.3, hence a complete loss of H3K4 methylation, did not hamper active transcription (Hödl and Basler, 2012). Also, the loss of H4K20 methylation upon *H4K20R* mutation unexpectedly supports development and does not phenocopy cell cycle and gene silencing defects reported upon the loss of the H4K20 methylase PR-Set7 (McKay et al., 2015). Here, by comparing the phenotype of Aurora B knock-down and *H3S28A* mutation in vivo, together with in vitro HMT assay, we specifically attribute the requirement of the unmodified H3S28 residue in supporting PRC2 deposition of H3K27 methylation.

Whereas the published data suggest that H3S28 phosphorylation might be important for eviction of PcG components for derepression of PcG target genes upon stimulatory cues (Gehani et al., 2010; Lau and Cheung, 2011; Sawicka et al., 2014), our data reveal a so far unacknowledged function of the unphosphorylated state of H3S28. We show that serine 28 is required to enable proper methylation of H3K27 by PRC2 and thus to establish polycomb-dependent gene silencing. Serine 28 of histone H3 is universally conserved in species that display canonical PRC2-dependent silencing mechanisms. Given the fact that we find no major mitotic defects upon its mutation, we propose that the major role of this residue is to ensure optimal PRC2 function while facilitating the removal of polycomb proteins in response to signals that induce phosphorylation.

Supplemental Information for this article is available online.

EXPERIMENTAL PROCEDURES

Plasmids

Construction of integration plasmids p ϕ C31attB3xHisGU carrying either wild-type H3, H3K27R, or H3S28A mutation followed published procedures (Günesdogan et al., 2010). Entry vectors pENTR221-HisGU, pENTRL4R1-HisGU, pENTRR2L3-HisGU, and their H3K27R mutant derivatives, together with the destination vector pDESTR3R4- ϕ C31attB, were obtained from Alf Herzig (Max-Planck-Institut für Infektionsbiologie). Gene synthesis (Eurofins

Scientific SE) of a DNA fragment carrying the codon exchange AGT→GCC introduced the H3S28A mutation. Subcloning of the H3S28A DNA fragment into entry vectors and subsequent recombineering followed the procedures published for generating the *H3K27R* mutant (Günesdogan et al., 2010).

Drosophila Stocks

Site-specific transgenesis and fly genetics were performed as previously described (Günesdogan et al., 2010). The histone deficiency mutant (Δ HisC) used in this study (*Df(2L)BSC104*) was obtained from Konrad Basler (Institute of Molecular Life Sciences) in the form of *Df(2L)BSC104, FRT40A* recombinant (Hödl and Basler, 2012). The *dUtxΔ* line was obtained from Jürg Müller (The Max Planck Institute of Biochemistry) in the form of *dUtxΔ, FRT40A* (Copur and Müller, 2013) recombinant.

Mosaic Analysis and Immunostaining

Egg depositions were allowed over a 24-hr time window at 25°C. Larvae developed for 2 days before they were heat shocked at 37°C for 1 hr. L3 larva emerged 3 days after heat shock, and they were selected for the lack of green balancer before dissection. Inverted larvae heads were fixed in 4% formaldehyde/PBS for 20 min at room temperature. After washing with PBS, the samples were permeabilized twice with 0.3% Triton X-100 in PBS for 30 min at room temperature. After blocking with 3% BSA 0.03% Triton X-100 in PBS for 1 hr, the samples were incubated with primary antibodies in 0.03% Triton X-100 in PBS overnight at 4°C. Samples were then incubated secondary antibodies conjugated to Alexa (Invitrogen) fluorophores before DAPI staining. Thorough washes were applied in between incubations. Imaginal discs were carefully removed and mounted with ProLong Gold (Invitrogen) antifade reagent. Micrographs were acquired on a Zeiss LSM 780 inverted confocal microscope. Raw micrographs were processed with ImageJ and assembled using Adobe Illustrator.

Scanning Electron Microscopy

Adult fly heads were fixed as described (Wolff, 2011) using the critical-point-dried method. They were then sputter-coated with a 10-nm-thick gold film and examined under a scanning electron microscope (Hitachi S4000) using a lens detector with an acceleration voltage of 10 kV at calibrated magnifications.

In Vitro PRC2 HMT Assay

Nucleosomes and chromatin arrays were reconstituted by the salt gradient dialysis method as described (Luger et al., 1999) using recombinant *Xenopus laevis* core histones. Nucleosomes contained a 187-bp-long DNA fragment with the 601 DNA sequence at its center (Munari et al., 2012). The histone mutant H3S28A was generated by site-directed mutagenesis. All reconstitutions were analyzed by native gel electrophoresis.

H3 peptides corresponding to H3 amino acids 21–33 were synthesized according to standard procedures. A non-natural tyrosine was added at the C terminus for quantification.

HMT reactions were performed using 1- μ g nucleosomes in a total volume of 15 μ l for 1 hr at 30°C. HMT reactions were carried out in presence of 0.8 μ Ci S-[methyl-³H]-Adenosyl-L-methionine (³H-SAM) and 325 ng PRC2 (75 nM) in HMT buffer (50 mM Tris-HCl [pH 8.8], 5 mM MgCl₂, and 4 mM DTT). Recombinant *Drosophila melanogaster* PRC2 complex, comprised of Su(z)12, E(z), Nurf55, and Esc, was a kind gift of Nicolas Thomä (Friedrich Miescher Institute) and was purified as described (Schmitges et al., 2011). PRC2 HMT assays in presence of H3_{21–33} peptides in trans contained 5 or 40 μ M of peptide. Reactions were stopped by addition of SDS sample buffer followed by separation by SDS-PAGE. Reactions were analyzed by fluorography.

More details on the experimental procedures can be found in the [Supplemental Information](#).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2015.04.055>.

AUTHOR CONTRIBUTIONS

P.Y.K.Y., A.-M.M., and G.C. conceived and designed the *Drosophila* experiments. A.S. performed the in vitro PRC2 HMT assays with input from W.F. P.Y.K.Y. performed all other experiments. All authors analyzed the data. P.Y.K.Y. and G.C. wrote the manuscript with help from A.S. and W.F.

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