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CBP-mediated acetylation of histone H3 lysine 27 antagonizes *Drosophila* Polycomb silencing

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Trimethylation of histone H3 lysine 27 (H3K27me3) by Polycomb repressive complex 2 (PRC2) is essential for transcriptional silencing of Polycomb target genes, whereas acetylation of H3K27 (H3K27ac) has recently been shown to be associated with many active mammalian genes. The Trithorax protein (TRX), which associates with the histone acetyltransferase CBP, is required for maintenance of transcriptionally active states and antagonizes Polycomb silencing, although the mechanism underlying this antagonism is unknown. Here we show that H3K27 is specifically acetylated by *Drosophila* CBP and its deacetylation involves RPD3. H3K27ac is present at high levels in early embryos and declines after 4 hours as H3K27me3 increases. Knockdown of E(Z) decreases H3K27me3 and increases H3K27ac in bulk histones and at the promoter of the repressed Polycomb target gene *abd-A*, suggesting that these indeed constitute alternative modifications at some H3K27 sites. Moderate overexpression of CBP in vivo causes a global increase in H3K27ac and a decrease in H3K27me3, and strongly enhances Polycomb mutant phenotypes. We also show that TRX is required for H3K27 acetylation. TRX overexpression also causes an increase in H3K27ac and a concomitant decrease in H3K27me3 and leads to defects in Polycomb silencing. Chromatin immunoprecipitation coupled with DNA microarray (ChIP-chip) analysis reveals that H3K27ac and H3K27me3 are mutually exclusive and that H3K27ac and H3K4me3 signals coincide at most sites. We propose that TRX-dependent acetylation of H3K27 by CBP prevents H3K27me3 at Polycomb target genes and constitutes a key part of the molecular mechanism by which TRX antagonizes or prevents Polycomb silencing.

KEY WORDS: CBP (NEJ), Histone H3K27, Acetylation, Trimethylation, Polycomb silencing, *Drosophila*

INTRODUCTION

The evolutionarily conserved Polycomb group (PcG) proteins, acting together in several complexes, are required for silencing of the homeotic genes and many other genes (Schwartz and Pirrotta, 2007). Polycomb repressive complex 2 (PRC2), which contains the H3K27-specific histone methyltransferase (HMTase) E(Z) and the other PcG proteins SU(Z)12 and ESC (or ESCL), as well as the histone H4-binding protein p55, is responsible for mono-, di- and trimethylation at histone H3K27 (H3K27me1/2/3). Only H3K27me3 is known to be required for Polycomb silencing and has a genome-wide distribution pattern distinct from that of H3K27me1/2 in human T cells (Wang et al., 2008).

PRC2 functions together with the PRC1 complex, which binds sites containing H3K27me3 via the chromodomain of its Polycomb (PC) subunit, and mono-ubiquitylates histone H2AK119 via its RING (also known as SCE) subunit (Cao et al., 2005; Wang et al., 2004). Both PRC2 and PRC1 associate, perhaps transiently, with the histone deacetylase RPD3, which is also required for Polycomb silencing (Chang et al., 2001; Tie et al., 2001; van der Vlag and Otte, 1999).

Polycomb silencing is antagonized by Trithorax group (TrxG) proteins (Klymenko and Muller, 2004; Poux et al., 2002; Ringrose and Paro, 2004), which are required for maintenance of active transcriptional states. Members of the TrxG are involved in chromatin remodeling, transcription initiation and elongation, and in post-translational modification of histones. TRX itself is a HMTase that specifically trimethylates histone H3 on lysine 4 (H3K4me3) (Smith et al., 2004), a modification generally associated with the promoter regions of active genes (Barski et al., 2007; Bernstein et al., 2005; Kim et al., 2005). Expression of the homeotic genes is greatly reduced in *trx* mutants (Breen and Harte, 1993; Mazo et al., 1990), but is less affected in *trx* PcG double mutants (Klymenko and Muller, 2004; Saleh et al., 2007), suggesting that Polycomb silencing occurs by default in the absence of TRX and thus that TRX is not simply a coactivator, but also an anti-silencer, actively preventing the silencing of Polycomb target genes in cells in which they must be expressed (Klymenko and Muller, 2004).

A TRX complex purified from *Drosophila* embryos contains the CREB-binding protein (CBP, encoded by *nej*) (Petruk et al., 2001), a conserved histone acetyltransferase (HAT) that is involved in many developmental processes (Goodman and Smolik, 2000; Smolik and Jones, 2007). This complex exhibits HAT activity in vitro, although the specific histone H3 residues targeted for acetylation and its functional consequences remain to be determined. Genetic studies suggest that CBP and TRX collaborate in preventing Polycomb silencing and in maintaining the expression of Polycomb target genes. Mutations in *trx* and *nej* (CBP) cause a similar loss of *Ubx* expression at the onset of Polycomb silencing (Petruk et al., 2001). Like TRX, CBP is recruited to the *bxd* PRE (Polycomb-response element) of the *Ubx* gene, and antagonizes Polycomb silencing of *bxd* PRE-mini-*white* reporter transgenes (Petruk et al., 2001).

The molecular basis of this antagonism is unknown, but is often assumed to involve the H3K4 methylation activity of TRX. However, recent evidence from genome-wide mapping of the sites of various histone modifications indicates that although H3K4me3 is widely associated with active promoters, a subset of transcriptionally inactive genes in human embryonic stem (ES) cells

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and various differentiated cells (Barski et al., 2007; Bernstein et al., 2005; Kim et al., 2005; Roh et al., 2006; Wang et al., 2008) contain both H3K27me3 and H3K4me3 in their promoter regions, indicating that the presence of H3K4me3 is not incompatible with H3K27me3 or silencing. This suggests that some other activity/function associated with TRX might be primarily responsible for preventing trimethylation of H3K27 sites targeted by PRC2 and thereby for antagonizing Polycomb silencing. One possibility is acetylation of H3K27 (H3K27ac), as acetylation and trimethylation of the same lysine are mutually exclusive.

H3K27ac was first identified in yeast (Suka et al., 2001) and more recently in mouse and human cells (Garcia et al., 2007). A recent genome-wide survey of 18 different acetyl-histone modifications in human T cells reported that H3K27ac is highly enriched at promoter regions of transcriptionally active genes (Wang et al., 2008), including Polycomb target genes (see Fig. S9A in the supplementary material). Here we show that the H3K27ac modification also occurs in *Drosophila* and that CBP is responsible for acetylation of H3K27. We provide evidence that at least a subpopulation of the H3K27 sites that are trimethylated by PRC2 are also alternatively acetylated, indicating that H3K27ac prevents trimethylation of H3K27 at sites in Polycomb target genes. Consistent with this, we find that H3K27ac and H3K27me3 have dynamic and complementary temporal profiles during embryogenesis: H3K27ac is present at high levels in early embryos and decreases as H3K27me3 increases after 4 hours (the onset of Polycomb silencing). Importantly, we show that TRX is also required for normal H3K27ac levels, suggesting that the anti-silencing function of TRX depends on its ability to promote and/or maintain H3K27 acetylation.

MATERIALS AND METHODS

Antibodies

Guinea pig anti-CBP antibodies were generated against six-histidine-tagged CBP (residues 1-287) and were purified on Protein A Sepharose. Rabbit anti-H3K27ac (ab4729) and goat anti-H3 antibody (ab12079) against a C-terminal peptide of H3 were from Abcam. Rabbit anti-H3K27me3 antibody was a generous gift from Thomas Jenuwein (Peters et al., 2003) or purchased from Upstate (07-449). Rabbit anti-acetyl-H3K18 (ab1191), anti-acetyl-H3K23 (ab46982) and anti-trimethyl-H3K4 (ab8580) antibodies were from Abcam. Rabbit anti-acetyl-H3K9 (07-352) and anti-acetyl-H3K14 (06-911) antibodies were from Upstate. Goat anti-GST antibody was from Amersham. Rabbit anti-E(Z), anti-ESC and anti-RPD3 antibodies, and guinea pig anti-SU(Z)12 and anti-ESCL antibodies were described previously (Tie et al., 2001; Tie et al., 2003; Tie et al., 2005; Tie et al., 2007). Rabbit anti-GCN5 antibody was kindly provided by Jerry Workman (Kusch et al., 2003). Anti-β-tubulin monoclonal antibodies were obtained from the Developmental Studies Hybridoma Bank at the University of Iowa.

Constructs

A PCR product encoding CBP residues 1-287 was inserted into a modified pET-11d vector at *Nde*I and *Nsi*I sites to yield pET-CBP(1-287)-H_6. A 5.2 kb CBP cDNA fragment (cut by *Nhe*I and *Nsi*I), encoding C-terminal residues 1476-3222, was inserted into the pMT-FLAG vector to yield pMT-FLAG-CBPΔN. An *Eco*RV fragment (encoding residues 1566-2142), which contains a portion of the HAT domain, was removed from this plasmid to yield pMT-FLAG-CBPΔ(N+HAT). A PCR product encoding GST-H3 was digested with *Sal*I and partially digested with *Sac*I (to avoid cutting at the *Sac*I site in the H3 cDNA) and was inserted into a modified pAct-5c vector to generate pAct-GST-H3. pAct-GST-H3(K27Q), containing a K27Q point mutation (AAG/CAG), was generated using the QuikChange Site-Directed Mutagenesis Kit from Stratagene. The plasmid pMT-CBP (full length) was kindly provided by S. M. Smolik (Ludlam et al., 2002).

Preparation of protein and histone extracts

Adult flies (0.2-0.3 g) were placed in a 1.5-ml tube, frozen in liquid nitrogen and then ground. For histone extraction, 0.2 M H_2SO_4 was used. For protein extraction, 0.4-0.6 ml of nuclear extraction buffer (containing 0.25 M NaCl, 0.11 M KCl, protease inhibitors and 10 mM sodium butyrate) was used. Supernatants (protein extracts) were collected after centrifugation (30,000 ***g*** for 1 hour at 4°C). Histone extractions in acid or high salt buffer from embryos were carried out as described (Shechter et al., 2007). Briefly, after preparation of embryo nuclear extracts (Tie et al., 2001), nuclear pellets were washed once in nuclear extraction buffer and then resuspended in 0.2 M H_2SO_4 or high salt buffer (50 mM Tris-HCl pH 8.0, 2.6 M NaCl, 0.05% NP40). Supernatants were collected by centrifugation. Histones were precipitated from acid extracts with acetone (5× volume). High salt extracts were desalted by Centrifugal Filter Tube (4 ml) with a 5-kDa nominal molecular weight limit (Millipore). Recombinant *Drosophila* histone H3 was expressed in BL-21 cells from pET-H3-H4 [from Jim Kadonaga (Levenstein and Kadonaga, 2002)] and extracted in high salt buffer.

Western analysis of histones in whole-cell extracts

Drosophila S2 cells were collected and resuspended in one volume of extraction buffer (8 M urea, 4.0% CHAPS, 40 mM Tris pH 7.4) and one volume of 2× SDS sample buffer at a final concentration of ~$0.5\text{-}1.0\times10^5$ cells/μl. The mixture was heated to 95°C for 5-10 minutes and 5-10 μl was electrophoresed in a 15% SDS-PAGE gel followed by western analysis using a 0.2 μm pore size nitrocellulose membrane. Various anti-H3 antibodies were used at dilutions ranging from 1:500 to 1:2000. Enhanced chemiluminescent (ECL) detection and West Dura Extended Duration Substrate (Pierce) were used.

Transient transfection assay

Drosophila S2 Cells were transiently transfected with plasmid DNA (pMT-CBP and pAct-GST-H3) using SuperFect Transfection Reagent (Qiagen) as described (Tie et al., 2005). After a 24-hour transfection, cells were treated with a final concentration of 0.5 mM $CuSO_4$ and 5 mM sodium butyrate for 2 days, then whole-cell extracts were prepared for western analysis.

Purification of FLAG-CBPΔN from S2 cells

S2 cells (from the *Drosophila* Genomics Resource Center) in six 60-mm dishes were transiently transfected with pMT-FLAG-CBPΔN. To induce overexpression of FLAG-CBPΔN, a final concentration of 0.4 mM $CuSO_4$ and 0.04 mM $ZnSO_4$ was added to the medium for 1-2 days at 25°C. Protein extracts from control and FLAG-CBPΔN-expressing S2 cells were prepared in 1 ml 50 mM Tris-HCl pH 7.4, 0.1 M NaCl, 10 mM KCl, 5% glycerol, 1 mM PMSF, and were incubated with 0.1 ml anti-FLAG M2 Agarose for 2 hours at 4°C. The beads were then washed extensively in 50 mM Tris-HCl (pH 7.4) buffer containing 0.3 M NaCl. Ten microliters of 2× SDS sample buffer was added to 5 μl of beads and used for western analysis with anti-FLAG M5 monoclonal antibodies (for FLAG-CBPΔN) and anti-GCN5 antibodies (negative control) (see Fig. S1A in the supplementary material). Thirty microliters of beads was used for SDS-PAGE when Coomassie Blue staining was performed (see Fig. S1B in the supplementary material).

In vitro acetylation assay

FLAG-CBPΔN was purified from transiently transfected S2 cells with anti-FLAG M2 Agarose (see Fig. S1 in the supplementary material). The acetylation reaction was initiated by mixing 30 μl FLAG-CBPΔN, bound to agarose beads, with 30 μl acetylation buffer (40 mM Tris-HCl pH 8.0, 0.1 M NaCl, 10% glycerol, 0.1 mM EDTA, 1 mM DTT, 1 mM PMSF, 5 mM sodium butyrate, 0.1 mM acetyl-CoA) and 10 μl (~10 μg) recombinant histone H3 (from *E. coli*). After a 1.5-hour incubation at 30°C, the reaction was quenched by adding 40 μl 2× SDS sample buffer. Samples were boiled and 10 μl of each was electrophoresed in a 15% SDS-PAGE gel followed by western analysis with anti-acetyl-H3 antibodies.

Human p300, purified from Sf9 insect cells infected with a recombinant baculovirus expressing FLAG-tagged p300, was a generous gift from Cheng-Ming Chiang (Thomas and Chiang, 2005). Ten or 20 ng of p300 was used in an in vitro acetylation reaction as above.

Mass spectrometric identification of histone H3 modification

Nuclei were isolated from 0-18 hour embryos and washed in nuclear extraction buffer (plus protease inhibitors and 10 mM sodium butyrate). *Drosophila* histones were extracted from nuclei by high salt buffer and were separated on a 15% SDS-PAGE gel (12 cm length) and stained with Coomassie Blue. The 17 kDa H3 band (~10 μg) was excised and washed, and then subjected to propionylation (of monomethylated and unmodified lysines) and digestion with trypsin (Peters et al., 2003), which cleaves on the C-terminal side of lysines that have been propionylated. Peptides were processed using LC-MS/MS on an Orbitrap XL platform. Two different queries (with ion scores over 30) identified H3 peptides corresponding to 27-KSAPATGGVKKPHR-40 and containing acetyl-K27. MS/MS fragmentation spectra of acetyl-K27 peptides were further manually validated (see Fig. S3 in the supplementary material).

Quantitative western analysis using Li-Cor imager

For quantitative western analysis, blots were blocked for 1 hour at room temperature using a solution of 1.5% cold water fish skin gelatin (Sigma) and then incubated overnight at 4°C with antibodies against specific histone H3 modifications. Following incubation with primary antibody, blots were washed and incubated with goat anti-rabbit IRDYE 680CW (Li-Cor) secondary antibody (1:20,000) for 1 hour at room temperature. Blots were washed and scanned using the Odyssey Infrared Imager (Li-Cor). After scanning, blots were stripped using 0.1 M NaOH, blocked as above and incubated with goat anti-H3 primary antibody overnight at 4°C. Blots were then incubated with donkey anti-goat IRDYE 800CW (Li-Cor) secondary antibodies (1:20,000), washed and scanned as above. Integrated signal intensity for histone modifications and total H3 was acquired using Odyssey Software. Data were exported to Excel and the ratios of signals from different histone modifications to total H3 obtained. This ratio was set to 1 for each control. Data displayed are the means and standard deviations from three independent samples (see Fig. S5 in the supplementary material).

Double-stranded RNA (dsRNA) and RNA interference (RNAi)

dsRNAs corresponding to portions of the *E(z)* (700 bp), *esc* (490 bp) and *escl* (619 bp) mRNA sequences were previously described (Kurzhals et al., 2008). Similarly, dsRNAs of *Su(z)12* (700 bp), *Rpd3* (950 bp, containing 223 bp of 5′ untranslated region and 727 bp of 5′ coding region), *CBP* (739 bp of 5′ coding region), *Sir2* (702 bp of coding region), *Gcn5* (662 bp of coding region), *Creb2* (759 bp of 5′ coding region) and *GFP* (green fluorescent protein) (700 bp of coding region) were synthesized and used for treatment of *Drosophila* S2 cells (Tie et al., 2007). For knockdown of E(Z) (or other PRC2 subunits), S2 cells were treated with dsRNA (15 μg/ml) for 7-8 days with addition of dsRNA four or five times for efficient knockdown, but only three times in 8 days for partial knockdown of E(Z). Cells were collected after RNAi. Whole-cell extracts were used for western analysis as described above. At least two separate RNAi treatments and western blots were carried out to confirm knockdown effects. S2 cells treated with dsRNA for *Su(z)12*, *CBP* and *Rpd3* appeared to grow slowly, yielding about a third as many cells as control S2 cells (either untreated or treated with GFP dsRNA).

Chromatin immunoprecipitation (ChIP) and real-time PCR analysis

ChIP was performed as described (Tie et al., 2007) using S2 cells with or without *E(z)* dsRNA treatment for 8 days. Protein G and A beads, 3 μg of rabbit anti-H3K27ac (ab4729) and anti-H3K27me3 (07-449), 8 μl of pre-immune, anti-E(Z) and anti-CBP sera were used for immunoprecipitations. Real-time PCR reactions were performed on a 7300 Real-Time PCR System (Applied Biosystems) with SYBR Green and 125 nM primers for *abd-A*. PCR products (93 or 106 bp) were obtained from two pairs of primers (sequences available upon request) located at the *abd-A* promoter and transcribed region (spanning 232-325 bp upstream or 329-435 bp downstream of the *abd-A* transcription start site). Each primer pair yielded a single peak in the dissociation curve, a slope of –3.5 and an R^2 value of 0.99 from the six-point standard curve.

ChIP coupled with DNA microarray (ChIP-chip) analysis

The ChIP-chip experiment described here was adapted from previous studies (Odom et al., 2004; Scacheri et al., 2006). Briefly, 1-2×10^8 human HEK293 cells were cross-linked with 1% formaldehyde for 10 minutes at room temperature. Cell nuclei were isolated and sonicated. DNA fragments were enriched by immunoprecipitation with antibodies against H3K4me3, H3K27ac or H3K27me3. After heat reversal of the cross-links, the enriched DNA was amplified by ligation-mediated PCR (LM-PCR) and then fluorescently labeled with Cy5 dUTP (Amersham). A sample of DNA (input) that was not enriched by immunoprecipitation was subjected to LM-PCR and labeled with Cy3 dUTP. ChIP-enriched and unenriched (input) labeled samples were co-hybridized to ENCODE microarrays (NimbleGen). Raw array data from the NimbleGen arrays were normalized using bi-weight mean using the NimbleGen software (version 2.3). Log_2 ratios corresponding to ChIP DNA/total genomic DNA were calculated. Ratio data from individual ChIP-chip experiments were quantile normalized and median smoothed.

ChIP-chip experiments using *Drosophila* S2 cells (cultured at 25°C in Schneider's *Drosophila* medium supplemented with 10% FBS) were carried out similarly, with minor modifications. Cy3 and Cy5 random primer labeling for input and ChIP-enriched samples was carried out using a CGH Labeling Kit for Oligo Arrays (Enzo, 42671). Labeled samples were purified using Centrisep spin columns (Princeton Separations, CS-901) and co-hybridized to 2.1 M feature *Drosophila melanogaster* tiling microarrays (NimbleGen, DM_5_Catolog_tiling_HX1) in a MAUI Hybridization System at 42°C for 18-20 hours. Sonicated salmon sperm DNA (20 μg) instead of human Cot-1 DNA was used for blocking during hybridization. NimbleScan software and Integrated Genome Browser (1.5 GB; Affymetrix) were used for analysis. Two separate ChIP-chip experiments were performed for confirmation.

Genetic crosses

For CBP overexpression, *UAS-CBP* flies (Kumar et al., 2004) were crossed to *hsp70-GAL4* and maintained continuously at 29°C to increase GAL4 expression from the leaky *hsp70* promoter. The *hsp70-GAL4* and *UAS-CBP* adults served as controls. Temperature-sensitive trx^1 (Breen, 1999) homozygotes were raised at 18°C (permissive temperature) and adults shifted to 29°C (restrictive temperature) for 4 days before collecting eggs and allowing progeny to develop at 29°C.

To moderately knock down CBP, *hsp70-GAL4*/*CyO* flies were crossed to the *UAS-CBP* RNAi line (Kumar et al., 2004) and progeny allowed to develop at 29°C.

A homozygous *UAS-CBP* transgenic line was crossed to a homozygous *GMR-GAL4* driver to overexpress CBP in the eye, which causes a small, rough eye phenotype (Kumar et al., 2004). For simultaneous overexpression of CBP and E(Z), *GMR-GAL4*/*CyO*; *UAS-CBP*/*TM3* was crossed to homozygous *hs-E(z)* (Laible et al., 1997) and progeny raised continuously at either 29°C, which promotes a moderate increase in *E(z)* expression from the leaky *hsp70* promoter (and also enhances GAL4-induced activation of *UAS-CBP*), or at 18°C as a control, which allows little, if any, *E(z)* expression from the *hsp70* promoter (and also somewhat reduces GAL4 activation activity, causing a slightly weaker CBP overexpression phenotype).

To examine whether CBP overexpression enhances Polycomb phenotypes, *hsp70-GAL4*/*CyO*; *UAS-CBP*/*UAS-CBP* was crossed to Pc^3/*TM3* at 29°C and scored for extra sex combs on T2 and T3 legs of male progeny of genotype *hsp70-GAL4*/+; *UAS-CBP*/Pc^3. Controls included the *CyO*/+; *UAS-CBP*/Pc^3 siblings from the same cross and *hsp-GAL4*/+; Pc^3/+.

For TRX overexpression, EP3541 flies (from the Szeged Stock Center) were crossed to a *da-GAL4* line or *hsp70-GAL4/CyO*.

Immunostaining of polytene chromosomes

Polytene chromosomes from wandering third instar larvae were immunostained with primary antibodies (anti-CBP, anti-TRX, anti-H3K4me3 and anti-H3K27ac), and FITC-labeled or Texas Red-labeled secondary antibodies as described (Tie et al., 2001; Tie et al., 2003). Chromosomes from homozygous trx^1 mutants were co-stained with anti-CBP (guinea pig) and anti-H3K27ac (rabbit) antibodies.

RESULTS

Histone H3K27 is acetylated by CBP in *Drosophila*

The H3K27ac modification has been found in yeast and mammals. As shown in Fig. 1A, it is also detected by western analysis in *Drosophila* embryos (lanes 3 and 4), but not in

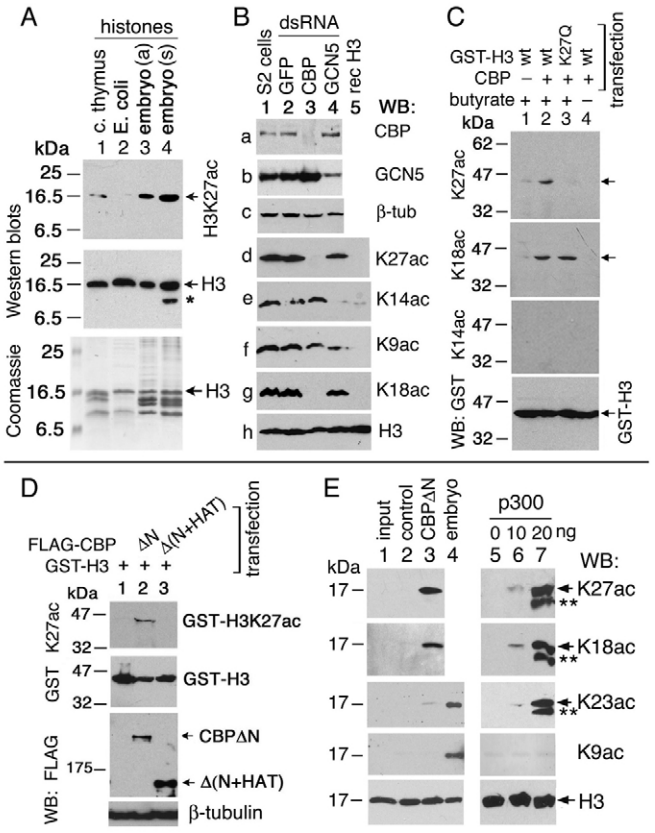

Fig. 1. H3K27 is acetylated by *Drosophila* CBP. (**A**) Western blots (top two panels) and Coomassie Blue staining (bottom) of calf thymus histones (lane 1), recombinant *Drosophila* H3 and H4 from *E. coli* (lane 2) and *Drosophila* embryo histones (0-22 hour, lanes 3 and 4) extracted with acid (a) or high salt (s). The asterisk marks N-terminally degraded H3. (**B**) Western blots of whole S2 cell extracts after a 5-day treatment with CBP or GCN5 dsRNAs (lanes 3 and 4). Recombinant (unmodified) H3 from *E. coli* in lane 5 serves as a general control for the modification-specific antibodies used. β-tubulin and histone H3 (c and h) serve as loading controls. (**C**,**D**) Western blots of whole S2 cell extracts after transient transfection to co-express (C) full-length CBP and GST-H3 (wild-type or K27Q mutant) or (D) truncated FLAG-CBP [CBPΔN and CBPΔ(N+HAT)] and GST-H3. Endogenous H3K27ac was strongly detected in lanes 1-4 (not shown). (**E**) Immunoblots of in vitro acetylation assays using purified FLAG-CBPΔN (lane 3) and FLAG-p300 (lanes 6 and 7). Lane 1 is input substrate (unmodified H3 from *E. coli*) and lane 2 is a parallel control using an anti-FLAG-purified fraction from non-transfected S2 cells. Lane 4 (embryo histones) serves as a positive control. The two asterisks mark a C-terminally degraded H3.

recombinant (unmodified) H3 (lane 2). These signals were completely abolished by pre-incubation of anti-H3K27ac antibodies with an H3K27ac peptide, but not with an H3K9ac or H3K14ac peptide (see Fig. S2 in the supplementary material), confirming that these antibodies are highly specific for H3K27ac. The relative amount of H3K27ac was much higher in *Drosophila* embryos than in calf thymus histones (lane 1) or in human HEK293 cells (not shown). To confirm the presence of H3K27ac in *Drosophila*, we analyzed histone H3 isolated from embryos by mass spectrometry. Acetyl-K27 was unequivocally identified in two peptides corresponding to H3 residues 27-40 (see Fig. S3 in the supplementary material).

To identify the *Drosophila* HAT responsible for the acetylation of H3K27, we used RNA interference (RNAi) in S2 cells to knock down CBP (the ortholog of mammalian CBP and p300) and also GCN5 (encoded by *Pcaf*) (the ortholog of mammalian GCN5 and PCAF), which has been reported to be required for H3K27 (and H3K14) acetylation in yeast (Suka et al., 2001) and plants (Benhamed et al., 2006). As shown in Fig. 1B, knockdown of CBP resulted in a substantial reduction in K27ac and K18ac (lane 3, panels d and g), indicating that CBP is required for the acetylation of H3 on K27 and K18 in S2 cells. Knockdown of GCN5 decreased K14ac as expected (lane 4, panel e), but had little if any effect on K27ac (lane 4, panel d).

To further confirm that CBP acetylates H3K27, we used a transient co-transfection assay in S2 cells. As shown in Fig. 1C, acetylation of GST-H3 on K27 was readily detected by western analysis with anti-H3K27ac antibodies when cells expressed both GST-H3 and CBP (lane 2, top panel), but was barely detectable when cells expressed GST-H3 alone (lane 1). Substitution of a glutamine for K27 (H3K27Q) in GST-H3 abolished the H3K27ac signal (lane 3, top panel). Acetylation of GST-H3 on K18 was also detected in cells co-transfected with CBP (lanes 2 and 3, second panel), but acetylation of K14 (third panel), K9 and K23 (data not shown) was not. CBP lacking the N-terminal 1475 residues (CBPΔN) retained the H3K27 acetylation activity, but further deletion of residues 1652-2232 from CBPΔN, which removes part of the HAT domain, resulted in loss of GST-H3K27 acetylation (Fig. 1D, lanes 2 and 3, top panel). These data strongly suggest that CBP acetylates H3K27 and that its HAT domain is required.

To determine whether CBP directly acetylates H3K27, we used recombinant *Drosophila* CBP and unmodified H3 as a substrate in an in vitro acetylation assay. Consistent with the in vivo results above, K27 and K18 (but not K9 or K14) were selectively acetylated by purified FLAG-CBPΔN (Fig. 1E, lane 3). A weak signal was detected with an H3K23ac-specific antibody. This could be due to a non-specific activity of CBP in vitro, as H3K23ac was readily detected in S2 cells after CBP knockdown (see lane 2 in Fig. 2B), or might reflect weak CBP HAT activity on K23 in vivo. Thus, results from CBP knockdown, S2 cell transfection and the in vitro enzyme assay indicate that *Drosophila* CBP directly and selectively acetylates H3K27 and H3K18.

To determine whether the H3K27 acetylation activity of *Drosophila* CBP has been evolutionarily conserved in humans, we also carried out in vitro acetylation using purified recombinant human p300, one of two closely related human homologs of *Drosophila* CBP. As shown in Fig. 1E (lanes 6 and 7), human p300 also selectively acetylates H3 on K27 and K18, and also on K23, but not on K9 or K14 (not shown).

RPD3 is involved in the deacetylation of H3K27ac

To identify the histone deacetylase(s) responsible for deacetylation of H3K27ac, we used RNAi to knock down two candidates, SIR2 and RPD3, both of which are associated with some PcG protein complexes and are required for robust Polycomb silencing (Chang et al., 2001; Furuyama et al., 2004; Tie et al., 2003; van der Vlag and Otte, 1999). Knockdown of RPD3, but not SIR2, elevated the H3K27ac level (Fig. 2B, lane 5, top two panels), indicating that *Drosophila* RPD3 is involved in the deacetylation of H3K27ac. Knockdown of RPD3 also increased H3K9ac and H3K14ac and appeared to slightly elevate H3K18ac and H3K23ac (Fig. 2B, lane 5), indicating that *Drosophila* RPD3 has broad substrate specificity, similar to yeast RPD3 (Suka et al., 2001). We cannot rule out the possibility that there might be additional deacetylases that target

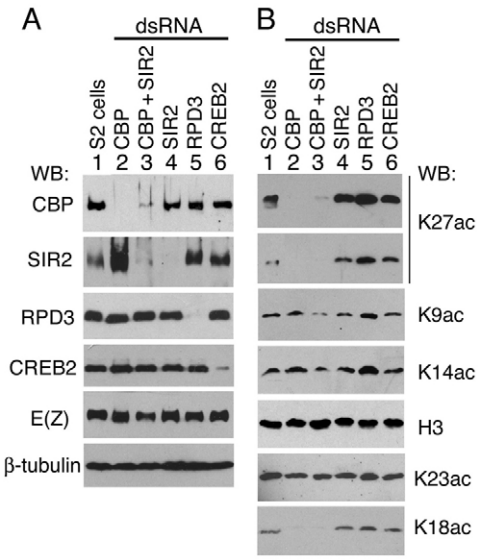

Fig. 2. RPD3 is involved in deacetylation of histone H3K27ac. Western analysis of S2 cell extracts, carried out as described in Fig. 1B, after knockdown of CBP, SIR2, RPD3 and CREB2 (as indicated above each lane 2-6), demonstrating the effects of each knockdown on (**A**) levels of proteins listed on left and (**B**) levels of histone H3 modifications listed on right. The top two panels in B show different exposures of the same anti-H3K27ac western. β-tubulin and H3 serve as loading controls.

H3K27ac, but these data raise the possibility that one role of the RPD3 associated with PcG complexes may be to deacetylate H3K27ac at Polycomb target genes to allow its subsequent trimethylation by the E(Z) complex. An analogous role in deacetylating H3K9 to allow its methylation has been proposed for the RPD3 present in SU(VAR)3-9 HMTase complexes (Czermin et al., 2001).

We also examined the effect of CBP knockdown on RPD3 and SIR2 levels, as elevation of either might account for the observed decrease in H3K27ac (Fig. 1B, lane 3). The RPD3 level was unaffected, but the SIR2 level was elevated in CBP-depleted S2 cells (Fig. 2A, lane 2). However, simultaneous knockdown of CBP and SIR2, or of CBP alone, reduced H3K27ac to similar extents (Fig. 2B, lanes 2 and 3, top panel), indicating that the decrease in H3K27ac is due to depletion of CBP itself and not to the elevated SIR2 level.

The developmental profiles of H3K27me3 and H3K27ac are dynamic and complementary

Polycomb silencing of the homeotic genes begins after the first 4 hours of embryogenesis, as the transiently expressed transcription factors encoded by the segmentation genes disappear. To determine whether the onset of Polycomb silencing is correlated with global changes in H3K27me3 and H3K27ac levels, we examined their temporal profiles during embryogenesis by western analysis of bulk histones. As shown in Fig. 3, relative to later stages, the H3K27me3 level was extremely low in early embryos (0-4 hours). It increased gradually between 4 and 16 hours (lanes 3-5), reached a plateau at a relatively high level between 12 and 16 hours, and was maintained at this level during the rest of embryogenesis (16-24 hours, lanes 6 and 7). H3K27ac exhibited a complementary temporal profile: it was present at a higher level in early stages, decreased progressively between 7 and 16 hours (Fig. 3, lanes 4 and 5), reached a plateau at a relatively low level between 16 and 20 hours (lane 6), and was maintained at this low level during the rest of embryogenesis (20-24 hours, lane 7). We extended the western analysis to several other modifications on H3. Interestingly, unlike H3K27me3, H3K27me2 had a similar temporal profile to K27ac, whereas K27me1, K18ac, K9ac, K36me3 and K4me3 exhibited different patterns that appeared to be less dynamic (Fig. 3). The remarkably complementary temporal profiles of H3K27ac and H3K27me3, which change more than 3-fold, are consistent with the possibility that they are alternative antagonistic modifications on the same H3 molecules. Furthermore, the presence of substantially greater amounts of H3K27ac in very early embryos suggests that the establishment of Polycomb silencing might require deacetylation of existing H3K27ac.

H3K27ac and H3K27me3 are alternative modifications on Polycomb target genes

To further explore whether H3K27ac and H3K27me3 are indeed alternative antagonistic modifications on the same H3 molecules, we examined their genome-wide distributions in *Drosophila* S2 cells by ChIP-chip. The results indicated that H3K27ac and H3K27me3 are mutually exclusive genome-wide (see Fig. S8 in the supplementary material). H3K27ac was absent when H3K27me3 was present on many previously identified Polycomb target genes (i.e. those marked by H3K27me3 and PC binding) (Schwartz et al., 2006), including the Antennapedia Complex genes (see Fig. S8A in the supplementary material) and the Bithorax Complex genes *abd-A* and *Ubx* (see Fig. S8B in the supplementary material). Interestingly, although H3K27me3 is absent within the transcribed region of *Abd-B* in a different *Drosophila* cell line (Schwartz et al., 2006), it was present across the entire *Abd-B* gene (but H3K27ac was absent) in the S2 cells used here (see Fig. S8B in the supplementary material).

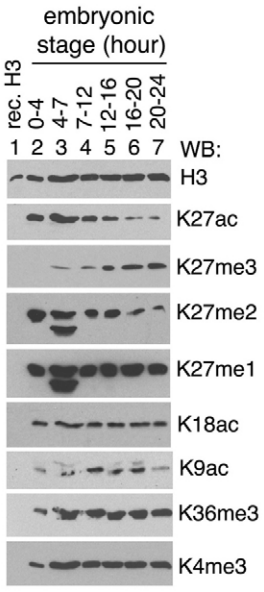

Fig. 3. Complementary changes in H3K27ac and H3K27me3 during embryogenesis. Western analysis of histone H3 extracted from *Drosophila* embryos in high salt as described in Fig. 1B. Embryo ages (hours after egg lay) are indicated above each lane. The band below K27me2 and K27me1 in lane 3 is partially degraded H3. In 0-4 hour embryos, H3K27me3 could be detected only in 4× concentrated extracts (data not shown).

Importantly, we identified a group of Polycomb target genes that contain H3K27me3 in a different *Drosophila* cell line (Schwartz et al., 2006), but lacked H3K27me3 in the S2 cells used here and instead contained H3K27ac (see Fig. S8C in the supplementary material). These cases strongly suggest that K27me3 and K27ac are alternative modifications on the same sites in Polycomb target genes.

Knockdown or overexpression of E(Z) has complementary effects on H3K27me3 and H3K27ac levels

If K27ac and K27me3 are alternative antagonistic modifications on the same H3 molecules in Polycomb target genes, we would expect that reducing H3K27me3 would elevate H3K27ac globally. To determine whether this is the case, we knocked down PRC2 subunits by RNAi in S2 cells. As expected, depletion of E(Z) by RNAi (Fig. 4A, lanes 4-6) caused a substantial global reduction in H3K27me3 and H3K27me2 levels in bulk histones. In addition, the H3K27ac level also increased ~3-fold (Fig. 4A, compare H3K27ac in lanes 4-6 with lanes 1-3), which is somewhat less than the fold change in H3K27ac levels observed in wild-type embryos between early and late embryogenesis (Fig. 3). The levels of H3K18ac and of other histone modifications did not increase. Similar increases in H3K27ac levels were observed in knockdowns targeting SU(Z)12 or the ESC and ESCL subunits of PRC2 (see Fig. S4 in the supplementary material), greatly reducing the likelihood that they are due to RNAi off-target effects. Furthermore, as expected, single knockdown of ESC or ESCL did not significantly decrease H3K27me3 or H3K27me2 or increase H3K27ac (see Fig. S4 in the supplementary material), as ESC and ESCL can substitute for one another in PRC2 complexes in S2 cells (Kurzhals et al., 2008).

We previously observed that H3K27me3, the least abundant of the three methyl-K27 isoforms, is the most sensitive to PRC2 subunit knockdown (Kurzhals et al., 2008; Tie et al., 2007). K27me3 can be depleted by a partial RNAi knockdown of E(Z) that leaves K27me2 and K27me1 levels unaffected. We took advantage of this to show that H3K27ac increases predominantly at the expense of H3K27me3 and not H3K27me2 (Fig. 4B). When E(Z) was only partially depleted in S2 cells (Fig. 4B, lane 3), so that H3K27me3 was dramatically reduced but the H3K27me2 level remained similar to that in control cells (lanes 1 and 2), the H3K27ac level still increased ~3-fold, just as it did following more complete knockdown of E(Z), which substantially decreased both K27me2 and K27me3 levels (Fig. 4A). Conversely, moderate overexpression of E(Z) in vivo, which also increases the SU(Z)12 level (Fig. 4B, lane 5) and presumably the amount of PRC2 complex, increased H3K27me3 and decreased H3K27ac (Fig. 4B, top two panels, lanes 4 and 5), but caused no obvious change in H3K27me2, H3K18ac or H3K4me3. These results further suggest that a substantial fraction of the H3K27ac and specifically the H3K27me3 isoform occur as alternative modifications on the same subpopulation of H3. They thus suggest that H3K27 acetylation might function as part of a regulatory mechanism for directly antagonizing or preventing Polycomb silencing, and may also be required for the developmentally programmed reversal of silencing. Of course, given the general association of histone acetylation with active genes, we cannot rule out the possibility that part of the increase in H3K27ac observed after E(Z) knockdown might be an indirect effect of activating genes that are not direct targets of Polycomb silencing.

To determine whether the increase in H3K27ac in E(Z)-depleted S2 cells (Fig. 4A) reflects acquisition of H3K27ac at the promoters of Polycomb target genes, we used ChIP to assay the presence of

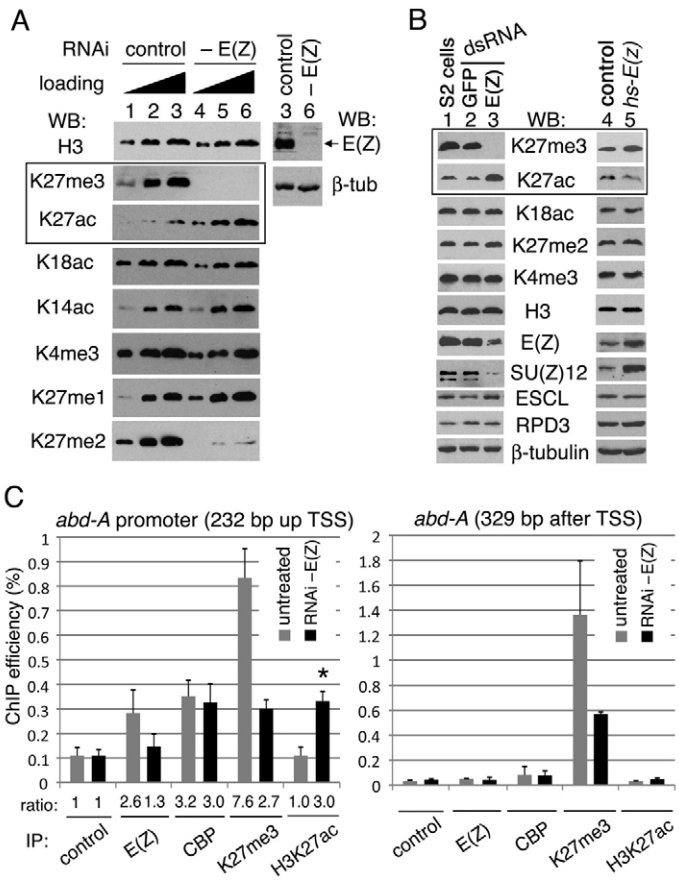

Fig. 4. Knockdown of E(Z) leads to reciprocal changes in H3K27me3 and H3K27ac. (**A**) Western analysis of different numbers of S2 cells (0.15, 0.3 and 0.45 million cells), either untreated (lanes 1-3) or after E(Z) knockdown (lanes 4-6). Note the decrease in H3K27me3 and H3K27me2 and the increase in H3K27ac after knockdown. The two lanes to the right show efficient depletion of E(Z). (**B**) Western analysis of whole S2 cell extracts (lanes 1-3) and adult fly extracts from controls (w^{1118}, lane 4) and flies constitutively overexpressing E(Z) from a *hs-E(z)* transgene raised at 29°C (lane 5). Lane 1, untreated cells; lane 2, control knockdown (GFP); lane 3, partial knockdown of E(Z). Note the residual E(Z) and that H3K27me2 was present at the control level. (**C**) Reduction of H3K27me3 and appearance of H3K27ac on the *abd-A* promoter after knockdown of E(Z). ChIP efficiency, in terms of the percentage of input DNA recovered by immunoprecipitation, was determined by real-time PCR and is shown for E(Z), CBP, H3K27me3 and H3K27ac. Rabbit pre-immune serum was used as a background control. The ratio of signal to background is indicated at the bottom of each column (left panel). Means and s.d. from two separate ChIP real-time PCRs are shown. Note that E(Z) and H3K27me3 signals at the *abd-A* promoter (left) were above background (2.6 and 7.6 times, respectively) in untreated S2 cells (gray), but were decreased to 1.3 and 2.7 times background after E(Z) knockdown (black). In the transcribed region of *abd-A* in untreated and E(Z) knockdown S2 cells, signals for E(Z), CBP and H3K27ac were not significantly above background (right-hand panel), indicating their absence.

H3K27ac and H3K27me3, as well as CBP and E(Z), at the promoter of the Bithorax Complex gene *abd-A*, which is repressed in the S2 cells used [H3K27ac is predominantly located in the promoter regions of active genes in human cells (Wang et al., 2008), suggesting that this localization might be the most important for the active state]. As shown in Fig. 4C, H3K27me3 but not H3K27ac

was present at the promoter of the repressed *abd-A* gene. After E(Z) knockdown, the H3K27me3 signal at the *abd-A* promoter decreased (down by ~65%), and H3K27ac was now present (at three times background level) at the promoter (Fig. 4C, asterisk in left panel) but not in the transcribed region (right-hand panel) of *abd-A*. These data indicate that H3K27ac and H3K27me3 occur as alternative modifications in the promoter region of *abd-A* and suggest that H3K27 acetylation can occur by default upon loss of E(Z). Interestingly, CBP appeared to be weakly present at the promoter of *abd-A* in control (untreated) and E(Z) knockdown S2 cells (Fig. 4C, left panel), suggesting that its H3K27 acetylation activity at promoters is likely to be regulated by other factors in vivo.

Altering CBP levels in vivo has complementary effects on H3K27ac and H3K27me3 levels

To further explore the potential for reciprocal antagonism between H3K27 acetylation and trimethylation, we examined the effect of CBP knockdown and overexpression in vivo. Whereas *CBP*-null mutants arrest in early embryogenesis (Akimaru et al., 1997), partial knockdown of CBP in vivo using an *hs-GAL4*-driven *UAS-CBP* RNAi transgene (Kumar et al., 2004) and continuous culture at 29°C allows survival to adulthood and led to a substantial decrease in H3K27ac, an increase (by ~49%) in H3K27me3 (Fig. 5A, top two panels; see quantitative western analysis in Fig. S5 in the supplementary material), but to no change in H3K4me3 or H3K27me2 levels in bulk histones. These adults exhibited partial transformation of abdominal segment A5 to A4 (42%, *n*=61; controls 0%, *n*=68), a characteristic phenotype of *trx* mutants that is due to reduced expression in A5 of *Abd-B*, the homeotic gene most sensitive to reduced *trx* function (Breen, 1999; Ingham, 1985). This suggests that the global reduction in H3K27ac caused by CBP knockdown leads to the silencing of *Abd-B* in A5 by allowing its default trimethylation by PRC2. A similar loss of expression of *Ubx*, also characteristic of *trx* mutants, was previously reported in *CBP* null mutant embryos (Petruk et al., 2001). Like *trx* mutations, mutations in *CBP* also act as enhancers of the dominant *Ubx* and *Dfd* mutant phenotypes in adults (Florence and McGinnis, 1998).

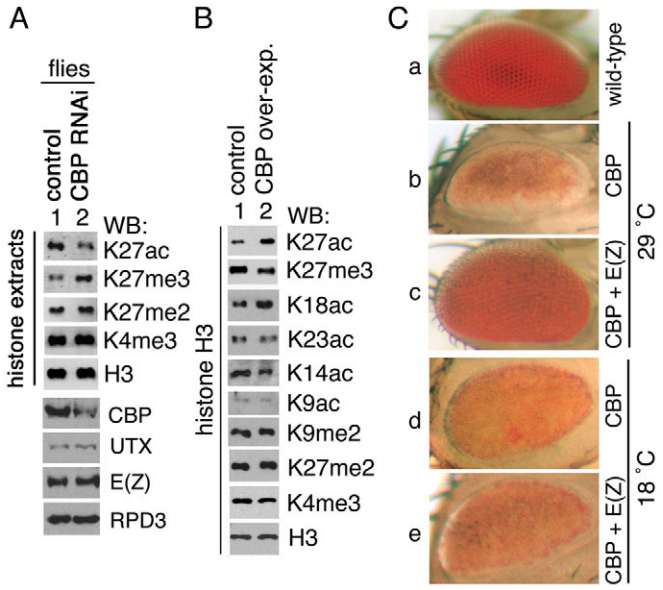

Fig. 5. Knockdown or overexpression of CBP leads to reciprocal changes in H3K27ac and H3K27me3. (**A**,**B**) Western analysis of histones and proteins from adult flies in which CBP has either been (A) moderately knocked down by RNAi (*hsp70-GAL4/+*; *UAS-CBP RNAi/+*) or (B) moderately overexpressed (*hsp70-GAL4/+*; *UAS-CBP/+*) (lane 2). Flies expressing GAL4 alone (*hsp70-GAL4*) serve as a control (lane 1). The H3K27me3 level (relative to total H3) was decreased by ~30% by CBP overexpression, as determined by a Li-Cor imager. Consistent with the CBP knockdown results, moderate CBP overexpression also increased the level of H3K18ac (lane 2 in B), but not of K23ac, K14ac or K9ac. (**C**) The small rough eye phenotype caused by stronger eye-specific overexpression of CBP is suppressed by simultaneous overexpression of E(Z). Eyes are shown from (a) a wild-type control (w^{1118}), (b,d) a CBP overexpresser and (c,e) a CBP+E(Z) overexpresser at 29°C and at 18°C as indicated. Homozygous *hs-E(z)* flies, raised at 29°C, have wild-type eyes (not shown).

Overexpressing CBP ubiquitously at high levels causes lethality (Ludlam et al., 2002), but overexpressing it constitutively at moderate levels from a *UAS-CBP* transgene permits survival to adulthood and led to an increase in H3K27ac and to a moderate reduction in H3K27me3 (by ~30%) as compared with controls expressing GAL4 alone (Fig. 5B, lanes 1 and 2). The reciprocal decrease in H3K27me3 further suggests that at least some H3K27 acetylation occurs on the same sites that are trimethylated by E(Z) and could thus antagonize Polycomb silencing (see below).

Although these moderate CBP overexpressers exhibited increased H3K27ac at the expense of H3K27me3 (Fig. 5B), they did not exhibit overt Polycomb phenotypes. This is not surprising because their H3K27me3 level was reduced by only ~30%, similar to that of heterozygous $E(z)^{63}$ null mutants (data not shown), which also do not exhibit any Polycomb phenotypes, suggesting that a greater reduction is required to detect adult Polycomb phenotypes in a wild-type genetic background. Nevertheless, this moderate CBP overexpression strongly enhanced the weak dominant silencing defects of heterozygous Pc^3 mutants, indicating that Polycomb silencing is indeed perturbed by CBP overexpression. Both the frequency and severity of the characteristic extra sex combs phenotype seen in Pc^3/+ males was enhanced (see Fig. S6 in the supplementary material). For example, 100% (*n*=82) of *hs-GAL4/+*; *UAS-CBP*/Pc^3 males raised at 29°C exhibited sex comb bristles (typically five or six) on their T3 legs, as compared with 36% (*n*=76) (typically only one or two bristles) of their *CyO/+*; *UAS-CBP*/Pc^3 sibling controls and 36% (*n*=80) (typically only one or two bristles) of the *hs-GAL4/+*; +/Pc^3 controls (see Table S1 in the supplementary material). This phenotype results from derepression in T2 and T3 leg imaginal discs of *Scr*, a direct target of Polycomb silencing, the expression of which is normally restricted to the T1 and cephalic imaginal discs (Pattatucci and Kaufman, 1991). Thus, CBP overexpression appears to antagonize silencing of the *Scr* gene in vivo.

To further explore this antagonism, we also determined whether simultaneous E(Z) overexpression could compensate for the effects of CBP overexpression. Although a high level of ubiquitous CBP overexpression is lethal, when targeted to the eye imaginal disc using the strong *GMR-GAL4* driver it reduces adult eye size and causes a rough eye phenotype (Kumar et al., 2004), similar to homozygous *E(z)* mutant clones in the eye (Janody et al., 2004). This phenotype is substantially suppressed, although not completely, if the CBP HAT activity is inactivated by mutation (Kumar et al., 2004), indicating that some, but not all, effects of CBP overexpression are dependent on its HAT activity. As shown in Fig. 5C, flies in which CBP and E(Z) were simultaneously overexpressed at 29°C [*GMR-GAL4/+*; *UAS-CBP*/*hs-E(z)*] had a normal eye size and a much milder rough eye phenotype (panel c) than their sibling controls (*GMR-GAL4/+*; *UAS-CBP/TM3*) (panel b), similar to that achieved by deleting or mutating the HAT domain in the *UAS-CBP*

transgene (Kumar et al., 2004). However, when expression from the *hs-E(z)* transgene was minimized, by culturing the same cross at 18°C, the small rough eye phenotype was not alleviated (Fig. 5C, panels d and e). This suggests that increased H3K27me3 associated with E(Z) overexpression antagonizes the increased H3K27ac caused by CBP overexpression.

TRX is required for normal H3K27ac levels

The functional collaboration between CBP and TRX in maintaining *Ubx* expression and antagonizing Polycomb silencing (Petruk et al., 2001) suggested that TRX might affect H3K27 acetylation. As shown in Fig. 6A, when the temperature-sensitive *trx*^{1} mutant is grown at a restrictive temperature (29°C), not only is the amount of TRX and H3K4me3 detectable on polytene chromosomes substantially reduced, as expected (Chinwalla et al., 1995), but the H3K27ac level is also reduced, whereas the CBP level appears unchanged (right-hand two panels). Western analysis of histones extracted from *trx*^{1} adults confirmed that they have reduced levels of H3K4me3 and H3K27ac, as well as of H3K18ac. They also had increased H3K27me3 (Fig. 6B, lane 2). The *trx*^{1} mutant thus exhibits the same changes in H3K27ac and H3K18ac levels as are caused by CBP knockdown in S2 cells, suggesting that the acetylation of H3K27 and H3K18 by CBP in vivo requires TRX.

Conversely, TRX overexpression under the control of a *da-GAL4* driver caused a substantial increase in the H3K27ac level detected on polytene chromosomes (Fig. 6C). Western analysis of

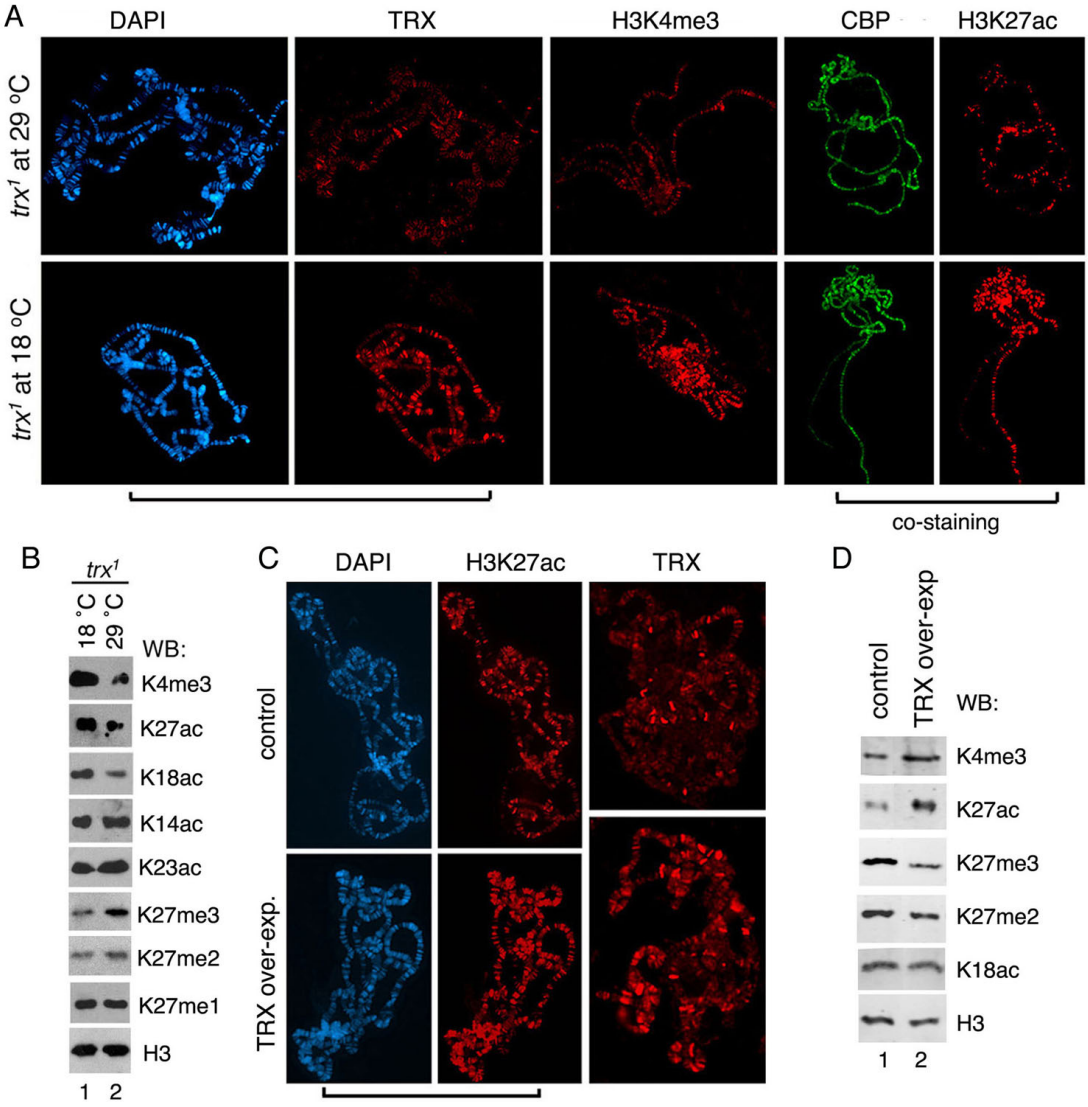

Fig. 6. TRX is required for H3K27 acetylation. (**A**) Polytene chromosomes from the temperature-sensitive *trx*^{1} mutant grown at permissive (18°C) or restrictive (29°C) temperature were stained under identical conditions with the antibodies indicated at the top of each panel. DAPI co-staining (left column) of the chromosomes stained with anti-TRX antibody (second column), indicates that the general chromosome structure appears unperturbed by the *trx*^{1} mutation. The two right-hand panels show simultaneous co-staining with guinea pig anti-CBP and rabbit anti-H3K27ac antibodies; the two signals appear similar to those in preparations stained with either anti-CBP or anti-H3K27ac alone (data not shown). (**B**) Western analysis of histones extracted from adult *trx*^{1} flies with antibodies specific for total H3 and post-translationally modified H3 as indicated. The CBP level appeared unchanged in the *trx*^{1} mutant at 29°C (data not shown). (**C**) Polytene chromosomes from control (top) and TRX-overexpressing (*da-GAL4* driver, bottom) larvae stained as in A. (**D**) Western analysis of histones extracted from control flies (*hs-GAL4*, lane 1) and flies overexpressing TRX under control of the *hs-GAL4* driver at 29°C (lane 2).

bulk histones confirmed the increases in H3K27ac and H3K4me3, and also revealed a corresponding decrease in H3K27me3 (see similar result in Fig. 6D). These adult TRX overexpressers exhibited phenotypes that are typically seen in Polycomb mutants (see Fig. S7 and Table S2 in the supplementary material), clearly indicating that TRX overexpression antagonizes Polycomb silencing in vivo.

Co-occurrence of H3K27ac with H3K4me3 in promoter regions

The above results suggest that the TRX dependence of H3K27 acetylation might reflect the coordinate action of TRX and CBP to trimethylate H3K4 and acetylate H3K27 at the same specific sites. Since the human TRX homolog, MLL, is also physically associated with CBP (Ernst et al., 2001), we carried out ChIP in human HEK293 cells coupled with a partial human genome tiling array (ChIP-chip) to determine whether the H3K4me3 and H3K27ac profiles are similar, and, at same time, to determine whether the mutually exclusive distributions of K27me3 and K27ac are conserved in humans. We found that H3K4me3 and H3K27ac coincide at most sites (see Fig. S9A in the supplementary material). Both are enriched at the 5′ regions of many genes, but in a subset of genes, including the Hox genes (top panel in Fig. S9A in the supplementary material), both H3K4me3 and H3K27ac were also present in both the promoter and transcribed regions. This remarkable similarity in their genome-wide profiles is consistent with the possibility that both modifications result from the coordinated action of TRX and CBP at the same sites (Jiang et al., 2007; Taverna et al., 2007). Furthermore, the H3K27ac and H3K27me3 distributions were also mutually exclusive in human cells (see Fig. S9C in the supplementary material). This result also indicates that H3K27ac and H3K27me3 do not occur on adjacent nucleosomes in the same promoter regions [as is the case for H3K4me3 and H3K4ac (Wang et al., 2008)], consistent with H3K27ac acting to prevent H3K27me3 on the same sites at Polycomb target genes. That deposition of H3K27ac in the promoter region is crucial for preventing Polycomb silencing is also suggested by the observation that the H3K27me3 found throughout the large 5′ regulatory region of the repressed *Ubx* gene remains unchanged when *Ubx* is active: H3K27me3 is lost only from the promoter region, where H3K4me3 appears (Papp and Muller, 2006).

DISCUSSION

The major findings of this work are: (1) that *Drosophila* CBP acetylates H3K27; (2) that this acetylation requires TRX; and (3) that it prevents H3K27 trimethylation by E(Z) at Polycomb target genes and antagonizes Polycomb silencing. The remarkably complementary developmental profiles of H3K27ac and H3K27me3 (but not H3K27me2) during embryogenesis suggest that the deposition of H3K27me3, which increases steadily after ~4 hours with the onset of Polycomb silencing, occurs at the expense of a substantial fraction of the H3K27ac already present. This suggests that the establishment of Polycomb silencing might require active deacetylation of this pre-existing H3K27ac. The reciprocal effects of knockdown and overexpression of CBP and E(Z) on H3K27 trimethylation and acetylation in bulk chromatin further suggest that the two modifications constitute alternative chromatin states associated with active and inactive genes. Consistent with this, ChIP-chip experiments (see Figs S8 and S9 in the supplementary material) revealed that H3K27me3 and H3K27ac are mutually exclusive genome wide. Moreover, in S2 cells, the inactive *abd-A* gene does not have the H3K27ac modification in its promoter region, but acquires it upon RNAi knockdown of E(Z). It will be important to determine whether such a modification switch occurs genome wide after loss of E(Z).

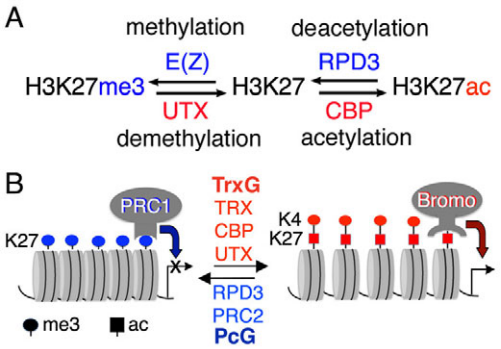

Fig. 7. Model of Polycomb silencing and its dynamic regulation. (**A**) Summary of enzymatic reactions on H3K27. (**B**) PRC2 and RPD3 promote Polycomb silencing by deacetylating H3K27ac and trimethylating H3K27 (blue oval) at promoter nucleosomes, which are bound by PC-containing PRC1. TRX, CBP and UTX collaborate to prevent Polycomb silencing by demethylating and acetylating H3K27 (red square) at promoter nucleosomes and stimulating transcription by trimethylation of H3K4 (red oval). The five nucleosomes displayed represent either inactive (left) or active (right) genes.

The ability of E(Z) overexpression to suppress the small rough eye phenotype of CBP overexpressers (Fig. 5C) further supports the conclusion that H3K27 trimethylation by E(Z) antagonizes H3K27 acetylation by CBP and suggests that deacetylation of H3K27 by RPD3, and possibly other deacetylases, might be a prerequisite for subsequent methylation by E(Z) and therefore important for reversal of an active state. Conversely, the ability of CBP and TRX overexpression to increase the global H3K27ac level at the expense of H3K27me3 suggests that either active demethylation of H3K27me3 by the H3K27-specific demethylase UTX (Agger et al., 2007; Lee et al., 2007; Smith et al., 2008), or histone replacement (Ahmad and Henikoff, 2002), might be a prerequisite to acetylation by CBP. Indeed, depletion of *Drosophila* UTX in vivo using a GAL4-inducible UTX RNAi transgene line results in an increase in H3K27me3, as previously reported (Smith et al., 2008), and in a marked decrease in H3K27ac (R.B., F.T. and P.J.H., unpublished). We summarize the enzymatic reactions responsible for depositing and removing these antagonistic modifications on H3K27 in Fig. 7A. These data, together with the evidence of developmentally programmed reversal of Polycomb silencing (Chen et al., 2005), now suggest that the widely accepted stability of Polycomb silencing during development might be more dynamically regulated than previously appreciated.

H3K27 acetylation

This is the first report, to our knowledge, that CBP/p300 acetylates H3K27. We show that recombinant *Drosophila* CBP acetylates H3K27 and K18 in vivo and in vitro (Fig. 1C,E). The greatly reduced H3K27ac levels in CBP-depleted S2 cells (Fig. 1B, Fig. 2B) also strongly suggest that CBP is the major H3K27 acetylase in *Drosophila*. The conservation of H3K27 acetylation by human p300, together with the reported association of CBP with the TRX homolog MLL in humans (Ernst et al., 2001), suggest that it is likely to play a similar role in antagonizing Polycomb silencing in mammals.

The genome-wide distribution of H3K27ac, as estimated from our human ChIP-chip experiments, appears very similar to that of H3K4me3. This suggests that H3K27ac is much more widely distributed than just at Polycomb target genes, which are estimated to number several thousand in mammalian cells and hundreds in *Drosophila*. Although these numbers could grow with the identification of additional Polycomb-silenced genes in additional cell types, the recently reported strong correlation of H3K27ac with active genes (Wang et al., 2008) suggests that it plays an additional role(s) in promoting the transcription of active genes, including those that are never targets of Polycomb silencing. (Note that the H3K27ac at non-Polycomb target genes will not be directly affected by global changes in H3K27me3.) Interestingly, like H3K27me3, H3K27ac appears on the transcribed regions of Polycomb target genes (see Fig. S8C,D and Fig. S9A, top panel, in the supplementary material), which might reflect a role for H3K27ac in facilitating transcriptional elongation, and, conversely, a role for H3K27me3 in inhibiting elongation. In addition to its anti-silencing role in preventing H3K27 trimethylation, H3K27ac may also serve as a signal for recruitment of other proteins with additional enzyme activities that alter local chromatin structure further to facilitate or promote transcription. Prime candidates are those containing a bromodomain, a conserved acetyl-lysine-binding module present in several dozen chromatin-associated proteins, including a number of TrxG proteins that also antagonize Polycomb silencing.

TRX dependence of H3K27 acetylation

The results presented here provide new insight into how TRX and CBP function together to antagonize Polycomb silencing. Robust H3K27 acetylation by CBP is dependent on TRX (Fig. 6A,B), suggesting that H3K27ac plays a crucial role in the anti-silencing activity of TRX. Consistent with this, preliminary genetic evidence suggests that the Polycomb phenotypes caused by TRX overexpression are dependent on CBP, as they are suppressed by RNAi knockdown of CBP (R.B. and P.J.H., unpublished). The nature of this dependence is currently unknown, but could involve targeting of CBP by TRX or regulation of the H3K27 acetylation activity of CBP by TRX.

The physical association of TRX and CBP and the widespread coincidence of H3K27ac and H3K4me3 sites in the human ChIP-chip data further suggest that the two modifications might be coordinately executed by TRX and CBP. However, our results also raise the possibility that H3K4 trimethylation by TRX itself might be less important for antagonizing Polycomb silencing than H3K27 acetylation. This possibility is also suggested by the discovery of Polycomb-silenced genes in ES and human T cells that contain 'bivalent' marks (both H3K4me3 and H3K27me3) in their promoter regions (Bernstein et al., 2006; Wang et al., 2008) [although the H3K4me3 levels at these inactive genes are typically lower, on average, than they are at active genes (Guenther et al., 2007), hinting at the possible importance of quantitative effects of the two marks (Papp and Muller, 2006)].

We propose a speculative model for the regulation of Polycomb silencing (Fig. 7B) that incorporates the activities of TRX, CBP, E(Z), RPD3 and UTX. Repressed genes are marked with H3K27me3. H3K27 trimethylation by PRC2 [which can also control DNA methylation in mammals (Vire et al., 2006)] requires RPD3 (and possibly other histone deacetylases) to deacetylate any pre-existing H3K27ac. H3K27me3 promotes binding of PC-containing PRC1 complexes, which may inhibit H3K27 acetylation and maintain silencing through 'downstream' events, including those promoted by the H2AK119 mono-ubiquitylation mediated by its RING subunit (Cao et al., 2005; Wang et al., 2004). Conversely, active genes are marked with H3K4me3 and H3K27ac. H3K27 acetylation by CBP is dependent on TRX and possibly other TrxG proteins, as suggested by a recent report that H3K27me3 levels are significantly increased on salivary gland polytene chromosomes from *trx*, *ash1* and *kis* mutants (Srinivasan et al., 2008). Our results predict that this increase will be accompanied by a decrease in H3K27ac. Interestingly, *ash1* encodes another HMTase that also interacts with CBP and antagonizes Polycomb silencing. Acetylation of H3K27 is likely to also require the K27-specific demethylase UTX when removal of pre-existing H3K27me3 is a prerequisite for acetylation, e.g. for developmentally regulated reversal of Polycomb silencing at the onset of differentiation. H3K27ac prevents H3K27 trimethylation and might also serve as a signal for recruitment of other TrxG proteins with additional chromatin-modifying activities that may protect the H3K27ac modification and also alter local chromatin structure to promote transcription and further inhibit Polycomb silencing.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/136/18/3131/DC1

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Table S1. CBP overexpression strongly enhances the *Pc³* mutant phenotype

Genotype	*n*	Extra sex combs (%)	
		T2	T3
CyO/+; *UAS-CBP*/*Pc³*	76	65	36
hsp-GAL4/+; *Pc³*/+	80	59	36
hsp-GAL4/+; *UAS-CBP*/*Pc³*	82	100	100

hsp70-GAL4/*CyO*; *UAS-CBP*/*UAS-CBP* females were crossed to *Pc³*/*TM3* males at 29°C. The presence of extra sex combs on T2 and T3 legs was scored in *hsp-GAL4*/+; *UAS-CBP*/*Pc³* males, and *CyO*/+; *UAS-CBP*/*Pc³* and *hsp-GAL4*/+; *Pc³*/+ males (controls).

Table S2. Overexpression of TRX causes Polycomb phenotypes

Genotype	*n*	Extra sex combs (%)	
		T2	T3
da-GAL4/+; +/+	58	0	0
EP3541/EP3541	60	0	0
da-GAL4/+; *EP3541*/+	66	92	85

da-GAL4/da-GAL4 females were crossed to *EP3541/EP3541* males at 25°C. The presence of extra sex combs on T2 and T3 legs was scored in *da-GAL4*/+; +/+ (control) and *da-GAL4*/+; *EP3541*/+ males. Homozygous *EP3541* adults showed no signs of Polycomb phenotypes.