

## RESEARCH ARTICLE

# Trithorax monomethylates histone H3K4 and interacts directly with CBP to promote H3K27 acetylation and antagonize Polycomb silencing

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**ABSTRACT**

Trithorax (TRX) antagonizes epigenetic silencing by Polycomb group (PcG) proteins, stimulates enhancer-dependent transcription, and establishes a ‘cellular memory’ of active transcription of PcG-regulated genes. The mechanisms underlying these TRX functions remain largely unknown, but are presumed to involve its histone H3K4 methyltransferase activity. We report that the SET domains of TRX and TRX-related (TRR) have robust histone H3K4 monomethyltransferase activity *in vitro* and that Tyr3701 of TRX and Tyr2404 of TRR prevent them from being trimethyltransferases. The *trx*<sup>Z11</sup> missense mutation (G3601S), which abolishes H3K4 methyltransferase activity *in vitro*, reduces the H3K4me1 but not the H3K4me3 level *in vivo*. *trx*<sup>Z11</sup> also suppresses the impaired silencing phenotypes of the *Pc*<sup>3</sup> mutant, suggesting that H3K4me1 is involved in antagonizing Polycomb silencing. Polycomb silencing is also antagonized by TRX-dependent H3K27 acetylation by CREB-binding protein (CBP). We show that perturbation of Polycomb silencing by TRX overexpression requires CBP. We also show that TRX and TRR are each physically associated with CBP *in vivo*, that TRX binds directly to the CBP KIX domain, and that the chromatin binding patterns of TRX and TRR are highly correlated with CBP and H3K4me1 genome-wide. *In vitro* acetylation of H3K27 by CBP is enhanced on K4me1-containing H3 substrates, and independently altering the H3K4me1 level *in vivo*, via the H3K4 demethylase LSD1, produces concordant changes in H3K27ac. These data indicate that the catalytic activities of TRX and CBP are physically coupled and suggest that both activities play roles in antagonizing Polycomb silencing, stimulating enhancer activity and cellular memory.

**KEY WORDS:** Trithorax, H3K4 monomethylation, CBP, *Drosophila*

**INTRODUCTION**

Polycomb group (PcG) and Trithorax group (TrxG) proteins play evolutionarily conserved roles in the epigenetic regulation of gene expression. Originally identified in *Drosophila* by their role in maintaining the spatially restricted patterns of homeotic (HOX) gene expression during development, they regulate many other genes that encode transcription factors and signaling factors that act as ‘master’ regulators of the many distinct cell identities found in multicellular organisms (Schwartz and Pirrotta, 2007; Schuettengruber et al., 2011). The mutually antagonistic activities of PcG and TrxG

proteins promote the stable, mitotically heritable maintenance of repressed and active transcriptional states, respectively.

Maintenance of transcriptionally silent states of PcG-regulated genes requires Polycomb repressive complexes 1 and 2 (PRC1 and PRC2) (Margueron and Reinberg, 2011), which are recruited to Polycomb response elements (PREs) (Müller and Kassis, 2006). PRC2 trimethylates histone H3 lysine 27 (H3K27me3), a mark that is distributed in broad domains over inactive PcG-regulated genes, encompassing promoters, flanking regulatory regions, including PREs and enhancers, as well as transcribed regions (Schwartz et al., 2006). PRC1 binds H3K27me3 via its PC subunit, and has several other activities essential for silencing (Margueron and Reinberg, 2011).

*Drosophila* Trithorax (TRX) is best known for its role in antagonizing transcriptional silencing by PcG proteins, stimulating enhancer-dependent transcription (Poux et al., 2002), and maintaining a mitotically heritable ‘cellular memory’ of prior transcriptional activity of PcG-regulated genes (Schuettengruber et al., 2011). TRX binds constitutively to PREs, apparently even through DNA replication (Petruk et al., 2012), which are thus also TRX response elements (TREs). TRX is also found at promoters of PcG-regulated genes (Schwartz et al., 2010; Enderle et al., 2011). Tethering a GAL4-TRX fusion protein to a *Ubx* reporter transgene revealed that TRX can boost enhancer-dependent reporter expression but has no intrinsic transcription-activating activity in the absence of enhancers. Stimulation of enhancer-dependent transcription by endogenous TRX requires the presence of a PRE/TRE (Pirrotta et al., 1995; Poux et al., 2002). Thus, although TRX is recruited to PRE/TREs, surrounding enhancers may be important targets of TRX catalytic activity.

TRX is a large multifunctional protein with a SET domain, four PHD fingers, and FYRN and FYRC domains (Ringrose and Paro, 2004), which are conserved in its mammalian orthologs MLL1 and MLL4. The TRX SET domain has been shown to have lysine methyltransferase activity with substrate specificity for histone H3K4 (Smith et al., 2004); however, its product specificity has not been definitively demonstrated (Smith et al., 2004; Ardehali et al., 2011). H3K4 is present in mono-, di- and tri-methylated isoforms *in vivo*. H3K4me3 is a signature mark of active promoters, whereas H3K4me1 is a signature mark of enhancers (Heintzman et al., 2007). Both TRX and its mammalian ortholog MLL1 were, until recently, thought to be responsible for deposition of the H3K4me3 mark at promoters of active TRX-regulated genes (Petruk et al., 2012). However, *Drosophila* SET1 (and mammalian SET1A and SET1B, also known as SETD1A and SETD1B) now appears to be the principal H3K4 trimethyltransferase responsible for the H3K4me3 at promoters of active genes (Ardehali et al., 2011; Hallson et al., 2012). This prompted us to reexamine the intrinsic

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catalytic activity of the TRX SET domain. We report here that both the TRX and TRR SET domains (and their mammalian orthologs) have only robust H3K4 monomethyltransferase activity *in vitro* and that a recombinant TRX core complex [TRX SET domain + WRAD (WDR5, RBBP5, ASH2L, DPY30)] has only a greatly enhanced H3K4 monomethyltransferase activity. Moreover, the genome-wide distribution of TRX is highly correlated with H3K4me1 (but not H3K4me3) at PcG-regulated genes. Consistent with this, the catalytically inactive *trx<sup>Z11</sup>* and *trr<sup>3</sup>* mutants have reduced H3K4me1 levels but normal H3K4me3 levels *in vivo*. Furthermore, *trx<sup>Z11</sup>* suppresses the Polycomb phenotype of *Pc<sup>3</sup>/+* mutant adults, implicating its catalytic activity in antagonizing Polycomb silencing.

We previously reported that the histone acetyltransferase (HAT) CBP (encoded by the *nejire* gene) antagonizes Polycomb silencing by acetylating histone H3K27 (H3K27ac), which prevents trimethylation of H3K27 by PRC2 (Tie et al., 2009). We also showed that H3K27ac levels are reduced in *trx<sup>l</sup>* mutants and elevated in TRX overexpressers (Tie et al., 2009), suggesting that TRX might promote acetylation of H3K27 by CBP at PcG-regulated genes. H3K27ac is highly correlated with actively transcribed genes, including many that are not PcG regulated, and is found at both their enhancers and promoters (Wang et al., 2008; Karličić et al., 2010). A TRX complex purified from *Drosophila* embryos was previously reported to contain CBP (Petruk et al., 2001). However, CBP was not found in TRX (or TRR) complexes subsequently purified from *Drosophila* S2 cells (Ardehali et al., 2011; Mohan et al., 2011), or in the orthologous human MLL1 complex (Dou et al., 2005). However, human CBP has been shown to bind directly to MLL1 and this appears to be required for MLL1-dependent transcription (Ernst et al., 2001; Goto et al., 2002; De Guzman et al., 2006; Arai et al., 2010). We show that CBP binds directly to TRX near its SET domain, physically coupling their H3K4 monomethylation and H3K27 acetylation activities and suggesting a molecular basis for TRX-dependent H3K27 acetylation. We discuss the implications of these new findings for the function of TRX in antagonizing silencing, stimulating enhancer-dependent transcription and maintenance of cellular memory.

## RESULTS

### The catalytically inactive *trx<sup>Z11</sup>* and *trr<sup>3</sup>* mutants have reduced H3K4me1 but normal H3K4me3 levels *in vivo*

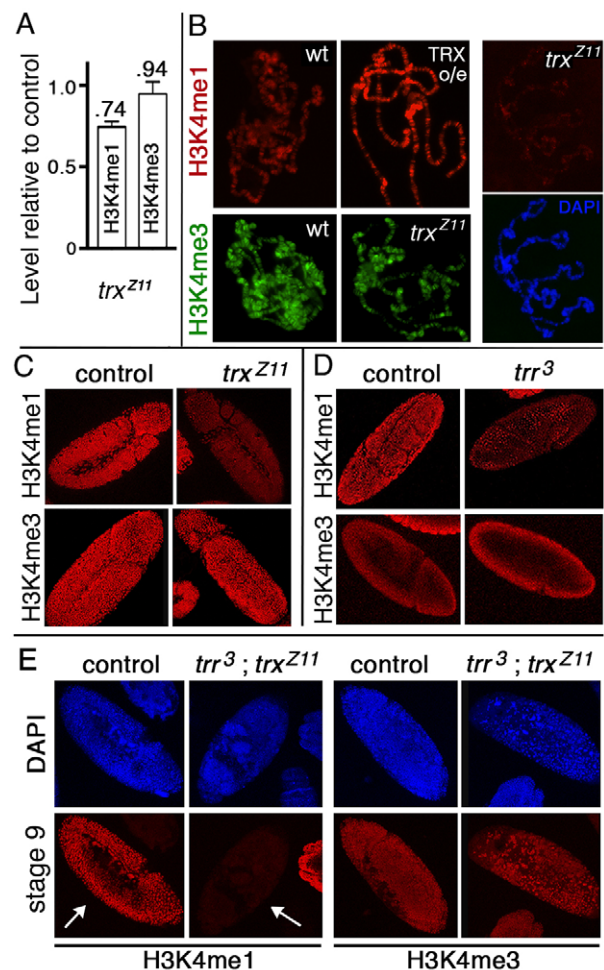
To determine the effect of selective loss of TRX catalytic activity on H3K4 methylation *in vivo*, we examined H3K4me1 and H3K4me3 levels in two *trx* mutants: the catalytically inactive *trx<sup>Z11</sup>* (Smith et al., 2004) and the hypomorphic temperature-sensitive *trx<sup>l</sup>* (Ingham and Whittle, 1980), a spontaneous mutation associated with a 9 kb insertion in the first intron (Breen and Harte, 1991). When raised at 29°C, *trx<sup>l</sup>* homozygotes have a substantially reduced amount of TRX protein (Tie et al., 2009) and so are expected to be impaired in all TRX functions. The catalytically inactive *trx<sup>Z11</sup>* missense mutation (G3601S) alters a single invariant SET domain residue (Stassen et al., 1995). It does not appear to affect the stability of the TRX protein (Chinwalla et al., 1995; Smith et al., 2004).

In late third instar salivary glands from *trx<sup>l</sup>* homozygotes (raised at 29°C) the H3K4me1 level was reduced (by 30%) in bulk histones compared with wild-type controls (29°C) (supplementary material Fig. S1A). The H3K27ac level was also reduced (by 24%), as was the H3K4me3 level (by 26%) (supplementary material Fig. S1A), consistent with our previously reported results from *trx<sup>l</sup>* adults (Tie et al., 2009). Conversely, the H3K4me1 level was increased by 40% in TRX overexpressers, as were the H3K27ac (by 28%) and H3K4me3 (by 34%) levels (supplementary material Fig. S1B).

RNAi knockdown of TRX in *Drosophila* S2 cells has similar effects on bulk H3K4me1 and H3K4me3 levels (Ardehali et al., 2011).

*trx<sup>Z11</sup>* homozygotes, which can survive into the pupal stage (Mortin et al., 1992; Breen, 1999), also have a reduced H3K4me1 level (by 26%) (Fig. 1A), but, in contrast to *trx<sup>l</sup>* mutants, their H3K4me3 level is not appreciably different from that of wild type (Fig. 1A). The H3K4me1 level was also substantially reduced on salivary gland polytene chromosomes of *trx<sup>Z11</sup>* homozygotes and increased on those of TRX overexpressers (Fig. 1B).

Previous temperature shift experiments with the *trx<sup>l</sup>* temperature-sensitive mutant identified a critical requirement for *trx* expression in 0- to 4-hour embryos (Ingham and Whittle, 1980). Consistent with this, a reduced H3K4me1 level (but normal H3K4me3 level) is also readily detectable in *trx<sup>Z11</sup>* embryos by stage 10 (4.3-5.3



**Fig. 1. The catalytically inactive *trx<sup>Z11</sup>* and *trr<sup>3</sup>* mutants exhibit reduced H3K4me1.** (A) Quantitative westerns of H3K4me1 and H3K4me3 levels, normalized to total H3, in third instar salivary glands from *trx<sup>Z11</sup>* homozygotes and wild-type (Oregon-R) controls. Standard errors were calculated from three independent samples. (B-E) Immunostaining of H3K4me1 and H3K4me3 (B) on polytene chromosomes from wild type (wt, Oregon-R), *trx<sup>Z11</sup>* mutant and TRX overexpressers (o/e), (C,D) in stage 10 *trx<sup>Z11</sup>* and *trr<sup>3</sup>* mutant embryos and wild-type controls (Oregon-R in C, *w<sup>1118</sup>* in D) and (E) in *trr<sup>3</sup>*; *trx<sup>Z11</sup>*/*trr<sup>3</sup>* double-mutant embryos. Arrows point to wild-type (*w<sup>1118</sup>*) and double-mutant embryos. DAPI staining for *trx<sup>Z11</sup>* (B, bottom right) indicates embryo position. Note that *trx<sup>Z11</sup>* polytene chromosomes in B appear under-replicated compared with wild type. Images were taken under the same settings as wild-type control. See also supplementary material Fig. S1D for H3K4me3 immunostaining in *trr<sup>3</sup>* mutant and wild-type embryos (stage 13).



hours) (Fig. 1C), around the same time that *trx* null mutants begin to show reduced levels of homeotic gene expression (Breen and Harte, 1993; Sedkov et al., 1994). This suggests that TRX catalytic activity is required for at least a portion of the total H3K4me1 *in vivo*. The apparently normal H3K4me3 level in *trx<sup>Z11</sup>* embryos (Fig. 1C) further suggests that the monomethyltransferase activity of TRX might be its principal activity *in vivo*.

*Drosophila* TRR is another SET domain protein with H3K4 methyltransferase activity, which functions as a co-activator of the Ecdysone receptor (EcR) (Sedkov et al., 2003). We examined H3K4me1 and H3K4me3 levels *in vivo* in two *trr* nonsense mutants: *trr<sup>1</sup>* and *trr<sup>3</sup>*, both of which are embryonic lethal. *trr<sup>1</sup>* appears to be a protein-null allele, and *trr<sup>3</sup>* truncates the SET domain and is expected to be catalytically inactive (Sedkov et al., 2003). The *trr<sup>1</sup>/Y* null mutant embryos had decreased levels of both H3K4me1 and H3K4me3, like *trx<sup>1</sup>* (supplementary material Fig. S1C). The *trr<sup>3</sup>/Y* mutant embryos had a decreased H3K4me1 level but apparently normal H3K4me3 level (Fig. 1D), like *trx<sup>Z11</sup>*.

Since H3K4me1 is only partially reduced in *trx* and *trr* mutants, we examined *trr<sup>3</sup>/Y; trx<sup>Z11</sup>* double mutants to determine whether H3K4me1 is further reduced. Although these embryos arrest earlier and appear to have more severe defects than either single mutant, the H3K4me3 level in individual nuclei appears to be similar to that of wild-type controls, whereas H3K4me1 was undetectable (Fig. 1E). These data strongly suggest that TRX and TRR are the principal H3K4 monomethyltransferases *in vivo* and that together they are responsible for the bulk of the H3K4me1 present in embryos. They further suggest that the reduced H3K4me3 in *trx<sup>1</sup>* and *trr<sup>1</sup>* null mutants is not due to the loss of their methyltransferase activities, but to the loss of some other function of TRX and TRR that remains intact in the *trx<sup>Z11</sup>* and *trr<sup>3</sup>* mutant proteins. One possibility is the third PHD finger of TRX, which binds to H3K4me3 (Chang et al., 2010; Park et al., 2010; Wang et al., 2010) and might shield it from demethylation.

### TRX H3K4 methyltransferase activity is required for antagonizing PcG silencing

We previously showed that TRX-dependent H3K27 acetylation by CBP antagonizes Polycomb silencing by preventing H3K27 trimethylation by PRC2 (Tie et al., 2009). Whether the H3K4 methyltransferase activity of TRX is also required for this antagonism has not been determined. We compared the ability of the catalytically inactive *trx<sup>Z11</sup>* and the protein-null *trx<sup>B11</sup>* mutations to suppress the extra sex combs phenotype of *Pc<sup>3</sup>/+* mutant adults, a characteristic PcG mutant phenotype due to derepression of *Scr* in mesothoracic (T2) and metathoracic (T3) legs of adult male flies. We found that *trx<sup>Z11</sup>* and *trx<sup>B11</sup>* mutations suppress the T2 extra sex combs of *Pc<sup>3</sup>/+* similarly (Table 1), indicating that TRX catalytic activity does play a role in antagonizing Polycomb silencing. Consistent with this, both TRX and H3K4me1 (but not H3K4me3) are present at all known PRE/TREs in the *Bithorax* and *Antennapedia* complexes (supplementary material Fig. S2 and see below).

**Table 1. *trx<sup>Z11</sup>* suppresses the *Pc<sup>3</sup>* phenotype**

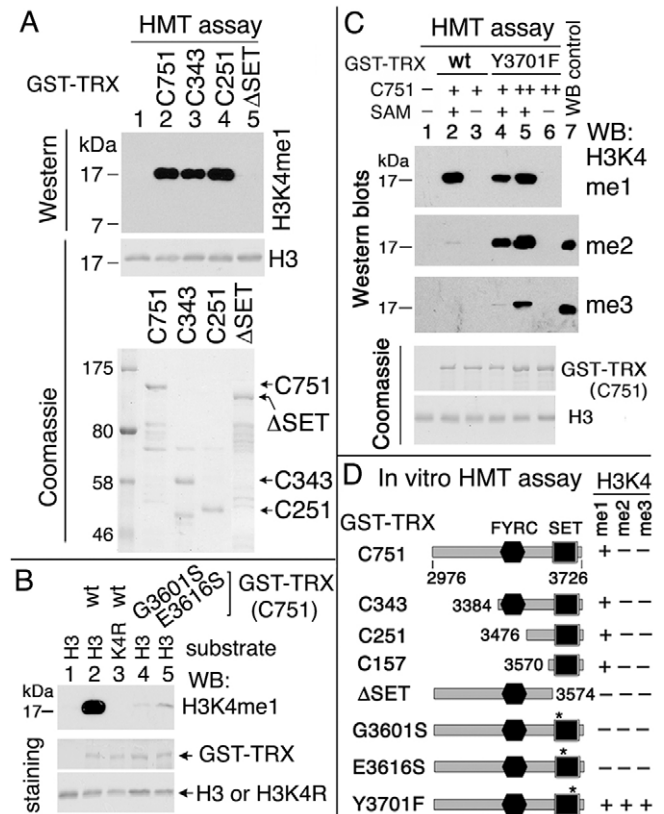
Genotype	T2 to T1 transformation % (scored flies)*
<i>Pc<sup>3</sup>/TM6B</i> (control)	33.3 (24/72)
<i>trx<sup>Z11</sup>/Pc<sup>3</sup></i>	0 (0/75)
<i>Pc<sup>3</sup>/TM6B</i> (control)	29.5 (26/88)
<i>trx<sup>B11</sup>/Pc<sup>3</sup></i>	0 (0/84)

\*T2 legs of adult male progeny were scored for the presence of extra sex combs. Similar results were observed from reciprocal crosses.

### Robust H3K4 monomethyltransferase activity of the TRX SET domain

The reduced H3K4me1 and normal H3K4me3 levels in the *trx<sup>Z11</sup>* mutant suggest that TRX might be predominantly an H3K4 monomethyltransferase. To examine this, we tested GST-TRX-C fusion proteins (see constructs in Fig. 2D) containing the C-terminal SET domain and additional adjacent residues (that mediate interaction with WRAD core subunits) in histone methyltransferase (HMT) assays. Reaction products were examined by western blot with antibodies that specifically recognize H3K4me1, H3K4me2 or H3K4me3. As stringent controls for the specificity of these antibodies, on each blot we included full-length recombinant H3 proteins with single chemically introduced methyl-K analogs of K4me1, K4me2 or K4me3 (Simon et al., 2007). These serve as sensitive controls for false-positive western signals caused by cross-reactivity that can appear as a result of using excess antibody or sample overloading.

We found that larger SET domain-containing fragments ( $\geq 251$  residues) have only a robust H3K4 monomethyltransferase activity on a recombinant (unmodified) free histone H3 substrate (Fig. 2A, lanes 2-4 and 2C, lane 2; supplementary material Fig. S6). Deletion of the SET domain from TRX-C751 abolished its enzymatic activity



**Fig. 2. The TRX SET domain is a histone H3K4 monomethyltransferase.** (A-C) Western blots (WB) of H3K4me1/2/3 reaction products of HMT assays conducted with purified wild-type and mutant GST-TRX-C (see constructs in D) and recombinant histone H3 and H3K4R mutant substrates. Coomassie Blue-stained GST fusion proteins and H3 substrates are shown below westerns. In lane 7 of C, recombinant H3K4me2 or H3K4me3 (20 ng/lane) was used as positive antibody control (additional controls in supplementary material Fig. S6). Note that use of the H3K4R mutant as substrate or the absence of the methyl donor SAM in HMT assays completely abolished the H3K4me1 signal (lane 3 in B and C), indicating the specificity of the HMT assay. (D) Summary of GST-TRX-C fragments and mutants tested in HMT assays.

(Fig. 2A, lane 5). The TRX SET domain also monomethylated H3K4 on an H3(1-20) peptide, which was confirmed by MALDI TOF mass spectrometry (supplementary material Fig. S3). We also examined the activity of TRX-C751 in complex with WRAD core subunits. A recombinant human MLL1-WRAD core complex assembled *in vitro* was previously shown to monomethylate but not trimethylate H3K4 by quantitative MALDI-TOF mass spectrometry. When TRX was substituted for MLL1 in this complex, we detected exclusively H3K4me1 and no H3K4me3 product in the HMT assay (supplementary material Fig. S4) indicating that, although the WRAD subunits may greatly increase the reaction rate, they do not convert the TRX SET domain to a trimethyltransferase.

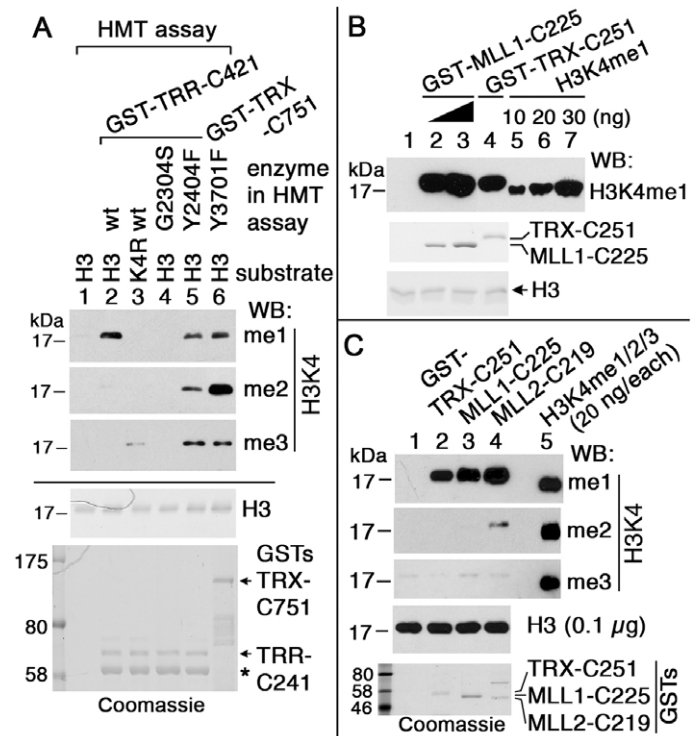
The *trx<sup>Z11</sup>* missense mutation (G3601S) alters a residue that is invariant in all SET domains (supplementary material Fig. S5) and is part of the conserved SAM-binding site (Cheng et al., 2005). It was previously reported to abolish catalytic activity (Smith et al., 2004). As expected, it abolishes the H3K4 monomethyltransferase activity of the TRX SET domain (Fig. 2B, lane 4), which was confirmed by MALDI-TOF mass spectrometry (supplementary material Fig. S3). An E3616S substitution, which alters an invariant Glu residue that lies next to an invariant Tyr in the predicted catalytic site (supplementary material Fig. S5), also severely impairs the H3K4 monomethyltransferase activity (Fig. 2B, lane 5).

The product specificity of SET domain methyltransferases (e.g. H3K4me1/2/3) depends on several key residues in the active site, including a so-called 'Phe/Tyr switch' position. Di- and trimethyltransferases tend to have a Phe at this position, whereas monomethyltransferases tend to have the bulkier Tyr residue; changing Tyr (Y) to Phe (F) can convert monomethyltransferases to di- and trimethyltransferases (Collins et al., 2005). To confirm that the absence of H3K4 trimethylation by the TRX SET domain is not due to the reaction conditions used or to loss of enzyme activity during the reaction, we changed the corresponding Tyr in TRX-C to Phe (Y3701F), which converted it to a di- and trimethyltransferase, yielding H3K4me2 and H3K4me3 at the expense of H3K4me1 (Fig. 2C, lanes 4 and 5). This indicates that Y3701 prevents H3K4 di- and trimethylation by the isolated TRX SET domain and further suggests that its principal intrinsic activity is H3K4 monomethylation. The corresponding substitution in the MLL1 SET domain (Y3942F) also converts it to a di- and trimethyltransferase (Patel et al., 2009). The results of the *in vitro* HMT assays are summarized in Fig. 2D.

### Conserved monomethylation of H3K4 by TRR and human MLL1 and MLL2

RNAi-mediated knockdown of the H3K4 methyltransferase TRR in S2 cells and wing imaginal discs was reported to reduce bulk H3K4me1 (Ardehali et al., 2011; Herz et al., 2012) and the TRR SET domain was also shown to have H3K4 monomethyltransferase activity *in vitro* (Ardehali et al., 2011). We confirmed that purified GST-TRR-C421 (C-terminal 421 residues) has only a robust H3K4 monomethyltransferase activity (Fig. 3A, lane 2). A G2304S substitution, corresponding to the *trx<sup>Z11</sup>* mutation (supplementary material Fig. S5), also abolished TRR methylation activity (lane 4) and the Y2404F substitution at the TRR Phe/Tyr switch position also converts it to an H3K4 di- and trimethyltransferase (Fig. 3A, lane 5). We conclude that the TRR SET domain also has only an intrinsic H3K4 monomethyltransferase activity.

MLL1 and MLL4 are human orthologs of TRX, and MLL2/ALR and MLL3 are human orthologs of TRR (Ardehali et al., 2011; Chauhan et al., 2012) (supplementary material Fig. S5). We compared human MLL1-C225 with TRX-C251 in our HMT assay and found that MLL1-C225 also has a robust H3K4



**Fig. 3. The SET domains of TRR and human MLL1 and MLL2 are robust H3K4 monomethyltransferases.** *In vitro* HMT assays (as in Fig. 2) of wild-type and mutant GST-TRR-C421 (A), human GST-MLL1-C251 (B) and GST-MLL2-C219 (C). Asterisk indicates the degraded form of GST-TRR-C421 in Coomassie Blue-stained gel (bottom of A). Lane 6 in A contains the GST-TRX-C751 Y3701F switch mutant for comparison with the corresponding TRR-C Y2404F mutant. Recombinant H3K4me1, H3K4me2 or H3K4me3 was used as positive control in B and C. The weak western signal in lane 3 of A (third panel down) is likely to be due to non-specific binding of the anti-H3K4me3 antibody to H3K4R.

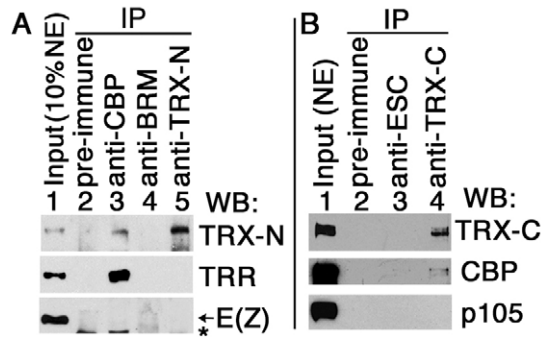
monomethyltransferase activity (Fig. 3B, lanes 2 and 3) as previously reported (Patel et al., 2009). The SET domain of MLL2 also has only a robust H3K4 monomethyltransferase activity (Fig. 3C, lane 4), indicating that this activity has been evolutionarily conserved in the human TRX and TRR orthologs.

### TRX and TRR are physically associated with CBP *in vivo*

We previously showed that *in vivo* some H3K27 acetylation by CBP depends on TRX (Tie et al., 2009). Given the conflicting claims about the presence of CBP in TRX complexes, we re-examined the association of CBP with TRX (and TRR) by co-immunoprecipitation (co-IP) assay. TRX and MLL1 are cleaved by Taspase 1 into N- and C-terminal fragments that remain physically associated *in vivo* (Hsieh et al., 2003a; Hsieh et al., 2003b; Capotosti et al., 2007). Using antibodies that specifically recognize TRX-N, we found that TRX-N and TRR (Fig. 4A, lane 3) were present in anti-CBP IPs from embryo nuclear extract (NE). CBP was also present in reciprocal anti-TRX-C IPs (Fig. 4B, lane 4). We conclude that TRX and TRR are each independently physically associated with CBP *in vivo*.

### TRX C-terminus contains a CBP-interacting domain (CID)

To determine whether TRX and CBP interact directly, we initially used GST-TRX-C751 in a GST pulldown assay with embryo NE. CBP (but not BRM, UTX or TRR) was strongly pulled down by



**Fig. 4. TRX and TRR physically associate with CBP *in vivo*.**

(A) Immunoblots of TRX-N, TRR and E(Z) after IP from nuclear extract (NE) by anti-CBP (lane 3), anti-BRM (lane 4) and anti-TRX-N (lane 5) antibodies. The asterisk indicates serum background. (B) Immunoblots of TRX-C, CBP and p105 after IP by anti-TRX-C (lane 4) and anti-ESC (lane 3) antibodies. Pre-immune serum in lane 2 (A,B) and the p105 subunit of the CAF1 complex (bottom of B) serve as negative controls.

GST-TRX-C751 (Fig. 5, lane 3). As a positive control, SNR1, which is a subunit of *Drosophila* BRM (SWI/SNF) complexes that was previously shown to bind to the TRX SET domain (Rozenblatt-Rosen et al., 1998), was also pulled down (lane 3 at bottom). The *trx<sup>Z11</sup>* missense mutation (G3601S) in the SET domain did not abolish pulldown of CBP or SNR1 (lane 4). Interestingly, GST-TRR-C421 also pulled down SNR1 (Fig. 5A, lane 5) but not CBP.

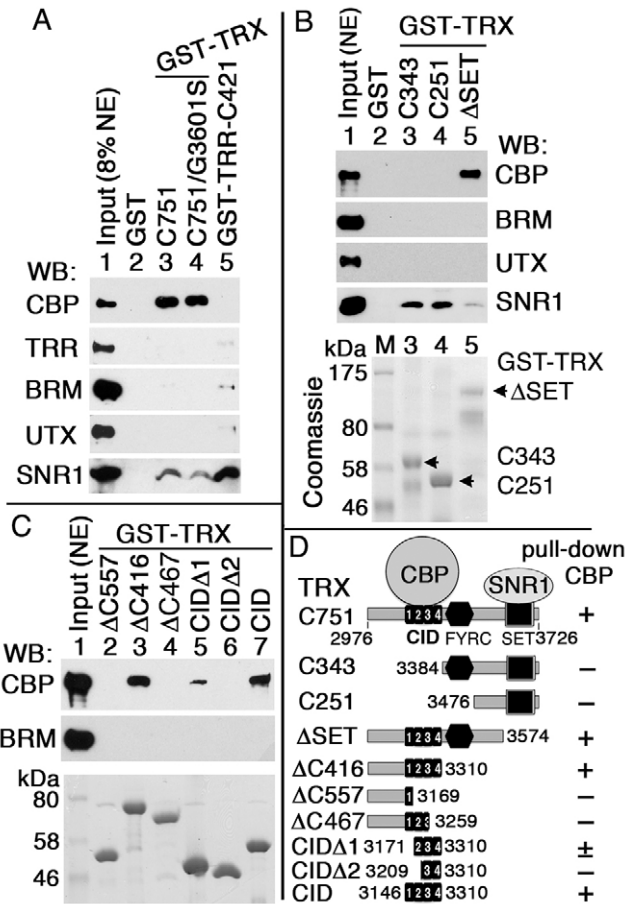
To map the region of TRX-C751 that mediates its physical association with CBP, we tested a set of subfragments of TRX-C751 (see constructs in Fig. 5D). The C-terminal fragments TRX-C343 and C251, which contain the SET domain, failed to pull down CBP but retained SNR1 binding (Fig. 5B, lanes 3 and 4), suggesting that the deleted region (TRX2976-3383) is required for the interaction with CBP. Consistent with this, deletion of the SET domain from TRX-C751 severely impaired SNR1 binding but retained CBP binding (lane 5). TRX- $\Delta$ C416, which deletes both the SET domain and FYRC (Hsieh et al., 2003b) from TRX-C751, also did not affect the CBP interaction (Fig. 5C, lane 3). However, deletion of an additional 141 residues (TRX- $\Delta$ C557) abolished CBP binding (lane 2). We assayed the GST-TRX-C pulldown in Fig. 5 and found that it has both robust H3K4 monomethylation and H3K27 and H3K18 acetylation activities *in vitro* (supplementary material Fig. S6).

To further delimit the CID, we used an additional set of TRX fragments (bottom four constructs in Fig. 5D) in GST pulldown assays. TRX- $\Delta$ C467, a deletion of just 51 additional C-terminal residues from TRX- $\Delta$ C416, abolished CBP binding (Fig. 5C, lane 4). TRX3146-3310 retained CBP binding (Fig. 5C, lane 7) and was defined as the CID. Deletion of 25 (CID $\Delta$ 1) and 63 (CID $\Delta$ 2) residues from the N-terminus of CID respectively impaired and abolished CBP binding (lanes 5 and 6). GST pulldown results are summarized in Fig. 5D. We conclude that TRX3146-3310 is the CID.

#### The KIX domain of CBP interacts directly with the TRX CID

To map the TRX-interacting region in CBP, we used GST-CBP fragments to pull down purified TRX CID *in vitro*. The TRX CID binds to the KIX domain (Fig. 6A, lane 5) but not to other conserved domains of CBP (lanes 3, 4, 6 and 7). These results are summarized in Fig. 6B.

We identified four KIX-binding motifs [ $\Phi$ XX $\Phi$  (Lee et al., 2009; Wang et al., 2012)] in the TRX CID (Fig. 6C). The KIX domain has two distinct binding surfaces that can simultaneously bind two  $\Phi$ XX $\Phi$  motifs (De Guzman et al., 2006; Wang et al.,



**Fig. 5. The C-terminus of TRX contains the CBP-interacting domain (CID).** (A-C) Immunoblots of endogenous TrxG proteins (CBP, BRM, UTX and SNR1) pulled down from NE by GST-TRX-C (or TRR-C421 in lane 5 of A). Coomassie Blue-stained purified GST fusion proteins used in pulldown assays are shown at the bottom of B and C. (D) Summary of GST-TRX fragments tested and results of CBP pulldowns. Numbers within the CID refer to four KIX-binding motifs (see Fig. 6C).

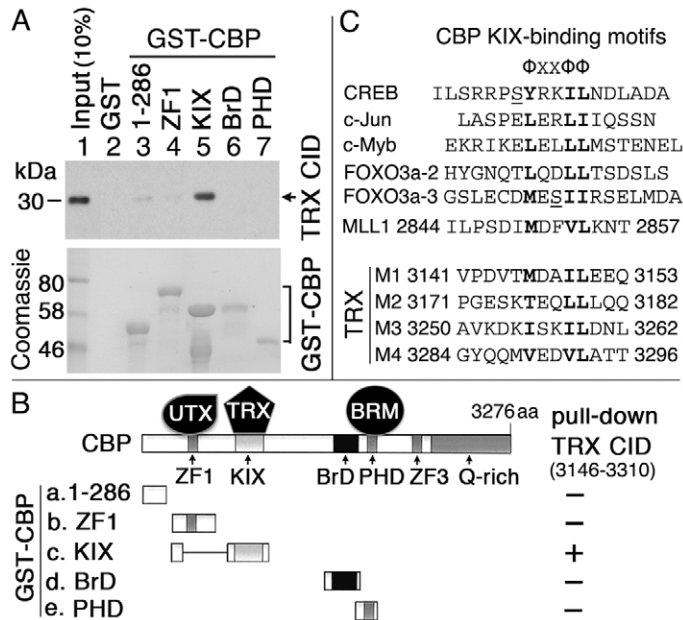
2012) and this capability mediates cooperative binding of MLL1 and CREB to CBP (Ernst et al., 2001). As shown in Fig. 5C, deletion of motif 1 (CID $\Delta$ 1) reduced binding by more than two-thirds, and deletion of motifs 1 and 2 (CID $\Delta$ 2) or motifs 3 and 4 ( $\Delta$ C467) abolished binding to the KIX domain. These data suggest that multiple KIX-binding motifs are required for robust binding of the TRX CID to CBP.

#### H3K4me1 enhances H3K27 acetylation by CBP

We previously showed that some H3K27 acetylation is dependent on TRX (Tie et al., 2009) and some has also recently been shown to depend on TRR (Herz et al., 2012). This suggests that monomethylation of H3 by TRX and TRR might influence H3K27 acetylation by CBP. To test this, we compared unmodified and K4me1-containing H3 as substrates for *in vitro* acetylation by recombinant CBP(1603-2678) (Tie et al., 2012). H3K27 (and H3K18) acetylation was enhanced 2- to 3-fold on the H3K4me1-containing substrate in this assay (Fig. 7A, compare lane 2 with lane 1). Other H3 modifications (K4me2/3, K9me1, K36me1) had no enhancing effect (Fig. 7B, lanes 2, 3, 5 and 6).

TRX overexpression *in vivo* elevates both H3K4me1 and H3K27ac levels (supplementary material Fig. S1B) and perturbs Polycomb silencing, causing homeotic derepression phenotypes



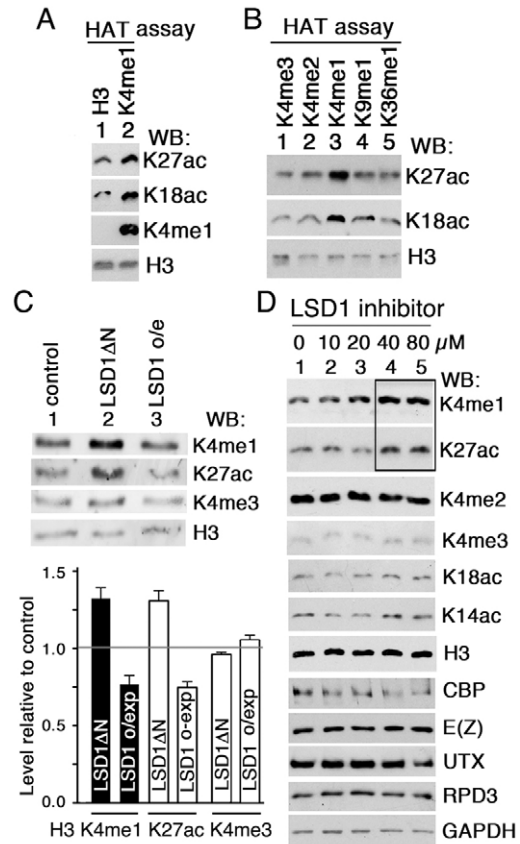


**Fig. 6. TRX binds directly to the CBP KIX domain.** (A) GST-CBP fragments were used to pull down purified TRX-CID (with a C-terminal His tag) and were Coomassie Blue stained (bottom). The TRX CID was detected by anti-His antibody (top). (B) Summary of GST-CBP fragments tested and results of TRX CID pull-down. Conserved domains including two zinc fingers (ZF1 and ZF3), KIX (also called CREB-binding domain) and bromodomain (BrD) are shown. The three enzymes TRX, UTX (H3K27 demethylase) and BRM (chromatin remodeler) in TrxG have been found to interact with CBP at KIX, ZF1 and PHD finger, respectively (Tie et al., 2012). (C) Sequence alignment of known KIX-binding motifs in MLL1 and transcription factors (Wang et al., 2012) and four putative KIX-binding motifs identified within the TRX CID.  $\Phi$  is a hydrophobic residue (in bold) and X is an arbitrary residue. Phosphorylated serines in CREB (S133) and FOXO3a-CR3 (S626) are underlined.

characteristic of PcG mutants. Moderate RNAi-mediated knockdown of CBP, using two independent *da*-GAL4-induced UAS-CBP RNAi transgenic lines, suppresses the extra sex comb phenotype of TRX overexpressers (Table 2). This indicates that CBP is required for the impaired silencing induced by TRX overexpression. Together with the suppression of *Pc*<sup>3</sup> phenotypes by *trx*<sup>Z11</sup> (Table 1), it also is consistent with the possibility that the elevated H3K4me1 in TRX overexpressers may antagonize silencing by locally enhancing H3K27 acetylation by TRX-associated CBP.

To test whether the H3K27ac level depends specifically on H3K4 monomethylation, we independently altered the H3K4me1 level by genetic manipulation of the *Drosophila* H3K4me1/2 demethylase LSD1 [encoded by the *Su(var)3-3* gene] and examined H3K4me1 and H3K27ac levels in adults. As previously reported (Di Stefano et al., 2007), the H3K4me1 level was increased (by 30%) in protein-null *lsd1*<sup>AN</sup> mutants compared with wild-type controls (Fig. 7C, top panel, lane 2) and decreased (by 25%) in LSD1 overexpressers (lane 3), while the H3K4me3 levels in both genotypes were little changed (Fig. 7C, third panel). The H3K27ac level was also increased in *lsd1*<sup>AN</sup> mutants and decreased in LSD1 overexpressers (Fig. 7C, second panel), consistent with an enhancing effect of H3K4me1 on H3K27 acetylation.

Chemically inhibiting LSD1 activity had a similar effect on the H3K27ac level. Incubating *Drosophila* S2 cells for 3 days in media containing 40  $\mu$ M and 80  $\mu$ M bisguanidine polyamine analog, which inhibits human LSD1 in breast cancer cells (Zhu et



**Fig. 7. H3K4me1 enhances acetylation of H3K27 by CBP.** (A, B) *In vitro* CBP HAT assays were performed using recombinant H3, either unmodified or containing single mono-, di- or trimethyl-K analogs (Simon et al., 2007) at K4, K9 or K36 as substrate. Western blots were used to assay the H3K27ac reaction product (top) and H3 (bottom, loading control). All conditions in HAT assays were identical except for the histone H3 substrate. (C) Immunoblots of various histone H3 modifications in salivary gland extracts from control (*w*<sup>1118</sup>; lane 1), *lsd1*<sup>AN</sup> homozygotes (lane 2) and LSD1 overexpresser (expressed under the control of *da*-GAL4) (lane 3). Quantitative westerns of histone H3 K4me1, K27ac, K27me3 and K4me3 are shown at the bottom with the control value set as 1. Error bars indicate s.d. of normalized means from three independent replicates. (D) S2 cells were treated with LSD1 inhibitor (10, 20, 40 and 80  $\mu$ M) for 3 days and then analyzed by western blot. Note that lower concentrations (10  $\mu$ M and 20  $\mu$ M) of inhibitor had no detectable effect on bulk H3K4me1 and H3K27ac levels (lanes 2 and 3 in B).

al., 2012), significantly increased their H3K4me1 level, as expected, and also increased their H3K27ac level, while CBP and H3K4me3 levels were unchanged (Fig. 7D, lanes 4 and 5). Together, these results further suggest that the TRX- and TRR-dependence of the H3K27ac level might, at least in part, reflect dependence on the H3K4 monomethylation activities of TRX and TRR.

### TRX is highly correlated genome-wide with H3K4me1, CBP and TRR at PRE/TREs and enhancers

To determine the extent to which TRX, TRR, CBP, H3K4me1 and H3K27ac colocalize on chromatin, we used our previous ChIP-chip data for CBP, H3K27ac, H3K27me3 and PC (Tie et al., 2012) and recent TRX-C, TRR, H3K4me1 and H3K4me3 ChIP-seq data (Enderle et al., 2011; Herz et al., 2012) from *Drosophila* S2 cells and generated an unbiased heat map clustered by all peaks. As shown in Fig. 8A, strong TRX-C peaks are highly correlated with CBP and TRR peaks (left three columns) in regions highly enriched

**Table 2. CBP RNAi suppresses the Polycomb phenotype caused by TRX overexpression**

Genotype	T2 to T1 transformation % (n)*
<i>Hsp70-Gal4/+; trx<sup>EP3541/+</sup></i> (control)	15.4 (10/65)
<i>Hsp70-Gal4/+; trx<sup>EP3541</sup>/CBP RNAi-1</i>	3 (2/62)
<i>Hsp70-Gal4/+; trx<sup>EP3541/+</sup></i> (control)	13.5 (10/74)
<i>Hsp70-Gal4/+; trx<sup>EP3541</sup>/CBP RNAi-2</i>	0 (0/68)

\*T2 legs of adult male progeny were scored for presence of extra sex combs.

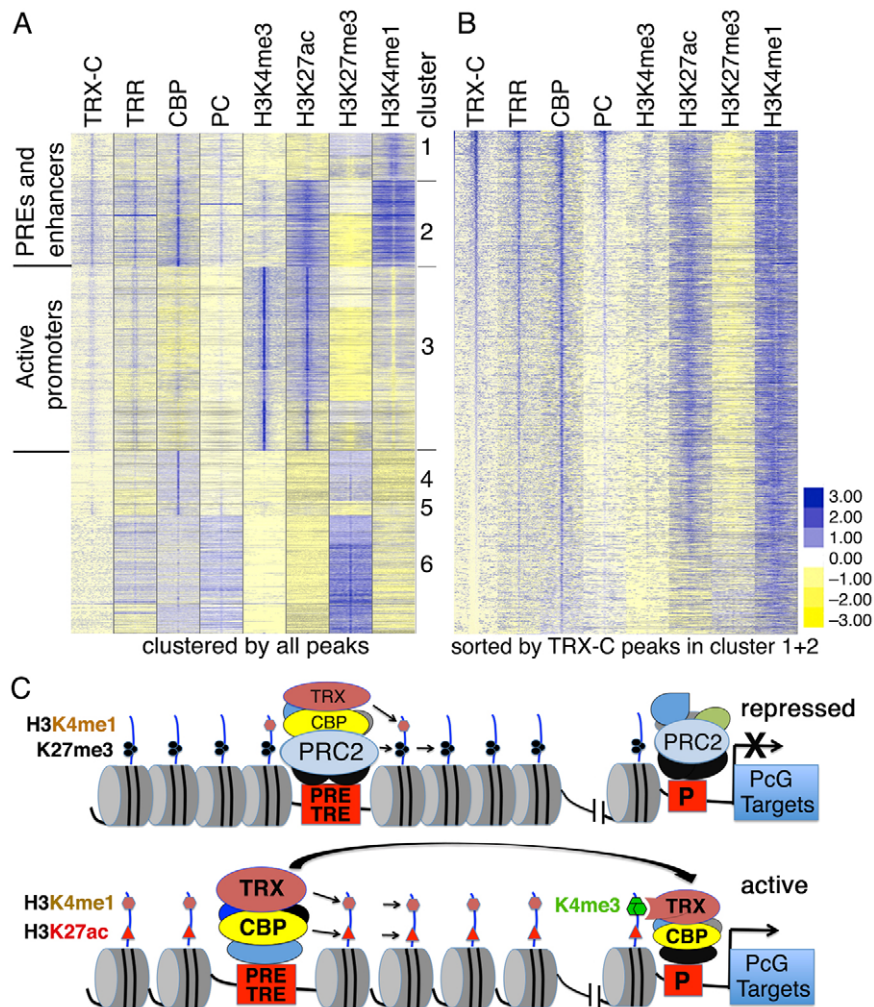
for H3K4me1 (clusters 1 and 2), consistent with the H3K4 monomethyltransferase activity of TRX and TRR and with the observed physical association of both TRX and TRR with CBP. The cluster 2 profile, containing CBP, H3K4me1 and H3K27ac (absence of H3K27me3), is similar to the signature of so-called ‘active’ enhancers. The cluster 1 profile, containing CBP, H3K4me1 and H3K27me3 marks (absence of H3K27ac), is similar to the signature of so-called ‘poised’ enhancers in mammals. Note that the H3K4me1 peaks in cluster 2 form broad domains spread over a much larger region than in cluster 1, and these are accompanied by similarly broad H3K27ac peaks, suggesting that this is a feature associated with genes in a transcriptionally active state. Known and presumed PREs (marked by very high sharp peaks of PC) and also enhancers of homeotic genes and other PcG-regulated genes are found in these two clusters (supplementary material Figs S7-S9). Some TRX binding sites correspond to functionally defined

enhancers recently identified in a novel genome-wide screen by STARR-seq (supplementary material Fig. S2) (Arnold et al., 2013). TRX-C and TRR are also present at active promoters marked by very strong H3K4me3 and H3K27ac peaks (Fig. 8A, cluster 3), but their signal intensities are weaker than in clusters 1 and 2. These data suggest that TRX and TRR are associated predominantly with H3K4me1-marked *cis*-regulatory elements including PREs and enhancers.

We generated an additional heat map (Fig. 8B) from clusters 1 and 2 (TRX- and TRR-enriched, H3K4me1-marked enhancers and PREs) sorted by the intensity of TRX-C peaks, which reveals that TRX-C is highly correlated with PC peaks and a portion of TRR, CBP and H3K4me1 peaks (Fig. 8B, top). TRR peaks also coincide with CBP, H3K27ac and H3K4me1 peaks, but some of them lack TRX-C and PC peaks (Fig. 8B, bottom), suggesting that TRR regulates both PcG and non-PcG target genes (supplementary material Fig. S10) (Herz et al., 2012), whereas TRX only regulates PcG target genes. We propose a model for epigenetic regulation of PcG gene expression by TRX and CBP in Fig. 8C.

## DISCUSSION

The major findings presented here are: (1) TRX and TRR are monomethyltransferases and together account for the bulk of the H3K4me1 *in vivo*; (2) the catalytic activities of both TRX and CBP are required to antagonize PcG silencing; (3) TRX and TRR are physically associated with CBP *in vivo* and TRX binds directly to



**Fig. 8. TRX-C colocalizes with H3K4me1, CBP and TRR at putative PRE/TREs and enhancers.** (A) Heat map clustered by peaks of TRX-C, TRR, CBP, PC, H3K4me3, H3K27ac, H3K27me3 and H3K4me1 from published ChIP-chip and ChIP-seq data in *Drosophila* S2 cells. Each column is centered on the peak midpoint within a 10 kb region ( $\pm 5$  kb from midpoint). (B) Heat map of clusters 1 and 2 (putative PRE/TREs and enhancers) in A sorted by TRX-C peak intensity. Weak H3K4me3 peaks in cluster 2 suggest that some promoters might be within 5 kb of putative enhancers or PREs (see *prn* and *zfh1* in supplementary material Fig. S9). (C) Model of epigenetic regulation of PcG target gene expression by TRX and CBP. TRX and CBP are physically associated and constitutively present on PRE/TREs. In PcG-repressed genes (top), TRX (and TRR)-dependent H3K4me1 is only present at PRE/TREs and H3K27ac deposition is inhibited by the broad domains of H3K27me3 that spread from the PRE to promoter (P). In active genes (bottom), H3K4me1 spreads from PRE/TREs to form broad domains over neighboring *cis*-regulatory regions, perhaps assisted by interactions between TRX/CBP and enhancer-bound transcriptional activators. The coupling of TRX and CBP catalytic activities and the stimulatory effect of H3K4me1 on acetylation by CBP promotes the formation of similar broad domains of H3K27ac, which might also be dependent on TRX/CBP interactions with enhancer-bound activators. This prevents PRC2-mediated deposition of H3K27me3 at the PRE and its propagation from the PRE over the same broad region. TRX (and CBP) also acts at promoters (Enderle et al., 2011) of active genes. Binding of the third PHD finger of TRX to promoter-associated H3K4me3 is required for transcription and might have an indirect effect on the H3K4me3 level.



the CBP KIX domain via a region that contains multiple KIX-binding motifs; (4) TRX and TRR colocalize genome-wide with H3K4me1 and CBP at PREs and enhancers; and (5) H3K4me1 enhances histone acetylation by CBP. Together, these data suggest that the primary target of TRX monomethyltransferase activity is not promoters but PREs and neighboring enhancers. They suggest a new model (Fig. 8C) for how TRX antagonizes Polycomb silencing, stimulates active enhancers, and establishes a cellular memory of active transcription. This differs significantly from the previous view that TRX trimethylates H3K4 (Petruk et al., 2012).

### Monomethylation activity of TRX and TRR

The evidence presented here indicates that the SET domains of TRX, TRR and their human orthologs possess intrinsic H3K4 monomethyltransferase activities (Figs 2, 3) and are prevented from being trimethyltransferases by the presence of the bulkier Tyr residue at their respective F/Y switch positions, as previously shown for MLL1 (Patel et al., 2009). Although these data do not rule out the possibility that TRX and TRR complexes might have some H3K4 trimethylation activity *in vivo* in some chromatin contexts, the reduced H3K4me1 and apparently normal H3K4me3 levels in the catalytically inactive *trx<sup>Z11</sup>* and *trr<sup>3</sup>* mutants (Fig. 1) strongly suggest that H3K4 monomethylation is the predominant activity of TRX and TRR *in vivo*. Moreover, the absence of detectable H3K4me1 in *trr<sup>3</sup>*; *trx<sup>Z11</sup>* double-mutant embryos (Fig. 1E) suggests that they are the principal H3K4 monomethyltransferases *in vivo*, consistent with their genome-wide colocalization with H3K4me1 at PREs and enhancers. Suppression of *Pc<sup>3</sup>* mutant phenotypes by *trx<sup>Z11</sup>* further suggests that the monomethyltransferase activity of TRX plays a role in antagonizing Polycomb silencing.

### Direct interaction and functional collaboration between TRX and CBP

We found that TRX and TRR are physically associated with CBP in embryo extracts, confirming a previous report for TRX (Petruk et al., 2001). The direct binding of TRX to the CBP KIX domain (Fig. 6) and the genome-wide correlation of H3K27ac with H3K4me1 on active genes suggests that their activities are coupled *in vivo*. Consistent with this, TRX-CBP complexes pulled down from embryo extracts have both H3K4 monomethyltransferase and H3K27 acetyltransferase activities (supplementary material Fig. S6). Moreover, the impaired Polycomb silencing caused by TRX overexpression *in vivo* (which elevates both H3K4me1 and H3K27ac levels) requires CBP (Table 2) and presumably the TRX-CBP interaction. Mutating the CID will be required to show this conclusively. No direct interaction between CBP and the TRR C-terminus was found, but we previously reported that CBP interacts directly with the H3K27 demethylase UTX (Tie et al., 2012), which is another subunit of the TRR complex (Mohan et al., 2011). Together, these data suggest that these direct interactions are required for TRX- and TRR-dependent H3K27 acetylation and further suggest that TRX and TRR complexes function by fundamentally similar mechanisms.

The enhanced *in vitro* acetylation of H3K27 on K4me1-containing recombinant H3 substrates (Fig. 6) suggests that H3K4me1 might be a preferred CBP substrate *in vivo*. Consistent with this, altering the H3K4me1 level *in vivo* by manipulating LSD1 causes concordant changes in H3K27ac in adults (Fig. 7C). Moreover, a genome-wide analysis of hundreds of bona fide enhancers in purified mesodermal cells from *Drosophila* embryos revealed that H3K27ac is not present on enhancers without H3K4me1, whereas H3K4me1 is present

without H3K27ac prior to enhancer ‘activation’ (Bonn et al., 2012). This suggests that the presence of H3K4me1 might be a prerequisite for the deposition of H3K27ac at enhancers. Interestingly, some of the catalytically inactive *trx<sup>Z11</sup>* mutants survive until the late pupal period and exhibit strong homeotic transformations. This suggests that TRX catalytic activity might be more important for stimulating enhancers that drive robust homeotic gene expression, whereas the physical association of TRX with CBP, which is intact in *trx<sup>Z11</sup>*, is more important for preventing silencing of normally active PcG-regulated genes in the embryo.

### TRX and TRR at PRE/TREs and enhancers

H3K4me1 and CBP are part of a conserved chromatin ‘signature’ of enhancers and H3K27ac marks ‘active’ enhancers (Heintzman et al., 2007; Wang et al., 2008). Our data strongly suggest that TRX and TRR are responsible both for the H3K4me1 on enhancers and, via their physical association with CBP, for the H3K27ac on active enhancers. Determining which H3K4me1 is TRX dependent will require ChIP-seq analysis of *trx<sup>Z11</sup>* mutant cells.

Like TRX, H3K4me1 and CBP are also present at PRE/TREs of both active and inactive genes, suggesting that PRE/TREs have a functional connection to enhancers. Functional analyses of the strong *bxd* PRE/TRE *in vivo* suggest that PRE/TREs are distinct from enhancers, do not possess enhancer activity, but can boost enhancer-dependent transcription in a TRX-dependent manner. A GAL4-TRX fusion protein tethered to a transgene reporter exhibits these same properties (Pirrotta et al., 1995; Poux et al., 2002).

TRR was recently shown to occupy many presumed enhancers (Herz et al., 2012). We find that TRR binds more sites than TRX and also co-occupies most TRX binding sites genome-wide, including PRE/TREs. This raises the possibility that both TRX and TRR regulate many PcG-regulated genes, perhaps in different contexts or in response to different signals. The presence of UTX in the TRR complex suggests that TRR can facilitate switching of PcG-regulated genes from silent to active, whereas TRX might only be capable of maintaining the expression of genes activated prior to the onset of Polycomb silencing in the early embryo, or genes subsequently derepressed by the UTX activity associated with the TRR complex. This might explain the previously reported critical requirement for TRX in early embryogenesis (0-4 hours; i.e. prior to the onset of Polycomb silencing) for later robust expression of the homeotic genes in imaginal discs. Absence of TRX in 0- to 4-hour embryos cannot be compensated by its subsequent restoration (Ingham and Whittle, 1980; Poux et al., 2002). Further investigation will be required to determine whether and in what contexts there is functional collaboration or division of labor between TRX and TRR.

### Mechanism of action of TRX and CBP

Although it is required continuously, the critical early requirement for TRX might provide an important clue to its function. This suggests that TRX and CBP, bound to PRE/TREs, might be required for de novo ‘priming’ of surrounding enhancers with H3K4me1 and H3K27ac in the early embryo (Bonn et al., 2012), prior to the onset of Polycomb silencing and perhaps even prior to transcriptional activation of the zygotic genome.

There is little detectable H3K27me3 in 0- to 4-hour embryos, whereas the H3K27ac level is already high relative to later embryonic stages (Tie et al., 2009). H3K4me1 is already present during syncytial stages (not shown). We speculate that before zygotic genome activation, TRX and CBP are constitutively bound to PRE/TREs and deposition of H3K4me1 and H3K27ac might



initially be restricted to nucleosomes adjacent to PRE/TREs. Binding of activators to early-acting enhancers promotes spreading of H3K4me1 and H3K27ac from PRE/TREs across adjacent *cis*-regulatory regions to form broad domains, perhaps facilitated by interactions between activators and TRX/CBP complexes. Spreading of H3K27ac initially proceeds unchecked by H3K27me3, encompassing all surrounding enhancers, including those that will be 'activated' later (e.g. the imaginal disc enhancers) and protects them from subsequent deposition of H3K27me3 by PRC2 at the onset of Polycomb silencing. PcG-regulated genes that are not activated in the early embryo become subject to deposition/spreading of H3K27me3 in similar broad domains, blocking subsequent H3K27 acetylation. There might also be some active removal of pre-existing H3K27ac by PRC1/PRC2-associated RPD3 (Tie et al., 2009). Subsequent activation requires removal of H3K27me3 by UTX, and thus might require TRR, which is also present at PRE/TREs and so is poised to respond to the binding of TRR-dependent activators, such as EcR.

Other functions of H3K4me1 and H3K27ac at PREs and enhancers are not yet understood, but they might (1) recruit H3K4me1 and H3K27ac 'readers' that further stimulate/maintain the active transcriptional state, (2) facilitate the targeting of enhancers to promoters (Ong and Corces, 2011) and (3) perpetuate the broad domains of H3K4me1 and H3K27ac by enhancing their own deposition by TRX and CBP, as suggested by the enhancing effect of H3K4me1 on H3K27 acetylation *in vitro*. Perpetuation of the broad domains of H3K4me1 and possibly H3K27ac through replication and mitosis could also constitute the elusive cellular memory of past transcriptional activity.

## MATERIALS AND METHODS

### Antibodies

Rabbit anti-TRX-N antibodies were generated against TRX residues 670-997. Rabbit anti-TRX-C antibodies were made previously (Chinwalla et al., 1995). Rabbit anti-CBP antibodies were generated against CBP residues 1-286 and purified on CBP(1-286)-coupled Sepharose. Rabbit anti-TRR and rat anti-SNR1 antibodies were kindly provided by A. Mazo (Sedkov et al., 2003) and A. Dingwall (Dingwall et al., 1995). Rabbit anti-H3K27ac (Abcam, ab4729), anti-H3K4me1 (Abcam, ab8895), anti-H3K4me2 (Abcam, ab11946), anti-H3K4me3 (Active Motif, 39159) antibodies were used in this study. Guinea pig anti-CBP and other antibodies were described previously (Tie et al., 2009; Tie et al., 2012).

### Constructs

The cDNA clones of TRX (LD39445) and TRR (SD13650) were obtained from the *Drosophila* Genomics Resource Center (DGRC). The SET domain-containing TRX-C751 (residues 2976-3726) (and others) and TRR-C421 (residues 2011-2431) were generated by PCR and inserted into modified pGEX-2T vector by *NdeI* and *NsiI* sites. Constructs pGEX-TRX(2976-3574), deleting the TRX SET domain (ΔSET), and pGEX-TRX(2976-3310) (ΔC416), were generated from pGEX-TRX-C751 by removing the C-terminal *NcoI-NsiI* and *XbaI-NsiI* fragments of TRX, then end-filling to produce blunt ends and recircularizing. Construct pGEX-TRX(2976-3169) (ΔC557) was generated from pGEX-TRX-C751 by removing the C-terminal *EcoRI-EcoRI* fragment of TRX and recircularizing. TRX mutations, *trx<sup>Z11</sup>* or G3601S (GGC/AGC), E3616S (GAA/TCA), Y3701F (TAC/TTC), the *trx<sup>Z11</sup>* corresponding substitution in TRR G2304S (GGC/AGC) and Y2404F (TAC/TTC) were generated using the QuikChange Site-Directed Mutagenesis Kit (Agilent). All mutations are confirmed by sequencing. PCR fragments coding wild-type histone H3 and mutant H3K4R were inserted into modified pET-11 vector (with a six-histidine tag at C-terminus) by *NdeI* and *NsiI* sites.

Human MLL1 construct pGEX-MLL1-C225 (residues 3745-3969) was described previously (Patel et al., 2009) and pGEX-MLL2-C219 (residues 5319-5537) was generated similarly.

### Histone H3 and GST fusion protein purification

GST-TRX-C proteins (wild type and mutants) were induced by 0.2 mM IPTG for 2 hours at gradual decreasing temperature from 35°C to 28°C in BL-21 cells and were purified on Glutathione Sepharose 4B (GE Healthcare). Histone H3 and H3K4R (with a 6xHis tag at C-terminus) were induced by 0.5 mM IPTG for 2 hours in BL-21 cells and were purified on Ni-NTA His Bind Resin (Qiagen) in 6-8 M urea. Purified histones were dialyzed in PBS and stored at -80°C.

### *In vitro* histone methyltransferase (HMT) assay

GST-TRX-C (1 μg) in 2× HMT buffer (Patel et al., 2009) (0.1 M Tris pH 8.2, 0.4 M NaCl, 6 mM DTT, 10 mM MgCl<sub>2</sub>, 10% glycerol) was mixed with recombinant histone H3 (1-4 μg) and 0.5 mM S-adenosyl-methionine (SAM) in a total volume of 30 μl. The reactions were incubated at 30°C (or 28°C) for 1 hour and stopped by adding one volume of 2× SDS sample buffer. H3 products were separated by SDS-PAGE (15%) and analyzed by western blot.

### Cell culture and treatment with LSD1 inhibitor

*Drosophila* S2 cells (DGRC) were cultured at 25°C in Schneider's *Drosophila* Medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS). S2 cells were seeded in 25 cm<sup>2</sup> flasks with 5 ml medium. When they had grown to 50-60% confluency, LSD1 inhibitor (a biguanidine polyamine analog from Calbiochem, catalog number 489476) was added to the medium at a final concentration of 10, 20, 40 or 80 μM. Cells were grown for 3 days and harvested for analysis of histone H3 modifications and proteins.

### Quantitative western blot analyses

For TRX mutants and over-expressors, protein extracts were prepared from larval salivary glands of *trx<sup>1</sup>*, *trx<sup>Z11</sup>*, *lsd1<sup>AN</sup>* mutants and *UAS-lsd1/da-GAL4* and *trx<sup>EP3541</sup>/da-GAL4*, which overexpress LSD1 and TRX (Tie et al., 2009). Homozygous *trx<sup>Z11</sup>* larvae from a *trx<sup>Z11</sup>/TM6B*, *Tb* stock were identified as non-*Tubby* (*Tb*). Third instar larval salivary glands were dissected in PBS and homogenized in equal volumes of PBS plus 8 M urea and 2× SDS sample buffer. For SDS-PAGE, 5-10 μl of the extracts were used. Quantitative western analyses were performed (Tie et al., 2009) using various anti-H3 antibodies. A GE Typhoon Trio system was used for data acquisition. Data were exported to Excel and the ratios of signals from different histone modifications to total H3 level were calculated.

### *Drosophila* strains

*trr<sup>3</sup>* and *trr<sup>1</sup>* stocks were obtained from A. Mazo (Sedkov et al., 2003). *UAS-lsd1* and *lsd1<sup>AN</sup>* stocks were obtained from N. Dyson (Di Stefano et al., 2007). CBP-RNAi lines 1 and 2 were from S. Smolik (#32577) (Ludlam et al., 2002) and J. Kumar (Kumar et al., 2004). The GAL4-inducible *trx<sup>EP3541</sup>* line used for TRX overexpression and other stocks were obtained from the Bloomington *Drosophila* Stock Center (BDSC).

### Genetic crosses

To determine whether the catalytic activity of TRX is required for antagonizing Polycomb silencing, we compared the suppression of the dominant extra sex comb phenotype of *Pc<sup>3</sup>/+* males by the protein-null *trx<sup>B11</sup>* and the catalytically inactive *trx<sup>Z11</sup>* mutations. *trx<sup>Z11</sup>/TM6B* and *trx<sup>B11</sup>/TM6B* females were crossed to *Pc<sup>3</sup>/TM3* males. Adult male progeny were scored for the presence of extra sex combs on the second thoracic (T2) leg, a *Pc<sup>3</sup>* phenotype resulting from derepression of *Scr* in T2 and indicative of homeotic transformation of T2 to T1 leg.

To determine whether moderate RNAi knockdown of CBP suppresses the Polycomb phenotypes caused by GAL4-induced overexpression of TRX, *trx<sup>EP3541</sup>/trx<sup>EP3541</sup>* females were crossed to wild-type males (control) and males from two homozygous CBP RNAi lines that target different portions of the *CBP* transcript: *Hsp70-Gal4; CBP RNAi-1* and *Hsp70-Gal4; CBP RNAi-2*. Adult male progeny were scored for the presence of extra sex combs on the T2 leg. Crosses were at 29°C, which promotes substantial leaky expression of *Hsp70-Gal4*.

For LSD1 overexpression, *UAS-lsd1* flies were crossed to *da-GAL4* (BDSC stock 8641) and maintained continuously at 25°C.

## Embryo immunostaining

*trx<sup>z11</sup>*, *trr<sup>1</sup>*, *trr<sup>3</sup>* single and *trr*; *trx<sup>z11</sup>* double-mutant embryos were obtained from the following stocks: (1) *trx<sup>z11</sup>/TM3, ftz-lacZ*, (2) *trr<sup>1</sup>* or *trr<sup>3</sup>/FM7c*, [*Kr-GAL4, UAS-GFP*] and (3) *trr<sup>1</sup>* or *trr<sup>3</sup>/FM7c*, [*Kr-GAL4, UAS-GFP*]; *trx<sup>z11</sup>/TM3, ftz-lacZ*. Hemizygous *trr/Y*, homozygous *trx<sup>z11</sup>* and *trr<sup>3</sup>*; *trx<sup>z11</sup>* double-mutant embryos were identified by the absence of GFP,  $\beta$ -galactosidase ( $\beta$ -gal), or both. Anti-H3K4me1 and anti-H3K4me3 antibodies (1:1000 dilution) were used for embryo staining as previously described (Tie et al., 2009).

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## Competing interests

The authors declare no competing financial interests.

## Author contributions

F.T., M.S.C. and P.J.H. designed experiments; F.T., R.B., B.H. and K.E.M. performed experiments; F.T., R.B., M.S.C. and P.J.H. analyzed data; A.R.S. and P.C.S. generated heat maps; F.T. and P.J.H. wrote the paper.

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## Supplementary material

Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.102392/-/DC1>

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