The histone methyltransferases Trithorax and Ash1 prevent transcriptional silencing by Polycomb group proteins

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Transcriptional on and off states of HOX genes and other developmental control genes are maintained by antagonistic regulators encoded by trithorax group (trxG) and Polycomb group (PcG) genes. The trxG proteins Ash1 and hTRX and the PcG repressor E(z) are histone methyltransferases (HMTases) that methylate distinct lysine residues in the N-terminal tail of histone H3. trxG proteins are generally thought to function as activators of HOX genes, but how histone methylation by Ash1 and Trx promotes HOX gene transcription is not clear. Here, we show that in *ash1* and *trx* mutants expression of HOX genes is lost within their normal expression domains, but we find that, contrary to expectation, this expression is restored in *ash1* and *trx* mutants that also lack PcG gene function. Moreover, such trxG PcG double mutants show severe misexpression of HOX genes and, hence, ectopic activation of HOX genes caused by the removal of PcG gene function also occurs in the absence of *ash1* and *trx* function. Together, these results suggest that the Ash1 and Trx HMTases are not 'coactivators' required for transcriptional activation of HOX genes, but function specifically as anti-repressors. We propose that histone methylation by Ash1 and Trx is required continuously throughout development to prevent inappropriate PcG silencing of HOX genes in cells in which they must stay transcriptionally active.

Keywords: polycomb; trithorax; histone methylation

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INTRODUCTION

Regulation of homeotic gene expression in *Drosophila* represents a paradigm for understanding how heritable transcriptional states are established and maintained during development. In the early embryo, activators and repressors encoded by segmentation genes determine in which cells homeotic genes are expressed and in

which cells these genes must remain inactive. These factors are, however, only transiently present and, during subsequent development, the on and off states of homeotic genes are then heritably maintained by two different groups of regulatory proteins. Polycomb group (PcG) gene products repress homeotic genes in cells in which these genes must stay inactive, whereas trithorax group (trxG) gene products maintain expression of homeotic genes in appropriate cells (reviewed in Francis & Kingston, 2001; Simon & Tamkun, 2002).

Recent progress in understanding the PcG/trxG system has come from the purification and characterization of PcG and trxG protein complexes. Two different PcG protein complexes with distinct biochemical activities have been characterized: Polycomb repressive complex 1 (PRC1) and the Esc–E(z) complex. PRC1 does not contain any enzymatic activity, but in *in vitro* assays it inhibits chromatin remodelling by SWI–SNF chromatin remodelling complexes (Shao *et al*, 1999; Francis *et al*, 2001; Saurin *et al*, 2001). In contrast, the Esc–E(z) complex functions as a histone methyltransferase (HMTase) that selectively methylates K27 and, to a minor extent, also K9 in the N-terminal tail of histone H3 (Cao *et al*, 2002; Czermin *et al*, 2002; Kuzmichev *et al*, 2002; Müller *et al*, 2002). Recent biochemical studies on trxG proteins showed that *Drosophila* Ash1 and mammalian ALL-1/MLL, the homologue of the *Drosophila* trithorax (Trx) protein, also function as HMTases. Ash1 selectively methylates K4 and K9 on histone H3 and K20 on histone H4 (Beisel *et al*, 2002; Byrd & Shearn, 2003), whereas ALL-1/MLL selectively methylates K4 in histone H3 (Milne *et al*, 2002; Nakamura *et al*, 2002).

What is the role of the Ash1 and Trx methyltransferases in maintaining expression of homeotic genes? The predominant view is that these trxG proteins function as transcriptional coactivators that are needed for expression of homeotic genes (Petruk *et al*, 2001; Beisel *et al*, 2002; Poux *et al*, 2002). Experiments in tissue culture cells appear to support this coactivator model and suggest that transcriptional activation by Ash1 and Trx requires their HMTase activity (Beisel *et al*, 2002; Milne *et al*, 2002). More recently, it has been proposed that Ash1 and Trx may not only function as coactivators but could also have a role in preventing the establishment of PcG silencing (Milne *et al*, 2002;

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Poux *et al*, 2002). At present, there is only very little evidence to support this view. One circumstantial argument is provided by the observation that expression of homeotic genes in *ash1* mutants (Lajeunesse & Shearn, 1995) or expression of a homeotic reporter gene in *trx* heterozygotes (Poux *et al*, 2002) is stochastically lost in some cells but is maintained in others. Lajeunesse & Shearn (1995) and Poux *et al* (2002) proposed that this variegated all-or-none loss of expression might reflect ectopic repression by PcG proteins, an assumption that was, however, not tested in either of these studies.

Here, we analysed HOX gene expression in different *trxG* PcG double-mutant combinations. Unexpectedly, these experiments reveal that *ash1* and *trx* are not required for transcriptional activation of HOX genes, but are needed throughout development to prevent the establishment of PcG silencing on HOX genes. We discuss and contrast these results with those obtained in earlier studies that analysed the antagonistic relationship between *trxG* and PcG proteins.

RESULTS AND DISCUSSION

To assess the role of the *trxG* proteins Trx and Ash1 in the maintenance of HOX gene transcription, we first analysed HOX gene expression in clones of cells that lack *trx* or *ash1* function in imaginal discs. Clones of mutant cells were induced by *Flp*-mediated recombination, and mutant cells were identified by the lack of a GFP-expressing marker gene. We analysed the effect of eliminating *ash1* or *trx* function by monitoring expression of the HOX gene *Ultrabithorax* (*Ubx*) in thoracic imaginal discs using an antibody against Ubx protein. In wild-type animals, *Ubx* is expressed in the haltere and third leg imaginal disc, but is repressed in the wing imaginal disc. We first analysed *ash1* and *trx* mutant clones, and we find that *trx* mutant clones in the haltere and third leg disc show complete loss of Ubx signal (Fig 1). *ash1* mutant clones also show complete loss of Ubx signal, but most haltere and third leg discs also contain a substantial fraction of mutant cells with apparently undiminished expression of *Ubx* (Fig 2). These results, together with earlier studies on *ash1* mutant larvae (Lajeunesse & Shearn, 1995) and studies on *trx* mutant clones in the adult epidermis (Ingham, 1981), show that both *ash1* and *trx* are critically needed for the maintenance of HOX gene expression.

We next analysed *Ubx* expression in *ash1* and *trx* mutant clones that also lack PcG function. Using null mutations in the PcG genes *Sex combs on midleg* (*Scm*) and *Enhancer of zeste* (*E(z)*), we generated *Scm trx* and *E(z) ash1* double-mutant clones and compared them with the corresponding single-mutant clones. As reported previously (Beuchle *et al*, 2001; Müller *et al*, 2002), *Scm* and *E(z)* single-mutant clones in the wing disc show severe misexpression of *Ubx*, whereas mutant clones in the haltere and third leg disc show wild-type levels of *Ubx* expression (Figs 1 and 2). Strikingly, *Scm trx* and *E(z) ash1* double-mutant clones show the same phenotype as *Scm* and *E(z)* single-mutant clones (Figs 1 and 2). First, *Scm trx* and *E(z) ash1* double-mutant clones in the wing disc show strong misexpression of *Ubx*, comparable to *Scm* and *E(z)* single-mutant clones in wing discs. Second, clones in the haltere and third leg disc show wild-type levels of *Ubx* expression. *Ubx* expression is thus restored in *trx* and *ash1* mutant cells when PcG gene function is removed.

Fig 1 | HOX gene expression in *trx* and *Scm* single-mutant clones and in *Scm trx* double-mutant clones. Wing, haltere (H) and third leg (L) imaginal discs stained with antibodies against GFP (green) and Ubx protein (red); clones of mutant cells (negative for GFP) were induced 96 h before analysis. In wild-type animals, *Ubx* is expressed in haltere and third leg discs but not in the wing disc. Top: Most *trx* single-mutant clones in haltere and third leg discs show complete loss of Ubx signal (empty arrowhead); in rare cases, some *trx* mutant cells within a clone maintain Ubx expression at wild-type levels (small arrow). Middle: *Scm* mutant clones in wing discs show strong misexpression of *Ubx* (filled arrowheads); clones in haltere and third leg discs show wild-type expression levels. Bottom: *Scm trx* double-mutant clones in wing discs show strong misexpression at levels comparable to *Scm* single-mutant clones. Note that *Scm trx* double-mutant clones in haltere and third leg discs show wild-type levels of *Ubx* signal (compare with *trx* single-mutant clones).

The strong misexpression of HOX genes in these PcG *trxG* double-mutant clones was unexpected, as a study by Ingham (1983) reported that the morphological defects observed in embryos mutant for the PcG gene *extra sex combs* (*esc*) are suppressed in *esc trx* double-mutant embryos. On the basis of the cuticle phenotype of such *esc trx* double mutants, Ingham (1983) concluded that HOX genes would be differentially expressed in the absence of PcG and *trxG* gene function. This notion could not be tested at that time owing to the lack of molecular probes. We therefore analysed HOX gene expression in *esc trx* and *Scm trx* double-mutant embryos and in the corresponding single mutants.

Embryos lacking *trx* function show a reduction of *Ubx* and *Abd-B* expression (Fig 3 and data not shown; see also Mazo *et al*, 1990; Breen & Harte, 1993), and *Scm* or *esc* mutant embryos show severe misexpression of both *Ubx* and *Abd-B* (Fig 3A and data not shown; see also Struhl & Akam, 1985; McKeon & Brock, 1991; Simon *et al*, 1992). Strikingly, *Scm trx* double-mutant embryos show the same strong misexpression of *Ubx* and *Abd-B* as *Scm* single-mutant embryos and the same severe PcG phenotype in the embryonic cuticle (Fig 3A). Thus, in embryos, as in imaginal discs, the *Scm* mutant phenotype is not suppressed

Fig 2 | HOX gene expression in *ash1* and *E(z)* single-mutant clones and in *E(z) ash1* double-mutant clones. Wing, haltere (H) and third leg (L) imaginal discs stained with antibodies against GFP (green) and Ubx protein (red); clones of mutant cells (negative for GFP) were induced 96 h before analysis. The Minute technique was used in this experiment, resulting in larger clones. Top: *ash1* single-mutant clones in haltere and third leg discs show complete loss of Ubx signal (empty arrowhead), but a significant fraction of mutant cells shows undiminished Ubx expression (small arrows). Middle: *E(z)* mutant clones in the wing disc show strong misexpression of Ubx (filled arrowheads); clones in the haltere and third leg disc show wild-type expression levels. Bottom: *E(z) ash1* double-mutant clones in wing discs show strong misexpression at levels comparable to *E(z)* single-mutant clones. Note that *E(z) ash1* double-mutant clones in haltere and third leg discs all show wild-type levels of Ubx signal (compare with *ash1* single-mutant clones).

in the absence of *trx* function. In the case of *esc trx* double-mutant embryos, the severe PcG phenotype that is observed in the cuticle of *esc* mutant embryos is partially suppressed, although most segments still show some posteriorly directed homeotic transformations (Fig 3A). At the level of HOX gene expression, we find that *esc trx* double-mutant embryos show the same widespread misexpression as *esc* single mutants. However, we find that the onset of misexpression in these *esc trx* double mutants is delayed and that the level of misexpression is lower compared to *esc* single mutants (Fig 3A and data not shown); this might explain the partial suppression of the PcG phenotype in the embryonic cuticle. Note that *Scm trx* double mutants show no such reduction of HOX gene expression compared with *Scm* single mutants (Fig 3A). Taken together, the widespread misexpression of HOX genes in *Scm trx* and *esc trx* double-mutant embryos shows that ectopic activation occurs in the absence of *trx* function. Nevertheless, it appears that *trx* function is needed for maximal levels of HOX gene transcription in *esc* mutant embryos. Surprisingly, the level of *Abd-B* expression in parasegments (ps) 13 and 14 of *esc trx* double mutants is even lower than in *trx* single mutants (Fig 3A); the reason for this is not clear.

Human Trx methylates K4 in the N-terminal tail of histone H3 (Milne *et al*, 2002; Nakamura *et al*, 2002), whereas Ash1

methylates K4 and K9 in H3 and K20 in H4 (Beisel *et al*, 2002; Byrd & Shearn, 2003). The loss of HOX gene expression in either *trx* or *ash1* mutant clones suggests that, despite this overlap in enzymatic specificity, both HMTases are required for maintaining HOX gene expression. To test a possible functional redundancy between Trx and Ash1, we next analysed *ash1 trx* double mutants and *ash1 Scm trx* triple mutants. *ash1* homozygous embryos that are derived from heterozygous parents show virtually normal HOX gene expression (data not shown), but *ash1* homozygous embryos that are derived from *ash1* mutant germ cells show reduction of HOX gene expression similar to *trx* homozygotes (Fig 3B). *ash1 trx* double homozygotes show slightly more extensive reduction of HOX gene expression than either *trx* or *ash1* single mutants, suggesting that these two HMTases synergize only to a minor extent to maintain HOX gene expression (Fig 3B and data not shown). Nevertheless, we find that *ash1 Scm trx* triple-mutant embryos show severe misexpression like *Scm trx* or *Scm* mutant embryos (Fig 3B). Thus, even the removal of both H3 K4-specific HMTases does not result in suppression of the PcG phenotype.

Two main conclusions can be drawn from the results reported here. First, *E(z) ash1* and *Scm trx* double mutants show misexpression of HOX genes outside and wild-type levels of HOX gene expression within HOX expression domains. This suggests that the Trx and Ash1 HMTases are not generally required for transcriptional activation of HOX genes. From our data set, the reduced levels of HOX gene misexpression in *esc trx* double-mutant embryos compared to *esc* single-mutant embryos provide the only indication that Trx may, under some circumstances, be required for transcriptional activation. The second main conclusion comes from the observation that removal of PcG gene function in *trx* and *ash1* mutant cells in imaginal discs restores HOX gene expression. This implies that in the absence of Trx or Ash1, PcG proteins repress HOX genes also within their normal expression domains. Taken together, our findings therefore suggest that Trx and Ash1 are not transcriptional 'coactivators', but function primarily as 'anti-repressors' that prevent PcG silencing of HOX genes within their normal expression domains. We note that other *trxG* regulators in *Drosophila* such as the SWI/SNF complex components Brahma (Brm), Osa and Moira (Mor) may have a much more direct role in the transcriptional activation process (e.g. Kal *et al*, 2000). The role of SWI/SNF proteins is therefore distinct from the anti-repressor function of Ash1 and Trx that we propose here. Unfortunately, the pleiotrophic phenotype of SWI/SNF mutants did not allow us to analyse their specific role in HOX gene regulation. Finally, it is interesting to note that in mice that are double mutant for the *trx* homologue *Mll* and the PcG gene *bmi-1*, the reduction of *HOX c8* expression observed in *Mll* single mutants is at least partly rescued (Hanson, 1999). This observation would be consistent with the interpretation that, in mice, *Mll* could exert a similar anti-repressor function to prevent HOX gene silencing by PcG proteins.

How do Ash1 and Trx counteract PcG repression? One possible scenario is that binding and/or histone methylation by Ash1 and Trx prevents the binding of PcG proteins to HOX genes within normal HOX expression domains. For example, methylation of the H3/H4 tails by Ash1 and/or Trx could prevent methylation of K27 in the H3 tail by the *E(z)* HMTase (Cao *et al*, 2002; Czermin *et al*, 2002; Kuzmichev *et al*, 2002; Müller *et al*,

Fig 3 | HOX genes are misexpressed in PcG *trxG* double-mutant embryos. Ventral views of embryonic cuticles (A, left column) and stage-16 embryos stained with antibody against Abd-B protein (A (right column), B). In cuticle images, the white bar marks the boundary between thoracic and abdominal segments, and the arrow in *wt* marks the eighth abdominal segment. In stained embryos, the anterior margin of parasegment 10 is marked by a black bar. Genotypes are as indicated; '*m-z-*' lack maternal and zygotic product, '*z-*' lack zygotic product. (A) In *wt* embryos, denticle belts in each segment have a characteristic shape (left) and Abd-B is expressed from ps 10 to ps 14 (right). Cuticles of *trx^{m-z-}* and *trx^{m-z-}* embryos are indistinguishable and show only mild homeotic transformations (for details, see Ingham, 1983); Abd-B expression is reduced in the CNS (empty arrowhead) and epidermis (not visible in this focal plane). Cuticles of *Scm^{m-z-}*, *Scm^{m-z-} trx^{m-z-}* and *esc^{m-z-}* embryos are indistinguishable; they show complete homeotic transformation of all segments into copies of the eighth abdominal segment and strong misexpression of Abd-B in all segments. *esc^{m-z-} trx^{m-z-}* embryos show partial suppression of homeotic transformations in the cuticle (i.e. thoracic denticle belts are comparable to those in *wt* embryos), but the head is abnormal and abdominal segments are still partly transformed towards the eighth abdominal segment; Abd-B is misexpressed in all segments, but expression levels are reduced compared with *esc^{m-z-}* embryos. For unknown reasons, Abd-B expression in ps 13 and 14 of *esc^{m-z-} trx^{m-z-}* mutant embryos is lower than in *wt*, *trx^{m-z-}* or *esc^{m-z-}* single-mutant embryos. (B) *ash1^{m-z-}* embryos show reduction of Abd-B expression similar to *trx^{m-z-}* mutant embryos (empty arrowheads). *ash1^{m-z-} trx^{m-z-}* mutant embryos show slightly more extensive reduction of Abd-B expression (empty arrowheads). *ash1^{m-z-} Scm^{m-z-} trx^{m-z-}* triple-mutant embryos show severe misexpression of Abd-B comparable to *Scm^{m-z-}* or *Scm^{m-z-} trx^{m-z-}* mutant embryos, but note that expression levels in the epidermis are slightly reduced.

2002) or prevent the binding of PcG protein complexes such as PRC1 (Shao *et al*, 1999; Fischle *et al*, 2003; Min *et al*, 2003). It is also possible that methylation by Trx and Ash1 does not prevent the binding of PcG proteins but interferes at a subsequent step by blocking their silencing function.

Methylation of histone tails appears to be a modification that is able to persist, at least for some time, even after dissociation of the HMTase (Ng *et al*, 2003). We previously proposed that H3 K27 methylation by E(z) may serve as a molecular mark of the silenced state of HOX genes (Müller *et al*, 2002). Similarly, histone methylation by Trx and Ash1 may provide a mark that allows the propagation of the active state of HOX genes through replication and mitosis. In particular, the methylation

mark created by Trx and Ash1 may be critical to prevent PcG silencing during stages of the cell cycle when no transcription is taking place.

METHODS

The following fly strains were used in this study: *w*; *FRT82B trx^{e2}/TM6C*, *w*; *FRT82B Scm^{D1}/TM6C*, *w*; *FRT82B Scm^{D1}trx^{e2}/TM6C*, *w*; *ash1²²FRT2A/TM6C*, *w*; *E(z)⁷³¹FRT2A/TM6C*, *w*; *E(z)⁷³¹ash1²²FRT2A/TM6C*, *esc⁶/CyO*; *FRT82B trx^{e2}/TM2*, *w*; *ash1²²FRT2A trx^{e2}/TM6C*, *w*; *ash1²²FRT2A Scm^{D1}trx^{e2}/TM6C*.

The PcG and *trxG* mutant alleles used in this study are all genetically or molecularly defined as null alleles (<http://flybase.bio.indiana.edu/>). Marked clones in imaginal discs were

induced and stained as described (Beuchle *et al*, 2001). Germ-line clone analysis, cuticle preparations and embryo stainings were carried out following standard protocols; balancer chromosomes carrying GFP marker genes were used to genotype mutant embryos.

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