Development 140, 3478-3485 (2013) doi:10.1242/dev.097204


# The histone H3-K27 demethylase Utx regulates HOX gene expression in *Drosophila* in a temporally restricted manner

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## SUMMARY

Trimethylation of histone H3 at lysine 27 (H3-K27me3) by Polycomb repressive complex 2 (PRC2) is a key step for transcriptional repression by the Polycomb system. Demethylation of H3-K27me3 by Utx and/or its paralogs has consequently been proposed to be important for counteracting Polycomb repression. To study the phenotype of *Drosophila* mutants that lack H3-K27me3 demethylase activity, we created $Utx^{\Delta}$, a deletion allele of the single *Drosophila Utx* gene. $Utx^{\Delta}$ homozygotes that contain maternally deposited wild-type Utx protein develop into adults with normal epidermal morphology but die shortly after hatching. By contrast, $Utx^{\Delta}$ homozygotes that are derived from *Utx* mutant germ cells and therefore lack both maternal and zygotic Utx protein, die as larvae and show partial loss of expression of HOX genes in tissues in which these genes are normally active. This phenotype classifies *Utx* as a trithorax group regulator. We propose that Utx is needed in the early embryo to prevent inappropriate instalment of long-term Polycomb repression at HOX genes in cells in which these genes must be kept active. In contrast to PRC2, which is essential for, and continuously required during, germ cell, embryonic and larval development, Utx therefore appears to have a more limited and specific function during development. This argues against a continuous interplay between H3-K27me3 methylation and demethylation in the control of gene transcription in *Drosophila*. Furthermore, our analyses do not support the recent proposal that Utx would regulate cell proliferation in *Drosophila* as *Utx* mutant cells generated in wild-type animals proliferate like wild-type cells.

**KEY WORDS: Polycomb, PcG, Trithorax, trxG, Utx, H3-K27me3**

## INTRODUCTION

Enzymes that add or remove post-translational modifications at specific amino acid residues of histone proteins have emerged as an important class of transcriptional regulators. Among these regulators are the Polycomb group (PcG) proteins, which form several distinct multiprotein complexes with specific histone-modifying activities (reviewed by Schwartz and Pirrotta, 2008; Müller and Verrijzer, 2009; Beisel and Paro, 2011; Lanzuolo and Orlando, 2012; O'Meara and Simon, 2012; Scheuermann et al., 2012). PcG proteins were first identified through genetic studies in *Drosophila*, where they are required for the repression of HOX and other developmental regulator genes. In particular, Polycomb complexes are essential for keeping HOX genes inactive in the progeny of cells in which these genes were initially repressed by transcriptional regulators that were transiently present in the early *Drosophila* embryo. Repression by PcG complexes is therefore thought to comprise an epigenetic memory mechanism that permits the heritable propagation of this repression throughout development (reviewed by Ringrose and Paro, 2004). The discovery that Polycomb repressive complex 2 (PRC2) is a histone methyltransferase that specifically methylates lysine 27 of histone H3 (H3-K27) and that trimethylation of H3-K27 (H3-K27me3) is present across extended stretches of chromatin at PcG-repressed genes, has made this modification an attractive candidate for a chromatin mark that might be inherited through replication and mitosis (Cao et al., 2002; Czermin et al., 2002; Kuzmichev et al., 2002; Müller et al., 2002; Bernstein et al., 2006; Papp and Müller, 2006; Schwartz et al., 2006; Trojer and Reinberg, 2006; Mikkelsen et al., 2007; Schuettengruber et al., 2009; Hansen et al., 2008; Margueron et al., 2009; Filion et al., 2010). Recent studies in *Drosophila* showed that cells in which wild-type histone H3 has been replaced by the nonmethylatable mutant H3K27R fail to maintain Polycomb repression (Pengelly et al., 2013). This provides strong evidence that methylation of H3-K27 is indeed crucial for the repression of Polycomb target genes in *Drosophila*.

Earlier studies identified H3-K27-specific demethylases. In particular, Utx and the related JmjD3 protein were found to demethylate H3-K27me3 *in vitro* and *in vivo* (Agger et al., 2007; De Santa et al., 2007; Hong et al., 2007; Lan et al., 2007; Lee et al., 2007; Swigut and Wysocka, 2007). The discovery of these enzymes suggested that H3-K27me3 in chromatin might be added and removed in a dynamic fashion. This challenged the view of H3-K27me3 as a stable epigenetic mark that, once installed on an array of nucleosomes, could only be removed by eviction of the modified octamer or by dilution due to deposition of unmodified octamers during DNA replication.

To understand the role of H3-K27me3 demethylation, it is essential to determine the phenotype of animals that lack H3-K27me3 demethylase activity and to investigate how the expression of PRC2-regulated genes is affected in such animals. Mouse, worm and flies contain different numbers of H3-K27me3 demethylase paralogs that are all characterised by a catalytic JmjC domain with an adjacent zinc-binding domain that is important for substrate specificity (Sengoku and Yokoyama, 2011; Kim and Song, 2011).

The mouse genome encodes three H3-K27me3 demethylase family members: the *Utx* (*Kdm6a* – Mouse Genome Informatics) gene on the X chromosome, the *Uty* gene on the Y chromosome and JmjD3 (*Kdm6b* – Mouse Genome Informatics). Recent studies reported that *Utx* homozygous mutant female mice die during

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**Accepted 11 June 2013**

embryonic development with defects in heart and neural tube morphogenesis and that males lacking both Utx and Uty show the same phenotype but that males only lacking Utx develop into normal and fertile adults (Lee et al., 2012; Shpargel et al., 2012; Welstead et al., 2012; Wang et al., 2012; Thieme et al., 2013). Uty thus evidently compensates for the lack of Utx in male *Utx* mutants (Shpargel et al., 2012). However, there is currently no evidence that Uty has H3-K27me3 demethylase activity, even though the catalytic JmjC domain and the adjacent zinc finger are highly conserved (Hong et al., 2007; Shpargel et al., 2012). Moreover, in a differentiation assay *in vitro*, knock-in male embryonic stem cells (ESCs) that express full-length but catalytically inactive Utx instead of the wild-type protein show normal induction of mesodermal marker genes, whereas knockout cells lacking Utx protein fail to induce expression of these genes (Wang et al., 2012). Together, these studies led to the suggestion that the Utx/Uty proteins, but not their H3-K27me3 demethylase activity, are crucial for normal embryonic development (Shpargel et al., 2012; Wang et al., 2012). *Jmjd3* mutant mice have been reported to be perinatal lethal (Satoh et al., 2010). At present, it is unclear whether the H3-K27me3 demethylase activities of JmjD3 and Utx compensate for each other (Lee et al., 2012; Shpargel et al., 2012; Welstead et al., 2012; Wang et al., 2012). Studies in tissue culture cell models suggest that Utx demethylates H3-K27me3 in target gene chromatin through a mechanism that requires elongating polymerase (Seenundun et al., 2010), whereas JmjD3 has been implicated to be required for the release of poised RNA polymerase II into productive transcriptional elongation (Chen et al., 2012).

*C. elegans* contains *utx-1* and three JmjD3 genes (Vandamme et al., 2012). *utx-1* mutant worms that are derived from heterozygous mothers and therefore contain maternally deposited wild-type UTX-1 protein are viable and show no morphological abnormalities. However, the mutant progeny from *utx-1* homozygotes (i.e. lacking both zygotic and maternal UTX-1 protein) arrest development at the late embryonic stage (Vandamme et al., 2012). Intriguingly, this phenotype can be rescued not only by wild-type but also by a catalytically inactive UTX-1 protein, suggesting that UTX-1 protein, but not its H3-K27me3 demethylase activity, is essential for *C. elegans* development (Vandamme et al., 2012). Worms lacking all three JmjD3 genes are viable and fertile and there is no evidence for a major functional redundancy between UTX-1 and the three JmjD3 proteins (Vandamme et al., 2012).

*Drosophila* contains a single *Utx* gene, also called *dUtx* (Smith et al., 2008). Recent studies analysed the phenotype of *Drosophila Utx* mutants containing a premature termination codon in the C-terminal portion of the JmjC domain (Herz et al., 2010). A large proportion of the animals homozygous for this allele died during the pupal stages, but some were found to develop into adults that showed a spectrum of phenotypes, including rough eyes and mortality shortly after eclosion (Herz et al., 2010). Based on studies with this allele in mosaic animals, it was proposed that Utx acts as a tumour suppressor in *Drosophila* (Herz et al., 2010).

In this study, we generated a *Drosophila Utx* knockout allele carrying a deletion of the catalytic JmjC domain. Our analyses of *Utx* mutants provide no evidence for a tumour suppressor function of *Utx* and show that the zygotic Utx product is dispensable for the normal development of epidermal structures but is essential for adult viability. However, we found that maternally deposited Utx protein in the early embryo is crucial for the normal expression of multiple HOX genes. This work establishes Utx as a trithorax group (trxG) regulator that is specifically required early in embryogenesis to set up long-term stable HOX gene expression patterns.

## MATERIALS AND METHODS

### Fly strains

The following *Drosophila* strains were used in this study:
*y w;Utx$^{\Delta}$/CyO, twi:GAL4, UAS:GFP*;
*y w;Utx$^{\Delta}$ FRT40A/CyO*;
*y w hs-flp, tubP:GAL4, UAS:nGFP; tubP:GAL80 FRT40A/CyO*;
*y w hs-flp; ovo$^{D1}$ FRT40A/CyO, hs:hid*;
*w; Utx$^{1}$ FRT40A/CyO, twi:GAL4, UAS:GFP*;
*w; Df(2L)BSC143/CyO, twi:GAL4, UAS:GFP*;
*y w hs-flp; hs-nGFP FRT40A*;
*y w hs-flp; hs-nGFP F2A*;
*y w; ash1$^{22}$ F2A/TM6C*;
*y w hs-flp; [Utx$^{\Delta}$; hs-nGFP F2A]/SM5^TM6*;
*w; [Utx$^{\Delta}$; ash1$^{22}$ F2A]/SM5^TM6*; and
*w; FRT40A*.

### Generation of the *Utx$^{\Delta}$* allele

The ends-out recombination strategy was used to generate *Utx$^{\Delta}$* by replacing the *Utx* JmjC coding region (Utx$_{503\text{-}952}$) with a *miniwhite* marker gene following the strategy described (Gong and Golic, 2003). In brief, for the *Utx* disruption construct, 3.5 kb of *Utx* 3′ coding and downstream sequences (FlyBase 2L:10269938..10273485) and 3.5 kb of 5′ coding and upstream sequences (FlyBase 2L:10275043..10278555) were cloned into pw35 (Gong and Golic, 2003). In this construct, the coding region of Utx$_{503\text{-}952}$ [FlyBase 2L:10273487..10275042 (on the minus strand)] was thus replaced by the *miniwhite* gene from pw35; the AGC codon for Ser503 was replaced with an in-frame TAA termination codon after the His502 codon of *Utx*.

### Analysis of survival into adults

To determine the fraction of animals developing into adults, three independent batches of 100 first instar larvae of genotype wild type, *Utx$^{\Delta}$/Utx$^{\Delta}$*, *Utx$^{\Delta}$/Df(2L)BSC143*, *Utx$^{1}$/Utx$^{1}$* and *Utx$^{1}$/Df(2L)BSC143* were collected, transferred into a vial with food, and larvae were reared at 25°C. Flies that eclosed from the pupal case were counted.

### Western blot analysis

To analyse Utx protein in wild-type or mutant larvae, total extracts from imaginal discs and CNS tissues of 50 third instar wild-type, *Utx$^{\Delta}$/Utx$^{\Delta}$* or *Utx$^{1}$/Utx$^{1}$* larvae were prepared by resuspending the tissues in 100 µl 1×LDS buffer (Life Technologies), sonication and then heating at 75°C for 5 minutes. The tissue suspension was centrifuged and 20 µl supernatant of each preparation was run on an 8% SDS polyacrylamide gel. The membrane was probed with anti-Utx$_{420\text{-}633}$ antibody (Tie et al., 2012) or with anti-Utx$_{1\text{-}153}$ antibody (Herz et al., 2010). For the analysis of histone modifications, total extracts from imaginal discs and CNS tissues were prepared the same way, using 50 third instar larvae of genotype wild type, *Utx$^{\Delta}$/Df(2L)BSC143* or *Utx$^{1}$/Df(2L)BSC143*. Serial dilutions using 6, 3 and 1.5 µl of each extract were loaded on a 15% SDS polyacrylamide gel. Membranes were probed with anti-H3-K27me3 (1:5000) (Peters et al., 2003), anti-H3-K27ac (1:5000; Active Motif), anti-H3-K4me1 (1:1000; Upstate) or anti-H4 (1:5000; Abcam) antibodies.

### Immunostaining of embryos and imaginal discs and preparation of adult cuticles

Preparation of embryonic cuticles, staining of embryos and larval imaginal discs and clonal analysis were performed following standard protocols. The following antibodies were used: anti-H3-K27me3 (1:300) (Peters et al., 2003), anti-H3-K27ac (1:300; Active Motif), anti-Ubx (1:30) (White and Wilcox, 1984) and anti-Abd-B (1:100) (Celniker et al., 1990). DNA was stained with Hoechst.

## RESULTS

### Zygotic *Utx* function is dispensable for morphogenesis but essential for adult viability

To investigate the function of Utx in *Drosophila* development, we used a homologous recombination strategy (Gong and Golic, 2003) to generate *Utx$^{\Delta}$*, a deletion allele that lacks most of the JmjC catalytic domain and additional portions of the *Utx* coding region

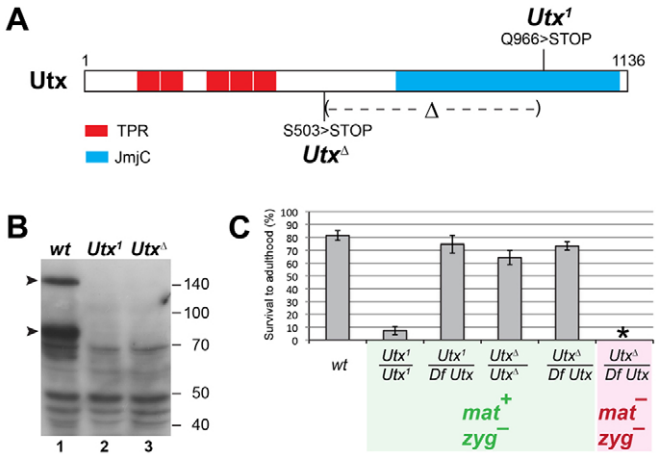


**Fig. 1. Analysis of Utx protein in *Utx* mutants and requirement for Utx in *Drosophila* development.** (**A**) Domain architecture of the *Drosophila* Utx protein showing the tetratricopeptide repeats (TPR) and the catalytic JmjC domain. The lesions of the previously described $Utx^{1}$ allele (Herz et al., 2010) and of the $Utx^{\Delta}$ deletion allele are indicated; in $Utx^{\Delta}$, a Ser503>stop mutation was introduced and a large portion of the JmjC coding region was deleted. (**B**) Total extracts from imaginal disc and CNS tissues from wild-type (wt, lane 1), homozygous $Utx^{1}$ (lane 2) and homozygous $Utx^{\Delta}$ (lane 3) larvae probed with an antibody against $Utx_{420\text{-}633}$. Note the lack of the 145 and 80 kDa bands (arrowheads) in the $Utx^{1}$ and $Utx^{\Delta}$ mutant extracts. Each lane contained the extract from ten third instar larvae and non-specific cross-reacting bands detected by the antibody provide an additional control for loading of comparable amounts of extract. (**C**) Survival of wild type and *Utx* mutants of the indicated genotypes to adults; *Df Utx* indicates *Df(2L)BSC143* and *mat+ zyg–* indicates that these animals were obtained from $Utx^{1}$ and $Utx^{\Delta}$ heterozygous mothers. *mat– zyg–* animals were obtained from mothers with $Utx^{\Delta}$ germline clones; they arrest development before the pupal stage (no adults were obtained, asterisk) (see main text). For each genotype, three independent batches with 100 first instar larvae each were collected, transferred into vials and the number of eclosed adults was determined. Error bars indicate s.d.

(Fig. 1A; supplementary material Fig. S1). $Utx^{\Delta}$ is thus a molecularly defined H3-K27 demethylase deficiency allele. We generated extracts from wild-type larvae and from larvae that were homozygous for $Utx^{\Delta}$ or $Utx^{1}$, a previously described point mutation (Fig. 1A) (Herz et al., 2010), and probed them with antibodies that had been raised against a central portion [anti-$Utx_{420\text{-}633}$ (Tie et al., 2012)] or the N-terminus [anti-$dUtx_{1\text{-}153}$ (Herz et al., 2010), referred to here as anti-$Utx_{1\text{-}153}$] of the Utx protein. In wild-type extracts, the anti-$Utx_{420\text{-}633}$ antibody detected two bands with apparent molecular weights of 145 and 80 kDa that were both absent in $Utx^{\Delta}$ or $Utx^{1}$ mutant extracts (Fig. 1B). This suggests that both the 145 and the 80 kDa proteins are Utx products. Although the 80 kDa band appears more intense than the 145 kDa band and could thus represent an alternate, shorter Utx product, it is also possible that the 80 kDa band represents a degradation product of the 145 kDa protein.

The anti-$Utx_{1\text{-}153}$ antibody detected the 145 kDa but not the 80 kDa band in wild-type extracts, suggesting that the 80 kDa Utx product lacks the N-terminus (data not shown). The anti-$Utx_{1\text{-}153}$ antibody also no longer detected the 145 kDa band in $Utx^{\Delta}$ extracts, but instead detected an extra band of ~55 kDa that is absent in wild-type extracts and is likely to correspond to the $Utx_{1\text{-}502}$ protein encoded by $Utx^{\Delta}$ (data not shown). In summary, the molecularly defined deletion of the JmjC domain in $Utx^{\Delta}$ and these western blot analyses demonstrate that $Utx^{\Delta}$ is a demethylase null mutation.

In a first set of experiments, we examined the phenotype of $Utx^{\Delta}/Df(2L)BSC143$ transheterozygotes that were derived from females with a $Utx^{\Delta}$ heterozygous germ line. Although these mutants do not express wild-type Utx protein from their genome, they still contain wild-type Utx product during the earliest stages of development, deposited in the egg by the mother. We refer to these as $Utx^{\Delta\ mat+\ zyg-}$ mutants to distinguish them from $Utx^{\Delta\ mat-zyg-}$ mutants that are derived from females with $Utx^{\Delta}$ germ cells and which therefore completely lack wild-type Utx protein (see below). The majority of $Utx^{\Delta\ mat+\ zyg-}$ animals develop into adults that are morphologically indistinguishable from wild-type flies, but die within a day after eclosion from the pupal case (Fig. 1C). Previous studies reported that animals homozygous for $Utx^{1}$ only rarely survive to adults and that surviving individuals show a rough eye phenotype and die shortly after eclosion (Herz et al., 2010). We found that $Utx^{1}/Utx^{1}$ homozygotes indeed only rarely survive to adults (Fig. 1C) but that $Utx^{1}/Df(2L)BSC143$ transheterozygotes develop into adults at a frequency comparable to $Utx^{\Delta}/Df(2L)BSC143$ transheterozygotes or $Utx^{\Delta}$ homozygotes and that these animals show no detectable morphological defects (Fig. 1C; data not shown). As in the case of $Utx^{\Delta}$ adults, $Utx^{1}/Df(2L)BSC143$ transheterozygous adults also die shortly after eclosion. We conclude that zygotic *Utx* function is not required for the development of morphologically normal epidermal structures but, for unknown reasons, it is essential for adult viability.

## Global H3-K27me3 and H3-K27ac levels are unaffected in *Utx* mutants

We next investigated how loss of Utx demethylase activity affects the global levels of H3-K27me3. Previous studies reported that bulk H3-K27me3 levels in $Utx^{1}$ homozygous larvae or in Utx RNAi-treated adults are increased compared with wild-type animals (Herz et al., 2010; Tie et al., 2012) and that bulk levels of histone H3 acetylation at lysine 27 (H3-K27ac) are decreased (Tie et al., 2012). We performed western blot analyses on serially diluted extracts from $Utx^{\Delta}/Df(2L)BSC143$ or $Utx^{1}/Df(2L)BSC143$ larvae with anti-H3-K27me3 and anti-H3-K27ac antibodies. We were unable to detect a change in the bulk levels of these modifications in *Utx* mutants compared with wild-type larvae (Fig. 2A). Probing the same material with an antibody against H3-K4me1, we found a very small but reproducible decrease in bulk H3-K4me1 levels in *Utx* mutants (Fig. 2A), consistent with a previous report (Herz et al., 2010). We also analysed H3-K27me3, H3-K27ac and H3-K4me1 levels by immunofluorescence labelling of imaginal wing discs with clones of $Utx^{\Delta}$ or $Utx^{1}$ cells. We found that H3-K27me3 and H3-K27ac, but also H3-K4me1, immunofluorescence signals in $Utx^{\Delta}$ or $Utx^{1}$ cells were comparable to those in neighbouring wild-type cells in the same tissue (Fig. 2B; data not shown). We conclude that lack of Utx has no major effect on global H3-K27me3 and H3-K27ac levels but results in a very small reduction in global H3-K4me1 levels.

## *Utx* mutant cells proliferate like wild-type cells

Previous studies reported that $Utx^{1}$ cell clones in imaginal discs show a growth advantage relative to neighbouring wild-type cells and proposed that *Utx* acts as a tumour suppressor (Herz et al., 2010). We used the exact same experimental conditions as Herz and co-workers (Herz et al., 2010) to analyse the size and growth of $Utx^{\Delta}$ or $Utx^{1}$ cell clones in the eye-antenna imaginal disc but found that both $Utx^{\Delta}$ and $Utx^{1}$ clones grow like control clones of wild-type

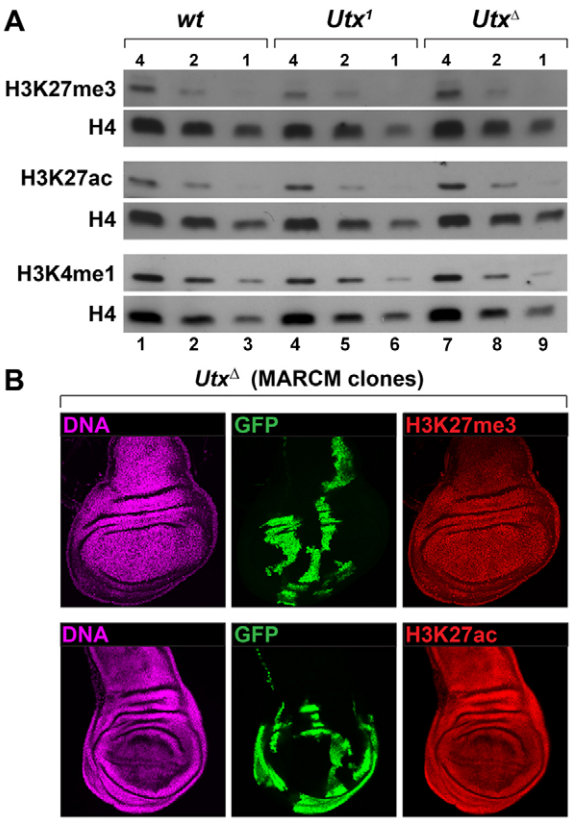


**Fig. 2. Bulk H3-K27me3 and H3-K27ac levels are unchanged in *Utx* mutants.** (**A**) Western blots of serial dilutions (4:2:1) of total extracts from imaginal disc and CNS tissues of wild-type (wt, lanes 1-3), *Utx*$^{1}$/*Df(2L)BSC143* (lanes 4-6) and *Utx*$^{\Delta}$/*Df(2L)BSC143* (lanes 7-9) larvae, probed with antibodies against H3-K27me3, H3-K27ac and H3-K4me1. In each case, the membrane was simultaneously probed with an antibody against histone H4 to control for extract loading and western blot processing but was exposed to film for a shorter period of time in order not to saturate the H4 signal. Note that H3-K27me3 and H3-K27ac levels are unchanged in *Utx* mutant extracts but that H3-K4me1 levels are very slightly (less than one-third) decreased in the mutants. (**B**) Wing imaginal discs with MARCM clones of *Utx*$^{\Delta}$ homozygous cells that are marked by the presence of GFP, stained with H3-K27me3 or H3-K27ac antibodies and Hoechst (for DNA). Note that the H3-K27me3 and H3-K27ac immunofluorescence signals in *Utx*$^{\Delta}$/*Utx*$^{\Delta}$ clone cells are comparable to those in the neighbouring *Utx*$^{\Delta}$/+ cells. Discs were analysed 96 hours after clone induction.

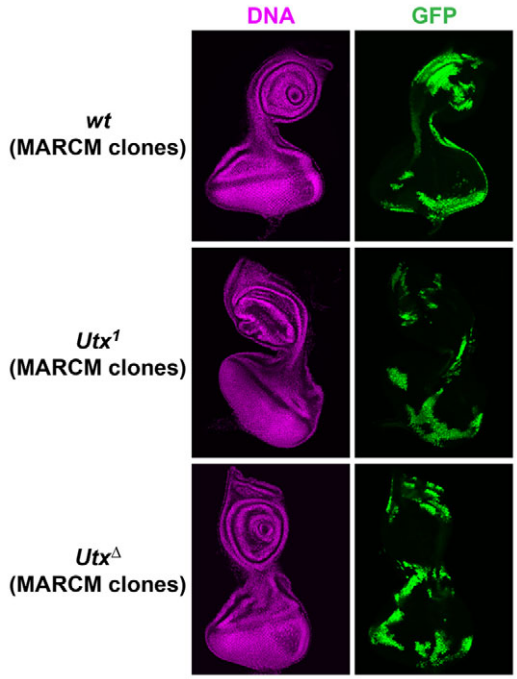


**Fig. 3. *Utx*$^{1}$ and *Utx*$^{\Delta}$ clones do not show an overproliferation phenotype.** Eye-antenna imaginal discs with MARCM clones of *Utx*$^{1}$ or *Utx*$^{\Delta}$ homozygous cells marked by the presence of GFP and stained with Hoechst. As reference, clones of a wild-type 2L chromosome arm (wt) were generated the same way and are also positively marked by GFP (top). In all cases, discs were analysed 96 hours after clone induction. Note that the clone sizes are comparable in all three genotypes and that the clone cells populate a comparable area of disc tissue.

cells that we induced as reference in a parallel experiment (Fig. 3). Using the *ey-flp* driver to induce clones across the entire eye-antenna imaginal disc, we found that adults with *Utx*$^{1}$ eyes show a rough eye phenotype similar to that reported for surviving *Utx*$^{1}$ homozygotes (Herz et al., 2010) (data not shown). Since *Utx*$^{1}$/*Df(2L)BSC143* transheterozygotes and *Utx*$^{\Delta}$ homozygotes do not show this phenotype, it seems likely that this rough eye phenotype is caused by a second site mutation on the 2L chromosome arm carrying the *Utx*$^{1}$ allele. In summary, these analyses provide no evidence that *Utx* mutant cells would have a growth advantage compared with wild-type cells.

## Maternally deposited Utx is essential for stably activated HOX gene expression

We next analysed the phenotype of mutants that lack both maternal and zygotic Utx product. We generated *Utx*$^{\Delta}$/*Df(2L)BSC143* animals from females with *Utx*$^{\Delta}$ germ cells. These *Utx*$^{\Delta\ mat-\ zyg-}$ animals develop to the larval stages; 85% die before reaching the third larval instar and the remaining larvae die during the third larval instar, with only a very small fraction (0.6%) developing to form white prepupae, which then also die (Fig. 1C). Thus, complete absence of Utx does not permit animals to develop beyond the third larval instar or to undergo metamorphosis, suggesting an essential function of maternally supplied Utx products. Nevertheless, if *Utx*$^{\Delta}$ oocytes (i.e. derived from females with a *Utx*$^{\Delta}$ germ line) are fertilised by wild-type sperm, zygotic expression of Utx protein rescues these *Utx*$^{\Delta\ mat-\ zyg+}$ animals into viable and fertile adults. The majority of such *Utx*$^{\Delta\ mat-\ zyg+}$ adults show wild-type morphology but a substantial fraction shows homeotic transformations that are reminiscent of *trithorax* (*trx*) mutants (Ingham and Whittle, 1980) and are likely to be caused by a reduction or loss of expression of HOX genes (see below). Specifically, 30% of *Utx*$^{\Delta\ mat-\ zyg+}$ males ($n$=382) show a patchy loss of pigmentation in the tergite of the fifth abdominal segment (A5), which represents a transformation of the affected tissue into a more anterior abdominal segment and is caused by loss of *Abd-B* expression in A5 (Fig. 4A). At very low frequency (2.8%, $n$=758), *Utx*$^{\Delta\ mat-\ zyg+}$ individuals also show transformation of parts of the haltere into wing tissue (not shown), a homeotic transformation caused by the loss of *Ultrabithorax* (*Ubx*) expression in this body segment.

The *trx*-like phenotypes in *Utx*$^{\Delta\ mat-\ zyg+}$ adults prompted us to analyse the expression of HOX genes in *Utx*$^{\Delta\ mat-\ zyg-}$ animals. *Utx*$^{\Delta\ mat-\ zyg-}$ mutant embryos show a strong reduction of Abd-B expression in parasegment (ps) 10 (Fig. 4B), from which the A5 segment of adults (Fig. 4A) is derived. Abd-B expression in the

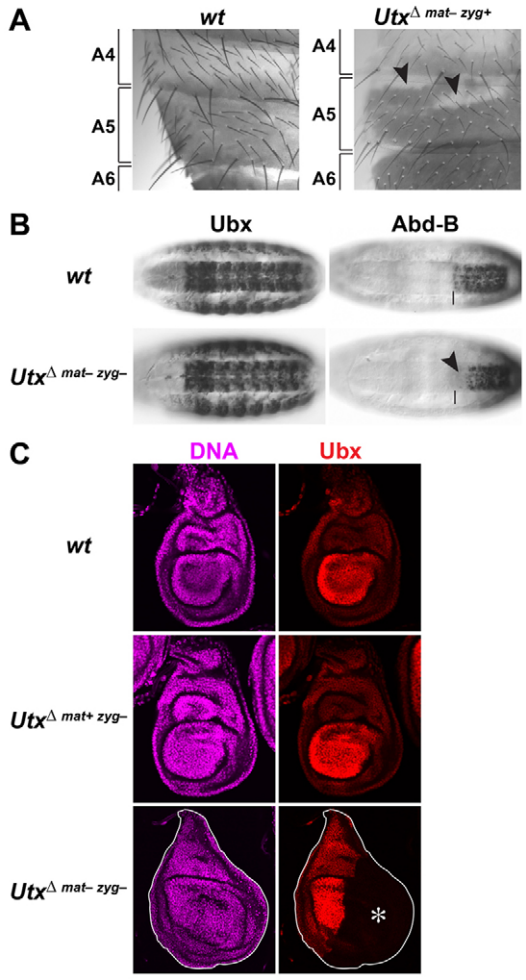


**Fig. 4. Trithorax-like homeotic transformations and loss of HOX gene expression in *Utx*$^{\Delta}$ mutants.** (**A**) Portions of the dorsal abdomen of adult males of the indicated genotype, showing the tergites of abdominal segments A4, A5 and A6. Note the patchy loss of pigmentation in the anterior part of A5 of the *Utx*$^{\Delta\, mat-\, zyg+}$ male (arrowheads), suggesting a transformation of this tissue to A4 segment identity. (**B**) Ventral views of stage 16 embryos stained with antibodies against the HOX proteins Ubx and Abd-B. The *Utx*$^{\Delta\, mat-\, zyg-}$ genotype refers to *Utx*$^{\Delta}$*/Df(2L)BSC143* embryos derived from females with a *Utx*$^{\Delta}$ germ line. Ubx expression is indistinguishable from wild type (wt). Abd-B expression is strongly reduced in parasegment (ps) 10 (arrowhead) but appears largely undiminished in more posterior parasegments; a vertical bar marks the anterior margin of the normal Abd-B expression domain in ps 10 in the CNS. (**C**) Haltere imaginal discs from third instar larvae stained with Ubx antibody and Hoechst. Mutant genotypes are *Utx*$^{\Delta}$*/Df(2L)BSC143* derived from a *Utx*$^{\Delta}$ heterozygous mother (*Utx*$^{\Delta\, mat+\, zyg-}$, middle) and from a female with a *Utx*$^{\Delta}$ germ line (*Utx*$^{\Delta\, mat-\, zyg-}$, bottom). Note the complete loss of Ubx expression in a large fraction of cells in the *Utx*$^{\Delta\, mat-\, zyg-}$ disc (asterisk) and that Ubx expression in the *Utx*$^{\Delta\, mat+\, zyg-}$ disc is indistinguishable from the wild type. The loss of Ubx expression is not compartment specific and always occurs in a clone-like pattern with large patches of Ubx-negative cells.

more posterior ps 11-14 appears largely undiminished with the exception of occasionally reduced expression in a few cells in ps 11 (Fig. 4B). We were unable to detect a reduction of *Ubx* expression in *Utx*$^{\Delta\, mat-\, zyg-}$ embryos compared with wild-type embryos (Fig. 4B).

We next analysed HOX gene expression in imaginal discs of *Utx*$^{\Delta\, mat-\, zyg-}$ larvae that survived to the third larval instar (see above). In wild-type animals, *Ubx* is expressed in all cells of the haltere and third leg discs, *Abd-B* is expressed in a characteristic pattern in the genital disc, and *Scr* is expressed in all cells of the first leg disc (Fig. 4C; data not shown). *Utx*$^{\Delta\, mat-\, zyg-}$ mutant larvae show complete loss of Ubx expression in a patchy pattern in haltere and third leg discs (Fig. 4C). Even though Ubx protein is undetectable in a substantial fraction of cells in these discs, its expression level in the remaining cells in these tissues appears undiminished compared with wild-type animals (Fig. 4C). The patchy nature of the Ubx-negative tissue, as opposed to a salt-and-pepper distribution of Ubx-negative and -positive cells across the tissue, suggests that the Ubx-negative cells are of clonal origin, a notion that we have not been able to test. This Ubx loss of expression phenotype was observed in all third instar larvae analysed, but the fraction of cells that show complete loss of expression varied between individuals. Abd-B protein expression in the genital disc of *Utx*$^{\Delta\, mat-\, zyg-}$ larvae appeared indistinguishable from that of wild-type animals (not shown). It should be noted that the genital disc represents the primordium for abdominal segment A8, A9 and A10 structures and that loss of Abd-B expression in *Utx*$^{\Delta\, mat-\, zyg-}$ embryos was restricted to ps 10, which is the primordium of abdominal segment A5 in adults (Fig. 4B). Similarly, the homeotic transformations in *Utx*$^{\Delta\, mat-\, zyg+}$ adults were also limited to A5 (Fig. 4A). In *Utx* mutants, *Abd-B* expression is thus only affected in a subset of cells of the *Abd-B* expression domain. Finally, we found Scr protein expression in first leg discs of *Utx*$^{\Delta\, mat-\, zyg-}$ larvae to be indistinguishable from that of wild type (data not shown). We also note that, in imaginal discs of *Utx*$^{\Delta\, mat+\, zyg-}$ animals, expression of all three HOX genes (*Ubx*, *Abd-B* and *Scr*) was indistinguishable from wild type, consistent with the lack of homeotic phenotypes in adults of this genotype (Fig. 4C; data not shown). Taken together, these data show that *Utx* function in the very early *Drosophila* embryo is required for the stable expression of the HOX genes *Ubx* and *Abd-B*, but that zygotic *Utx* function is dispensable for normal HOX gene expression.

## Distinct temporal requirements for the trxG regulators Utx, Trx and Ash1

The observation that Utx is required for the long-term expression of multiple HOX genes classifies Utx as a trxG regulator (Kennison and Tamkun, 1988; Kennison, 1995). However, the temporal requirement for Utx during the *Drosophila* life cycle is very distinct from that of other trxG proteins such as Trx and Ash1. Specifically, maternally deposited Utx product is sufficient to permit *Drosophila* to develop into adults with morphologically normal epidermal structures (i.e. in *Utx*$^{\Delta\, mat+\, zyg-}$ animals). Maternal Utx product becomes extensively diluted during embryonic and larval cell divisions and *Utx*$^{\Delta\, mat+\, zyg-}$ third instar larvae indeed lack detectable levels of wild-type Utx protein (Fig. 1B). For the regulation of HOX gene expression – though not for viability of adults – Utx therefore appears to be required only early in development. This stands in clear contrast to the requirement for Trx and Ash1; the function of these proteins is needed throughout development and removal of either protein from wild-type cells during the larval stages results in loss of HOX gene expression (Ingham, 1981; Klymenko and Müller, 2004).

One possible explanation for the lack of a trxG phenotype in animals lacking zygotic Utx protein (i.e. in *Utx*$^{\Delta\, mat+\, zyg-}$ mutants) could be that *Utx* function during larval development is redundant in the presence of Ash1 or Trx. We investigated this possibility as follows. Clones of cells that are homozygous for the protein-null mutation *ash1*$^{22}$ in haltere or third leg imaginal discs show loss of *Ubx* expression in the majority of cells but a fraction of cells

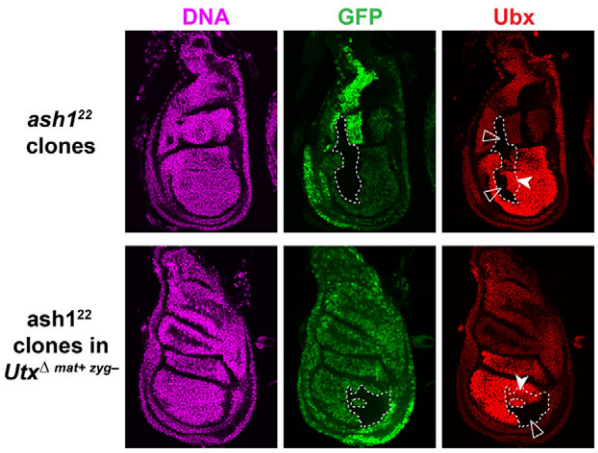


**Fig. 5. Lack of Utx function does not exacerbate loss of HOX gene expression in *ash1* mutants.** Haltere imaginal discs from third instar larvae with clones of *ash1*$^{22}$ homozygous cells were stained with Ubx antibody and Hoechst. *ash1*$^{22}$ mutant cells are marked by the absence of GFP and were induced in a wild-type genetic background (*ash1*$^{22}$, top) or in the background of *Utx*$^{\Delta\ mat+\ zyg-}$ homozygous larvae (*Utx*$^{\Delta}$, *ash1*$^{22}$, bottom). Discs were analysed 96 hours after clone induction. In each sample, the boundary of one clone is outlined. Note that in both genotypes a fraction of cells in the clone shows complete loss of Ubx expression (open arrowheads), whereas other cells maintain high to moderate levels of Ubx expression (white arrowheads).

nevertheless maintains high or moderate levels of *Ubx* expression (Fig. 5) (see Klymenko and Müller, 2004). This all-or-none loss of expression phenotype is also observed in *ash1*$^{22}$ homozygotes, and it correlates with the accumulation of H3-K27me3 at the *Ubx* promoter and coding regions in haltere and third leg imaginal disc cells (Papp and Müller, 2006). If zygotically expressed Utx protein synergised with Ash1 in imaginal disc cells to maintain HOX gene expression, one might expect a more extensive loss of *Ubx* expression in clones of *Utx*$^{\Delta}$*; ash1*$^{22}$ double-mutant cells. We therefore induced clones of *ash1*$^{22}$ homozygous cells in *Utx*$^{\Delta\ mat+\ zyg-}$ animals (Fig. 5). However, we found that the fraction of cells that maintain high levels of *Ubx* expression in these *ash1*$^{22}$ *Utx*$^{\Delta}$ double-mutant clones was comparable to that in *ash1*$^{22}$ single-mutant clones (Fig. 5). Thus, even in a genetic background in which another trxG regulator is removed, there is no evidence for a role of zygotic Utx protein in HOX gene regulation.

## DISCUSSION

In this study, we used a *Utx* allele with a deletion of the catalytic domain to investigate the role of Utx and its H3-K27 demethylase activity in *Drosophila* development. Analyses of animals that lack maternal and zygotic Utx product uncovered that Utx protein is crucial for the long-term stable expression of multiple HOX genes. By contrast, animals that lack only zygotic expression of Utx develop into adults with morphologically normal epidermal structures, suggesting that zygotic Utx function is not needed for HOX gene regulation. Moreover, in animals lacking maternally deposited Utx protein, zygotic Utx product alone is unable to fully rescue normal HOX gene expression and these animals show homeotic transformations (i.e. in *Utx*$^{\Delta\ mat-\ zyg+}$ animals). Together, these data suggest that Utx function is crucial for HOX gene regulation right at the onset of zygotic transcription in the early embryo. Even though zygotic Utx expression is apparently dispensable for normal morphogenesis of epidermal structures, *Utx*$^{\Delta\ mat+\ zyg-}$ adults nevertheless die shortly after eclosion from the pupal case. It is possible that this lethality is caused by defective morphogenesis of internal tissues or organs. Alternatively, Utx might be needed for some other vital function in adult *Drosophila*. Below, we discuss in turn the conclusions that can be drawn from these results and consider possible mechanisms as to how Utx might regulate HOX gene expression.

### The requirement for Utx function is more limited than for PRC2

Considering that Utx is the only known H3-K27 demethylase in *Drosophila*, it is interesting to compare the requirement for Utx with that for PRC2 during *Drosophila* development. First, germ cells with null mutations in different PRC2 subunits fail to develop (Phillips and Shearn, 1990; Birve et al., 2001), but *Utx*$^{\Delta}$ mutant germ cells develop into oocytes that support embryonic development (this study). PRC2 is thus essential for germ cell development whereas Utx is not. Second, removal of PRC2 subunits in somatic cells at any time point during development (e.g. in imaginal disc cells in larvae) results in misexpression of PRC2 target genes and cell fate changes (Birve et al., 2001; Müller et al., 2002), but animals lacking zygotic Utx expression develop into adults with normal epidermal morphology. PRC2 is thus continuously required during embryonic and larval development. By contrast, Utx is crucial during embryogenesis and in adults but development from the third larval instar onwards, through pupal development and metamorphosis, appears to occur largely normally in the absence of detectable Utx protein (Fig. 1B).

Taken together, this argues against a view that transcriptional regulation of PRC2 target genes during development involves a constant interplay between H3-K27 methylation and demethylation. In this context, it is interesting to note that, in second instar larvae that lack zygotic PRC2 function but had survived up to this stage because of maternally supplied PRC2, H3-K27me3 levels are comparable to those in wild-type larvae at the same stage (Nekrasov et al., 2007). This suggests that H3-K27me3 that was generated by maternally supplied PRC2 remains stable when the supply of maternal PRC2 disappears and *de novo* deposition of this modification ceases. This further supports the notion that H3-K27me3 is a stable modification in *Drosophila* that is apparently not subject to constant turnover.

### Possible molecular mechanisms of HOX gene regulation by Utx

We envisage two possible mechanisms of how Utx could function to activate the expression of HOX genes, one of which depends on the H3-K27me3 demethylase activity of Utx and another that acts independently of this enzymatic activity.

A first scenario would be that H3-K27me3 demethylation by Utx in the early embryo removes ‘ectopic’ H3-K27 trimethylation by PRC2 at HOX genes in cells in which these genes need to be expressed. According to this view, the loss of HOX gene expression in *Utx*$^{\Delta\ mat-\ zyg-}$ animals would be due to ectopic placement of H3-K27me3 and, as a consequence, PcG silencing of HOX genes within their normal expression domains. H3-K27me3 demethylation by Utx would thus act to antagonise the establishment of PcG repression. Recent studies reported the genome-wide binding profile of Utx in *Drosophila* tissue culture cells and found that Utx binding colocalises tightly with the binding of PcG protein complexes at Polycomb response elements (PREs) of HOX genes (Tie et al., 2012). This supports the idea that the function of Utx in HOX gene

chromatin is intimately linked to that of PRC2. Below, we discuss the possible relationship between this proposed role of Utx in antagonising H3-K27me3 deposition by PRC2 and the role of Ash1 and Trx in the same process.

As discussed in the Introduction, previous studies reported that Utx protein, but not its H3-K27me3 demethylase activity, is essential for normal *C. elegans* development (Vandamme et al., 2012) and for the correct differentiation of murine ESCs *in vitro* (Wang et al., 2012). An alternative scenario would thus be that *Drosophila* Utx promotes HOX gene transcription by a demethylase-independent mechanism. How could this work? As in worms and mammals, *Drosophila* Utx is a component of an MLL3/4-like complex that includes the H3-K4 methyltransferase Trithorax-related (Trr) (Cho et al., 2007; Issaeva et al., 2007; Lee et al., 2007; Patel et al., 2007; Mohan et al., 2011; Vandamme et al., 2012). Recent studies in *Drosophila* proposed that Trr is required for genome-wide H3-K4 monomethylation at enhancers and that H3-K27me3 demethylation of enhancers by Trr-associated Utx would be a general mechanism to convert inactive to active enhancers (Herz et al., 2012). Although we have been unable to detect changes in bulk H3-K27me3 and H3-K27ac levels in *Utx* mutants, we found a slight decrease in bulk H3-K4me1 levels (Fig. 2A), consistent with earlier studies (Herz et al., 2010). This decrease in H3-K4me1 is not simply due to a reduction in Trr protein levels because these were found to be undiminished after RNAi-mediated knockdown of Utx (Herz et al., 2012). One possibility would thus be that Utx is needed for H3-K4 monomethylation by Trr and thereby contributes to the activation of HOX gene expression in the early embryo. *trr* zygotic mutants die as embryos without any obvious trxG mutant phenotypes (Sedkov et al., 1999), but the phenotype of embryos lacking maternal and zygotic Trr protein is not known and so it is currently unclear whether Trr plays a role in HOX gene regulation in the early embryo. In the context of this scenario, we would emphasize that $Utx^{\Delta\ mat-\ zyg-}$ mutants develop to the larval stages and, with some notable exceptions (i.e. the regulation of Abd-B expression in ps 10 in the embryo), the enhancer-mediated activation of genes required for embryonic patterning must therefore by and large occur normally in the absence of Utx. Similarly, $Utx^{\Delta\ mat+\ zyg-}$ larvae develop into morphologically normal adults and the reduction of H3-K4me1 levels in these animals does not therefore seem to compromise enhancer activity in any drastic manner.

## Relationship between Utx, Ash1 and Trx in maintaining HOX gene expression

Utx, Ash1 and Trx are all required for the normal stable expression of multiple HOX genes. Previous studies provided several independent lines of genetic and biochemical evidence that Trx and Ash1 keep HOX genes active by antagonising PRC2 activity. First, HOX gene expression is lost in *ash1* mutants but is restored in *ash1* mutants that also lack E(z), the catalytic subunit of PRC2, suggesting that Ash1 is not needed for HOX gene transcription but rather to prevent PRC2 from shutting down HOX gene expression (Klymenko and Müller, 2004). Second, chromatin immunoprecipitation studies of the HOX gene *Ubx* in imaginal discs have shown that although PRC2 is also bound at *Ubx* in cells in which it is transcribed, Ash1 inhibits deposition of H3-K27me3 at the promoter and coding region in these cells (Papp and Müller, 2006). Third, on recombinant nucleosomes, the histone modifications H3-K36me2/3 and H3-K4me3 that are generated by Ash1 and Trx, respectively, strongly inhibit PRC2 from methylating H3-K27 on the same histone tail (Schmitges et al., 2011; Yuan et al., 2011).

Here, we found that removal of Utx in imaginal disc cells has no effect on HOX gene expression. Moreover, in *ash1* mutant haltere and third leg imaginal disc cells where PRC2 deposits faulty H3-K27 trimethylation in the *Ubx* promoter and coding region (Papp and Müller, 2006), and where *Ubx* expression is lost in many although not all cells, removal of Utx does not exacerbate this loss of *Ubx* expression (Fig. 5). This suggests that, at least in larvae, H3-K27me3 demethylation is not crucial for antagonising the formation of repressive H3-K27me3 chromatin on active HOX genes. During larval development, H3-K4 and H3-K36 trimethylation by Trx, Ash1 and possibly other H3-K4 and H3-K36 histone methyltransferases therefore appears to be the predominant mechanism that prevents HOX gene-bound PRC2 from depositing H3-K27me3. This might, however, be different in the early embryo. It is possible that during the establishment of HOX gene expression domains at the blastoderm stage, H3-K4me3 and H3-K36me2/3 marks are not yet fully installed on HOX gene chromatin and that it is during this critical time window that demethylation by Utx is needed to remove faulty H3-K27me3 deposition from HOX genes in cells in which they need to be expressed.

**Acknowledgements**
We thank A. Bergmann, P. Harte and Ali Shilatifard for the generous gift of fly strains and antibodies; Andres Gaytan de Ayala and Tony Chen for help with generating the $Utx^{\Delta}$ disruption construct; and Sandra Müller for generation of transgenic flies.

**Funding**
This work was supported by funds from the MPI of Biochemistry. Deposited in PMC for immediate release.

**Competing interests statement**
The authors declare no competing financial interests.

**Author contributions**
O.C. and J.M. designed the experiments; O.C. carried out the experiments; J.M. supervised the work and wrote the paper.

**Supplementary material**
Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.097204/-/DC1

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