Genome-wide RNAi screen in *Drosophila* reveals Enok as a novel trithorax group regulator

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**Abstract**

**Background:** Polycomb group (PcG) and trithorax group (trxG) proteins contribute to the specialization of cell types by maintaining differential gene expression patterns. Initially discovered as positive regulators of HOX genes in forward genetic screens, trxG counteracts PcG-mediated repression of cell type-specific genes. Despite decades of extensive analysis, molecular understanding of trxG action and regulation are still punctuated by many unknowns. This study aimed at discovering novel factors that elicit an anti-silencing effect to facilitate trxG-mediated gene activation.

**Results:** We have developed a cell-based reporter system and performed a genome-wide RNAi screen to discover novel factors involved in trxG-mediated gene regulation in *Drosophila*. We identified more than 200 genes affecting the reporter in a manner similar to trxG genes. From the list of top candidates, we have characterized Enoki mushroom (Enok), a known histone acetyltransferase, as an important regulator of trxG in *Drosophila*. Mutants of *enok* strongly suppressed extra sex comb phenotype of *Pc* mutants and enhanced homeotic transformations associated with *trx* mutations. Enok colocalizes with both TRX and PC at chromatin. Moreover, depletion of Enok specifically resulted in an increased enrichment of PC and consequently silencing of trxG targets. This downregulation of trxG targets was also accompanied by a decreased occupancy of RNA-Pol-II in the gene body, correlating with an increased stalling at the transcription start sites of these genes. We propose that Enok facilitates trxG-mediated maintenance of gene activation by specifically counteracting PcG-mediated repression.

**Conclusion:** Our ex vivo approach led to identification of new trxG candidate genes that warrant further investigation. Presence of chromatin modifiers as well as known members of trxG and their interactors in the genome-wide RNAi screen validated our reverse genetics approach. Genetic and molecular characterization of Enok revealed a hitherto unknown interplay between Enok and PcG/trxG system. We conclude that histone acetylation by Enok positively impacts the maintenance of trxG-regulated gene activation by inhibiting PRC1-mediated transcriptional repression.

**Keywords:** Epigenetic cellular memory, Gene regulation, trithorax group, Polycomb group, Enok, Genome-wide RNAi screen, Histone modifications, H3K23 acetyl transferase
Background
In multicellular eukaryotes, specialization of cell types is initiated by the onset of differential gene expression patterns in response to specific signals during early development. These differential gene expression profiles contribute to cell fate determination and differentiation during subsequent development. Thus, cell type-specific gene expression patterns are transmitted through cell divisions to daughter cells by a process known as epigenetic transcriptional cellular memory. In plants and mammals, DNA methylation, together with specific covalent modifications of histones, ensures faithful inheritance of cell type-specific gene expression patterns [1–3]. Genetic analyses in Drosophila discovered two groups of genes, the Polycomb Group (PcG) and the trithorax Group (trxG), that contribute to the maintenance of cellular memory [4–8]. The PcG maintains genes in a repressed state whereas trxG proteins act as anti-silencing factors and ensure activation of cell type-specific genes. Proteins encoded by the PcG and trxG genes act in different multiprotein complexes and modify local properties of chromatin to maintain transcriptional repression or activation of their target genes, respectively [9]. The PcG complexes, Polycomb Repressive Complex 1 and 2 (PRC1 and PRC2), are linked to histone H2A lysine 118 mono-ubiquitination (H2AK118ub1) [10] and histone H3 lysine 27 trimethylation (H3K27me3) [11–14], respectively, to maintain heritable patterns of repression. In contrast, different trxG complexes are known to deposit histone H3 lysine 4 trimethylation (H3K4me3) [15] and histone H3 lysine 27 acetylation (H3K27ac) [16], known hallmarks of active gene expression. Besides histone-modifying proteins, trxG also includes ATP-dependent chromatin remodeling factors that achieve an open conformation of DNA to allow transcription [9]. Molecular and biochemical characterization has revealed that the heterogeneous group of trxG proteins not only contributes to epigenetic cellular memory but also plays a role in general transcriptional activation [8].

In Drosophila, PcG/trxG proteins bind to specialized cis-acting elements called PReS/TREs (Polycomb Response Elements/Trithorax Response Elements) [17, 18] to maintain transcriptional cellular memory [19]. Several PRE/TRE elements exist within homeotic gene clusters (i.e., Bithorax Complex and Antennapedia Complex) and non-homeotic targets of PcG/trxG. High-resolution mapping of PcG-binding sites in Drosophila has identified hundreds of PREs genome wide [20–22]. In addition, several PREs have been shown to maintain stable and heritable gene expression of reporter genes in transgene reporter assays. Transgenic flies carrying either iab-7PRE or bxd-PRE fused to reporter genes have been extensively used to characterize mitotic and meiotic inheritance of PcG/trxG-dependent cellular memory [19].

Initially, trxG genes were identified as positive regulators of HOX genes in forward genetic screens. Numerous other trxG members were identified as suppressors of PcG-dependent homeotic phenotypes or as mutations that mimic loss of function of HOX genes in Drosophila [8]. Here, we have developed a cell-based reporter assay which is sensitive to the changing levels of PcG and trxG. This reporter was used to perform a large-scale genome-wide RNAi screen to discover new trxG genes using Drosophila cell culture. Employing stringent criterion, more than 200 genes were identified as potential trxG regulators, including known members of trxG and chromatin modifiers. Using a range of in vitro and in vivo assays, we have validated Drosophila Enok as a trxG regulator that strongly suppresses Pc mutant phenotype and enhances trx mutant phenotype. Further, we show that Enok colocalizes with TRX and its depletion results in enhanced levels of PC at chromatin and consequent downregulation of trxG target genes. Moreover, reduced expression of trxG targets after depletion of Enok correlates with an increased mono-ubiquitination of histone H2A at lysine 118 (H2AK118ub1) and an increase in stalled RNA Polymerase II. Our results suggest that Enok counteracts PcG-mediated repression and influences the ability of trxG to maintain transcription of its target genes.

Results
A cell-based reporter system for RNAi screen to discover novel trxG regulators
Although several PRE-based transgenes have been shown to maintain stable and heritable gene expression of reporter genes in Drosophila [19], their analyses involve time-consuming dissections and staining that cannot be easily optimized for a genome-wide RNAi screen. We aimed at developing a robust cell-based reporter assay in Drosophila, sensitive to the levels of PcG/trxG, that could help us discover novel regulators of trxG. To this end, we adapted the PBX-bxd-IDELacZ reporter that has been previously characterized in flies [23]. In this reporter, bacterial LacZ gene is regulated by Drosophila Ubx (Ultrabithorax) promoter [24] and bxd-PRE [25] along with PBX (postbithorax) and IDE (Imaginal Disc Enhancer) enhancers of Ubx [26]. We modified PBX-bxd-IDELacZ by replacing LacZ with Enhanced Green Fluorescent Protein (EGFP) (hereafter referred to as PRE-EGFP) (Additional file 1: Fig. S1a) to monitor EGFP expression in fly cells. We hypothesized that in Drosophila cells transfected with the PRE-EGFP reporter, depletion of trxG or over-expression of PcG genes would result in a decreased EGFP expression.
To validate the sensitivity of our reporter, PRE-EGFP was transiently transfected in Drosophila cells alone (Fig. 1a) or with constructs over-expressing either Pc (Polycomb) (Fig. 1b) or E(z) (Enhancer of Zeste) (Fig. 1c). To rule out any variations in transfection efficiency, Actin promoter-driven RFP (pActin-RFP) was also co-transfected with PRE-EGFP (Fig. 1a–d). As compared to cells transfected with PRE-EGFP alone (Fig. 1a), there was a marked decrease in the number of EGFP positive cells when either Pc (Fig. 1b) or E(z) (Fig. 1c) were over-expressed while the number of RFP-positive cells remained constant. To evaluate the specificity of the reporter, DNApol-a50, a nuclear factor not involved in PcG-mediated repression, was co-transfected with

![Fig. 1](image-url)

**Fig. 1** Proof of concept for PcG/trxG-mediated regulation of PRE-EGFP and PRE-FLuc reporters. a Drosophila Schneider (S2) cells co-transfected with PRE-EGFP and pActin-RFP show high levels of EGFP and RFP expression. Co-transfection of PRE-EGFP + pActin-RFP reporters with Pc b or E(z) c over-expressing constructs resulted in strong repression of EGFP but had no effect on RFP. Over-expression of a non-PcG protein, DNApol-a50 d shows no effect on PRE-EGFP as both EGFP and RFP expression levels are comparable to those of a. Merge images show comparable RFP signal and cell density. e Total cell lysates from transfected cells a–d were probed with anti-FLAG, anti-GFP and anti-tubulin antibodies on a Western blot. Over-expression of both PC and E(z) shows a drastic reduction in EGFP expression while DNApol-a50 did not significantly change EGFP levels. f Over-expression of PC represses PRE-FLuc in a dose-dependent manner. Increased repression of FLuc was observed with increasing amounts (1, 2, 4 ng) of transiently transfected Pc over-expression construct, pActin-Pc. g Knockdown of known members of trxG (trx, ash1, brm, mor) leads to decrease in FLuc expression in cells transiently transfected with PRE-FLuc along with pActin-R.Luc (Renilla Luciferase) used as a normalization control. Knockdown of FLuc revealed strong repression of reporter. dsRNA against LacZ was used as a negative control. Relative FLuc values, normalized against R.Luc, were recorded at 72 and 96 h after transfection are shown. All knockdowns of trxG genes resulted in significant downregulation of relative FLuc levels (*p < 0.0001) at both time points. Experiments shown in f and g were performed in triplicates in two different sets and were analyzed by student t test f or one-way ANOVA g (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 or ****p ≤ 0.0001). Error bars represent SEM.
PRE-EGFP (Fig. 1d). Over-expression of DNApol-a50 had no effect on PRE-EGFP. This was further confirmed by Western blot of total cell lysates from the transfected cells with anti-GFP antibody (Fig. 1e). To monitor the impact of perturbations in trxG more quantitatively and to develop a robust reporter for high-throughput RNAi screens, Firefly Luciferase (FLuc) gene was cloned instead of EGFP in the PRE-EGFP construct (hereafter referred to as PRE-FLuc) (Additional file 1: Fig. S1a). Drosophila cells transfected with PRE-FLuc together with increasing amounts of Pc over-expression construct showed a dose-dependent decrease in the amount of relative FLuc activity (Fig. 1f). To further validate that PRE-FLuc could be used to discover factors that impact trxG-mediated gene regulation, cells transfected with PRE-FLuc were subjected to knockdown of known trxG members and the resultant FLuc activity was monitored (Fig. 1g). Treatment of cells with dsRNAs against trithorax (trx), absent small or homeotic discs1 (ash1), brahma (brm) and moira (mor) resulted in a significant decrease in the amount of FLuc expression as compared to cells treated with dsRNA against LacZ, indicating the specific silencing of PRE-FLuc reporter. The sensitivity of PRE-FLuc reporter to the changing levels of PcG and trxG corroborated with our hypothesis and allowed us to use PRE-FLuc in a high-throughput cell-based RNAi screen to identify novel players in trxG-mediated gene regulation.

Genome-wide RNAi screen reveals novel trxG-like factors
A genome-wide RNAi screen was carried out using dsRNAs from Drosophila Heidelberg 2 (HD2) library, covering about 98% of the annotated Drosophila genome [27, 28]. Importantly, dsRNAs against known trxG members (trx, ash1) and specific reporter (FLuc) were used as positive controls, whereas dsRNA against LacZ was used as a negative control in all plates. Along with PRE-FLuc reporter construct, Actin promoter-driven Renilla Luciferase (pActin-RLuc) was co-transfected as a normalization control to exclude possible artifacts such as cell death and effect on general transcription. The experimental strategy followed for the screen is summarized in Additional file 2: Fig. S2a (for details see “Materials and methods”). Based on the Z scores obtained from positive controls (trx, ash1), cut-offs were defined for the screen (Additional file 2: Fig. S2b, c) and a list of 217 potentialtrxG candidates was generated (Additional file 3: Table S1). Importantly, several known members of trxG appeared in the list of candidate genes, thus validating the efficiency of our assay. A high-resolution interaction map of the candidate trxG genes identified in our screen highlights the presence of trxG interacting partners including MRG15 [29], Pontin [30, 31], smc3 [32] and wapl [33] (Fig. 2). Further analysis revealed our candidate genes to be involved in a multitude of cellular processes including cell division, cell fate determination, development and cell signaling. From our top scoring candidates, we selected enoki mushroom (enok) for detailed genetic and molecular analyses because of its known interaction with PC [34], Ash1 [35] and involvement in gene activation during oogenesis in flies [36].

Mutants of enok behave like trxG mutants
Although Drosophila Enok interacts with PcG [34, 35] and is involved in acetylation of histone H3K23 [36], the physiological relevance of Enok in epigenetic cellular memory remained elusive. To investigate whether enok genetically interacts with the PcG/trxG system, two mutant alleles of enok were crossed to two different alleles of Pc (1, XLS). Pc heterozygous mutants show a strong extra sex comb phenotype. Importantly, both mutant alleles of enok strongly suppressed the extra sex comb phenotype (Fig. 3a, b). Since enok mutants strongly suppressed extra sex comb phenotype, we examined the genetic interaction of enok with trx by crossing enok mutant flies with two different alleles of trx (1, E2). As compared to wild type, trx heterozygous mutants (trx1/+ or trxE2/+) show A5 to A4 transformation (Fig. 3c) indicated by loss of pigmentation in A5 [37]. As compared to trx heterozygotes, a strong A5 to A4 transformation was observed in enok/+; trx+/+ double-mutant flies (Fig. 3c). Both the alleles of enok strongly enhanced A5 to A4 transformations when crossed with either trx1 or trxE2. These genetic analyses suggest that enok, like classic trxG genes, strongly suppresses homeotic transformations caused by Pcp and enhances those caused by trx mutations.

To characterize enok at the molecular level, we questioned if mutations in enok alter gene expression patterns of homeotic and non-homeotic targets of trxG in fly embryos because homozygous enok mutants do not survive to adulthood. As compared to w1118 embryos, enok1 and enok2 homozygous embryos showed a drastic reduction in expression of abdominal-A (abd-A), Abdominal-B (Abd-B), Ultrabithorax (Ubx), Deformed (Dfd) and pointed (pnt) in real-time PCR analysis (Fig. 3d). Additionally, stage 15 homozygous enok mutant embryos showed aberrant patterns of Ubx and Abd-B staining as compared to w1118 embryos (Fig. 3e–l). At this stage, Ubx expression is weak in parasegment 5 (PS5), highest in PS6 and progressively decreases from PS7-12 (Fig. 3e, f). However, homozygous enok mutant embryos displayed strongly diminished Ubx expression in all these regions (Fig. 3g, h), similar to trx mutations [38, 39]. Moreover, stripes of Ubx expression appear misaligned and merged across different parasegments (Fig. 3g, h). Normal expression of Abd-B progressively
increases from PS10 to PS14 (Fig. 3i, j), whereas enok mutant embryos displayed loss of expression of Abd-B in PS10–PS12, resulting in a shift of Abd-B expression boundary to PS13–PS14 only (Fig. 3k, l). The decreased expression of Abd-B correlates with the increased A5 to A4 transformation observed in Fig. 3c and is also known to be associated with trxG mutations [40, 41]. These results indicate that enok interacts with trxG at the genetic level and is involved in the regulation of trxG target genes.
Enok maintains active state of trxG targets by inhibiting PC
To investigate a potential molecular link between Enok and trxG, we generated a Drosophila transgene expressing enok coding sequence fused with Myc tag, under UAS promoter (Additional file 4: Fig. S3a). Immunostaining of polytene chromosomes from third instar larvae of UAS-enok-Myc crossed with sgs-GAL4 revealed association of Enok with polytene chromatin primarily at the interband regions (Fig. 4a–c). Importantly, there was a significant overlap between TRX and Enok-binding sites. In addition, a significant overlap between PC and Enok was also observed on polytene chromosomes (Additional file 4: Fig. S3b–e).

Chromatin-binding profile of Enok on TRX-binding sites was validated by ChIP analysis of chromatin from cells expressing FLAG-tagged Enok (Additional file 4: Fig. S3f). FLAG-Enok was observed at the transcription start sites (TSS) of pipsqueak (psq), pannier (pnr), pointed (pnt) and disconnected (disco) which are known binding sites of PcG/trxG [42–45]. Moreover, Enok was also found to be associated with iab-7PRE, bxd and Dfd regulatory regions of homeotic genes (Fig. 4d), known to be bound by both PC and TRX proteins [46]. In contrast, ChIP from empty vector control cells resulted in negligible enrichment at all the regions analyzed. Enok binding on TRX-binding sites was further validated by ChIP using anti-Enok antibody on wild-type Drosophila S2 cells. A similar pattern, albeit with a stronger enrichment, was seen on all the targets analyzed (Fig. 4d). Importantly, Enok was absent on an intergenic region that is not bound by TRX [47].

Next, we questioned how Enok facilitates trxG-mediated gene activation. Similar to the effect of trx knockdown, depletion of enok in Drosophila cells (Additional file 4: Fig. S3g) resulted in a significant decrease in mRNA levels of pnr and pnt (Fig. 4e) that are known non-homeotic targets of trxG [16]. Knockdown of enok, however, did not significantly alter the amount of trx mRNA in the cells (Additional file 4: Fig. S3h). Depletion of enok also did not cause a change in the enrichment of TRX protein (Additional file 4: Fig. S3i) or trxG-associated histone modification, H3K27ac (Additional file 4: Fig. S3j), on trxG target genes when analyzed by ChIP. We, therefore, hypothesized that Enok might be essential for trxG-mediated gene activation not by enhancing trxG function but by specifically antagonizing the activity of PcG. Hence, we analyzed the association of PC at active (pnr, pnt) and silent (bxd, Dfd) targets after the knockdown of enok in cells (Fig. 4f). Depletion of enok, resulting in diminished H3K27ac (Additional file 4: Fig. S3k), showed a greater enrichment of PC at all sites analyzed by ChIP (Fig. 4f). Moreover, enhanced levels of PRC1-associated H2AK118ub1 were also found at active and silent loci in cells with enok knockdown (Fig. 4g). Interestingly, in enok-depleted cells, there was no difference in the enrichment of E(z) and H3K27me3 levels when compared to LacZ dsRNA-treated cells (Additional file 4: Fig. S3 l, m). Together, these results highlight a PRC1-specific role of Enok in counteracting repression to maintain active gene expression.

To further investigate the role of Enok in antagonizing PRC1-mediated repression, cells were treated with either enok dsRNA alone or in combination with dsRNA against Pc (enok + Pc) or E(z) (enok + E(z)) (Fig. 4h–j). Interestingly, gene expression levels of pnr and pnt were significantly reduced in enok and enok + E(z) depleted cells when compared to LacZ dsRNA-treated cells. In contrast, enok + Pc knockdown cells showed pnr and pnt expression levels comparable to the LacZ dsRNA-treated control (Fig. 4h). Since depletion of enok and Pc together resulted in near wild-type levels of gene expression, we concluded that Enok works specifically to counteract PRC1 and contributes to anti-silencing act of trxG.

Next, we determined the occupancy of RNA Polymerase II (Pol-II) at pnt and pnr. We found an increased enrichment of Pol-II at the TSS of pnt and pnr in enok and enok + E(z)-depleted cells (Fig. 4i), when compared to LacZ dsRNA-treated cells. This increase in Pol-II
occupancy at the TSS was accompanied by decreased Pol-II enrichment in the gene body of *pnt* and *pnr* (Fig. 4j). These findings, along with the decreased expression of *pnt* and *pnr* (Fig. 4h), indicate stalling of Pol-II [48, 49] at the TSS of these trxG targets. In contrast, as compared to cells with knockdown against *enok* alone or *enok+E(z)*, cells treated with dsRNA against *enok*+*Pc* showed a decreased enrichment of Pol-II at the TSS (Fig. 4i) and an increased enrichment in the gene body (Fig. 4j). This result, along with the restoration of expression levels of *pnt* and *pnr* in *enok*+*Pc* (Fig. 4h)-depleted cells, indicated a release of Pol-II from the paused state. Taken together, our results suggest that depletion of *enok* leads to repression of trxG targets due to increased PRC1 recruitment which prevents Pol-II from transcribing its target genes.
Discussion

We have developed an ex vivo approach that led to the discovery of several new genes regulating trxG-mediated gene activation. Using a well-characterized bxd-PRE-reporter [23], comprising of Ubx promoter and enhancers, we developed a cell-based assay and performed a genome-wide RNAi screen in Drosophila. Based on the Z scores of trx and ash1 knockdown, we defined a stringent cut-off and identified more than 200 genes affecting the reporter in a manner similar to trxG genes. Identification of known members of trxG and their interactors as well as chromatin modifiers in the genome-wide RNAi
screen validated our reverse genetics approach and efficacy of the reporter system to discover new regulators of trxG. Moreover, presence of chromatin modifiers like members of TIP60 complex and proteins associated with RNA polymerase II, known to interact with trxG, further substantiates that regulators of gene activation were predominantly identified. Although we identified only a subset of known trxG members in our screen, failure to identify all can be attributed to the highly context-dependent working of PcG/trxG system [50]. Since two specific enhancers of Ubx drive the expression of our reporter, it might be regulated by only a subset of trxG members, which could further validate the failure to identify all members of trxG. Interestingly, some of the top scoring candidates in our screen were also recently found to be a part of the interaction network of GAGA factor, a known trxG member [51].

We characterized trxG-like behavior of Enok, and established its genetic and molecular link with trxG. Although Drosophila Enok has previously been shown to interact with PC [34] and Ash1 [35], its physiological relevance with PcG/trxG or epigenetic cellular memory remained elusive. Our results demonstrate that enok behaves like a trxG gene, by antagonizing PcG, and is essential for maintaining active gene expression in Drosophila. Appearance of extra sex combs in Pc heterozygous males is a consequence of ectopic activation of homeotic genes which relies upon the trxG. However, depletion of trxG proteins counteracts the reduced dose of PC, restoring normal regulation of homeotic genes and suppressing the extra sex comb phenotype [8]. Strong suppression of extra sex comb phenotype by two different mutants of enok illustrates that it acts as a trxG gene, consequently counteracting repression maintained by PcG. This finding is further supported by the fact that both mutant alleles of enok strongly enhance trx mutant phenotype, which also corroborates with drastic reduction in transcript levels of trxG target genes in embryos lacking functional enok. A significant overlap between Enok and TRX at chromatin further validates our genetic analysis. Since depletion of enok led to increased PC binding and enhanced H2AK118ub1 at trxG targets, we suggest that Enok may specifically inhibit PRC1 and facilitate anti-silencing activity of trxG. In contrast, no change in enrichment of E(z) and its associated mark, H3K27me3, was observed at TSS of trxG targets in cells with reduced enok, indicating recruitment of PRC1 in a potentially H3K27me3-independent manner. Such PRC2-independent recruitment of PRC1 has also been reported previously [52].

In light of our results, we propose that Enok counteracts PRC1-mediated block of transcription, evident in the form of stalled Pol-II at the TSS of pnr and pnt in cells with depleted enok. Molecular interaction of Enok with PRC1 on developmental genes in flies and humans [35] further supports the notion that Enok facilitates trxG by inhibiting PRC1. In mice, MOZ (homolog of Enok) is known to play an antagonistic role to PcG member BMI1 in regulating Hox genes [53]. In agreement with the finding that PC chromodomain binding to H3K27me3 requires an unmodified H3K23 [54], our data suggest that Enok-mediated H3K23ac inhibits binding of PC to its target genes. We propose that in the presence of Enok at active loci, acetylated H3K23 inhibits binding of PRC1 leading to increased transcriptional activity of Pol-II (Fig. 5a). On the other hand, loss of Enok leads to decreased H3K23ac, thus allowing PRC1 binding and consequent stalling of Pol-II at TSS (Fig. 5b). Since Enok was also found to associate with silent loci (bsd, Dfd, iab-7) and interact with PRC1, we suggest that Enok is kept in an inactive state on these loci by PC in a manner similar to the inhibitory interaction between PC and CBP [55]. Further molecular and biochemical characterization of this intricate relationship between PcG and Enok will help discover how trxG maintains dynamic gene expression patterns during development.

Conclusion

In summary, we have developed a cell-based assay for an ex vivo genome-wide RNAi screen to identify potential trxG regulators in Drosophila. Our RNAi screen led to the discovery of more than 200 genes which perturbed our luciferase-based reporter in a manner similar to known trxG members. We have also provided evidence that Enok, a top trxG candidate in our screen, contributes to anti-silencing action of trxG by counteracting PcG proteins. We propose that H3K23 acetylation by Enok counteracts PcG-mediated suppression by inhibiting PRC1 recruitment, contributing to gene activation. Genetic and molecular evidence obtained suggests that Enok interacts with trxG and as a result with their major developmental regulatory targets, thus providing a possible molecular link through which it could influence epigenetic cell memory.

Materials and methods

Antibodies

Following antibodies were used in this study: mouse α-GFP (Roche, 11814460001, WB:1:5000), mouse α-Tubulin (Abcam, ab44928, WB: 1:2000), mouse α-Ubx (DSHB, Fp3.38, IF: 1:20), mouse α-Abd-B (DSHB, 1A2E9, IF: 1:40), rabbit α-TRX (gift from R. Paro, IF: 1:20, ChIP: 5 µl), rabbit α-PC (Santa Cruz, D220, IF: 1:20, ChIP: 2 µl), rabbit α-E(z) (gift from R. S. Jones, ChIP: 2 µl), rabbit α-H3K27ac (Abcam, ab4729, ChIP: 2 µl), mouse α-H3K27me3 (Abcam, ab6002,
ChIP: 2 µl), mouse α-Myc (Santa Cruz, 9E10, WB: 1:1000, IF: 1:50), mouse α-FLAG M2 (Sigma Aldrich, WB: 1:2000, ChIP: 5 µl), rabbit α-Enok (gift from J. Workman, WB: 1:1000, ChIP: 5 µl), mouse α-H2A-ubi (Millipore, 05-678, ChIP: 5 µl), rabbit α-H3K23ac (Millipore, 07-355, WB: 1:10,000) and mouse α-RNA polymerase II (Abcam, ab5408, ChIP: 1 µl).

**Drosophila cell culture**

*Drosophila* S2 cells were cultured in Schneider's *Drosophila* medium (Gibco, ThermoFisher Scientific), supplemented with 10% fetal bovine serum (Gibco, ThermoFisher Scientific) and 1% penicillin–streptomycin (Gibco, ThermoFisher Scientific) at 25 °C. Schneider S2 cells adjusted to serum-free growth medium (D.Mel-2, Invitrogen) were cultured in Express Five SFM (Gibco, ThermoFisher Scientific) supplemented with 20 mM GlutaMAX (Gibco, ThermoFisher Scientific) and 1% penicillin–streptomycin.

**Construction of reporter plasmid**
The Carnegie 20 fly transformation vector, PBX-PRE1.6-IDE-Ubx promoter-LacZ was a kind gift from Jürg Müller [23]. The plasmid was modified to replace LacZ with

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**Fig. 5** Proposed model depicting antagonistic effect of Enok on PRC1. **a** At active trxG target genes, Enok acetylates H3K23 which inhibits PRC1 binding and consequent transcriptional block of RNA-Pol-II. As a consequence, genes remain in an active state. **b** In the absence of Enok, H3K23 remains unmodified which allows PRC1 recruitment to the chromatin and addition of H2AK118 mono-ubiquitination. Subsequently, PRC1 holds RNA-Pol-II in a stalled state, leading to gene silencing.
EGFP or F.Luc (Firefly Luciferase) using Sal I restriction sites. Since the ~24 kb plasmid was not suitable for efficient transfection in cell culture, the cassette containing PBX-PRE1.6-IDE-Ubx promoter was cloned into a smaller cell culture vector pCoBlast (ThermoFisher Scientific) using the following strategy: a Sal I restriction site was added downstream of Not I in the Multiple Cloning Sites (MCS) of pCoBlast using linkers. The PBX-PRE1.6-IDE-Ubx promoter was excised from the fly transformation vector by digesting with Not I and Sal I and cloned into the modified pCoBlast at Not I and Sal I sites. The reporter genes EGFP and F.Luc were then PCR amplified from the respective fly vectors, along with the Hsp70-PolyA tail, using primers with an Xho I restriction site which were cloned at Sal I in the pCoBlast-PBX-PRE1.6-IDE-Ubx vector. pActin-RFP and pActin-R.Luc (Renilla Luciferase) were used as internal control reporter plasmids. Primer details can be found in Additional file 5: Table S2.

PRE-EGFP reporter assay

S2 cells (1x10⁶) were transfected with PRE-EGFP reporter alone (0.5 µg) or together with constructs expressing full-length Pc [pMT-FLAG-His-Pc] (0.5 µg), full-length E(z) [pMT-FLAG-His-E(z)] (0.5 µg) or full-length DNAPol-a50 [pMT-FLAG-His-DNAPol-a50] (0.5 µg) using Effectene reagent as per recommended protocol (Qiagen). Expression of Pc, E(z) and DNAPol-a50 was induced with 500 µM CuSO₄. After 72 h of induction, EGFP and RFP signals were monitored using a confocal microscope (Nikon C2). Total cell lysates were analyzed by Western blot.

PRE-F.Luc reporter assay
dsRNA was introduced into cells by the bathing protocol described previously [56]. 250 ng dsRNA was preloaded into 384-well plates (Greiner). Each plate had positive and negative controls (see results). Subsequently, 10,000 (~30 µl) D.Mel-2 cells were dispensed per well using a MultiDrop (ThermoLab systems). After 24-h incubation at 25 °C, the cells were transfected with 0.005 µg of a DNA mix (0.002 µg of PRE-F.Luc, and 0.003 µg of pActin-R.Luc) per well using Effectene reagent. 3 days post-transfection, cells were lysed to measure F.Luc and R.Luc (Renilla Luciferase) activities using a dual luminescence assay on a Mithras LB940 plate reader (Berthold Technologies). Each experiment was performed in triplicates within two different replicates.

For the over-expression assay of Pc, 0.005 µg of a DNA mix (0.002 µg of PRE-F.Luc and 0.003 µg of pActin-R.Luc) was transfected alone or with increasing concentrations (0.001, 0.002 and 0.003 µg) of pActin-FLAG-Myc-Pc.

dsRNA synthesis

Templates for the preparation of dsRNAs were amplified by PCR from cDNA or genomic DNA using T7-tailed oligonucleotides as primers. These templates were then used for in vitro transcription to synthesize dsRNA using T7 Megascript kit (Ambion) following the manufacturer’s instructions. Primers for all the known members of trxG were chosen from second-generation Drosophila dsRNA library (Heidelberg 2) [27]. Complete primer and amplification sequence information can be found at http://rnan.dkfz.de.

Genome-wide RNAi screen
dsRNA was synthesized using the second-generation Drosophila dsRNA library (Heidelberg 2) as described [57]. Bathing of D.Mel-2 cells with dsRNA against whole genome [57] was carried out as described above. Each gene in the HD2 library was knocked down in triplicates and the entire experiment was carried out in duplicates. Each plate contained controls (see “Results”) in triplicates. 5 days from the start of the experiment, F.Luc and R.Luc values were recorded using a dual luminescence reader as described [58]. The ratio of the experimental reporter F.Luc to the invariant co-reporter R.Luc values was calculated to exclude possible artifacts, such as cell death and effect on general transcription. Knockdown of genes that affected both F.Luc and R.Luc was removed from further analysis. Relative F.Luc and R.Luc expressions were averaged for both replicates. Results were exported into excel sheets and were analyzed using the guidelines described previously [59]. Instead of using 99.99% confidence values as cut-offs, more stringent cut-offs were defined based on trx and ash1 Z scores (higher Z score). A number of ribosomal proteins, translational initiation or elongation factors were also found affecting PRE-F.Luc. Since these genes are known to appear in multiple genome-wide RNAi screens as an unexplained group of hits [60], all such factors were excluded from the list of candidates (Additional file 3: Table S1).

Protein interaction analysis

An interaction map of candidates identified as potential trxG regulators in the genome-wide RNAi screen was made using the STRING database [61]. A custom confidence value of 0.300 was used. The network was grouped into six clusters using k-means. Since many proteins were involved in multiple processes, the clusters were then manually modified to refine the functional grouping of the proteins.
Western blotting
Cells were lysed in cold lysis buffer (150 mM NaCl, 0.05 M Tris, 1% Triton X-100) supplemented with protease inhibitors, supernatants were collected in fresh tubes and mixed with 2X reducing sample buffer and boiled at 95 °C for 5 min. The proteins were resolved on 12% SDS-PAGE and transferred onto nitrocellulose membranes that were blocked with 5% milk for 2 h before probing with the appropriate antibodies overnight at 4 °C. Secondary antibodies, HRP conjugated, were used at 1:10,000 dilution and blots were developed using ECL reagents (GE Healthcare).

Fly strains
The following fly strains were obtained from Bloomington Drosophila Stock Center: enok1 (BN 6284), enok2 (BN 6285), PcXL5/TM3Ser,Sb, P{1}/TM3Ser, trx1 (BN 2114), trxE2 (BN 24160).

The following strains were used for staining and gene expression analysis of embryos:
1. enok1/Cyo, P{GAL4-Kr.C}DC3, P{UAS-GFP:S65T} DC7 (referred to as enok1).
2. enok2/Cyo, P{GAL4-Kr.C}DC3, P{UAS-GFP:S65T} DC7 (referred to as enok2).

The following strains were used for polytene staining:
1. P[w+mcUASp-Myc-enok] (generated in this study).
2. P[Sgs3-GAL4.PD] (BN 6870).

Genetic analysis
Mutant flies of selected candidates and w1118 were crossed to Pc alleles (Pc1 and Pc2LS) at 25 °C. Males in the progeny of these crosses were scored for extra sex combs as described previously [62]. enok2 and w1118 were crossed to trx alleles (trx1 and trx2E) at 29 °C. enok2 was also crossed to w1118. The progeny of these crosses were scored for mutant phenotypes.

For analyzing the expression of homeotic genes in vivo, embryos homozygous for enok1 or enok2 mutations were collected at 20 h post-laying from the progeny of a cross between heterozygous mutant alleles, balanced with GFP-marked balancer. Absence of GFP in embryos was an indicator of the required genotype. Same-aged embryos from w1118 were used as control. Embryos were flash frozen in liquid nitrogen and total RNA was isolated using Trizol Reagent (Ambion). The total RNA was treated with TURBO DNase (Ambion) and was used to make cDNA using SuperScript III First-Strand Synthesis System (Invitrogen) following the manufacturer’s instructions. Further, expression levels of abd A, Abd-B, Dfd, Ubx and pnt (with RP49 as normalization control) were quantified with real-time PCR (Applied Biosystems Inc, 7500) using SYBR Green (Applied Biosystems Inc). Primer details can be found in Additional file 5: Table S2.

Generation of stable cell lines and transgenic flies
To generate vectors expressing tagged proteins, RNA extracted from S2 cells or w1118 embryos was used to make cDNA. Primers designed for Gateway Cloning were used to amplify enok CDS from cDNA which was then cloned into pENTR-dTOPO vector (ThermoFisher Scientific, K240020). LR Clonase reactions (ThermoFisher Scientific, 11791100) were set with DGVC (Drosophila Gateway Vector Collection) vectors containing either Myc or FLAG tags to prepare epitope tagged Enok for both cell culture and fly transformation. Primer details can be found in Additional file 5: Table S2.

For the generation of stable cell lines, pMT-FLAG-enok plasmid was transfected into S2 cells using Effectene transfection reagent (Qiagen). Transfected cells were selected using Hygromycin B (final concentration 250 µg/mL). Finally, cells were induced with 500 µM CuSO4 for 48 and 72 h and stable cells were confirmed for the expression of our protein by Western blotting with antibody against the FLAG tag.

Fly transformation vector expressing Myc-Enok, under the control of UASp, was used to generate transgenic fly lines by injecting w1118 embryos using standard protocol [63]. Transgenic flies were confirmed by Western blotting with α-Myc antibody (Additional file 4: Fig. S3a).

Immunohistochemistry
Transgenic flies carrying UAS-Myc-enok were crossed with sgs-GAL4 driver line. Salivary glands from third instar larvae were isolated and polytene chromosomes were stained with α-TRX and α-Myc using standard protocol [64].

For embryonic staining, stage 15 embryos were dechorionated and GFP-negative embryos were separated under an epifluorescent stereo microscope (Nikon, C-SS230) and were stained using standard protocol [64]. All images were acquired using the Nikon C2 Confocal Microscope.

Chromatin immunoprecipitation (ChIP)
ChIP was performed from either stable cell lines induced with CuSO4 for 72 h or S2 cells as described previously [46]. Purified ChIP DNA from each reaction was quantified using real-time PCR. Chromatin enrichment as
percentage of input was calculated using ΔΔCT method as described previously [65]. For ChIP after knockdown of enok, cells were treated with 10 µg/mL of dsRNA for 4–6 days. Knockdown was confirmed by Western blotting. ChIP was then performed with 1 × 10⁷ cells.

Briefly, 3 × 10⁷ cells were fixed at room temperature with 1% formaldehyde for 10 min. Cross-linking was stopped by the addition of glycine to a final concentration of 0.125 M. Cells were washed with 1× PBS and lysed with Buffer A (10 mM Tris, pH 8, 0.25% Triton X-100, 10 mM EDTA, 0.5 mM EGTA) followed by two washes with Buffer B (10 mM Tris, pH 8, 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA). Cells were sonicated in 300 µl of sonication buffer (10 mM Tris, pH 8, 1 mM EDTA, 0.5 mM EGTA) using Bioruptor (Diagenode) at high setting for 15–25 min (30 s on, 30 s off) such that chromatin fragment sizes were between 100 and 500 bp. Sonicated chromatin was centrifuged at 13,000 rpm for 10 min and the cleared chromatin was stored at −80 °C. Chromatin was diluted with 2× RIPA buffer (20 mM Tris, 2 mM EDTA, 280 mM NaCl, 2% Triton X-100, 0.2% SDS, 0.2% sodium deoxycholate) and precleared by incubating with DYNA beads (Invitrogen) for 2 h at 4 °C with 20 rpm rotation. Precleared chromatin was incubated with the appropriate antibody overnight at 4 °C with 20 rpm rotation. Immunocomplexes were pulled down with DYNA beads. The beads were washed five times with 1x RIPA, once with 1× LiCl Buffer (10 mM Tris, pH 8, 250 mM LiCl, 1 mM EDTA, 0.5% NP-40, 0.5% sodium deoxycholate) and twice with 1× TE (10 mM Tris pH 8, 1 mM EDTA). Chromatin was eluted by incubating beads at 65 °C with 500 µl of freshly made elution buffer (0.1 M sodium bicarbonate, 1% SDS) for 15 min. Reverse cross-linking of chromatin was carried out overnight with 5 M NaCl at 65 °C followed by proteinase K treatment for 2 h at 45 °C and reverse cross-linked chromatin was extracted using phenol–chloroform followed by ethanol precipitation. All buffers were supplemented with PMSF, aprotinin, leupeptin and pepstatin protease inhibitors (ThermoFisher Scientific).

Supplementary information

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Additional file 1: Fig. S1. (a) PRE-Reporter system, with EGFP or Luciferase as read outs, comprised of Ubx (Ultrathoraxa) promoter along with a 1.6 kb bxd-PRE, flanked by PBX (postbithorxa—embryonic enhancer) and IDE (Imaginal Disc Enhancer). (b) Schematic of working hypothesis for validating PcgTrxnG responsive reporter system in Drosophila cells. Transient transfections of PRE-Reporter constructs (EGFP/Luc) along with either overexpression of Pcg or depletion of trxnG by RNAi would diminish the reporter gene (EGFP/Luc) expression and could potentially be used to discover novel players involved in epigenetic cellular memory through genome-wide RNAi screen.
manuscript. AM analyzed the screen data. All authors read and approved the final manuscript.

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Availability of data and materials
All data generated and analyzed in the study are available in the main or additional files provided.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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