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Genome-wide binding of *Drosophila* Mask reveals its role in maintenance of gene activation by Trithorax group

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Abstract

The Trithorax group (trxG) proteins counteract repressive effect of Polycomb group (PcG) complexes and maintain transcriptional memory of active states of key developmental genes. Although, chromatin structure and modifications appear to play a fundamental role in this process, it is not clear how trxG prevents PcG-silencing and heritably maintain an

active gene expression state. Here, we report a hitherto unknown role of *Drosophila* Mask which emerged as one of the candidate trxG genes in our screen. The genome-wide binding profile of Mask correlates with known Trithorax binding sites across *Drosophila* genome. Association of Mask at chromatin overlaps with H3K27ac and depletion of Mask results in strongly diminished H3K27ac levels. Moreover, Mask predominantly associates with actively transcribed genes and its depletion results in downregulation of trxG targets. Importantly, strong suppression of extra sex comb phenotype by *mask* mutant provides physiological relevance that Mask is required for maintenance of gene activation by trxG.

Introduction

At the heart of development in multicellular eukaryotes is the specialization of cells. Differential gene expression profiles established during the patterning processes lead to specialization of cells during early embryonic development. In order to grow and maintain a specialized state, the particular expression profiles of genes need to be transmitted to daughter cells in all cell lineages. The Polycomb (PcG) and Trithorax (trxG) group proteins maintain the transcriptional states of important developmental regulators like the *Hox* genes which ensure that determined cell fates are faithfully propagated during cell proliferation[1-5]. Both the PcG and trxG proteins act in large multi-protein complexes and modify the local properties of chromatin to maintain transcriptional states of repression and activation of their target genes, respectively[6]. The association of PcG and trxG proteins with chromatin is evolutionarily conserved and defects in PcG/trxG system lead to severe morphological abnormalities during development[7,8]. Although, chromatin structure and modifications appear to be important, genome organization has emerged as an important factor in transcriptional regulation by PcG/trxG[9].

In *Drosophila*, PcG and trxG proteins are known to exert their functions by binding to specific chromosomal elements known as *PREs* (Polycomb Response Elements)[10,11]. Gene silencing by the Polycomb Repression Complex 1 (PRC1) and Polycomb Repression Complex 2 (PRC2) employs an intricate interplay of different components that regulate the target genes in a spatio-temporal manner during development. Importantly, histone H3 lysine 27 trimethylation (H3K27me3), histone H2A lysine 118 ubiquitination (H2AK118ub) and stalled RNA polymerase II at gene promoters are considered hallmarks

of PcG-silencing. Although the trxG proteins are known to directly counteract repression by the PcG, the maintenance of gene activation by trxG is seen as a constant battle against the default state of silencing by PcG. Details of the molecular mechanism employed by trxG to prevent PcG-silencing still remain elusive[12,13]. The trxG proteins display a wide range of functional diversity which includes covalent modifications of histones, chromatin remodeling and general transcriptional processes. It can be envisaged that the function and composition of trxG complexes is modulated in various cell types at specific target genes[14]. Due to the intricate nature of gene activation by trxG, it is important to identify the as yet unknown accessory proteins that may interact with the trxG in maintenance of gene activation.

Our laboratory employed an *ex-vivo* genome-wide RNAi approach to identify novel trxG factors involved in maintaining gene activity. More than 200 different genes were identified as potential trxG regulators. Multiple ankyrin repeats single KH domain (Mask), a large scaffold protein, was also among the potential trxG candidate genes[15]. Initially, Mask was discovered in *Drosophila* as a regulator of Epidermal Growth Factor (EGF) signaling. It was reported to play an important role in photoreceptor differentiation, cell survival and proliferation[16]. *Drosophila* Mask is a large nuclear as well as cytoplasmic protein which contains 4001 amino acids harboring different functional domains. Notably, Mask has been linked to different cell signaling pathways and various cellular processes in proliferating[17-19] and non-proliferating cells[20]. Besides EGF signaling, Mask is known to act downstream of Hippo signaling pathway as a co-transcription factor, required for organ size regulation during development[17-19].

Here we report a hitherto unknown role of Mask in the maintenance of gene activation by trxG in *Drosophila* and humans. We discovered that the genome-wide binding profile of Mask massively overlaps with Trx protein binding sites in *Drosophila*. Importantly, Mask associates with known *PREs* within Antennapedia complex (ANT-C) and Bithorax complex (BX-C) regions, which are canonical targets of trxG/PcG[21]. Mask is also enriched at histone H3 lysine 27 acetylation (H3K27ac) marked chromatin, a hallmark of trxG mediated gene activation[22]. Depletion of Mask in *Drosophila* and mammalian cells resulted in a drastic reduction in global H3K27ac levels. The molecular interaction

between Mask and CREB binding protein (CBP), a trxG protein that catalyzes H3K27ac, further corroborates the effect of Mask on H3K27ac levels. Finally, strong suppression of PcG associated extra sex comb phenotype by *mask* mutant and the effect of Mask depletion on expression of *engrailed* (*en*) gene, a known target of trxG, in flies highlight the physiological relevance of Mask function in maintenance of gene activation by trxG.

Results

Mask regulates trxG target genes and associates with polytene chromosomes

Since Mask appeared as a potential trxG gene in an *ex-vivo* genome-wide RNAi screen based on luciferase reporter in *Drosophila*[15], we first validated whether Mask is able to regulate expression of endogenous trxG target genes. To this end, we knocked down *mask* and analyzed the expression of, *pannier* (*pnr*), *pointed* (*pnt*) and *pipsqueak* (*psq*), known targets of trxG[22]. Depletion of Mask resulted in downregulation of trxG targets similar to the effect of *trx* knockdown in these cells (Figure 1A, Supplementary Figure 1B). This highlights a potential role of Mask in maintenance of gene activation by trxG in *Drosophila*. Since trxG/PcG proteins influence gene expression through their association with chromatin[5,23], it was investigated if Mask also binds to chromatin. To determine the chromatin association of Mask, we isolated salivary glands from third instar larvae of w^{1118} flies and stained polytene chromosomes with anti-Mask antibody. Importantly, Mask was found to localize on inter-band regions on polytene chromosomes, which indicates its predominant binding on potentially actively transcribed regions (Figure 1B-D).

Genome-wide binding profile of Mask overlaps with Trx binding at active genes

After observing binding of Mask on polytene chromosomes, the genome-wide binding profile of Mask on chromatin was investigated. To this end, chromatin immunoprecipitation sequencing (ChIP-seq) from *Drosophila* cells was performed using anti-Mask antibody. Mask was found to bind 2848 regions and 69% of these binding sites were in promoters, whereas few sites covered introns and distal intergenic regions (Figure 2A). The genome-wide binding profile of Mask further indicated that Mask occupies chromatin regions mainly near TSS (Transcription Start Site) of genes (Figure 2B). Heat

maps of genome-wide binding profiles of Mask and Trx within ±10 kb of TSSs of genes bound by Trx indicate overlap in their binding pattern (Figure 2C). Our ChIP-seq data shows a significant overlap of Mask-bound sites with previously reported Trx[24] and Pc[25] binding profiles (Figure 2D, F). In addition, a strong correlation of Mask binding with H3K27ac marked chromatin was observed (Figure 2E). In contrast, only a small number of Mask binding sites overlap with H3K27me3 marked regions (Figure 2G). These results clearly indicate a potential role of Mask in gene activation which was validated by comparison of Mask binding sites with previously reported actively transcribed genes in *Drosophila* cells[26,27] (Figure 2H). Additionally, Mask enrichment at known enhancers, such as *Ptx1* and *Optix*[24], also suggests its potential role in gene activation (Supplementary Figure 2A,B). A comparison of short motifs enriched in ChIP-seq data of Mask and previously reported ChIP-seq data of Trx revealed similarity in top-scoring motifs. Most importantly, GAGA motif, a known GAGA factor (GAF) binding site and GCCAT motif, a known PHO binding motif were enriched in case of both Mask and Trx bound chromatin regions[28] (Figure 2I). Importantly, an overwhelming overlap of Mask binding sites with previously reported genome-wide binding profiles of Trx and Pc within BX-C (Figure 3A) and ANT-C (Figure 3B) was observed where Mask was found to occupy majority of all known *PREs*[21,29]. Analysis of Mask binding sites at cell signaling genes revealed that Mask associates with thirty three different genes belonging to Wnt signaling pathway, including *en* (Supplementary Table 1). The Trx and Pc binding sites at *PREs* near *en* promoter[30-32] also show a strong overlap between Mask, Trx and Pc (Figure 3C).

Mask positively regulates H3K27ac and interacts with CBP

A strong correlation was observed between Mask and H3K27ac across the genome as exemplified by the *CCT3* locus adjacent to BX-C (Figure 3A, Supplementary Figure 2C). Additionally, Mask binding at *pnr*, *pnt* and *psq* loci was investigated (Figure 3C), the genes downregulated after depletion of either Mask or Trx (Figure 1A). It was observed that Mask was strongly enriched on these actively transcribed genes which correlates with higher enrichment of H3K27ac. To dissect the molecular interaction between Mask and H3K27ac, we decided to look at the effect of depletion of Mask on global levels of H3K27ac. After knocking down *mask* in *Drosophila* cells, H3K27ac levels were found to

be strongly diminished (Figure 3D). Such a drastic effect of impaired Mask function on H3K27ac encouraged us to investigate conservation of this interaction in mammalian cells. To this end, we prepared HEK293T cells that stably expressed shRNA against *ANKHD1*, a mammalian homologue of *Drosophila* Mask. Moreover, we also generated *ANKHD1* knockout (KO) cells using CRISPR/CAS system in HEK293T cells. Strongly diminished levels of global H3K27ac were observed in response to depletion of *ANKHD1* (Figure 3E). These results correlate with downregulation of highly acetylated genes after depletion of Mask and provide clues towards mechanistic interactions of Mask with CBP, a known trxG member that catalyzes H3K27ac (Figure 1A, Figure 3C). We next investigated if Mask protein interacts with CBP in *Drosophila*. To this end, we performed co-immunoprecipitation (Co-IP) experiments from *Drosophila* cells that stably expressed FLAG-tagged full-length Mask protein. As compared to empty vector (EV) control, immunoprecipitation (IP) of Mask-FLAG using anti-FLAG antibody resulted in enrichment of endogenous CBP in Co-IP (Figure 3F). Interaction between Mask and CBP supports the notion that Mask positively regulates H3K27ac (Figure 3E). Together, these results suggest that Mask is required in maintenance of gene activation by trxG.

Mask exhibits trxG like behavior and regulates trxG targets in *Drosophila*

Since Mask was found to bind at multiple sites within homeotic genes in BX-C and ANT-C (Figure 3A, B) in *Drosophila* cells, we questioned if Mask association at homeotic genes has any physiological relevance. To this end, we crossed a *mask* mutant ($mask^{EY09448}$) with two different *Pc* mutants (Pc^{1}, Pc^{XL5}) to observe effect of *mask* mutation on extra sex comb phenotype in *Pc* mutants. Normally, *Pc* heterozygous mutant flies show extra sex comb phenotype due to de-repression of *Sex comb reduced* (*Scr*) gene in second and third pair of legs[33]. In the *Pc* heterozygous mutant background, $mask^{EY09448}$ strongly suppressed the extra sex comb phenotype, indicating loss of *Scr* expression (Figure 4A, Supplementary Figure 3). Strong suppression of extra sex comb phenotype by $mask^{EY0944}$ suggests a trxG-like behavior which correlates with Mask binding on *Scr* locus in ChIP-seq data (Figure 3B). Moreover, genetic interaction between *mask* and *trithorax* (*trx*) mutants was analyzed by crossing *mask* mutant ($mask^{EY09448}$) with *trx* mutant (trx^{1}) flies. The *trx* heterozygous mutant male flies show loss of pigmentation on the fifth abdominal

segment (A5) due to decreased *Abdominal-B* (*Abd-B*) expression that controls dark pigmentation in male posterior body segments[34,35]. As compared to heterozygous trx^{1} mutant males used as a control, $mask^{EY09448}/trx^{1}$ trans-heterozygote male flies showed a significantly higher percentage of A5 to A4 transformation (Figure 4B). To further confirm this physiological evidence at the molecular level, we investigated if depletion of Mask in flies affects *en* gene expression, a known trxG/PcG target[30-32]. To this end, we crossed $UAS\text{-}mask^{RNAi}$ flies with a *GAL4* driver line (*en-GAL4,UAS-GFP*) to knockdown *mask* and observe its effect on *en* expression in the imaginal discs isolated from the third instar larvae. A cross between *en-GAL4,UAS-GFP* and w^{1118} served as control. The control flies expressing *en-GAL4,UAS-GFP* alone show expression of GFP that overlaps with en staining in posterior compartment of imaginal discs where en is normally expressed[36] (Figure 4C). As compared to the control flies, expression of $UAS\text{-}mask^{RNAi}$ driven by *en-GAL4,UAS-GFP* driver resulted in drastic reduction in en staining with a concomitant reduction in GFP expression. Since GAL4 was under *en* enhancer, the decrease in both endogenous en expression and *en-GAL4* driven GFP, upon *mask* knockdown can be explained (Figure 4C). These findings were further confirmed by crossing $UAS\text{-}mask^{RNAi}$ with *GawB-GAL4* driver which is normally expressed in pouch region of wing, leg and haltere discs (Supplementary Figure 4A). As compared to control flies that expressed *GawB-GAL4* driver alone, a drastic reduction in *en* expression was observed as a consequence of *mask* knockdown in progeny of flies expressing $mask^{RNAi}$ driven by *GawB-GAL4* driver (Supplementary Figure 4B). It is pertinent to mention that genome-wide binding profile of Mask revealed its association with known trxG/PcG binding sites within *en PRE* (Figure 3C), which further supports the drastic reduction of *en* expression after depletion of Mask in flies. Together, our results suggest that Mask is required for maintenance of gene activation of key developmental genes by trxG.

Discussion

Our results demonstrate that *Drosophila* Mask exhibits trxG like behavior and plays an important role in maintenance of gene activation. Genome-wide binding profile of Mask has revealed key evidence about how Mask may contribute to the process of cell fate

maintenance and epigenetic cell memory. Presence of Mask at *PREs* and other known Trx and Pc binding sites within BX-C and ANTP-C regions highlights the possibility of close interactions between Mask and trxG/PcG system. Moreover, association of Mask near TSS at known trxG targets across the genome strongly supports the notion that Mask is likely required to maintain gene activation. In corroboration, Mask genome-wide binding profile massively overlaps with Trx binding sites and also associates with actively transcribed genes in *Drosophila* cells. The fact that Mask positively regulates expression of trxG targets (Figure 1A and Figure 4C) and also co-occupies Trx binding sites on these genes (Figure 3C), supports our hypothesis that Mask is required to maintain gene activation. Moreover, the suppression of extra sex comb phenotype by *mask* mutant suggests that Mask antagonizes PcG repression and acts as an anti-silencing factor. Notably, presence of Mask at *Scr* and other homeotic genes found in Mask ChIP-seq data may explain why *mask* mutant suppresses extra comb phenotype and enhances *trx* phenotype. However, it is not clear how Mask specifically interacts with trxG members to counteract PcG repression and maintains gene activation. The role of Mask in gene activation can be explained by its biochemical interaction with CBP and association with CBP-mediated H3K27ac on chromatin. It is substantiated by the fact that depletion of Mask leads to a drastic reduction in global H3K27ac levels in *Drosophila* and humans. Interestingly, we recently reported a histone kinase Ballchen (Ball)[37] as a positive regulator of H3K27ac in *Drosophila*[38]. Importantly, Ball is known to biochemically interact with Mask via Obscurin, a muscle sarcomere structural protein in *Drosophila*[39]. Both Ball[38] and Mask also interact with CBP, highlighting a possible Mask-Ball-CBP nexus which may act to maintain gene activation. However, this potential involvement of Ball and Mask in regulation of gene expression through CBP is most likely different from their role in muscle development through their interactions with Obscurin.

In Hippo signaling pathway, Mask acts as a transcriptional co-activator of Yorkie (Yki) by shuttling Yki from cytoplasm to nucleus which is crucial for activation of Yki target genes[17-19]. Whether Mask plays a similar role in shuttling of CBP or other trxG members to the nucleus must be investigated to decipher how exactly Mask contributes to the process of gene activation by trxG. Mask has also been shown to bind Yki target genes, however, no direct transcriptional activation function of Mask has been identified[18]. The fact that

expression of trxG targets like *pnr*, *pnt*, *psq* (Figure 1A) and *en* (Figure 4C) was drastically reduced after depletion of Mask as well as presence of Mask at Trx binding sites in these genes strongly supports our hypothesis that Mask is required to maintain gene activation in flies.

Mask is a large scaffold protein which contains two multiple ankyrin repeat domains and a KH domain. These domains are also conserved in mammalian orthologues of Mask[18,40]. KH domains are commonly found in proteins involved in transcription regulation and nucleic acid binding, while ankyrin repeats are required for protein-protein interactions[41,42]. Histone methyltransferases, G9a and GLP, contain ankyrin repeats and these proteins bind to H3K9me1 and H3K9me2 marks via these domains[43]. Swi6, a homolog of the *Drosophila* heterochromatin protein 1 (HP1), is another ankyrin repeat domain containing chromatin regulator in yeast which is important for gene regulation[44,45]. The ankyrin repeat domains in Mask may facilitate a potential multivalent interaction by which Mask promotes clustering of multiple trxG epigenetic factors and contributes to genome organization of transcriptionally active regions. However, further work is required to explore possible involvement of Mask in genome organization of chromatin domains of actively transcribed regions.

The multifaceted role played by Mask during fly development can be explained by the fact that it is associated with genomic loci of various components of different cell signaling pathways (Supplementary Table1). Mask is reported to be a modulator of receptor tyrosine kinase (RTK) signaling pathways as it genetically interacts with Sevenless (SEV) and Epidermal growth factor receptor (EGFR), contributing to proliferation, cell survival and differentiation of photoreceptors[40]. Mask also plays a pivotal role in JAK/STAT signaling by positively regulating dimerization of its receptor, Domeless[46]. Moreover, *Drosophila* Mask is required for normal microtubule (MT) organization in both neurons and muscles[20]. Notably, Wnt signaling and FoxO pathways control synaptic terminal growth by targeting MT stability[47]. It is important to highlight that components of RTK, Wnt, JAK/STAT and Hippo signaling were enriched in our Mask ChIP-seq data (Supplementary Table1). We speculate that specific signaling pathways may bring Mask and trxG together to regulate genes involved in cell fate determination, maintenance of cell viability and cell proliferation.

Methodology

Cell culture

Schneider's *Drosophila* medium (Gibco, ThermoFisher Scientific), containing 10% fetal bovine serum (Gibco, ThermoFisher Scientific) and 1% penicillin–streptomycin (Gibco, ThermoFisher Scientific) was used to culture *Drosophila* S2 cells at 25°C. Schneider S2 cells adjusted to serum-free growth medium (Invitrogen), known as D.Mel-2 cells were cultured in Express Five SFM (Gibco, ThermoFisher Scientific) containing 20mM GlutaMAX (Gibco, ThermoFisher Scientific) and 1% penicillin–streptomycin. DMEM (ThermoFisher Scientific) medium containing 10% fetal bovine serum (Gibco, ThermoFisher Scientific) and 1% penicillin–streptomycin (Gibco, ThermoFisher Scientific) was used to culture mammalian HEK293T cells at 37°C with 5% CO_2.

Synthesis of dsRNA and RNAi

Templates for dsRNA synthesis were amplified by PCR from cDNA samples using primers containing T7 promoter sequence. These amplicons were used to prepare dsRNA through *in vitro* transcription using T7 Megascript kit (Ambion) following the manufacturer's instructions. Primers for *trx* were taken from second-generation *Drosophila* dsRNA library (Heidelberg 2)[48]. Further information about these primers can be found at [http://rnai.dkfz.de]. Primer for *mask* and *LacZ* dsRNA synthesis are given in (Supplementary Table 2). For *ex-vivo* RNAi, cells were treated with 20µg of respective dsRNA in 6-well plate as described previously[15,37] and harvested after 5 days of treatment for further experiments.

Generation of stable cell line expressing *ANKHD1* shRNA

To knockdown *ANKHD1,* 0.6×10^6 HEK293T cells were seeded in a six well plate and transfected with 1ug of *ANKHD1* shRNA plasmid (Santa Cruz, sc-92073-SH) using Lipofectamine LTX Reagent with PLUS Reagent (ThermoFisher Scientific) following manufacturer's protocol. Untreated cells were labeled as mock. Media was removed 48 hours post-transfection, and replaced with fresh medium supplemented with 3ug/ml

puromycin. Both the treated and untreated cells were passaged 5-6 times in 6 well plate and media was replaced with freshly prepared puromycin selection media every 2 days. Stable HEK293T cell expressing *ANKHD1* shRNA were lysed and analyzed by western blotting.

Generation of CRISPR/Cas9 knockout of *ANKHD1* in HEK293T cells

HEK293T cells, $0.6x10^6$ per well in a 6 well plate, were transfected with a mixture of 1ug *ANKHD1* CRISPR/Cas9 KO (Knockout) plasmid (Santa Cruz, sc-405893-KO-2) and 1ug *ANKHD1* HDR (Homology Directed Repeat) plasmid (Santa Cruz, sc-405893-HDR-2) using Lipofectamine LTX Reagent with PLUS Reagent (ThermoFisher Scientific) following manufacturer's protocol. Untreated cells were labeled as mock. *ANKHD1* HDR plasmid confer puromycin resistance and RFP (Red Fluorescent Protein) signal for stable cells selection. After 24 hours, cells were visualized under fluorescent microscope for RFP signal. Media was removed 48 hours post-transfection, and replaced with fresh medium supplemented with 3ug/ml puromycin. Both the treated and untreated cells were passaged into $10cm^3$ cell culture dish and media was replaced with freshly prepared puromycin selection media every 2 days. After seven selections, there were no colonies in mock cells while discrete colonies in *ANKHD1* knockout cells were trypsinized and transferred to a 12 well plate and allowed to grow for further analysis.

RNA isolation and relative gene expression analysis

Total RNA was isolated using TRIzol and cDNA was synthesized using Invitrogen Superscript III First-Strand Synthesis System for RT-PCR following manufacturer's protocol. qPCR was performed to analyze the relative expression of target genes as described previously[15]. Expression of target genes in test and control samples was normalized with expression of endogenous control, Actin. Relative expression was determined by ΔΔCT method as described previously[49]. The control sample was set to value one for analyzing effect on the test sample.

Western blotting

Drosophila S2 cells were harvested by centrifugation and lysed in lysis Buffer (150mM NaCl, 0.05M Tris, 1% Triton X-100) supplemented with following protease inhibitors:

pepstatin (0.5 µg/ml), leupeptin (0.5 µg/ml), aprotinin (0.5 µg/ml), and phenylmethylsulfonyl fluoride (PMSF) (1 mM). After transferring the supernatant to fresh tubes, 2X reducing sample buffer was added and samples were boiled at 95°C for 5 minutes. Protein samples were resolved on Novex precast Tris-acetate 3-8% gradient gels (ThermoFisher Scientific). Proteins transfer onto nitrocellulose membranes was carried out at 140V for 2 hours. Blocking was done using 5% skimmed milk prepared in PBST (PBS containing 0.1% Tween-20). Blots were probed overnight with relevant antibodies at 4°C. HRP conjugated secondary antibodies were used at 1:10,000 dilution and blots were developed with ECL reagent (GE Healthcare).

Generation of S2 stable cell line expressing FLAG-tagged Mask

cDNA prepared from S2 cells was used as template to amplify *mask* CDS. To clone 12006 bp long coding sequence of *mask*, it was divided it into three overlapping contigs. Fregment-1 of *mask* CDS was amplified and cloned in *pENTR-d-TOPO* entry vector (ThermoFisher Scientific) to generate *mask*-Fragment1-p-ENTR™/D-TOPO vector. This vector was linearized using HindIII and AscI restriction enzymes to clone the remaining fragments in frame. Fragment-2 and fragment-3 were PCR amplified in such a way that they had 15bp overlap with the adjacent fragment. Fragment-3 particularly had 15bp 3'-end overlap with linearized *mask*-Fragment1-p-ENTR/D-TOPO vector to make intact full-length *mask* entry clone. Fragments and the vector were combined using In-Fusion HD cloning kit (TaKaRa) following manufacturer's protocol. For epitope tagging of full-length *mask*, CDS was sub-cloned in *pMTFHW* destination vector[50], by setting up LR Clonase reaction following manufacturer's protocol (ThermoFisher Scientific). The resulting vector *pMT-FLAG-mask* was transfected in *Drosophila* S2 cells using Effectene transfection reagent (Qiagen) and cells were cultured in hygromycin supplemented media up to 8 consecutive passages. A stable cell line was confirmed by inducing the cells with $CuSO_4$ and subsequent western blotting using anti-FLAG antibody (Supplementary Figure 5A, B).

Antibody generation

His-tagged-Mask fusion protein, containing amino acids 1047 to 1171 of the full-length Mask, was purified using HPLC (High-performance liquid chromatography) based Ni-

Column for antibody production. Mask antibody was generated by injecting rabbits with 450ug of purified His-tagged-Mask followed by two 100ug booster injections administered with 14 days gap each. Serum was collected after 2 months and antibody was confirmed by using anti-Mask serum for Western blotting (Supplementary Figure 1A, B).

Co-Immunoprecipitation (Co-IP)

S2 cells expressing FLAG-tagged Mask or empty vector (EV) were harvested by centrifugation at 3000rpm for 5 minutes and washed with PBS twice. Cells were suspended in 1mL lysis buffer (140mM NaCl, 20mM Tris (7.4 pH), 1mM EDTA, 0.5% NP40, 10% glycerol, 0.2mM Na2VO4, pepstatin 0.5µg/mL, leupeptin 0.5µg/mL, aprotinin 0.5µg/mL,1mM PMSF) and incubated on ice for 30 minutes. The lysate was centrifuged at 14,000rpm for 15 minutes and the supernatant was transferred to a tube containing pre-washed 40µL of M2-FLAG agarose beads (Sigma-Aldrich). After one hour of incubation, beads were gently washed thrice with lysis buffer and resuspended in 30µL Laemmli loading buffer. IP samples were heated at 95°C for 5 minutes and resolved on Novex precast Tris-acetate 3-8% gradient gels (ThermoFisher Scientific) followed by Western blot using specific antibodies.

ChIP-seq Analysis

Chromatin immunoprecipitation (ChIP) from *Drosophila* D.Mel-2 cells was carried out using anti-Mask antibody as described previously[15]. Sequencing of purified DNA was performed using BGISEQ-500 at BGI Genomics Co., Ltd. BGI SOAPnuke filter was utilized to trim low-quality reads and adaptors[51,52]. The ChIP-seq data was analyzed using Galaxy web public server[53]. Sequencing reads were mapped to *Drosophila* genome (dm6) using Bowtie version 2[54]. Peak calling from alignment results was performed using MACS version 2 with minimum FDR (q-value) cut-off set at 0.05 for peak detection [55]. Using ChIPseeker from Galaxy server, a list of annotated peaks was generated from peak file for further analysis[56]. Comparative heat maps of Mask and Trx were generated using deepTools[57], (bamComapre, computeMatrix and plotHeatmap). Heat maps of Mask and Trx binding profiles were centered on Trx peaks with coverage of ±10Kb around TSSs. MEME-ChIP - motif discovery, enrichment analysis and clustering on large nucleotide datasets (Galaxy Version 4.11.2+galaxy1) was utilized to generate Discriminative

Regular Expression Motif Elicitation (DREME) output of Mask and Trx ChIP-seq profiles for de-novo motif discovery[58]. List of actively transcribed genes in S2 cells was used from[38] and compared with Mask bound genes to create Venn diagram. ChIP-seq data of Trx, H3K27ac and H3K27me3 were used from GSE81795[24] while Pc ChIP-seq data was retrieved from GSE24521[25].

Fly strains and genetic analysis

Following flies were obtained from Bloomington *Drosophila* Stock Center:

*Pc^{XL5}/TM3Ser,Sb, Pc^{1}/TM3*Ser, *trx^{1}* (BN 2114), *y^{1}w^{67c23};P(EPgy2)mask^{EY09448}/TM3,Sb^{1} Ser^{1}* (17569), *P{UAS-Dcr-2.D}1,w1118;P{en2.4-GAL4}e16E,P{UAS-2xEGFP}AH2* (BN 25752), *w[*];P{w[+mW.hs]=GawB}c804* (BN 33824), *w^{1118}*.

For sex comb analysis, *mask* mutant *mask^{EY09448}* was crossed with *Pc* mutant alleles (*Pc^{1} and Pc^{XL5}*). As a control, *w^{1118}* flies were also crossed with *Pc^{1} and Pc^{XL5}* for comparison. All crosses were maintained at 25°C and male progeny of these crosses were scored for extra sex comb phenotype as described previously[59]. The *mask^{EY09448}* flies were crossed with *trx* mutant (*trx*^{1}) and *w^{1118}*. These crosses were kept at 29°C, and male F1 progeny from these crosses were scored for A5-A4 transformation phenotype. For, an *in-vivo mask* knockdown, *mask$^{UAS-RNAi}$* fly line P{GD9632}v33396 obtained from VDRC stock center was crossed with *UAS-Dicer;en-GAL4,UAS-GFP* fly line and wing imaginal discs were immunostained with anti-engrailed antibody. For further confirmation, *mask$^{UAS-RNAi}$* fly was crossed with a fly line (*w[*];P{w[+mW.hs]=GawB}c804*) which express GAL4 in pouch regions of wing, leg and haltere imaginal discs. Progeny from this cross was dissected at third instar larval stage and imaginal discs were stained with DAPI and anti-engrailed antibody using standard protocol described[60]. For immunostaining of polytene chromosome, polytene squashes were prepared from salivary glands of third instar larvae of *w^{1118}* fly. Immunostaining was performed using DAPI and anti-Mask antibody following standard protocol[61].

Antibodies

Antibodies used during this study are as following: Rabbit anti-Polycomb (Santa Cruz Biotechnology, sc-25762 (d-220) Polycomb, diluted 1:500 for western blotting), mouse

monoclonal anti-EZH2 (Sigma Aldrich, CS203195, stock concentration: 1 mg/mL diluted 1:1000 for western blotting), mouse anti-engrailed (Developmental Studies Hybridoma Bank, AB_528224, diluted 1:50 for immunofluorescence), mouse anti-FLAG M2 (Sigma Aldrich, F3165, stock concentration: 4 mg/mL diluted 1:2,000 for western blotting), rabbit anti-H3K27ac (Abcam, Ab4729, stock concentration: 1 mg/mL, diluted 1:2,000 for western blotting), mouse anti-H3 (Abcam, ab10799, stock concentration: 1 mg/mL, diluted 1:10,000 for western blotting), mouse anti-Tubulin (Abcam, ab44928, WB: 1:2000), Rabbit anti-Mask serum, ChIP: 7μL, WB: 1:250, rabbit anti-CBP serum (gift from Alexander M. Mazo, WB: 1:3,000).

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Figure Legends

Figure 1: Mask regulates trxG targets and binds to interband regions on polytene chromosomes. (A) Relative gene expression of *pnr, pnt* and *psq* measured by qRT-PCR, after knockdown of *mask* in D.Mel-2 cells. Cells treated with *LacZ* dsRNA served as normalization control (dashed line) while Trx depleted cells were used as positive control. Depletion of Mask results in downregulation of *pnr, pnt* and *psq*, similar to the *trx* knockdown. Experiment was repeated three times and independent t-tests were performed for each target gene (*$p \leq 0.05$, **$p \leq 0.01$). **(B-D)** Immunostaining of polytene chromosomes from 3rd instar larvae of w^{1118} with DAPI **(B)** and anti-Mask antibody **(C)** reveals binding of Mask mainly on interband regions in polytene chromosomes.

Figure 2: Genome-wide binding profile of Mask overlaps with Trx binding at active genes. (A) ChIP-seq data of Mask shows that Mask binding sites are mainly promoter regions across genome. Besides promoter regions, Mask was observed at very few UTR's, introns, downstream and distal intergenic regions. **(B)** Analysis of Mask enrichment across ±1Kb of TSS highlights its association predominantly at TSS of target genes. **(C)** Heat maps of enrichment patterns of Mask and Trx within ±10 Kb of TSSs of genes bound by Trx, elucidate overlap in Mask and Trx binding near TSS. **(D-G)** Venn diagrams showing overlap of genome-wide binding sites of Mask with previously known genome-wide profiles of Trx, Pc, H3K27ac and H3K27me3. Substantial overlaps were observed between Mask, Trx **(D)**, Mask, H3K27ac **(E)** and Mask, Pc **(F).** Relatively little overlap between Mask binding and H3K27me3 was observed **(G)**. **(H)** Venn diagram representing Mask bound genes superimposed with the list of actively transcribed genes in S2 cells. Most of the Mask associated genes were found to be transcriptionally active. **(I)** Top scoring short DNA binding motifs of Mask, generated from ChIP-seq data using MEME-ChIP database, show very high similarity with the DNA binding motifs of Trx. The frequency with which each nucleotide occurs is determined by the proportion of the total height of each letter. Motifs are presented with their respective E-values.

Figure 3: Mask associates with known Trx/Pc binding sites in homeotic/non-homeotic genes and positively regulates H3K27ac. (A-C) Integrated genome browser view of ChIP-seq data of Mask in comparison with Trx, Pc, H3K27ac and H3K27me3 at

various loci known to be bound by Trx and Pc. Mask occupies several known binding sites of Pc and Trx in Bithorax complex (BX-C) **(A)** and Antennapedia complex (ANT-C) **(B).** All the peaks marked with red asterisk represent known *PREs* in BX-C and ANT-C[62] **(A-B). (C)** Mask occupies known Trx and Pc binding sites in *engrailed (en)* locus which corresponds to known *PREs* (marked with asterisk) in *en* gene. Mask was also highly enriched at regions where H3K27ac was present (highlighted regions), including *pnr, pnt* and *psq* loci. **(D)** Western blot analysis of whole cell lysates from *Drosophila* D.Mel-2 cells, treated with dsRNA against *mask*, exhibit strongly diminished levels of H3K27ac. Cells treated with dsRNA against *LacZ* served as control while total levels of histone H3 were used as a normalization control. **(E)** Western blot analysis of HEK293T cells stably transfected with *ANKHD1* shRNA and *ANKHD1* CRISPR knockout (KO) plasmids, exhibit drastically reduced H3K27ac levels as compared to untreated cells. Total levels of histone H3 served as loading control in immunoblot. **(F)** Western blots of endogenous CBP and FLAG-tagged Mask after co-immunoprecipitation using anti-FLAG antibody in *Drosophila* S2 cells stably expressing FLAG-tagged Mask. IP from S2 cells expressing empty vector (EV) served as negative control. As compared to control cells, CBP co-precipitated along with FLAG-Mask in (IP), confirming their biochemical interaction.

Figure 4: Mask exhibits trxG like behavior and regulates trxG targets in *Drosophila.* **(A)** Mutant of *mask* (*mask*^{EY09448}) was crossed with *Pc* mutant (*Pc*^{1}) and males in the progeny with *mask*^{EY09448}/*Pc*^{1} genotype were scored for extra sex comb phenotype. Males from *Pc*^{1} flies crossed with *w*^{1118} served as control. *Pc*^{1}/+ exhibited a strong extra sex comb phenotype in contrast to *mask*^{EY09448}/*Pc*^{1}. Total 200 male flies of the desired genotype from progeny of each cross were analyzed and based on the presence of extra sex combs on 2nd and 3rd pair of legs, flies were categorized according to the severity of phenotype. These categories are: –, no extra sex combs; +, 1–2 hairs on 2nd leg; ++, more than three hairs on 2nd leg; +++, more than 3 hairs on 2nd leg and 1–2 hairs on 3rd leg; ++++, strong sex combs on both 2nd and 3rd pairs of legs as described previously[59]. The experiment was repeated three times independently and individual t-test was used to compare each phenotype category. **(B)** *mask* mutant, *mask*^{EY09448}, was crossed with *trx* mutant (*trx*^{1}) and males in the progeny with *mask*^{EY09448}/*trx*^{1} genotype were scored for A5 to A4 transformation. Loss of pigmentation in A5 segment of adult male flies (marked

by asterisk) was used as readout for A5 to A4 transformation[35]. As a control, progeny from a cross between *trx*^{1} and *w*^{1118} was scored for the phenotype. High percentage of *mask*^{EY09448}/*trx*^{1} male flies exhibited A5 to A4 transformation as compared to control heterozygous *trx*^{1}/+ male flies. Representative image of A5 to A4 transformation phenotype in *mask*^{EY09448}/*trx*^{1} male flies as compared to the wild type are shown. All crosses were performed in triplicates and independent t-test was applied for analysis (ns; non-significant, *; $p \leq 0.05$, **; $p \leq 0.01$). **(C**) Depletion of Mask results in drastic reduction in en expression**.** As a control, wing imaginal discs from progeny of *w*^{1118} flies crossed with *en-GAL4,UAS-GFP* show expression of GFP overlap with en expression. As compared to the control, wing imaginal disc from the progeny of *UAS-mask*^{RNAi} fly crossed with *en-GAL4,UAS-GFP* show drastically reduced level of en along with diminished levels of GFP. This indicates effect of *mask* knockdown on endogenous (anti-engrailed staining) as well as on *en-GAL4* driven GFP.

Figure: 1

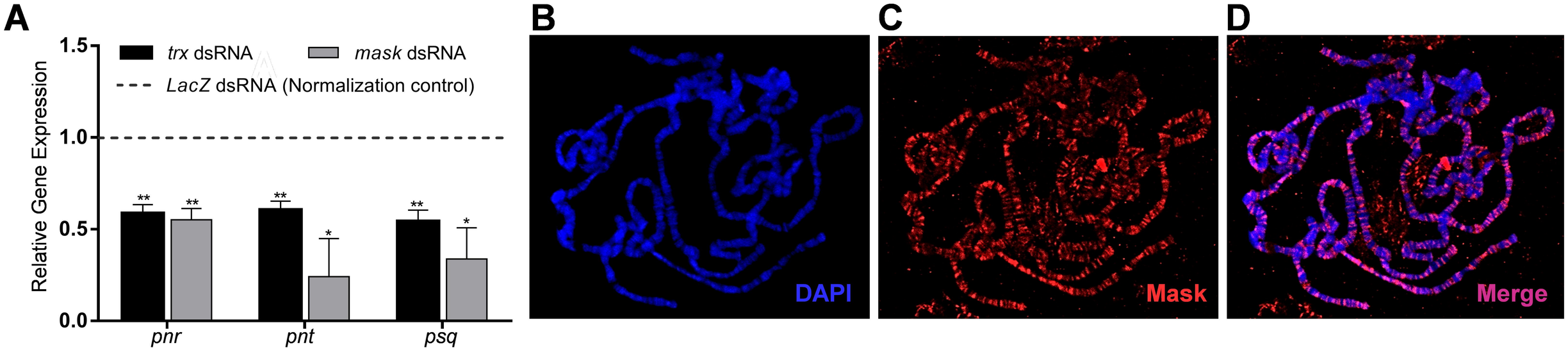

A
B
C
D
trx dsRNA
mask dsRNA
LacZ dsRNA (Normalization control)
Relative Gene Expression
1.5
1.0
0.5
0.0
**
**
**
*
**
*
pnr
pnt
psq
DAPI
Mask
Merge

Figure: 2

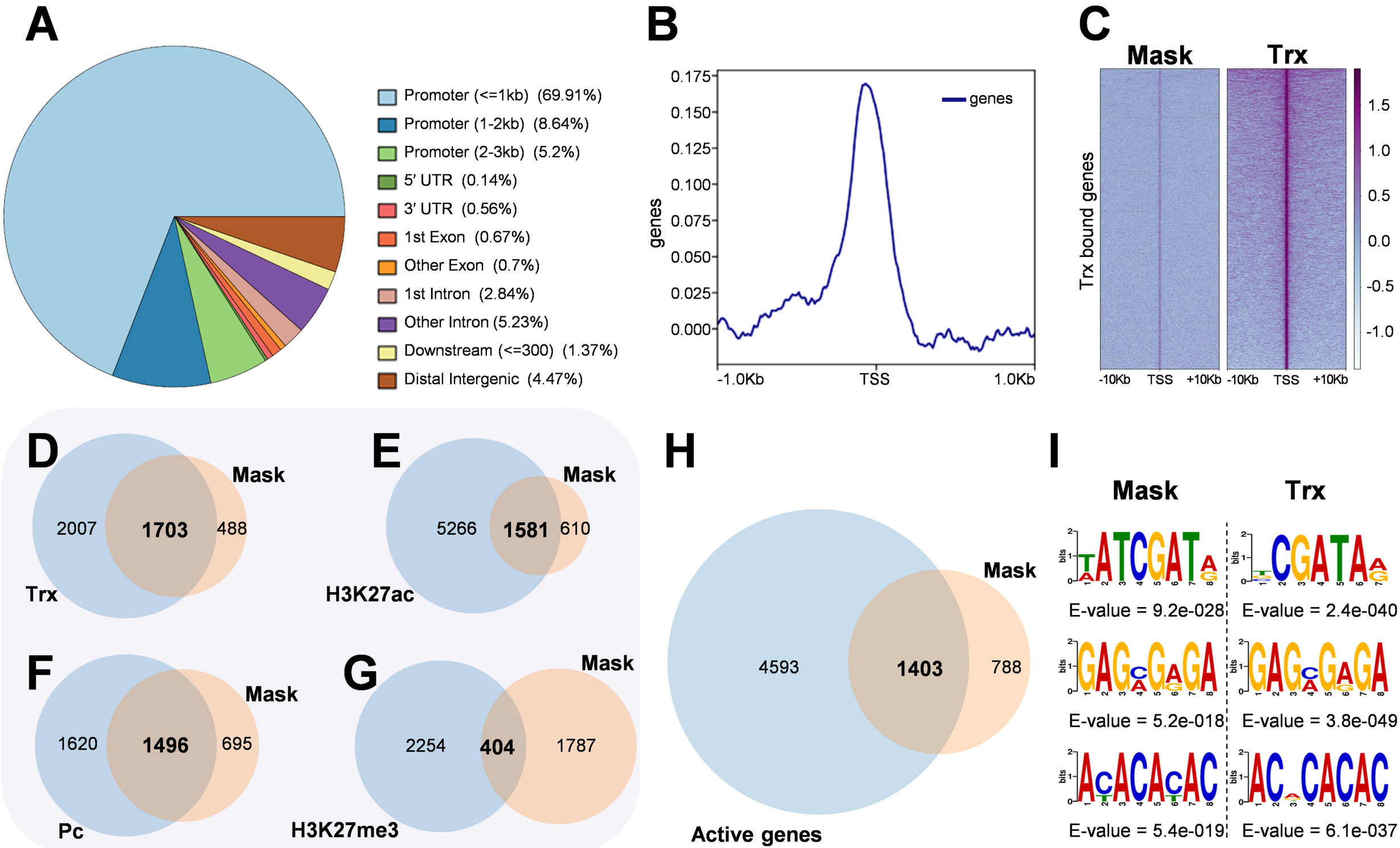

A
Promoter (<=1kb) (69.91%)
Promoter (1-2kb) (8.64%)
Promoter (2-3kb) (5.2%)
5′ UTR (0.14%)
3′ UTR (0.56%)
1st Exon (0.67%)
Other Exon (0.7%)
1st Intron (2.84%)
Other Intron (5.23%)
Downstream (<=300) (1.37%)
Distal Intergenic (4.47%)
B
genes
0.175
0.150
0.125
0.100
0.075
0.050
0.025
0.000
-1.0Kb
TSS
1.0Kb
C
Mask
Trx
Trx bound genes
1.5
1.0
0.5
0.0
-0.5
-1.0
-10Kb
TSS
+10Kb
D
Mask
2007
1703
488
Trx
E
Mask
5266
1581
610
H3K27ac
F
Mask
1620
1496
695
Pc
G
Mask
2254
404
1787
H3K27me3
H
Mask
4593
1403
788
Active genes
I
Mask
Trx
E-value = 9.2e-028
E-value = 2.4e-040
E-value = 5.2e-018
E-value = 3.8e-049
E-value = 5.4e-019
E-value = 6.1e-037

Figure: 3

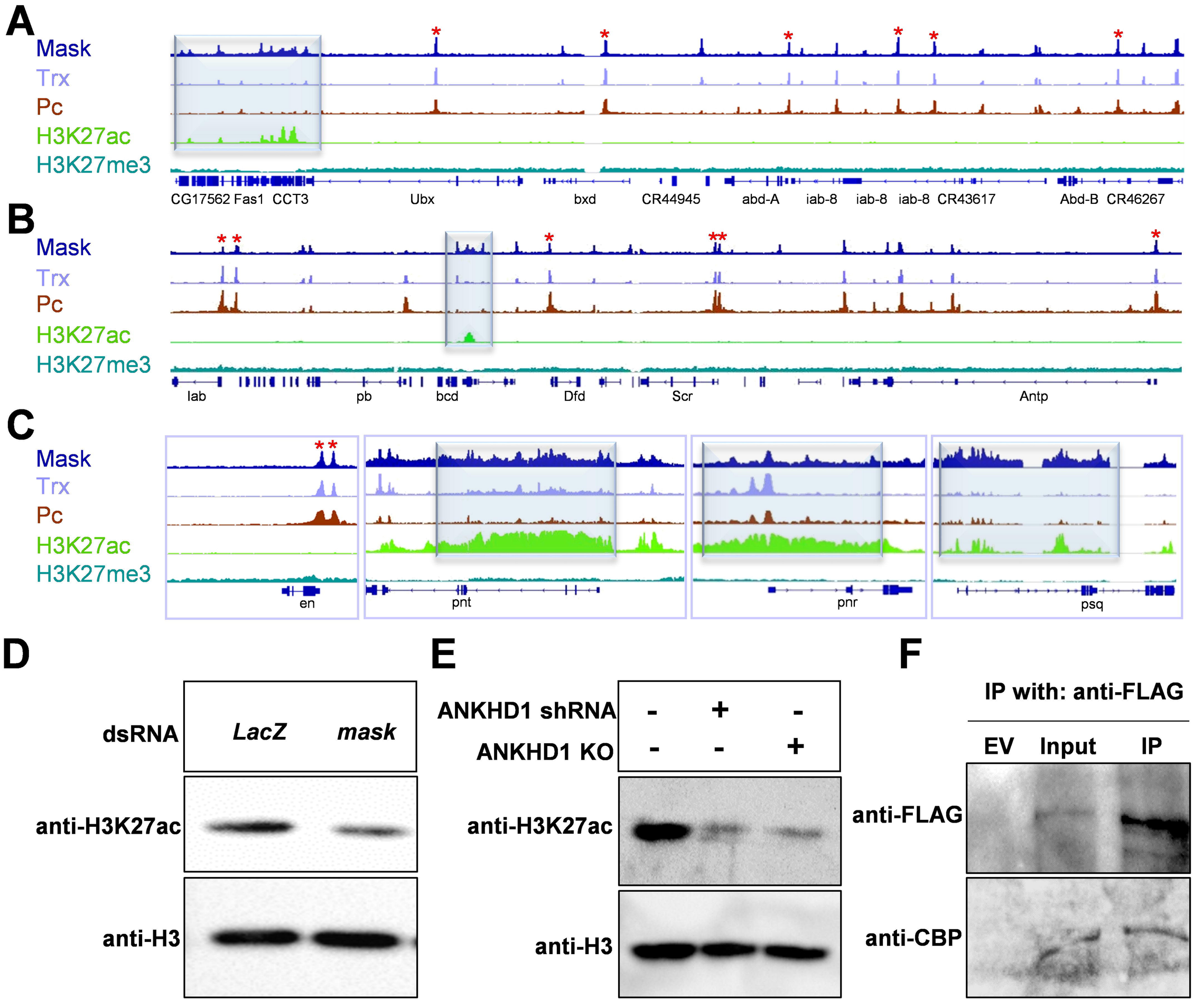
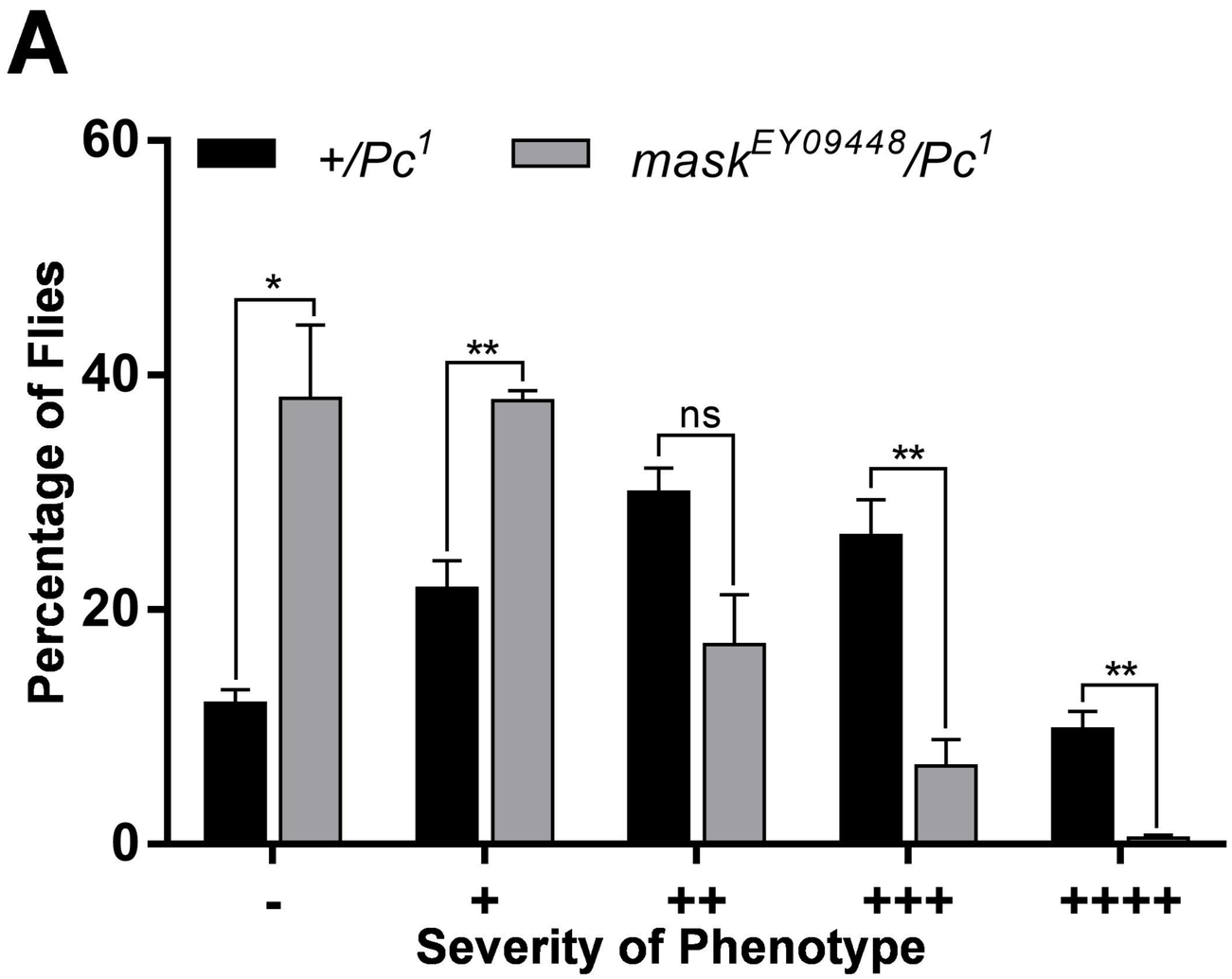
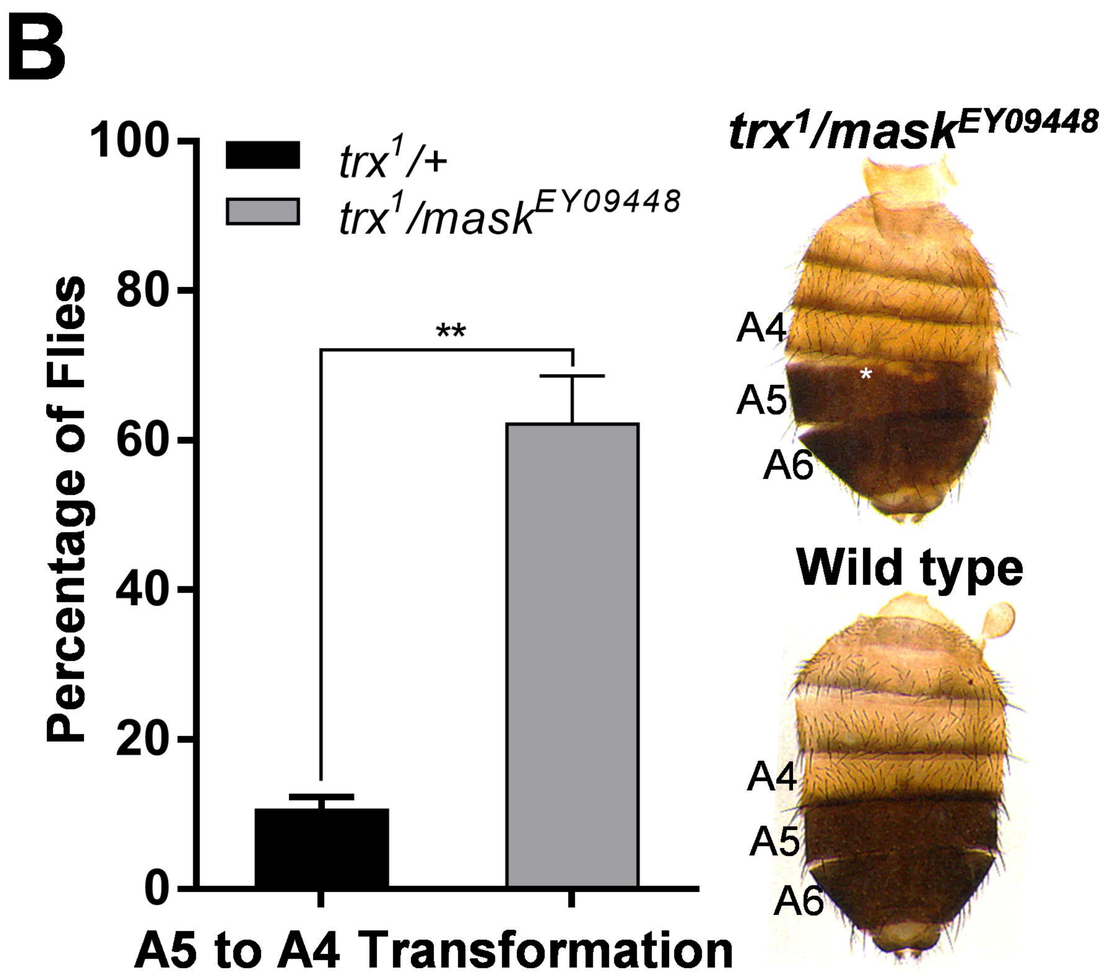
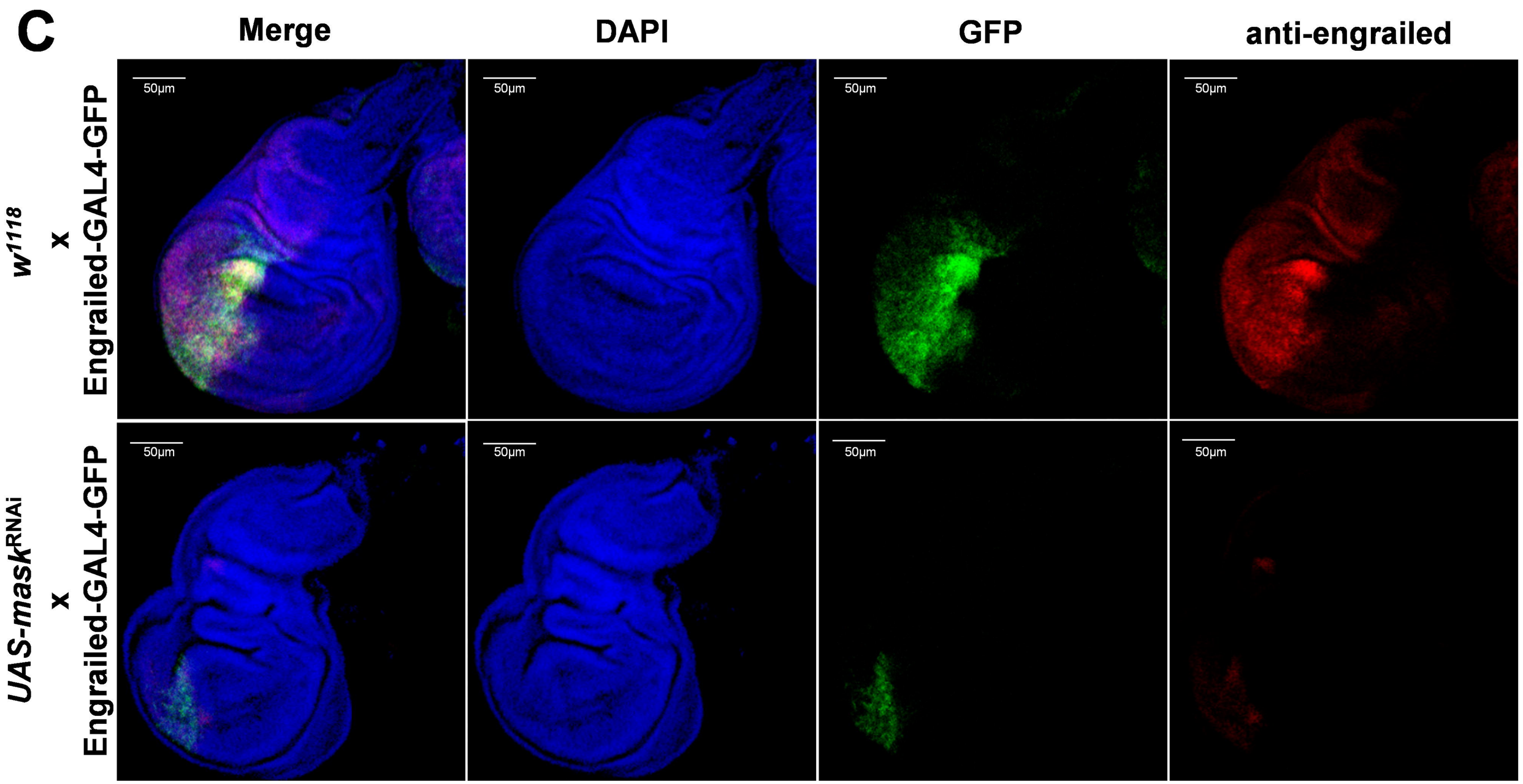

A
Mask
Trx
Pc
H3K27ac
H3K27me3
CG17562 Fas1 CCT3 Ubx bxd CR44945 abd-A iab-8 iab-8 iab-8 CR43617 Abd-B CR46267
B
Mask
Trx
Pc
H3K27ac
H3K27me3
lab pb bcd Dfd Scr Antp
C
Mask
Trx
Pc
H3K27ac
H3K27me3
en pnt pnr psq
D
dsRNA
LacZ
mask
anti-H3K27ac
anti-H3
E
ANKHD1 shRNA - + -
ANKHD1 KO - - +
anti-H3K27ac
anti-H3
F
IP with: anti-FLAG
EV Input IP
anti-FLAG
anti-CBP

Figure: 4


A
+/Pc1
maskEY09448/Pc1
Percentage of Flies
60
40
20
0
*
**
ns
**
**
-
+
++
+++
++++
Severity of Phenotype


B
trx1/+
trx1/maskEY09448
Percentage of Flies
100
80
60
40
20
0
**
A5 to A4 Transformation
trx1/maskEY09448
A4
A5
A6
*
Wild type
A4
A5
A6


C
Merge
DAPI
GFP
anti-engrailed
w1118
x
Engrailed-GAL4-GFP
UAS-maskRNAi
x
Engrailed-GAL4-GFP
50µm