Topoisomerase 3β interacts with RNAi machinery to promote heterochromatin formation and transcriptional silencing in *Drosophila*

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Topoisomerases solve topological problems during DNA metabolism, but whether they participate in RNA metabolism remains unclear. Top3β represents a family of topoisomerases carrying activities for both DNA and RNA. Here we show that in *Drosophila*, Top3β interacts biochemically and genetically with the RNAi-induced silencing complex (RISC) containing AGO2, p68 RNA helicase, and FMRP. Top3β and RISC mutants are similarly defective in heterochromatin formation and transcriptional silencing by position-effect variegation assay. Moreover, both Top3β and AGO2 mutants exhibit reduced levels of heterochromatin protein HP1 in heterochromatin. Furthermore, expression of several genes and transposable elements in heterochromatin is increased in the Top3β mutant. Notably, Top3β mutants defective in either RNA binding or catalytic activity are deficient in promoting HP1 recruitment and silencing of transposable elements. Our data suggest that Top3β may act as an RNA topoisomerase in siRNA-guided heterochromatin formation and transcriptional silencing.
Topoisomerases, known as the “magicians of the DNA world”, can catalyze strand passage reactions for DNA, leading to relaxation of supercoils generated during replication or transcription, and decatenation of tangled DNA during recombination and chromosome segregation. Topoisomerase inactivation can lead to abnormal development, shortened lifespan, lethality, and human diseases.

Unlike the well-characterized DNA topoisomerases, RNA topoisomerases have drawn little attention for many years. The first eukaryotic RNA topoisomerase, human Top3β, was discovered only recently. Since then, RNA topoisomerase activity has been observed in Type IA topoisomerases from all three domains of life. The prevalence of this activity implies that it can provide growth advantage to its host, so that it is retained through millions of years of evolution. The findings also indicate that many Type IA topoisomerases are dual-activity enzymes, capable of solving topological problems for both DNA and RNA. In human, only one of the two Type IA enzymes, Top3β, possesses dual activities, whereas its paralog, Top3α, contains only DNA activity. Top3β but not Top3α contains a conserved RNA-binding domain, RGG-box; and it strongly depends on this domain to bind mRNAs in cells, catalyze RNA topoisomerase reactions, and promote synapase formation. Top3β has been purified in a complex with TDRD3 (Tudor domain-containing 3); and this complex biochemically and genetically interacts with FMRP, an RNA-binding protein (RBP) that is inappropriately silenced in Fragile X syndrome and known to modulate translation of mRNAs important for neurodevelopment and autism. Interestingly, Top3β gene mutation has been linked to schizophrenia and autism, suggesting that Top3β and FMRP may work together to prevent mental dysfunction. Top3β and FMRP bind common mRNAs, associate with mRNA translation machinery, and regulate gene expression at synapse.

In addition to regulating mRNA translation, FMRP also interacts with RNAi machinery to facilitate both transcriptional and post-transcriptional silencing of genes and transposable elements (TEs) in mammals and Drosophila. In Drosophila, FMRP has been purified as a component of an RNAi-induced silencing complex (RISC), containing FMRP, Argonaute 2 (AGO2), p68 RNA helicase (encoded by Rm62), Vg (an RBP), and Tudor-staphylococcal nuclease (Tudor-SN). Mutations in RISC and other components of the RNAi machinery disrupt heterochromatic silencing of genes and TEs.

Here we report the purification of the Top3β–TDRD3 complex from Drosophila S2 cells, and find that it stably associates with RISC. We demonstrate that Top3β mutants display defective heterochromatin formation and transcriptional silencing, which resemble RISC mutants. Moreover, Top3β genetically interacts with RISC to promote heterochromic gene silencing and recruitment of heterochromatin protein HP1. Our data reveal a function for a dual-activity topoisomerase in RNA metabolism.

**Results**

*Drosophila* Top3β–TDRD3 complex associates with FMRP and RISC. We used two different antibodies against *Drosophila* TDRD3, and immunoprecipitated its complex with Top3β from S2 cell lysates. Both antibodies (TDRD3-A and C) isolated two major polypeptides with about equal molar ratio, which were identified as TDRD3 and Top3β by mass spectrometry (MS) (Fig. 1a) and immunoblotting (Fig. 1b). These results are identical to our previous findings for the human complex, and suggest that the complex is conserved in animals.

To identify proteins that associate with the complex, we analyzed the entire TDRD3 immunoprecipitants by MS. We identified peptides from not only FMRP, but also other RISC components—AGO2, p68, and VIG; as well as other FMRP-associated proteins (Fig. 1a). However, the number of peptides derived from RISC were fewer than from either Top3β or TDRD3; and with the exception of FMRP, RISC components were not detectable in the TDRD3 immunoprecipitate by silver-stained analysis. These data suggest that a minor fraction of Top3β–TDRD3 in cells associates with RISC. No peptides from p68 were detected in the immunoprecipitate of TDRD3-A antibody. This could be due to antibody disruption of their association, because this antibody was raised against the region of TDRD3 that binds p68 (see Figs. 1a and 2a). It should be cautioned that although the number of peptides recovered from MS often reflect the protein abundance, they are not always reliable for quantitative comparisons between different samples. We therefore performed IP-Western, which confirmed the presence of several RISC components in TDRD3 immunoprecipitates (Fig. 1b). These data are consistent with our previous findings that Top3β–TDRD3 associates with FMRP in Drosophila, and further suggest that a fraction of Top3β–TDRD3 interacts with the FMRP-containing RISC.

To verify the association, we performed immunoprecipitation (IP) by transfecting Flag-tagged Top3β, a Flag-Top3β mutant deleted of its RGG-box, as well as Flag-TDRD3, into S2 cells; and immunoprecipitated the complex with the Flag antibody. MS detected the peptides from not only Top3β and TDRD3, but also FMRP, AGO2, and p68 (Supplementary Fig. 1A and B). As a control, these RISC proteins were absent in mock-IP using the Flag antibody from S2 cells lacking Flag-Top3β or Flag-TDRD3 (Supplementary Fig. 1B). The results indicate that the association between RISC and Top3β–TDRD3 is specific.

We investigated whether the observed association is mediated by RNA by IP–MS using cell extracts untreated or treated with RNase A. We observed a 10-fold reduction in number of peptides derived from FMRP and p68 in TDRD3 IP from RNase A-treated than -untreated extracts (Supplementary Fig. 1C). Similar reduction in RNase-treated extract was also observed in IP using Flag antibodies (Supplementary Fig. 1B). These results differ from those of human studies, where RNase treatment has little effect on the association between Top3β–TDRD3 and FMRP, suggesting that the Top3β complex may have diverged interactions and functions with RNA during evolution. We noted that in IP–MS by Flag-Top3β or Flag-Top3βΔRGG mutant, the RNase treatment had no significant effect on the number of peptides derived from AGO2, even though the treatment completely eliminated the number of peptides from FMRP and p68 IP (Supplementary Fig. 1B). In IP–MS by Flag-TDRD3 and the two TDRD3 antibodies, the number of AGO2 peptides was reduced by more than 50%, while the number of FMRP and p68 (about 90% or more). The data suggest that AGO2 may have direct protein–protein interactions with Top3β and/or TDRD3 that are not mediated by RNA.

A fraction of FMRP associates with RISC and Top3β–TDRD3. We next performed reciprocal IP–MS with an FMRP antibody, and obtained three abundant polypeptides, which were identified as FMRP and its two known interactors, Caprin and Rin (Fig. 1a). MS analyses of the entire immunoprecipitate obtained peptides from RISC (AGO2, p68), Top3β and TDRD3, supporting the notion that these proteins associate (Fig. 1a; table). Because the number of peptides from RISC, TDRD3, and Top3β were fewer than that of FMRP (about 5% or less), only a small fraction of FMRP may associate with RISC and Top3β–TDRD3.

We also transfected epitope-tagged RISC components into S2 cells, and performed reciprocal IP with the antibody for the
epitope. Silver-staining revealed TDRD3 as a minor polypeptide in the immunoprecipitates of two p68 isoforms (RE and RC) (Fig. 1a). MS identified peptides from RISC, as well as TDRD3 and Top3β (Fig. 1a; table). Immunoblotting further confirmed the presence of these proteins (Fig. 1c). We noted that a previous IP–MS study detected Top3β and TDRD3 in the immunoprecipitate of Flag-AGO2 from S2 cells22. In addition, the Drosophila Protein Interaction Map (DPIM) project reported Top3β and TDRD3 peptides in IP by tagged VIG. Consistent with these results, transfection of epitope-tagged-AGO2 and VIG into S2 cells followed by IP-Western obtained TDRD3 (Fig. 1c). Overall, these data support the notion that a fraction of Top3β–TDRD3 associates with RISC. Because the number of TDRD3 peptides is higher than that of Top3β in the immunoprecipitates of FMRP

Fig. 1 Top3β–TDRD3 stably associates with RISC in Drosophila. a Silver-stained SDS-PAGE analysis of the immunoprecipitated complexes of Top3β–TDRD3, FMRP, p68, and AGO2 from S2 cells; and a partial list of the number of peptides identified by MS analysis of each immunoprecipitate (bottom). The antibodies used for immunoprecipitation are indicated on the top, which include two against TDRD3 (A and C), one for FMRP, and HA antibodies for tagged p68-isoforms (RE and RC) and AGO2. Top3β, TDRD3, components of RISC, and FMRP-associated proteins are indicated by arrows on top and by brackets at the bottom. b IP-Western confirms co-IP of Top3β–TDRD3 with different components of RISC by two TDRD3 antibodies. c Reciprocal IP-Western shows that Top3β–TDRD3 co-IPs with HA-tagged p68, VIG, and AGO2 using transfected S2 cells and HA antibodies. Note that the level of TDRD3 in each IP is higher than that of Top3β, which is consistent with the MS data.
Fig. 2  TDRD3 acts as a scaffold linking Drosophila Top3β to RISC.  

(a) A cartoon shows similarity and differences between human and Drosophila Top3β-TDRD3 complexes (see Results for details).  

(b) Summary of domain-mapping experiment to show different mutants of TDRD3 (left), and their interactions with Top3β and components of RISC (right). The experiment was based on co-IP between different Flag-TDRD3 constructs and their interacting partners from transfected S2 cells. The detailed IP-Western data are in Supplemental figure (Supplementary Fig. 2A). The presence (+), absence (−), or reduction (down arrow), of interactions are indicated.  

c) IP-Western shows that FLAG-Top3β co-IPs with reduced amount of FMRP and p68 in S2 cells depleted with TDRD3 by RNAi. A control experiment was included using RNAi that targets GFP.  

d) IP-Western shows that FMRP co-IPs with reduced amount of Top3β, but normal amount of p68, in S2 cells inactivated of TDRD3 by CRISPR.
and p68 (about 3–10-fold) (Fig. 1a; table), RISC may have more direct interactions with TDRD3 than Top3β.

**Drosophila** TDRD3 acts as a scaffold linking Top3β to RISC. Human TDRD3 acts as a scaffold, interacting with Top3β and FMRP with its N and C terminal domains, respectively (Fig. 2a)3,4. We studied whether Drosophila TDRD3 acts similarly by mutating different regions of Flag-TDRD3 (Fig. 2b), transfecting them into S2 cells, and testing their association with Top3β and RISC by co-IP. First, we found that Drosophila TDRD3 was identical to its human homolog in requiring the same intervening loop within its OB-fold to associate with Top3β (Fig. 2b; Supplementary Fig. 2A and B; Δloop mutant). Second, Drosophila TDRD3 was similar to its human homolog in partial dependence on its Tudor domain for FMRP association (Fig. 2b, Supplementary Fig. S2B; E772K mutant). However, Drosophila TDRD3 was independent of its C-terminal domain (CTD) for FMRP association (Fig. 2a, Supplementary Fig. 2B; N790 (ΔCTD)), whereas human TDRD3 was dependent3,4. Interestingly, the FMRP-interacting motif (FIM) in human TDRD3-CTD was absent in Drosophila TDRD3-CTD. Instead, a region at the N-terminus of Tudor, named TYD motif, was found to have sequence similar to human FIM (Fig. 2b, Supplementary Fig. S2C). Deletion of this motif (ΔTYD) reduced the amount of FMRP that co-IPs with Flag-TDRD3 (Supplementary Fig. 2D), suggesting that this motif could be the Drosophila FIM. Further mapping revealed that TDRD3 has another region that contributes to FMRP association (between 501 and 547) (Fig. 2b, Supplementary Fig. 2A, E, F; see N500 and N547). These data suggest that TDRD3 in human and flies use different mechanisms to bind FMRP.

We mapped an AGO2-binding domain (ABD) to residues 501–547 of TDRD3 (Fig. 2b; Supplementary Fig. 2A, E, F), the same region that also contributes to FMRP association. This region is well conserved in TDRD3 from different fly species, but poorly in vertebrates (Supplementary Fig. 2G). We also mapped a p68-binding domain (PBD) to residues between 281 and 440 (Fig. 2b; Supplementary Fig. 2A, E, F). This region contains a LFDL motif, which is conserved among TDRD3 homologs from flies to human (Fig. 2b, Supplementary Fig. 2H), hinting that the association between Top3β–TDRD3 and the RNA helicase may be conserved in animals.

We performed co-IP using Drosophila TDRD3-deficient cells5, and found that Flag-Top3β co-IPed with reduced amount of FMRP and p68 in TDRD3-depleted cells (Fig. 2c). In addition, FMRP co-IPed with reduced amount of Top3β but comparable amount of p68 in TDRD3-knockout cells (Fig. 2d). These data mimics the findings for human TDRD33,4, and suggest that Drosophila TDRD3 acts as a scaffold linking Top3β to FMRP and p68; but it is dispensable for FMRP–p68 association.

**Top3β and RISC promote heterochromatic gene silencing.** RISC suppresses heterochromatic gene silencing in position effect variegation (PEV) assay15,19–21,24. This prompted us to study if Top3β functions similarly using the same assay (Fig. 3a). Briefly, a PEV reporter that encodes white gene and localizes near heterochromatin can result in variegated red eyes due to spreading of heterochromatin from regions nearby and subsequent transcriptional silencing in some but not all cells. Mutations in histone methyltransferase (Su(var)3-9), RISC, and RNAi biogenesis enzyme Dcr-2, can dominantly suppress this phenotype by inhibiting heterochromatin spreading and gene silencing, leading to eyes with more red cells even uniform red eyes. Consistent with earlier reports, introducing a single mutant allele of AGO2, Rm62 (encoding p68), or Su(var)3-9, into the w1118 reporter line suppressed PEV as they produced uniform red eyes (Fig. 3b, top). Importantly, introducing a single copy of any one of the three Top3β knockout alleles into this line produced similar uniform red eyes (Fig. 3b, top; Supplementary Fig. 3A), indicating that Top3β acts as a dominant suppressor of PEV as does RISC. As a negative control, introducing a wildtype Top3β allele in w1118 white eye mutant background into w1118 line did not alter the eye phenotype (Fig. 3b, Supplementary Fig. 3A; Top3β mutants were derived from w1118).

We then introduced one Top3β-knockout allele into two additional reporter lines in which the white gene is located in different pericentric heterochromatin, and observed similar suppression of PEV (Fig. 3b, middle and bottom). We further tested Top3β using PEV assays based on two non-white reporters, LacZ and S8, which have a distinct location in heterochromatin compared to the white reporters. The advantage of the new reporters is that they can detect modifiers of PEV in tissues and developmental stages different from adult eyes. Notably, a Top3β knockout allele increased the reporter expression and thus suppressed PEV in both assays (Fig. 3c and Supplementary Fig. 3B). Together, Top3β mutation suppressed five PEV reporters in different heterochromatin regions and in different tissues (Fig. 3d), suggesting that Top3β may promote heterochromatin gene silencing throughout Drosophila development.

**Top3β has no effect on post-transcriptional silencing.** We examined whether Top3β can act like RISC in RNAi-mediated post-transcriptional silencing by using a reporter line that induces RNAi to post-transcriptionally silence the white gene expression, leading to white eye phenotype25,26. We found that introducing Top3β mutant in the reporter line did not alter the white eye phenotype, whereas introducing the Dcr-2 mutant produced uniform red eyes (Supplementary Fig. 3C), suggesting that Top3β is dispensable for RNAi-mediated post-transcriptional silencing. The fact that Top3β-KO flies have normal post-transcriptional silencing also argue that the observed effect of Top3β on heterochromatic gene silencing in PEV assay is not due to defective RNAi biogenesis.

**Top3β and RISC genetically interact in PEV assay.** We next investigated if Top3β and RISC genetically interact to suppress PEV by epistasis analysis. We chose the 39C-2 as the reporter, because PEV suppression in this line by Top3β mutant generated about 50% of red cells, suitable for detecting either a suppressor or enhancer by the second mutation (Fig. 4a).

First, the introduction of the double heterozygous mutant of Top3β−/−;AGO2−/− into 39C-2 line enhanced PEV, evidenced by a decrease of the percentage of red cells and appearance of the white eye (Fig. 4b). This result contrasts with those of their single heterozygous mutants, which produce more red cells and thus suppression of PEV (Fig. 4a, b). Extraction of the eye pigment and subsequent measurement confirmed that the red color was decreased in the double but increased in each single mutant (Fig. 4b, right graph). These data suggest that Top3β and AGO2 interact genetically during heterochromatin silencing. We also analyzed interactions between Top3β and AGO1, which is a homolog of AGO2 but functions in the miRNA pathway. We found that the Top3β−/−;AGO1−/− double mutant suppressed PEV similarly as the AGO1−/− single mutant did (Fig. 4c). This result differs from that of the Top3β−/−;AGO2−/− double mutant that enhanced PEV, indicating that the genetic interaction between Top3β and AGO2 is specific.

Second, the introduction of Top3β−/−;Rm62−/− double heterozygous mutant into the reporter line enhanced PEV (Fig. 4d). This phenotype differs from that of their respective single mutants, but resembles that of Top3β−/−;AGO2−/− double
mutant, suggesting that Top3β genetically interacts with both AGO2 and Rm62 in heterochromatin silencing.

Third, the introduction of Top3β<sup>−/−</sup>;Fmr1<sup>−/−</sup> double mutant into 39C-2 line suppressed PEV similarly as their respective single mutants (Fig. 4e), suggesting that Fmr1 does not have the same genetic interactions with Top3β as AGO2 and Rm62 do, and that the function of FMRP in RISC is different from that of the other two proteins.

Fourth, the introduction of Top3β<sup>−/−</sup>;Dcr-2<sup>−/−</sup> double mutant into the reporter line enhanced PEV (Fig. 4f). This phenotype
differs from their respective single mutants, but mimics those of Top3β−/+;AGO2−/+ and Top3β−/-;Rm62−/+ double mutants. These results support a model that siRNAs produced by Dcr-2 form complexes with AGO2, and these siRNA-loaded RISC complexes genetically interact with Top3β to promote heterochromatin formation and gene silencing.

**Heterochromatin is abnormal in Top3β mutant flies.** RISC mutants have aberrant cellular distribution of heterochromatin markers, including HP1 and H3K9me2. We analyzed these markers by immunostaining salivary glands of the Top3β mutant. In agreement with previous data, both markers displayed concentrated staining in the control flies (Supplementary Fig. 4A, B),
which are due to clustering of pericentric heterochromatin of different chromosomes. Notably, both markers showed less concentrated staining in Top3β mutant. As a control, histone H3 showed similar staining in both mutant and the control flies. Quantification confirmed that the ratios between the signals of HP1 or H3K9me2 versus that of H3 were reduced in Top3β mutant. Immunoblotting revealed that Top3β mutant had normal levels of the two markers (Supplementary Fig. 4C), indicating that Top3β mutation does not affect HP1 synthesis or global H3K9 methylation. Together, the results imply that Top3β mutation may disrupt HP1 recruitment and H3K9 methylation in pericentric heterochromatin, which is consistent with the PEV data.

Top3β and RISC promote HP1 recruitment to heterochromatin. We next performed HP1 ChIP-seq (chromatin immunoprecipitation followed by high-throughput sequencing) to identify the defective heterochromatin in Top3β and AGO2 mutants at the molecular level. Our analyses were focused on chromosomes 2 and 3, because their pericentric heterochromatin is long and distinguishable from euchromatin.

Consistent with earlier reports, the HP1 signals of HP1 were enriched in pericentric heterochromatin in control and Top3β mutant flies, after normalization with their respective input signals (Supplementary Fig. 5A, top). Then we calculated the difference in HP1 signals between each mutant and the control (w1118) using the SICER (Spatial-clustering method for Identification of CHIP-Enriched Regions) program, and displayed the HP1-decreased islands (HDIs) and HP1-increased islands (HIIs) by blue or red lines, respectively, in bedgraphs. The analysis revealed much more HDIs (blue) than HIIs (red) in pericentric heterochromatin of both Top3β and AGO2 mutants (Fig. 5a; Supplementary Fig. 5B). There was also an HDI at telomeric heterochromatin of chromosome 3L (Fig. 5a). Bedgraphs of sequencing reads in 100-bp windows across two telomeric and one representative pericentric heterochromatin regions revealed that many HP1 peaks present in the control flies are concomitantly reduced in both Top3β and AGO2 mutants (Fig. 5b–d), which is consistent with the SICER data and suggests that Top3β and RISC can work together to recruit HP1 to the same loci. The reduction of HP1 signals was confirmed by ChiP-qPCR for two representative pericentric heterochromatin loci (see Fig. 6d, e).

More defective HP1 recruitment in AGO2 than Top3β mutant. We observed that in pericentric heterochromatin, there are more HDIs in AGO2 than Top3β mutant (Fig. 5a, Supplementary Fig. 5B). In addition, the height of HP1 peaks was reduced to a greater extent in the AGO2 than Top3β mutant (Fig. 5a, Supplementary Fig. 5B). These data suggest that the effect of RISC on HP1 recruitment is stronger than that of Top3β.

Quantification further revealed the differential effects of the two mutants on HP1 recruitment. First, the sum of HDIs represent 23.6% and 43.3% of all HP1 islands in Top3β and AGO2 mutants, respectively; which are about 500- and 100-fold higher than the percentages of HIIs (0.04% and 0.4%, Fig. 5e), respectively; indicating that the predominant effect of Top3β and RISC on heterochromatin is to increase HP1 recruitment. However, over 55% of HP1 islands showed no change in either mutant, arguing that Top3β and RISC are necessary for recruiting HP1 to some but not all loci. Second, the sum of HDIs accounted for 13.6% and 37.0% of the entire length of pericentric heterochromatin in Top3β and AGO2 mutants, respectively (Fig. 5f), consistent with the SICER bedgraphs showing more HDIs in AGO2 than Top3β mutant (Fig. 5a, Supplementary Fig. 5B). Of all the HDIs, 3.6% was present in Top3β mutant only, 27% in AGO2 mutant only, whereas 10% were present in both (Fig. 5f). The HDIs present in both mutants represented 74% and 27% of the total HDIs in Top3β and AGO2 mutants, respectively. The data suggest that a large fraction of Top3β and a small fraction of RISC may work together to recruit HP1 to the same loci, whereas a small fraction of Top3β and majority of RISC can act independently to recruit HP1.

Top3β and AGO2 genetically interact for HP1 recruitment. To study whether Top3β and AGO2 genetically interact in pericentric heterochromatin formation as suggested by PEV data, we analyzed their double mutant by HP1 ChIP-seq. The number of HDIs (blue) in pericentric heterochromatin was fewer in the double mutant than those of their single mutants (Fig. 5a, Supplementary Fig. 5B). In addition, many HP1 peaks reduced in each single mutant were not reduced in the double mutant (Fig. 5d, Supplementary Fig. 5C). Quantification of SICER data showed that while HDIs in each single mutant accounted for >20% of total HP1 islands, the corresponding percentage in the double mutant was 1.2%, a decrease of 20-fold or more (Fig. 5e). Concomitantly, the percentage of HP1 islands of no change was increased in the double mutant (98.6%) when compared to their single mutants (76.4% and 56.3%). Moreover, the ratio between HDIs and HIIs is about 6 for the double mutant, which is considerably smaller than those of Top3β and AGO2 single mutants (about 500 and 100, respectively) (Fig. 5e). These data suggest that the defective heterochromatin phenotype is largely suppressed in the double mutant. The data are reminiscent of those of the PEV assay (Fig. 4a, b), suggesting that Top3β and RISC genetically interact to promote HP1 recruitment to pericentric heterochromatin.

Top3β, AGO2, and HP1 binding sites overlap in heterochromatin. If Top3β and RISC directly promote HP1 recruitment, their binding sites on chromatin may overlap with those of HP1. To test this hypothesis, we identified genome-wide Top3β and AGO2 binding sites in fly heads by ChIP-seq. For AGO2 ChIP, we utilized a fly line that has a Flag-tag knocked-in and fused in-frame to the endogenous AGO2. SICER analysis showed that AGO2 and Top3β ChIP islands are present in heterochromatin at much lower frequency and scores than those of HP1 (Supplemental Fig. 6A; HP1 islands used a scale 100-fold higher than the others). Specifically, the percentages of AGO2 and Top3β ChIP islands in total heterochromatin were about 0.04% and 0.4%, respectively; indicating that the predominant effect of Top3β and RISC on heterochromatin is to increase HP1 recruitment. However, over 55% of HP1 islands showed no change in either mutant, arguing that Top3β and RISC are necessary for recruiting HP1 to some but not all loci. Second, the sum of HDIs accounted for 13.6% and 37.0% of the entire length of pericentric heterochromatin in Top3β and AGO2 mutants, respectively (Fig. 5f), consistent with the SICER bedgraphs showing more HDIs in AGO2 than Top3β mutant (Fig. 5a, Supplementary Fig. 5B). Of all the HDIs, 3.6% was present in Top3β mutant only, 27% in AGO2 mutant only, whereas 10% were present in both (Fig. 5f). The HDIs present in both mutants represented 74% and 27% of the total HDIs in Top3β and AGO2 mutants, respectively. The data suggest that a large fraction of Top3β and a small fraction of RISC may work together to recruit HP1 to the same loci, whereas a small fraction of Top3β and majority of RISC can act independently to recruit HP1.

**Fig. 4** Top3β and RISC genetically interact to promote heterochromatin gene silencing by PEV assay. a Representative eye images show suppression of PEV reporter 39C-2 by heterozygous mutation of Top3β or AGO2. b Representative eye images (left), and pigment quantification assay (right), show that double heterozygous mutants of Top3β and AGO2 enhance PEV (white eyes) for the 39C-2 reporter line. c Same as b, except that mutants of Top3β and AGO2 were analyzed. d Same as a, except that Top3β and Rm62 mutants were analyzed. e Same as b, except Top3β and fmr1 mutants were analyzed. f Same as b, except Top3β and Dcr-2 mutants were analyzed. Several images of control and single heterozygous mutants were copied from Fig. 3b for the convenience of readers. The asterisks in graphs mark p-values less than 0.05 by Student T-test. n.s. represents statistically non-significant p values (p > 0.05). The error bars are obtained by standard error method. The results are obtained from three independent experiments.
0.3% and 1.4%, respectively (Fig. 5g), which are substantially smaller than that of HP1 (72%), as well as that of HDIs in total heterochromatin of AGO2 and Top3β mutants (37% and 13.6%, respectively; Fig. 5f). The data are consistent with a mechanism of "nucleation and spreading": RISC and Top3β may bind a small number of loci to nucleate the initial assembly of heterochromatin, which may then recruit additional components to spread to other regions.

Interestingly, almost all AGO2 islands (98%) in pericentric heterochromatin overlap with those of HP1 (Fig. 5g), supporting a role of chromatin-bound RISC in HP1 recruitment. In contrast, a much smaller percentage of Top3β islands (8%) overlap with those of HP1 (Fig. 5g) or AGO2 (Supplemental Fig. 6B), arguing that a minor fraction of chromatin-bound Top3β may be involved in the same process. Inspection of selected regions in heterochromatin revealed that some HP1-reduced peaks in Top3β
and AGO2 mutants overlap with AGO2 and Top3β binding sites (Fig. 5b–d), supporting the notion that RISC and Top3β work together to promote HP1 recruitment. 

**Top3β and AGO2 recruit HP1 to some loci in euchromatin.** Our HP1 ChIP assay detected a small number of HDIs and HIIs in euchromatin of Top3β and AGO2 mutants (Fig. 5a and Supplementary Fig. 5B). Notably, the percentage of HDIs (1.3–2.1%) was about 40–50-fold higher than HIIs (0.03–0.04%) (Supplementary Fig. 5D), suggesting that Top3β and RISC can also promote HP1 recruitment to euchromatin, albeit at fewer loci than heterochromatin. Some HDIs were concomitantly present in single and double mutants of Top3β and AGO2 (Fig. 5a and Supplementary Fig. 5B–C), suggesting that Top3β and RISC may work in the same pathway in HP1 recruitment at these loci. In agreement with this notion, a fraction of AGO2 and Top3β ChIP islands were found to overlap with those of HP1 and each other in euchromatin (Supplementary Fig. 6C and D); and inspection of representative euchromatin regions revealed that some AGO2 and Top3β binding sites overlap with HP1-reduced islands in Top3β and AGO2 mutants (Supplementary Fig. 5C).

We noticed that majority of Top3β and AGO2 ChIP islands are localized in euchromatin (Supplementary Fig. 6A). This is in contrast to HP1 islands, most of which are in heterochromatin, implying that majority of Top3β and RISC have functions in euchromatin that are unrelated to HP1 recruitment. Indeed, only a minor fraction (16%) of AGO2 islands overlap with those of HP1 in euchromatin (Supplementary Fig. 6C), which is much smaller than the 98% observed in heterochromatin, consistent with previous findings that AGO2 can function independent of HP1 recruitment.

**Top3β promotes H3K9 methylation.** HP1 is known to bind H3K9me3 in heterochromatin. Our data that H3K9me2 immunostaining is reduced in the Top3β mutant prompted us to investigate whether H3K9 methylation is decreased in pericentric heterochromatin by ChIP-seq. We observed enrichment of H3K9me2 and H3K9me3 in pericentric heterochromatin, which is similar to that of HP1 (Supplementary Fig. 5A). SICER analysis revealed more decreased (blue) than increased (red) islands for both methylation marks in pericentric heterochromatin of the Top3β mutant (Fig. 6a and Supplementary Fig. 7), indicating that Top3β is needed for normal H3K9 methylation in heterochromatin. The percentages of islands with decreased H3K9me2 and H3K9me3 signals were 7.0% and 4.6%, respectively, among all their respective islands (Fig. 6b), which are about 18- and 23-fold higher than percentages of the islands with increased signals (0.4% and 0.2%, respectively; Fig. 6b, c). These differences are smaller than that of HP1 (~500-fold) (Fig. 5e), suggesting that Top3β is more important for HP1 recruitment than H3K9 methylation.

We then selected two representative loci from pericentric heterochromatin of 3L and 2R based on the presence of HDIs (Fig. 6d, e; left), and found that both loci exhibited concomitant reduction of H3K9me2 and H3K9me3 signals by bedgraph analyses and ChIP-qPCR (Fig. 6d, e; right). Together, these data suggest that Top3β is needed for HP1 recruitment and H3K9 methylation at the same loci in heterochromatin. We observed smaller changes in H3K9me2 and H3K9me3 islands in euchromatin when compared to those in heterochromatin (Fig. 6a, b; Supplementary Fig. 7). The ratios between the percentages of decreased and increased islands are 0.7 and 1.1 (Fig. 6c), respectively, which are considerably smaller than the 18- and 23-fold ratios for the two marks in heterochromatin (Fig. 6b). These data suggest that the major effect of Top3β on HP1 recruitment and H3K9 methylation is in heterochromatin. 

**Top3β mutant displays defective transcriptional silencing.** As defective heterochromatin formation often leads to defective silencing of genes and TE, we investigated whether Top3β mutant has this defect at several heterochromatin loci where HP1 level is reduced. In the telomeric region of chromosome 3L, three neighboring genes (Lsp1y, CG13405, and mthl8) were found to be upregulated by RNA-seq, microarray assays (Fig. 7a), and RT-qPCR (Fig. 7b). In addition, seven TEs located between mthl8 and Lsp1y were also found to be upregulated in the Top3β mutant (Fig. 7c). As a control, a gene located outside of this locus (pk61c) showed no obvious change. Similarly, in the telomeric region of chromosome X, two neighboring genes were upregulated (Supplementary Fig. 8A, 8B). Moreover, RNA-seq and RT-qPCR revealed that two TEs located in pericentric heterochromatin, Doc and gypsy1, are consistently de-silenced in Top3β mutant flies (Fig. 7d, e). Together, these data support our PEV data that Top3β is needed for transcriptional silencing.

**Top3β function requires RNA binding and catalytic activity.** We have previously shown that Top3β depends on both its RNA binding and catalytic activities to promote synapse formation using transgenic rescue experiment. Here we used the same strategy to study whether Top3β depends on the same activities to mediate heterochromatin formation and transcriptional silencing. Briefly, we transgenically expressed in the Top3β mutant background the wildtype and two mutant versions of Top3β-a catalytic point mutant (Y332F) that contains normal mRNA binding
activity but lacks topoisomerase activity for both DNA and mRNA, and an RGG box-deletion mutant (ΔRGG) that has strongly reduced mRNA binding, as well as decreased topoisomerase activity for both nucleic acids (Fig. 8a, b)⁴,⁸. ChIP assays revealed that the reduced HP1 levels at the two representative loci in pericentric heterochromatin in the Top3β mutant (Fig. 6d, e) were rescued by the wildtype but not two mutants of Top3β (Fig. 8c, d). In addition, Top3β wildtype transgene increased HP1 signals at a telomeric locus of X chromosome to a level that is higher than that of the control line, whereas its two mutants

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**Fig. 6** Top3β promotes H3K9 methylation and HP1 recruitment in heterochromatin. a Bedgraphs show differences of SICER scores of H3K9me2 and H3K9me3 ChIP-seq data between Top3β mutant versus the control (w¹¹¹⁰) for chromosome 3 (Y-axis). Islands with decreased and increased signals are marked by blue and red, respectively. b A graph shows the percentage of H3K9me2 or H3K9me3 islands that are decreased (blue), increased (red), or of no change (gray) in heterochromatin (Het) or euchromatin (Eu) of Top3β mutant when compared to the control. The quantification is based on SICER analysis from 3 independent ChIP experiments. c A graph shows the ratio of islands with decreased over those with increased signals of HP1, H3K9me2, and H3K9me3 in heterochromatin (red) and euchromatin (blue) of Top3β mutant when compared to the control. The quantification for HP1 is based on 3 ChIP-seq experiments. d, e Bedgraphs of ChIP-seq data (left) and graphs of RT-qPCR (right) show that two representative pericentric heterochromatin regions on chromosome 3L and 2R have decreased signals of HP1, H3K9me2, and H3K9me3 in Top3β mutant when compared to the control. The red box marks the region in which 2 primer pairs for qRT-PCR were selected. A pair of control primers was selected from the euchromatin of the corresponding chromosomes. The results of RT-qPCRs were from triplicates. The error bars are obtained by standard error method.
were defective (Fig. 8e). These data suggest that Top3β depends on both RNA-binding and catalytic activities to promote HP1 recruitment to specific loci in heterochromatin.

We also found that the two TEs de-silenced in the Top3β mutant (Doc and gypsy1, Fig. 7c) were largely repressed by transgenic expression of the wildtype Top3β (Fig. 8f). In contrast, this repression was reduced by transgenic expression of the two mutant proteins (Fig. 8f). These data suggest that Top3β depends on its RNA-binding and catalytic activities for transcriptional silencing.

**Discussion**

Recent evidence suggests that Top3β has two functions: one for DNA, where it reduces negative supercoiling to resolve R-loops; and the other for mRNAs, where it associates with polyribosomes and FMRP to regulate translation. Here we show that Top3β has an additional function—it interacts with RISC to facilitate heterochromatin formation and transcriptional silencing. Because Top3β is a dual-activity enzyme, one key question is whether it acts on DNA or RNA during this process. Our findings that Top3β mutant deleted of its RNA-binding domain (RGG-box) is defective in promoting HP1 recruitment to heterochromatin and in silencing of TE s suggest that Top3β acts on RNA. This notion is further supported by the data that Top3β biochemically and/or genetically interacts with an RNA slicer (AGO2), an RNA helicase (p68), and an RNase (Dcr-2). However, because the RGG-box deletion mutant not only has defective RNA-binding activity, but also reduced catalytic activity for both DNA and RNA, it is possible that the reduced catalytic activity on either DNA, or RNA, or both, is responsible for the defective heterochromatin formation. A mutation that inactivates the catalytic activity of Top3β on one nucleic acid but not the other is needed to clarify this issue.

Another question is whether Top3β acts as a topoisomerase or an RNA-binding protein. Our findings that Top3β-Y332F mutant, which has normal RNA binding but no catalytic activity, is defective in promoting HP1 recruitment and TE silencing suggest that Top3β acts as a topoisomerase. The next question is where the topological problem may come from? The current hypothesis on heterochromatin formation postulates that siRNA-loaded RISC may guide histone methyltransferases and HP1 to
their targets by base-pairing interactions with nascent RNAs transcribed from the heterochromatin locus. We predict that this process may produce RNA topological problems at some loci, which depend on Top3β to resolve. Possibly, the nascent RNA interacts with histone methyltransferase and is needed for heterochromatic gene silencing. The biochemical and genetic interactions between a Type 1A topoisomerase and helicase have been similarly observed for Top3α and BLM: their enzymatic activities are coupled to resolve complex DNA structures. Based on this similarity, we hypothesize that Top3β and p68 may employ a mechanism mimicking their DNA counterpart—their enzymatic activities may be coupled to resolve complex RNA structures during heterochromatin formation (Fig. 9). p68 may unwind secondary structures in nascent RNA transcribed at heterochromatin. This unwinding may generate topological
stress, which may be relieved by Top3β to enable p68 to continue the unwinding reaction, making nascent RNA accessible to binding by siRNA-loaded RISC, and enabling subsequent recruitment of histone methyltransferases and HP1. When either p68 or Top3β is mutated, this process is defective, leading to defective heterochromatin, and increased white gene expression in PEV assay. However, this hypothesis is difficult to explain why the double mutant between Rmi62 and Top3β exhibits suppression of the PEV phenotype observed in each single mutant. We speculate that when both p68 and Top3β are mutated, alternative pathways may be activated to form heterochromatin. One alternative pathway may be mediated by PIWI44, which can interact with FMRP to promote heterochromatin silencing in somatic cells45. Future work is needed to show whether activities of Top3β and p68 are coupled, and to identify the alternative pathway(s).

Finally, our findings that Top3β depends on both RNA binding and catalytic activities for recruiting HP1 and silencing TE’s are reminiscent of our previous data that Top3β requires the same two activities to promote synapse formation5, suggesting that these Top3β-mediated processes may be connected. Indeed, misregulation of heterochromatin and TEs has been linked to abnormalities in brain development, schizophrenia, other neurological disorders46, and aging47. Notably, individuals carrying Top3β mutations have increased risks of schizophrenia and other mental disorders48, and Top3β mutant animals exhibit abnormal neurodevelopment and shortened lifespan48. Thus, regulation of heterochromatin formation and transcription silencing could be one mechanism used by Top3β to prevent mental dysfunction and shortened lifespan.

Methods

*Drosophila* stocks and genetics. Flies were cultured on corn syrup-soy recipe food from Archon Scientific at 25 ± 1 °C and 60 ± 5% humidity, under a 12 h/12 h light/dark cycle, except for the PEV assay, which was performed at 22 °C. Top3β e-p element remobilized mutants Top3βp1, Top3βp16, and Top3βp17 were a generous gift from T. Hsueh55. Su(var)3-9(T00616, Dcr-2(RGG00045; 3ChIP-seq) PEVE line 39C-2, 39C-12, 118E-10, and 118E-28 were a generous gift from S. Elgin, yw[Dat(Y)]BLYL2/PiHS-lacZ66; w1118,AGO2[2]/TM3, SbR(23)StR69, Ir[3]M0.362 w/+/TM3, S. Serl, cr[4]P{f7-17Z}II=(P2)AGO-p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AGO}p{AG}
Unwinding of the nascent RNA by p68 helicase generates physical stress that blocks the unwinding

Fig. 9 Top3β relieves the stress and enables unwinding by p68

siRNA-loaded RISC binds nascent RNA and recruits heterochromatin components

Some Nascent RNAs form higher order structures that prevent base-pairing with siRNA-loaded RISC.

Unwinding of the nascent RNA by p68 helicase generates physical stress that blocks the unwinding of heterochromatin formation by interacting with RNAi machinery. A model illustrating how Top3β works with RISC in heterochromatin formation and transcriptional silencing. The nascent RNA transcribed from heterochromatin may form secondary and other structures by base-pairing with itself, which prevents access by siRNA-loaded RISC. These RNA structures are unwound by p68 helicase of RISC, and this process may produce torsional stress. Top3β may relieve this stress by facilitating the unwinding process and makes nascent RNA accessible to base pairing by siRNA-loaded RISC. Binding of RISC to the nascent RNA may recruit HP1 and H3K9 methyltransferases to assemble heterochromatin. The coordinated actions of p68-Top3β in resolving complex RNA structures may resemble those of their homologs, BLM-Top3α, in resolving complex DNA structures that prevent base-pairing with siRNA-loaded RISC. These RNA structures are unwound by p68 helicase of RISC, and this process may produce torsional stress. Top3β may relieve this stress by facilitating the unwinding process and makes nascent RNA accessible to base pairing by siRNA-loaded RISC.
Immunostaining. The wandering third instar larvae were washed in 1× PBS and dissected for the salivary glands. The glands were fixed in 4% paraformaldehyde (pH 7.6) for 20 min at room temperature. After washing four times, the samples were incubated in 0.1% Triton X100 and 1% normal goat serum in 1× PBS for 30 min at room temperature for permeabilization. The primary antibodies (α-mouse HP1—DSHB CA19-1250; α-mouse H3K9me2—abcam ab12120 1:200; α-rabbit H3—abcam ab1791 1:200) and secondary antibodies (1:400) were incubated in 0.1% Triton X100 and 1% NGS overnight at 4°C. Washing was performed three times in 10 min intervals after each incubation with 0.1% Triton X100 and 1% NGS. Preparations were examined with Zeiss LSM-710 confocal laser scanning microscopy. For quantification of HP1 and H3K9me2, respective channels were separated and the intensity per area was measured using ImageJ.

PEV, eye pigment, LacZ, and bristle quantification. PEV assays using different white reporter have been described18,58,59. All crosses were performed at 25 °C except for w*64b at 22 °C. The phenotypes were examined 48 h after eclosion. For eye color quantification, 10 fly heads were homogenized in 0.1% HCl in methanol. After incubating overnight at 4 °C, the absorbance of the cleared supernatant was measured at 480 nm. The data were obtained from at least three independent experiments.

The PEV assay with a LacZ reporter was performed using a published protocol60. To summarize, crawling 3rd instar larvae were heat-shocked at 37 °C for 30 min and at least 10 larvae per sample were analyzed each time. The PEV assay based on the SB reporter was performed using a published protocol18. w1118 and Top3p26 females were crossed to male T(2;3)SbV, In(3R)Mo, SbV sr1/TM3, Ser1,c n1. The male progenies were analyzed by counting 7 pairs of major dorsal macrochaetes. The assay was done three times counting at least 20 flies for each trial. The RNAi-mediated post-transcriptional silencing assay was performed using a published protocol25. The reporter line (GMR-wir), which expresses an inverted repeat targetting white, was crossed with Top3p or Dcr-2 mutant, and the eye phenotype was examined for more than 20 flies in each genotype.

Data availability
All relevant data supporting the key findings of this study are available within the article and its Supplementary Information files or from the corresponding author upon upon reasonable request. The next-generation sequencing and microarray data have been deposited at GEO database (GSE119736 and GSE119185).

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