Supplementary information

Materials and Methods

Plasmid construction

DNA fragments corresponding to Atro, ATN1, RERE, RERE (281-1566), RERE (361-1566), and dG9a were cloned into the EcoRV site of the pCMX-Flag or pCMX-NFlag vector to generate the corresponding plasmids. Flag-RERE (481-1566), -RERE (281-360), -RERE (361-480), -RERE (281-480), -MTA1 (161-339), -MTA2 (141-319), and -MIER1 (199-364) were generated by inserting the corresponding DNA fragment into the EcoRI and BamHI sites of the pCMX-NFlag vector. In pCMX-NFlag, Flag is tagged with a nuclear localization signal. EcoRI and SalI sites were used to generate NFlag-MTA3 (144-322). Both G9a and SET9 were cloned into the EcoRI and BamHI sites of pCMX-Flag vector. Atro was cloned into the SmaI site of the pCMX-CFP vector. RERE (281-360), RERE (361-480), and RERE (281-480) were cloned into the EcoRI and SmaI sites of the pGEX-4T1 vector to generate the corresponding plasmids for expressing GST-fused proteins. Constructs for *Atro.IR1 and Atro.IR2* were generated by cloning *XbaI* digested fragments corresponding to the 301-1098 and 1129-1689 regions of *Atro* into the pWIZ vector (Lee and Carthew, 2003). All of the DNA fragments were obtained by PCR reaction, using either EST clones purchased from Open Biosystems or cDNA libraries from Clontech/BD Biosciences as templates.

Coimmunoprecipitation

10ug of plasmid encoding each Flag-tagged protein was transfected into HEK293 cells by lipofectamine 2000 (Invitrogen). 24 hours after transfection, the cells were lysed with lysis buffer (50mM Tris-Cl pH8.0, 150mM NaCl, 10% glycerol, 0.5% NP-40, complete protease inhibitor (Roche)) at 4°C for 30 minutes. After a 30-second sonication, the lysate was cleared by centrifugation at 14000rpm at 4°C for 15 minutes and was incubated with anti-Flag-M2 agarose affinity gel (Sigma) overnight. The agarose gel was washed 6 times with washing buffer (50mM Tris pH8.0, 150mM NaCl, 0.5% NP-40, complete protease inhibitor) and resuspended with SDS loading buffer for western blot. Antibodies used in western blot are as follows: anti-G9a (gift from Dr. Nakatani) (Ogawa et al., 2002), anti-SET9 (Upstate Biotechnology, 07-314), anti-HDAC1 (Santa Cruz, sc-7872), anti-HDAC2 (Santa Cruz, sc-7899), anti-GFP (Invitrogen, A-11122), anti-Flag (Sigma, F7425).

Coimmunoprecipitation on salivary gland

Two hundred pairs of salivary glands from the third instar larvae of *Hsp70::Atro* flies were dissected in PBS with 0.1% Triton X-100, and then homogenized in lysis buffer (50mM Tris-Cl pH8.0, 150mM NaCl, 10% glycerol, 0.5% NP-40, complete protease inhibitor). After sonication for 30 seconds, the lysate was cleared by centrifugation at 14000rpm for 15 minutes at 4^{o}C. The antibody bound protein A agarose was prepared by mixing with rabbit IgG or rabbit anti-Atro in lysis buffer at 4^{o}C for 4 hours, and was incubated with the precleared salivary gland lysate overnight at 4^{o}C. After washing with washing buffer (50mM Tris-Cl pH8.0, 150mM NaCl, 0.5% NP-40, complete protease inhibitor) for 6 times, the protein A agarose was resuspended with SDS loading buffer,

and subject to western blot analysis, using anti-Atro or Rpd3 (Pile and Wassarman, 2000) antibodies.

Histone methylation assay

After the coimmunoprecipitation steps, the agarose gel-bound immunoprecipitate was incubated with 1uCi ^{3}H-labeled S-adenosyl-methionine (Amersham) and 2ug recombinant histone octamers (kindly provided by Dr. Danny Reinberg's lab), or 0.5ug histone H3 peptide containing residue 1-21, residue 21-44, or residue 1-21 with dimethylated lysine 9 (all from Upstate Biotechnology) in 1X methylation buffer (0.05M Tris-HCl pH 8.5, 5mM $MgCl_2$, 4mM DTT) at 30 ^{o} C for 1 hour. The reaction was stopped by adding SDS loading buffer and was subject to electrophoresis. The gel was fixed, dried and exposed for fluorography. For the TSA treatment experiments, the immunoprecipitate was incubated as above except with 0.5ug histone H3 peptide (1-21) with acetylated lysine 9 (Upstate Biotechnology) in the presence of DMSO or 10uM TSA (Sigma Aldrich, T8552). The recombinant SET9 was purchased from Upstate Biotechnology.

Glutathione S-transferase (GST) pull-down assay

Bacterially expressed GST and GST-fused proteins were purified, immobilized by glutathione-agarose beads, and incubated in buffer (50mM Tris-Cl pH8.0, 150mM NaCl, 10% glycerol, 0.5% NP-40, Complete protease inhibitors) with ^{35}S-labeled *in vitro* translated proteins prepared according to the manufacturer's instructions (Promega- TNT, T7 Quick coupled Transcription/Translation systems). After incubation for 1 hour at 4^{o} C, the beads were washed three times with washing buffer (50mM Tris-Cl pH8.0, 150mM NaCl, 0.5% NP-40, Complete protease inhibitors) and resuspended with SDS loading buffer followed by electrophoresis. The gel was dried and exposed to X-ray film.

Immunostaining on salivary gland cells

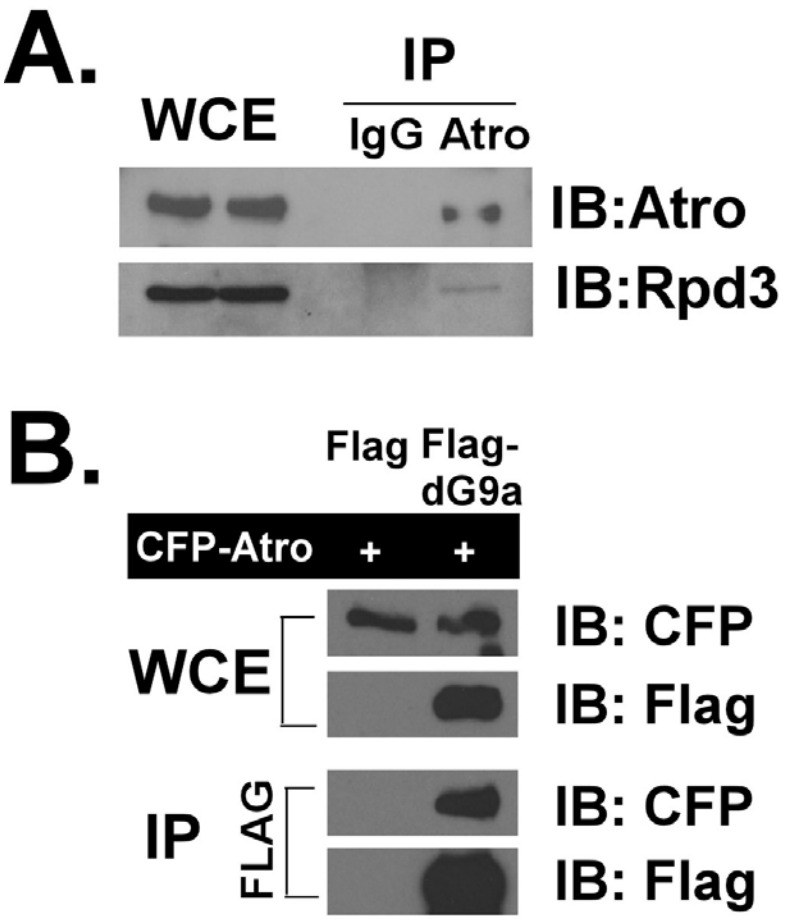
Salivary glands were dissected from third instar *w^{1118}* or *Hsp70::Atro.IR1* larvae in PBS with 0.1% Triton X-100, and fixed with 3.7% formaldehyde in PBS. After the salivary glands were pressed, they were stained with guinea pig anti-Atro, and rabbit anti-*Drosophila* HDAC1/Rpd3 (Abcam) antibody followed by FITC conjugated donkey anti-guinea pig IgG and Texas-red conjugated donkey anti-rabbit IgG (Jackson Laboratories).

Polytene chromosome staining

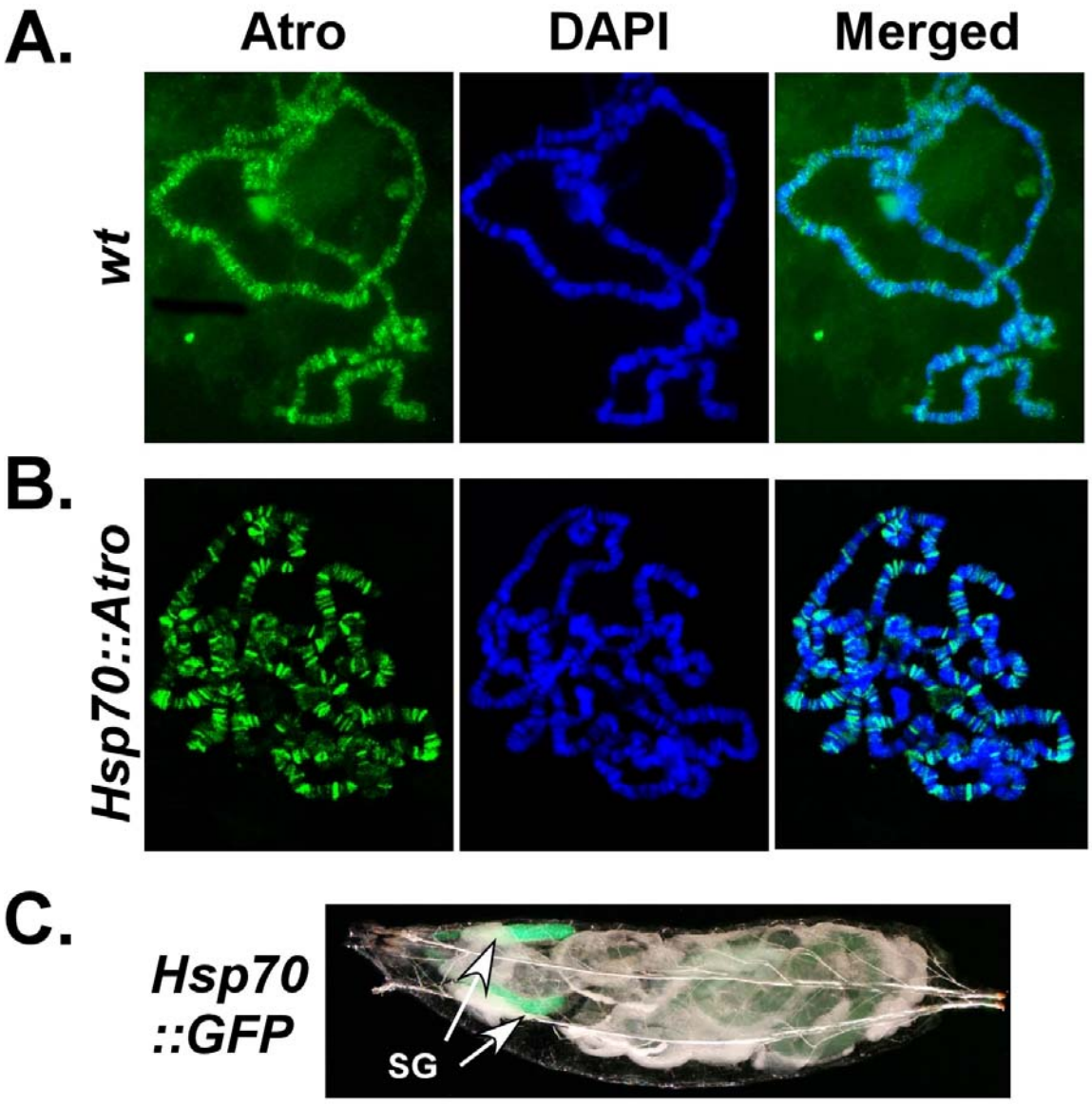
Salivary glands from third instar *w^{1118}* or *Hsp70::Atro* larvae were dissected in PBS with 0.1% Triton X-100. They were placed in fixative containing 3.7% formaldehyde, 1% Triton X-100 in PBS for 20 seconds, and then transferred to fixative containing 3.7% formaldehyde and 50% acetic acid for 3 minutes. After being pressed and spread, the chromosomes were stained with guinea pig anti-Atro and rabbit anti-Rpd3 (Abcam), followed by FITC conjugated donkey anti-guinea pig IgG (Jackson Laboratories) and Alexa 546 conjugated goat anti-rabbit IgG (Invitrogen). For the Atro/dG9a spreads, the chromosomes were stained with rabbit anti-Atro and guinea pig anti-dG9a, followed by FITC conjugated donkey anti-rabbit IgG, biotin conjugated donkey anti-guinea pig IgG, and finally with Texas red conjugated streptavidin (all from Jackson Laboratories).

Silver staining

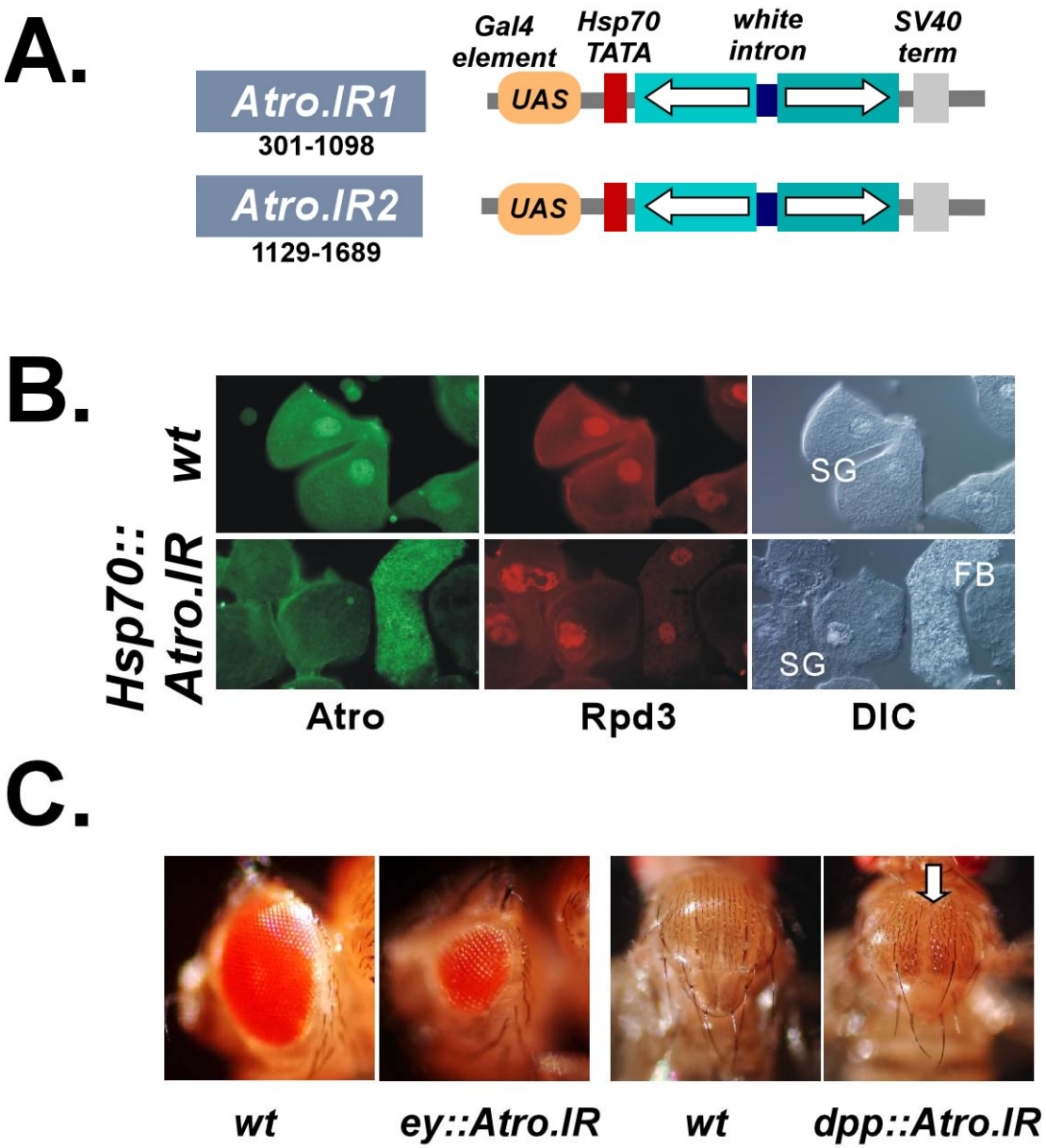
The immunoprecipitate of NFlag, Flag-ATN1 and NFlag-RERE (281-480) was loaded onto and separated by 4%-15% gradient gel (Bio-Rad). The gel was then silver stained using the silver stain kit from Bio-Rad (161-0443).



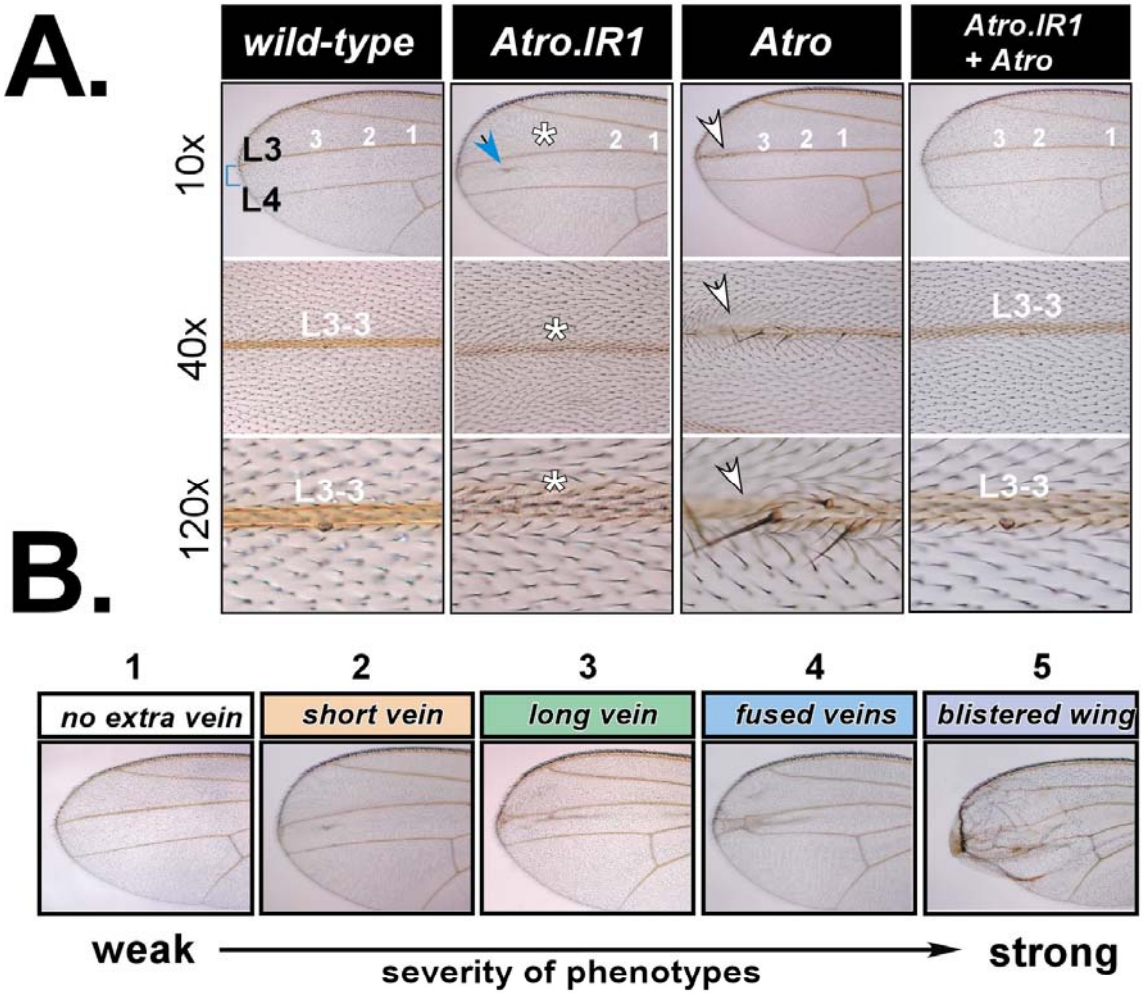
Supplementary Figure 1. Atro interacts physically with Rpd3 and dG9a. (A) Immunoprecipitation (IP) and western blot experiments to demonstrate the interaction between Atro and Rpd3 in *Drosophila*. Cell extracts prepared from ~200 pairs of salivary glands from third instar *Hsp70::Atro* larvae were subject to IP experiments, using anti-Atro antibody and the control IgG, respectively. The IP protein complexes were immuno-blotted (IB) with anti-Atro and anti-Rpd3 antibody. WCE: whole cell extract. Note: dG9a antibody was not included in this study, because none of the tested dG9a antibodies works well for western blot analysis. **(B)** IP and western blot analysis to show the interaction between Atro and dG9a. HEK293 cells were transfected with plasmids expressing a combination of Flag and CFP-Atro or Flag-dG9a and CFP-Atro, respectively. The cell extracts were IP with anti-Flag antibody and immunoblotted with anti-Flag and anti-CFP antibody, respectively.



Supplementary Fig 2. Atro binds distinct chromosomal regions. Immunostaining experiments performed on polytene chromosomes to determine whether Atro binds chromosomes. Salivary gland cells prepared from *wild-type* larvae **(A)** or from *Hsp70::Atro* larvae **(B)** were immunostained with anti-Atro antibody. Chromosomes were counterstained with DAPI (4',6-diamidino-2-phenylindole) to mark condensed chromosomal regions. **(C)** GFP expression showing that *Hsp70-Gal4* is a salivary gland-specific Gal4 driver. The image combines light microscopy and immunofluorescent images of *Hsp70::GFP* third instar larvae. SG: salivary glands.



Supplementary Fig 3. Generation and characterization of two *Atro* dsRNA-expressing fly lines. (A) Diagram showing two *Atro* dsRNA-expressing constructs used to generate *Atro.IR1* and *Atro.IR2* fly lines, respectively. The DNA fragments used to generate these fly lines are indicated. The orientation of each insert is indicated by an arrow. **(B)** Co-immunostaining experiments performed on squashed salivary glands to show the knockdown effect by *Atro* dsRNA. Salivary glands derived from *wild-type* or *Hsp70::Atro.IR1* larvae were immunostained with Atro (FITC) and Rpd3 (Texas-Red) antibodies. SG: salivary gland; FB: fat body; DIC image is shown on the right. **(C)** Light microscopy images showing the eye and notal phenotypes displayed by *wild-type*, *ey::Atro.IR1*, and *dpp::Atro.IR1* flies, respectively. The cleft phenotype is marked with an arrow.



dpp::Atro.IR1-		n	1	2	3	4	5
w (1118)	control	67	4.5%	80.6%	14.9%		
Egfr (t1)	hypomorph	56	71.4%	28.6%			
Egfr (E1)	hypermorph	57				96.5%	3.5%
Atro (35)	null	54			7.8%	70.3%	21.9%
Rpd3 (04556)	loss of function	62			54.8%	32.2%	13%
Rpd3 (313)	hypomorph	42		9.8%	71.4%	18.8%	
dG9a (RG5)	null	66		18.1%	60.6%	21.3%	
dG9a (Del34)	loss of function	34		23.5%	61.8%	14.7%	

n: number of flies scored.

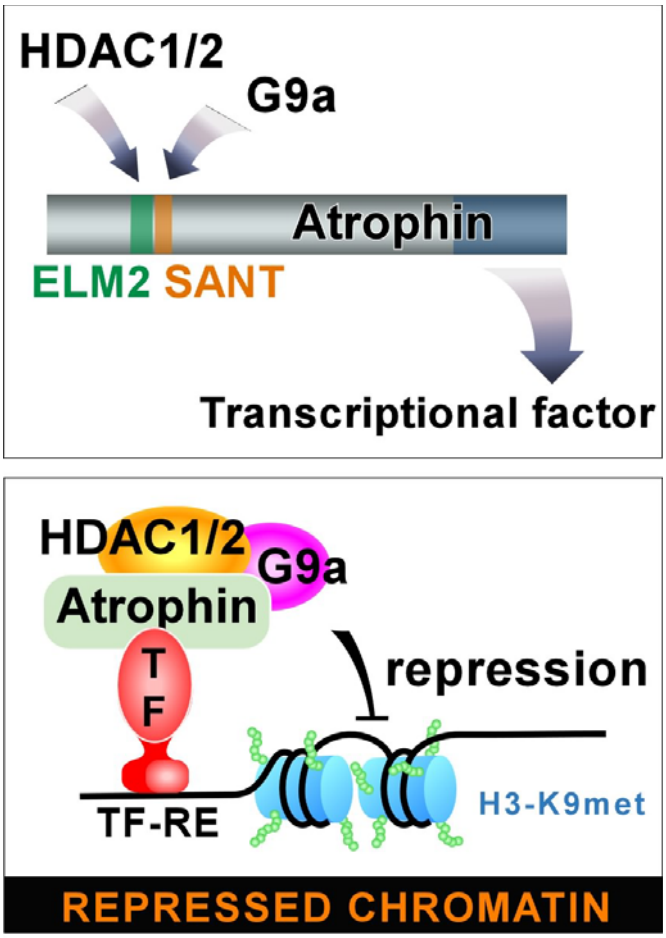
Supplementary Fig 4. *Atro, dG9a,* and *Rpd3* act in concert to repress wing vein formation in *Drosophila*. (A) Expressing *Atro* dsRNA, *Atro*, or both in the *Drosophila* wing to test their effects on wing vein formation. All indicated UAS-based fly lines were individually crossed with the *dpp-Gal4* line, which directs *Atro* dsRNA or Atro protein expression in the intervein region between the L3 and L4 veins (marked by a bracket).

Atro.IR1: *Atro* dsRNA expressing fly line. A blue arrow marks the appearance of ectopic wing veins; an asterisk marks the loss of the L3-3 campaniform sensillum; a white arrow head marks the loss of wing vein and the appearance of slender bristles. The lower panels show enlarged views centering on L3. **(B)** Genetic experiments to determine whether *Atro* dsRNA-mediated wing vein phenotypes are modulated by mutations of *dG9a* or *Rpd3*. A recombined *dpp::Atro.IR1* fly line was individually crossed with each indicated fly line. The progenies from each of these crosses were scored for the severity of their wing vein phenotypes according to the 5 shown wing defects. The percentage of flies displaying each indicated wing phenotype is presented in the bottom panel. w^{1118} and $EGFR^{t1}$, $EGFR^{E1}$, $Atro^{35}$ lines were used as negative and positive controls, respectively. The classification of each tested mutation is shown. Experiments were performed at 24.5 ^{o}C.

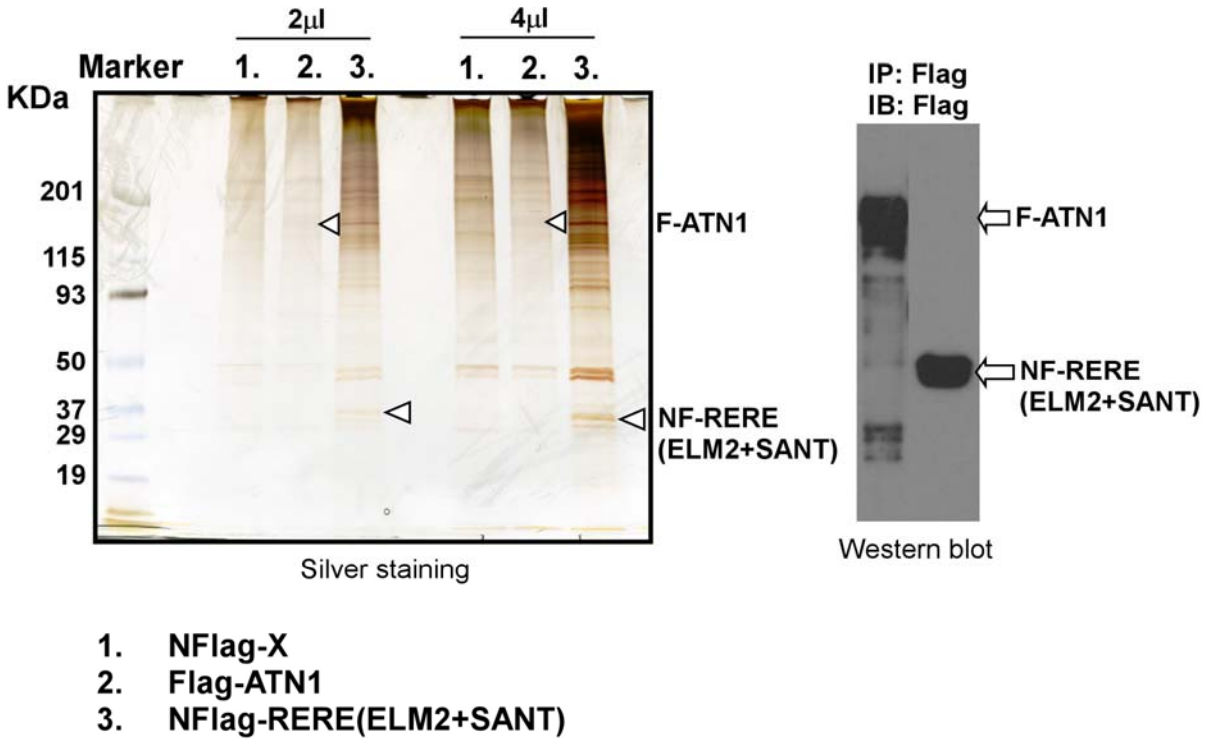
Description and interpretation for the experiments and results

Directed expression of *Atro* dsRNA in the L3 and L4 inter-vein region, using a *dpp-Gal4* driver, causes ectopic wing vein formation. This result is consistent with the report that Atro, by antagonizing the activity of EGFR, can suppress wing vein formation (Charroux et al., 2006). In these flies, we also observed loss of the third campaniform sensillum on vein L3 (L3-3), which is a mechanosensory organ. Therefore, in addition to suppressing wing vein formation, Atro also participates in maintaining or promoting sensory organ development in *Drosophila*. Overexpression of Atro in the wings of *dpp::Atro* flies leads to opposite effects, including a partial loss of wing vein and ectopic formation of slender bristles (sensory organs) at the distal region of L3. Expressing both *Atro* dsRNA and Atro together restored wings to a wild-type form, indicating that the observed *Atro* dsRNA-mediated phenotypes are specific.

To facilitate our genetic investigations, we generated a recombined *dpp::Atro.IR1* fly line and crossed it with w^{1118}, $EGFR^{t1}$, $EGFR^{E1}$, $Atro^{35}$, $Rpd3^{04556}$, $Rpd3^{313}$, $dG9a^{RG5}$, and $dG9a^{Del34}$, respectively. The resulting progenies derived from these genetic crosses were scored for the severity of wing-vein defects. In these genetic experiments, $EGFR^{t1}$, and $EGFR^{E1}$ were used as controls, because *EGFR* is known to interact with *Atro* genetically in the wing (Charroux et al., 2006). No wing phenotype was observed for all the tested heterozygous mutant flies, nor for homozygous *dG9a* mutant flies, which have been recently reported to have no visible phenotype (Seum et al., 2007). As expected, $EGFR^{t1}$ (hypomorph) acts as a strong suppressor, whereas both $EGFR^{E1}$ (hypermorph) and $Atro^{35}$ (null) behave as strong enhancers of the *Atro* dsRNA-mediated wing phenotype. In the latter cases, the number of flies showing elongated, thickened, and fused wing veins, or blistered wing, is significantly increased. $Rpd3^{04556}$, which is a loss-of-function allele, also acts as a strong enhancer. In comparison, consistent but less pronounced enhancing effects were seen when $Rpd3^{313}$ (hypomorph), $dG9a^{RG5}$ (null), and $dG9a^{Del34}$ (loss of function) flies were tested. Since *Atro* dsRNA-mediated wing phenotypes are enhanced by mutations of *Rpd3* or *dG9a*, we conclude that *Rpd3* and *dG9a* collaborate with *Atro* to suppress wing vein formation in *Drosophila*.



Supplementary Fig 5. A model depicting Atrophin-HDAC1/2-G9a complex actions on chromosomes. Atrophin proteins, such as RERE and Atro, recruit HDAC1/2 and G9a through their ELM2 and SANT domains, respectively. Through the coordinated actions of HDAC1/2 and G9a, H3-K9 is methylated at the chromatin locus where Atrophin and its associating transcriptional factors bind. Stable methylation of H3-K9 at these specific chromatin regions results in the formation of repressed chromatin, leading to gene silencing. TF: transcriptional factor; TF-RE: transcriptional factor responsive element.



Supplementary Fig. 6. Silver staining showing additional proteins are associated with the ELM2 and SANT domains of RERE. Silver staining was performed on a 4%-15% gradient gel loaded with 2ul or 4ul of immunoprecipitates prepared from cells expressing NFlag, Flag-ATN1, and NFlag-RERE (281-480), respectively. Western blot shows the amount of Flag-ATN1 and NFlag-RERE (281-480) immunoprecipitates detected by Flag antibody.

References:

Charroux, B., Freeman, M., Kerridge, S. and Baonza, A. (2006) Atrophin contributes to the negative regulation of epidermal growth factor receptor signaling in Drosophila. *Dev Biol*, **291**, 278-290.

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Pile, L.A. and Wassarman, D.A. (2000) Chromosomal localization links the SIN3-RPD3 complex to the regulation of chromatin condensation, histone acetylation and gene expression. *Embo J*, **19**, 6131-6140.

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