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Atrophin recruits HDAC1/2 and G9a to modify histone H3K9 and to determine cell fates

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Atrophin family proteins, including the vertebrate arginine–glutamic acid dipeptide repeats protein (RERE) and *Drosophila* Atrophin (Atro), constitute a new class of nuclear receptor corepressors. Both RERE and Atro share the ELM2 (EGL-27 and MTA1 homology 2) and SANT (SWI3/ADA2/N-CoR/TFIII-B) domains, which are also present in other important transcriptional cofactors. Here, we report that the SANT domain in RERE binds to the histone methyltransferase G9a, and that both the ELM2 and SANT domains orchestrate molecular events that lead to a stable methylation of histone H3-lysine 9. We establish the physiological relevance of these interactions among Atrophin, G9a, and histone deacetylases 1 and 2 in *Drosophila* by showing that these proteins localize to overlapping chromosomal loci, and act together to suppress wing vein and melanotic-mass formation. This study not only shows a new function of the SANT domain and establishes its connection with the ELM2 domain, but also implies that a similar strategy is used by other ELM2–SANT proteins to repress gene transcription and to exert biological effects.

Keywords: Atrophin; SANT domain; ELM2 domain; G9a; HDAC1/2
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INTRODUCTION

Atrophin proteins are conserved nuclear receptor corepressors (Wang *et al*, 2006; Zhang *et al*, 2006) and include vertebrate atrophin 1 (ATN1; Koide *et al*, 1994; Nagafuchi *et al*, 1994), vertebrate arginine–glutamic acid dipeptide repeats protein (RERE, also known as atrophin 2; Yanagisawa *et al*, 2000) and *Drosophila* Atrophin (Atro, also known as Grunge; Erkner *et al*, 2002; Zhang *et al*, 2002). Human ATN1 is known to cause neurodegenerative dentatorubral–pallidoluysian atrophy when its glutamine-repeat tract is expanded (Koide *et al*, 1994; Nagafuchi *et al*, 1994). The neurotoxicity caused by glutamine-repeat-expanded ATN1 is not due to the loss of ATN1 functions, as *Atn1*-knockout mice show no detectable phenotype (Shen *et al*, 2007). By contrast, mutations of murine *Rere* cause embryonic lethality and severe developmental defects (Zoltewicz *et al*, 2004). The apparently divergent effects of RERE and ATN1 on animal development suggest that their functions are not equivalent.

The functional differences between RERE and ATN1 can be attributed to structural divergence between these two proteins. Notably, ATN1, which resembles a truncated version of RERE, lacks several conserved domains, including the BAH (bromo-adjacent homology), ELM2 (EGL-27 and MTA1 homology 2) and SANT (SWI3/ADA2/N-CoR/TFIII-B) domains, which are all located at the amino terminus of RERE. The ELM2 and SANT domains are also present in Atro and several other important transcriptional repressors such as MTA (metastasis-associated family) proteins, CoREST (REST corepressor) and MIER1 (mesoderm induction early response 1; Ding *et al*, 2003). The conjoined conservation of these two domains in various transcriptional repressors suggests that they have important functions.

Ours and earlier studies on the ELM2 domain have shown its direct involvement in binding to histone deacetylases 1 and 2 (HDAC1/2; Ding *et al*, 2003; Wang *et al*, 2006). In comparison, less is known about the exact function of the SANT domain of RERE/Atro, although previous studies have implicated the SANT domain in binding to histone tails (Boyer *et al*, 2002; Yu *et al*, 2003), activating the enzymatic activity of HDAC3 (Guenther *et al*, 2001) and stimulating the histone demethylation activity of lysine-specific histone demethylase 1 (LSD1; Shi *et al*, 2005). SANT is evidently a versatile domain and its properties depend not only on its coding sequences, but also on other regions of the proteins within which it resides.

Here, we expand our knowledge about the SANT domain by reporting that the SANT domain of RERE/Atro recruits the histone methyltransferase (HMTase) G9a and that it enables RERE to stably methylate lysine 9 of histone H3 (H3K9) through cross-talk with the ELM2 domain. We find a parallel relationship among Atro, dG9a and Rpd3 (the fly homologue of HDAC1/2) in *Drosophila*. By using the fly system, we show that Atro, dG9a and Rpd3 bind to overlapping chromosomal regions, and act together to suppress wing vein and melanotic-mass formation in adult flies. Our results

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indicate that recruiting HDAC1/2 and G9a is an important mechanism used by ELM2–SANT domain proteins to modify histone tails, regulate gene transcription and control animal development.

RESULTS AND DISCUSSION

RERE and Atro, but not ATN1, recruit G9a in human cells

There is increasing evidence to indicate that, in addition to histone deacetylation, histone lysine methylation (HMT) is another mechanism used by transcriptional regulators to repress gene transcription (Sims *et al*, 2003). For example, methylation on K9 and K27 of histone H3, and K20 of histone H4 has been shown to contribute to large-scale chromosomal repression (Lachner *et al*, 2003). As both the ELM2 and SANT domains are found in several transcriptional regulators known to have potent repressive activities (Ding *et al*, 2003), we suggested that, in addition to their known associations with HDAC1/2 (Wang *et al*, 2006), these two conserved domains are also connected with HMT activity.

To test whether ELM2/SANT-mediated transcriptional repression involves HMT, we expressed Flag-tagged ELM2 and SANT domains of RERE (hereafter called RERE^{ELSA}; Fig 1A) in human embryonic kidney cells (HEK293) and performed *in vitro* HMT assays on the immunoprecipitated RERE^{ELSA} complex. The RERE^{ELSA} complex exerts HMT activity (Fig 1B), although weaker than that of the control G9a. This result was expected because RERE is not by itself an HMTase. Therefore, we speculated that RERE^{ELSA} acquires its HMT activity by associating with HMTases.

Our HMT assays also showed that the RERE^{ELSA} complex preferentially methylates histone H3. We came to this conclusion on the basis of the following evidence: (i) the size of the methylated histone matches that of histone H3 (Fig 1B); (ii) the methylated histone migrates with G9a-methylated histone H3 (Fig 1B); and (iii) the RERE^{ELSA} complex also methylates a synthetic peptide that encompasses only the first 21 amino acids of histone H3, H3(1–21) (Fig 1C). By contrast, the RERE^{ELSA} immunoprecipitation complex fails to methylate H3(21–44), suggesting that the two lysine residues (K4 and K9) located within H3(1–21) are potential targets for RERE^{ELSA}. Therefore, we tested an H3 (1–21)K9met2 peptide in further HMT assays. If H3K9 is a target of the RERE^{ELSA} complex, prior methylation should prevent it from being methylated by the RERE^{ELSA} complex. As we predicted, H3(1–21)K9met2 cannot be methylated by the RERE^{ELSA} complex. In comparison, robust methylation was achieved by the control SET9, which is an H3K4 HMTase (Fig 1D). Thus our data indicate that the RERE^{ELSA} complex primarily targets H3K9, but not H3K4, for methylation.

Subsequently, we investigated which HMTase lends Atrophin proteins (and RERE^{ELSA}) the ability to methylate H3K9. We focused on G9a because G9a is an important HMTase known to catalyse mono- and dimethylation of H3K9 in euchromatin (Rice *et al*, 2003). Furthermore, both HDAC1, which binds to RERE strongly (Wang *et al*, 2006), and G9a have been found in the same protein complexes (Shi *et al*, 2003; Duan *et al*, 2005). A combination of co-immunoprecipitation and western blot analysis was performed on extracts derived from cells expressing Flag-tagged ATN1, RERE and Atro (Fig 1A,E). The assays confirmed that G9a is present in the immunoprecipitation complexes associated with RERE and Atro, but not with ATN1. The interaction between G9a and RERE or Atro is specific, as SET9 was not found in any of the immunoprecipitation complexes.

The physical association between RERE/Atro and G9a was further validated in HEK293 cells by using a cell biology approach. We performed co-immunostaining experiments on cells expressing green fluorescent protein (GFP)-tagged RERE or cyan fluorescent protein (CFP)-tagged Atro. These assays showed that endogenous G9a, which is known to form nuclear speckles, is recruited to the RERE/Atro-mediated nuclear foci (Fig 1F). These results provide further support to our conclusion that G9a is an associating factor of RERE and Atro. A similar interaction might occur between Atro and dG9a, the *Drosophila* homologue of G9a (Mis *et al*, 2006; Stabell *et al*, 2006).

The SANT domain of RERE binds to G9a directly

Both RERE and Atro have ELM2 and SANT domains, which are absent in ATN1. As ATN1 fails to bind to G9a (Fig 1E), we predicted that either or both of the ELM2 and SANT domains are involved in G9a association. A series of constructs expressing Flag-RERE variants with or without the ELM2 and SANT domains (Fig 1A) were generated and used for co-immunoprecipitation and western blot experiments. As shown in Fig 2A, G9a associates only with those RERE variants that contain the SANT domain (compare lanes 2–4,6,8 with lanes 5,7). The SANT domain of RERE is therefore involved in recruiting G9a.

Glutathione-*S*-transferase (GST) pull-down assays were then performed to investigate whether the observed interaction between the SANT domain of RERE (RERE^{SANT}) and G9a is direct or indirect. In our assays, GST-RERE (361–480) and GST-RERE (281–480), both of which contain the SANT domain, pulled down G9a (Fig 2B). By contrast, GST-RERE (281–360), which lacks the SANT domain, failed to do so. The direct interaction observed between SANT domain and G9a is specific because none of the GST-RERE variants tested pulled down the control SET9.

A SANT domain similar to that of RERE/Atro has been found in several other transcriptional regulators, many of which also contain the ELM2 domain. For example, MTA proteins, which are intrinsic components of the nucleosome remodelling and histone deacetylase (NuRD/Mi-2) complex (Tong *et al*, 1998; Xue *et al*, 1998; Zhang *et al*, 1998), and MIER1, which is encoded by a fibroblast growth factor-responsive gene (Paterno *et al*, 1997), contain both ELM2 and SANT domains (Ding *et al*, 2003). Our findings led us to investigate whether these other ELM2–SANT domain proteins also show an affinity towards G9a. By using both immunoprecipitation and western blot experiments, we first confirmed the known associations between MTAs or MIER1 and HDAC1/2, and subsequently showed that the four tested ELM2–SANT domain proteins also recruit G9a (Fig 2C). Association with both HDAC1/2 and G9a is therefore a shared property of several ELM2–SANT domain proteins.

The ELM2 and SANT domains modify histone H3K9

Acetylation and methylation on H3K9 are known to be mutually exclusive. In order for H3K9 to be methylated, the prior removal of the acetyl groups by HDACs is essential. Having linked the functions of the ELM2 and SANT domains to both HDAC1/2 and G9a, we hypothesized that coordinated actions by these two domains might be responsible for stable methylation of H3K9. To test this hypothesis, we treated the RERE^{ELSA} complex with trichostatin A (TSA), a potent HDAC inhibitor. We reasoned

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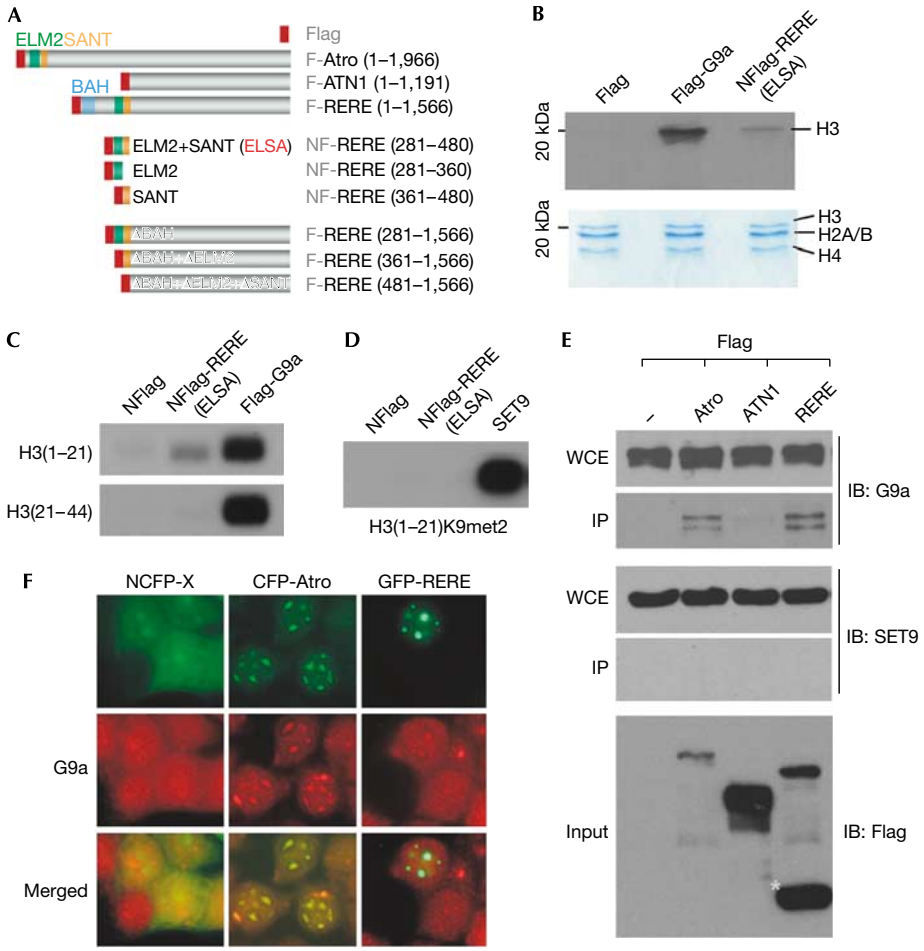

Fig 1 | Arginine–glutamic acid dipeptide repeats protein exerts histone methylation activity and associates with G9a. (**A**) Diagram showing the Flag-tagged Atrophin (Atro) proteins used in immunoprecipitation experiments. The conserved BAH (bromo-adjacent homology), ELM2 (EGL-27 and MTA1 homology 2) and SANT (SWI3/ADA2/N-CoR/TFIII-B) domains in each protein are highlighted in different colours. (**B**) Histone methylation (HMT) assays were performed on recombinant histone octamers to determine whether the ELM2–SANT domains of arginine–glutamic acid dipeptide repeats protein (RERE) mediate HMT activity. The identity of each histone is determined by its size in the Coomassie blue-stained gel; G9a, a known histone H3-specific histone methyltransferase (HMTase), was used as a control. (**C**,**D**) HMT assays were performed on the indicated peptides to determine which specific lysine residue is a target of the RERE^{ELSA} complex. Recombinant SET9, a known H3K4 HMTase, was used as a control. (**E**) Western blot experiments were performed on the indicated immunoprecipitation products to test whether G9a is associated with Atro proteins. SET9 was used as a negative control. A cleaved product of RERE is marked with an asterisk. (**F**) Immunostaining experiments were performed on cells expressing green fluorescent protein (GFP)-tagged RERE or cyan fluorescent protein (CFP)-tagged Atro to determine whether endogenous G9a is recruited to the RERE/Atro-mediated nuclear foci. Nuclear localization signal-tagged CFP (NCFP) was used as a control. ELSA, ELM2 + SANT domains; F, Flag; H3K9met2, dimethylated H3K9; IB, immunoblot; IP, immunoprecipitation; NF, nuclear localization signal-tagged Flag; WCE, whole-cell extract.

that if deacetylation of histone H3K9 is a prerequisite for its methylation, then blocking the HDAC activity of the RERE^{ELSA} complex would inevitably affect its ability to methylate an acetylated form of H3K9. As shown in Fig 2D, treating the RERE^{ELSA} complex with TSA, but not the control DMSO, impaired its ability to methylate H3(1–21)K9Ac. Integrating both HDAC and HMT activities therefore seems to be a strategy used by RERE, and perhaps by other ELM2–SANT domain proteins, to methylate H3K9.

Atro–dG9a–Rpd3 binds overlapping chromosomal regions

Building on the physical connections that we established among RERE, HDAC1/2 and G9a in human cells, we investigated next the biological relevance of their interactions. We focused on the fly system, because Atrophin, HDAC1/2 and G9a are all conserved in *Drosophila*. Furthermore, by using immunoprecipitation experiments, we also succeeded in showing parallel interactions between Atro and Rpd3 (the fly homologue of HDAC1/2), and between Atro and dG9a (supplementary Fig 1 online).

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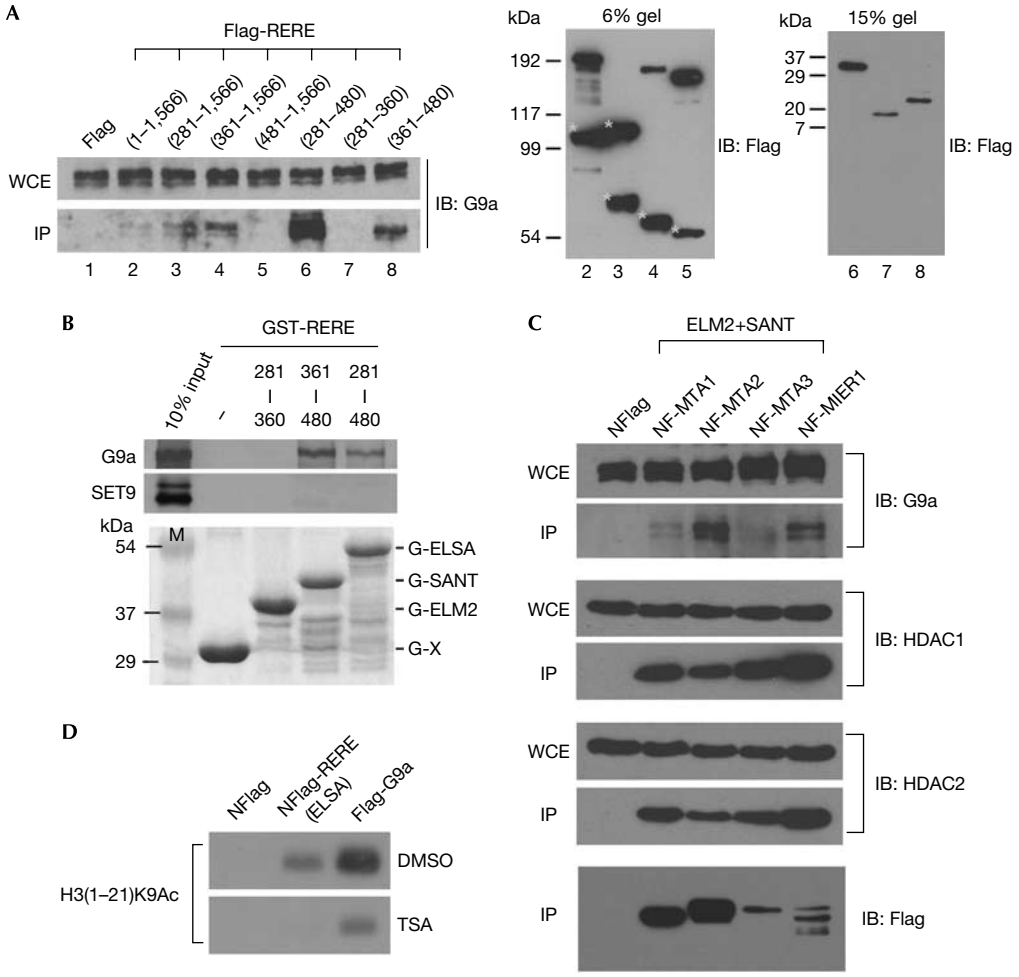

Fig 2 | The SANT domain of arginine–glutamic acid dipeptide repeats protein binds to G9a and coordinates with the ELM2 domain to methylate H3K9. (**A**) Western blot experiments were performed on the indicated immunoprecipitation products to determine which region of arginine–glutamic acid dipeptide repeats protein (RERE) is responsible for G9a association. The input proteins are shown in the right panels. Cleaved products of RERE are marked with asterisks. Based on the molecular weights of the shown cleaved products of RERE, they all contain both ELM2 and SANT domains. (**B**) Glutathione-*S*-transferase (GST) pull-down experiments were used to determine whether the SANT domain of RERE binds to G9a directly. G9a and SET9 were labelled with [^{35}S]methionine. The expression of GST-RERE proteins used in the pull-down assays is shown by Coomassie blue staining. (**C**) Western blot experiments were performed on the indicated immunoprecipitation products to test whether G9a is associated with additional ELM2–SANT domain proteins. Histone deacetylases 1 and 2 (HDAC1/2) were used as positive controls. (**D**) Histone methyltransferase assays were performed on an acetylated form of H3(1–21)K9 to determine whether RERE^{ELSA}-mediated histone methylation is sensitive to trichostatin A (TSA) treatment. TSA is an HDAC inhibitor. ELM2, EGL-27 and MTA1 homology 2; ELSA, ELM2 + SANT domains; G, GST; G9a, a known histone H3-specific histone methyltransferase; IB, immunoblot; IP, immunoprecipitation; MIER1, mesoderm induction early response 1; MTA1–3, metastasis-associated family proteins 1–3; NF, nuclear localization signal-tagged Flag; SANT, SWI3/ADA2/N-CoR/TFIII-B; SET9, an H3-K4 histone methyltransferase; WCE, whole-cell extract.

As Atro, dG9a and Rpd3 are all expressed in the nucleus of salivary gland cells, we used these cells to investigate, by means of their mutual interactions, whether these three proteins localize to overlapping chromosomal regions. Immunostaining experiments were performed on squashed polytene chromosomes derived from wild-type and *Hsp70*::*Atro* (Hsp for Heat-shock protein) third instar larvae (*Hsp70-Gal4* is a salivary gland-specific driver (Tsai *et al*, 2004; Mizutani *et al*, 2005), which allows us to induce Atro expression in salivary gland cells). In keeping with its role as a chromatin-binding factor, both endogenous and overexpressed Atro bind to distinct chromosomal loci that are primarily within the inter-band regions (supplementary Fig 2 online). As *Hsp70*::*Atro* larvae gave a stronger staining pattern, these larvae were used for further analysis.

We performed co-immunostaining experiments on the polytene chromosomes from the salivary gland cells of *Hsp70*::*Atro* larvae by using antibodies directed against Atro and against dG9a, Rpd3 or RNA polymerase II-phosphorylated-Ser5, which is a

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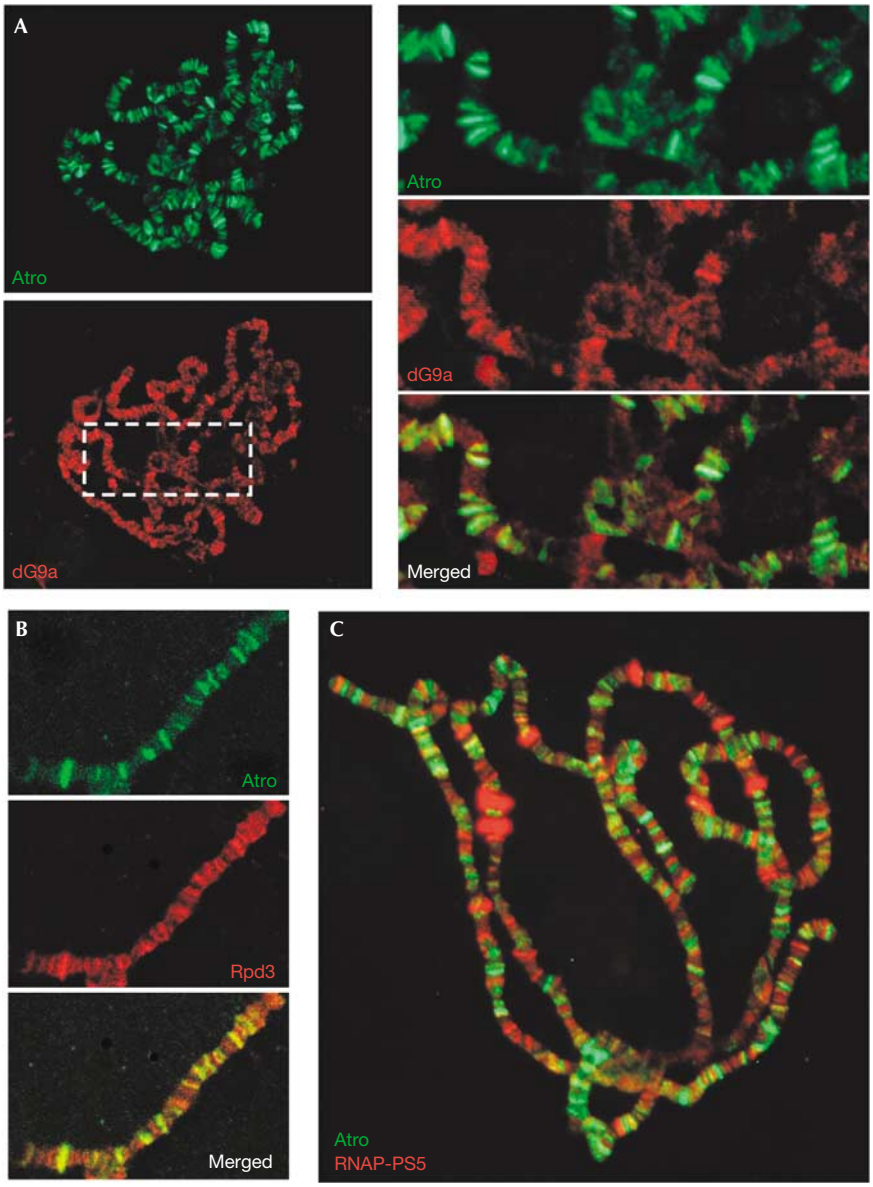

Fig 3 | Atro, dG9a and Rpd3 bind to overlapping chromosomal regions in *Drosophila*. A co-immunostaining experiment was performed on salivary gland cells to determine the relative positions of Atro and dG9a, Rpd3 or a marker for transcriptional initiation (RNA polymerase II-phosphorylated-Ser5 (RNAP-p-S5)) on polytene chromosomes. Salivary gland cells derived from *Hsp70*::*Atro* late third instar larvae were subjected to co-immunostaining using (**A**) Atro and dG9a antibodies, (**B**) Atro and Rpd3 antibodies, and (**C**) Atro and RNAP-p-S5 antibodies. *Hsp70-Gal4* is a salivary gland-specific Gal4 driver without heat-shock treatment. The enlarged images shown in the right panels of (**A**) correspond to the boxed area in the lower panel. Atro, *Drosophila* Atrophin; dG9a, the *Drosophila* homologue of G9a; Hsp70, Heat-shock protein 70; Rpd3, the fly homologue of histone deacetylases 1 and 2.

marker for transcriptional initiation. As expected, many—although not all—chromosomal regions that are enriched in Atro are also positive for dG9a or Rpd3 (Fig 3A,B). By contrast, the regions bound by Atro show little gene transcriptional initiation activity (Fig 3C). On the basis of these results, we propose that Atro, dG9a and Rpd3, by means of their mutual interactions, bind to specific chromosomal loci, where they act together to repress gene transcription.

Atro–dG9a–Rpd3 represses melanotic-mass formation

Next, we investigated, through genetic interactions, whether *Atro*, *dG9a* and *Rpd3* participate in overlapping pathways to control *Drosophila* development. For this purpose, we generated two fly lines expressing *Atro* double-stranded (ds) RNA, *Atro.IR1* and *Atro.IR2*, which allowed us to use the Gal4-UAS system to knock down Atro expression in a tissue-specific manner (supplementary Fig 3A,B online). Directed expression of either form of *Atro* dsRNA

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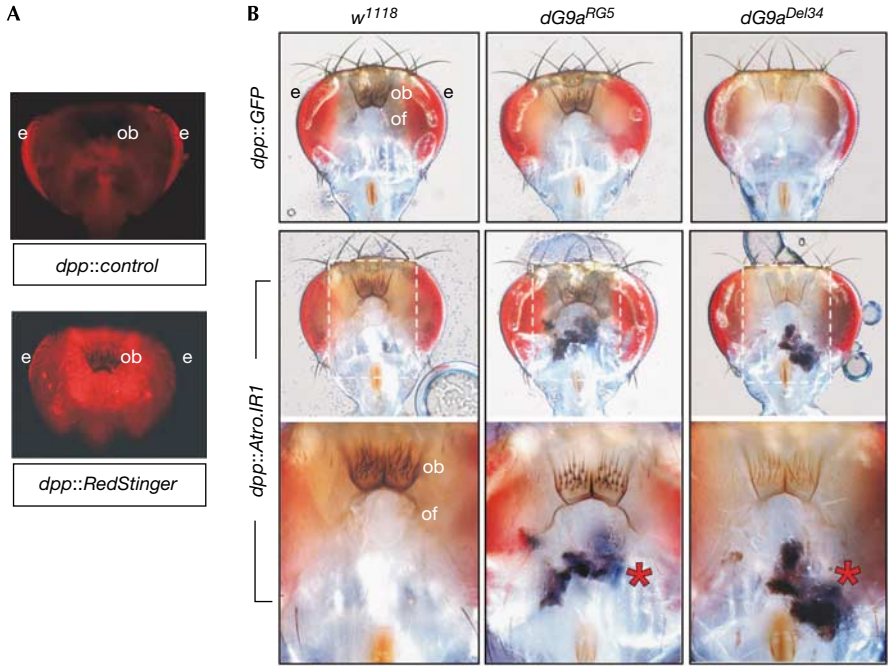

C

Flies displaying melanotic masses in the head

	dpp::*GFP*		*dpp*::*Atro.IR1*	
w^{1118}	0%	(*n*=37)	0%	(*n*=49)
$Atro^{35}$	0%	(*n*=35)	0%	(*n*=36)
$Rpd3^{313}$	0%	(*n*=41)	0%	(*n*=55)
$Rpd3^{04556}$	0%	(*n*=52)	6.1%	(*n*=49)
$dG9a^{RG5}$	0%	(*n*=27)	37.5%	(*n*=32)
$dG9a^{Del34}$	0%	(*n*=32)	30.7%	(*n*=39)

n=number of male flies scored

Fig 4 | *Atro*, *dG9a* and *Rpd3* act together to suppress melanotic-mass formation in the adult head of *Drosophila*. (**A**) The expression of RedStinger was used to determine that *dpp-Gal4* is active in the adult head. The images show posterior views of the heads of *dpp-Gal4* and *dpp*::*RedStinger* flies. RedStinger is a DsRed variant. (**B**) Examination of adult heads to determine whether *Atro* interacts genetically with *dG9a* and *Rpd3*. Images show posterior views of the heads from flies with the indicated genotypes. *dpp*::*Atro.IR1* is an *Atro* double-stranded RNA-expressing line, w^{1118} and *dpp*::*GFP* were used as negative control lines, $dG9a^{RG5}$ is a deletion line generated by homologous recombination and $dG9a^{Del34}$ is an imprecise P-element excision line. The bottom panel contains enlarged images corresponding to the boxed areas shown in the middle panel. The melanotic masses are indicated by asterisks. (**C**) Genetic data showing the number and percentage of flies with melanotic masses in their heads. The genotype of each examined fly line is indicated. $Atro^{35}$ is a null allele, $Rpd3^{04556}$ is a loss-of-function allele and $Rpd3^{313}$ is a hypomorphic allele. Atro, *Drosophila* Atrophin; dG9a, the *Drosophila* homologue of G9a; e, eye; ob, occipital bristles; of, occipital foramen; Rpd3, the fly homologue of histone deacetylases 1 and 2.

in the L3 and L4 inter-vein region, by using a *dpp-Gal4* driver, causes ectopic wing vein formation (supplementary Fig 4A online). This result is consistent with the report that Atro, by antagonizing the activity of epidermal growth factor receptor (Egfr), can suppress wing vein formation (Charroux *et al*, 2006). The observed *Atro* dsRNA-mediated phenotype is specific because it can be fully rescued when both *Atro* dsRNA and Atro protein are simultaneously expressed in the wing (supplementary Fig 4A online).

Subsequently, we generated a recombined *dpp*::*Atro.IR1* line and tested it against a series of mutations, including those of *Rpd3* and *dG9a*. Specifically, we wished to discover whether any *Atro* dsRNA-mediated phenotype can be modulated by the further mutation of *Rpd3* or *dG9a*. The wing vein phenotype is enhanced when *Rpd3* or *dG9a* is mutated, although, in comparison, *Rpd3* seems to have a more prominent role than *dG9a* in assisting *Atro* to suppress wing vein formation (detailed information about these genetic experiments and results is provided in the supplementary information online).

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We discovered a more pronounced genetic interaction between *Atro* and *dG9a* in the adult head, where *dpp-Gal4* is also active (Fig 4A). Melanotic masses, a possible consequence of aggregated haemocytes, were found in the heads of approximately 37.5% of adult $dG9a^{RG5}/Y$; *dpp::Atro.IR1/+* flies and in approximately 30.7% of adult $dG9a^{Del34}/Y$; *dpp::Atro.IR1/+* flies (Fig 4B,C; *dG9a* is an X-chromosome-linked gene, $dG9a^{RG5}$ is a null allele and $dG9a^{Del34}$ is a loss-of-function allele (Seum *et al*, 2007)). By contrast, only approximately 6.1% of *dpp::Atro.IR1/* $Rpd3^{04556}$ (loss-of-function allele) adult flies were afflicted with melanotic masses, and none of the other fly lines tested produced melanotic lesions (Fig 4C). These genetic data for *Atro*, *dG9a* and *Rpd3* observed in the *Drosophila* head differ from those found in the *Drosophila* wing (supplementary Fig 4C online), suggesting that various tissues show different sensitivity to loss of Atro, dG9a and Rpd3 in *Drosophila*. As no melanotic masses were detected in the heads of *dG9a* mutant or *dpp::Atro.IR1/*$Atro^{35}$ flies, we conclude that the formation of melanotic masses is due to the combined loss of Atro and *dG9a* or *Rpd3* in the adult head.

Prospects and implications

The results of this study suggest that RERE and Atro use their ELM2 and SANT domains to recruit HDAC1/2 and G9a, respectively. The recruited HDAC1/2 and G9a, in turn, orchestrate sequential molecular events that lead first to the deacetylation and then to the methylation of H3K9 at the chromosomal loci where RERE or Atro resides (see a model in supplementary Fig 5 online). A similar mode of action might apply to other ELM2–SANT domain proteins, such as MTA1–3 and MIER1, as these factors also show affinity towards both HDAC1/2 and G9a (Fig 2C). Although not tested in this study, another ELM2 and SANT domain protein, CoREST, has been identified in a large carboxy-terminal binding protein complex (with approximately 20 associated polypeptides), which also contains G9a and HDAC1/2 (Shi *et al*, 2003). Our results led us to propose that a stable CoREST–HDAC1/2–G9a protein complex forms as a result of the binding of both HDAC1/2 and G9a to the ELM2–SANT domains of CoREST.

MTA proteins and CoREST have been shown to be components of large protein complexes that show properties ranging from chromatin remodelling and histone deacetylation to transcriptional repression (Shi *et al*, 2003; Bowen *et al*, 2004). As the ELM2 and SANT domains of RERE/Atro resemble those of MTA and CoREST, we predict that RERE or Atro might also be subunits of large protein complexes. In support of this prediction, silver staining of the $RERE^{ELSA}$ immunoprecipitation complex showed that $RERE^{ELSA}$ is associated with multiple proteins (supplementary Fig 6 online). It is therefore conceivable that the ELM2–SANT domains, by acting as binding scaffolds for various transcriptional cofactors, allow proteins such as RERE, Atro, MTAs or CoREST to modify chromatin structures and to silence gene transcription efficiently.

Atrophin proteins have already been shown to interact with various transcriptional regulators, including homeobox protein (Zhang *et al*, 2002), nuclear receptors (Wang *et al*, 2006; Zhang *et al*, 2006) and ETO/MTG8 (which causes acute myeloid leukaemia 1; Wood *et al*, 2000). MTA proteins are implicated in various cancers and CoREST is involved in regulating neuronal cell fate. Our findings about the properties of the ELM2 and SANT domains suggest that the recruitment of HDAC1/2 and G9a is an important strategy used by these various transcriptional regulators to repress gene transcription; perturbation of this process might cause diseases and defects such as melanotic masses. As specific inhibitors of G9a have recently been identified (Kubicek *et al*, 2007), our results raise the possibility that G9a inhibitors might be useful, either alone or together with HDAC inhibitors, as therapeutic agents for treating diseases such as neurological disorders or cancers that involve Atrophin, CoREST or MTA proteins.

METHODS

***Drosophila* stocks and experiments.** Stable *Atro.IR1 and Atro.IR2* fly lines were generated by injecting constructs into w^{1118} embryos (Duke University Non-Mammalian Model Systems Fly-shop). The lines w^{1118}, *dpp-Gal4*, *Hsp70-Gal4*, *ey-Gal4*, *UAS-GFP*, *UAS-RedStinger*, $Rpd3^{04556}$, $Egfr^{t1}$ and $Egfr^{E1}$ were obtained from the Bloomington Stock Center. $Rpd3^{313}$ was obtained from T. Grigliatti (University of British Columbia; Mottus *et al*, 2000); $dG9a^{BG5}$ and $dG9a^{Del34}$ were gifts from Dr P. Spierer (University of Geneva; Seum *et al*, 2007). *UAS-Atro* and $Atro^{35}$ lines were described previously (Erkner *et al*, 2002; Charroux *et al*, 2006); *dpp::Atro.IR1* was generated by chromosomal recombination. Genetic experiments were carried out at 24.5 °C.

Antibody generation. Polyclonal Atro antibodies were developed both in guinea-pigs and rabbits by using KLH-conjugated synthetic polypeptide ADTPALRQLSEYARPHVA. The polyclonal dG9a antibody was developed in guinea-pigs by using KLH-conjugated combined synthetic polypeptides AMEADRRTDDSYYFDLDN and GEEICFDYGEKFWRVEHR.

Additional materials and methods are described in the supplementary information online.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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