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Mutation of *Drosophila Lsd1* disrupts H3-K4 methylation resulting in tissue specific defects during development

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Summary

Histone tail modifications play a fundamental role in the processes that establish chromatin structure and determine gene expression [1–4]. One such modification, histone methylation was considered irreversible until the recent discovery of histone demethylases. Lsd1 was the first demethylase to be identified [5]. Lsd1 is highly conserved from yeast to humans but its function has primarily been studied using biochemical approaches. The mammalian ortholog has been shown to demethylate mono and dimethyl K4 and K9 residues of histone H3 [5,6]. Here we describe the effects of *Lsd1* mutation in *Drosophila*. The inactivation of *dLsd1* strongly affects the global level of mono and dimethyl-H3-K4 methylation and results in elevated expression of a subset of genes. *dLsd1* is not an essential gene, but animal viability is strongly reduced in mutant animals in a gender specific manner. Interestingly, *dLsd1* mutants are sterile and possess defects in ovary development, indicating that dLsd1 has tissue specific functions. Mutant alleles of *dLsd1* suppress positional effect variegation, suggesting a disruption of the balance between euchromatin and heterochromatin. Taken together, these results show that dLsd1-mediated H3-K4 demethylation has a significant and specific role during *Drosophila* development.

Results and Discussion

Originally Lsd1 was found as a component of co-repressor complexes [7–11]. Lsd1 demethylase activity was only discovered recently [5] and was found to be modulated by its associated proteins, such as CoREST [12,13]. Lsd1 depletion in mammalian cells correlates with increased gene expression and elevated levels of H3-K4 methylation at target promoters [5]. However Lsd1 can also act as a co-activator and demethylates H3-K9, a repressive mark [6].

Lsd1 is evolutionary conserved [5] but little is known about its biological function. To address this question we have generated flies carrying a mutation in the sole *Drosophila* gene, *CG17149/dLsd1*, that exhibits high homology to Lsd1 (Fig. S1A). *dLsd1* contains both a putative amine oxidase domain and a SWIRM domain (Fig. S1B). In the Exelixis collection of mutants [14] we found two piggyBac insertions in the vicinity of *CG17149/dLsd1: f03544* (designated as $dLsd1^1$) and f00678 ($dLsd1^2$). Using FRT sites in the piggyBac transposon [15] to promote trans recombination between $dLsd1^1$ and $dLsd1^2$ we generated a deletion allele of *dLsd1*, $dLsd1^{\Delta N}$ (Fig. S1B,C).

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Southern blot analysis confirmed the authenticity of the *dLsd1* alleles (Fig. S2A, S2B). *dLsd1*$^{\Delta N}$ lacks the presumptive promoter region and the N-terminal portion of the gene, including the SWIRM domain (Fig. S1B). Quantitative PCR analysis using primers specific for the 5′ end of *dLsd1* confirmed the absence of these sequences in *dLsd1*$^{\Delta N}$ homozygous flies (Fig. S2C). Low levels (<20%) of 3′ transcripts persist in the mutant animals (data not shown) but any potential products would lack the putative nuclear localization signal and the SWIRM domain and are unlikely to be functional. The SWIRM domain is thought to function in protein-protein interactions, DNA protein interaction and enzyme catalysis [16–19]. Inactivation of this domain greatly reduces the stability and demethylase activity of Lsd1 [16,17]. Western blot analysis showed that dLsd1 is expressed at high levels in wild-type (wt) flies but no dLsd1 protein was detected in *dLsd1*$^{\Delta N}$ homozygous flies (Fig. S2D). Hence *dLsd1*$^{\Delta N}$ is, most likely, a null allele.

This collection of mutant alleles provided us with the opportunity to study the biological function of *dLsd1* in an animal model system.

First, we assessed the effects of *dLsd1* mutation on viability. Crosses of *dLsd1*$^{\Delta N}$ heterozygous animals gave only one-third of the expected number of homozygous *dLsd1*$^{\Delta N}$ progeny (Table S1). Interestingly, this reduction in viability is more dramatic in the male progeny (approximately 90% of the viable *dLsd1*$^{\Delta N}$ homozygotes were females) (Table S2).

dLsd1$^{\Delta N}$ mutants are sterile. In these animals, ovary development is severely impaired (Fig. 1C,D). The *Drosophila* ovary consists of approximately 16 ovarioles, chains of developing egg-chambers with a germarium at the anterior tip. The germarium contains germline stem cells (GSC) and somatic stem cells (SSC) which give rise respectively to the germline cysts and to follicle cells (Fig. 1C,E,G,I,M) [20]. Interestingly, DNA staining shows that *dLsd*$^{\Delta N}$ mutant ovaries lack proper ovariole structures (Fig. 1D,F) and the formation of egg chambers is abnormal at very early stages. Both the germline and follicle cells appear abnormal (Fig. 1H, L, N) and, strikingly, the 16 cells cysts fail to be properly encapsulated by follicle cells (Fig. 1H). In males, the testes are morphologically intact, but DNA staining suggests defects during spermatogenesis (data not shown). Interestingly, *dLsd1*$^{\Delta N}$ homozygotes also have a held-out wing phenotype (Fig. 1B) that renders them unable to fly.

To confirm that these defects are due specifically to *dLsd1* loss and are not the result of secondary mutations, we performed complementation tests with a deficiency (*Df(3L) ED4858)* that uncovers the *dLsd1* gene (Table 3). Trans-heterozygotes carrying *dLsd1*$^{\Delta N}$ and *Df(3L)ED4858* recapitulated the phenotypes observed in *dLsd1*$^{\Delta N}$ homozygous flies (Table S3).

We conclude that *dLsd1* mutation reduces viability in a gender-dependent manner, causes abnormal ovary development, and results in animal sterility. Collectively, these results point to important roles for *dLsd1* in the late stages of development. dLsd1 levels are highest in the embryonic stages (Fig. S2E,F), suggesting that dLsd1 might also have functions during early stages of development that may be masked in the homozygous *dLsd1* mutants by maternally supplied products.

Biochemical studies with human Lsd1 have led to reports that Lsd1 can act both as a co-repressor of transcription by demethylating H3-K4 [5], and as a co-activator by demethylating H3-K9 [6]. To ask which of these potential activities is predominant in *Drosophila*, we examined the levels of histone modification in *dLsd1* mutant flies. The global level of mono and dimethyl-H3-K4 was considerably higher in *dLsd1* mutants than in wild-type flies (Fig. 2A,B); this effect was particularly striking in adult males (Fig. 2B). In contrast, we found no increase in the global levels of methyl-H3-K9, indeed the level of dimethyl and trimethyl-H3-K9 decreased slightly in *dLsd1* mutants (Fig. 2A,B). The levels of monomethyl-H4-K20,

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dimethyl-H3-K27, dimethyl-H4-K20, trimethyl-H3-K36 and acetyl-H3-K9 were unaltered in *dLsd1*$^{\Delta N}$ mutant flies (Fig. 2A). Interestingly, ovaries contain a higher level of dLsd1 and a lower level of monomethyl-H3-K4 than the rest of the adult female (Fig. 2C), potentially explaining why *dLsd1* mutation selectively perturbs ovary development.

As a further test, we generated *UAST-dLsd1* transgenic flies and found that increased levels of dLsd1 reduced di and monomethyl-H3-K4 (Fig. 2D), confirming that dLsd1 is a critical determinant of the global level of H3-K4 methylation. Surprisingly, these animals lacked any clear developmental defects.

H3-K4 methyl residues are highly enriched in euchromatin. To test whether the elevated levels of mono and dimethyl-H3-K4 in *dLsd1* mutants alter the balance between euchromatin and heterochromatin, we used three variegating systems (*T(2;3)Stubble*^{variegated} *(Sb*^{v}*)*, *In(1) y*^{3P}*yellow* and *In(1)white*^{m4h}) and asked whether *dLsd1* alleles modify positional effect variegation (PEV). PEV is the mosaic pattern of gene silencing observed when genes that are normally located in euchromatin regions are placed into a heterochromatic environment [21]. Suppressors of PEV include mutants of heterochromatin-associated proteins, such as HP1 and the histone H3-K9 methyltransferase *Su(var)3–9* [22].

T(2;3)Sb^{v} translocation juxtaposes the *Sb* mutation and the centric heterochromatin of the second chromosome, resulting in mosaic flies with both *Sb* and normal bristles [23,24]. Activation of dominant *Sb* results in stubble bristles. When *T(2;3)Sb*^{v} was crossed to *dLsd1*$^{\Delta N}$, we observed a significant increase in the frequency of *Sb* bristles (Fig. 3A). Similar results were found using the *yellow* locus [25,26]. Analysis of bristles in the wing of *In(1) y*^{3P}*/+; +; dLsd1*$^{\Delta N}$*/+* flies (Fig. 5B) showed that a single allele of *dLsd1*$^{\Delta N}$ or *dLsd1*^{2} suppressed *yellow* variegation, giving a 25% reduction of yellow bristles (Fig. 3C). Suppression of variegation was also observed using *w*^{m4h} (Fig. S3). These results indicate that *dLsd1* alleles are potent suppressors of PEV and suggest that the absence of *dLsd1* alters chromatin structure.

Mono and dimethyl forms of H3-K4 are linked to transcriptional activation; the increased level of these modifications in *dLsd1*$^{\Delta N}$ mutants suggests that dLsd1 normally represses transcription. Previous studies with human cells have shown that Lsd1 regulates the expression of neuron-specific genes [12]. To ask whether this function is conserved, we depleted dLsd1 from SL2 cells using RNAi and examined the expression of neuron-specific genes, such as Nicotinic Acetylcholine Receptor beta (nAcrβ) and Na channel (Nach). dLsd1 depletion increased the level of monomethyl-H3-K4 and increased the expression of both nAcrβ and Nach (Fig. 4A), indicating that Lsd1's role in the repression of neuron-specific genes is conserved between *Drosophila* and humans.

The homeobox (Hox) gene locus is subject to extensive H3-K4 methylation by Trithorax group proteins [27]. We therefore asked whether the expression level of the *Hox* genes Ultrabithorax (Ubx) and abdominal-A (abdA), is affected by dLsd1 depletion. Ubx and abdA mRNA levels increased two-fold in SL2 cells treated with dLsd1 double stranded RNA (dsRNA) (Fig. 4A). These changes were specific and were not seen with other control genes (dDP and Hid). To verify the relevance of these observations *in vivo* we compared the expression of these genes in wild-type and *dLsd1*$^{\Delta N}$ mutant flies. A significant upregulation of each of these targets was found in *dLsd1*$^{\Delta N}$ mutant flies (Fig. 4B and data not shown), confirming the importance of dLsd1-mediated repression *in vivo*. Intriguingly we observe that this upregulation is age dependent: the difference in gene expression is minimal in larval stages, consistent with this the Hox gene expression pattern in imaginal discs from *dLsd1*$^{\Delta N}$ mutant larvae and in embryos is largely unaltered (Fig. S4). However the level of nAcrβ, Ubx and Abd-B gradually and significantly increases with age after eclosion (Fig. 4B), suggesting that dLsd1 function is especially important for the regulation of gene expression in adult tissues.

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Using *Drosophila melanogaster*, we describe the consequences of eliminating Lsd1 function in an animal model. The results help to place the previously described biochemical activities of Lsd1 into a biological context and show that dLsd1 is a key determinant of the global levels of mono and dimethyl-H3-K4 *in vivo. dLsd1* mutation impacts heterochromatin homeostasis and leads to ectopic expression of a subset of genes in flies. Curiously we observe that the consequences of the global upregulation of H3-K4 methylation on animal development are restricted to a few specific organs. One of the clearest defects is in the ovary where $dLsd1^{\Delta N}$ mutant egg chambers are abnormal early in development and follicle cells fail to encapsulate the cyst. This defect is consistent with the observation that dLsd1 is highly expressed in the ovary, and indicates that dLsd1 mediated demethylation of H3-K4 plays a crucial role in this organ. We suggest that, in $dLsd1^{\Delta N}$ mutants, the elevated expression level of dLsd1 target genes causes tissue specific defects. We note that both Hox genes and neuron-specific genes are among the target upregulated in $dLsd1^{\Delta N}$ mutants. The upregulation occurs late in development and this may explain the lack of homeotic phenotypes or other early developmental defects in $dLsd1^{\Delta N}$ mutants. The stronger changes in histone modification seen in $dLsd1^{\Delta N}$ mutant males compared to females and the sex dependent lethality caused by *dLsd1* mutation raise the possibility that dLsd1 may have a sex-specific distribution on chromatin alternatively its mutation may alter the chromatin distribution of the male specific lethal complex.

Whereas mammalian Lsd1 has been shown to be able to demethylate H3-K9 [6], we saw no increase in the global level of dimethylated H3-K9. This indicates that this activity is either not conserved in *Drosophila*, or that is redundant with other demethylases, or that is restricted to specific loci and/or specific tissues.

This study opens the road to further studies aimed at delineating the specific functions of dLsd1 in the control of gene expression. Genome-wide studies will be necessary to identify all the dLsd1 regulated genes and to identify the target genes responsible for each of the developmental defects.

One important implication of the tissue specific defects seen in the $dLsd1^{\Delta N}$ mutant animals is the possibility that dLsd1 may be manipulated *in vivo* to modulate specific biological processes. The results support the idea that global changes in the levels of histone methylation can impact specific developmental processes. These results also highlight the need for additional studies to understand how histone methylation is dynamically regulated *in vivo* and how these changes contribute to normal development and disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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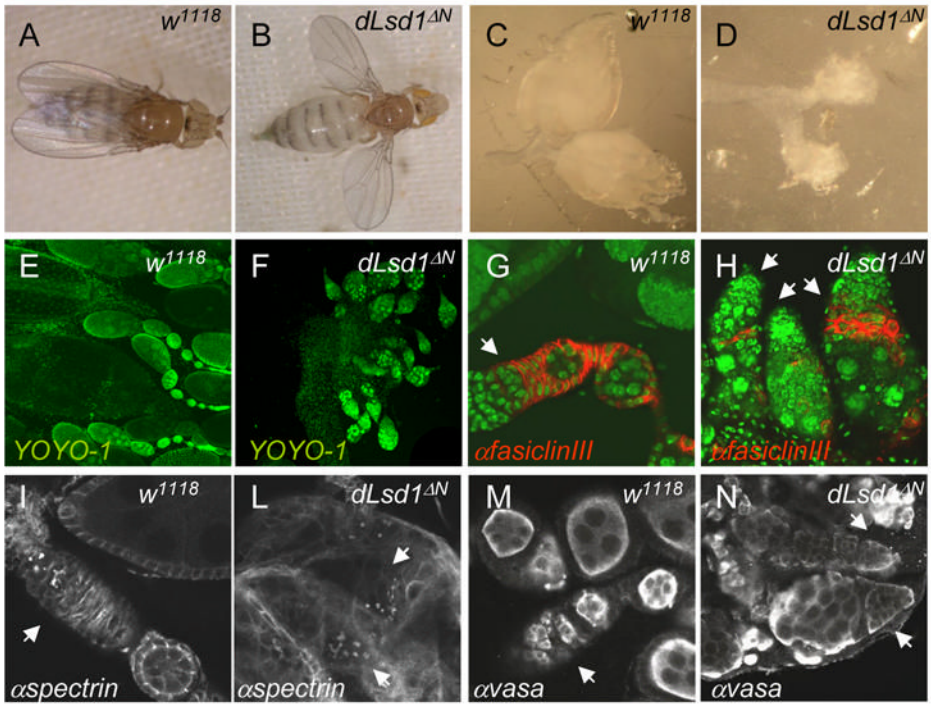

Figure 1. *dLsd1$^{\Delta N}$* mutant animals have developmental defects

A, B: *dLsd1$^{\Delta N}$* mutant flies have a held-out wing phenotype. Images of wild-type (*w^{1118}*) and *dLsd1$^{\Delta N}$* flies. **C, D**: *dLsd1$^{\Delta N}$* mutant ovaries are smaller than wild-type ovaries. Images of the ovaries of wild-type and *dLsd1$^{\Delta N}$* flies. **E, F**: *dLsd1$^{\Delta N}$* mutant ovaries lack proper ovariole structures. DNA staining (YOYO-1) of a wild-type ovary versus the ovary of *dLsd1$^{\Delta N}$* flies. **G, H:** In *dLsd1$^{\Delta N}$* mutant ovaries follicle cells fail to encapsulate the cyst. Wild-type and *dLsd1* mutant ovarioles were stained with YOYO-1 (green) and anti-fasiclin III (red) to outline somatic cells. **I, L, M, N**: Egg chambers of *dLsd1$^{\Delta N}$* mutant ovaries are abnormal at very early stages. Wild-type and *dLsd1$^{\Delta N}$* mutant ovarioles were stained with anti-αspectrin (white), to show the fusomes (I,L) and anti-vasa (white) to label the germ cells (M,N). The germarium is indicated by arrows.

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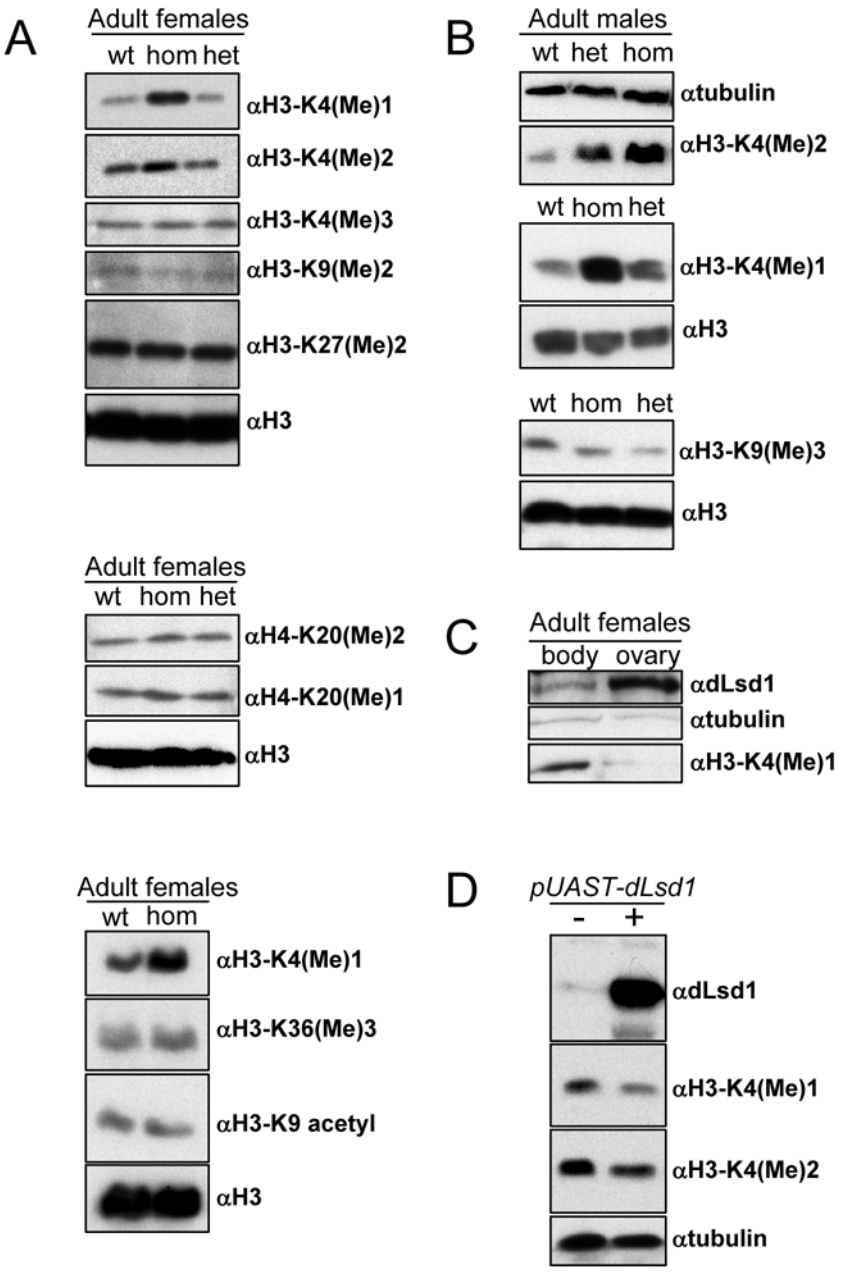

Figure 2. dLsd1 specifically affects global levels of H3-K4 mono and dimethylation

A: Western blot analysis of protein extracts from w^{1118}*(wt)*, $dLsd1^{\Delta N}/dLsd1^{\Delta N}$ *(hom)* and $dLsd1^{\Delta N}/TM3$ *(het)* females using antibodies specific for different modified histone H3 and H4 residues as indicated on the right. Equal loading was assessed by blotting with either a histone H3 specific antibody or a tubulin specific antibody. **B:** Western blot analysis of protein extracts from w^{1118}, $dLsd1^{\Delta N}/dLsd1^{\Delta N}$ and $dLsd1^{\Delta N}/TM3$ males. **C:** dLsd1 is highly expressed in the ovary. Western blot analysis of dLsd1 expression and of the level of H3-K4 monomethylation in the ovary. dLsd1 protein level are higher in the ovary than in the rest of the body and this correlates with lower levels of H3-K4 monomethylation. **D:** Ectopic expression of dLsd1 affects H3-K4 methylation levels and gene expression. Ectopic expression of the *pUAST-dLsd1* transgene was driven by the *Actin5C-GAL4* driver. dLsd1 expression was verified by Western blot analysis. The level of mono and dimethyl H3-K4 in w^{1118} versus

dLsd1 overexpressing flies are shown. Equal loading was assessed using a tubulin specific antibody.

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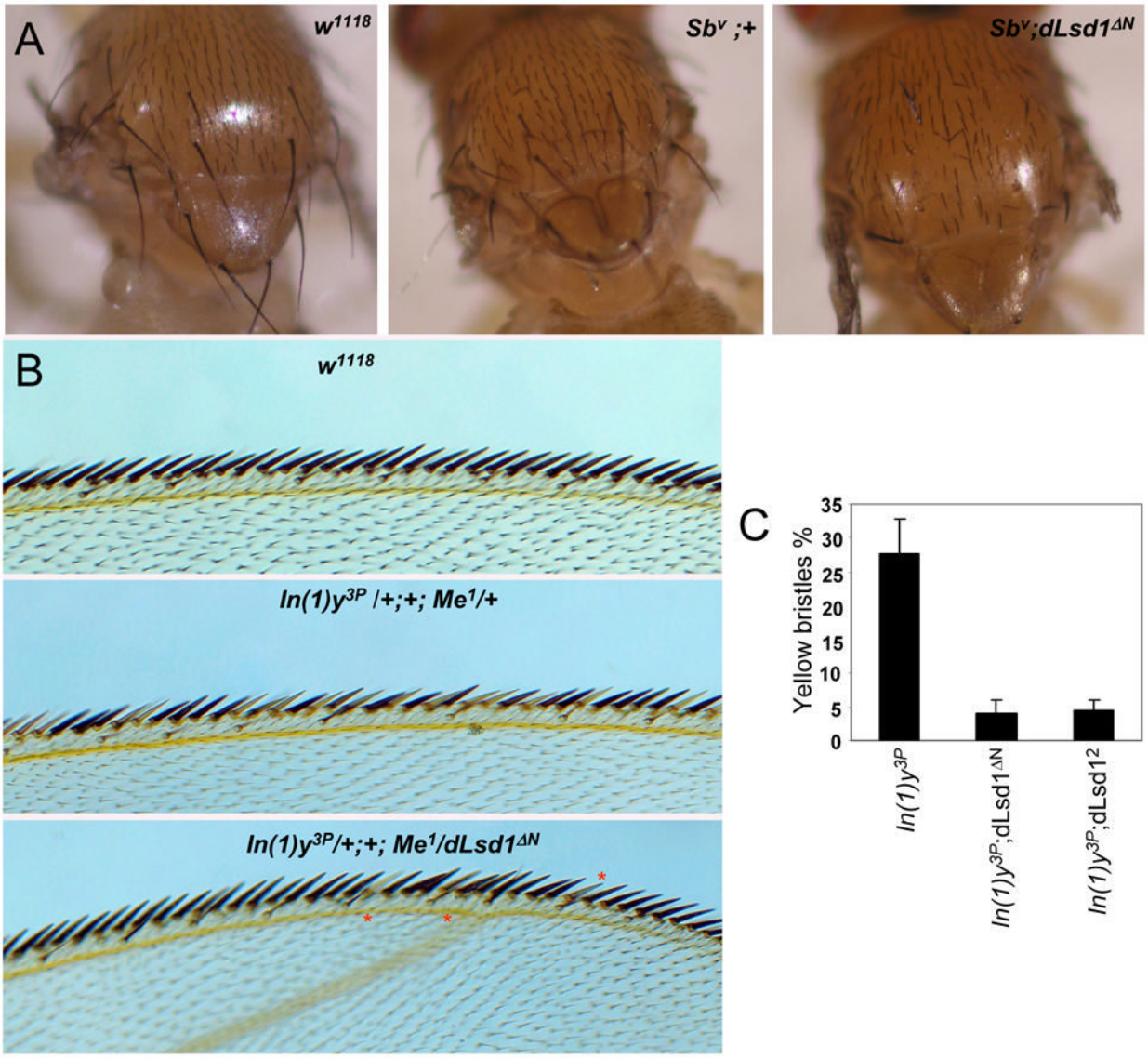

Figure 3. dLsd1 is a suppressor of PEV

Heterozygotes for the *dLsd1*$^{\Delta N}$ allele were crossed to either *T(2;3)Sb*^{v} or *In(1)y*^{3P}. The effects of *dLsd1*$^{\Delta N}$ on *Stubble* variegation of *T(2;3)Sb*^{v} are shown in **A**. The effects of *dLsd1*$^{\Delta N}$ on *Yellow* variegation are shown in **B**. Red asterisks indicate the yellow bristles in *In(1)y*^{3P}*/+;+; Me*^{1}*/dLsd1*$^{\Delta N}$ flies. **C:** Quantification of the percentage of yellow bristles in the indicated genotypes.

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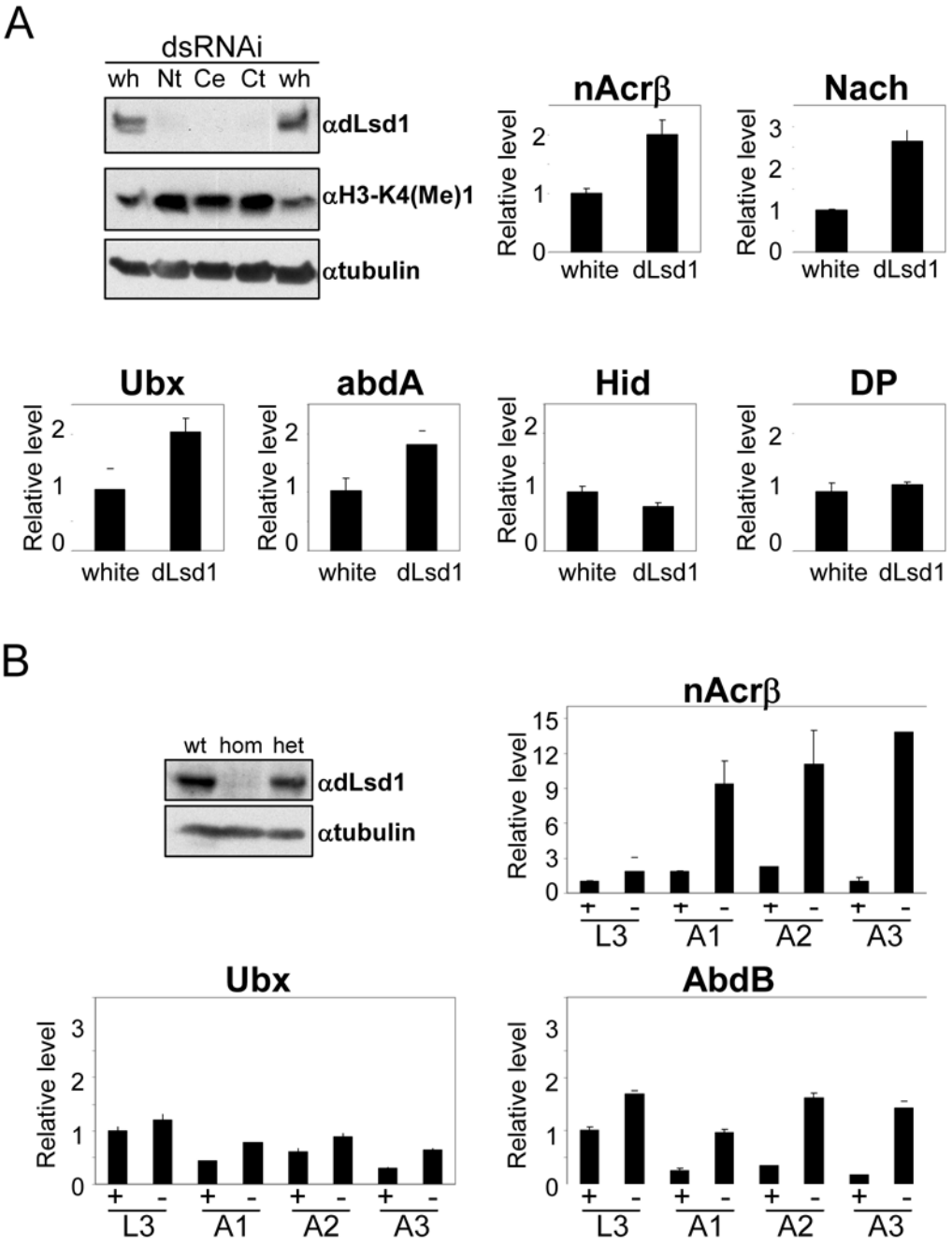

Figure 4. Neuron specific genes and *Hox* genes are misexpressed in SL2 cells depleted for dLsd1 and in *dLsd1*$^{\Delta N}$ mutant animals

A: Western blot analysis of SL2 cells treated for 8 days with white and dLsd1 dsRNA. Three different dsRNAs directed against the N-terminal (Nt), Central (Ce) or C-terminal (Ct) region target dLsd1 protein with the same efficacy while dsRNA against the *white* gene (wh), used as negative control, does not affect dLsd1 protein expression. qPCR analysis of the expression of the indicated genes 8 days after dsRNA treatment of SL2 cells. The expression level is normalized against the white dsRNA treated control. Experiments were performed in triplicate and standard deviation of the mean is indicated. **B:** Western blot analysis of wild-type (wt), *dLsd1*$^{\Delta N}$/*dLsd1*$^{\Delta N}$ (hom) and *dLsd1*$^{\Delta N}$/*TM3* (het) flies with an antibody specific for dLsd1 and

with anti-tubulin (loading control). qPCR analysis of the expression of the indicated genes in wild-type (+) and *dLsd1* mutant (−) third instar larvae (L3) and adult flies respectively 1, 2 and 3 days after eclosion (A1, A2, A3). Rp49 was used as a control (data not shown). Experiments were performed in triplicate and standard deviation of the mean is indicated.

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