Depletion of histone deacetylase 3 antagonizes PI3K-mediated overgrowth of Drosophila organs through the acetylation of histone H4 at lysine 16

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Summary
Core histone modifications play an important role in chromatin remodeling and transcriptional regulation. Histone acetylation is one of the best-studied gene modifications and has been shown to be involved in numerous important biological processes. Herein, we demonstrated that the depletion of histone deacetylase 3 (Hdac3) in Drosophila melanogaster resulted in a reduction in body size. Further genetic studies showed that Hdad3 counteracted the organ overgrowth induced by overexpression of insulin receptor (InR), phosphoinositide 3-kinase (PI3K) or S6 kinase (S6K), and the growth regulation by Hdad3 was mediated through the deacetylation of histone H4 at lysine 16 (H4K16). Consistently, the alterations of H4K16 acetylation (H4K16ac) induced by the overexpression or depletion of males-absent-on-the-first (MOF), a histone acetyltransferase that specifically targets H4K16, resulted in changes in body size. Furthermore, we found that H4K16ac was modulated by PI3K signaling cascades. The activation of the PI3K pathway caused a reduction in H4K16ac, whereas the inactivation of the PI3K pathway resulted in an increase in H4K16ac. The increase in H4K16ac by the depletion of Hdad3 counteracted the PI3K-induced tissue overgrowth and PI3K-mediated alterations in the transcription profile. Overall, our studies indicated that Hdad3 served as an important regulator of the PI3K pathway and revealed a novel link between histone acetylation and growth control.

Key words: Drosophila, Histone deacetylase 3, Body size, H4K16 acetylation, PI3K signaling

Introduction
Core histone modifications are known to play an essential role in the regulation of chromatin organization and transcription. These modifications include acetylation, methylation, phosphorylation, ubiquitination, sumoylation and poly(ADP-ribosylation) (Berger, 1999; Jenuwein and Allis, 2001; Kouzarides, 2007). Histone acetylation is one of the best-studied modifications and is thought to be involved in both the initiation and elongation steps of transcription (Vogelauer et al., 2000; Hassan et al., 2001; Wang et al., 2009). The acetylation of the core histone tails alters the folding dynamics of nucleosomal arrays and 30-nm chromatin fibers (Luger and Richmond, 1998; Annunziato and Hansen, 2000) and recruits specific chromatin remodeling complexes that exert the specific function(s) of chromatin (Turner, 2000; Shabbazian and Grunstein, 2007).

The acetylation of histones is regulated by two highly conserved classes of histone enzymes, histone acetyltransferases (HATs) and histone deacetylases (HDACs) (Peterson and Laniel, 2004), which catalyze the addition and removal, respectively, of acetyl groups on histone lysine residues (Roth et al., 2001; Struhl, 1998). Reversible histone acetylation and deacetylation are highly regulated processes that are crucial for chromatin reorganization and the regulation of gene transcription in response to extracellular conditions. The balance between the acetylation and deacetylation of histones serves as a key regulatory mechanism for gene expression and governs numerous developmental processes and disease states (Marks et al., 2001; Butler and Bates, 2006).

HDACs have been classified into four subfamilies based on their homologs and functional similarities (Witt et al., 2009). Hdad3 is a class I HDAC that shares homology with yeast Rpd3. This protein is reportedly present in the nuclear, cytoplasmic and membrane fractions (Yang et al., 2002). The knockout of Hdad3 in mice leads to embryonic lethality before day 9.5 (Bhaskara et al., 2008). The inactivation of Hdad3 has been shown to delay cell cycle progression and result in cell cycle-dependent DNA damage, inefficient repair and increased apoptosis in mouse embryonic fibroblasts (Montgomery et al., 2008; Knutson et al., 2008; Zampetaki et al., 2010). Hdad3 has also been shown to be upregulated in various tumor types (Spurling et al., 2008; Wu et al., 2010; Wilson et al., 2006). However, the precise function and underlying molecular mechanism of Hdad3 in these processes remain largely unknown.

The Drosophila melanogaster (D. melanogaster) ortholog to human Hdad3 is known to be Hdad3 or dHdad3 (Johnson et al., 1998). Herein, we used D. melanogaster to investigate the function of Hdad3 during development. We found that the depletion of Hdad3 in D. melanogaster results in a reduction in both organ and body sizes. Hdad3 controls growth through the regulation of H4K16 deacetylation. Alterations in H4K16ac through the ectopic expression of MOF, a histone acetyltransferase that specifically
targets H4K16, result in changes of cell/body size. We also found that H4K16ac is modulated by PI3K signaling. Increasing the level of H4K16ac by depleting Hdac3 effectively reverses the PI3K-induced tissue overgrowth and alterations in the transcription profile.

**Results**

The depletion of Hdac3 results in a reduction in both organ and body sizes in *Drosophila melanogaster*

Hdac3 is conserved between fruit flies and mammals (supplementary material Fig. S1). To investigate the role of Hdac3 during development, transgenic flies expressing double-stranded (ds) *D. melanogaster* Hdac3 RNA were produced using a UAS/Gal4 system as previously described (Liu et al., 2005). An Hdac3 cDNA fragment (between 691 and 1198 bp in its open reading frame) was cloned into a SympUAS vector, which allowed the Hdac3 fragment to be transcribed bi-directionally, thereby producing dsRNA and silencing the endogenous gene targets. The ubiquitous expression of Hdac3 RNAi using the actin-Gal4 driver resulted in lethality before the 3rd-instar larval stage. Tissue-specific expression of Hdac3 RNAi was achieved by crossing the flies carrying the RNAi transgene with flies carrying eyeless-Gal4 (ey-Gal4) or engrailed-Gal4 (en-Gal4), which resulted in a reduction in the size of the eye (Fig. 1B) and the posterior compartment of the wing (Fig. 1C). When Hdac3 RNAi was expressed in the fat body using pumpless-Gal4 (ppl-Gal4), the progeny of the *Hdac3* RNAi flies were partially lethal (supplementary material Table S1), and the surviving progeny had a small body size at the 3rd-instar larval, pupal and adult stages (Fig. 1D). The average weight of the *Hdac3* RNAi female progeny was ~16% less than that of the controls (ppl-Gal4/+); female flies (*P*<0.05; *n*=5 groups per genotype); 100 flies in each group, with the same quantity used in each of the following groups, unless specified), and the average weight of the male progeny was reduced by ~14% (*P*<0.05, *n*=5 groups) compared with that of the controls (ppl-Gal4/+, *n*=5 groups). Two independent transgenic *Hdac3* RNAi lines were both found to affect body size, implying that the defect was not due to the location of the transgene in the genome.

To confirm the effect of Hdac3 on growth, we also produced additional transgenic fly lines that overexpress Hdac3. Progeny ectopically expressing Hdac3 with the actin-Gal4 driver indeed exhibited slightly increased body size compared with that of the controls (supplementary material Fig. S2). Compared with the controls (actin-Gal4/+, *n*=5 groups), the body weight of the female

![Bar diagram showing the average weight of Hdac3 RNAi adult progeny relative to the parental control.](image)

Fig. 1. The depletion of Hdac3 results in reduced organ and body size. (A) The expression level of Hdac3 in transgenic flies ppl >Hdac3 Rl (ppl-Gal4/+) was determined by qRT-PCR. Rp49 was used as an internal control. ppl/+ (ppl-Gal4/+), and +/Hdac3 Rl (UAS-Hdac3 RNAi/+), are the respective parental controls. (B) The depletion of Hdac3 in the eye reduces the size of the eye. The images shown are representative of male fly eyes taken at the same magnification. The scale bars indicate 0.15 mm. ey/+ (eyeless-Gal4/+, ey >Hdac3 Rl (eyeless-Gal4/+, UAS-Hdac3 RNAi/+), +/Hdac3 Rl (UAS-Hdac3 RNAi/+). (C) The depletion of Hdac3 in the wing reduces the size of the posterior wing compartment. Representative images of male fly wings are shown. The scale bars indicate 0.2 mm. en/+ (en-Gal4/+, en >Hdac3 Rl (en-Gal4/+, UAS-Hdac3 RNAi/+), +/Hdac3 Rl (UAS-Hdac3 RNAi/+). (D) The depletion of Hdac3 in the fat body results in a reduction in body size in the 3rd-instar larval (a), pupal (b) and adult (c, female; d, male) progeny. (e) The bar diagrams show the average weight of the Hdac3 RNAi adult progeny (middle) relative to the ppl/+ parental control (left, set at 100%). A total of 100 flies were used in each group, *n*=5 groups per genotype. The data are represented as the means ± standard deviation (SD). The *P*-values were calculated by one-way analysis of variance (ANOVA). 1, ppl/+ (ppl-Gal4/+); 2, ppl >Hdac3 Rl (ppl-Gal4/+, UAS-Hdac3 RNAi/+); 3, +/Hdac3 Rl (UAS-Hdac3 RNAi/+).
Hdac3 inhibits the PI3K pathway via H4K16ac

progeny increased by 6% \((P<0.05, n=5 \text{ groups})\), and the body weight of the male progeny increased by 5% \((P<0.05, n=5 \text{ groups})\). The phenotype resulting from the depletion of Hdac3 was rescued by the co-expression of Hdac3 (data not shown), implying that the growth defects specifically result from a reduction in Hdac3.

**Hdac3 controls growth by regulating cell size and number**

To examine whether the reduction in organ/body size after the depletion of Hdac3 was due to the decrease in cell size, cell number or both, the phenotype of depleted Hdac3 was characterized by quantifying the area and number of the ommatidia, which reflect cell size and cell number, respectively. Scanning electron micrographs (SEMs) of whole eyes showed that the eyes of Hdac3 RNAi flies were smaller than those of the controls. The average area of the male fly eyes was reduced by 30% \((P<0.01, n=20)\) when compared with those of the controls \((n=18)\) (Fig. 2).

The size of the ommatidia was determined by counting the number of ommatidia in a specific square \((0.13 \text{ mm} \times 0.13 \text{ mm})\). The depletion of Hdac3 resulted in an 11% reduction in ommatidial size \((P<0.05, n=20)\) when compared with that of the controls \((n=18)\) (Fig. 2). The total number of ommatidia in Hdac3-depleted eyes revealed a 24% reduction \((P<0.01, n=20)\) when compared with that of the controls \((n=18)\) (Fig. 2). The 11% decrease in cell area and 24% decrease in cell number can account for the 30% reduction in eye area observed in the Hdac3-depleted flies. Therefore, these results suggest that Hdac3 controls organ/body size via the alteration of both cell size and cell number.

**PI3K pathway activation is impaired in Hdac3-depleted flies**

The InR/PI3K pathway is reported to be important in regulating growth (Chen et al., 1996; Leevers et al., 1996; Montagne et al., 1999). The inactivation of positive regulatory components of the pathway has been shown to decrease organ size, whereas the overexpression of these regulatory components can result in tissue overgrowth. Given the similar phenotypes between the depletion of Hdac3 and the inactivation of the pathways, we suspected that Hdac3 is a component of these pathways. To test this possibility, we then studied the subcellular localization of the pleckstrin homology (PH) domain using a transgenic fly expressing a GFP-PH domain fusion protein \((\text{tGPH})\) (Britton et al., 2002).

In the InR/PI3K signaling pathway, insulin, or an insulin-like ligand, binds to and activates the insulin receptor tyrosine kinase \((\text{InR})\) on target cells (Chen et al., 1996), resulting in the activation of phosphatidylinositol 3-kinase \((\text{PI3K})\) (Leevers et al., 1996). Activated PI3K generates the second messenger phosphatidylinositol-3,4,5-trisphosphate, which in turn binds to the PH domain-containing proteins and transports it from the cytosol to the plasma membrane (Lizcano and Alessi, 2002). Therefore, the activation of PI3K signaling is often evaluated by the subcellular localization of the \(\text{tGPH}\) reporter. If Hdac3 depletion is associated with the activation of PI3K signaling, the \(\text{tGPH}\) reporter may be relocated. Indeed, we found that the \(\text{tGPH}\) reporter showed a marked reduction of plasma membrane-bound \(\text{tGPH}\) in the larval fat body cells of Hdac3 RNAi flies, whereas in the control larvae, \(\text{tGPH}\) was mainly localized at the plasma membrane (Fig. 3A).

We next measured the level of phospho-Akt and phospho-S6K in the Hdac3-depleted flies using western blot analysis. The results showed that the depletion of Hdac3 caused a slight decrease in the level of phospho-Akt and phospho-S6K (Fig. 3B), supporting an interaction between Hdac3 and the PI3K-Akt pathway. In addition, we tested the interaction of Hdac3 with the JAK-STAT and the EGFR pathways. Neither the JAK-STAT nor the EGFR pathways was obviously affected in the Hdac3-depleted flies (supplementary material Fig. S3). These observations support that the depletion of Hdac3 negatively regulates PI3K pathway, but not others.

**The depletion of Hdac3 counteracts the tissue overgrowth induced by InR, PI3K or S6K**

InR, PI3K and S6 kinase \((\text{S6K})\) (Montagne et al., 1999) are positive regulatory components of the InR/PI3K/S6K pathways. The inactivation of these positive regulatory components has been verified to decrease organ size, whereas their overexpression results in tissue overgrowth. To further evaluate the functional role of Hdac3 in the InR/PI3K signaling pathways, we performed genetic experiments in the eye using \(\text{GMR-Gal4}\).

The overexpression of InR is known to cause large, disorganized eyes (Chen et al., 1996). However, these overgrowth phenotypes were strongly suppressed when Hdac3 was depleted in the eye in conjunction with InR overexpression (Fig. 4). Notably, the progeny co-expressing InR and Hdac3 RNAi had a phenotype that resembled those observed in flies expressing Hdac3 RNAi alone. Similar results were also obtained when PI3K or S6K was co-expressed with Hdac3 RNAi (Fig. 4). These results suggest that Hdac3 is also functionally involved in the organ growth regulated by InR, PI3K or S6K.
The depletion of Hdac3 leads to an increase in histone H4 acetylation at lysine 16

Loss-of-function mutations in *Hdac3* are known to affect histone acetylation and position-effect variegation (Zhu et al., 2008), a heterochromatin-associated transcriptional gene silencing phenomenon (Eissenberg and Elgin, 2000). We were interested in exploring whether Hdac3 regulates organ/body size through the regulation of histone acetylation. To test this hypothesis, we performed immunostaining to examine the effects of Hdac3 depletion on histone acetylation. We expressed *Hdac3* RNAi in wing discs with *en-Gal4*, which activated the Gal4 driver in the posterior wing compartment. The wing discs were dissected and stained using antibodies against the specific acetylated lysines on the core histones H3/H4. The stained wing discs showed an increase in H4K16ac where Hdac3 was depleted (Fig. 5A, circled area). In contrast, H4K12 acetylation was not remarkably affected. Western blots showed the same results (Fig. 5A).

We also used *hsFLP/FRT*-mediated mitotic recombination to generate Hdac3-depleted clones in the fat body (Manfruelli et al., 1999). Larvae with GFP fluorescent signals were selected and wing discs with *en-Gal4*, which activated the Gal4 driver in the posterior wing compartment. The wing discs were dissected and stained using antibodies against the specific acetylated lysines on the core histones H3/H4. The stained wing discs showed an increase in H4K16ac where Hdac3 was depleted (Fig. 5A, circled area). In contrast, H4K12 acetylation was not remarkably affected. Western blots showed the same results (Fig. 5A).

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dissected. The fat body tissues were stained using antibodies against H4K16ac and discs large (Dlg, a septate junction protein that labels membranes). Compared with the control cells adjacent to the induced clones, the Hdac3-depleted clones exhibited elevated H4K16ac levels (Fig. 5A). These results indicate that Hdac3 depletion triggers an increase in H4K16ac levels.

Histone H4K16 acetylation is known to play an important role in numerous biological processes, such as chromatin remodeling, the DNA damage response, double-strand break (DSB) repair and the cellular life-span (Shogren-Knaak et al., 2006; Krishnan et al., 2011; Sharma et al., 2010; Dang et al., 2009). To investigate whether H4K16ac is involved in Hdac3-mediated growth control processes, we next generated transgenic flies overexpressing H4K16A, an H4 mutant in which the lysine at residue 16 (K16) is mutated into an alanine (K16A), which makes the residue incapable of being acetylated. The results showed that the overexpression of the H4K16A mutant in the Hdac3 RNAi background partially attenuated the Hdac3 depletion-induced decrease in eye size (Fig. 5B). These results indicate that Hdac3 depletion triggers an increase in H4K16ac levels.

Hdac3 regulates organ/body size via the regulation of H4K16ac

Histone H4K16 acetylation is modulated by the PI3K signaling pathway

PI3K signaling is a key signal transduction pathway with many important physiological responses (Cantley, 2002). However, both the precise role of PI3K signaling and the mechanism by which the PI3K signaling cascades regulate gene transcription in the nucleus remain to be determined. Chromatin remodeling events, especially histone posttranslational modifications, provide an epigenetic mechanism for the regulation of many nuclear events, including gene transcription. The genetic relationship between Hdac3 and the PI3K pathway prompted us to test whether PI3K signaling is also associated with H4K16ac. We performed immunostaining in wing discs from progeny that overexpressed either wild type PI3K (en >PI3K) or a dominant negative PI3K mutant (en >PI3KDN) using antibodies against H4K16ac and H4K12ac. The results indicated that the activation of PI3K (en >PI3K) in the posterior wing compartment (circled
The increase in H4K16ac by Hdac3 depletion counteracts the PI3K-induced tissue overgrowth and global transcriptional profile

Given that H4K16ac is involved in growth and that PI3K regulates both the growth of tissues and the level of H4K16ac, we explored whether Hdac3 inhibits PI3K signaling via the regulation of H4K16ac. Immunoassays were performed in wing discs. The results showed that the depletion of Hdac3 rescued the decrease in H4K16ac caused by the overexpression of PI3K (Fig. 8A). The PI3K-induced overgrowth phenotypes were also suppressed by the depletion of Hdac3 (Fig. 8B). Notably, the hyperphosphorylation of Akt following PI3K activation appeared not to be affected after the depletion of Hdac3 (Fig. 8A).

To confirm the role of H4K16ac in the PI3K-mediated growth regulation, the co-expression of H4K16Ac and PI3K was performed under the control of the GMR-Gal4 driver. As previously reported, the overexpression of PI3K resulted in large, disorganized eyes (Leevers et al., 1996). The presence of H4K16Ac further enlarged the PI3K-mediated increase in ommatidial size (supplementary material Fig. S4), confirming that Hdac3 antagonizes PI3K signaling via its regulatory effect on the acetylation of histone H4K16.

To further investigate the molecular mechanisms underlying the effect of Hdac3 depletion on the PI3K pathway, both microarray analysis and quantitative real-time PCR were conducted to compare the transcriptional activity in flies overexpressing PI3K with that in flies co-expressing PI3K and Hdac3 RNAi. The gene expression profile analysis revealed that a large proportion of upregulated genes observed in the PI3K overexpressing flies were downregulated in the Hdac3 RNAi flies and that a number of genes downregulated in the PI3K overexpressing flies were upregulated in the Hdac3 RNAi flies (Fig. 8C). Notably, the depletion of Hdac3 in the PI3K overexpressing flies restored ~85% (288 out of the 339 genes) of the twofold difference in gene transcription induced by the activation of the PI3K pathway (Fig. 8C). The transcriptional changes in the genes involved in the regulation of size (Puig et al., 2003; Huang et al., 1999; Bender et al., 1997) were further detected using quantitative real-time PCR. As shown in Fig. 8D, the negative regulators of growth, such as FOXO (forkhead box, sub-group O), PTEN (phosphatase and tensin...
homolog), EcR (Ecdysone receptor) and Eip74EF (Ecdysone-induced protein 74EF), that were downregulated in the PI3K overexpressing flies were rescued by Hdac3 depletion. These results demonstrated that the depletion of Hdac3 also counteracted the PI3K-mediated gene expression.

Discussion
A fundamental question in biology is how organisms are able to grow to an appropriate body and organ size and what are the mechanisms that control the process of growth. Drosophila melanogaster is an ideal model system for examining the mechanisms of growth regulation. Previous studies have identified several genes associated with growth, but the molecular mechanisms have not been fully understood. In this paper, we utilized a Drosophila UAS/Gal4 system and characterized the function of Hdac3 in Drosophila. The results suggest that Hdac3 is a critical player in both organ and body growth. Hdac3 depletion caused a reduction in both organ and body size due to fewer and smaller cells (Fig. 1; Fig. 2).

Hdac3 is a component of the nuclear receptor co-repressor complex containing N-CoR (nuclear receptor corepressor) and SMRT (silencing mediator for retinoid and thyroid hormone receptors), both of which are recruited by nuclear hormone receptors to regulate gene transcription (Li et al., 2000; Guenther et al., 2001). Several substrates were found to be targets of Hdac3, including histones (Bhaskara et al., 2010; Johnson et al., 2002) and non-histone proteins (Yang et al., 1997; Zampetaki et al., 2010). Among the targets affected by Hdac3, we found that H4K16ac is a critical epigenetic modification associated with animal growth, as demonstrated not only by the finding that alterations in H4K16ac were closely associated with Hdac3-induced organ/body growth but also by the finding that mutating H4K16 directly affected Hdac3-induced growth (Fig. 5).

Histone H4K16 acetylation is known to function as a dual switch for higher-order chromatin and protein–histone interactions (Shogren-Knaak et al., 2006) and has been shown to regulate embryonic stem cell self-renewal (Li et al., 2012) and cellular life span (Dang et al., 2009). Recent work in our laboratory has suggested that H4K16ac in Drosophila not only is critical for the acetylation of H4K5, H4K8 and H3K9, which are hallmarks of active chromatin, but also exerts an effect on H3K9 methylation and the association of HP1 with chromatin, which are hallmarks of heterochromatin (H.-M.W. and F.-L.S., unpublished data, report in preparation). We therefore presume...
that the changes in H4K16ac affect higher-order chromatin and alter the transcription of genes related to growth. However, the exact mechanism by which H4K16ac regulates the transcription of genes related to growth needs to be further investigated.

One of the main findings in this work is the genetic interaction between Hdac3/H4K16ac and the PI3K pathway. The PI3K pathway is a highly conserved signal transduction cascade from flies to humans. Previous studies have identified a number of the components of this signaling pathway. However, the mechanisms by which this pathway regulates nuclear events, such as gene transcription, remain largely unknown. In this work, we showed for the first time that PI3K signaling modulates the acetylation of H4K16ac. This finding was supported by our results showing that the activation of PI3K in the transcription profile caused by PI3K overexpression. The figure shows the results of the unsupervised cluster (hierarchical cluster, uncentered correlation, average linkage) of 7785 genes with an intensity filter (the probe intensities were greater than 50 on at least three arrays) and a variance filter (hybridization signal intensities vary across the samples). Each row represents one gene. Red and green indicate up- and downregulation, respectively. (D) The Hdac3- and PI3K-induced alterations in gene transcription were tested by quantitative real-time PCR. The data are expressed as the means ± s.d. from three independent experiments. TSC1 was used as a negative control, and Rp49 was used as an internal control.

Although the exact mechanism by which PI3K regulates H4K16ac is still unknown, we demonstrated that the loss of Hdac3 inhibited PI3K-mediated overgrowth, thus suggesting that PI3K targets the activity of Hdac3 and subsequently affects H4K16ac. This hypothesis is supported by the observations that Drosophila Hdac3 can form a complex with Akt (W.W.L. and F.L.S., unpublished data) and that the complex of human Hdac3 with the deacetylase activation domain (DAD), the human SMRT co-repressor and inositol tetraphosphate is required for the activation of Hdac3 enzymatic functionality (Watson et al., 2012). Our observation that the depletion of Hdac3 decreased the level of phospho-Akt and affected the subcellular localization of GFP-PH (Fig. 3) also supported this possibility. However, the observation that Hdac3 depletion failed to counteract the PI3K-induced hyperphosphorylation of Akt while completely rescuing the decrease in H4K16ac and the tissue overgrowth induced by the PI3K overexpression (Fig. 8) indicated that Hdac3 likely counteracts the PI3K-induced tissue overgrowth by modulating the level of H4K16ac.

The hyperactivation of the PI3K pathway is known to be associated with many types of human cancer (Ghayad and Cohen,
2010; Martelli et al., 2009; Kawauchi et al., 2009). A number of HDAC inhibitors have been developed and applied in clinical trials to inhibit tumor growth (Witt et al., 2009). However, the molecular mechanisms of these HDAC inhibitors in cancer prevention remain to be elucidated. In the present study, we found that the overexpression of PI3K decreases H4K16ac in vivo (Fig. 6). Further studies have shown that increasing the level of H4K16ac by depleting Hdac3 can antagonize the PI3K-induced tissue overgrowth (Fig. 8). This finding, therefore, may provide further insight into the mechanisms by which the HDAC inhibitors inhibit tumor growth.

Materials and Methods

Drosophila stocks and genetic crosses

The flies were cultured on standard cornmeal, sucrose and yeast agar medium at 25°C unless specified. The following fly lines were used in this study: actin-Gal4, GMR-Gal4, engrailed-Gal4 (en-Gal4), eyeless-Gal4 (ey-Gal4), OK107-Gal4, pupiless-Gal4 (ppl-Gal4) (Zinke et al., 1999), hs-FLP, [Act5C <y-<Gal4]/UAS-GFP(nls)/cyo, UAS-PI3K (BL#8286), UAS-Inr (BL#8262), UAS-Six (BL#6910) and UAS-PKA(UAS-PKA) (BL#8289), w1118 was used as the wild-type (WT) control. Detailed genetic information about these stocks can be found in FlyBase.

We used hsFLP/FRT-mediated mitotic recombination (Xu and Rubin, 1993) to generate the desired mosaic clones in the fat bodies of flies as previously described (Manfrelli et al., 1999). Female transgenic flies were crossed with male flies (yw1/1, hs-FLP; [Act5C <y-<Gal4]/UAS-GFP(nls)/cyo) and cultured at 25°C. The 2–6-hour embryos were collected and heat-shocked at 37°C for 1 hour. The embryos were then transferred to 25°C and cultured for several days until they reached the 3rd larval stage. The larvae with GFP fluorescence signals were selected and dissected.

Con structs and production of transgenic flies

Transgenic flies were produced using the pUAS system (Rubin and Spradling, 1982). To produce the Hdac3 and MOF RNAi constructs, ~500 bp of the coding sequences of the genes were amplified by RT-PCR and subcloned into the Sym-pUAST vector, which has two inverted UAS sequence elements (Giordano et al., 2002). This construct allows the inserts to be transcribed bi-directionally, thereby producing dRNA that silences endogenous gene targets.

The full-length cDNAs encoding the MOF and Hdac3 genes were amplified by RT-PCR and cloned into the pUAST vector to generate the MOF and Hdac3 overexpression vectors. Each vector contains a GFP tag at the C-terminus of the protein.

The full-length cDNAs encoding the histone H4 were amplified by RT-PCR and cloned into the pCR7/CT-TOPO vector to generate the pCR7-H4 vector. Starting with the pCR7-H4 vector, we used a PCR-based site-directed mutagenesis kit (TaKaRa, D401) to introduce the H4K16 point mutation, Lys (K16) to Ala (K16A). The mutant H4 was subcloned into the pUAST vector to generate the mutant H4K16A overexpression vector, which contains a GFP tag at the C-terminus of the protein.

All of the constructs were confirmed by double-stranded DNA sequencing. The PCR primer sequences are provided in supplementary Material Table S2. The purified plasmid was injected into w1118 embryos together with a helper plasmid that expressed transposase to generate transgenic flies according to the standard germline transformation procedure.

Real-time PCR

Total RNA was isolated using the TRIzol reagent according to the manufacturer’s instructions (Invitrogen). Complementary DNA (cDNA) was reverse transcribed using the M-MLV reverse transcriptase enzyme (Promega). RealMasterMix (SYBR Green; Tiangen FP202) was used in the subsequent real-time PCR analyses. MRNA (mRNA) levels were normalized against the housekeeping gene (SYBR Green; Tiangen FP202) was used in the subsequent real-time PCR analyses. MRNA (mRNA) levels were normalized against the housekeeping gene. The expression profiling data were analyzed by the CapitalBio Corporation (Irrazary et al., 2003). A probe set with intensities that were higher than 50 on at least three arrays was selected and subjected to hierarchical cluster analysis. In the unsupervised analysis, the dataset was processed with a variation filter to eliminate probe sets with hybridization signal intensities that did not show large variability. The hierarchical cluster analyses (uncentered correlation and average linkage) were performed using log2-transformed data in which the median value of each probe set was set to zero, and the data were viewed with the algorithms in the software package R biocomputer. The data and the expression profiles were deposited in NCBI’s Gene Expression Omnibus and can be accessed through GEO Series accession number GSE38552.

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Supplementary material available online at


References


Fig. S1. Hdac3 is a highly conserved protein present in various species. Alignment of Hdac3 protein sequences in *Drosophila*, mice and humans using the DNAMAN software.
Fig. S2. Ubiquitous expression of Hdac3 increased the body size in the 3rd-instar larvae, pupae and adults.

Bar diagrams showing the average weight of the Hdac3 over-expressing adults, act>Hdac3 ov (act-Gal4/+; UAS-Hdac3 ov/+), relative to that of the act/+ parental controls (act-Gal4/+, set at 100%). Hdac3 ov/+ (UAS-Hdac3 ov+) is another parental control. A total of 100 flies were weighed in each group, n = 5 groups per genotype. The data are expressed as the means ± SD. The p-values were calculated by ANOVA.
Fig. S3. Neither the JAK-STAT nor the EGFR pathway was affected by Hdac3.

**A.** The EGFR pathway was not affected by the depletion of Hdac3. Immunostaining analysis was performed in wing discs with antibodies against ERK and ERK-p. The circled areas show the posterior wing compartment expressing en-Gal4.

**B.** The expression pattern of STAT-GFP failed to show an obvious change in the Hdac3-depleted cells. The STAT-GFP reporter was used in this experiment. Hdac3 dsRNA was expressed in the dorsal region of the wing disc using the ap-Gal4 driver and in the posterior compartment of the wing using the en-Gal4 driver. The enclosed areas (with arrows), where the H4K16ac levels are increased, mark the Hdac3-depleted compartments.
**Fig. S4.** H4K16A mutation enhances the PI3K-mediated tissue overgrowth phenotypes.

SEMs showing compound eyes from female flies under the control of the GMR-Gal4 driver. The scale bars indicate 50 µm. The bar diagram shows the quantification of the PI3K-induced ommatidial area with and without the H4K16A mutant. The data are expressed as the means ± SD, n = 12 per genotype.
Table S1. Fat body-specific depletion of Hdac3 causes partial lethality in *Drosophila melanogaster*.

The transgenic line names and progeny numbers are shown. Female transgenic flies were crossed with male *ppl-Gal4/cyo* driver line. The error denotes the range of at least two independent experiments.

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<th>Lines</th>
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<th>Viability</th>
<th>Average viability (± SD)</th>
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Table S2. The sequences of the primers used for the amplification of the cDNA fragments and quantitative real-time PCR.