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Research Article

The histone deacetylase inhibitor trichostatin A influences the development of *Drosophila melanogaster*

L. A. Pile, F. W.-H. Lee and D. A. Wassarman*,+

National Institutes of Health, National Institute of Child Health and Human Development, Cell Biology and Metabolism Branch, Building 18T, Room 101, Bethesda, Maryland 20892 (USA)

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Abstract. We examined the consequences of the deacetylase inhibitor trichostatin A (TSA) on the development of *Drosophila melanogaster*. When fed to flies, TSA caused lethality and delayed development at concentrations as low as 5 μM, had stronger effects on males than females, and acted synergistically with mutations in the gene encoding the RPD3 deacetylase to cause notched wings, but did not appear to affect a SINA signaling pathway that is normally repressed by the SIN3 corepressor. These findings suggest that deacetylated histones play an important role in normal developmental progression and establish parameters for genetic screens to dissect the role of deacetylases in this process.

Key words. Trichostatin A; RPD3; SIN3; HDAC; deacetylase; SINA; *Drosophila.*

Acetylation of core histones (H2A, H2B, H3, and H4) plays an essential role in modulating gene expression [1]. For example, alterations in histone acetylation are associated with hyperactivation of the male X chromosome during dosage compensation and silencing of euchromatic genes by position-effect variegation (PEV) in *Drosophila* [2, 3]. Histone acetylation is a reversible process that is regulated by the opposing activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs) [1]. HATs catalyze addition while HDACs catalyze removal of an acetyl group from the ε-amino group of lysine side chains in the N-terminal tails of core histones.

Inhibitors of HDACs, such as n-butyrate and trichostatin A (TSA), have been used to probe the role of HDACs and histone hyperacetylation on gene expression and development. Treatment of flies with n-butyrate suppresses PEV [3], treatment of frog embryos with TSA leads to embryonic lethality and defects in the head and tail regions [4, 5], treatment of starfish embryos with TSA leads to developmental arrest during the early gastrula stage [6], and treatment of mammalian tissue culture cells with TSA alters the expression of ~ 5% of cellular genes [7].

The *Drosophila* genome encodes five HDACs, including TSA-sensitive dHDAC1 (referred to as RPD3), and five Sir2-like proteins that may possess NAD-dependent histone deacetylase activity [8, 9]. RPD3 is the catalytic subunit of the SIN3/RPD3 complex that interacts with sequence-specific DNA-binding proteins and nuclear hormone receptors to repress transcription [10]. Repression of genes in yeast is associated with localized SIN3- and RPD3-dependent deacetylation of histones [11, 12]. Mutations in *Drosophila rpd3* are lethal and suppress the silencing of genes affected by PEV [13].

Mutations in *Drosophila sin3* are also lethal and were identified as enhancers of the rough eye phenotype caused by ectopic expression of *seven in absentia* (*sina*) [14]. Presumably, a reduction in the activity of the

* Corresponding author.
+ Present address: University of Wisconsin Medical School, Department of Pharmacology, 1300 University Avenue, Madison, Wisconsin 53706 (USA), e-mail: dawassarman@facstaff.wisc.edu

SIN3/RPD3 complex increases the activity of SINA, which in *Drosophila* and mammals involves binding ubiquitin-conjugating enzymes and targeting proteins such as Tramtrack [14], DCC (deleted in colon cancer) [15], N-CoR (nuclear receptor corepressor) [16], and c-Myb [17] for degradation. We were interested in understanding the role of SIN3/RPD3-mediated deacetylation in SINA signaling pathways and, more generally, the role of histone deacetylation during development.

To initiate these studies, we set out to determine the phenotypic consequences of inactivating HDACs during *Drosophila* development and then to determine if mutations in *sin3* or *rpd3* modify these phenotypes. A previous study in *Drosophila* used the HDAC inhibitor n-butyrate [3], but we chose to use TSA because, unlike n-butyrate, concentrations of TSA that inhibit histone deacetylation do not affect other enzymatic activities [18, 19].

Materials and methods

Flies were cultured on medium containing agar, cornmeal, yeast, and molasses but no propionic acid, which has been shown to be a deacetylase inhibitor [3]. The medium was cooled to 55 °C before adding TSA (Wako Pure Chemical Industries) to a final concentration of 0, 5, 10, 15, or 20 µM. The stock solution of TSA was 1 mg/ml in dimethyl sulfoxide (DMSO; Sigma). To ensure that the flies were developmentally synchronized, 0- to 1.5-h embryos of the indicated genotype were collected on standard egg-laying medium. Fifty embryos were transferred, on a slab of egg-laying medium that did not contain TSA, to each vial containing either control DMSO-containing food or TSA-containing food and cultured at 25 °C. The number, sex, and external phenotype of eclosed adult flies was scored each day from 10 to 21 days post egg laying (PEL). An experiment consisted of six vials (i.e., 300 total embryos) of each of the TSA concentrations. A minimum of three experiments (i.e., 900 total embryos) was performed for each TSA concentration and genotype. Assays were performed on w^{1118} (referred to as wild type), *l(3)04556/TM3 Sb* (referred to as *rpd3/+*), $l(3)04556^{X5}$/*TM3 Sb* (referred to as *rpd3-X5/+*), and $sin3^{e374}$/*CyO* (referred to as *sin3/+*) flies. $l(3)04556^{X5}$ flies were generated by mobilizing the w^{+} P element by crossing *l(3)04556/TM3 Sb* flies with flies carrying a source of transposase. Southern blot analysis revealed that the *rpd3* gene is deleted in $l(3)04556^{X5}$ (data not shown). $sin3^{e374}$ is probably a null allele as it contains an 8-base pair deletion approximately 50-base pairs downstream of the translation initiation codon [E. M. Schlag and D. A. Wassarman, unpublished observation].

Results

The percentage of wild-type males and females that eclosed after being cultured on food containing various concentrations of TSA is presented in figure 1A. Three major conclusions can be drawn from this data. First, both male and female flies displayed a dose-sensitive response to TSA. The viability of flies raised on 20 µM TSA was reduced more than 20-fold relative to flies raised in the absence of TSA. Second, males were more sensitive than females to TSA. Males and females raised in the absence of TSA eclosed at approximately the same rate, while

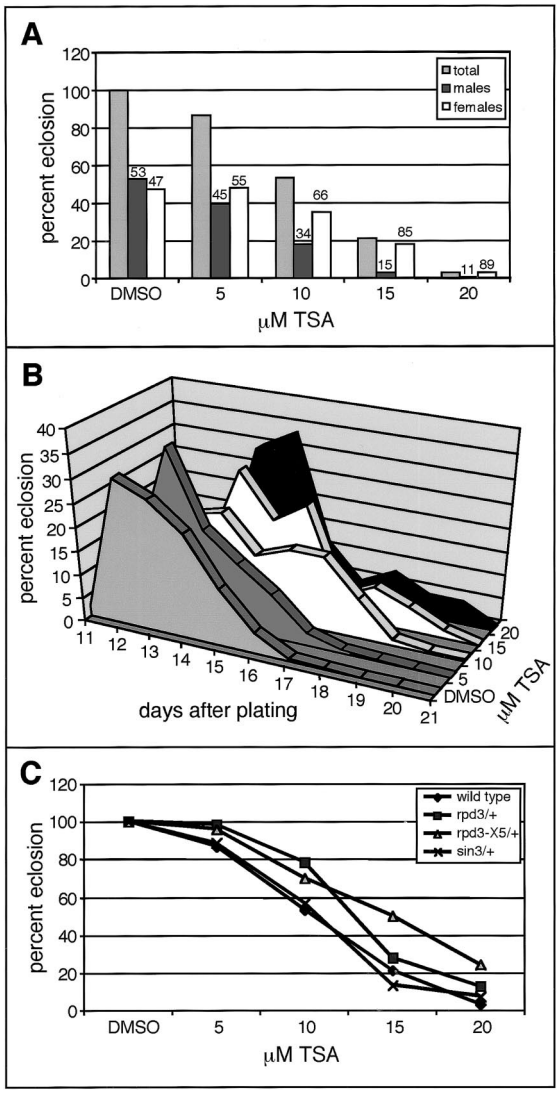

Figure 1. (*A*) Viability of male versus female flies on media with TSA. Percent eclosion is equal to the number of observed flies divided by the number of expected flies times 100. (*B*) Timing of development on media with TSA. Percent eclosion is equal to the number of flies that eclosed on a given day divided by the total number of flies that eclosed times 100. (*C*) Viability of *rpd3* and *sin3* mutant flies on media with TSA. Percent eclosion was calculated as in (*A*).

two-fold and five-fold fewer males than females eclosed when raised on 10 and 20 µM TSA, respectively. Third, TSA probably caused lethality during first-, second- or early third-instar larval development, since embryos developed on media that did not contain TSA and dead wandering (late) third-instar larvae and pupae were rarely observed (data not shown). No gross phenotypic abnormalities were observed that would provide insight into the TSA-sensitive developmental process that caused larval lethality (data not shown).

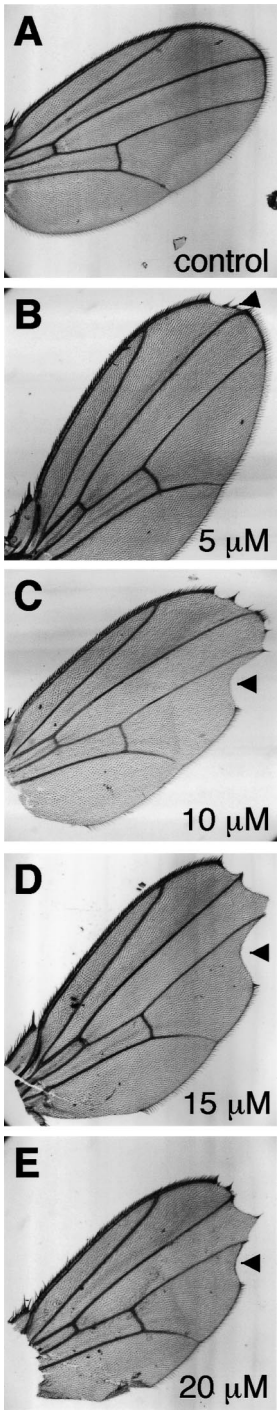

Figure 2. Micrographs of wings from heterozygous *rpd3* adult flies that were raised on media with TSA.

The developmental timing of flies cultured on food containing various concentrations of TSA is presented in figure 1B. These data indicate that TSA causes a dose-dependent delay in development. All flies raised in the absence of TSA eclosed prior to 17 days PEL, but ~20% of flies raised on 10 µM or higher TSA eclosed 17 days or more PEL. The average time of development was 12.6, 13.2, 13.7, 15.1, 15.3, and 15.3 days for flies raised on normal food (without DMSO), DMSO, and 5, 10, 15, and 20 µM TSA, respectively. Thus, TSA caused a significant delay in the normal progression of development. However, developmentally delayed flies, even those that eclosed after 20 days, appeared phenotypically normal.

A comparison of the effects of TSA on flies that carry mutations in genes encoding components of the SIN3/RPD3 HDAC complex is presented in figure 1C. Mutation of one of the two copies of *rpd3* or *sin3* did not enhance the rate of lethality or the developmental delay caused by TSA (fig. 1C and data not shown). In fact, *rpd3* mutations weakly suppressed the lethality caused by TSA, with a null allele of *rpd3* (*rpd3-X5*) having a greater effect than a hypomorphic allele (*rpd3*). In contrast, *rpd3* flies, but not other flies tested, displayed a notched wing phenotype (fig. 2). Wild-type flies have a continuous border along the wing margin, while *rpd3* flies had notches that increase in number and size with increasing TSA concentration (compare fig. 2A to B–E). Finally, the rough-eye phenotype caused by ectopic expression of SINA was not altered when flies were raised on medium containing 10 µM TSA, a TSA dose that caused 50% lethality (data not shown).

Discussion

We have presented evidence that in *Drosophila*, the deacetylase inhibitor TSA (1) causes lethality and delays development at concentrations as low as 5 µM, (2) has stronger effects on males than on females, and (3) acts synergistically with mutations in *rpd3* to cause notched wings but does not affect a SINA signaling pathway that is normally repressed by SIN3.

The lower survival rate of males than females treated with TSA may be attributed to inactivation of RPD3, as opposed to other deacetylases, since *rpd3* mutant males have been shown to survive at significantly lower rates than females [13]. Differential sensitivity of males and females to deacetylated histones may reflect the presence of the Y chromosome in males or the dosage compensation mechanism that hyperactivates the transcription of genes on the X chromosome in males. A deacetylase, like RPD3, is possibly required to maintain the heterochro-

matic structure of the Y chromosome or to modulate the level of acetylation of lysine 16 on histone H4 along the male X chromosome as part of the dosage compensation mechanism [2].

The finding that TSA caused notched wings in *rpd3* mutant flies suggests that the Notch signal pathway is regulated by histone deacetylation. In *Drosophila*, *Notch* mutants show wing notching, and in *Xenopus* and mammals, RPD3/HDAC1 has been implicated as a component of Notch signaling pathways [20, 21]. In contrast, reducing the dose of *rpd3* suppressed (did not enhance) TSA toxicity, suggesting that TSA-inhibited RPD3 protein has some other, possibly non-catalytic, activity that is toxic. The lack of an effect of TSA on the rough eye caused by misexpression of *sina* is in accord with the finding that, unlike mutations in *sin3*, mutations in *rpd3* do not enhance the *sina* phenotype (data not shown). This suggests that SIN3 may function independently of a deacetylase in some contexts.

Lethal effects of TSA have been observed in developmental studies conducted in other metazoan organisms [4–6]. In *Drosophila*, n-butyrate and propionic acid also prolong development, as do mutations in the gene encoding the $TAF_{II}250$ HAT [22]. Thus, *Drosophila* may serve as a model system to probe the role of regulated histone acetylation and deacetylation in executing the normal developmental program. The dose sensitivity of the TSA-induced lethality (fig. 1) and wing phenotype (fig. 2) suggests that screens for modifiers of these phenotypes may identify genes that regulate the acetylation status of histones or genes that are differentially expressed in response to the inhibition of histone deacetylases.

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1 Kou M.-H. and Allis C. D. (1998) Roles of histone acetyltransferases and deacetylases in gene regulation. BioEssays **20:** 615–626
2 Bone J. R., Lavender J., Richman R., Palmer M. J., Turner B. M. and Kuroda M. I. (1994) Acetylated histone H4 on the male X chromosome is associated with dosage compensation in *Drosophila*. Genes Dev. **8:** 96–104
3 Mottus R., Reeves R. and Grigliatti T. A. (1980) Butyrate suppression of position-effect variegation in *Drosophila melanogaster*. Mol. Gen. Genet. **178:** 456–469
4 Almouzni G., Khochbin S., Dimitrov S. and Wolffe A. P. (1994) Histone acetylation influences both gene expression and development of *Xenopus laevis*. Dev. Biol. **165:** 654–669
5 Damjanovski S., Sachs L. M. and Shi Y.-B. (2000) Multiple stage-dependent roles for histone deacetylases during amphibian embryogenesis: implications for the involvement of extracellular matrix remodeling. Int. J. Dev. Biol. **44:** 769–776
6 Ikegami S., Ooe Y., Shimizu T., Kasahara T., Tsuruta T., Kijima, M. et al. (1993) Accumulation of multiacetylated forms of histones by trichostatin A and its developmental consequences on early starfish embryos. Rouxs Arch. Dev. Biol. **202:** 144–151
7 Van Lint C., Emiliani S. and Verdin E. (1996) The expression of a small fraction of cellular genes is changed in response to histone hyperacetylation. Gene Expr. **5:** 245–253
8 Adams M. D., Celniker S. E., Holt R. A., Evans C. A., Gocayne J. D. Amanatides P. E. et al. (2000) The genome sequence of *Drosophila melanogaster*. Science **287:** 2185–2195
9 Barlow A. L., van Drunen C. M., Johnson C. A., Tweedie S., Bird A. and Turner B. M. (2001) dSIR2 and dHDAC6: two novel, inhibitor-resistant deacetylases in *Drosophila melanogaster*. Exp. Cell Res. **265:** 90–103
10 Ayer D. E. (1999) Histone deacetylases: transcriptional repression with SINers and NuRDs. Trends Cell Biol. **9:** 193–198
11 Kadosh D. and Struhl K. (1998) Targeted recruitment of the SIN3-RPD3 histone deacetylase complex generates a highly localized domain of repressed chromatin in vivo. Mol. Cell. Biol. **18:** 5121–5127
12 Rundlett S. E., Carmen A. A., Suka N., Turner B. M. and Grunstein M. (1998) Transcription repression by Ume6 involves deacetylation of lysine 5 of histone H4 by RPD3. Nature **392:** 831–835
13 Mottus R., Sobol,R. E. and Grigliatti T. A. (2000) Mutational analysis of a histone deacetylase in *Drosophila melanogaster*: missense mutations suppress silencing associated with position effect variegation. Genetics **154:** 657–668
14 Neufeld T. P., Tang A. H. and Rubin G. M. (1998) A genetic screen to identify components of the *sina* signaling pathway in *Drosophila* eye development. Genetics **148:** 277–286
15 Hu G., Zhang S., Vidal M., La Baer J., Xu T. and Faeron E. R. (1997) Mammalian homologs of seven in absentia regulate DCC via the ubiquitin-proteosome pathway. Genes Dev. **11:** 2701–2714
16 Zhang J., Guenther M. G., Carthew R. W. and Lazar M. A. (1998) Proteosomal regulation of nuclear receptor corepressor-mediated repression. Genes Dev. **12:** 1775–1780
17 Tanikawa J., Ichikawa-Iwata E., Kanei-Ishii C., Nakai A., Matsuzawa S.-I., Reed J. C. et al. (2000) p53 suppresses the c-Myb-induced activation of heat shock transcription factor 3. J. Biol. Chem. **275:** 1557–15585
18 Yoshida M., Kijima M., A., Akita M. and Beppu T. (1990) Potent and specific inhibition of mammalian histone deacetylases both in vivo and in vitro by trichostatin A. J. Biol. Chem. **265:** 17174–17179
19 Kruh J. (1982) Effects of sodium butyrate, a new pharmacological agent, on cells in culture. Mol. Cell. Biochem. **42:** 65–82
20 Bray S. (1998) Notch signaling in *Drosophila*: three ways to use a pathway. Semin. Cell Dev. Biol. **9:** 591–597
21 Kao H.-Y., Ordentlich P., Koyano-Nakagawa N., Tang Z., Downes M., Kitner C. R. et al. (1998) A histone deacetylase corepressor complex regulates the Notch signal transduction pathway. Genes Dev. **12:** 2269–2277
22 Wassarman D. A., Aoyagi N., Pile L. A. and Schlag E. (2000) TAF250 is required for multiple developmental events in *Drosophila*. Proc. Natl. Acad. Sci. USA **97:** 1154–1159