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Dissecting the Biological Functions of *Drosophila* Histone Deacetylases by RNA Interference and Transcriptional Profiling*[S]

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Zinc-dependent histone deacetylases (HDACs) are a family of hydrolases first identified as components of transcriptional repressor complexes, where they act by deacetylating lysine residues at the N-terminal extensions of core histones, thereby affecting transcription. To get more insight into the biological functions of the individual HDAC family members, we have used RNA interference in combination with microarray analysis in *Drosophila* S2 cells. Silencing of *Drosophila* HDAC1 (DHDAC1), but not of the other DHDAC family members, leads to increased histone acetylation. Silencing of either DHDAC1 or DHDAC3 leads to cell growth inhibition and deregulated transcription of both common and distinct groups of genes. Silencing DHDAC2 leads to increased tubulin acetylation levels but was not associated with a deregulation of gene expression. No growth of phenotype and no significant deregulation of gene expression was observed upon silencing of DHDAC4 and DHDAC*X*. Loss of DHDAC1 or exposure of S2 cells to the small molecule HDAC inhibitor trichostatin both lead to a G_2 arrest and were associated with significantly overlapping gene expression signatures in which genes involved in nucleobase and lipid metabolism, DNA replication, cell cycle regulation, and signal transduction were over-represented. A large number of these genes were shown to also be deregulated upon loss of the co-repressor SIN3 (Pile, L. A., Spellman, P. T., Katzenberger, R. J., and Wassarman, D. A. (2003) *J. Biol. Chem.* 278, 37840–37848). We conclude the following. 1) DHDAC1 and -3 have distinct functions in the control of gene expression. 2) Under the tested conditions, DHDAC2, -4, and *X* have no detectable transcriptional functions in S2 cells. 3) The antiproliferative and transcriptional effects of trichostatin are largely recapitulated by the loss of DHDAC1. 4) The deacetylase activity of DHDAC1 significantly contributes to the repressor function of SIN3.

Chromatin modifications such as acetylation, methylation, phosphorylation, ubiquitination, and sumoylation of histones play an important role in the regulation of transcription (1–5). Together with DNA methylation, these modifications are part of a broad and multifaceted strategy whereby eukaryotes regulate gene expression at the epigenetic level (6–8).

Acetylation of histones is tightly controlled by the activity of histone acetyltransferases and histone deacetylases (HDACs)[2] (9, 10). Histone deacetylation may repress transcription by different mechanisms. On the one hand, this process increases the charge density on the N termini of the core histones thereby strengthening histone tail-DNA interactions and blocking access of the transcriptional machinery to the DNA template. In addition, histone modifications are specifically recognized by chromatin-interacting proteins thus favoring the formation of higher order chromatin structures (heterochromatin).

There is ample evidence that aberrations in the epigenetic regulation of gene expression at the levels of DNA methylation and histone acetylation or histone methylation are an important component in the process of malignant transformation of human cells (11). A number of histone acetyltransferase mutations were identified in cancer, and HDACs were found to be overexpressed or aberrantly recruited in several human malignancies (12). Small molecule HDAC inhibitors induce cell cycle arrest, differentiation, and apoptosis in cancer cells with a significant window over normal cells, and some molecules have already entered clinical trials and show promising results (13).

Higher organisms have evolved a considerable complexity in the histone deacetylase family; thus, in mammals, 17 different HDAC subtypes were identified, which were grouped into three classes according to their sequence homologies with the yeast proteins Rpd3 (class I), HDA1 (class II), and Sir (class III) (14–16). Class I and class II proteins are evolutionarily related and share a common enzymatic mechanism, the Zn-catalyzed hydrolysis of the acetyl-lysine amide bond. The HDAC inhibitors that are presently in clinical trials are rather nonselective and are thought to inhibit most or many of the class I and II proteins. Class III proteins are evolutionarily unrelated to class I or class II and catalyze the transfer of the acetyl group onto the sugar moiety of NAD (16). Profiling of yeast HDAC knock-out strains has shown that the yeast HDACs have very distinct biological functions and are recruited to different regions of the yeast genome (17, 18).

An even greater differentiation of functions seems to have occurred in HDACs of higher eukaryotes through a multitude of mechanisms as follows: the recruitment into different co-repressor complexes (19), the modulation of deacetylase activity by protein-protein interactions (20) or by post-translational modifications (21, 22), tissue-restricted expression patterns, differing and often stimulus-responsive subcellular localizations, and splice variants (15). An additional level of complexity is added by the finding that several HDACs, besides their effects on histones, seem to influence gene expression by histone modification independent mechanisms, namely by affecting the acetylation status and the

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1.

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[2] The abbreviations used are: HDAC, histone deacetylases; DHDAC, *Drosophila* HDAC; RNAi, RNA interference; dsRNA, double-stranded RNA; TSA, trichostatin A; RT, reverse transcription; GO, gene ontology; FACS, fluorescence-activated cell sorter.

activity of transcription factors (23–25). More recently, extra-transcriptional activities that impinge on processes such as protein folding or modifications of cytoskeleton proteins (26) have been added to the biological curricula of some HDACs.

Our focus presently concentrates on the biological functions of class I and class II HDACs, which are the targets of present antineoplastic drug candidates. Among mammalian class I deacetylases (subtypes 1–3 and 8), HDACs 1 and 2 are closely related and found in the ubiquitously expressed mSin3a, NURD/Mi2/NRD, and CoRest corepressor complexes (19). HDAC3 associates to and is activated by SMRT and NCoR co-repressors that play an important role in regulation of gene expression by nuclear hormone receptors (19). HDAC8 was recently found to be expressed in smooth muscle where it is required for muscle contractility (27). The class IIa HDACs (subtypes 4, 5, 7, and 9) are characterized by tissue-specific expression and stimulus-dependent nucleocytoplasmic shuttling (15). They are the target of several kinases, and some phosphorylated forms are confined to the cytosol by interaction with 14-3-3 proteins. In the nucleus they associate with transcription factors, notably of the MEF and Runx families, and control differentiation and cellular hypertrophy in muscle and cartilage tissues (28, 29). HDAC7 has a specific role in clonal expansion of T-cells by suppressing Nur77-dependent apoptosis (30). Class IIb subtypes 6 and 10 have a duplication of their catalytic domains, but the second catalytic domain is thought to be dysfunctional in HDAC10. HDAC6 is the only deacetylase known to act on tubulin (31, 32). Tubulin deacetylation is required for disposal of misfolded proteins in aggresomes (33). HDAC6 also deacetylates Hsp90, pointing to a broader role of this subtype in protein folding (34). Finally, very little is known about HDAC11 that cannot be clearly assigned to either class I or class II HDACs based on sequence motifs (35).

We were interested to gain more insight into the division of labor among HDACs in higher eukaryotes. In particular, we wanted to identify functional clusters of genes regulated by individual subtypes. We would furthermore like to correlate those gene clusters with the antiproliferative activity of nonselective small molecule inhibitors as a strategy to identify the subtypes that mediate the antiproliferation effect. To this purpose, we have used *Drosophila* S2 cells as a model system. This system is attractive because insects have only five class I/II HDACs with orthologs to mammalian HDACs 1 and 2 (DHDAC1 = CG7471), 3 (DHDAC3 = CG2128), 4, 5, 7, and 9 (DHDAC4 = CG1770), 6 (DHDAC2 = CG6170), and 11 (DHDAC*X* = CG31119) (for further details see the Supplemental Material). By using RNAi in combination with microarray analysis, we show that only DHDAC1 and DHDAC3 detectably affect transcription in S2 cells pointing to either very specialized or nontranscriptional roles of the other subtypes. Furthermore, a substantial phenotypic and transcriptional overlap between DHDAC1 RNAi and the HDAC inhibitor trichostatin A (TSA) points to this subtype as an important mediator of the antiproliferative effects of TSA.

EXPERIMENTAL PROCEDURES

Cells—Drosophila Schneider cell lines (S2), obtained from Patrizia Somma (University of Rome "La Sapienza"), were cultured at 25 °C in Shield and Sang M3 Insect Medium (Sigma) containing 20% fetal bovine serum (Sigma).

RNA Interference—To generate double-stranded RNA (dsRNA) for RNAi, sequences directed against the protein to be silenced were amplified by RT-PCR from S2 total RNA. Each primer used in the PCR contained a 5′ T7 RNA polymerase-binding site (GGATCCTAATACGACTCACTATAGGGAGG) followed by sequences specific for the targeted genes.

The following primer sets (oriented 5′ → 3′) were used for each protein (T7 promoter sequence is underlined): DHDAC1 for GGATCCTAATACGACTCACTATAGGGAGGATCCGTAGCTGCGGC and rev GGATCCTAATACGACTCACTATAGGGAGGATGCCTTGTTGCTGT; DHDAC2 for GGATCCTAATACGACTCACTATAGGGAGGTCCCAAGGTGCTCTACATCAGCTT and rev GATCCTAATACGACTCACTATAGGGAGGGCCATCCTCGCGCCACGCAC); DHDAC3 for GGATCCTAATACGACTCACTATAGGGAGGGAGCCCAGAAGCTGAAC and rev GGATCCTAATACGACTCACTATAGGGAGGCGGGGTCTGCACCATTTGGACGCTGG); DHDAC4 for GGATCCTAATACGACTCACTATAGGGAGGCCCACGCACATCCACATCCACATG and rev GGATCCTAATACGACTCACTATAGGGAGGTGACCTTGTGCGGCGGTGAA); DHDAC*X* for GGATCCTAATACGACTCACTATAGGGAGGGCCCAACTGCAGCTCGACGACGGC and rev GGATCCTAATACGACTCACTATAGGGAGGGCGCAGCTCCACCGCACACCGAATGC); and new DHDAC*X* for GGATCCTAATACGACTCACTATAGGGAGGGTTGCTCGTACAGAGGCGGT and rev GGATCCTAATACGACTCACTATAGGGAGGGCCACCACTCAGCAGCATCAC). dsRNA molecules directed against DHDAC1, DHDAC2, and DHDAC4 were 1000 bp in length, and the sequence against DHDAC3 was 861 bp. Two different sequences against DHDAC*X* of 500 bp each were also synthesized. PCR products were used as templates for *in vitro* transcription using the Megascript RNAi kit (Ambion). 500 μg of PCR product was first purified and concentrated using Amicon microcon-PCR filters (Millipore) and then used as template for 2–4-h *in vitro* transcription reaction. Nuclease digestion to remove DNA and single-stranded RNA was carried out in accordance with the manufacturer's instructions. Finally, the dsRNA was purified on columns provided by the kit. RNAi was carried out according to the protocol developed by Clemens *et al.* (36). 3×10^6 S2 *Drosophila* tissue culture cells were plated into a 100-mm dish in 6 ml of medium without fetal bovine serum. Approximately 90 μg of dsRNA was added and mixed by swirling. After 30 min, 12 ml of media containing 20% fetal bovine serum was added. The cells were kept in culture and analyzed for up to 5 days.

TaqMan RT-PCR—Total RNA was extracted from S2 cells the 3rd and the 5th day following addition of dsRNA by using the RNeasy mini kit (Qiagen). Total RNA was DNase I-treated (Qiagen), quantified spectrophotometrically, and subjected to one-step real time RT-PCR (Taqman One-Step RT-PCR Master Mix, Applied Biosystems) on an ABI Prism 7700 machine (Applied Biosystems). The thermal cycling conditions used were as follows: RT for 30 min at 48 °C and 10 min at 95 °C, followed by 40 cycles of amplification at 95 °C for 15 s and 60 °C for 1 min. Normalization for RNA levels was based on glyceraldehyde-3-phosphate dehydrogenase and α-tubulin expression. For amplification of each gene the following primers and probe sets were used: DHDAC1, sense 5′-TGCGCCAAAGTGCCAAA-3′, antisense 5′-GGATGAACTTGCGAGCTCAGA-3′, and FAM probe 5′-TCGGACCGGCACCAAAGTAAACCA-3′; DHDAC2, sense 5′-CCAACGGCACTGATCTACGA-3′, antisense 5′-TGAAGCGCTCGGGACACT-3′, and FAM probe 5′-CCAGCACTGTTGCCTGTGGGACAA-3′; DHDAC3, sense 5′-CGGACCGTAGGGTGTCGTACT-3′, antisense 5′-CATCAACCAGCTTGTCCTCATC-3′, and FAM probe 5′-CTACAACGCGGACGTGGGCAACTT-3′; DHDAC4, sense 5′-GAATGGCACAACGACAACGT-3′, antisense 5′-TCGCCCGAGGAGCTCTT-3′, and FAM probe 5′-CCCTACAGGAATCGCGGCGT-3′; DHDAC*X*, sense 5′-CGACCCATGCGCTTTCA-3′, antisense 5′-AACCATAATCCAATGCCAGCTT-3′, and FAM probe 5′-CAGCCGGTTCCATTTTGGCCG-3′; glyceraldehyde-3-phosphate dehydrogenase, sense 5′-CGACCCATGCGCTTTCA-3′, antisense 5′-AACCATAATCCAATGCC-

AGCTT-3′, and FAM probe 5′-CAGCCGGTTCCATTTTGGCCG-3′; and α-tubulin, sense 5′-CCCCGTCACGGCAAGTAC-3′, antisense 5′-ACGTCCTTGGGCACAACATC-3′, and FAM probe 5′-TGG-CCTGCTGCATGCTGTACCG-3′.

Cell Growth Assay—To determine the growth curves cells were resuspended by gentle mixing and counted each day following the addition of dsRNA for a total of 5 days. Trypan blue staining was used to assess cell viability.

FACS Analysis—To prepare cells for FACS analysis, 5×10^5 cells were washed twice with phosphate-buffered saline and resuspended in 500 μl of phosphate-buffered saline, 0.1% Triton X-100, 20 μl of propidium iodide (1 mg/ml), and 10 μl of RNase A (10 mg/ml). Stained cells were analyzed with a FACScan apparatus (BD Biosciences), and the data were quantified using Cellquest software.

Immunoblotting Analysis—Whole-cell SDS lysates were resolved by electrophoresis on 4–12% SDS-PAGE pre-cast gels (Bio-Rad) and transferred to nitrocellulose (Schleicher & Schuell). Filters were blocked in 10 mM Tris-HCl, pH 8, 150 mM NaCl, 0.1% Tween 20 (TBST) with 5% nonfat dried milk for 1 h at room temperature. Filters were washed once in TBST and then incubated with primary antibodies diluted 1–5 μg/ml in 5% nonfat dried milk in TBST overnight at 4 °C. Filters were washed five times in TBST and then incubated with horseradish peroxidase-coupled secondary antibodies at a dilution of 1:5000 in 5% nonfat dried milk-TBST for 1 h at room temperature. Filters were washed five times in TBST as for primary antibody incubations and processed for chemiluminescent detection using an ECL kit (Amersham Biosciences). Anti-DHDAC1 serum (diluted 1:1000) was a gift from Alexander Brehm (Adolf-Butenandt-Institut Molekularbiologie LMU München), and a polyclonal anti-dHDAC4 serum was made by immunizing rabbits with the peptide MSSPDDRIPIHDLPSEAGGC-OH (Anaspec, San Josè, CA). The following antibodies were commercially available: monoclonal anti-α-tubulin (clone DM 1A; Sigma), monoclonal anti-acetylated-tubulin (clone 6-11B-1; Sigma), monoclonal anti-actin (clone C4; NeoMarkers), polyclonal anti-acetylhistone H3 (06-599; Upstate), polyclonal anti-mouse peroxidase conjugate (31444; Pierce) and polyclonal anti-rabbit peroxidase conjugate (A6154; Sigma).

Analysis of mRNA Expression Using Oligonucleotide Arrays—Microarray analysis was performed with GeneChip *Drosophila* arrays (Affymetrix) using as probes cRNAs prepared from samples 3 and 5 days after addition of dsRNA or after exposure to TSA (6, 12, and 24 h and 3 and 5 days post-treatment). We hybridized two replicate samples on distinct chips for each condition. Every experiment was repeated at least two times and processed separately. cRNA was prepared and labeled following protocols listed the GeneChip Expression Analysis Technical Manual (available online). Total RNA was isolated using the RNeasy mini kit (Qiagen). Double-stranded cDNA was synthesized from 16 to 24 μg of RNA and was used as a template to synthesize biotin-labeled cRNA by *in vitro* transcription using the BioArray High Yield RNA transcript labeling kit (Enzo). Amplified cRNA was fragmented and hybridized to arrays according to the manufacturer's procedures. Probe hybridization and data collection were performed by the Genopolis Consortium at the University of Milano, Bicocca.

Annotation and Statistical Analysis of Microarray Expression Data—Data were analyzed using Rosetta Resolver 5.0 (Rosetta Biosoftware) in combination with MatLab 7.0. Raw data resulting from the scans of Affymetrix GeneChip microarrays monitoring 13,500 *Drosophila* transcripts, according to sequences from version 1.0 of Flybase, were preprocessed (image processing and normalization) using the Rosetta Biosoftware proprietary algorithm (refer to the Rosetta Biosoftware instruction manual for technical details). A "Rosetta *p* value" (see manual) was calculated for each gene probe set. For series-specific ratio experiments, single channel intensity replicates within the same series were combined and compared against the combination of control experiments at the same time point. The resulting series-specific ratio experiment replicates were combined into "global ratio experiments." Global ratio experiments were then used for the selection of genes differentially expressed. Error weighted average using error values associated to each data point was used to combine the replicates. Hierarchical clustering using cosine correlation as a similarity measure was used to cluster genes and samples. The unweighted average link was used to calculate the distance from a cluster to all remaining unclustered points. Genes to be clustered were pre-selected using the following criteria: a log ratio associated *p* value <0.01 and absolute log ratio >0.3 (2-fold change) in at least two experiments. The following criteria were used for the selection of differentially expressed genes: up-regulated genes, log ratio *p* value <0.01 and fold change >2; down-regulated genes, log ratio *p* value <0.01 and fold change less than -2. Gene ontology annotations and classification were performed on the selected genes using the NetAffx™ web-based software. *p* values relative to the overlap between gene sets and to the analysis of gene ontology annotations were calculated using the Fisher exact test. No correction for the number of categories was made because the same genes may fall under different GO annotations. Microarray data were deposited in the GEO data base.

RESULTS

To selectively knockdown DHDACs function in S2 *Drosophila* cells, we took advantage of RNA interference using dsRNA molecules with an approximate length of 500–1000 bp, derived from the coding region of each DHDAC. Quantitative real time PCR analysis was done to assess the kinetics of mRNA knockdown. DHDAC mRNA levels displayed a sharp reduction up to 2–3 days, depending on the subtype, and only slightly declined thereafter for up to 5 days following addition of dsRNA (Fig. 1*A*). At 5 days, silencing of DHDAC1, DHDAC3, and DHDAC*X* was very efficient, and mRNA levels decreased to 10% or less with respect to untreated or unspecific dsRNA-treated cells. For the remaining subtypes, lower efficiency was observed, and upon RNAi, DHDAC2 and DHDAC4 transcripts decreased only to 40 and 20% the level of control samples, respectively. The knockdown efficiency was not significantly improved by increasing the amount of dsRNA, by repeated addition of the same dsRNA, by changing the dsRNA sequence, or by transfection with small, 21-nucleotide short interfering RNAs (not shown). To assess the selectivity of DHDAC silencing, we performed RT-PCR TaqMan analysis on transfected cells 2, 3, and 5 days following addition of dsRNA. No unintended alterations were noticed at day 2 (not shown), whereas at longer incubation times, a mutual influence was observed only between DHDAC1 and -3 (Fig. 1*B*). Knockdown of either of these DHDACs leads to a decrease in the mRNA levels of the other subtype. This phenomenon might be related to the high degree of sequence homology between DHDAC1 and -3 and a resulting off-target silencing by the RNAi machinery. Still, the specific silencing was 10- to 5-fold more efficient at any time point. To investigate whether the depletion of mRNA correlated with a reduction in the protein level, we analyzed whole-cell extracts from transfected cells with antiserum directed against DHDAC1 or DHDAC4. Immunoblotting analysis showed that dsRNA treatment resulted in both proteins being significantly down-regulated after 5 days (Fig. 2). Unfortunately, we failed to obtain reliable antisera against the other DHDAC subtypes.

Effects on Histone and Tubulin Acetylation—RNAi of either DHDAC1 but not of the other DHDACs leads to increased histone H3 acetylation (Fig. 3). TSA-treated cells also showed a significant increase

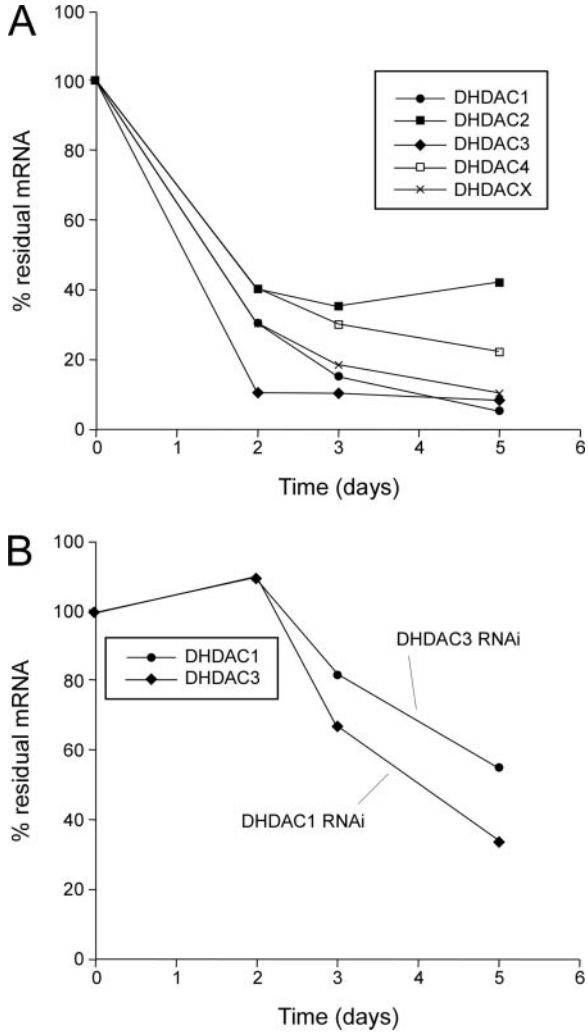

FIGURE 1. **Expression levels of DHDAC subtypes upon RNAi.** *A,* dsRNAs homologous to each of the DHDAC subtypes was added to S2 cells, and specific mRNA levels were determined by real time Taqman PCR after 2, 3, and 5 days. *B,* using Taqman PCR, DHDAC1 mRNA levels were determined in cells in which HDAC3 was down-regulated by RNAi, and DHDAC3 mRNA levels were determined in cells with down-regulated DHDAC1. Averages from 2 to 3 experiments are shown between which variability was less than 20%.

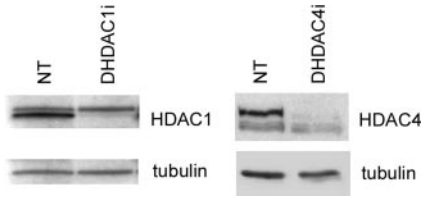

FIGURE 2. **Protein expression levels of DHDAC1 and -4 upon RNAi.** Proteins were extracted from S2 cells 5 days after triggering RNAi by addition of specific dsRNAs directed against DHDAC1 or DHDAC4. DHDAC protein levels were detected by Western blot analysis using antisera directed against DHDAC1 or DHDAC4. Extra bands are because of nonspecific reactivity of the polyclonal antisera. *NT* indicates nontransfected.

in acetylated histone H3 (Fig. 3). In contrast, only DHDAC2 RNAi gave an increase in acetylated tubulin. This increase is in agreement with the known function of the mammalian ortholog of DHDAC2 (HDAC6), which is the only reported tubulin deacetylase. These data suggest that even the lower knockdown efficiency obtained with DHDAC2 RNAi was sufficient to elicit biologic effects.

DHDAC RNA Effects on Proliferation of S2 Cells—HDAC inhibitors have pronounced anti-proliferative effects on mammalian cells. To

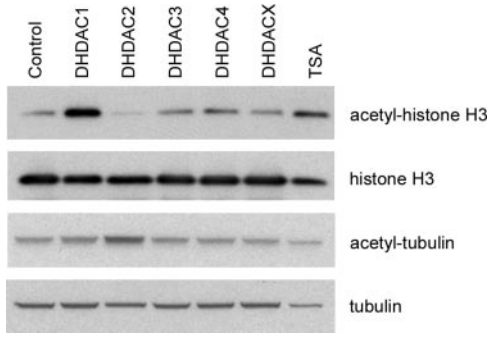

FIGURE 3. **Histone and tubulin acetylation changes upon loss of DHDACs or TSA treatment.** S2 cell extracts were made 5 days after addition of dsRNAs directed against each DHDAC or of 75 nM TSA. Western blots were developed using antisera against acetylated histone H3 (*top panel*) or acetylated tubulin (*3rd panel*).

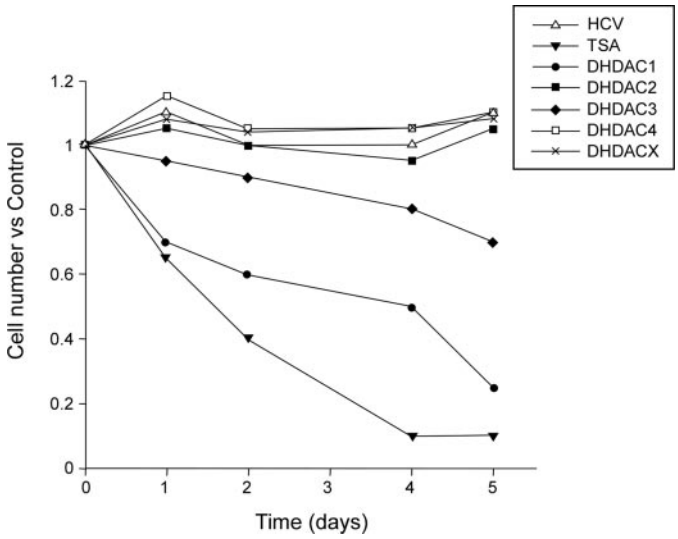

FIGURE 4. **Growth curves of S2 cells upon RNAi or TSA treatment.** S2 cells were plated at a density of 200,000 cells/ml and either treated with dsRNAs directed against DHDACs or an unrelated gene (hepatitis C virus (*HCV*) open reading frame) or with 75 nM TSA. Cells were counted at the indicated time points. Cell counts were divided by the counts of untreated (control) cells. An experiment representative of three different growth curve determinations is shown in which the same trend was observed.

determine whether S2 cells are also growth-arrested, we determined [^{14}C]thymidine incorporation for 48 h after incubation with increasing concentrations of TSA. We observed a dose-dependent inhibition of thymidine incorporation with an IC_{50} of 75 nM (not shown). We next compared the effect of 75 nM TSA on growth curves to that elicited by DHDAC RNAi in a time course experiment over 5 days (Fig. 4). Under these conditions, TSA leads to an almost complete growth arrest after 3 days. DHDAC1 RNAi, and to a lesser extent DHDAC3 RNAi, also affected cell growth, and DHDAC1-interfered cells were almost completely arrested 5 days post-transfection. These cells retained their viability as judged by trypan blue exclusion (not shown), whereas TSA treatment reproducibly resulted in an increased amount of trypan blue-positive cells. In contrast, no significant inhibition of cell growth was observed in cells in which DHDAC2, -4, or -*X* had been knocked down.

FACS analysis showed that the anti-proliferative effect of DHDAC1 RNAi was associated with a redistribution of the cell population with a notable shift from the G_1 to the G_2/M phases of the cell cycle. This effect, detectable since the first 72 h after interference (not shown), became stronger at day 5 (Fig. 5). We notice that our data are at variance with those published by Pile *et al.* (40), who failed to observe cell cycle effects upon silencing of DHDAC1. Possibly the reason for these differences is attributable to different extents in knockdown efficiency. In our

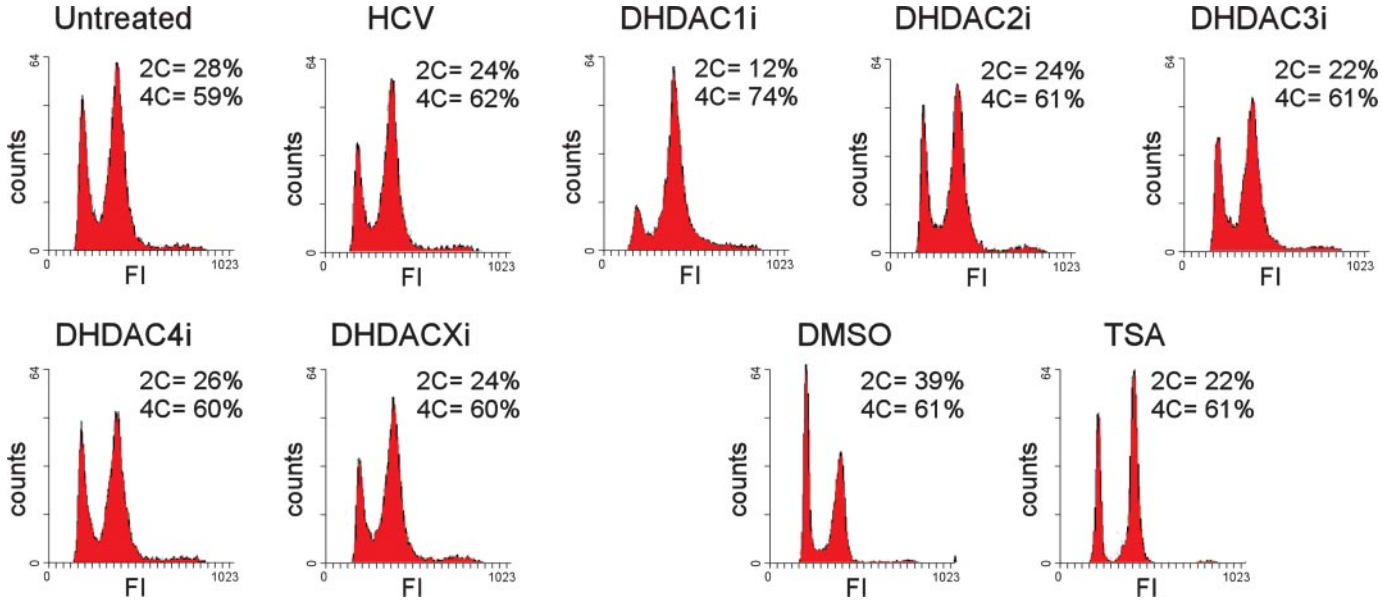

FIGURE 5. **Cell cycle analysis of S2 cells grown in the presence of DHDAC short interfering RNAs or treated with TSA.** S2 cells were harvested 5 days after addition of dsRNAs against DHDACs or 48 h after addition of 250 nM TSA. Cells were processed as described under "Experimental Procedures" and analyzed on a FACScan machine (BD Biosciences). The *2C* and *4C* cell populations, corresponding to cells in the G_1 or G_2/M phases of the cell cycle, were quantified using Cellquest.

hands, TSA-treated S2 cells also showed a strong increase in the G_2/M population already after 48 h. Very few mitotic events were detectable in DHDAC1-interfered or TSA-treated S2 cells, indicating that the cell cycle block most likely occurred in G_2 (not shown). Silencing of DHDAC3 or of the other DHDACs had no detectable effect on the cell cycle distribution. To assess whether the cell death observed in TSA-treated S2 cells was because of the simultaneous inhibition of more than one DHDAC, we performed double knockdown experiments of DHDAC1 and the other DHDACs. However, no significant effect on cell viability was observed in any of these experiments (not shown).

Microarray Analysis—To analyze the influence of individual DHDAC silencing on gene expression, we decided to perform a microarray analysis on Affymetrix arrays hybridized with cRNAs from DHDAC-interfered S2 cells, along with TSA-treated and nonspecific dsRNA-treated cells. A heat map showing a time course experiment can be found in the Supplemental Material. By using the criteria outlined under "Experimental Procedures," we identified treatment-related, specific signatures for TSA-treated cells ($n = 625$ and 1046 at 3 and 5 days, respectively) and upon DHDAC1 RNAi ($n = 177$ and 832 at 3 and 5 days, respectively) or DHDAC3 RNAi ($n = 10$ and 64 at 3 and 5 days, respectively). No significant gene expression signatures could be detected from cells in which DHDAC2, -4, or *-X* were silenced. A representative experiment is shown in Fig. 6.

A large overlap, affecting 56% of the genes deregulated upon loss of DHDAC1, was observed between the TSA and DHDAC1 signatures at 5 days post-RNAi (Fig. 7). 36% of the genes regulated by DHDAC3 were also changed in TSA-treated cells (Fig. 8). These data indicate that the transcriptional roles of both DHDACs are accomplished to a significant extent through their catalytic activities. A significant overlap between the DHDAC1 and DHDAC3 signatures generated 5 days after RNAi was also observed that involved 17 genes (26.5% of the DHDAC3 signature) (Fig. 7). Clustering of the regulated genes by GO according to biological processes revealed both differences and functional similarities between DHDAC1 and -3 (Table 1).

Genes involved in the metabolism of nucleic acids and their components, DNA replication, regulation of cell cycle, chromosome segregation, cytokinesis, and mitotic spindle organization, were significantly enriched ($p < 0.1$) in the DHDAC1 signature. More specifically, RNAi of DHDAC1 leads to down-regulation of a number of genes involved cell cycle progression and DNA replication (*Cdc45, double parked, gnf1,* and *dpa,* and the origin recognition complex genes *orc1, orc2, orc5,* DNA *pol-73,* and *Mcm2/3/3AP/5/6/7*), purine/pirimidine biosynthesis (*ade5, Ts, Rnrs, RMr2,* and *DPYs*), mitotic spindle organization or chromosome segregation *(cap, cap-G, CAP-D2, Pavarotti, fascetto, polo, sak,* and *cmet*) and cytokinesis (*pbl* and *scra*). These changes are in line with the growth arrest observed in cells with decreased DHDAC1 levels and could be secondary signatures of this phenotype. Furthermore, DHDAC1 silencing leads to decreased expression of positive regulators of G_2/M transition such as *cdc2c, polo* kinase, cyclin-dependent kinases, *and string*. All of these genes also decrease in TSA-treated samples. The decrease of *string*, which encodes the *Drosophila* homolog of the CDC25 phosphatase, may be particularly relevant as its elimination was shown to be sufficient to cause G_2 arrest in S2 cells. In addition, down-regulation of *string* and G_2 arrest was also observed by others upon RNAi of the HDAC1-recruiting SIN3 co-repressor (39, 40) (also see below).

RNAi of either DHDAC1 or DHDAC3 affects regulators of transcription from pol II promoters, although these genes are not significantly enriched in either signature. Most interestingly, only three genes in this category, *sox14* and the ecdysone-induced *eip74ef* (up-regulated) and *nvy* (down-regulated), were common to both DHDAC1 and DHDAC3 RNAi experiments. *smr*, encoding the SMRTER co-repressor, was regulated only by the latter and not by DHDAC1. *SMRTER* is structurally divergent but functionally similar to the vertebrate nuclear co-repressors SMRT and N-CoR that are known to interact with HDAC3 (20). The retinoblastoma-related genes *rbf* and *rbf2* were instead selectively down-regulated by DHDAC1. In mammalian cells HDAC1 interacts with retinoblastoma to silence E2F target gene expression (37, 38). This suggests the existence of DHDAC subtype-specific feedback loops affecting components of different transcriptional repressor complexes.

Proteolysis and peptidolysis genes are affected by RNAi of either DHDAC1 or DHDAC3, although a statistically significant enrichment of this category is observed only in the DHDAC3 signature (p value = 0.05). DHDAC1 seems to be involved in the repression of several me-

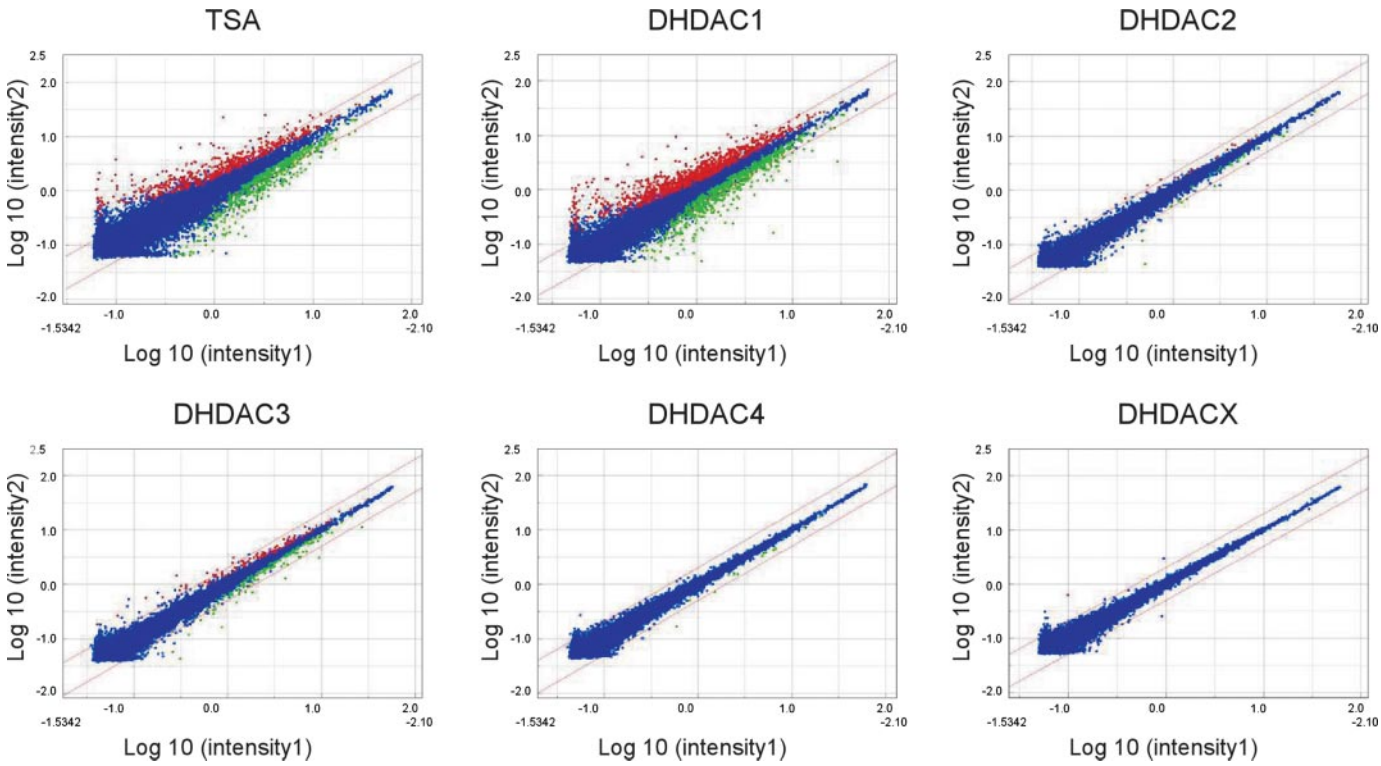

FIGURE 6. **Gene expression changes in S2 cells upon loss of DHDACs or TSA treatment.** Microarray analysis was done 5 days after triggering of RNAi or 24 h upon addition of 75 nM TSA to S2 cells. Plots show *log 10* (*intensity 1*) (untreated samples) *versus log 10* (*intensity 2*) (treated samples). Significant ($p < 0.01$) changes are in *red* (up-regulated) or *green* (down-regulated). The two lines indicate a 2-fold change in gene expression. One representative experiment is shown.

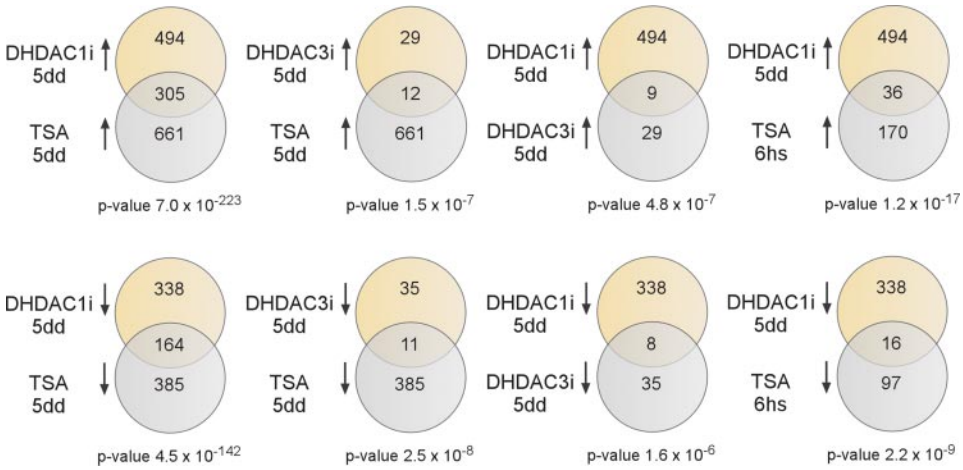

FIGURE 7. **Venn diagrams comparing sets of up- and down-regulated genes from DHDAC1 or DHDAC3 RNAi and TSA treatment of *Drosophila* S2 cells.** Statistical significance of the intersections was assessed using Fisher exact test.

talloprotease and serine proteases, whereas DHDAC3 positively regulates a group of serine proteases. Genes involved in protein folding and phospholipid metabolism are specifically enriched in the DHDAC3 signature, whereas genes involved in lipid metabolism were enriched in the DHDAC1 signature.

We next decided to further investigate the overlap between the DHDAC1 and the TSA signatures. It is likely that both primary and secondary transcriptional effects contribute to the signatures observed upon RNAi of DHDAC1 at 3 and 5 days post-transfection. To narrow down this signature and to enrich it for genes that are likely primary targets of DHDAC1, we determined the overlap between the TSA 6-h post-treatment samples with DHDAC1 silencing 5 days post-treatment (Fig. 7).

36 up-regulated and 16 down-regulated genes were identified in this way. Among the latter, 10 genes have a GO biological process assignment and of these 4 are involved in purine or pyrimidine metabolism (see Supplemental Material). Most strikingly, thymidylate synthase is among these negatively regulated genes.

Down-regulation of this gene by pharmacologically relevant HDACi has also been described in mammalian cells (41), and histone deacetylase inhibitors were shown to increase the cytotoxicity of the thymidylate synthase inhibitor 5-fluorouracil (42). Our data suggest that regulation of thymidylate synthase by HDAC1 is evolutionarily conserved from *Drosophila* to mammals.

Among the up-regulated genes, 23 could be assigned a GO biological process annotation. Among these, seven (30%) are predicted to be involved in energy metabolism (carbohydrate, lipid, polysaccharide, and pyruvate), and eight (35%) have carrier or transport functions, suggesting an important primary transcriptional role of HDAC1 in the regulation of genes involved in energy metabolism and mitochondrial functions.

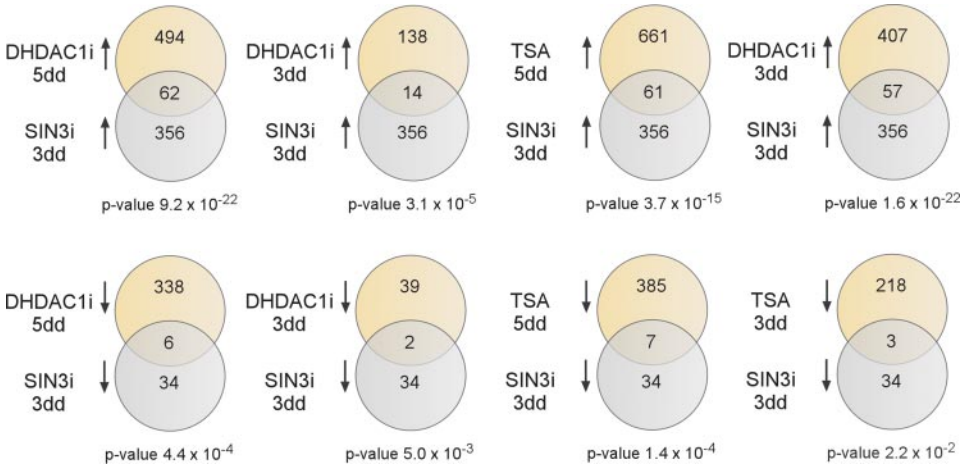

FIGURE 8. **Venn diagrams comparing the DHDAC1 and TSA signatures described in this work with the Sin3-regulated genes described by Pile and co-workers (40).** Probes ($n = 1065$, columns) have been selected for being overexpressed in at least two experiments and clustered according to cosine correlation. The color map refers to experiment log ratio values measured against a combined set of control experiments used as base line.

TABLE 1

Gene expression signatures resulting from loss of DHDAC1 or DHDAC3 grouped by biological process gene ontology annotations

Biological process	No. of genes	One-side Fisher test p value
DHDAC 1 $n = 832$		
Not annotated	323	
Nucleobase, nucleoside, nucleotide and nucleic acid metabolism	142	0.06
Signal transduction	84	0.96
Lipid metabolism	46	0.03
Proteolysis and peptidolysis	40	0.94
Regulation of transcription regulation from PolII promoter	35	0.89
Carbohydrate metabolism	34	0.5
DNA replication	32	0.02
Intracellular protein transport	32	0.82
Cation transport	28	0.49
Regulation of cell cycle	23	0.01
Chromosome segregation	22	0.01
Protein targeting	18	0.5
Cytokinesis	11	3×10^{-8}
Positive regulation of signal transduction	5	0.01
Mitotic spindle organization and biogenesis	3	0.05
Negative regulation of transcription from RNA polymerase II promoter, mitotic	2	0.05
DHDAC 1 $n = 64$		
Not annotated	22	
Proteolysis and peptidolysis	8	0.05
Regulation of transcription regulation from pol II promoter	6	0.13
Protein folding	3	0.03
Phospholipid metabolism	3	0.01

Notably, these genes include glycogen phosphorylase, involved in glycogen turnover, malate dehydrogenase, a component of the Krebs cycle, acetyl-coenzyme A acyltransferase, involved in mitochondrial fatty acid β-oxidation, as well as CG6608, a putative mitochondrial cation transporter.

In addition to these genes, *dcp1*, the *Drosophila* homolog of caspase 6 and *wunen*, a phosphatidate phosphatase are also up-regulated. The latter protein could participate in the generation of pro-apoptotic lipids such as ceramide known to be involved in HDAC inhibitor-mediated cell death (43).

The DHDAC1-SIN3 complex was found by others to be essential for *Drosophila* G_2 phase progression (39) and RNAi of SIN3 also lead to G_2 arrest. This similarity is not unexpected because DHDAC1 is thought to be involved in the transcriptional repressor functions of SIN3. We therefore compared the published microarray profile of SIN3 RNAi (40) with our TSA or DHDAC1 RNAi data. Significant overlaps were detected between the published SIN3 and our DHDAC1 and TSA signatures (Fig. 8). It is important to note that the published Sin3 RNAi data represent common changes observed in both S2 and Kc cells. Possibly an even better overlap may be obtained if only Sin3 S2 data were considered.

Among the genes commonly up-regulated upon SIN3 or DHDAC1 silencing, 42 had a biological function GO annotation, and of those eight are involved in ion, metabolite, or protein transport; seven are involved in amino acid, glucose, or lipid metabolism; and five are involved in nucleotide or nucleoside metabolism.

In contrast to DHDAC1 RNAi, the knockdown of SIN3 predominantly resulted in gene up-regulation (361 up-regulated *versus* 35 down-regulated genes), leading to a very modest overlap of only 6 commonly down-regulated genes. Notably, among these common genes are the already discussed genes thymidylate synthase and *string*.

DISCUSSION

The aim of this work was to gain more insight into the role of individual zinc-dependent histone deacetylase subtypes in higher eukaryotes using RNAi in *Drosophila* S2 cells as a model system. Remarkably, only silencing of DHDAC1, but not of other DHDACs, resulted in increased histone acetylation, and only loss of DHDAC1 and DHDAC3 led to a significant gene expression signature on Affymetrix microarrays. The picture is complicated by the finding that RNAi of either DHDAC1 or -3 caused some off-target silencing of the other subtype. We believe that this off-target silencing is contributing very little to the overall biological data given its low efficiency, the different growth and cell cycle phenotypes, and the different transcriptional signatures observed upon loss of DHDAC1 or -3. Still, we cannot exclude that some commonly regulated genes reflect off-target silencing rather than a truly overlapping biological role (see also below). We interpret the lack of transcriptional effects upon loss of the other DHDACs as indicative of DHDAC2, -4, and -*X* having mainly nontranscriptional functions in S2 cells, possibly acting on non-histone substrates. In fact, RNAi of DHDAC2, the *Drosophila* homolog of HDAC6, resulted in a significant increase in tubulin acetylation levels, consistent with the known role of HDAC6 as tubulin deacetylase (31, 32). The lack of a transcriptional profile associated with DHDAC2 knockdown is also consistent with the observation that tubacin, a specific small molecule inhibitor of HDAC6, fails to produce a significant gene expression change in human A549 cells under condi-

tions where tubulin acetylation is significantly enhanced (44). The mammalian homologs of DHDAC4, HDACs 4, 5, 7, and 9, are widely documented to be involved in the regulation of gene expression, but they do so in a very tissue-specific way (15). This also emerges from the specific phenotypes shown by knock-out animals (28, 29). It is thus conceivable that these proteins might exert nontranscriptional functions in other tissues. Finally, very little is known about the biology of DHDAC*X* or its mammalian homolog HDAC11. The fact that it associates with HDAC6 (35) may indicate a possible role in those processes in which HDAC6 is involved, namely in the regulation of protein folding (via deacetylation of HSP90) or disposal of protein aggregates (33, 34). In the light of the increasing number of non-histone substrates that are being identified, the term "histone deacetylase" may turn out to be a misnomer for many members of this family of proteins. Functional redundancy could be an alternative explanation for the lack of transcriptional effects upon loss of DHDAC2, -4, and -*X*. This redundancy may involve HDACs or other functionally related proteins.

DHDAC1 was the only member of the family whose RNAi gave the "classical" picture of increased histone acetylation levels in conjunction with changes in gene expression profile. DHDAC1 and its close relative DHDAC3 are recruited into different co-repressor complexes (such as SIN3-DHDAC1 and possibly SMRTER-DHDAC3). Several lines of evidence, however, suggest that these complexes may cooperate in mediating transcriptional repression. Thus, SMRTER was shown to interact with SIN3 to mediate repression of ecdysone-regulated genes (45). In line with this notion, the ecdysone-induced *eip74ef* gene was up-regulated upon either DHDAC1 or DHDAC3 RNAi. Further evidence for this cooperation came from the significant overlap of the DHDAC1 and DHDAC3 signatures and from the regulation of gene categories with similar functions. Still, even in cases where gene ontology suggests an involvement of both DHDACs in the same biological processes (such as regulation of expression of pol II-associated proteins or of proteases/peptidases), they influence the expression of different subsets of genes within these functional categories, suggesting a more subtle functional differentiation. It is actually possible that the control of a number of biological processes at the level of histone acetylation has been evolutionarily conserved but that, within these processes, a division of labor among different HDAC subtypes has ensued to allow for a larger flexibility and a more differentiated regulation. As stated above, off-target silencing prevents us from giving an unambiguous interpretation for those genes that are commonly regulated upon loss of either DHDAC.

A significant overlap was detected between the published SIN3 signature (40) and our DHDAC1 signature. The common gene categories identified (transport proteins and enzymes involved in energy metabolism) were compatible with the recently reported function of SIN3 in the regulation of mitochondrial energy metabolism and suggest that many of these genes are actually regulated by SIN3 via recruitment of DHDAC1. In this context also the phenotype observed by us upon DHDAC1 RNAi, namely a G_2 arrest, is in nice agreement with the published phenotype of SIN3 knockdown in S2 cells (39). Most intriguingly, in both experiments a down-regulation of the positive G_2/M regulator *string* was observed, whose knockdown was reported by others to be sufficient to induce a G_2 block (39). These results point to string down-regulation as a likely cause of cell cycle arrest. It is interesting to compare these data to the recently published results on the conditional mSIN3A deletion in mouse embryonic fibroblasts (46). This deletion also leads to a G_2 arrest accompanied by deregulation of genes involved in cell cycle progression, DNA replication, apoptosis, and mitochondrial metabolism, suggesting a substantial evolutionary conservation of the function of this co-repressor despite the additional diversification that occurred in the HDAC family in mammals and the existence of multiple, distinct Sin3 complexes in higher eukaryotes.

The small molecule HDAC inhibitor TSA recapitulated to a large extent the phenotypic and gene expression changes observed upon DHDAC1 RNAi. This compound leads to a G_2 arrest in S2 cells and deregulated a number of genes that were common to those influenced by DHDAC1 knockdown. Still, TSA did induce significant cell death at longer incubation times that was not observed upon silencing of DHDAC1. It is not clear whether these differences arise from a quantitatively larger extent of DHDAC1 inhibition obtained with the small molecule inhibitor or from its ability to simultaneously inhibit multiple HDAC subtypes. It has to be mentioned that we were not able to induce increased cell death by double knockdown experiments of DHDAC1 and the other *Drosophila* HDACs. We conclude that DHDAC1 certainly is an important mediator of the antiproliferative effects of TSA in S2 cells, but we cannot exclude that other mechanisms contribute to it.

We used the overlap of the TSA signature at early time points with the DHDAC1 signature to generate a data set that is enriched for those genes that have a high probability to be directly regulated by DHDAC1. Among these were several genes encoding for transporters and proteins involved in mitochondrial energy metabolism as well as the pro-apoptotic genes *DCP1* (caspase 6 homolog) and *wunen* (phosphatidate phosphatase). The very early deregulation of these genes is striking in the light of recently emerging aspects of the mechanism of action of small molecule HDAC inhibitors. Despite the fact that several compounds are in advanced clinical testing for different forms of cancer, the mechanistic rationale for their tumor cell selectivity remains poorly understood (43). This is particularly true for what concerns the mechanism of apoptosis induction. There are several reports suggesting that mitochondria-mediated generation of reactive oxygen species contributes to the mechanism of tumor cell killing by HDAC inhibitors (47–49). From our data and those generated on SIN3 in *Drosophila* and mouse cells, it appears that this co-repressor complex exerts an important and evolutionarily conserved role in repressing mitochondrial metabolism and controlling mitochondrial size and number and that the deacetylase activity of HDAC1 plays a pivotal role in this function. It is tempting to speculate that reactive oxygen generation by HDAC inhibitors might arise from a deregulated activation of mitochondrial metabolism and the uncoupling of mitochondrial electron transport chains. The link between HDAC inhibition and mitochondrial function certainly deserves a more detailed study with a particular focus on its role in inducing apoptosis in human cancer cells.

While this manuscript was in preparation, Cho and co-workers (50) reported the gene expression profiles of S2 cells overexpressing individual DHDACs. We notice very little overlap between our data and those reported by Cho *et al.* (50). A possible explanation for these differences is that the transient decrease in expression produced by RNAi and reported in this work leads to a qualitatively different perturbation with respect to the stable overexpression approach pursued by Cho *et al.* (50). In this latter case, adaptive gene expression changes might have been selected that heavily contribute to the overall signature.

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Dissecting the Biological Functions of *Drosophila* Histone Deacetylases by RNA Interference and Transcriptional Profiling

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