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Inhibition of Histone Deacetylase 3 Produces Mitotic Defects Independent of Alterations in Histone H3 Lysine 9 Acetylation and Methylation[S]

Robyn Warrener, KeeMing Chia, William D. Warren, Kelly Brooks, and Brian Gabrielli

Diamantina Institute, University of Queensland, Princess Alexandra Hospital, Brisbane, Queensland, Australia (R.W., K.M.C., K.B., B.G.); and Comparative Genomics Centre, James Cook University, Townsville, Queensland, Australia (W.D.W.)

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ABSTRACT

The constitutive heterochromatin of the centromere is marked by high levels of trimethylated histone H3 lysine 9 (H3K9) and binding of the heterochromatin protein 1 (HP1), which are believed to also have an important role in mitosis. Histone deacetylase inhibitors (HDACis) are a class of anticancer agents that affect many cellular processes, including mitosis. Here we examine the mechanism by which these drugs disrupt mitosis. We have used *Drosophila melanogaster* embryos to demonstrate that treatment with the HDACi 100 μg/ml suberic bishydroxamic acid (IC_{50} 12 μg/ml), conditions that induce extensive H3K9 acetylation and aberrant mitosis in mammalian cells, induced aberrant mitosis in the absence of de novo transcription. We have examined the effect of the same treatment on the levels of H3K9 modification and HP1 binding in human cancer cells and found only minor effects on H3K9 methylation and HP1 binding. Complete loss of trimethylated H3K9 or depletion of HP1α and β had no effect on mitosis, although specific depletion of histone deacetylase 3 (HDAC3) replicates the mitotic defects induced by the drugs without increasing H3K9 acetylation. These data demonstrate that H3K9 methylation and HP1 binding are not the targets responsible for HDACi-induced aberrant mitosis, but it is a consequence of selective inhibition of HDAC3.

Introduction

Histone deacetylase inhibitors (HDACis) are a class of antitumor drugs that are proving to have clinical efficacy in a number of tumor types as either single agents or as part of a combination therapy. These drugs produce a range of effects on tumor cells, including promoting the expression of differentiation markers, cell cycle effects, and induction of apoptosis in tumor cells, but have little cytotoxic effect on normal cells (Lindemann et al., 2004; Bolden et al., 2006). HDACi treatment induces a range of transcriptional changes, although these are dependent on the HDACi and cell line used (Glaser et al., 2003; Peart et al., 2005). It has been generally believed that the antitumor effects observed in response to HDACi are a consequence of transcriptional changes. However, histone deacetylases (HDACs) are associated with a range of other functions, including the protein chaperone 90-kDa heat shock protein (Aoyagi and Archer, 2005) and DNA damage recognition in association with Ku70 (Subramanian et al., 2005).

The antiproliferative activity of HDACi results from a combination of inhibiting cell-cycle progression and promoting cell death, the latter being more efficient in cycling cells (Bernhard et al., 2001; Peart et al., 2003; Burgess et al., 2004). HDACi-induced cell-cycle effects include arrest at the G_1/S transition, which is primarily a consequence of increased $p21^{CIP1}$ expression (Archer et al., 1998; Burgess et al., 2001); a G_2-phase checkpoint arrest, observed in small number of cell lines (Qiu et al., 2000); and mitotic defects, including mitotic arrest and mitotic slippage, observed to occur in most cell lines in response to a broad range of HDACis (Warrener et al., 2003; Stevens et al., 2008). Progression through the HDACi-induced aberrant mitosis induces cell death (Warrener et al., 2003); thus, understanding

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ABBREVIATIONS: HDACi, histone deacetylase inhibitor; H3K9, histone H3 lysine 9; HP1, heterochromatin protein 1; SBHA, suberic bishydroxamic acid; HDAC, histone deacetylase; Jmjd, Jumonji; PBS, phosphate-buffered saline; DAPI, 4′-6′diamindino-2-phenylindole; siRNA, short interfering RNA; GFP, green fluorescent protein; HA, hemagglutinin; DD, demethylase dead; ACA, anticentromere.

the mechanism(s) by which HDACi induces the aberrant mitosis is critical to understanding how these drugs promote cell death.

HDACi-induced mitotic defects are observed after relatively short-term treatment with the drugs and seem to be a consequence of exposure during the S-phase before the defective mitosis (Blagosklonny et al., 2002; Warrener et al., 2003; Dowling et al., 2005; Robbins et al., 2005). HDACi treatment causes some disruption to the normal microtubule spindle architecture, failure of normal chromosome congression, extended duration of mitosis, and mitotic slippage (Qiu et al., 2000; Taddei et al., 2001; Warrener et al., 2003; Dowling et al., 2005; Stevens et al., 2008). These mitotic defects are independent of the HDACi used and are observed in multiple cell lines, indicating that the effects are a common response to inhibition of these drugs. The mitotic defects produced phenocopy defects of kinetochore and centromeric components, and this has suggested the possibility that HDACi-induced hyperacetylation of the normally hypoacetylated centromeric heterochromatin may be a primary effector of HDACi-induced mitotic defects (Taddei et al., 2001; Robbins et al., 2005).

Heterochromatin is specialized chromatin that is transcriptionally silenced by a range of epigenetic mechanisms, including DNA methylation and histone modification. Key histone methylation marks defining heterochromatin are di- and trimethylated histone H3 Lys9 (H3K9), which acts as a binding site for the chromodomain of heterochromatin protein 1 (HP1) (Eissenberg and Elgin, 2000; Bannister et al., 2001; Nakayama et al., 2001). Genetic knockout of the SUV39H1 and two methyltransferases responsible for H3K9 di- and trimethylation results in mitotic defects and genomic instability (Peters et al., 2001). Extended long-term treatment with low doses of HDACi also results in a loss of HP1 binding to the centromeric heterochromatin and abnormal mitosis (Taddei et al., 2001). Together, these observations suggested that short-term treatment with higher doses of HDACi may generate mitotic defects by disrupting HP1 binding and normal centromeric heterochromatin function. The disrupted heterochromatin may underlie the defective kinetochore-associated spindle assembly checkpoint in response to HDACi. To investigate this possibility, we have examined how S-phase treatment with HDACi, under conditions that produce aberrant mitosis and mitotic slippage, affect the centromeric heterochromatin and whether this is responsible for the aberrant mitosis observed.

Materials and Methods

Materials. Suberoyl bishydroxamic acid (SBHA) was purchased from Sigma-Aldrich (St. Louis, MO). SBHA was dissolved in dimethyl sulfoxide.

***Drosophila melanogaster* Embryos.** *D. melanogaster* strain w^{1118} was grown at 25°C on standard cornmeal-treacle media. Embryos were collected, dechorionated in 50% bleach for 2 min, then rinsed thoroughly in PBS. After treatment in 100 μg/ml SBHA in PBS for 1 h, embryos were fixed in 1:1 solution of 3.7% paraformaldehyde (in PBS)/heptane for 30 min and devitenillized by the addition of an equal volume of methanol and shaken vigorously for 30 s. DNA was detected by staining with 4′-6′diamindino-2-phenylindole (DAPI; Sigma-Aldrich) and immunofluorescent detection of α-tubulin was performed by using a monoclonal α-tubulin antibody (Sigma-Aldrich) and an Alexa Fluor 555 couple secondary antibody (Invitrogen, Carlsbad, CA).

Cell Culture, Transfection, and Drug Treatment. All human cell lines used in this work, HeLa, HeLa Tet-On, Bcl2-HeLa, and MCF7, were cultured in a humidified incubator at 37°C with 5% CO_2. Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) Serum Supreme (Lonza Walkersville, Inc., Walkersville, MD). Cells were synchronized to the G_1/S-phase boundary by the addition of 2.5 mM thymidine to the culture media for 16 h. Treatment with HDACi was performed at synchrony release, and cells were collected at the times indicated. Knockdown of HP1α and/or HP1β was achieved by transfection with 50 μM concentration of each siRNA using Dharmafect I reagent (Dharmacon RNA Technologies, Lafayette, CO), according to manufacturer's recommendations. The following siRNAs were used: HP1α 1, HP1β1, HP1β2 (ON-TARGETplus; Dharmacon), and HP1α2 (5′-CCUGAGAAAAACUUGGAUUTT-3′); HDAC1, HDAC2, and HDAC3 (ON-TARGETplus SMARTpools; Dharmacon). Cells transfected with siRNA were allowed to grow for 24 h before being synchronized in G_1/S phase with 2.5 mM thymidine for 16 h. SBHA (100 μg/ml) was added to the cells upon release, and cells were harvested 9 h after release in G_2/M. Silencer Select Negative Control siRNA 1 (Ambion, Austin, TX) was used as a scrambled siRNA. Inducible GFP-tagged Jumonji (Jmjd)2C histone demethylase-expressing plasmids were a kind gift from T. Jenuwein Laboratory (Vienna, Austria) (Fodor et al., 2006). HeLa Tet-On cells were transfected using Lipofectamine 2000 (Invitrogen). After transfection, cells were synchronized overnight with 2.5 mM thymidine, and plasmid expression was induced upon release with 2 μg/ml doxycycline. Jmjd expression was confirmed by GFP detection. HA-tagged Jmjd2B (GASC1)-expressing plasmids were a kind gift from K. Helin (Copenhagen, Denmark) (Cloos et al., 2006). Bcl2-HeLa-expressing cells were transfected using Lipofectamine 2000 (Invitrogen), and Jmjd2B expression was confirmed by detection of the HA-tagged protein.

Immunoblotting. Cell pellets were lysed in 1× SDS lysis buffer (0.4% SDS, 2% glycerol, 2.5 mM Tris, pH 6.7, and 0.3 M 2-mercaptoethanol), and cell lysates were quantified using Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). Samples (20–40 μg) were resolved by 12% SDS-polyacrylamide gel electrophoresis and transferred electrophoretically to membrane. Membranes were immunoblotted with primary antibodies against HP1α, HP1β (Millipore Bioscience Research Reagents, Temecula, CA), HDAC1, HDAC2, HDAC3, acetyl lysine, di- and trimethylated H3K9, acetylated H3K9, and histone H3 antibodies (Cell Signaling Technology, Danvers, MA), proliferating cell nuclear antigen (Dako Denmark A/S, Glostrup, Denmark), and α-tubulin (Sigma-Aldrich). These were detected with the appropriate horseradish peroxidase-conjugated secondary antibodies (Zymed Laboratories, South San Francisco, CA) and detected by chemiluminescence.

Immunofluorescent Staining. Cells grown on poly(L-lysine)-coated coverslips were fixed in ice-cold methanol overnight at −20°C. Coverslips were washed twice in PBS and then incubated in cell-blocking buffer [3 mg/ml bovine serum albumin (Sigma-Aldrich) in PBS with 0.05% (v/v) Tween 20] for 30 min at room temperature before immunostaining with antibodies against α-tubulin, human ACA autoimmune serum, HP1α, and HP1β for 1 h at room temperature. For HP1 staining, cells were permeabilized before fixing with 50 μg/ml digitonin, 130 mM sucrose, 50 mM KCl, 50 mM sodium acetate, 20 mM Hepes, pH 7.5, 5 mM $MgCl_2$, and 2 mM EGTA for 90 s before washing twice with PBS and fixing with −20°C methanol. Coverslips were washed twice in PBS and immunostained with the corresponding Alexa Fluor 488 and/or Alexa Fluor 555 (Invitrogen) secondary antibodies for 30 min at room temperature. DNA was counterstained with DAPI (Sigma-Aldrich). Fluorescent microscopy was carried out with Axioskop 2 plus (Carl Zeiss, Thornwood, NY).

Chromosome Spreads. Nocodazole (0.5 μg/ml) was added to cell cultures 1 h before harvest. Mitotic cells were mechanically dis-

lodged and then washed with PBS and resuspended in hypertonic buffer (10 mM Tris, pH 7.4, 10 mM NaCl, and 5 mM $MgCl_2$) at 2×10^5/ml for 10 min. Cell suspension (50 μl) was spun onto microscope slides using a Shandon Cytospin at 5000 rpm for 2 min. Samples were fixed with 4% paraformaldehyde diluted in PBS for 10 min and permeabilized with 0.1% Nonidet P-40 in PBS for 10 min. Samples were blocked with 2% bovine serum albumin/0.1% saponin for 30 min, and antibodies were applied in the same manner for 16 h at 4°C. Secondary antibodies and DAPI applied in 0.1% (w/v) skim milk powder in PBS for 2 h. Samples were mounted under coverslips with ProLong Gold antifade reagent (Invitrogen).

Fluorescence-Activated Cell Sorting. Cells were fixed in ice-cold 70% ethanol in 1× PBS and stored at −20°C. Samples were washed in 1× PBS and resuspended in 0.5 mg/ml RNaseA and 5 μg/ml propidium iodide in PBS. Cells were filtered through 37 μm gauze, and single-cell suspensions were analyzed on a FACSCalibur system (BD Biosciences, San Jose, CA) using CellQuest and Modfit data analysis software (Verity Software House, Topsham, ME).

Results

HDACis Affect Mitosis without Transcription. To determine whether HDACi-induced mitotic defects can occur in the absence of transcription, we turned to the *D. melanogaster* embryo. In *D. melanogaster,* the first 12 embryonic cell cycles after fertilization consist of alternating S and M phases driven by maternally contributed mRNAs and protein. These cycles occur synchronously and do not require zygotic transcription, because de novo transcription does not commence until after cycle 13 (Foe et al., 1993). This provides an excellent model system to examine whether transcription is necessary for the HDACi-induced mitotic defects. For this study, the hydroxamic acid class of HDACi, SBHA, was used. Previous studies have demonstrated that all classes of HDACi have similar effects on mitosis (Warrener et al., 2003; Stevens et al., 2008). SBHA (IC_{50} 12 μg/ml) rapidly induced maximal histone acetylation when used at a concentration of 100 μg/ml (Brinkmann et al., 2001; Gabrielli et al., 2004), and was used at this concentration throughout this study as a generic HDACi. Exposure of early embryos to HDACi resulted in mitotic defects, with loss of the uniform distribution of nuclei in the embryo, a phenotype similar that resulting from exposure to paclitaxel (Taxol; Bristol-Meyers Squibb, Princeton, NJ) (Fig. 1A). Embryos stained for microtubules and DNA revealed extensive numbers of nuclei with hypercondensed DNA, which is indicative of mitotic delay, failure of chromosome migration to the midline of the spindle, and giant nuclei that arise from mitotic slippage (Fig. 1, A and B), a phenotype similar to that reported in human tumor cells (Warrener et al., 2003; Stevens et al., 2008). This indicated that the mitotic defects induced by HDACi treatment were unlikely to be due to transcriptional changes alone. Because many of the mitotic defects caused by HDACi resemble defects in kinetochore/centromeric proteins, one possible target for the HDACi was the normally hypoacetylated centromeric heterochromatin.

HDACi Treatment Has Only a Small Effect on H3K9 Methylation in Mitosis. Key histone methylation marks defining heterochromatin are di- and trimethylated histone H3K9. This site can be acetylated or methylated; the modifications are mutually exclusive. Di- and trimethylated H3K9 act as the binding site for HP1, and it is possible that HDACi can disrupt normal centromeric heterochromatin structure and kinetochore function by altering the association of HP1 with H3K9. To investigate whether short-term HDACi treatment administered in S phase influenced H3K9 modification, the normal dynamics of H3K9 acetylation and methylation through the cell cycle were assessed in synchronized HeLa cells. The level of H3K9 acetylation across the normal cell cycle was very low and could be detected only with very long exposures, whereas the total H3 acetylation measured using a generic antiacetylated lysine antibody, and both di- and trimethylated H3K9 were readily detected. There was an increase in the dimethylated H3K9 as cell progressed through S into G_2 phase, although little obvious difference in the trimethylated H3K9 was observed (Fig. 2A). The effect of HDACi addition on H3K9 acetylation and methylation was examined in a similar experiment. When 100 μg/ml SBHA was added to synchronized HeLa cells in early S phase, the duration of G_2/M phase was extended, mainly due to increased time in mitosis as reported previously (Stevens et al., 2008). A rapid increase in H3K9 acetylation with HDACi addition was noted, which was mirrored by the increased total H3 acetylation (Fig. 2, B and C). H3K9 trimethylation was unaffected by HDACi treatment during progression into mitosis, marked by the increased level of mitogen-activated protein kinase kinase-1 phosphorylated on Thr286 by cyclin B/cdk1 and a marker of mitosis (De Boer et al., 2008), but it blocked the increase of H3K9 trimethylation observed in the following G_1 phase, marked by decreased cyclin A and increased cyclin E (Fig. 2C and Supplementary Fig. 1). HDACi treatment blocked the increased H3K9 dimethylation during

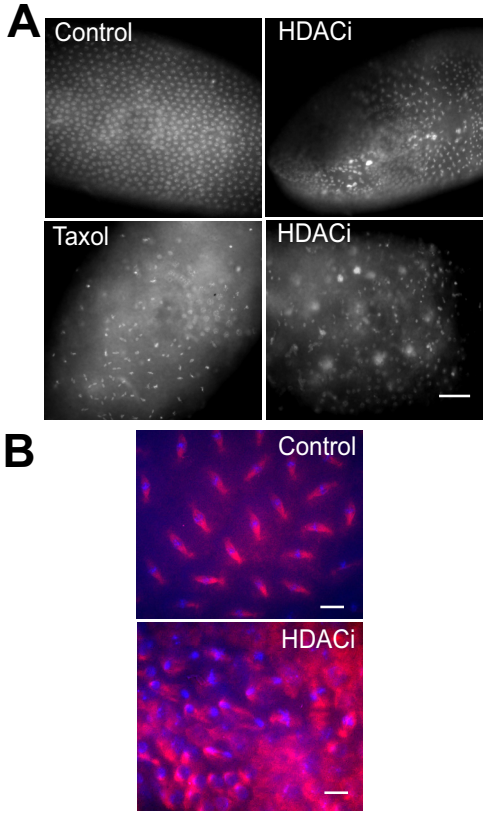

Fig. 1. HDACis induce aberrant mitosis in *D. melanogaster* embryos independent of transcription. *D. melanogaster* embryos isolated immediately after fertilization were either untreated or treated with 100 μg/ml SBHA or 10 ng/ml paclitaxel for 1 h, and then fixed and stained for either DNA (A) or DNA (blue) and α-tubulin (red). Scale bars, 50 μM (A) and 10 μM (B).

S phase, and the level of dimethylation was further reduced during the subsequent G_1 phase, paralleling the decrease in the trimethyl mark. The reduction in H3K9 methylation seemed to be independent on the level of H3K9 acetylation, because treatment with 100 μg/ml SBHA increased acetylation without reducing the methylation significantly more than observed with 10 μg/ml (Fig. 2C). This suggests that the deacetylases acting on the H3 that is targeted for dimethylation were more sensitive to SBHA than those acting on the pool of H3 that is not targeted for K9 methylation.

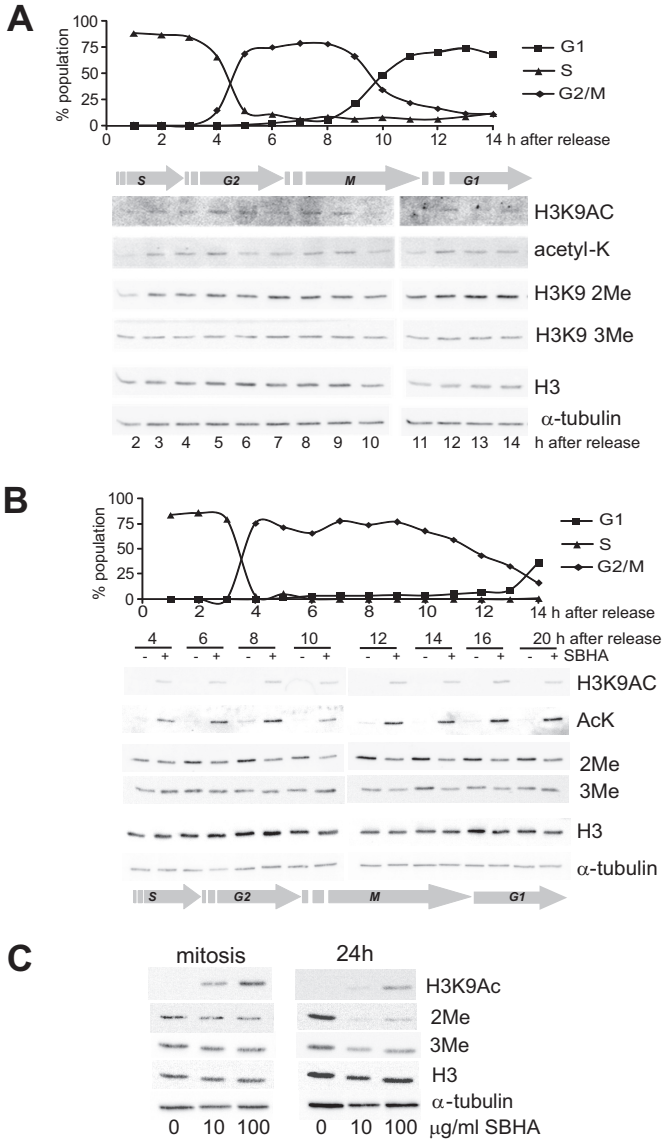

Fig. 2. Cell cycle-dependent effects of HDACi treatment on H3K9 modification. Synchronized HeLa cells were treated without (A) or with 100 μg/ml SBHA (B) at time of release from the thymidine synchrony arrest. Samples were collected at the indicated times for analysis of H3K9 modification. The cell cycle positions were determined by flow cytometric analysis of the DNA content and phases indicated by the arrows at the bottom. Cell lysates were immunoblotted for H3K9 acetylation (Ac), dimethylation (2Me), trimethylation (3Me), total acetylation with an acetyl lysine antibody (AcK), and total histone H3. α-Tubulin was used as a loading control. C, synchronized HeLa cells were treated as in A, either untreated or treated with 10 or 100 μg/ml SBHA. Cells were harvested either at mitosis or 24 h after drug addition when the cells were arrested in late G_1 phase, and lysates were immunoblotted for the indicated proteins and modifications.

To examine whether the gross changes in H3K9 acetylation and methylation observed were uniform across the chromosomes or localized to centromeric heterochromatin, mitotic chromosome spreads were performed to assess their chromosomal distribution. Little H3K9 acetylation was detectable in the untreated controls, whereas a general increase in this modification was observed across the chromosomes, including the centromeric regions, after HDACi treatment (Fig. 3). H3K9 dimethylation seemed relatively uniform across control chromosomes, and no regions of the chromosomes were specifically affected by HDACi treatment. The trimethyl H3K9 was more localized at the centromeric regions, marked by costaining with human ACA serum, as well as in the telomeric regions, but again, no discernible changes were observed after HDACi treatment (Fig. 3).

The lack of change in H3K9 trimethylation and small decrease in dimethylation suggested that there was likely to be little change in HP1 binding after HDACi treatment. Examination of HP1α and β, the primary centromeric binding HP1 isoforms in G_2/M phase (Hayakawa et al., 2003), revealed little change in either protein's association with the centromeric regions. To more readily detect HP1α and β binding to the chromatin, cells were briefly permeabilized before fixation to remove HP1 not tightly associated with the chromatin (Schmiedeberg et al., 2004) (see *Materials and Methods*). A punctate staining pattern was observed with HP1β after permeabilization (Supplementary Fig. 2). Little change was observed in HP1α or HP1β binding in G_2/M phase cells with HDACi treatment (Fig. 4, A and B). The lack of effect on HP1 binding was also observed in normally fixed cells, although the foci were less prominent (Fig. 4C), and in MCF7 cells in which the foci were very prominent without permeabilization (Supplementary Fig. 3). In cells that had progressed through mitosis and into the subsequent G_1 phase in the presence of HDACi, there was the expected loss of HP1 foci corresponding to the reduction in H3K9 methylation at this time (Fig. 2 and Supplementary Fig. 4). Thus, S-phase treatment with HDACi did not seem to affect HP1 chromatin association.

Demethylation of H3K9 and Depletion of HP1 Does Not Affect Mitosis. HDACi treatment reduced H3K9 dimethylation and to a lesser extent trimethylation before entry into aberrant mitosis (Fig. 2). To determine whether reduced H3K9 methylation was responsible for the aberrant mitosis observed, the H3K9 demethylase Jmjd was transiently expressed, and its effect on mitosis was assessed. Jmjd2C was expressed as an inducible GFP-tagged protein, either as the active or as a demethylase dead (DD) mutant form. Jmjd2C was an efficient trimethyl H3K9 demethylase and had detectible activity against the dimethyl mark (Fig. 5A and Supplementary Fig. 5) (Cloos et al., 2006; Fodor et al., 2006). Overexpression of active Jmjd2C markedly reduced cell viability and the number of cells entering mitosis. To overcome this problem, the closely related Jmjd2B (also called Gasc1) was transiently overexpressed in HeLa cells stably overexpressing the antiapoptotic protein Bcl-2 (Burgess et al., 2004). Whereas this increased the numbers of cells overexpressing Jmjd proteins, few Jmjd2B (Fig. 5B) or Jmjd2C (data not shown) overexpressing cells were detected in mitosis. Those detected revealed the presence of normal metaphase and anaphase cells. This contrasted with HDACi-treated cells

in which no metaphase or anaphase figures were observed (Stevens et al., 2008) (Fig. 7C).

Although HDACi treatment had little effect on HP1 α or β binding to chromatin, siRNA depletion of both HP1 isoforms was performed to determine whether there were subtle effects mediated through HP1 that could account for the aberrant mitosis observed. Two independent siRNAs were used to knock down each isoform, and these reduced the protein levels efficiently by 24 h after transfection. All four siRNAs were combined to efficiently deplete HP1 α and β together (Fig. 6A). Examination of mitotic cells depleted for both isoforms revealed no defects and no quantitative differences in the distribution of cells across the phases of mitosis observed (Fig. 6, B and C). This contrasts to HDACi treatment, in which the majority of cells arrested in prometaphase (Fig. 7C). In addition, overexpression of a dominant-negative mutant form of HP1β deleted for the chromo shadow domain that reduced HP1 foci did not affect normal mitosis (data not shown). In addition, codepletion of HP1 α and β had no effect on the aberrant mitosis promoted by HDACi treatment (Fig. 6D), indicating that HP1 α and β are not required for the drug-induced mitotic defects.

Depletion of HDAC3 Mimics HDACi Effects on Mitosis. The minor effects of HDACi treatment on either H3K9 methylation or HP1 binding, and lack of effect of directly modifying H3K9 methylation and HP1 binding on mitosis, demonstrated that the mitotic defects observed with drug treatment were independent of this heterochromatin marker. HDAC3 has been demonstrated to localize to the mitotic spindle in association with the nuclear receptor corepressor N-Cor, and depletion of HDAC3 induced mitotic defects that seemed similar to those observed with HDACi treatment (Ishii et al., 2008). We examined the effect of depletion of individual class 1 HDAC isoforms and examined their effects on mitosis. Individual deletion of either HDAC1 or HDAC2 had no detectible effect on mitosis (data not shown). Codepletion of HDAC1 and HDAC2 was sufficient to increase H3K9 acetylation but had no effect on the proportion of mitotic cells in each phase of mitosis (Supplementary Fig. 6, A and B). By contrast, depletion of HDAC3 had no effect on H3K9 acetylation or acetylation of other sites on H3 detected using a generic acetyl lysine antibody and little effect on either H3K9 di- or trimethylation. It did result in an accumulation of the mitotic marker phosphorylated B23 to an extent similar to HDACi treatment, indicating that it caused a delay in mitosis similar to HDACi treatment (Fig. 7A and Supplementary Fig. 6a). Immunofluorescence staining for mitotic cells revealed marked increase in the proportion of cells in with a prometaphase morphology, a formed bipolar spindle but with chromosomes not aligned at the metaphase plate. Costaining with human ACA serum to detect centromeres revealed that centromeres had migrated to the astral side of the spindle poles, a feature commonly observed with HDACi treatment (Fig. 7b). Quantitation revealed a significant increase in the proportion of cells with noncongressed chromosomes (identified as prometaphase cells), from 24% in the scrambled siRNA transfected cells to 47% in HDAC3 depleted cells ($p < 0.0001$), although this was still less than the 90% prometaphase cells seen with HDACi treatment (Fig. 7c).

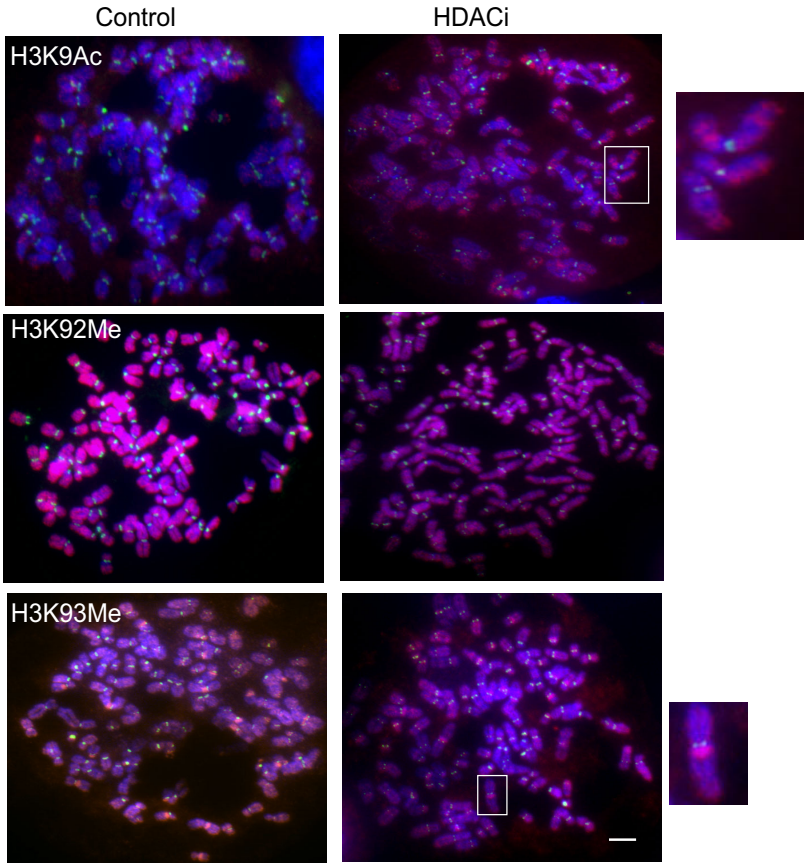

Fig. 3. Chromosomal spreads show little change in trimethyl H3K9 centromeric staining. Mitotic chromosome spreads were performed on either untreated or cells treated with 100 μg/ml SBHA from the beginning of S phase as in Fig. 2 and harvested as cells entered mitosis (10 h after synchrony release). Chromosomes were stained for the indicated modifications (red), DNA (blue), and ACA to mark the centromeres (green). Higher magnification of the white boxed regions are shown at right. Scale bar, 5 μM.

The increased proportion of cells in prometaphase suggested that cells were delaying in mitosis. Time-lapse microscopy revealed the average time taken for HDAC3-depleted cells to traverse from prophase to anaphase was delayed, increasing from 31 to 44 min ($p < 0.0001$; Fig. 8A). The increased mitotic transit time suggested that either the mitotic aberrations were readily resolved or the cells failed to maintain the spindle assembly checkpoint-dependent mitotic arrest and exited prematurely without correctly partitioning their genome-producing multinuclear cells. An increased proportion of cells with multiple nuclei were observed in HDAC3 depleted cells to a level comparable with HDACi treatment (Fig. 8, B and C). Thus, HDAC3 depletion induced aberrant mitosis and premature exit similar to the effect of HDACi.

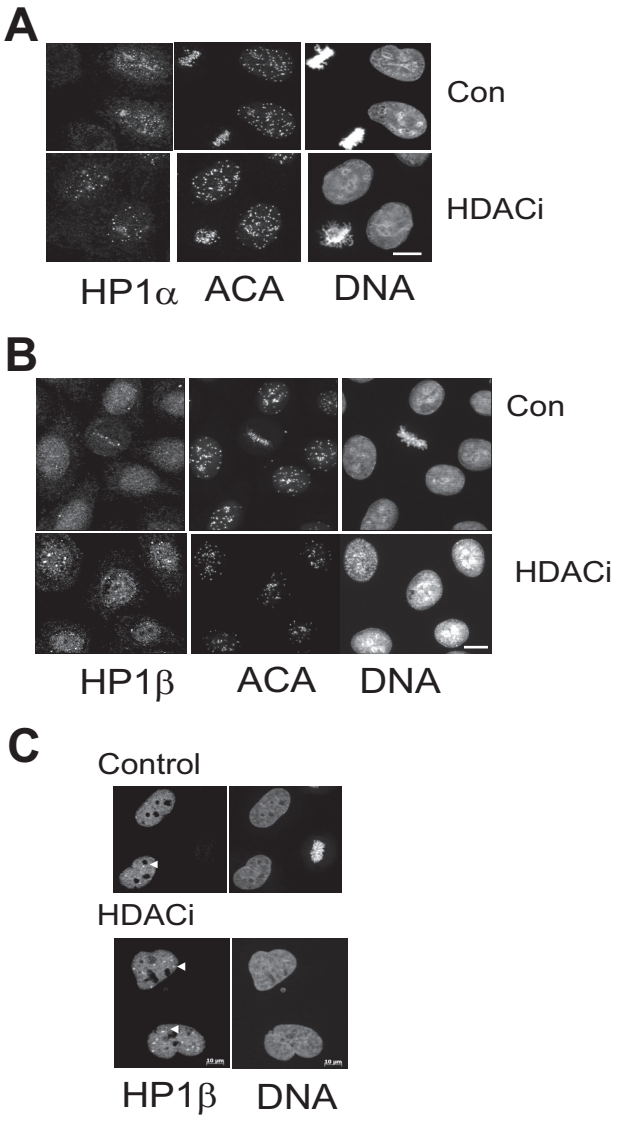

Fig. 4. HDACi treatment has little effect on HP1 centromeric association. A and B, synchronized HeLa cells, either untreated (Con) or treated with 100 μg/ml SBHA (HDACi) in S phase were harvested in G_2/M phase (7 h after synchrony release), permeabilized, and then fixed and stained for HP1α or HP1β, ACA, and DNA. C, HeLa cells were treated as in B, harvested in G_2 phase and fixed without permeabilizing first, and stained for HP1β and DNA. Scale bar, 10 μm.

Discussion

HDACis have marked effects on mitosis, although the mechanism by which they produce these effects is at present unclear. We have demonstrated that HDACis can induce aberrant mitosis in *D. melanogaster* early embryos, which run off maternal mRNA and protein stores, indicating that HDACis cause significant mitotic effects independent of transcription. Microarray analysis of various cell lines treated with a range of HDACi has failed to identify common gene expression changes able to account for the mitotic defects observed (Glaser et al., 2003; Peart et al., 2005), whereas we and others have observed these mitotic defects in a number of cell lines with a range of HDACi (Qiu et al., 2000; Taddei et al., 2001; Warrener et al., 2003; Dowling et al., 2005; Robbins et al., 2005; Stevens et al., 2008). The lack of consistent transcriptional changes in response to HDACi treatment contrasts strongly with the consistent mitotic defects observed and provides further support that the HDACi effect on mitosis is independent of transcriptional changes.

Previous studies have demonstrated that the mitotic effects induced by HDACi treatment required the presence of the drug through S phase (Warrener et al., 2003). This timing coincides with changes in histone acetylation required for deposition of chromatin histones onto the newly replicated DNA (Taddei et al., 1999; Tyler et al., 1999; Dillon and Festenstein, 2002). The acetylation state of the euchromatin is rapidly affected by HDACi treatment independent of the cell cycle stage, but histones deposited in newly replicated heterochromatic regions are only acetylated during S phase (Taddei et al., 1999). This had suggested that increased acetylation of heterochromatin H3K9, blocking methylation of this site, may underlie the aberrant mitosis observed. We found that H3K9 methylation increased moderately during S phase, ensuring the preservation of the heterochromatin in the newly repli-

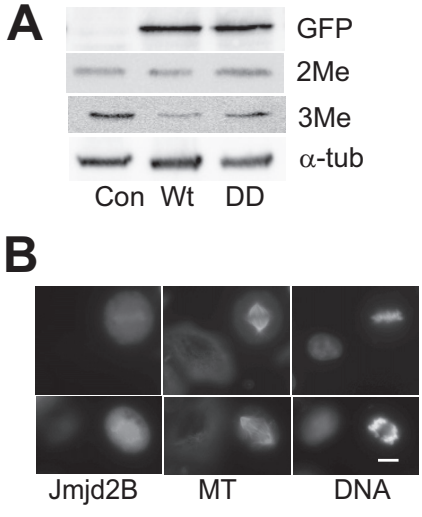

Fig. 5. Jmjd2C demethylation of H3K9 does not affect mitosis. A, HeLa cells were transfected with either Jmjd2C or an inactive mutant (DD). Twenty-four house after transfection, cells were harvested and immunoblotted for GFP to define Jmjd2C overexpression, dimethyl (2Me) or trimethyl (3Me) H3K9, and α-tubulin as a loading control. B, HeLa cells were transfected with HA-tagged Jmjd2B, harvested at 24 h after transfection, and stained for the HA-tagged Jmjd2B, α-tubulin (MT), and DNA. Scale bar, 10 μm.

cated DNA. Surprisingly, HDACi treatment had a stronger effect on the dimethyl H3K9 mark than the trimethyl mark, suggesting that H3K9 dimethylation is more dynamic than trimethylation before mitosis. Both methylation marks were strongly reduced after transit through mitosis. This may be a consequence of dynamic exchange of free and chromatin-associated H3 in G_1 phase as reported in *Xenopus laevis* (Stewart et al., 2006), with the inhibition of HDACs blocking methylation of the newly deposited histone, or be related to the loading of CENPA, the H3 variant that specifically marks the centromeric heterochromatin, which also occurs in G_1 (Jansen et al., 2007).

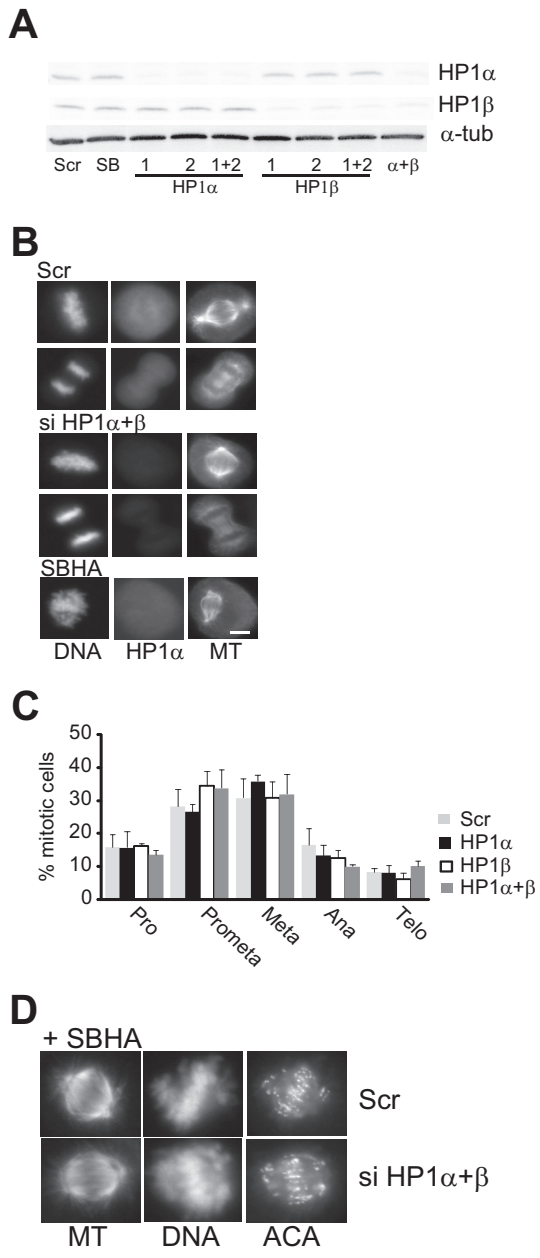

Fig. 6. HP1 depletion does not affect mitosis. A, HeLa cells were transfected with either scrambled control siRNA (Scr), HP1 α an β, or α and β siRNA. Forty-eight hours after transfection, cells were harvested and immunoblotted for HP1α and β and α-tubulin as a loading control. HeLa cells treated with 100 μg/ml SBHA (SB) are shown; as a further control (B), HeLa cells were treated with either scrambled control (Scr) or HP1α and β siRNA, synchronized, and harvested at G_2/M (9 h after synchrony release). In parallel, synchronized HeLa cells were treated with 100 μg/ml SBHA in S phase and then harvested in G_2/M. Cells were fixed and stained for DNA, HP1α, and α-tubulin (MT). Scale bar, 10 μm. C, cells treated with either scrambled (Scr), HP1α and β, or α and β siRNA, synchronized, and then harvested in G_2/M, fixed, and stained as in B. The percentage of mitotic cells in each phase of mitosis, prophase, prometaphase, metaphase, anaphase, and telophase was quantified. The data are from counting >100 cells for each experiment from three experiments. D, HeLa cells were transfected with either scrambled (Scr) or HP1 α and β siRNA, synchronized, and then treated with 100 μg/ml SBHA in S phase. Cells were harvested at G_2/M (9 h after synchrony release), fixed, and stained for α-tubulin (MT), DNA, and ACA. Scale bar, 10 μm.

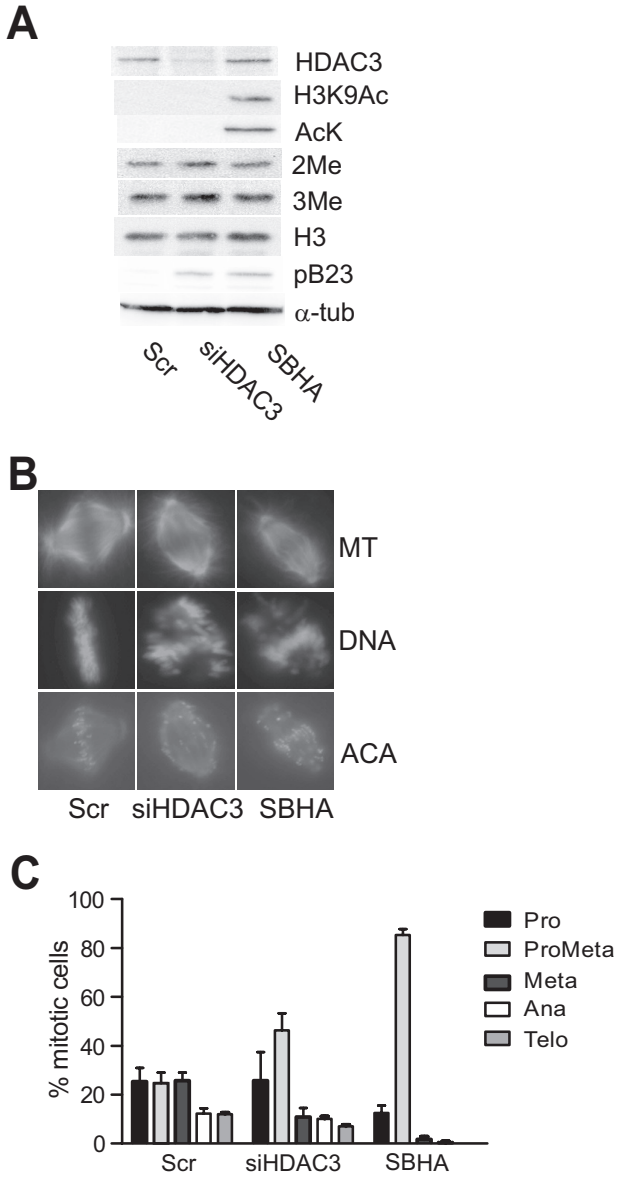

Fig. 7. Depletion of HDAC3 delays cells in prometaphase. HeLa cells were treated with scrambled (Scr) or HDAC3 siRNA, synchronized, and then harvested at G_2/M (9 h after synchrony release). In parallel, synchronized HeLa cells were treated with 100 μg/ml SBHA in S phase and harvested at G_2/M. Cells were analyzed by immunoblotting for HDAC3, H3K9 acetylation (H3K9Ac), generic acetyl lysine antibody that detects acetylated H3 (AcK), dimethylation (H3K92Me), trimethylation (H3K93Me) total H3 protein, phosphorylated B23 (pB23) as a marker of mitosis, and α-tubulin (α-tub) as a loading control. B, HeLa cells treated identically as in A were fixed at G_2/M and stained for DNA, α-tubulin (MT), and ACA. C, the percentage of mitotic cells in each phase of mitosis, prophase, prometaphase, metaphase, anaphase, and telophase were quantified. The data are from counting >100 cells for each experiment from three experiments.

The relative lack of effect of HDACi treatment on HP1 binding in G_2/M phase cells is not surprising given the very minor reduction in the trimethylated H3K9, which seems to be the primary binding motif for HP1 given their colocalization at particularly the centromeric regions. The reported loss of HP1 binding with HDACi treatment is a consequence of long-term exposure to low doses of HDACi over multiple cell cycles rather than short-term exposure as examined in the current study (Taddei et al., 2001, 2005). We did observe reduced HP1 heterochromatin binding, but only after the cells have transited through mitosis, correlated with the strong reduction in H3K9 di- and trimethylation. The surprising lack of effect of the near complete loss of trimethylated H3K9 by Jmjd2B and C on mitosis despite the loss of HP1 binding after expression of these proteins (Fodor et al., 2006) supports our HP1 siRNA depletion studies, demonstrating that HP1 binding is not required for normal mitosis. Moreover, HP1 binding is normally reduced in mitosis by the phosphorylation of H3S10 (Fischle et al., 2005; Hirota et al., 2005), suggesting that HP1 association with the centromeric heterochromatin is not a normal requirement for mitosis.

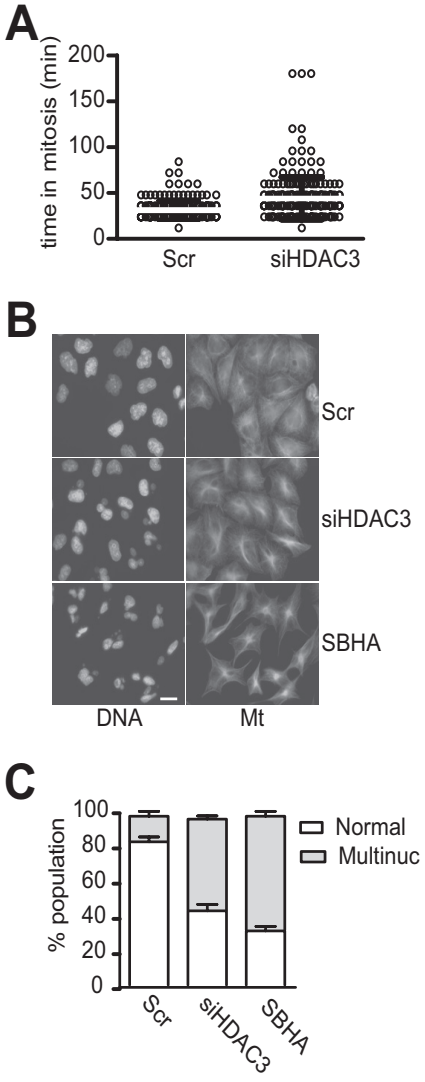

Fig. 8. HDAC3 depletion promotes premature mitotic exit. A, HeLa cells were transfected with either scrambled control (Scr) or HDAC3 siRNA and then synchronized and followed after release from the synchrony arrest as they progressed through mitosis. The time for mitotic progression from prophase to anaphase was assessed for >200 cells for each condition. B, HeLa cells were transfected with either scrambled (Scr) or HDAC3 siRNA, synchronized, and then fixed at 24 h after synchrony release after the cells had exited mitosis. In a parallel experiment, cells were treated with 100 μg/ml SBHA and fixed as above. Cells were stained for α-tubulin and DNA. Scale bar, 10 μm. C, quantitation of the proportion of cells with a single nucleus (normal) or multiple nuclei (multinuc) as a marker of premature mitotic exit. The data are from counting more than 100 cells in each experiment in three independent experiments.

Our findings that alterations to H3K9 methylation or HP1 binding had little effect on mitosis suggested that the mitotic effects observed with HDACi are not due to increased H3K9 acetylation, other histone or protein acetylation, or non-HDAC targets of the drugs. The last of these possibilities can be readily dismissed because all of the HDACis used, representing examples from each class of HDACi, induce the same spectrum of mitotic defects (Cimini et al., 2003; Warrener et al., 2003; Dowling et al., 2005; Robbins et al., 2005; Stevens et al., 2008). The increased H3K9 acetylation is also unlikely to contribute to the aberrant mitosis observed because depletion of HDAC 1 and 2 increased this modification without any effect on mitosis. HDAC3 depletion has no effect on H3K9 acetylation, and we have confirmed that it induces aberrant mitosis (Ishii et al., 2008).

HDAC3 depletion has also been reported to cause sister chromatid dissociation associated with modification of H3K4, a modest increase in acetylation and reduction in the methylation of this residue, the latter seemed to be primarily the centromeric levels of this modification (Eot-Houllier et al., 2008). This was associated with a loss of sister chromatid cohesion and loss of centromeric localization of Shugoshin (Sgo1), a PP2A targeting protein that regulates sister chromatid separation (Wang and Dai, 2005). Extended treatment of mitotic-arrested cells with HDACi produces a similar loss of sister chromatid cohesion proportion of cells (Magnaghi-Jaulin et al., 2007). This effect is at odds with the observations reported here, which have shown that rather than loss of cohesion, the mitotic chromosomes have a "closed arm" appearance similar to Haspin overexpression, which results in increased histone H3Thr3 phosphorylation (Dai et al., 2006), which was also reported with HDAC3 depletion (Eot-Houllier et al., 2008). We observed little effect on total H3K4 methylation with HDAC3 depletion, and HDACi treatment induced an increase in methylation of this residue (Supplementary Fig. 7). The latter had been reported previously with other HDACis, and it was suggested that increased acetylation of H3K9 was coordinated with increased H3K4 methylation (Nightingale et al., 2007).

Selective inhibition of HDAC3 seems to be responsible for at least some of the mitotic defects observed with HDACi treatment, although the exact mechanism is still unclear. It is evident, however, that this does not involve effects on H3K9 acetylation, methylation, or HP1 binding. Other nonhistone protein acetylations may contribute to the defective mitosis observed with HDACi treatment. BubR1, a kinetochore associated protein and key regulator of mitosis, is regulated by acetylation, although in this case, acetylation activates the protein (Choi et al., 2009), whereas the normal mitotic activation of BubR1 is blocked

by HDACi treatment (Stevens et al., 2008). Other potential targets are the Aurora kinases, which have critical roles in normal mitosis, and inhibition of these kinases promote premature mitotic exit (Vader and Lens, 2008). HDAC3 depletion has been reported to promote proteasome-dependent destabilization of Aurora A and B (Cha et al., 2009), which may contribute to the aberrant mitosis and premature exit we have observed. However, we have reported previously little effect of HDACi treatment on Aurora B levels in mitosis (Stevens et al., 2008), and although we have observed reduced Aurora A levels, complete inhibition of Aurora A with a selective inhibitor initiated a strong mitotic arrest rather than bypass of mitotic checkpoint observed with HDACi (K. M. Chia and B. Gabrielli, unpublished observations).

In summary, we have found that HDACi effects on mitosis seem to be independent of transcriptional changes induced by drug treatment. Surprisingly, HDACi treatment has only a minor effect on H3K9 methylation and HP1 binding to the centromeric heterochromatin before mitosis, and depletion of trimethylated H3K9 or HP1 does not influence mitosis adversely, indicating that the mitotic effects of HDACi are independent of this heterochromatin marker. The primary target of HDACi seems to be HDAC3, and selective depletion of this HDAC isoform mimicked many of the effects of HDACi treatment on mitosis and promoted premature mitotic exit. These findings demonstrate that the mitotic effect of HDACi is at least in part mediated by inhibition of HDAC3 function at the kinetochore and suggests that drugs specifically targeting HDAC3 may have value as anticancer drugs.

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Address correspondence to: Dr. Brian Gabrielli, University of Queensland Diamantina Institute, Princess Alexandra Hospital, Brisbane, Queensland, Australia 4102. E-mail: briang@uq.edu.au